

# Signaling Takes Shape in the Immune System

## Review

Michael L. Dustin\*† and Andrew C. Chan\*††

\*Center for Immunology and  
Department of Pathology and Immunology  
†Howard Hughes Medical Institute and  
Division of Rheumatology  
Department of Medicine  
Washington University School of Medicine  
St. Louis, Missouri 63110

### Introduction

The adaptive branch of the immune system is built on the principle that the enemy can take any shape or form, but will display different protein sequences from the host. A highly flexible mechanism has evolved to recognize foreign protein segments based on a developmentally programmed series of transient cell–cell synapses between T cells armed with T cell antigen receptors (TCR) and antigen-presenting cells (APC) decorated with peptide ligands. The ligands are composed of peptides held in a surface groove on major histocompatibility complex molecules (MHC–peptide) that together form an interacting surface for the TCR (Garcia et al., 1996). Immature T lymphocytes (thymocytes) develop with each cell expressing one of a vast diversity of TCRs. Thymocytes interact with epithelial and dendritic APC in the thymus to test the interaction of the TCR with self-MHC–peptide complexes. Thymocytes undergo apoptosis if they express a TCR with a “strong” interaction with any self-protein segment, or if there is no interaction with self-MHC peptide complexes. Conversely, thymocytes proliferate and differentiate into mature T cells if they express a TCR that has a “weak” interaction with self-protein sequences and, hence, provide the potential for stronger recognition of foreign protein sequences in the future. Defining “strong” and “weak” in this context requires an understanding of how TCR interact with MHC–peptide complexes and how these interactions are linked to downstream events in T cell activation.

The activation of the mature T cell requires interaction of the TCR and MHC–peptide complexes in a specially organized cell–cell junction between the T cell and the APC that has been aptly described as an immunological synapse (Norcross, 1984; Paul and Seder, 1994). The physical interaction of TCR with MHC–peptide complexes is unique among signaling systems in that it takes place over a continuum of kinetic parameters with different sensitivity thresholds at different points in development. When appropriately engaged, the TCR becomes the epicenter for assembly of an intensively studied signaling complex. The immediate and long-term response of T cells to MHC–peptide complexes is subject to tunable thresholds that are partly set in the synapse and determine the outcomes of T cell development and the immune response. The tuning of signaling thresholds in

this system is vitally important to the host since overreaction to self-proteins can lead to crippling autoimmune disease and underreaction to pathogen derived foreign sequences leads to equally disastrous susceptibilities to infection or tumors. Immature and mature T cells exhibit differences in signaling requirements, currently the role of the immunological synapse remains less defined for the function of immature T cells.

Recent studies have emphasized the importance of cell asymmetry, cytoskeletal dynamics, membrane organization and molecular patterning in setting thresholds for the T cell activation process. Dynamic molecular patterns can be visualized by fluorescence microscopy in the immunological synapse (Monks et al., 1998; Wülfing et al., 1998; Grakoui et al., 1999). Shortly after contact between the T cell and APC, a bull’s-eye pattern forms with a central region dominated by integrins surrounded by a ring of engaged MHC–peptide complexes (Figure 1A). Over a period of minutes this bull’s-eye inverts resulting in a mature immunological synapse characterized by a central group of activated TCRs (the central supramolecular activation cluster or cSMAC) that is surrounded by a large ring of adhesion receptors (the peripheral supramolecular activation cluster or pSMAC) (Figure 1B). Maintenance of this stable bull’s-eye pattern correlates well with multiple parameters of effective activation of the T cell and the immune response. The clarity with which the T cell uses signaling to build large supramolecular assemblies and the potential for differential assembly of these complexes to dictate distinct biological outcomes of TCR activation provide a unique opportunity to further understand this process.

Currently there are a limited number of observations of molecular events in immunological synapse formation and our understanding of the molecular basis of this process is based on an array of model systems where the relationship to the entire process of immunological synapse formation is not entirely clear. Nonetheless, it is useful that the physiological T cell activation process can be broken down into a series of discrete steps. In some cases the model systems used to test molecular requirements for TCR-induced morphologic alterations relate only to one or two of these steps, rather than the entire process. Hence, the best way to organize diverse information about the molecular basis of immunological synapse formation and full T cell activation is to use these steps as an initial framework. Below we discuss the morphologically defined stages of T cell activation and then provide a framework for the molecular basis of this process in terms of basic building blocks of cytoskeleton, membrane structure, and signaling.

### Steps of Physiological T Cell Activation

The current paradigm for physiological T cell activation can be broken down into several steps. The T cells are first polarized (1) and attracted to potential APC by chemoattractants. Next, polarized T cells enter into non-antigen-specific adhesion (2) with the APC. Adhesion receptors bring TCR and MHC into proximity in a manner

†E-mail: dustin@pathbox.wustl.edu [M. L. D.], achan@im.wustl.edu [A. C. C.]

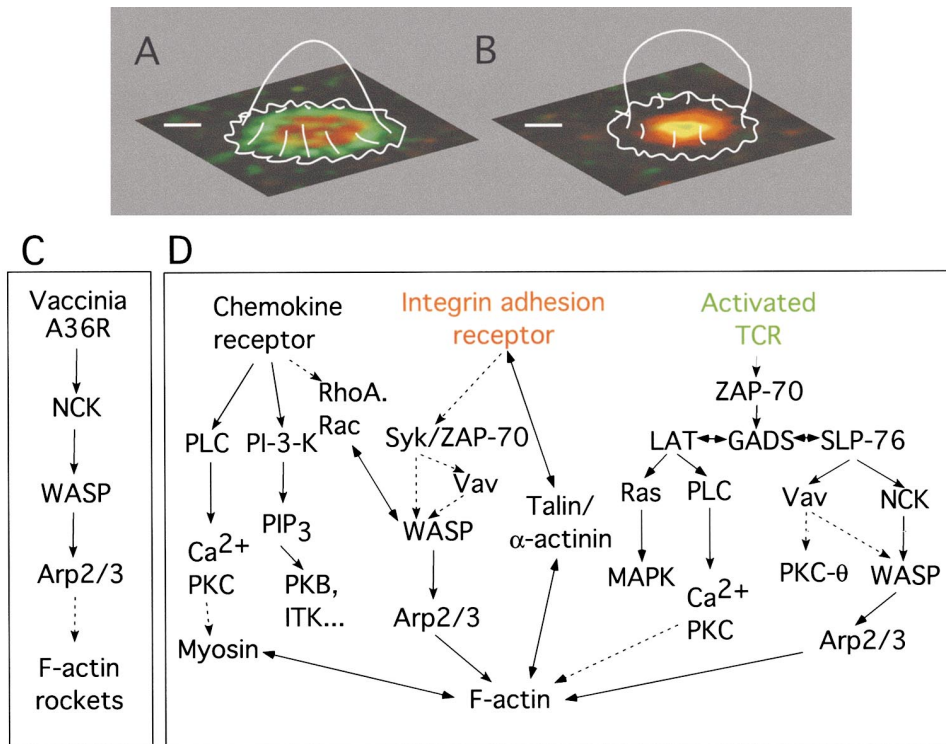


Figure 1. Immunological Synapse Formation and Some Basic Signaling Networks Connecting Signaling and Actin Polymerization (A and B) The pattern of MHC–peptide complexes (green) and ICAM-1 (red) in a model immunological synapse at  $<30\text{ s}$  (A) and  $1\text{ hr}$  (B) (Grakoui et al., 1999). The white outline is drawn to provide a scale for the T cell. Scale bar =  $2\ \mu\text{m}$ . (C) Vaccinia signaling pathway leading to actin polymerization. (D) A simplified view of signaling pathways leading from surface receptors to actin. The arrows reflect binding or inductive interactions in the pathway. PLC = phospholipase C, PI-3-K = phosphatidylinositol-3-kinase; PIP<sub>3</sub> = phosphatidylinositol-3,4,5-trisphosphate.

that determines subsequent TCR engagement and signaling thresholds. The TCR is then engaged (3) by MHC–peptide complexes. If the interaction of TCR and MHC–peptide complexes exceeds developmentally and environmentally determined kinetic and numerical thresholds, then signaling and immunological synapse formation (4) are initiated. The synapse must be stabilized (5) for several hours for full T cell activation. Termination (6) of the immunological synapse results from repolarization of the T cell to disassemble the cSMAC and migrate away from the APC. We will discuss our current understanding of the mechanisms of these steps below following introduction of two broad concepts.

### The Cytoskeletal Renaissance

An important building block of the immunological synapse are components of the cytoskeleton. Models for TCR collaboration with actin have been profoundly influenced by a recent surge in understanding of how surface receptors are linked to actin polymerization. This renaissance has been fueled by years of biochemical and genetic studies and in the last 10 years by an *in vitro* model based on the rocketing locomotion of intracellular pathogens and viruses in the cytoplasm of infected cells. These studies have identified a common pathway for actin polymerization based on recruitment and activation of the Arp2/3 complex to the membrane surface (Higgs and Pollard, 2000). Vaccinia virus employs a tyro-

sine phosphorylated membrane protein that links to a complex of the adaptor protein Nck and WASP, the protein deficient in Wiskott-Aldrich syndrome, to activate the Arp2/3 complex (Frischknecht et al., 1999) (Figure 1C). As we will discuss below, the Vaccinia system has several parallels to TCR-mediated signaling. WASP and Arp2/3 are basic components of the actin polymerizing machinery present in all nucleated cells. However, it is the unique ways in which these molecules are recruited to receptors at the surface, and the regulation of an array of actin binding proteins, that shape the resulting assemblies.

