MOLECULAR DYNAMICS AT THE IMMUNOLOGICAL SYNAPSE

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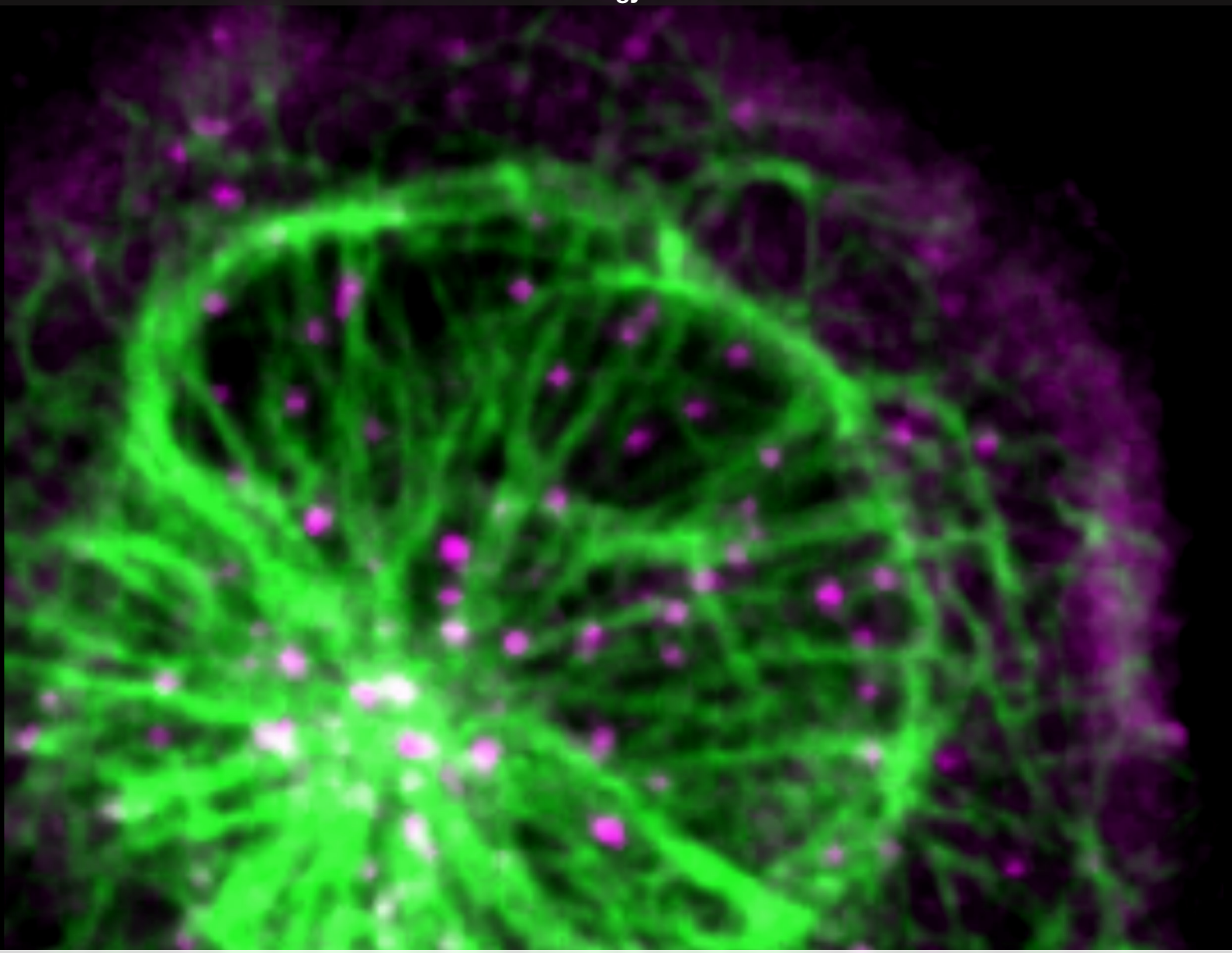

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MOLECULAR DYNAMICS AT THE IMMUNOLOGICAL SYNAPSE

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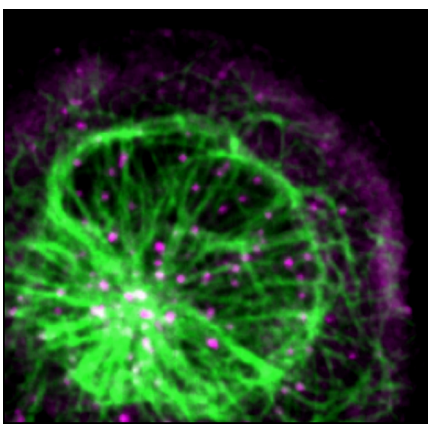

Immunological synapses are regulated by the interplay between the actin cytoskeleton, which polymerizes at the synapse periphery, microtubules, which form radial arrays from the center to the edges of the synapse, and signaling protein complexes that need both actin and microtubule dynamics to move centripetally and control T cell receptor signal transduction. The image shows signaling microclusters containing the adaptor SLP76 (magenta) aligned on microtubules (green) in a synapse formed by a Jurkat T cell spread on anti-CD3-coated coverslip.

Picture by R. Lasserre and V. Di Bartolo, Institut Pasteur.

The immunological synapse (IS) is a specialised cell-cell adhesion that mediates antigen acquisition and regulates the activation of lymphocytes. Initial studies of the IS showed a structure composed of stable supra-molecular activation clusters (SMAC) organised during the interaction of helper T lymphocytes with B lymphocytes, working as antigen presenting cells. A central SMAC of coalesced T cell receptors (TCRs) and a peripheral SMAC for cell-cell adhesion were observed. IS with similar structure was later described during antigen acquisition by B cells and during the interaction of NK cells with target and healthy cells. More recent research developed with microscopy systems that improve the spatial and temporal resolution has showed the complex molecular dynamics at the IS that governs lymphocyte activation. Currently, the IS is seen as a three-dimensional structure where signalling networks for lymphocyte activation and endosomal and cytoskeleton machinery are polarised. A view has emerged in which dynamic microclusters of signalling complexes are composed of molecular components attached to the plasma membrane and other components conveyed on sub-synaptic vesicles transported to the membrane by cytoskeletal fibers and motor proteins. Much information is nonetheless missing about how the dynamics of the endosomal compartment, the cytoskeleton, and signalling complexes are reciprocally regulated to achieve

the function of lymphocytes. Experimental evidence also suggests that the environment surrounding lymphocytes exposed to different antigenic challenge regulates IS assembly and functional output, making an even more complex scenario still far from being completely understood. Also, although some signalling molecular components for lymphocyte activation have been identified and thoroughly studied, the function of other molecules has not been yet uncovered or deeply characterised. This research topic aims to provide the reader with the latest information about the molecular dynamics governing lymphocyte activation. These molecular dynamics dictate cell decisions. Thus, we expect that understanding them will provide new avenues for cell manipulation in therapies to treat different immune-related pathologies.

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EDITORIAL
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Editorial: Molecular Dynamics at the Immunological Synapse

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Editorial on the Research Topic

Molecular Dynamics at the Immunological Synapse

The immunological synapse (IS) is a specialized cell–cell adhesion that mediates antigen acquisition, lymphocyte activation, and effector function. Seminal studies showed a structure composed of stable central and peripheral supramolecular activation clusters (cSMAC and pSMAC) organized at the interface of interacting helper T lymphocytes and B lymphocytes. The T cell receptor (TCR) and signaling molecules were found accumulated at the cSMAC, whereas the integrin LFA-1 and cytoskeleton components distributed at the pSMAC (1). The dynamics of these clusters at the IS was further tracked on the imaging plane by using antigen-presenting planar lipid bilayers (2). Currently, the IS is seen as a three-dimensional structure where signaling networks and components of the cellular machinery, including the endosomal compartment and the cytoskeleton, are polarized and reciprocally regulated to achieve proper T cell activation [Martin-Cofreces et al.; (3, 4)]. It has been also proved an important role of the IS in intercellular communication, being a local target for cytokine secretion and for the delivery of exosomes probably conveying important regulatory clues to antigen-presenting cells (APCs) (5, 6). The advent of new microscopy systems that improve the spatial and temporal resolution has shown the complex molecular dynamics at the IS. Early signaling is organized in dynamic microclusters at the periphery of the IS and, concomitantly, subsynaptic vesicles transport to these sites molecular components of signaling complexes (7–9). Much information is nonetheless missing about how this interplay between signaling and dynamic vesicular traffic and cytoskeleton is regulated.

This research topic (RT) contains 10 articles that cover different aspects of the molecular dynamics at the IS. Data are contributed on the spatial regulation of the signaling molecule Lck, an important molecular requirement for TCR triggering. With the assistance of super-resolution microscopy, Kapoor-Kaushik et al. provide a piece of original data to discuss how the spatial organization of Lck is regulated in activated T cells. Although the open conformation promotes clustering, signaling downstream the TCR further controls the spatial organization of Lck. Regarding the role of integrins in the triggering of the TCR and T cell activation, Hashimoto-Tane and Saito have recently demonstrated the existence at the IS of adhesion rings of integrins and focal adhesion molecules surrounding TCR-containing microclusters. This so-called microsynapse is proposed to support weak TCR activation *via* cell–cell local adhesion signals.

One novel and timely aspect discussed by Comrie and Burkhardt is how mechanotransduction, the transformation of mechanical forces into biochemical modifications, contributes to the TCR triggering and the intracellular signaling. The authors focus on the role of mechanical forces directed by filamentous actin (F-actin). The review contributed by Hivroz and Saitakis focuses on other mechanical clues that regulate T cell activation, including the effect of membrane protrusions and oscillations, cell mobility and spreading, the TCR engagement itself, or the engagement of LFA-1 during the IS formation.

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Regarding the regulation of F-actin regulators, Ramirez-Munoz et al. propose that a local action of the cofilin activator Slingshot-1 at the IS might mediate an ultrasensitive/bistable response of the cofilin signaling module. This signaling module might then contribute to the specific and sensitive responses of naïve T cells and the more efficient and faster activation of antigen-experienced T cells.

The relevance of the cytoskeleton remodeling at the dendritic cell (DC) side is discussed by Benvenuti, who focuses her attention on the role of actin regulators, such as fascin and WASp, among others. The author also discusses about DC polarity and secretion induced by maturation stimuli. For example, the Cdc42-mediated polarization of the MTOC controls the delivery of IL-12 to the DC-T cell IS, a process mediated by VAMP7. Thus, it is envisaged that the activating signal three (inflammatory cytokines) is coupled at the IS to the activating signals one (TCR) and two (costimulation).

The dynamics of the endosomal compartment is discussed in the review by Onnis et al.. The authors revise the different Rab GTPases controlling the recycling routes targeting different receptors, such as the TCR and CXCR4, to the IS. They also highlight the recently noticed role of components of the intraflagellar transport system in controlling the traffic of the TCR to the IS downstream the centrosome polarization. This contribution poses the notion that IS and cilium constitute functional homologs. Important mechanisms of cell–cell communication are also described, including the trogocytosis and the local delivery of exoxomes and microvesicles.

Spatial organization of the IS also resembles the phagocytic cup, leading to the concept of the phagocytic synapse. Niedergang et al. remark this parallelism and discuss the organization, mechanism of assembly, and regulation of both structures. They pay attention to immune and phagocytic receptors, the interplay of the actin and tubulin cytoskeleton and the vesicular traffic. Discussion is provided about the role of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors and Rab GTPases in polarized vesicular traffic.

The structure and function of costimulatory and coinhibitory receptors upon the engagement of B7 molecules expressed on APCs are discussed by Brzostek et al.. They describe the function of CD28 and CTLA4 in the immune response, the regulatory role in the cytoskeleton dynamics and signaling and the distribution to the IS in effector and regulatory T cell.

Rocha-Perugini et al. discuss the role of tetraspanin-enriched microdomains in the local accumulation of receptors, adhesion molecules, and integrins at the IS. Associations are described between IS-located tetraspanins, several signaling molecules, and the actin cytoskeleton.

In summary, this RT highlights the fine-tuned molecular dynamics at the IS that allows proper T cell activation and effector functions. Methodological and technical advances in microscopy techniques improving spatial and temporal resolution are helping us to understand how the dynamics of the cytoskeleton and the endosomal compartment reorganizes micro and nanodomains of signaling complexes that, in turn, mediate lymphocyte immune responses. In addition, complementary biophysical approaches as well as the comparison with biological systems mentioned in this collection may provide useful hints to unravel the complexity of ISs.

AUTHOR CONTRIBUTIONS

PR-N wrote the first draft of the manuscript and updated the last version. AA and VB corrected and completed the initial draft.

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PERSPECTIVE
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Ultrasensitivity in the Cofilin Signaling Module: A Mechanism for Tuning T Cell Responses

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Ultrasensitivity allows filtering weak activating signals and responding emphatically to small changes in stronger stimuli. In the presence of positive feedback loops, ultrasensitivity enables the existence of bistability, which convert graded stimuli into switch-like, sometimes irreversible, responses. In this perspective, we discuss mechanisms that can potentially generate a bistable response in the phosphorylation/dephosphorylation monocycle that regulates the activity of cofilin in dynamic actin networks. We pay particular attention to the phosphatase Slingshot-1 (SSH-1), which is involved in a reciprocal regulation and a positive feedback loop for cofilin activation. Based on these signaling properties and experimental evidences, we propose that bistability in the cofilin signaling module might be instrumental in T cell responses to antigenic stimulation. Initially, a switch-like response in the amount of active cofilin as a function of SSH-1 activation might assist in controlling the naïve T cell specificity and sensitivity. Second, high concentrations of active cofilin might endow antigen-experienced T cells with faster and more efficient responses. We discuss the cofilin function in the context of T cell receptor triggering and spatial regulation of plasma membrane signaling molecules.

Keywords: ultrasensitivity, bistability, cofilin, Slingshot-1, T cell receptor

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ACTIN DYNAMICS AND ACTIVATION OF T CELLS

Initial signaling events triggered by the T cell receptor (TCR) after the specific engagement of antigenic peptide–MHC complexes (pMHC) occur in dynamic TCR microclusters organized at the periphery of the immunological synapse (IS) (1). TCR microclusters migrate to the center of the IS, where they are endocytosed for signaling downmodulation (2). The actin cytoskeleton is essential for the early signaling and centripetal movement of TCR molecules and integrins that precedes TCR downmodulation (1, 3, 4). TCR early signaling promotes the formation of a dynamic network of filamentous actin (F-actin), which, in turn, mediates the maturation of the IS with the formation of a central and a peripheral supramolecular activation cluster (cSMAC and pSMAC, respectively) (5).

Beyond the function in the initial signaling events and IS maturation, actin dynamics have been suggested to regulate the kinetics of the TCR/pMHC engagement. Experiments based on Förster resonance energy transfer (FRET) in live cells have demonstrated that the affinity of the TCR/pMHC interaction is higher but yet short-lived than previously detected by *in vitro* experiments

Abbreviations: ac, active cofilin; Ag-e, antigen-experienced; APCs, antigen-presenting cells; F-actin, filamentous actin; pCof, phospho-cofilin; pMHC, peptide–MHC complex; SEE, staphylococcal E enterotoxin; tCof, total cofilin.

(6). The actin cytoskeleton was proved to promote a high dissociation rate. These data pose the question about how brief TCR interactions can efficiently activate T cells that are scanning antigen-presenting cells (APCs), which frequently contain low densities of surface antigenic pMHC compared with endogenous pMHC. High affinity and brief interactions might assist the serial-specific engagement of TCR molecules compacted together in surface oligomers, so-called nanoclusters or "protein islands" (6, 7). TCR clustering can also help to keep specificity while raising sensitivity of T cells by ensuring the effective half-life or "confinement time" of a TCR–pMHC interaction as predicted by the rebinding model that was recently proposed (8, 9). Antigen-experienced (Ag-e) T cells exhibit bigger TCR nanoclusters that parallel a lower activation threshold than the observed in naïve T cells (10). Thus, it seems that an avidity-maturation process mediates enhanced responses seen in effector or memory T cells (10, 11). The mechanism regulating the organization of cell surface nanoclusters is nonetheless not known. Interestingly, it has been recently proposed that dynamic short actin filaments promote the formation of surface protein oligomers (12). Thus, in addition to controlling kinetic parameters of the TCR/pMHC engagement and the molecular dynamics during early T cell activation, actin dynamics might also be involved in the spatial and temporal organization of cell surface oligomers of signaling molecules.

REGULATION OF ACTIN DYNAMICS BY COFILIN

Cofilin depolymerizes and severs F-actin, being in this way one of the major regulators of actin dynamics in the cell. Activity of cofilin is regulated by a phosphorylation/dephosphorylation monocycle of the serine residue in position 3 (Ser-3) (**Figure 1A**). Phosphorylation of Ser-3 by LIM kinases 1 and 2 (LIMK1 and LIMK2) and testicular protein kinases 1 and 2 (TESK1 and TESK2) inactivates cofilin. By contrast, activation of cofilin is mediated by several phosphatases, including serine–threonine phosphatases PP1 and PP2A, chronophin, and a subfamily of dual-specific phosphatases, called Slingshots (SSH-1, SSH-2, and SSH-3) (13). Among Slingshots, SSH-3 does not bind F-actin and shows a less efficient cofilin-phosphatase activity (14). Beyond the regulation by phosphorylation cofilin is also inactivated by PIP_2 binding at membranes (15) and by oxidative stress conditions (16). Cofilin action on F-actin generates both new barbed ends ready to polymerize and a pool of globular actin to feed polymerization (17–19). In this way, cofilin promotes the formation of a dynamic network of F-actin (20), which is essential for the stimulation of T cells (21). In fact, agents that perturb cofilin dynamics inhibit IS assembly and T cell effector functions (22). Despite the significance of cofilin recruitment to the IS (22), there is no information about the molecular dynamics of cofilin regulators during IS assembly and T cell activation.

ULTRASENSITIVITY IN THE COFILIN SIGNALING MODULE

Signaling modules based on opposing enzymes, such as the cofilin phosphorylation/dephosphorylation monocycle (**Figure 1A**), can exhibit different steady-state response functions (**Figures 1B–D**). When enzymes are working far from saturation and mass action kinetics are assumed, the steady-state response function exhibits a Michaelian shape, which is linear at low stimulatory inputs and tends to a plateau when the amount of substrate decreases with stronger stimulation (**Figure 1B**). However, properties, such as reciprocal regulations, positive feedback loops, and multiphosphorylation reactions, are known to generate ultrasensitive responses (23), which are characterized by a sigmoidal, switch-like relation between the stimulus and the response, frequently described by the cooperative Hill equation (**Figure 1C**) (24). In addition to the above-mentioned properties, ultrasensitivity is also generated when substrate levels make both the inhibitory and the activating enzyme to operate close to saturating conditions (so-called zero-order ultrasensitivity) (24), and when a signaling molecule and its activator are concomitantly located to a particular cell compartment (25). Thus, both enzyme levels and molecular dynamics (spatial and temporal regulation) are essential for the output of signaling modules and, consequently, for the cell response. One of the benefits of ultrasensitivity is that it enables cells to filter low stimulatory inputs and to get fast and efficient responses as the stimulus increases. Most importantly, in the presence of positive feedback or double negative feedback loops, ultrasensitivity can also facilitate bistable responses, which constitute real switches in which two stable steady-states are possible (low/"off" and high/"on") for one particular stimulatory input and an intermediate response cannot take place (**Figure 1D**) (26). As soon as a threshold is reached, the system turns to the "on" state, where it stays even when the stimulus falls under the threshold level, a property called hysteresis. When positive feedback loops are very strong, bistable responses can be irreversible. In this situation, the "on" state is maintained even when the stimulus is completely depleted. Bistability indicates the existence of a molecular memory controlling the response of the signaling module.

The cofilin signaling module has several of the above-mentioned properties that generate ultrasensitivity (**Figure 1A**), including (i) a reciprocal regulation mediated by Slingshot-1 (SSH-1) activation, which activates cofilin and inactivates LIMK1 (27); (ii) a positive feedback loop on SSH-1, which can be self-activated by auto-dephosphorylation (28); and (iii) a positive feedback loop due to the enhanced (1200-fold) cofilin-phosphatase activity of SSH-1 when it is bound to F-actin networks (29), whose organization is promoted by cofilin action (20). These positive feedback loops could promote a bistable response showing hysteresis, especially at high local concentrations of active cofilin that would make LIMK1 to work close to saturation (26). It seems then probable that any stimulus triggering SSH-1 activation will generate an ultrasensitive or bistable response. These regulatory mechanisms of SSH-1 have not been established in either SSH-2 or SSH-3. Supporting a TCR-mediated activation of SSH-1, we found that although an even distribution, with partial colocalization with F-actin, was found in non-stimulated cells (data not shown), GFP-SSH-1 accumulated at peripheral sites in the IS (**Figure 1E**), as previously described for cofilin (22). These data support the notion that TCR signals could generate an ultrasensitive response of the cofilin signaling module due to the accumulation of cofilin and its activator SSH-1

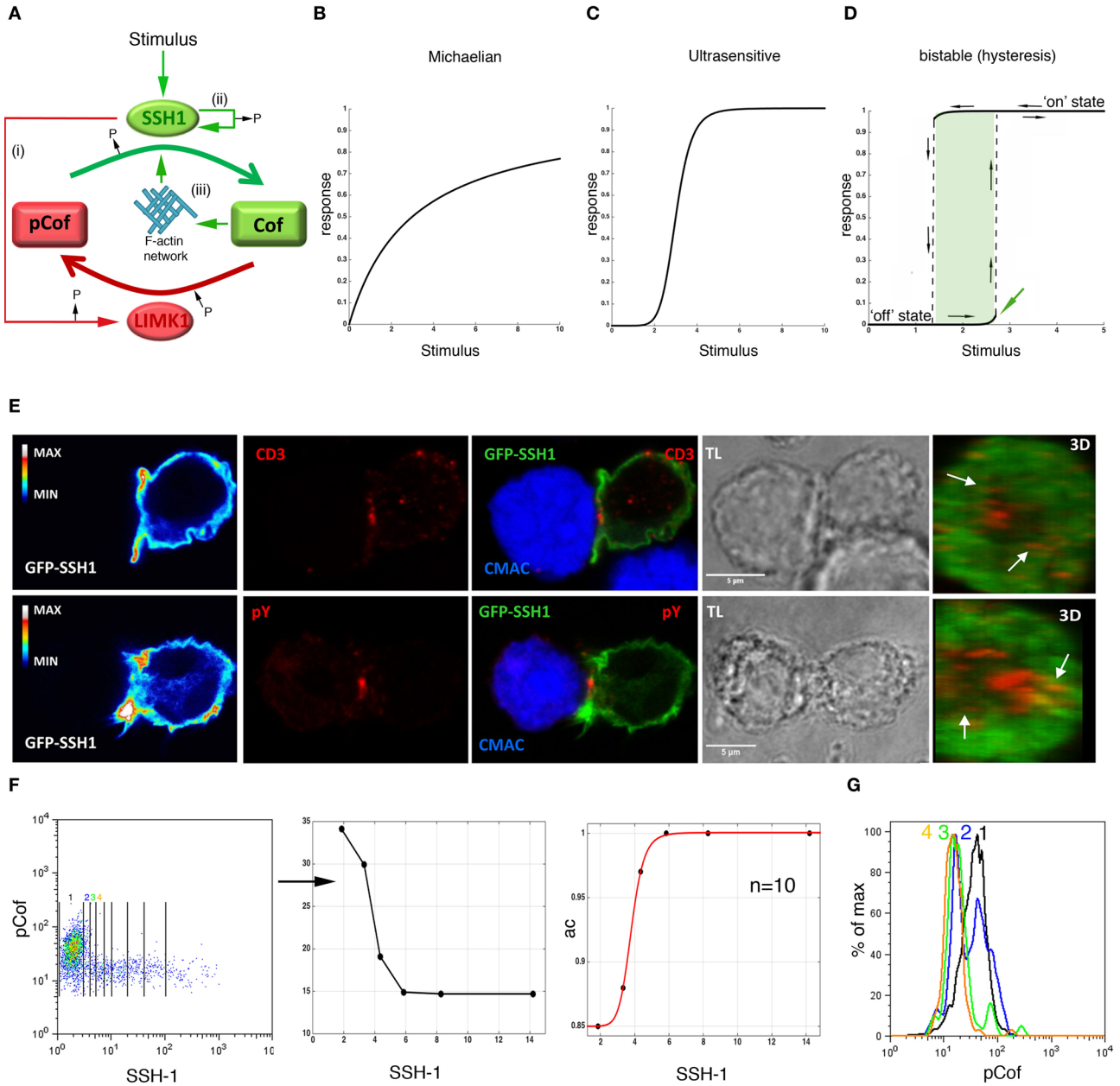

FIGURE 1 | **Ultrasensitivity in the cofilin phosphorylation/dephosphorylation monocycle**. **(A)** Regulation of cofilin activity by SSH-1 and LIMK1. Red and green lines indicate negative and positive regulations, respectively. (i)–(iii) label the reciprocal regulation and positive feedback loops on SSH-1 activation as indicated in the main text. Schematics are shown of graded Michaelian **(B)**, ultrasensitive **(C)**, and bistable **(D)** steady-state response functions. Dashed lines represent intermediate, not possible, states. The green area labels the window of stimulatory inputs generating two stable steady states. A green arrow labels the threshold for switching the module on. Black arrows indicate the going-up and going-down responses characteristic of hysteresis. **(E)** Confocal microscopy of Jurkat CD4 T cells transiently transfected with GFP-SSH-1 and interacting with Raji cells presenting staphylococcal E enterotoxin (SEE). Confocal sections of the green, red, and merged channels as well as three-dimensional (3D) reconstructions of interaction sites are shown. Arrows indicate CD3 clusters and sites of early phosphotyrosine (pY) signaling. Calibration bars quantify the intensity of GFP-SSH-1 (left panels). APCs are identified by staining with 7-amino-4-chloromethylcoumarin (CMAC). TL: transmission light. **(F)** Levels of endogenous phospho-cofilin (pCof) as a function of ectopically expressed GFP-SSH-1 in non-stimulated Jurkat CD4 T cells assessed by flow cytometry (FACS). Lines delimit the regions obtained to plot the mean of pCof versus the mean of GFP-SSH-1 levels (middle panel). Right panel: active cofilin (ac), calculated as [total cofilin(tCof) − pCof]/tCof, as a function of the mean of GFP-SSH-1 levels as before. The tCof was obtained from FACS data (not shown). Black dots are experimental data fitted to a four-parameter Hill equation (red line). Hill exponent (*n*) is indicated. Goodness of fit: SSE 2.2e−6, adjusted *R*-square: 0.9998, and RMSE: 0.00085. **(G)** Histograms of pCof in each of the four regions labeled in **(F)** with color-coded numbers. Note the bimodal distribution of pCof in region 2. Panels show one representative experiment out of three.

at the IS. Nonetheless, cofilin-activating signals, such as costimulation (30), might also regulate the dynamics of SSH-1 during T cell activation. It is also plausible that local SSH-1 will be on its highest activation state bound to cortical F-actin. Thus, an efficient inhibition of LIMK1 and, consequently, a reciprocal regulation, is expected at these sites. Consistent with ultrasensitivity, we have found a sharp decrease in the mean of phospho-cofilin amount in T cells as a function of SSH-1 levels (**Figure 1F**; Figures S1 and S2 in Supplementary Material). Interestingly, the detection at the lowest SSH-1 levels of two discrete populations of active and inactive cofilin with no intermediate states suggests the existence of a bistable response (**Figure 1G**; Figure S2 in Supplementary Material). In agreement with irreversible bistability and hysteresis, we have found a higher proportion of active cofilin in Ag-e than in resting T cells, even when they were deprived from the antigenic stimulus for 6 days (**Figure 2A**). In order to further demonstrate irreversibility, it will be needed to design experiments for the complete deprivation of peptide antigenic stimulation by, for example, combining antigen washing and pharmacological inhibition of early TCR signals as previously done (31).

PHYSIOLOGICAL RELEVANCE

We propose that the steady-state response of active cofilin as a function of SSH-1 activation may be an irreversible bistable switch (**Figure 2B**). A fast increase in the actin dynamics is expected once an activation threshold is reached due to TCR and costimulatory signals. Other environmental clues may participate in the regulation of active cofilin during T cell activation. For example, the local reducing environment promoted by dendritic cells at inflammatory sites (32) has been proposed to prevent the inhibition of cofilin activity by PIP_2 in antigen-specific T cells (33). Interestingly, this might also prevent inhibition of SSH-1 by reactive oxygen species (34). Cofilin ultrasensitive response in coordination with other signaling modules may have potential effects in the sensitivity, specificity, rapidity, and efficiency of switch-like T cell responses.

In naïve T cells, ultrasensitivity might be instrumental in maintaining the peripheral tolerance to low signals emanating from self-peptides while endowing cells with enough sensitivity to foreign antigens. Switching the module to the "on" state will

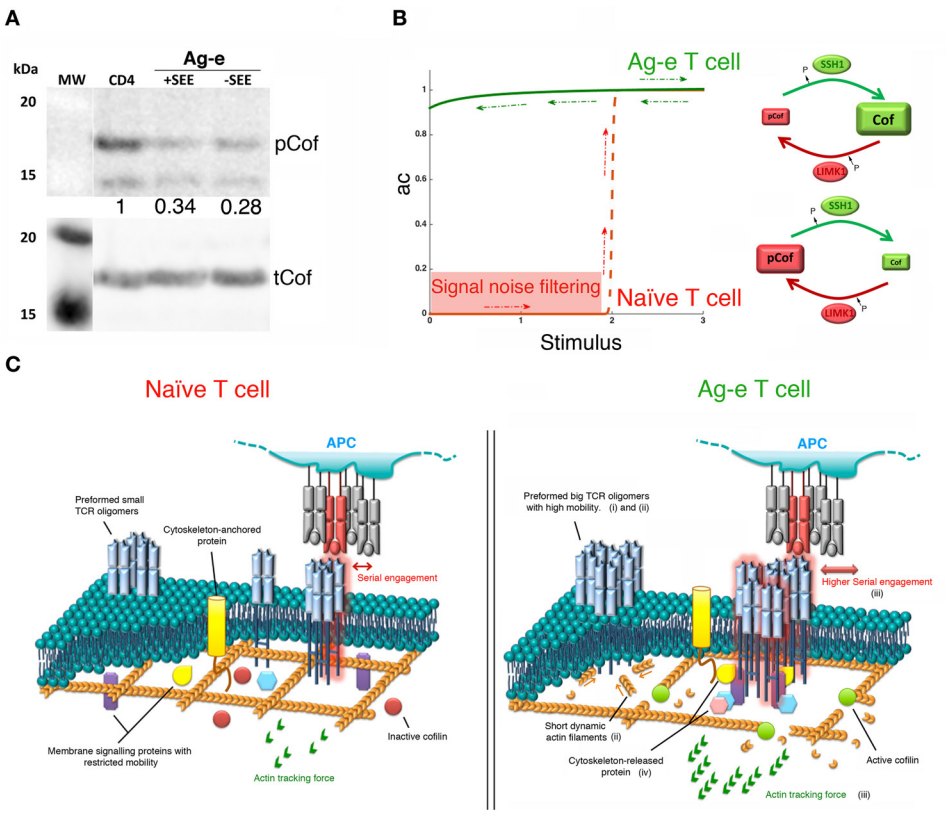

FIGURE 2 | Bistability in the cofilin signaling module. **(A)** Western blot showing levels of phospho-cofilin (pCof) and total cofilin (tCof) in resting peripheral blood CD4 T cells and in Ag-e CD4 T cell blasts obtained by stimulating peripheral blood mononuclear cells with staphylococcal E enterotoxin (SEE) for 7 days (labeled as +SEE). In some samples (labeled as −SEE), antigenic stimulation was applied for only 24 h and then washed away leaving cells without the stimulus for 6 days. The western blot of one representative experiment out of five is shown. Numbers indicated the ratio of pCof/tCof normalized to resting cells. **(B)** Left panel: schematic of the proposed steady-state response of active cofilin (ac) as a function of T cell stimulation. The red line shows the ultrasensitive response expected in naïve T cells. Lower stimuli would be filtered out as noise (red area). The green line shows the "on" state of the module proposed for Ag-e T cells. Note, hysteresis (red and green arrows). Right panels: schematics of the cofilin module in naïve and Ag-e T cells. **(C)** Effects of high levels of active cofilin on T cell stimulation in Ag-e cells versus naïve T cells, as explained in the main text by points (i)–(iv).

rapidly increase actin dynamics to assist on the early assembly of TCR microclusters after the engagement of antigenic pMHC. This will enable an efficient organization of initial signaling complexes.

Slingshot-1 may participate in the molecular memory that keeps the cofilin signaling module in the "on" state in Ag-e T cells even when the antigenic stimulus is depleted. Although recent findings challenge the notion that Ag-e T cells have lower activation thresholds (35), there is a general agreement about the faster and more efficient responses seen in these cells when compared to their naïve counterparts. High levels of active cofilin will increase the depolymerization and severing of actin. This might promote the following effects in tuning TCR triggering and signaling during the activation of Ag-e T cells (**Figure 2C**): (i) a higher mobility of TCR nanoclusters. This may raise the chance of finding and engaging antigenic pMHC; (ii) the formation of big TCR oligomers at the cell surface due to abundant short dynamic actin filaments, as described for other surface molecules (12). A bistable response of the cofilin module might then represent a mechanism for the avidity-maturation or for the rebinding model mentioned above; (iii) the generation of stronger actin tracking forces. This would ensure both, more efficient T cell responses by promoting enough short-lived serial engagements of TCR molecules at the larger nanoclusters and the discrimination of low quality ligands; and (iv) a more efficient release of molecules participating in initial events of signaling by the TCR. Recently, it has been shown that TLR signaling in B cells increases the cofilin-dependent actin dynamics and, consequently, reduces the BCR confinement enhancing signaling (36). The same mechanism might also operate in T cells.

In summary, while the cofilin signaling module at the "off" state may guarantee the auto-tolerance and sensitivity of naïve T cells, the "on" state may mediate faster and more efficient responses exerted by Ag-e T cells. Regulation of SSH-1 activity is expected to be essential for switching the cofilin signaling module to the on state. Thus, regulators and dynamics of SSH-1 during the activation of primary T cells should be investigated. A described regulator of SSH-1 during insulin signaling is PI3K (37). PI3K is an effector of ras (38), whose activation is characterized by a bistable response (31). Thus, it seems that T cell responses are tuned by bistability in several signaling modules operating in early steps downstream the TCR (31, 39–41). In order to formally demonstrate the existence of hysteresis, we will need models that enable us to reach maximal stimulation of primary T cells with physiological antigenic peptides and then be able to go down in the stimulation before re-testing the activation state of the signaling module (31). Acute perturbation of signaling components and the cell machinery (such as cytoskeleton and endosomal compartment) should inform about the key players and the dynamics controlling the ultrasensitive response. In this context, dynamic regulation of cofilin by slingshots is under further investigation in our group.

AUTHOR CONTRIBUTIONS

RR-M performed experiments, analyzed data, and revised the manuscript. PC-S setup experimental protocols, analyzed data, and revised the manuscript. PR-N contributed to the research design, analyzed data, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00059

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Distinct Mechanisms Regulate Lck Spatial Organization in Activated T Cells

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Phosphorylation of the T cell receptor (TCR) by the kinase Lck is the first detectable signaling event upon antigen engagement. The distribution of Lck within the plasma membrane, its conformational state, kinase activity, and protein–protein interactions all contribute to determine how efficiently Lck phosphorylates the engaged TCR. Here, we used cross-correlation raster image correlation spectroscopy and photoactivated localization microscopy to identify two mechanisms of Lck clustering: an intrinsic mechanism of Lck clustering induced by locking Lck in its open conformation and an extrinsic mechanism of clustering controlled by the phosphorylation of tyrosine 192, which regulates the affinity of Lck SH2 domain. Both mechanisms of clustering were differently affected by the absence of the kinase Zap70 or the adaptor Lat. We further observed that the adaptor TSAd bound to and promoted the diffusion of Lck when it is phosphorylated on tyrosine 192. Our data suggest that while Lck open conformation drives aggregation and clustering, the spatial organization of Lck is further controlled by signaling events downstream of TCR phosphorylation.

Keywords: Lck, T cell signaling, assembly of signaling complexes, membrane organization, super-resolution fluorescence microscopy, image correlation spectroscopy

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INTRODUCTION

T lymphocytes participate in an immune response when they become activated through the T cell receptor (TCR). However, despite the identification of the major players and sequences of events involved in T cell signaling pathways, the question of "How does T cell receptor signaling begin?" remains poorly understood (1, 2). TCR signaling is initiated when peptides bound to major histocompatibility complexes (pMHC) engage the TCR. The first detectable signaling event is the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) on TCR/CD3 subunits by the Src kinase Lck. Lck is attached to the plasma membrane through the myristoylation and palmitoylation of residues at its amino terminus. Next to the membrane anchor are a Src homology 3 (SH3) and a SH2 domains, followed by a catalytic tyrosine kinase domain and a short carboxy-terminal tail. Phosphorylation and dephosphorylation of a carboxy-terminal inhibitory tyrosine (Y505) and an activating tyrosine (Y394) in the catalytic domain regulate Lck kinase activity.

Lck activity is directly linked to its conformation, as phosphorylated Y505 binds intramolecularly to the SH2 domain, thereby promoting a closed state that prevents substrate access to the kinase domain. A large percentage of Lck is already phosphorylated on Y394 in resting cells and the proportion of active Lck is not dramatically increased upon TCR activation (3), although the opening of Lck is locally promoted at TCR engagement sites (4). To phosphorylate the TCR, the kinase and substrate must be in close proximity in order to interact, initiate, and sustain signaling; yet the underlying mechanisms for this molecular process are unknown.

The spatial organization of Lck is regulated by several different mechanisms. Lck can bind to and diffuse with the coreceptor CD4, which in turn binds to the pMHC complex on the antigen-presenting cell (1, 2). This association is thought to deliver Lck to the TCR and facilitate the phosphorylation of intracellular domains on the TCR–CD3 complex by Lck (5–7). The role of CD4 in facilitating TCR phosphorylation by Lck is ambiguous and complex. Indeed, while TCR signaling can occur in the absence of coreceptors (8, 9), CD4 association with Lck seems to be crucial for MHC restriction during thymic selection. The initial recruitment model was proposed based on the observations of Xu and Littman, in which initial TCR phosphorylation is mediated by coreceptor-independent Lck, while the coreceptor recruitment to TCR–CD3 complex occurs in a subsequent step (10). More recent studies support a model, in which CD4 sequesters most of the Lck molecules, thereby limiting the pool of Lck available to phosphorylate TCR that have not engaged a MHC molecules (11, 12). However, the work of Stepanek et al. suggest that only very few CD4 molecules are coupled to Lck and that TCR scans multiple CD4 to find one that is coupled to Lck (13).

The SH2 and SH3 domains of Lck mediate intramolecular interactions and the binding to a great variety of signaling proteins, such as TCRζ, Zap70, Csk, and CD45, as well as adaptor proteins, such as LIME and TSAd (14). These interactions may potentially modulate Lck diffusion or distribution within the membrane. Diffusing Lck can also be trapped in protein microdomains (15). We have previously shown that TCR activation triggers the clustering of Lck. Interestingly, this clustering was controlled by the conformation of the kinase, with the open/active form inducing clustering and the inactive/closed form preventing it, thereby establishing a link between the distribution of Lck and its kinase activity (16).

Our previous results suggest a relationship between the clustering of Lck and a local increase in signaling ability. Because T cells tightly regulate the strength and extent of TCR signaling, it is likely that the molecular processes following TCR activation impact on Lck distribution, thereby retroactively modulating Lck activity. Such a feedback mechanism has already been shown for the regulation of Zap70 clustering by SLP-76 (17). The SH2 domain of Lck represents a privileged candidate to facilitate such a feedback mechanism. Indeed, not only does this single binding domain connect Lck to a great variety of adaptors and effectors, more importantly, its binding affinity is regulated by phosphorylation on an adjacent tyrosine 192 (Y192). Activation of TCR triggers phosphorylation on Y192 (18, 19), which modifies the binding of the SH2 domain to Lck substrates and correlates to reduced signaling downstream of the TCR (20, 21). Thus, phosphorylation on Y192 can induce a switch in Lck-binding partners and may also affect the distribution of the kinase in the membrane.

Two recent studies illustrate the role of Y192 in regulating Lck activity and interactions. First, Granum et al. showed that preventing phosphorylation of Y192 (Lck Y192F mutation) led to a greater extent of tyrosine phosphorylation, including CD3ζ. This study also identified various proteins, including the adaptor protein TSAd, which displayed a greater affinity for the SH2 domain of Lck when Y192 was phosphorylated (21). TCR activation promotes the phosphorylation of TSAd by Lck as well as their association, which potentially inhibits Lck activity (22–24) and further enhances Y192 phosphorylation (21).

The second study by Sjölin-Goodfellow et al. demonstrated that selective inhibition of the kinase Zap70 led to a pronounced decrease in Y192 phosphorylation on Lck in resting and activated cells. This coincided with an increased phosphorylation of the Lck-activating tyrosine 394 (Y394) (25). Lck and Zap70 functions and activities are tightly intertwined in T cell signaling, making Zap70 another likely candidate for regulating Lck distribution. Zap70 binds to the intracellular ITAM domains of the TCR complex after they are phosphorylated by Lck. Finally, Lck further binds to the phosphorylated tyrosine 319 on Zap70, an event that stabilizes the activated conformation of Lck and facilitates the activation of Zap70 (26). Interestingly, Zap70 inhibition does not affect Lck phosphorylation on the activating Y394 (27), suggesting that if Zap70 can regulate Lck activity, it is likely to do so through the control of Lck localization. Once recruited to the ITAMs of TCR and fully activated, Zap70 phosphorylates the adaptor protein Lat. Lat too is susceptible of modifying Lck distribution, as it interacts with Lck upon TCR activation (28, 29) and preferentially associates with the open form of Lck (30). Lat also contributes to Lck phosphorylation at Y394 upon TCR stimulation (29).

Hence, we set out to quantify the contribution of the phosphorylation on Y192 Lck, Zap70, and Lat to the spatial organization of Lck in activated Jurkat T cells using cross-correlation raster image correlation spectroscopy (ccRICS) and photoactivated localization microscopy (PALM). We used an open mutant, Lck(Y505F), as well as a mutant that cannot be phosphorylated on Y192, Lck(Y192F), and measured their diffusion and propensity to aggregate and cluster in Lck-deficient Jurkat T cells reconstituted with Lck and in Jurkats lacking Zap70 or Lat. Our results show that similarly to locking Lck in an open conformation, preventing phosphorylation on Y192 promotes Lck clustering, albeit *via* a fundamentally different mechanism. While clustering of open Lck was found to be intrinsic and only attenuated by the absence of Zap70 and Lat, clustering of Lck(Y192F) was not associated with self-aggregation and was dramatically modified in cells lacking Zap70 or Lat. These data suggest that while Lck open conformation drives aggregation and clustering, Lck spatial organization is further controlled by signaling events happening downstream of TCR ITAMs phosphorylation.

RESULTS

Interaction of Diffusing Proteins in Jurkat T Cells Measured with ccRICS

We used raster image correlation spectroscopy (RICS) to extend the data on Lck spatial organization obtained previously in fixed and live cells using PALM (16). The RICS method derives information on protein diffusion and binding dynamics in live cells by spatiotemporal correlation analysis of fluctuations in fluorescence intensity acquired within the pixels of a time series of images (31). If the acquisition is extended to a two-color experiment, then a ccRICS analysis can be carried out between the two channels to extract the fraction of interacting molecules, based on the principle that proteins moving as part of the same complex will give rise to fluctuations in fluorescence intensity that positively cross correlate (32).

To test the validity of this approach for studying Lck dynamic and interactions in activated Lck-deficient Jurkat cells – JCaM1 – we transiently expressed or co-expressed the following constructs: Lck–EGFP and Lck–mCherry, Lck–EGFP–mCherry, and Lck10–EGFP and Src15 mCherry, i.e., the two unrelated membrane anchors of Lck and Src, respectively. Cells were activated on coverslips coated with antibodies against CD3ϵ and CD28 and imaged between 10 and 40 min of activation as described in Section "Materials and Methods." We quantified the percentage of EGFP and mCherry proteins diffusing together from the amplitude of the ccRICS function, which is a measure of their interaction (**Figure 1A**). About half of the Lck molecules were interacting when the two fluorescent proteins were on separate copies of Lck (52 ± 13%), while 80 ± 10% of EGFP and mCherry diffused together when the fluorescent proteins were fused together in the positive control Lck–EGFP–mCherry. By contrast, only 26 ± 15% of Lck10 molecules were associated with Src15 (**Figure 1B**). These results confirm that ccRICS identified the EGFP and mCherry labels attached to the same Lck molecules as diffusing together and two unrelated membrane anchors as predominantly not associated. The diffusion coefficients were as expected similar for the single- or double-labeled Lck, while Lck10 diffused significantly faster (0.7 ± 0.2, 0.8 ± 0.2, and 1.4 ± 0.5 $\mu m^2/s$, respectively). These values reflected the fact that Lck10 only contained the membrane anchor that cannot interact with other proteins while full-length Lck is larger and has the potential for protein–protein interactions. These values are also within the same range than those previously measured by single particle tracking (15, 33, 34).

Together these data demonstrate that approximately half of the population of Lck molecules interacts with each other in activated Jurkat cells and that ccRICS is a suitable methodology to measure Lck interactions and diffusion.

Lck Clustering Was Facilitated by Two Distinct Mechanisms

To compare the contribution of two different mechanisms – conformational state of Lck versus protein–protein interactions mediated by the SH2 domain of Lck upon TCR activation – to the spatial distribution of Lck, we expressed WT Lck, Lck(Y505F), and Lck(Y192F), fused to either EGFP or mCherry in the Lck-deficient Jurkat T cell line JCaM1. Lck(Y505F) cannot be phosphorylated on the inhibitory Y505 and is therefore locked into an open conformation. When Lck cannot be phosphorylated on Y192, its SH2 domain is prevented from binding to many potential interactors, including the kinases Pyk2 and Itk, the phosphatase SHP-1, and the adaptor protein TSAd (21). We co-expressed each Lck variant labeled with EGFP and with mCherry to investigate their self-association. As for **Figure 1**, live cells were imaged between 10 and 40 min of activation on glass coverslips coated with antibodies against CD3ϵ and CD28.

Around 74% of Lck(Y505F) and 55% of Lck(Y192F) were found to self-associate (**Figure 2A**), which corresponds to the values for the EGFP–mCherry positive control and WT Lck, respectively (**Figure 1**). The high propensity of Lck(Y505F) to self-aggregate was also reflected in a low diffusion coefficient that was of about half that of WT Lck (0.38 ± 0.1 versus 0.7 ± 0.2 $\mu m^2/s$, respectively). Interestingly, the tendency of Lck(Y505F)–EGFP to diffuse with WT Lck–mCherry was similar to that measured for WT Lck, suggesting that open Lck only interacts with open Lck and does not recruit WT Lck into clusters of open Lck (Figure S1 in Supplementary Material). In parallel to the ccRICS experiments, we expressed WT Lck, Lck(Y505F), and Lck(Y192F) fused to the photo-switchable fluorescent protein PS-CFP2 in JCaM1 cells and imaged them after 10 min of activation using PALM. As previously described (16), the open Lck mutant Lck(Y505F) displayed a very high level of clustering [quantified by the Ripley K function, $L(r) - r$], assembling in clusters that were denser, larger, and less numerous than WT Lck (**Figures 2B,C**). Lck(Y192F) also clustered significantly more than WT Lck, albeit in a very different way than Lck(Y505F), forming more clusters of lower density (**Figures 2B,C**). Level of clustering of WT Lck and Lck(Y192F) were similar in resting cells (Figure S1B in Supplementary Material). Finally, expression of CD4 in JCaM1, which do not express endogenous CD4, did not have a significant impact on either Lck diffusion or clustering (Figure S2 in Supplementary Material).

These data point toward two different mechanisms for the clustering of the constitutively open versus the low-affinity SH2 mutants of Lck. On the one hand, locking Lck in the open conformation intrinsically increased its affinity for itself, as illustrated by ccRICS, which led to a low number of very dense clusters. On the other hand, preventing the TCR activation-induced affinity of Lck SH2 domain did not affect its affinity for self but somehow unexpectedly led to the formation of many clusters of low density. This suggests that the interactions mediated by the Lck SH2 domain when Y192 is phosphorylated somehow prevents the close packing of Lck in clusters and correlates to the inhibitory effect of Y192 phosphorylation on TCR signaling (20, 21).

Zap70 Enhanced the Clustering of Lck(Y505F) and Was Required for Lck(Y192F) Clustering

Zap70 is essential for T cell signaling, acting immediately downstream of Lck. Zap70 can bind to the SH2 domain of Lck (26) and is essential for Y192 phosphorylation on Lck (25).

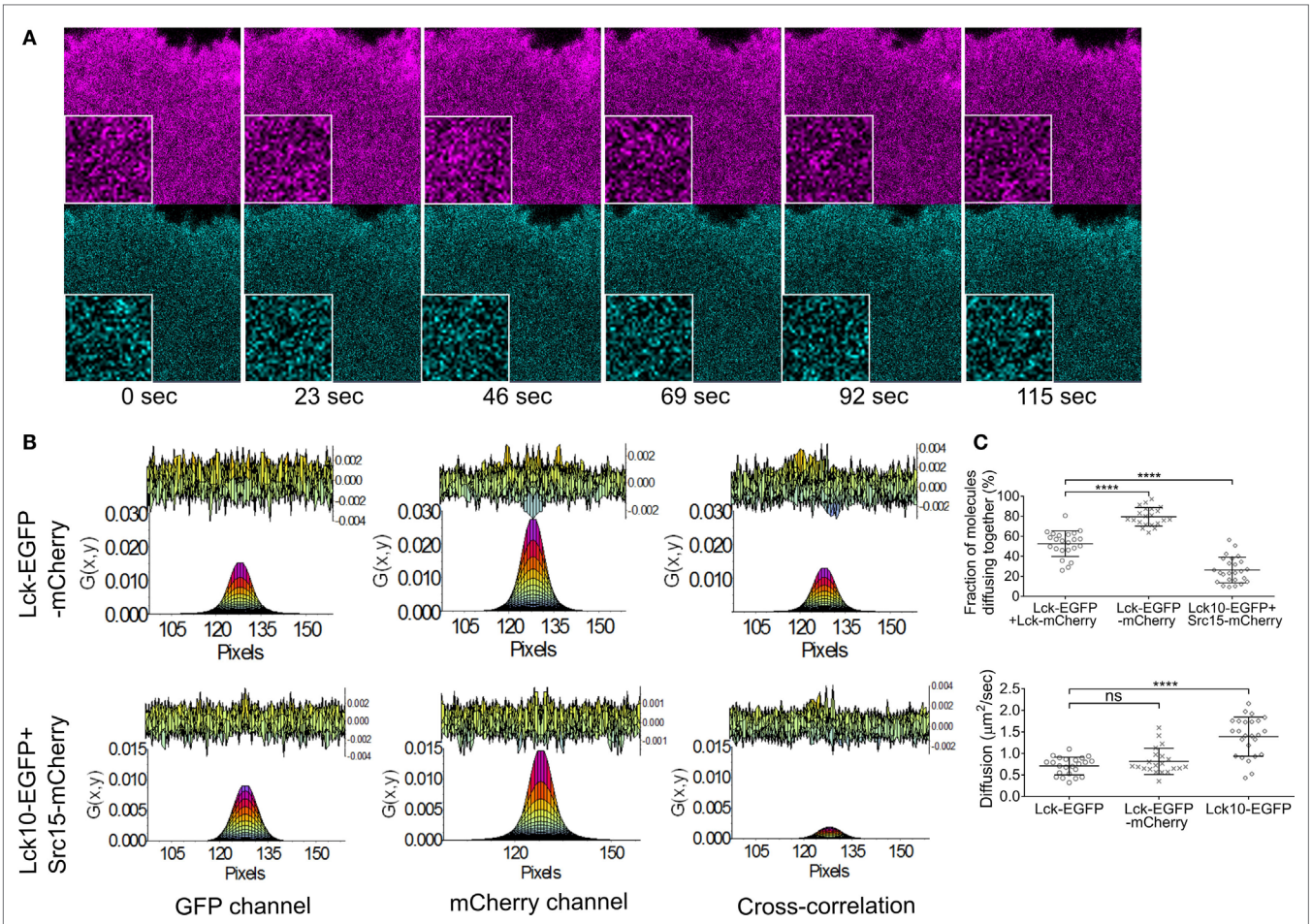

FIGURE 1 | Validity of the ccRICS approach to measure the interaction of diffusing proteins in Jurkat T cells. JCaM1 cells transfected, respectively, with (1) WT Lck–EGFP and WT Lck–mCherry, (2) Lck–EGFP–mCherry (positive control), and (3) Lck10–EGFP and Src15–mCherry (negative control) were pre-activated for 10 min on glass coverslips coated with activating anti-CD3 and anti-CD28, and then imaged for approximately 3 min (100 frames) between 10 and 40 min of activation. **(A)** Example of images used for ccRICS – cells transfected with Lck–EGFP–mCherry. Inset: zoom of a 32 × 32 pixels region. **(B)** One-component fitting of the RICS function in the EGFP and mCherry channels and the ccRICS function between the two channels for the positive (upper row) and negative controls (lower row). The top of the charts shows the residual component of the fit. **(C)** Top: fractions of molecules diffusing together. Bottom: diffusion coefficients for WT Lck, the positive control and the negative control extracted, respectively, from the cross-correlation and EGFP autocorrelation functions. Each symbol in **(B)** represents one cell; small horizontal lines indicate mean (±SEM). ns, not significant; $^{**}P < 0.005$ and $^{****}P < 0.0005$ (unpaired *t*-test). Data are from three independent experiments with at least 21 cells.

