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REVIEW

Multiple actin networks coordinate mechanotransduction at the immunological synapse

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Activation of naive T cells by antigen-presenting cells (APCs) is an essential step in mounting an adaptive immune response. It is known that antigen recognition and T cell receptor (TCR) signaling depend on forces applied by the T cell actin cytoskeleton, but until recently, the underlying mechanisms have been poorly defined. Here, we review recent advances in the field, which show that specific actin-dependent structures contribute to the process in distinct ways. In essence, T cell priming involves a tug-of-war between the cytoskeletons of the T cell and the APC, where the actin cytoskeleton serves as a mechanical intermediate that integrates force-dependent signals. We consider each of the relevant actin-rich T cell structures separately and address how they work together at the topologically and temporally complex cell–cell interface. In addition, we address how this mechanobiology can be incorporated into canonical immunological models to improve how these models explain T cell sensitivity and antigenic specificity.

Introduction

T cells play a central role in the adaptive immune system. Cytotoxic T cells directly kill virally infected cells and cancers cells, while helper and regulatory T cells activate and tune the effector functions of other cells of the immune system. In both cases, T cells must distinguish rare foreign antigens from abundant, harmless, self-proteins, a task that requires exquisite sensitivity and specificity (Courtney et al., 2018). Because T cells must interpret subtle antigenic differences and subsequently effect or suppress an immune response, even comparatively modest defects in T cell activation machinery can result in immunodeficiency on one hand and autoimmunity on the other (Comrie and Lenardo, 2018; Janssen et al., 2016). T cell activation requires direct cell–cell contact with antigen-presenting cells (APCs). Initial priming of naive T cells, usually by dendritic cells (DCs), induces proliferation and differentiation of T cells, amplifying and tuning the immune response. Later, these primed T cells interact with target cells or other cells of the immune system to carry out their effector functions. In both cases, the T cell receptor (TCR) interacts with major histocompatibility complex molecules loaded with antigenic peptides (pMHCs). Initial TCR binding to cognate pMHCs induces a signaling cascade that results in massive reorganization of the T cell cortical actin cytoskeleton, forming a specialized cell–cell interface termed the immunological synapse (IS; Dustin et al., 2010). At the IS, additional receptor–ligand pairs interact, relaying signals that prime and shape the T cell response.

To initiate a protective T cell response, several challenges must be overcome. First, T cells must seek out MHCs bearing rare antigenic peptides amid a sea of complexes containing self-peptides. To achieve this, T cells must rapidly scan numerous MHCs on the APC surface. Second, since each T cell clone recognizes a single, specific antigen, this process must be simultaneously performed by many different T cells, scanning many different APCs. Finally, T cell recognition must be tightly controlled to avoid mistaken responses to self-peptides that could lead to autoimmunity. The ability of T cells to overcome these challenges has fascinated the scientific community for many years. Intensive research efforts have led to a relatively mature understanding of the biochemical cues that T cells sense at the IS and the downstream signaling cascades through which T cells interpret these cues to launch an appropriate response. Recently, however, it has become clear that mechanical cues are also required. Force application by the T cell cytoskeleton on an interacting APC is essential for appropriate T cell activation and discrimination between self- and nonself-antigens (Das et al., 2015; Hong et al., 2015; Hu and Butte, 2016; Li et al., 2010; Liu et al., 2014; Pryshchep et al., 2014; Sawicka et al., 2017). Although there is now a consensus that force is needed for T cell activation, the concept is fairly new, and the field is struggling to understand the mechanisms of mechanotransduction in the context of canonical immunological models derived from earlier biochemical analyses. Moreover, most of what we know about

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the mechanobiology of the immune response comes from reductionist experimental systems, which highlight individual features of the biology at the expense of an integrated understanding that spans the scale and complexity of living systems. Current efforts in the field are aimed at understanding how mechanical force functions at the single-molecule level, such as during initial TCR triggering, as well as during higher-order cell biological events involving the dynamic topology of the T cell–APC interface. Here, we will review recent progress in the field, linking what we know about T cell mechanobiology to the discrete actin networks that organize force-generating structures at the IS. In addition, we will address the ways in which cellular mechanobiology complements the canonical immunological models that are used to explain T cell activation.

Three discrete actin networks collaborate to shape IS function

Following initial TCR-ligand interaction, at least three discrete actin networks form and maintain the shape and function of the IS (Fig. 1; Hammer et al., 2019). Although there are variations in actin architecture in different model systems, the basic elements are conserved (Box 1). For the purposes of this review, we will focus on the lamellipodial branched actin network, the lamellar actomyosin network, and actin foci, which are thought to be related to invadosome-like protrusions (ILPs). These three networks are organized by three different actin polymerization pathways, regulated by WAVE2, formins, and WASp, respectively. While WASp and WAVE2 serve as nucleation-promoting factors that activate the Arp2/3 complex to generate branched actin networks, formin family proteins generate linear actin filaments either de novo or on the barbed ends of existing branched actin networks. Inhibitor studies indicate that these discrete actin networks largely function independently of one another, although there is some coordinate control due to competition for free actin monomer (Chan et al., 2019; Fritzsche et al., 2013, 2016; Isogai and Danuser, 2018; Rotty and Bear, 2014; Suarez et al., 2015). Whether there is additional, higher-order crosstalk remains to be established. Importantly, each of these actin networks generates a distinct structure that serves a specific functional role during T cell activation; these roles will be detailed later in this review.

