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THE IMMUNOLOGICAL SYNAPSE

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■ **Abstract** The adaptive immune response is initiated by the interaction of T cell antigen receptors with major histocompatibility complex molecule-peptide complexes in the nanometer scale gap between a T cell and an antigen-presenting cell, referred to as an immunological synapse. In this review we focus on the concept of immunological synapse formation as it relates to membrane structure, T cell polarity, signaling pathways, and the antigen-presenting cell. Membrane domains provide an organizational principle for compartmentalization within the immunological synapse. T cell polarization by chemokines increases T cell sensitivity to antigen. The current model is that signaling and formation of the immunological synapse are tightly interwoven in mature T cells. We also extend this model to natural killer cell activation, where the inhibitory NK synapse provides a striking example in which inhibition of signaling leaves the synapse in its nascent, inverted state. The APC may also play an active role in immunological synapse formation, particularly for activation of naïve T cells.

INTRODUCTION

T cell activation requires sustained T cell receptor (TCR) interaction with MHC-peptide complexes in the immunological synapse (IS) between the T cell and antigen-presenting cell (APC) (1, 2). The required duration of signaling is on the order of hours, while the activating and inhibitory molecular interactions in the IS have half-lives on the order of seconds (3, 4). Imaging studies of the IS have revealed a remarkable organization that may help account for the longevity and specificity of signaling. The mature IS has been defined by the bull's eye arrangement of supramolecular activation clusters (SMACs) that form within a few minutes of T cell–APC contact (5–7). SMACs are detected by fluorescence microscopy and ap-

pear as increased densities of specific molecules (Figure 1). The center of the bull's eye or cSMAC is enriched for TCR and MHC-peptide complexes. The ring of the bull's eye or pSMAC contains the integrin LFA-1 and its major counterreceptor ICAM-1. The cSMAC also includes the signaling molecules on the T cell cytoplasmic side of the IS, including protein kinase C θ (PKC θ) and the src family kinase lck. The T cell side of the pSMAC contains the integrin-associated cytoskeletal proteins including talin. The formation of the IS requires an intact T cell cytoskeleton and actually begins as an inverted structure with a central adhesion cluster surrounded by a ring of engaged TCR (5). The APC can be replaced by an artificial phospholipid bilayer. Therefore, activity of the APC is not absolutely required but may regulate IS formation. The final stage of IS formation is the stabilization of the central cluster of MHC-peptide complexes, which correlates with sustained parallel engagement of TCR by at least 60 MHC-peptide complexes. Maintenance of the stabilized IS for greater than 1 hr is well correlated with full T cell activation.

Physiological T cell activation can be divided into a series of temporal stages: T cell polarization, initial adhesion, IS formation (initial signaling), and IS maturation (sustained signaling) (5). The IS embodies a concept for understanding the high-order temporal and spatial cooperation of multiple biochemical and genetic elements known to be required for T cell activation. We discuss membrane organization at the outset due to the fundamental nature of this information and the need to incorporate recent advances in this area into an overview of how signaling complexes form in the IS. We then outline the temporal stages in formation of the IS and their relationship to signaling. The natural killer cell IS is presented as a showcase for the integration of positive and negative signals. Finally, evidence for an active role for the APC in IS formation is considered.

MEMBRANE RAFTS

Intrinsic Organization of the Plasma Membrane

Since our goal is to describe the highly organized structure that forms at the contact between T cell and APC, it is appropriate to first review concepts about preexisting structures within the plasma membrane. Cell biologists have long acknowledged that the plasma membrane is not homogenous (8, 9). This concept is currently associated with the word and concept of a raft. This topic has been extensively reviewed (10, 11). A particularly useful, broader, and more succinct overview of the subject is available (12). We focus on aspects that are most relevant to IS function.

The physical basis of rafts has been extensively discussed over many years as "membrane domains," and the process that produces them has been termed "lateral phase separation" (8, 9). Model membranes of pure phospholipids display a characteristic melting temperature with acyl chains ordered below and disordered above this temperature. In a membrane composed of several different types of phospholipids, the various lipids may not mix, and laterally segregated domains may form with relatively stable ordered and disordered regions. While the effect