Hence, we repeated the ccRICS and PALM measurements of WT Lck, Lck(Y505F), and Lck(Y192F) in a Zap70-deficient Jurkat T cell line, P116, in order to determine how the absence of Zap70 impacts on the spatial organization of Lck. Locking Lck in an open conformation still promoted Lck self-aggregation compared to WT Lck, albeit to a much lower extent than in JCaM1 cells (from 41 ± 9 to 53 ± 14%). It is possible that the presence of endogenous unlabeled open Lck reduced the detected fraction of co-diffusing Lck molecules. As in JCaM1 cells, we observed no difference in self-aggregation for Lck(Y192F) relative to WT Lck (**Figure 3A**). Diffusion of Lck(Y505F) was decreased to the same extent than self-association and diffusion of Lck(Y192F) was not affected (**Figure 3A**). The increase of Lck(Y505F) clustering compared to WT Lck as measured by PALM followed the trend observed in the ccRICS measurement, displaying the same but attenuated changes in density, size, and number of clusters in P116 cells as in JCaM1 cells (**Figures 3B,C**). In contrast, absence of Zap70 did not have any significant effect on Lck(Y192F) clustering compared to WT Lck (**Figures 3B,C**). Finally, the comparison of WT Lck clustering in JCaM1 cells and in P116 cells revealed that the absence of Zap70 clearly promote Lck clustering trough a mechanism that does not rely on self-association (Figure S3 in Supplementary Material). However, this observation has to be moderated by the fact that JCaM1 and P116 cells may have different homeostasis to compensate for the lack of Lck and Zap70, respectively.

These data suggest that Zap70 was differentially involved in the two type of Lck clustering as we observed in **Figure 2**: Zap70 only had modest impact on conformation-induced clustering but severely impacted on Lck clustering facilitated by the high affinity state of the Lck SH2 domain. Because of the differential effect, the data support the idea that two distinct mechanisms exist for Lck clustering.

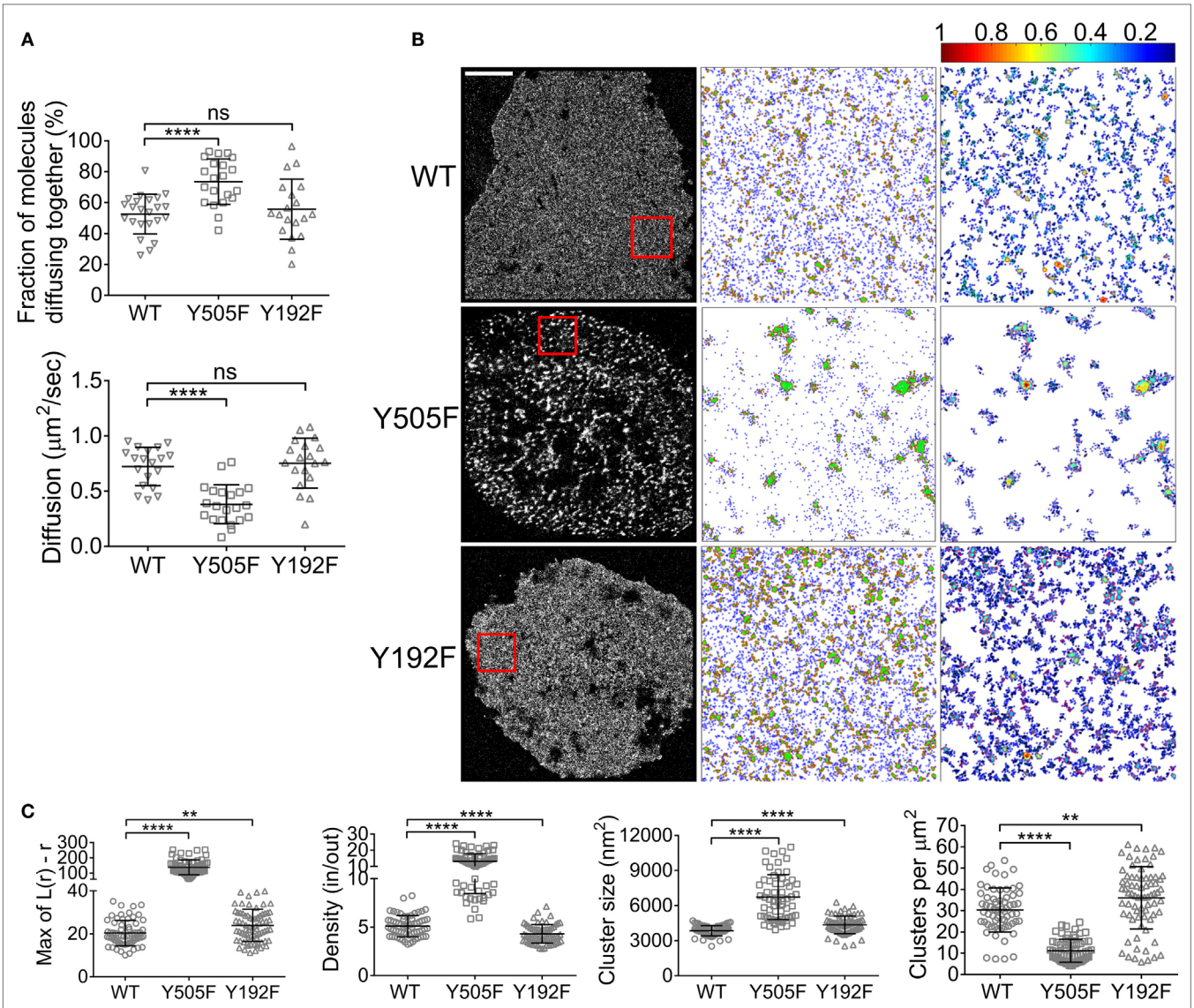

FIGURE 2 | Intrinsic and SH2-mediated Lck clustering. **(A)** JCaM1 cells expressing (1) WT Lck–EGFP and WT Lck–mCherry, (2) constitutively open Lck(Y505F)–EGFP and Lck(Y505F)–mCherry, or (3) SH2-binding mutant Lck(Y192F)–EGFP and Lck(Y192F)–mCherry were activated and imaged as in **Figure 1**. Top: fractions of molecules diffusing together. Bottom: diffusion coefficients for WT Lck, open Lck, and the SH2 Lck mutant. Data for WT Lck are the same than data plotted in **Figure 1**. **(B)** First column: single-molecule PALM images of WT Lck–PS-CFP2, Lck(Y505F)–PS-CFP2, and Lck(Y192F)–PS-CFP2 in JCaM1 cells incubated on glass coverslips coated with activating anti-CD3 and anti-CD28 and fixed after 10 min. Scale bars, 5 μm. Middle column: cluster maps generated by DBSCAN analysis from the 4 μm × 4 μm regions highlighted in red. Last column: maps showing the clusters identified by DBSCAN and color-coded for relative density (0–1). **(C)** From left to right: maxima (Max) of Ripley's *K* function curves of image regions and relative density in clusters, cluster size, and number per area obtained from DBSCAN analysis. Each symbol represents one cell **(A)** or one image region **(C)**; small horizontal lines indicate mean (±SEM). ns, not significant; $^{**}P < 0.001$ and $^{****}P < 0.00005$, unpaired *t*-test for the ccRICS data **(A)** and Mann–Whitney test for the PALM data **(C)**. Data are from three to five independent experiments with a total of at least 19 cells.

Lat Contributed to the Clustering of Open Lck But Repressed Lck(Y192F) Clustering

In the canonical model of the TCR signaling cascade, the primary target of Zap70 kinase activity is the adaptor protein Lat (1, 26). It has also been demonstrated that Lat interacts with Lck with a predilection for the open conformation (28–30). In order to evaluate whether Lat influences Lck spatial organization, we performed the same ccRICS and PALM experiments in Jurkat T cells where Lat expression had been knocked out with CRISPR/Cas9 gene editing (**Figure 4A**). In contrast to what our observation in Zap70-deficient cells (**Figure 3A**), the intrinsic tendency of Lck(Y505F) to self-aggregate more than WT Lck was similar to what we measured in cells expressing Lat (**Figure 4B**, 68 ± 16 and 43 ± 15%, respectively). The diffusion coefficient of open Lck was also decreased compared to WT Lck (**Figure 4B**, 0.76 ± 0.2 and 0.54 ± 0.2 $\mu m^2/s$, respectively), although to a slightly lower

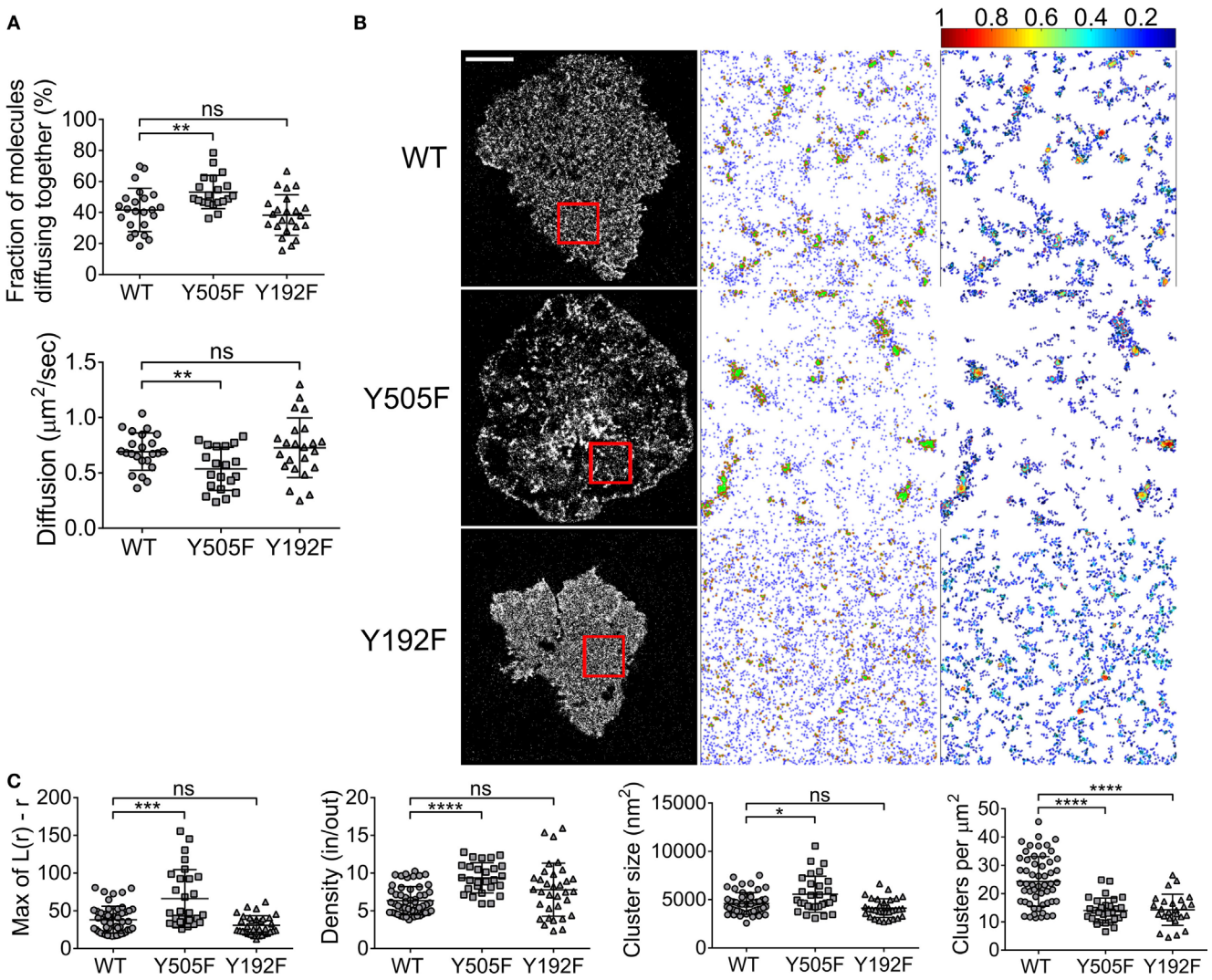

FIGURE 3 | Zap70 promotes intrinsic clustering of open Lck and is required for clustering of SH2 mutant Lck. **(A)** Zap70-deficient P116 cells expressing (1) WT Lck–EGFP and WT Lck–mCherry, (2) constitutively open Lck(Y505F)–EGFP and Lck(Y505F)–mCherry, or (3) SH2-binding mutant Lck(Y192F)–EGFP and Lck(Y192F)–mCherry were activated and imaged as in **Figure 1**. Top: fractions of molecules diffusing together. Bottom: diffusion coefficients for WT Lck, open Lck, and the SH2 Lck mutant. **(B)** First column: single-molecule PALM images of WT Lck–PS-CFP2, Lck(Y505F)–PS-CFP2, and Lck(Y192F)–PS-CFP2 in P116 cells incubated on glass coverslips coated with activating anti-CD3 and anti-CD28 and fixed after 10 min. Scale bars, 5 μm. Middle column: cluster maps generated by DBSCAN analysis from the 4 μm × 4 μm regions highlighted in red. Last column: maps showing the clusters identified by DBSCAN and color-coded for relative density (0–1). **(C)** From left to right: maxima (Max) of Ripley's *K* function curves of image regions and relative density in clusters, cluster size, and number per area obtained from DBSCAN analysis. Each symbol represents one cell **(A)** or one image region **(C)**; small horizontal lines indicate mean (±SEM). ns, not significant; $^{*}P < 0.05$, $^{***}P < 0.0005$, and $^{****}P < 0.00005$, unpaired *t*-test for the ccRICS data **(A)** and Mann–Whitney test for the PALM data **(C)**. Data are from three independent experiments with a total of at least 20 cells.

extent than what we observed in the presence of Lat. Lck(Y192F) self-aggregation and diffusion coefficient were not affected by the absence of Lat (**Figure 4B**).

Similarly to the ccRICS data, the PALM data showed that open Lck(Y505F) was more clustered than WT Lck in Lat-deficient cells, although to a much lesser extent to what we observed in cells expressing Lat (**Figures 4C,D**). More interestingly, knocking out Lat boosted the SH2-related clustering of Lck(Y192F), mostly by drastically increasing the density of Lck(Y192F) clusters (**Figures 4C,D**). Comparing WT Lck clustering in Lat-deficient cells and JCaM1 showed that Lat promotes Lck clustering of WT Lck and Lck(Y505F) (Figure S3A in Supplementary Material). Bypassing Lat signaling by stimulating Lat KO cells with PMA + ionomycin did return clustering levels of WT Lck to the values observed in cells expressing Lat, but not of Lck(Y505F), suggesting that Lat might regulate the clustering of WT and open Lck through different mechanisms or that clustering of WT Lck is more sensitive to the ionic strength of the cytoplasm (Figure S3B in Supplementary Material).

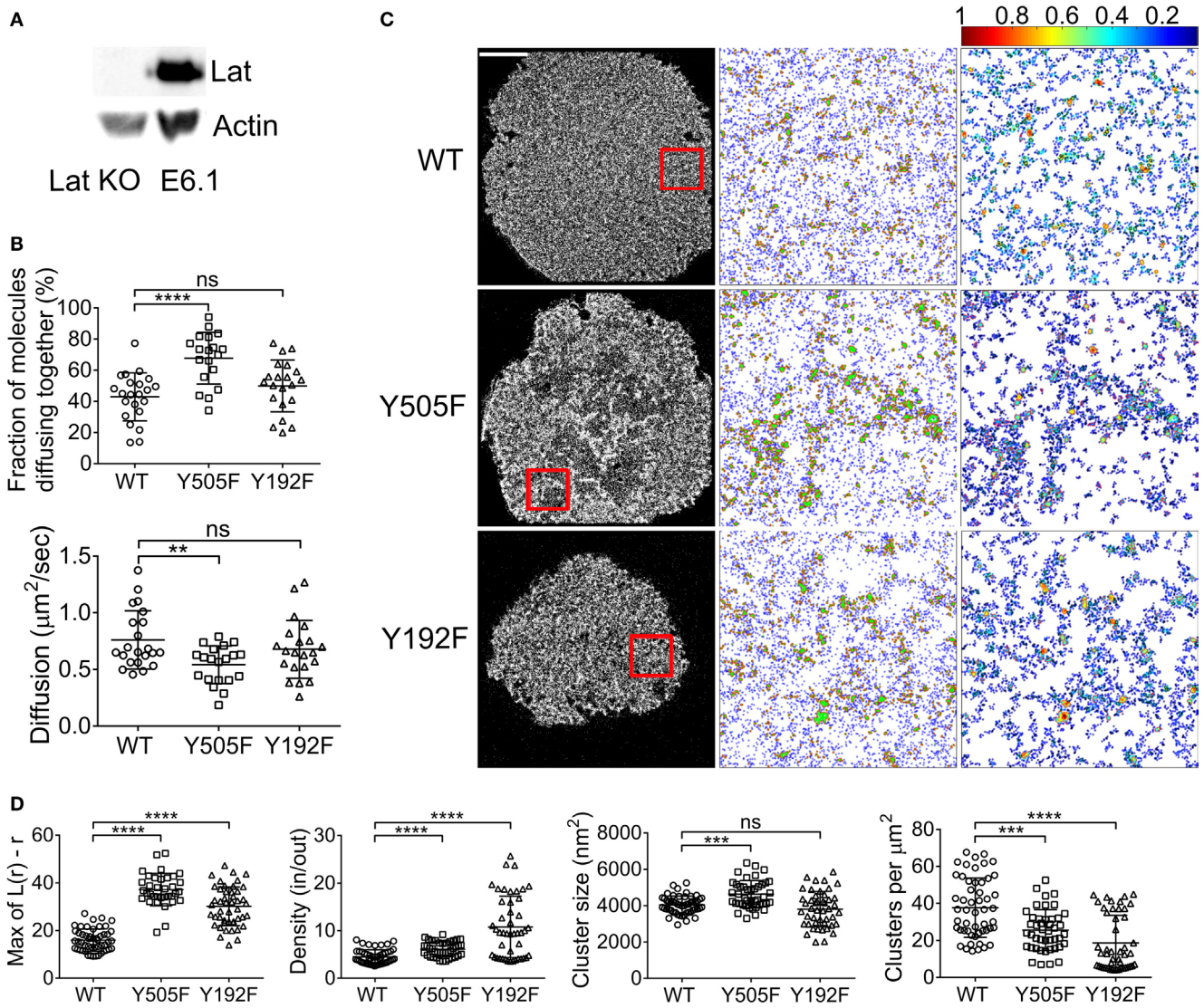

FIGURE 4 | Lat contributes to the clustering of open Lck but represses the clustering of the SH2 mutant Lck. **(A)** Immunoblot of Lat KO cells and wild-type E6.1 Jurkat cells. **(B)** Lat KO cells expressing (1) WT Lck–EGFP and WT Lck–mCherry, (2) Lck(Y505F)EGFP and Lck(Y505F)–mCherry, or (3) Lck(Y192F)–EGFP and Lck(Y192F)–mCherry were activated and imaged as in **Figure 1**. Top: fractions of molecules diffusing together. Bottom: diffusion coefficients for WT Lck, open Lck, and the SH2 Lck mutant. **(C)** First column: single-molecule PALM images of WT Lck–PS-CFP2, Lck(Y505F)–PS-CFP2, and Lck(Y192F)–PS-CFP2 in Lat KO cells incubated on glass coverslips coated with activating anti-CD3 and anti-CD28 and fixed after 10 min. Scale bars, 5 μm. Middle column: cluster maps generated by DBSCAN analysis from the 4 μm × 4 μm regions highlighted in red. Last column: maps showing the clusters identified by DBSCAN and color-coded for relative density (0–1). **(D)** From left to right: maxima (Max) of Ripley's *K* function curves of image regions and relative density in clusters, cluster size, and number per area obtained from DBSCAN analysis. Each symbol represents one cell **(A)** or one image region **(B)**; small horizontal lines indicate mean (±SEM). ns, not significant; $^{**}P < 0.005$, $^{***}P < 0.0005$, and $^{****}P < 0.00005$, unpaired *t*-test for the ccRICS data **(B)** and Mann–Whitney test for the PALM data **(D)**. Data are from three to five independent experiments with a total of at least 20 cells.

In light of these results, it appears that Lck(Y192F) was allowed to "cluster freely" even more in the absence of Lat, suggesting that the protein network organized by Lat contributes to restraining Lck clustering *via* SH2 domain interacting partners. Additionally, Lck(Y505F) clustered less in absence of Lat and Zap70; however, the potentiating effects of Zap70 on the clustering of open Lck were far greater than those of Lat on open Lck clustering. This is in agreement with a TCR network topology, where Zap70 is more closely located to Lck than Lat.

TSAd Bound to Lck and Promoted Its Diffusion

It was unexpected that preventing the TCR-dependent increase of the affinity of the Lck SH2 domain in the Lck(Y192F) mutant enhanced its clustering. It is logical to assume that the association of Lck with a protein network would rather immobilize Lck and promote cluster formation. In an attempt to understand this apparent contradiction, we focused on the adaptor protein TSAd, which was recently shown to associate with Lck upon

Y192 phosphorylation (21). Lck–EGFP or Lck(Y192F)–EGFP were transiently co-expressed in JCaM1 cells together with TSAd–mCherry. Cells were imaged by ccRICS as for the Lck aggregation experiments to measure the association of Lck or Lck(Y192F) with TSAd. In accordance with the data of Granum et al., we observed that the fraction of Lck WT associating with TSAd was almost twofold higher than for Lck(Y192F) (**Figure 5**, 42 ± 12 and 22 ± 6%, respectively). Importantly, the diffusion measurements revealed that (a) the diffusion coefficient of WT Lck was increased in cells overexpressing TSAd, (b) but the diffusion coefficient of Lck(Y192F) was not affected by TSAd overexpression. The latter had a similar diffusion coefficient in these cells as WT Lck had in JCaM1 cells (**Figure 5**). The diffusion of TSAd followed the exact opposite trend, being slower when Lck could bind to TSAd and faster for the Lck(Y192F) mutant. These data suggest that upon phosphorylation of Y192, Lck bound to and co-diffused with the fast moving TSAd, which consequently decreases the probability of Lck to be immobilized in clusters.

DISCUSSION

We have demonstrated previously that TCR activation leads to an increase in Lck clustering and that this clustering is driven by the open/active conformation of Lck (16). Here, we confirmed that open Lck has an intrinsic tendency to assemble into clusters, as it displayed a higher affinity for self and diffused slower than WT Lck. Our data further indicated that signaling proteins downstream of TCR contributed to regulating Lck distribution in the plasma membrane of activated T cells, however, through mechanisms that were not related to conformation-induced clustering. Indeed, preventing the phosphorylation of Y192 by a tyrosine to phenylalanine point mutation (Y192F) also resulted in a significant increase in Lck clustering. Phosphorylation on Y192 is triggered by TCR activation (18, 19) and is associated with the downregulation of TCR signaling (20, 21). Functionally, Y192 phosphorylation represents a signaling switch that controls the affinity of the Lck SH2 domain for tyrosine-phosphorylated-interacting partners. The phosphatase SHP-1 is among the proteins that display a greater affinity for Lck upon Y192 phosphorylation (21) and is at the same time the central element of a negative feedback mechanism that dephosphorylates Lck and TCR upon TCR activation (35). One could thus speculate that the Y192-mediated SHP-1 deactivation of Lck favors the closed conformation and consequently prevents Lck clustering. Preventing Y192 phosphorylation would then lead to more activated Lck and in turn enhance conformation-induced clustering. However, we found identical levels of self-association for Lck(Y192F) and WT Lck with ccRICS and Lck(Y192F) clusters had very different characteristics than the Lck(Y505F) clusters when measured with PALM, being in much higher number and having a very low density. Hence, the clustering that we observed when we prevented phosphorylation of Y192 was fundamentally different from that of Lck(Y505F)-induced clustering and was more likely to be related to direct modifications of Lck spatial organization through protein–protein interactions mediated by its SH2 domain. Note that despite the differences in their cluster properties, Lck clustering induced by Y505F and Y192F correlate to an increased phosphorylation of the TCR (21, 36), suggesting that Lck is more efficient at phosphorylating TCR when in clusters.

In cells lacking Zap70, conformation-induced clustering of Lck was significantly attenuated compared to what we observed in JCaM1 cells. If we put the possible contribution of untagged open Lck in these cells aside, this suggests that Zap70 further promotes intrinsic clustering of open Lck. This could be achieved by favoring the confinement of Lck. Indeed, Zap70 kinase activity is essential to the assembly of the protein network downstream of Lat, which in turn is directly linked to actin regulation at the immunological synapse. Interactions both with the protein network installed by Lat and with the actin cytoskeleton could regulate Lck confinement. Zap70 can also have a kinase-independent scaffolding function (27), which could contribute to regulating Lck distribution through the direct binding of Lck to Zap70 (26). The picture gets even more complex when considering (a) that the absence of Zap70 in P116 cells reversed Y192F-induced changes in clustering, despite the fact that Y192 phosphorylation does not modify the affinity of Lck for Zap70 (21) and (b) that WT Lck clustering was greatly

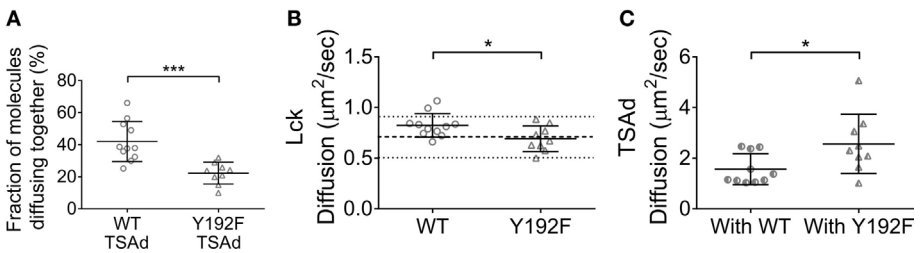

FIGURE 5 | TSAd binding to Lck promotes Lck diffusion. JCaM1 cells transfected, respectively, with (1) WT Lck–EGFP and TSAd–mCherry, or (2) Lck(Y192F)–EGFP and TSAd–mCherry were activated and imaged as in **Figure 1**. **(A)** Fraction of TSAd–mCherry molecules diffusing together with either WT Lck–EGFP or Lck(Y192F)–EGFP. **(B)** Diffusion coefficients for WT Lck–EGFP and Lck(Y192F) in cells expressing TSAd–mCherry. **(C)** Diffusion coefficients for TSAd–mCherry in cells expressing WT Lck–EGFP or Lck(Y192F)–EGFP. Each symbol represents one cell; small horizontal lines indicate mean (±SEM). *$P < 0.05$ and ***$P < 0.0005$, unpaired *t*-test. Data are from three independent experiments with at least nine cells.

increased in P116 cells relative to cells expressing Zap70. All in all, the intricate relationship between Zap70 and Lck spatial distribution likely reflects the versatile role played by Zap70 in T cell signaling. Indeed, while Zap70 is essential for the propagation of TCR signaling (26) as a kinase and as an adaptor protein (27), it also mediates a negative feedback signaling that directly moderates Lck activity (25).

Lat interacts with Lck (28) and this interaction could be involved in the contribution of Lat to Lck(Y505F) clustering. However, this interaction cannot explain the link between Lat and Lck(Y192F) clustering as it is not mediated by an SH2 domain–phosphotyrosine association. Additionally, Y192 phosphorylation does not modify the affinity of Lck for Lat (21). It has been shown recently that preventing tyrosine phosphorylation on SLP-76, a scaffold protein downstream of Lat in the TCR signaling cascade, led to constitutively increased phosphorylation of Y192. On the other hand, knock-out of SLP-76 led to a constitutive decrease in Y192 phosphorylation (37). This suggests that the protein network assembled by Lat is susceptible of regulating Lck distribution through Y192 phosphorylation. Interestingly, while interactions with Lat recruit SLP-76 to the membrane and TCR complex, SLP-76 is phosphorylated by Zap70 (26, 38). Thus, the opposite effects we observed in Zap70- and Lat-deficient cells on the clustering of Lck(Y192F) could relate either to the lack of phosphorylation of SLP-76 tyrosine – in Zap70-deficient cells – or to the reduced recruitment of SLP-76 to the plasma membrane – in Lat-deficient cells.

We further observed that upon TCR activation, Y192 phosphorylation contributes to promoting Lck association with TSAd, an adaptor protein lacking enzymatic activity (21–23). There is conflicting evidence on the role of TSAd in T cell signaling (39), as knock-out of TSAd (22, 40) or its overexpression (23, 41) both impair T cell activation. However, TSAd-deficient mice had a higher susceptibility to T-cell-related autoimmune diseases (39), which rather supports the hypothesis of a moderating role for TSAd in T cell signaling. Our data showed that diffusing Lck and TSAd associated when Y192 could be phosphorylated and that this association was impaired in the Lck(Y192F) mutant. Given the fast diffusion of TSAd, it is possible that this SH2 domain-mediated association of Lck prevents the formation of dense Lck clusters and consequently downregulates Lck activity. Interestingly, Lat also interacts with TSAd (39), which could potentially favor the recruitment of TSAd to the plasma membrane. In this context, the absence of Lat might result in a lower probability of TSAd binding to Lck and explain why Lck(Y192F) clustering is boosted in Lat-deficient cells.

It has been previously suggested that binding to TSAd would promote the open conformation of Lck by breaking the SH2–pY505 intramolecular interaction (42). However, our data do not support this model, as the reduced association of TSAd and Lck(Y192F) versus WT Lck that we observed in ccRICS correlates to a higher level of clustering of Lck(Y192F) versus WT Lck. Nevertheless, the affinity of Lck SH2 domain for pY505 is indeed relatively low (43), and it is generally assumed that an engaged SH2 domain would promote the open conformation of Lck. In that respect, it would be very interesting to determine if phosphorylation on Y192 affects the affinity of SH2 domain for Y505, thereby establishing a link between the two mechanisms observed in this study.

Finally, Couture et al. reported that when phosphorylated on Y192, Lck bound to much less proteins, although these proteins were not identified (20). Hence, we cannot exclude that the increase in clustering observed for Lck(Y192F) is the consequence of Lck being engaged in more protein–protein interactions.

In conclusion, when bearing in mind the inhibitory effect of Y912 phosphorylation, we could speculate that the "declustering" of Lck when Y192 is phosphorylated is a way of downregulating Lck signaling, similarly to what has been described for Zap70 clusters (17). It could also be a way of "recycling" the Lck population engaged in clusters, either in order to allow Lck molecules to search for more triggered TCRs or to allow Lck to engage in other processes related to later T cell signaling. For instance, once released from clusters, Lck molecules would be more likely to bind Itk for later signaling events as suggested previously (21).

MATERIALS AND METHODS

Plasmids and CRISPR/Cas9

Mammalian expression constructs encoding full-length wild-type human Lck and the constitutively open Lck(Y505F) mutant were a gift from T. Harder. PS-CFP2 expression backbone was obtained from Evrogen. The Y192F single point-substitution mutants of Lck were made by site-directed mutagenesis. The 10- and 15-amino acid N-terminus regions of Lck and Src were fused to EGFP and mCherry, respectively, *via* a short 4 amino acid (GGGG) linker. Lck–EGFP–mCherry was made by cloning the mCherry coding sequence into pm-Lck–EGFP-N1 using *Age*I.

For the knocking out of Lat, Jurkat cells were transfected with two gRNAs (guide RNA) that were specifically designed to target genomic Lat DNA, together with cas9 expression plasmid. Twenty-four hours post-transfection, transfected single cells were FACS sorted and seeded into 96-well plates. Cell clones were screened by using western blotting with a Lat antibody (9166, Cell Signaling Technology) and clones lacking Lat eventually grown to an appropriate population for around 20 days.

Sample Preparation

E6.1, JCaM1, P116, and Lat KO cells were cultured in RPMI media (Gibco) supplemented with 10% fetal calf serum (FCS) and transfected by electroporation (NEON, Invitrogen) to express WT and mutant Lck, Sr15, Lck10, and TSAd fused to EGFP, mCherry, or PS-CFP2. For ccRICS experiments, cells were activated on anti-CD3ε (16-0037, eBioscience) and anti-CD28 (16-0289, eBioscience) antibody-coated coverglass by allowing the cells to settle upon the activating surface for 10 min at 37°C prior to imaging. For PALM experiment, cells were activated for 10 min and subsequently fixed in 4% paraformaldehyde for 13 min. Antibody was adsorbed onto surfaces by incubating

clean glass coverslips with 10 μg/ml antibody for at least 1 h at 37°C.

Cross-Correlation Raster Image Correlation Spectroscopy

The ccRICS measurements were performed on a Zeiss LSM780 laser scanning microscope, using a LCI Plan-Neofluar NA = 1.3 water immersion 63× objective (Zeiss, Germany). Lck–GFP was excited with the 488-nm emission line of an Argon laser. Lck–mCherry was excited with the 561 nm emission line of a diode pump solid state (DPSS) laser. Lck–GFP and Lck–mCherry were measured simultaneously with GaAsP detectors using the 493–556-nm and 613–696-nm collection ranges, respectively. For each channel, the pinhole was set to 1 AU. For each ccRICS experiment, we acquired a stack of 100 frames in a selected field next to the cell edges. The pixel frame size of the image field was set to 256 × 256 and collected at an electronic zoom that resulted in a pixel size of 50 nm. The pixel dwell time was set to 12.61 μs/pixel, which resulted in a line time of 7.56 ms and frame time of 1.15 s. The acquired ccRICS data were processed and analyzed by the SimFCS software developed at the Laboratory for Fluorescence Dynamics (www.lfd.uci.edu) as described in the previously published papers (31, 32).

Briefly, for each two-color experiment, the RICS function was calculated in channels 1 and 2 for the entire image stack, with a moving average applied to remove slow cell movements. The resulting 3D RICS profile was then fit to a one-component diffusion model in each channel and the $G(0)$ values and diffusion coefficients were derived from the fits recorded. The cross RICS function was then calculated between the two channels fit to a one-component diffusion model and the cross $G(0)$ value and diffusion coefficient derived from the fit recorded. The fraction of molecules bound was then derived by taking the ratio of $G(0)_{CROSS}/G(0)_{CH1}$ if $G(0)_{CH1} < G(0)_{CH2}$ or $G(0)_{CROSS}/G(0)_{CH2}$ if $G(0)_{CH2} < G(0)_{CH2}$.

PALM Imaging

Photoactivated localization microscopy images were acquired on a TIRF microscope (ELYRA; Zeiss) with a 100×, NA = 1.46 oil-immersion objective. For PS-CFP2, photoconversion was performed with 8 μW of 405-nm laser radiation and imaging of green-converted PS-CFP2 with15–30 mW of 488-nm light. For PALM, 15,000–20,000 images were acquired per sample using a cooled, electron-multiplying CCD (EMCCD) camera (iXon DU-897D, Andor) with an exposure time of 18 ms. Recorded images were analyzed using Zeiss ZEN software. Drifting of the sample during acquisition was corrected relative to the position of surface-immobilized 100 nm colloidal gold beads (BBInternational, UK) that were placed on each sample.

PALM Data Processing

SMLM data were analyzed using custom software written in MATLAB (MathWorks) for detection of clusters and extraction of clustering parameters. Typically, for each cell, one to five non-overlapping representative regions of 4 μm × 4 μm were selected for analysis.

First, we used Ripley's K function as previously described (44) to determine the extent of clustering of a population of molecules compared to a randomly distributed set of molecules. This was calculated using SpPack, an add-in for Microsoft Excel (45), as well as a custom MATLAB version optimized for larger data sets. In short, the Ripley's K function calculates for each molecule the number of neighbor molecules within a given radius r corrected by the total density; finally, for each radius, the average is calculated over all molecules. The Ripley's K function provides ensemble information on the whole region of interest; it provides information on the level of clustering of molecules in a region; however, no analysis is performed at the cluster level, and therefore, no information is available on individual clusters.

To retrieve information on individual clusters, we used density-based spatial clustering application with noise (DBSCAN) analysis (46) to identify individual clusters. The DBSCAN method detects clusters using a propagative method, which links points belonging to the same cluster based on two parameters: the minimum number of neighbors ϵ ($\epsilon = 3$) in the radius r ($r = 20$ nm). The DBSCAN routine is implemented in MATLAB and subsequently coded in C++ and compiled in a Matlab executable (MEX) file to improve the speed of processing.

Statistics

Statistical significance of the means of two data sets was assessed with unpaired t-test with Welch's correction for the ccRICS data sets, which displayed normal distributions, and with a Mann–Whitney test for the PALM data sets, which did not all have normal distributions.

AUTHOR CONTRIBUTIONS

NK-K, FK, EC, and YY did and analyzed ccRICS and PALM experiments; EH conceived and analyzed ccRICS experiments; ZY and JL contributed to molecular biology; TT designed the analysis of PALM data; KG and SP contributed to the manuscript preparation; JR was responsible for conceptualization and the manuscript preparation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00083

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REVIEW
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Action and Traction: Cytoskeletal Control of Receptor Triggering at the Immunological Synapse

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It is well known that F-actin dynamics drive the micron-scale cell shape changes required for migration and immunological synapse (IS) formation. In addition, recent evidence points to a more intimate role for the actin cytoskeleton in promoting T cell activation. Mechanotransduction, the conversion of mechanical input into intracellular biochemical changes, is thought to play a critical role in several aspects of immunoreceptor triggering and downstream signal transduction. Multiple molecules associated with signaling events at the IS have been shown to respond to physical force, including the TCR, costimulatory molecules, adhesion molecules, and several downstream adapters. In at least some cases, it is clear that the relevant forces are exerted by dynamics of the T cell actomyosin cytoskeleton. Interestingly, there is evidence that the cytoskeleton of the antigen-presenting cell also plays an active role in T cell activation, by countering the molecular forces exerted by the T cell at the IS. Since actin polymerization is itself driven by TCR and costimulatory signaling pathways, a complex relationship exists between actin dynamics and receptor activation. This review will focus on recent advances in our understanding of the mechanosensitive aspects of T cell activation, paying specific attention to how F-actin-directed forces applied from both sides of the IS fit into current models of receptor triggering and activation.

Keywords: immunological synapse, actin, cytoskeleton, mechanotransduction, integrin, T cell receptor, adhesion, costimulation

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INTRODUCTION

During their circulation through blood, lymphoid tissues, and peripheral sites of inflammation, T cells encounter and respond to a variety of environmental stimuli. Several of these responses are dependent on the application of external forces. A good example of this is the shear flow-induced activation of cell adhesion molecules during the slow rolling and firm adhesion steps of diapedesis, the process that brings cells from the bloodstream into tissues. Following diapedesis, T cells generate internal forces that drive their migration through the tissue stroma, in search of antigen-presenting cells (APCs) bearing major histocompatibility complex molecules loaded with their cognate peptides (pMHC). When T cells recognize these APCs, a specialized adhesive contact known as the immunological synapse (IS) is formed. The IS promotes sustained T cell/APC interactions and serves as a platform for exchange of information between the two cells. As with diapedesis and migration, T cell/APC adhesion and signal transduction at the IS depend on physical forces exerted by actin

cytoskeletal dynamics. As detailed further below, actin-dependent protrusive forces drive close apposition of the two cells, bringing receptors on the T cell in contact with ligands on the APC. In addition, some IS-associated signaling molecules are physically linked to actin filaments; forces exerted on these molecules by the actin network result in conformational changes needed for full T cell activation.

Signaling at the IS takes place in dynamic microclusters containing surface receptors and downstream signaling molecules. These microclusters form at the periphery of the IS, within a region rich in branched actin filaments, reminiscent of the lamellipodium found at the leading edge of a migrating cell, and then move toward the center of the IS in parallel with centripetal flow of the actomyosin network (1). Importantly, ongoing actin flow is needed to sustain TCR signaling; if flow is arrested, intracellular Ca^{2+} levels drop, and early signaling intermediates are rapidly dephosphorylated (2). Although the signaling events that direct F-actin polymerization and cytoskeletal flow at the IS are well understood, the mechanism by which actin flow enhances T cell activation has remained elusive. Recent studies point to the involvement of force-induced receptor activation (3), as well as force-driven formation and centralization of signaling microclusters (1, 2, 4, 5). According to this paradigm, early signaling events drive the robust polymerization of F-actin at the IS, which in turn functions to enhance signal transduction events leading to full T cell activation. In this review, we will focus on the mechanisms through which cytoskeletal dynamics in T cells and APCs serve to control mechanosensitive signaling events at the IS and consider how cytoskeletal function can be included in current models of receptor triggering.

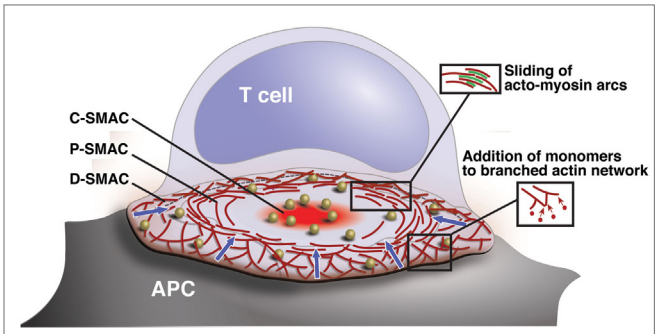

FIGURE 1 | Organization and actin dynamics within the IS. Diagram showing the architecture of a radially symmetric "bulls-eye" IS such as that formed between a B cell and an antigen-specific mature T cell. Based on molecular segregation, the IS can be divided into three regions: (1) a peripheral actin-rich region termed the distal supramolecular activation cluster (D-SMAC), (2) a deeper region rich in LFA-1 and actomyosin arcs termed the peripheral supramolecular activation cluster (pSMAC), and (3) a central region rich in PKCθ and other signaling molecules termed the central supramolecular activation cluster (cSMAC). Signaling microclusters containing TCR and other signaling molecules (gold balls) form and begin to signal in the IS periphery and are transported by the cytoskeleton toward the cSMAC region, where signal extinction takes place. Microcluster movement is coupled to centripetal flow of the actin network (blue arrows). Actin flow is driven primarily by addition of actin monomers to the barbed ends of branched actin filaments, which lie just under the plasma membrane. This generates a pushing force that drives the network inward. In addition, myosin-driven sliding of actin filaments causes contraction of the network. This provides a pulling force that stabilizes the network and maintains radial symmetry.

F-ACTIN DYNAMICS ON THE T CELL SIDE OF THE IS

During stimulation by an APC, T cells exhibit robust actin polymerization in the periphery of the contact area, centripetal (retrograde) flow, and eventual disassembly of F-actin filaments near the center of the contact (2, 4) (**Figure 1**). Consistent with this, actin filaments are generally shorter, more branched, and more dynamic in the periphery of the IS, where nucleation of new actin filaments and polymerization of monomers onto the growing ends of existing filaments are occurring (6). Centripetal flow of the actomyosin network is primarily driven by the polymerization of F-actin, which continuously pushes on the plasma membrane (2, 4). This process is accompanied by the contractile activity of non-muscle myosin IIA, which organizes actin filaments into arcs within the lamellar region. This process stabilizes the network and maintains radial symmetry. Under conditions where F-actin depolymerization is blocked, myosin activity results in network constriction. Simultaneous inhibition of F-actin polymerization, F-actin depolymerization, and myosin contractility results in complete inhibition of lamellipodial actin flow (2, 4). Recently, it has become evident that there are actually two pools of dynamic actin filaments at the IS. In addition to the prominent lamellipodial pool, actin polymerization also takes place in smaller actin foci, structures that are closely associated with newly formed TCR microclusters (7). These foci are likely equivalent to the podosome- or invadopod-like protrusions (ILPs) first visualized in T cells interacting with endothelia, and later also found at the T cell/APC interface (8, 9). Although it has not been directly demonstrated, it seems likely that the conditions shown to arrest lamellipodial actin flow also arrest dynamics of these TCR-associated actin foci.

Actin-Regulatory Pathways Downstream of the TCR

Within the T cell, multiple signaling pathways, including those downstream of the TCR, CD28, and the integrin lymphocyte function-associated antigen 1 (LFA-1), lead to the activation of actin-regulatory proteins (**Figure 2**). The relevant signaling pathways downstream of the TCR have been reviewed extensively (10–12) and will only be briefly discussed here. Following TCR engagement, several protein tyrosine kinases, including Lck and ZAP-70, are activated, leading to the phosphorylation of multiple effectors. One key effector is the scaffold protein linker for activation of T cells (LAT). LAT phosphorylation recruits SLP-76 to the IS, and with it the Rho-family GTPase exchange factor (GEF) Vav1, the adapter Nck, and the IL-2-inducible T cell kinase (Itk). Activation of Vav1 and other GEFs, such as PIX and SLAT (13–15), leads to GTP loading and activation of the small GTPases CDC42 and Rac1. These GTPases, in turn, recruit and activate the actin nucleation promoting factors WASp and WAVE2, which work in concert with the related protein HS1

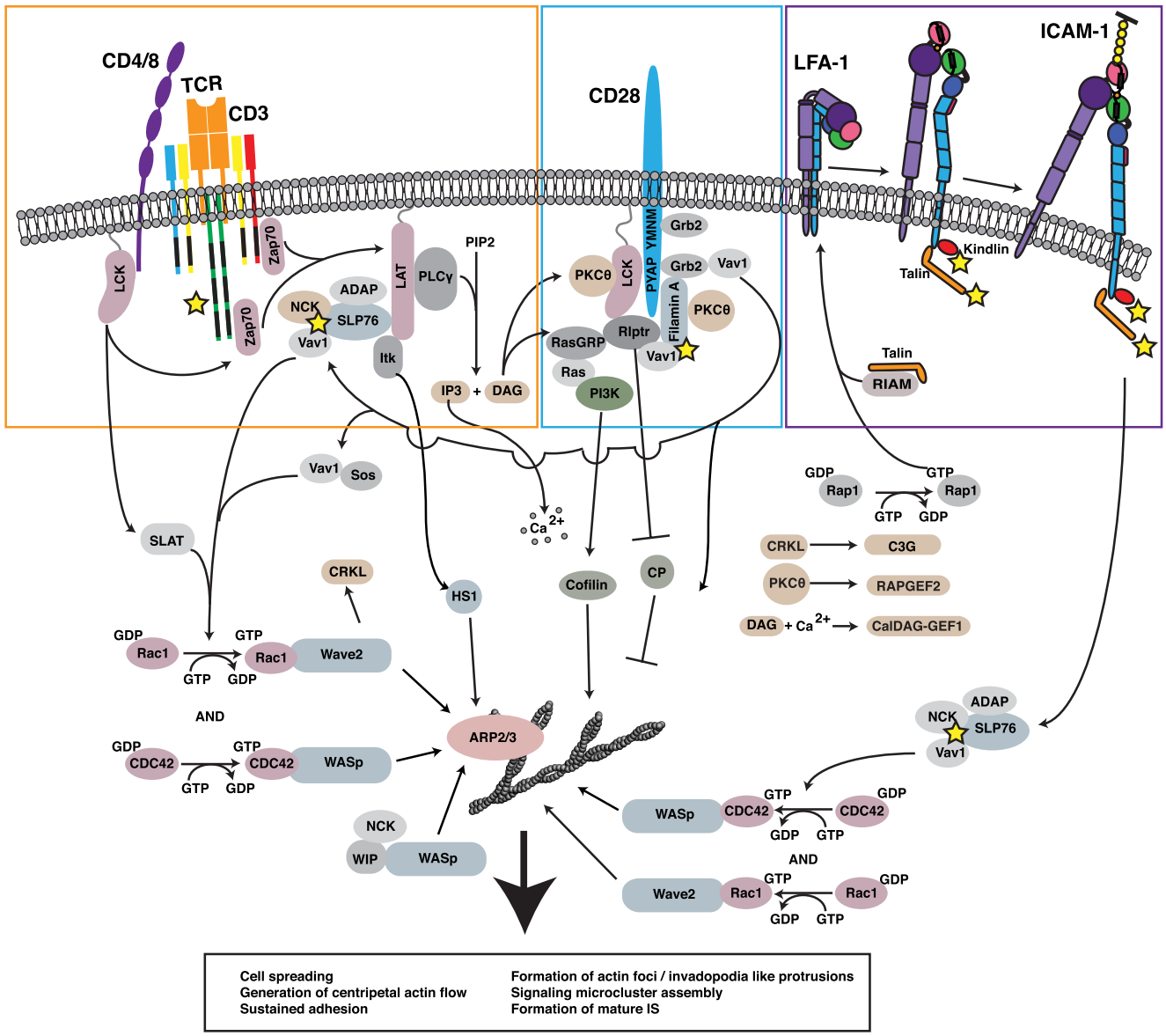

FIGURE 2 | Regulation of the F-actin network at the IS. Ligation of multiple receptors, including the TCR, the costimulatory molecule CD28, and the adhesion molecule LFA-1, results in the induction of robust actin polymerization at the IS. The pathways that mediate F-actin polymerization are highly interdependent. For example, the TCR-dependent activation of Lck is involved in initiating CD28-mediated signaling. Moreover, both TCR-induced activation and CD28-induced recruitment of PKCθ contribute to LFA-1 activation and downstream signaling. Vav1, a GEF for the critical actin regulators Rac1 and CDC42 and their respective effectors WAVE2 and WASp, is triggered in a co-operative fashion downstream of each of these key surface receptors. In addition, signaling events downstream of CD28 lead to inhibition of capping protein and activation of cofilin, events that allow growth and remodeling of the branched actin network. Together with HS1, WAVE2 and WASp activate ARP2/3 complex-dependent growth of branched actin filaments, forming lamellipodial protrusions and invadopodium-like protrusions, respectively. Collectively, these events lead to cell spreading, retrograde actin flow, and formation of the mature IS, along with assembly and centripetal flow of TCR-associated signaling microclusters. In addition to triggering actin nucleation, TCR, CD28, and LFA-1 all associate with the F-actin network (proteins known to interact with F-actin are denoted by yellow stars). In many cases, these interactions with the F-actin network serve to drive additional signaling events *via* actin-dependent feedback loops.

to orchestrate Arp2/3 complex-dependent polymerization of branched actin filaments (16–18).

Interestingly, WASp, WAVE2, and HS1 play distinct roles in organizing lamellipodial actin and actin foci. WAVE2 localizes strongly to lamellipodial protrusions and is essential for their generation (17, 19), whereas WASp is largely dispensable for generation of these structures (20). Instead, WASp localizes to and is essential for the formation of TCR-associated actin foci (7), further extending the similarity between these structures and podosomes in other hematopoietic cells (21, 22). The role of WAVE2 in generating actin foci cannot be meaningfully tested because WAVE2-deficient T cells do not spread in response to

TCR engagement, but WAVE2 is absent from these structures (7). HS1 can be found in both lamellipodia and actin foci, and in its absence, both sets of structures are disordered (7, 16). Thus, it appears that WAVE2 organizes lamellipodia that result in T cell spreading on the APC, WASp organizes TCR-associated foci that protrude into the APC, and HS1 augments and organizes both sets of actin-rich structures.

Integrin-Mediated Organization of the T Cell F-Actin Network

Another effect of TCR signaling is to induce conformational changes in LFA-1, an integrin that mediates IS formation and firm adhesion (23). LFA-1 engagement initiates a signaling cascade that parallels and intersects with the TCR-triggered cascade. This process has been termed "outside-in" signaling to distinguish it from "inside out signaling" events that trigger initial integrin activation downstream of TCR or chemokine receptor engagement. Molecules activated downstream of LFA-1 engagement include FAK, ERK1/2, JNK, and PLCγ1 (24–26). LFA-1 regulates F-actin through the ADAP-mediated activation of SLP-76 (27–29). This results in F-actin polymerization, likely through the Vav-mediated activation of Rac1, CDC42, WASp, and WAVE (**Figure 2**) (30–32). Recruitment of the Arp2/3 complex to the site of integrin engagement is enhanced by interactions of the complex with the talin-binding protein vinculin (32–34). As discussed later, integrin activation and vinculin binding to talin are dependent on the interaction of talin with the F-actin network and on ongoing F-actin flow. This suggests a robust feed-forward loop whereby integrin activation is dependent on F-actin-generated forces and results in increased activation of F-actin nucleating factors and polymerization at the IS.

Although integrin engagement can induce actin polymerization, it can also modulate F-actin flow rates. Engagement of VLA-4, a β1 integrin expressed on activated T cells, by immobilized VCAM-1 greatly decreases the centripetal flow of F-actin at the IS (35). This likely occurs through the interaction of multiple actin-binding proteins with the β chain of VLA-4, thus linking the ligand-immobilized integrin to the F-actin network and retarding network flow (35, 36). So, while integrins are capable of nucleating F-actin polymerization, the overall effect on the F-actin network will depend on the strength of the outside-in signal, the interaction between the integrin cytoplasmic domain and the actin network, the viscoelastic properties of the network itself, and the mobility of the integrin ligand (since only immobilized ligand could oppose forces on the integrin tail).

Costimulatory Signals Leading to F-Actin Remodeling

Coligation of the costimulatory molecule CD28 with the TCR leads to robust IL-2 production, activation, and expansion of naive T cells (37). The classical pathways involved with CD28 costimulation have been extensively reviewed (38–41). As part of this process, CD28 signaling regulates F-actin dynamics. CD28 can interact with F-actin through binding to filamin A (**Figure 2**). By binding to the adapter protein Grb-2, CD28 also promotes the formation of Vav 1/SLP-76 complexes and initiates downstream signaling (42–44). In cells in which Csk, a negative regulator of Lck, has been inhibited, CD28 binding to CD80/86 can mediate robust F-actin polymerization (45). CD28-dependent F-actin polymerization occurs through Vav-mediated activation of CDC42 and is enough to initiate cell spreading, though the appearance of the F-actin network is not as symmetrical as with TCR stimulation (46). CD28 costimulation has also been shown to induce the dephosphorylation and activation of the actin-severing protein cofilin (47). Somewhat counter-intuitively, actin severing by cofilin can increase rates of actin polymerization by providing actin monomer and freeing otherwise capped barbed ends (48). The overall effect of increasing both F-actin severing and polymerization is to create a highly branched F-actin network, a process that can strengthen lamellipodial protrusions and contribute to F-actin flow. Another molecule that is likely to participate in CD28-dependent actin responses is the lymphoid cell-specific actin-uncapping protein, Rltpr. As detailed in Section "Regulation of CD28 Signaling by the F-Actin Network," Rltpr interacts with CD28 and plays an essential role in costimulatory signaling (49). It remains to be determined if Rltpr functions to remove capping protein from barbed ends of actin filaments at the IS, but if so, this will also be important for F-actin remodeling.

In addition to CD28, it is likely that many other costimulatory proteins also modulate the T cell actin response. One protein known to interact extensively with F-actin is CD2. CD2 is expressed on the surface of NK cells and T cells, and it can mediate cell adhesion and induce signaling events that promote T cell activation (50, 51). Through the cytoplasmic adaptor molecule CD2AP/CMS, CD2 engagement can recruit and activate capping protein, cortactin and WASp, facilitating the formation of a short, branched actin network (52–56).