The lamellipodial branched actin network

Visually, the most prominent actin network at the IS is the dense actin network that defines the distal part of the IS (dSMAC). This network, which corresponds to the lamellipodial region of a migrating cell, forms within minutes after initial TCR engagement. It is composed mainly of branched actin filaments generated by WAVE2 and the Arp2/3 complex (Nolz et al., 2006). The signaling pathway responsible for creating this network involves TCR-induced activation of class I phosphoinositide 3-kinase, which generates phosphatidylinositol (3,4,5) phosphate on the plasma membrane and creates binding sites for the Rac guanine nucleotide exchange factor DOCK2. DOCK2 then activates Rac1, the main Rho-GTPase modulator of WAVE2 protein function (Nishikimi et al., 2013; Sanui et al., 2003). After its recruitment to the IS periphery, WAVE2 activates Arp2/3 complex–dependent polymerization of branched actin at the membrane, facilitating T cell spreading (Le Floc'h et al., 2013;

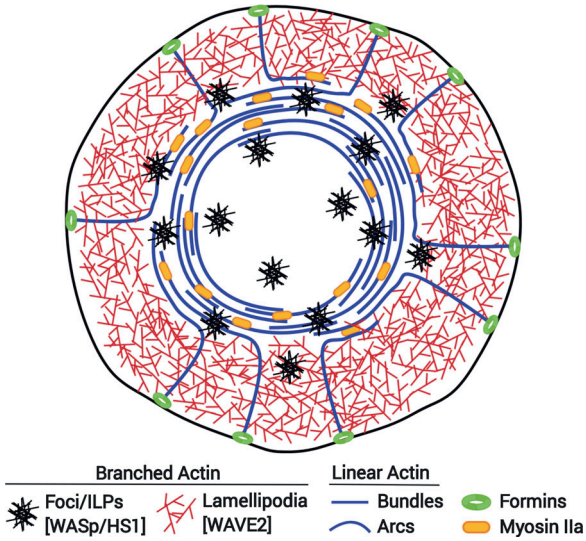

Figure 1. **Three discrete actin networks collaborate to create distinct functional regions within the canonical "bullseye" IS.** The outer ring of the IS (distal supramolecular activation cluster [dSMAC]) corresponds to the lamellipodium of a migrating cell. This region contains a prominent branched actin network (red) generated by the Arp2/3 complex activator WAVE2. Actin polymerization at the edge of the spreading T cell pushes this network inward, along with associated TCR signaling complexes. Radially arrayed within the dSMAC region are bundles of linear actin filaments, generated by formin activity near the edge of the spreading cell. These bundles bend as they move inward and are cross-linked by myosin IIA, forming actomyosin arcs (blue). This network defines the peripheral supramolecular activation cluster (pSMAC) region, which is enriched in integrins, and corresponds to the lamellar region of a migrating cell. Inward movement of actomyosin arcs is driven primarily by myosin contractility. Disassembly of this network leads to an actin-poor region in the center of the IS known as the central supramolecular activation cluster (cSMAC). The cSMAC is associated with receptor internalization and signal extinction and provides a site for exocytosis of secretory granules. The third actin network consists of actin foci (black), which are related to protrusive structures termed ILPs. These form a punctate pattern within the central regions of the IS and contain branched actin filaments generated by WASp, with help from HS1. These structures are closely associated with sites of TCR-induced tyrosine phosphorylation.

Nolz et al., 2006). Once the cell is fully spread, continued actin polymerization functions to fuel centripetal (retrograde) actin flow. Importantly, this network can be rapidly blocked using the membrane-permeant small-molecule inhibitor of the Arp2/3 complex, CK666 (Murugesan et al., 2016). Apart from its role in activating de novo polymerization of actin branched networks downstream of TCR activation, WAVE2 also recruits vinculin to the IS. Vinculin is an actin binding protein that links the actin cytoskeleton and integrin clusters through interaction with talin (Ziegler et al., 2006). The VCA domain of WAVE2 mediates the formation of WAVE2–Arp2/3–vinculin complexes, which in turn recruit talin to the IS to create a direct connection between the actin machinery and integrins. This direct link is necessary for induction of integrin affinity maturation and clustering (Comrie et al., 2015a; Nolz et al., 2007, 2008).

The actomyosin network

The actomyosin network consists of linear actin filaments within the dSMAC (lamellipodial) region of the IS, which

Box 1. Cytoarchitecture of T cell lines and primary T cells

Much of the early work characterizing actin dynamics at the IS was performed using Jurkat T cells, a transformed human thymoma cell line, because these cells are large and readily genetically manipulated (Bunnell et al., 2001). Spreading Jurkat cells are very round, their lamellae are very stable, and the cytoskeletal elements within them are highly ordered, greatly facilitating quantitative analysis of protein dynamics. However, Jurkat T cells lack several important molecules, including the inositol phosphatases PTEN and SHIP and the mechanosensitive adapter protein CasL, which has led to concerns about their use (Abraham and Weiss, 2004; Kamiguchi et al., 1999). Although there are some morphological differences between Jurkat cells and primary human and mouse T cells (Colin-York et al., 2019b), molecular analyses reveal that many of the actin structures and the regulatory pathways that generate them are conserved (Barda-Saad et al., 2005; Hong et al., 2017; Nolz et al., 2006). One notable exception to this is the presence of actin foci/ILPs, which are readily observed in primary cells but not apparent in Jurkat cells (Kumari et al., 2015). Notably, variations in IS architecture are also observed among primary T cell subtypes and depending on the stimulatory APC (Kumari et al., 2019), probably reflecting the different biophysical properties of the two interacting cells.

transition into actomyosin arcs within the more centrally located pSMAC region (corresponding to the lamellar region of a migrating cell; Yi et al., 2012). This network is generated by membrane-bound formins, primarily Dia1 (Murugesan et al., 2016). Situated at the dSMAC, Dia1 polymerizes radially distributed actin filaments that extend toward the center of the IS. After traversing the lamellipodial branched actin network, these linear filaments are bent and organized into antiparallel concentric arcs by myosin IIA (Murugesan et al., 2016; Yi et al., 2012). These arcs continue to flow inwards toward the center of the IS. They are dismantled at the pSMAC-cSMAC interface, creating a region of low actin density at the center of the IS (Yi et al., 2012). Concentric flow of actomyosin arcs sweeps high-affinity integrins to the pSMAC–cSMAC interface, and indirectly drives TCR microclusters (MCs) toward the cSMAC (Murugesan et al., 2016), where signaling is extinguished (Varma et al., 2006). The actomyosin network is disrupted by the pan-formin inhibitor SIMFH2, by RNAi-mediated suppression of Dia1 (Rizvi et al., 2009), or by inhibition of myosin IIA function with blebbistatin. Loss of arcs leads to reduced centralization of LFA-1 and TCR MCs, resulting in decreased T cell adhesion and signaling (Murugesan et al., 2016). Interestingly, inhibiting the WAVE2-dependent lamellipodial network does not affect arc formation, consistent with the view that these two networks have distinct, independent functions. The signaling pathways that control the actomyosin network are incompletely understood. In other cell types, formins are anchored to the membrane through interactions with phosphatidylinositol 4,5-bisphosphate (Ramalingam et al., 2010), and are activated there by Rho GTPases. For Dia1, the relevant GTPase is typically RhoA (Kühn and Geyer, 2014). Interestingly, RhoA also functions through Rho-associated protein kinase, which is known to control myosin activity at the IS (Babich et al., 2012; Yi et al., 2012).