### Excitement about Membrane Domains

Membrane domains have become a focus in many areas of cell biology. Ever since the introduction of fluid mosaic models of membranes, there has been discussion of the idea that membranes may be organized into distinct lateral domains with enormous potential to organize processes such as signaling. Early evidence for membrane domains came from fluorescence lifetime measurements that identified at least two distinct environments for fluorescent membrane probes in the plasma membrane of live cells, corresponding to lipid phases with different degrees of order of their hydrocarbon chains (Klausner et al., 1980). Perturbation of these domains resulted in defects in T cell activation (Richieri et al., 1990). However, there was no concept of domain size

or associated protein composition and these findings were not widely exploited. Membrane domains have taken on renewed interest in recent years due to methods for biochemical analysis, the association of membrane domains with specific functions, and the ability to manipulate the domains (Brown and London, 2000). A particularly interesting type of membrane domain termed membrane rafts, detergent insoluble domains (DIGs), or glycolipid enriched microdomains (GEMs) is relatively ordered compared to the bulk of the plasma membrane. This domain is enriched in cholesterol, glycosphingolipids, glycosylphosphatidyl inositol linked membrane proteins, and a variety of acylated cytoplasmic proteins, and occupies roughly 10% of the cell surface. Many important molecules in T cell signaling are localized to these domains as will be discussed below. Notably, 3,4,5 and 4,5 phosphorylated forms of phosphatidylinositol that form binding sites for pleckstrin homology (PH) domains may also be concentrated in GEMs (Bunnell et al., 2000). One current idea is that the GEMs are very small in resting cells with only a few membrane and peripheral proteins on board each GEM that may readily coalesce during signaling in response to receptor crosslinking (Sheets et al., 1999). The energetics of forming these domains is likely to be highly dependent on proteins in a manner that is still not well understood. However, GEMs have become an important organizational principle in many signaling models and will be discussed extensively below in terms of TCR signaling and immunological synapse stabilization.

#### Step 1: T Cell Polarization

The immunological synapse is a provisional structure that is transiently established in response to the appropriately processed antigen in the form of an MHC-peptide complex on activated APC, such as a dendritic cell. The T cell must first encounter the dendritic cell, usually in a secondary lymphoid tissue. Our current understanding of the migration patterns of mature T cells and activated dendritic cells provides a mechanism for this encounter (Forster et al., 1999). Chemokines, such as secondary lymphoid tissue chemokine (SLC), trigger both naïve and some memory T cells to pass through high endothelial venules to enter the T cell area of lymph nodes. At the same time, SLC attracts activated dendritic cells from the tissues into lymphatics that allow these cells access to T cell areas of lymph nodes where the dendritic cells extend an elaborate web of membrane processes to facilitate contact with many T cells. This migration pattern brings these cells into the same arena, but chemokines also induce important changes in the T cell that are critical for immunological synapse formation.

Chemokine receptor signaling shares components with TCR signaling, but is very different in its kinetics and function (Figure 1D). Chemokine receptor signaling in the context of polarity and migration is fast and transient, in contrast to TCR signaling that is sustained over hours. Chemokine receptors are members of the transmembrane serpentine family of receptors, which are linked to heterotrimeric G proteins. Chemokine binding releases the  $G_{\alpha}$  subunit and unmask an effector binding site on  $G\beta\gamma$  subunits. The activated  $\beta\gamma$  subunits

recruit a number of signaling components involved in stimulating  $Ca^{2+}$  mobilization (phospholipase C), lipid phosphorylation (phosphatidylinositol-3-kinase), and activation of Rho family G proteins involved in actin cytoskeletal regulation (Servant et al., 2000). The duration of  $G\beta\gamma$  activation and effector function is regulated by the time required for guanine nucleotide hydrolysis by the  $G_{\alpha}$  subunit, which in turn is regulated by regulators of G protein signaling (RGS) proteins. The G protein subunits are differently lipid modified. The  $G_{\alpha}$  subunit is palmitoylated so that it may preferentially partition into ordered phases like GEMs, whereas the  $\gamma$  subunit is prenylated so that it may preferentially partition into disordered domains (Moffett et al., 2000). The functional significance of this differential partitioning is not known.

The initial response to chemokine receptor signaling is rapid actin polymerization and myosin II activation, which has the effect of increasing cytoskeletal tension (Elson et al., 1990). However, this initial “cringe” response polarizes the cell such that the microvilli and large glycoproteins such as CD43 are transported to the trailing pole of the cell known as the uropod along with the microtubule organizing center (MTOC). As importantly, dynamic actin-based protrusions emerge from the gradient front (Weiner et al., 1999). In the presence of an adhesive substrate, activation of adhesion receptors may stabilize cell polarization and locomotion. The actin-based protrusions that define the leading edge of the polarized T cell have the same, if not less, numbers of TCR than the trailing edge, but the TCR in the leading structures is vastly more sensitive to engagement by either MHC-peptide complexes or anti-TCR antibodies (Wei et al., 1999). Thus, polarized T cells have actin-based protrusions that are highly reactive to MHC-peptide complexes. The step of cell polarization prepares the T cells for antigen recognition even before they touch an APC.

#### Step 2: Adhesion

It is well documented *in vitro* and *in vivo* that T cell activation requires activity of adhesion receptors. Adhesion receptors are used for physical anchorage and to provide feedback to the cell about its environment, thus adhesion and signaling are essentially inseparable (Springer, 1990). The abundance of appropriate MHC-peptide complexes is too low to mediate significant adhesion. Therefore, adhesion receptors are required for efficient TCR engagement. There are several classes of adhesion receptors that are thought to be important on T cells. These include integrin family members like LFA-1 interacting with binding partner ICAM-1 and VLA-4 interacting with VCAM-1 and immunoglobulin superfamily members like CD2 interacting with CD58 (human) or CD48 (rodent), and CD28 interacting with CD80 or CD86. All of these molecules are implicated in both physical adhesion and signaling in multiple studies. The coreceptors CD4 and CD8 are also members of the immunoglobulin superfamily that appear to have roles in both adhesion and signaling by binding to nonpolymorphic regions of MHC class II and I molecules, respectively, following TCR engagement. The activation of adhesion receptors is also related to cellular polarization. It is most likely that T cells are more sensitive to MHC-peptide complexes at

the leading lamellipodium because adhesion receptors are also more active in these regions (Schmidt et al., 1993). This increased activity can be attributed to an accelerated cytoskeletal response to ligand-coated surfaces as revealed in studies on fibroblasts.

Adhesion receptors interact with ligands with very low affinity, so effective interaction is dependent on alignment of apposing membranes to achieve a high two-dimensional affinity (Dustin et al., 1997). Two-dimensional affinity is the physiological affinity for an adhesion receptor or TCR interaction that is expressed in units of surface density such as molecules/ $\mu\text{m}^2$ . The ratio of the two-dimensional affinity to the entropy-corrected solution affinity is the confinement length ( $\sigma$ ).  $\sigma$  is the average fluctuation in the distance between apposed membranes in the contact area. For example, the interaction of CD2 and CD58 adhesion receptors holds membrane at an optimal distance of approximately 15 nm based on the crystal structure (Wang et al., 1999), but with a  $\sigma$  of 5 nm.  $\sigma$  can be smaller or larger than the optimal separation between membranes based on the relative order of the interacting membranes and the forces that act to prevent the optimal spacing between membranes such as glycocalyx repulsion. A corollary of two-dimensional affinity is that adhesion receptors that are of similar size may cooperate locally and coexist in the same domain, while adhesion receptors of different sizes must segregate laterally (van der Merwe et al., 2000). To take the CD2 example again, it was predicted that the 15 nm span of CD2/CD48 interaction would establish the ideal environment for efficient TCR interaction with ligands and that disrupting this relationship by making CD48 4–8 nm larger would decrease T cell sensitivity to antigen. It was found that normal CD48 increased T cell sensitivity to antigen, while larger forms of CD48 were potent inhibitors to T cell activation and productive TCR interaction with MHC–peptide complexes (Wild et al., 1999). These experiments indicate that the unnaturally long CD2–CD48 interaction “stiff-arms” the TCR away from the MHC–peptide complexes, thus blocking the initial TCR engagement. It has been demonstrated that sites of LFA-1/ICAM-1 interaction (nominal distance 40 nm) and CD2/CD58 interaction (nominal distance of 15 nm) segregate in contact areas (Dustin et al., 1998). Thus, adhesion receptor size defines an architectural element of the immunological synapse.

Another effect of adhesion receptors is in the linkage of actin-based protrusions to integrin-mediated adhesion. We have observed that integrin-mediated adhesion is linked to generation of protrusions adjacent to the adhesive sites that force close contact between plasma membrane and the substrate (Grakoui et al., 1999). The molecular mechanism of this process may involve actin binding proteins of the Ena/VASP family as demonstrated for establishment of cell–cell contacts in epithelial cells (Vasioukhin et al., 2000). These proteins have been implicated in slowing fibroblast migration, and this may have to do with the use of these proteins to divert adhesive energy to sensory, rather than movement, activities (Machesky, 2000). It is of interest then that Ena/VASP family proteins are linked to the TCR by specific proteins, including the src family kinase fyn and the fyb/SLAP adaptor molecule (Krause et al., 2000).

