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Review

Signaling microdomains in T cells

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ABSTRACT

Sub-micron scale signaling domains induced in the plasma membrane of cells are thought to play important roles in signal transduction. In T cells, agonist MHC-peptide complexes induce small diffraction-limited domains enriched in T cell receptor (TCR) and signaling molecules. These microclusters serve as transient platforms for signal initiation and are required for sustained signaling in T cells, although each microcluster functions for only a couple of minutes. How they are formed, and what mechanisms promote and regulate signaling within TCR microclusters is largely unknown, although it is clear that TCR engagement and dynamic reorganization of cortical actin are involved. Here, we review current understanding of signaling within microclusters in T cells, and speculate on how these structures may form, initiate biochemical signals, and serve as sites of both signal integration and amplification, while also facilitating appropriate termination of TCR and related signaling.

1. Introduction

T cells play a central part in mounting effective immune responses by recognizing peptide fragments of infecting organisms and orchestrating a coordinated response against them. They express somatically rearranged antigen receptors (TCR) that recognize peptide antigens bound to major histocompatibility complex molecules (pMHC) on the surface of antigen presenting cells (APC). Engagement of the TCR leads to phosphorylation of its cytoplasmic ITAMs (Immunoreceptor Tyrosine-based Activation Motifs) by the tyrosine kinase Lck, allowing recruitment of the Syk family kinase ZAP-70. Recruitment and consequent transactivation of ZAP-70 allows it to phosphorylate multiple tyrosine residues on the membrane-tethered adapter protein LAT, which leads to cooperative assembly of LAT-nucleated multimolecular complexes. The assembled LAT signalosome propagates biochemical signals to the interior of the cell. A hallmark of this process is the early rise in intracellular calcium concentration following TCR ligation, mediated by recruitment and activation of PLCγ1 to the LAT signalosome. In addition, a number of accessory molecules including the coreceptor CD4 or CD8, the costimulatory molecule CD28, and the adhesion molecules CD2 and LFA-1, are co-engaged by their counter-receptors during antigen recognition. Signaling by these receptors is affected by, and in turn can modulate, TCR signals, resulting in a finely calibrated biochemical relay that encodes the strength of pMHC binding in the context of the presenting surface (APC) and the tissue microenvironment. Integration of these signals allows the T cell to make appropriate context-specific responses such as cytokine secretion, proliferation, cytolysis, phenotypic differentiation, provision of 'help' for B cell activation, or apoptosis.

Antigen recognition and signaling take place at the contact interface between the T cell and APC known as the immunological synapse (IS) [1,2]. This radially symmetrical cellular junction, which reaches its mature state after prolonged interaction (~10–30 min) with an antigen-bearing partner cell, is organized into a distinct central zone containing TCR, CD28 and PKCθ (central supramolecular activating cluster or cSMAC), and a peripheral adhesive zone containing LFA-1, talin, and ERM proteins (peripheral supramolecular activating cluster or pSMAC) (reviewed in [3]). Since many of the T cell's activating receptors and signaling molecules are present in the cSMAC, and its formation strongly correlates with effective T cell stimulation, this supramolecular domain was initially thought to mediate cell activation. More recent studies demonstrate that early signaling associated with the TCR, such as ZAP-70 activity, is largely downregulated before the cSMAC forms, and that TCR present there is dephosphorylated [4,5]. These observations, along with the identification of lysosomal compartments that are close to the plasma membrane in the cSMAC [4], now suggest that this region serves mainly downregulatory and degradative functions. Specialized effector

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and downregulatory functions of the cSMAC are also suggested by its absence or modification according to cell-type. Prototypical cSMAC/pSMAC patterning, as originally described in T–B cell interfaces, is not commonly observed in T-cell interfaces with dendritic cells [6], and are absent from thymocyte interfaces [7]. Cytotoxic T cells appear to form a modified IS, in which the central region is divided between a TCR-rich cSMAC and a secretory domain for delivery of lytic granules (reviewed in [8]).

TCR proximal signaling is thought to occur in TCR-rich submicrometer-scale plasma membrane domains which form almost instantaneously upon T cell contact with antigen-bearing surfaces in all T cell subtypes studied. These TCR microclusters form throughout the contact interface following TCR ligation [4], and after a brief stationary phase, translocate centripetally to assemble into the cSMAC. Signaling microdomains have recently emerged as a general organizing principle of spatially regulated signaling in lymphocytes and other cells [9,10], leading to the suggestion that ligand-induced microclusters may constitute the basic signaling unit that initiates and organizes immune cell signaling [4,11,12].