Actin foci at the dSMAC and pSMAC

This actin network consists of discrete actin structures termed foci that are spread throughout the dSMAC and pSMAC regions of primary T cells activated on stimulatory surfaces (Kumari et al., 2015). The existence of these structures was missed for many years, because they are not apparent in Jurkat T cells, where most of the early work was done (Box 1). Similar to the lamellipodial network, actin foci are made of branched filaments polymerized by Arp2/3, as demonstrated by loss of both networks upon treatment with CK666. Importantly, although the two networks are spatially overlapping, they are generated by different actin nucleators. While the lamellipodial network is dependent on WAVE2 activation, actin foci are WASp driven (Kumari et al., 2015). Indeed, their discovery explains why WASp-deficient T cells show defects in TCR signaling but no disruption of the lamellipodial actin network (Cannon and Burkhardt, 2004; Gomez et al., 2007). Interestingly, WASp knockout (KO) T cells show no reduction in total F-actin content, in part because these structures represent a minor subset of IS-associated actin filaments, but perhaps also due to compensatory increases in actin polymerization by other nucleation promoting factors. Inhibition of myosin II or formins has no effect on actin foci, indicating that formation of actin foci is independent of the actomyosin arc network. Hematopoietic lineage cell-specific protein 1 (HS1), another important actin regulatory protein, is recruited to the foci by WASp (Kumari et al., 2015). Although HS1 serves to stabilize foci, their continued presence in HS1 KO T cells demonstrates that HS1 is not necessary for their formation (Kumari et al., 2015). The upstream signaling pathways needed for formation of actin foci has not been tested directly, but earlier work shows that WASp recruitment and activation at the IS involves signaling through phosphatidylinositol 4,5-bisphosphate, the Rho guanine nucleotide exchange factor Vav1, and the Rho GTPase Cdc42, with feedback enhancement by the kinase Itk (Burkhardt et al., 2008; Labno et al., 2003).

Force contributes to T cell activation in multiple ways

Following the initial contact between a T cell and an APC, the T cell actively applies force on the APC surface (Bashour et al., 2014; Hu and Butte, 2016; Hui et al., 2015; Husson et al., 2011; Sawicka et al., 2017). Whole-cell experiments have shown that this occurs in two distinct, consecutive phases. Initially, the T cell pushes against an interacting cell in an antigen-independent manner. Then, after recognition of cognate antigen, the T cell pulls the APC back to form a close interaction. The two phases show characteristic speeds and are separated by a well-defined time interval (Husson et al., 2011; Sawicka et al., 2017). Importantly, application of both pushing and pulling forces is actin dependent, and abolishing the actin network eliminates both (Hu and Butte, 2016). When considered in the context of an intact IS, it is clear that these pushing and pulling forces can contribute to TCR signaling in several ways. Indeed, as detailed below, these forces can be readily incorporated into the models that immunologists use to describe specific aspects of the T cell priming process (Box 2).

TCR deformation: Translation of mechanical forces into biochemical signals

The most straightforward way in which mechanical forces promote T cell activation is at the receptor level. Several studies indirectly pointed to a role for mechanical force in initial TCR triggering (Basu et al., 2016; Hong et al., 2015, 2018; Hu and

Box 2. Models for TCR signaling: Essential concepts

Kinetic segregation (Anton van der Merwe et al., 2000; Davis and van der Merwe, 2006)
The kinetic segregation model posits that size-based sorting of cell-surface proteins at the IS can initiate TCR signaling. According to this model, in resting cells, TCR complexes diffuse freely in the T cell membrane together with kinases and phosphatases that maintain the basal (tonic) signaling needed for T cell survival without triggering a full response. When the T cell interacts with an APC, shorter receptor–ligand pairs, such as CD2–CD48/CD58 and TCR–pMHC, segregate into close-contact zones, which exclude bulkier molecules, including the phosphatases CD45 and CD148. This tips the balance toward phosphorylation of TCR complexes and enhances the formation of additional antigen-specific TCR–pMHC bonds within the close-contact zones.

Kinetic proofreading (McKeithan, 1995)
The kinetic proofreading model was proposed to explain how T cells can sample numerous MHCs bearing harmless self-peptides and respond selectively to rare agonist pMHCs. The model posits that initial TCR engagement is not sufficient to initiate productive T cell activation. Instead, several consecutive steps involving tyrosine phosphorylation and molecular complex assembly must occur. During the resulting lag period, disassociation of the TCR–pMHC bond allows reversal of these modifications, usually through the activity of phosphatases. Thus, short-lived nonspecific TCR–pMHC bonds disassociate before achieving T cell activation, whereas longer-lived specific TCR–pMHC bonds support the formation of stable signaling complexes that support full T cell activation.

Serial triggering (Valitutti, 2012; Valitutti and Lanzavecchia, 1997)
The serial triggering model addresses the problem that agonist pMHCs are very rare, and TCR–pMHC bonds exhibit low affinities and high off-rates, making T cell activation unlikely. This model incorporates the finding that a single pMHC can serially engage and trigger ∼200 TCRs (Valitutti et al., 1995; recall that every TCR on the surface of a T cell has the same specificity). It posits that high TCR–pMHC off-rates contribute to T cell activation by allowing a single pMHC to interact with many TCR molecules in a serial fashion. Provided that the duration of each individual binding event is sufficient to allow kinetic proofreading, even a rare pMHC can provide a sufficiently robust stimulus to activate the T cell. Thus, a direct prediction of this model is that strong antigens have bond half-times that are long enough to allow kinetic proofreading but not too long to prevent serial triggering.

