

Modulation of T cell signaling by the actin cytoskeleton

Yan Yu^{1,*}, Alexander A. Smoligovets^{2,3,4} and Jay T. Groves^{2,3,5,*}

¹Department of Chemistry, Indiana University, Bloomington, IN 47405-7102, USA

²Howard Hughes Medical Institute, Department of Chemistry, University of California, Berkeley, CA 94720, USA

³Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA

⁴Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA

⁵Mechanobiology Institute, National University of Singapore, 117411, Singapore

*Authors for correspondence (yy33@indiana.edu; jtgroves@lbl.gov)

Journal of Cell Science 126, 1049–1058

© 2013. Published by The Company of Biologists Ltd

doi: 10.1242/jcs.098210

Summary

The actin cytoskeleton provides a dynamic framework to support membrane organization and cellular signaling events. The importance of actin in T cell function has long been recognized to go well beyond the maintenance of cell morphology and transport of proteins. Over the past several years, our understanding of actin in T cell activation has expanded tremendously, in part owing to the development of methods and techniques to probe the complex interplay between actin and T cell signaling. On the one hand, biochemical methods have led to the identification of many key cytoskeleton regulators and new signaling pathways, whereas, on the other, the combination of advanced imaging techniques and physical characterization tools has allowed the spatiotemporal investigation of actin in T cell signaling. All those studies have made a profound impact on our understanding of the actin cytoskeleton in T cell activation. Many previous reviews have focused on the biochemical aspects of the actin cytoskeleton. However, here we will summarize recent studies from a biophysical perspective to explain the mechanistic role of actin in modulating T cell activation. We will discuss how actin modulates T cell activation on multiple time and length scales. Specifically, we will reveal the distinct roles of the actin filaments in facilitating TCR triggering, orchestrating ‘signalosome’ assembly and transport, and establishing protein spatial organization in the immunological synapse.

Key words: T cell activation, T cell receptor, Actin, Antigen-presenting cell, Microcluster, Immunological synapse, Membrane spatial organization

Introduction

T cells have a central role in adaptive immunity, and their activation involves many spatially and temporally coordinated signaling processes on multiple time and length scales. There are three distinct stages during the activation process: T cell receptor (TCR) triggering, signal persistence and signal termination. A crawling T cell constantly scans the surfaces of antigen-presenting cells (APCs) in search of antigen peptides that are bound to major histocompatibility complex proteins (pMHCS). Upon recognition of such peptides, ligation of integrin molecules facilitates the initial close cell–cell contact, and engagement between TCRs and agonist pMHCS subsequently triggers the initial TCR signaling across the plasma membrane. TCRs and their co-receptors, other accessory proteins, the microtubule organization center (MTOC) and secretory organelles all reorient to a position near the cell–cell contact site. At the same time, the T cell membrane protrudes over the APC surface, leading to the formation of a specialized membrane junction, namely the immunological synapse (Fig. 1A). Here, we focus our discussion on the canonical pattern of concentric protein domains, but it is important to note that a variety of protein patterns exist for different subsets of T cells as well as in other immune cells (Davis and Dustin, 2004). A hallmark of TCR triggering is the rapid increase in the concentration of intracellular calcium ions (Lewis, 2001). Following TCR triggering, signaling is amplified and sustained in microclusters (Seminario and Bunnell, 2008; Choudhuri and Dustin, 2010; Dustin and Groves, 2012). TCRs in the cell periphery rapidly assemble into microclusters consisting of tens

to hundreds of molecules. Concurrently, other signaling factors (Box 1) are recruited to the macromolecular signaling complex to propagate downstream signaling events, including kinases such as ζ -chain-associated tyrosine-protein kinase 70 (ZAP70) (Wang et al., 2010; Sherman et al., 2011) and the lymphocyte-specific tyrosine-protein kinase Lck (Nika et al., 2010), adaptor proteins such as the linker for activation of T cells family member 1 (Lat) (Lillemeier et al., 2010) and the SH2-domain-containing leukocyte protein of 76 kDa [SLP-76 (also known as lymphocyte cytosolic protein 2, LCP2)] (Barda-Saad et al., 2010), as well as actin-polymerizing factors such as Wiskott–Aldrich syndrome protein (WASp) (Gomez and Billadeau, 2008; Reicher and Barda-Saad, 2010). These signaling microclusters undergo continuous translocation and become spatially organized in the synapse to form a ‘bull’s eye’ pattern of several distinct concentric domains, which are known as supramolecular activation clusters (SMACs; Fig. 1B) (Monks et al., 1998; Grakoui et al., 1999). TCR microclusters are transported such that they accumulate in the central SMAC (cSMAC). Distinct from the newly formed microclusters in the cell periphery, these centralized microclusters are mostly associated with receptor internalization and signaling degradation (Mossman et al., 2005; Varma et al., 2006), thus making the term ‘supramolecular activation cluster’ a misnomer. Integrins, such as lymphocyte function-associated antigen 1 (LFA-1), together with many cytoskeletal linker proteins, reorganize to form a ring structure surrounding the cSMAC, forming the peripheral SMAC (pSMAC). The distal SMAC (dSMAC) is enriched with proteins with large extracellular domains, including the protein-tyrosine phosphatase

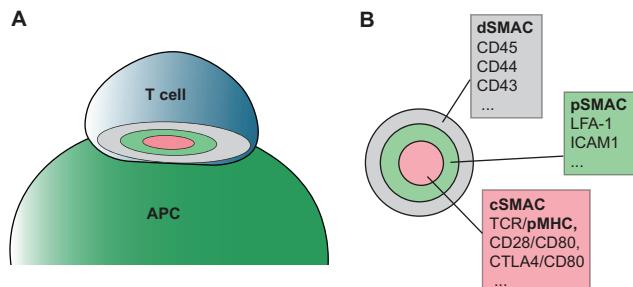


Fig. 1. Schematic diagrams of the immunological synapse at the junction between a T cell and an antigen-presenting cell (APC). (A) T cell activation leads to large-scale protein segregation in the immunological synapse, which is a specialized cell–cell junction. (B) Proteins are translocated and differentially sorted in the immunological synapse, forming a ‘bull’s eye’ pattern of several distinct concentric domains, which are known as supramolecular activation clusters (SMACs). The central SMAC (cSMAC; pink) is enriched with TCR microclusters, co-stimulatory receptors and protein-tyrosine kinases. Peripheral SMAC (pSMAC; green) contains integrins, such as lymphocyte function-associated antigen 1 (LFA-1) that binds to intercellular adhesion molecule 1 (ICAM1), and many cytoskeletal linker proteins (such as talin). Proteins with large extracellular domains, including protein-tyrosine phosphatase CD45 and glycoproteins CD44 and CD43, accumulate in the distal SMAC (dSMAC; grey). Other than the canonical form of protein patterns illustrated here, a variety of immunological synapse patterns exist for different subsets of T cells as well as in other immune cells (Davis and Dustin, 2004).

CD45 (also known as receptor-type tyrosine-protein phosphatase C). All signaling events must be coordinated in time and space to achieve accurate T cell activation, and each of these activities is dependent on the actin cytoskeleton.

The actin cytoskeleton is a filamentous network known to provide mechanical forces for cell polarization and motility, to scaffold proteins for binding and assembly and also to transport molecules. Its importance in T cell activation has long been established, ever since the first studies of the actin cytoskeleton in T cell activation (Geiger et al., 1982; Ryser et al., 1982). However, the nature of the mechanisms linking actin with T cell activation has only recently begun to be unveiled (Fig. 2). Actin drives the process of cell polarization and maintains the cell–cell contact – the very first steps for T cell activation; it also likely provides a scaffold for clustering, translocation and spatial segregation of proteins, key steps to amplify and sustain T cell signaling (Bardia-Saad et al., 2005; Billadeau et al., 2007; Dustin, 2007; Gomez and Billadeau, 2008; Beemiller and Krummel, 2010). It has been proposed that mechanical forces from actin might be required in TCR triggering, although the exact mechanism underlying this is still debated (van der Merwe and Dushek, 2011). In addition, studies also suggest that T cell activation requires actin retrograde flow, instead of static filaments (Kaizuka et al., 2007; Yu et al., 2010; Babich et al., 2012). An emerging picture is that the actin cytoskeleton provides a dynamic framework for the spatial and temporal regulation of both mechanical and biochemical signaling pathways in T cell activation. Here, we discuss, with an emphasis on the mechanistic aspects, how the actin cytoskeleton regulates T cell activation during TCR triggering, assembly and translocation of signaling proteins and the formation of the immunological synapse.

The role of actin in T cell receptor triggering

TCR triggering is the process by which TCR–pMHC ligation leads to intracellular signaling. It is not only the initial step of T cell

Box 1. Key proteins involved in T cell signaling

TCR (T cell receptor) is a cell-surface heterodimer that recognizes antigen peptides bound to major histocompatibility complex proteins (pMHCS) on antigen-presenting cells (APCs). TCR heterodimers associate constitutively with multiple CD3 proteins [γ , δ , ε and ζ] in the T cell membrane for signal transduction. The term ‘TCR’ is sometimes applied to this larger complex. All CD3 subunits in the complex contain immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic domains, whose phosphorylation leads to signaling downstream of TCR triggering (for details, see van der Merwe and Dushek, 2011).

Lck (lymphocyte-specific tyrosine-protein kinase) is a membrane-tethered kinase that phosphorylates tyrosine residues in the ITAMs in the TCR–CD3 complex. Doubly phosphorylated ITAMs are the docking sites for ZAP70 and other TCR signaling-associated proteins. Lck is often associated with the CD4 or CD8 co-receptors, which might potentiate its activity by bringing it into the proximity of the CD3 chains (for details, see Davis and van der Merwe, 2011).