2. T cell signaling occurs in dynamic microclusters

Assembly of TCRs into clusters prior to SMAC formation was first described by wide-field or confocal fluorescence imaging of T cells interacting with antigen presenting cells or planar substrates [1,13,14]. These methods demonstrated that TCR-evoked signaling at the polarized T cell interface was spatially organized at early times. They often benefited from the use of high pMHC or anti-CD3 densities that drive rapid formation of large TCR clusters with very high contrast. They were less successful in resolving TCR rich structures formed at more physiological densities or that sustain signaling after SMAC formation. This created a dilemma when it was discovered that TCR signaling appeared to be terminated in the cSMAC [15] and no structure associated with sustained TCR signaling after SMAC formation was observed. This dilemma was resolved by the combined application of supported planar bilayer technology and TIRFM (Total Internal Reflection Fluorescence Microscopy). Glass-supported planar-bilayers allow tethering of correctly oriented and laterally mobile proteins such as pMHC, ICAM-1, CD48/CD58 and CD80, to form chemically defined flat surrogate presenting surfaces, while TIRFM provides exceptional contrast for visualizing their organization at submicrometer resolution at the T cell-planar bilayer junction [16].

Clustering of TCR can be detected within a few seconds of contact with antigen-bearing bilayers [17,18]. They are initially small, containing on average ~20 TCRs, and form throughout the contact interface (Fig. 1B). Much of the early antigen-induced signaling in T cells, described in detail by bulk biochemical methods, occurs at TCR microclusters (Fig. 1D–F). They are sites of increased Lck activity [17], and recruit ZAP-70 [12,17], presumably through Lck mediated phosphorylation of TCR ITAMs. The important costimulatory molecule CD28 also clusters with TCR microclusters upon binding its counter-receptor CD80 [19]. The accessory/adhesion molecule CD2, that markedly reduces the threshold for TCR signaling [20], is also enriched within TCR microclusters. Moreover, CD2 is able to form microclusters, and initiate transient intracellular calcium flux, even in the absence of TCR engagement [21]. Notably however, this is insufficient for cell activation, underscoring the importance of TCR engagement for appropriate T cell activation. The surface-expressed tyrosine phosphatase CD45, which can dephosphorylate TCR ITAMs and other tyrosine phosphorylated substrates, is strongly excluded from TCR microclusters (Fig. 1A–C) [4,22]. This appears to be specific to microclusters, as the conditions that lead to CD45 segregation are absent by the time they reach the cSMAC, where TCR and CD45 are colocalized [4,23].

Perhaps surprisingly, it is not yet known how the TCR coreceptor (CD4 or CD8) organizes in relation to TCR microclusters beyond the first seconds of T cell-APC interaction. The coreceptor is known to be closely associated with TCR [14,24,25], and is present in early large clusters prior to cSMAC formation, but its location relative to sites of sustained signaling is not known [14]. Notably, the coreceptor binds pMHC molecules independently of TCR and with low affinity [26], lending kinetic and stoichiometric flexibility to its participation in the tripartite TCR-pMHC-CD4/8 interaction. It is intriguing that TCR engagement by antibodies, where coreceptor-pMHC interactions cannot occur, enhances coreceptor association with TCR [27]. Since this requires the cytoplasmic portion of the coreceptor [28], which is constitutively associated with Lck [29,30], it seems likely that coreceptor recruitment may in part be dependent on TCR signaling. Establishing the precise arrangement, dynamics, and numbers of coreceptors incorporated within TCR microclusters will provide important information on how they affect the threshold for TCR signaling [20]. For instance, transient and rapidly exchanging coreceptor interactions within TCR microclusters might suggest that this is achieved through signal amplification by supra-stoichiometric recruitment of coreceptor and associated Lck to sites of TCR engagement (Fig. 2C–D). Such interactions may conform to TCR pseudodimerization models, where agonist pMHC engagement leads to disproportionate recruitment of 'self' pMHC. Alternatively, stable incorporation of coreceptors into TCR microclusters, as suggested by measurements at the plasma membrane [31], may point to a more structural role in determining the supramolecular configuration of TCR microclusters.

The transmembrane adapter molecule LAT is thought to associate with TCR microclusters within seconds of their formation [12,18]. Biochemical experiments clearly show that ZAP-70 (associated with phsophorylated TCR) is the critical kinase that phosphorylates LAT, although it is unclear what intermolecular associations induce its incorporation in TCR microclusters. Presumably, kinase activity alone may increase residency in TCR microclusters, if substrate release is slow. Such a mechanism would also allow time for recruitment of kinases and adapters to LAT, which may stabilize its association with the TCR. Very little is known about the kinetics and spatial behavior of enzymatic reactions constrained to two-dimensional biological membranes, which merit investigation in this context. Another possibility arises from recently described nanoscale clustering of both TCR and LAT in resting T cells [32]. These nanosclusters appear to coalesce, without mixing, into conglomerations of TCR and LAT following TCR engagement. This view of pre-existing 'protein islands' containing receptors and signaling proteins as the constituent parts of the T cell signaling machinery will require further experimental characterization. It is worth pointing out that 'preclustering' of TCR has not yet been convincingly demonstrated on the surface of live T cells [33,34], and is not suggested by the subunit stoichiometry of the assembled TCR [35]. An alternative view, supported by studies of Fc receptors, is that cortical actin may act to confine groups of independently diffusing TCR [36,37]. This concept can reconcile the discrepancy between electron microscopy and dynamic measurements of membrane protein domains [38].