THE F-ACTIN CYTOSKELETON AND THE CONTROL OF MOLECULAR ACTIVATION AT THE T CELL–IS

As detailed above, multiple signaling cascades converge to initiate and control F-actin flow at the IS. Conversely, however, F-actin dynamics are critical for proper signal transduction. Thus, a positive feedback loop exists whereby initial signaling events induce F-actin restructuring, which in turn reinforces and sustains signaling. In the following sections, we will describe the mechanisms by which the F-actin network can control or mediate signaling events on the T cell side of the IS.

Maintaining Quiescence in Resting Cells

The maintenance of T cells in a quiescent state in the absence of cognate antigen is critical for the prevention of autoimmunity and the proper regulation of the immune response as a whole. To maintain quiescence, T cells make use of several mechanisms. Based on work in B cells, one likely mechanism involves segregation of signaling molecules into separate cell surface compartments. As has been shown for the B cell receptor (BCR) (57), the T cell actin cytoskeleton may limit baseline signaling by preventing clustering of the TCR or downstream signaling intermediates. In fact, one way that antigen experienced cells maintain increased

sensitivity to antigen is through the oligomerization and clustering of the TCR, suggesting that this process is, in fact, regulated (58). Additionally, it has been reported that large clusters of TCR and LAT are maintained separately in resting cells, and only overlap upon activation (59). Although LAT clusters are maintained by the actin cytoskeleton, it remains possible that actin also separates LAT and TCR clusters in resting T cells (60). Reorganization of the actin network following stimulation could then permit or drive cluster growth and molecular interactions. In B cells, actin-binding proteins of the ezrin, radixin, moesin (ERM) family limit BCR cluster formation, preventing aberrant signaling through the maintenance of diffusional barriers (57). BCR signaling transiently deactivates ERM proteins, allowing for increased BCR diffusion and cluster formation. This cycle is required for antigen capture, as both constitutively active and dominant negative ERM proteins interfere with this process (61). This shows that while ERM-mediated diffusional barriers may aid in maintaining a quiescent state, these barriers also undergo a dynamic cycle of activation and deactivation. A similar process may be occurring in T cells, since TCR stimulation also causes ERM protein dephosphorylation and cytoskeletal relaxation (62).

T Cell Migration, Initial Antigen Scanning, and the Conversion to a Stable IS

In many experimental systems, T cells are introduced to stimulatory surfaces from suspension, such that initial TCR-induced actin polymerization is required for cell spreading and synapse formation. *In vivo*, however, initial contact between T cells and APCs occurs within the context of T cell migration. T cell migration requires actin-mediated protrusion of the leading edge and myosin-mediated contraction of the trailing uropod (63–65). Initial T cell scanning is characterized by short-lived T cell/APC interactions. During these interactions, T cells form mobile synapses known as kinapses, which exhibit protein segregation patterns analogous to those seen in mature synapses, but are not radially symmetrical (66, 67). In essence, then, the conversion between kinapse and synapse entails altering the symmetry of the actomyosin network. This appears to be determined, at least in part, by the strength of TCR signaling. In support of this, the balance between PKCθ signaling and WASp activity determines if cells are likely to break or maintain symmetry (68). Although additional details of how T cells maintain this balance are yet to be worked out, it has been proposed that PKCθ fosters symmetry breaking by activating localized myosin contractility (67). In addition, there is evidence that intracellular calcium levels also play an important regulatory role (69–71).

Coupling of Signaling and Actin-Driven Microcluster Dynamics

Following the formation of a stable, symmetric synapse, microclusters of TCR and downstream signaling components, such as Zap70 and SLP76, form in the periphery of the IS (peripheral supramolecular activation cluster, pSMAC) and undergo transport to the center of the contact zone (central supramolecular activation cluster, cSMAC). Depolymerization of F-actin abolishes the generation of new TCR microclusters, as well as inward movement of existing TCR microclusters (1, 72), but the mechanisms linking the actin cytoskeleton to microcluster formation and movement have yet to be fully worked out. Since microtubules and cytoplasmic dynein have been implicated in microcluster movement toward the cSMAC (73), one could imagine a model in which the actin network functions as a static scaffold for microcluster nucleation, with subsequent microtubule-dependent microcluster transport contingent upon maintenance of this actin scaffold. However, this model has been ruled out; when cells are treated with an inhibitor cocktail that arrests actin dynamics but leaves the network intact, the formation and translocation of SLP76 microclusters are blocked (2). Furthermore, actin flow rates are locally perturbed at TCR microclusters that encounter a barrier to inward transport (74), suggesting direct interactions between the TCR and the actin network. Although it remains unclear exactly how actin dynamics promote continued signaling from individual microclusters, arresting F-actin dynamics interrupts phosphorylation of PLCγ, resulting in a rapid drop in intracellular Ca^{2+} levels (2). Actin foci are likely to be the relevant actin-rich structures in this context, since loss of WASp (or HS1) inhibits PLCγ1 activation and associated Ca^{2+} signaling, while loss of WAVE2 affects Ca^{2+} signaling at the level of CRAC channel coupling, leaving PLCγ1 activation intact (17).

In addition to driving microcluster formation and sustaining signaling, IS-associated F-actin flow sets a molecular countdown for signal termination. Tyrosine phosphorylation of early signaling intermediates typically occurs in microclusters located in the pSMAC (72), whereas the cSMAC is an area of protein dephosphorylation, ubiquitinylation, and internalization to form IS-associated microvesicles (75, 76). Prolonging the time microclusters spend in the cell periphery actually prolongs signaling lifetime (72, 77). For example, recruitment of TCR into the cSMAC is dependent on the ubiquitin-binding protein TSG101, and knockdown of TSG101 inhibits cSMAC formation and increases microcluster lifetime and total phosphotyrosine levels at the IS (76). Thus, while dynamic actin filaments first initiate the formation of active signaling microclusters, they subsequently lead to their deactivation by driving their accumulation at the cSMAC. Interestingly, formation of TCR-enriched microvesicles occurs as a linear function of MHC density (75). Moreover, the amount of active signaling that occurs within the cSMAC varies with peptide dose and agonist strength (78). Thus, signal activation and extinction can be modulated at the level of microcluster dynamics, to tune T cell responses over a broad range of antigenic signals.

Force Generation and T Cell Activation

During the initial contact between a migrating T cell and an APC, and in the radially symmetric mature synapse, multiple forces are applied to the molecular contacts between the two cells. As T cells migrate on the APC surface, actin polymerization at the leading edge and myosin contractility at the trailing uropod provide this force, while at the mature IS, the retrograde F-actin flow provides a similar force. With this in mind, molecular contacts between TCR and pMHC, integrins and integrin ligands, and costimulatory

molecules and their ligands must persist and signal under constant strain. Interestingly, the generation of molecular forces at the IS downstream of pMHC–TCR interactions is directly correlated with the antigenicity of a given pMHC (79), and T cells respond differently depending on the mechanical properties of the stimulatory surfaces they encounter (80, 81). Over the stiffness ranges tested so far, it has been shown that human T cells respond better to substrates of increasing stiffness, and this corresponds to an increased ability to generate force at the IS along with increasing substrate stiffness (81, 82). Additionally, migrating T cells are far more sensitive to antigen when encountered at the leading edge, rather than at the less dynamic uropod, suggesting that the forces at the leading edge prime the TCR to respond to cognate antigen (83, 84). This evidence suggests that mechanical force is integrally involved in T cell activation. If this is the case, then studying the mechanical forces on the TCR and other receptors at the IS and the relevant mechanosensitive signaling pathways becomes critically important in gaining a complete understanding of T cell activation.

Cytoskeletal Forces and the Initiation and Maintenance of TCR Signaling

Although the molecular interactions between the TCR and pMHC have been extensively characterized, the mechanism by which information on receptor ligation is transmitted across the plasma membrane and transformed into the biochemical signals associated with TCR triggering is unknown and hotly debated. Several challenges unique to the TCR/pMHC interaction must be overcome in order to initiate signaling, and any model proposed to describe TCR triggering must take these into account (85). First, TCR triggering must be extraordinarily sensitive, as there are typically only a few molecules of cognate pMHC on the surface of a given APC. Indeed, TCR triggering and T cell activation can occur in response to a single pMHC complex (86). Second, the TCR must efficiently discriminate between rare agonist and plentiful non-agonist pMHC molecules. Finally, TCR triggering must occur despite a near limitless diversity in the binding of pMHC and TCR. Several models have been proposed to account for these requirements. It is illuminating to consider these models in terms of the potential role of forces generated by F-actin at the IS. It is important to note that many observations that support a role for cytoskeletal force can be explained within the context of multiple models, and it is likely that several mechanisms are working together to initiate TCR triggering.

The Kinetic-Segregation Model

The kinetic-segregation model was proposed, in part, to account for the large proportion of Lck that is phosphorylated on the activating tyrosine, Y394, even in the absence of TCR stimulation (87). It is likely that this active Lck is continuously opposed by the action of CD45 and other phosphatases, since pharmacological phosphatase inhibition induces T cell activation in the absence of TCR stimulation (88–90). Additionally, removal of the Lck negative regulator C-src tyrosine kinase (Csk) results in the activation of proximal TCR triggering events (45). It is therefore unsurprising that the balance between tonic signaling and activation of TCR signaling depends on the expression of Csk, inhibitory phosphatases such as CD45, and kinases such as Lck (91). The kinetic-segregation model proposes that close membrane apposition enforced by the TCR/pMHC bond length (~15 nm) is too small to allow colocalization of proteins with large extracellular domains, such as CD45. In the model, the TCR is a passive player in this process, and close membrane apposition is driven entirely by the affinity of TCR for pMHC and the formation of multiple bonds leading to stochastic size-based sorting and exclusion of large molecules from pMHC/TCR rich areas. Exclusion of CD45 then allows the system to be dominated by the constantly active Lck, and TCR triggering ensues (92). Indeed, *in vitro* modeling of the TCR signaling network on reconstituted liposomes has shown that clustering of CD3ζ and Lck is enough to overcome even high concentrations of CD45 and induce signaling (93), and large ectodomain proteins have been shown to enhance clustering of smaller proteins and their ligands in live cells (94). Signaling can continue following dissociation of TCR/pMHC as phosphorylated ITAMs can be protected by interaction with their specific binding partners (93).

The strongest evidence for the kinetic-segregation model is based on observations that truncation of the CD45 ectodomain (creating a shorter molecule) impairs TCR-mediated signaling, and that full function can be restored by simply adding any large ectodomain to truncated CD45 (95, 96). Additionally, the size of the ectodomain influences segregation of CD45 and TCR into separate protein islands, with larger ectodomains resulting in greater separation. Moreover, extending the length of the extracellular domain of pMHC by the addition of various length tethers results in poor T cell activation corresponding to greater distances between the APC and T cell, and poor exclusion of CD45 from both the interfaces and from CD3 clusters (97, 98). Though elongated pMHC does not affect TCR or coreceptor binding or TCR clustering, it remains possible that elongation of pMHC affects the force transduced to the TCR, an idea that will be considered below.

Despite the evidence in favor of the kinetic-segregation model, several key problems have arisen in the literature. First, some authors have found that small ectodomains can result in CD45 exclusion from TCR and CD2 microclusters, as well as the total IS interface, suggesting that ectodomain size may not be the only contributing factor in this process (91, 99). Additionally, truncation of the intracellular domain of CD43 results in poor exclusion from the IS, suggesting that segregation based on size is not enough to determine molecular sorting at the IS for all large molecules (100). In these instances, molecular crowding and active transport may also be involved. Second, in NK cells, where similar molecular sorting events separate inhibitory and activating receptors based on ectodomain size (101), it has been found that segregation depends largely on the surface expression level of the small ectodomain protein and its receptor; more expression (and more receptor–ligand engagement) leading to greater segregation (102). This makes sense in that, in order to exclude large ectodomain proteins, the combined bond strength between shorter molecules and their ligands must be strong enough to deform the local plasma membrane and bring cells into close proximity, overcoming resistance posed by the entire glycocalyx.

Given the generally low affinity of the TCR for pMHC, multiple interactions would be needed to provide this force. This idea is troublesome given recent evidence that only one pMHC can induce the formation of a microcluster containing hundreds of TCRs, presumably excluding CD45, on a responding T cell (86). Finally, it has been shown that TCR microclusters that exclude CD45 can form in the absence of agonist pMHC, and even in the complete absence of MHC on artificial surfaces coated with ICAM-1 (103). This observation necessitates a different mechanism besides stochastic exclusion of large molecules following TCR/pMHC bond formation to explain any size-based exclusion of CD45 from TCR microclusters.

The polymerization of the F-actin network and forces generated by the network may be enough to overcome these limitations. During kinapse formation, the T cell actin network is undergoing dynamic regulation through a combination of chemokine receptor, costimulatory molecule, and integrin-mediated signaling. This reorganization of the F-actin network may be enough to push the T cell and APC membranes close together, overcoming the charge repulsion presented by the glycocalyx (104). This force could initiate CD45 exclusion from the TCR in areas of close membrane apposition, even in the absence of TCR/pMHC interactions. Following TCR engagement, forces generated by F-actin polymerization could work in concert with the close membrane apposition enforced by TCR/pMHC interactions to propagate this process, further separating CD45 and other large ectodomain proteins from TCR microclusters, and ultimately excluding them from the mature IS. In line with this idea, it has recently been found that T cells can produce invadosome-like protrusions into the membrane of an APC. These protrusions can form in the absence of antigen (though their frequency and longevity are increased in the presence of antigen) and induce extremely close membrane apposition, overcoming charge repulsions mediated by the glycocalyx (8). Critically, these protrusions (which presumably correspond to the WASp-dependent actin foci described above) are completely dependent on the F-actin network, occur in multiple T cell/APC models, and precede the onset of early TCR triggering. This phenomenon likely explains why disruption of the F-actin network prevents the formation of new TCR microclusters, even in the continued contact between the T cell and an artificial APC (1), since receptor clustering would depend on proximity to pMHC, and CD45 exclusion. Based on this evidence, the kinetic-segregation model can be modified to account for the contribution of F-actin-generated force in initiating close membrane apposition, particularly in the presence of low pMHC concentration, thus contributing to CD45 exclusion form sites of TCR–pMHC binding (**Figure 3A**).

The Kinetic Proofreading Model

The kinetic proofreading model of TCR triggering, initially proposed by McKeithan, posits that TCR triggering requires individual bond lifetimes above a certain threshold duration, and longer than the dissociation time (105). Furthermore, if unbinding occurs prior to this threshold being reached then no signaling occurs and the TCR resets itself. This model was later refined to allow for retention of TCR signaling intermediates, so that rebinding of pMHC to the same TCR would continue from where the previous interaction left off (106). This fits well with the observations that fast pMHC on rates can overcome low dwell times/high off rates and lead to high apparent affinities and TCR triggering. That is, if a pMHC rebinds prior to diffusing away from the TCR, it could induce TCR triggering by reaching the threshold even when any given interaction is particularly short (107, 108). In fact, in 2D experimental paradigms, k_{on} has been shown to be one of the best predictors of pMHC agonist strength (109, 110).

Force produced by the F-actin network may play an interesting role in the kinetic proofreading model. It has recently been shown that the TCR can engage in catch-bond molecular interactions, in which applied force prolongs the interaction time with cognate pMHC (111). In that study, Liu et al. show that in the absence of force on the TCR/pMHC bond, there is an inverse relationship between the average lifetime of the bond and pMHC potency. However, following the application of 10 pN of exogenous force to the bond, agonist pMHC bond lifetimes increase, behaving like catch-bond molecular interactions, while antagonist bond lifetimes decrease, behaving like more traditional slip-bond type molecular interactions. This leads to a 57-fold increase in the ratio of bond lifetimes between strong agonist and strong antagonist peptides following the application of force. Additionally, catch-bond behavior correlated strongly with the strength of the agonist (as measured by T cell stimulatory capacity) such that the strongest agonist pMHC had the largest increase in bond lifetime following the application of force and required the greatest force to induce the catch-bond behavior. Interestingly, it has been shown that at the IS, the actin cytoskeleton acts to decrease the half-life of some TCR/pMHC bonds (109). Thus, internally generated force provided by the F-actin network could function similarly to the external force applied in the study by Liu et al. In terms of the kinetic proofreading model, force would thus allow for increased specificity and greater bond lifetimes for agonist vs. antagonist pMHC, enhancing sensitivity and diminishing noise during TCR signal acquisition (**Figure 3B**).

The Serial Triggering/Serial Engagement Model

The serial engagement model was proposed as a way of accounting for the high specificity of the TCR, despite low 3D affinities and high off rates, and low numbers of agonist pMHC on the APC surface. In this model, pMHC serially engages with multiple TCRs, triggering each one individually before moving onto another, and thereby taking advantage of the high off rate to trigger multiple receptors (112). Later studies have confirmed that a single pMHC is capable of recruiting hundreds of TCRs into a complex, initiating T cell activation and cytokine production (86). It has previously been proposed that actin-induced apposition of the T cell and APC membranes would bring the TCR into close proximity to pMHC complexes, accommodating the fast on-rates characteristic of agonist pMHC (113). It is possible that in addition to facilitating single pMHC/TCR interactions, the actin cytoskeleton also serves to bring additional TCRs into the immediate vicinity of ligated TCR/pMHC pairs. This would increase activation efficiency by reducing the time it would take for pMHC to encounter another TCR. The actin cytoskeleton is critical for the formation of TCR and signaling microclusters

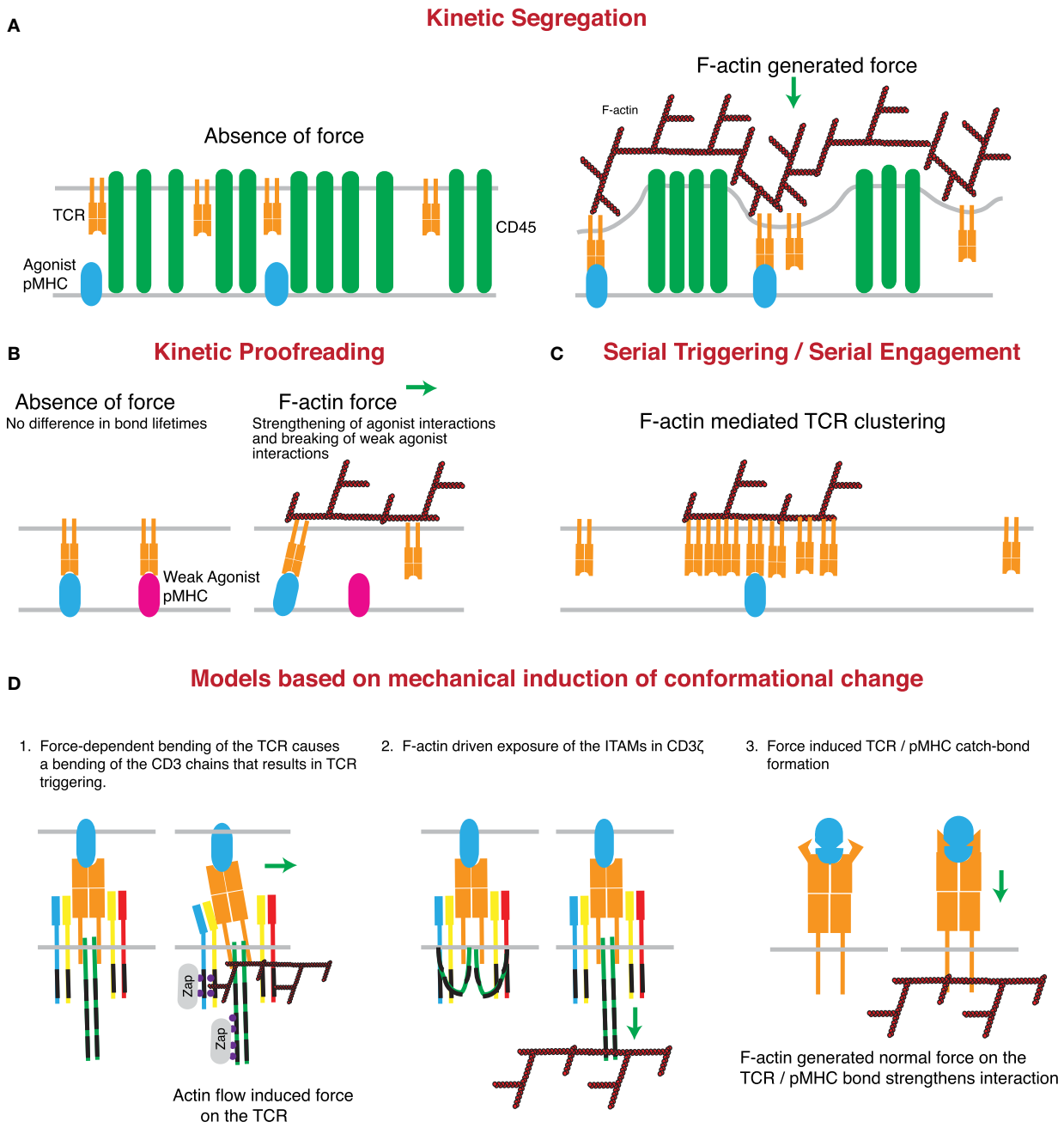

FIGURE 3 | Mechanisms through which actin-dependent forces can contribute to proposed models controlling TCR triggering. (A) The kinetic-segregation model of TCR signaling is dependent on the separation of molecules with small extracellular regions, such as the TCR, from those with large extracellular regions, such as the phosphatase CD45. Actin-dependent protrusions would serve to bring the T cell and APC plasma membranes into close proximity, thereby driving molecular segregation. This should occur even in the presence of low numbers of cognate pMHC on the APC surface. **(B)** The kinetic proofreading model proposes that TCR triggering is based on longer bond lifetimes for strong agonists than weak agonists. The force-dependent catch-bond behavior of the TCR with strong, but not weak, agonist pMHC complexes can enhance bond lifetime for strong agonists, while serving to rupture the slip-bonds formed by TCRs engaging weak or non-agonist pMHC. **(C)** The serial triggering/serial engagement molecule could benefit from the presence of multiple F-actin interactions with the TCR. Though it may not be force dependent, the association of the TCR with the F-actin network could lead to clustering of the TCR on the plasma membrane, allowing for rapid successive unbinding and rebinding, and serial triggering of multiple TCRs by a single pMHC complex. **(D)** Several conformational changes that have been described for the TCR may be induced or enhanced by the application of force by the T cell actin cytoskeleton. The first posits a mechanical lever-type action of the TCR under the application of a tangential force. According to this model, bending of the stiff CD3 chains propagates to the intracellular domain and results in signal initiation. The second model suggests that actin associations with the CD3 complex help to pull the CD3 chains away from the inner leaflet of the plasma membrane, thus exposing the ITAMs for phosphorylation and binding of essential regulators such as the kinase ZAP70. The third model is based on catch-bond molecular interactions between TCR and cognate pMHC complexes. According to this variant of the kinetic proofreading model, cytoskeletal force causes a conformational change in the TCR that results in stronger pMHC binding and prolonged or enhanced signaling.

following simultaneous engagement of multiple TCR molecules (1, 2). It is therefore possible that TCR clustering induced by a single pMHC is also induced or stabilized by the F-actin network, thereby leading to enhanced TCR triggering (**Figure 3C**). In other systems, direct tethering of transmembrane proteins to cortical actin induces nanoclustering (114). The TCR associates with the F-actin network through both ITAM-dependent and -independent mechanisms (115–117). Although the ITAM-dependent mechanism requires phosphorylation by Lck and is therefore likely to take place after initial TCR triggering, the ITAM-independent mechanism is mediated by two RRR sequences in the CD3ζ chain and causes the constitutive association with F-actin. This association is essential for clustering of the TCR, IS formation, and T cell activation following TCR engagement. Thus, the constitutive and inducible interactions between TCR and F-actin could produce localized increases in TCR concentration, thereby facilitating serial engagement.

Conformational Change and the Mechanical Induction of TCR Triggering

Recently, the idea that conformational change and mechanosensing may play a critical role in TCR triggering has gained significant traction (3, 12, 118). Structural studies demonstrate the existence of several conformational changes that can occur upon pMHC binding (119–122). In many cases, however, the documented changes in TCR structure did not propagate away from the pMHC-binding site. Conformational changes in the constant domain, away from the antigen-binding site, were subtle, and it remains unclear if these represent conserved changes found in all triggering interactions. Furthermore, it is unclear how such small changes can propagate to the intracellular portion of the CD3 chains. This brings us to the one key problem faced by models proposing conformational changes initiated in the TCR by pMHC binding alone. Specifically, any conformational change must be present in all TCR/agonist pMHC interactions and absent from TCR/non-agonist pMHC interactions. Given the near limitless variation in the TCR- and pMHC-binding sites, it is hard to imagine that all productive interactions occur with a given binding geometry necessary to initiate the same structural changes. In support of this, activating antibodies can perform their function in the absence of any overt structural change to the TCR structure in solution (123). Further complicating the matter, multiple groups have observed that soluble monomeric pMHC is poorly suited to activating T cells, even at extremely high concentrations (124–128), despite TCR/pMHC half-lives otherwise associated with TCR triggering in a 2D environment (109). Finally, as mentioned earlier, simple elongation of the pMHC reduces TCR triggering despite maintaining efficient binding to the TCR, again suggesting that binding-induced conformational change is unlikely to represent a complete TCR triggering mechanism. Interestingly, by incorporating slight modifications to the conformational change model that take into account the cell biology of TCR/pMHC interactions at the IS, one can overcome all of these problems (**Figure 3D**).

Within the IS, the TCR is dynamically associated with the F-actin network through multiple direct and indirect interactions (**Figure 2**) (6, 74, 116, 117, 129, 130). These interactions allow F-actin-generated force to be applied to the TCR *via* the actin–TCR linkage. Any resistance to this force provided by surface-bound pMHC could then be converted into a conserved conformational change in the TCR. One key result of refocusing the driver of conformational change from molecular interactions occurring at the site of pMHC engagement to mechanical force applied on the TCR is that these changes in protein structure can occur regardless of the specific molecular contacts occurring between the TCR and pMHC. As long as the interaction is of sufficient affinity to stay bound in the presence of force, productive TCR triggering will ensue, thus overcoming the challenge created by the diversity in pMHC/TCR interactions. Additionally, this mechanism does not require that conformational changes occur within the ectodomains of the TCR subunits; it works equally well for changes in ITAM-containing intracellular domains (131).

Several lines of evidence support the existence of a mechanotransduction-based mechanism for TCR triggering. As mentioned earlier, observations that soluble monomeric pMHC cannot initiate efficient TCR triggering pose a particular problem for the conformational change model (124). Interestingly, surface anchoring of monomeric pMHC overcomes this limitation as low numbers of surface-bound monomeric pMHC can initiate TCR triggering (86, 132, 133). In part, this sensitivity to pMHC and continued signaling is dependent on an intact cytoskeleton, as addition of actin depolymerizing agents causes rapid loss of calcium flux without loss of IS formation (1, 132, 134). This effect of actin inhibition is specific to 2D stimulatory settings, since actin depolymerization when the TCR is cross-linked in solution leads to prolonged calcium responses (135). Moreover, inhibition of cytoskeletal dynamics under conditions that retain the actin scaffold also results in a drop in intracellular calcium (2), showing that dynamic actin plays an active role. A likely explanation for these observations is that in the 2D setting, as opposed to monomer in solution, the dynamic actin filaments can generate force on the pMHC/TCR bond, thereby initiating signaling. Supporting this idea, tangential force applied on the TCR through non-activating antibodies can result in initiation of calcium flux. Strong stimulatory anti-CD3ε antibodies may mimic this force by binding to the side of the complex in a way that induces a bending of the CD3 molecule, in contrast to non-activating antibodies that bind more perpendicularly at the membrane-distal portion of the CD3 complex (136). These data support a model in which force on the TCR/pMHC complex applied tangentially, and not normally, makes the TCR act as a lever, bending and activating the associated CD3 complexes (**Figure 3D**, model 1) (137). Critically, the actin flow at the IS is radially symmetric and directed toward the center of the IS. Force vector measurements at the IS show that this actin flow results in a similarly directed force applied to the substratum through the TCR (138, 139). This force is consistent with the F-actin-driven centralization of TCR microclusters and would apply a tangential force on the TCR/pMHC bond.

Interestingly, an external normal force can also initiate TCR triggering, though whether normal and tangential forces act by the same or different conformational triggering mechanisms is unknown (140). Critically, it was shown that simple contact between the TCR and pMHC probe was insufficient to induce

signaling. Instead, continuous force was required to maintain calcium flux; signaling stopped and resumed with the cessation and reapplication, respectively, of external force. Consistent with this, the loss of TCR triggering that occurs when the extracellular domain of the pMHC is artificially elongated, usually used as evidence for the kinetic-segregation model, can be overcome through the application of tangential or normal force to the TCR/pMHC bond. This finding is important in that it suggests that prior findings, interpreted as support for the kinetic-segregation model, can be reevaluated to fit into a coherent theory of TCR triggering based on the application of force on the TCR/pMHC bond. Although these findings strongly suggest the existence of conformational changes induced through the application of normal and/or tangential force, the structural nature of these changes with each type of force is still unclear. This is complicated by the fact that conformational changes under strain are particularly difficult to study, as they are not likely to exist with pMHC binding to purified, soluble, TCR components.

How exactly force is applied to the TCR is an important question. As already mentioned, the TCR can interact both directly and indirectly with the actin cytoskeleton. The direct association of CD3ζ is mediated by two stretches of basic amino acids, and mutation of these residues results in decreased synapse formation and T cell activation (116). Interestingly, these same amino acid stretches also mediate binding of the CD3ζ to the negatively charged inner leaflet of the plasma membrane, limit the phosphorylation of ITAMs (141, 142), and are required for synaptic recruitment of CD3 (143). The dual role of the basic stretch suggests a possible competition of binding for the basic residues in the CD3ζ cytoplasmic domain, with binding to the inner leaflet acting as a negative regulator for activation, and binding to the actin cytoskeleton acting as a positive regulator. It also raises the possibility that following ligand binding, force exerted by the actin cytoskeleton on CD3ζ may physically disrupt the association of CD3 chains with the plasma membrane, helping to expose the ITAMs for subsequent phosphorylation (**Figure 3D**, model 2). Similar binding of the CD3ϵ chain to the plasma membrane also restricts phosphorylation of ITAMs within the ϵ chain by Lck (144). Though no direct CD3ϵ/F-actin interaction has been discovered, it is known that CD3ϵ can bind directly to Nck following TCR engagement prior to detectable phosphorylation of ITAMs, and the Nck-binding site is exposed following TCR engagement, and independently of TCR signal initiation (131, 145). This interaction is critical for the initiation of TCR triggering at very early steps, since mutating the residues involved in Nck binding or blocking the interaction with cell permeant peptides results in greatly diminished phosphorylation of CD3ζ, CD3ϵ, and Zap70, reduced recruitment of CD3 to the synapse, and inhibition of proliferation and effector function (146, 147). Nck is linked to actin polymerization through recruitment and binding of N-WASp and WASp (148, 149). Since an N-WASp-mediated linkage between actin and p130 Cas has been proposed to cause the force-dependent activation of p130 Cas (150), it is likely that the connection of CD3ϵ to the F-actin network through Nck can transduce a similar force. Therefore, CD3ϵ ITAM phosphorylation could be regulated by actin-generated force in a way similar to the one proposed for CD3ζ.

An alternate model involving normal force stems from recent work by Liu et al. demonstrating that TCR/pMHC interactions show catch-bond behavior (111). Since many of the theoretical mechanisms for catch-bond formation require an accompanying conformational change (151), this study provides strong circumstantial evidence for the existence of an as-yet undefined conformational change at the site of TCR/pMHC interaction. In the study by Liu et al., a tensile normal force was applied by retraction of an extracellular probe bearing pMHC, though it has been theorized that a similar normal force can be generated internally through the action of the F-actin network (118). In this case, the F-actin flow at the IS would pull on the TCR, inducing a conformational change in the ectodomain that would strengthen the interaction with bound pMHC complexes (**Figure 3D**, model 3). Even if this conformational change does not initiate signaling, it could enhance the probability of TCR triggering, as in the kinetic proofreading model.

Regulation of Integrin Function by Cytoskeletal Forces

In T cell/APC contacts, integrins are primarily responsible for the adhesive interactions that maintain cell–cell contact (152, 153). Each integrin consists of an α and a β subunit, paired as shown in **Figure 4A**. In T cells, integrins are required for firm adhesion to endothelium during diapedesis and for formation of stable T cell/APC interactions, resulting in T cell activation or effector function. As such, integrins must function in a variety of extracellular environments, even under the extraordinary strain placed on the integrin–ligand bonds by the shear flow in blood vessels. Furthermore, integrin activation must be tightly regulated to prevent improper lymphocyte function. In general, integrins are regulated at two distinct levels: affinity (the strength of interaction between each individual integrin molecule and its ligand) and valency (integrin density at the cell–cell interface). Both valency and affinity contribute to adhesion (154). Therefore, the overall strength of interaction, or avidity, is a product of valency, affinity, and relative contact area (155). In T cells, intracellular signals emanating from chemokine receptors or the TCR have been shown to increase the activation state of integrins on the cell surface. This "inside out" signaling can result in either changes in valency or affinity, and a large body of work has accumulated defining the relevant biochemical pathways (155–157). Recently, new data have emerged demonstrating the regulation of integrin activation through applied forces (158–160). In the following section, the signaling pathways governing integrin activation at the IS will be covered, with a particular focus on the role of cytoskeletal forces in initiating and sustaining changes in integrin valency and affinity.

The αLβ2 (CD11a/CD18) integrin LFA-1 is expressed exclusively in leukocytes and is essential for T-cell trafficking and IS formation. In resting T cells, LFA-1 is maintained in an inactive, bent conformation with very low ligand-binding capacity (**Figure 4D**). Signaling through TCR and CD28 results in the activation of the small GTPase Rap1 downstream of PLCγ, PKCθ, and CrkL (161–164) (**Figure 2**). Following Rap1 activation, talin is recruited to the IS through the action of RIAM, which links talin to

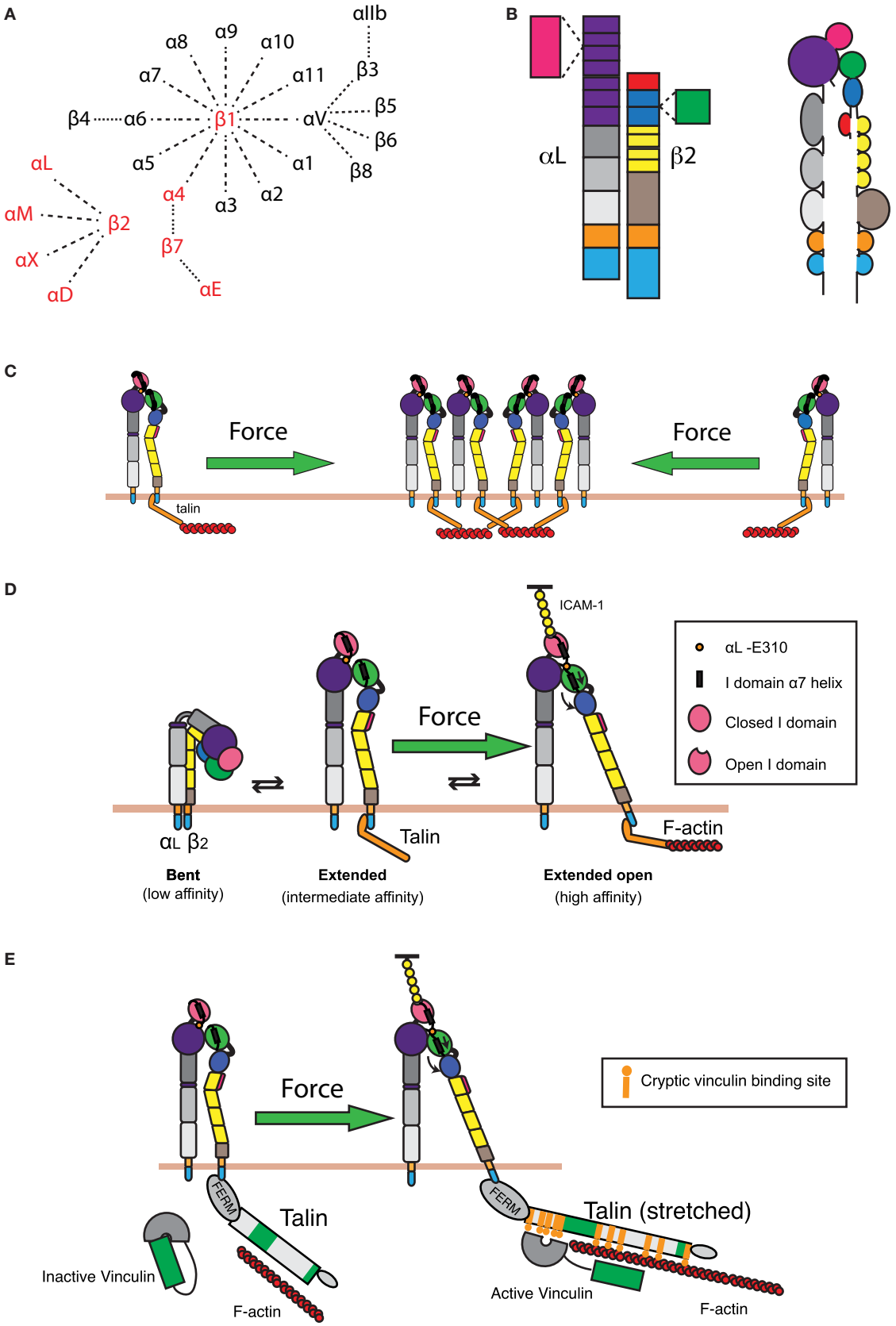

FIGURE 4 | Integrin regulation by cytoskeletal forces.

(Continued)

FIGURE 4 | **Continued**
(A) Known α and β integrin chains and pairings. Integrin pairs expressed in leukocytes are depicted in red. **(B)** Domain structure of the integrin LFA-1. The α chain consists of an intracellular tail (cyan), a transmembrane domain (orange), two calf and one thigh domains (gray), and a β-propeller domain (purple) with an inserted ligand-binding I domain (pink). The β chain consists of an intracellular tail (cyan), a transmembrane region (orange), a β tail domain (brown), four EGF repeats (yellow), a hybrid domain (blue) with an inserted βI domain (green), and a PSI domain (red). **(C)** Retrograde actin flow drives LFA-1 into the IS from the cell periphery. This increases local concentrations of LFA-1, thereby increasing the valency of the interaction and strengthening cell–cell adhesion. **(D)** LFA-1 can exist in roughly three conformations: a bent, low affinity conformation; an extended intermediate affinity conformation; and an extended conformation, where the hybrid domain on the β chain is swung outward, allowing for downward movement of the α7 helix in the βI domain. This downward movement allows the βI domain to bind an internal ligand in the αI domain, causing downward movement of the αI domain α7 helix and opening of the ligand-binding site. These changes generate a high affinity, extended-open conformation. Maintenance of this conformation at the IS is dependent on ongoing actin flow, presumably because connection of the β chain intracellular domain to the dynamic F-actin network is enough to drive swing-out of the hybrid domain. The resulting force-dependent increase in affinity would promote and augment changes induced by ligand binding. **(E)** In addition to regulating LFA-1 affinity for ligand, applied force can also strengthen the connection of LFA-1 to the underlying actin cytoskeleton. Talin, a key protein that links integrins to the actin network, can stretch upon the application of force. This stretching reveals up to 11 cryptic vinculin-binding sites. Vinculin, itself an actin-binding protein, then binds to the exposed sites and reinforces linkage to the F-actin network.

the membrane targeting CAAX domain of activated Rap1 (165). Recruitment of talin to the IS is required for LFA-1 affinity and valency modulation as well as conjugate formation (166). Binding of the talin head domain to the cytoplasmic domain of the integrin β chain causes alterations to the β transmembrane domain, thereby relieving interactions between the α and β chains that maintain the bent conformation. This process allows unfolding of LFA-1 and the adoption of an intermediate conformation with 10-fold increased affinity for ligand over the bent conformation (167–175). This "switch-blade" like unfolding occurs in the presence of activating antibodies or ligand-mimetic peptides and exposes epitopes that report on integrin activation (176, 177). Importantly, overexpression of the talin head domain is enough to result in extension of the majority of LFA-1 molecules on the cell surface, but it does not fully rescue cell adhesion, suggesting that the actin-binding domain of talin is essential for full LFA-1-mediated cell adhesion (166, 178). In addition to talin, other proteins, including members of the kindlin family of adaptors, are known to bind to the cytoplasmic domains of integrin β chains and link them to the cytoskeleton, further emphasizing that cytoskeletal linkage is essential for proper integrin function (160, 179).

The role of the cytoskeleton in mediating changes in LFA-1 valency is not straightforward. Early studies proposed a mechanism whereby cytoskeletal restraints limited the mobility of LFA-1 in resting cells, thus preventing clustering. Upon activation, cytoskeletal restraints were released, allowing the free diffusion and coalescence of LFA-1, thus increasing valency (180–182). In this model, the increased association of high affinity LFA-1 with the cytoskeleton limits the ability to support firm adhesion (183). In support of this idea, low dose Cytochalasin D increases LFA-1 mobility and clustering and increases cell adhesion to ICAM-1-coated surfaces. These changes do not induce, and function independently of, changes to integrin affinity and conformational change (154, 184). Importantly, later studies demonstrated that the integrin clustering mediated by actin depolymerization only occurs in the presence of ligand, and suggested a trapping mechanism aided by the increased diffusivity of LFA-1 in the absence of the cytoskeleton (184). This would indicate that LFA-1 interaction with the cytoskeleton limits valency and is in contradiction with the finding that talin, the main link between LFA-1 and the cytoskeleton, is required for LFA-1 synaptic accumulation (166). Further complicating the picture is the observation that transport of microclusters containing LFA-1 and ICAM-1 at the IS is dependent on an intact actomyosin network (4, 185). One of the key confounding factors in this literature is that the studies that identified the actin cytoskeleton as a negative regulator of LFA-1 valency were not carried out within the context of an IS. In an IS setting, actin retrograde flow can actively draw LFA-1 into the IS, increasing the valency of LFA-1/ICAM-1 interactions (**Figure 4C**).

In addition to the regulation of valency, integrin avidity can be regulated at the level of affinity. Changes in integrin affinity are generally associated with conformational changes (158) (**Figure 4D**). As previously mentioned, "inside out" regulation of integrin extension mediates the transition from the low affinity to the high affinity conformation. Conformational change from the intermediate to the high affinity state results in a further 100-fold increase in affinity for ligand and has been proposed to be mediated by forces generated by the T cell actin cytoskeleton (159, 175). Structural changes associated with integrin activation have been characterized using activating mutations and antibodies (176, 186–188). Typically, integrin activation and ligand binding are associated with a lateral swing-out of the hybrid domain and downward movement of the α7 helix in the βI domain. This induces the high affinity conformation of the βI domain and has been shown to occur through a series of conformational intermediates (189). In αI domain-containing integrins such as LFA-1, the activated βI domain binds an invariant glutamate residue in the C-linker region between the αI- and β-propeller region. This results in downward movement of the αI domain α7 helix and adoption of the extended open, high affinity, αI domain. Importantly, antibodies that stabilize the extended and extended-open conformations greatly increase LFA-1's affinity for ligand, resulting in a near 1000-fold dynamic affinity range from the bent to the extended-open conformations (175, 190). Furthermore, shortening of the C-linker region to mimic the downward motion exerted by the βI domain results in constitutively active LFA-1 (187). Steered molecular dynamic simulations have demonstrated that conversion between different integrin conformations can occur through the application of physical forces. Pulling on the headpiece or on bound ligand can overcome interactions between the hybrid and β-tail domains that help maintain the bent conformation, resulting in integrin extension. Importantly, forces applied to the headpiece were not sufficient in these simulations to induce the opening of the headpiece or separation of the integrin legs (191). Interestingly, similar simulations have shown that a tensile force applied parallel to the membrane on

the β cytoplasmic tail can be propagated along the β chain, resulting in hybrid domain swing-out and affinity modulation (188). Since talin binds to the integrin β cytoplasmic domain, any force applied on LFA-1 through talin's linkage to the retrograde actin flow at the IS would result in a similar tensile force, and should mediate integrin affinity maturation (**Figure 4E**). Intriguingly, it is known that high affinity LFA-1 is more tightly bound to the actin cytoskeleton than intermediate or low affinity LFA-1, supporting the idea that linkage to the underlying cytoskeleton is involved in conformational regulation (192). Our recent work has demonstrated that, indeed, the force provided by retrograde actin flow is critical for maintaining LFA-1 in the high affinity conformation, ligand binding, and clustering of LFA-1 at the IS (193). Thus, connection of LFA-1 to the dynamic actin network provides the force required to initiate integrin recruitment to and clustering within the IS, thereby increasing valency, and also provides the force to induce conformational change to the high affinity state (**Figure 4**, green arrows).

Consistent with the prediction that force can enhance LFA-1 affinity, integrins engage in catch-bond interactions (194–196). As with other adhesion molecules, such as selectins, integrin bond lifetime increases as tensile normal force is applied, until a threshold known as critical force is reached, where bonds are rapidly ruptured (151, 197). Importantly, blocking binding of the αI internal ligand by the open βI domain inhibits catch-bond behavior, suggesting that conformational change initiated by hybrid domain swing-out is required to initiate catch-bond interactions. Furthermore, it has been shown that α5β1 and LFA-1 bond lifetimes are increased following a short, transient, period of high force application. For LFA-1/ICAM-1 interactions, loading and then unloading of applied force stabilizes the integrin/ligand bond, increasing the average bond lifetime from 1.5 to over 35 seconds (198).

So far, we have discussed the mechanosenstive aspects of LFA-1 regulation with a focus on integrin–ligand interactions. Importantly, the connection between the T cell and the APC mediated by the integrin–ligand bond is only as strong as the weakest link in the pathway (199). Whereas catch-bond interactions between integrins and their ligands exhibit increased affinity with the application of force, the links that connect integrins to the cytoskeleton are thought to behave as more conventional slip bonds, where force decreases bond lifetime. Nonetheless, the talin-mediated linkage of LFA-1 to the actin cytoskeleton is regulated by the application of force. Once talin binds to the integrin β tail through its head domain and to F-actin through its rod domain, actin–myosin-mediated force pulls on talin. This causes talin to unfold like an uncoiling spring, thereby exposing up to 11 cryptic vinculin-binding sites (200, 201) (**Figure 4E**). Binding of vinculin to the talin rod domain then allows vinculin to bind to F-actin and enforces the integrin linkage to the actin cytoskeleton (160, 201–204). Although this process is reversible, such that loss of force leads to diminished vinculin binding, vinculin binding can stabilize the unfolded conformation of talin (205). In T cells, vinculin is recruited to the IS and is required for talin recruitment and conjugate formation, suggesting that the destabilized talin–F-actin bond is not enough to maintain LFA-1 activation (206). Thus, talin–vinculin binding represents another force-dependent step in the pathway leading to integrin activation, and another mechanism through which cell adhesion can be enhanced by F-actin flow (**Figure 4E**).

Integrin-mediated outside-in signaling has been mentioned earlier as a driver of multiple pathways of T cell activation. Importantly, the conformational changes that mediate LFA-1 affinity maturation are also required for outside-in signal initiation. Blocking LFA-1 affinity maturation leads to decreased IL-2 production and T cell proliferation (207). Likewise, inhibition of the separation of the transmembrane domains through addition of an inter-subunit disulfide bond results in the loss of outside-in induced stress fiber formation and cell spreading in CHO cells (208). Conversely, affinity modulation through the addition of conformational change-inducing antibodies results in the same pattern of outside-in tyrosine phosphorylation as actual ligand binding. This suggests that integrin conformational changes are necessary and sufficient to induce outside-in signaling. Therefore, forces on the integrin–ligand bond that induce and stabilize integrin conformational change are likely to also be required to initiate and sustain outside-in signal transduction.

Given the accumulating evidence that physical force exerted by the actin cytoskeleton drives conformational changes that mediate LFA-1 activation and stabilize this active conformation, we must re-evaluate our understanding of TCR-mediated integrin activation. Under this new paradigm, forces generated by the retrograde flow of the T cell actin cytoskeleton act as a key component of inside-out signaling and are a critical allosteric regulator of integrin activation at the IS.

Regulation of CD28 Signaling by the F-Actin Network

As with the TCR and LFA-1, there is strong evidence that F-actin contributes to costimulatory signaling at the IS. Here, we will focus on CD28, although signaling through CD2 and other costimulatory molecules also involves the actin cytoskeleton (52–55, 209–211). Microclusters of CD28 form concomitantly with TCR microclusters and then segregate into their own domain outside of the cSMAC, but the role of F-actin in the formation and centralization of these microclusters is unknown. Somewhat surprisingly, CD28 microclusters only require the presence of ligand to form and centralize and will do so even in the absence of the CD28 cytoplasmic tail, though differences in the rates were not addressed (212). This suggests that CD28 microcluster formation is primarily the result of kinetic segregation. If so, F-actin dynamics could contribute to this process, as diagrammed in **Figure 5**. Regardless of the effects of F-actin on CD28 microclusters, several studies indicate that propagation of signals downstream of CD28 is dependent on F-actin dynamics. First, the F-actin-uncapping protein Rltpr, which interacts with CD28, is absolutely required for CD28 signaling. Rltpr-deficient mice mimic the phenotype of CD28 knockout mice, and Rlptr is required for the CD28-mediated focusing of PKCθ and Carma 1 within the central region of the IS (49). Second, Tan et al. showed in thymocytes that activation of Src kinases by acute inhibition of Csk recapitulates many early signaling events in the TCR signaling pathway, but does not allow elevation of intracellular Ca^{2+} or ERK phosphorylation (45). Intriguingly, this blockade in

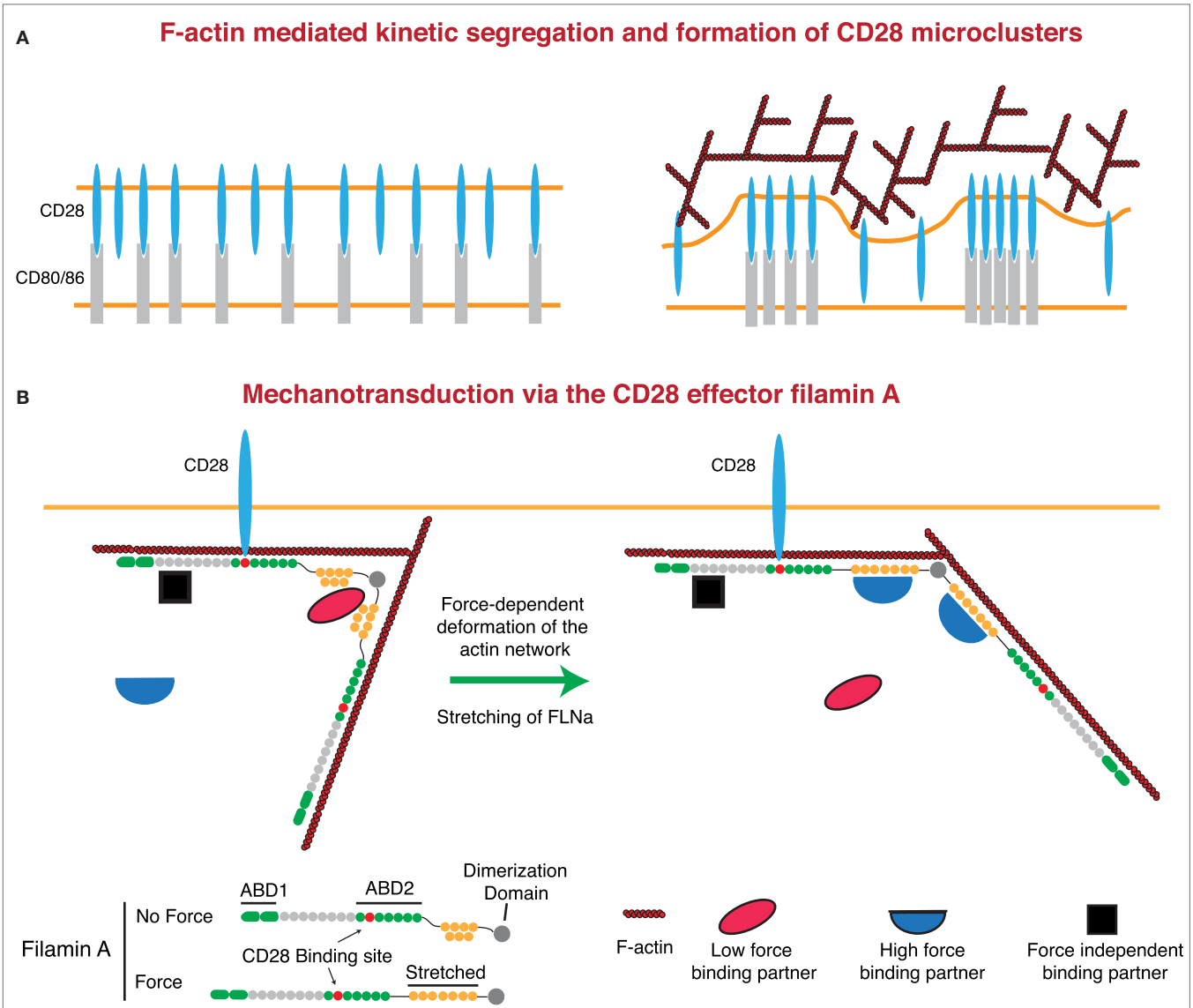

FIGURE 5 | Regulation of CD28 signaling by applied forces. (A) CD28 clustering at the IS may occur in a signaling-independent manner through the kinetic segregation of bound and unbound molecules. Actin polymerization could contribute to this process by generating protrusions that bring the T cell plasma membrane into close proximity with that of the APC. In the case of a receptor and a surface bound ligand, the free receptor would occupy less extracellular space than the bound receptor–ligand pair, such that bound receptors would be forced to cluster within areas of low membrane proximity. **(B)** Filamin A (FLNa) is a scaffolding protein and an essential CD28 effector that can undergo force-dependent conformational change. Force causes the extension of the second rod domain of filamin A, eliminating binding sites that exist in the relaxed protein, and exposing a different set of binding sites that only exist in the extended protein. Thus, under tension, FLNa can release low force-binding partners and recruit new proteins to the CD28 signaling complex.

signaling could be overcome by perturbing the actin cytoskeleton or by stimulating CD28-mediated F-actin rearrangement. These data support a model in which cortical actin forms a functional barrier between active PLC-γ1 and its substrate, and engagement of CD28 remodels actin architecture to allow signaling to proceed. Finally, it has been shown that costimulatory signaling by CD28 can induce greater force on stimulatory surfaces than TCR triggering alone (presumably through regulation of the F-actin network). This additional force is not applied through CD28 itself. Instead, force is applied through the TCR, at least in the absence of integrin engagement (138). Perhaps CD28 costimulation can lead to greater forces on other mechanosensitive receptors, including integrins. This idea is consistent with the finding that CD80 and CD86 on DCs increase the strength of cell–cell interactions with a responding T cell (213).

