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Molecular mechanisms and functional implications of polarized actin remodeling at the T cell immunological synapse

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Abstract

Transient, specialized cell-cell interactions play a central role in leukocyte function by enabling specific intercellular communication in the context of a highly dynamic systems level response. The dramatic structural changes required for the formation of these contacts are driven by rapid and precise cytoskeletal remodeling events. In recent years, the immunological synapse that forms between a T lymphocyte and its antigen-presenting target cell has emerged as an important model system for understanding immune cell interactions. In this review, we discuss how regulators of the cortical actin cytoskeleton control synaptic architecture and in this way specify T cell function.

Keywords

Cytoskeleton; Actin; T cells; Immunological synapse; T cell receptor; cytotoxicity; signal transduction

Introduction

Effective immune responses against pathogenic agents and cancer require rapid and specific threat detection and intercellular communication. In many cases, immune cells accomplish these goals by building specialized, transient interactions with each other and also with other cells in the surrounding tissue. This often requires them to completely reorganize their cellular architecture in a matter of minutes. Lymphocytes like T cells, B cells, and natural killer cells (NK) exemplify this dramatic structural plasticity by adopting drastically different structural configurations depending on their location, motility, and activation state. This belies their common depiction in textbooks as round and featureless spheres.

Over the past 15 years, the rapid architectural changes that accompany T cell activation have emerged as an important model system for understanding cell-cell interactions in the immune system. All T cells express a unique T cell receptor (TCR), which is designed to recognize peptides (typically 9–11 amino acids in length) derived from foreign organisms. These peptides are presented by major histocompatibility complex (MHC) proteins on the surfaces of other cells. Engagement of cognate peptide-MHC (pMHC) by the TCR induces the robust proliferative, transcriptional, and secretory responses that are generally associated with T cell activation. It also promotes a dramatic structural change, as the T cell gloms onto

the side of the antigen-presenting cell (APC), forming a stereotyped contact known as an immunological synapse (IS)[1]. The IS has long been characterized by the concentric architecture it adopts during the sustained phase of its maturation (Fig. 1). The central domain, also called the central supramolecular activation cluster (cSMAC), contains spent TCR molecules in the process of downregulation. This is surrounded by a peripheral SMAC (pSMAC), which is dominated by a ring of the $\alpha_L\beta_2$ integrin LFA-1. This integrin ring is generally thought to be crucial for mediating adhesion with the APC. Surrounding the pSMAC at the very edge of the contact is the distal SMAC (dSMAC), which consists of a circular array of filamentous actin (F-actin). Together, these radially symmetric zones provide the context within which T cell activation and sustained intercellular communication occur. Although this review will focus on T cells, it is important to note that both B cells and NK cells also form synaptic contacts with APCs and target cells, respectively, and that these contacts bear striking similarities to the T cell IS[2,3].

Actin dynamics play a central role in the formation and the maintenance of the IS[4] (Fig. 1). Initially, a uniform sheet of F-actin powers radially symmetric expansion over the surface of the APC. Once IS growth has stabilized, cortical F-actin reorganizes into the peripheral ring that will become the dSMAC. Continuous retrograde flow within the dSMAC promotes adhesion by clustering LFA-1 in the pSMAC[5]. This flow also regulates TCR signaling and maintains IS symmetry. Finally, F-actin depletion from the center of the IS generates an “actin hypodense” region that is thought to facilitate secretion toward the APC by enabling the fusion of intracellular compartments with the synaptic membrane[6–8]. Both CD4⁺ helper T cells and CD8⁺ cytotoxic T lymphocytes (CTLs) use soluble cytokines to communicate with other immune cells. In addition, CTLs kill infected or transformed cells by secreting cytotoxic perforin and granzymes. The ability to release these factors directionally enhances the specificity and perhaps also the potency of these secretory responses[9,10]. Hence, the annular F-actin configuration that defines the IS also serves as a structural foundation for its function.

Despite years of research, our knowledge of the molecular mechanisms that guide actin dynamics at the IS remains incomplete. TCR activation triggers a very complex network of signaling events, and it has been difficult to tease apart which of these events regulate the cytoskeleton directly and which influence it only secondarily. These ambiguities at the level of molecular cause and effect have complicated efforts to assess the roles played by specific F-actin structures during T cell activation. In this review, we will discuss recent advances in our understanding of how F-actin is remodeled at the IS and how that remodeling contributes to T cell function.

Tools for imaging the IS

Lymphocytes are challenging experimental systems for cell biologists because they are small, highly dynamic, and lack the beautifully distributed organelles often seen in adherent cell types. Over the past ten years, the study of IS formation has been revolutionized by developments in single cell imaging. Standard 3-dimensional epifluorescence or confocal microscopy lacks the spatial and temporal resolution required to dissect the rapid signaling events and shape changes associated with T cell activation. To circumvent this issue, a

number of labs have developed oriented approaches in which the APC is replaced by a glass surface or supported lipid bilayer containing stimulatory ligands[11,12]. T cells form IS-like contacts with these surfaces that are oriented perpendicular to the imaging axis. As such, they are amenable to total internal reflection fluorescence (TIRF) microscopy, a high-resolution technique that focuses on the first 100 nm of the imaging sample[13]. Because the section illuminated by TIRF encompasses the synaptic membrane and a thin slice of cytoplasm above it, it is well suited for the analysis of signaling dynamics at the membrane and F-actin remodeling in the cortex just beneath it. Using this approach, it is relatively straightforward to monitor the early stages of IS growth and the emergence of the synaptic F-actin ring.

More recently, the oriented IS approach has been merged with so-called super-resolution imaging modalities, including structured illumination microscopy (SIM), stimulated emission depletion (STED), and photoactivated localization/stochastic optical reconstruction microscopy (PALM/STORM)[6,7,14–16]. These technologies, in particular the PALM/STORM approach, enable investigators to probe the organization of signaling complexes at a truly molecular scale. This level of resolution has already proved to be exceedingly useful for the analysis of early TCR signaling[16,14,15], and will no doubt influence future research into the T cell cytoskeleton.

The mechanistic analysis of IS formation has also benefited from advances in our ability to control TCR activation. Much of the intracellular signaling triggered by TCR engagement take place within seconds, making it very difficult to establish a reliable order of events. To address this issue, we have developed a photoactivation system for triggering TCR signaling in defined regions of the T cell membrane[17]. T cells are first attached to glass surfaces containing a photoactivatable pMHC reagent that is nonstimulatory to its cognate TCR until it is irradiated with UV light. Then, a micron-scale area beneath the T cell is UV-irradiated, creating a region of agonist pMHC that the T cell can recognize. Subsequent signaling events are monitored by TIRF or epifluorescence microscopy. The spatiotemporal control afforded by this approach has enabled us to assemble well-defined pathways linking TCR stimulation to cytoskeletal regulators.

Although oriented single cell imaging systems have substantially improved our understanding of T cell activation and IS formation, it is important to keep in mind that they cannot recapitulate all aspects of the bona fide T cell-APC contact. Electron microscopy and tomography has revealed, for instance, that both CD4⁺ and CD8⁺ T cells form complex, undulating contacts with the APC characterized by extensive interdigitation of T cell and APC membrane[18,19]. Furthermore, certain APCs, in particular dendritic cells (DCs), appear to play a very active role in the IS assembly process, polarizing both their cytoskeleton and certain intracellular compartments toward the contact site[20–22]. These complex dynamics cannot be replicated by inanimate, planar surfaces. Hence, future progress will depend in part on the development of higher resolution imaging approaches capable of monitoring three-dimensional T cell-APC interactions both in vitro and in vivo.