An important aspect of adhesion receptor function is that it is regulated by TCR (Dustin and Springer, 1989). The mechanism of this positive feedback is still not completely understood. Initially it was thought that signals from the cytoplasm would directly regulate integrin affinity through a process of inside-out signaling. However, studies from many labs have failed to identify such signaling for the key adhesion receptor LFA-1. It appears now that adhesion receptor regulation by chemokine or TCR signals is quite subtle, but provides an even more powerful mechanism for integrating adhesion and signaling. In the current model LFA-1 on the resting lymphocytes is held still on the surface by an actin-based cytoskeletal interaction that prevents it from cooperating with its neighbors to form adhesive clusters. This is the low-avidity form of LFA-1 that maintains the nonadhesive state of circulating lymphocytes. Upon activation of the T cell by chemokines, the LFA-1 is freed from this interaction and can diffuse locally, an unexpected form of inside-out signaling based on manipulation of membrane protein dynamics (Kucik et al., 1996). This allows molecules to congregate at ICAM-1-rich interfaces with other cells to nucleate adhesive clusters (Lub et al., 1997). Interaction with ICAM-1 induces a conformational change in LFA-1 that initiates a poorly understood signaling cascade, an outside-in signal, that induces new cytoskeletal interactions that reinforce adhesion. The ligand-induced conformational change also produces a higher affinity for ligand, which may provide more time for initial pioneering interactions to recruit receptors and binding partners into an adhesive cluster. The physical mechanism by which the new cytoskeletal interactions stabilize adhesion may include promoting increased membrane alignment and thus higher two-dimensional affinity. Ligand-induced cytoskeletal association has not been demonstrated in lymphocytes due to the technical difficulty of working with these cells, but has been shown in fibroblasts that offer experimental advantages (Felsenfeld et al., 1996). In the current model, the affinity change is not used to increase ligand binding directly at the level of single receptor–ligand pairs, but is used to link ligand binding to signaling and a cytoskeletal response to promote adhesion at the higher organizational level of forming an adhesive cluster. These adhesive clusters are the raw material for cell locomotion and the adhesive element of the immunological synapse.

The specific cytoplasmic proteins that mediate the cellular response to ligand-induced changes in integrins are not well defined. Protein tyrosine kinases (PTKs) of the Syk family and the Rho family guanine nucleotide exchange factor Vav are implicated in regulation of integrin activity (Mainiero et al., 2000) (Figure 1D). Integrin-based adhesive structures known as podosomes are enriched in actin polymerizing machinery including WASP and the Arp2/3 complex, which are likely to be downstream of Syk and Vav, and will induce local actin polymerization (Linder et al., 2000). Finally, PI3K is required to generate PIP<sub>2</sub> for activation of WASP and actin polymerization. A complex of Src family PTKs, PI3K, and the tyrosine phosphatase SHP-1 has been implicated in regulation of integrin function in leukocytes (Roach et al., 1998). The genetic deficiency of SHP-1 in motheaten mice results in hyperadhesiveness of leukocytes and a profound inflammatory disease. Many of these same

molecules are implicated in TCR signaling and dynamics following activation.

### Step 3: Initial TCR Engagement

TCR engagement is both remarkably sensitive and flexible. A few foreign MHC-peptide complexes are sufficient to activate a T cell. These interactions have affinities in the  $10^{-6}$  M range and half-lives on the order of 5 s (Matsui et al., 1994). The interactions between TCR and self-MHC that are required for positive selection of thymocytes and survival of mature T cells are more transient and are very difficult to measure (Boniface et al., 1999). Explaining how these very weak molecular interactions lead to biological responses is likely to require more than membrane alignment and simple two-dimensional affinity. One mechanism to increase sensitivity is to increase the valency of the interactions.

Multivalent interactions are likely to have an advantage in terms of affinity and dissociation kinetics in cell-cell interfaces. It is well known that the multivalency of soluble receptors, such as antibodies, increases the binding avidity for cell surface ligands by suppressing dissociation of the antibody from the ligand-coated surface. Although there are many molecular interactions linking two adherent surfaces, transient molecular interactions in the interface are not protected from dissociation as long as they are moving independently in the membrane. This opens the possibility of increasing interaction strength by preclustering receptors. If, for example, the TCR and MHC-peptide complexes were initially located in complementary preformed clusters on the surface of the T cells and APC, respectively, then much weaker interactions could produce stable binding of one TCR cluster to an apposing MHC-peptide cluster. This is because following association, the dissociation of the clusters from each other would require simultaneous release of all interactions. The time required for dissociation of the entire array would then be the key parameter that determines the response. Positive selection or survival signals would then result from a basal multivalent interaction that might induce some local rearrangements to assemble signaling complexes. The inclusion of even one foreign MHC-peptide complex with a longer half-life in the self MHC-peptide cluster could induce a significant increase in the time the TCR and MHC clusters remain associated to assemble more complete signaling complexes toward fully activating the T cell. This would suggest an important role for self-MHC-peptide complexes in the sensitive response to agonist MHC-peptide complexes. Currently, there is no direct support for this, but there are provocative early indications. There is evidence that MHC class I and class II molecules exist in arrays on the surface of APC based on GEMs (Szollosi et al., 1996; Anderson et al., 2000). It has recently been suggested that these arrays include the ligands for CD28 (Turley et al., 2000). There is also evidence that TCR and CD2 are associated with GEMs (Yashiro-Ohtani et al., 2000). Understanding the molecular organization of the TCR and MHC-peptide complexes on the surface will be an area of intense activity in the near future.

Models for TCR engagement initially focused on the idea that each receptor was internalized following en-

gagement (Valututti et al., 1995). This model explains the ability of a small number of MHC-peptide complexes to cause degradation of a larger number of TCR in the responding T cell. An alternative model has emerged, based on observations of stable MHC-peptide and TCR accumulations in the immunological synapse and SMACs (Monks et al., 1998; Grakoui et al., 1999; Johnson et al., 2000). This model is based on formation of a metastable structure in which many TCR are engaged in parallel. Parallel engagement provides a clear mechanism for sustained signaling that is essential for full T cell activation (Dustin and Cooper, 2000). The use of GFP tagged MHC molecules has permitted the tracking of molecules on the surface of interacting cells (Wülfing et al., 1998; Krummel et al., 2000). However, the distribution of these molecules is regulated by multiple factors and likely is more complex than any particular binary interaction. This problem will be even more complex if it turns out that arrays of receptors are the basic unit of interaction. However, fluorescence imaging technologies such as resonance energy transfer, in conjunction with fluorescent proteins and non-function-blocking Fab fragments of monoclonal antibodies should facilitate dynamic analysis of these membrane assemblies.

### Step 4: TCR Signaling and Immunological Synapse Formation

Engagement of the TCR by MHC-peptide to assembly of signaling complexes, generates multiple second messengers, and induces cytoskeletal changes required to stop the migration of the T cells (the stop signal). The connection between T cell movement and signaling is likely to be critical for an *in vivo* response where the contact between rare antigen-specific T cells and APC that have encountered antigens in the periphery must be preserved (Ingulli et al., 1997). Therefore, some early second messengers including  $Ca^{2+}$  increases and changes in inositol phospholipid metabolism may focus on control of immunological synapse formation to lay the foundation for sustained signaling and full T cell activation, which requires transcriptional activation (Figure 1D, Figure 2). The cytoskeletal events linked to early TCR signaling and the stop signal are the polymerization of actin at the interface between the T cell and APC and reorientation of the MTOC from the uropod to just under the contact interface. Movement of the MTOC is also a marker for movement of the Golgi apparatus so that constitutive and regulated secretion are directed at the immunological synapse (Kupfer et al., 1991). We would argue that proximal early signaling events not only initiate communication with nuclear processes, but regulate the actin cytoskeleton, which in turn lays a foundation for sustained signaling and IL-2 production based on cell polarization and formation of well defined supramolecular activation clusters within the immunological synapse.

Generation of these second messengers involves the sequential activation of three distinct families of protein tyrosine kinases (PTKs)—the Src, Syk, and Tec families of PTKs. The CD4 and CD8-associated Lck, as well as the Fyn PTK (members of the Src family of PTKs), phosphorylate the two tyrosine residues within the immunoreceptor tyrosine-based activation motif (ITAM)

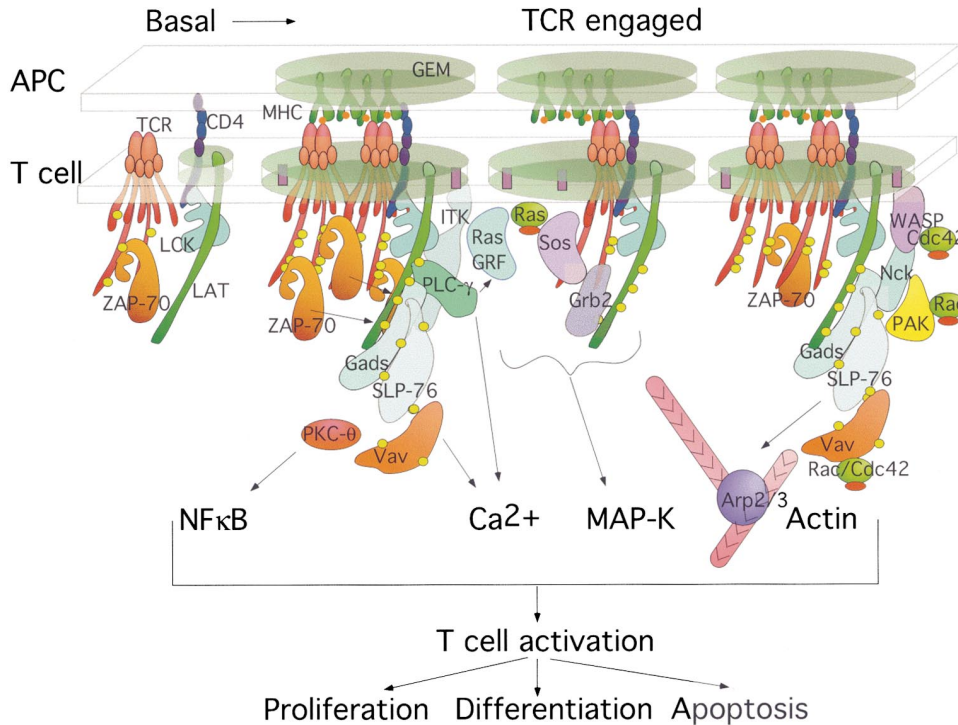


Figure 2. Model for Molecular Assembly in the Immunological Synapse

The bottom membrane is the T cell plasma membrane and the top membrane is the APC membrane. Green regions of membrane are rafts, which are initially very small. Engagement by MHC-peptide complexes induces TCR oligomer formation and coalescence of rafts into larger structures incorporating CD4 and LAT. Transphosphorylation by Lck and ZAP-70 initiate assembly of a signaling complex with ties to second messengers and actin. The signals originating from the immunological synapse are integrated at the level of the plasma membrane and in the nucleus to determine key events of T cell activation. Small yellow circles represent tyrosine phosphorylated residues, the small red ovals are GTP bound to GTPases, and the purple rectangle in the membrane are inositol phospholipids. The processes depicted are spread over different TCR complexes for pictorial clarity. The actual organization of complexes regulating Ca<sup>2+</sup>, NF-κB, Map kinases (MAP-K), and Actin remains to be determined.