CD28 interacts with the F-actin cross-linking protein filamin A (FLNa) through the PxxPP motif in the CD28 cytoplasmic tail and domains 10–12 of FLNa. FLNa is recruited to the IS in a CD28-dependent manner following TCR stimulation, and CD28 interaction with FLNa is required for T cell costimulation (214). FLNa is a large, rod-like protein that is composed of an N-terminal actin-binding domain and 24 Ig-like domains. Ig-like domains

1–15 are referred to as Rod 1, while domains 16–23 make up Rod 2, with the 24th domain allowing for homodimerization. In addition to binding actin, FLNa is a prolific scaffolding protein with over 90 known binding partners, including intracellular signaling molecules, receptors, ion channels, transcription factors, and adhesion proteins (215, 216). Critically, many of these interactions can be regulated through the application of either external or internally generated force. Cryptic binding sites in the compact Rod 2 domain are exposed, and binding sites in the normal state are abolished following the application of force. This could occur through stretching of the Rod 2 domain under conditions where the F-actin network is under stress (216–218). This is particularly relevant at the IS, where the robust F-actin flow is likely to apply considerable stress to the network and may represent an important force-dependent aspect of CD28-mediated costimulation (**Figure 5**). Indeed, the mechanical regulation of FLNa-binding partners sets up several potential signaling mechanisms. Molecules that are recruited under force could allow for localized signal activation and signal amplification. Conversely, molecules that are released following force application could act as soluble signaling factors, exerting their function on areas distant from the IS. FLNa has been shown to be important for PKCθ recruitment to, and NF-κB-inducing kinase (NIK) activation at, the IS. Interestingly, while NIK is constitutively associated with FLNa, interaction with PKCθ requires CD3/CD28 signaling. Thus, it will be interesting to see if FLNa-mediated CD28 recruitment of PKCθ is force dependent.

Force-Based Activation of Other Mechanosensitive Molecules at the IS

The preceding sections address force-induced activation of surface expressed receptors and their ligands, but F-actin-generated forces affect cytoplasmic molecules as well. One prime example is CasL, a lymphocyte-specific member of the Crk-associated substrate (Cas) family of proteins. Members of the Cas family contain a highly conserved Src kinase substrate domain with multiple phosphorylatable YxxP motifs. For the non-hematopoietic isoform p130Cas, it has been shown that these motifs are exposed by mechanical stretching of the protein (219, 220). Stretching of p130Cas and exposure/phosphorylation of the Crk-binding site is dependent on integrin binding to an immobilized substrate. In T cells, phosphorylation of CasL allows binding to Crk and the associated GEF C3G, leading to the activation of the small GTPase Rap1. Since Rap1 activation induces the recruitment of talin and the affinity maturation of multiple integrins, it seems likely that CasL functions in a positive feedback loop linking mechanical forces on engaged integrins to additional integrin activation.

Two independent studies have demonstrated that myosin contractility is required for maximal CasL phosphorylation, though in both studies there was significant CasL phosphorylation left at the IS following myosin inhibition (221, 222). Interestingly, phosphorylation of Cas within the substrate domain is largely independent of myosin contractility, but completely dependent on F-actin polymerization (150). It is therefore likely that even in the absence of myosin contractility, continued polymerization-driven F-actin flow at the IS provides sufficient force to drive stretch-dependent Cas/CasL activation.

Cytoskeletal Forces May Create Signaling Rich and Poor Zones at the IS

Different mechanosensitive receptors can apply varying amounts of force on their ligands (223), and the maximum amount of force that can be applied is only as strong as the weakest link in the complex that links ligand to receptor and receptor to the actin network (199). Because of this, the force that allows for the greatest signaling for each receptor could be very different depending on the strength of these varying molecular interactions. In terms of the IS, TCR–pMHC catch-bonds can withstand a maximum force of roughly 10 pN. Unfolding of domain 20 in the CD28-binding protein FLNa occurs with the application of roughly 15 pN (224), while the integrin–ligand bonds are capable of withstanding maximum force of 30 pN (111, 194). Even the linkage of vinculin to the talin rod domains has a maximum force (~25 pN) that can be applied before unbinding occurs, since the talin helices become unstable (225). Since we have shown that the F-actin network slows as it moves toward the center of the IS, peak force is likely to decrease concomitantly [this assumes that force is directly proportional to the rate of F-actin flow, though reality may be more complex (199)]. If so, then signaling from force-resistant molecules would be initiated and sustained in regions of high and moderate F-actin dynamics (i.e., dSMAC and pSMAC regions), while molecular interactions with low force resistance would only occur in areas of moderate F-actin dynamics (i.e., the pSMAC region). This could set up intrinsic areas of maximal signaling for each receptor as microclusters form and traverse the IS. Additionally, since F-actin dynamics are poor or non-existent in the cSMAC, and the cSMAC represents an area of low force generation during the stable phase of IS formation (138), this region should not support force-dependent signaling. In keeping with this model, TCR microclusters retain their phosphorylation in the pSMAC, but become poorly phosphorylated in the cSMAC (77). Thus, the distribution of F-actin-generated forces at the IS may serve both to initiate signaling and to limit ongoing signaling by sweeping microclusters into areas of poor F-actin dynamics. In keeping with this, limiting the centralization of microclusters and maintaining them in the peripheral F-actin-rich compartments of the IS enhance microcluster lifetime and signaling (77). Additionally, for molecules that engage in catch-bond molecular interactions, the bond strength and half-life may also be regulated across the IS radius, with stronger interactions occurring in areas of higher F-actin-generated force, as long as that force is not above the rupture force. To really understand how changing actin rates and actin-generated forces affect T cell signaling differentially across the IS, careful measurements of traction forces on different molecules across the IS radius will be required.

THE CONTRIBUTION OF THE DENDRITIC CELL CYTOSKELETON TO T CELL PRIMING

Although a great deal is known about the mechanisms through which T cell signaling affects actin dynamics on the T cell side of the IS, and *vice versa*, much less is known about the APC side

of the synapse. The APC has long been assumed to be a passive partner in IS organization, and until recently, little attention has been paid to the possible contribution of the APC cytoskeleton. However, recent evidence indicates that at least for DCs, the F-actin network plays a critical role in regulating IS-associated signaling events leading to T cell activation.

DCs Form Barriers to Lateral Diffusion and Control Synaptic Patterns

One area where the DC cytoskeleton is likely to play an active role is in regulating IS formation and structure (226). T cells responding to B cells or stimulatory supported lipid bilayers form the classical mature synapse with a characteristic annular pSMAC and cSMAC pattern (227, 228). This shows that in the absence of barriers to ligand mobility, TCR and LFA-1 microclusters will be driven toward the IS center in a T cell autonomous fashion. The DC/T cell IS lacks this annular pattern and is instead characterized by multiple patches of protein segregation. Even at late time points, there is no central accumulation of CD3 (229). Therefore, it is highly likely that the DC forms barriers to the free diffusion of T cell ligands that are either cytoskeletal or topological in nature. Importantly, these two possibilities are not mutually exclusive and the lateral mobility of some proteins could be regulated through linkage to the DC actin cytoskeleton, while others could be restricted by topological barriers. In either case, the DC actin cytoskeleton would play a crucial role in defining and maintaining these diffusive barriers. The existence of barriers at the DC/T cell IS has important implications for the mechanical regulation of signaling. As detailed below, we found that the T cell actin cytoskeleton activates mechanosensitive molecules, such as integrins, by applying force to the receptor–ligand bond, while barriers to diffusion provided by the DC cytoskeleton provide a retentive counter force on the ligand, thereby increasing tension at the molecular level. Through a similar mechanism, regulation of ligand mobility could prevent microcluster centralization and deactivation, thereby enhancing T cell activation. Just as upregulation of T cell ligands enhances T cell priming by mature DCs, control of molecular mobility would serve as a second mechanism through which DCs could modulate their T cell stimulatory capacity.

The DC Cytoskeleton Plays a Critical Role in T Cell Priming

The DC F-actin network has been observed to polarize toward a cognate T cell in an MHC-dependent manner (230), and treatment of DCs with actin depolymerizing agents impairs the DCs ability to prime T cell responses (231). Polarization of the DC actin network is mediated by Rho-family GTPases and is required for proper conjugate formation, IL-2 production, and T cell proliferation (232–234). Similarly, the ARP2/3 complex activator WASp promotes the maintenance of T cell–DC interactions; WASp-deficient DCs exhibit fewer and shorter-lived contacts with cognate T cells, and a diminished ability to prime T cell proliferation (235, 236). Although it is clear that the F-actin network on the DC side of the synapse plays a key role in T cell conjugate formation and T cell activation, how changes in the DC cytoskeleton are regulated, and their role in IS-mediated signal transduction are open questions in the field.

Maturation-Associated Changes in the DC Actin Network

Following recognition of danger signals through pattern recognition receptors, DCs undergo a maturation process that increases their stimulatory potential as APCs. Maturation is associated with an increase in F-actin content and increased plasma membrane ruffling, as well as major changes in actin-regulatory proteins. The resulting cytoskeletal changes alter antigen uptake and migratory behavior and increase the stimulatory potential of DCs by modifying the F-actin network at the DC–IS. Among the actin-regulatory proteins that are upregulated during maturation are the actin bundling protein fascin, which is greatly increased in expression (237, 238), the actin-severing protein cofilin, which is activated by dephosphorylation (239), and the actin-binding protein moesin, which is increased both by enhanced expression and phosphorylation-dependent activation (240). Interestingly, there is evidence that fascin and cofilin can work together to remodel the F-actin network (241). Moreover, fascin polarizes to the site of T cell engagement on DCs (231), and we have observed that T cells preferentially bind to pre-formed moesin-rich regions (240). It is important to point out that in addition to changes in proteins such as fascin, cofilin, and moesin that directly bind to actin filaments, DC maturation is associated with changes in regulatory molecules that control actin dynamics. For example, the activation of the Rho-family GTPase CDC42 is diminished during DC maturation (242). Similarly, the semaphorin receptor plexin-A1 is upregulated during maturation and is recruited to the DC–IS, where it mediates Rho activation, F-actin polarization, and T cell activation (234, 243). Going forward, it will be important to define how these changes in cytoskeletal proteins and their regulators impact DC–IS function. Likely mechanisms include physical reorganization of the DC membrane and generation of cytoskeletal tethers or corrals that limit the lateral mobility of T cell ligands.

Regulated Mobility of T Cell Ligands on the DC Surface

Consistent with the idea that activation of T cell surface receptors is force dependent, *in vitro* analysis shows that modulation of ligand mobility can influence T cell activation (244). Though comparatively few studies have addressed ligand mobility on the DC surface, there is already evidence that ligand mobility is regulated in ways that are important for T cell priming.

Control of MHC Lateral Mobility

The strongest evidence that ligand mobility is important for TCR triggering comes from studies in which diffusion of an αCD3 antibody in stimulatory bilayers is limited by a physical barrier. Under these conditions, TCR microclusters are trapped in the periphery of the IS, resulting in increased microcluster phosphorylation and

cellular activation (77). Interestingly, limiting the forward mobility of TCR microclusters causes local deformation of the F-actin network (6, 74, 245), suggesting that molecular forces between the TCR and the viscoelastic actin network are increased.

Though MHC class II lateral mobility is not constrained by the F-actin network in B cells or DCs (57, 240), the lateral mobility of MHC class I is restricted by the actin cytoskeleton (246–248). It is worth noting that many of the studies documenting TCR catch-bonds were done using the OTI or 2C CD8 TCR transgenic models, suggesting the possibility that TCR mechanotransduction and control of MHC lateral mobility may be more important in MHCI/TCR than MHCII/TCR interactions. It will be interesting to examine the lateral mobility of MHCI in professional APCs and to ask if the control of MHCI lateral mobility correlates with TCR triggering.

Control of Integrin Ligand Lateral Mobility

As mechanosenstive proteins, integrins respond to the physical properties of their ligand-binding environment. In fact, integrin-mediated cell spreading does not occur unless the ligand can withstand roughly 40 pN of applied force (223). In line with this idea, stiffness of the extracellular matrix correlates with outside in signaling (249), and surface immobilization of ICAM-1 is required for TCR-induced LFA-1 conformational change (193, 250). Importantly, the lateral mobility of ICAM-1 can have dramatic effects on immune cell function. In particular, NK cells adhere firmly to target cells in which ICAM-1 lateral mobility is low, and increasing ICAM-1 mobility decreases the efficiency of conjugate formation and lytic granule polarization (251). This suggests that restriction of the lateral mobility of integrin ligands increases tension on the ICAM-1/LFA-1 bond. In endothelial cells, members of the actinin and ERM family of actin-binding proteins limit the lateral mobility of ICAM-1 through interactions with a concerted polybasic region on the ICAM-1 cytoplasmic tail. Importantly, the constrained lateral mobility of ICAM-1 greatly increases the efficiency of T cell diapedesis, suggesting that this is a critical determining factor for LFA-1 adhesiveness (252, 253). We have recently shown that during DC maturation, ICAM-1 undergoes a specific decrease in lateral mobility mediated by interactions with moesin and α-actinin, which are upregulated and activated in response to inflammatory stimuli (240). This decrease in ICAM-1 lateral mobility enhances conjugate formation and LFA-1 affinity maturation, and ultimately contributes to T cell priming. This evidence indicates that DC maturation is associated with biophysical changes that constrain ligand mobility and promote mechanical integrin activation.

Control of CD80/86 Lateral Mobility

Constrained lateral mobility of the CD28 ligands CD80 and CD86 on the APC surface may also be important for APC function. Consistent with this idea, the cytoplasmic tails of CD80 and CD86 are essential for their costimulatory activity and mediate the separation of CD28 microclusters from TCR microclusters (254). These tails contain a highly conserved poly-basic motif that mediates protein clustering and cytoskeletal interactions (255, 256). This motif resembles known ERM-binding sites in other proteins, including ICAM-1 (253, 257). Given the importance of F-actin linkage and reorganization to CD28 function, it will be interesting to see if DCs can modulate costimulatory signals by regulating the lateral mobility of CD80 and 86.

T Cells and DCs Coordinately Regulate the Activation of LFA-1 at the IS

Although there is increasing evidence that the TCR, CD28, and integrins can be activated by application of external forces on individual receptors, it is critically important to know if the molecular forces generated internally at the IS can drive these same activation pathways. We have recently shown that T cell actin retrograde flow drives conformational change of the integrin LFA-1 into the high affinity form, as well as its accumulation and organization at the IS (193). In a reciprocal process, DCs actively limit ICAM-1 lateral mobility on the plasma membrane through upregulation of the proteins moesin and α-actinin-1 (240). The limitation on ICAM-1 lateral mobility opposes the forces applied by the T cell actin cytoskeleton, thus enhancing tension and promoting LFA-1 affinity maturation, T cell adhesion, and priming. Thus, the F-actin networks in the T cell and the interacting DC work in concert to efficiently activate the integrin LFA-1. Interestingly, coordinated regulation of force application on LFA-1 and constrained ICAM-1/ligand lateral mobility is also at play during T cell migration on and diapedesis through inflamed endothelium (253, 258, 259). The theme that appears is that T cell adhesion to either APCs or endothelial cells, whether that is brought on by antigen recognition or exposure to chemokine, involves both a concerted change in the T cell and a reciprocal change in the interacting partner. Thus, for mechanosensitive molecules involved in T cell function, the physical properties of the ligand on the surface of interacting cells must be considered along with the forces applied to the receptor itself.

FUTURE DIRECTIONS FOR BASIC AND TRANSLATIONAL RESEARCH

Our understanding of the mechanobiology associated with signaling events at the IS is in its infancy. In particular, we lack concrete information on how the relevant forces are generated at the IS, which molecules act as true mechanosensors, and how the function of these molecules is coordinated to tune the immune response. Pioneering studies using biophysical approaches, such as modulation of substrate stiffness, physical manipulation using micropipettes, and traction force measurements, have all been informative (81, 138, 260), and there is much work yet to do in this arena.

To relate these biophysical measurements to T cell signaling, we also need to develop additional probes for protein conformation and activation state. For example, antibodies specific for integrin activation intermediates have been quite valuable (175, 193), as have antibodies that detect stretching of Cas (150, 222). Conformational probes for integrins and Cas family members have been successful because these proteins undergo large scale, concerted, changes as part of their

regulated function. Given the paucity of robust conformational changes that are detectible in the ectodomains of the TCR, it is unsurprising that similar reagents do not exist for the study of TCR mechanosensing. One conformation-specific antibody has been reported for the TCR, though this epitope is exposed upon pMHC binding even in force-free conditions (131). Building upon work showing that agonist mAbs bind to the membrane-distal CD3ϵ lobe and exert torque on the CD3 complex (136), it may be possible to ask if cytoskeletal forces on TCRs engaged to pMHC have a similar effect by measuring changes in binding of anti-CD3ϵ Fabs in the presence or absence of F-actin dynamics. In any case, it seems likely that analysis of TCR force sensing will continue to be challenging, and that novel tools that detect changes transmitted across the cell membrane will be needed.

One caveat to the use of conformation-specific antibodies is that they often induce or stabilize the conformations they detect. This considerably limits their use, especially in live cell experiments. Thus, future studies are likely to require fluorescent tension biosensors for live cell microscopy similar to those that have been used to detect force on vinculin (261). This technology is evolving rapidly (262, 263). FRET-based biosensors have already been used to detect activation of VLA-4 in migrating T cells (263), and similar biosensors have been used to create integrin ligands that register force applied by spreading cells (264, 265). Another ligand-based approach involves a tension gauge tether consisting of a piece of double stranded DNA attached to an integrin ligand on one strand and to the substratum on the other such that the DNA strands separate with applied force, which can be varied over a range of 12–56 pN (223). Related ligand-based force sensors could be extremely useful in measuring the forces applied to specific receptor–ligand pairs, and the effects of cytoskeletal perturbations on these forces.

As a closing note, it is worth considering how new knowledge about the mechanobiology of T cell signaling could influence immunotherapy. Recent clinical successes have generated great interest in T cell-based immunotherapies for cancer and infection (266, 267). Although many approaches exist for adoptive T cell therapy, a common first step involves the *ex vivo* expansion of T cell subsets, typically through the use of stimulatory beads. Much thought has gone into optimizing the antibody cocktails used for bead coating, but relatively little attention has been paid to their physical properties. Yet, these properties are likely to be highly significant. For example, most stimulatory magnetic beads commercially available for T cell activation are ~4 μm in diameter; yet, human peripheral blood T cells engaging APCs readily spread to a diameter of 12 μm and resting blasts can spread to over 20 μm. A bead that is not large enough to induce robust spreading is not likely to induce the symmetric actin flow associated with mechanical signaling. Thus, beads with a larger surface area may enhance T cell activation and expansion. Another key parameter is the rigidity of the stimulatory surface. Human T cells reportedly respond better on softer surfaces, with increased IL-2 secretion, proliferation, and effector function increasing upon decreased cell surface rigidity (81). Interestingly, the substrate rigidity range used in this study was 100–1000 kPa, several orders of magnitude above physiological values for soft lymphoid tissues. Further research aimed at optimizing surface area and rigidity may prove valuable in creating artificial APCs for clinical applications.

AUTHOR CONTRIBUTIONS

WC and JB reviewed and interpreted the literature, drafted and revised the manuscript, and approved the final version for publication. They both agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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REVIEW
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Biophysical Aspects of T Lymphocyte Activation at the Immune Synapse

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T lymphocyte activation is a pivotal step of the adaptive immune response. It requires the recognition by T-cell receptors (TCR) of peptides presented in the context of major histocompatibility complex molecules (pMHC) present at the surface of antigen-presenting cells (APCs). T lymphocyte activation also involves engagement of costimulatory receptors and adhesion molecules recognizing ligands on the APC. Integration of these different signals requires the formation of a specialized dynamic structure: the immune synapse. While the biochemical and molecular aspects of this cell–cell communication have been extensively studied, its mechanical features have only recently been addressed. Yet, the immune synapse is also the place of exchange of mechanical signals. Receptors engaged on the T lymphocyte surface are submitted to many tensile and traction forces. These forces are generated by various phenomena: membrane undulation/protrusion/retraction, cell mobility or spreading, and dynamic remodeling of the actomyosin cytoskeleton inside the T lymphocyte. Moreover, the TCR can both induce force development, following triggering, and sense and convert forces into biochemical signals, as a *bona fide* mechanotransducer. Other costimulatory molecules, such as LFA-1, engaged during immune synapse formation, also display these features. Moreover, T lymphocytes themselves are mechanosensitive, since substrate stiffness can modulate their response. In this review, we will summarize recent studies from a biophysical perspective to explain how mechanical cues can affect T lymphocyte activation. We will particularly discuss how forces are generated during immune synapse formation; how these forces affect various aspects of T lymphocyte biology; and what are the key features of T lymphocyte response to stiffness.

Keywords: T lymphocytes, immune synapse, force control, TCR, LFA-1, biomechanics, stiffness

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INTRODUCTION

T lymphocytes are motile small cells, which play a key role in adaptive immune responses against pathogens and tumor cells. T lymphocyte activation is triggered by the recognition *via* the T-cell receptor (TCR) expressed at the surface of T lymphocytes, of antigenic peptides, derived from pathogens or tumors and associated with major histocompatibility complex (MHC) molecules exposed at the surface of antigen-presenting cells (APCs). Numerous costimulatory or co-inhibitory receptor/ligand pairs present at the plasma membrane of both cells can also modulate T lymphocyte activation (1). Thus, T lymphocyte activation is crucially dependent on the close interaction between both plasma membranes. This interaction is organized in time and space by the formation of structures,

termed immune synapses, in which molecules are unevenly distributed and segregated while remaining mobile (2–4).

Thanks to increasingly sophisticated visualization techniques, more and more information is accumulated on the organization of both plasma membrane receptors and signaling molecules at the immune synapses. Visualization of T lymphocyte interactions with APCs showed that these cellular partners were submitted to pulling, pushing, and shearing forces due to cell motility relative to each other (5); continuous spontaneous motion of plasma membrane (6); and cytoskeletal remodeling (7–9). A specific function of mechanical forces in T lymphocyte activation was even proposed in the first study showing the dynamic formation of immune synapse (10). Forces exerted by T lymphocytes during these contacts have only been quantified recently (11–13). The TCR itself was shown to be a mechanosensor, i.e., able to convert the mechanical forces exerted during TCR binding to peptide–MHC complexes into a biochemical signal (14–16). Finally, at the resting T lymphocyte membrane, there are organized complexes of receptors and signaling molecules, maintained in a state of basal activity where the membrane receptors are readily available to interact with their ligands on an APC surface and to induce a signaling cascade. This dynamic organization resembles a buffer condition that is able to respond to a minute amount of agonist pMHC in a sea of endogenous pMHCs and is optimized not only for the identification of antigen but also for the initiation and amplification of signals following successful antigen recognition (17–19).

Although formation of the immune synapse has been extensively studied, information on the mechanical properties of the microenvironment and on how these properties affect T lymphocyte functions has only recently become available. We will thus review herein recent advances on the knowledge of how T lymphocytes generate or respond to forces during antigen recognition and immune synapse formation.

FORCES IN T CELLS

When interacting with an APC, T lymphocyte morphology changes drastically: the cell moves on the APC surface, develops invadosome-like structures which push into the cortex of the APC (20–22), spreads on the APC, and eventually stops. During each of these steps, T lymphocytes exert and/or are submitted to forces, which can affect receptor/ligand bonds. We will discuss the pathways involved in the generation of these forces.

Spontaneous Membrane Oscillations and Formation of Protrusions

Lymphocytes, such as other cell types, display membrane undulations with amplitude of several tens of nanometer and frequency ranging between 0.2 and 30 Hz (23). Moreover, when interacting with a surface, T lymphocytes rapidly develop protrusions and retractions that are organized in lateral waves along the cell membrane (24). Filopodia or microvilli are protrusive structures with a length between 0.1 and several micrometers that display receptors at their tips and present cycles of protrusions/retractions, which allow them to sense both the mechanical and biochemical environments (25). In order to grow, filopodia have to develop protrusion forces against the membrane that are mainly produced by actin polymerization at the filopodial tip (26–28). Filopodial diameter is in the same range as the diameter of membrane tubes, the generation of which requires forces ranging from 5 to 30 pN (29). Filopodia not only exert protrusive/pushing forces but also retracting/pulling ones, which have been measured using traction force microscopy, i.e., by recording the local deformation of a soft substrate of known stiffness in which fluorescent beads are embedded (29, 30). In neuron cells, the pulling forces developed by filopodia have been shown to be in the order of 1 nN (31). During their migration on endothelial cells, T cells can form F-actin-based protrusions, termed invadosome-like protrusions (ILPs) (32). These structures are small (diameter of ~0.2 μm) and transient (half-life of ~20 s) and physically push against the endothelial cell surface (20, 33). It has been postulated that ILPs can sense the stiffness of endothelial cells by "tiptoing" their surface (32, 34, 35). More recently, Yang et al. (36) described the forces developed by chemotactic T lymphocytes. A laser trap was used to position two beads, one as source of chemokine gradient and the other to measure the forces exerted by the migrating T lymphocytes (in their case, the Jurkat leukemic T cell line). The protrusion forces measured at the leading edge of Jurkat cells migrating in a gradient of SDF-1 were as high as 1000 pN and increased in parallel to the chemoattractant gradient. Moreover, the forces required to stop the migrating cells ranged from 100 to 300 pN. Finally, tensile forces may also be present at the membrane when short molecule bridges at the T lymphocyte/APC interface (i.e., TCR/pMHC or CD2/CD58 pairs) exclude other larger molecules (LFA-1/ICAM-1 pairs or CD45) (37). These results (see **Figure 1** for summary) demonstrate that spontaneous undulations of the T lymphocyte membrane and formation/retraction of filopodia and other cellular protrusions can generate forces that facilitate probing of the biomechanical microenvironment (38). Meanwhile, receptor/ligand bonds are submitted to a wide range of forces during cell migration. This will result in the modulation of signaling cascades induced by mechanosensitive molecules.

TCR Engagement Induces Force Generation

Despite the increasing knowledge of signaling pathways engaged after recognition of pMHC by the TCR, the triggering mechanism of the signaling cascade still remains controversial (39). Several mechanisms have been proposed, which involve aggregation, conformational changes, and segregation (40). This matter has been reviewed extensively and will not be addressed further. Yet, studies aiming to investigate if and how receptor engagement generates forces that might then be converted in biochemical signal are sparse.

In one study, Hosseini et al. used atomic force microscopy (AFM) to measure the adhesion forces between a T cell hybridoma and a B cell line used as APC (41). Results showed that in the presence of antigen, adhesion forces built up with time of conjugate formation, starting from 1 to 2 nN at the beginning of the interaction to 14 nN after 30 min. The adhesion forces were mainly due

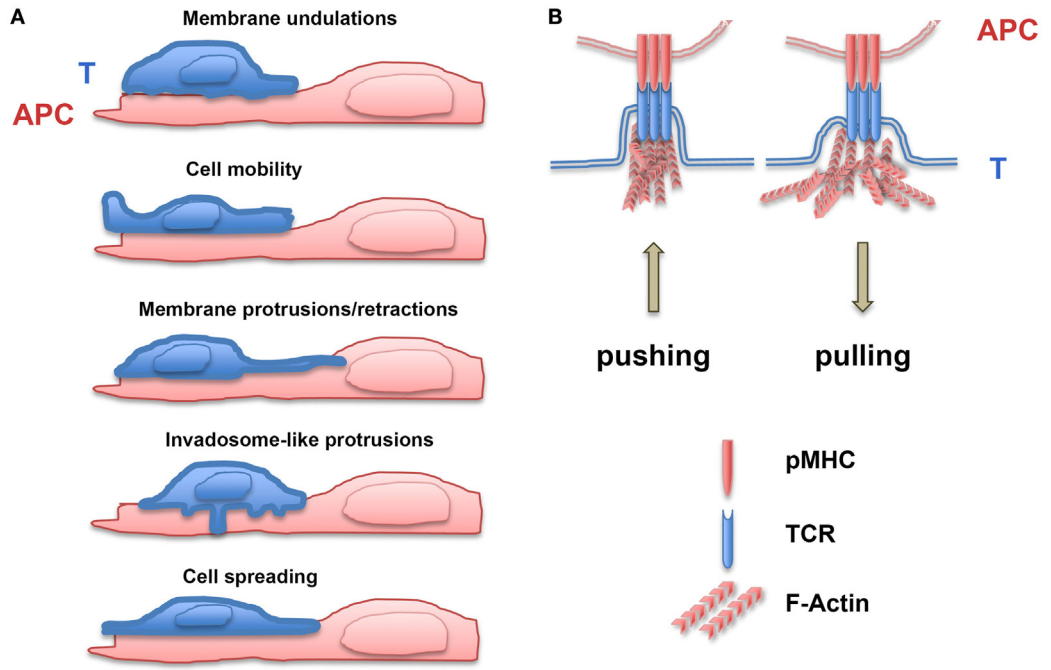

FIGURE 1 | Generation of forces during T lymphocyte/APC contacts. **(A)** Forces are exerted on receptor/ligand bonds by membrane T lymphocyte undulations, cell mobility, membrane protrusions/retractions, invadosome-like protrusions, and cell spreading on antigen-presenting cells (APC). **(B)** Upon TCR triggering, T lymphocytes develop pushing and pulling forces on TCR/pMHC bonds, which depend on polymerization of F-actin.

to lymphocyte function-associated antigen-1 (LFA-1)-mediated adhesion, since the integrin inhibitor BIRT377 almost completely abolished forces in the conjugates (41). Similar experiments were performed on conjugates formed between mouse primary T lymphocytes expressing the OT1 transgenic TCR and a mouse dendritic cell line presenting OVA peptides of different affinities (42). In this experimental model, adhesion forces between cellular partners were smaller (up to 1.5 nN) and correlated to the ability of the different peptides to activate the T lymphocytes; better agonist peptides induced stronger adhesion forces (42).

Even though the above studies provided values for interaction forces between T lymphocytes and APCs, they could neither address the question of the relative contribution of different molecules to the forces measured nor the question of the contribution of each cell partner in force generation. Therefore, we adapted the biomembrane force probe (BFP) technique, which was developed to probe molecular adhesion (43), in order to assay the generation of forces by T lymphocytes. The BFP consisted of a red blood cell (RBC), which was on one side coupled to a bead coated with antibodies and held on the other side by a pipette. A human primary $CD4^+$ T lymphocyte held by a second pipette was brought into contact with the BFP. Activation of the T lymphocyte was monitored by imaging increases in the intracellular Ca^{2+} concentration, $[Ca^{2+}]i$, and forces exerted by the T lymphocyte on the BFP were measured on time lapse stacks of images by determining the elongation of the RBC with respect to the position of the fixed micropipette (11). When the bead was coated with anti-CD3 antibodies, three consecutive phases were observed following T lymphocyte contact with the BFP: a latency phase, which lasted less than a minute during which no force and no $[Ca^{2+}]i$ increase were observed; a pushing phase consisting of the growth of a directional cell protrusion characterized by an initial axial compression of the RBC and a peak in $[Ca^{2+}]i$ increase; and, in most cases, a pulling phase characterized by protrusion/retraction and generation of pulling forces, as witnessed by the elongation of the BFP. The initial forces exerted by T lymphocytes on the RBC were around 25 pN for a probe stiffness of 50 pN/μm. Measurement of elongations showed that CD3 engagement on T lymphocytes triggered a constant pulling loading rate of ~2 pN/s. These characteristic three phases were not observed when the bead was coated with anti-MHC-I antibodies, showing that the mere binding of the bead to the T lymphocyte membrane is not sufficient to induce forces (11). Engagement of LFA-1 together with CD3 modified the forces exerted compared to CD3 alone: when the bead coupled to the BFP was coated with an anti-CD18 antibody (specific against the β_2 chain of LFA-1), a clear decrease in growth velocity and protrusion length during the pushing phase was observed. Moreover, the pulling phase started earlier and the protrusion morphology was changed from a "tube-like" to a "cup-like" structure resembling the phagocytic cup. Engagement of LFA-1 alone on resting primary T lymphocytes did not generate any pushing phase. It also generated 100-fold lower pulling loading rates (0.2 pN/s for a probe stiffness of 50 pN/μm) than the pulling loading rates induced by CD3 engagement alone (25 pN/s for the same probe stiffness). Absence of force generation in response to just LFA-1 triggering can be attributed to the fact

that T lymphocytes were not pretreated for inside-out signaling induction (i.e., pretreatment with chemokines, anti-CD3, or phorbol-ester). Thus, integrins can generate traction forces and modify the forces induced upon CD3/TCR triggering. Indeed, force measurements performed on human neutrophils submitted to chemotactic gradients on hydrogel substrates revealed that neutrophils also generated traction forces, which were dependent on β_2 integrin engagement and signaling (44). This was not specific to LFA-1 engagement since binding of $\alpha_5\beta_1$ integrins to fibronectin and activation of these integrins by addition of Mn^{2+} were also been shown to induce traction forces (45).

Two more studies confirmed that T lymphocytes generate significant forces upon CD3 engagement. In the first one, Bashour et al. used elastomer pillar arrays of known spring constant coated with activating antibodies (12). In this experimental setting, each pillar tip deflection caused by cell attachment and spreading is monitored using live cell videomicroscopy (46, 47). Human primary $CD4^+$ T lymphocytes were put on micropillars coated with anti-CD3 antibodies and several phases were observed (12). In the first phase, cell spreading generated only minor forces. After this phase, cells ceased to spread and started to exert significant traction forces, which were essentially centripetal and exerted mostly at the cell periphery. The forces generated per pillar were around 50 pN. In the same study, forces exerted by mouse primary $CD4^+$ T lymphocytes on the same pillars were fourfold higher (200 pN/pillar). No forces were measured when pillars were coated with an antibody against the costimulatory molecule CD28 alone. However, the dual presence of anti-CD3 and anti-CD28 antibodies resulted in doubling the traction forces exerted by T lymphocytes on the micropillars. This was observed when the anti-CD28 Ab was present on the pillar together with anti-CD3 or when added in solution (12). These results suggest that the traction forces induced by CD28 engagement are not directly generated through the CD28 receptor. They are rather due to signaling-dependent amplification of the forces triggered by TCR engagement.

In another study, Hui et al. used traction force microscopy to measure the forces exerted by Jurkat cells during TCR activation (13). Jurkat cells were put on polyacrylamide gels coated with anti-CD3 antibodies and embedded with fluorescent beads at the top surface. The traction forces exerted by the cells were measured by tracking fluorescent bead displacement. In the presence of anti-CD3 and for a substrate stiffness rigidity of 1–2 kPa, traction forces were in the order of 2 nN, whereas forces exerted on substrates coated with a non-activating antibody were below 1 nN (13).

From the above results, it is evident that TCR–CD3 engagement can generate forces in T lymphocytes. These forces can be modified by the engagement of costimulators, such as LFA-1 and CD28. We will now discuss the potential outcomes of forces on T cell activation.

EFFECT OF FORCES ON T CELL ACTIVATION

We have seen in the previous paragraphs that membrane undulations, protrusions and retractions, cell migration, and TCR triggering can generate forces that can be exerted on receptor/ligand bonds. In the next section, we will discuss the effect of these forces on specific receptor/ligand pairs at the T lymphocyte/APC interface, i.e., TCR/pMHC and LFA-1/intercellular adhesion molecule-1 (ICAM-1), and on overall T lymphocyte activation.

Forces Exerted on TCR/pMHC Bonds

T lymphocytes typically recognize peptides of 8–11 amino acids presented by MHC molecules. The TCR can "sense" a single amino acid substitution and translate it in a different functional response. Moreover, T lymphocytes can precisely discriminate a small number (2–10) of pMHC complexes for which they are specific within a sea of self or foreign peptide-MHC molecules (17–19). How this exquisite specificity and sensitivity is achieved is still a matter of investigation. Forces exerted on the TCR–pMHC bonds may have a key role in these processes. Indeed, it has been shown that the TCR functions as a mechanosensor, i.e., it can convert mechanical cues into biochemical signals (16). The first direct evidence was obtained by E. Reinherz's group, who used optically trapped beads coated with non-activating anti-CD3ϵ antibodies or pMHC to apply forces on the TCR and monitored T lymphocyte activation by measuring $[Ca^{2+}]i$ increase (14). They showed that T cells were triggered mechanically, since application of a tangential force (50 pN) to the coated bead induced calcium signaling. Force application on beads coated with pMHC complexes that did not bind TCR had no effect on calcium flux. In another study, Li et al. used a fibroblast cell line expressing a single chain Fv anti-CD3ϵ antibody elongated by a tether (15). Binding to CD3 did not induce calcium signaling unless a mild perpendicular shear stress or a normal pulling force on the T lymphocyte bound to the surrogate APC was applied (15). By contrast, pulling forces applied on CD28 or CD62L did not increase intracellular calcium levels. These studies demonstrated that the TCR could transform a mechanical signal (force) into a biochemical one ($[Ca^{2+}]i$ increase). Yet, several questions still remain unresolved and particularly whether forces applied on TCR/pMHC bonds can affect T cell antigen recognition and discrimination. Work from the group of C. Zhu elegantly demonstrated that mechanical forces applied using a BFP on TCR/pMHC-I (48) and TCR/pMHC-II (49, 50) affected dissociation kinetics in a peptide-specific way. Forces applied to the bonds prolonged the lifetimes of single TCR–pMHC bonds for agonists (catch bonds) but shortened those for antagonists (slip bonds). When forces of 10 pN were applied by BFP on OT1 TCR/pMHC bonds, the ratio of OT1 TCR–pMHC bond lifetimes for the agonist peptide versus a weaker altered peptide grew 57-fold compared to when no force was applied (49, 50), demonstrating that forces can increase the power of antigen discrimination. The functional outcome of different peptides recognized by the same TCR was also shown to be coupled with the cumulative lifetime of the TCR–pMHC bonds (49).

A TCR deformation model where mechanical stress could induce conformational changes that would unmask sites of phosphorylation and allow TCR signaling was also proposed (51). Application of forces on the TCR would expose the immunoreceptor tyrosine-based activation motifs present in the CD3ϵ and ζ chains, otherwise buried into the hydrophobic core of the

membrane lipid bilayer (52, 53). More recently, it was proposed that the structural features of TCR–CD3 complexes are adapted to permit sensing and discrimination of the forces to which TCR/pMHC bonds are submitted (54). Das et al. used optical tweezers and DNA tether spacer technology, which allow for application of forces in the order of piconewton with a spatial precision of nanometer, in order to address the mechanisms involved in the control of strength and lifetime of the TCR–pMHC-I bonds (55). They confirmed that forces applied on TCR–pMHC-I bonds increased the lifetime of the bond and showed that the state of the CβFG loop region, a 12-amino acid peptide present in the constant region of the β chain of the TCR of all mammalian αβTCRs (56), is involved in the increased lifetime of TCR–pMHC-I bonds submitted to tensile forces (55). This study suggests that forces physically modify the αβTCR by switching it from an "extended form" that binds weakly to a "compact form" that binds more robustly. The conformational changes of the TCR would then be transmitted to the CD3 signaling complexes associated with the TCR through mechanisms that have yet to be discovered. Finally, a recent study showed that the juxtamembrane region of ζ–ζ homodimers are divaricated within the TCR–CD3ζ complexes and that TCR engagement drives the intra-complex juxtaposition of the ζ–ζ juxtamembrane regions (57). This mechanical switch might thus couple TCR engagement with CD3ζ-dependent signaling.

Forces Exerted on Integrin/Ligand Bonds

Integrins are heterodimeric transmembrane proteins that mediate interactions in-between cells and interactions between cells and the extracellular matrix. Avidity of integrins is regulated by changing their valency, i.e., by changing their density at the cell/cell interface and/or changing their affinity for ligands (58). The LFA-1 integrin plays an essential role for T lymphocyte trafficking, immune synapse formation, and T lymphocyte activation (59). In resting T lymphocytes, LFA-1 is in an inactive bent conformation state, which binds with low avidity to its ligand ICAM-1. TCR stimulation induces a change in LFA-1 conformation, resulting in a more extended conformation of the integrin with an intermediate affinity (60). Finally, binding of LFA-1 to its ligand modifies further its conformation with further increase in its affinity (61, 62). Forces have been shown to play a role in affinity maturation of integrins. Indeed, application of tensile forces on integrin/ligand bonds increases bond strength and longevity (63). This was also reported for LFA-1/ICAM-1 interactions, indicating that, as for TCR/pMHC, these molecules form catch bonds (64). Moreover, it has been shown that the integrin bonds "remember" the history of the forces they have been submitted to. This phenomenon was called "cyclic mechanical reinforcement," as the bond strength accumulates over repeated cycles of forces and is maintained after force removal (65). For instance, fibronectin/$\alpha_5\beta_1$ integrin bonds dissociate within 1 s at a force of 5 pN, while upon cyclic mechanical reinforcement, the bond lifetimes can be extended to 14 s. Similar mechanisms apply to LFA-1/ICAM-1-specific bonds (65). Although head rearrangements of integrins are induced by ligand binding, this might take seconds to happen in the absence of force (66). Application of forces on the bonds would thus shorten the time required for conformational change. Moreover, cyclic mechanical reinforcement would strengthen the bonds by easing and accumulating the reversible conformational change of integrins with multiple force cycles. Therefore, during immune synapse formation, dynamic cyclic traction forces are exerted on LFA-1/ICAM-1 bonds by cycles of membrane undulations, protrusions, and retractions or by direct LFA-1 engagement, since, as described above, this can lead to force generation in T lymphocytes. By inducing conformational changes of integrins, forces during immune synapse formation can facilitate adhesion between T lymphocytes and APCs and probably participate to the costimulatory activity of LFA-1, although this remains to be tested.

THE ACTOMYOSIN CYTOSKELETON: A FORCE GENERATOR AT THE IMMUNE SYNAPSE

Forces experienced by T lymphocytes during synapse formation can come from the exterior but can also come from the interior generated by the cell's own cytoskeleton. Many reviews have described and discussed remodeling of the cytoskeleton at the immune synapse and its potential role. We will herein concentrate on the role of the actomyosin cytoskeleton on the generation of forces. In the first dynamic study of immune synapse formation on artificial lipid bilayer, Grakoui et al. proposed a model of synapse formation in three stages (10): in the first stage, LFA-1 binding in the center of nascent synapse would provide "a fulcrum for cytoskeletal protrusive mechanisms that force an outermost ring of T cell membrane into close apposition with the substrate"; in the second stage, the transport of TCR–pMHC pairs to the center of the synapse would be actin driven; and in the last stage, the forces exerted would equilibrate, leading to stabilization (10). This model already proposed that forces generated by the T lymphocyte cytoskeleton would play a key role in immune synapse formation. It is remarkable to note that this model fitted so well to the experimental data obtained later on. Actin cytoskeleton has long been known to control T lymphocyte activation at different levels, such as adhesion to APC, early signaling through the TCR, and release of cytolytic granules or cytokines (67–71). T lymphocytes, when activated by the TCR–CD3, spread rapidly (in 2–4 min) on the activating substrate or cell they interact with, they stabilize (for 15–20 min), and then retract (10, 21, 72–74). These phases are reminiscent of the phases observed when adherent cells spread on their substrate (75). Indeed, the different zones of the immune synapse or supramolecular activation clusters (SMACs) have been compared to the lamellipodium (for the distal SMAC), the adhesive lamella (for the peripheral SMAC), and the uropod (for the non-adhesive central SMAC) of a mobile adherent cell (76). During synapse formation, microclusters of receptors form in the periphery and then move toward the center of the synapse (77). LFA-1 clusters stop in the pSMAC lamella zone, whereas TCR microclusters follow their path toward the cSMAC where they are endocytosed (78, 79) or secreted (80).

In the context of spreading described earlier, the centripetal movement of receptor clusters has been proposed to be driven by a combination of pushing forces originating from actin retrograde flow in the lamellipodium and pulling forces generated in the lamella by myosin-based contraction. Indeed, the inward flow of cortical F-actin at the immune synapse has been shown to be the major driving force behind microcluster movement (67, 81–84). The role of myosin II-based contractions at the lamella in microcluster movement, although more controversial (85), has also been shown to control the centripetal movement of both TCR and LFA-1 microclusters (86–88). One can speculate that the resistance of TCR, LFA-1, and other receptors to this mobilization would generate traction forces on the receptor/ligand bonds. Thus, coupling of receptors with the actin cytoskeleton together with mobility of the ligands at the membrane of the APC would be key elements in force generation on receptors. Adaptor molecules, such as talin, mediate interaction of LFA-1 with the actin cytoskeleton (89). The generation of localized traction forces by actin retrograde flow has indeed been shown to regulate adhesion (90, 91) in many cell types, including T lymphocytes forming immune synapses (92). In contrast, coupling of TCR to the actin cytoskeleton remains elusive. Yet, interactions of TCR clusters with actin have been revealed in experiments that introduced selective barriers, which altered TCR microcluster transport to the central SMAC (82, 93). Association of signalosomes with tyrosine-phosphorylated CD3 complexes may contribute to dynamic coupling of TCR–CD3 complexes with actin flow. The mobility of ligands on the surface of APC is another parameter to take into account into the generation of forces on receptor/ligand bonds (92, 94, 95) (**Figure 2** and see later discussion in Section "T Lymphocytes Interact with Cells That Have Different Mechanical Properties"). More studies and modeling analysis are required to address these specific aspects.

EFFECT OF SUBSTRATE STIFFNESS ON FORCE DEVELOPMENT AND T CELL ACTIVATION

The mechanical behavior of solid materials, such as plastic and glass, can be described as purely elastic. This means that their stiffness can be expressed as the ratio of the applied stress and the resulting deformation, which is termed elastic (or Young's) modulus. On the other hand, cells and tissues display viscous properties in addition to their elastic ones and are, hence, viscoelastic materials. Two components can describe the mechanical properties of viscoelastic materials, one elastic and the other viscous, referred to as storage and loss moduli. In viscoelastic materials, the duration of force/stress application is also important, resulting in time-dependent deformations. Storage and loss moduli are different from the elastic or Young's modulus that is more often reported in literature, since calculation of the latter is not taking into account the duration of force application. Henceforth, we refer to elastic modulus as a measure of stiffness, unless otherwise mentioned.

The effects of substrate stiffness can be as diverse as growth, differentiation, migration, and survival (96–99). Particularly, it was demonstrated that cells display differential spreading (100), velocities (101), traction forces (102), and physiological behavior (103) in response to variations in stiffness. In a seminal study (104), Discher and co-workers showed that stem cell fate could be influenced just by the stiffness of culture substrates. Another

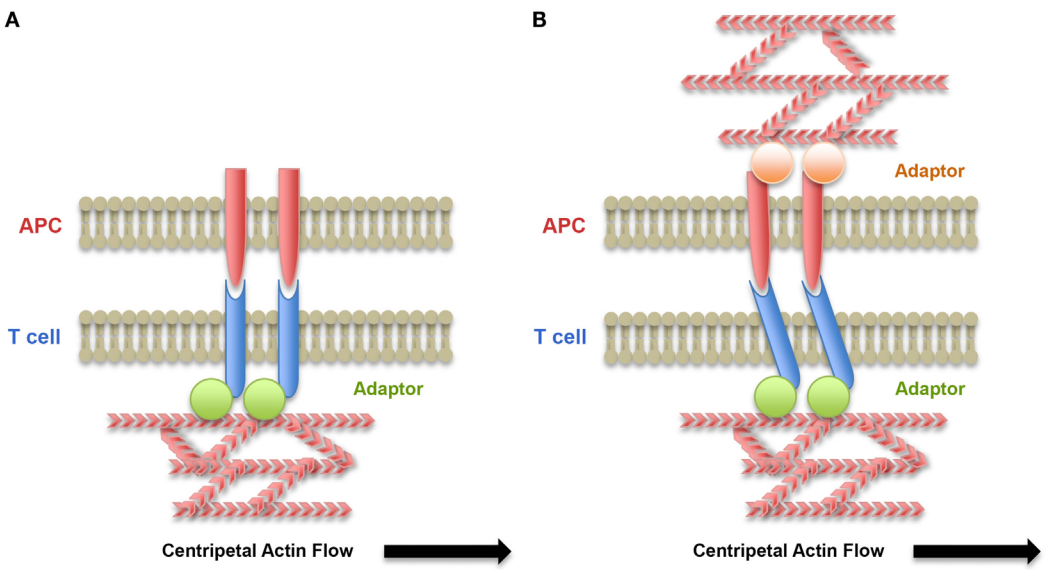

FIGURE 2 | Role of the cytoskeleton of the T lymphocyte and the APC on force exertion on receptor/ligand bonds. (A) Centripetal flow of actin exerts forces on receptor/ligand bonds when receptors are coupled by an adaptor to the cytoskeleton. These forces may lead to conformational changes of the receptors and signaling. **(B)** When ligands are associated with the APC cytoskeleton, forces on receptor/ligand bonds are submitted to resistance due to reduced mobility of the ligands on the APC surface and the forces exerted on bonds are increased.

important observation was that cells match their stiffness to that of the environment by regulating their actin cytoskeleton (105). Moreover, several cell types have been reported to display durotaxis, i.e., migration from soft toward stiff substrates (101, 106).

In this section, we will report on recent studies that have begun to shed light on the mechanical properties of T lymphocyte environment and on T cell responses to these properties.

T Lymphocytes Interact with Cells That Have Different Mechanical Properties

T lymphocytes are mobile cells that are exposed to different chemical and mechanical environments. Inside lymph nodes, T lymphocytes interact transiently with a number of different APCs, each of them potentially activated by different stimuli and presenting a varying repertoire of agonist/non-agonist peptides on MHC molecules (5, 107). In blood vessels, T lymphocytes interact with endothelial cells, and inside tissues, effector T lymphocytes interact with their targets, i.e., infected or tumor cells. Much of the studies on immune synapse formation and T cell activation have been performed on plastic or glass surfaces or on planar lipid bilayers supported on glass. Even though these surfaces provide an ideal substrate to follow receptor/ligand interactions and rearrangements, they are flat and rigid with no topological variation. Moreover, plastic and glass display stiffness in the ranges of gigapascal. In contrast, cells in the body generally display stiffness in the range of 50 Pa–40 kPa (97) with primary human T lymphocytes and Jurkat cells being at the soft end of this range (108, 109) with their stiffness ranging from 50 to 90 Pa. Therefore, in order to really study the effect of mechanical properties on T lymphocyte biology, it is vital to know the mechanical landscape that the cells encounter *in vivo* and use substrates with stiffness values inside this physiological range. Using a single-cell rheometer (110), we recently showed that different human myeloid APCs have different viscoelastic properties and that their Young's modulus values vary from 500 Pa for monocytes and DCs to 900 Pa for macrophages (109). Moreover, inflammatory conditions modified the viscoelastic properties of myeloid cells, which were halved or doubled when cells were treated with a TNFα/PGE_2 cocktail or IFNγ, respectively (109). These results suggest that viscoelastic properties of myeloid cells are additional parameters of inflammation that can be integrated with biochemical factors to generate an adapted T lymphocyte response. Other studies have also reported variations in myeloid cell mechanical properties (111–113). Finally, it is worth noting that endothelial cells have also been shown to change their viscoelastic properties in response to inflammation (35), suggesting that this might be a more general process.

In our study, the viscoelastic properties of human myeloid cells were dependent on myosin IIA activity and correlated to the F-actin content in each type of cells (109). These results suggest that the actomyosin cytoskeleton of myeloid cells is responsible for their mechanical properties. Interestingly, older reports have shown that DC cytoskeleton was indispensable for priming of T cells since following DC treatment with actin depolymerizing drugs, naïve $CD4^+$ T cells were unable to proliferate (114). DCs were shown to polarize their cytoskeleton toward the immune synapse only upon successful antigen recognition by the T cell, and this was critical for TCR triggering and IL-2 production (115, 116). Maturation of DCs has also been associated with remodeling of their cytoskeleton, leading to development of projections directed toward T lymphocytes to optimize cell/cell interactions (117, 118). More recently, it has been shown that the cortical actin network of DCs regulated ICAM-1 lateral mobility at the cell surface and that DC maturation regulated mobility and clustering of ICAM-1 (95). The constrained ICAM-1 mobility associated with DC maturation was shown to promote formation of T lymphocyte/DC conjugates as well as T lymphocyte proliferation. On the T lymphocyte side, it was shown that LFA-1 affinity maturation correlated to ICAM-1 lateral mobility on the DC surface, i.e., low mobility of ICAM-1 induced high-affinity conformational changes of LFA-1 (95). The same group showed that actin flow in T lymphocytes was indispensable to maintain LFA-1 in the high-affinity conformation at the immune synapse and that ICAM-1 mobility directly affected distribution of high-affinity LFA-1 on the surface of engaged T lymphocytes (92). These results suggest a model in which ICAM-1 mobility on APC surface modulates resistance to tensile forces applied by the T lymphocyte actin cytoskeleton on LFA-1/ICAM-1 bonds, highlighting the role of mechanotransduction in cell conjugate formation and T lymphocyte activation (**Figure 2**). These mechanisms may apply to other receptor/ligand pairs, such as CD28/CD80-CD86, which are also coupled to the actin cytoskeleton (119–122). They may also apply to other cell types, such as endothelial cells. Therefore, these studies show that APCs can contribute both biochemical and mechanical cues to T cell activation. Overall, the above findings are (1) highlighting the requirements for APC/T lymphocyte crosstalk (123) for immune synapse formation and T lymphocyte activation and the need for more studies focusing on the mechanical properties of both sides of the immune synapse and (2) stressing the importance for T lymphocytes to sense the mechanical and topological properties of their environment in order to locate a specific target and respond.

It is worth noting that the mechanical properties of tissues and organs can be also modified in normal and pathological conditions. For example, it was recently shown that the contractility of fibroblastic reticular cells is regulated upon inflammation by the expression of CLEC-2 on mature dendritic cells (124, 125). CLEC-2, by interacting with podoplanin expressed on fibroblastic reticular cells, induces the relaxation of these cells that leads to a decrease of the lymph node stiffness that is probably important for its expansion (125). Moreover, tumor mechanics, and in particular the rigidity of tumoral tissues, has been shown to play a role in tumor development (126). These changes in mechanical properties of tissues and organs might also affect overall T lymphocyte activity.