TCR microclusters translocate medially and incorporate into the cSMAC within 2–3 min, where they are dephosphorylated [39]. The continuous generation of TCR microclusters in the IS periphery allows sustained TCR signaling despite their short life-span, while transport and dephosphorylation in the cSMAC allows abrupt termination of TCR signals. Under conditions where pMHC are limiting it is likely that they will be recycled and used to form TCR microclusters in a serial manner, although this has not been directly observed. It is known however, that TCR is present within recycling endosomes within the IS [40]. In contrast, within minutes following their coassembly, LAT and it's associated signaling

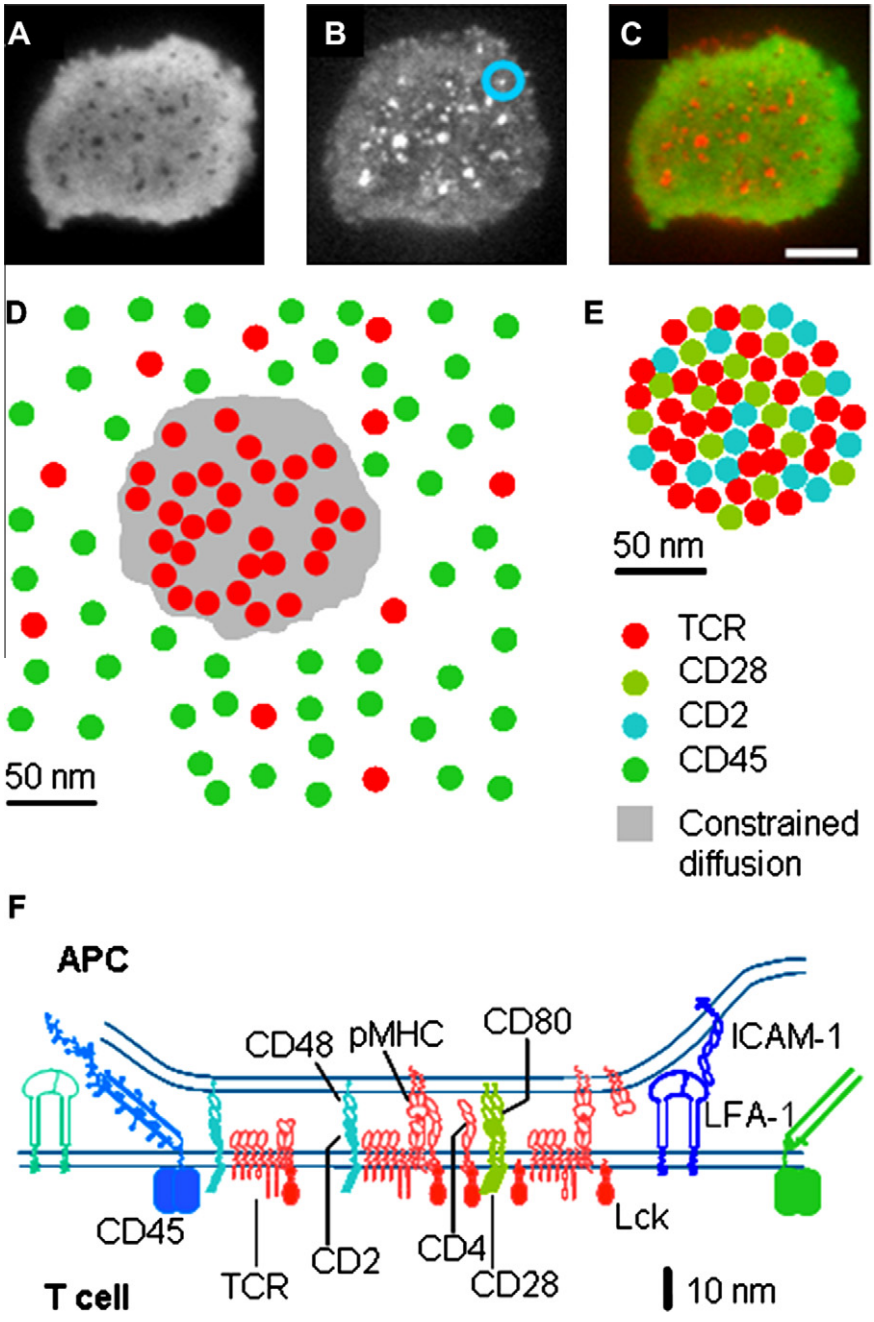

Fig. 1. Early T cell signaling occurs within submicron-scale plasma membrane domains (A–C). TIRFM image of a murine CD4+ T cell, which transgenically expresses the AND TCR, interacting with a glass supported planar bilayer containing agonist pMHC ligands (MCC-I-E^k) and ICAM-1. CD45 was visualized using an antibody Fab' fragment labeled with Alexa 488 (A), and TCR microclusters were detected using a murine TCRβ chain-specific Fab' fragment labeled with Alexa 568 (B). The merged channel (C) shows segregation of CD45 (green) from microclusters (red). Scale bar represents 4 μm. Adapted from [4] (D). Depiction of the distribution TCR and CD45 in the region of a microcluster (blue circle in (B)). Surface densities are approximately representative from estimates of total T cell surface TCR [85] and CD45 [86]. The gray zone depicts a putative area of constrained lateral diffusion of TCR (and other transmembrane proteins) in which TCRs are enriched by ~fivefold (E and F). Cartoon of the molecular components of a TCR-enriched microcluster, depicting the extent of lateral packing (E) and membrane topography (F) that might be expected based on reported constituents present within TCR microclusters, their dimensions and binding interactions.