Butte, 2016; Hui et al., 2015; Husson et al., 2011; Li et al., 2010; Pryshchep et al., 2014; Sawicka et al., 2017). One of the first studies to demonstrate this directly made use of a long tether to dampen forces applied by the T cell in order to ask whether ligand engagement alone, in the absence of tension on the TCR–ligand bond, is sufficient to initiate signaling (Li et al., 2010). No activation was detected when T cells were stimulated using this tether under static conditions, but when external forces were introduced, signaling was detected based on calcium flux. Other studies subsequently confirmed this finding, and showed that forces in the piconewton range are needed to trigger TCR signals (Feng et al., 2017; Hu and Butte, 2016; Hui et al., 2015; Liu et al., 2016; Sawicka et al., 2017). In keeping with this, early tyrosine phosphorylation events downstream of TCR engagement occur at sites of maximal applied force (Bashour et al., 2014; Hui et al., 2015), and lytic granule secretion occurs at these sites as well (Basu et al., 2016).

Exactly how mechanical forces on the TCR are translated into biochemical signals (mechanotransduction) remains controversial. Classical mechanotransduction models involve force-induced conformational changes in the extracellular domain of a receptor, which are transmitted across the cell membrane, inducing structural changes in the cytoplasmic domain and, ultimately, downstream signaling events. In the case of the TCR, however, the situation is less clear, because the ligand-binding α and β chains have very short cytoplasmic domains that lack signaling motifs. Signals are instead transmitted via the associated CD3 chains, which are phosphorylated at immunoreceptor tyrosine-based activation motif (ITAM) sites by Src family nonreceptor tyrosine kinases. A series of studies has suggested a mechanism through which forces on the extracellular TCRαβ chains can lead to CD3 phosphorylation (Fig. 2). In resting T cells, the cytoplasmic domains of most CD3ζ chains are associated with the inner leaflet of the plasma membrane (Fig. 2 A; Aivazian and Stern, 2000). Nuclear magnetic resonance structures show that intracellular CD3 domains are tightly associated with membrane lipids, making them inaccessible for phosphorylation by Src family kinases (Duchardt et al., 2007; Xu et al., 2008). Ligand binding by the TCR complex induces conformational changes that expose CD3 ITAM sites for phosphorylation, which prevents reassociation with the membrane (Fig. 2 C; Lee et al., 2015; Swamy et al., 2016). Therefore, once phosphorylated, the CD3 ITAMs are exposed to the T cell cytoplasm to allow increased binding by ZAP70, leading to propagation of downstream signaling (Fig. 2 D). Although there is no direct evidence linking the application of mechanical forces on TCRαβ to conformational changes in CD3 molecules, some evidence supports this idea. Laser trap experiments showed that application of force on TCRαβ elicits a conformation change, which was attributed mainly to the extension of the CβFG loop region within TCRβ (Das et al., 2015). The location of the CβFG loop in relation to the CD3ε chain supports the idea that it serves as a lever to push down on the CD3 complex (Sun et al., 2001), exposing the ITAM sites for phosphorylation (Xu et al., 2008). In support of this idea, stabilization of the FG loop increased bond lifetime and inhibited TCR signaling (Feng et al., 2017). While future work will undoubtedly add additional mechanistic details, these studies demonstrate that mechanical deformation of the TCR αβ chains can, in fact, lead to phosphorylation of ITAM sites and initiation of downstream signals. As detailed further in the sections that follow, mechanical force also contributes to TCR signaling via higher-order structures, but in each case, these processes are almost certainly coupled to this fundamental mechanism of receptor deformation–based mechanotransduction.

Protrusive forces overcome the physical barrier to TCR–pMHC interactions

Moving up from the molecular to the cell biological level, mechanical force plays an important role in the earliest events associated with T cell activation. The surfaces of the T cell and APC each bear a glycocalyx composed of large, heavily glycosylated proteins, which serve as a physical barrier to interactions between the smaller TCR and pMHC molecules (Shaw and Dustin, 1997; Springer, 1990). Moreover, two major components of the T cell glycocalyx are the receptor tyrosine phosphatases CD45 and CD148, which maintain TCR-dependent phosphorylation