ZAP70 (ζ -chain-associated protein of 70 kDa) is a tyrosine-protein kinase from the Syk family that docks at phosphorylated ITAMs of the TCR. Docking, subsequent phosphorylation by Lck and trans-autophosphorylation all increase kinase activity. ZAP70 phosphorylates a number of signaling proteins, including LAT and SLP-76 (for details, see Wang et al., 2010).

LAT (linker for activation of T cells) is a scaffold protein that assembles key effectors of T cell activation, such as SLP-76, PLC γ 1, Grb2 and others. After its phosphorylation by ZAP70 and Lck, LAT recruits many other signaling proteins to form protein microclusters that are distinct from TCR microclusters (for details, see Balagopalan et al., 2010).

SLP-76 (SH2 domain-containing leukocyte protein of 76 kDa, also known as LCP2), an adaptor that mediates interactions between a host of proteins, including LAT and the actin-regulatory proteins Nck and Vav1. SLP-76 is activated through phosphorylation by Lck and ZAP70 as well as through TCR-independent pathways (for details, see Jordan and Koretzky, 2010).

Myosin is a large family of actin-associated, ATP-powered motor proteins, of which non-muscle myosin IIA is one predominant isoform in T cells. Myosin IIA generates tension in the actin cytoskeleton by crosslinking filaments and sliding them with respect to one another (for details, see Vicente-Manzanares et al., 2009).

activation but is also extremely sensitive and specific – signaling can be triggered by extremely low densities of activating agonist peptides in the presence of high levels of self-pMHCS (stochastic noise); T cells must also differentiate agonists of various potency. The TCR complex consists of a TCR heterodimer (TCR $\alpha\beta$) that is responsible for ligand recognition and multiple CD3 molecules (CD3 γ , CD3 δ , CD3 ϵ and CD3 ζ) that mediate all proximal signaling events. TCR engagement with the antigen peptide-MHC protein complexes triggers tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs), which are present in the cytoplasmic domains of all CD3 subunits in the TCR complex (van der Merwe and Dushek, 2011). Three main models have been proposed so far to understand how TCR engagement leads to biochemical signaling across the plasma membrane: conformational change, kinetic segregation and receptor aggregation. We discuss below the possible roles of actin in each mechanism.

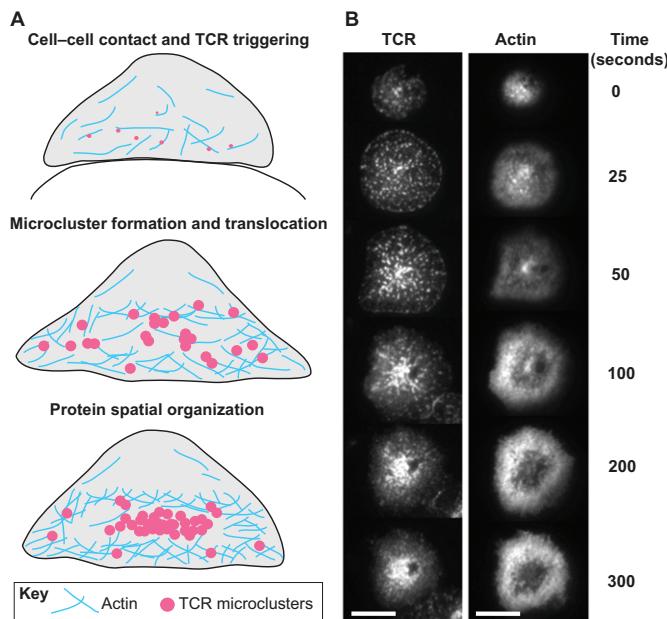


Fig. 2. A mechanistic overview of actin in T cell activation. (A) After T cell triggering, the actin cytoskeleton promotes cell polarization, maintains cell–cell contact and facilitates TCR signaling across the plasma membrane. TCR signaling is amplified and sustained in distinct microclusters (also known as ‘signalosomes’). Actin acts as a scaffold for the clustering of proteins, drives their centripetal translocation and spatially organizes the microclusters to different domains to form the immunological synapse. In a mature immunological synapse, actin is depleted from the central area where TCR microclusters accumulate. (B) Transport of TCR microclusters and actin retrograde flow are temporally coordinated during T cell activation. In a T cell triggered by a stimulatory supported lipid bilayer, the TCRs (here labeled with anti-TCR Fab conjugated to Alexa Fluor 594) and actin (labeled with EGFP conjugated to actin-binding UtrCH) are imaged simultaneously by using total internal reflection fluorescence microscopy (TIRFM). Scale bars: 5 µm. Images in panel B were adapted, with permission, from images published previously (Yu et al., 2012).

Actin in the conformational change model

The conformational change model explains T cell triggering at the level of the single TCR. The central hypothesis of this model is that TCR–pMHC binding alone, without contribution from other forces, exerts a pulling force on the TCR, leading to conformational changes in the CD3 subunits and thus downstream biochemical signaling (Sun et al., 2001; van der Merwe, 2001; Beddoe et al., 2009). As a result, the proline-rich sequence in the CD3 ϵ cytoplasmic tail becomes accessible to interact with a cytoskeletal adaptor protein, the non-catalytic region of the tyrosine kinase cytoplasmic protein Nck1 (Gil et al., 2002). These observations serve as indirect evidence to support the involvement of actin in the conformational change model. A more significant role for actin was proposed in a modified version, called the receptor deformation model (Ma et al., 2008; Ma and Finkel, 2010). In this model, TCR–pMHC binding itself does not initiate TCR signaling. Instead, as the T cell moves in an actin-dependent manner with respect to its attached APC, it generates tension across its membrane. The effect of this tension is a pulling force that is transmitted to the TCR, thereby inducing a conformational change in the TCR–CD3 complex and making the cytoplasmic ITAMs more accessible to the tyrosine-protein kinase Lck.

As the structure of an intact TCR–CD3 complex is still unsolved, there have been limited structural data to prove the conformational changes directly. But considerable evidence pinpoints mechanical effects from the actin cytoskeleton as an important factor in TCR triggering. First of all, actin might be essential to ensure the specificity and sensitivity of T cell triggering, by enabling the rapid association–dissociation of TCR–pMHC binding. The pulling force from actin proposed in the receptor deformation model is expected to cause an increase in the dissociation of TCR–pMHC, and this has been demonstrated experimentally in recent studies. Huppa and colleagues used single-molecule fluorescence resonance energy transfer (FRET; Box 2) to measure the TCR–pMHC binding

Box 2. Interdisciplinary physical techniques for studying actin in T cell activation

Glass-supported lipid bilayer is a planar lipid bilayer structure formed by self-assembly of lipids on pre-cleaned glass substrates. Proteins can be tethered to the lipid bilayer by various types of linkages, including streptavidin–biotin binding, polyhistidine nickel-chelating lipid linkage or glycosylphosphatidylinositol (GPI) linker. Glass-supported lipid bilayers have proven useful for the microscopic study of the spatial organization of proteins in cell–cell junctions (for details, see Groves and Dustin, 2003).

Total internal reflection fluorescence microscopy (TIRFM) is an optical technique to achieve single-molecule fluorescence imaging at interfaces. When an excitation laser is totally reflected at the interface between glass and cell media, only fluorescent molecules located within ~200 nm of the interface are illuminated. TIRFM is a powerful technique to visualize single molecules in or near cell membranes (for details, see Axelrod, 2003).

Super-resolution fluorescence imaging is a type of optical microscopy to observe single-molecule fluorescence below the optical diffraction limit. Several different types of super-resolution imaging techniques have been developed, such as photoactivation localization microscopy (PALM), stochastic optical reconstruction microscopy (STORM) and stimulated emission depletion (STED) microscopy. Super-resolution microscopes can be employed to resolve molecular structures that are indistinguishable by conventional fluorescence microscopes owing to the diffraction limit, such as protein nanoclusters and actin structures (for details, see Huang et al., 2009; Illingworth and van der Merwe, 2012).

Fluorescence speckle microscopy is a technique to analyze the dynamics and assembly of macromolecular structures such as actin filaments. When a small fraction of molecules within a macromolecular structure are fluorescently labeled, their fluorescence appears as distinct puncta, also called speckles. Movements of those speckles reveal the motion and turnover of the macromolecular structure they are located in (for details, see Danuser and Waterman-Storer, 2006).

Fluorescence (or Förster) resonance energy transfer (FRET) is an optical technique to quantify, for example, molecular interactions, protein structural dynamics and protein binding kinetics by measuring the distance between two fluorophores. When energy is non-radically transferred from a donor fluorophore to a receptor, the energy transfer efficiency is dependent on their distance. FRET has been employed to study TCR–pMHC binding kinetics (Huppa et al., 2010), protein–protein interactions (Zal and Gascoigne, 2004) and tyrosine phosphorylation (Randriamampita et al., 2008) in T cell activation (for details, see Wouters et al., 2001).

kinetics between TCRs in the T cell membrane and pMHCs on glass-supported lipid bilayers (Box 2) (Huppa et al., 2010). The dissociation rate (k_{off}) at the membrane junction (two-dimensional) was faster than that obtained from measurements in solution (three-dimensional) – a key feature that is required for T cells to sample rapidly a small number of cognate pMHCs among a high level self-pMHCs on an APC. Interestingly, k_{off} decreased significantly when actin filaments were depolymerized. A similar effect of the actin cytoskeleton on TCR–pMHC binding was also independently observed by Huang and colleagues, who used a micropipette and a biomembrane force probe to measure the binding kinetics between a T cell and an antigen-presenting red blood cell (Huang et al., 2010). Both studies demonstrate that actin is crucial to ensure rapid TCR–pMHC binding and possibly for the high specificity and sensitivity of T cell activation. Second, the model postulates that the mechanical force generated by actin must be transmitted to the TCR–CD3 complex by means of TCRs. This is structurally plausible as the part of the TCR β chain is in close proximity to the relatively rigid CD3 ϵ ectodomain (Ghendler et al., 1998; Kim et al., 2010). More importantly, mechanical forces applied on the TCR heterodimer can trigger T cell signaling (Kim et al., 2009; Li et al., 2010; Husson et al., 2011). Additional evidence to support the necessity of actin-generated force for TCR triggering is the observation that monomeric agonist pMHC can trigger T cell signaling when anchored on the cell surface, but the same agonist in solution cannot.