molecules dissociate from TCR microclusters and remain as persistent and active signaling domains in the IS periphery [12,41]. Dissociation from TCR microclusters is also observed for CD28, which appears to translocate with TCR but fails to enter the cSMAC, continuing to signal within an annular structure at its border [19,39]. Although the mechanisms that separate TCR and LAT microdomains remain unclear, the dissociation of TCR from CD28 involves ubiquitin-mediated recognition and directed sorting of TCR by the ESCRT I member TSG101 [39]. It seems likely that ubiquitination and ESCRT complexes also play a role in separation and sorting of other molecules associated with TCR microclusters, including LAT.

The abundant adhesion and signaling molecule LFA-1, which clusters upon binding ICAM-1, is segregated from TCR microclusters at the outset, and assembles independently into the ring-like pSMAC [42]. How this segregation occurs and what couples TCR and LFA-1 signals in inside-out (avidity maturation of LFA-1 clusters) and outside-in (lowering TCR activation threshold) signaling is unclear. One possibility is that LFA-1 microclusters may promote the stability of TCR microclusters by enhancing cortical actin

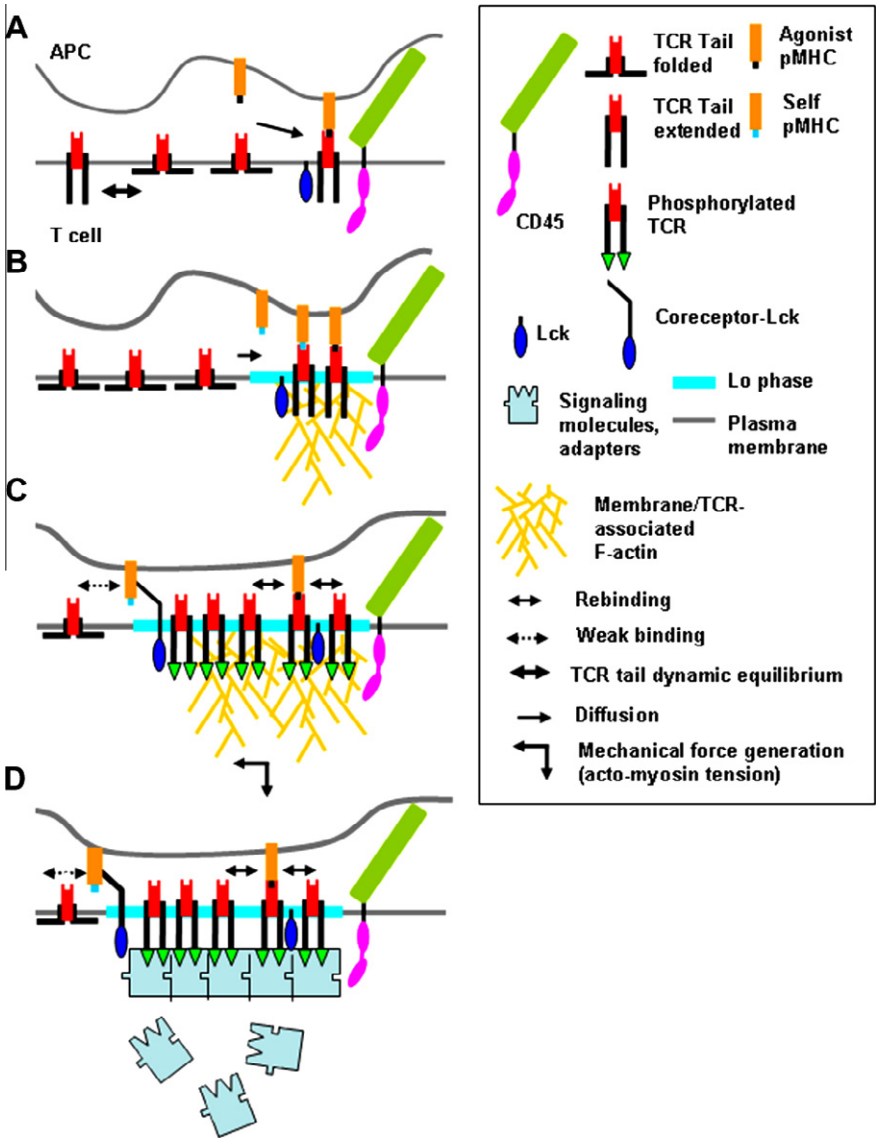

Fig. 2. Model of TCR microcluster formation and signaling (A). TCR engagement by pMHC agonist shifts the equilibrium of its cytoplasmic tail conformations from 'folded' states associated with the plasma membrane to 'extended' states [60], allowing closer *cis* associations with other plasma membrane receptors. (B) Unengaged TCRs may more closely associate with agonist-bound TCR though diffusion–collision, assisted by transient binding to self pMHC. These encounters may also be assisted by coreceptor binding to self pMHC [58]. Although TCR tail conformational changes have not been observed in response to low-affinity pMHC, these may be very transient, requiring assays capable of detecting very fast kinetic processes for detection. Agonist pMHC-nucleated accumulation of several TCRs may result in membrane condensation around their transmembrane segments [47]. Negatively charged lipids may be sequestered in these regions, inducing plasma membrane/TCR coupling to F actin. Both liquid-ordered lipid phase changes and actin reorganization may constrain diffusion of accumulated TCRs, trapping them within close contact areas. For clarity T cell accessory molecules, which are present in these microclusters, have been omitted. However, binding of accessory receptors by their ligands may be critical for optimal alignment of apposed membranes for TCR binding [87]. Although this may reduce access of the CD45 ectodomain to nascent clusters, its phosphatase domains may still have access to TCR cytoplasmic ITAMs, so preventing triggering (C). Accumulation of TCR (and accessory molecules) may extend regions of close contact that is sufficient to segregate CD45 phosphatase activity from microcluster resident TCRs, allowing stable phosphorylation of TCR ITAMs by Lck. Very few agonist pMHC may be needed to maintain tail conformational changes and triggering within microclusters as the local increase in TCR density may allow rapid rebinding [73,74,88], setting up conditions for serial triggering of TCR. Coreceptor-mediated recruitment may also operate at this stage. Myosin IIA mediated tension on actin filaments may exert mechanical forces on TCR-pMHC (and other receptor–ligand) bonds, which may be necessary for triggering. (D) Recruitment of signaling molecules and adapters to phosphorylated TCR ITAMs may lead to cooperative stabilization of microclusters, reducing their dependence on actin to maintain domain integrity [17].

