# Signaling and Polarized Communication Across the T Cell Immunological Synapse

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Annu. Rev. Cell Dev. Biol. 2016. 32:303–25

First published online as a Review in Advance on August 3, 2016

The *Annual Review of Cell and Developmental Biology* is online at cellbio.annualreviews.org

This article's doi: 10.1146/annurev-cellbio-100814-125330



## Keywords

immunological synapse, T cell, polarization, microvesicles, signaling

## Abstract

T cells express a somatically recombined antigen receptor (αβTCR) that is calibrated during development to respond to changes in peptides displayed by major histocompatibility complex proteins (pMHC) on the surface of antigen-presenting cells (APC). A key characteristic of pMHC for adaptive immunity is the ability to sample internal states of cells and tissues to sensitively detect changes associated with infection, cell derangement, or tissue injury. Physical T cell–APC contact sets up an axis for polarization of TCR, adhesion molecules, kinases, cytoskeletal elements, and organelles inherent in this mode of juxtacrine signaling. The discovery of further lateral organization of the TCR and adhesion molecules into radially symmetric compartments, the immunological synapse, revealed an intersecting plane of symmetry and potential for regulated symmetry breaking to control duration of T cell–APC interactions. In addition to organizing signaling machinery, the immunological synapse directs the polarized transport and secretion of cytokines and cytolytic agents across the synaptic cleft and is a site for the generation and exocytic release of bioactive microvesicles that can functionally affect recipient APC and other cells in the environment. This machinery is coopted by retroviruses, and human immune deficiency virus-1 may even use antigen-specific synapses for infection of healthy T cells. Here, we discuss recent advances in the molecular and cell biological mechanisms of immunological synapse assembly and signaling and its role in intercellular communication across the synaptic cleft.

## Contents



## INTRODUCTION: CELLULAR CONTEXT OF THE αβTCR-pMHC RECOGNITION SYSTEM

Adaptive immunity is characterized by de novo assembly of host protective receptors that can bind a diverse array of natural or manmade molecular patterns referred to as antigens. The cells that bear these receptors are referred to as lymphocytes. In gnathostomes (jawed vertebrates), three adaptive lymphocyte types—αβT cells, γδT cells, and B cells—utilize somatic gene rearrangement by recombinase-activating gene products to construct repertoires of antigen receptors that are based on an immunoglobulin (β-sandwich) fold (Davis et al. 1984). Both types of T cells express cell surface T cell receptors (TCR) that recognize antigens through immunological synapses with an antigen-presenting cell (APC), whereas B cells produce cell surface and secreted forms of the same antigen receptor (BCR) and can acquire antigen through an immunological synapse or from solution. The necessity of an immunological synapse for T cells arises from the nature of their development and origin of the preferred ligands of the TCR.

Nascent T cells migrate to the thymus, where the repertoire of developing T cells undergoes a calibration process referred to as clonal selection. The αβT cells survive if they recognize ligands, which are peptides generated by cytoplasmic or endosomal degradation and bound to major histocompatibility complex (MHC) products on the surface of APC, in an appropriate avidity range (Little & Parham 1999). These pMHC complexes juxtapose polymorphic self-restriction elements and self-peptides to generate a highly personalized αβ T cell repertoire that is tuned to the cytoplasmic and tissue steady state of the individual (Kosmrlj et al. 2010). In contrast, γδT cells appear to be selected on specific host surface proteins that display one state in the thymus and can display another state in conditions of stress or infection (Corbett et al. 2014, Sandstrom et al. 2014). The result of thymic education for αβT and γδT cells is a repertoire of cells that

balances competing requirements to recognize diverse pathogens and avoid autoimmunity. T cell development requires an immunological synapse (Bousso et al. 2002, Hailman et al. 2002, Richie et al. 2002).

Adaptive immunity evolved in common ancestors of gnathostomes and agnathans (jawless fish) at least 480 million years ago (Hirano et al. 2013). This realization is based on the study of an alternative system for adaptive immunity discovered recently in agnathans (Holland et al. 2014). These animals utilize a gene conversion mechanism mediated by activation-induced cytidine deaminase to produce three types of variable lymphocyte receptors (VLR) built from tandem leucine-rich repeats (Boehm et al. 2012). There is a class of cells that make secreted, polyvalent antibody molecules, termed VLR-B, that seem analogous to B cells. Furthermore, VLR-A- and VLR-C-bearing cells express only transmembrane forms of these receptors and correspond transcriptionally to αβT cells and γδT cells of gnathostomes. Importantly, no MHC class I or II equivalent has been found in agnathans (Deng et al. 2013). Consistent with this, VLR-A can recognize intact foreign antigens with high affinity, which seems more similar to a gnathostome antibody than to the αβTCR (Deng et al. 2010). Thus, VLR-A- and VLR-C-bearing cells may be fully capable of responding to soluble foreign antigens, unlike gnathostome T cells. VLR-A- and VLR-C-bearing lymphocytes may nonetheless recognize their antigens on other cells in some contexts, because antigen either is an integral membrane protein or is associated with a cell surface (Batista et al. 2001). Although adaptive immunity is a relatively new innovation on an evolutionary timescale, its cellular context predates the evolution of the antigen receptors. In this context we focus on how evolutionarily conserved strategies for polarized cell communication contribute to immune defense by αβT cells (which we refer to below as T cells) through immunological synapse formation.

## THE IMMUNOLOGICAL SYNAPSE: POLARIZATION AND SYMMETRY

### Hair Trigger Signaling with a Safety Mechanism

The immunological synapse describes an entire cell-cell interface covering 50–100 $\mu m^2$ of close approach. This area is only approximately 10–20% of the T cell surface area, so setting up this interface can generate an axis of polarity perpendicular to the plane of contact (**Figure 1**). Seminal studies from Kupfer and colleagues revealed the dramatic polarization of receptors and signaling machinery in T cells interacting with APC (Kupfer & Singer 1989, Kupfer et al. 1987, Monks et al. 1997). Improved resolution through 3D deconvolution revealed a radially symmetric interface in which the TCR-pMHC interactions clustered in the center and the integrin family adhesion molecule LFA-1 organized into a radially symmetric bull's-eye pattern (Monks et al. 1998). Kupfer and colleagues referred to these microscale domains as supramolecular activation clusters (SMACs). Kupfer and coauthors defined three SMACs in two early papers: the central (c)SMAC enriched in TCR and protein C-θ, the peripheral (p)SMAC enriched in LFA-1 and talin, and the distal (d)SMAC enriched in CD45 and F-actin (**Figure 1**) (Freiberg et al. 2002, Monks et al. 1998). Reconstitution of T cell activation by supported lipid bilayers (SLBs) that present bona fide ligands in a laterally mobile single plane provided complementary dynamic information on immunological synapse assembly (Grakoui et al. 1999, McConnell et al. 1986). SLBs presenting the CD2 ligand CD58 and the LFA-1 ligand intercellular adhesion molecule-1 (ICAM-1) demonstrated striking segregation of these adhesion systems during adhesion of activated T cells (Dustin et al. 1998), confirming Springer's (1990) earlier speculation. SLB systems containing only pMHC and ICAM-1 induced T cell proliferation and recapitulated the SMAC pattern (Dustin et al. 1998, Grakoui et al. 1999). More importantly, the SLB model allowed the dynamics of the immunological synapses to be quantitatively studied, revealing phases of rapid