encoded within each of the TCR signaling modules. Crosslinking of chimeric receptors with cytoplasmic domains solely encoding the TCRζ or ε ITAMs is sufficient not only for the generation of second messengers that are induced following TCR crosslinking, but also for MTOC polarization and actin polymerization (Letourneur and Klausner, 1992; Lowin-Kropf et al., 1998).

Src family PTKs are regulated by an intramolecular interaction between the SH2 domain and a C-terminal phosphotyrosine that keeps the kinases in an inactive conformation. The C-terminal inhibitory site (505 in Lck) is phosphorylated by the c-Src kinase (Csk), and this phosphate is removed by the CD45 protein tyrosine phosphatase. Tyrosine phosphatases are not highly specific for particular substrates, so CD45 also inactivates Lck by dephosphorylating the 394 autophosphorylation site (D'Oro and Ashwell, 1999). CD45 is likely to be regulated by two processes: dimerization and localization. Dimerization of CD45 inhibits phosphatase activity (Mejetti et al., 1998). CD45 is excluded from GEMs based on biochemical studies (Rodgers and Rose, 1996). Beyond GEMs, the location of CD45 in the immunological synapse has been controversial and highlights the complexity of assigning subcellular localization based on fluorescence microscopy alone. Two studies with cell-cell systems have demonstrated that CD45 is

present in the immunological synapse at some times (Sperling et al., 1998), while being excluded from the synapse at other times (Leupin et al., 2000). A kinetic study revealed initial exclusion of CD45 from the central area of integrin engagement, followed by transport of CD45 back to the vicinity of the cSMAC, possibly in endosomal structures (Johnson et al., 2000). The mechanism of CD45 movement in the immunological synapse is not known. One possibility is that CD45 may be excluded from the contact based on the size of its ectodomain (van der Merwe et al., 2000).

There is also an indication that Csk compartmentalization may play an important role in T cell activation based on studies of the docking protein Phosphoprotein-associated with glycosphingolipid-enriched microdomains (PAG) or Csk binding protein (CBP) (Brdicka et al., 2000; Kawabuchi et al., 2000). This GEM localized docking protein is phosphorylated in resting T cells and binds to the SH2 domain of Csk. PAG/CBP is dephosphorylated following TCR engagement and presumably releases Csk from the plasma membrane/GEM compartment (Brdicka et al., 2000). In turn, Csk would be sequestered away from Lck and other Src-PTK targets. Consistent with this model, the Csk SH2 domain is required for its ability to attenuate TCR activation, where cells expressing a mutant Csk molecule with a nonfunctional SH2

domain can be rescued by targeting the SH2 mutant to the plasma membrane (Cloutier et al., 1995; Brdicka et al., 2000; Kawabuchi et al., 2000). Thus, at least two factors may modulate Lck activation in forming the immunological synapse: CD45 exclusion from the contact site and Csk release from its GEM localized docking site.

Phosphorylation of both tyrosine residues within the TCR ITAM mediates the binding of ZAP-70 and Syk (members of the Syk-family of PTK) with the phosphorylated receptor. TCR activation through formation of a TCR-MHC-CD4/CD8 ternary complex, in turn, colocalizes Src and Syk, as well as Tec, families of PTKs with the activated receptor to induce their enzymatic activation. Loss of any of the three families of PTKs abrogates normal T cell development and function (Cheng and Chan, 1997; Yang et al., 2000). Similarly, T cell lines lacking Lck or expressing a dominant-negative form of ZAP-70 are unable to polarize the MTOC following TCR crosslinking (Lowin-Kropf et al., 1998). Thus, it appears that the sequential activation for Src and Syk families of PTKs is required not only for the generation of second messengers, but also for normal cytoskeletal reorganization following T cell activation. While Syk protein tyrosine kinases interact with the phosphorylated receptor encoded ITAMs, their subcellular localization relative to the maturing synapse remains unclear. Furthermore, while membrane localization of the PTKs is similarly required for efficient TCR activation, additional studies are required to define their localization relative to the immunological synapse.

The enzymatic activities of these three PTK families are required to phosphorylate a growing number of linker proteins that function as scaffolds to localize and assemble signaling complexes. Most notable are the SH2-containing leukocyte protein of 76 kDa (SLP-76) and the transmembrane Linker for Activation of T cells (LAT) (Myung et al., 2000; Zhang et al., 2000). Moreover, these linker proteins are required for the coordinate activation of a diverse number of signaling pathways. Phosphorylation of multiple tyrosine residues within the cytoplasmic tail of LAT provides docking sites for the binding of PLC- $\gamma$ 1 (required for Ca<sup>2+</sup> increases), the Grb2 and GRAP adaptor molecules (implicated in Ras activation), and the Gads adaptor protein (also known as GrpL, Mona, Grid, Grblg, Grb2L, and Grp40) (Liu and McGlade, 1998; Zhang et al., 2000). The latter interacts with a number of proteins including SLP-76 and facilitates the translocation of SLP-76 containing signaling complexes to the GEMs (Liu et al., 1999). Tyrosine phosphorylation of SLP-76 also permits the assembly of macromolecular complexes involving the Vav guanine nucleotide exchange factor and the Nck adaptor protein (see below). Hence, the appropriate tyrosine phosphorylation of these linker proteins by the TCR-activated PTKs is required to coordinate signaling pathways and normal T cell function.

TCRs are transported from the initial site of engagement to the center of the synapse by a remarkable, active process. This process proceeds from the first seconds following contact to 5 or 10 min when formation of the cSMAC is complete. This process overlaps almost completely in time with the period of most profound tyrosine phosphorylation of TCR-associated compo-

nents and the highest levels of Ca<sup>2+</sup> signaling. The formation of the cSMAC has a basic resemblance to the process of capping first described in the 1970s (Unanue et al., 1972). The major difference is that immunological synapse formation requires continued coordination of TCR engagement and adhesion during formation of the cSMAC, while capping is triggered by high-affinity antibody cross-linking and typically does not require adhesion receptor engagement. It should be noted that in vitro coengagement of adhesion receptors and TCRs with antibodies can enhance signaling, but this process is most likely different from immunological synapse formation. At this time, most of the studies that dissect the molecular mechanism of TCR movement during signaling have focused on the more accessible capping of TCRs with antibody crosslinking. This is likely to provide a good model for the generation of actin and myosin activation by TCR signals, but less physiologic for TCR engagement or the coordination of the TCR signaling with adhesion.

The movement of TCR on the surface at the peak of TCR signaling is correlated with the linkage of TCR signaling to actin polymerization and cytoskeletal regulation. This is likely to involve activation of Rho family GTPases by guanine nucleotide exchange factors (GEFs), such as Vav, which is tyrosine phosphorylation by the Src and Syk PTKs rapidly after TCR engagement (Crespo et al., 1997). Using purified Vav and heterologous expression systems it has been shown that tyrosine phosphorylation of Vav contributes to the activation of Rac and Cdc42 GDP/GTP exchange (Han et al., 1997). Rac plays a critical role in actin polymerization and the formation of lamellipodia and membrane ruffles; Cdc42 mediates the formation of filopodia. Consistent with the importance of Rho family GTPases in cytoskeletal reorganization, *vav1*<sup>-/-</sup> T cells are unable to form actin patches or receptor caps following TCR crosslinking (Fischer et al., 1998; Holsinger et al., 1998). In addition, expression of mutant Cdc42 molecules in T cells interferes with MTOC formation and actin polymerization following receptor activation (Stowers et al., 1995). The activation of members of the Rho family of GTPases is an essential component for actin polymerization and receptor aggregation. Recent data indicates that Rho family members may have additional roles in regulating T cell differentiation and development. For example, Rac2 has been shown to be specifically expressed during differentiation of T cells to a Th1 proinflammatory phenotype (Li et al., 2000). In these cells, Rac2 has a specific role in transcriptional activity of interferon- $\gamma$  promoters and *rac2*<sup>-/-</sup> mice demonstrate attenuated production of interferon- $\gamma$ . In addition, inhibition of Rho function in the thymus results in an arrest at the earliest stages of T cell development (Costello et al., 2000). Additional studies will be required to determine the role of these small G proteins in immunological synapse formation.