Finally, it is possible that viscoelastic properties of T lymphocytes themselves are also modified by activation. The strength of TCR signaling may induce changes in T lymphocyte stiffness, which in turn may affect their interactions with APC and target cells as well as their migratory properties. Along this line, it is worth noting that T lymphocytes can adopt two types of migratory behavior (5, 127). Strong TCR stimulation can lead to complete arrest of T lymphocyte migration and stable conjugation

with an APC, which can last several hours (128), while when interacting with TCR ligands of low potency or low affinity, T lymphocytes do not completely stop migrating and establish brief dynamic contacts with the APC (129), termed kinapses (127). TCR signaling strength modifies the actomyosin cytoskeleton of T lymphocytes (130, 131), which may lead to an alteration of their mechanical properties. It would thus be interesting to measure the effect of TCR signaling strength and also cytokine environment on T lymphocyte viscoelastic properties.

T Lymphocytes Sense and Adapt to Substrate Stiffness

As discussed above, forces exerted by T lymphocytes may be important to probe their environment and particularly to test the stiffness, as we do when exerting pressure with our finger on a substrate. For example, it was proposed that "T lymphocytes are guided by the mechanical 'path of least resistance' as they transverse the endothelium" (34). In fact, T lymphocytes develop ILPs that physically push against the endothelial cell surface (20, 33, 34), suggesting that the role of these protrusions is to test the stiffness of endothelial cells in order to find "soft" areas to cross through (35). It is worth noting that these protrusions have also been proposed to facilitate the activation of memory/effector T cells to pMHC exposed on endothelial cells (22). Thus, T lymphocytes can sense the stiffness of the substrate they interact with. We have shown that not only T lymphocytes sense stiffness but also adapt to it. The pulling forces exerted by T lymphocytes upon TCR–CD3 triggering increased with the stiffness of the BFP used (11). This adaptation of forces to stiffness was not found in another study (12). Yet, the stiffness range used in each study might be very different.

Recent studies have addressed the effect of substrate stiffness on T lymphocyte activation. Using polyacrylamide gels with varying stiffness (range from 2 to 200 kPa) coated with an activating anti-CD3ϵ antibody, it was shown that mouse naïve $CD4^+$ T lymphocytes modulated their response according to the stiffness of gel substrates (132). Production of IL-2 and early phosphorylation of Zap70 and Src family kinases was higher on "stiff" (100–200 kPa) substrates. This response to substrate stiffness was observed only when the anti-CD3ϵ antibody was attached to the gel and was abrogated in the presence of the myosin inhibitor blebbistatin (132). These results suggest that the mechanotransduction involved in T cell activation requires coupling of the TCR–CD3 to the substrate and intact myosin II activity. In another study, human naïve $CD4^+$ and $CD8^+$ T lymphocytes were cultured on poly-dimethoxysilane (PDMS) substrates with stiffness ranging from 100 to 10,000 kPa. The "soft" substrates (~100 kPa) induced higher IL-2 and IFNγ production as well as more T lymphocyte proliferation (133). These results seem inconsistent with the previous study (132). Yet, for poly-acrylamide gel substrates, immobilization was performed by coupling of biotinylated activating antibodies on acrylamide-conjugated streptavidin. In contrast, coating of PDMS substrates was performed by passive adsorption of antibodies on the hydrophobic surface (133), possibly resulting in both loss of immobilized material over time and passive adsorption of proteins from the culture medium. In a third study, human $CD4^+$ T lymphoblasts were activated on PDMS substrates of varying stiffness, which presented anti-CD3 antibodies either alone or together with ICAM-1 molecules (134). In this study, "soft" (5 kPa) substrates induced less tyrosine phosphorylation than the "rigid" (2000 kPa) ones, and ICAM-1 increased the response to "stiff" substrates (134).

Even though the aforementioned reports provide very interesting results, their focus is on a stiffness range (2–10,000 kPa) that is non-physiological for T cells in the body, since APCs were shown to display stiffness ranging from 0.19 to 1.45 kPa (109). A recent study (13) looked at the response of Jurkat cells to substrates of a more physiologically relevant stiffness range (0.2–6 kPa). The authors used polyacrylamide gels, treated with hydrazine hydrate and coated with poly-L-lysine and an activating anti-CD3 antibody. They quantified the effect of substrate stiffness on CD3-induced signaling by following tyrosine phosphorylation by immunoblotting and microscopy (13). They showed that tyrosine phosphorylation peaked higher and more rapidly on "stiff" gels (5 kPa) but decreased more rapidly than on "soft" gels (1 kPa).

Although these studies (summarized in **Table 1**) are difficult to directly compare because they use different cell types, substrate chemistry, antibody immobilization, and stiffness ranges, they overall reveal that T lymphocytes are indeed mechanosensitive. It is not entirely clear what is the mechanosensing mechanism, yet, as summarized above, it requires TCR-dependent actomyosin remodeling. One explanation for the effects on TCR triggering and subsequent activation could be the local spreading and

TABLE 1 | T lymphocyte response to substrate stiffness.

Cell type	Substrate chemistry	Activators coated	Stiffness range	T cell functions measured	Response to stiffness	Reference
Mouse naïve $CD4^+$ T cells	Polyacrylamide gels containing streptavidin	Biotinylated anti-CD3, anti-CD28	10–200 kPa	IL-2 production, phosphorylation of SFK and Zap70	↑ activation with ↑ stiffness	Judokusumo et al. (132)
Human naïve $CD4^+$ and $CD8^+$ T cells	PDMS, passive adsorption of proteins	Anti-CD3, anti-CD28	100 kPa–2 MPa	IL-2 and IFNγ production, cell proliferation	↑ activation with ↓ stiffness	O'Connor et al. (133)
Jurkat T cells	Polyacrylamide gels treated with hydrazine hydrate	Poly-L-lysine and anti-CD3	1–5 kPa	Phosphorylation of Zap70, Lat, SLP76	↑ peak activation with ↑ stiffness, ↑ sustained activation with ↓ stiffness	Hui et al. (13)
Human primary $CD4^+$ T cell blasts	PDMS, passive adsorption of proteins	Anti-CD3, ICAM-1-Fc	5 kPa–2 MPa	Tyrosine phosphorylation	↑ activation with ↑ stiffness	Tabdanov et al. (134)

deformation of the T cell membrane in contact with substrate of different stiffness. In the kinetic-segregation model (37), local membrane deformation and segregation of large glycoproteins have to occur before TCR and pMHC can come close to one another and interact (40). It was proposed that this process required cytoskeleton-derived force (135). One could postulate that this could occur at the tips of ILPs or other small protrusions of the cell, i.e., short filopodia. Regarding the deformability of the substrate, "stiff" substrates would allow more deformation of the T lymphocyte membrane and better molecular segregation at the T cell protrusions compared to "soft" substrates and this, in turn, would result in an increased number of successful interactions between TCR and ligands (anti-CD3 antibodies or pMHC complexes). The increased number of TCR engagements would induce increased intracellular signaling that would then activate the actin cytoskeleton to produce larger cell protrusions and generate forces (11–13). This process can eventually result in increasing bond lifetimes of TCR and LFA-1 for their ligands. By inducing more conformational changes, it would lead to increased T lymphocyte activation. Thus, "stiff" substrates would display a kind of mechanical signal amplification. This mechanism has already been proposed for fibroblast adhesion on substrates of varying stiffness (100). Further work will be required to test this hypothesis for T lymphocytes.

CONCLUSION AND PERSPECTIVES

Recent evidence has shown that TCR signaling and T lymphocyte activation are not solely regulated by chemical signals of the environment but also by mechanical cues. Forces exerted by the exterior or the T lymphocyte itself regulate the lifetime of receptor/ligand bonds. This, in turn, increases adhesion of T lymphocytes to APCs and allows for better discrimination of agonist pMHC. Forces exerted by T lymphocytes also help the cells probe the substrates they interact with by testing their stiffness, which might be a key parameter of T cell activation. We now need to explore further the viscoelastic properties of cells and tissues in physiological and pathological conditions in order to develop experimental models that better mimic the mechanical landscape of T lymphocytes. At the molecular level, we need to study the role of known costimulators or co-inhibitors of T lymphocyte activation in force development and force sensing and find out potential mechanical crosstalk between receptors. Finally, at the cell level, it would be interesting to study if and how mechanical cues can modulate the functions of different T lymphocyte subsets. It would be particularly important to see if mechanics can modulate naïve T lymphocyte priming or effector T lymphocyte functions (cytotoxicity and cytokine secretion). Elucidating these issues will provide further insight into T lymphocyte activation under normal and pathological conditions that could be translated in novel therapeutic strategies.

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CH wrote the review, edited the manuscript, and designed table and figures. MS wrote part of the review and edited the manuscript and figures and table.

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REVIEW
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Vesicular Trafficking to the Immune Synapse: How to Assemble Receptor-Tailored Pathways from a Basic Building Set

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The signals that orchestrate T-cell activation are coordinated within a highly organized interface with the antigen-presenting cell (APC), known as the immune synapse (IS). IS assembly depends on T-cell antigen receptor engagement by a specific peptide antigen-major histocompatibility complex ligand. This primary event leads to polarized trafficking of receptors and signaling mediators associated with recycling endosomes to the cellular interface, which contributes to IS assembly as well as signal termination and favors information transfer from T cells to APCs. Here, we will review recent advances on the vesicular pathways implicated in IS assembly and maintenance, focusing on the spatiotemporal regulation of the traffic of specific receptors by Rab GTPases. Based on accumulating evidence that the IS is a functional homolog of the primary cilium, which coordinates several central signaling pathways in ciliated cells, we will also discuss the similarities in the mechanisms regulating vesicular trafficking to these specialized membrane domains.

Keywords: immune synapse, receptor trafficking, Rab GTPases, IFT, primary cilium

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INTRODUCTION

Adaptive immunity relies on the presentation of major histocompatibility complex-associated peptide ligand (pMHC) by an antigen-presenting cell (APC) to a cognate T cell to allow for its activation. This process is coordinated by a highly specialized membrane domain that forms at the interface between T cell and APC, known as the immune synapse (IS), which ensures the long-lasting signaling required for T cell activation (1, 2).

T cell responses are finely regulated by the dynamic modulation of the levels of surface T cell receptor (TCR) (3). In quiescent T cells, TCR expression is dependent on a balance of *de novo* synthesis, endocytosis, recycling, and degradation, recycling between the plasma membrane and the cytoplasmatic pool being a major determinant (4). Constitutive endocytosis of the TCR requires protein kinase C (PKC)-dependent phosphorylation of a di-leucine motif on the CD3γ chain, which enables the CD3 complex to interact with the clathrin adaptor protein 2 (AP-2) and to be directed to recycling endosomes for returning to the plasma membrane (5). Constitutive TCR recycling subserves a dual function. First, it acts as a quality control mechanism allowing for the identification and degradation of TCR complexes that have lost their integrity. Second, it permits the formation of an intracellular pool of functional TCRs that can be rapidly polarized to the IS in response to engagement of plasma membrane-associated TCRs.

T-cell antigen receptor triggering induces receptor internalization, which is followed by either polarized recycling to the plasma membrane or receptor degradation (6). The pathway of ligand-dependent TCR internalization is mediated by the lymphocyte-specific protein tyrosine kinase (Lck) (7), the membrane-remodeling GTPase dynamin 2 (8), and PKC-regulated activation of CD3γ, which routes the internalized TCRs to the recycling compartment (9). Alternatively, activated TCRs may undergo degradation to allow for signal termination. The E3 ligase casitas B-lineage lymphoma (Cbl-b), which is upregulated in response to the interaction of programed death 1 ligand 1 (PD-L1) on APC and programed death 1 (PD-1) on $CD8^+$ T cells, plays a key role in this process (10). TCR triggering induces CD3ζ ubiquitination by Cbl-b, which is recruited to the engaged TCRs by the protein tyrosine kinases Lck and ζ-chain-associated protein kinase 70 (ZAP-70) (10–12). Ubiquitinated TCRs are recognized by tumor susceptibility gene 101 (Tsg101) and sorted to multivesicular bodies (MVBs) for degradation (13), thereby making space for incoming TCRs and turning off signaling. Interestingly, recent evidence indicates that internalized TCRs continue to signal, thereby contributing to sustained signaling from their endosomal localization (8, 14). Moreover, internalized TCRs are in part delivered to the APC as microvesicles (15), highlighting a role for TCR endocytosis beyond the canonical function of signal termination.

Here, we will summarize of our current understanding of the recycling pathways that regulate the traffic of endosomal TCRs as well as of other receptors, including the C-X-C chemokine receptor type 4 (CXCR4) and the transferrin receptor (TfR), and of membrane-associated signaling mediators, such as Lck and linker for activation of T cells (LAT), which participate as key players in IS assembly and function. We will also discuss the emerging role of the IS as a platform for vesicular traffic-mediated transcellular communication beyond its established role in the secretion of soluble effectors.

POLARIZED TCR RECYCLING AT THE IS: SEEING THE TIP OF THE ICEBERG

Rab GTPases and Their Effectors in TCR Trafficking to the IS

Recycling receptors traffic through at least two temporally and spatially distinct highly conserved pathways orchestrated by members of the Rab GTPase family: a short-loop and a long-loop. Following internalization, receptors are delivered to early endosomes, marked by Rab5, and rapidly returned to the plasma membrane under the control of Rab4 (short-loop). Alternatively, recycling receptors may transit from early endosomes to the pericentrosomal endocytic recycling compartment and return to the plasma membrane *via* a Rab11-dependent route, thus completing the long-loop recycling (16–18). Intracellular TCRs have been found associated with both $Rab4^+$ and $Rab11^+$ endosomes, with the $Rab11^+$ compartment centrally implicated in endosome recycling to the IS (19, 20). In addition to these universally used recycling Rabs, more specific Rab GTPases and traffic regulators have been mapped to the TCR recycling pathway. One example is Rab35 and its GTPase-activating protein (GAP) EPI64C (21). Rab35 is a Rab GTPase implicated in cytokinesis in *Drosophila* (22) and in the regulation of endosomal trafficking as well as actin polymerization in several insect and mammalian cell lines (23). In T lymphocytes, Rab35 colocalizes with the TCR at the pericentrosomal compartment, wherefrom it is recruited at the IS, thus regulating polarized TCR recycling, and IS formation in concert with EPI64C (21). We have moreover recently identified Rab29, an as yet poorly characterized Rab GTPase, as a new component of the TCR recycling pathway. The Rab29 subfamily, which also includes Rab32 and Rab38, has been implicated in the traffic of melanosomes (24), as well as of the mannose-6-phosphate receptor (MPR) in epithelial and neuronal cells (25, 26). We found that in T cells, Rab29 acts as a complex with Rab11 to control TCR delivery to the IS membrane through microtubule-dependent polarized recycling. In Rab29-depleted cells, recycling TCRs accumulate indeed in $Rab11^+$ endosomes that fail to polarize to the IS notwithstanding a correct positioning of the centrosome due to defective recruitment of the dynein microtubule motor (27).

Recycling endosomes are associated not only with microtubules but also with actin that generates force for vesicle movement along the microtubule tracks. Both early and recycling endosomes polarizing to the IS during T cell activation have been shown to colocalize with the nucleation promoting factor WASP and SCAR homolog (WASH) (28), which mediates local actin polymerization by recruitment of the Arp2/3 actin adapter complex. WASH is required for TCR trafficking following T cell stimulation (29). Accordingly, activated WASH-deficient T cells express reduced TCR levels, which is likely to lead to a defect in sustained signaling, accounting for their proliferation defect (29). WASH also contributes to maintain the levels of the costimulatory receptor CD28 and the integrin lymphocyte function-associated antigen 1 (LFA-1), as well as of the glucose transporter 1 (GLUT1), at the surface of activated T cells (29), which indicates that the role of this adaptor in receptor trafficking is not restricted to the TCR.

A previously uncharacterized role in T cell activation has been recently ascribed to the sorting nexins (SNX), which regulate several steps in vesicular trafficking. Both SNX17 and SNX27 have been shown to accumulate at the IS, where they play opposite roles in T cell activation. SNX17 colocalizes with endosomal TCRs and is required for their recycling to the plasma membrane (30). At variance, SNX27 interacts with diacyl glycerol kinase ζ (DGKζ), which negatively controls TCR signaling, at early and recycling endosomes, and traffics to the IS to blunt the Ras–Erk pathway (31).

Control of TCR Trafficking to the IS by Regulators of Primary Cilium Assembly

Further insights into the pathway of TCR recycling have emerged with the unexpected identification in this pathway of components of the intraflagellar transport (IFT) system, which regulates the assembly of the primary cilium, an organelle of which T cells are normally devoid (32, 33). IFT particles are responsible for cargo movement into the cilium and back to the cell body through their interaction with the microtubule motors kinesin-2 and

cytoplasmic dynein-2, respectively (34, 35). We have shown that in T cells, IFT20 cooperates with the IFT proteins IFT88, IFT57, and IFT52 to promote TCR recycling to the IS downstream of centrosome polarization (32, 36). IFT20 participates early in the trafficking pathway by forming a complex with Rab5 and the TCR on early endosomes. In IFT20-depleted cells, recycling TCRs accumulate in Rab5$^+$ endosomes, which fail to cluster at the IS despite a normal polarization of the centrosome, indicating that IFT20 controls TCR traffic from early to recycling endosomes (36).

The finding that proteins implicated in ciliogenesis are exploited by T cells to assemble the IS has provided support to the emerging notion that these specialized structures are functional homologs. In addition to their morphological similarities, underscored by the polarized arrangement of the centriole and Golgi apparatus beneath the respective membrane domains, both the primary cilium and the IS act as signaling platforms as well as sites of intense vesicular trafficking and polarized exocytosis (37–40). These similarities can be exploited to identify new components of the pathways governing IS assembly, as witnessed by our recent implication of the small GTPase Rab8 in polarized TCR recycling. Growth of the ciliary membrane and targeting of specific receptors to this location is orchestrated by a Rab11–Rab8 cascade, which interfaces with a multimolecular complex known as the Bardet–Biedl syndrome complex (BBSome) (41). In this cascade, serum starvation represents the signal that promotes the centrosomal trafficking of the Rab8 guanine nucleotide exchange factor Rabin8 through its association with Rab11 and the transport protein particle II (TRAPPII) complex. At the centrosome, the BBSome associates with Rabin8 and activates Rab8 to allow ciliary membrane biogenesis (42). We have recently demonstrated that in T cells, Rab8 colocalizes with IFT20, Rab11, and Rab29 and acts downstream of these trafficking mediators to regulate polarized TCR recycling and T cell activation (27, 43). Of note, Rab8, at variance with IFT20 and Rab29, is not required for the polarization of TCR$^+$ endosomes to the IS (**Figure 1**). Rather, Rab8 controls the docking/fusion step at the plasma membrane of TCR$^+$ endosomes that have clustered beneath the IS membrane by recruiting vesicle-associated membrane protein-3 (VAMP-3) (43), a vesicular soluble NSF attachment protein (v-SNARE), which had been previously reported to regulate docking and fusion of the TCR$^+$ endosomes with the IS membrane (44). Independent evidence for a role for Rab8 in TCR trafficking has been provided by Soares et al., who showed that vesicular TCRζ colocalizes with both Rab8b and Rab3d (45).

It is noteworthy that the elucidation of the pathways controlling receptor traffic to the IS has helped furthering our understanding of the mechanisms that orchestrate ciliogenesis. We have recently found that ciliated cells express Rab29 which, similar to T cells, participates in a complex that includes Rab8, Rab11, and IFT20, as well as the molecular motors kinesin and dynein. Rab29-depleted cells show defects in ciliogenesis, with a reduction in the number of cells forming a cilium and, where present, a reduced ciliary length. Ultrastructural analysis reveal that these cells have no alterations in the structure of the cilium but a significant vesicle enrichment around the ciliary base indicating that Rab29 controls ciliary assembly by favoring cargo trafficking to the cilium, a central one being the Hedgehog-associated transmembrane receptor Smoothened (Smo) (46). Additionally, we demonstrated that, similar to T cells, VAMP-3 interacts with Rab8 in ciliated cells, promoting the delivery of Smo to the ciliary membrane (43). VAMP-7, another v-SNARE implicated in traffic to the IS, has also been recently implicated in ciliogenesis (47). Hence, studying IS assembly and ciliogenesis provides a unique opportunity of cross-feeding, as recently highlighted by the implication of the Hedgehog pathway, one of the major signaling pathways orchestrated by the primary cilium, in the release of cytotoxic granules at the synapse of cytotoxic T cell effectors (48).

A COMBINATORIAL STRATEGY FOR THE DELIVERY OF SPECIFIC RECYCLING RECEPTORS TO THE IS

Signaling at the IS to promote T cell activation, while triggered by the TCR, requires the coengagement of several other receptors as well the recruitment of key membrane-bound signaling mediators. Emerging evidence highlights the existence of individual trafficking modules, which ensure cargo specificity within the universal recycling pathways orchestrated by Rab4 and Rab11. In this section, we will present some examples that illustrate the versatility of the vesicular pathways that control endosomal trafficking to the IS.

C-X-C Chemokine Receptor Type 4

Among the receptors known to become enriched at the IS is CXCR4, a ubiquitously expressed heterotrimeric G protein-coupled receptor, which regulates T cell development, migration, and activation (49, 50). CXCR4 participates in IS assembly, signaling through Gi and the janus-activated kinases 1/2 (JAK1/2) to maintain the T cell:APC contact (51) and promoting local actin polymerization and centrosome polarization (51). Ligand binding induces CXCR4 internalization through clathrin-coated pits *via* a PKC-mediated, β-arrestin-dependent pathway leading to CXCR4 sorting either to lysosomes (52–54) or to Rab11$^+$ recycling endosomes containing TCR cargo (19, 55). The colocalization of CXCR4 with TCR$^+$ endosomes, which depends on actin polymerization mediated by Gα13 and Rho (19), may reflect the ability of CXCR4 to heterodimerize with the TCR both at the plasma membrane and in endosomes (49). The pathway that controls CXCR4 targeting for lysosomal degradation, which involves its ubiquitination by the E3 ligase atrophin interacting protein 4 (AIP4) (56) and its interaction with the endosomal-sorting complex required for transport (ESCRT) (57), has been extensively characterized. Conversely, while CXCR4 deubiquitination has been identified as one of the factors that favor CXCR4 recycling (57), relatively little is known about the molecular mechanisms mediating this process. In T cells, CXCR4 surface expression is dependent on IQ motif-containing GTPase-activating protein 1 (IQGAP1), a cytoskeleton-interacting scaffold protein that is required for tethering CXCR4$^+$ early endosomes to microtubules to redirect their receptor cargo to the plasma membrane (58). We have recently provided further insights into the pathway that controls the traffic of endosomal CXCR4 by identifying Rab29

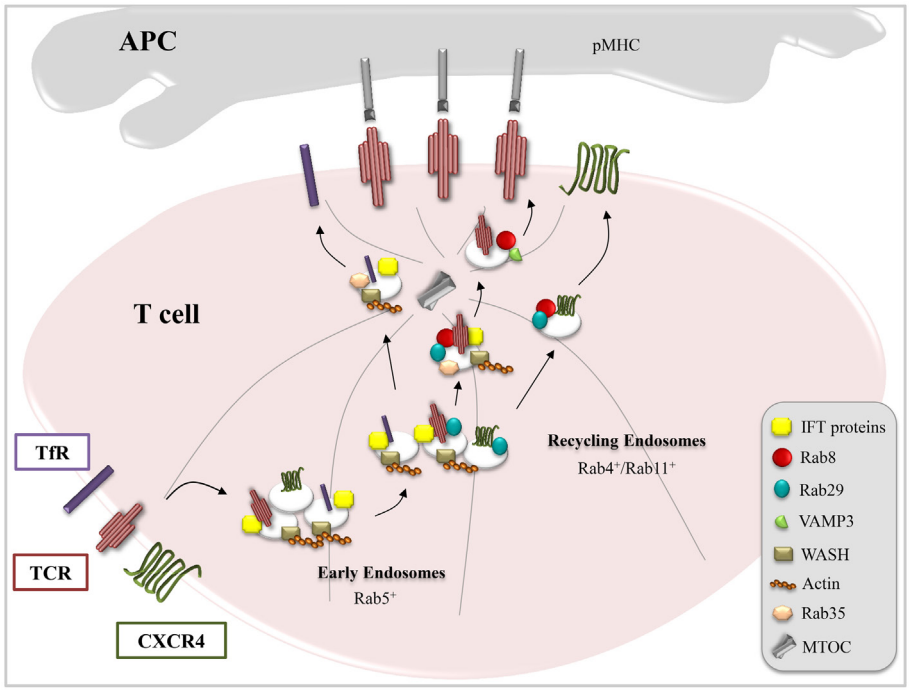

FIGURE 1 | Combinatorial usage of trafficking regulators in receptor targeting to the IS. T cell activation requires IS delivery of receptors (e.g., TCR, TfR, and CXCR4) and membrane-associated signaling mediators (e.g., Lck and LAT) associated with endosomal pools. This is achieved through tailor-made trafficking pathways characterized by unique combinations of common (Rab5, Rab4, and Rab11) and specific (IFT proteins, Rab8, Rab29, VAMP3, WASH, and Rab35) endosomal regulators, as exemplified here for TCR, TfR, and CXCR4.

and Rab8 as new regulators of both constitutive and polarized recycling of this receptor to the IS. It is noteworthy that the IFT proteins do not participate in this pathway, notwithstanding their functional interplay with both Rab29 and Rab8 in TCR recycling (**Figure 1**) (27, 36, 43).

Transferrin Receptor

Interestingly, these same traffic regulators are used in a different combination for the traffic of yet another receptor, the TfR, to the IS. The TfR, which plays a central role in iron homeostasis, is one of the best characterized recycling receptors. Upon binding iron-loaded transferrin, the TfR enters the cell through clathrin-mediated endocytosis, which is regulated by the clathrin adaptor AP-2, the phosphoinositide PtdIns(4,5)P_2, the membrane-remodeling GTPase dynamin 2, Rab5, the cortical actin regulator cortactin, and the kinase Src (59, 60). It subsequently accumulates in recycling endosomes, wherefrom it returns to the plasma membrane both through the short-loop (Rab4-dependent) and the long-loop (Rab11-dependent) pathways (61, 62). In T cells, the function of the TfR goes beyond controlling iron uptake. It has indeed been demonstrated that the TfR interacts with CD3ζ and promotes its tyrosine phosphorylation following binding of holotransferrin (63), suggesting that it might participate in lymphocyte activation by modulating TCR signaling. In support of this notion, surface expression of the TfR increases upon TCR stimulation and the receptor polarizes to the IS. Blocking the TfR using a neutralizing antibody results in defective T-cell:APC conjugate formation and TCR clustering at the IS, underscoring a function for this receptor in IS assembly (44, 64). Although several Rabs and trafficking mediators other than Rab4 and Rab11 (e.g., Rab12, Rab22, Rab8, and Arl13b) have been implicated in the regulation of TfR recycling in other cells types (65–68), the pathway controlling polarized recycling of this receptor to the T cell IS is only beginning to be elucidated. Similar to the TCR, the TfR requires Rab35 and its GAP EPI64C as well as the actin adaptor WASH to recycle to the T cell plasma membrane (21, 28). Moreover, we have recently demonstrated that IFT20 interacts with the TfR and is implicated in its recycling to the IS. Remarkably, IFT20, while regulating TCR and TfR recycling, is not involved in CXCR4 recycling. At variance, Rab8 and Rab29, while participating in TCR and CXCR4 recycling, are dispensable for TfR recycling. This suggests a scenario where different receptors (TCR, CXCR4, and TfR), while sharing some components of the universal short-loop and long-loop pathways, adopt personalized routes by combining individual traffic regulators, such as IFT20, Rab8, and Rab29, allowing specificity to be achieved during polarized recycling to the IS (**Figure 1**).

Lck and LAT

The combinatorial strategy used by T cells to coordinate the traffic of specific receptors or membrane-associated signaling mediators to the IS has been recently shown to be also exploited to generate signaling nanodomains at the TCR activation sites (45, 69). Similar to the TCR, the initiating protein tyrosine kinase

Lck and the transmembrane adaptor LAT are present in T cells as two cellular pools, of which one is associated with the plasma membrane and the other with recycling endosomes, the latter becoming polarized to the IS upon TCR triggering (70–72). Sorting of Lck and LAT to the central domain of the IS, known as central supramolecular activation cluster (cSMAC), is regulated by the lipid raft-associated myelin and lymphocyte (MAL) protein, whose rapid accumulation at the raft-enriched IS promotes the recruitment of the microtubule and transport vesicle docking machinery (73). Moreover, both LAT phosphorylation and the recruitment of LAT^+ vesicles to TCR activation sites critically depend on the v-SNARE VAMP7 (74). In their recent report, Soares et al. (45) provided evidence for the existence of traffic modules specifically tailored to promote the synaptic transport of Lck and LAT versus TCRζ in response to TCR engagement. They found that $Rab11^+$ vesicles containing Lck rapidly localize to the IS. Subsequently, in response to increased calcium levels, $Rab27a^+Rab37^+$ vesicles containing LAT and $Rab3d^+$ $Rab8b^+$ vesicles containing TCRζ are delivered to the IS. This is achieved through the interaction of VAMP-7 associated to both LAT^+ and $TCR\zeta^+$ vesicles with the calcium sensor synaptotagmin-7. This report not only provides new insights into the modularity of the traffic pathways exploited by T cells to target specific molecules to the IS but also supports the notion that the synaptic membrane is a mosaic of nanodomains generated with the central contribution of vesicular traffic that coordinate signaling to promote the assembly of a fully competent IS.

The combinatorial assembly of unique trafficking modules within the common basic recycling pathways orchestrated by Rab4 and Rab11 provides an explanation to emerging evidence generated in several different cell types that the endosome pools marked by these GTPases are actually mosaics of $Rab4^+$ or $Rab11^+$ endosome subpopulations characterized by specific arrays of traffic regulators and carrying distinct receptor cargoes. For instance, in epithelial-like CHO cells, the TfR and the glucose transporter GLUT4 transit through distinct pools of $Rab4^+$ endosomes (75). Similarly, while the majority of ciliary proteins share the Rab8-Rab11 cascade, the traffic of specific receptors to the ciliary membrane is controlled by unique mediators. For example, Rab23 is specifically required for the ciliary traffic of Smo and the dopamine receptor but is dispensable for ciliary targeting of the receptor protein Kim1 and of the microtubular tip end-binding protein 1 (EB1), notwithstanding their common requirement for Rab8 (76, 77). IFT25 and IFT27 have been specifically implicated in ciliary trafficking of Smo, without affecting either the formation of the ciliary axoneme or the localization of other ciliary membrane-associated proteins, such as ADP-ribosylation factor-like protein 13B (ARL13B) and adenylate cyclase 3 (ADCY3) (78–81). We have moreover provided evidence that while required for Smo trafficking, Rab29 is dispensable for the ciliary localization of β1 integrin (27). Hence, the trafficking machinery is emerging as a combinatorial system of dynamic modules that ensure the specificity of receptor/cargo transport (81). This system, of which we are only beginning to fathom the complexity, is further complicated by indications that the traffic modules that have been identified may be tissue specific. For example, at variance with epithelial cells, the TfR is associated in neuronal cells with a distinct subpopulation of $Rab11^+$ endosomes marked by ADP-ribosylation factor 6 (Arf6) (82). Unraveling this complexity is a major future challenge.

VESICULAR TRAFFICKING AS A MEANS OF TRANSCELLULAR COMMUNICATION

The role of the IS as a platform for focalized exocytosis of cytokines and/or lytic granules by effector T cells is well established and has been extensively reviewed (40). Emerging evidence indicates however that vesicular traffic at the IS subserves important regulatory functions during the interactions that occur between T cell and cognate APC. In this context, the IS is exploited as a means of cell-to-cell communication to fine tune both the T cell and the APC (83).

T cells are able to extract surface molecules from other cells with which they establish contacts upon dissociation (84). This process is known as trogocytosis and leads to intercellular exchange of membrane patches. T cells take up into their plasma membranes costimulatory molecules, adhesion molecules, and pMHC expressed on APCs probably as a consequence of coincidental T cell phagocytosis of APC membrane during TCR downmodulation (85). It has been proposed that phagocytosed APC membrane fragments fuse with the endosomal compartment and recycle to the T cell plasma membrane, conferring to T cells the capacity to directly activate other $CD4^+$ T cells, which allows for an increase in the number of APCs presenting cognate antigen and facilitates the activation of effector T cells (86). In addition, trogocytosis has been linked to sustained T cell signaling since the pMHCs extracted from the APC remain associated with the engaged TCRs, resulting in elevated levels of ZAP-70 and phosphorylated proteins and thus prolonging the presentation step (87, 88).

The second example of transcellular communication involving vesicular traffic at the IS is the release by T cells of exosomes that are taken up by the APC (89). Exosomes are formed by inward budding of the limiting membrane of MVBs which, upon TCR triggering, are polarized toward the APC and fuse with the plasma membrane to release the vesicles. The polarization of MVBs is regulated by phospholipids (diacylglycerol and PIP3), the lipid kinase DGKα, and the serine/threonine protein kinases 1/2 (PKD1/2) (89, 90). Among the proteins that are involved in exosome biogenesis and release, an important role is played by the ESCRT complex as well as by several Rab GTPases (e.g., Rab4, Rab11, Rab27a, Rab27b, and Rab35) (91). It will be interesting to address the potential implication of the recently identified regulators of vesicular trafficking to the T cell IS in exosome secretion. Important insights into the function of the exosomes released at the IS have emerged from the analysis of their contents. Mittelbrunn et al. showed indeed that these exosomes are loaded with microRNAs that are able to regulate gene expression following their uptake by the APC, one being the Sry-box transcription factor 4 (Sox-4) (92). This may affect the ability of the APC to shape the differentiation program of the engaged T cell. Of note, since the MVBs in the APC do not polarize to the IS (92), the current model posits a unidirectional transfer of exosomes from the T lymphocyte to the APC.

It has been recently shown that the ESCRT-I protein Tsg101 is involved in targeting internalized ubiquitinated TCRs to microvesicles that are subsequently released at the IS through a mechanism regulated by vacuolar protein sorting 4 (VPS4). These synaptic microvesicles are delivered to the APC bearing cognate pMHC where they initiate intracellular signals, thereby acting as a means of transcellular communication (15). Interestingly, synaptically released TCR-enriched microvesicles are able to activate signaling in B cells-presenting specific pMHC, suggesting a novel mechanism of T cell help where the amount of help is adjusted to the density of pMHC at the B cell surface (15).

The role of extracellular vesicles as a means of cell-to-cell communication is now well established, particularly in immune cells, where they act as vehicles for the transfer of immunomodulatory molecules (89, 93). The focused release of extracellular vesicles at the IS ensures a cellular confinement to allow for their uptake by the APC with minimal diffusion. Of note, similar to the IS, the primary cilium has been identified as a site of focused release of membrane vesicles. Vesicle secretion from the distal ends of the cilium has been reported in vertebrate retinal photoreceptors as well as in epithelial cells lining the urinary lumen (94). In the model organism, *Chlamydomonas reinhardtii*, these ciliary ectosomes carry a protease implicated in the liberation of the daughter cell following mitosis (95), underscoring the shared informative role of the IS and the cilium achieved through the release of extracellular vesicles.

FUTURE PERSPECTIVES

Emerging evidence of a role for post-translational modifications other than phosphorylation in the regulation of the molecular machinery that orchestrates vesicular traffic is adding a further layer of complexity to this biological process (96, 97). While receptor ubiquitination has long been known to act in concert with kinases and β-arrestins to regulate the trafficking of G protein-coupled receptors (GPCRs) (57), specific components of the pathways that control receptor traffic have turned out to be regulated by ubiquitination. For example, the GPCR β2 adrenoceptor (β_2AR) is able to regulate its own trafficking by ubiquitination and activation of Rab11a (98). Moreover, the activity of the F-actin-nucleating protein WASH is fine tuned through K63-linked ubiquitination by the MAGE-L2-TRIM27 ubiquitin ligase and by the ubiquitin-specific peptidase 7 (USP7) deubiquitinating enzyme to prevent its overactivation, thus ensuring a proper WASH-mediated endosomal actin assembly and protein recycling (99, 100). In T cells, ubiquitination has recently been implicated in the traffic not only of receptors, such as the TCR and CXCR4, but also of signaling mediators that traffic to the IS, as recently exemplified by LAT. This adaptor has been shown to recruit to the IS the E3 ligase TNF receptor-associated factor 6 (TRAF6), which is essential for its ubiquitin-dependent phosphorylation (101), highlighting this cooperation between LAT and TRAF6 as a new regulatory mechanism in T-cell activation. Sumoylation, a reversible post-translational process mediated by small ubiquitin-like modifier (SUMO), has also been recently implicated in IS assembly and function, as documented for PKC-θ, which has been shown to be sumoylated is response to TCR engagement (102). These new findings underscore post-translational modifications as a new important area of study for the dissection of the traffic-related mechanisms that regulate IS assembly and T cell activation. Again, the primary cilium, where recent evidence has been provided for the regulation of ciliary trafficking by ubiquitination or sumoylation (103, 104), may provide interesting candidates to further our understanding of the post-translational control of traffic to the IS.

The identification of vesicular traffic as a central regulator of IS assembly and function highlights another important emerging area of investigation, namely the causative implication of traffic defects in T cell-mediated diseases. Abnormalities in TCR and CD4 trafficking have been associated to enhanced signaling in systemic lupus erythematosus (SLE) (105, 106). SLE T cells show enhanced endocytic trafficking due to overexpression of Rab5 and HRES-1/Rab4, a small GTPase encoded by the HRES-1 human endogenous retrovirus, which is essential for the targeting of TfR, CD4, and TCRζ for lysosomal degradation in T cells (105, 107). Paradoxically, these trafficking abnormalities, which are likely to account for the reduced expression of TCRζ at the surface of SLE T cells, have been associated with enhanced signaling. Alterations in the intracellular localization and degradation of signaling mediators, such as LAT, have also been associated to the signaling abnormalities observed in SLE T cells (108). While implicating trafficking defects in the hypersensivitity of SLE T cells, these data underscore the importance of elucidating the underlying mechanisms.

Interestingly, HRES-1/Rab4 appears to play a role also in the context of human immunodeficiency virus (HIV) pathogenesis. Overexpression of HRES-1/Rab4 in T cells has been shown to abrogate HIV infection by inhibiting surface expression of CD4 and targeting it for lysosomal degradation (107). Since expression of the HIV coreceptor CXCR4 is crucial to mediate viral entry (109), modulation of CXCR4 internalization and recycling may also contribute to HIV infection (110). In addition, mutations in CXCR4 that impair its intracellular traffic, resulting in impaired receptor recruitment to the IS (111), have been associated to the development of the warts, hypogammaglobulinemia, infections, and myelokathexis (WHIM) immunodeficiency syndrome.

Collectively, these results point to abnormalities in vesicular traffic as an important determinant in T cell-related diseases. We expect that furthering our understanding of the pathways that control this process may be exploited to identify relevant molecular targets for which existing approved drugs might already be available or new drugs might be designed.

AUTHOR CONTRIBUTIONS

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Comparative Anatomy of Phagocytic and Immunological Synapses

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The generation of phagocytic cups and immunological synapses are crucial events of the innate and adaptive immune responses, respectively. They are triggered by distinct immune receptors and performed by different cell types. However, growing experimental evidence shows that a very close series of molecular and cellular events control these two processes. Thus, the tight and dynamic interplay between receptor signaling, actin and microtubule cytoskeleton, and targeted vesicle traffic are all critical features to build functional phagosomes and immunological synapses. Interestingly, both phagocytic cups and immunological synapses display particular spatial and temporal patterns of receptors and signaling molecules, leading to the notion of "phagocytic synapse." Here, we discuss both types of structures, their organization, and the mechanisms by which they are generated and regulated.

Keywords: phagocytosis, immunological synapse, immune receptor, signal transduction, actin, microtubules, exocytosis, endocytosis

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INTRODUCTION

Immunological synapses are organized cell–cell contacts shaped at the interface between T cells and antigen-presenting cells (APCs) (**Figure 1**). They are triggered by the binding of T cell antigen receptors (TCR) to their ligands, peptide antigens associated with major histocompatibility complex molecules (pMHC) expressed on the surface of APCs. TCR engagement induces the polarization of the T cell toward the APC and a coordinated reorganization of various T cell components, including receptors, signaling and adhesion molecules, the actin and microtubule cytoskeleton, and intracellular vesicle traffic. Thus, the TCR and its proximal signaling molecules (e.g., protein kinases and phosphatases, signaling adapters, and effectors molecules) form dynamic signaling complexes at the immunological synapse that drive T cell activation. Moreover, TCR signaling triggers the fine reorganization of the actin and microtubule cytoskeleton that ensures synapse architecture and signaling complex dynamics, critical for TCR signal regulation. Finally, various intracellular compartments polarize toward the immunological synapse, including the Golgi apparatus, early and late endosomes, and mitochondria. Importantly, the TCR signaling machinery, actin and microtubule cytoskeleton, and intracellular vesicle traffic interplay at the synapse to sustain and regulate T cell activation (1).

Phagocytic cup formation mirrors a large number of events occurring during immunological synapse formation, before leading to a productive engulfment of the target (**Figure 1**). First, clustering of phagocytic receptors induced by particle-associated ligands triggers signal transduction pathways similar to those engaged by the TCR. In particular, a similar spatial and temporal segregation of tyrosine kinases and phosphatases was observed at both immunological synapses and phagocytic cups, leading to the notion of "phagocytic synapse" (2). Second, phagocytic receptor signaling triggers

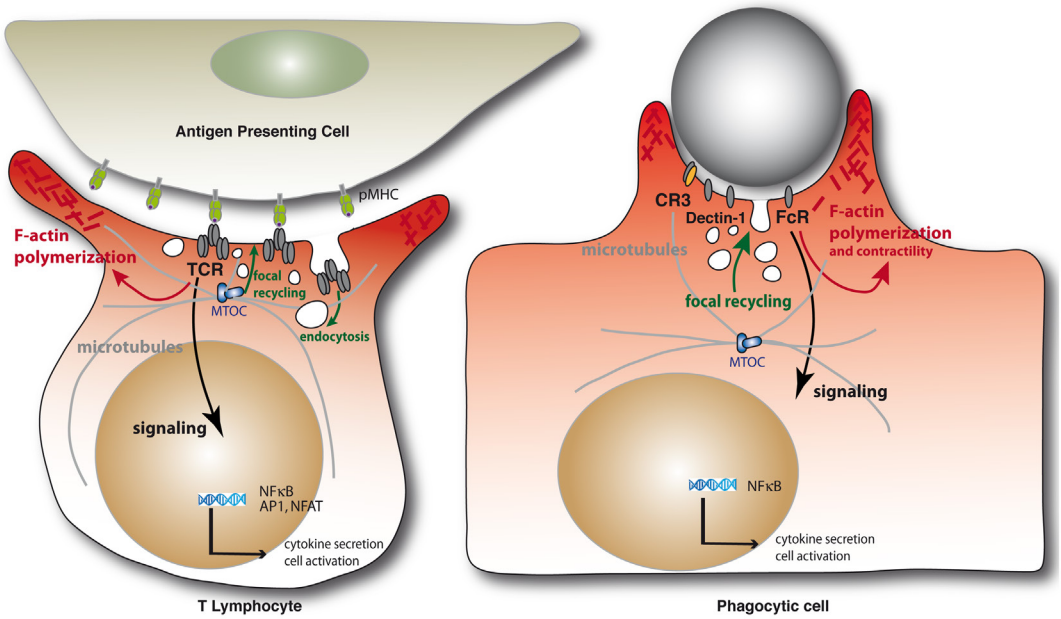

FIGURE 1 | Schematic representation of the immunological synapse and the phagocytic cup formation. Immunological synapse formation is initiated by the engagement of TCRs on the surface of the T lymphocytes by peptide antigen–MHC complexes on the APC (left). Similarly, engagement of phagocytic receptors by multiple ligand binding on a target particle drives the formation of phagocytic synapses (right). In both settings, receptor engagement leads to F-actin polymerization and membrane deformation at contact sites. Polarization of the MTOC and microtubule network toward at the IS are important for the delivery of vesicles containing cytokines or lytic enzymes in helper or cytotoxic T cells, respectively, but also to deliver TCR-signaling components during immunological synapse formation. Microtubules also contribute to F-actin remodeling in complement-mediated phagocytosis. Internalization of cell surface TCRs by endocytosis and their focal recycling participate in the regulation of T cell activation. Finally, in either system, triggering of multiple signaling pathways downstream of the surface receptors leads to *de novo* transcriptional programs controlling cell survival, activation, and cytokine production.

a profound reorganization of the actin cytoskeleton that is similar to the one induced by the TCR, generating large membrane extensions rich in filamentous (F)-actin. Third, microtubule dynamics are also important for some receptor-mediated phagocytosis. Fourth, intracellular traffic involving several vesicular compartments reorients toward the phagocytic cup. Fifth, internalization of the triggered receptors together with their ligands occurs and may lead to their degradation or recycling back to the plasma membrane. Interestingly, the TCRs may be phagocytosed from the immunological synapse internalizing with them their pMHC ligands together with portions of the APC membrane (3). Finally, a series of downstream signaling events lead to cytokine gene activation in both cases.

We review here the molecular and cellular events taking place in both phagocytic and immunological synapses, highlighting their mechanisms of regulation.

RECEPTOR SIGNAL TRANSDUCTION IN T CELLS AND PHAGOCYTES

T cell receptor engagement induces a series of molecular reorganization events that stabilize T cell–APC interactions and optimize signal transduction. Several other receptors are recruited to the immunological synapse and contribute to the activation process. These include the co-receptors CD4 and CD8, co-stimulatory receptors such as CD28, or adhesion proteins such as the integrins $\alpha_L\beta_2$ (LFA1) or $\alpha_4\beta_1$ (VLA4) [reviewed in Ref. (4)].

One of the earliest events elicited by antigen recognition is the sequential activation of protein tyrosine kinases belonging to the Src and Syk families. The Src-family kinases Lck and/or Fyn, phosphorylate several TCR complex subunits, namely CD3 (ε, γ, and δ) and ζ (5). These subunits are all endowed with one or more consensus sequences called immunoreceptor tyrosine-based activation motif (ITAM), each containing two phosphorylatable tyrosine residues. Doubly phosphorylated ITAMs then recruit Syk-family kinases, either ZAP-70 or Syk (6), whose tandem SH2 domains provide specific, high-affinity binding to ITAM phosphotyrosines. Src kinases may be further required to phosphorylate and activate Syk kinases, in particular ZAP-70. The interplay between these two families of tyrosine kinases is crucial for transmitting downstream signals. Thus, Syk family kinases phosphorylate adaptor proteins, such as LAT and SLP-76 that in turn gather signaling effectors within multiprotein complexes, or signalosomes (6). Moreover, both Src- and Syk-family kinases activate several enzymes recruited in these signalosomes that are responsible for the generation of intracellular second messengers, such as Ca^{2+} or phosphoinositides. Collectively, these early steps, induced within seconds after TCR engagement, initiate a cascade of downstream events leading to cytoskeletal rearrangement and cellular polarization. Concomitantly, various serine–threonine kinases, including MAP kinases, are activated, regulating the

activation of several transcription factors that will drive in turn T cell growth and differentiation and the production of effector cytokines (7).

Detection and engulfment of bacteria or fungi by phagocytic cells are triggered by a similar sequence of early events. However, multiple unrelated ligands trigger phagocytosis by engaging distinct receptors. Indeed, phagocytic receptors can recognize their target by binding either to specific molecules expressed on the target's surface or to opsonizing antibodies or complement subunits previously bound to the target. For instance, phagocytosis of IgG-coated pathogens is triggered upon antibody recognition by Fcγ receptor (FcγR), whereas integrins, such as $\alpha_M\beta 2$ (also known as Mac-1 or CR3), can recognize complement-coated particles. Finally, phagocytosis of fungi expressing β-glucans on their cell wall is triggered by Dectin-1 receptor (8).

Phagocytic Fc receptors (FcγRII and FcγRIII) belong to the immunoreceptor family and are structurally related to antigen receptors. Importantly, they transmit activating signals using ITAM motifs that are either built in the receptor intracellular tail or in the associated common γ-chain (9). Hence, early signaling events involve Src- and Syk-family kinases, similarly to what explained above for the TCR. In macrophages, the Src kinases Lyn, Hck, and Fgr are involved in FcR-induced phagocytosis. However, phagocytosis was significantly reduced but not abolished in cells of triple knockout mice, suggesting the existence of further redundancy or alternative triggering mechanisms (10). In contrast, Syk knockout resulted in a complete block of phagocytosis, indicating the indispensable role of this kinase (11). Since Syk, but not ZAP-70, has been shown to phosphorylate ITAM motifs (12), it can be envisaged that Syk can trigger some phagocytic activity in the absence of Src kinases.

The β-glucan receptor Dectin-1, a member of the C-type lectin receptor (CLR) family, also induces sequential activation of Src and Syk kinases. Dectin-1 displays in its cytoplasmic domain ITAM-like sequences named hem-ITAM, each containing a single tyrosine-based motif. Once phosphorylated by Src kinases, they are able to bind Syk and trigger downstream activation (13). Since Dectin-1 is a dimer, it has been proposed that Syk binds in trans to two phosphorylated hem-ITAMs on adjacent subunits in order to be recruited to the activated receptor (13). However, this model has not been validated experimentally. Furthermore, a potential alternative mechanism for Syk recruitment has been revealed recently, implying a scaffolding role of the protein tyrosine phosphatase SHP-2 in bridging Syk to Dectin-1 and other CLRs (14).

The molecular mechanisms underlying integrin-dependent phagocytosis, such as that elicited by complement-coated particles binding to CR3, are more complex than those described for FcRs and Dectin-1. Importantly, integrin binding to their ligand requires prior activation *via* a conformational change induced by "inside-out signaling." This priming phase is induced by inflammatory or pathogen-specific signals, such as those triggered by G-protein-coupled (GPCRs) or toll-like receptors (TLRs). These proteins initiate different signaling cascades converging on a common effector, the GTPase Rap1 (15). Active Rap1 induces the recruitment of RapL, RIAM, and talin to integrin cytoplasmic tails, thus promoting the switch of integrins to their extended conformation that can bind ligands with high affinity (16). Then, ligand-bound integrins transmit "outside-in" signals that drive actin polymerization and downstream activation. These steps involve several effectors including the protein kinases FAK (or Pyk2) and ILK, non-muscle myosin II, and Rho GTPases (17). Nonetheless, the fact that Syk inhibition impairs CR3-mediated phagocytosis demonstrates the existence of some crosstalk between integrin activation and ITAM-bearing receptors or adaptors (18). Interestingly, FcRs have confined mobility in the plasma membrane, in fenestrated cortical actin structures that depend on the activity of Src- and Syk-family kinases (19). Integrins or pattern recognition receptors, such as the scavenger receptor CD36, are potentially initiating Syk activation, leading to FcR increased mobility and engagement (8). However, further work is needed to define the molecular basis of integrin interplay with ITAM-dependent signaling.

SPATIOTEMPORAL ORGANIZATION OF IMMUNE AND PHAGOCYTIC RECEPTORS AND THEIR SIGNALING MACHINERIES

How early signals are elicited by antigen or phagocytic receptors engagement is still a matter of debate. One model proposed for TCR activation postulates that initial triggering is achieved when key inhibitory proteins, such as the tyrosine phosphatase CD45, are segregated away from the engaged TCR and the proximal tyrosine kinase Lck. This segregation is mainly driven by the size of membrane protein ectodomains. Indeed, the length of the TCR–pMHC pairs is relatively small (7 nm) compared to that of CD45 ectodomain (28–50 nm); hence, TCR engagement by pMHC induces the formation of areas of close juxtaposition of T cell and APC membranes from which phosphatases are excluded (20, 21). As a consequence, local activity of tyrosine kinases would be favored, leading to an increase in net phosphorylation of TCR downstream effectors and T cell activation. Interestingly, a similar mechanism was observed during Dectin-1-dependent phagocytosis, leading to the "phagocytic synapse" model. Indeed, Dectin-1 engagement by β-glucan-bearing particles results in local exclusion of phosphotyrosine phosphatases CD45 and CD148 from receptor-enriched areas containing phosphotyrosine, thus triggering downstream signaling (e.g., Syk phosphorylation) and phagocytic cup formation (2). Importantly, several results suggest that this mechanism also concerns FcRs (22, 23); hence, it may be relevant for all phagocytic receptors.

Concomitantly to initial kinase and phosphatase segregation, T cell receptor subunits, the tyrosine kinases Lck and ZAP70, and the adapters LAT and SLP76 associate into dynamic signaling complexes that nucleate at the periphery of immunological synapses and then migrate toward its center, where they concentrate or vanish (24–26). Interestingly, centripetal dynamics of signaling complexes at the immunological synapse and their concentration in the center is a regulatory mechanism that depends on actin and microtubule cytoskeleton and is meant to downregulate proximal TCR signaling (27–29). Various mechanisms have been proposed for TCR signal downregulation at the synapse. These include relocalization to membrane regions containing the tyrosine

phosphatase CD45 (28), internalization and degradation of TCR and signaling complexes (30–32), post-translational modification of signaling adapters leading to signalosome disassembly (33), or the extracellular release of vesicles containing TCR (34). Of note, in FcR-mediated phagocytosis, receptors are engaged sequentially in a receptor-guided, zipper-like advance of the membrane over the particle surface, and there is no evidence for a movement of the receptors toward the base of the phagocytic cup. Receptors are downregulated from the surface with the engulfment of the particle. Thus, the late events in the mature immunological synapse differ from those observed in phagosome completion and closure.

ACTIN AND MICROTUBULE CYTOSKELETON INTERPLAY

Signaling downstream of the TCR and phagocytic receptors leads to intense and transient actin polymerization that relies on the activation of Rho family GTPases (35). In T cells and phagocytes, Rho GTPase activation occurs to a large extent *via* tyrosine phosphorylation and activation of the Rac1 and Cdc42 guanine exchange factor (GEF) Vav (36, 37). In addition, Rac1 can be activated by other GEFs, including DOCK2, DOCK8, Tiam1, and Trio. DOCK2 is involved in Rac1 activation downstream of the TCR and in lymphocyte migration in response to chemokines. DOCK2 and DOCK8 physiological relevance has been underscored by the discovery of human-inherited immunodeficiencies caused by *DOCK2* or *DOCK8* gene mutations. B and T cells from these patients display impaired actin polymerization and migration in response to chemokines, as well as impaired lytic granule release by NK cells (38, 39). DOCK family proteins are also involved in phagocytosis as regulators of Rac1 (40).