of cholesterol on membrane structure is variable depending on the specific lipid and its phase, it is generally true that cholesterol induces the formation of liquid-ordered structures, which segregate from disordered structures. These cholesterol induced liquid-ordered regions are enriched in cholesterol itself and appear to also include gangliosides like GM1 and the glycosylphosphatidylinositol (GPI) moieties of glycolipid anchored proteins that are incorporated into the liquid-ordered structures. Interestingly, the liquid-ordered structures are relatively insoluble in Triton X-100 detergent at low temperature, a property shared with biological rafts, which are also referred to as detergent-insoluble, glycolipid-enriched domains (DIGs) or glycosphingolipid enriched membranes (GEMs) (12). Hence, detergent insolubility of proteins is used as a biochemical marker for inclusion in rafts. The presence of many intrinsic membrane proteins in biological membranes may perturb or reinforce lipid packing and have a profound influence on the size and stability of lipid-based structures. The idea that proteins selectively associate with different lipid structures or may themselves organize lipid structures is central to the proposed roles of rafts in signaling and the IS.

EARLY EVIDENCE FOR RAFTS

T cell activation by cross-linking of rafts was described nearly a quarter century ago. These experiments were based on cholera toxin, a pentavalent molecule that binds with high affinity to GM1 and that upon further cross-linking induces capping of this lipid (13). Up to that point, capping had been a phenomenon performed using antibodies and related reagents to aggregate proteins (14). Capping of gangliosides suggested a "possible direct or indirect association between surface gangliosides and submembraneous cytoskeletal assemblies that control modulation of these surface components and may transmit stimuli to the interior of the cell" (15). Within the following decade, Spiegel and coworkers showed that cross-linking of gangliosides in thymocyte membranes resulted in their patching and induced DNA replication. The authors suggested "that gangliosides may self-associate in the plasma membrane, which may explain the basis for ganglioside redistribution and capping" (16).

A decade ago, two works appeared that seem to have set the stage for the current interest in rafts among cell biologists and immunologists. Brown & Rose indicated a role of lateral phase separation of glycosphingolipids and cholesterol in apical targeting of GPI-anchored proteins in polarized epithelial cells (17). Stefanova and coworkers demonstrated that lck could be co-immunoprecipitated with GPI-anchored proteins providing evidence for lipid-based structures spanning the plasma membrane (18). The wider impact of these ideas on several cell biological problems has been summarized (19).

Controversy over the Physical Nature of Rafts

The putative rafts are too small for visualization by light microscopy. The distribution of raft markers is homogenous in resting cells. For example, prior to exposing

a mast cell line to its antigen, the Fc-receptor and GM1 are homogeneously distributed (20, 21). The same applies to the TCR and GM1 in studies of T cells (22). In general, cross-linking has been required to produce large enough aggregates to be visualized using epifluorescence microscopy.

A number of groups have attempted imaging methods that extend the resolution of fluorescence microscopy. Fluorescence resonance energy transfer (FRET) is sensitive to distances on the order of a few nanometers. Because FRET between GPI-anchored transferrin receptors interacted with natural ligands, Varma & Mayor concluded that rafts exist, but they are *smaller* than 70 nm in diameter (23). Using a similar approach and observing four distinct constructs interrogated with larger probes, Edidin and colleagues found no evidence of microdomains enriched in either GPI-anchored proteins or GM1 (24). These disparate results could be reconciled if the unperturbed rafts were indeed at a nanometer scale.

Baird and coworkers propose that prior to cross-linking, the rafts are sufficiently small that each contains a single molecule of the tyrosine kinase lyn or FcRε (20). Cross-linking the FcR creates a larger aggregate that can then recruit the small monomeric rafts, each containing a single molecule of lyn. This coalescence of rafts allows lyn to phosphorylate a large number of FcRs, thus allowing phosphorylation and signaling. The model reflects a much older concept of annular lipid, where rafts would represent the ability of specific proteins and lipoproteins to recruit a surround of liquid-ordered lipids that would then be predisposed to coalesce following either cross-linking or changes in conformation. Therefore, the initial size of rafts may be quite small, but the regulation of raft aggregation by cross-linking or lateral protein interactions in membranes is likely central in signaling and IS formation.

IMMUNOLOGICAL SYNAPSE FORMATION

T Cell Polarization and Initial Adhesion

Circulating T cells are rounded and nonpolarized, with a uniform radial distribution of membrane domains, receptors, and microvilli on the cell surface (25). These cells are relatively nonmotile and integrin adhesion molecules are maintained in a low-activity state (26). Thus, initial adhesion between the naive T cell and APC might require an innate signal that sets the stage for IS formation, for example, exposure to chemokines. T cells encounter chemokine gradients as they extravasate into lymph nodes and inflamed tissues. Therefore, T cells will be exposed to chemokines before they encounter APC.