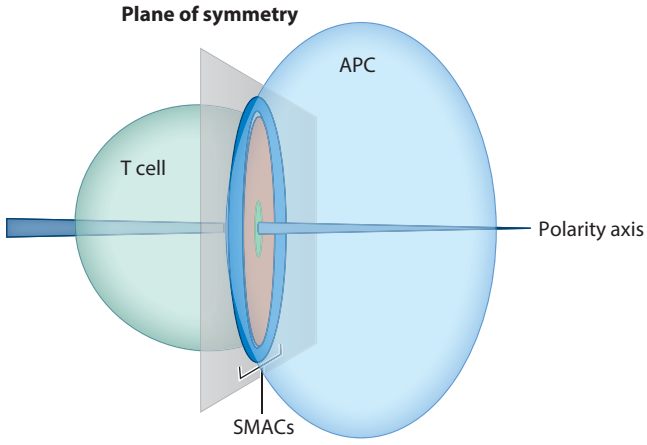


**Figure 1**

Polarity and symmetry in the mature immunological synapse. Supramolecular activation clusters (SMACs) are shown (cSMAC, *green*; pSMAC, *red*; dSMAC, *blue*). The axis of directed secretions is the spear passing through the cSMAC. The plane of symmetry is the plane of the immunological synapse, which is radially symmetric when stable. The T cell will migrate if the adhesion ring breaks symmetry. Abbreviations: APC, antigen-presenting cell; cSMAC, central SMAC; dSMAC, distal SMAC; pSMAC, peripheral SMAC.

spreading with peripheral TCR engagement followed by centralization of the TCR and formation of the adhesion ring (Grakoui et al. 1999). The SLB system also allowed for estimation of 2D dissociation constants and the number of TCR and LFA-1 engaged. On the basis of these results, we proposed the terms nascent immunological synapse and mature immunological synapse to describe the pattern evolution, as the nature of the SMACs was transitional and evolved over time. This radially symmetric structure is organized to move receptor clusters toward the center by using F-actin-based motility in a dynamic balance. Thus, the immunological synapse is a symmetric interface that centers the perpendicular secretory polarity axis toward the APC (**Figure 1**). More recent work has tracked activation events to microclusters in the periphery of the mature immunological synapse, and we start with a discussion of this fundamental signaling unit (Bunnell et al. 2002, Campi et al. 2005).

In the context of feed-forward amplification from regulated adhesion and costimulatory systems, T cells achieve single-molecule sensitivity to pMHC (Bachmann et al. 1997, Dustin & Springer 1989, Irvine et al. 2002, Kupfer & Singer 1989). This performance is dependent on an intact actin cytoskeleton (Valitutti et al. 1995, Varma et al. 2006). The minimal components needed for regulated T cell signaling were identified in the late 1980s (Weiss 2010). A formal demonstration of the minimal requirements for T cell signaling in live cells was recently accomplished by reconstitution of TCR signaling components in the 293T stromal cell line (James & Vale 2012). The TCR complex lacks intrinsic catalytic activity but has 10 cytoplasmic immunotyrosine activation motifs, which have pairs of tyrosines. The TCR complex associates with three non–receptor tyrosine kinases to phosphorylate these sites and transduce signals into the cell. However, coexpression of the TCR complex with two of these kinases, lymphocyte kinase (Lck) and ζ-associated kinase of 70 kDa (ZAP-70), results in spontaneous activation. Such activation can then be turned off by (*a*) the Lck negative regulatory kinase C-terminal Src kinase (Csk), (*b*) a docking protein needed to bring Csk to the membrane, and (*c*) the transmembrane tyrosine phosphatase CD45 (**Figure 2**). CD45 counteracts the inhibitory activity of Csk by dephosphorylating the inhibitory C-terminal tyrosine and locally suppresses the tyrosine kinase cascade. Thus,

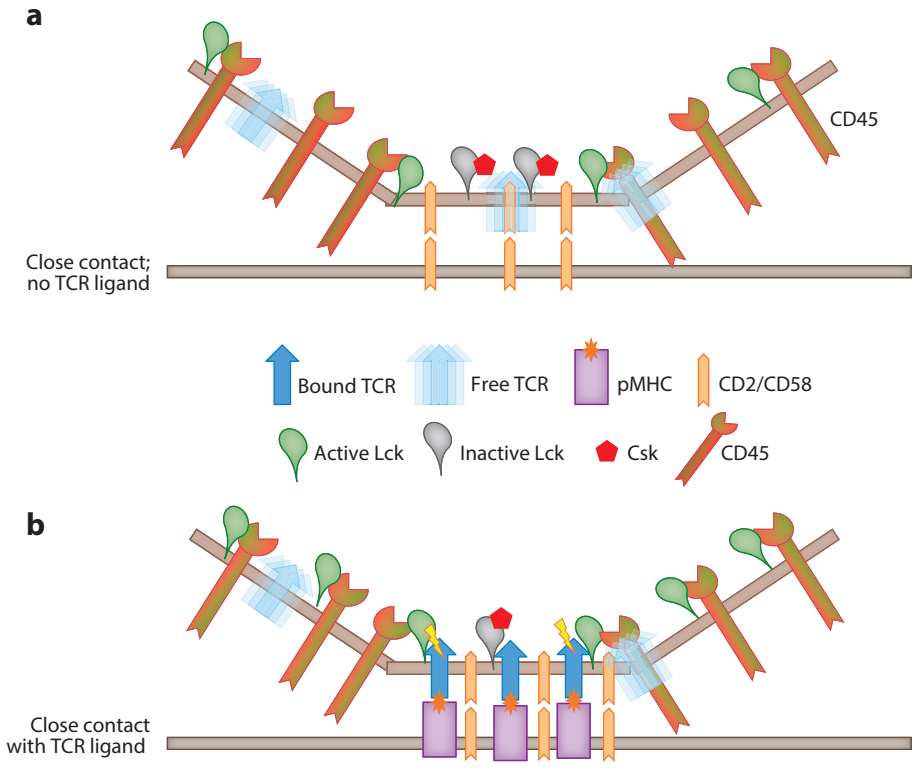


**Figure 2**

Csk kill switch for close contacts. A potential problem with kinetic segregation–based triggering models is that small adhesion molecules like CD2 and CD58 (*light orange links*) may signal aberrantly. Csk (*red pentagons*) may act as a kill switch to prevent such aberrant signaling. (*a*) Outside contact areas, CD45 (*red and green lollipop shapes*) keeps Lck (*teardrop shape*) in active form (*green*) by removing C-terminal phosphates. CD45 prevents this active Lck from initiating signaling with free TCR (*blurred blue arrow*) by dephosphorylating immunotyrosine activation motifs (ITAMs). Formation of close contacts by CD2 and CD58 (*light orange links*) excludes CD45 and traps some active Lck and free TCR. Aberrant signaling is prevented by Csk, which kills activity of Lck (*gray*) that does not have immediate access to CD45. TCR that is near enough to the edges of the close contact to access active Lck also has access to CD45, and signaling is attenuated. (*b*) When the TCR is engaged, the Lck near the outer 0.5 μm of the cluster has ready access to CD45, which counteracts Csk, but phosphorylated TCR ITAMs are kept away from CD45 by interaction with the pMHC (*orange star and purple rectangle*) such that the tyrosine kinase cascade can be activated (*lightning bolts*).