In phagocytes, the pioneering description of the involvement of Rho family proteins initially led to the classification of type I phagocytosis implicating Rac1 and Cdc42 downstream of FcR and type II phagocytosis relying on RhoA downstream of CR3 (41). More recently, RhoG has been shown to act as regulator for both FcR and CR3-mediated phagocytosis (42). As RhoG is also critical for phagocytosis of apoptotic bodies (43), and for the nibbling of MHC-associated portions of APC membrane by T cells (3), it could well act as a still ill-defined "master regulator" in immunological synapse and phagosome formation. Dynamic studies by fluorescence resonance energy transfer (FRET) revealed different patterns of activation for Rac and Cdc42 downstream of FcR. Active GTP-Cdc42 is present at the tip of the advancing pseudopod where it colocalizes with polymerizing actin, while Rac1 activation is biphasic. GTP-Rac1 is induced at a low level early after particle binding and peaked at the time of pseudopod fusion (44). Cdc42 activation and phosphatidylinositol-4,5-bisphosphate PI(4,5)P_2 accumulation in the nascent phagocytic cup activate effectors among which the actin nucleation promoting factor (NPF) N-WASP that acts on the Arp2/3 actin nucleation complex. Rac1 is then essential for F-actin polymerization to complete extension and closure, through activation of another NPF, the WAVE complex. In CR3-mediated phagocytosis, RhoA is critical for the signaling to actin polymerization as it activates the Rho-Kinase (ROCK), the formin mDia1, and myosin II that are implicated in polymerization and contraction of F-actin around the particles (41, 45–47). The microtubules are important for this pathway, and CLIP1 (CLIP-170), a microtubule plus-end protein, is especially required for efficient recruitment of mDia1 downstream of CR3 and therefore for efficient phagocytosis (48, 49), showing crosstalk between microtubules and actin.

Immunological synapse formation and function require the coordinated activation of RhoA after initial LFA-1 clustering and Rac1 and Cdc42 activation downstream of the TCR (35). Active Cdc42 and its effector WASP are independently recruited to the synapse. WASP seems not to be necessary for broad actin polymerization at the synapse, but rather for the generation of dynamically polymerizing actin foci that facilitate PLCγ activation and calcium flux (50). Consistently, WASP is necessary for efficient IL2 production (51, 52). In contrast, WAVE2, Arp2/3, and the cortactin homolog HS1 are required for T cells to regulate actin polymerization at the synapse (53–55). In turn, actin dynamics is necessary for triggering and sustaining T cell activation (56). This occurs in various concomitant ways, including the regulation of T cell–APC conjugate formation *via* integrin clustering (57), the interplay between actin cytoskeleton regulators and the calcium second messenger (58), or the regulation of immunological synapse architecture and its interplay with the TCR signaling machinery (59). Finally, the formation of signaling microclusters around the synapse periphery and their convergence toward the center depends on actin dynamics and F-actin inward flows (24, 60).

Cortical actin-associated proteins, such as ezrin and moesin, play important roles in building an activation competent immunological synapse. These proteins connect the cortical cytoskeleton with membrane components. Thus, moesin supports CD43 exclusion from the center of the synapse, a mechanism proposed to remove the CD43-dependent steric inhibition and to facilitate synapse formation (61–63). Moreover, ezrin and moesin contribute to the architecture of the immunological synapse, cell cortex rigidity, and T cell activation as well as differentially regulate early and late activation events (64–67).

Microtubules are finely reorganized at the immunological synapse bringing the microtubule-organizing center (MTOC) close to T cell–APC contact (67–69). Microtubule polarization depends on TCR-induced signaling (70, 71) and the microtubule-driven molecular motor dynein (72). Interestingly, ezrin plays a critical role in driving the MTOC close to the synapse, in controlling microtubule network organization, and in signaling microcluster dynamics at the synapse. Ezrin does so *via* its association with the polarity regulator Dlg1 (67). Moreover, the actin-nucleating proteins Diaphanous 1 (mDia1) and formin-like 1 (FMNL1) are also necessary to polarize MTOC to the synapse (53). The involvement of ezrin and formins in MTOC polarization highlights that actin and microtubule network organization at the synapse are tightly connected. Microtubule stability modulated by the HDAC6 deacetylase is also regulated during immunological synapse formation and necessary for synapse formation and T cell activation (73). Actin and microtubule interplay is also

critical for T cell effector function, such as polarized secretion of helper cytokines, since it is necessary for Golgi complex polarization toward the APC (74).

As mentioned above, microtubule–actin interplay is also necessary for efficient phagocytosis (48). Of note, the MTOC has also been reported to be relocated at the site of phagosome formation (75), but given that multiple targets are often phagocytosed at the same time, how this applies to uptake in physiological situations is uncertain. Similarly, when a cytotoxic T cell is engaged in multiple contacts, the antigen-specific delivery of lytic granules occurs independently of centrosome positioning (76).

Microtubule dynamics and organization ensure the delivery of TCRs and signaling molecules to the synapse *via* recycling endosomes (77–79). Moreover, microtubules, together with actin flows, drive signaling microcluster centripetal movement at the synapse (67, 80). Therefore, microtubules drive the arrival and removal of TCRs and signaling molecules in a way to sustain and regulate TCR signaling at the synapse.

ACTIN DYNAMICS AND CLEARANCE

Actin polymerization is crucial to achieve efficient pseudopod extension and phagosome formation, but actin turnover and depolymerization is as important. This turnover, which occurs at the base of the phagocytic cup (81), is directly dependent on the hydrolysis of $PI(4,5)P_2$ (82), which is mediated by several effectors including phosphatases that hydrolyze $PI(4,5)P_2$, such as phospholipase C, PI3 kinase, and 5′ phosphatases, such as Inpp5b or oculocerebrorenal syndrome of Lowe (OCRL) (81, 83–85). In addition, the severing protein cofilin is recruited to the site of phagocytosis and its activity is regulated by LIM kinase (86). Interestingly, the presence of OCRL at sites of phagocytosis was shown to depend on vesicular recruitment of AP1 and EpsinR adaptors, which is under the unexpected control of the NF-kB signaling protein Bcl10 (81), showing how interconnected the signaling and trafficking events are. Inactivation of Rho GTPases is also achieved by several Rho GAP proteins, such as ARHGAP12, ARHGAP25, and SH3BP1, that are recruited under the dependence of PI3K and synergistically inactivate Rac and Cdc42 (87). Actin clearance from the base of the phagocytic cup, which is required for large but not small particle internalization (87), is then necessary for vesicles to make their way to the plasma membrane (81).

Actin clearance is also observed in immunological synapses, and it is thought to be important to facilitate vesicle fusion at the synapse, particularly in cytotoxic T cells, which destroy target cells by the polarized secretion of lytic granules (88). F-actin relocalization at the immunological synapse depends on $PI(3,4,5)P_3$ (89) and modulates cytotoxicity. Actin and $PI(4,5)P_2$ are cleared from the site of secretion, indicating a tight interplay between actin cytoskeleton reorganization and phospholipid second messenger at the synapse (68, 90).

Therefore, the reorganization of the actin and microtubule cytoskeleton is triggered by TCR and phagocytic receptors and is the key to maintain the structure and function of phagocytic cups and immunological synapses.

VESICLE TRAFFIC DURING PHAGOCYTIC CUP AND IMMUNOLOGICAL SYNAPSE FORMATION

Phagocytic cup formation generates membrane protrusions capable of engulfing particles of different sizes. Instead of a decrease in membrane surfaces after internalization of the phagosomes, an increase in cell surface was reported during phagosome formation using capacitance measurements (91). This is in agreement with the concept of membrane remodeling and "focal delivery" of intracellular compartments at the site of phagosome formation (92, 93). The requirement for focal vesicle fusion in optimal phagocytosis of large targets came from studies interfering with the fusion machineries composed of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs). These are membrane fusion regulatory proteins that form a tri-party complex composed of one vesicle (v)-SNARE and two target membrane (t)-SNAREs. SNARE complex formation helps bringing together the two membranes to facilitate their fusion. SNAREs act with various regulatory proteins, such as Rab GTPases, Munc proteins, and the calcium sensors synaptotagmins to bring together, dock, tether, and fuse vesicles with target membranes, either the plasma membrane or other vesicles (94). Several intracellular vesicular compartments have been implicated in focal recruitment and fusion concomitant with phagosome formation (95–97). These include recycling endosomes bearing the v-SNARE VAMP3 on their surface (98–100) and late endocytic compartments displaying the v-SNARE VAMP7 or lysosomes (101, 102). The endocytic compartments also harbor the adaptor proteins AP1 and EpsinR, both implicated in efficient phagosome formation, while the AP2 complexes and the clathrin-related endocytic machinery are not involved (81, 100). Interestingly, $VAMP3^+/AP1^+$ endosomes also partially colocalize with TNFα, a cytokine that is delivered at the site of forming phagosomes (103).

Similarly, different endosomal compartments and vesicle traffic regulators are involved in immunological synapse formation. These compartments differentially transport TCRs, the tyrosine kinase Lck, and the adapter LAT to the synapse by recycling these proteins back and forth between their plasma membrane location and endosomes. These endosomal compartments display different traffic regulators, such as Rab GTPases (i.e., Rab4, Rab8, Rab11, Rab27, and Rab35), transport proteins (i.e., MAL, intraflagellar transport proteins), or vesicle fusion regulators (i.e., VAMP3, VAMP7, Synaptotagmin-7, and Munc13) (77, 78, 104–108). The immunological synapse clusters the t-SNAREs SNAP23 and syntaxin 4 preparing the zone for active vesicle fusion activity. It is still a matter of debate whether vesicles transporting the signaling adapter LAT only dock and stay as subsynaptic vesicles (106, 109, 110) or fuse with the plasma membrane driving LAT clustering at the synapse (77, 78, 111, 112). The regulated exocytosis of vesicular compartments in T cells might also be important during the early stages of synapse formation when a large lamellipodium-like membrane structure is formed over the APC. Finally, vesicle traffic is important for T cell effector functions, such as polarized secretion of cytokines or cytotoxic granules in helper and cytotoxic cells, respectively (88, 113).

During phagosome formation, the recruitment of compartments and their fusion are regulated by small GTPases of the Rab and ARF families. Rab11, localized on the recycling compartments, is implicated in efficient phagocytosis (114–116). ARF6 was shown to be activated during phagosome formation and to control the delivery of $VAMP3^+$ recycling endosomes (99, 117, 118). Rab35 regulates actin-dependent phagosome formation by recruiting ACAP2, an ARF6 GTPase-activating protein (119), or by regulating the localization of Rac1 and Cdc42 (120). In addition, Rab11 and ARF6 activities might be coordinated *via* common effectors, such as the Rab11-FIP3/4/RIP/RCP (Rab-coupling proteins), also named arfophilins, which were implicated in phagosome formation and maturation (115). Rab31 (Rab22b) recruits the adaptor APPL2 that participates in PI3K/Akt signaling and phagosome completion (121). As Rab35 recruits the OCRL phosphatase during cytokinesis (122), it could also be implicated together with Rab5 (85) in OCRL recruitment during phagocytosis, although this has not been demonstrated. There are therefore multiple levels of regulation that implicate tight coordination between the signaling platforms and their subcellular localization, and further investigations are required to dissect them both in the context of the immunological synapse and the phagocytic cup.

CONCLUSION

Although we have largely progressed in our understanding of the mechanisms underlying the membrane and cytoskeletal reorganization that support phagosome and immunological synapse formation, there are still a number of issues that need further in-depth investigation. These issues may be different in the phagocytosis and the immunological synapse fields, but a comparison of the two systems may help solve these different questions faster. These include how some phagocytic receptors get engaged and the type of signals they generate? What is the phospholipid chemistry of each of the systems and its influence on cytoskeleton organization? What is the precise time and space organization of signaling complexes and vesicular compartments? Interestingly, we have recently described several examples of "*ménage à trois*" between receptor signals, vesicle traffic, and cytoskeletal structures in both processes; for instance, the involvement of the proinflammatory signaling pathway NFκB in the control of vesicle trafficking and actin clearance in nascent phagosomes *via* the signaling protein Bcl10 (81), or the orchestrated action of the TCR signaling machinery, the actin and microtubule cytoskeleton, and intracellular vesicle traffic in ensuring immunological synapse architecture and function in T cell activation and effector functions, such as polarized secretion of cytokines or cytotoxic granules (1). Collectively, the vast majority of data presented here emphasize the similarities between immunological and phagocytic synapses formation and suggest a possible evolutionary link between these two structures, whereby the phagocytic synapse of innate immune cells would be an ancestor of the immunological synapse in the adaptive immune system (123).

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FN, VDB, and AA contributed equally to this review.

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The Dendritic Cell Synapse: A Life Dedicated to T Cell Activation

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T-cell activation within immunological synapses is a complex process whereby different types of signals are transmitted from antigen-presenting cells to T cells. The molecular strategies developed by T cells to interpret and integrate these signals have been systematically dissected in recent years and are now in large part understood. On the other side of the immune synapse, dendritic cells (DCs) participate actively in synapse formation and maintenance by remodeling of membrane receptors and intracellular content. However, the details of such changes have been only partially characterized. The DCs actin cytoskeleton has been one of the first systems to be identified as playing an important role in T-cell priming and some of the underlying mechanisms have been elucidated. Similarly, the DCs microtubule cytoskeleton undergoes major spatial changes during synapse formation that favor polarization of the DCs subcellular space toward the interacting T cell. Recently, we have begun to investigate the trafficking machinery that controls polarized delivery of endosomal vesicles at the DC–T immune synapse with the aim of understanding the functional relevance of polarized secretion of soluble factors during T-cell priming. Here, we will review the current knowledge of events occurring in DCs during synapse formation and discuss the open questions that still remain unanswered.

Keywords: immune synapse, dendritic cells, actin cytoskeleton, polarized secretion, antigen presentation

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INTRODUCTION

Once in the lymph node, dendritic cells (DCs) have few hours to transfer the information gathered in the periphery to T cells. Live imaging of the lymph node unveiled that, depending on the inflammatory context and antigen density, T-cell activation can ensue from long-lasting interactions or by collection of signals from subsequent repetitive contacts (1). Recent technologies to visualize recruitment and activation of signaling molecules *in vivo* within individual DC–T contacts is beginning to shed light on the actual functional meaning of various types of intercellular encounters (2, 3). Nevertheless, our present understanding of the subcellular events underlying informational transfer at the synapse and signal decoding by T cells mostly comes from *in vitro* studies. As largely discussed in this topic, sophisticated analysis unveiled the mechanism by which the antigen-presenting cell (APC)-derived flow of information is turned into fine tuned T-cell activation. Less is known, instead, about the events that control efficient delivery of antigen-derived signals to T cells. Among APCs, DCs are uniquely potent in their ability to launch adaptive responses. They are composed of a complex network of different subsets that differentially control T-cell functions. The classical $CD8\alpha^+$ with the ontogenetically related tissue resident $CD103^+$ cells and the Cd11b subsets share some general principles, i.e., the ability to migrate from tissue to regional lymph nodes charged

with antigens, but are specialized in the activation of $CD8^+$ and $CD4^+$ T lymphocytes, respectively (4). Yet, this paradigm is still evolving and these distinctions may turn out to be less strict.

In the following paragraphs, the events that takes place on the DCs side of the immune synapse (DC-IS) refer mostly to studies conducted using DCs models, i.e., cells differentiated from monocytes in the human system or from bone marrow precursors in the mouse system and unfortunately do not yet take into account such complexity. Still, common themes have emerged that will certainly be translated into more physiological cell types in the near future.

SURFACE RECEPTORS

To simplify and categorize events occurring at the DC-IS, it is useful to proceed from the surface inward to the cytoplasm. Remodeling of the plasma membrane architecture and redistribution of the surface receptors have been documented in DCs from the earliest instance upon T-cell contact and even before the two cell bodies become adherent. Some conserved mechanisms to support intercellular interactions described in the neuronal synapse operate in DCs and are essential for contact formation, including semaphorins, plexins, and neuropilins (5). Plexin-1A, a receptor for class-3 semaphorins is highly expressed in mature DCs, cluster at the IS, and controls T-cell priming by regulating cytoskeletal remodeling, possibly *via* Rho activation (6, 7). A second receptor for semaphorins, neuropilin-1, is expressed on both sides of the synapse and can control DC–T encounter during priming and during instruction of regulatory T cells (8, 9). The way DCs interact with T cells is peculiar in terms of global geometry and microdomains organization, as compared with the synapse between B cells and T cells. Unlike B cells, DCs possess a highly dynamic membrane with projections such as veils and ruffles that are increased during the process of maturation (10, 11) and further modify when an approaching T cell is sensed by chemokine receptors (12, 13). In human DCs, a peculiar microvilli-like structure was shown to be the preferential site of association with T cells leading to multiple aggregates of TCR/CD28 signaling complexes in T cells, as opposed to the concentrically structured immune synapse formed by B cells (14). A multifocal synapse is frequently formed also in murine T cells interacting with DCs, further strengthening that antigen presentation by DCs is more dynamic than by B cell (14, 15). Mirroring TCR clustering, MHC class-II and costimulatory molecules such as CD86 are recruited at the DC contact area very early during initial DC–T scanning. This recruitment is driven by ICAM-1/3 interactions with LFA-1 *via*, a mechanism that depends on an intact actin cytoskeleton and the Vav1/Rac1 effectors (16). Delivery of MHC class-II at the plasma membrane depends on long tubules of late endosomal origin that contain MHC–peptide complexes and move directionally toward the contact zone. This process is conserved in murine and human DCs both during antigen presentation and cross-presentation (17, 18) and ensures continuous fueling of TCR ligands in the IS region. Other costimulatory molecules undergo spatial reorganization on the APC. CD40 clusters at the synapse in B cells during delivery of T-cell help and it is stored in intracellular vesicles and rapidly released upon contact with an allogeneic CD4 T cells in rat lymph DCs (19, 20). Mirroring CD40 clustering, CD40L in T cells is recruited at the IS with human DCs and this polarization is important for DCs–T cross-talk and T-cell induced DCs maturation as it triggers IL-12 production by DCs (21). T-cell-induced DC maturation provides an important demonstration of the mutual exchange that occurs between the two cells [reviewed in Ref. (22)]. A further costimulatory molecule CD70, a TNF-family member receptor with critical roles in T-cell priming, is concentrated at the DC-IS. Interestingly, CD70 is found in intracellular compartments that overlap with class-II positive vesicles, and this localization depends on the Invariant chain (Ii), suggesting a shared control of antigen peptides and costimulatory molecules transport to the synaptic area (23, 24). Similarly, ICAM-1 is recycled at the interaction zone through recycling compartments where it intersects MHC class-II molecules (25). ICAM-1 mobility on the DCs membrane is a critical factor controlling activation of T cells. Besides being the main adhesive force to stabilize the interaction, LFA-1 is important to modulate signaling from the TCR. Binding of LFA-1 to its ligand, in fact, enhances signaling for key TCR signal transduction molecules, such as PI3K, PLC-γ, MAP kinases, and SLP-76, reinforcing IL-2 production and T-cell proliferation (26–29). A recent study revealed that cytoskeletal remodeling during DC maturation causes a decrease in the lateral mobility of ICAM-1 in the membrane. Laterally confined ICAM-1 imposes forces on the interacting LFA-1 that promote ligand-dependent activation and increase T-cell priming (30).

SIGNALING MOLECULES

Underneath the plasma membrane and concomitantly to redistribution of membrane receptors, several events take place in the cytoplasm of DCs, suggesting that the DC-IS is an active signaling zone. Spinophilin is a PDZ domain-containing protein that is highly expressed in the dendritic spines of neurons where it controls interactions with the underlying cytoskeleton and with membrane trafficking proteins. Spinophilin was shown to be expressed in DCs and to cluster at the DC membrane in antigen-specific conjugates, and its depletion caused a strong inhibition of T-cell priming (31). This study did not address the consequences of spinophilin loss in terms of cytoskeletal proteins distribution and overall cell symmetry. It is likely that spinophilin participates in the network of PDZ domain-containing proteins regulating cell polarity, indicating the importance of asymmetric distribution of functional subdomains for antigen presentation.

Durable presentation of antigens to T cells, either *via* long-lasting contacts or cumulative interactions, is essential for efficient priming of T cells in lymph nodes (1). The life span of DCs is short, and it is therefore essential to control their fitness when they come to accomplish their ultimate task in the lymph node. Intriguingly, it was demonstrated that synapse formation provides antiapoptotic signals to DCs. Formation of the synapse induces recruitment and activation of the antiapoptotic protein Akt at the DC-IS followed by activation of prosurvival signals, including inhibition of FOXO and activation of NF-κB [(32) and reviewed in Ref. (33)]. Given the extensive remodeling of actin and microtubule cytoskeleton in DCs engaged in synapses (see

below), it is likely to expect that other large signaling complexes become selectively recruited and activated at the site of interaction. However, the membrane receptors primarily involved, the composition of these signaling platforms, and the hierarchy of their assembly still remains to be elucidated.

THE ACTIN CYTOSKELETON

All synaptic events, from intercellular contact formation and adhesion to signal transduction, are orchestrated by the actin and microtubules cytoskeleton, their upstream regulators and downstream effectors in a continuous feedback loop.

Soon after the discovery of the supramolecular activation cluster in T cells, researchers discovered that DCs actively participate in synapse formation by polarizing the actin cytoskeleton, unlike B cells. The causal link was strong as F-actin pharmacological inhibitors inhibited the T-cell priming capacity of DCs (34). Actin remodeling is controlled by a set of proteins that promote actin nucleation and polymerization, in turn controlled by Rho GTPases. Studies to decipher the molecular pathways involved in the control of actin remodeling in DCs showed, at first, an involvement of the actin-bundling protein, fascin. This protein is highly expressed selectively in DCs, upregulated during maturation, and necessary for DCs full antigen-presenting function (35). Few years later, we found that one essential mediators of actin remodeling during synapse formation in DCs is the small GTPases, Rac. Genetic deletion of Rac1 and 2 rendered DCs unable to establish productive interaction with T cells, leading to extremely inefficient T-cell activation (36). Two main classes of actin nucleating factors, formins and WASp family, involved, respectively, in the formation of long actin polymer elongation and branched actin networks were both shown to play a role in DCs migration and interaction with T cells. Deletion of mDia, a formin family member, inhibits DCs migration to LNs and decreases the capacity to establish long contacts with T cells and to induce their activation (37). WASp, expressed selectively in hematopoietic cells and target of mutations in Wiskott–Aldrich syndrome (38), controls various DCs functions, including the capacity to form stable interactions with T cells and the stability and structure of the synapse. *In vivo* two-photon microscopy analysis in the 3D environment of the lymph node showed decreased contact time between WASp null DCs and $CD8^+$ T cells. Closer inspection *in vitro* revealed a defect in accomplishing the complete cycle of movements required to establish a full contact. As a consequence, WASp null conventional DCs often undergo repetitive contacts of short duration, resulting in decreased T-cell proliferation and IFN-γ production (39). Similarly, CD4 T cells are inefficiently primed by WASp null DCs *in vivo* and *in vitro* (40). In this last work, Ca^{2+} signaling and clustering of signaling molecules were shown to be decreased in T cells interacting with mutant cells, indicating that failure to sustain proximal events represents an important contribution to the overall immunodeficiency in WAS. Recently, a novel cell-free system to mimic the T-cell surface was used to address the role of WASp at the DC-IS with increased resolution. The results show that a spatially organized structure containing MHC-II, ICAM-1, and actin forms at the DC-IS, and it is stabilized in a WASp- and Arp2/3-dependent manner (41). It would be interesting to investigate whether WASp is involved in controlling the lateral mobility of ICAM-1, a parameter recently emerged to be critically during antigen presentation by DCs (30). Interestingly, the function of WASp goes beyond the control of motility and signaling at the IS as it also acts in tuning innate responses by toll-like receptors in plasmacytoid DCs (42).

The role of actin and actin regulatory proteins involved in synapse formation in DCs is summarized in **Figure 1**. Despite these insights, the triggers and the players of actin remodeling during synapse formation in DCs are not yet understood in sufficient details. As shown in a recent elegant study, DCs switch between different actin nucleating machineries during the process of migration and antigen uptake (43). What type of actin machinery prevails during antigen presentation at the IS and how the switch to this third DCs' specific function is achieved remains an interesting open question.

POLARITY AND SECRETION ON THE APC SIDE OF THE IMMUNE SYNAPSE

Polarity is a highly conserved mechanism to spatially organize different functions within a cell, common to many processes, including the regulation of immune cells functions. The importance of polarity is well characterized in T cells where centrosome polarization in CTLs and T-helper cells is a fairly well-understood mechanism to optimize release of cytotoxic granules and cytokines, respectively (44–46). In B cells, centrosome polarization at the synapse is a mechanism to orient the secretion of

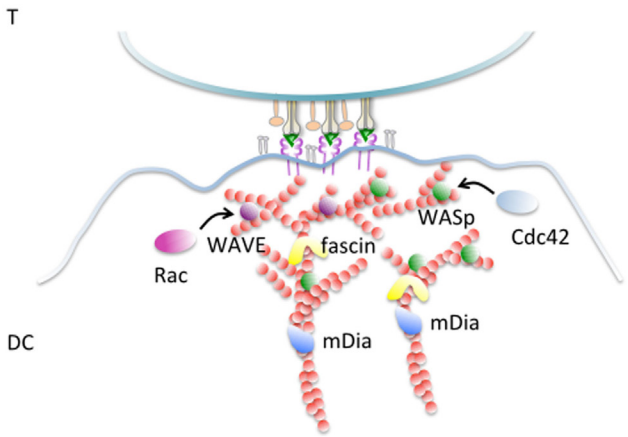

FIGURE 1 | Actin remodeling at the DC-IS. F-actin filaments become polarized and cluster enriched at the junction with an antigen-specific T cells. Fascin, an actin-bundling protein implicated in extension of membrane protrusion and development of dendrites is enriched in this area and controls the capacity of DCs to activate T cells. The Rho GTPase Rac is required for synapse formation and T-cell activation, likely *via* WAVE-mediated actin remodeling. WASp is a further activator of the Arp2/3 complex that is essential to support the antigen-presenting activities of DCs by promoting actin branching, thereby stabilizing the synaptic structure. WASp activation is mediated by cdc42 that is also found enriched at the DC-IS. The formin family mDia, involved in elongation of long actin filaments is important to establish and stabilize DC–T contacts and to support T-cell activation.

lysosomes and to promote the extraction of immobilized BCR ligands (47). B cells were shown to polarize also in the context of a B–T cell synapse with both the centrosome and the Golgi apparatus reoriented toward the interaction zone (48).

Many evidences exist that cell polarity and polarized membrane trafficking establishes also in DCs upon formation of antigen-specific conjugates. For instance, tubules of MHC class-II emanating in the direction of the interacting T cell indicate that remodeling of membrane trafficking is induced by antigen-specific contacts (17). The ability to polarize the tubules is acquired upon signals from toll-like receptors and depends on microtubules integrity (49, 50). DCs contacting NK cells were shown to polarize cytokines in the synaptic cleft, further suggesting directional vesicular transport (51, 52). Indeed, it was formally demonstrated that DC, following induction by a microbial maturation stimuli, polarizes the microtubule-organizing center (MTOC) toward the DC–T interface (53). This process depends on cdc42 as genetic deletion of this key polarity protein hampers the ability of DCs to reposition the centrosome underneath the synaptic membrane. Cdc42 is indeed highly enriched at the contact zone in DCs indicating its possible role as a WASp activator as well. Vesicles carrying newly synthesized interleukin 12, a key mediator of T-cell priming, clustered around the Golgi and were rapidly repositioned at the DC-IS, resulting in polarized secretion and local induction of IL-12-dependent signaling in T cells (53) (**Figure 2**). Thus, similarly to what have been shown in T cells, polarized transport of soluble mediators is a fundamental process that contributes to the antigen-presenting properties of DCs as ablation of cdc42-mediated polarization inhibits T-cell proliferation (53). The notion that the so-called signal 3 (proinflammatory cytokines) can be locally coupled to antigenic and costimulatory signals, and it can be delivered in *cis* may have important consequences for the fate imprinted to different T cells that interact sequentially with the same APC.

These findings raise the question of the link between microtubules and membrane trafficking proteins that support the directional transport of mediators en route to exocytosis. In macrophages and in T cells, members of the SNARE and Rab family were shown to control selectively directional secretion of various cytokines (44, 54). In DCs, we have recently discovered that the SNARE VAMP-7 plays a key role in the intracellular trafficking and secretion of newly synthesized IL-12. VAMP-7 not only controls the multidirectional transport of the cytokine toward the plasma membrane but, most importantly, it is absolutely required for clustering and secretion of IL-12 at the DC-IS (55). These findings reveal an usual transport route *via* late endosomes and represent the first insight into the molecular mechanism that orchestrate trafficking and secretion of soluble factors in DCs.

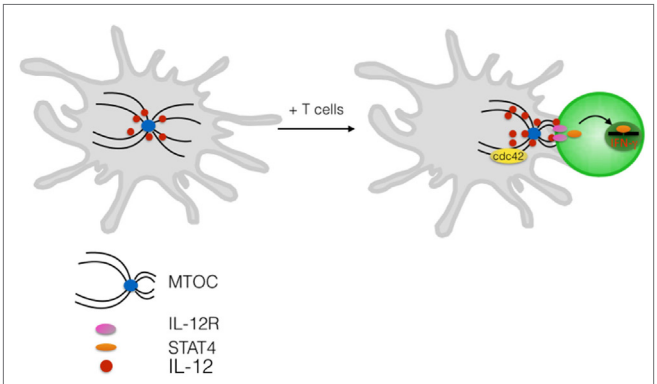

FIGURE 2 | Polarized delivery of cytokine at the DC-IS. Upon contact with antigen-specific T cells DCs reorient the centrosome toward the interacting T cell. Polarization depends on the Rho GTPase cdc42. Newly synthesized IL-12 is contained in intracellular vesicles that cluster in the Golgi area and are readily redistribute at the IS upon antigen-specific contact formation. Release of IL-12 in the synaptic cleft induces activation of STAT4 and triggering of IFN-γ in the interacting T cell.

CONCLUSION

Both synaptic partners contribute to the successful outcome of the intercellular interaction between an activated DCs and a naive T cell, i.e., T-cell activation.

It is becoming obvious that a complex cross-talk between the two cells exists and that understanding T-cell activation cannot do without a deep understanding of the DCs synapse. Further efforts are envisaged in at least two directions. The first is the systematic dissection of the receptors and the downstream effectors operating sequentially in DCs and their functional significance. This goal will be achieved, thanks to the recent development of cell-free system to reconstitute the T-cell membrane. Second, to increase physiological relevance, future studies should take into account the complexity of the DCs system, which is composed of various DCs subsets that are most likely equipped with different tools to transmit information to their partners.

AUTHOR CONTRIBUTIONS

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

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Dynamic Regulation of TCR–Microclusters and the Microsynapse for T Cell Activation

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The interaction between a T cell and an antigen-presenting cell is the initiating event in T cell-mediated adaptive immunity. The Immunological Synapse (IS) is formed at the interface between these two cell types, and is the site where antigen (Ag)-specific recognition and activation are induced through the T cell receptor (TCR). This occurs at the center of the IS, and cell adhesion is supported through integrins in the area surrounding the TCR. Recently, this model has been revised based on data indicating that the initial Ag-specific activation signal is triggered prior to IS formation at TCR–microclusters (MCs), sites where TCR, kinases and adaptors of TCR proximal downstream signaling molecules accumulate as an activation signaling cluster. TCR–MCs then move into the center of the cell–cell interface to generate the cSMAC. This translocation of TCR–MCs is mediated initially by the actin cytoskeleton and then by dynein-induced movement along microtubules. The translocation of TCR–MCs and cSMAC formation is induced upon strong TCR stimulation through the assembly of a TCR–dynein super complex with microtubules. The Ag-specific activation signal is induced at TCR–MCs, but the adhesion signal is now shown to be induced by generating a "microsynapse," which is composed of a core of TCR–MCs and the surrounding adhesion ring of integrin and focal adhesion molecules. Since the microsynapse is critical for activation, particularly under weak TCR stimulation, this structure supports a weak TCR signal through a cell–cell adhesion signal. The microsynapse has a structure similar to the IS but on a micro-scale and regulates Ag-specific activation as well as cell–cell adhesion. We describe here the dynamic regulation of TCR–MCs, responsible for inducing Ag-specific activation signals, and the microsynapse, responsible for adhesion signals critical for cell–cell interactions, and their interrelationship.

Keywords: immunological synapse, microcluster, microsynapse, dynein, cSMAC, cytoskeleton, F-actin, LFA1

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TCR–MICROCLUSTERS AND THE IMMUNE SYNAPSE

T Cell Activation Signaling through TCR–Microclusters

Acquired immunity is exemplified by antigen (Ag)-specific responses, which are initiated by specific recognition of Ag by T or B cells. In the case of T cells, an Ag-specific cell encounters and interacts with Ag-bearing dendritic cell (DC) in the draining lymph node and uses its T cell receptor (TCR) to recognize the Ag peptide–MHC complex on the DC. This interaction induces an Immunological Synapse (IS) at the interface between the T cell and DC. The initial finding of this structure by

Kupfer's group was mainly based on microscopic visualization of the *z*-axis of the interface using advanced microscopy and deconvolution. They observed that the interface had a specifically segregated bulls-eye structure with a centralized TCR (with PKCθ) surrounded by the integrin LFA-1 (with Talin) (1). This segregation was achieved by accumulation of TCRs and adhesion molecules together with certain signaling molecules. Thus, the structure appeared to be related to T cell activation, and they termed the structure, the Supra Molecular Activation Cluster (SMAC), the central area for TCR accumulation as the cSMAC, and the peripheral LFA-1 accumulated area as the pSMAC. The initial analysis already noted the size difference of molecules in the c- and p-SMAC, i.e., that smaller molecules with one or two Ig domains in their extracellular region tended to accumulate in the cSMAC, while larger molecules such as integrins or CD45 accumulated in the pSMAC or distal dSMAC surrounding the cSMAC (2). These observations led to the segregation kinetics model of T cell activation (3), which proposes that the segregation in the periphery of large sized phosphatases such as CD45 from the central region of TCR engagement allows for activation of Lck kinase, followed by induction of the initial downstream signals for T cell activation (4).

The mature IS, supported by cellular adhesion through LFA-1, was thought to be an ideal structure for inducing an activation signal upon Ag recognition through the TCR. However, it was noticed that IS formation can be variable, depending on the cell types and stimulation conditions; some T cells do not form IS, nor do T cells with B cells rather than DC as antigen-presenting cell (APC) (5, 6). It was also proposed that only strong stimulation induced cSMAC formation (these situations are discussed later in terms of their relationship with the microsynapse). Furthermore, since the generation of the cSMAC, even on a supported planar bilayer, took about 10 min after interaction of the TCR and pMHC, it was noted that this would be too late for triggering the initial TCR signals (7). Analysis of very early activation after the interaction of Ag-specific T cells and a planar bilayer containing specific Ag peptide–MHC revealed that the TCR begins clustering immediately after T cells recognize the peptide–MHC on the planar bilayer, prior to mature IS formation. We stated to call these initial clusters TCR–microclusters (TCR–MC) as a minimal unit of clusters mediating both initial and sustained TCR signaling (8–12). MCs were described by Krummel and Davis as small clusters of CD3ζ accumulating at the center of the interface upon stimulation, and which were synchronized with the calcium response (13, 14). Quantification analysis of the TCR–MCs revealed that each cluster contains approximately one hundred (50–300) TCR molecules. This TCR accumulation immediately upon peptide/MHC stimulation was found to be associated with the simultaneous accumulation of the kinase ZAP70 and adaptor proteins LAT and SLP76 in the same TCR–MC. Upon stimulation, every TCR–MC is stained by Abs against phospho-ZAP70, phospho-SLP-76, and phospho-tyrosine. Thus, a TCR–MC is generated by accumulation of a hundred TCR–CD3 complexes, kinases and adaptors and induces immediate phosphorylation of these molecules. TCR–MCs also contain a substantial quantity of the known proximal signaling intermediates including ZAP70, LAT, SLP-76, PLCγ, and cytoskeleton-related molecules Nck and Vav (15, 16), which further induce triggering of a Ca^{2+} flux and activation of downstream effector molecules. TCR–MCs are generated first at the center of the interface between the T cell and the planar bilayer or APC, and then are increased over the entire interface as the T cells spread. The initial activation signal is therefore induced in the newly generated TCR–MCs on the cell surface. Regarding the relationship of TCR–MC and the IS, TCR–MCs move toward the center of the interface after maximum cellular spreading, and the accumulated TCRs generate the cSMAC of the IS. It was noticed that only the TCR–CD3 complexes move to the center to form the cSMAC, while other signaling molecules such as ZAP70 and SLP-76 move only a short distance toward the center but do not accumulate in the cSMAC. These molecules disappear during their transport toward the center, probably by endocytosis. It has been noticed that TCR–MCs do not generate a cSMAC in some T cells, such as thymocytes and hybridomas, or under certain conditions, including weak Ag stimulation. However, even under conditions without cSMAC formation, T cells generate TCR–MCs to induce activation signals.

Signaling clusters induced upon TCR stimulation had been previously demonstrated when Jurkat cells were stimulated by immobilized anti-CD3 Ab (15). In this situation, TCR-CD3 appeared to be fixed and immobilized on the cover slip, but clusters of LAT, SLP76, and PLCγ, which induce the phosphorylation and activation of downstream signaling molecules, were generated. In this system, distal signaling intermediate molecules become dissociated from the immobilized TCR and move to intracellular compartments; SLP76 moves to a perinuclear structure and Nck and WASP to an actin-rich compartment and the immobilized TCRs do not move to the center or make the cSMAC (15, 16). Although there are some differences between pMHC-induced TCR–MC in normal T cells and Ab-stimulated signaling clusters in Jurkat cells, a general common feature is that, prior to the IS formation, TCR–MCs composed of TCR-CD3, kinases and adaptors are generated at the interface upon Ag recognition, which induces the initial signal for T cell activation. Later, though depending on stimulation conditions, the TCR–MCs move to the center of the interface to generate the cSMAC of the mature IS.

Recent imaging analysis using super-resolution microscopy as well as EM analysis revealed that several molecules of TCR or LAT are pre-clustered prior to Ag stimulation as "nanoclusters," which are then assembled together upon stimulation to form a MC (17, 18). In this regard, it is noted that the dynamics of signaling molecules within TCR–MCs are not uniformly regulated, and the signaling components within the cluster are variable and dynamic in their behavior.

A transient initial activation signal is not sufficient for full activation of T cells to induce cytokine production and cell proliferation, instead sustained activation for several hours is at a minimum required (19). Not only initial activation but also sustained continuous activation is induced through TCR–MCs at the peripheral region of the interface (9). TCR–MCs are continuously generated at the cellular edge with lamellipodial structures and move inward to the cSMAC. When the generation of the peripheral MCs is interrupted by the addition of anti-pMHC Ab, the formation of peripheral TCR–MCs is immediately halted, but the cSMAC is maintained (10). Moreover, the blockade of newly

generated TCR–MCs functionally inhibited activation signals. Therefore, the continuous generation of TCR–MCs is critical for inducing sustained activation signals.

Compartmentalization of TCR Signaling at the IS

The cSMAC, as the representative structure of the original description of the IS, has several specific functions: (a) increasing the cell adhesion between the T cell and APC. Since the affinity of the individual TCR and pMHC interaction is so low, on the order of 10^{-4}M (20), the accumulation of thousands of TCRs increases the avidity for pMHC for cellular adhesion between the T cell and APC, although the adhesion is mainly supported by integrin binding in the pSMAC. (b) directing the targeted secretion of cytotoxic granules and cytokines toward APC (21, 22). (c) serving as the site for endocytosis and/or exocytosis (23, 24) of the TCR complex, which functions to negatively regulate T cell activation. (d) inducing co-stimulation signals as described below.

In the case of the IS formed between cytotoxic T cells and target cells, the cSMAC area is further segregated into two functional domains, a signaling domain through the TCR and a secretory domain, through which cytotoxic granules are secreted onto target cells (23, 25). These functional domains are present even in $CD4^+$ T cells.

Because the TCR/CD3 complex accumulates in the cSMAC, whereas upon Ag recognition, the majority of the downstream kinases and adaptors do not (9, 10), and little phosphorylation of these signaling molecules was observed in the cSMAC, it is thought to be inactive in signaling. Rather, the cSMAC is thought to be responsible for endocytosis and degradation of the TCR, which consequently contributes to negative regulation of T cell activation by decreasing the TCR complex. Indeed, a large invagination of the TCR was observed (26), and endocytic/degradation machinery such as TSG101 (27) and a lipid for multivesicular body formation for degradation, lysobisphosphatidic acid (LBPA), is assembled in the cSMAC (10), indicating that the TCR complex is endocytosed at the cSMAC and targeted for degradation. In contrast to its function in TCR endocytosis, it was recently reported that vesicles containing the TCR are secreted from the cSMAC (24). Thus, the contribution of endocytosis vs. exocytosis of TCR-containing vesicles in the cSMAC has to be better understood. When the cSMAC area was precisely analyzed by microscopy, two distinct areas were found – $CD3^{hi}$ and $CD3^{lo}$ (**Figure 1**) Bleaching experiments revealed that the $CD3^{hi}$ area is rigid whereas the $CD3^{lo}$ area is very mobile and dynamically regulated. Using planar membranes containing MHC class II with covalently linked peptide, the $CD3^{lo}$ area but not the $CD3^{hi}$ region was found to be associated with pMHC, suggesting that the $CD3^{lo}$ region is actively participating in binding to pMHC, but the $CD3^{hi}$ region may contain TCR complexes that are either in the process of dissociation from pMHC or have already done so and are on the path to endocytosis and/or exocytosis (28, 29).

In contrast to these data showing that the cSMAC is in general a negative regulatory site through TCR endocytosis/degradation, imaging analysis of co-stimulation signals indicated that a sustained co-stimulation signal is induced through a part of the cSMAC (30, 31). We demonstrated that upon Ag stimulation, the positive co-stimulatory receptor CD28 is first co-localized in the peripheral TCR–MC (recall that a co-stimulation signal is induced through the TCR–MC in the initial phase of activation) and then moves to and later accumulates in the cSMAC region, particularly in the $CD3^{lo}$ area (we call this area the "signaling cSMAC") (**Figure 1**). A search for the molecules whose behavior is similar to CD28 identified PKCθ and CARMA1, which also accumulated in a similar region of the cSMAC. A CTLA4–Ig fusion protein is used to inhibit CD28 co-stimulation since it binds more strongly than CD28 to the common ligand CD80/86. Addition of CTLA4–Ig blocks the association between CD28 and its ligands and results in no accumulation of CD28 in the signaling cSMAC. At the same time, PKCθ was also no longer found in the cSMAC, indicating that CD28 recruits PKCθ to the signaling cSMAC, probably to mediate co-stimulation. Using a biochemical approach, CD28 was found to be physically associated with PKCθ through association with Lck. The V3 region of PKCθ binds to the SH3 region of Lck and the SH2 region of Lck binds to the proline-rich region of CD28 (32). The accumulated CD28 recruits PKCθ and then CARMA1 into the $CD3^{lo}$ signaling cSMAC region, where sustained co-stimulation signals are induced, including NF-κB activation.

The analysis of TCR–MC and the cSMAC has revealed spatially distinct signaling compartments within a single T cell. These might be structural correlates corresponding to the old idea that both signal 1 and signal 2 are required for full T cell activation, i.e., TCR-induced Ag-recognition signal as signal 1 is mediated through the TCR–MC whereas the CD28-induced sustained co-stimulation signal as signal 2 is mediated through the signaling cSMAC (**Figure 1**). Recently, an actin-uncapping protein Rltpr was shown to be essential for CD28-mediated co-stimulation, a finding that connects CD28 and PKCθ/CARMA1 (33). Rltpr is also localized in the same signaling cSMAC upon TCR stimulation, where it may mediate the co-stimulation signaling function.

Negative regulation of T cell activation by the inhibitory co-stimulation receptor CTLA-4 is also induced at the same cSMAC region. Because CD28 and CTLA4 share the same ligands CD80/CD86 and CTLA4 has a much higher (20-fold) affinity for these ligands, even low expression of CTLA4 on the T cell surface can compete with CD28 for ligand binding, which is the major mechanism for CTLA4-mediated inhibition (34, 35). CTLA-4 mostly accumulates in the intracellular secretory lysosomes and, upon TCR stimulation (36), it moves toward the plasma membrane at the cSMAC, particularly to the $CD3^{lo}$ signaling cSMAC, the same region where CD28 accumulates. Accumulated CTLA4 at the signaling cSMAC locally competes with CD28 for ligand binding. Therefore, CTLA-4-mediated inhibition is induced by ejecting the CD28-PKCθ–CARMA1 signaling machinery from the signaling cSMAC (11).

Thus, the current view of the cSMAC has evolved. It is not merely a site for negative regulation through TCR endocytosis/degradation, but instead a particular region within the cSMAC is the site for inducing activation signals and is also a regulatory site by virtue of its inhibitory co-stimulation.

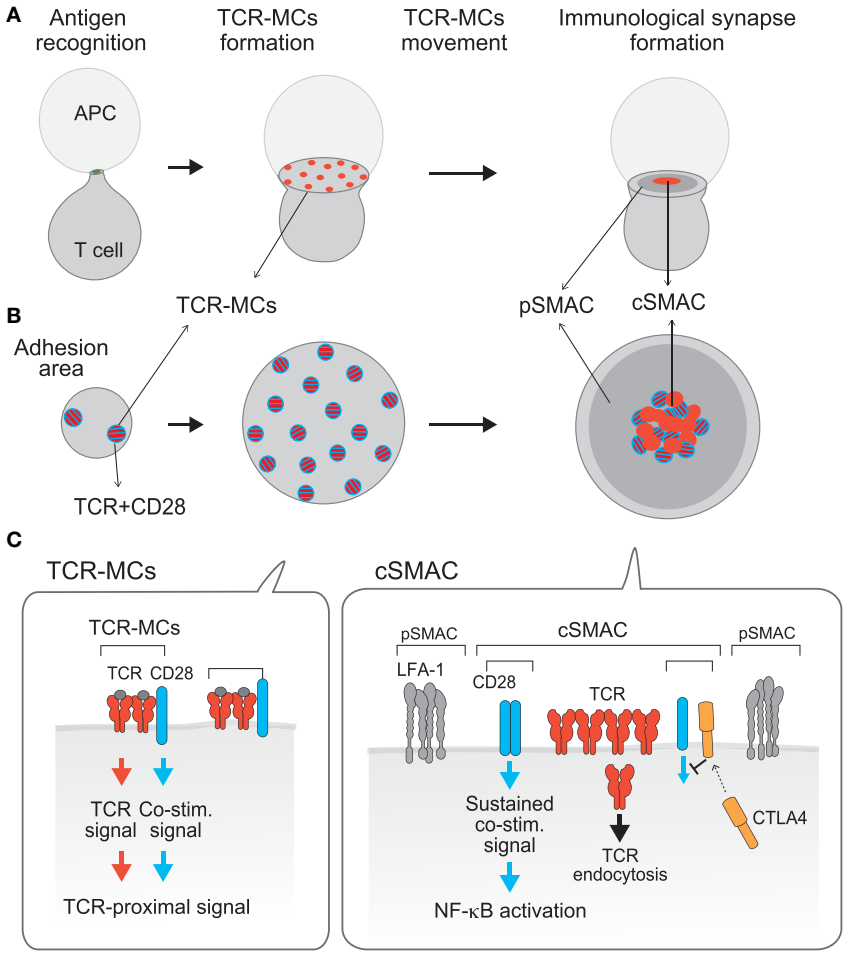

FIGURE 1 | Compartmentalization of TCR activation signals: TCR–microcluster and cSMAC. [**(A)**, *x–y* axis; **(B)**, *z*-axis; **(C)**, a model for the assembly on the membrane] Upon Ag recognition, T cells form a conjugate with the APC (left) and generate TCR–microclusters (MCs) at the interface with the APC. TCR–MCs contain TCRs (red) and the proximal signaling molecules as well as the CD28 co-stimulation receptor (blue), and induce the initial activation signal (middle). After maximum spreading, TCR–MCs begin to move toward the center of the interface to form the cSMAC (right). It was noted that there is a $CD3^{hi}$ region (red) and a $CD3^{lo}$ region (mixture of red and blue) within the cSMAC; the $CD3^{hi}$ region is rigid and may represent the site for TCR endocytosis, whereas the $CD3^{lo}$ region is dynamically regulated and various costimulation molecules as CD28 and CTLA-4 are co-localized. Thus, we named this $CD3^{lo}$ region the "signaling cSMAC." In the cSMAC, the TCR complex is subjected to endocytosis/degradation for negative regulation, whereas CD28 recruits PKCθ and CARMA1 to induce a sustained co-stimulation signal leading to downstream events such as of NF-κB activation. The inhibitory co-stimulation receptor CTLA4 is translocated to the same cSMAC area as CD28 and competes with CD28 to eject CD28-PKCθ from the cSMAC, resulting in inhibition of activation. Thus, the TCR activation signal is regulated by spatially distinct signals: The Ag recognition signal as "Signal 1" is mediated by TCR–MCs and a sustained co-stimulation signal as "Signal 2" is mediated by the signaling region of the cSMAC.

CYTOSKELETAL REGULATION OF TCR–MICROCLUSTER MOVEMENT

When T cells are stimulated with different concentrations of peptide/MHC on a planar bilayer, the cSMAC is formed with relatively high concentrations of antigen (>1 μM) but cannot be formed with low concentrations (<10 nM). Stimulation with low doses of Ag induces TCR–MCs, but they do not move toward the center of the interface and do not form the cSMAC. Considering that the cSMAC negatively regulates T cell activation through TCR endocytosis/degradation, weak stimulation to trigger weak signals may not require such an inhibitory mechanism. In contrast, upon strong stimulation with high doses of Ag, TCR–MCs move to and accumulate in the center, generating a cSMAC. Therefore, the movement of TCR–MCs is regulated by activation signal strength. At the steady state before stimulation, the TCR forms small clusters on the cell surface consisting of a few to ten molecules as "nano-clusters" (17, 37), as described above. Some signaling molecules such as LAT have been shown to be in nano-clusters distinct from the TCR. However, upon TCR stimulation, these distinct nano-clusters begin to form larger clusters by coalescing with signaling molecules such as LAT (17). These coalesced clusters are likely to be equivalent to TCR–MCs as a signaling unit. The size of TCR–MCs to be translocated centripetally into the cSMAC should be minimum (38).

The translocation and function of TCR–MCs is dependent on the actin cytoskeleton. Upon TCR stimulation, the actin cytoskeleton dynamically changes the cell morphology to promote centripetal flow at the periphery (39, 40). Inhibition of actin polymerization at the initial stage of T cell activation resulted in blockade of T cell adhesion, generation of TCR–MCs and activation. Addition of the actin inhibitor during the initial formation of TCR–MCs inhibits the generation of additional TCR–MCs, and consequently inhibits activation signals (8, 10). F-actin is initially generated upon TCR activation as a distal ring at the peripheral edge of the cell along with cell spreading, and then forms a large peripheral ring. In addition to this distal lamellipodial ring, small foci of F-actin have been found in co-localization with TCR–MCs (41). The peripheral actin exhibits retrograde flow toward the center of the interface. The new TCR–MCs, which are generated at the lamellipodial edge in a random manner upon interaction with peptide–MHC, then start to move toward the center, coincident with the actin retrograde flow (42). Since the interaction of TCR–MCs and actin appears to be weak and the actin centripetal flow is faster than the movement of TCR–MCs, TCR–MCs may be propelled by transient linkage to the actin retrograde flow (42, 43). However, the actin retrograde flow can only reach to about the middle of the path to the center, and the central/peripheral areas are free of actin (44). This raises the question of how are TCR–MCs translocated further to the cSMAC? We found that TCR–MCs translocate further into the central region along microtubules by assembly with the microtubule-associated motor protein dynein (45) (**Figure 2**). Dynein generally transports various cellular cargos by walking along cytoskeletal microtubules toward the minus-end of the microtubule. Indeed, we could co-immunoprecipitate the dynein–dynactin complex with the TCR complex upon TCR stimulation. When T cells were treated to (a) down-modulate dynein expression by siRNA-mediated knockdown, (b) inhibit dynein kinase activity, or (c) inhibit microtubule formation, TCR–MCs failed to move to the center and did not form the cSMAC. Consequently, these treatments resulted in augmented activation signals, resulting in enhanced phosphorylation of downstream signal molecules, such as SLP76, Vav and Erk, and elevated cytokine production. The finding that inhibition of cSMAC formation resulted in augmented activation indicates that the cSMAC functions as negative regulator, as previously shown similarly in CD2AP-deficient mice (46).