The conformational change model is particularly appealing to explain the possible role of actin in the high sensitivity and specificity of T cell triggering. By regulating the TCR–pMHC binding strength, actin enables T cells to detect signals rapidly at extremely low densities and differentiate agonists of different potency. This mechanism is supported by compelling evidence, but thus far no structural data have been obtained to demonstrate structural changes directly in the intact TCR–CD3 complex upon the application of physical forces or upon binding of agonist pMHC. The conformational change concept is also challenged by some recent studies (Fernandes et al., 2012; James and Vale, 2012) and, furthermore, it is unclear how the pulling force is transmitted from the TCR to the CD3 cytoplasmic domains and whether actin has any role in that step.

Actin in the kinetic segregation model

In addition to its role in directly influencing single TCR–pMHC complexes, actin might also have a role in TCR triggering at the level of TCR clusters. The kinetic segregation model and receptor aggregation model (discussed below) are better suited to describe the possible underlying mechanisms. Proposed in 1996, the kinetic segregation model postulates that TCR triggering is caused by size-dependent segregation of kinases from phosphatases present at the membrane junction between T cell and APC (Davis and van der Merwe, 1996; Davis and van der Merwe, 2006). Membrane receptors with small extracellular domains, including the TCR–pMHC pair and kinases, accumulate in many small ‘close-contact zones’ (~15 nm apart), from which large glycoproteins such as the inhibitory tyrosine phosphatase CD45 are excluded owing to steric effects. In resting T cells, the ITAMs have a low phosphorylation level because they are constantly phosphorylated by tyrosine kinase Lck and dephosphorylated by phosphatase CD45. However, the size-dependent exclusion of CD45 shifts the kinase–phosphatase balance, leading to prolonged phosphorylation of the ITAMs and

thus TCR triggering (Choudhuri et al., 2009). This mechanism is supported by mounting evidence. For example, exclusion of phosphatases from areas of TCR triggering has been confirmed experimentally (Leupin et al., 2000; Lin and Weiss, 2003; Varma et al., 2006). Moreover, T cell signaling is reduced when protein segregation is disrupted by truncation of the extracellular domains of CD45 and CD148 (Irles et al., 2003; Lin and Weiss, 2003) or elongation of the ectodomain of agonist pMHCs (Choudhuri et al., 2005; Choudhuri et al., 2009).

Actin probably has important roles in many aspects of the kinetic segregation model. First of all, actin is required for forming and maintaining the ‘close-contact zones’ (Valitutti et al., 1995a; Bunnell et al., 2001; Cannon and Burkhardt, 2002), the very first step of TCR signaling. Second, actin and actin-based molecular motors are coupled to the plasma membrane and regulate membrane tension (Nambiar et al., 2009; Skruzny et al., 2012), a potential facilitator of protein segregation in the ‘close-contact zones’ during TCR triggering. A recent study by James and Vale suggests that protein segregation is not solely driven by the extracellular sizes of cell-surface molecules but also by a certain membrane force (James and Vale, 2012). Intriguingly, by depolymerizing actin with pharmacological agents, they observed a negligible role for actin in protein segregation in the small population of cells that did form cell–cell contacts out of the large population of cells that did not. The observation is surprising and might be attributable to the heterogeneity of cell response to pharmacological treatment. Finally, as the kinetic segregation model is also applicable to the triggering of other membrane receptors such as the co-stimulatory molecules (Davis and van der Merwe, 2006), actin might further modulate TCR triggering by transducing co-stimulatory signals to lower the threshold number of agonist pMHCs required for activation (Bachmann et al., 1997; Suzuki et al., 2007).

Actin could also modulate TCR triggering at the cluster level by promoting formation of lipid rafts. Lipid rafts are defined as nanometer-sized membrane domains that are enriched in cholesterol, sphingolipids and signaling proteins (Lingwood and Simons, 2010). In this model, it is hypothesized that the lipid rafts, formed upon the TCR–pMHC interactions, trigger TCR signaling by facilitating the association of the TCR–CD3 complexes with tyrosine kinases while excluding inhibitory phosphatases such as CD45 (Harder et al., 2007; Filipp et al., 2012). The actin cytoskeleton might be crucial in transporting TCRs into the kinase-rich lipid rafts and maintaining the lipid raft environment. Evidence to support the role of actin in this mechanism includes studies in which disruption of the actin cytoskeleton leads to disappearance of lipid rafts and CD45 in the immunological synapse (Valensin et al., 2002; Chichili et al., 2010; Chichili et al., 2012). However, a recent study suggests that formation of lipid rafts is not sufficient for Lck clustering (Rossy et al., 2013). It should also be noted that the existence of lipid rafts is controversial, mainly because many of the experimental approaches used were found to be questionable and some groups were unable to observe these small and dynamic membrane domains directly (Shaw, 2006; Leslie, 2011).

Actin in the receptor aggregation model

The receptor aggregation model simply states that binding between TCRs and pMHCs leads to aggregation of TCR–CD3 complexes and thus allows for enhanced local TCR signaling. The model stemmed from the longstanding observations that

forced aggregation of TCRs, using antibodies or soluble multimeric forms of agonist pMHCs, can trigger T cell activation. In addition to the role of actin in maintaining cell–cell contact, actin might also contribute to TCR–CD3 aggregation by acting as a scaffold to stabilize and support protein aggregation – which will be discussed further in the next section. However, the receptor aggregation model has many limitations. One major problem is that the model cannot explain why T cell signaling can be triggered by extremely low densities of pMHCs, at which TCR microclusters are small, sparse or unobservable (Sykulev et al., 1996; Irvine et al., 2002; Manz and Groves, 2010). As different modifications have been made to the original model, the TCR aggregation model looks more plausible when other mechanisms such as conformational change (Reich et al., 1997; Alam et al., 1999) and TCR serial triggering (Valitutti et al., 1995b) are incorporated.

Taken together, the actin cytoskeleton is likely to be indispensable for the generation of signals from TCRs regardless of the models proposed. All three models – conformational change, kinetic segregation and receptor aggregation – are not mutually exclusive. They describe the TCR triggering process on different length scales: the conformation change model focuses on the level of single TCRs, whereas the kinetic segregation and receptor aggregation models concern the molecular cluster level. In fact, many experimental observations support more than one model. Therefore, it is possible that actin has multiple roles in a combination of these mechanisms at various stages in TCR triggering.

The role of actin in formation of the immunological synapse

After TCR triggering, TCRs and other receptors as well as intracellular proteins cluster into distinct domains, creating large-scale spatial organization in the immunological synapse. The entire process of TCR clustering, translocation and large-scale segregation is correlated with the T cell biochemical signaling and thus provides a direct readout of T cell activation in time and space.

The role of actin in signaling in microclusters

TCRs, together with other signaling molecules, form microclusters following their ligation with the agonist pMHCs. Although TCR microclusters that accumulate in the cSMAC were originally believed to be the site for T cell signaling, it has become clear that, under normal triggering conditions, the newly formed microclusters in the periphery of the cell contact area are the active signaling units (Varma et al., 2006; Cemerski et al., 2008), also referred to as ‘signalosomes’ (Werlen and Palmer, 2002). Actin is required for signaling at the microcluster level because either disruption or over-stabilization of actin abrogates the formation of TCR microclusters, Ca^{2+} signaling (Campi et al., 2005; Varma et al., 2006) and tyrosine phosphorylation (Shen et al., 2005). One important question that many recent studies aimed to answer is the exact nature of the contribution of actin to T cell signaling within the microclusters.