293T cells with all these components at physiological levels have a quiescent TCR that is poised to respond to pMHC on an APC in the context of adhesion molecules, but this TCR does not respond upon adhesion to an APC lacking the cognate pMHC (James & Vale 2012). The absence of CD45 or Csk, even in the absence of pMHC ligands, results in runaway signaling. Although it has been shown that events downstream of TCR triggering result in the release of Csk from the T cell membrane, the mechanism by which TCR engagement brings about this release is not clear (Brdicka et al. 2000). These reconstitution results emphasize the critical importance of the regulatory triad of Lck, CD45, and Csk in explaining how the TCR might be triggered.

The recent elucidation of the structure of the CD45 extracellular domain provides a specific mechanism for triggering by local exclusion of CD45 from close contacts formed by the αβTCR

interacting with pMHC (Chang et al. 2016). CD45 has a large extracellular domain, and the smallest splice variant projects ~22 nm from the cell surface, as revealed by structural studies and biophysical measurements of the CD45 extracellular domain on a membrane surface (Chang et al. 2016). Thus, CD45 is quantitatively excluded from areas in which the T cell and APC membranes are close enough to allow TCR-pMHC interaction consistent with the kinetic segregation model proposed by Davis & van der Merwe (2006) and supported by experimental observations (Choudhuri et al. 2005, Varma et al. 2006). Physical experiments in model membranes demonstrate that the thermodynamic drive to exclude molecules just slightly larger than the intermembrane space is surprisingly strong due to thermal fluctuations in the membranes, entropic penalties for restricting the tilting of the larger domain, and lateral crowding (Schmid et al. 2016). Experiments manipulating the extracellular domains of adhesion molecules, pMHC, and CD45 support the importance of CD45 exclusion in TCR triggering (Choudhuri et al. 2005, James & Vale 2012, Wild et al. 1999). In vitro reconstitution studies also support the conclusion that reduction of CD45 density in the presence of TCR cytoplasmic domains and Lck leads to increased signaling (Hui & Vale 2014). A challenge raised by Chang et al. (2016) is whether cells can closely adhere to each other without triggering signaling due to the size differential between CD45 and the TCR. CD2 is a particularly problematic adhesion molecule in this regard, and even integrins can exclude CD45 in the context of immunological synapses (Freeman et al. 2016, Graf et al. 2007). A kill-switch mechanism is needed to prevent adhesion events in vivo from nonspecifically activating T cells.

We propose that Csk is the kill switch that keeps the TCR from going off "half-cocked" without a pMHC ligand. The kinetic segregation model incorporates the requirement that the diffusing TCR needs to be held in a close contact for a period of time (on the order of 2 s) (Dushek et al. 2012). The measured diffusion coefficient of the TCR is 0.05 $\mu m^2/s$, and if the close contact area has a radius of 0.1 μm, then the average dwell time of the TCR in the close contact is 0.1 s, which then increases with the square of the close contact radius. A variety of measurements, including the time required for a T cell to initiate signaling in response to agonist pMHC, suggest that the critical dwell time to generate a signal must be less than 2–3 s (Huse et al. 2007, Stepanek et al. 2014). Thus, antigen specificity is maintained if close contacts are either transient (<1 s) or small (<0.3-μm radius). The spatiotemporal dynamics of spontaneous T cell probing of neutral surfaces in vitro are consistent with T cells forming close contacts over small areas (~0.2 $\mu m^2$) for short times (<1 s) (Brodovitch et al. 2015). Erroneous signaling in larger close contacts mediated by small adhesion molecules or activated integrins without pMHC may be prevented by the requirement of CD45 to maintain Lck activity in the face of phosphorylation of the inhibitory C-terminal tyrosine by Csk (Tan et al. 2014). Large close contacts mediated by CD2 do generate partial signals in T cells (Kaizuka et al. 2009), but Csk is critical for preventing this effect from spiraling out of control. Other safety mechanisms may include the tendency of the ITAM tyrosines to be buried in the bilayer in the resting state and thus not be readily accessible to kinases (Xu et al. 2008). Cytoplasmic $Ca^{2+}$ elevation is a key signal that releases these interactions and so could be seen as an amplification mechanism (Gagnon et al. 2012, Shi et al. 2013). These triggering processes will thus be most efficient when the TCR is trapped by its interaction with pMHC in submicrometer microclusters for >2 s. If the TCR is trapped in small close contacts by other mechanisms, the T cell will likely be activated. The ability of glycan-binding lectins to orchestrate close contacts that trap TCR may be why some relatively nonspecific reagents are such potent mitogens. Similarly, we anticipate that the signaling process triggered by adhesion to glass (described in Chang et al. 2016) is due to the physical trapping of the TCR in the close contacts.

Lck movement is in part controlled by coreceptors that bind to the MHC class I or II molecules, referred to as CD8 and CD4, respectively. The interaction of coreceptors with MHC molecules is particularly weak, and obtaining accurate measurements has been challenging. Recent studies

utilizing SLBs have succeeded in measuring the 2D $K_d$ for interaction of CD4 and MHC class II in the context of CD2-CD58-mediated close contacts (Jönsson et al. 2016). These studies demonstrate that coreceptors have ~100-fold-weaker interactions than do bona fide adhesion molecules (Dustin et al. 2007). Mathematical modeling suggests that such weak interaction can contribute to a log increase in the rate of signaling complex assembly (Jönsson et al. 2016), which is consistent with data on the contribution of CD4 to helper T cell functional responses to pMHC.

## The Connection to F-Actin

Immunological synapse formation is F-actin dependent (Grakoui et al. 1999, Valitutti et al. 1995), whereas microtubules and intermediate filaments are largely dispensable for radial pattern formation. T cell activation is also favored by presentation of pMHC on surfaces with dimensions similar to that of an APC (Mescher 1992). The coupling of antigen receptors to F-actin and myosin II contractility was noted in the 1970s through fluorescence microscopy analysis of patching and capping of polyvalent mitogens (lectins) and antibodies to antigen receptors (Braun et al. 1978). Analysis of patching was further extended by electron microscopy in membrane sheets, which revealed distinct patching of cross-linked Fc receptor (a type of antigen receptor that binds soluble antibodies or immune complexes) into osmium-dense structures (enriched in unsaturated lipids) that also recruited clathrin-coated pits for internalization (Wilson et al. 2004). TCR can also be triggered by cross-linking with antibodies, and this approach is still common in signaling studies. Nanoparticles coated with multiple pMHC were recently found to induce T cell tolerance in vivo (Clemente-Casares et al. 2016), demonstrating interesting new properties at this intermediate scale between soluble antibody cross-linking and antigen presentation by APC.