Chemokine receptor signaling results in the rapid polarization of T lymphocytes. Exposure to a chemokine gradient induces the formation of a front end, or lamellapodium, and a back end, or uropod (25). Chemokine receptors are members of the serpentine family that link to heterotrimeric G proteins. Interestingly, chemokine receptor and TCR signaling share a number of components but differ greatly with respect to time. Effective chemokine signals are transient and determine the

differential of signal strength as a function of time or distance. In contrast, TCR signals are maintained for hours and appear to integrate MHC-peptide and adhesion molecule signals to determine if they reach or exceed thresholds set during T cell differentiation.

Interaction with chemokines induces conformational changes in chemokine receptors that lead to the dissociation of the heterotrimeric G protein into active α (GTP bound) and $\beta\gamma$ subunits. While the role of rafts in G-protein signaling is not clear, it is interesting that $\beta\gamma$ subunits are prenylated, which favors exclusion from raft lipids, while the α subunit is acylated, which favors association with raft lipids (27). The time taken by the α subunit to hydrolyze GTP defines the time window of $\beta\gamma$ subunit activity. This time period is influenced by a regulator of G-protein signaling (RGS) proteins that act as GTPase activating proteins (GAPs) for the α subunits (28). In addition to this intrinsic timing mechanism, chemokine receptors are desensitized by G-protein receptor kinase/arrestin mechanisms acting on the receptor itself (29). The active $\beta\gamma$ subunits interact with phospholipase C (PLC) and phosphatidylinositol-3-kinase (PI-3K). PLCγ and PI-3K in turn activate protein kinase C (PKC) and generate ligands for pleckstrin homology (PH) domains, respectively. The Vav protooncogene and WASP, the protein deficient in the X-linked immunodeficiency Wiskott-Aldrich syndrome, have functionally relevant PH domains. Vav is a rho family guanine nucleotide exchange factor (GEF) that activates CDC42 and rac (30). WASP is activated by phosphatidylinositol (PtdIns)-4,5-bisphosphate and CDC42, and in turn WASP recruits and activates the ARP2/3 complex (31). The activated ARP2/3 complex nucleates new actin polymerization, a fundamental process for cell locomotion.

The response to chemokines is itself a polarized process. In granulocytes responding to a gradient of the chemoattractant fMLP, the PH domain of the Akt kinase, which interacts with phosphatidylinositol-3,4,5-trisphosphate, is transiently accumulated in the leading lamellipodium (32). Finally, neutrophils permeabilized after exposure to chemokine gradients demonstrated that new actin filaments were selectively generated at the edge of the cell near the chemoattractant source (33). These studies suggest that the leading edge of the migrating leukocyte is itself a specialized supramolecular compartment even before IS formation is initiated.

These changes at the leading edge of polarized lymphocytes lead to increased sensitivity to antigen. This has been directly demonstrated by studies in which polarized lymphocytes were contacted with APC on the leading membrane projections or on their uropods, where the leading edge was found to be greater than tenfold more sensitive than the uropod to antigen (34). The same signals that enhance actin polymerization at the leading edge are also implicated in integrin activation, possibly because integrin regulation requires coordinated actin polymerization. Enhanced integrin engagement at the leading edge may trigger actin-based protrusions tipped by TCR-enriched membranes that could form the basis of sensory contacts involved in MHC-peptide sampling and the initial stage of IS formation (35). An actin-based program for developing adhesion molecule–tipped protrusion has been vividly described for epithelial cell contacts (36). Conversely,

the uropod is important as a site at which adhesive interactions are broken and as a depot for molecules that might inhibit interactions. For example, the mucin CD43 is sequestered in the uropod of polarized T cells, which may reduce the antiadhesive effects of this protein at the leading edge (25). Thus, polarization sets the stage for effective adhesion, TCR engagement, and IS formation.