T cell receptor stimulation induces two events in relation to dynein-mediated translocation of TCR–MCs; one is the assembly of the TCR complex with the dynein/dynactin complex, and the other is the translocation of the MTOC (microtubule organization center, or centrosome) to the vicinity to the engagement site on the membrane at the interface. Kinetic studies revealed that MTOC translocation takes place first, followed by the translocation of TCR–MCs (45). Thus, TCR–MCs move along the microtubules, which are localized close to the plasma membrane after the MTOC moves to the site of TCR engagement. The TCR complex is assembled with the dynein complex and associates with microtubules after the MTOC and microtubules become

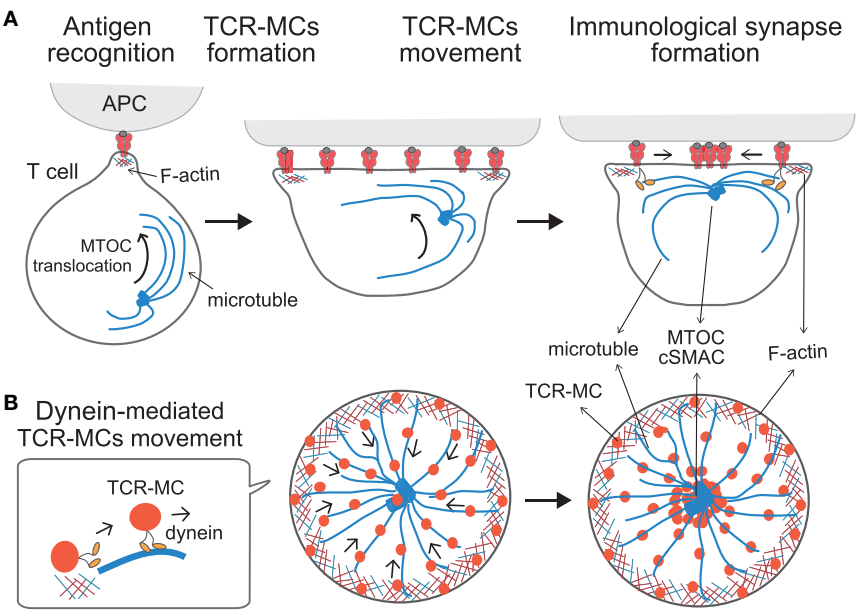

FIGURE 2 | Translocation of TCR–microclusters through cytoskeletal regulation. (A,B) depict the *x–y* axis and the *z* axis, respectively. Upon Ag recognition, T cells generate TCR–MCs (red) all over the interface. During this time, the MTOC (blue dot) is quickly translocated to the vicinity to the plasma membrane and finally to the TCR engagement site. Initially, TCR–MCs generated in the peripheral area move toward the center, coincident with actin retrograde flow (mesh structure). Thereafter, TCR–MCs are translocated to the cSMAC along the microtubules (blue line), which are translocated together with the MTOC into close proximity to the membrane in a dynein-mediated manner. The TCR/CD3 complex associates with the dynein–dynactin complex upon TCR stimulation, and then further assembles with microtubules. The dynein-mediated translocation of TCR–MCs regulates T cell activation because blockade of microtubules or dynein function prevents cSMAC formation and enhances T cell activation.

localized to the membrane. This interplay leads the movement of the TCR–MCs toward the center along microtubules in a dynein-mediated fashion, thus generating the cSMAC. Therefore, the translocation of TCR–MCs is regulated cooperatively both through F-actin retrograde flow initially, and then later by dynein-mediated movement along microtubules, ultimately leading to formation of the cSMAC (**Figure 2**).

The translocation of the MTOC to the interface of the TCR engagement site is regulated by TCR signals upon pMHC stimulation (22, 47, 48). TCR engagement upon triggering with weak stimulation induces neither MTOC translocation nor the translocation of the TCR–MCs to generate the cSMAC. Such a weak stimulatory signal, which is induced at the TCR–MCs, may not need negative regulation at the cSMAC.

MICROSYNAPSES SUPPORT ADHESION AND SIGNALING

Ag recognition and subsequent activation of T cells requires strong contact and adhesion with APC for a certain extended time period to induce full activation. Because the affinity of the TCR–pMHC interaction is very low, Ag recognition by the TCR is supported by strong cellular adhesion through specific adhesion molecules, particularly the integrin LFA-1 binding to its ligand ICAM-1/ICAM-2. The TCR-induced activation signal and the LFA1-mediated adhesion signal are mutually regulated. The TCR signal induces a specific LFA-1 conformational change that results in high affinity binding to the ligand, a process known as "inside-out signaling" (49, 50). This inside-out signal involves the activation of SLP76, ADAP, RIAM, and Rap1/RapL. Furthermore, the high affinity configuration of LFA-1 is acquired through an LFA-1-mediated downstream signal (51, 52), known as "outside-in signaling." This outside-in signal induces activation of kinases and clustering of SLP76/ADAP (53, 54).

In the mature IS, the cSMAC as the TCR-enriched central region is surrounded by LFA-1 at the peripheral region as the pSMAC, which forms a "bulls-eye" shaped structure. During the course of IS formation, the cSMAC is generated by the translocation of peripherally induced TCR–MCs into the center of the interface. Then how is LFA-1 accumulated into the pSMAC? We found that LFA-1, as well as focal adhesion molecules represented by Pyk2, Paxillin, and vinculin, accumulate just around the TCR–MC and form a kind of "adhesion-ring" in micro scale during the very initial stage of T cell activation (55). The formation of the micro adhesion ring is dependent on LFA1, because no adhesion ring is formed in the absence of the LFA1-ICAM1 interaction on a planar bilayer lacking ICAM-1. The micro adhesion-ring is induced transiently after the initial formation of TCR–MCs, and disappears before the TCR–MCs move to the center to form a cSMAC (**Figure 3**) (55). The bulls-eye shaped structure with the central TCR–MCs surrounded by the micro adhesion-ring of LFA1, Pyk2, and Paxillin resembles the structure of the mature IS, represented by the central TCR surrounded by LFA1, therefore we suggest naming this structure the "microsynapse" (**Figure 3**). In addition to LFA1 signals, microsynapse formation is totally dependent on F-actin, since inhibitors of both F-actin and Arp2/3 block the formation of the adhesion ring, but this treatment had

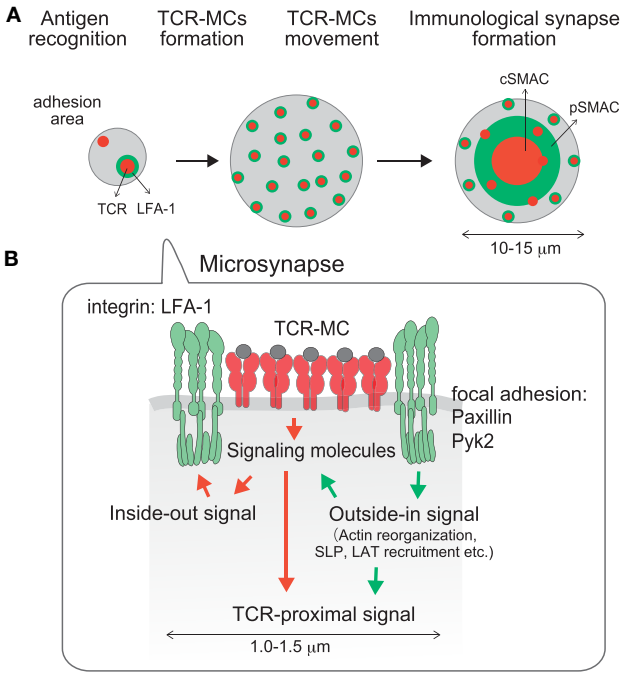

FIGURE 3 | A microsynapse composed of a core TCR–MC and a surrounding micro adhesion-ring. (A) time course of microsynapse generation, **(B)** molecular assembly in the microsynapse. Immediately after TCR–MCs are formed, an adhesion-ring composed of integrin LFA-1 and focal adhesion molecules such as Paxillin and Pyk2 is generated around the TCR–MC. Because the structure resembles the mature Immunological Synapse in a micro scale, this structure was designated the microsynapse. The adhesion-ring is dependent on LFA-1 outside-in signaling and is supported by F-actin and Myosin II. Cluster formation by LAT and SLP76, but not the TCR or ZAP70, is supported by the microsynapse. The adhesion-ring is a transient structure and disappears before cSMAC formation. The microsynapse is sustained upon weak TCR stimulation, whereas it disappears quickly upon strong stimulation, suggesting that it functions to augment the TCR activation signal upon weak stimulation.

no effect on TCR–MC formation. The involvement of Myosin II as an F actin-related effector molecule in microsynapse formation was also analyzed. Treatment with a Myosin II inhibitor reduced microsynapse formation while retaining TCR–MC formation. These observations all indicate that F-actin supports the formation and function of the microsynapse. The functional importance of the microsynapse was revealed by the observation that it is maintained for a longer period when T cells are stimulated with low doses of Ag or weak stimulation, whereas it exists only transiently upon strong stimulation. This was similarly observed upon T cell stimulation with low affinity Ag peptide, such as altered peptide ligand (APL) or with T cells whose TCR had low affinity for the Ag–MHC. These data suggest that the microsynapse structure functions to enhance cellular adhesion to support TCR–MCs, which generate initial activation signals particularly upon weak stimulation. Weak interaction between the TCR and pMHC may require stronger cell adhesion mediated by the microsynapse to induce Ag recognition, followed by triggering initial activation signals. On the other hand, strong TCR engagement can induce

sufficient signals for activation by itself in the relative absence of such strong cellular adhesion or co-stimulation.

The requirement for F-actin in microsynapse formation is consistent with the formation of F-actin clusters in TCR–MCs, which are localized at the center of the microsynapse. The F-actin at the TCR–MC is clearly distinct from the peripheral large actin ring and is specialized to support the microsynapse. Whereas the clustering of TCR, CD3, and ZAP70 is relatively independent of F-actin, clustering of LAT and SLP76, as well as molecules in the adhesion-ring such as LFA1, Pyk2 and paxillin, is strongly dependent on F-actin. Therefore, the components of the microsynapse induce two different types of clusters; F-actin dependent clusters (LAT, SLP76, adhesion-ring) and relatively F-actin independent clusters (TCR, ZAP70, etc.). Dependency of F-actin was found to parallel the dependency on TCR signal strength. Whereas TCR and ZAP70 cluster formation is dependent on stimulation signal strength, LAT and SLP76 clusters are relatively independent of signal strength. Such differences in the molecular dynamics of LAT and SLP76 from TCR and ZAP70 are evidence that LAT and SLP76 clustering are dependent on the microsynapse, which is supported by F-actin. Recently it was reported that the phospho-PLCγ cluster is formed in an F-actin- and WASP-dependent manner (41). Since the "actin foci" supporting phospho-PLCγ are quite similar to the F-actin cluster at the center of the microsynapse, the PLCγ cluster may also be supported by the microsynapse.

CONCLUDING REMARKS

Initial contact of a T cell with a cognate Ag-bearing APC induces T cell activation. This critical interaction creates the IS to deliver signals into T cells. The activation unit leading to T cell activation is the TCR–MC, which recruits downstream signaling molecules and mediates the activation signal. The TCR–MC is supported by a ring of adhesion molecules as the microsynapse. Since the cSMAC is formed mainly upon strong stimulation and under limited circumstances *in vivo*, microsynapses generated even upon weak stimulation may play more general and critical roles for early T cell activation in physiological situations. Although at present, the fine analyses described here can be achieved only by using a planar bilayer system, the technique should be extended to the analysis of cell–cell interactions *in vivo* by using *in vivo* imaging microscopy with better resolution. To get a clear picture of the signal events at the IS, first, the cooperative signaling between the TCR–MC signal and other signals such as co-stimulation, adhesion, cytokine, and innate signals should be clarified, and second, individual signaling pathways downstream of the TCR–MC, e.g., Ras-MAPK and PI3K-mTOR, should be analyzed in a spatial-temporal manner, i.e., both the timing and cellular compartmentalization of positive vs. negative signaling molecules need to be studied. The dynamics of these opposing signals may fine tune the activation signals, which controls the direction of cell fate.

AUTHOR CONTRIBUTIONS

AH-T performed experiments, and TS and AH-T wrote the review article.

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Cell Type-Specific Regulation of Immunological Synapse Dynamics by B7 Ligand Recognition

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B7 proteins CD80 (B7-1) and CD86 (B7-2) are expressed on most antigen-presenting cells and provide critical co-stimulatory or inhibitory input to T cells via their T-cell-expressed receptors: CD28 and CTLA-4. CD28 is expressed on effector T cells and regulatory T cells (Tregs), and CD28-dependent signals are required for optimum activation of effector T cell functions. CD28 ligation on effector T cells leads to formation of distinct molecular patterns and induction of cytoskeletal rearrangements at the immunological synapse (IS). CD28 plays a critical role in recruitment of protein kinase C (PKC)-θ to the effector T cell IS. CTLA-4 is constitutively expressed on the surface of Tregs, but it is expressed on effector T cells only after activation. As CTLA-4 binds to B7 proteins with significantly higher affinity than CD28, B7 ligand recognition by cells expressing both receptors leads to displacement of CD28 and PKC-θ from the IS. In Tregs, B7 ligand recognition leads to recruitment of CTLA-4 and PKC-η to the IS. CTLA-4 plays a role in regulation of T effector and Treg IS stability and cell motility. Due to their important roles in regulating T-cell-mediated responses, B7 receptors are emerging as important drug targets in oncology. In this review, we present an integrated summary of current knowledge about the role of B7 family receptor–ligand interactions in the regulation of spatial and temporal IS dynamics in effector and Tregs.

Keywords: Treg, PKC-theta, PKC-eta, CTLA-4, costimulation, immunological synapse, co-inhibition

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INTRODUCTION

The adaptive immune system must distinguish between self and non-self in order to provide protection from pathogenic challenges while sparing the organism's own tissues. Recognition of B7 ligands (CD80 and CD86, also known as B7-1 and B7-2, respectively) by co-stimulatory CD28 and co-inhibitory CTLA-4 (cytotoxic T-lymphocyte-associated protein 4, also known as CD152) receptors plays a critical role in regulation of effective self versus non-self discrimination. CD28 signaling is required for optimum proliferation and function of effector T cells, whereas CTLA-4 plays a critical role in negative regulation of immune responses, as it is required for turning off effector T cell signaling and regulatory T cell (Treg) development and suppressive functions. These opposing immunomodulatory roles of CTLA-4 and CD28 are of considerable clinical significance. CTLA-4 was the first immune checkpoint receptor targeted for cancer immunotherapy, and the anti-CTLA-4 antibody ipilimumab is used in the clinic for treatment of advanced melanoma (1).

CD28 co-stimulatory function is also relevant for cancer immunotherapy, as chimeric antigen receptors (CARs) containing CD28 cytoplasmic regions have been shown to induce efficient T cell effector functions (2). However, targeting CD28 with the superagonistic monoclonal antibody TGN1412 was a tragic failure, when administration of the antibody during a phase I clinical trial induced severe systemic inflammatory responses in healthy volunteers (3). Therefore, a comprehensive understanding of expression patterns, signaling pathways, and functional roles of CD28 and CTLA-4 on effector and Treg subsets can have significant medical impact.

CD28 and CTLA-4 recognize their B7 ligands in the context of the cell-to-cell interface, termed the immunological synapse (IS), formed between a T cell and an antigen-presenting cell (APC). Receptor ligation at the IS leads to accumulation of interacting molecules at different regions of the synapse, forming distinct molecular patterns known as supramolecular activation clusters (SMAC) (4–6). The canonical mature T cell IS consists of a central SMAC (cSMAC) containing TCR (on the T cell) and pMHC (on the APC) molecules, surrounded by the peripheral SMAC (pSMAC) containing LFA-1 (on T cell) and ICAM-1 (on APC) adhesion molecules as well as F-actin. The outer ring of the IS, known as the distal SMAC (dSMAC) contains molecules with large ectodomains, such as CD45 and CD43. The SMAC regions contain smaller microdomains, known as microclusters (7). The IS is highly dynamic, with movement of TCR microclusters toward the center of the synapse, where they undergo endocytosis. Antigen recognition under physiological conditions does not always result in formation of this canonical IS structure; nevertheless this model provides a useful framework for understanding spatial dynamics of molecular interactions at the interface between T cell and APC membranes. The IS is the main site of immune receptor triggering and recruitment of signaling intermediates, leading to signal initiation and integration. B7 ligand recognition leads to distinct localization of CD28 and CTLA-4 receptors at the SMAC, modulation of cytoskeletal dynamics as well as recruitment of protein kinase C (PKC) isoforms to the IS. The effect of B7 ligand recognition on the IS dynamics is cell type specific, with effector T cells and Tregs displaying different CD28 and CTLA-4 localization, leading to differential recruitment of PKC-θ and PKC-η to the effector T cell and Treg synapses. This review presents a brief outline of the roles of CD28 and CTLA-4 in the immune system, followed by a more detailed discussion of CD28 and CTLA-4 localization patterns in the IS, and the consequences of B7 ligand recognition on IS structure and stability in T effector and Tregs.

B7 LIGAND RECOGNITION: STRUCTURAL FEATURES AND EXPRESSION PATTERNS

B7-1 and B7-2 (CD80 and CD86) molecules share a similar structure, consisting of one membrane-distal variable domain-like and one membrane-proximal constant domain-like immunoglobulin superfamily (IgSF) domain. Purified CD80 crystallizes in a dimeric form, and undergoes spontaneous homodimerization in solution (8), whereas CD86 crystalizes as a monomer (9). The two different oligomeric states of B7 were also observed using Forster resonance energy transfer (FRET) analysis on the surface of APCs, with CD80 present on the cell surface mainly in the form of dimers, and CD86 being monomeric (10, 11). CD80 and CD86 are expressed on dendritic cells (DCs), macrophages, and B cells, with CD86 displaying higher constitutive expression and more rapid upregulation after activation. B7 molecules are also expressed on activated mouse and human effector T cells (12–14). CD80 and CD86 bind to CTLA-4 with significantly higher affinity than to CD28. CD80 is a stronger ligand, with K_D 0.2 μM for CTLA-4 and 4 μM for CD28 interaction, whereas the K_D for CD86 binding to CTLA-4 is 2 and 20 μM for CD28 (15).

CD28 monomers consist of a V-like IgSF extracellular domain, transmembrane regions, and a short cytoplasmic tail with no enzymatic activity. CD28 is expressed on the cell surface as a glycosylated, disulfide-linked homodimer of 44 kDa chains. In adult humans, CD28 is constitutively expressed on approximately 80% of CD4+ and 50% of CD8+ T lymphocytes. Loss of CD28 expression, most marked in the CD8 compartment, has been observed in humans during aging and autoimmune diseases (16–18). CD28 is expressed on all mouse T cells, and it is not downmodulated during aging (19). Repeated *in vitro* antigenic stimulation (20, 21) and exposure to common-γ chain cytokines or type I interferons (22) leads to downregulation of CD28 expression on human T cells. However, *in vivo* antigenic stimulation has been reported to increase CD28 surface levels on mouse T cells (23).

CTLA-4 shares structural similarity with CD28, forming homodimers of V-like IgSF monomers. CTLA-4 contains a 36-amino-acid-long cytoplasmic tail with no enzymatic activity. CTLA-4 is not expressed on the surface of resting effector T cells (24, 25), but is expressed constitutively in Tregs (26) under control of Foxp3 and NFAT (27–29). In both conventional T cells and Tregs, surface CTLA-4 is continuously endocytosed via a clathrin- and dynamin-mediated pathway, and recycled to the plasma membrane (30–34). Activation of effector and Tregs leads to upregulated levels of CTLA-4 on the cell surface. CTLA-4 internalization is mediated by the heterotrimeric adapter protein AP-2 (30, 34, 35) [regulation of CTLA-4 trafficking is the subject of an excellent recent review in Ref. (36)], whereas CTLA-4 trafficking from the trans-Golgi network to the cell surface involves formation of a multimeric complex consisting of transmembrane adapters TRIM and LAX, as well as small GTPase Rab8 (37, 38). CTLA-4 present in recycling endosomes is protected from lysosomal targeting through interaction between LRBA protein (lipopolysaccharide-responsive and beige-like anchor protein) and CTLA-4's tail region (39). Since its lysosomal degradation involves interaction with another clathrin adaptor complex AP-1 that binds to the same tyrosine-based motif (Y201) of CTLA-4 as LRBA (35) (the interaction motifs in CTLA-4 cytoplasmic region are summarized in **Figure 1**), it has been suggested that the binding of LRBA may prevent interaction with AP-1 and thereby protect the protein from degradation (39).

Both CTLA-4 and CD28 rely on the amino acid motif MYPPPY in the vicinity of Y139 in human CTLA-4 and Y123 in CD28 for binding to the B7 proteins (46–48). Importantly, despite the identical amino acid sequence of the interaction site, CTLA-4 and CD28 are capable of effectively discriminating between B7 proteins. A key study from the Allison lab (48) reported that

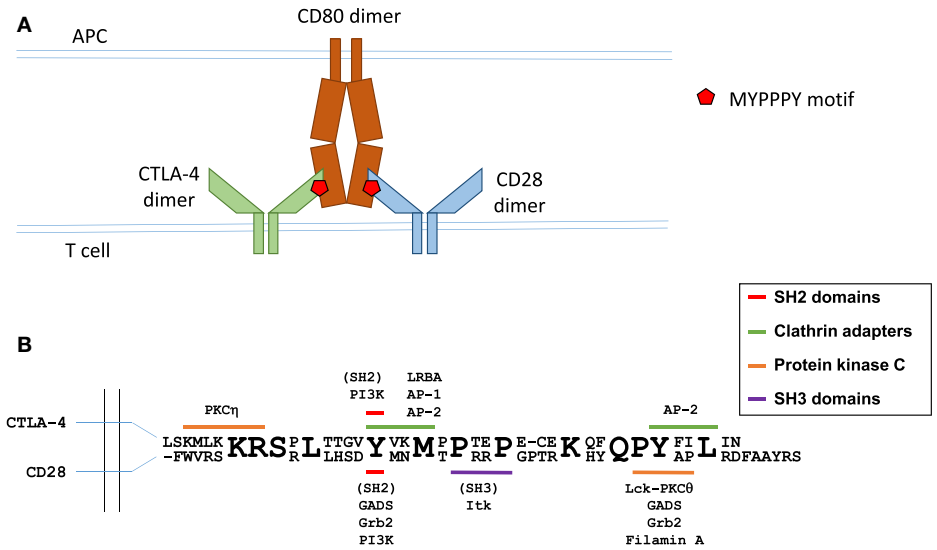

FIGURE 1 | Molecular interactions in B7 ligand recognition. (A) Schematic representation of CD28 and CTLA-4 binding to the B7 ligands. **(B)** Schematic representation of the cytoplasmic regions of CTLA-4 (top sequence) and CD28 (bottom sequence). Known interaction partners of CTLA-4 are shown above and of CD28 below the alignment, and the motifs implicated in these interactions are color coded as indicated. Figure based on Hou et al. (40), Isakov and Altman (41, 42), Margulies (43), Schneider and Rudd (36), Sharpe and Freeman (44), and Stamper et al. (45).

the binding of a B7 ligand was critical for the concentration of CTLA-4 at the IS and contributed to the concentration of CD28, and that CD86 was a preferred ligand for CD28 and CD80 for CTLA-4. Antigen-pulsed B cells expressing CD80 effectively concentrated CTLA-4 at the synapse. Furthermore, in synapses formed by B cells expressing only CD80, there was evidence for competition between CTLA-4 and CD28 for ligand binding, as CD28 accumulation was reduced even further when CTLA-4 was present at the IS. Conversely, peptide-pulsed B cells expressing only CD86 strongly increased the accumulation of CD28 at the synapse, but failed to recruit CTLA-4 (48).

CD28 IN REGULATION OF THE IMMUNE RESPONSE

CD28 is the prototypic co-stimulatory molecule, and CD28 ligation leads to enhanced cytokine production, cell survival, and proliferation of effector T cells. The critical role of CD28-mediated signaling in optimum T cell responses is demonstrated by the T cell effector functions afforded to second-generation CARs containing cytoplasmic regions of CD28 and CD3ζ, but not by first-generation CARs lacking CD28 sequences (2). The cytoplasmic region of CD28 contains two main signaling motifs (summarized in **Figure 1**): a proximal YMNM motif and a distal proline-rich PYAP motif (49). The YMNM motif mediates phosphatidylinositol 3-kinase (PI3K) binding (50–52), leading to Akt activation; YMNM can also bind to GRB2/GADS adaptor proteins (51, 53) and the PYAP motif binds to Lck (54), filamin A, and GRB2/GADS (53, 55). The YMNM motif is followed immediately by another poly-proline motif PRRP, reported to bind the kinase Ikt (56). Analysis of knock-in mutant mice revealed that the PYAP motif is critical for IL-2 production and proliferation *in vitro*, as well as for *in vivo* antibody production and germinal center formation (57), whereas YMNM plays a role in augmenting T cell proliferation (58). Interestingly, knock-in T cells with both their YMNM and PYAP motifs mutated display less severe activation defects than CD28-deficient T cells, suggesting some functional role for the PRRP motif and/or yet unidentified cytoplasmic sequences.

CD28 is required for the thymic generation and peripheral maintenance of a functional Treg population. CD4+ Foxp3+ Tregs are key negative regulators of T cell-mediated immunity and are required for the control of spontaneous responses to self through several mechanisms (59, 60). Contact-mediated suppression relies on CTLA-4 interactions with its ligands and is discussed in detail below. Bystander suppression is mediated by suppressive cytokines, mainly IL-10 (61) and TGF-β (62) produced by activated Tregs, and by induction of cytokine starvation in target cells by IL-2 clearance (63). B7 ligand recognition plays an important role in Treg development and function, summarized in **Table 1**. In CD28-deficient NOD mice, the percentage of peripheral Tregs is strongly reduced (64). Similar reductions are observed in NOD mice lacking both CD80 and CD86, leading to the conclusion that the B7–CD28 interaction is required for the formation of the full Treg repertoire. The reduction in the percentage of Tregs in NOD mice treated with B7-blocking CTLA-4-Ig correlates with a higher incidence of spontaneous autoimmune diabetes (64). Subsequent analysis revealed that Treg deficiency in $CD28^{-/-}$ mice can be traced back to thymic development. The percentage of Treg precursors among thymic CD4 single-positive cells is significantly reduced in $CD28^{-/-}$ mice as well as in NOD mice injected with anti-CD80 and CD86 antibodies (65), and in B7 double knockout mice (66).

TABLE 1 | B7 ligand recognition in Treg synapse formation and suppressive functions.

Surface interactions	Biological significance for Tregs	Reference
CD28–B7	Motility Tonic signals necessary for survival	Lu et al. (160), Thauland et al. (163) Zhang et al. (71)
CD28–B7	Antigen recognition Motility stop signal	Apostolou et al. (176), Jordan et al. (68), Knoechel et al. (177), Walker et al. (178) Lu et al. (160), Thauland et al. (163)
TCR–pMHC	Synapse formation and stabilization Activation	Onishi et al. (161) Schmidt et al. (179), Zhang et al. (71)
LFA-1–ICAM-1	Proliferation Surface accumulation of CTLA-4	Walker et al. (178), Zheng et al. (155) Catalfamo et al. (170)
CTLA-4–B7 TCR–pMHC	Synapse stabilization	Onishi et al. (161), Zanin-Zhorov et al. (151)
LFA-1–ICAM-1	Contact suppression	Kong et al. (152), Qureshi et al. (146)

Peripheral homeostatic expansion of Tregs – but not effector T cells – in normal syngeneic hosts is also strongly suppressed by anti-CD80 and CD86 antibodies (66). A mechanistic explanation for the thymic requirement for CD28 was proposed by Tai et al. (67) who examined the consequences of CD28 deletion in a TCR-transgenic model. Mice expressing the AND TCR and its agonist ligand, a pigeon cytochrome *c* peptide, were found to effectively induce thymic Tregs only in the presence of CD28. This means that while a strong selection signal through TCR is indeed required (68), it is not sufficient for the full initiation of the agonist selection program leading to the generation of Tregs, and that a co-stimulatory signal from mTEC-expressed B7 molecules through CD28 is also required. It is noteworthy that a small proportion of regulatory phenotype T cells were still generated in the absence of CD28 but these cells lacked suppressive capacity (67). Earlier data from the same group indicated that CD28 is also required for the deletion of thymocytes by negative selection (69, 70).

A study using mice in which CD28 was selectively deleted in cells expressing Foxp3 (Cd28-ΔTreg), reported only a minor decrease in the percentage of thymic Treg precursors (71). This is in line with previous observations that CD28 is involved in the generation of early, Foxp3-negative Treg precursors (72). However, in stark contrast to earlier studies, homeostatic expansion of Tregs in the periphery was reported to occur independently of CD28 (73). Tregs from CD28-ΔTreg mice displayed reduced suppressive capacity, and consequently CD28-ΔTreg animals developed spontaneous autoimmunity (71). Costimulation through CD28 is required for *in vivo* expansion of Tregs in the presence of TCR stimulation and IL-2 (74). CD28 stimulation is also required for the conversion of naïve CD4 T cells into Tregs *in vivo* (75, 76) and *in vitro* (77).

CTLA-4 IN REGULATION OF THE IMMUNE RESPONSE

CTLA-4 is a critical negative regulator of the immune response. Germline CTLA-4 knockout in mice results in massive lymphoproliferation (78), and is lethal at 3–4 weeks of age (78, 79). The peripheral T cell profile in these animals is strongly skewed toward CD4 cells that rapidly proliferate in a CTLA-4-Ig-sensitive manner – indicating the dependence on B7–CD28 interaction – and infiltrate non-lymphoid tissues (78, 80). Introduction of CTLA-4-sufficient Tregs reverts the lymphoproliferative disorder and prevents early lethality in CTLA-4 knockout mice (81), whereas blocking of CTLA-4 on Tregs completely abrogates their suppressive function (62, 66, 82). CTLA-4-deficient Tregs are unable to control lymphopenia-driven homeostatic expansion of conventional CD4 cells (83). Importantly, interaction between CTLA-4 and B7 expressed on effector T cells was found to be dispensable for the control of the latter in mixed bone marrow chimera experiments as both $B7^{-/-}CTLA\text{-}4^{-/-}$ and $B7^{+/+}CTLA\text{-}4^{-/-}$ effector T cells were efficiently suppressed by CTLA-4-sufficient Tregs (66). B7 expression is also not required on Tregs themselves (66). These data indicate that B7 expressed on a cell subset distinct from effector and Tregs mediates interactions with Treg-expressed CTLA-4 and immune suppression. CTLA-4-deficient Tregs are characterized by similar expression of CD25, PD1, GITR, and of suppressive cytokines IL-10 and IL-35 (83). Foxp3 promoter-controlled deletion of CTLA-4 in Tregs resulted in lymphoproliferative disease and tissue infiltration, and was lethal at ~7–8 weeks of age [i.e., somewhat delayed compared to germline knockout Ref. (84)]. Similarly to Foxp3-driven CD28 deficiency, thymic development of Tregs was normal, as was their survival in the periphery. However, cells lacking CTLA-4 were unable to control proliferation of target cells stimulated by anti-CD3 antibody and DC, and to induce tumor rejection (84).

Unlike CD28, CTLA-4 is not required for Treg development in the thymus. CTLA-4 is expressed by a subset of thymocytes predominantly residing at the corticomedullary junction (85) and is strongly upregulated upon induction of negative selection (86). There is no requirement for CTLA-4 expression to initiate central Treg development and peripheral expansion, as CTLA-4 knockout mice exhibit elevated percentage of Tregs and increased Ki67 expression, indicative of their active proliferation (87). Moreover, deletion of CTLA-4 in TCR-transgenic mice increases the frequency of Foxp3-positive Treg precursors in the thymus and leads to the formation of a specific population of Foxp3-positive DP thymocyte subsets in the thymic cortex (85). However, CTLA-4 can play a role in formation of the induced Treg population, as CTLA-4 has been shown to induce expression of Foxp3 and Treg conversion in the intestine (88).

CD28 AND IMMUNOLOGICAL SYNAPSE ARCHITECTURE IN EFFECTOR T CELLS

CD28 shows a unique cSMAC localization pattern that is important for its efficient co-stimulatory functions. CD28 co-localizes with TCR microclusters at the earliest observable time-point after

agonist pMHC recognition (89, 90), and the early accumulation of CD28 at the IS shows similar kinetics and localization as the TCR complex. In a mature IS, CD28 is present at the cSMAC, but segregates away from TCR (90, 91). This segregation of CD28 from TCR at the IS is required for optimum T cell activation, as shown in a study comparing different anti-CD3 and CD28 micropatterns on planar stimulatory surfaces (92). The spatial separation of TCR and CD28 at the mature IS is regulated by localization of CD28 ligands, as full length CD80 separates from TCR at the IS, but CD80 with deleted cytoplasmic region localizes with TCR (93). Moreover, the tailless CD80 molecule does not provide an optimum co-stimulatory signal and does not show efficient accumulation at APC: T cell contact interface (94, 95). This suggests a role for B7 interactions with cytoskeleton and/or other cytoplasmic components in regulation of IS architecture. CD28 recruitment and maintenance at the synapse requires both CD28 and TCR ligand binding (90, 96). CD28 accumulation at the synapse has been shown to be independent of antigenic pMHC affinity to TCR, with weak and strong agonist pMHC complexes inducing similar levels of CD28 recruitment (97). The role of CD28-mediated signaling and interactions in regulation of CD28 localization at the synapse is somewhat controversial, with a report indicating unperturbed IS localization of CD28 with mutated or deleted cytoplasmic region (90), whereas another study observed impaired IS localization of CD28 with deleted cytoplasmic domain or with a mutation at Y188 within the CD28 PYAP motif (96).

CD28 ligation has been shown to induce rapid internalization of the receptor, with half of the endocytosed fraction degraded in lysosomes and half recycled back to the cell surface (98). CD28 downregulation depends on PI3K (73), with preferential endocytosis of CD28 molecules associated with PI3K (98). CD28 is endocytosed via clathrin-coated pits, and this process requires coupling of WASP to PI3K and CD28 via sorting nexin 9 (73). CD28 downregulation from the synapse can also be influenced by stoichiometry of its B7 ligands (11). FRET analysis of B7 fluorescent protein fusions demonstrated that CD80 is present at the cell surface as a mixed population of dimers and monomers, with CD86 predominantly present in monomeric form (10). Experimental increase in CD80 dimerization resulted in enhanced T cell: APC conjugate formation and more sustained accumulation of Lck and PKC-θ at the IS (11).

Co-stimulatory signals play a critical role in regulation of cytoskeleton dynamics at the IS during T cell interaction with APC (summarized in **Table 2**). CD28 ligation induces movement of actin cytoskeleton toward the IS (99), and CD28 engagement is required for sustained actin accumulation at the IS (100). CD28 stimulation alone leads to actin polymerization and recruitment of actin at the IS (101). CD28 signaling is important in multiple pathways involved in actin filament nucleation, elongation, and depolymerization. The guanine nucleotide exchange factor Vav1 controls the activity of small Rho GTPases Cdc42 and Rac1 that regulate actin polymerization activity of WASP and WAVE2, respectively. WASP and WAVE2 are actin nucleation-promoting factors that, together with the Arp2/3 complex, facilitate formation of new actin filaments.

CD28 ligation induces tyrosine phosphorylation of Vav1 (102), and CD28-dependent actin remodeling requires Cdc42 (103) and Rac1 (104). The molecular interactions linking CD28 to Vav1 phosphorylation are not yet fully elucidated. CD28-dependent Vav1 phosphorylation has been shown to require binding of the adaptor protein GRB2 to CD28 (105, 106), but a recent report provided evidence for GRB2-independent Vav1 binding to CD28 and a role of PIP5K1A (phosphatidylinositol 4-Phosphate 5-Kinase α) and Vav1 cooperation in regulation of actin, downstream of CD28 (107). Jurkat cells expressing CD28 with mutated C-terminal PYAP motif, important for GRB2 binding, failed to recruit Vav1 to the IS or to rearrange actin after CD28 ligation (107); however, Vav1 phosphorylation in response to CD28 ligation was not assessed in this study, and in another report the PYAP motif was shown to be dispensable for CD28-dependent Vav1 phosphorylation (108). The Arp2/3 actin nucleation complex cooperates with filamins, large multidomain proteins with a role in actin crosslinking (109), to establish actin structure. Filamin A is phosphorylated (110) and recruited to the IS (108) after CD28 ligation in T cells, with the PYAP motif on the cytoplasmic region of CD28 mediating the interaction with filamin A (108, 111). Knockdown of filamin A expression did not affect CD28-mediated Vav1 phosphorylation, but reduced Cdc42 activity and impaired CD28-mediated costimulation (108). However, changes

TABLE 2 | CD28 in regulation of cytoskeleton dynamics at the immunological synapse.

Cytoskeletal regulator	CD28-induced modification	Effect on cytoskeleton	Reference
Vav1	Phosphorylation, leading to activation	Vav1 controls activity of small Rho GTPases Cdc42 and Rac1 that regulate actin polymerization activity of WASP and WAVE2, respectively	Nunes et al. (102), Raab et al. (104), Salazar-Fontana et al. (103), Schneider and Rudd (106)
Filamin A	Direct interaction with CD28, phosphorylation	Filamin A has a role in actin crosslinking	Muscolini et al. (111), Tavano et al. (108)
Cofilin	Dephosphorylation, leading to activation	Actin severing protein. Blocking cofilin–actin interaction reduces T cell:APC conjugation	Lee et al. (119), Wabnitz et al. (120)
Rltpr	Unknown	Actin-uncapping protein. Wild-type Rltpr is required for CD28-dependent costimulation, but this seems to be independent of its actin-uncapping function	Liang et al. (113)
CapZIP	Phosphorylation	Actin-uncapping protein. CapZIP is required for CD28-dependent costimulation, but its effect on T cell cytoskeleton are unknown	Tian et al. (110)

in actin structure or dynamics at the IS of filamin A knockdown cells have not been reported. Moreover, knocking down filamin A did not impair ezrin accumulation at the IS (108).

Actin filaments contain a fast growing barbed end, which can be bound to actin capping protein. Capping protein binding to the barbed end prevents addition of new actin subunits, limiting the filament elongation. Actin capping and subsequent actin polymerization can be regulated by actin-uncapping proteins (112). An actin-uncapping protein Rltpr is required for CD28-dependent costimulation (113), and Rltpr colocalizes with CD28 in CD80-dependent signaling microclusters (113), suggesting a role of Rltpr in CD28-mediated actin rearrangement at the synapse. However, a direct role of Rltpr for CD28-dependent actin modification is unclear. Rltpr does not immunoprecipitate with CD28 (113) and is not phosphorylated after CD28 ligation (110). Moreover, an Rltpr mutation that abolishes CD28-mediated costimulation does not impair Rltpr's actin-uncapping ability or CD28-dependent actin rearrangements at the synapse (113). The Rltpr mutation that reduced CD28-dependent costimulation abrogates CD28-dependent recruitment of PKC-θ and Carma1 to the IS (113) through a yet unidentified molecular mechanism, suggesting that Rltpr acts as an adaptor at the IS independently of its actin-uncapping functions. A recent phosphoproteomic screen identified actin-uncapping CapZIP as part CD28 signaling network (110). Importantly, CapZIP is required for CD28-dependent costimulation of cytokine production (110). However, it has not yet been reported if CapZIP can directly interact with CD28 and if it is required for CD28-dependent changes in actin dynamics. In summary, the current evidence suggests that CD28-dependent signaling may regulate actin capping through actin-uncapping proteins CapZIP and potentially Rltpr.

CD28 signaling regulates actin dynamics through control of activity of the actin-severing protein cofilin. Cofilin is a ubiquitously expressed 19 kDa protein that cleaves actin filaments, thus, promoting actin depolymerization, but also creating new barbed ends for filament elongation (114). Cofilin's actin binding capacity is negatively regulated by its phosphorylation at serine 3 (115, 116), and binding to phospholipids (117). Blocking cofilin interaction with actin reduces T cell proliferation and cytokine production, as well as conjugation with APCs (118). In resting human T cells, cofilin is present mainly in the inactive phosphorylated form, and CD28 or CD2 signal together with TCR, but not TCR signal alone, induces cofilin dephosphorylation and actin binding (119, 120). The precise sequence of signaling events linking CD28 ligation to cofilin activation is unknown. Cofilin is dephosphorylated by serine phosphatases PP1 and PP2A (121), and CD3/CD28-induced cofilin dephosphorylation requires Ras (120). Additionally, CD28 may regulate cofilin activity through control of levels of membrane phospholipids (114).

There is strong evidence that CD28-dependent regulation of actin dynamics is important for the effector T cell functions. CD28 enhances T cell:APC conjugate formation *in vitro* (122, 123). Knock-in mice with mutated PYAP motif show reduced IL-2 production and proliferation *in vitro*, and impaired *in vivo* antibody production and germinal center formation (57). This could be a result of impaired cytoskeletal rearrangement, as the PYAP motif is implicated in Vav1 and filamin A recruitment. However, the effects of PYAP mutations on cytoskeletal dynamics and synapse stability have not yet been reported for primary T cells, and this motif is also important for binding to Lck (54), GRB2/GADS (53, 55), and PKC-θ (124), as discussed below. Analysis of a mouse mutant with inducible inhibition of Csk, a negative regulator of Src family kinases, strongly suggested that CD28-dependent actin remodeling is critical for initiation of full TCR signal in thymocytes (125). However, thymocytes from PYAP mutant knock-in mice do not show obvious phenotypic defects (57), suggesting that CD28-independent pathways can regulate actin cytoskeleton dynamics during thymocyte development.

CD28 AND REGULATION OF PKC-θ LOCALIZATION AT THE EFFECTOR T CELL IS

CD28 plays a critical role in regulation of the IS localization of the novel protein kinase C (nPKC) isoform PKC-θ (summarized in **Table 3**). The PKC family consists of 10 serine/threonine kinase isoforms, with important roles in regulation of multiple cellular processes in different cell types. All nPKC isoforms (PKC-θ, PKC-δ, PKC-ϵ, and PKC-η) require diacylglycerol (DAG), but

TABLE 3 | Molecular determinants of PKC-θ localization at the immunological synapse.

Interaction/activity	Molecular determinants	Effect on immunological synapse	Reference
PKC-θ–CD28	Polyproline motif within the PKC-θ V3 hinge region and PYAP motif in CD28; Lck-mediated interaction	PKC-θ V3 hinge and CD28 PYAP motif are required for CD28 cSMAC localization	Kong et al. (124)
PKC-θ–CD28	Sumoylation of PKC-θ at lysines 325 and 506	Abrogated PKC-θ sumoylation reduces PKC-θ localization at the IS and its colocalization with CD28, induces colocalization of PKC-θ and filamin A at periphery of the IS	Wang et al. (139)
PKC-θ–DAG	C1 domains of PKC-θ	C1 domains mediate initial PKC-θ recruitment to the synaptic membrane, but they do not support PKC-θ central accumulation at the synapse	Basu et al. (134), Carrasco and Merida (136), Quann et al. (135)
PKC-θ kinase activity	Unknown, possibly through autophosphorylation at threonine 219 between the tandem C1 domains	PKC-θ kinase activity is required for its recruitment to the IS	Cartwright et al. (138), Thuille et al. (137)
Rltpr	Unknown, no interaction between Rltpr and PKC-θ has been detected	Wild-type Rltpr is required for PKC-θ and CARMA1 recruitment to cSMAC	Liang et al. (113)

not Ca^{2+}, for activation, and are expressed in T cells and play multiple roles in regulation of T cell signaling and effector functions (126). Central localization of PKC-θ is one of the hallmarks of the mature effector T cell IS. A seminal study by Monks at al. identified PKC-θ as the only PKC isoform recruited to effector T cell IS (127). However, more recent studies show that PKC-η and PKC-ϵ are also recruited (128–130), with some evidence that their recruitment precedes that of PKC-θ (129). PKC-ϵ and PKC-η display homogeneous distribution over the entire synapse, whereas PKC-θ displays discrete cSMAC localization contained within the peripheral actin ring (128–132).

An important study using lipid bilayers presenting antigen and co-stimulatory signal, and TIRF microscopy to examine PKC-θ localization at the effector T cell IS, revealed initial colocalization of PKC-θ with TCR/CD28 microclusters (90). This was followed by PKC-θ recruitment to the cSMAC, where it segregated, together with CD28, to TCR^{low} regions in the periphery of cSMAC (90). The initial stages of PKC-θ recruitment to the effector T cell IS do not depend on CD28 ligand binding, but CD28 ligation is required for sustained PKC-θ localization at the synapse and colocalization of PKC-θ with CD28 (90, 133). PKC-θ interacts with CD28 after PMA treatment (90) (which induces PKC activation) and TCR/CD28 stimulation (124).

The molecular determinants of PKC-θ synapse localization have been mapped to the V3 hinge region and C1 domains (132). nPKCs share a conserved structure, with an amino-terminal C2 domain, tandem C1 domains, and V3 hinge linked to a carboxyl-terminal kinase domain. PKC-θ interaction with CD28 and cSMAC localization requires a polyproline motif within the V3 hinge region (124), and V3 hinge regions from PKC-ϵ and PKC-η mediate their diffuse accumulation at the synapse (134). A carboxyl-terminal poly-proline motif (PYAP) in the CD28 cytoplasmic tail is required for its interaction with PKC-θ, with strong evidence suggesting that this is an indirect interaction mediated through Lck, with the Lck SH3 domain binding to the polyproline motif in PKC-θ V3 and the Lck SH2 domain binding a phosphorylated tyrosine within the CD28 PYAP motif (124). Tyrosine 188 within the PYAP motif was also identified as critical for CD28 and PKC-θ central synapse localization in an earlier study (96).

Additionally, C1 domains of PKC-θ also play a role in its synapse localization, through interaction with DAG at the synapse membrane (134, 135). C1 domains can mediate initial PKC-θ recruitment to the synaptic membrane (135), but they do not support PKC-θ central accumulation and retention and the membrane (136), and nPKC C1 domains are not sufficient to determine the respective synapse localizations of PKC-θ versus PKC-ϵ and PKC-η (134). Phosphorylation of PKC-θ threonine 219 (T219), in a hinge region between the tandem C1 domains, is required for PKC-θ localization at the IS (137). Moreover, sustained synapse localization is dependent on PKC-θ kinase activity (137, 138), most likely through a requirement for PKC-θ autophosphorylation at T219 (132, 137). PKC-θ recruitment to the IS also requires expression of wild-type Rltpr actin-uncapping protein (113). T cells from mice expressing an Rltpr mutant could not recruit PKC-θ to the IS (113). The precise role of Rltpr in the regulation of PKC-θ synapse localization is unknown but seems to be independent of Rltpr actin-uncapping function, and no direct interactions between Rltpr and PKC-θ have been observed (113).

A recent report identified a novel activation-dependent post-translational modification of PKC-θ that modulates CD28–PKC-θ interactions and IS architecture (139). TCR stimulation of resting murine and human T cells leads to conjugation of SUMO1 (small ubiquitin-like modifier) to PKC-θ lysine (K) 325 and K506 by SUMO E3 ligase PIASxβ (139). Importantly, TCR and CD28 costimulation resulted in stronger PKC-θ sumoylation than TCR stimulation alone. Sumoylation-resistant PKC-θ with mutated K325 and K506 residues showed reduced interaction with CD28 and filamin A, and diffuse localization at the membrane in the IS (139). Inhibiting PKC-θ sumoylation through PIASxβ knockdown or overexpression of a desumoylating enzyme also abrogated PKC-θ localization at the IS, and reduced its colocalization with CD28 (139). Wild-type PKC-θ segregated from filamin A at the IS, with mainly pSMAC localization of filamin A. Inhibition of PKC-θ sumoylation altered the IS architecture, inducing colocalization of PKC-θ and filamin A at the periphery of the synapse (139).

The localization of PKC-θ to the center of the IS is critical for its functions in effector T cells. Mutations of the poly-proline motif in the V3 region of PKC-θ reduced activation of primary effector CD4+ T cells (124). Critically, overexpression of murine V3 domain sequesters PKC-θ from CD28 and cSMAC in mouse CD4+ T cells, and reduces PKC-θ-dependent gene expression *in vitro*, as well as CD4+ Th2 and Th17 immune responses *in vivo* (124). Similarly, expression of sumoylation-resistant PKC-θ mutants, with impaired synapse localization, does not rescue defects in cytokine production, activation of PKC-θ dependent transcription factors, and Th2 differentiation of human T cells with downregulated expression levels of endogenous PKC-θ (139). Additionally, mutations in the CD28 PYAP motif, required for PKC-θ interaction with CD28 and for IS localization, severely impaired effector T cell functions *in vivo* (57). However, it must be noted that PKC-θ synapse localization seems to be inseparable from its interaction with CD28, and the observed functional effects of impaired PKC-θ synapse recruitment could also be caused by reduced CD28 interactions with PKC-θ, Lck, and/or filamin A.

CTLA-4 DYNAMICS AT THE EFFECTOR AND Treg IS

Recognition of B7 ligands by CD28 and CTLA-4 at the effector T cell IS leads to competitive displacement of CD28 and PKC-θ from its central region. In the absence of stimulation, regulatory and conventional T cells express similar levels of CD28, but CTLA-4 expression is significantly higher in unstimulated Tregs (71, 140, 141). TCR signaling induces polarization of both intracellular (142) and membrane pools of CTLA-4 toward the IS of effector T cells, and TCR signal strength determines CTLA-4 localization at the IS (97). CTLA-4 is recruited to the effector T cell cSMAC with delayed kinetics relative to that of TCR and CD28, segregates away from $CD3^{high}$ regions and forms a ring-like

structure (141). CTLA-4 recruitment to and stabilization at the IS depends on its ligand binding, but occurs at both high and low B7 ligand densities (141). Critically, recruitment of CTLA-4 to the IS influences CD28 localization, due to competition for ligand binding. At high ligand densities, CTLA-4 recruitment leads to exclusion of CD28 from the cSMAC and its accumulation outside the pSMAC (141). At low ligand densities, CTLA-4 prevents formation of CD28 clusters at the T effector IS (141). Importantly, CTLA-4-mediated displacement of CD28 from the cSMAC leads to impaired synaptic localization of PKC-θ (141). CTLA-4 ligation has also been reported to reduce the size of T cell: APC contact interface and to reduce ZAP70 microcluster formation (143).

The localization of CTLA-4 in the T effector synapse depends on the molecular dimensions of the extracellular region of the protein, as CTLA-4 molecules with elongated ectodomains failed to accumulate at cSMAC despite unimpaired ligand binding (141). However, it has not been reported if CTLA-4 with elongated ectodomains affected CD28 clustering at the synapse, and it is not known whether similar dimensions of CD28 and CTLA-4 receptor–ligand complexes are important for efficient regulation of co-stimulatory signal and/or competition for ligand binding at the synapse. The matching sizes of activating and inhibitory receptor–ligand complexes are critical for signal integration and regulation of NK-cell functions (144, 145), and it is plausible that a similar requirement exists for co-stimulatory and co-inhibitory signaling in effector T cells.

A molecular mechanism for CTLA-4 involvement in the downregulation of CD80/CD86 has been established in the seminal work by Qureshi et al. (146). Using co-cultured CHO cells expressing either human CTLA-4 or GFP-tagged human CD86, they observed transfer of GFP signal into CTLA-4 expressing cells, and its accumulation in the endolysosomal system, indicative of CD86 trans-endocytosis. Endocytosis-deficient CTLA-4 failed to induce trans-endocytosis of CD86-GFP and resulted in the accumulation of CD86 at cell contacts. These findings were confirmed using purified human Tregs incubated with DC where CD86 expression on the surface of the DC was reduced in the presence of Tregs but not effector T cells, and TCR stimulation increased the rate of trans-endocytosis (146). The most direct consequence of reduction of B7 proteins on the surface of APC is manifested in fewer and less prolonged interactions between APC and effector T cells (147, 148) reduced PKC-θ recruitment and activation in these cells (149) and, consequently, repression of IL-2 production by effector T cells (150). Recently, it has been shown that surface expression of CTLA-4 on effector T cells is sufficient for downregulation of CD86 expression from APCs (40).

Tregs display radically different synapse localization of CD28 and PKC-θ than effector T cells (summarized in **Figure 2**). In a stimulating planar lipid bilayer system, the recruitment of CD28 to the Treg IS is barely detectable, whereas CTLA-4 is recruited robustly, in stark contrast to conventional CD4 T cells (141). Displacement of CD28 from Treg synapses by CTLA-4 coincides with the absence of PKC-θ clusters in the cSMAC zone of Treg synapses. Similarly, the switch of developmental program during the *in vitro* conversion of naïve CD4 T cells into Tregs results

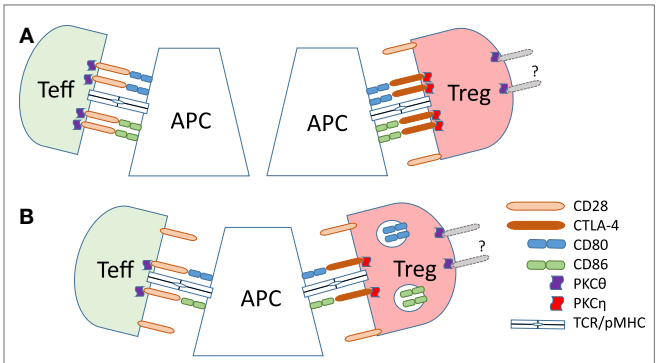

FIGURE 2 | Dynamics of B7 ligand recognition at effector and Treg IS. **(A)** B7 (CD80 or CD86) ligation leads to accumulation of CD28 and associated PKC-θ at the T effector cell IS. High-affinity B7 binding by CTLA-4 on Tregs leads to accumulation of CTLA-4 and the associated PKC-η at the Treg IS, and exclusion of CD28 and PKC-θ from the IS. **(B)** CTLA-4 ligand binding in Tregs results in the trans-endocytosis of the B7 ligands. This reduces the amount of the B7 ligands on the surface of the APC, leading to reduced co-stimulatory signals delivered to effector T cells.

in a loss of PKC-θ signal at the synapse. Correct localization of CTLA-4 to the IS is functionally important, as elongation of the extracellular domain of CTLA-4 resulted in a loss of its concentration in the synapse and reduction of suppressive activity of Tregs (141). In a lipid bilayer system, addition of CD80 or ICAM-1 to the bilayer increases the recruitment of PKC-θ to the synapse in both effector and Tregs, but stimulation through TCR strongly decreases the recruitment in Tregs (151). Reduction of PKC-θ activity results in increased Treg proliferation and elevated suppressive capacity (151).

An important insight into the signaling mechanism downstream of CTLA-4 recruitment to the Treg synapse was provided in a recent study by Kong et al. (152) which identified PKC-η as the only PKC isoform physically interacting with CTLA-4. In Tregs, a phosphorylated form of PKC-η binds constitutively to CTLA-4. PKC-η localizes to the IS in close proximity to the TCR (152). Interaction between PKC-η and CTLA-4 was found to be critical for Treg function. Phosphorylated serine residues S28, S32, and S317 of PKC-η are responsible for the interaction with CTLA-4, and loss of S28 or S32 results in a strong inhibition of some Treg suppressive functions. The importance of PKC-η was further emphasized by the finding that, although PKC-η-deficient Tregs expressed normal levels of functional LFA-1 required for the stabilization of contacts with APC, they showed a marked decrease in their ability to continuously clear CD86 from the APC surface. While CD86 clearance on first contact with APC was unaffected by the loss of PKC-η, the reduction of CD86 on reintroduced APC was substantially delayed (152). These findings suggest that PKC-η is not directly involved in CTLA-4-induced trans-endocytosis, and that a feedback signaling mechanism from PKC-η may be required for the recycling of CTLA-4 from the endolysosomal system. It remains to be elucidated whether or not the amount of surface-expressed CTLA-4 is reduced and its intracellular retention or lysosomal degradation is accelerated in the absence of PKC-η. In an earlier study, a deletion of amino

acids 191–223 of the intracellular domain of CTLA-4 did not substantially affect the *in vitro* suppression of target T cell proliferation in the presence of CD3 crosslinking antibody and APC, or *in vivo* suppression in a colitis model (26). Kong et al. have shown that this deletion-mutant of CTLA-4 retains its association with PKC-η, suggesting that the remaining cytoplasmic portion (amino acids 182–191) of CTLA-4 is sufficient for suppressive signals using PKC-η.