Actin might regulate T cell signaling by maintaining the association between TCRs and other signaling proteins. That explains the observation that, upon actin depolymerization by pharmacological drugs, the concentration of intracellular Ca^{2+} is reduced within one minute and no new TCR clusters form, but

existing TCR microclusters remain intact for at least a few further minutes (Varma et al., 2006). Interestingly, if T cells are treated with jasplakinolide, a pharmacological drug that interrupts actin depolymerization, Ca^{2+} signaling also stops, but phosphorylation of ZAP70 in the TCR microclusters remains (Babich et al., 2012). This observation suggests that the integrity of signaling clusters only requires the scaffolding function of actin, but keeping the dynamic association of molecules inside microclusters for sustained signaling requires actin retrograde flow. Indeed, the scaffolding effects appear to be reciprocal as one of our recent studies has suggested that microclusters possess the capacity to organize the actin cytoskeleton locally around themselves (Smoligovets et al., 2012). The scaffolding properties of actin might be mediated by its regulatory proteins (Fig. 3). The guanine-nucleotide-exchange factor (GEF) Vav1 and Wiskott-Aldrich syndrome protein WASp, both key regulators for F-actin nucleation and remodeling, have been observed to be recruited to TCR–pMHC binding sites (Sasahara et al., 2002; Zeng et al., 2003; Barda-Saad et al., 2005; Tybulewicz, 2005; Yokosuka et al., 2005; Miletic et al., 2006; Miletic et al., 2009). It has been suggested that Vav1 associates with the Lat–SLP-76 adaptor complex (Sylvain et al., 2011; Pauker et al., 2012) and that WASp binds to the proline-rich sequences in CD3 chains (Gil

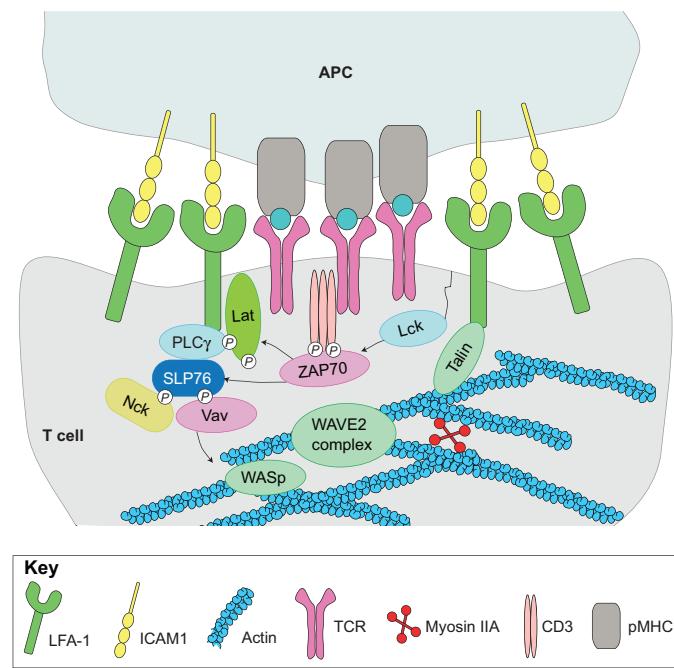


Fig. 3. Schematic illustration of some of the key proteins involved in linking actin to TCR signaling. Engagement of TCRs with agonist pMHC molecules leads to phosphorylation of the cytoplasmic domains of CD3 by lymphocyte-specific tyrosine-protein kinase (Lck). Subsequently, tyrosine kinases such as ζ -chain-associated tyrosine-protein kinase 70 (ZAP70), actin adaptor proteins such as cytosolic protein Nck1 (Nck1), the scaffold protein SH2-domain-containing leukocyte protein of 76 kDa (SLP-76), and actin polymerization-regulatory molecules such as Wiskott-Aldrich syndrome protein (WASp) and the Wiskott-Aldrich syndrome protein family member 2 (WAVE2, also known as WASF2) complex, are recruited to the TCR–CD3 complexes to regulate localized actin polymerization. Integrins such as leukocyte function-associated antigen 1 (LFA-1) also regulate the actin cytoskeleton through linker proteins such as talin.

et al., 2002). Moreover, Vav1 was shown to stabilize signaling microclusters during T cell activation independently of its intrinsic GEF activity (Miletic et al., 2009; Sylvain et al., 2011), suggesting that it functions as a linker protein to load microclusters onto the actin cytoskeleton.

Actin might also modulate T cell signaling by stabilizing preformed protein clusters in quiescent T cells. With the help of high-resolution imaging techniques (Box 2), Lillemeier and colleagues revealed that TCR and the adaptor protein Lat exist in separate membrane domains with a diameter of 70–140 nm (Lillemeier et al., 2010), whereas Sherman and co-workers reported pre-existing Lat nanoclusters as small as dimers in resting T cells (Sherman et al., 2011). The difference could in part be due to the differences in sample preparation, but both studies confirmed that actin is required for nanocluster stability. As protein preclustering has been proposed as a regulatory ‘safety-check’ mechanism in T cell activation (Lillemeier et al., 2010; Chung et al., 2012), the prescaffolding effect exerted by actin probably has an important role. However, the idea that pre-existing protein clusters contribute to T cell activation was recently challenged by the finding that Lat molecules participate in early T cell signaling by means of subsynaptic vesicles instead of preclusters (Williamson et al., 2011).

In addition, as discussed in the context of the kinetic segregation and receptor aggregation models, actin might also modulate TCR microcluster formation and signaling by controlling the mechanical properties of the cell membrane or promoting membrane compartmentalization. Besides the aforementioned lipid raft hypothesis, an alternative mechanism for the role of actin in the assembly of microclusters is the so-called ‘picket fence model of diffusion restriction’ (Kusumi et al., 2005). This model posits that the high local density of actin and actin-associated membrane proteins restricts the diffusion of other membrane-associated or membrane-proximal cytoplasmic proteins and thus promotes assembly of microclusters. Although this model is supported by some data (Andrews et al., 2008; Treanor et al., 2010; Treanor et al., 2011), it does not explain why proteins can rapidly coalesce or dissociate from the clusters or how TCR microclusters are transported from the cell periphery to the cSMAC.

Actin in microcluster translocation and spatial organization of the immunological synapse

Within a few minutes of cluster formation, TCR microclusters are centripetally translocated to coalesce into cSMACs, and ligated integrins and associated proteins accumulate around the periphery of the synapse. It has long been recognized that receptor transport and spatial organization in the immunological synapse require retrograde flow of actin (Dustin and Cooper, 2000; Burkhardt et al., 2008; Gomez and Billadeau, 2008; Beemiller and Krummel, 2010). To reveal the physical interactions between TCR clusters and actin, Kaizuka and colleagues showed that TCR microclusters and integrins move centripetally together with the actin cytoskeleton by using imaging of fluorescently labeled protein clusters in Jurkat cells that are triggered by a glass-supported lipid bilayer containing stimulatory ligands (Kaizuka et al., 2007). Based on the observation that TCR microclusters move slower than the underlying retrograde actin flow, they proposed that a ‘stick-slip’ movement of TCR microclusters occurs on the actin cytoskeleton (Kaizuka et al., 2007). However, considering that each TCR microcluster contains tens to hundreds of molecules,

simultaneous breakage of all the molecular linkages to actin in the ‘slip’ phase seems unlikely. The Groves group later proposed a frictional coupling mechanism (Fig. 4), based on a study that employed physical barriers on glass-supported lipid bilayers (DeMond et al., 2008). When the centripetal transport of TCR microclusters was hindered by a physical barrier, the microclusters did not stop but continued their motion with a different direction (DeMond et al., 2008). This suggests that the coupling between microclusters and actin is rather transient – multiple weak links form and break at different times to generate a frictional force between microclusters and actin. Two subsequent studies provided further support for the idea of the frictional coupling model and showed that actin itself slows down as it passes over mechanically trapped TCRs (Yu et al., 2010; Smoligovets et al., 2012).

The transient coupling mechanism predicts that the strength of attachment to the actin cytoskeleton correlates positively with the number of molecular linkages. This could explain why TCR and LFA-1 microclusters, although both coupled with actin, are transported differentially to destinations that are micrometers apart. Indeed, it has been found that, when the LFA-1 clusters are crosslinked by using bivalent and tetravalent antibodies, larger clusters that presumably have more linkages to actin are transported closer to the center of the immunological synapse and that tetravalently crosslinked LFA-1 clusters are able to reach the cSMAC (Fig. 4B) (Hartman et al., 2009). This observation cannot be explained by the size-exclusion mechanism in the kinetic segregation model. Instead, it suggests that spatial sorting of proteins in the immunological synapse can be driven by their specific coupling interactions to the actin cytoskeleton.

Formation of the immunological synapse is tightly associated with signaling activities: signaling is sustained during microcluster formation (Bunnell et al., 2002; Lee et al., 2003; Yokosuka et al., 2005) and attenuated (Varma et al., 2006) or amplified under conditions of weak agonist (Cemerski et al., 2008) in cSMACs. Therefore, actin-mediated protein cluster formation, translocation and spatial sorting might provide a dynamic framework for T cells to regulate signaling activities in time and space.

Interplay between actin and myosin IIA in TCR signaling

Compelling evidence indicates that the retrograde flow of actin is an essential part of the formation of immunological synapses and of T cell activation, and multiple processes might generate and control this flow. Actin retrograde motion is likely to be driven by the polymerization of its filaments (Henson et al., 1999). Additionally, the actin-associated molecular motor myosin II has been found to contribute to actin flow by exerting contractile forces on actin filaments (Vicente-Manzanares et al., 2009; Betapudi, 2010; Arii et al., 2010; Bhuwania et al., 2012). Non-muscle myosin IIA is the dominantly expressed isoform of myosin II in T cells (Jacobelli et al., 2004). The importance of non-muscle myosin IIA in T cell polarity and migration is well established (Smith et al., 2003; Sanchez-Madrid and Serrador, 2009; Jacobelli et al., 2009; Jacobelli et al., 2010), but its role in formation of the immunological synapse and T cell activation is still controversial. Studies from some groups have shown that myosin IIA is necessary for TCR microcluster movement and immunological synapse organization (Wülfing and Davis, 1998; Ilani et al., 2009; Yu et al., 2012; Yi et al., 2012; Kumari et al., 2012), whereas other groups have found that neither TCR cluster