Phosphorylation of multiple tyrosine residues on SLP-76 creates docking sites that bind distinct SH2 domain containing molecules, including Vav and Nck (Bubeck Wardenburg et al., 1998). The activation of Vav and the subsequent generation of GTP-bound forms of Rho-GTPases regulates a number of proteins that mediate reorganization of the actin cytoskeleton. Since Nck interacts with both Pak and WASP, two proteins that are

dependent on Rho family GTPase activation, this scaffolding is required for the colocalization of the activated GEF, Vav, with the Rho-GTPase-dependent Pak and WASP molecules to regulate Pak activation and actin polymerization. While Pak has been implicated in cytoskeletal reorganization and colocalizes with focal adhesions in fibroblast cell lines (Harden et al., 1996), its function in T cell cytoskeletal reorganization remains undefined. A potentially important pathway involves activation by Pak of LIM-kinase and myosin light chain kinase to modulate the actin cytoskeleton.

WASP also functions in a Rho-dependent fashion (Rohatgi et al., 1999). As mentioned above, WASP, the molecular defect in the Wiskott-Aldrich syndrome (WAS), interacts with WIP and the Arp2/3 complex to induce actin nucleation, a process which can be regulated by both Cdc42, PI(4,5)P<sub>2</sub> and Grb2. Patients with Wiskott-Aldrich syndrome as well as *wasp*<sup>-/-</sup> murine T cells demonstrate defective T cell proliferative capacities and receptor capping following TCR crosslinking (Snapper et al., 1998). In addition, missense mutations within WASP that interfere with WIP-WASP interactions have been described in WAS patients and further support the functional significance of the WASP/WIP complex in T cell function (Stewart et al., 1999). While the GEF that contributes to WASP function has not been fully defined, Vav appears a likely candidate as *vav*<sup>-/-</sup> T cells also demonstrate capping defects following TCR crosslinking, though other Pak-associated GEFs, such as PIX, may also regulate the cytoskeleton (Manser et al., 1998). In addition, SLP-76, which interacts with Vav and Nck, also interacts with tyrosine phosphorylated FYB/SLAP-130 (da Silva et al., 1997). The latter has been described to localize at the receptor cap with WASP and Arp2/3, and inhibition of binding between FYB/SLAP-130 and Eva/VASP or WASP/Arp2/3 complexes impairs TCR-dependent actin polymerization (Krause et al., 2000). However, while *vav*<sup>-/-</sup> T cells also exhibit multiple signaling defects in Ca<sup>2+</sup>, Erk, JNK, and NF-κB activation (Costello et al., 1999), *wasp*<sup>-/-</sup> T cells exhibit normal Erk and JNK activation, and only a 20% reduction in TCR-induced increase in Ca<sup>2+</sup>. Hence, there appear to be a subset of Vav effectors that selectively regulates the T cell cytoskeleton.

The initial observation that PKCθ is preferentially detected in the center of the SMAC following TCR engagement raised the possibility of its role in T cell function (Monks et al., 1998). In fact, PKCθ is biochemically and functionally associated with Vav (Villalba et al., 2000). Whereas Vav plays an important role in activation of multiple TCR-mediated signaling pathways, PKCθ appears to be involved primarily in transcriptional regulation. *PKCθ*<sup>-/-</sup> T cells demonstrate a selective defect in NF-κB activation (Sun et al., 2000). Though cytoskeletal reorganization in *PKCθ*<sup>-/-</sup> T cells was not examined, a recent study using overexpression of constitutively active and dominant-negative mutants of PKCθ in Jurkat T cells failed to demonstrate any defects in TCR-induced actin polymerization (Villalba et al., 2000). Therefore, it appears unlikely that PKCθ is required for synapse formation. On the other hand, it is possible that immunological synapse formation will be required to activate PKCθ. Further study is required to better define the mechanism

of PKCθ activation and recruitment to the immunological synapse and its potential selective targets.

The other cytoskeletal component that is likely to play an important role in transport of TCR to the center of the immunological synapse is myosin II. Activation of myosin II is required for antibody dependent capping and for actin based cell locomotion (Braun et al., 1978). These actions of myosin II are based on its ability to contract actin gels in a manner that can move actin-associated receptors in a polarized fashion in the plane of the plasma membrane. Myosin II is activated by myosin light chain kinase which, in turn, is activated by a Ca<sup>2+</sup>/calmodulin based mechanism. Myosin light chain kinase is also influenced by other kinases; for example, it is inhibited by Pak. Myosin II also contributes to long range receptor transport from the free surface of the T cell into the contact area within a few minutes of initial T cell activation (Wülfing and Davis, 1998). Therefore, there are ingredients in the acutely activated T cell to orchestrate cSMAC formation based on actin polymerization from the engaged TCR and myosin II activation.

#### Step 5: Stabilization

Duration of signaling is also a critical parameter for T cell activation. The maintenance of the immunological synapse over many hours may be promoted by molecular changes during maturation of the immunological synapse. For example, the MHC-peptide complexes accumulated in the cSMAC are held tightly as measured by fluorescence photobleaching recovery experiments (Grakoui et al., 1999). This was surprising since the intrinsic interaction of the TCR and MHC-peptide complex is transient and would not be expected to prevent MHC-peptide exchange in the immunological synapse. This locking of the MHC-peptide complex suggests formation of a higher order structure and may be related to the lateral interactions of TCR and MHC molecules following ligand binding (Reich et al., 1997). Alternatively, the recruitment of more rigid lipid rafts to the T cell and APC sides of the immunological synapse may contribute to stabilization (Figure 2). Stabilization of phosphotyrosine signals has been associated with the recruitment of GEMs to the interface between T cells and anti-TCR coated beads triggered by coligating the costimulatory molecule CD28 (Viola et al., 1999). While the nature of the signals that are generated from the stable immunological synapse is not as well understood as the early signals described above, it is clear that sustained signaling (up to 20 hr) is necessary for full T cell activation (Iezzi et al., 1998).

The association of TCR signaling components with GEMs may provide insights into requirements for stabilization of the immunological synapse. The more rigid platform provided by GEMs may increase the stability of signaling complexes by increasing cooperation of the multiple transient interactions on which these complexes are built (Felder et al., 1993). While the TCR in resting cells are weakly associated with GEMs, accumulating evidence suggests that molecules important in T cell activation, as well as the TCR itself, traffic into these microdomains following receptor engagement. Moreover, molecules such as CD4, PAG, Lck and LAT, which are palmitoylated, are preferentially detected within

GEMs. Expression of a nonpalmitoylated mutant of Lck or a transmembrane chimera with a non-S-acylated form of Lck, and hence unlikely to reside in GEMs, is unable to mediate TCR-activation of a number of downstream signaling pathways (Janes et al., 1999). Similarly, GEM localization of LAT is also required for TCR-mediated signaling events (Zhang et al., 1998). The ability of tyrosine phosphorylated LAT to bind and assemble signaling complexes as discussed above within these GEMs suggests that targeting of signaling complexes to these domains represents an important feature of T cell activation. Two recent studies of thymocytes demonstrate that LAT tyrosine phosphorylation and the movement of the TCR and ZAP-70 into GEMs is correlated with Erk activation and T cell development (Werlen et al., 2000).

While providing a more rigid platform, the GEMs may also help organize competing or antagonistic pathways to sustain TCR signaling and maintenance of the immunological synapse. The localization of signaling complexes to these microdomains may sequester signaling complexes from negative regulatory proteins, such as protein phosphatases including CD45. Additionally, these domains are enriched for a variety of cytoskeletal components including actin. Hence, the assembly of signaling complexes within these domains may also localize proteins such as Vav and WASP with their binding partners to facilitate cytoskeletal reorganization. It has been demonstrated recently that GEMs may also nucleate actin polymerization in a manner dependent on PIP<sub>2</sub>, tyrosine phosphorylation, WASP, and the Arp2/3 complex (Rozelle et al., 2000).

GEMs may also provide a structural basis for formation of TCR and MHC-peptide arrays to enhance the avidity of interactions as described above. While more ordered lipids are a prominent feature of GEMs, it is likely that proteins also regulate the formation of these structures. For example, these proteins may organize the TCR and CD2 on the T cell and MHC-peptide complexes and GPI-anchored proteins like CD48 on the APC to provide a more stable platform for the receptor interactions. It has been suggested that antigen processing in the APC may lead to organization of MHC class I and class II molecules into GEMs along with CD80, the binding partner for the T cell signaling molecule CD28. While there is currently no evidence that the CD28 coexists with GEMs in the T cell membrane, the interaction of CD28 with CD80/MHC arrays may translocate CD28 into proximity of the TCR within GEMs to initiate CD28 synergism with the TCR. CD28 coengagement with the TCR results in more sustained tyrosine phosphorylation of TCR itself and of Vav (Tuosto and Acuto, 1998). The localization of adhesion receptors in the mature immunological synapse may have a profound impact on the potential of these receptors to regulate T cell activation. Interestingly, CD28 interactions with CD80 are frequently clustered in the center of the immunological synapse, while CD2 interactions with CD48 often accumulated in a unique supramolecular cluster (Grakoui et al., 1999).

#### Step 6: Termination

The termination of immunological synapse formation occurs in the time frame of 24–48 hr in vivo (Ingulli et

al., 1997). Effector T cells in vitro terminate interactions more rapidly and this process has been observed. The ability to sustain the immunological synapse appears to depend not only on the number of MHC-peptide complexes engaged, but on how they are organized. Poorly organized synapses are observed to spontaneously terminate in the time frame of 30 min. After which the T cell acquires a new polarity or direction that can be identified based on the pattern of adhesion receptor engagement. The T cell moves away from the site and the cSMAC is degraded or shed. The signals that focus T cell polarity on the synapse, involving proteins like Cdc42 (Stowers et al., 1995), are likely to be critical to sustain synapses in vivo. In support of this it has been observed that integrin-mediated adhesion at points on the T cell outside the synapse can disengage the synapse when polarity is not maintained by active TCR signaling.