Synaptic actin remodeling

Although other cell surface receptors, in particular integrins like LFA-1 and VLA-4 ($\alpha_4\beta_1$), can influence cytoskeletal architecture at the IS, the TCR plays the predominant role. Signal transduction from the TCR has been studied intensely for many years, and as a result we know a fair amount about the early steps in the pathway[23] (Fig. 2). Engagement of pMHC by the TCR induces the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) within the associated CD3 chains by the Src family protein tyrosine kinase (PTK) Lck. Phosphorylated ITAMs recruit and activate the Syk family kinase ZAP-70, which together with Lck mediates the phosphorylation of two scaffolding proteins, the linker for activation of T cells (LAT) and SH2 domain-containing leukocyte protein-76 (SLP76). LAT and SLP76 then form a complex at the plasma membrane that functions as a platform for a number of important signaling enzymes. The guanine nucleotide exchange factor (GEF) SOS is recruited via its associated adaptor protein Grb2 to LAT, where it triggers Ras activation and the MAP kinase pathway. The LAT-SLP76 complex also recruits phospholipase C γ 1 (PLC γ 1), which hydrolyzes phosphatidylinositol 4,5, bisphosphate (PIP $_2$) to generate two second messengers: inositol trisphosphate (IP $_3$) and diacylglycerol (DAG). IP $_3$ diffuses into the cytoplasm to trigger calcium (Ca $^{2+}$) signaling while DAG activates protein kinase C (PKC) and other enzymes such as the Ras guanyl releasing protein 1 (RasGRP1), another Ras specific GEF. As discussed later in this review (see *Signaling pathways controlling centrosome polarization*), synaptic DAG accumulation drives TCR-induced remodeling of the microtubule cytoskeleton. The LAT-SLP76 complex also couples TCR activation to actin remodeling, both by physically recruiting actin regulators and also by activating second messenger pathways that coordinate actin regulators at a distance. Our knowledge of these relationships will be discussed in more detail below. We will begin with the proteins that directly nucleate actin polymerization and then discuss the pathways that link these proteins to the TCR signaling machinery.

Nucleation-promoting factors and Rho-family GTPases

Nucleation-promoting factors (NPFs) play a central role in T cell actin dynamics. They form a rather structurally diverse family that can be divided into two subclasses based on domain structure[24,25]. Type I NPFs include the Wiskott-Aldrich syndrome protein (WASp), neural-WASp (NWASp), WASp family verprolin-homologous protein (WAVE 1–3, or SCAR), WASp and SCAR homolog (WASH), WASp homologue associated with actin, membranes and microtubules (WHAMM), and junction-mediating regulatory protein (JMY). Type II NPFs, for their part, include cortactin and the leukocyte-specific homolog of cortactin, HS1.

Class I NPFs induce robust actin polymerization through the Arp2/3 complex, a 7-protein assembly that drives the growth of branched F-actin arrays[25]. Suppression of Arp2/3 components blocks TCR-induced lamellipodia formation and inhibits spreading over the surface of the APC, demonstrating the importance of the complex for IS growth[26]. The two predominant class I NPFs in T cells are WASp and WAVE2[4]. Both contain a conserved VCA (Verprolin homologous, central hydrophobic, and acidic) region that mediates interaction with Arp2/3. Each has a distinct N-terminus, however, that defines its

unique function by specifying localization and interaction with other proteins. WASp associates constitutively with the WASp interacting protein (WIP), which functions both as a chaperone and regulatory partner. WAVE2, for its part, incorporates into a large multiprotein complex that contains PIR121, NAP125, ABI, and HSPC300.

Both WASp and WAVE2 are coupled to upstream signals by members of the Rho family of GTPases, which function as master regulators of the actin cytoskeleton in most cell types[27]. Like all small GTPases, Rho family members cycle between an inactive, GDP-bound state and an active, GTP-bound form that is capable of interacting with downstream effectors. Cdc42, the Rho-GTPase most often associated with linear actin structures like filopodia, activates WASp by binding to a conserved motif within its N-terminal region[28]. This relieves autoinhibitory interactions, releasing the VCA domain to engage Arp2/3. By contrast, it is the GTPase Rac, best known for its role in driving sheet-like lamellipodial growth, that activates WAVE2[24]. Rac does not bind to WAVE2 directly, but rather the PIR121 component of the WAVE complex. Importantly, both Rac and Cdc42 are rapidly activated by TCR engagement[29–31], consistent with a role for both proteins in synaptic actin dynamics. Importantly, WASp can also be activated by PIP₂, which functions both to localize WASp to the plasma membrane and also to destabilize autoinhibitory interactions between its N- and C-terminal domains[32,33].

Humans lacking WASp develop a severe immune deficiency (Wiskott-Aldrich syndrome, or WAS) that is accompanied by thrombocytopenia and eczema[34]. Because T cells from WAS patients display defects in antigen-induced proliferation, differentiation, and cytokine secretion[35,36], there has been a great deal of interest in the role of WASp and its regulators during T cell activation. Mechanistic experiments have demonstrated that WASp physically interacts with LAT-SLP76 via the adaptor protein Nck[37] (Fig. 2). This would place it in close proximity to Vav1, an activator of Cdc42 that also associates with SLP76[38]. Hence, one could easily imagine a signaling pathway moving from TCR activation through WASp that might be responsible for driving TCR-induced F-actin growth at the IS. Consistent with this notion, T cells from Wiskott-Aldrich Syndrome (WAS) patients and WASp knock-out (KO) mice show cytoskeletal defects, including the formation of unstable synapses [39–42].

In other studies, however, WASp was found to be essentially dispensable for synaptic F-actin dynamics[43,44]. The relative importance of the protein for IS formation may depend on the strength of TCR signaling. In T cells with a low affinity receptor or under conditions of low pMHC ligand density, WASp is required for amplifying signals to the cytoskeleton[43]. This role is superfluous, however, in the context of abundant ligand or a high affinity TCR. Interestingly, WASp also appears to be necessary for the maintenance of radially symmetric IS architecture. WASp KO T cells initially form normal synapses on stimulatory lipid bilayers, but these contacts quickly break down as the cells begin to migrate over the surface[45]. Taken together, these results suggest that WASp is more likely to function as a TCR signal amplifier and contact stabilizer, rather than as a direct mediator of synaptic F-actin accumulation. Consistent with this idea, recent studies indicate that loss of Cdc42, the upstream regulator of WASp, has little to no effect on the overall level of synaptic actin polymerization[46].

The ambiguity of the Cdc42-WASp loss-of-function data has led investigators to assess the role of the Rac-WAVE2 module as an alternative master regulator of synaptic F-actin. The importance of both Rac and WAVE for sheet-like actin polymerization in other systems implies that they might be good candidates, given that the IS resembles a radially symmetric lamellipodium[47]. Indeed, loss-of-function studies demonstrated a clear role for both proteins in IS growth and F-actin ring formation. Jurkat T cells lacking WAVE2 displayed dramatic defects in adhesion and cell spreading on stimulatory surfaces, despite the fact that a number of other events in the TCR signaling network were intact[48]. Single cell imaging studies of WAVE2 dynamics were consistent with these results[49]. During IS growth, WAVE2 localizes to the leading edge of the radial lamellipodium. It then shifts into a thicker annular pattern overlying the region of eventual F-actin ring formation. Taken together, these data are indicative of a central role for WAVE2 in both the establishment and maintenance of synaptic F-actin architecture. Similar results were obtained in loss-of-function experiments targeting Rac. T cells express two Rac isoforms: the ubiquitous Rac1 and the hematopoietic-specific Rac2. shRNA-mediated suppression of either protein significantly inhibits both cell spreading and F-actin ring formation on stimulatory lipid bilayers[49], which is consistent with other work documenting a TCR-induced actin polymerization defect in Rac2 KO T cells[50]. Importantly, simultaneous suppression of both Rac1 and Rac2 leads to a much stronger defect than loss of either isoform alone[49], indicating that the two proteins contribute additively in this context. The importance of robust Rac expression for T cell function was recently highlighted by the discovery that blocking Hedgehog signaling in cytotoxic T lymphocytes (CTLs) impairs their cytotoxic potential by reducing Rac levels[51].

The type II NPF HS1 has also been implicated in synaptic actin dynamics, although its role is less well defined. Like WASp and WAVE2, HS1 contains an acidic domain that can recruit and activate the Arp2/3 complex[52]. TCR activation induces tyrosine phosphorylation of HS1 by Lck and ZAP-70 [53]. This promotes recruitment of the protein to the IS, where it interacts directly with the tyrosine kinase ITK, a component of the LAT-SLP76 complex[54]. Loss-of-function studies indicate that while HS1 is not required for TCR-stimulated actin polymerization and lamellipodial protrusion, it is involved in organizing and maintaining these structures[53]. T cells lacking HS1 also display defective TCR-induced Ca^{2+} responses, indicative of a role for the protein in receptor proximal signaling[54]. HS1 has been described to associate with WASp in DCs [55], and it is tempting to speculate that HS1 contributes to IS architecture and sustained TCR signaling by stabilizing the branched-actin filaments generated by WASP and other Arp2/3 complex activators.