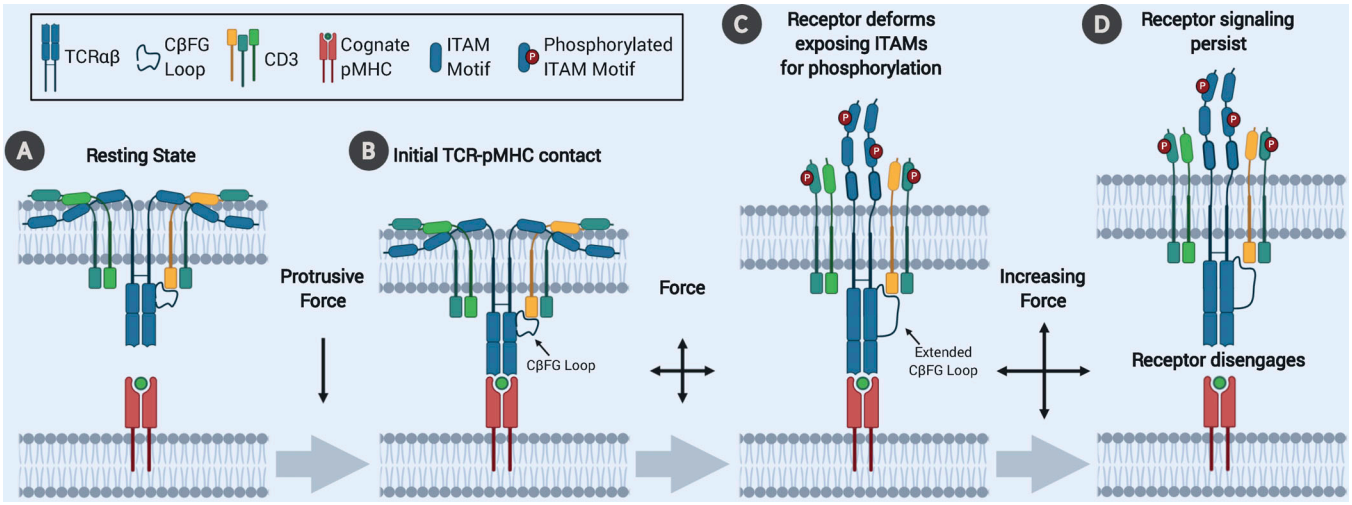

Figure 2. **TCR deformation translates mechanical forces into biochemical signals. (A)** In the resting state, ITAM motifs within the cytoplasmic domains of CD3 molecules interact with the inner leaflet of the T cell plasma membrane. **(B)** Protrusive forces applied by the T cell bring the TCR into contact with its cognate pMHC ligand. Importantly, although the TCR is bound to its ligand, no signaling is initiated at this stage. **(C)** Force application by the T cell puts the TCR–pMHC bond under tension. In the case of a high-affinity cognate ligand, a catch bond forms, allowing the transduction of force from the T cell cytoskeleton onto the TCR itself. Deformation of the TCRαβ chains extends the FG loop, which acts as a lever to push against the CD3 molecules and exposes ITAMs for phosphorylation by the Src family kinase Lck. Once ITAMs are phosphorylated, the cytoplasmic domains are unable to interact with the inner leaflet of the membrane. The ITAMs remain exposed and recruit ZAP70 to promote downstream TCR signaling. **(D)** Increasing force eventually breaks the TCR–pMHC bond, allowing the pMHC molecule to interact with additional TCRs to support serial triggering.

below a triggering threshold for activation (Imbert et al., 1994; O'Shea et al., 1992; Secrist et al., 1993). Therefore, to initiate TCR signaling, it is necessary to both overcome the glycocalyx barrier and segregate the TCR from tyrosine phosphatases. This tips the balance in favor of the tyrosine kinases that phosphorylate the TCR complex, initiating downstream signaling. According to the kinetic segregation model (Box 2; van der Merwe et al., 1995), T cells overcome this barrier by forming close contact sites within the larger T cell–APC contact area. These close-contact regions are initially formed by the small signal-independent adhesion molecule CD2 and later become enriched in TCR and coreceptors such as CD28 (Shaw and Dustin, 1997). Based on steric hindrance, they exclude large molecules like CD45 and CD148, thereby permitting initiation of TCR signaling. Importantly, stabilization of these close-contact zones long enough to allow productive signaling is heavily dependent on the TCR–pMHC bond lifetime, ensuring that signaling will occur only upon interaction with agonist peptides.

Although experimental evidence supporting the kinetic-segregation model has been accumulating, there are still several key issues that the model does not resolve. First, TCR–pMHC binding is generally low affinity, and therefore multiple bonds will be needed in order to stabilize the close-contact regions. However, it is known that under some conditions, a single pMHC molecule is sufficient to initiate the formation of active TCR MCs containing hundreds of TCR molecules (Huang et al., 2013). Second, it has been shown that TCR MCs can form and exclude CD45 while interacting with antagonist (low affinity) pMHC, or (if ICAM-1 is included on the stimulatory surfaces) in the total absence of pMHC molecules (Crites et al., 2014). These issues with the kinetic segregation model are easily solved if, instead of relying on stochastic interactions of adhesive molecules followed by TCR–pMHC interactions, the model is modified to incorporate the observation that T cells actively push against the APC.

The T cell surface is covered in microvilli, which range in length from 100 nm to several micrometers. The median length of these structures is ∼380 nm, long enough to penetrate beyond the ectodomains of most glycocalyx proteins (Jung et al., 2016; Weinbaum et al., 2007). T cell microvilli are highly dynamic, even in the absence of an external stimulus, moving over the total area of the T cell in ∼1 min (Cai et al., 2017). The actin regulatory proteins responsible for generating and maintaining T cell microvilli have yet to be identified, although there is evidence that ezrin and moesin are involved (Brown et al., 2003). WASp is not required, since T cells from WASp KO mice and human Wiskott-Aldrich syndrome patients show normal microvilli structures (Majstoravich et al., 2004). Importantly, microvillar tips show a four- to sixfold enrichment in both TCR and CD3 molecules as compared with other areas of the T cell membrane. This enrichment is lost upon actin depolymerization (Jung et al., 2016). Thus, T cell microvilli are ideally suited to provide the missing component for the kinetic segregation model. First, microvilli can penetrate the glycocalyx, creating close-contact zones even in the absence of agonist pMHC. Second, initial low-affinity binding of a TCR to an agonist pMHC can be further stabilized by protrusive forces, thereby diminishing the need for multiple stabilizing TCR–pMHC bonds. Finally, microvilli contain preclustered TCRs that can amplify an initiating signal, and their highly dynamic nature allows them to quickly scan an interacting APC for rare agonist pMHCs (Fig. 3 B). At present, the best direct evidence that T cell microvilli actually play this role comes from a series of compelling experiments whereby T cells were activated on lipid bilayers