There have been a number of in vitro observations that report mobility of T cells over APC during antigen presentation (Underhill et al., 1999). This movement suggests that the structure of the immunological synapse is not well maintained in these interactions, although this has not been directly examined. It is possible that the behavior of T cells on professional APC may be regulated by a number of local factors. For example, chemokine gradients can disrupt immunological synapse formation and it is possible that T cells attaching to cultured macrophages may face a complex landscape of TCR interactions and local chemokine gradients (Bromley et al., 2000). This could be an important factor if a T cell enters a tissue with many antigen-positive cells. The ability of the T cell to navigate in such a tissues space would be dependent on the ability of some guidance signals to override the TCR stop signal. In addition to chemokines, collagen extracellular matrix also promotes more dynamic interactions of T cells and APC (Gunzer et al., 2000). In lymph nodes key chemokine receptors are likely to be desensitized as the T cell passes through the high endothelial venules and the T cell-APC interaction is sequestered from extracellular matrix (Ebnet et al., 1996). Therefore, lymph nodes may present an environment for formation of prolonged (hours) T cell-APC interactions.

The termination of this type of immunological synapse may be as simple as the entry of the T cell into mitosis, which has profound effects on adhesion and cytoskeletal assemblies. If one daughter cell maintained the synapse, this could form a basis for asymmetric cell division to generate a daughter cells with predispositions toward different developmental fates. This could be useful for dividing cells into effector and memory phenotypes in the appropriate ratios (Sallusto et al., 1999). Alternatively, one possibility is that death or migration of the dendritic cell may also limit the duration of immunological synapse formation (Ingulli et al., 1997).

#### Conclusions

Focusing on the individual steps of physiological T cell activation allows application of a number of genetic and biochemical studies on model systems to be applied to understanding the integrated process of T cell activation. The flow of events from initial polarization of the T

cell to formation and termination of the synapse may span a period of 2 days *in vivo*. What are the determinants that control this process? We know that the kinetics of the TCR interaction with the MHC-peptide complex, the number of MHC-peptide complexes, and the presence of specific adhesion receptors are all important. Additional molecules and environments are likely to play an important role in formation of stable synapses. These determinants appear to regulate the interaction of signals and morphogenic processes such as actin polymerization and myosin II activation in the synapse. The process of immunological synapse formation has been studied most extensively in mature effector T cells. Further study will be required to determine the role of immunological synapse formation for different populations of T lymphocytes *in vivo*, including naïve T cells and memory T cells, which have different activation requirements in terms of number of MHC-peptide complexes and duration of signaling. Within the effector and memory populations there are further functional divisions of T cells into phenotypes that stimulate phagocytic antimicrobial responses (Th1) or stimulate allergic type hypersensitivity reactions (Th2). While Th1/Th2 choice is determined by cytokines, response of T cells to cytokines may be modulated by the immunological synapse just as the response of fibroblasts to growth factors is modulated by focal adhesions. The immunological synapse concept is also important in understanding directed cytokine secretion (Paul and Seder, 1994). T cells secreting different cytokines at different targets may form different kinds of synapses. Further study is required to determine the role of synapse formation in T cell differentiation and in the distinct effector functions of these cells. Finally, the area of T cell development is largely unexplored with respect to immunological synapse formation. It will be exciting to determine how temporal changes in gene expression in the developmental lineage alter cell interaction and the underlying molecular patterns. Is the bull's-eye pattern of the immunological synapse (Figure 1B) the supramolecular shape of things to come in signaling? While the concept of the synapse is over a hundred years old, studies in both the nervous system and immune systems on molecular organization of synapses are just beginning. It is likely that many surprises are still in store.

#### Acknowledgments

We thank Jerri Smith for preparation of the manuscript and Daved Fremont for help with the graphics. We also thank the reviewers and editor for their suggestions on improving the manuscript. We apologize to colleagues whose work could not be directly cited due to length constraints.

#### References

- Anderson, H.A., Hiltbold, E.M., and Roche, P.A. (2000). Concentration of MHC class II molecules in lipid rafts facilitates antigen presentation. *Nat. Immunol.* 2, 156–162.
- Boniface, J.J., Reich, Z., Lyons, D.S., and Davis, M.M. (1999). Thermodynamics of T cell receptor binding to peptide-MHC: evidence for a general mechanism of molecular scanning. *Proc. Natl. Acad. Sci. USA* 96, 11446–11451.
- Braun, J., Fujiwara, K., Pollard, T.D., and Unanue, E.R. (1978). Two distinct mechanisms for redistribution of lymphocyte surface macromolecules. I. Relationship to cytoplasmic myosin. *J. Cell Biol.* 79, 409–418.
- Brdicka, T., Pavlistova, D., Leo, A., Bruyns, E., Korinek, V., Angelisova, P., Scherer, J., Shevchenko, A., Hilgert, I., Cerny, J., et al. (2000). Phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG), a novel ubiquitously expressed transmembrane adaptor protein, binds the protein tyrosine kinase csk and is involved in regulation of T cell activation. *J. Exp. Med.* 191, 1591–1604.
- Bromley, S.K., Peterson, D.A., Gunn, M.D., and Dustin, M.L. (2000). Cutting edge: hierarchy of chemokine receptor and TCR signals regulating T cell migration and proliferation. *J. Immunol.* 165, 15–19.
- Brown, D.A., and London, E. (2000). Structure and function of sphingolipid- and cholesterol-rich membrane rafts. *J. Biol. Chem.* 275, 17221–17224.
- Bubeck Wardenburg, J., Pappu, R., Bu, J.Y., Mayer, B., Chernoff, J., Straus, D., and Chan, A.C. (1998). Regulation of PAK activation and the T cell cytoskeleton by the linker protein SLP-76. *Immunity* 9, 607–616.
- Bunnell, S.C., Diehn, M., Yaffe, M.B., Findell, P.R., Cantley, L.C., and Berg, L.J. (2000). Biochemical interactions integrating Itk with the T cell receptor-initiated signaling cascade. *J. Biol. Chem.* 275, 2219–2230.
- Cheng, A.M., and Chan, A.C. (1997). Protein tyrosine kinases in thymocyte development. *Curr. Opin. Immunol.* 9, 528–533.
- Cloutier, J.F., Chow, L.M., and Veillette, A. (1995). Requirement of the SH3 and SH2 domains for the inhibitory function of tyrosine protein kinase p50csk in T lymphocytes. *Mol. Cell. Biol.* 15, 5937–5944.
- Costello, P.S., Walters, A.E., Mee, P.J., Turner, M., Reynolds, L.F., Prisco, A., Sarner, N., Zamoyska, R., and Tybulewicz, V.L. (1999). The Rho-family GTP exchange factor Vav is a critical transducer of T cell receptor signals to the calcium, ERK, and NF-kappaB pathways. *Proc. Natl. Acad. Sci. USA* 96, 3035–3040.
- Costello, P.S., Cleverley, S.C., Galandrini, R., Henning, S.W., and Cantrell, D.A. (2000). The GTPase rho controls a p53-dependent survival checkpoint during thymopoiesis. *J. Exp. Med.* 192, 77–86.
- Crespo, P., Schuebel, K.E., Ostrom, A.A., Gutkind, J.S., and Bustelo, X.R. (1997). Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the vav proto-oncogene product. *Nature* 385, 169–172.
- da Silva, A.J., Li, Z., de Vera, C., Canto, E., Findell, P., and Rudd, C.E. (1997). Cloning of a novel T-cell protein FYB that binds FYN and SH2-domain-containing leukocyte protein 76 and modulates interleukin 2 production. *Proc. Natl. Acad. Sci. USA* 94, 7493–7498.
- D'Oro, U., and Ashwell, J.D. (1999). Cutting edge: the CD45 tyrosine phosphatase is an inhibitor of Lck activity in thymocytes. *J. Immunol.* 162, 1879–1883.
- 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 Springer, T.A. (1989). T cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. *Nature* 341, 619–624.
- Dustin, M.L., Golan, D.E., Zhu, D.M., Miller, J.M., Meier, W., Davies, E.A., and van der Merwe, P.A. (1997). Low affinity interaction of human or rat T cell adhesion molecule CD2 with its ligand aligns adhering membranes to achieve high physiological affinity. *J. Biol. Chem.* 272, 30889–30898.
- Dustin, M.L., Olszowy, M.W., Holdorf, A.D., Li, J., Bromley, S., Desai, N., Widder, P., Rosenberger, F., van der Merwe, P.A., Allen, P.M., and Shaw, A.S. (1998). A novel adapter protein orchestrates receptor patterning and cytoskeletal polarity in T cell contacts. *Cell* 94, 667–677.
- Ebnet, K., Kaldjian, E.P., Anderson, A.O., and Shaw, S. (1996). Orchestrated information transfer underlying leukocyte endothelial interactions. *Annu. Rev. Immunol.* 14, 155–177.
- Elson, E.L., Pasternak, C., Liu, Z.Y., Young, J.I., Schwab, B., Worthen, G.S., Downey, G., Michaels, R., McConnaughey, W.B., McDan-