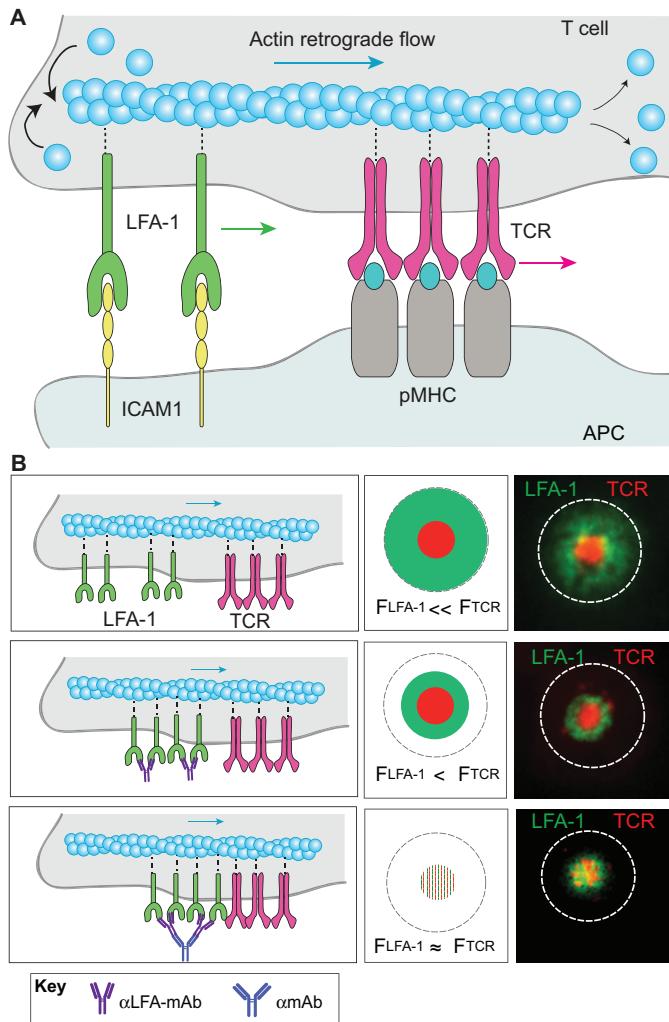


Fig. 4. The frictional coupling model for the translocation and spatial segregation of microclusters. (A) Schematic illustration of the frictional coupling model, in which microclusters of membrane receptors are translocated towards the center of an immunological synapse between a T cell and an antigen-presenting cell (APC) by multiple transient linkages to the underlying centripetal actin flow. One major driving force for the actin retrograde flow comes from actin polymerization pushing against the cell membrane. Non-muscle myosin IIA might also exert contractile forces on the actin filaments to contribute to the actin flow, but its exact role is unclear. (B) Experimental results suggest that differential frictional coupling between protein microclusters and actin drives their spatial segregation in the immunological synapse. Here, LFA-1 integrins were crosslinked by either monomeric or multimeric primary antibodies to simulate microclustering in T cells that were activated by stimulatory supported lipid bilayers. LFA-1 molecules alone (green) do not form clusters as large as those formed by TCRs (red) in control cells and accumulate in the peripheral SMAC (pSMAC; top row). As a result, the frictional coupling of LFA-1 clusters to actin (F_{LFA-1}) is much smaller than that of TCR clusters (F_{TCR}): $F_{LFA-1} \ll F_{TCR}$. Crosslinking of LFA-1 with monomeric antibodies ($\alpha\text{LFA-mAb}$) results in clusters that are transported closer to the center of the immunological synapse owing to their relatively stronger association with the actin cytoskeleton, in which case $F_{LFA-1} < F_{TCR}$ (middle row). When the multimeric crosslinker (αmAb linked to $\alpha\text{LFA-mAb}$) is used, the resulting LFA-1 clusters are able to reach the central SMAC (cSMAC), where TCR microclusters accumulate (bottom row). Fluorescence images were adapted, with permission, from figures published previously (Hartman et al., 2009).

transport nor Ca^{2+} signaling is dependent on myosin IIA function (Jacobelli et al., 2004; Babich et al., 2012; Beemiller et al., 2012). It has been found that myosin drives a rapid inward translocation of TCR microclusters only during the early stage of signaling, whereas actin polymerization provides a slower basal rate of motion that persists throughout the entire life span of an immunological synapse (Yu et al., 2012). Consequently, actin retrograde flow alone is able to organize the microclusters spatially in response to myosin inhibition, albeit at a slower rate than in the presence of uninhibited myosin IIA (Yu et al., 2012). In agreement with these observations, Yi and colleagues reported that contraction of myosin II and actin retrograde flow together transport microclusters, but that myosin II is not required for formation of immunological synapses. In their study, the specific locations of myosin II and the actin filaments were also nicely examined. By contrast, others have recently reported that actin retrograde flow is driven solely by actin polymerization that pushes against the edge of the cell membrane (Babich et al., 2012; Beemiller et al., 2012). Interestingly, Babich and colleagues also observed that, although myosin IIA is not required, actin retrograde flow is not the only driving force for microcluster centralization (Babich et al., 2012).

The discrepancies arising from all these studies could be due to a combination of factors. First, different T cell types were used: primary murine cells (Jacobelli et al., 2004; Yu et al., 2012; Beemiller et al., 2012), human Jurkat cells (Ilani et al., 2009; Babich et al., 2012; Yi et al., 2012) or primary human cells (Ilani et al., 2009; Babich et al., 2012). Although no detailed comparison across different cell types is available, there might be some heterogeneity with regards to the expression and requirement of motor proteins, or cell responses to drug treatment. Second, the discrepancy might be due to the use of different stimulatory surfaces, such as surfaces with immobilized anti-CD3 versus supported lipid bilayers with agonist pMHCS, as T cell activation is known to be sensitive to the type, concentration and mobility of agonists (van der Merwe and Dushek, 2011; Hsu et al., 2012). Third, different actin labeling methods might restrict quantification of actin in certain locations (i.e. pSMAC versus dSMAC) or even interfere with actin polymerization (Burkhardt et al., 2007; Riedl et al., 2008; Milroy et al., 2012).

Concluding remarks

Since the first study of the role of actin in T cell activation more than three decades ago, tremendous progress has been made towards identifying individual factors that link the complex signaling pathways and cytoskeletal elements of T cells through the use of traditional biochemical studies (Burkhardt et al., 2008; Reicher and Barda-Saad, 2010). Many recent breakthroughs, such as those that relate actin dynamics to TCR activation in real-time, were made possible by employing interdisciplinary approaches such as high-resolution imaging techniques. It has now been established that the importance of the actin cytoskeleton in T cell activation transcends the maintenance of cell morphology and transport of proteins. In fact, actin regulates T cell activation by maintaining cell–cell contact, facilitating TCR triggering and scaffolding protein assemblies and organization. In spite of our progress towards understanding of the complex role of actin in T cell signaling, many important questions remain. For example, an emerging idea is that the physical forces from actin and actin-associated proteins also modulate T cell activation, but we do not

know the exact mechanisms or proteins involved that transduce mechanical forces to biochemical signaling pathways. The distinct filamentous structures of actin and the spatial distribution of molecular motors in T cells need to be distinguished with high resolution, possibly by using transmission electron microscopy (TEM) or super-resolution imaging techniques. Another key focus of research is to understand the role of actin in temporally coordinating different signaling pathways. Additionally, the question of actin integration in T cell co-stimulation remains to be answered. New regulatory proteins will doubtless be identified and novel strategies must be developed to dissect the spatial and temporal connectivity between signaling pathways in order to answer all these questions – only then will a more comprehensive understanding of the role of actin in T cell functions be achieved.

Funding

Supported by the Director, Office of Science, Office of Basic Energy Sciences, of the US Department of Energy (contract number DE-AC02-05CH11231 to Y.Y., A.A.S and J.T.G.). A.A.S was partially supported by the Office of the Congressionally Directed Medical Research Program Idea Award BC102681 under US Army Medical Research Acquisition Activity no. W81XWH-11-1-0256.