There is still much that we do not understand about how NPFs and Rho GTPases shape F-actin at the IS. On balance, however, the data suggest that Rac-WAVE2, Cdc42-WASp, and HS1 have distinct and complementary roles in the process. Whereas Rac-WAVE2 is critical for the initiation of synaptic actin polymerization and growth of the F-actin ring, WASP and HS1 appear to be involved in TCR-induced signal transduction and maintenance of the mature IS (Fig. 2).

The role of Rho GEFs

Rho-GTPase activation is mediated by Rho GEFs, which promote the exchange of GTP for GDP within the GTPase active site. Classical Rho GEFs contain a catalytic Dbl-homology (DH) region, which is typically situated just N-terminal to a regulatory pleckstrin homology (PH) domain[56]. Among these DH-PH proteins, Vav1 has been considered to be the major regulator of actin polymerization following TCR activation[38]. It can activate both Rac and Cdc42 *in vitro*, and studies have indicated that it is required for full activation of WASp via the recruitment of GTP-bound Cdc42 [57]. Furthermore, loss of Vav1 results in defective TCR capping and T cell activation due to impaired cytoskeletal reorganization[58–60,44,61].

That being said, the degree to which Vav1 contributes to actin cytoskeleton remodeling directly remains unclear. It is a large, multisubunit protein that contains, in addition to its DH-PH region, a number of domains that mediate protein-protein interactions. These domains confer scaffolding function to Vav1, and studies suggest that it plays an important role in stabilizing the LAT-SLP76 complex and thereby promoting early TCR signaling[62,63]. Vav also plays a crucial role in the inside-out activation of integrins, independently of WASp[58,44]. The inability of Vav1^{-/-} T cells to upregulate integrin affinity, and thereby adhesion to the APC, could explain the defect in IS formation previously observed. Furthermore, Vav1 has been shown to influence actin remodeling in an indirect manner by inactivating ezrin-radixin-moesin (ERM) proteins after TCR activation[64]. ERM proteins act as crosslinkers between the cortical F-actin and proteins in the plasma membrane[65], and their inactivation promotes transient relaxation of the cytoskeleton during T cell-APC contact formation. The complexity of the Vav1 loss-of-function phenotype is consistent with the idea that it operates early in the TCR signaling cascade and that its functions encompass more than just the activation of Rho GTPases. Indeed, it was recently shown that a Vav1 point mutant lacking GEF activity could completely rescue the signaling and developmental phenotype of Vav deficient T cells[66]. Although this result is somewhat controversial, it does suggest that the nucleotide exchange activity of Vav is of secondary importance during T cell activation.

T cells also express various atypical Rho GEFs of the CDM (CED-5, Dock180, Myoblast City) family[67]. Proteins in this family catalyze nucleotide exchange via a conserved dedicator of cytokinesis (Dock) homology region 2 (DHR2) located near their C-terminus. Many CDM GEFs also interact constitutively with the engulfment and cell motility-1 (Elmo1) adaptor, which confers stability and regulates GEF activity. Accumulating evidence indicates that CDM proteins control actin polymerization in multiple cell types. The Dock2 isoform, which is a Rac-specific GEF, is particularly important for shaping polarized actin-based structures in leukocytes[68]. Dock2 is recruited to the leading edge of migrating neutrophils, suggesting that it drives protrusive actin polymerization in lamellipodial structures[69,70]. Consistent with this idea, T cells and B cells lacking Dock2 exhibit profound migration defects toward chemokines both *in vitro* and *in vivo*[71,72]. Dock2 deficiency also inhibits IS formation; T cells lacking the protein fail to spread properly on stimulatory bilayers and form miniaturized contacts characterized by thin peripheral F-actin rings and poor TCR accumulation in the cSMAC[49,30]. Interestingly, LFA-1 accumulation

is intact in these cells[30], implying that the IS size defect is independent of integrin-mediated adhesion. Expression of a constitutively active form of Rac restores cell spreading[49], indicating that Dock2 operates through Rac in this context. Indeed, TCR-induced Rac activation is essentially abrogated in Dock2^{-/-} T cells, despite the fact that other signaling responses are largely intact[49,30]. TIRF imaging has revealed that both Dock2 and Elmo colocalize with F-actin during the growth and subsequent consolidation of the IS[49]. They display particularly strong overlap with the F-actin ring, implying that continuous Rac activation is required for the maintenance of IS size. Taken together, these data indicate that the Dock2-Elmo complex is critical for driving protrusive F-actin polymerization in the synaptic lamellipodium. This is remarkably similar to the role it plays at the leading edge of migrating leukocytes, and highlights the previously noted similarities between the two structures[47].

Recent studies have implicated another CDM family member, the Cdc42-specific GEF Dock8, in the regulation of lymphocyte development and function[73–76]. In humans, inactivating Dock8 mutations cause an immunodeficiency syndrome characterized by severe cutaneous viral infections and allergies[77,78]. Studies using Dock8^{-/-} mice have revealed specific defects in lymphocyte function that manifest during the later stages of the adaptive immune response. B cells lacking Dock8 proliferate normally in response to antigen, but then they fail to form robust germinal centers and produce affinity-matured antibodies[76]. Similarly, whereas primary T cell expansion and differentiation are unaffected in Dock8^{-/-} mice, memory T cell persistence is markedly impaired[74].

The immunological phenotypes observed in Dock8^{-/-} animals are associated with changes in IS architecture. LFA-1 accumulation is reduced in both B cell and T cell synapses, and in T cells this integrin-clustering defect is accompanied by decreased F-actin accumulation[74,76]. NK cells lacking Dock8 display similar deficiencies in LFA-1 and F-actin enrichment. In addition, lytic granules containing perforin and granzyme fail to polarize toward the synaptic membrane[73]. Proteomic studies in this system have revealed that Dock8 associates with both WASp and the integrin regulator talin, indicating that it could function as a structural and/or functional bridge that couples target cell adhesion to F-actin dynamics. In light of this mechanistic insight, it is perhaps not surprising that loss of Dock8 inhibits target cell killing by NK cells[73]. Precisely how Dock8 contributes to T cell and B cell maturation is less clear, however. It is possible that the synaptic defects occurring in the absence of Dock8 could affect asymmetric cell division, which has been linked to both memory T cell formation and competition between B cells for T cell help[79,80]. Impaired synapse formation could also lead to selective defects in signal transduction to the nucleus. Indeed, the memory phenotype seen in Dock8^{-/-} T cells is markedly similar to that observed in T cells bearing a specific point mutation in the TCR transmembrane domain[81]. Interestingly, this mutation impairs NF-κB activation without altering other important transcriptional responses. A possible link between the Cdc42-Dock8 axis and NF-κB signaling in lymphocytes remains to be explored.

Taken together with what is known about Dock2, these results imply a conserved role for CDM family members in building transient cell-cell interactions. Leukocytes express a number of other CDM proteins, including Dock1, Dock5, Dock10, and Dock11. Their

precise functions are, for the most part, poorly understood, and represent interesting areas for future investigation. Moving forward, it may be useful to keep in mind that CDM GEFs are large proteins that could also function as scaffolding molecules. Indeed, recent work has revealed an alternative role for Dock8 as an adaptor in the TLR9-MyD88 pathway[82]. Hence, CDM GEFs could conceivably influence cellular behavior in a manner independent of Rho GTPases.

PI3K dependent activation of the Rac-Dock2 module

Although it has been known for some time that Dock2 is crucial for TCR-induced Rac activation, precisely how TCR signaling engages Dock2 was only recently elucidated. All CDM proteins contain a DHR1 domain that mediates recruitment to membranes containing phosphoinositides[67]. The DHR1 domain of Dock2 binds with particularly high affinity to phosphatidylinositol (3,4,5)-phosphate (PIP₃)[69], which is generated from PIP₂ by members of the phosphoinositide 3-kinase family of enzymes. Using a fluorescent biosensor derived from the PIP₃ specific PH domain of Grp1, we found that PIP₃ form an annular gradient at the IS that overlies the peripheral F-actin ring[49]. The close correspondence between this accumulation pattern and that of Dock2-Elmo suggested that PIP₃ drives Dock2-Elmo1 recruitment to the IS. Indeed, pretreatment of T cells with the PI3K-specific inhibitor wortmannin abrogated Dock2-Elmo1 enrichment at the T cell-APC contact and also inhibited TCR-induced activation of Rac. This phenotype was accompanied by dramatic reductions in both IS size and synaptic F-actin enrichment. Conversely, shRNA-mediated suppression of PTEN, a lipid phosphatase that antagonizes PI3K signaling, markedly increased Rac activation and IS growth. Importantly, a Dock2 mutant lacking the DHR1 domain failed to accumulate in an annular configuration, confirming the importance of PIP₃ recognition for synaptic recruitment. Collectively, these data demonstrate that PIP₃ induces plasma membrane translocation of Dock2 upon T cell activation, thereby targeting its activity to the periphery of the IS (Fig. 2). A recent study from Fukui and colleagues demonstrated that PIP₃ also recruits Dock2 to the NK cell IS[83], suggesting that this pathway is conserved among all lymphocytes.