- iel, M., et al. (1990). Activation of mechanical responses in leukocytes. *Biorheology* 27, 849–858.
- Felder, S., Zhou, M., Hu, P., Urena, J., Ullrich, A., Chaudhuri, M., White, M., Shoelson, S.E., and Schlessinger, J. (1993). SH2 domains exhibit high-affinity binding to tyrosine-phosphorylated peptides yet also exhibit rapid dissociation and exchange. *Mol. Cell. Biol.* 13, 1449–1455.
- Felsenfeld, D.P., Choquet, D., and Sheetz, M.P. (1996). Lingand binding regulates the directed movement of beta1 integrins on fibroblasts. *Nature* 383, 438–440.
- Fischer, K.D., Kong, Y.Y., Nishina, H., Tedford, K., Marengere, L.E., Kozieradzki, I., Sasaki, T., Starr, M., Chan, G., Gardener, S., et al. (1998). Vav is a regulator of cytoskeletal reorganization mediated by the T-cell receptor. *Curr. Biol.* 8, 554–562.
- Forster, R., Schubel, A., Breitfeld, D., Kremmer, E., Renner-Muller, I., Wolf, E., and Lipp, M. (1999). CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* 99, 23–33.
- Frischknecht, F., Moreau, V., Rottger, S., Gonfloni, S., Reckmann, I., Superti-Furga, G., and Way, M. (1999). Actin-based motility of vaccinia virus mimics receptor tyrosine kinase signalling. *Nature* 401, 926–929.
- Garcia, K.C., Degano, M., Stanfield, R.L., Brunmark, A., Jackson, M.R., Peterson, P.A., Teyton, L., and Wilson, I.A. (1996). An  $\alpha\beta$  T cell receptor structure at 2.5Å resolution and its orientation in the TCR-MHC complex. *Science* 274, 209–219.
- 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.
- Gunzer, M., Schäfer, A., Borgmann, S., Grabbe, S., Zänker, K.S., Bröcker, E.B., Kämpgen, E., and Friedl, P. (2000). Antigen presentation in extracellular matrix: interactions of T cells with dendritic cells are dynamic, short lived, and sequential. *Immunity* 13, 323–332.
- Han, J., Das, B., Wei, W., Van Aelst, L., Mosteller, R.D., Khosravi-Far, R., Westwick, J.K., Der, C.J., and Broek, D. (1997). Lck regulates Vav activation of members of the Rho family of GTPases. *Mol. Cell. Biol.* 17, 1346–1353.
- Harden, N., Lee, J., Loh, H.Y., Ong, Y.M., Tan, I., Leung, T., Manser, E., and Lim, L. (1996). A Drosophila homolog of the Rac- and Cdc42-activated serine/threonine kinase PAK is a potential focal adhesion and focal complex protein that colocalizes with dynamic actin structures. *Mol. Cell. Biol.* 16, 1896–1908.
- Higgs, H.N., and Pollard, T.D. (2000). Activation by Cdc42 and PIP(2) of Wiskott-Aldrich syndrome protein (WASP) Stimulates Actin Nucleation by Arp2/3 Complex. *J. Cell Biol.* 150, 1311–1320.
- Holsinger, L.J., Graef, I.A., Swat, W., Chi, T., Bautista, D.M., Davidson, L., Lewis, R.S., Alt, F.W., and Crabtree, G.R. (1998). Defects in actin-cap formation in Vav-deficient mice implicate an actin requirement for lymphocyte signal transduction. *Curr. Biol.* 8, 563–572.
- Iezzi, G., Karjalainen, K., and Lanzavecchia, A. (1998). The duration of antigenic stimulation determines the fate of naive and effector T cells. *Immunity* 8, 89–95.
- Ingulli, E., Mondino, A., Khoruts, A., and Jenkins, M.K. (1997). In vivo detection of dendritic cell antigen presentation to CD4(+) T cells. *J. Exp. Med.* 185, 2133–2141.
- Janes, P.W., Ley, S.C., and Magee, A.I. (1999). Aggregation of lipid rafts accompanies signaling via the T cell antigen receptor. *J. Cell Biol.* 147, 447–461.
- Johnson, K.G., Bromley, S.K., Dustin, M.L., and Thomas, M.L. (2000). A supramolecular basis for CD45 regulation during T cell activation. *Proc. Natl. Acad. Sci. USA* 97, 10138–10143.
- Kawabuchi, M., Satomi, Y., Takao, T., Shimonishi, Y., Nada, S., Nagai, K., Tarakhovskiy, A., and Okada, M. (2000). Transmembrane phosphoprotein Cbp regulates the activities of Src-family tyrosine kinases. *Nature* 404, 999–1003.
- Klausner, R.D., Kleinfeld, A.M., Hoover, R.L., and Karnovsky, M.J. (1980). Lipid domains in membranes. Evidence derived from structural perturbations induced by free fatty acids and lifetime heterogeneity analysis. *J. Biol. Chem.* 255, 1286–1295.
- Krause, M., Sechi, A.S., Konradt, M., Monner, D., Gertler, F.B., and Wehland, J. (2000). Fyn-binding protein (Fyb)/SLP-76-associated protein (SLAP), Ena/Vasodilator-stimulated phosphoprotein (VASP) proteins and the Arp2/3 complex link T cell receptor (TCR) signaling to the actin cytoskeleton. *J. Cell Biol.* 149, 181–194.
- Krummel, M.F., Sjaastad, M.D., Wulfig, C., and Davis, M.M. (2000). Differential clustering of CD4 and CD3zeta during T cell recognition. *Science* 289, 1349–1352.
- Kucik, D.F., Dustin, M.L., Miller, J.M., and Brown, E.J. (1996). Adhesion activating phorbol ester increases the mobility of leukocyte integrin LFA-1 in cultured lymphocytes. *J. Clin. Invest.* 97, 2139–2144.
- Kupfer, A., Mosmann, T.R., and Kupfer, H. (1991). Polarized expression of cytokines in cell conjugates of helper T cells and splenic B cells. *Proc. Natl. Acad. Sci. USA* 88, 775–779.
- Letourneur, F., and Klausner, R.D. (1992). Activation of T cells by a tyrosine kinase activation domain in the cytoplasmic tail of CD3 epsilon. *Science* 255, 79–82.
- Leupin, O., Zaru, R., Laroche, T., Muller, S., and Valitutti, S. (2000). Exclusion of CD45 from the T-cell receptor signaling area in antigen-stimulated T lymphocytes. *Curr. Biol.* 10, 277–280.
- Li, B., Yu, H., Zheng, W., Voll, R., Na, S., Roberts, A.W., Williams, D.A., Davis, R.J., Ghosh, S., and Flavell, R.A. (2000). Role of the guanosine triphosphatase rac2 in T helper 1 cell differentiation. *Science* 288, 2219–2222.
- Linder, S., Higgs, H., Hufner, K., Schwarz, K., Pannicke, U., and Aepfelbacher, M. (2000). The polarization defect of Wiskott-Aldrich syndrome macrophages is linked to dislocalization of the Arp2/3 complex. *J. Immunol.* 165, 221–225.
- Liu, S.K., and McGlade, C.J. (1998). Gads is a novel SH2 and SH3 domain-containing adaptor protein that binds to tyrosine-phosphorylated Shc. *Oncogene* 17, 3073–3082.
- Liu, S.K., Fang, N., Koretzky, G.A., and McGlade, C.J. (1999). The hematopoietic-specific adaptor protein gads functions in T-cell signaling via interactions with the SLP-76 and LAT adaptors. *Curr. Biol.* 9, 67–75.
- Lowin-Kropf, B., Shapiro, V.S., and Weiss, A. (1998). Cytoskeletal polarization of T cells is regulated by an immunoreceptor tyrosine-based activation motif-dependent mechanism. *J. Cell Biol.* 140, 861–871.
- Lub, M., van Kooyk, Y., van Vliet, S.J., and Figdor, C.G. (1997). Dual role of the actin cytoskeleton in regulating cell adhesion mediated by the integrin lymphocyte function associated antigen-1. *Mol. Biol. Cell* 8, 341–351.
- Machesky, L.M. (2000). Putting on the brakes: a negative regulatory function for Ena/VASP proteins in cell migration. *Cell* 101, 685–688.
- Mainiero, F., Soriani, A., Strippoli, R., Jacobelli, J., Gismondi, A., Piccoli, M., Frati, L., and Santoni, A. (2000). RAC1/P38 MAPK signaling pathway controls beta1 integrin-induced interleukin-8 production in human natural killer cells. *Immunity* 12, 7–16.
- Manser, E., Loo, T.H., Koh, C.G., Zhao, Z.S., Chen, X.Q., Tan, L., Tan, I., Leung, T., and Lim, L. (1998). PAK kinases are directly coupled to the PIX family of nucleotide exchange factors. *Mol. Cell* 1, 183–192.
- Matsui, K., Boniface, J.J., Steffner, P., Reay, P.A., and Davis, M.M. (1994). Kinetics of T-cell receptor binding to peptide/IEK complexes: correlation of the dissociation rate with T cell responsiveness. *Proc. Natl. Acad. Sci. USA* 91, 12862–12866.
- Mejete, R., Bilwes, A.M., Noel, J.P., Hunter, T., and Weiss, A. (1998). Dimerization-induced inhibition of receptor protein tyrosine phosphatase function through an inhibitory wedge. *Science* 279, 88–91.
- Moffett, S., Brown, D.A., and Linder, M.E. (2000). Lipid-dependent targeting of G proteins into rafts. *J. Biol. Chem.* 275, 2191–2198.
- Monks, C.R., Freiberg, B.A., Kupfer, H., Sciaky, N., and Kupfer, A. (1998). Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* 395, 82–86.
- Myung, P.S., Boerthe, N.J., and Koretzky, G.A. (2000). Adapter pro-