Once the TCR-proximal kinase cascade is ignited, the engaged TCR needs to recruit a palmitoylated adaptor, linker of activated T cells (LAT), to propagate signaling downstream (Samelson 2002). The TCR and LAT are basally segregated in submicrometer domains referred to by Lillemeier et al. (2006) as protein islands. Progressive LAT phosphorylation serves as a scaffold for the cooperative assembly of a macromolecular signaling complex (known as the LAT signalosome) by recruitment of PLC-$\gamma$1, Grb2/Sos, and Gads/SLP-76/Vav/NCK/WASP (Barda-Saad et al. 2005). A third non–receptor tyrosine kinase, Itk, phosphorylates and contributes to the activation of PLC-$\gamma$1. Assembly of the LAT signalosome is central to signal transmission into the cell interior by generating the second messengers inositol trisphosphate and diacylglycerol. In vitro reconstitution of the LAT signalosome reveals that the multicomponent signal complex represents a separate fluid phase at a membrane interface and has an intimate reciprocal interaction with F-actin (Su et al. 2016). Such observations are consistent with PLC-$\gamma$ activation by WASP-dependent F-actin foci associated with TCR and LAT microclusters (Kumari et al. 2015).

## Polarity in the Search for pMHC

Many metazoan cells exhibit stable polarity in the distribution of molecules in cell membranes and the cytoplasm and in the position of cellular organelles; such positioning is often determined by developmental and environmental cues (Bryant & Mostov 2008, Nelson 2003). Cell polarity is established by the asymmetric distribution and signaling of canonical polarity proteins such as the PAR, Scribble, and Crumbs complex, which acts through small GTPases to direct organelle distribution, cytoskeleton organization, and vesicle transport (Arsenio et al. 2015, Krummel & Macara 2006, Russell 2008). An archetypal example of cell polarization is the stable apical-basal polarization of epithelial cells. These cells form planar sheets lining luminal surfaces that are

attached to a basement membrane made of actively deposited extracellular matrix. Cell adhesion to the extracellular matrix provides critical polarity cues for establishing planar cell polarization. Polarization is achieved by repositioning of the actin and microtubule cytoskeleton and cellular organelles and by redirection of vesicle trafficking and endo/exocytosis to establish and maintain asymmetric apical-basal protein, cytoskeleton, and organelle distributions, which are important for secretory functions and cell adhesion. A more dynamic polarization occurs in migrating cells during embryonic development or as part of tissue remodeling. In migrating keratinocytes, for instance, a front-rear polarity is maintained to drive motility in response to extracellular gradients and anatomical cues. T cells not only reversibly utilize front-rear polarity during motility in their search for antigen in lymphoid tissues (Krummel & Macara 2006), but also undergo periods of motility arrest upon detecting antigen, to form an immunological synapse polarized toward the APC surface (**Figure 1**) (Mempel et al. 2004, Sims et al. 2007).

T cells are highly polarized and motile cells that relentlessly scan lymphoid tissues in search of antigen (Miller et al. 2002). G protein–coupled chemokine receptor sensing of stromal cell chemokines drives this search with relatively low adhesion of T cells to surrounding stromal cells and potential APC (Woolf et al. 2007). In lymphoid tissues, the key chemokines are produced by fibroblastic reticular cells (FRC) that form 3D networks with many branch points (Bajenoff et al. 2006). FRC secrete CCL19 and -21, which confine T cell migration territories and contribute to T cell segregation from B cell zones, as T cells prefer to remain in contact with the CCL19/21-bearing surfaces (Cremasco et al. 2014, Malhotra et al. 2012). Deletion studies show that FRC are essential for antiviral T cell responses and control B cell survival and antibody responses through generation of B cell–activating factor (Cremasco et al. 2014). The decision of T cells to turn or persist at branch points is controlled, at least in part, by a type I myosin, Myog1, which promotes turning behavior in T cells at the expense of overall speed and increases search efficiency (Gerard et al. 2014). Short bursts of fast, linear motility, in combination with random turns, promote more efficient antigen search strategies in peripheral tissues. For instance, $CD8^+$ T cell migration at sites of infection exhibits so-called Lévy random walk motility, which is superior to purely Brownian random walk motility in maximizing the search volume (Harris et al. 2012).

## Antigen-Induced Motility Arrest and Symmetry Breaking

Agonist pMHC can arrest T cells through a $Ca^{2+}$-dependent mechanism (Negulescu et al. 1996, Skokos et al. 2007) or a $Ca^{2+}$-independent mechanism (Dustin et al. 1997, Waite et al. 2013), depending upon the differentiated state of the T cells. The LAT signalosome generates intracellular inositol trisphosphate through the recruitment and activation of PLC-$\gamma$, which triggers the release and depletion of endoplasmic reticulum $Ca^{2+}$ stores. $Ca^{2+}$ store depletion leads to clustering of the endoplasmic reticulum $Ca^{2+}$ sensor Stim1 and promotes the association of Stim with Orai dimers to form the tetrameric capacitative release–activated $Ca^{2+}$ (CRAC) channels at the T cell plasma membrane (Penna et al. 2008), resulting in prominent elevation of intracellular $Ca^{2+}$ concentration. Capacitative $Ca^{2+}$ entry is a hallmark of early antigen-induced T cell activation and is essential for transcriptional activation of nuclear factor of activated T cells (NFAT) (Oh-hora 2009). Periods of T cell motility arrest correspond to formation of immunological synapses. However, with APC such as dendritic cells (Tseng et al. 2008) and in vivo, the symmetry is not as apparent (Dustin 2007, Sims et al. 2007).

The transition from rapid scanning motility ($\sim$12 μm/min) to stable immunological synapses ($<$2 μm/min) can take hours for naive T cells and may proceed through a mobile close contact referred to as a kinapse (Dustin 2007). Although methods to categorize this semimotile signaling

state in vivo is still a work in progress, kinapses are readily observed on SLBs or glass substrates in vitro, where they exhibit a characteristically large contact area, high levels of LFA-1/ICAM-1 interaction, and lack of symmetry (Sims et al. 2007).

It has been proposed that the immunological synapse can set up asymmetry in the first cell division of naive or memory $CD8^+$ T cells and may constitute a deterministic mechanism for the generation of effector cells while also generating or maintaining a pool of memory T cells (Chang et al. 2007, Ciocca et al. 2012). Asymmetric partitioning of proteasomes organized by the immunological synapse, and more recently components needed for energy metabolism, may also promote antiviral fates on the basis of the selective degradation of transcription factors (Chang et al. 2011, Verbist et al. 2016). However, T cell transfer experiments in mice have generated different outcomes in different labs with respect to the requirement for asymmetric division in establishment of $CD8^+$ T cell memory (Buchholz et al. 2013, Lemaitre et al. 2013). Thus, although asymmetric division organized by the immunological synapse has been demonstrated, how it contributes to in vivo immune responses requires additional study.