polymerization, and effectively corralling TCR microclusters within gaps in the resulting F-actin meshwork. This may explain the requirement for dynamic actin remodeling for TCR microcluster formation, without the need for direct TCR interactions with actin.

3. Actin coupling and transport of signaling microclusters

The initial coassembly of signaling molecules in early TCR microclusters is dependent on dynamic remodeling of cortical actin [17]. Once formed, these microclusters undergo centripetal migration towards the IS center, which is also dependent on actin flow. The involvement of actin in TCR activation has been widely reported (reviewed in [43]), although the majority of studies employ global actin depolymerization, which does not easily suggest a molecular basis for its function. Only one biochemical study demonstrates that the TCR itself physically associates with actin or an actin modulating complex [44]. Several molecules within the TCR microcluster signaling complex could indirectly link to F-actin. The obvious candidate is Vav1, a Rho family GEF (guanine nucleotide exchange factor) that is recruited to the LAT signalosome,

through its interaction with the adapter proteins SLP76 and Nck [45,46]. A second possibility is tethering of cortical actin to sites of active phosphoinositol lipid production at the plasma membrane. This seems plausible as long as TCR microclusters contain LAT and/or CD28, as DAG and IP3 are generated by LAT-recruited PLCγ1, while CD28 engagement is known to recruit and activate PI3K, which catalyzes PIP3 production. Tightly regulated generation of phosphoinositol lipids, which are well known to recruit PH domain-containing actin nucleating molecules (eg. N-WASP), in the proximity of TCR/LAT/CD28 within microclusters, may thus provide a dynamic mechanism for actin coupling (Fig. 2B–D). Since Vav1 can activate PLCγ1, and is also recruited by CD28, the Nck-SLP76-Vav1 complex may be particularly important for both coupling TCR signaling assemblies to actin and directing their lateral organization through lipid mediators. Clustering of TCR and other receptors in microdomains could directly enrich for phosphoinositol lipids. This could occur through transmembrane segment length-mediated effects on lipid phase, resulting in liquid-ordered (Lo) lipid domains [47] (PIP, PIP2, and PIP3 preferentially partition into the Lo phase), or through attraction of these negatively charged lipids to clustered juxtamembrane polybasic regions in the cytoplasmic portions of assembled receptors [48]. This may result in the induction of liquid-ordered lipid shells around microclusters. In either case, these microdomains could enrich for negatively charged lipids that promote actin nucleation (Figs. 1D, 2).