References

- Alam, S. M., Davies, G. M., Lin, C. M., Zal, T., Nasholds, W., Jameson, S. C., Hogquist, K. A., Gascoigne, N. R. and Travers, P. J. (1999). Qualitative and quantitative differences in T cell receptor binding of agonist and antagonist ligands. *Immunity* **10**, 227-237.
- Andrews, N. L., Lidke, K. A., Pfeiffer, J. R., Burns, A. R., Wilson, B. S., Oliver, J. M. and Lidke, D. S. (2008). Actin restricts Fc epsilon RI diffusion and facilitates antigen-induced receptor immobilization. *Nat. Cell Biol.* **10**, 955-963.
- Arii, J., Goto, H., Suenaga, T., Oyama, M., Kozuka-Hata, H., Imai, T., Minowa, A., Akashi, H., Arase, H., Kawaoka, Y. et al. (2010). Non-muscle myosin IIA is a functional entry receptor for herpes simplex virus-1. *Nature* **467**, 859-862.
- Axelrod, D. (2003). Total internal reflection fluorescence microscopy in cell biology. *Methods Enzymol.* **361**, 1-33.
- Babich, A., Li, S., O'Connor, R. S., Milone, M. C., Freedman, B. D. and Burkhardt, J. K. (2012). F-actin polymerization and retrograde flow drive sustained PLC γ 1 signaling during T cell activation. *J. Cell Biol.* **197**, 775-787.
- Bachmann, M. F., McCall-Faienza, K., Schmits, R., Bouchard, D., Beach, J., Speiser, D. E., Mak, T. W. and Ohashi, P. S. (1997). Distinct roles for LFA-1 and CD28 during activation of naive T cells: adhesion versus costimulation. *Immunity* **7**, 549-557.
- Balagopalan, L., Coussens, N. P., Sherman, E., Samelson, L. E. and Sommers, C. L. (2010). The LAT story: a tale of cooperativity, coordination, and choreography. *Cold Spring Harb. Perspect. Biol.* **2**, a005512.
- Barda-Saad, M., Braiman, A., Titterton, R., Bunnell, S. C., Barr, V. A. and Samelson, L. E. (2005). Dynamic molecular interactions linking the T cell antigen receptor to the actin cytoskeleton. *Nat. Immunol.* **6**, 80-89.
- Barda-Saad, M., Shirasu, N., Pauker, M. H., Hassan, N., Perl, O., Balbo, A., Yamaguchi, H., Houtman, J. C., Appella, E., Schuck, P. et al. (2010). Cooperative interactions at the SLP-76 complex are critical for actin polymerization. *EMBO J.* **29**, 2315-2328.
- Beddoe, T., Chen, Z., Clements, C. S., Ely, L. K., Bushell, S. R., Vivian, J. P., Kjær-Nielsen, L., Pang, S. S., Dunstone, M. A., Liu, Y. C. et al. (2009). Antigen ligation triggers a conformational change within the constant domain of the alphabeta T cell receptor. *Immunity* **30**, 777-788.
- Beemiller, P. and Krummel, M. F. (2010). Mediation of T-cell activation by actin meshworks. *Cold Spring Harb. Perspect. Biol.* **2**, a002444.
- Beemiller, P., Jacobelli, J. and Krummel, M. F. (2012). Integration of the movement of signaling microclusters with cellular motility in immunological synapses. *Nat. Immunol.* **13**, 787-795.
- Betapudi, V. (2010). Myosin II motor proteins with different functions determine the fate of lamellipodia extension during cell spreading. *PLoS ONE* **5**, e8560.
- Bhuwania, R., Cornfine, S., Fang, Z., Krüger, M., Luna, E. J. and Linder, S. (2012). Supervillin couples myosin-dependent contractility to podosomes and enables their turnover. *J. Cell Sci.* **125**, 2300-2314.
- Billadeau, D. D., Nolz, J. C. and Gomez, T. S. (2007). Regulation of T-cell activation by the cytoskeleton. *Nat. Rev. Immunol.* **7**, 131-143.
- Bunnell, S. C., Kapoor, V., Trible, R. P., Zhang, W. and Samelson, L. E. (2001). Dynamic actin polymerization drives T cell receptor-induced spreading: a role for the signal transduction adaptor LAT. *Immunity* **14**, 315-329.
- Bunnell, S. C., Hong, D. I., Kardon, J. R., Yamazaki, T., McGlade, C. J., Barr, V. A. and Samelson, L. E. (2002). T cell receptor ligation induces the formation of dynamically regulated signaling assemblies. *J. Cell Biol.* **158**, 1263-1275.
- Burkel, B. M., von Dassow, G. and Bement, W. M. (2007). Versatile fluorescent probes for actin filaments based on the actin-binding domain of utrophin. *Cell Motil. Cytoskeleton* **64**, 822-832.
- Burkhardt, J. K., Carrizosa, E. and Shaffer, M. H. (2008). The actin cytoskeleton in T cell activation. *Annu. Rev. Immunol.* **26**, 233-259.
- Campi, G., Varma, R. and Dustin, M. L. (2005). Actin and agonist MHC-peptide complex-dependent T cell receptor microclusters as scaffolds for signaling. *J. Exp. Med.* **202**, 1031-1036.
- Cannon, J. L. and Burkhardt, J. K. (2002). The regulation of actin remodeling during T-cell-APC conjugate formation. *Immunol. Rev.* **186**, 90-99.
- Cemerski, S., Das, J., Giurisato, E., Markiewicz, M. A., Allen, P. M., Chakraborty, A. K. and Shaw, A. S. (2008). The balance between T cell receptor signaling and degradation at the center of the immunological synapse is determined by antigen quality. *Immunity* **29**, 414-422.
- Chichili, G. R., Westmuckett, A. D. and Rodgers, W. (2010). T cell signal regulation by the actin cytoskeleton. *J. Biol. Chem.* **285**, 14737-14746.
- Chichili, G. R., Cail, R. C. and Rodgers, W. (2012). Cytoskeletal modulation of lipid interactions regulates Lck kinase activity. *J. Biol. Chem.* **287**, 24186-24194.
- Choudhuri, K. and Dustin, M. L. (2010). Signaling microdomains in T cells. *FEBS Lett.* **584**, 4823-4831.
- Choudhuri, K., Wiseman, D., Brown, M. H., Gould, K. and van der Merwe, P. A. (2005). T-cell receptor triggering is critically dependent on the dimensions of its peptide-MHC ligand. *Nature* **436**, 578-582.
- Choudhuri, K., Parker, M., Milicic, A., Cole, D. K., Shaw, M. K., Sewell, A. K., Stewart-Jones, G., Dong, T., Gould, K. G. and van der Merwe, P. A. (2009). Peptide-major histocompatibility complex dimensions control proximal kinase-phosphatase balance during T cell activation. *J. Biol. Chem.* **284**, 26096-26105.
- Chung, W., Abel, S. M. and Chakraborty, A. K. (2012). Protein clusters on the T cell surface may suppress spurious early signaling events. *PLoS ONE* **7**, e44444.
- Danuser, G. and Waterman-Storer, C. M. (2006). Quantitative fluorescent speckle microscopy of cytoskeleton dynamics. *Annu. Rev. Biophys. Biomol. Struct.* **35**, 361-387.
- Davis, D. M. and Dustin, M. L. (2004). What is the importance of the immunological synapse? *Trends Immunol.* **25**, 323-327.
- Davis, S. J. and van der Merwe, P. A. (1996). The structure and ligand interactions of CD2: implications for T-cell function. *Immunol. Today* **17**, 177-187.
- Davis, S. J. and van der Merwe, P. A. (2006). The kinetic-segregation model: TCR triggering and beyond. *Nat. Immunol.* **7**, 803-809.
- Davis, S. J. and van der Merwe, P. A. (2011). Lck and the nature of the T cell receptor trigger. *Trends Immunol.* **32**, 1-5.
- DeMond, A. L., Mossman, K. D., Starr, T., Dustin, M. L. and Groves, J. T. (2008). T cell receptor microcluster transport through molecular mazes reveals mechanism of translocation. *Biophys. J.* **94**, 3286-3292.
- Dustin, M. L. (2007). Cell adhesion molecules and actin cytoskeleton at immune synapses and kinapses. *Curr. Opin. Cell Biol.* **19**, 529-533.
- Dustin, M. L. and Cooper, J. A. (2000). The immunological synapse and the actin cytoskeleton: molecular hardware for T cell signaling. *Nat. Immunol.* **1**, 23-29.
- Dustin, M. L. and Groves, J. T. (2012). Receptor signaling clusters in the immune synapse. *Annu. Rev. Biophys.* **41**, 543-556.
- Fernandes, R. A., Shore, D. A., Vuong, M. T., Yu, C., Zhu, X., Pereira-Lopes, S., Brouwer, H., Fennelly, J. A., Jessup, C. M., Evans, E. J. et al. (2012). T cell receptors are structures capable of initiating signaling in the absence of large conformational rearrangements. *J. Biol. Chem.* **287**, 13324-13335.
- Filipp, D., Ballek, O. and Manning, J. (2012). Lck, membrane microdomains, and ter triggering machinery: defining the new rules of engagement. *Front. Immunol.* **3**, 155.
- Geiger, B., Rosen, D. and Berke, G. (1982). Spatial relationships of microtubule-organizing centers and the contact area of cytotoxic T lymphocytes and target cells. *J. Cell Biol.* **95**, 137-143.
- Ghendler, Y., Smolyar, A., Chang, H. C. and Reinherz, E. L. (1998). One of the CD3epsilon subunits within a T cell receptor complex lies in close proximity to the Cbeta FG loop. *J. Exp. Med.* **187**, 1529-1536.
- Gil, D., Schamel, W. W., Montoya, M., Sánchez-Madrid, F. and Alarcón, B. (2002). Recruitment of Nck by CD3 epsilon reveals a ligand-induced conformational change essential for T cell receptor signaling and synapse formation. *Cell* **109**, 901-912.
- Gomez, T. S. and Billadeau, D. D. (2008). T cell activation and the cytoskeleton: you can't have one without the other. *Adv. Immunol.* **97**, 1-64.
- Grakoui, A., Bromley, S. K., Sumen, C., Davis, M. M., Shaw, A. S., Allen, P. M. and Dustin, M. L. (1999). The immunological synapse: a molecular machine controlling T cell activation. *Science* **285**, 221-227.
- Groves, J. T. and Dustin, M. L. (2003). Supported planar bilayers in studies on immune cell adhesion and communication. *J. Immunol. Methods* **278**, 19-32.
- Harder, T., Rentero, C., Zech, T. and Gaus, K. (2007). Plasma membrane segregation during T cell activation: probing the order of domains. *Curr. Opin. Immunol.* **19**, 470-475.
- Hartman, N. C., Nye, J. A. and Groves, J. T. (2009). Cluster size regulates protein sorting in the immunological synapse. *Proc. Natl. Acad. Sci. USA* **106**, 12729-12734.
- Henson, J. H., Svitkina, T. M., Burns, A. R., Hughes, H. E., MacPartland, K. J., Nazarian, R. and Borisy, G. G. (1999). Two components of actin-based retrograde flow in sea urchin coelomocytes. *Mol. Biol. Cell* **10**, 4075-4090.