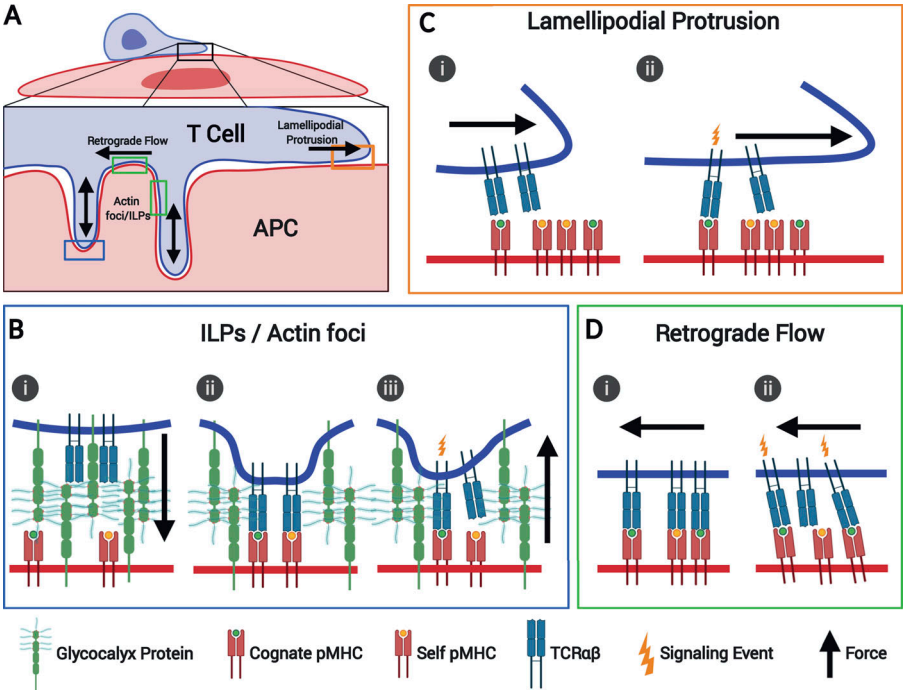

Figure 3. **Force contributes to T cell activation through several distinct actin structures.** **(A)** Overview of a T cell scanning an APC by migrating on its surface. During this process, discrete actin structures apply force at different areas of the T cell–APC interaction, facilitating T cell activation. **(B)** Upon initial contact between a T cell and an APC, ILPs/actin foci push into the APC to overcome the glycocalyx and create close-contact areas between the two membranes. Exclusion of bulky glycocalyx proteins allows for TCR–pMHC interaction. Subsequent retraction of these dynamic structures creates tension on the TCR–pMHC bonds that facilitates antigen discrimination and TCR activation. **(C)** At the leading edge of the migrating T cell, actin-driven lamellipodial protrusions apply force on the interacting APC, allowing for TCR triggering and signal accumulation. The same mechanism is in play in early stages of the formation of a stable synapse, where initial triggering induces spreading of the T cell on the APC surface. **(D)** Actin retrograde flow (or centripetal flow in a stable synapse) sweeps TCR MCs toward the center of the cell, promoting serial triggering of many TCR molecules by a single-agonist pMHC.

coated with agonist pMHC and ICAM-1, together with fluorescent quantum dots (Qdots) of varying size (Cai et al., 2017). 16-nm Qdots were excluded from sites of TCR–pMHC interactions, but 13-nm Qdots were not. Since the TCR–pMHC bond is ∼15 nm, this demonstrates that scanning microvilli are sufficient to foster this interaction. Notably, Qdot exclusion was detected even in the absence of agonist peptide, consistent with the idea that this process precedes and initiates TCR signaling. Interestingly, modeling studies support a mechanical feedback mechanism in which microvillar movement is slowed by the formation of catch-bond interactions, a process that could promote antigen discrimination (Pullen and Abel, 2019).

Force affects TCR binding kinetics, facilitating antigen discrimination

After TCR engages pMHCs on the APC surface, mechanical force again plays a role, in allowing the T cell to assess the quality of the TCR–pMHC interaction. This process underlies the exquisite ability of T cells to identify agonist peptides in a sea of non-agonist self-peptides. This aspect of T cell function has been explained by the kinetic proofreading model (Box 2; McKeithan, 1995), which posits that following TCR engagement, a sequence of receptor-proximal events must occur before transmission of the signal to downstream intermediates. Simply put, if the lifetime of a TCR–pMHC bond is shorter than the time needed for these proximal events to take place, downstream signaling will not occur. The model was later refined to allow serial, consecutive interactions between the same TCR–pMHC pair, where every interaction "picks up" at the same spot the previous one ended (Dushek et al., 2009). The kinetic proofreading model was consistent with the 3D binding kinetics of TCRs and their ligands derived from solution binding measurements. In those studies, binding on-rates for most ligands were found to be fairly similar, and off-rates were inversely correlated with agonist strength (Huang et al., 2010). However, T cells detect their ligands on cell surfaces, so 2D kinetics provides a better representation of TCR–pMHC interactions. Moreover, TCR molecules are clustered on the T cell membrane, which greatly affects net 2D binding kinetics (Wang and Reinherz, 2012). When 2D binding kinetics was measured, it became apparent that both on- and off-rates were considerably higher than in 3D conditions (Hong et al., 2015; Huang et al., 2010). These shorter-lived bonds created problems for the kinetic proofreading model. In fact, direct single-molecule measurements of TCR–ligand bond lifetime under static (no force) 2D conditions showed an inverse correlation between bond lifetime and agonist strength. Interestingly, this inverse relationship was quickly reversed with the application of ∼10 pN of force on the TCR–ligand bond (Hong et al., 2015; Liu et al., 2014). Under force, binding to high-affinity ligands induces a conformational change in the TCR to lock the bond in place (a catch bond). Binding to low-affinity ligands does not induce this effect, and the bond is broken (a slip bond). This "catch/slip" bond behavior is well known in other receptor–ligand pairs (Kong et al., 2013; Marshall et al., 2003). When DNA-based tension sensors were used to address this biology in intact T cells, it was found that T cells indeed exert piconewton forces on TCR–pMHC bonds (both agonists and antagonists; Liu et al., 2016; Ma et al., 2019). These findings fit well into a revised version of the kinetic proofreading model that incorporates mechanical force (Brockman and Salaita, 2019). According to this revised model, when T cells interact with agonist pMHCs, forces applied by the T cell actin cytoskeleton induce catch-bond behavior, prolonging TCR–pMHC interaction. These same forces eventually break the TCR–pMHC bond, but only after allowing

enough time for signaling to proceed past the proofreading step. Severing of the bond, in conjunction with TCR clustering, then facilitates binding of the same pMHC molecule to other TCR molecules (Liu et al., 2014). Note that TCR triggering can be achieved even under actin-depolymerizing conditions if external force is applied to the pMHC–TCR bond (Hu and Butte, 2016). Thus, while this mechanism for antigen discrimination relies on the T cell actin cytoskeleton, it is the force exerted by actin dynamics that is required.