In the first few minutes of contact between T cells and antigen-bearing surfaces, actin polymerization is accelerated in the peripheral lamellipodial perimeter of the IS. Its inward extension, and depolymerization near the cSMAC border, results in retrograde actin 'flow'[42]. TCR and other microclusters appear to use this actin treadmill for centripetal transport. The rate of microcluster movement is slower than the underlying actin flow, suggesting that they are loosely coupled, or that microclusters encounter significantly greater barriers to movement within the plasma membrane in the direction of actin flow. In either case, the resultant influence of retrograde actin flow conforms to a frictional 'stick-slip' motion of microclusters, representing phases of strong actin coupling under tension, followed by release and acceleration [49]. Although this is generally observed to be the case, the remarkably linear tracks of inward-moving TCR microclusters suggests that other cytoskeletal or plasma membrane structures may guide their translocation. This could involve movement along actin cables, although no firm evidence for such structures exist, but seem not to be guided by underlying microtubules, as they are dispensable for both microcluster formation and transport ([50] and Dustin et al., unpublished observations). Recently, another dimension to actin-mediated transport has been added by the demonstration that myosin IIA, which is involved in generating tension along actin fibers, greatly accelerates actin-mediated transport and signaling within TCR microclusters [51]. The role of traction forces in determining signaling within microclusters is consistent with recent demonstrations of TCR signaling by the application of mechanical forces approximately lateral to the plasma membrane (Fig. 2C [52,53].

4. How do microclusters form?

Although TCR microclusters are induced by pMHC engagement, once formed, they do not require further TCR-pMHC interactions to maintain their organization [4]. Early microcluster size is roughly proportional to the locally available pMHC density as revealed by experiments with partitioned bilayers [54]. One scenario by which microclusters could form is through initial (monomeric) engagement of multiple TCRs by cognate pMHC, leading to binding-induced *cis* oligomerization. Such a mechanism would likely involve incorporation of un-engaged TCRs into microclusters in order to account for the very small numbers of pMHC (1–200) [55–57], representing very low density at the IS, that can fully activate T cells. One mechanism that might allow capture of nearby TCRs around an engaged TCR is suggested by the pseudodimerization model [58]. This model proposes that cognate (high affinity) TCR binding leads to recruitment of other TCRs through coreceptor interactions with TCR-bound cognate pMHC. If this interaction is transient, as is thought to be the case, many surrounding TCRs could be recruited in this way (Fig. 2). However, accumulation of multiple TCRs through weak *cis* interactions is difficult to imagine under these conditions unless some local diffusional constraints also operate (discussed above) around engaged TCR. Evidence for plasma membrane phase change to liquid-ordered states at sites of TCR engagement makes such a scenario at least plausible [47]. A prediction of such a model would be that TCR microcluster formation is sensitive to disruption of coreceptor interactions. Mechanisms based on recruitment and retention of TCR do not easily account for their uniform size however, as TCR clusters formed in this way would tend to grow unless some other cellular process intervened. One possibility is that nascent (early) TCR clusters are unable to engage underlying cortical actin until they reach a particular size, when multiple TCRs can contribute sufficient avidity for actin-coupling and transport. This is consistent with recent observations linking cluster size and their ability to translocate [59].

A second, and complementary, mechanism that might generate conditions that favor binding-induced TCR clustering is the removal of constraints that limit *cis* association of engaged TCRs (Fig. 2A and B). The TCR cytoplasmic tails are thought to be closely associated with the plasma membrane in the unengaged state [60]. As there are six ITAM bearing cytoplasmic chains for each TCR, these are envisaged to occupy a significant area of the cytoplasmic face of the plasma membrane, preventing close lateral interactions with other membrane tethered proteins, including TCRs. Upon TCR binding to pMHC ligands (or binding of the CD3ε ectodomain by antibodies), the tails undergo a conformational change leading to their release from the plasma membrane [60,61]. Although it is unclear how ligand binding results in conformational changes in the cytoplasmic portions of the TCR, it seems that some can occur solely through TCR binding, without the need for Lck activity [61,62]. This binding-induced change could therefore precede Lck-mediated TCR phosphorylation. It is less clear whether conformational changes induced in the TCR cytoplasmic portions also renders them more amenable to phosphorylation by Lck. This seems to be the case in cell-free systems [60], although CD3ε ITAMs appear readily susceptible to phosphorylation in the unengaged state when phosphatase activity is inhibited [63,64]. It is now becoming clear that, like some conformational changes in the TCR cytoplasmic portions, TCR clustering does not require Lck activity, but is dependent on TCR engagement [17,65]. It is therefore conceivable that these phenomena are related but occur independently of and/or before TCR phosphorylation (Fig. 2A and B). Conformational changes in the cytoplasmic domains of the TCR could, along with mechanisms discussed above, increase the propensity for pMHC-induced clustering, but may nevertheless remain accessible to the phosphatase activity of CD45, until binding-induced receptor recruitment and signaling consolidates their organization (Fig. 2D).