## ANTIGEN-INDUCED CYTOSKELETAL POLARIZATION

### Actin Polymerization and Mechanical Force

There has been recent interest in mechanical forces and how they impact signaling. There are two classes of mechanisms—one in which the force induces a conformational change in the TCR that is part of the triggering process (Das et al. 2015) and another in which the force results in catch bond behavior that is important for determining the TCR:pMHC dwell time—but otherwise no explicit conformation change is evoked to achieve triggering (Liu et al. 2014). Similarly, it has been proposed that forces in the immunological synapse have a role in control of integrin activation (Zhu et al. 2008). The force required for these transitions is generally on the order of 10 pN. Very little force is needed to move CD45 laterally in the cell membrane, but the F-actin-dependent transport process may involve protrusions that help to drive close contact (Sage et al. 2012).

T cells encounter antigen in highly specialized environments that combine a confining 3D scaffold with abundant chemokinetic cues to drive rapid amoeboid migration (Bajenoff et al. 2006, Gerard et al. 2014, Worbs et al. 2007). The leading-edge protrusions driven by F-actin polymerization generate forces on the order of 25–75 pN, and the leading edge is the most sensitive pole of the T cell for detection of pMHC (Negulescu et al. 1996, Yang et al. 2015). T cells on nanopillar substrates coated with antibodies to the TCR tend to first push and then pull with forces on the order of 25–100 pN (Bashour et al. 2014). Traction force microscopy suggests that T cells generate integrated forces of 1–2 nN, which is consistent with the nanopillar results focusing on force generated at single points of contact (Hui et al. 2015). Recent studies with a DNA-based force gauge further confirm that T cells exert forces in the 10–20-pN range on TCR-pMHC interactions (Liu et al. 2016). The pushing force triggered by TCR signaling is mediated by WASP-dependent actin polymerization at TCR microclusters, which also appears to have a role in amplification of PLC-$\gamma$ recruitment and activation (Kumari et al. 2015). Dendritic cells are much stiffer than T cells such that T cell protrusive forces will likely be translated into rearrangement of surface proteins, which can be achieved at lower forces for laterally mobile proteins like CD45 (Bufi et al. 2015, Jönsson et al. 2012). Along these lines, the tissue microenvironment changes during an immune response. For example, CLEC-2 on activated dendritic cells induces relaxation of fibroblastic reticular scaffold cells through binding to podoplanin (Acton et al. 2014, Astarita et al. 2015). This relaxation of the stroma allows expansion of lymph nodes to accommodate more T

and B cells but may also make the environment less stiff. Forces generated by cells may alter the kinetics of the TCR-pMHC interaction, and thus alter the dwell time, leading to changes in T cell sensitivity to pMHC in the context of infection.

One way to approach the question of force sensing by T cells has been to use elastic substrates of varying mechanical stiffnesses. When polydimethylsiloxane (PDMS) was used to vary the stiffness of the substrate, T cells made more IL-2 and divided more times in response to the softer PDMS substrates (O'Connor et al. 2012), which approached the stiffness of a dendritic cell. However, even softer hydrogels were less effective at activating T cells than were stiffer hydrogels (Judokusumo et al. 2012). In this context we should make a distinction between the lateral mobility of lipid-anchored molecules in SLBs and elastic substrates such as hydrogels, PDMS, and PDMS pillars. SLBs are mechanically complex in that lateral movement of lipid-anchored proteins is controlled by the viscosity of the bilayer. Thus, with a liquid disordered bilayer, the force required to change the path of a diffusing protein is only ~5 fN (Jönsson et al. 2012). In contrast, vertical movement in SLBs is linked to the rigid glass substrate and eventually requires ~100 pN force over a distance of 1–2 nm to extract the lipid from the bilayer, which is similar to the case for the most rigid PDMS substrates. The use of more viscous liquid ordered bilayer phases in SLBs creates some useful effects, but care is needed in their interpretation. In these systems, lateral mobility can be very slow on the timescale of biological experiments, but cellular force generation can still move molecules laterally, leading to the formation of clusters. This effect was recently exploited to generate unique cellular responses to reconstituted adhesion molecules that could not be triggered by more fluid liquid disordered or completely immobile gel-phase SLBs (Biswas et al. 2015). Molecules covalently attached to elastic substrates will be physically displaced with force-distance relationships in the 2–30 pN/nm range (Ghassemi et al. 2012), but the molecules should return to their original position when the force is released, which is not the case for high-viscosity SLBs, in which clusters formed by cellular forces remain even when force is released. Therefore, the forces experienced by T cells at the molecular and cellular levels are likely to be very different for viscous SLB substrates and elastic hydrogels or PDMS substrates. Artificial substrates tend to isolate individual components of the physical repertoire of a dendritic cell (Comrie et al. 2015). Although different materials allow for systematic variation of stiffness, other parameters change at the nanoscale and can impact interpretation (Trappmann et al. 2012). For example, hydrogels are much less interactive with cell surfaces than silicates like PDMS. Thus, the signaling activity on close approach of cells to glass reported by Chang et al. (2016) may also promote T cell responses to PDMS substrates. It will be important to determine whether there is any nonspecific interaction of TCR with softer PDMS that could enhance signaling by a mechanism independent of mechanical effects. Hydrogels are not immune to these issues; mesh size of less cross-linked hydrogels may differ in ability to exclude molecules like CD45 from contact areas, as the surfaces are porous on a molecular length scale, and molecules like CD45 may be able to enter close contacts by a hop diffusion process (Kusumi et al. 2005). Measurements in supported planar bilayers suggest that cytoskeletal force decreases the half-life of the TCR-pMHC interaction (Huppa et al. 2010), but a second study did not observe accelerated dissociation (O'Donoghue et al. 2013). The TCR and major integrin family adhesion molecules in T cells have very distinct mechanisms of interaction with the actin cytoskeleton but likely integrate F-actin networks to control synapse stability (Sims et al. 2007, Tabdanov et al. 2015).

## MTOC Reorientation

The movement of the microtubule-organizing center (MTOC) to the interface between cytotoxic T cells and target cells is one of the seminal observations leading to the concept of a polarized

immunological synapse (Geiger et al. 1982). The MTOC, also referred to as the centrosome, includes two centrioles and associated pericentriolar material serving as a nucleation center for microtubules. The microtubules' minus ends are attached to the MTOC, and the plus ends radiate out to the cell periphery. As the name centrosome implies, the MTOC is a distribution center of the cell and organizes transport of vesicles and organelles within the cytoplasm. That T cells are relatively small cells does not divorce them from the need for such organization. The mechanism by which the MTOC moves to the synapse requires both the minus end–directed motor dynein and the ability to depolymerize microtubules (Yi et al. 2013). This scenario suggests an end-capture-shrinkage mechanism anchored to the center of the nascent synapse. The oscillation of the MTOC between two immunological synapses formed with different target cells has also been observed (Kuhn & Poenie 2002). Dynein is recruited to TCR signalosomes, raising the possibility that the TCR signalosome may be directly responsible for MTOC movement to the synapse (Hashimoto-Tane et al. 2011).