- teins in lymphocyte antigen-receptor signaling. *Curr. Opin. Immunol.* **12**, 256–266.
- Norcross, M.A. (1984). A synaptic basis for T-lymphocyte activation. *Ann. Immunol.* **135D**, 113–134.
- Paul, W.E., and Seder, R.A. (1994). Lymphocyte responses and cytokines. *Cell* **76**, 241–251.
- Reich, Z., Boniface, J.J., Lyons, D.S., Borochoy, N., Wachtel, E.J., and Davis, M.M. (1997). Ligand-specific oligomerization of T cell-receptor molecules. *Nature* **387**, 617–620.
- Richieri, G.V., Mescher, M.F., and Kleinfeld, A.M. (1990). Short term exposure to cis unsaturated free fatty acids inhibits degranulation of cytotoxic T lymphocytes. *J. Immunol.* **144**, 671–677.
- Roach, T.I., Slater, S.E., White, L.S., Zhang, X., Majerus, P.W., Brown, E.J., and Thomas, M.L. (1998). The protein tyrosine phosphatase SHP-1 regulates integrin-mediated adhesion of macrophages. *Curr. Biol.* **8**, 1035–1038.
- Rodgers, W., and Rose, J.K. (1996). Exclusion of CD45 inhibits activity of p56lck associated with glycolipid-enriched membrane domains. *J. Cell Biol.* **135**, 1515–1523.
- Rohatgi, R., Ma, L., Miki, H., Lopez, M., Kirchhausen, T., Takenawa, T., and Kirschner, M.W. (1999). The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. *Cell* **97**, 221–231.
- Rozelle, A.L., Machesky, L.M., Yamamoto, M., Driessens, M.H., In-sall, R.H., Roth, M.G., Luby-Phelps, K., Marriotti, G., Hall, A., and Yin, H.L. (2000). Phosphatidylinositol 4,5-bisphosphate induces actin-based movement of raft-enriched vesicles through WASP-Arp2/3. *Curr. Biol.* **10**, 311–320.
- Sallusto, F., Lenig, D., Forster, R., Lipp, M., and Lanzavecchia, A. (1999). Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* **401**, 708–712.
- Schmidt, C.E., Horwitz, A.F., Lauffenburger, D.A., and Sheetz, M.P. (1993). Integrin-cytoskeletal interactions in migrating fibroblasts are dynamic, asymmetric, and regulated. *J. Cell Biol.* **123**, 977–991.
- Servant, G., Weiner, O.D., Herzmark, P., Balla, T., Sedat, J.W., and Bourne, H.R. (2000). Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. *Science* **287**, 1037–1040.
- Sheets, E.D., Holowka, D., and Baird, B. (1999). Critical role for cholesterol in Lyn-mediated tyrosine phosphorylation of FcεpsilonR1 and their association with detergent-resistant membranes. *J. Cell Biol.* **145**, 877–887.
- Snapper, S.B., Rosen, F.S., Mizoguchi, E., Cohen, P., Khan, W., Liu, C.H., Hagemann, T.L., Kwan, S.P., Ferrini, R., Davidson, L., et al. (1998). Wiskott-Aldrich syndrome protein-deficient mice reveal a role for WASP in T but not B cell activation. *Immunity* **9**, 81–91.
- Sperling, A.I., Sedy, J.R., Manjunath, N., Kupfer, A., Ardman, B., and Burkhardt, J.K. (1998). TCR signaling induces selective exclusion of CD43 from the T cell-antigen-presenting cell contact site. *J. Immunol.* **161**, 6459–6462.
- Springer, T.A. (1990). Adhesion receptors of the immune system. *Nature* **346**, 425–433.
- Stewart, D.M., Tian, L., and Nelson, D.L. (1999). Mutations that cause the Wiskott-Aldrich syndrome impair the interaction of Wiskott-Aldrich syndrome protein (WASP) with WASP interacting protein. *J. Immunol.* **162**, 5019–5024.
- Stowers, L., Yelon, D., Berg, L.J., and Chant, J. (1995). Regulation of the polarization of T cells toward antigen-presenting cells by ras-related GTPase CDC42. *Proc. Natl. Acad. Sci. USA* **92**, 5027–5031.
- Sun, Z., Arendt, C.W., Ellmeier, W., Schaeffer, E.M., Sunshine, M.J., Gandhi, L., Annes, J., Petrzilka, D., Kupfer, A., Schwartzberg, P.L., and Littman, D.R. (2000). PKC-θ is required for TCR-induced NK-κB activation in mature but not immature T lymphocytes. *Nature* **404**, 402–407.
- Szollasi, J., Horejsi, V., Bene, L., Angelisova, P., and Damjanovich, S. (1996). Supramolecular complexes of MHC class I, MHC class II, CD20, and tetraspan molecules (CD53, CD81, and CD82) at the surface of a B cell line JY. *J. Immunol.* **157**, 2939–2946.
- Tuosto, L., and Acuto, O. (1998). CD28 affects the earliest signaling events generated by TCR engagement. *Eur. J. Immunol.* **28**, 2131–2142.
- Turley, S.J., Inaba, K., Garrett, W.S., Ebersold, M., Untermaier, J., Steinman, R.M., and Mellman, I. (2000). Transport of peptide-MHC class II complexes in developing dendritic cells. *Science* **288**, 522–527.
- Unanue, E.R., Perkins, W.D., and Karnovsky, M.J. (1972). Ligand-induced movement of lymphocyte membrane macromolecules. I. Analysis by immunofluorescence and ultrastructural radioautography. *J. Exp. Med.* **136**, 885–906.
- Underhill, D.M., Bassetti, M., Rudensky, A., and Aderem, A. (1999). Dynamic interactions of macrophages with T cells during antigen presentation. *J. Exp. Med.* **190**, 1909–1914.
- Valututti, S., Müller, S., Cella, M., Padovan, E., and Lanzavecchia, A. (1995). Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature* **375**, 148–151.
- van der Merwe, P.A., Davis, S.J., Shaw, A.S., and Dustin, M.L. (2000). Cytoskeletal polarization and redistribution of cell-surface molecules during T cell antigen recognition. *Semin. Immunol.* **12**, 5–21.
- Vasioukhin, V., Bauer, C., Yin, M., and Fuchs, E. (2000). Directed actin polymerization is the driving force for epithelial cell-cell adhesion. *Cell* **100**, 209–219.
- Villalba, M., Coudronniere, N., Deckert, M., Teixeira, E., Mas, P., and Altman, A. (2000). A novel functional interaction between Vav and PKCθ is required for TCR-induced T cell activation. *Immunity* **12**, 151–160.
- Viola, A., Schroeder, S., Sakakibara, Y., and Lanzavecchia, A. (1999). T lymphocyte costimulation mediated by reorganization of membrane microdomains. *Science* **283**, 680–682.
- Wang, J.H., Smolyar, A., Tan, K., Liu, J.H., Kim, M., Sun, Z.Y., Wagner, G., and Reinherz, E.L. (1999). Structure of a heterophilic adhesion complex between the human CD2 and CD58 (LFA-3) counterreceptors. *Cell* **97**, 791–803.
- Wei, X., Tromberg, B.J., and Cahalan, M.D. (1999). Mapping the sensitivity of T cells with an optical trap: polarity and minimal number of receptors for Ca(2+) signaling. *Proc. Natl. Acad. Sci. USA* **96**, 8471–8476.
- Weiner, O.D., Servant, G., Welch, M.D., Mitchison, T.J., Sedat, J.W., and Bourne, H.R. (1999). Spatial control of actin polymerization during neutrophil chemotaxis. *Nat. Cell Biol.* **1**, 75–81.
- Werlen, G., Hausmann, B., and Palmer, E. (2000). A motif in the alpha T-cell receptor controls positive selection by modulating ERK activity. *Nature* **406**, 422–426.
- Wild, M.K., Cambiaggi, A., Brown, M.H., Davies, E.A., Ohno, H., Saito, T., and van der Merwe, P.A. (1999). Dependence of T cell antigen recognition on the dimensions of an accessory receptor-ligand complex. *J. Exp. Med.* **190**, 31–41.
- Wülfing, C., and Davis, M.M. (1998). A receptor/cytoskeletal movement triggered by costimulation during T cell activation. *Science* **282**, 2266–2269.
- Wülfing, C., Sjaastad, M.D., and Davis, M.M. (1998). Visualizing the dynamics of T cell activation: intracellular adhesion molecule 1 migrates rapidly to the T cell/B cell interface and acts to sustain calcium levels. *Proc. Natl. Acad. Sci. USA* **95**, 6302–6307.
- Yang, W.C., Collette, Y., Nunes, J.A., and Olive, D. (2000). Tec kinases: a family with multiple roles in immunity. *Immunity* **12**, 373–382.
- Yashiro-Ohtani, Y., Zhou, X.Y., Toyo-Oka, K., Tai, X.G., Park, C.S., Hamaoka, T., Abe, R., Miyake, K., and Fujiwara, H. (2000). Non-CD28 costimulatory molecules present in T cell rafts induce T cell costimulation by enhancing the association of TCR with rafts. *J. Immunol.* **164**, 1251–1259.
- Zhang, W., Triple, R.P., and Samelson, L.E. (1998). LAT palmitoylation: its essential role in membrane microdomain targeting and tyrosine phosphorylation during T cell activation. *Immunity* **9**, 239–246.
- Zhang, W., Triple, R.P., Zhu, M., Liu, S.K., McGlade, C.J., and Samelson, L.E. (2000). The association of Grb2, Gads and phospholipase C-γ1 with phosphorylated LAT tyrosine residues: the effect of tyrosine mutations on T cell antigen receptor-mediated signaling. *J. Biol. Chem.* **275**, 23355–23361.