TCR activation elicits rapid and robust PIP₃ production in the synaptic membrane[84–87]. In addition to its role in structuring the IS, this PIP₃ also promotes cell survival, proliferation, and a range of transcriptional responses[88]. PIP₂ is converted into PIP₃ by class I PI3Ks, which function as obligate heterodimers containing one catalytic and one regulatory subunit. Class IA catalytic subunits (p110 α , p110 β , and p110 δ) pair with regulatory subunits of the p85/p55 family, which contain SH2 domains that mediate interactions with phosphotyrosine containing signaling complexes. Conversely, the lone class IB catalytic subunit (p110 γ) binds to regulatory subunits of the p101/p87 family, which associate with activated G-protein coupled receptors. p110 α is ubiquitously expressed, while p110 β , p110 δ , and p110 γ are more restricted to leukocytes[89]. There is some controversy about the relative contribution of these different isoforms to TCR-induced PI3K signaling. Studies using genetically modified mice that lack p110 δ activity have strongly implicated this isoform as the key player in the process[90,91]. Residual PI3K activity is still observed in p110 δ ^{-/-} T cells, however, suggesting that additional PI3K isoforms play a role[92]. Other studies have implicated p110 α and the even the class IB isoform p110 γ [93,94].

We initially adopted a pharmacological approach to address this question, using different combinations of selective small molecule inhibitors to block specific PI3K isoforms either alone or in combination[49]. Targeting p110 δ in this manner eliminated the majority of TCR-induced PI3K activity, stunted IS growth, and impaired synaptic F-actin ring formation. Combining p110 δ inhibition with agents targeting either p110 β or p110 α essentially abrogated PI3K signaling and induced dramatic defects in IS architecture. We obtained similar results using T cells that expressed shRNA against both p110 δ and p110 α , arguing that our pharmacological data were not artifactual. By contrast, inhibition of p110 γ had little to no effect on TCR-induced PI3K activity and IS formation. Together, these data indicate that class IA PI3Ks function in a partially redundant manner to control PIP₃ dependent F-actin dynamics at the IS.

It is worth noting that PIP₃ also organizes F-actin in the leading edge lamellipodium of migrating cells[95–97]. In this context, however, the primary source of PIP₃ is likely to be PI3K γ , which participates in most GPCR-induced responses, including those triggered by chemokines[88]. Chemokine receptor signaling has been found to both potentiate and inhibit TCR responses, depending on the experimental system[98,99]. The use of distinct PI3K isoforms by GPCRs and the TCR could ensure that these receptors do not compete for signaling components even during periods of concurrent stimulation. This would presumably allow them to engage in crosstalk in a variety of situations.

PI3K recruitment and activation

Upon engagement of cognate pMHC, TCRs coalesce into plasma membrane “microclusters” that can be visualized by TIRF microscopy[1,100] (Fig. 1). These structures contain 10–20 receptors each[101], along with a number of activated signaling molecules, including Lck, ZAP-70, and the LAT-SLP76 complex. Microclusters are thought to play an important role in signal propagation by bringing kinases into close apposition with their substrates and thereby enabling them to overcome cellular phosphatase activity. We recently demonstrated that fluorescently labeled p85/p55 subunits colocalize extensively with TCR microclusters[49], suggesting that class IA PI3Ks interact with receptor-proximal signaling components. Consistent with this interpretation, it has been shown that phosphorylated LAT and SLP76 both bind directly to the p85/p55 SH2 domains[102–104]. Importantly, phosphotyrosine recognition also allosterically activates class I PI3Ks[105,106]. Hence, interactions with the LAT-SLP76 complex could serve to both localize these enzymes and to stimulate their catalytic throughput.

Class I PI3Ks are also activated by the small GTPase Ras, which binds to a conserved domain within the p110 subunits[107,108]. Indeed, Ras appears to synergize with phosphotyrosine containing peptides in order to induce full PI3K activation[109]. Loss-of-function mutations targeting the Ras binding domains of p110 α and p110 γ dramatically attenuate PI3K signaling *in vivo*[110,111], validating the importance of this interaction. The Ras family comprises the three closely related isoforms, Hras, Kras, and Nras, each of which acts as a molecular switch cycling between inactive GDP-bound and active GTP-bound states[112]. In T cells, Ras signaling drives TCR-induced proliferative and transcriptional responses through both the PI3K and MAP kinase pathways[113,112]. Its role in

cytoskeletal remodeling, however, is less well understood. Using an shRNA-based approach, we found that depletion of *Nras*, the most highly expressed isoform in T cells, inhibits TCR-induced Rac activation, synaptic F-actin ring formation, and cell spreading[49]. Conversely, expression of a constitutively active mutant of Ras enhanced IS growth in a PI3K dependent manner. These results demonstrated that *Nras* controls synaptic architecture through the PI3K-Rac pathway (Fig. 2), establishing Ras signaling as an important cytoskeletal regulator in T cells.

All Ras proteins are C-terminally prenylated, and these modifications mediate localization to a number of different membrane compartments within the cell[112]. Studies have shown that these distinct pools of Ras are differentially sensitive to TCR and integrin dependent signals. Thus, whereas stimulation of the TCR induces Ras activation only in the Golgi apparatus, simultaneous engagement of the TCR and LFA-1 promotes Ras activation in both the Golgi and the plasma membrane[114]. Remarkably, combined stimulation of the TCR and LFA-1 is also required for sustained, symmetric spreading and F-actin ring formation on stimulatory bilayers[49]. That the criteria for annular synaptic architecture match the requirements for plasma membrane activation of Ras strongly suggests that the two processes are linked. This would make sense given that class IA PI3Ks, the downstream targets of Ras and the key mediators of F-actin ring formation, localize to plasma membrane microclusters[49]. In T cells, Ras activation is mediated by the combined activities of RasGRP1 and SOS[115]. Whereas SOS associates directly with the LAT-SLP76 complex, RasGRP1 is recruited into the TCR signaling cascade by interacting with DAG in the synaptic membrane (Fig. 2). It will be interesting to investigate how the distinct recruitment mechanisms used by these two GEFs affect the spatial pattern of Ras activation as the IS matures.

F-actin depolymerization and bundling

Although much attention has focused on the regulation actin polymerization by Rac, Cdc42, and the molecules that control them, these pathways alone cannot explain the complex F-actin dynamics observed at the IS. Indeed, it is becoming increasingly clear that F-actin severing and bundling factors also make key contributions to synaptic architecture and function. Deciphering how these proteins coordinate with Rho family members and other regulators in the context of TCR signaling represents an exciting area of study.

It has been known for some time that T cell activation is associated with the transient upregulation of cofilin, an actin depolymerization and severing factor[116,117]. Cofilin is constitutively phosphorylated by LIM kinase, a modification that inhibits its activity[118,119]. Engagement of the TCR in concert with costimulatory receptors such as CD28 or CD2 induces dephosphorylation of this site within minutes, which is most likely mediated by the related serine/threonine phosphatases PP1 and PP2A[120]. Dephosphorylated cofilin subsequently accumulates in the pSMAC and dSMAC[121], where it presumably regulates F-actin dynamics. Consistent with this idea, cell-permeable peptides that block the interaction between cofilin and actin perturb IS organization and also inhibit activation-induced cytokine secretion by CD4⁺ T cells[121]. In addition, a dominant negative cofilin construct was observed to block B cell spreading on antigen-coated

surfaces[122]. Although these results clearly imply a role for cofilin in IS assembly, resolving precisely how the protein functions in this context is complicated by the fact that severing and depolymerization can exert myriad effects on F-actin networks that depend on prevailing conditions in the cellular neighborhood. For example, if the local concentration of free monomeric actin is high, severing could actually induce a net increase in F-actin by generating additional barbed ends capable of seeding new filament growth. High-resolution live imaging experiments that better correlate cofilin activity with F-actin dynamics should begin to address this issue.