### Intraflagellar Transport Proteins

Most cells, with the exception of hematopoietic cells, have a primary cilium that is initiated by docking of one of the centrioles to the plasma membrane (Singla & Reiter 2006). The T cell side of the immunological synapse may capture machinery used by other cells in assembly and function of the primary cilium and flagella (Singla & Reiter 2006). Specifically, intraflagellar transport proteins play important roles in delivery of TCR to the immunological synapse (Finetti et al. 2009). The primary cilium is the site of Hedgehog signal reception, and recent studies have shown that T cells also receive signals from Indian Hedgehog to regulate Rac expression and sustained cytotoxic activity (de la Roche et al. 2013). Additional proteins are important for delivery and turnover of signaling proteins during sustained signaling, which is critical for T cell proliferation and differentiation (Iezzi et al. 1999). These events include delivery of TCR, LAT, and Lck in distinct vesicular carriers (Soares et al. 2013) and removal of TCR from the interface by the proteins TC21 and RhoG (Martinez-Martin et al. 2011).

## POLARIZED SECRETION

Polarized secretion is a hallmark of classical synapses and one of the defining characteristics of immunological synapses (Dustin & Colman 2002, Poo et al. 1988). Polarized secretion is particularly well studied in effector T cells, with cytotoxic T cells releasing prestored perforin and granzyme from large secretory lysosomes and helper T cells releasing a variety of cytokines that regulate immune responses.

### Secretory Granules in Cytolytic T Cells

$CD8^+$ cytotoxic T cells kill by two distinct mechanisms: (*a*) $Ca^{2+}$-dependent secretion of perforin and granzyme B stored in lysosome-like compartments and (*b*) $Ca^{2+}$-independent expression of Fas ligand. Cytotoxic T cells readily form immunological synapses with target cells and release granules into a well-defined secretory domain (Stinchcombe et al. 2006), but mature immunological synapse formation with MTOC polarization may not always be essential for target cell killing (Bertrand et al. 2013, Purbhoo et al. 2004). TCR signalosomes have characteristics of podosomes, which are secretory specializations in other cell types (Kumari et al. 2015, Sage et al. 2012). Regardless of whether a synapse and MTOC reorientation is required, the release of granules is highly regulated and appears to involve a priming step involving delivery of proteins from

earlier endosomal compartments to the plasma membrane (Marshall et al. 2015). There is also interest in how cortical F-actin in the immunological synapse accommodates directed secretion. In innate-like NK cells, there is evidence for a sparse cortical F-actin network with holes large enough for granules to fit through (Brown et al. 2012), similar to the concept of a synaptic grid in neurons. In contrast, a more extensive F-actin clearing may take place in cytotoxic T cell synapses (Ritter et al. 2015).

### Cytokine Secretion

$CD4^+$ helper T cells do not appear to store cytokines in granules as do cytotoxic T cells. Rather, helper T cells constitutively release the cytokines they are making through either polarized or nonpolarized pathways. Circumstantial evidence for polarized secretion on the basis of the proximity of the Golgi apparatus to the synapse has been called into question, as both proteins secreted in polarized pathways and those secreted in nonpolarized pathways pass through the Golgi apparatus. Janeway and colleagues utilized a model system based on trapping T cells in a filter, delivering anti-CD3 to one side and noting that IL-4 accumulated preferentially on the same side of the filter (Poo et al. 1988). An alternative approach based on a precision application of cytokine capture and detection on a stimulatory substrate demonstrated that interferon-$\gamma$ (IFN-$\gamma$) is also released from helper T cells in a polarized manner but that TNF and chemokines are released in a nonpolarized manner (Huse et al. 2006). The nondirectional release of chemokines by cytotoxic T cells can exacerbate immunopathology by recruiting myelomonocytic cells in the context of viral meningitis (Kim et al. 2009).

## POLARIZED VESICLE TRANSFER

### T Cell Microvesicle Biogenesis

Extracellular vesicles are typically thought to arise through budding and scission from the limiting membrane of specialized late endocytic compartments known as multivesicular bodies (MVBs) (Babst 2011). Nascent intraluminal vesicles (ILVs) at the MVB limiting membrane are formed by late-acting members of ESCRT (endosomal sorting complex required for transport) proteins (McCullough et al. 2013), which form distinct macromolecular assemblies that mediate outward membrane budding and bud neck scission, resulting in the accumulation of ~50–100-nm ILVs into the MVB lumen. Exocytosis of MVBs, through intracellular $Ca^{2+}$-dependent mechanisms, leads to release of their ILVs as extracellular vesicles, often termed exosomes. The release of extracellular vesicles by T cells has long been observed (Blanchard et al. 2002, Esposito et al. 2014), although the mechanisms that lead to the formation and release of such vesicles are still being worked out. Early studies in $CD8^+$ T cells detected the presence of TCR-containing membranous particles along with typical electron-dense cargo in cytolytic granules (Peters et al. 1989). Activation of $CD4^+$ T cells by anti-TCR antibodies on surfaces resulted in the release of vesicles containing $\alpha\beta$TCR and TSG101 (Blanchard et al. 2002). Polarized release and transfer of TCR by cytotoxic T cells to APC were recently observed during antigen-induced exocytosis of cytolytic granule contents (Ritter et al. 2015). The regulated secretory pathway in cytolytic T cells (Holt et al. 2006), controlled in part by Rab27 and Munc 13-4, is presumably also involved in vesicles released by this route.

However, the subcellular origin of T cell–derived microvesicles has been difficult to pin down, because definitive markers for MVB origin are yet to be identified (Cocucci & Meldolesi 2015). Nevertheless, more recent studies in $CD4^+$ T cell lines suggest that extracellular vesicles

containing tetraspanins (CD63), which are thought to be markers of exosomes, are released at the immunological synapse and are transferred to target cells by antigen-triggered exocytosis (Mittelbrunn et al. 2011). As with extracellular vesicles in cytolytic granules, these exosomes also contain TCR, although they appear not to be highly enriched in TCR, as is the case for tetraspanins and other markers. It is unclear whether the TCR contained in exosomes has any role in targeting the vesicles to cells bearing relevant pMHC.