ADHESION

Integrating signals delivered through multiple receptor systems is likely to be a key feature of the immunological synapse. Molecular segregation in the IS is a fundamental process in accomplishing integration. Topological models of T cell signaling predict that those molecules with large extracellular domains would be excluded from sites of TCR/peptide-MHC interactions due to the small distances between apposing membranes ($\sim$15 nm) (37). These close contact areas are established by adhesion molecules, which play an essential role in T cell activation. Close contacts may be achieved indirectly through cytoskeletal protrusions anchored to larger adhesion mechanisms like LFA-1 and ICAM-1 (5) or more directly by smaller adhesion receptor pairs like CD2 and CD58 that would work immediately beside the TCR. In agreement with this idea, increasing the size of the CD48 ligand for CD2 by adding additional Ig-like domains inhibits TCR engagement of MHC-peptide (38). Interestingly, both CD2 and its ligand CD48 are associated with rafts, as is the TCR (39). Depending upon the size of these structures, an initial close association of CD2 and TCR in rafts may explain the dominant negative effect of long forms of CD48, which may themselves be proximal to MHC molecules in rafts on the APC (40). The topological model is also consistent with observations that large mucin-like molecules including CD43 and CD45 are excluded from T cell/APC conjugates (41–43).

T CELL RECEPTOR SIGNALING

The mechanism of selective TCR triggering is a hotly debated area with three major models based on kinetic/oligomerization (44), conformational change (45), and kinetic/segregation (37). Current molecular data on IS formation cannot distinguish between these models, but it is certain that kinetic parameters are important. In particular, the off rate of the monomeric TCR-MHC/peptide interaction (46, 47) plays a decisive role in determining the final MHC density accumulated into the mature IS, and hence the extent of T cell activation (5). Likewise, the inhibitory effects of altered peptide ligands can manifest in a decreased MHC density in the cSMAC, consistent with a spoiler role where unproductive engagement of TCRs by antagonist peptide/MHC with fast off rates could dull the ability of the T cell to cluster MHC (48). Allosteric interactions may nevertheless have an impact on the stability of the central MHC cluster (49). Thermodynamic analysis of TCR/MHC-peptide binding has revealed a case for induced fit, which may explain the ability

of excess TCR to scan through MHC complexes collected in the IS to differentiate signal from noise (50).

IS formation is a clear example where kinetics of interaction is linked to molecular segregation in formation of the SMACs; however, the very earliest signals leading to formation of these micron scale domains are not readily observed by conventional fluorescence microscopy. An added complication is that much of our knowledge of TCR signaling mechanisms is based on antibody cross-linking in T cell lines and hybridomas as the triggering event in the absence of any adhesion or contact organization. Thus, the following discussion combines information from multiple types of experiments with a goal of generating a coherent and testable model for how IS formation integrates the TCR signal with multiple additional signals to set a robust threshold for full T cell activation.

The first biochemical event that can be detected upon TCR/CD3-ligation is the lck-mediated phosphorylation of tyrosine residues within the immunoreceptor tyrosine-based activation motifs (ITAMs) of the invariant CD3 and ζ-chains, and the recruitment and activation of ZAP-70 (51). Activated ZAP-70 then phosphorylates a series of adapter proteins that lead to recruitment of PLC-γ, PI-3K, Itk, and Ras to the activated TCR complex. Tyrosine phosphorylation, calcium flux, and the generation of inositol phospholipids are proximal intracellular events following MHC-peptide recognition by the TCR, maximally occurring within 30 sec to 5 min following activation. This is the time frame in which the IS forms (5) and in which the T cell undergoes internal cytoskeletal rearrangements that align the secretory apparatus to the IS (1). The maturation of the IS into a stable structure continues well beyond this time frame into a range of sustained signaling important in T cell activation. TCR may be desensitized by a process of downregulation leading to a serial engagement of multiple TCR, followed by internalization and degradation (52). However, the IS may initiate a process of parallel engagement of multiple TCR in the cSMAC that may forestall internalization and promote a nucleus of sustained signaling (35). How is the IS formed, what are the intracellular signals that regulate cell polarization and de novo gene transcription, and how are these functions coordinated and/or interdependent? Recent progress in the dissection of T cell signaling mechanisms has greatly increased our understanding of how the T cell orchestrates signaling networks to regulate these processes.