It is noteworthy that TCR engagement alone fails to induce cofilin dephosphorylation. Rather, costimulatory receptors like CD28 and CD2 appear to play the dominant role[116,117]. Biochemical studies in this area indicate that these molecules induce cofilin activation through Ras and PI3K[123]. CD28-induced dephosphorylation of cofilin is blocked by small molecule PI3K inhibitors and by overexpression of dominant negative Ras. Conversely, cofilin dephosphorylation is enhanced by a constitutively active Ras mutant. The parallels between this regulatory pathway and the mechanisms controlling Dock2-Rac signaling are striking, and they imply close coordination between actin polymerization and severing at the periphery of the IS.

Research in F-actin disassembly has also focused on coronin1, a conserved actin binding protein that is highly expressed in hematopoietic cells. Coronin1 has been found to antagonize F-actin growth by enhancing cofilin activity and also by directly inhibiting the Arp2/3 complex[124–126]. Point mutations in its coding sequence cause immunodeficiencies in humans that are characterized by susceptibility to viral infections[127–129]. Mice lacking coronin1 display dramatic defects in T cell persistence and trafficking, despite apparently normal thymic development[130]. These phenotypes were initially attributed increased F-actin levels, which were proposed to dysregulate cell migration and induce apoptosis via the mitochondrial pathway[130]. Other studies of coronin1^{-/-} T cells, however, failed to document a substantive defect in cytoskeletal dynamics, and instead identified an alternative function for the protein in facilitating TCR-induced PLC γ activation and Ca²⁺ influx[131,132]. This led to the alternative hypothesis that coronin1 dependent signal transduction (rather than actin dynamics) is required for peripheral T cell survival, and that the previously observed migration and trafficking defects resulted secondarily from rapid cell death[131,132]. Another study identified additional signaling phenotypes in coronin1^{-/-} T cells, including reduced NF- κ B signaling[133]. However, this work also documented enhanced F-actin accumulation and reduced dynamics at the IS, consistent with a role for coronin1-mediated F-actin disassembly in synaptic structure and function. Detailed mechanistic experiments that focus on the various functional domains of coronin1 will be required to resolve these apparently contradictory findings. The observed signaling defects in coronin1^{-/-} T cells could be secondary to cytoskeletal dynamics, or vice versa. It is also possible, however, that the contribution of coronin1 to downstream signaling is entirely separable from its role in shaping cortical F-actin.

Recent work has implicated the actin bundling protein L-plastin in IS formation and stability[135,136]. L-plastin contains four actin-binding calponin homology domains, which

crosslink F-actin into parallel arrays[137]. T cells lacking L-plastin exhibit reduced antigen-induced proliferation and cytokine production. Proximal TCR signaling in these cells is normal, however, implying a defect further downstream in the activation process[135,136]. Imaging studies have revealed that L-plastin deficiency impairs the formation and persistence of the IS. L-plastin^{-/-} T cells spread poorly on surfaces containing anti-CD3 antibodies and form smaller contacts with APCs than their wild type counterparts[136]. L-plastin colocalizes with LFA-1 in the pSMAC, and time resolved experiments suggest that, in the absence of L-plastin, LFA-1 is not maintained properly at the IS[135]. These data are consistent with the idea that L-plastin functions to stabilize integrin-mediated contacts, and that this stabilization is required for the maturation of TCR signals. Precisely how L-plastin controls integrin localization in this context is not well understood. Although L-plastin is known to interact with the cytoplasmic tail of $\beta 1$ integrins[138], a direct interaction with $\beta 2$ integrins like LFA-1 has not been documented, as yet. It is also unclear how L-plastin is regulated by upstream signals from the TCR and costimulatory receptors. The protein is phosphorylated at Ser5, which has been reported to modulate its localization and enhance F-actin binding[139]. However, Ser5 appears to be dispensable for L-plastin function at the IS, implying an alternative mechanism of regulation[135]. L-plastin also contains an N-terminal calmodulin binding domain that couples its activity to Ca^{2+} signaling. This domain was found to be required for L-plastin localization to the pSMAC, and pharmacological inhibition of calmodulin impaired LFA-1 accumulation at the IS, similar to the effect of L-plastin deficiency[135]. These results are particularly intriguing because they suggest a possible mechanism for how sustained Ca^{2+} signaling could promote persistent integrin mediated adhesion to the APC.

In many lamellipodial structures, nonmuscle myosin II (NMII) bundles F-actin at the lamellipodium-lamella boundary into arc-like structures oriented parallel to the leading edge. This is thought to contribute to both actin turnover and retrograde flow[140,141]. High-resolution imaging experiments have demonstrated that NMII also drives contractile arc formation at the IS[5]. Precisely how these F-actin arcs contribute to IS structure and function remains unclear and is quite controversial. Two studies have documented defects in TCR microcluster movement after pharmacological inhibition of NMII activity or suppression of NMII protein[142,5]. In one case, this trafficking phenotype was also associated with dramatic inhibition of receptor-proximal tyrosine phosphorylation and Ca^{2+} flux, suggesting that NMII-dependent control of synaptic F-actin is crucial for early TCR signaling[142]. Signaling defects of this kind were not observed in other studies, however[143,144,5]. Indeed, even the microcluster trafficking phenotype has been difficult to reproduce[143]. The possible reason(s) for these discrepancies will not be discussed here at great length, as they have been explored extensively in a recent review[145]. It is worth noting, however, that NMII operates in numerous intracellular locales, both within the IS and also outside of it. NMII at the sides and back of the T cell, for instance, is crucial for cell migration and also contributes to centrosome reorientation toward the APC [144,146] (see *Crosstalk between actin remodeling and microtubule network*). It is important to keep these and other NMII-dependent processes in mind, particularly when interpreting loss-of-function phenotypes resulting from global inhibition of this motor.

F-actin dynamics and the microtubule cytoskeleton

IS formation is accompanied by dramatic remodeling of the T cell microtubule cytoskeleton. Within minutes of antigen recognition, the centrosome (also known as the microtubule-organizing center, or MTOC) moves to a position just beneath the center of the IS[147]. Microtubules radiate from centrosome with their minus ends in and plus ends out. Hence, reorienting the centrosome in this manner sets up an axis of polarity within the T cell in which all microtubules point toward the center of the IS. A number of vesicular organelles, including the Golgi apparatus, the endosomal compartment, and secretory lysosomes, cluster around the centrosome in close apposition to the synaptic membrane. Because of this, it is generally thought that centrosome reorientation potentiates the directional secretion of cytokines and cytolytic factors toward the APC (see *Functional implications of the IS*) [148,10].

Synaptic F-actin ring formation occurs just prior to centrosome docking at the center of the IS[8]. This striking temporal correlation suggests that the two events may be causally linked. A number of studies, however, argue that actin dynamics and centrosome reorientation operate independently. Using specialized target cells that expressed either integrin ligands or pMHC alone, Burkhardt and colleagues demonstrated that robust F-actin accumulation at the T cell-target cell interface could be uncoupled from the position of the centrosome [149]. Subsequently, it was found that depletion of Arp2/3 or Dock2, which are both required for robust F-actin ring formation at the IS, has no effect on centrosome reorientation[26,49]. Hence, it remains unclear whether actin dynamics plays a direct role in the process, and if so, what that role might be. In the following sections, we will briefly discuss what is known about the mechanisms governing centrosome reorientation in T cells (this topic has been covered extensively in recent reviews)[148,147]. We will then discuss a potential role for actin dynamics in more detail.