- Hsu, C. J., Hsieh, W. T., Waldman, A., Clarke, F., Huseby, E. S., Burkhardt, J. K. and Baumgart, T. (2012). Ligand mobility modulates immunological synapse formation and T cell activation. *PLoS ONE* **7**, e32398.
- Huang, B., Bates, M. and Zhuang, X. (2009). Super-resolution fluorescence microscopy. *Annu. Rev. Biochem.* **78**, 993-1016.
- Huang, J., Zarnitsyna, V. I., Liu, B., Edwards, L. J., Jiang, N., Evavold, B. D. and Zhu, C. (2010). The kinetics of two-dimensional TCR and pMHC interactions determine T-cell responsiveness. *Nature* **464**, 932-936.
- Huppa, J. B., Axmann, M., Mörtelmaier, M. A., Lillemeyer, B. F., Newell, E. W., Brameshuber, M., Klein, L. O., Schütz, G. J. and Davis, M. M. (2010). TCR-peptide-MHC interactions in situ show accelerated kinetics and increased affinity. *Nature* **463**, 963-967.
- Husson, J., Chemin, K., Bohineust, A., Hivroz, C. and Henry, N. (2011). Force generation upon T cell receptor engagement. *PLoS ONE* **6**, e19680.
- Ilani, T., Vasiliver-Shamis, G., Vardhana, S., Bretscher, A. and Dustin, M. L. (2009). T cell antigen receptor signaling and immunological synapse stability require myosin IIa. *Nat. Immunol.* **10**, 531-539.
- Illingworth, J. J. and van der Merwe, P. A. (2012). Dissecting T-cell activation with high-resolution live-cell microscopy. *Immunology* **135**, 198-206.
- Irles, C., Symons, A., Michel, F., Bakker, T. R., van der Merwe, P. A. and Acuto, O. (2003). CD45 ectodomain controls interaction with GEMs and Lck activity for optimal TCR signaling. *Nat. Immunol.* **4**, 189-197.
- Irvine, D. J., Purhoo, M. A., Krogsbaard, M. and Davis, M. M. (2002). Direct observation of ligand recognition by T cells. *Nature* **419**, 845-849.
- Jacobelli, J., Chmura, S. A., Buxton, D. B., Davis, M. M. and Krummel, M. F. (2004). A single class II myosin modulates T cell motility and stopping, but not synapse formation. *Nat. Immunol.* **5**, 531-538.
- Jacobelli, J., Bennett, F. C., Pandurangi, P., Tooley, A. J. and Krummel, M. F. (2009). Myosin-IIA and ICAM-1 regulate the interchange between two distinct modes of T cell migration. *J. Immunol.* **182**, 2041-2050.
- Jacobelli, J., Friedman, R. S., Conti, M. A., Lennon-Dumenil, A. M., Piel, M., Sorensen, C. M., Adelstein, R. S. and Krummel, M. F. (2010). Confinement-optimized three-dimensional T cell amoeboid motility is modulated via myosin IIa-regulated adhesions. *Nat. Immunol.* **11**, 953-961.
- James, J. R. and Vale, R. D. (2012). Biophysical mechanism of T-cell receptor triggering in a reconstituted system. *Nature* **487**, 64-69.
- Jordan, M. S. and Koretzky, G. A. (2010). Coordination of receptor signaling in multiple hematopoietic cell lineages by the adaptor protein SLP-76. *Cold Spring Harb. Perspect. Biol.* **2**, a002501.
- Kaizuka, Y., Douglass, A. D., Varma, R., Dustin, M. L. and Vale, R. D. (2007). Mechanisms for segregating T cell receptor and adhesion molecules during immunological synapse formation in Jurkat T cells. *Proc. Natl. Acad. Sci. USA* **104**, 20296-20301.
- Kim, S. T., Takeuchi, K., Sun, Z.-Y. J., Touma, M., Castro, C. E., Fahmy, A., Lang, M. J., Wagner, G. and Reinherz, E. L. (2009). The alphabeta T cell receptor is an anisotropic mechanosensor. *J. Biol. Chem.* **284**, 31028-31037.
- Kim, S. T., Touma, M., Takeuchi, K., Sun, Z. Y., Dave, V. P., Kappes, D. J., Wagner, G. and Reinherz, E. L. (2010). Distinctive CD3 heterodimeric ectodomain topologies maximize antigen-triggered activation of alpha beta T cell receptors. *J. Immunol.* **185**, 2951-2959.
- Kumari, S., Vardhana, S., Cammer, M., Curado, S., Santos, L., Sheetz, M. P. and Dustin, M. L. (2012). T lymphocyte myosin IIA is required for maturation of the immunological synapse. *Front. Immunol.* **3**, 230.
- Kusumi, A., Nakada, C., Ritchie, K., Murase, K., Suzuki, K., Murakoshi, H., Kasai, R. S., Kondo, J. and Fujiwara, T. (2005). Paradigm shift of the plasma membrane concept from the two-dimensional continuum fluid to the partitioned fluid: high-speed single-molecule tracking of membrane molecules. *Annu. Rev. Biophys. Biomol. Struct.* **34**, 351-378.
- Lee, K. H., Dinner, A. R., Tu, C., Campi, G., Raychaudhuri, S., Varma, R., Sims, T. N., Burack, W. R., Wu, H., Wang, J. et al. (2003). The immunological synapse balances T cell receptor signaling and degradation. *Science* **302**, 1218-1222.
- Leslie, M. (2011). Mysteries of the cell. Do lipid rafts exist? *Science* **334**, 1046-1047.
- Leupin, O., Zaru, R., Laroche, T., Müller, S. and Valiutti, S. (2000). Exclusion of CD45 from the T-cell receptor signaling area in antigen-stimulated T lymphocytes. *Curr. Biol.* **10**, 277-280.
- Lewis, R. S. (2001). Calcium signaling mechanisms in T lymphocytes. *Annu. Rev. Immunol.* **19**, 497-521.
- Li, Y.-C., Chen, B.-M., Wu, P.-C., Cheng, T.-L., Kao, L.-S., Tao, M.-H., Lieber, A. and Roffler, S. R. (2010). Cutting Edge: mechanical forces acting on T cells immobilized via the TCR complex can trigger TCR signaling. *J. Immunol.* **184**, 5959-5963.
- Lillemeyer, B. F., Mörtelmaier, M. A., Forstner, M. B., Huppa, J. B., Groves, J. T. and Davis, M. M. (2010). TCR and Lat are expressed on separate protein islands on T cell membranes and concatenate during activation. *Nat. Immunol.* **11**, 90-96.
- Lin, J. and Weiss, A. (2003). The tyrosine phosphatase CD148 is excluded from the immunologic synapse and down-regulates prolonged T cell signaling. *J. Cell Biol.* **162**, 673-682.
- Lingwood, D. and Simons, K. (2010). Lipid rafts as a membrane-organizing principle. *Science* **327**, 46-50.
- Ma, Z. and Finkel, T. H. (2010). T cell receptor triggering by force. *Trends Immunol.* **31**, 1-6.
- Ma, Z., Sharp, K. A., Janmey, P. A. and Finkel, T. H. (2008). Surface-anchored monomeric agonist pMHCs alone trigger TCR with high sensitivity. *PLoS Biol.* **6**, e43.
- Manz, B. N. and Groves, J. T. (2010). Spatial organization and signal transduction at intercellular junctions. *Nat. Rev. Mol. Cell Biol.* **11**, 342-352.
- Miletic, A. V., Sakata-Sogawa, K., Hiroshima, M., Hamann, M. J., Gomez, T. S., Ota, N., Kloeppl, T., Kanagawa, O., Tokunaga, M., Billadeau, D. D. et al. (2006). Vav1 acidic region tyrosine 174 is required for the formation of T cell receptor-induced microclusters and is essential in T cell development and activation. *J. Biol. Chem.* **281**, 38257-38265.
- Miletic, A. V., Graham, D. B., Sakata-Sogawa, K., Hiroshima, M., Hamann, M. J., Cemerski, S., Kloeppl, T., Billadeau, D. D., Kanagawa, O., Tokunaga, M. et al. (2009). Vav links the T cell antigen receptor to the actin cytoskeleton and T cell activation independently of intrinsic Guanine nucleotide exchange activity. *PLoS ONE* **4**, e6599.
- Milroy, L. G., Rizzo, S., Calderon, A., Ellinger, B., Erdmann, S., Mondry, J., Verveer, P., Bastiaens, P., Waldmann, H., Dehmelt, L. et al. (2012). Selective chemical imaging of static actin in live cells. *J. Am. Chem. Soc.* **134**, 8480-8486.
- Monks, C. R., Freiberg, B. A., Kupfer, H., Scialyi, N. and Kupfer, A. (1998). Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* **395**, 82-86.
- Mossman, K. D., Campi, G., Groves, J. T. and Dustin, M. L. (2005). Altered TCR signaling from geometrically repatterned immunological synapses. *Science* **310**, 1191-1193.
- Nambiar, R., McConnell, R. E. and Tyska, M. J. (2009). Control of cell membrane tension by myosin-I. *Proc. Natl. Acad. Sci. USA* **106**, 11972-11977.
- Nika, K., Soldani, C., Salek, M., Paster, W., Gray, A., Etzensperger, R., Fugger, L., Polzella, P., Cerundolo, V., Dushenk, O. et al. (2010). Constitutively active Lck kinase in T cells drives antigen receptor signal transduction. *Immunity* **32**, 766-777.
- Pauker, M. H., Hassan, N., Noy, E., Reicher, B. and Barda-Saad, M. (2012). Studying the dynamics of SLP-76, Nck, and Vav1 multimolecular complex formation in live human cells with triple-color FRET. *Sci. Signal.* **5**, rs3.
- Randriamampita, C., Mouchacca, P., Malissen, B., Marguet, D., Trautmann, A. and Lellouch, A. C. (2008). A novel ZAP-70 dependent FRET based biosensor reveals kinase activity at both the immunological synapse and the antisynapse. *PLoS One* **3**, e1521.
- Reich, Z., Boniface, J. J., Lyons, D. S., Borochov, N., Wachtel, E. J. and Davis, M. M. (1997). Ligand-specific oligomerization of T-cell receptor molecules. *Nature* **387**, 617-620.
- Reicher, B. and Barda-Saad, M. (2010). Multiple pathways leading from the T-cell antigen receptor to the actin cytoskeleton network. *FEBS Lett.* **584**, 4858-4864.
- Riedl, J., Crevenna, A. H., Kessenbrock, K., Yu, J. H., Neukirchen, D., Bista, M., Bradke, F., Jenne, D., Holak, T. A., Werb, Z. et al. (2008). Lifeact: a versatile marker to visualize F-actin. *Nat. Methods* **5**, 605-607.
- Rossy, J., Owen, D. M., Williamson, D. J., Yang, Z. and Gaus, K. (2013). Conformational states of the kinase Lck regulate clustering in early T cell signaling. *Nat. Immunol.* **14**, 82-89.
- Ryser, J. E., Rungger-Brändle, E., Chaponnier, C., Gabbiani, G. and Vassalli, P. (1982). The area of attachment of cytotoxic T lymphocytes to their target cells shows high motility and polarization of actin, but not myosin. *J. Immunol.* **128**, 1159-1162.
- Sasahara, Y., Rachid, R., Byrne, M. J., de la Fuente, M. A., Abraham, R. T., Ramesh, N. and Geha, R. S. (2002). Mechanism of recruitment of WASP to the immunological synapse and of its activation following TCR ligation. *Mol. Cell* **10**, 1269-1281.
- Seminario, M. C. and Bunnell, S. C. (2008). Signal initiation in T-cell receptor microclusters. *Immunol. Rev.* **221**, 90-106.
- Shaw, A. S. (2006). Lipid rafts: now you see them, now you don't. *Nat. Immunol.* **7**, 1139-1142.
- Shen, A., Puente, L. G. and Ostergaard, H. L. (2005). Tyrosine kinase activity and remodelling of the actin cytoskeleton are co-temporally required for degranulation by cytotoxic T lymphocytes. *Immunology* **116**, 276-286.
- Sherman, E., Barr, V., Manley, S., Patterson, G., Balagopalan, L., Akpan, I., Regan, C. K., Merrill, R. K., Sommers, C. L., Lippincott-Schwartz, J. et al. (2011). Functional nanoscale organization of signaling molecules downstream of the T cell antigen receptor. *Immunity* **35**, 705-720.
- Skruzny, M., Brach, T., Ciuffa, R., Rybina, S., Wachsmuth, M. and Kaksonen, M. (2012). Molecular basis for coupling the plasma membrane to the actin cytoskeleton during clathrin-mediated endocytosis. *Proc. Natl. Acad. Sci. USA* **109**, E2533-E2542.
- Smith, A., Bracke, M., Leitinger, B., Porter, J. C. and Hogg, N. (2003). LFA-1-induced T cell migration on ICAM-1 involves regulation of MLCK-mediated attachment and ROCK-dependent detachment. *J. Cell Sci.* **116**, 3123-3133.
- Smoligovets, A. A., Smith, A. W., Wu, H. J., Petit, R. S. and Groves, J. T. (2012). Characterization of dynamic actin associations with T-cell receptor microclusters in primary T cells. *J. Cell Sci.* **125**, 735-742.
- Sun, Z. J., Kim, K. S., Wagner, G. and Reinherz, E. L. (2001). Mechanisms contributing to T cell receptor signaling and assembly revealed by the solution structure of an ectodomain fragment of the CD3 epsilon gamma heterodimer. *Cell* **105**, 913-923.
- Suzuki, J., Yamasaki, S., Wu, J., Koretzky, G. A. and Saito, T. (2007). The actin cloud induced by LFA-1-mediated outside-in signals lowers the threshold for T-cell activation. *Blood* **109**, 168-175.