Signaling, Rafts, and Segregation

The regulation of lck may be particularly responsive to membrane structure. Lck is an acylated protein that accumulates in DIGs and is visualized in aggregated rafts. In fact, membrane-bound lck that is excluded from DIGs is incapable of mediating full T cell activation (53). Lck is negatively regulated through the carboxyl-terminal tyrosine phosphorylation site (Y505), which mediates intramolecular binding to the SH2 domain that places the kinase in a closed conformation of decreased activity (54, 55). Conversely, a second site of tyrosine phosphorylation (Y394) found in the activation loop of the kinase domain is autophosphorylated to activate the kinase for

substrate phosphorylation. Phosphorylation of inhibitory Y505 in lck is controlled by the opposing actions of the tyrosine kinase csk and by the tyrosine phosphatase CD45. Interestingly, Y394 of lck is also dephosphorylated by CD45, as well as by PEP (56, 57). CD45 is excluded from DIGs and from aggregated rafts (22, 58), whereas the CSK docking protein Cbp/PAG is an acylated transmembrane protein that is concentrated in DIGs (59). Cbp/PAG is dephosphorylated by an unknown phosphatase upon TCR engagement and releases CSK from the rafts. Thus, raft dynamics during activation may profoundly influence net activity of src family kinases and the initiation and maintenance of TCR signals.

Several groups have demonstrated that CD45 is excluded from the developing IS, and this is consistent with proposed models of signaling initiation whereby the exclusion of tyrosine phosphatases from membrane compartments is required for triggering the kinase cascade (37, 42, 43). The large ectodomain of CD45 may be responsible for this initial exclusion. Thus, in addition to its exclusion from low-density rafts, the location of CD45 may also be regulated by the size of its ectodomain through alternative splicing in a cell-specific manner (60). This model would permit a greater accessibility to the IS by the smaller rather than the larger isoforms. Accordingly, CD45 isoforms have differences in their effects on T cell activation (61). Imaging of CD45 with antibodies interacting with all isoforms demonstrates decreased CD45 in areas of ICAM-1/LFA-1 accumulation, possibly contributing to LFA-1 activation (62). However, a discreet pool of CD45 moves back into the central region of the mature IS, adjacent to the area of TCR/MHC engagement (43). In fact, the CD45 in this location appears to be in a previously undescribed cytoplasmic compartment near the cSMAC. The positive or negative roles of this CD45 movement are unknown, but it may provide a mechanism to sustain lck activity near engaged TCR.

The TCR is only weakly associated with DIGs both in nonstimulated Jurkat T cells and in thymocytes (63–65). Following TCR activation the association of TCR complexes with the DIG fraction increases. Particularly, the hyperphosphorylated P-p23 form of the ITAM rich ζ-chain is preferentially associated with DIGs as compared to the less phosphorylated P-p21 form. Conversely, little phosphorylated ζ-chain is found outside DIGs, consistent with the preferential phosphorylation of proteins within the rafts (63). Coreceptors CD4 and CD8 are strongly associated with DIGs and may play an important role in the initial alternations in the lipid and signaling environment of the TCR.

Once recruited to the ITAMs, ZAP-70 phosphorylates the Linker for activation of T cells (LAT), a 36/38 kDa transmembrane protein that is a critical docking molecule for a number of TCR effectors and is localized to DIGs (66, 67). Following phosphorylation by ZAP-70, LAT recruits directly and indirectly a number of signaling proteins to DIGs, including PLC-γ1, members of the Grb2/Gads/Grap adapter family, PI-3K, SLP-76, and Vav (66). The SH3 domains of the Grb2-adaptors are associated with the Ras GEF, Sos, and with SLP-76, and they serve to recruit these molecules to the cell membrane. Both LAT-deficient and Slp76-deficient Jurkat T cells are unable to initiate significant calcium signaling and

display poor NF-AT activation following TCR ligation, an effect that correlates with the abrogation of PLC-γ1 tyrosine phosphorylation (67).

Importantly, the activation and compartmentalization of a number of critical signaling molecules, including PLC-γ1, Itk, and Vav, are influenced by the interaction of their PH-domains with PtdIns lipids (68, 69). PtdIns-4,5-P_2 is highly enriched within low-density rafts of Jurkat T cells; hence the recruitment of PLC-γ1 to low-density rafts may juxtapose PLC-γ1 to its substrate (64). Similarly, the localization of PI-3K to the nucleated signaling complex catalyzes the conversion of PtdIns-4,5-P_2 to PtdIns-3,4,5-P_3, which is an essential regulator of Vav GEF activity and Itk kinase activity (68).