It is now established that transduction of piconewton forces through the TCR is a requirement for proper T cell triggering and dependent on an intact actin cytoskeleton (Feng et al., 2017; Huang et al., 2010; Ma et al., 2019). But what is the specific actin machinery that provides this force? The answer is likely to involve ILPs. These structures were initially noticed in T cells scanning the endothelial monolayer for sites of transmigration, but were later also observed in T cells interacting with APCs (Carman, 2009; Carman et al., 2007). Similar to what has been proposed for microvilli, ILPs enforce close T cell–APC contacts (Sage et al., 2012). Unlike microvilli, which form independently of WASp and contain linear actin filaments (Majstoravich et al., 2004), ILPs are highly enriched in HS1, which interacts with the Arp2/3 complex in branched actin filaments (Sage et al., 2012). Treating T cells with WASp shRNA abrogated HS1 enrichment, and the Arp2/3 inhibitor CK666 completely abolished the formation of ILPs, suggesting that these structures are composed of Arp2/3-dependent, branched actin networks (Kumari et al., 2015).

ILPs are dynamic cell surface structures that continuously probe the APC, independently of TCR signaling (Sage et al., 2012). Since they are enriched in CD3 and TCR-proximal signaling molecules and exclude both CD43 and CD45, they are believed to be involved in TCR triggering (Fig. 3 B). There is a close relationship between ILPs and actin foci, which are also dependent on Arp2/3 complex activity, as well as WASp and HS1. Indeed, the difference may be a technical one; ILPs are protrusive structures that have been detected in T cells interacting with APCs and endothelial cells, whereas actin foci are flatter structures that are found in T cells interacting with stiff surfaces. It was therefore suggested that actin foci are, in fact, "frustrated" ILPs (Kumari et al., 2015). On the other hand, since ILPs form in the absence of antigenic stimulation but actin foci require it, the two structures may represent sequential stages of the same process. In support of this idea, TCR-induced calcium flux leads to an arrest in ILP dynamics (Sage et al., 2012), and the same process has been shown to promote the formation of actin foci at the location of TCR MCs (Kumari et al., 2015). Since ongoing WASp-dependent actin polymerization at foci is needed for later steps in the TCR signal transduction pathway, one can envision a process in which ILPs promote initial TCR–pMHC contact and subsequently transform into foci, where signals are amplified and sustained to promote full T cell activation. Regardless of whether they represent one structure or two sequential ones, ILPs/actin foci are good candidates to be the structures where force-dependent ligand discrimination takes place. Actin foci colocalize with signaling MCs containing TCR and tyrosine-phosphorylated signaling intermediates (Kumari et al., 2015). This is significant, since assembly and dissolution of these structures is thought to be the molecular basis of kinetic proofreading. Moreover, actin foci fail to form in WASp$^{-/-}$ T cells, and they are rapidly dissociated upon treatment with CK666. In both cases, this is accompanied by disruption of TCR-dependent signaling. Finally, although force generation by actin foci has not been directly detected, phosphorylation of the tension-sensing adapter protein CasL occurs in TCR-rich puncta, which resemble actin foci (Kumari et al., 2012; Santos et al., 2016). Going forward, it will be important to tease apart the relationship between ILPs and actin foci and test directly whether these structures correspond to sites where TCR–pMHC interactions are under tension.

Forces applied by lamellipodial networks control spreading, signaling, and IS formation

In addition to inducing polymerization of branched actin-rich foci, TCR activation also induces polymerization of lamellipodial branched actin networks, resulting in T cell spreading on the APC surface. As the cell reaches its maximal size, the same actin machinery drives retrograde flow, forming the well-known bullseye IS architecture. Both spreading and retrograde flow are dependent on Ca^{2+} flux and the actin nucleator WAVE2 (Babich et al., 2012; Bunnell et al., 2001; Murugesan et al., 2016; Yi et al., 2012), and eliminating CRAC channel–mediated Ca^{2+} flux results in rapid deterioration of the IS actin structure (Hartzell et al., 2016). The spreading process not only allows the T cell to scan a larger area of the APC surface but also applies force on receptor–ligand pairs, promoting further peptide discrimination and TCR activation (Fig. 3 C). During retrograde flow, TCR MCs are transported toward the center of the IS, where signaling is extinguished by endocytosis (Varma et al., 2006). This results in translation of force produced by the T cell actin machinery onto the TCR (Fig. 3 D). Since many pMHC molecules are partially confined on the APC membrane (Comrie et al., 2015b), TCR molecules that bind pMHC on the APC surface will experience drag forces created by the APC cytoskeleton, amplifying tension on the TCR–pMHC bond. Recent work has shown that antigen-binding kinetics influences actin flow rates, creating a feedback loop that can tune the forces experienced by the TCR (Colin-York et al., 2019a). Actin cytoskeletal dynamics drive centripetal flow of TCR MCs through two mechanisms. First, after TCR activation, the transmembrane adapter protein LAT forms condensates, which associate with actin through complexes containing Nck and WASp (Ditlev et al., 2019). This mechanism was shown to depend on linear actin polymerization by formins. Second, in more central (lamellar) regions of the IS, integrin-dependent actomyosin arcs sweep TCR MCs inward through frictional coupling (indirect physical interactions; Babich et al., 2012; DeMond et al., 2008; Smoligovets et al., 2012; Yi et al., 2012; Yu et al., 2010). Forces exerted by actin flow not only facilitate TCR triggering but also break TCR–pMHC bonds. Because TCR signaling and transport occurs in MCs containing numerous receptors, this bond breakage actually enhances signaling by allowing serial interactions of a single pMHC molecule with many TCR molecules.

An integrated view of force application in the context of overall synapse architecture

All of the mechanical processes described previously are happening more or less simultaneously at the T cell–APC interface. The cell biological features of this interface introduce important complexities that are not recapitulated by current experimental systems. Indeed, most of what we know about T cell mechanobiology comes from in vitro studies using planar stimulatory surfaces or artificial APCs coated with high concentrations of high-affinity ligands. These ligands are typically either completely immobile or, if lipid bilayers are used, infinitely mobile. In in vivo settings, T cell–APC interactions are quite different; APCs present very low numbers of ligand molecules of varying affinity in the presence of many other proteins that can influence TCR signaling. The mobility of molecules in the APC membrane is variable and the APC surface is not smooth. Indeed, due to the action of microvilli and ILP structures on the T cell side of the interaction, the membranes of two interacting cells become interdigitated (see electron micrographs in Carman, 2009; Carman et al., 2007; Sage et al., 2012). Finally, the APC is not a passive player in the cell–cell interaction. This is especially true for DCs, which use their own actin network to promote antigen presentation (Al-Alwan et al., 2001; Comrie et al., 2015b; Blumenthal et al., 2019 *Preprint*). Mature DCs assume a polarized morphology with their characteristic veils on one side of the cell and microvilli on the other. DC microvilli are rich in pMHC and costimulatory molecules and are the preferred site for T cell binding (Fisher et al., 2008). Moreover, DCs generate WASp-dependent structures that stabilize adhesive contacts with interacting T cells (Malinova et al., 2016). Given the topological and mechanical complexity of the T cell–DC interface, the distinction between pushing and pulling forces is lost, at least at the cell biological level. While a particular T cell structure may be pushing or pulling against the DC surface, the directionality of forces is not necessarily maintained at the level of individual receptor–ligand pairs (Fig. 3).