An attractive possibility for exclusion of CD45 from regions of TCR microclusters is the notion of size-based segregation, as articulated in the kinetic-segregation model [66]. This model proposes that the small size of TCR and accessory molecules, and their counter-receptors, would limit spacing of apposed membranes, where they are bound, to the span of the resulting receptor–ligand complexes (~14 nm) [67]. These regions of close contact are proposed

to be small (>200 nm diameter), and may be transient without TCR engagement, for example mediated by CD2-CD48 or CD28-CD80, or even pMHC-coreceptor interactions. Close contacts could physically segregate larger molecules, in particular, membrane-tethered CD45, which possesses a large and heavily glycosylated ectodomain, rendering these regions relatively deficient of phosphate activity (Figs. 1F and 2C). Diffusional trapping of TCR by cognate pMHC within these regions could lead to stable ITAM phosphorylation due to the basal activity of plasma membrane-tethered Lck [68], unopposed by CD45 mediated dephosphorylation. Although this model was constructed to account for triggering of individual TCRs, and growing evidence points to its importance in immunoreceptor triggering, it is unclear whether segregation of engaged TCR from CD45, at the scale of single receptors, has any consequence on accessibility of the cytoplasmic phosphatase domain to TCR-associated phosphorylated substrates. The most compelling observation of phosphatase segregation from TCR remains the striking exclusion of CD45 from TCR microclusters [4].

Mathematical models of membrane junctions containing receptor–ligand complexes of different lengths, also suggest that laterally organized microdomains bound by closely apposed membranes can form spontaneously, without the need for signaling or active transport [69]. A particularly compelling (and testable) model considers the influence of ligand–receptor complex dimensions, binding properties, thermal fluctuations of apposed membranes, and the fractional contact between membranes, in determining the propensity to form laterally organized domains [70]. This predicts both cooperativity in domain (microcluster) formation, and segregation of clusters of different complex dimensions as observed for TCR-pMHC and LFA-1-ICAM-1 complexes. Artificial junctions, formed between ruptured GUVs, (Giant Unilamellar Vesicles) containing lipids with large PEGylated headgroups, and supported planar bilayers, spontaneously organize into submicron-scale domains of close contact, remarkably similar in size and distribution to microclusters in T cells [71]. These are absent when only small molecules are tethered as receptors to apposed lipid-bound ligands, suggesting that large flexible molecules on membranes can influence junctional topography to form small discrete regions of close contact. Taken together, these studies suggest that many of the conditions necessary for microdomain formation and signaling may occur spontaneously at the IS, while conditions that prevent TCR clustering are only removed upon binding of cognate pMHC.

5. Why is signaling organized in microdomains?

TCR microclusters contain all the relevant molecules and provide the necessary physicochemical environment required for signal initiation in T cells. Although it is not clear whether the first TCR-evoked signals are produced within microclusters, their appearance correlates with signaling in the moments following TCR engagement. Moreover, their continued generation appears necessary for sustained signaling [4]. That they can form independently of triggering makes them attractive candidates as specialized domains that prepare the ground for efficient detection of antigens on appropriate presenting surfaces. The precise mechanism of TCR triggering is not known. Three models of triggering have gained prominence in the past decade – TCR-CD3 conformational change, co-receptor mediated pseudodimerization (or other forms of receptor oligomerization), and kinetic segregation – all of which almost certainly play a part (reviewed in [72]). The conditions observed within TCR microclusters may offer a clue as to why each of these mechanisms, when perturbed in isolation, points to a seemingly sufficient demonstration of its requirement for TCR triggering. If triggering were to take place within microclusters, all conditions postulated in the different triggering models (tail conformational change, *cis* di/oligo-merization, phosphatase exclusion) would be fulfilled simultaneously. If their *coincidence* is necessary for triggering, then abrogating any of these mechanisms would be sufficient to disrupt it.

It is notable that although ~10 or less agonist pMHC ligands can fully activate T cells, these ligands must trigger an order of magnitude more TCRs to achieve cell activation [73]. Moreover, signaling in response to these ligands – the total triggering activity – appears to be integrated by the T cell within seconds, making it unlikely that long-range lateral diffusion of TCR alone mediates the process of 'serial engagement'. It is therefore tempting to imagine that pMHC trapped within TCR microclusters significantly enhance serial engagement through rebinding (Fig. 2C and D), as suggested by theoretical calculations and recent experimental observations [74]. The effective coupling of triggering and serial engagement within microclusters would also explain the apparent on-rate acceleration (when compared to 3D measurements) identified in recent 2D measurements of TCR-pMHC binding [75,76].

Integration of signals from the TCR and accessory molecules is thought to occur at the level of the MAP kinase pathway leading to Erk1/2 activation and the generation of dynamic intracellular increases in calcium concentration, that results in selective transcriptional activation [46]. Much of the proliferative and transcriptional responses made by T cells are governed by activation of these signaling pathways, which are thought to be influenced by multiple proximal inputs including ZAP-70, PLCγ1, PKCθ and Grb2-SOS. Early TCR microclusters also contain CD28 and LAT, and recruit all of these kinases, enzymes and adapters. It seems probable that their concerted action within these spatially limited domains has some advantage in mediating efficient signaling to downstream pathways. The intimate connection between TCR and CD28 signals (especially in naïve T cells) may arise because of their location within the same signaling domains, leading to sharing and cooperative stabilization of key signaling molecules. The more global changes in intracellular calcium concentration may still require signaling from within microdomains, as sites of single receptor signaling may be too transient and of insufficient amplitude to easily allow endoplasmic reticulum or mitochondrial recruitment, organelles involved in calcium homeostasis. Mitochondrial recruitment to the IS appears necessary for buffering (and hence potentiating) intracellular calcium levels [77].