- Sykulev, Y., Joo, M., Vturina, I., Tsomides, T. J. and Eisen, H. N.** (1996). Evidence that a single peptide-MHC complex on a target cell can elicit a cytolytic T cell response. *Immunity* **4**, 565-571.
- Sylvain, N. R., Nguyen, K. and Bunnell, S. C.** (2011). Vav1-mediated scaffolding interactions stabilize SLP-76 microclusters and contribute to antigen-dependent T cell responses. *Sci. Signal.* **4**, ra14.
- Treanor, B., Depoil, D., Gonzalez-Granja, A., Barral, P., Weber, M., Dushek, O., Bruckbauer, A. and Batista, F. D.** (2010). The membrane skeleton controls diffusion dynamics and signaling through the B cell receptor. *Immunity* **32**, 187-199.
- Treanor, B., Depoil, D., Bruckbauer, A. and Batista, F. D.** (2011). Dynamic cortical actin remodeling by ERM proteins controls BCR microcluster organization and integrity. *J. Exp. Med.* **208**, 1055-1068.
- Tybulewicz, V. L.** (2005). Vav-family proteins in T-cell signalling. *Curr. Opin. Immunol.* **17**, 267-274.
- Valensin, S., Paccani, S. R., Ulivieri, C., Mercati, D., Pacini, S., Patrucci, L., Hirst, T., Lupetti, P. and Baldari, C. T.** (2002). F-actin dynamics control segregation of the TCR signalling cascade to clustered lipid rafts. *Eur. J. Immunol.* **32**, 435-446.
- Valitutti, S., Dessing, M., Aktories, K., Gallati, H. and Lanzavecchia, A.** (1995a). Sustained signaling leading to T cell activation results from prolonged T cell receptor occupancy. Role of T cell actin cytoskeleton. *J. Exp. Med.* **181**, 577-584.
- Valitutti, S., Müller, S., Cella, M., Padovan, E. and Lanzavecchia, A.** (1995b). Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature* **375**, 148-151.
- van der Merwe, P. A.** (2001). The TCR triggering puzzle. *Immunity* **14**, 665-668.
- van der Merwe, P. A. and Dushek, O** (2011). Mechanisms for T cell receptor triggering. *Nat. Rev. Immunol.* **11**, 47-55.
- Varma, R., Campi, G., Yokosuka, T., Saito, T. and Dustin, M. L.** (2006). T cell receptor-proximal signals are sustained in peripheral microclusters and terminated in the central supramolecular activation cluster. *Immunity* **25**, 117-127.
- Vicente-Manzanares, M., Ma, X., Adelstein, R. S. and Horwitz, A. R.** (2009). Non-muscle myosin II takes centre stage in cell adhesion and migration. *Nat. Rev. Mol. Cell Biol.* **10**, 778-790.
- Wang, H., Kadlecik, T. A., Au-Yeung, B. B., Goodfellow, H. E., Hsu, L. Y., Freedman, T. S. and Weiss, A.** (2010). ZAP-70: an essential kinase in T-cell signaling. *Cold Spring Harb. Perspect. Biol.* **2**, a002279.
- Werlen, G. and Palmer, E.** (2002). The T-cell receptor signalosome: a dynamic structure with expanding complexity. *Curr. Opin. Immunol.* **14**, 299-305.
- Williamson, D. J., Owen, D. M., Rossy, J., Magenau, A., Wehrmann, M., Gooding, J. J. and Gaus, K.** (2011). Pre-existing clusters of the adaptor Lat do not participate in early T cell signaling events. *Nat. Immunol.* **12**, 655-662.
- Wouters, F. S., Verveer, P. J. and Bastiaens, P. I.** (2001). Imaging biochemistry inside cells. *Trends Cell Biol.* **11**, 203-211.
- Wülfing, C. and Davis, M. M.** (1998). A receptor/cytoskeletal movement triggered by costimulation during T cell activation. *Science* **282**, 2266-2269.
- Yi, J., Wu, X. S., Crites, T. and Hammer, J. A., III** (2012). Actin retrograde flow and actomyosin II arc contraction drive receptor cluster dynamics at the immunological synapse in Jurkat T cells. *Mol. Biol. Cell* **23**, 834-852.
- Yokosuka, T., Sakata-Sogawa, K., Kobayashi, W., Hiroshima, M., Hashimoto-Tane, A., Tokunaga, M., Dustin, M. L. and Saito, T.** (2005). Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap70 and SLP-76. *Nat. Immunol.* **6**, 1253-1262.
- Yu, C. H., Wu, H. J., Kaizuka, Y., Vale, R. D. and Groves, J. T.** (2010). Altered actin centripetal retrograde flow in physically restricted immunological synapses. *PLoS ONE* **5**, e11878.
- Yu, Y., Fay, N. C., Smoligovets, A. A., Wu, H. J. and Groves, J. T.** (2012). Myosin IIa modulates T cell receptor transport and CasL phosphorylation during early immunological synapse formation. *PLoS ONE* **7**, e30704.
- Zal, T. and Gascoigne, N. R.** (2004). Using live FRET imaging to reveal early protein-protein interactions during T cell activation. *Curr. Opin. Immunol.* **16**, 418-427.
- Zeng, R., Cannon, J. L., Abraham, R. T., Way, M., Billadeau, D. D., Bubeck-Wardenberg, J., Burkhardt, J. K.** (2003). SLP-76 coordinates Nck-dependent Wiskott-Aldrich syndrome protein recruitment with Vav-1/Cdc42-dependent Wiskott-Aldrich syndrome protein activation at the T cell-APC contact site. *J. Immunol.* **171**, 1360-1368.