In addition to the topological complexity of the T cell–APC interface, it is important to keep in mind that this is often a moving contact. T cells scan APCs while migrating along their surfaces, forming dynamic cell–cell junctions termed "kinapses" (Dustin, 2008). This mode of interaction allows T cells to integrate signals from multiple locations on the APC and from different cells within a lymphoid organ (Mempel et al., 2004; Miller et al., 2004). In fact, in vivo imaging studies show that T cells scan APCs mostly through this kinapse mechanism (Mayya et al., 2018). In essence, kinapse and synapse structures are variations of the same actin architecture, but synapses retain radial symmetry while kinapses are polarized, resulting in net T cell movement. T cells oscillate between these two distinct modes, breaking and reforming radial symmetry as a result of signaling events, including elevation of intracellular Ca^{2+} levels, and the balance between WASp and PKCθ activity (Negulescu et al., 1996; Sims et al., 2007). The motile nature of T cell–APC contacts adds another level through which forces exerted at the cell biological level can contribute to mechanotransduction events associated with T cell activation. For example, forces associated with T cell motility, even those generated far from the cell–cell interface, can generate tension on TCR–pMHC bonds (Fig. 3).

Clearly, the textbook view of the IS is an oversimplification. The synapse is highly complex both in terms of dynamics and topology. Moreover, synapse architecture varies with the type of T cell–APC contact (Friedl et al., 2005). While T cells form a traditional bullseye IS with B cells, T cell–DC interactions result in the formation of a multifocal synapse (Fisher et al., 2008; Thauland and Parker, 2010). Thus, depending on the nature of the T cell and the interacting APC, T cells form different force-generating structures. Moreover, even within an individual T cell–APC contact, T cells simultaneously use different force-producing structures within different regions of the synapse, and this landscape changes as signaling progresses (Fritzsche et al., 2017).

The biophysical properties of the APC directly influence TCR signaling

To fully understand mechanotransduction at the IS, it is essential to consider the APC side of the interface. The biophysical properties of the APC cortex impact the ability of the T cell to use its force-producing structures to induce TCR deformation and signaling. Current measurements of force application by T cells come from studies in which T cells interact with very stiff surfaces in the gigapascal range (Callister, 2000). In contrast, cells in the body are much softer, with cortical stiffness values in the range of 5 Pa to 40 kPa (Janmey and McCulloch, 2007), and APCs exhibit stiffness values on the lower side of this range (Bufi et al., 2015; Blumenthal et al., 2019 *Preprint*). T cells sense substrate stiffness through the TCR (Judokusumo et al., 2012; Blumenthal et al., 2019 *Preprint*), and substrate stiffness has a direct effect on T cell priming (Judokusumo et al., 2012; O'Connor et al., 2012; Blumenthal et al., 2019 *Preprint*), gene expression (Saitakis et al., 2017), and effector functions (Basu et al., 2016; Saitakis et al., 2017). Interestingly, the stiffness of the interacting surface also directly influences the amount of force applied by the interacting T cell (Hui et al., 2015; Husson et al., 2011; Sawicka et al., 2017). Thus, it appears that mechanosensing through the TCR induces a mechanical feedback loop that influences T cell activation. The importance of stiffness sensing by T cells is highlighted by the fact that as part of the maturation program through which DCs differentiate and become optimized for T cell priming, they undergo an increase in cortical stiffness (from ∼2 kPa for immature DCs to 4–6 kPa for mature DCs). While this may seem a modest increase, T cells stimulated on surfaces of 2 kPa are nearly unresponsive, while T cells stimulated on surfaces of 4–8 kPa proliferate efficiently (Blumenthal et al., 2019 *Preprint*). Thus, this alteration in the biophysical properties of the APC surface is sensed by interacting T cells and serves, along with up-regulation of cytokines and stimulatory ligands, to control appropriate T cell priming.

In parallel with changes in cortical stiffness, maturing DCs also undergo changes in ligand mobility. Although the mobility of MHCs on the DC surface is not affected, maturation induces a dramatic actin-dependent decrease in the mobility of ICAM-1, the ligand for the T cell integrin LFA-1 (Comrie et al., 2015b). This decrease in ICAM-1 mobility on the DC side of the synapse

generates a counterforce for actin-dependent tension on LFA-1, promoting conformational changes in LFA-1 that lead to enhanced adhesion and T cell priming. Interestingly, the tension on LFA-1 also affects the dynamics of the T cell actin network (Jankowska et al., 2018). Since the TCR interacts with that same actin network, this can indirectly influence tension on the TCR, modulating TCR signaling. The details of how this process is used to modulate signaling events at the IS remains poorly understood. Nonetheless, this example highlights the fact that T cell priming involves a tug-of-war between the cytoskeletons of the T cell and the APC, which likely impacts the function of multiple receptor–ligand pairs.

Future outlook

Over the past several years, it has become clear that mechanobiology plays a key role in T cell activation. An important emerging concept is that the actin cytoskeleton serves as a mechanical intermediate that integrates force-dependent signals coming from distinct receptor–ligand pairs. This type of signal integration, which can coordinate nuanced responses over large distances, may be used by other cell types as well. Going forward, the challenge for cell biologists studying T cell activation is to understand how forces exerted on both sides of the IS influence receptor signaling and how these mechanical cues are integrated with biochemical signals to shape the immune response.

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