We recently uncovered a second route of extracellular vesicle generation in primary $CD4^+$ T cells; this route is induced by recognition of cognate pMHC on APC. These extracellular vesicles arise from the cSMAC of the immunological synapse, which forms as a result of actin-mediated surface transport and accumulation of TCR at the synapse center. These inactive TCR, which are dephosphorylated, were thought to be internalized at the cSMAC for degradation within lysosomes. In a surprising twist, we found that >80% of TCR at the cSMAC, formed with supported planar bilayers containing pMHC and ICAM-1, are released from the T cell surface as TCR-enriched, plasma membrane–derived extracellular vesicles (ectosomes) (Choudhuri et al. 2014). Not surprisingly, these T cell ectosomes are also generated by ESCRT-dependent mechanisms (Choudhuri et al. 2014, Vardhana et al. 2010). In contrast to the high TCR levels present in these ectosomes, they are not enriched in typical exosomal markers, reflecting their cell surface origin. Synaptic ectosomes have sufficient TCR to fasten themselves to surfaces bearing relevant pMHC and are thus antigen targeted. It is not known whether $\gamma\delta$T cells release ectosomes enriched for TCR, and there is no evidence that B cells release ectosomes with surface immunoglobulin even on planar bilayer supports. The latter is not expected, as B cells must be specialized to harvest antigens. Thus, we would caution against equating appearance of an antigen receptor–positive cSMAC in a fluorescence microscope with extracellular vesicle release without verification by electron microscopy or other high-resolution methods (**Figure 3**).

Taken together, these recent findings suggest that antigen-induced production and polarized release of exosomes and ectosomes are common processes in cytolytic and helper T cell immunological synapses. The many possibilities by which both membrane-associated and cytoplasmic cargo could be sorted to exosomes and ectosomes, combined with the different mechanisms of release, point to substantial diversity in composition that may be of functional importance. Much work remains to be done in dissecting the biogenesis of T cell–derived ectosomes and in uncovering mechanisms by which synaptic ectosomes influence immune function.

## Extracellular Vesicles in Intercellular Communication

T cell synaptic ectosomes are enriched in TCR due to their release from the cSMAC, where TCR accumulates, and remain engaged to cognate pMHC displayed on the apposed APC surface (Choudhuri et al. 2014). Consequently, released TCR-enriched ectosomes are transferred across the synaptic cleft to B cells bearing cognate pMHC at T cell–B cell synapses. Engagement of cognate pMHC on B cells by T cell ectosomes initiates early signaling in B cells, causing their internalization by pMHC-dependent endocytosis. Although the precise mechanisms are unclear, internalized T cell ectosomes continue to signal in B cells. Transsynaptic transfer and uptake of T cell ectosomes raise the possibility that they support B cell signaling from endocytic compartments well after T cells have disengaged and moved on in their search for other antigen-presenting targets. Numerous experiments show that, in permissive cytokine environments, multivalent pMHC engagement (cross-linking) supports proliferation and antibody production by activated (antigen-primed) B cells (Andre et al. 1994, Lang et al. 2001, Monroe & Cambier 1983).

Production of TCR on ectosomes and exosomes appears to reconcile the decades-old observation of soluble TCR present in supernatants of activated T cells. Strikingly, TCR-containing

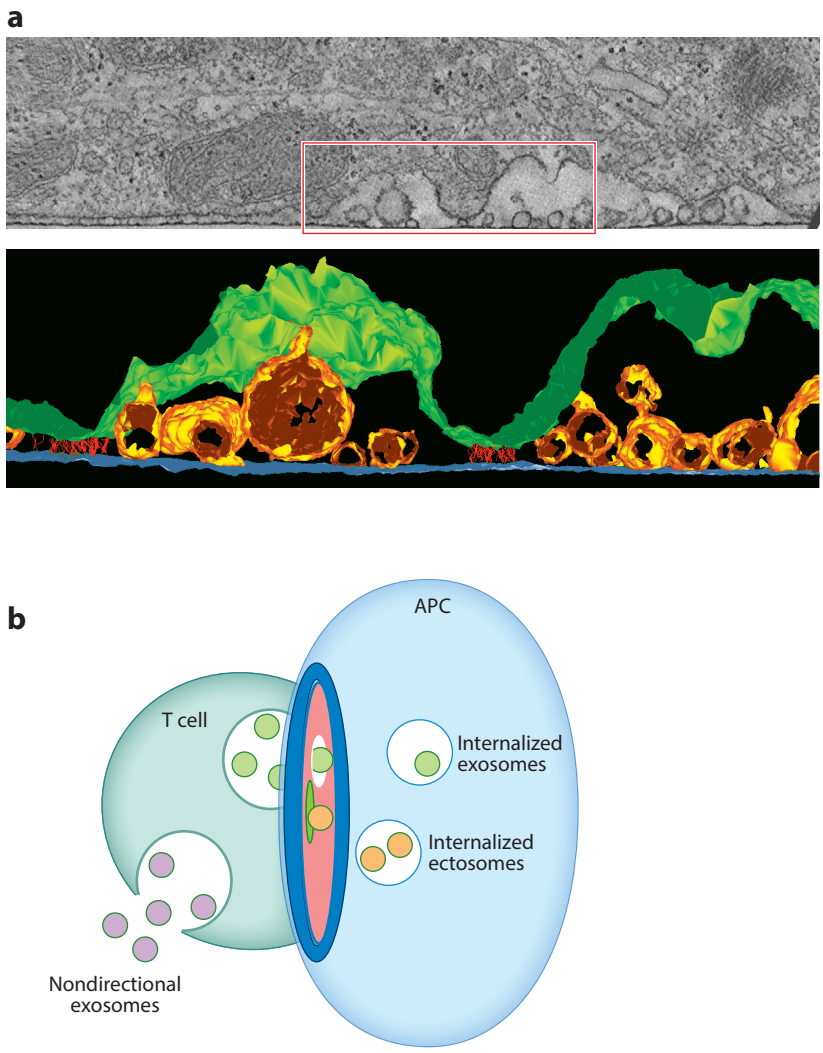


**Figure 3**

Synaptic ectosomes by electron tomography and modes of extracellular vesicle release. (*a*) (*Top*) A 50-nm-deep electron tomogram. (*Bottom*) A model generated by tracing the plasma membrane (*green*), vesicles (*gold*), and supported lipid bilayer (*blue*) of the boxed area of the tomogram in the top subpanel. This image clearly identifies extracellular vesicles between the $CD4^+$ T cells and the supported planar bilayer containing cognate pMHC and intercellular adhesion molecule-1. Images in panel *a* courtesy of David Stokes & Jaime Llodra. (*b*) Schematic showing three release routes for extracellular vesicles from T cells: exosomes that are released nondirectionally, exosomes released into the synapse and taken up by the antigen-presenting cell (APC), and ectosomes that bud from the central supramolecular activation cluster and are taken up by the APC. Vesicle colors reflect potentially different compositions, although whether these different compositions exist is not known.

supernatants were sufficient to induce antibody secretion by primed B cells displaying cognate pMHC antigens (Guy et al. 1989). No known mechanism exists for cleavage and release of TCR from the cell surface, raising the strong possibility that TCR released in culture supernatant are instead present on extracellular vesicles. Whether TCR-enriched ectosomes provide prolonged help in mobilizing antigen-specific B cell effector functions will require further investigation. T cell ectosome transfer to B cells may be critical in longer-term signal integration by APC that are visited for a few hours by helper $CD4^+$ T cells in vivo.