Responses to CD80 and CD86 signals in effector T cells are largely similar (153, 154). By contrast, addition of anti-CD80 antibody or CTLA-4 Fab fragments to the co-culture of target T cells, DC, and prestimulated Tregs ablated the suppressive function of the latter, whereas addition of anti-CD86 or anti-CD28 antibodies increased suppression to the same degree (155). Furthermore, blocking CD86 inhibited DC-induced division of Tregs, whereas blocking CD80 enhanced division (155). These data suggest that, in contrast to effector T cells, Tregs can effectively discriminate between CD80–CTLA-4 and CD86–CD28 signals.

CTLA-4 IN REGULATION OF REGULATORY AND EFFECTOR T CELL SYNAPSE STABILITY AND CELL MOTILITY

A growing body of evidence clearly suggests a role for CTLA-4 in regulation of synapse stability, duration of conjugation with APC and overall motility in Treg and Teff cells. Anti-CTLA-4 blocking antibody treatment has been shown to increase effector T cell motility *in vitro* (122, 156) and *in vivo* (157–159). Importantly, it has been reported that CTLA-4 ligation has different outcomes for synapse stability and motility of regulatory versus effector T cells (122, 160).

Regulatory T cells form a more stable IS than effector T cells, and this enhanced synapse stability has been implicated in CTLA-4-dependent downregulation of B7 cell surface expression by Tregs. In mixtures with conventional CD4 T cells of same specificity, TCR-transgenic Tregs preferentially bind to DCs and exclude conventional cells (161). Similarly, in a planar lipid bilayer system, Tregs form a more long-lived IS than do effector T cells of the same specificity (151). Addition of blocking CTLA-4 antibody does not overrule the competitive advantage of Tregs, but loss of LFA-1 results in its reversal, indicating that LFA-1 is at least partially responsible for the preferential binding of Tregs (161). Stimulated TCR-transgenic Tregs specifically reduce expression of both CD80 and CD86 on DC and to lesser degree B cells (84, 87, 161, 162). In the absence of stimulating peptide, the B7 molecules are not downregulated. CTLA-4-deficient Tregs as well as wild-type cells in the presence of CTLA-4 blocking antibody do not reduce B7 expression, and neither do Tregs from LFA-$1^{-/-}$ mice (84, 161), indicating that the formation of a stabilized, LFA-1 dependent, Treg-APC IS is important for the B7 downregulation.

B7 ligand recognition can modulate Treg motility. Tregs migrate rapidly on non-stimulating bilayers but slow down significantly, and increase contact time, upon encountering a TCR signal (TCR stop signal) (160, 163). Importantly, the stop signal required for the slowing down of Tregs is CTLA-4-independent, as CTLA-4-deficient TCR-transgenic Tregs slowed down as efficiently as CTLA-4-sufficient cells in mixed culture with antigen-pulsed DC (160). Similarly, addition of blocking CTLA-4 antibody to Tregs on lipid bilayers containing CD80, pMHC, and ICAM-1 did not affect their motility (163). Although active displacement of CD28 from the IS by CTLA-4 precludes an active role for the former in the establishment and stabilization of Treg–APC synapses, a growing body of data indicates that CD28 may be important for the orchestration of Treg motility and contact half-life with APC prior to mature synapse formation. However, data on involvement of CD28 in stop signaling remain contradictory. While CD28-deficient Tregs stop normally in mixed culture (160), CD28-blocking antibody interfered with the stop signal (163). Specific loss of CD28 in Tregs reduces surface expression of CTLA-4 on these cells and results in reduced suppressive capacity and systemic autoimmunity (71), indicating that tonic signaling input downstream of transient B7–CD28 interactions may regulate recycling of CTLA-4 protein. CD86 and CD28 input is also important for DC-induced proliferation of Tregs (155).

Unlike Tregs, effector T cells are sensitive to CTLA-4-dependent reversal of the TCR stop signal. In the first report of CTLA-4 dependent reversal of TCR stop signal, Schneider et al. (156) used anti-CTLA-4 stimulation and observed that it enhanced effector T cell motility on LFA-1-coated plates. Moreover, anti-CD3 antibody induced reduction in T cell motility, but a combination of anti-CD3 and anti-CTLA-4 did not elicit this stop signal. In the same study, CTLA-4 expression on effector T cells increased their motility and reduced their contact time with APCs in the context of antigen recognition in the lymph node (156). CTLA-4 was also shown to reverse the TCR stop signal in human effector T cell clones *in vitro* (122). Additionally, CTLA-4 antibody treatment enhanced effector T cell motility in the context of an anti-tumor response (158, 159). However, two other 2 photon imaging studies did not report this differential effect of CTLA-4 blockade on regulatory and effector CD4+ T cell populations (157, 164). A study using a mouse model of T cell responses in pancreatic islet grafts reported that CTLA-4 blockade slightly increased motility of both effector and Treg populations, suggesting that CTLA-4 marginally reduces CD4+ T cell motility *in vivo* (157). However, the imaging performed in this study was conducted in an immunoprivileged site (the islet grafts were injected into the anterior chamber of the eye), which could have affected the cellular motility observed. Moreover, the role of TCR signal in the reported effector and Treg motility changes is unknown, as this study observed direct interactions between pancreatic peptide-specific TCR-transgenic CD4 T cells and islet cells, which do not express MHC class II and, thus, cannot present antigenic peptide to T cells. Another study investigating motility of tolerized diabetogenic CD4+ T cells reported no effect of CTLA-4 blockade on T cell motility (164). However, this study did not differentiate between effector and Treg populations, and the effect on CTLA-4 on control, non-tolerized diabetogenic T cells was not reported (164). Overall, the results from these two studies indicate that CTLA-4 has limited effect on motility of self-antigen-specific CD4+ effector T cells,

similar to its relatively limited effect on Treg motility. Given that the natural Treg lineage consists of self-reactive T cells (165), this raises an interesting possibility that the role of CTLA-4 in regulation of synapse stability and cellular motility of CD4+ T cells depends on their TCR specificity.

Importantly, the different effects of CTLA-4 blockade on effector T cells and Tregs have also been observed in a recent study using 2 photon microscopy to examine the behavior of the two CD4+ T cell populations in intact lymph nodes (13). CTLA-4 blockade increased Treg motility but decreased effector T cell motility in the presence of antigen, consistent with the proposed role of CTLA-4 in reversal of TCR-induced motility stop in effector, but not regulatory, T cell populations. Anti-CTLA-4 antibody administration increased effector T cell contact time with DCs presenting antigen, but reduced Treg contacts with DCs, strongly suggesting that CTLA-4 has opposing effects on effector and Treg IS stability *in vivo*. However, as CTLA-4 blockade increased the steady-state motility of Tregs, but had no effect on effector T cell motility in the absence of antigenic stimulation, the reduced effector T cell motility and enhanced clustering with DCs after anti-CTLA-4 treatment could be the result of exclusion of Tregs from T cell: DC clusters, rather than a direct effect of CTLA-4 on effector T cells. Interestingly, this study also reported regulatory-effector T cell contacts that were dependent on Treg recognition of B7 expressed on activated T cells (13), suggesting that CTLA-4: B7 interaction plays a role in regulation of T:T cell synapse formation and facilitates Treg-mediated suppression.

The molecular mechanism of CTLA-4-dependent regulation of effector T cell synapse stability and cellular motility is unknown. The initial observation that anti-CTLA-4 treatment enhances effector T cell motility on LFA-1 coated slides and in response to TCR signal was originally interpreted as evidence for an as yet unidentified CTLA-4-induced signal overriding the TCR stop signal (156). CTLA-4 ligation was shown to reduce IS stability (122, 166) and decrease cytoskeletal rearrangements at the synapse (166) through an unknown molecular mechanism. CTLA-4 ligation was also shown to activate the small G protein Rap1 (167, 168), and CTLA-4-induced Rap1 activity was linked to destabilization of the IS (53). However, CTLA-4 mediated increase in Rap1 activity has also been linked to enhanced LFA-1 mediated adhesion (167–169), which is difficult to reconcile with the reduced synapse stability. Moreover, an *in vivo* study reported that intact anti-CTLA-4 antibody and its Fab fragments enhanced effector T cell motility equally well, suggesting that CTLA-4-dependent signaling did not play a role in the motility enhancement (158). Given the role of CD28 in regulation of cytoskeletal dynamics, it is plausible that CTLA-4 may reduce synapse stability and enhance T cell motility through counteracting CD28-mediated cytoskeletal rearrangement through competition for B7 ligand binding. However, there is conflicting evidence to support this hypothesis. It has been reported that the CTLA-4-dependent increase in motility does not require CD28 expression (160), and that the cytoplasmic region of CTLA-4 is required for regulation of T cell motility (166), suggesting a role for as yet unidentified CTLA-4-dependent signaling.

CONCLUSION

B7 ligand recognition plays an important role in orchestrating the IS architecture in both effector T cells and Tregs. During recognition of antigen, B7 ligand binding induces CD28 localization to distinct TCR^{low} clusters within the central region of the effector T cell IS. This CD28 recruitment can be counteracted by CTLA-4 through competition for ligand binding and/or by removal of co-stimulatory ligands through trans-endocytosis. CD28 recruitment to the IS induces PKC-θ localization to the center of the IS, through interactions between PKC-θ V3 hinge region and the proline-rich motif on the cytoplasmic tail of CD28. CD28 ligation leads to cytoskeletal rearrangements at the IS through CD28-dependent control of multiple pathways regulating cytoskeletal dynamics: Vav1 and cofilin activation, filamin A binding, and regulation of actin-uncapping proteins. CD28-dependent PKC-θ recruitment and modulation of cytoskeleton plays a critical role in regulation of effector T cell functions. CTLA-4 is a negative regulator of effector T cell functions, and there is evidence that CTLA-4 can reduce effector T cell IS stability through reversal of the TCR-induced stop signal.

While signaling through CD28 is important for steady-state homeostasis, motility, target recognition, and division of Tregs, their activation results in active exclusion of CD28 and PKC-θ and recruitment of CTLA-4 and PKC-η to the synapse. Both phenomena are required for the suppressive function. In contrast to conventional CD4 T cells, CTLA-4 and PKC-η act as positive regulators of Treg function. Since CTLA-4 binds to B7 proteins with significantly higher affinity than CD28 and exclusively activates PKC-η (152), it is reasonable to conclude that its affinity alone may be sufficient to initiate the exclusion of CD28 from potential B7 binding sites. Preferential activation of PKC-η is then a direct outcome of exclusive CTLA-4 recruitment. Higher affinity of CTLA-4–B7 interactions also explains why Tregs are capable of actively recruiting B7 proteins to the synapse, while effector T cells are not (170). Increased affinity of B7–receptor interaction and recruitment of B7 proteins to the synapse also contribute to more long-lived, stable synapses between Tregs and APCs as compared to conventional T cells.

B7 ligand recognition induces dissimilar immune synapse architecture in mature effector T cells and Tregs, but its role in regulation of the immune synapse dynamics at different stages of T cell development is poorly understood. It remains to be determined if the IS formed by pre-selection thymocytes shows the central localization of CD28 and/or PKC-θ, similar to that observed in effector T cells. Given that immature thymocytes do not show the centralized TCR accumulation at the immune synapse (171, 172), and that PKC-θ is not required for NFκB activation in thymocytes (173), it is likely that CD28/PKC-θ dynamics at the thymocyte IS are different to the dynamics in mature effector T cells. At the other end of the T cell's lifetime, CD28/PKC-θ immune synapse dynamics in exhausted T cells or T cells from aged individuals are very poorly understood. Anergic murine T cells were shown to display unimpaired PKC-θ recruitment (174), but loss of CD28 from human T cells due to repeated antigen exposure or aging may have implications on PKC-θ synapse localization, resulting in altered kinetics and architecture of

the synapse, and changes in downstream signaling. Furthermore, despite the massive amount of data on CTLA-4 biology and the growing importance of the CTLA-4 pathway in immunotherapy, its role in regulation of T cell functions and IS dynamics remains incompletely understood. Since activated effector T cells express CTLA-4 and since surface CTLA-4 is capable of B7 extraction from target membranes regardless of cell type (146), it will be intriguing further to explore the potential role of CTLA-4 in the effector T cell-intrinsic restriction of strength and/or duration of activation, independently of bystander suppression by Treg-expressed CTLA-4. At the signaling level, given the role of CD28 signaling in regulating cytoskeleton dynamics at the IS, CTLA-4 can likely counteract these CD28-mediated pathways, either indirectly through reducing levels of B7 proteins on APCs, or directly through interactions yet unidentified with other binding partners. PKC-η is a likely candidate, as Tregs lacking PKC-η showed enhanced conjugation with DCs, and the CTLA-4–PKC-η complex has been shown to interact with the focal adhesion complex components PAK2 and GIT2, as well as with the guanine nucleotide exchange factor αPIX (152), with a known role in regulating cytoskeletal dynamics (175). Better understanding of the effect of B7 ligand recognition on the IS dynamics at different stages of T cell development and in different T cell subsets is likely to have significant implications for the development of novel immunotherapy strategies.

AUTHOR CONTRIBUTIONS

VR and JB wrote the initial draft of this review. VR, NG, and JB rewrote and edited the article.

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REVIEW
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Function and Dynamics of Tetraspanins during Antigen Recognition and Immunological Synapse Formation

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Tetraspanin-enriched microdomains (TEMs) are specialized membrane platforms driven by protein–protein interactions that integrate membrane receptors and adhesion molecules. Tetraspanins participate in antigen recognition and presentation by antigen-presenting cells (APCs) through the organization of pattern-recognition receptors (PRRs) and their downstream-induced signaling, as well as the regulation of MHC-II–peptide trafficking. T lymphocyte activation is triggered upon specific recognition of antigens present on the APC surface during immunological synapse (IS) formation. This dynamic process is characterized by a defined spatial organization involving the compartmentalization of receptors and adhesion molecules in specialized membrane domains that are connected to the underlying cytoskeleton and signaling molecules. Tetraspanins contribute to the spatial organization and maturation of the IS by controlling receptor clustering and local accumulation of adhesion receptors and integrins, their downstream signaling, and linkage to the actin cytoskeleton. This review offers a perspective on the important role of TEMs in the regulation of antigen recognition and presentation and in the dynamics of IS architectural organization.

Keywords: tetraspanins, tetraspanin-enriched microdomains, adhesion receptors, immunological synapse, T-cell activation

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TETRASPANIN-ENRICHED MICRODOMAINS

Tetraspanins comprise a family of small proteins with four transmembrane domains and are present on the plasma membrane and intracellular vesicles of virtually all mammalian cells. The tetraspanins CD9, CD63, CD81, CD82, and CD151 have a broad tissue distribution, whereas others are restricted to particular tissues, such as TSSC6, CD37, and CD53, in hematopoietic cells (1). Tetraspanins have small and large extracellular loops (SEL and LEL, respectively) and short N- and C-terminal intracellular tails (2). The LEL domain mediates specific protein–protein interactions with laterally associated proteins and a few known ligands, while the cytoplasmic regions provide links to cytoskeletal and signaling molecules (3). Tetraspanins organize a type of cell surface membrane microdomain, known as tetraspanin-enriched microdomains (TEMs) (2, 4), based on their exceptional ability to form multimolecular complexes. Studies using novel advanced microscopy techniques in the intact membranes of living cells have provided a more complete picture of the supramolecular organization of these microdomains (5). The diversity of TEM composition is reflected by different interaction

levels, in which each tetraspanin recruits one or more partner proteins forming direct and stable primary complexes, which are assembled through tetraspanin–tetraspanin interactions to form larger complexes that can vary depending on the cell type (6). However, this classical view of TEMs has recently been challenged. Super-resolution microscopy has shown that, in B cells and dendritic cells (DCs), CD53 and CD37 single clusters overlap only to a minor extent with CD81 or CD82 clusters. Moreover, CD53 and CD81 nanoclusters are in closer proximity to their partners MHC class II (MHC-II) and CD19 than to other tetraspanins (7). Additional research using super-resolution microscopy is necessary to dissect the spatial and temporal organization of TEMs in different systems.

In the context of the immune system, TEMs regulate important processes including antigen (Ag) recognition and presentation, protein trafficking, cell proliferation, and leukocyte extravasation (1). All cells of the immune system express tetraspanins, although the tetraspanin repertoire differs between cell types (3). Several receptors responsible for immune cell functions, like the Ag receptors T-cell receptor (TCR) and B-cell receptor (BCR), pathogen receptors, and MHC molecules, are included in TEMs; furthermore, both ubiquitously expressed tetraspanins such as CD81 and immune cell-specific tetraspanins such as CD37 have been shown to be important for immunity (1). In human T lymphocytes, tetraspanins CD9, CD53, CD81, and CD82 act as costimulatory molecules (8–13), and this activity is independent of the classic CD28 costimulatory pathway (12–16). T cells from mice lacking tetraspanins CD81, CD151, CD37, or Tssc6 are hyperproliferative (17–20), and CD37- and CD81-deficient mice have impaired T-cell-dependent immune responses (17, 21–23). Moreover, CD81 expression in both T and B cells is essential for T-cell activation and proper Th2 responses (24–26).

Tetraspanins are also involved in the process of leukocyte extravasation. CD81 controls integrin α4β1 avidity, being essential for monocyte and B cell adhesion under shear flow (27), and CD9 regulates LFA-1-mediated T cell adhesion under flow conditions (28). Moreover, monocyte and T cell transmigration across brain endothelial cell monolayers is significantly reduced by monoclonal antibodies against CD81 in rodent and human *in vitro* models (29). This inhibitory effect was driven by CD81 expressed in both leukocytes and endothelial cells (29). Transmigrated eosinophils exhibit reduced CD9 expression levels, and their adhesion properties are inhibited by antibodies against CD9 (30, 31). In endothelial cells, various adhesion receptors are included in preassembled tetraspanin-based endothelial adhesive platforms; these platforms coalesce at docking structures for adherent leukocytes during the transmigration process (32, 33).

Immune cells, such as T cells, B cells, and DCs, can release extracellular vesicles that are an important vehicle for intercellular communication and have a role in the regulation of the immune response by different mechanisms (34). Tetraspanins, especially CD9, CD63, and CD81, are highly enriched in extracellular vesicles and have been widely used as exosomal markers. Importantly, growing evidence suggests a functional role for tetraspanins in the biogenesis, targeting, and function of extracellular vesicles (35). In particular, high throughput quantitative proteomic approaches have demonstrated that exosomes from $CD81^{-/-}$ mouse T lymphoblasts show an impaired inclusion of CD81 partners, including MHC molecules, BCR, ICAM-1, and Rac (36).

Together, all these observations indicate that tetraspanins influence many aspects of cellular immunity, sometimes exerting antagonistic roles, and may provide a means of manipulating the immune response for potential therapeutic applications.

THE IMMUNOLOGICAL RELEVANCE OF TETRASPANINS IN ANTIGEN-PRESENTING CELLS

TEMs and Antigen Recognition: Interaction with Pattern-Recognition Receptors

The plasma membrane of antigen-presenting cells (APCs) contains specialized membrane microdomains that organize the spatial distribution of MHC and associated proteins, pattern-recognition receptors (PRRs), and integrins, which are essential for efficient Ag recognition, presentation, and ultimately the activation of the T cell. APCs express a broad repertoire of specific receptors involved in the recognition and uptake of Ags from pathogens, damaged tissues, or tumor cells. In particular, pathogen-derived Ags are recognized by different PRRs that bind to conserved microbial structures called pathogen-associated molecular patterns (PAMPs) (37). The recent identification of specific PRR interactions with tetraspanins has provided new insights into the organization of Ag receptors at the APC membrane and their subsequent downstream signaling (38). In this part, we will revise the recent data that have demonstrated tetraspanin interactions with different receptors involved in Ag recognition (**Figure 1**).

Dectin-1 is a C-type lectin receptor (CLR) that recognizes β-glucans in fungal cell walls, triggering phagocytosis, and the antifungal immune response. Dectin-1 signaling is only activated by particulate β-glucans, which cluster the receptor in synapse-like structures from which regulatory tyrosine phosphatases are excluded (39). Two independent studies have demonstrated that CD63 and CD37 interact with dectin-1 on the APC membrane (**Figure 1A**; **Table 1**). CD63 associates with dectin-1 in immature DCs and promotes yeast phagocytosis (40) (**Table 1**). CD37 stabilizes dectin-1 at the APC surface, and this interaction has functional consequences since CD37 inhibits dectin-1-mediated IL-6 production in response to zymosan cell wall preparations (41) (**Table 1**). Moreover, $CD37^{-/-}$ mice are protected against systemic infection with *Candida albicans* (42). CD63 has also been reported to be selectively recruited to yeast-containing phagosomes (43) (**Table 1**), and this observation was subsequently extended to CD82 (44) (**Figure 1B**; **Table 1**). After pathogen uptake, CD82 is rapidly recruited to the membrane of nascent pathogen-containing phagosomes prior to fusion with lysosomes (44) (**Figure 1B**).

In addition to the reported recruitment of TLR2 and TLR4 to lipid rafts (59–61), other studies demonstrate that TLR4 associates with TEMs. In macrophages, CD9 partly colocalizes with CD14 regulating its expression, its association with TLR4, and

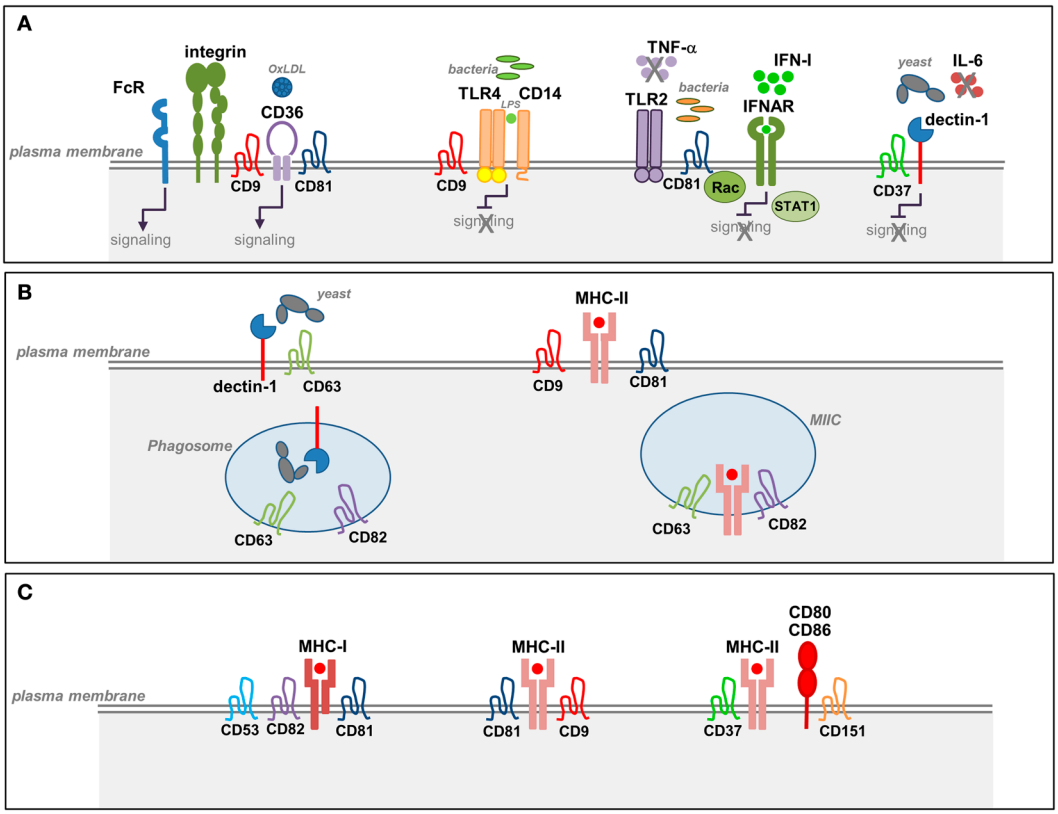

FIGURE 1 | Tetraspanins in the function of APCs. **(A)** Tetraspanin interactions with pathogen-recognition receptors (PRRs) in APCs. Tetraspanins interact with specific PRRs at the plasma membrane of macrophages and DCs. CD37 associates with dectin-1 and inhibits dectin-1 mediated IL-6 production triggered by the recognition of fungal cell walls. CD9 forms a complex with CD14 and TLR4 and negatively regulates TLR4 signaling in response to LPS. CD81–Rac interaction inhibits TLR2- and IFNAR-signaling pathways and prevents the subsequent activation of STAT1 in response to *Listeria monocytogenes*. CD36 associates with β1 and β2 integrins and tetraspanins CD9 and CD81 forming a complex that facilitates CD36-signaling and its interaction with FcγRs. Interaction between CD9 and FcγRs promotes phagocytosis and macrophage activation. **(B)** Tetraspanin interactions during Ag processing and MHC-II biosynthesis. CD63 interacts with dectin-1 in immature DCs and promotes yeast phagocytosis. Both CD63 and CD82 are selectively recruited to yeast-containing phagosomes. CD82 and CD63 are highly enriched in MIIC compartments that contain newly synthesized MHC-II and accessory proteins. **(C)** Tetraspanin interactions during Ag presentation. Several tetraspanins associate with MHC-I and MHC-II molecules on APCs. Tetraspanins CD9, CD53, CD81, and CD37 associate with MHC-II molecules preferentially at the plasma membrane. MHC-II molecules loaded with a restricted antigenic peptide repertoire are included in TEMs together with accessory molecules and costimulatory molecules. CD9 facilitates MHC-II clustering, and CD151 is involved in the clustering of costimulatory molecules.

the formation of the CD14–TLR4 complex necessary for LPS-induced signaling (45) (**Figure 1A**; **Table 1**). Using the *Listeria monocytogenes* infection model, we recently demonstrated that CD81 is able to interfere with TLR2- and interferon-α/β receptor (IFNAR)-mediated bacterial recognition in DCs, modulating the subsequent $CD8^+$ T cell response (53) (**Figure 1A**; **Table 1**). Importantly, $CD81^{-/-}$ mice are protected against lethal systemic *Listeria* infection. $CD81^{-/-}$ DCs show increased production of proinflammatory mediators and a more efficient activation of protective cytotoxic T cells. This effect is mediated specifically through direct interaction between CD81 and Rac. Indeed, inhibition of CD81–Rac interaction in wild-type DCs using CD81 C-terminal peptides, which block CD81-mediated signaling (62), promotes the same phenotype observed in $CD81^{-/-}$ DCs (53).

In macrophages, CD9 interacts with CD36, a scavenger receptor involved in the recognition of microbes or self-ligands, regulating CD36-mediated uptake of oxidized low-density lipoproteins (46) (**Figure 1A**; **Table 1**). CD36 clustering is necessary for the initiation of signal transduction and internalization of receptor–ligand complexes. CD36 was recently shown to form a heteromeric complex containing β1 and β2 integrins and the tetraspanins CD9 and CD81. CD36 inclusion in this complex facilitates its association with ITAM-bearing adaptor Fcγ receptors (FcγR), allowing CD36-dependent Syk activation and the internalization of ligand-bound CD36 (47) (**Figure 1A**; **Table 1**). In addition, CD9 functionally associates with FcγRs, modulating signals for phagocytosis, and FcγR-mediated immune responses (**Table 1**). Cross-linking of CD9-FcγRIII induces colocalization of CD9, αMβ2 integrin and F-actin, promoting macrophage activation (48) (**Figure 1A**). In human monocytes and skin-derived DCs, CD9 and CD81 are molecular partners of the trimeric form of FcεRI (**Figure 1**; **Table 1**), the high-affinity receptor for IgE, and are overexpressed in patients with atopic dermatitis (49).

TABLE 1 | Tetraspanin associations in pathogen-recognition receptors and APC functions.

Tetraspanin	Interacting molecule	Cell type	Function	Reference
CD9	TLR4–CD14 complex	Macrophages	Regulates LPS-induced signaling	(45)
	CD36	Macrophages	Mediates CD36–integrin complex formation and ligand-bound internalization and signaling	(46, 47)
	FcγR	Macrophages	Interacts with and regulates FcγR-mediated immune responses	(47, 48)
	FcεRI	Monocytes and DCs	Association at the membrane	(49)
	MHC-II	DCs	Association at the membrane	(50–52)
CD81	Rac1	DCs	Controls TLR2- and IFNAR-mediated bacterial recognition	(53)
	CD36	Macrophages	Regulates CD36–integrin complex formation, ligand-bound internalization and signaling	(46, 47)
	FcεRI	Monocytes and DCs	Association at the membrane	(49)
	BCR	B cells	Controls CD19 surface expression and BCR complex downstream signaling	(3, 54)
	MHC-II	DCs	Association at the membrane	(50, 51)
CD37	Dectin-1	Macrophages	Controls dectin-1 stabilization at the membrane and signaling triggered by dectin-1 recognition of yeast cell walls	(41)
	MHC-II	B cells and DCs	Associates with and regulates MHC-II-dependent antigen presentation	(55, 56)
CD63	Dectin-1	DCs	Associates with dectin-1 and regulates yeast phagocytosis	(40, 43)
	MHC-II	DCs	Associates with peptide-loaded MHC-II and controls its surface expression	(50, 51, 57, 58)
CD82	MHC-II	Macrophages and DCs	Association at MHC-II-enriched compartments, and fungal and bacterial phagosomes	(44, 50, 51, 57)
CD53	MHC-II	B cells, DCs	Association at the membrane	(50, 55)
CD151	CD80, CD86	DCs	Regulates costimulation during Ag presentation	(56)

The tetraspanin CD81 plays an important role in Ag-induced B cell activation, B cell development, and survival. It associates functionally with CD19 and CD21, which are members of the BCR complex (3, 54) (**Table 1**). CD81 deficiency in humans and mice leads to antibody deficiency syndrome by preventing CD19 surface expression (21, 63). Moreover, visualization of primary B cells by super-resolution microscopy shows that CD81-enriched microdomains and the actin cytoskeleton regulate CD19 mobility and organize CD19 and BCR interactions, controlling BCR downstream signaling (64).

In the context of viral infection, CD81 was identified as a receptor for hepatitis C virus (HCV) (65), not only in hepatocytes but also in B cells, T cells, NK cells, and DCs (66). The dynamic properties of CD81 at the membrane are essential for HCV infection (67). Anti-CD81-specific antibodies mediate protection against HCV infection *in vivo*, further demonstrating the functional consequences of this recognition (68). Tetraspanin dynamics at the membrane are also exploited by other viruses. For example, CD9 and CD81 negatively regulate human immunodeficiency virus 1 (HIV-1)-induced membrane fusion (69).

TEMs during Antigen Processing and Presentation

T cell recognition of specific antigenic peptides bound to MHC-I and MHC-II molecules on DCs leads to T cell activation and subsequent initiation of T cell-mediated immune responses. In DCs, mechanisms regulating MHC-II intracellular transport are well known (70), and tetraspanins have a role in this process since several tetraspanin family members associate with MHC-II molecules. Interactions between MHC-I molecules and tetraspanins CD53, CD81, and CD82 have been described (71) (**Figure 1C**). Moreover, tetraspanins CD9, CD81, CD82, CD63, CD53, and CD37 interact with MCH-II molecules (50, 55, 57, 72) (**Figures 1C** and **2**; **Table 1**). These interactions might lead to the regulation of MHC-II subcellular distribution. CD9, CD53, and CD81 associate with MHC-II at the plasma membrane (50) (**Figure 1C**; **Table 1**). In contrast, CD82 and CD63 are highly enriched in MHC-II-enriched compartments (MIIC) (**Figure 1B**; **Table 1**), particularly in intraluminal vesicles, where they associate with each other and with the chaperone HLA-DM, playing an important role in the late stages of MHC-II maturation (50, 57) (**Table 1**). Analysis of protein dynamics by Föster resonance energy transfer (FRET) in MIIC shows that CD63 stably associates with MHC-II and regulates MHC-II surface expression, whereas CD82 associates with HLA-DM without affecting MHC-II expression (58). Knockdown of CD63, CD82, CD9, or CD81 did not prevent MHC-II peptide loading (58). In addition, live cell imaging studies have shown differential CD63 and CD82 subcellular localization in the context of DC phagocytosis. Whereas CD63 and MHC-II are specifically recruited to yeast-containing phagosomes after phagosomal acidification (43), CD82 and MHC-II molecules are recruited to fungal and bacterial phagosomes before fusion with lysosomes and phagosomal acidification (44) (**Figure 1B**; **Table 1**). These results support a role for CD63 and CD82 in the dynamic intracellular trafficking of MHC-II after pathogen uptake, playing non-redundant roles in these processes.

Tetraspanins are also involved in the clustering of MHC molecules (**Figure 1C**). APCs express very small amounts of relevant MHC-II–peptide complexes on the plasma membrane. These MHC-II–peptide complexes are organized and clustered on the cell surface, allowing efficient cross-linking of TCRs and promoting Ag-specific T cell activation (73). It is widely accepted that MHC-II molecules are concentrated into two types of membrane microdomains, TEMs, and lipid rafts (74). The composition and dynamics of these microdomains are essential factors in the outcome of T cell activation. Evidence from a model of

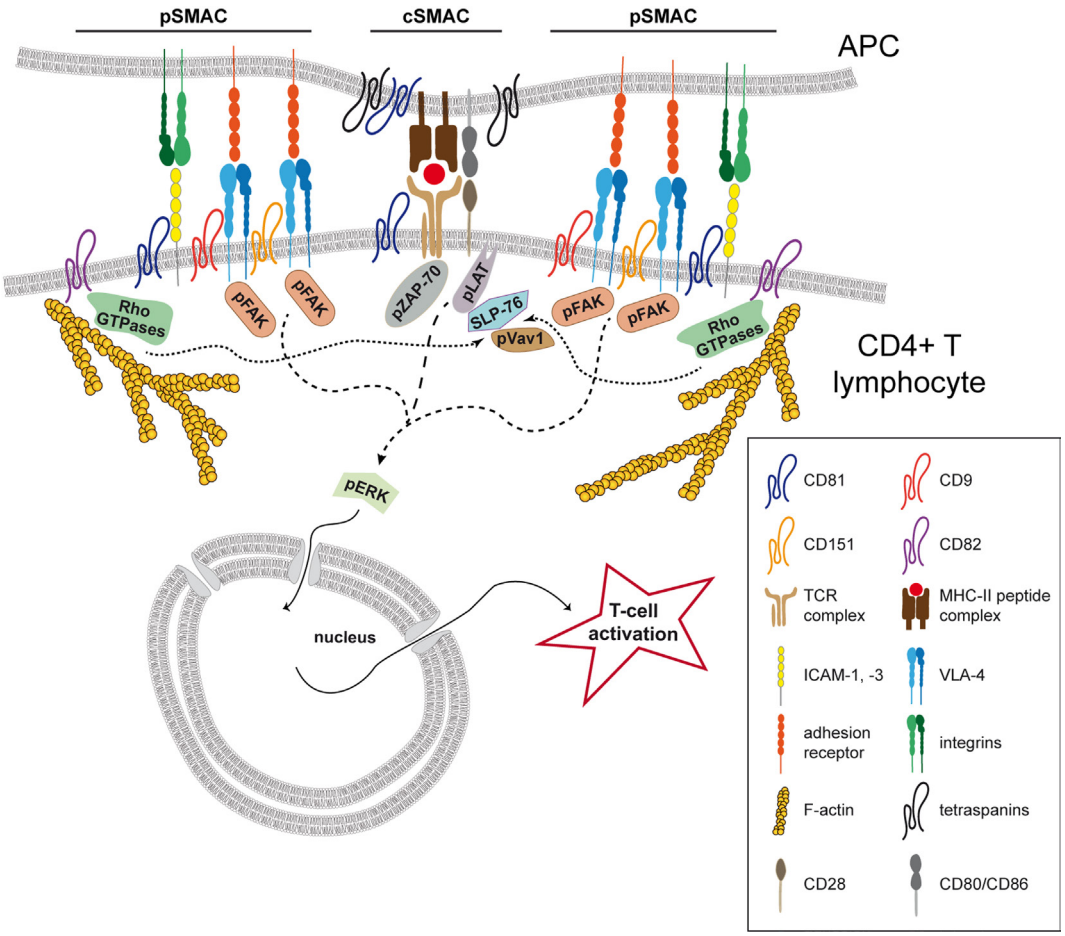

FIGURE 2 | Tetraspanins organize the T-cell immunological synapse. Tetraspanin CD81 regulates the organization of the immunological synapse (IS) in CD4+ T lymphocytes through the association with CD3ζ at the central SMAC (cSMAC). CD81 controls the localization of the TCR complex and its downstream signaling, positively modulating the phosphorylation of ZAP-70, LAT, and ERK1/2 (dashed line). At the peripheral area of the cell–cell contact (pSMAC), tetraspanins CD9, and CD151 are important for integrin VLA-4 relocalization and activation, positively regulating the integrin downstream phosphorylation of FAK and ERK1/2 (lines with small dashes). At this location, CD81 also interacts with the adhesion receptor ICAM-1, regulating its segregation during IS maturation. Tetraspanin CD82 accumulates at the pSMAC and triggers actin polymerization and the activation of the Rho GTPase pathway (RhoA, Rac1, and Cdc42). The activation of this pathway induces the phosphorylation and the association of Vav1 and SLP76 (dotted lines), potentiating the phosphorylation of the TCR signaling molecules LAT and ZAP-70. In APCs, CD81 is enriched at the IS and several tetraspanins are described to associate with MHC-II. Moreover, CD151, CD37, and Tssc6 were described to regulate antigen presentation by DCs.

raft disruption in B cells suggests that MHC-II association with lipid rafts is important for presentation of Ag at low concentrations (75). Other studies report that TEMs contain MHC-II molecules loaded with a restricted antigenic peptide repertoire, together with HLA-DM and the costimulatory molecule CD86. In contrast, raft-associated MHC-II molecules display a highly diverse set of peptides (51) (**Table 1**). However, these results are controversial, since the MHC-II determinant CDw78, which is used to identify selectively tetraspanin-associated MHC-II, also defines a conformation of peptide-bound MHC-II acquired through the trafficking to lysosomal compartments (76). Moreover, TEM-induced MHC-II clustering is also supported by evidence that CD9 is required to facilitate the formation of I-A/I-E MHC-II multimers, which are responsible for enhancing the T cell stimulatory capacity of DCs (52) (**Table 1**). However, a subsequent study showed that cholesterol depletion disrupts MHC-II I-A/I-E interactions, whereas the absence of CD9 or CD81 has no effect (77). This controversy might be due to the differential sensitivity of microdomains to cholesterol depletion. Although TEMs are more resistant to cholesterol depletion than lipid rafts, partial disruption is also observed under certain conditions. Therefore, it is possible that rafts and TEMs both contribute to MHC clustering.

Studies derived from tetraspanin-deficient mice have shown that certain tetraspanin members do not promote MHC multimerization, being rather involved in Ag presentation. DCs from CD37$^{-/-}$ or CD151$^{-/-}$ mice induce hyperstimulation of T cells (56), and similar results were obtained with DCs from Tssc6$^{-/-}$ mice and CD37$^{-/-}$ Tssc6$^{-/-}$ double knockout mice (78). CD37$^{-/-}$ DCs induce T cell hyperstimulation through a mechanism

that regulates MHC-dependent Ag presentation, whereas CD151 in DCs regulates T cell costimulation (56) (**Figures 1C** and **2**; **Table 2**). DC maturation is required for effective T-cell costimulation and involves the upregulation of costimulatory and adhesion molecules (79, 80). In contrast to conventional DCs, plasmacytoid DCs lack CD9 surface expression, which could be responsible for their significant low expression of MHC-II and limited T cell stimulatory potential (80). TEMs thus play a well-documented role in the regulation of different aspects of the MHC-II lifecycle in APCs, including MHC-II clustering and intracellular trafficking of peptide–MHC-II complexes to the APC plasma membrane.

ROLE OF TETRASPANINS IN THE ORGANIZATION OF T-CELL IMMUNOLOGICAL SYNAPSES

The Immunological Synapse

The initiation of T cell activation mediated by APCs, mainly DCs, requires the establishment of a dynamic structure formed at the cell–cell contact called the immunological synapse (IS) (**Figure 2**). This structure is characterized by a dynamic spatiotemporal recruitment of Ag receptors, costimulatory molecules, and adhesion proteins to specific zones at the T cell–APC interface. At the T cell side of mature IS, TCR microclusters are clustered together with costimulatory proteins, signaling molecules, and other signaling adaptors at the central supramolecular activation complex (cSMAC) (91–96). More specifically, preexisting TCR nanoclusters (97) concatenate into microclusters, as demonstrated with high-resolution imaging techniques like photoactivated localization microscopy (PALM) and stimulated emission depletion (STED) (98–100). These microclusters form in the periphery of the IS and are translocated toward the cSMAC in a process dependent on the actin cytoskeleton (93, 94, 98, 101, 102). The central area is surrounded by a peripheral SMAC (pSMAC), where integrins and adhesion receptors are localized (81, 91, 102–104). The super-resolution optical techniques near-field scanning optical microscopy (NSOM) and single-dye tracking (SDT) revealed that, like the TCR, LFA-1 is preorganized into nanoclusters that coalesce into microclusters after ligand binding (105, 106). The stability of the IS depends on the binding of integrins, not only lymphocyte function-associated antigen 1 (LFA-1; αLβ2) but also very late antigen 4 (VLA-4; α4β1), to their ligands, the adhesion receptors intercellular adhesion molecule-1, -3 (ICAM) in the case of LFA-1 (91, 103, 104, 107–109). The VLA-4 ligand at the T-cell–APC interface remains unknown (104). In resting T lymphocytes, integrins are mostly in an inactive bent conformation, with low affinity and avidity for ligands. TCR stimulation triggers intracellular signaling that leads integrins to adopt an intermediate-affinity conformation, and then the extended high-affinity conformation (110). These conformational changes induced by TCR signaling modify integrin avidity through a process called inside-out signaling (111), which ultimately regulates integrin affinity for their ligands (112, 113). LFA-1 engagement by its ligand ICAM-1 triggers outside-in signaling, inducing cytoskeletal reorganization that recruits T cell signaling proteins to the IS (113–115).

Both the TCR and LFA-1 modulate cytoskeletal dynamics. TCR signaling triggers actin polymerization enabling the extension of the actin network downstream of LFA-1 (116). VLA-4 costimulation regulates the cytoskeletal movements that drive TCR microclusters associated with signaling complexes to the central area of the IS (109). Moreover, it has been suggested that TCR microcluster formation is dependent on actin polymerization (94, 101); however, other investigators claim that actin is necessary only for microcluster maintenance (98). Continuous actin retrograde flow sustains T cell signaling and signal termination at the central area of the IS (94, 101, 102, 117). Actin filaments are also important for the segregation of adhesion molecules to the pSMAC (91, 102, 103), and actin centripetal flow is essential for the maintenance of LFA-1 in a high-affinity conformation at this location (118, 119).

The T cell cytoskeletal network thus plays an essential role in the spatial organization of the IS. However, the precise mechanisms by which molecules are specifically partitioned into central and peripheral areas of the IS remain an open question. It has been proposed that this segregation is supported by size

TABLE 2 | Tetraspanin associations in T cells and their role at the immunological synapse.

Tetraspanin	Associated proteins	Signaling pathway	Function	Reference
CD81	CD3ζ	ZAP-70, LAT, ERK1/2	Controls TCR relocalization to the IS and subsequent downstream signaling	(81)
	CD3δ, CD4, CD8		Association at the membrane	(82–84)
	VLA-4		Association at the membrane	(85)
	ICAM-1		Regulates ICAM-1 distribution at the IS	(81)
CD9	VLA-4	FAK, ERK1/2	Mediates VLA-4 accumulation at the IS and integrin downstream signaling	(86)
	LFA-1		Controls LFA-1-dependent adhesion	(28)
CD151	VLA-4	FAK, ERK1/2	Regulates VLA-4 accumulation at the IS and integrin downstream signaling	(86)
CD82	Actin	Rho GTPases, Vav1, and SLP76	Is enriched at the IS, regulating actin polymerization and TCR downstream signaling	(87–89)
	VLA-4		Association at the membrane	(85)
	CD4, CD8		Association at the membrane	(82–84)
CD53	VLA-4		Association at the membrane	(85)
	CD2		Association at the membrane	(90)
CD63	VLA-4		Association at the membrane	(85)

differences in the ectodomains of immune surface interacting proteins; e.g., LFA-1-ICAM-1 (40 nm) and CD2-CD58 (15 nm) (120–122). In agreement with this view, evidence suggests size-dependent exclusion from the cSMAC of large phosphatases such as CD45, thus allowing the initiation of TCR signaling (94, 123). Recent data show that CD45 is already excluded from preexisting TCR microclusters (124). Given that the TCR in naïve T cells is already clustered with signaling molecules, and that numerous proteins that are translocated to, rearranged and accumulated at the IS are known to associate with tetraspanins, we postulate that protein–protein interactions driven by TEMs actively contribute to IS architectural organization.

Tetraspanins and the Distribution of Receptors at the T-cell IS

Tetraspanin CD81 accumulates at the IS in both T lymphocytes and APCs (125) (**Figure 2**), and we recently found that CD81 is an important molecular organizer of the IS structure at the T cell side (81). Fluorescence recovery after photobleaching (FRAP) experiments indicate that CD81 is mostly confined to the cSMAC in the early IS (81), where it colocalizes with the CD3ζ component of the TCR complex (81, 125) (**Figure 2**). Analyses by phasor fluorescence-lifetime imaging microscopy (phasorFLIM)-FRET reveal that CD81 associates with CD3ζ at the cSMAC of the early IS (81) (**Figure 2**). In the late IS, CD81 and CD3ζ spread throughout the cell–cell contact and CD81 diffusion decreases, suggesting stable protein–protein interactions throughout the IS. In agreement with this view, CD81 and CD3ζ interaction increases with the IS maturation (81). As a molecular organizer, CD81 controls CD3ζ relocalization to the cSMAC, and the efficient maintenance of the CD3 signaling complex at the cell–cell contact (**Figure 2**). Hence, CD81 knockdown reduces the number of CD3ζ microclusters at the cSMAC, as detected by total internal reflection microscopy (TIRFM), and impairs TCR downstream signaling, reducing the phosphorylation of CD3ζ, ZAP-70, LAT, and ERK1/2 (81) (**Figure 2**; **Table 2**). Moreover, pretreatment of T cells with soluble GST-LEL-CD81 (81), which decreases membrane diffusion of the protein (33), increases T cell activation (81), further indicating that CD81 regulates T cell activation by controlling the duration of TCR signaling at the membrane. A direct CD81-mediated signaling does not seem to be involved in this process, since CD81 C-terminal peptides do not affect T cell activation (81). Thus, by organizing TEMs CD81 regulates spatial molecular organization during the maturation of the IS.

In T lymphocytes, different tetraspanins associate with receptors that are enriched at the IS. In addition to CD3ζ (81), CD81 also interacts with the CD3δ subunit of the TCR complex (84) (**Table 2**). CD9 localizes with TCR signaling molecules in lipid microdomains (10), CD81 and CD82 associate with CD4 and CD8 coreceptors (82, 83) (**Table 2**), and CD53 interacts with the costimulatory receptor CD2 (90) (**Table 2**). It is therefore conceivable that the IS architectural organization of these receptors depends on their inclusion in TEMs through interaction with different tetraspanins. Further research is required to address this notion.

Adhesion Molecules, Tetraspanins, and the Stabilization of the T-cell IS

Integrins and adhesion receptors are also included in TEMs. In T cells, CD9 interact with LFA-1 (28), CD81, CD82, and CD53 with VLA-4 (85), and CD81 with ICAM-1 (81) (**Table 2**). In the immune system, tetraspanins regulate cell–cell adhesion through LFA-1 and ICAM-1: CD81 and CD82 promote T-APC cell–cell interaction (126, 127); CD81 induces thymocyte aggregation (128); and CD53 modulates NK and B cell aggregation (129, 130). Conversely, leukocyte LFA-1-dependent adhesion is negatively regulated by CD9 (28) (**Table 2**). Integrin adhesiveness can be regulated by several mechanisms, such as alterations in the affinity of individual integrin molecules or changes in their clustering on the cell surface or their interactions with ligands. Tetraspanins can modulate integrin activity through various mechanisms. For example, CD81 modulates VLA-4 avidity for its ligand VCAM-1, and CD151 stabilizes α3β1 integrin in its active conformation and regulates α6 integrin diffusion at the plasma membrane (27, 131, 132). CD9 promotes β1 activation, LFA-1 aggregation, and in leukocytes it seems to be essential for a balanced regulation of β1 and β2 integrin activity: it increases β1 adhesion to fibronectin but diminishes LFA-1-mediated adhesion (28, 133).

At the IS, CD81 regulates pSMAC organization through association with the adhesion receptor ICAM-1, controlling ICAM-1 segregation at the cell–cell contact during IS maturation (81) (**Figure 2**; **Table 2**). CD81 knockdown decreases the proportion of early synapses, in which ICAM-1 is confined to the pSMAC, and increases the proportion of late synapses (81). During maturation of the IS, ICAM-1 redistributes throughout the entire cell–cell contact, with increasing colocalization and molecular interaction with CD81 (81). T cell activation is also regulated by other tetraspanins. CD9 and CD151 modulate VLA-4 accumulation at the IS (86) (**Figure 2**; **Table 2**). Interestingly, the IS enrichment of β1 integrins in a high-affinity conformation is impaired in T cells knocked-down for CD9 and CD151, suggesting that integrin activation upon IS formation occurs within TEMs (86). The conformational changes of β integrin extracellular domains can be controlled by the actin linker protein talin (134), which accumulates at the pSMAC (91) and is required for LFA-1 activation mediated by the TCR (135). However, CD9 and CD151 knockdown does not alter talin relocalization to the IS, indicating that these tetraspanins are not involved in the regulation of integrin inside-out signaling (86). Integrins and adhesion molecules can act as signaling receptors. Integrin or ICAM-1 costimulation triggers T cell activation (136–138), and LFA-1 coengagement with the TCR lowers the T cell activation threshold (139, 140). VLA-4 ligation also costimulates T cells in a TCR-dependent manner (141), and polarizes T lymphocytes toward Th1 responses (104). LFA-1 and VLA-4 activation is controlled by the interaction with a cascade of adaptor and signaling proteins (142, 143), and these downstream signaling can be modulated by tetraspanins. CD151 supports the phosphorylation of FAK, Src, and p130CAS (144) and promotes the activation of small GTPases and ERK1/2 in an integrin-dependent manner (145, 146). ERK1/2 signaling is also increased by CD9 (147). During T-APC cognate cell–cell interactions, CD9 and CD151 knockdown reduces FAK and ERK1/2

phosphorylation, and impairs the enrichment of phosphorylated FAK at the IS (86) (**Figure 2**; **Table 2**). Tetraspanins CD9 and CD151 are therefore important for integrin enrichment at the IS, modulating integrin downstream signaling.

As previously mentioned, the actin cytoskeleton plays a crucial role in the regulation of the spatial organization of TCRs and adhesion molecules at the IS. The links between tetraspanins, membrane receptors, adhesion proteins, and the actin cytoskeleton suggest a possible regulation of this process by TEMs. CD81 and CD9 are connected to the actin cytoskeleton through α-actinin and ezrin-radixin-moesin (ERM) proteins (148, 149). CD151, CD81, and CD82 regulate the actin cytoskeleton through RhoA and Rac1 signaling molecules (62, 150–152). In T lymphocytes, CD82 costimulation triggers actin polymerization and T-cell activation by stabilizing signaling downstream of TCR/CD3 (87, 88) (**Figure 2**; **Table 2**). T cell morphological changes induced by CD82 engagement depend on the activity of Rho GTPases (RhoA, Rac1, and Cdc42), involving the association of Vav1 and the adapter molecule SLP76 with the Rho GTPase pathway (88). Importantly, CD82 is enriched at the IS in an actin-dependent manner (89) (**Figure 2**; **Table 2**). CD82-dependent regulation of the actin cytoskeleton during T cell activation may involve its interaction with LFA-1. CD82 regulates T cell-APC adhesion-dependent signaling (153), through its interaction with LFA-1 (126), and like LFA-1, CD82 localizes at the pSMAC (89) (**Figure 2**). At the IS, CD82 seems to stabilize interactions with the actin cytoskeleton, favoring the formation of signaling complexes. It would be interesting to determine whether CD82 dynamics depend on its association with LFA-1, and whether CD82 can modulate LFA-1 functions.

Thus, at the IS, tetraspanins CD9, CD81, CD82, and CD151 mediate the connections between adhesion molecules, the actin cytoskeleton and signaling complexes. Increasing evidence highlights the importance of TEMs in the organization of the temporal and spatial molecular distribution at the IS, generating the context that allows full T cell activation.

CONCLUDING REMARKS

In APCs, different receptors involved in pathogen recognition and Ag presentation are associated with tetraspanins. Further investigations are necessary to determine the spatial distribution and segregation of receptors within TEMs, as well as the importance of these microdomains in the regulatory mechanisms of receptor functions and downstream signaling. The establishment of long-lasting T cell–APC contacts, which lead to the formation of the IS and ultimately promote an efficient T cell activation, are required for the initiation of T cell-mediated immune responses. IS stability depends on the binding of integrins to adhesion receptors upon TCR ligation, triggering downstream signaling. The complex IS architectural organization depends on the inclusion of the receptors concentrated at the IS into TEMs, through their dynamic and spatiotemporal interactions with different tetraspanins. The important role of TEMs in the regulation of the dynamic process of IS formation has been recently emphasized. These specialized membrane domains allow the compartmentalization of receptors and adhesion molecules and connect them to the cytoskeleton and signaling complexes that induce T cell activation. The development of advanced microscopy techniques will provide further insight into IS dynamics and the contribution of TEMs and other microdomains to this process. Considering the plasticity of the interactions that take place in TEMs, strategies that regulate IS organization by targeting tetraspanins could allow therapeutic manipulation of the final outcome of T cell activation and the subsequent immune response.

AUTHOR CONTRIBUTIONS

VR-P, FS-M, and GMH had scientific discussion for this work and wrote the manuscript.

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