Signaling pathways controlling centrosome polarization

TCR activation induces centrosome reorientation to the IS, and a number of receptor proximal signaling proteins, including Lck, ZAP-70, LAT, and SLP76, are absolutely required for the process[150,151]. Using the localized photoactivation system described earlier in this review (see *Tools for Imaging the IS*), we discovered a DAG dependent signaling pathway that couples early TCR signaling to centrosome dynamics[152,153] (Fig. 2). DAG, which is generated by PLC γ in the LAT-SLP76 complex, forms a sustained intracellular gradient that is centered at the IS[153]. This gradient functions by recruiting three members of the novel protein kinase C (nPKC) subfamily: PKC ϵ , PKC η , and PKC θ [152]. All three proteins contain a conserved tandem C1 domain module that mediates recruitment to membranes containing DAG. PKC ϵ and PKC η arrive at the IS first, ~15 seconds before the centrosome, and occupy the entire synaptic membrane. PKC θ is then recruited (5–10 seconds before the centrosome) to a more confined zone that falls within the F-actin ring. Depletion experiments have implicated all three nPKCs in the centrosome reorientation pathway; PKC θ appears to play a unique role, while PKC ϵ and PKC η operate in a redundant manner upstream of PKC θ . The precise function of each isoform awaits the identification of relevant substrates in this context, most of which remain unknown. Recent

work has also implicated PKC isozymes of the atypical subfamily (aPKCs) in the centrosome reorientation response[154,155]. These proteins (PKC ζ and PKC ι) lack DAG binding domains, and are regulated instead by protein-protein interactions. In adherent cell types such as fibroblasts and epithelial cells, aPKCs function as integral components of the Par (for partitioning defective) complex, which plays a central role in the establishment and maintenance of cell polarity over long timescales[156–158]. Components of this complex, including phosphorylated PKC ζ and the scaffolding protein Par-3, accumulate in the IS, and pharmacological inhibition of aPKC activity impairs centrosome reorientation[154,159]. If and how this aPKC pathway combines with DAG signaling during polarization responses is not known. It has been proposed, however, that the DAG-nPKC pathway mediates initial centrosome reorientation, after which the aPKC-Par complex stabilizes the polarized state[148]. Additional studies will be required to test this hypothesis rigorously.

In most cell types, large-scale organelle rearrangements are mediated by molecular motor proteins that move along actin or microtubules. Studies from multiple labs have implicated the minus end-directed microtubule motor dynein in the T cell centrosome reorientation response[160,146,161,162]. It has been known for some time that dynein accumulates in the synaptic membrane within minutes of TCR engagement. Localized in this manner, it would be well positioned to reel in the centrosome toward the IS by pulling on the microtubules that radiate from it. Consistent with this idea, depletion or pharmacological inhibition of dynein was found to inhibit centrosome polarization in a number of experimental systems[146,161,162]. Residual reorientation was generally observed, however, suggesting that other molecular motors might be involved in the process. Indeed, we recently showed that NMII, an actin-based motor, collaborates with dynein to move the centrosome in T cells[146]. Simultaneous suppression or inhibition of both motors elicits a much more dramatic defect in polarization than perturbation of either motor alone, indicative of a partially redundant relationship. Interestingly, NMII and dynein adopt reciprocal localization patterns during the reorientation response. Dynein accumulates at the IS, while NMII forms transient clusters in the cortex behind the advancing centrosome. These results suggest a model whereby dynein “pulls” the centrosome from the front while NMII “pushes” it from behind. nPKCs are crucial for establishing the reciprocal localization of dynein and NMII, and they appear to function at least in part by phosphorylating inhibitory sites within the myosin regulatory light chain (MyoRLC)[146]. This induces the depletion of NMII from the region of TCR stimulation, creating asymmetry in the overall distribution of the motor. How nPKCs regulate dynein accumulation is not known, and it is an area of active investigation.

Crosstalk between actin remodeling and microtubule network

The discovery that NMII, an actin-based motor, contributes to centrosome polarization suggests that actin dynamics may play an important role in the process. Ironically, the relevant actin-based structures in this context appear to be the contractile clusters induced by NMII in the sides and back of the T cell, well away from the IS and its stereotyped F-actin ring. It is not clear how the formation of these cortical clusters might influence centrosome movement. NMII dependent centrosome polarization is, however, abolished by the microtubule depolymerization agent nocodazole[146], suggesting that force generated by

NMII is transduced along microtubules to the centrosome. This result implies that stabilized microtubules capable of transmitting force might be a prerequisite for effective polarization.

In that regard, it is interesting to note that multiple formin family proteins, including DIA1, FMNL1, and INF2, have been implicated T cell centrosome reorientation[163,26]. Formins promote the accumulation of stabilized pool of microtubules containing C-terminally detyrosinated tubulin monomers[164,165]. TCR activation is associated with the formation of detyrosinated microtubules, and perturbations that block this process also inhibit centrosome reorientation[163]. Recent imaging experiments suggest that, in activated T cells, most detyrosinated microtubules orient their plus ends toward the distal pole, opposite the IS[166]. This rearward projecting pool would be ideally positioned to transduce forces from cortical NMII clusters forming in the sides and back of the cell. Formins are perhaps better known for their role in promoting actin polymerization[167]. They possess both actin nucleation and actin bundling activity, and they are particularly strong inducers of unbranched actin structures such as actin cables. Although it is not known if these functions contribute to centrosome polarization, it is tempting to speculate that they might synergize with NMII activity to generate contractile clusters in the cortex.

Another potential link between the centrosome and F-actin dynamics involves the relationship between PKC θ and WASp. In a very intriguing set of experiments, Dustin and colleagues demonstrated that PKC $\theta^{-/-}$ T cells form hyperstable synapses on stimulatory bilayers and maintain these symmetric contacts for extended periods[168,45]. By contrast, WASp $^{-/-}$ T cells form unstable synapses that transition quickly into asymmetric, migratory “kinapses”[45]. Importantly, pharmacological inhibition of PKC θ in WASp deficient cells restores IS stability, suggesting that the two proteins function antagonistically in this context. Although the basis for this antagonism is not well understood, it is worth noting that PKC θ can directly phosphorylate WIP[169,170]. Precisely how this phosphorylation event modulates WIP-WASp function, if at all, remains to be seen. Nevertheless, given the key roles played by nPKCs during centrosome polarization, it will be interesting to investigate whether the regulation of WASp activity is involved in the process.

Although these potential links between F-actin and microtubule dynamics are quite intriguing, we know very little about their molecular bases and functional relevance. This area of study has been complicated by the overall complexity of the F-actin cytoskeleton. There are numerous distinct pools of F-actin within activated T cells, and defining how each of these pools interacts with the TCR signaling network will be a key step toward understanding their roles in shaping cellular architecture.

Functional implications of synaptic actin remodeling

Although initially thought to induce and amplify T cell activation, it is now generally accepted that IS formation is the product of TCR signaling rather than its cause. Indeed, studies from multiple labs suggest that the IS functions as a specifier rather than an intensifier, constraining the TCR signaling network and focusing downstream effector responses onto the APC. Below, we will discuss the purpose of the IS, focusing on the role

of F-actin dynamics in regulating T cell activation, adhesion, and the targeted delivery of effector responses.

Tuning of TCR signaling

Over the past ten years, high resolution imaging studies of signaling components within the IS have dramatically altered our conception of its role in signal initiation and regulation. Within seconds of forming, TCR microclusters begin to move toward the center of the IS, where they coalesce into a larger cluster that will eventually become the cSMAC[171,172]. As the IS matures, new TCR microclusters continuously emerge in the periphery and move inward to become incorporated into the cSMAC. This stereotyped centripetal motion is driven largely by retrograde flow within the F-actin ring. Acute disruption of F-actin with depolymerization agents like latrunculin A rapidly arrests microcluster motility and also abrogates the formation of new microclusters[101]. As mentioned above, certain studies have indicated a role for NMII in microcluster motility, although these results are quite controversial[143,145,142,5]. Dynein has also been implicated in the centralization process, particularly during the final stage in which microclusters move through the F-actin hypodense zone at the center of the contact[173]. This area is rich in microtubules due to the close proximity of the reoriented centrosome, and microclusters have been observed to track along these microtubules to access the cSMAC. Studies indicate that TCR signaling from microclusters is extinguished during centralization[171,174,101]. Whereas peripheral microclusters are enriched in phosphorylated Lck and ZAP-70, these markers are conspicuously absent from the cSMAC[171]. Furthermore, perturbations that impair microcluster centralization markedly enhance TCR signaling responses[173,175]. Hence, TCR signaling is initiated in microclusters and extinguished by the incorporation of these microclusters into the cSMAC. Importantly, this implies that sustained TCR signaling depends on the continued formation of new TCR microclusters in the periphery.