Transsynaptic uptake of T cell ectosomes and exosomes at the immunological synapse likely has nonredundant functions in recipient cells. In human $CD4^+$ T cell lines, released exosomes contain micro-RNAs (miRNAs), including *miR-335*, that alter gene expression patterns in the recipient Raji B cell line following superantigen-induced conjugation (Mittelbrunn et al. 2011). Although these findings have not been confirmed in primary antigen-specific human T cell–B cell interactions, they provide an intriguing glimpse into the realm of extracellular vesicle–mediated immune modulation by transferred miRNAs. This exosome transfer is mechanistically distinct from ectosome transfer, as the former process is blocked by either sphingomyelinase treatment of Jurkat T cells or knockdown of hepatocyte growth factor–regulated tyrosine kinase substrate (Hrs) (involved in early-acting ESCRT complexes), neither of which impairs synaptic ectosome release by primary $CD4^+$ T cells. Intriguingly, a recent study reported that murine $Foxp3^+$ T regulatory cells (Tregs) engage in nondirected release of exosomes capable of regulating Th1 effector cells, which produce IFN-$\gamma$ in the context of viral and intracellular bacterial infections (Okoye et al. 2014). Suppression of Th1 cell proliferation and cytokine secretion by Treg-derived exosomes involved the transfer of miRNAs to Th1 cells, raising the possibility that exosomes contribute to paracrine suppression (O'Hara 1995). Thus, extracellular vesicles, like cytokines (Huse et al. 2006), either appear to function through the synapse to achieve greater specificity or can be released in a nondirected fashion to allow paracrine communication with many surrounding cells. As ectosomes are preassociated with pMHC, they may be less likely to escape the synapse. T cell ectosomes may induce signaling and modulate the function of recipient cells through surface receptor interactions. Whether such ectosomes contain miRNAs or other cargo that might contribute to immune cell regulation will require further investigation.

## HIV TRANSMISSION

The T cell tropic human immunodeficiency virus-1 (HIV-1) uses the trimeric envelope protein gp120 to target T cell surface receptors CD4, CXCR4, and CCR5 to gain entry into $CD4^+$ T cells, where they are able to replicate and integrate into the T cell genome (Berger et al. 1999). Upon production of viral proteins and RNA in infected T cells, viral components traffic to the plasma membrane, where ESCRT machinery, notably TSG101 and ALIX, is coopted for budding and release of viral particles (von Schwedler et al. 2003). Because nascent virus at the cell surface and cell-free virus particles are particularly susceptible to host-neutralizing antibodies, HIV-1 has evolved sophisticated mechanisms to avoid exposure to host immunity during transmission and propagation in the host. A key strategy used by HIV-1 is to hijack components of the immunological synapse to form virological synapses (VS) with uninfected cells (Piguet & Sattentau 2004). The VS sequesters nascent virus particles within tightly apposed cell-cell junctions and effectively shields released virions from host antibodies. Consequently, contact-dependent cell-to-cell viral transmission is as much as 1,000 times more efficient than cell-free infection and is a major mode of viral transmission in secondary lymphoid tissues.

In classical HIV-mediated T cell virological synapses, tentative cell-cell contacts are initiated by surface-assembled gp120 on HIV-infected T cells. This action results in the engagement of CD4 in *trans* on uninfected $CD4^+$ T cells, dendritic cells, and macrophages. Tentative contacts are consolidated into adhesive junctions by LFA-1, which binds to ICAM-1 on target cells (Vasiliver-Shamis et al. 2010). Integrin-mediated adhesion and signaling recruit the MTOC to the VS, which guides cytoskeletal polarization (particularly F-actin remodeling) and directed transport of viral components to the VS, resulting in the formation of a pSMAC-like adhesive ring and central organization of viral proteins. Although the TCR is not engaged in target cells, engagement of CD4 by gp120 appears to induce attenuated TCR-proximal signals—including phosphorylation of TCR-ζ, Lck, ZAP-70, LAT, and PLC-γ—although intracellular $Ca^{2+}$ elevation is not observed (Vasiliver-Shamis et al. 2009). The HIV structural polyprotein Gag is recruited to the VS by lateral surface transport and active trafficking from nearby intracellular stores. Recent reports also suggest that the nascent HIV Env protein utilizes the regulated secretion pathway of T cells (Jolly et al. 2011) and that trafficking and polarized exocytosis thus play a part in delivering Env to the VS. The role of microtubule-mediated transport in HIV transmission remains controversial, however, as several studies have found no effect of microtubule depolymerization. Gag is recruited to the T cell plasma membrane and assembles into a submembrane lattice in discrete puncta. Assembled Gag recruits TSG101 and ALIX, and subsequently members of the ESCRT III and VPS4 complexes, to promote outward budding and scission from the plasma membrane (Baumgartel et al. 2011, Jouvenet et al. 2011). The ESCRT-mediated mechanism of HIV budding from the surfaces of T cells is remarkably similar to the release of TCR-rich ectosomes at the immunological synapse, pointing to the possibility that HIV coopts this host cellular response for viral transmission.

## OUTLOOK

Above we review recent work on cell polarization and the T cell immunological synapse across scales of submicrometer molecular assemblies involved in TCR triggering, from subcellular organelle dynamics to tissue-scale processes in lymph nodes, during an immune response. Our review begins with a discussion of the evolutionary context of adaptive immunity and how major adaptive immune cell types appear to have been in place as carriers prior to the evolution of adaptive immunity, which suggests that there are highly conserved behaviors associated with these developmental lineages. These areas remain completely open to investigation, as no cell biology has yet been done with adaptive lymphocytes from agnathans or on the interactions of most gnathostome innate lymphocytes, other than the relatively well studied natural killer cells. Advances in the isolation and culture of sufficient numbers of these cells, combined with innovative single-cell imaging approaches that would reduce the numbers of cells needed for quantitative imaging, would allow for deeper exploration of these outstanding issues. There is also significant potential to use tools of the immunological synapse, including SLBs, to better understand the changes in organization of γδTCR ligands that are needed to induce triggering. With regard to conventional helper and cytotoxic T cells, basic mechanisms that initiate signaling are not fully defined and are open to investigation. The physiological role of extracellular vesicles is becoming clear with promising progress in defining exosomes in Treg cell function. Genetic or pharmacological methods to target different sources of extracellular vesicles will be critical for determining in vivo function. Finally, technological advances in light and electron microscopy will allow for a better understanding of how T cells and other immune cells utilize polarity and complex 3D structures like immunological synapses to mount effective immune responses.

## DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

## ACKNOWLEDGMENTS

This research was supported by Wellcome Trust Principle Research Fellowship grant 100262/Z/12/Z and by National Institutes of Health grants R37AI43542 to Michael L. Dustin and R00AI093884 to Kaushik Choudhuri.

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