Signaling within small plasma membrane domains appears to have another emergent property that may be valuable for cellular decision making, especially when pMHC ligands are limiting. Elegant cell-biological studies demonstrate that organization of Ras, the main effector of ERK activation, into submicron-scale domains, leads to conversion of analogue extracellular stimuli into digital intracellular signals, which serve to demarcate a sharp signaling threshold for coupling to downstream signaling (eg. ERK1/2) [78].). The observation that signaling within microdomains exhibits bistable or 'switch-like' behavior is particularly relevant to T cell signaling for two reasons. Firstly, these cells are often required to make decisions for activation based on engagement of a few pMHC ligands (sensitivity) but with high fidelity (specificity). Fulfilling these requirements is greatly facilitated by sharp signaling thresholds that activate effectively binary effector choices (lytic granule release, calcium or Ras mediated transcriptional activation, cell division). Second, bistable systems, which depend on positive and negative regulatory loops, also maintain signaling along the triggered biochemical trajectory on encountering further qualitatively similar stimuli (hysteresis). This has the advantage of generating relatively constant signaling output in the face of fluctuating stimuli (within certain limits). These phenomena have yet to be investigated in detail in T cells, although a number of negative regulatory feedback loops that impinge on TCR-associated activating

signals have been described (eg Dok and SHP1 pathways) [79,80]. It seems that Ras mediated signaling is bistable in T cells [81]. Whether this is due to intrinsic biochemical properties of Ras signaling and regulation, or is determined by its lateral organization at the plasma membrane will be interesting to investigate. In particular, determining whether Ras clustering, along with its regulatory GAP and GRP, coincides with TCR or other microclusters at the IS may help to distinguish these possibilities.

Clustering of receptors and signaling proteins may also facilitate their efficient sorting and downregulation. This is especially important for TCR and LAT signaling as their continued activation leads to pathological autoreactivity and inflammation [82,83]. Long-lived costimulatory signaling is thought to be important for naïve T cell activation, and cell proliferation, therefore requiring their sorting out of TCR microclusters which are subject to rapid downregulation. The principal method of downregulation and signal termination of TCR at the IS is by actin-mediated transport from the periphery to the cSMAC. As discussed above, TCR destined for downregulation by this process must first form microclusters, suggesting that this packaging of TCR provides a simple means of gathering actively signaling TCRs for downregulation. Their sorting into the deactivating synapse center is then mediated by ubiquitin-dependent recognition by TSG101 [39]. That CD28 is not subject to this process, despite partially sharing the actin-mediated translocation machinery [39,84], suggests that sorting at the level of microclusters may be an important cell-biological strategy for the spatial regulation of cell signals.

6. Future perspectives

The discovery of TCR enriched microclusters has lead to many exciting developments and advances in our understanding of how the immunological synapse works to generate signals in T cells. This has coincided with a broader cell biological effort in understanding the role of membrane microdomains in cell signaling, that is now leading to the development of general principles that govern the biophysical and biochemical behavior of membrane microdomains. Recently, these new ideas have begun to be applied to T cell signaling, placing well established biochemical processes within a spatially and temporally regulated molecular framework.

Many interesting questions arise from considering membrane microdomains as units of signaling in T cells. Some deserve particular mention, as they may lead to fundamental insights into so-far unresolved questions at the center of T cell biology. The first, relates to the mechanism of microcluster formation. A number of possibilities are discussed above, of which the influence of receptor–ligand dimensions on their propensity towards lateral organization within membrane junctions, is particularly exciting. Such phenomena, as suggested by model in vitro systems, if confirmed for *bona fide* receptor–ligand systems (such as TCR/pHMC and LFA-1/ICAM-1), promise to have broader implications for the biology of molecular organization at cellular junctions. Another exciting prospect is the potential for nanoscale molecular and structural characterization of signaling microdomains (for example by super-resolution imaging, and electron-cryotomography) to yield new insights into how TCR signaling is organized. The diverse and growing number of models that attempt to account for TCR triggering, may relate to the many processes that are simultaneously initiated within T cell surface microclusters, thus unifying many of these isolated mechanisms into an integrated signaling scheme. These processes need not be mechanistically related to one another, but rather operate in the same spatial and temporal locus due to the permissive biochemical and biophysical environment within microdomains. Indeed, they may all be important for triggering precisely because TCR microclusters are in themselves central to this process.

The assembly, sorting, transport and down-regulation of T cell signaling microdomains is only now beginning to be investigated in detail. The new disciplines of synthetic biology and optical nanoscopy are intersecting with more established cell biological and biophysical approaches to deepen our understanding of the composition and function of T cell membrane microdomains. These are exciting times for the field of T cell signaling, as we approach a level of experimental sophistication that may allow us to test the veracity of our findings by faithfully recreating these complex cellular processes in synthetic signaling systems.

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