The cSMAC appears to be a focal point for TCR internalization and ubiquitin dependent degradation. shRNA-mediated suppression of components of the endosomal sorting complex required for transport (ESCRT), which is required for receptor downregulation in many cell types, blocks cSMAC formation, TCR degradation, and signal attenuation[174]. cSMAC formation is also inhibited by small molecules targeting the ubiquitination reaction. Furthermore, lysobisphosphatidic acid, a marker for the multi-vesicular bodies that degrade internalized TCR, is highly enriched within the cSMAC[101]. Interestingly, efficient TCR internalization has also been found to require WASp, suggesting an important role for actin polymerization independent of the F-actin ring[39]. In other systems, class I NPFs have been shown to promote vesicle formation by inducing Arp2/3 dependent membrane tubulation[176], and the same process may occur at the cSMAC. Moving forward, it is also worth keeping in mind that receptor internalization does not necessarily mean that all signaling is extinguished. Indeed, recent work from Pierce and colleagues indicates that internalized B cell receptors continue to transduce signals from the endosomal compartment, and that these signals are qualitatively different from those emanating from receptors on the cell surface[177]. It will therefore be interesting to investigate the signaling contribution of downregulated TCR during IS maturation.

Regulation of integrin-mediated adhesion

Synaptic F-actin dynamics and the pathways that control it also play key roles in boosting integrin-mediated adhesion to the APC. Mature synapses are characterized by dramatic LFA-1 accumulation in the pSMAC[178], which is thought to promote APC adhesion by enhancing the avidity of integrin-ligand interactions. The formation of this annular cluster of LFA-1 is driven by retrograde F-actin flow in the dSMAC and NMII activity in the pSMAC-dSMAC boundary[5]. TCR signaling also enhances adhesion on a per molecule basis by altering integrin conformation[179,180]. Activating signals induce the conversion of integrins from a bent configuration with low affinity for ligand into an extended, high affinity state. A key regulator of this maturation process is the small GTPase Rap1, which drives integrin extension downstream of the TCR, GPCRs, and other activating receptors[180]. Billadeau and colleagues have demonstrated that the WAVE2 complex can directly regulate integrin affinity maturation by recruiting C3G, an exchange factor that catalyzes the formation of active, GTP-loaded Rap1[181]. WAVE2 may also contribute to the process by promoting F-actin growth, thereby creating a platform for the recruitment of other integrin activators, such as the scaffolding molecule talin[182]. Consistent with this notion, the Arp2/3 complex, which drives lamellipodial protrusion downstream of WAVE2, is required for integrin-mediated adhesion to activating surfaces and target cells[26]. Notably, WASp appears to be dispensable for this process[44], suggesting that either the composition of the WAVE2 complex or the type of F-actin growth it induces is specifically required for affinity maturation.

The relationship between IS formation and integrin function is likely to be quite complex. As described above (see ***PI3K recruitment and activation***), outside-in LFA-1 signaling is required for robust class IA PI3K activation and F-actin ring formation[49]. It has also been shown that engagement of VLA-4 can extend the life of signaling microclusters at the IS by retarding their centripetal flow[183]. Hence, while IS components induce integrin affinity maturation and clustering, integrins enhance TCR signaling and promote IS formation. This positive feedback relationship is undoubtedly quite important for T cell activation, and deciphering the molecular mechanisms that control it is an interesting goal for future research.

Regulation of cytotoxicity

CTLs play a crucial role in immune responses against intracellular pathogens (e.g. viruses) and cancer by selectively destroying infected or transformed target cells. The killing process is induced by TCR recognition of cognate pMHC on the surface of the target. This is followed by IS formation and then the directional secretion of cytotoxic perforin and granzyme molecules into the synaptic space[10]. These factors induce target cell apoptosis, after which the CTL continues its search for antigen elsewhere. As mentioned above, perforin and granzyme are stored in specialized secretory lysosomes known as lytic granules. TCR activation triggers the Ca²⁺ dependent migration of these granules along microtubules toward the centrosome, which is itself reorienting toward the target cell[184]. This concentrates the granules at the IS, where they undergo fusion with the synaptic membrane. Granule polarization is thought to enhance both the power and the specificity of the lytic response.

Synaptic F-actin dynamics contribute to directional killing in at least two ways. First, the depletion of cortical F-actin from the central IS promotes exocytosis in this region by facilitating lytic granule access to the synaptic membrane[8]. Super-resolution imaging studies in NK cells indicate that F-actin is thinned, rather than completely removed, at the center of the IS, creating hot spots for granule targeting[6,7]. Recent work has implicated coronin1 in this localized F-actin effacement. NK cells lacking coronin1 exhibit enhanced levels of synaptic F-actin, accompanied by defects in degranulation and killing[134]. Interestingly, loss of coronin1 in this context does not appear to cause any defects in activation-induced signaling, unlike what has been observed for T cells. Another recent study has suggested that Cdc42 may also be involved in creating secretory hot spots[46]. Depletion of Cdc42 in CD4⁺ T cells leads to an overabundance of F-actin at the IS and a concomitant defect in cytokine secretion. This effect can be reversed by treatment with low dose latrunculin, suggesting that Cdc42 functions by inducing F-actin disassembly. It will be interesting to see whether this same Cdc42 dependent pathway also potentiates lytic granule release from CTLs.

The second way in which F-actin dynamics contribute to CTL-mediated killing is by controlling the overall efficiency of cytotoxic interactions. This role became apparent during our studies of PI3K-Dock2 signaling and its effects on CTL function[49]. In the absence of Dock2, CTLs form small, structurally fragile synapses and display markedly impaired killing responses. By contrast, CTLs lacking PTEN exhibit enlarged, robust-looking synapses and are almost five times more effective than controls at destroying target cells. Importantly, depletion of either Dock2 or PTEN has no effect on TCR-induced degranulation, indicating that these phenotypes do not result from changes in lytic granule secretion. Rather, the data suggest that synaptic architecture, in particular the size and strength of the F-actin ring, plays an important role in modulating the lytic power of each individual degranulation event. Precisely how synaptic F-actin structure might function in this capacity is not clear. It is tempting to speculate, however, that the IS might orient or distort the target cell in such a way as to potentiate the effects of cytolytic secretion. Recent studies in B cells have highlighted the importance of mechano-transduction for synapse function[185]. It will be interesting to see if analogous features contribute to cytotoxic killing.

Concluding remarks

The speed and structural complexity of synaptic cytoskeletal dynamics continue to present challenging technical hurdles to experimentalists, and as a result there is still much that we do not understand about the process. How integrin signaling contributes to synaptic architecture, for example, remains mysterious, and the same can be said for the mechanisms controlling selective actin depolymerization at sites of secretion. Furthermore, we have barely scratched the surface with regard to how cytoskeletal dynamics potentiates and focuses T cell function. Nevertheless, some important mechanistic themes have emerged in recent years. First, lipid second messengers appear to play a central role in specifying the synaptic cytoskeleton, with PIP₃ shaping F-actin and DAG controlling centrosome reorientation. Signaling lipids are well suited for quickly building transient, polarized interfaces like the IS, and it is likely that they are used by other leukocytes to construct

analogous cellular structures. Second, feedback relationships within and between signaling pathways are required both to amplify polarized responses and to create well-defined architectural domains on the cell surface. The complex interactions between TCR and integrin signaling are an excellent example of this crosstalk behavior. In coming years, the continued development of higher resolution imaging modalities and the incorporation of improved tools for perturbation, such as CRISPR/Cas9 technology [186], will enable researchers to dissect the complex mechanisms governing synaptic cytoskeletal remodeling more effectively. We anticipate that these efforts will lead, in turn, to the identification of molecular strategies for assessing the functional importance of these structural transformations in more complex settings.

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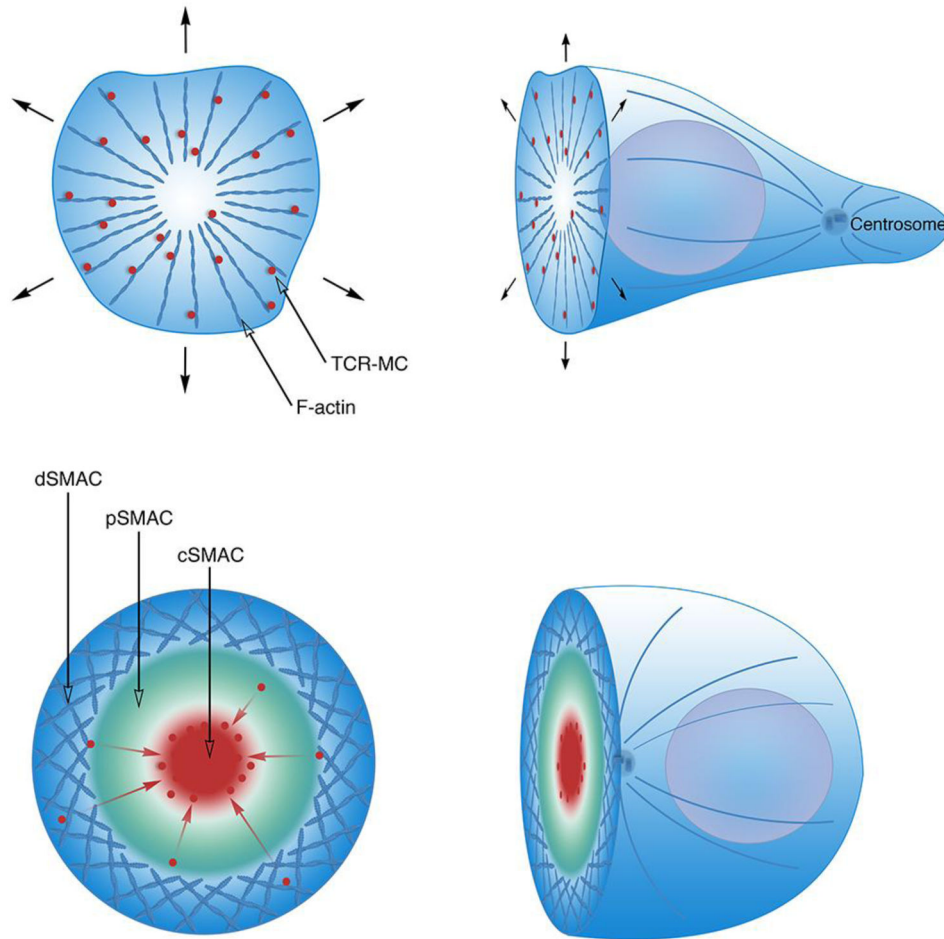


Figure 1. Cytoskeletal remodeling and the immunological synapse

Schematic diagram showing IS initiation (top) and maturation (bottom) both from the side (right) and from the perspective of the APC (left). Radially symmetric spreading over the surface of the APC is driven by protrusive actin polymerization, accompanied by the formation of TCR microclusters (TCR-MC, red dots) in the plasma membrane. As the IS matures, retrograde flow of F-actin drives TCR-MCs into the cSMAC, while F-actin is reorganized into a peripheral ring known as the dSMAC. Integrins, for their part, cluster in the pSMAC. Concomitantly, the centrosome reorients to a position just beneath the center of the IS.

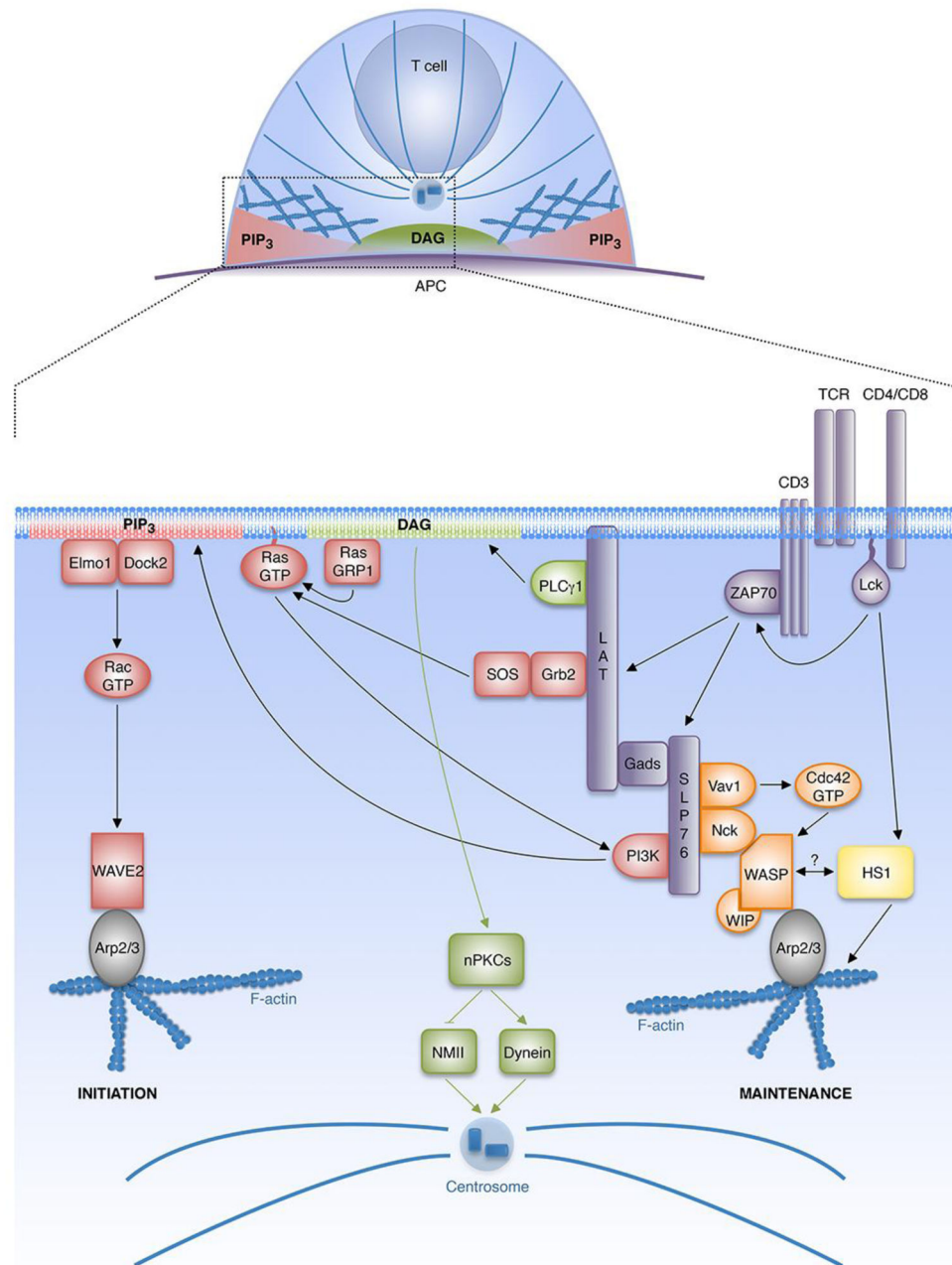


Figure 2. Signaling pathways coupling TCR activation to the cytoskeleton

TCR engagement triggers activation of the tyrosine kinases Lck and ZAP-70, which in turn leads to assembly of the LAT-SLP76 signalosome. This complex allows the induction of different signaling pathways leading to the activation of the NPFs WASp, HS1, and WAVE2, which induce local actin polymerization through the Arp2/3 complex. While WAVE2 appears to be critical for the initiation of synaptic actin polymerization, WASp and HS1 might collaborate for the stabilization and the maintenance of the IS. Class IA PI3Ks bind to LAT and SLP76 in a pTyr-dependent manner, and are then activated in a Ras-

dependent fashion. SOS, in complex with the adaptor Grb2, and RasGRP1 are two GEFs for Ras that operate downstream of the TCR. PI3K-mediated PIP₃ production allows the redistribution of the GEF Dock2 (in complex with Elmo1) to the periphery of the IS, where it drives F-actin remodeling through Rac and WAVE2. Meanwhile, WASp associates with the LAT-SLP76 complex via interaction with Nck and is activated by Cdc42. HS1 is thought to participate in actin dynamics by stabilizing branched-actin filaments generated by Arp2/3 complex activators. TCR engagement also drives centrosome reorientation towards the IS. PLC γ 1 is recruited via its interaction with LAT and generates DAG in the synaptic membrane. The DAG gradient induces the recruitment of nPKCs, which mediate centrosome polarization through the regulation of the motor proteins dynein and NMII.