Following TCR ligation, tyrosine phosphorylated SLP-76 binds to the SH2 domain of Vav, and Vav itself becomes tyrosine phosphorylated, further increasing its GEF activity (70). Vav is important for the reorganization of the actin cytoskeleton. Antibody-mediated TCR capping is abrogated in T lymphocytes from Vav-deficient mice, although the tyrosine phosphorylation of proximal signaling components appears normal (71, 72). However, Vav-deficient T cells are unable to polymerize actin and fail to induce the association of ζ-chain to the cytoskeleton (72). The capping phenotype of Vav-deficient T cells correlates with the inhibition in T cell activation caused by the cytoskeletal inhibitor Cytochalasin D. Like Vav-deficient T cells, Cytochalasin D–treated T cells are unable to induce IL-2 gene transcription but are fully able to phosphorylate proximal signaling proteins following TCR ligation (71, 73). Consistent with this model, a recent study shows that vav-deficient T cells have defects in MHC clustering in T cell–APC contacts induced by superantigen (74). This demonstrates the important concept that the earliest TCR signaling events do not require cytoskeletal change, but instead lead to cytoskeletal rearrangements.

SLP-76, Vav, and Nck form a trimolecular complex, which itself binds to PAK and WASP, both mediators of cytoskeletal rearrangement. The colocalization of tyrosine phosphorylated Vav with Nck enables GTP-bound Rac and Cdc42 to bind and activate proteins implicated in cytoskeletal rearrangement, notably PAK and WASP (35, 75). In addition, SLP-76 provides a further link to the T cell cytoskeleton through the adaptor protein SLAP-130/Fyb (76). With SLP-76, SLAP-130/Fyb localizes to the interface between T cells and anti-CD3 coated beads and binds Evl, a member of the Ena/VASP family associated with actin-based projections and required for actin remodeling of the T cell cytoskeleton following T cell activation (77). These proteins collectively provide mechanisms for linking TCR signaling to actin cytoskeletal changes that are likely to be required for IS formation (35).

Sustaining the Signal: Quantitative or Qualitative?

The majority of T cell signaling mechanisms studied to date have been analyzed within the 15-min period concurrent with the formation of the IS, but little is known about the signals emanating from the mature IS at later times. Although by the

time the IS is stabilized and the peak of phosphotyrosine signaling has subsided, detectable phosphorylation of tyrosine residues remains within the signaling cluster (42). The process of stabilization of the MHC-peptide cluster in the IS may signify the generation of signals both sustained and new.

Notably, PKCθ, but no other PKC isoform, translocates to the central region of the IS in the time frame of IS stabilization concordant with an increase in its catalytic activity (6, 78) (Figure 2). Detectable recruitment of PKCθ to the cell membrane is observed only with T cell activation induced by agonist ligands; it does not occur with suboptimal partial or antagonist ligands (6). Recruitment of PKCθ may define the signaling threshold necessary to form a stable IS and fully activate the T cell. PKCθ is critically important for the regulation of IL-2 in T cells. The overexpression of PKCθ, but not PKCα, in T cell lines dramatically increases both AP-1 and NFκB activity following TCR ligation (79). The activation of Vav and signaling through JNK appear necessary for efficient PKCθ activation and translocation (80). The overexpression of Vav increases the amount of F-actin at the cell membrane and induces the specific recruitment of PKCθ to these patches, while dominant negative forms of Vav inhibit this translocation. Thus, the Vav/Rac pathway of T cell signaling appears to be critically required for the recruitment and activation of PKCθ, potentially forming a unique signal in the mature IS.

Costimulation

The two-signal model for T cell activation proposes that signal one is provided by the TCR/CD3 complex, while signal two is generated by engagement of T cell costimulatory receptors (81). The unification of the two signal model with the IS model is a current challenge in the field. Although numerous molecules have been implicated as costimulatory receptors, CD28 is the classical example.

It was proposed that CD28 activates the jun kinase, JNK, which is required for the induction of cytokine gene transcription (82). Experiments demonstrating trans-costimulation—in which the defective costimulatory activity of fixed APC could be restored by the addition of third party normal APC (83) or B7-transfected L cells (84)—bolsters this argument. However, coengagement of the TCR is still necessary. Furthermore, the ability to activate T cells from CD28-deficient mice demonstrates that CD28 is not absolutely required (85). In fact, double mutant mice lacking CD28 and CD2 are more impaired in T cell activation than are the corresponding single mutant mice (86). This suggests a degree of redundancy among adhesion/costimulation molecules. The only unique phenotypes associated with the CD28-deficient mice are the inability to respond to a wide range of peptide antigens, in contrast to lacking CD2 and LFA-1, which could only alter the quantitative aspects of the T cell response to a given MHC-peptide complex. These different attributes correlate with the location of these molecules in the immunological synapse; only CD28 is localized in the cSMAC, whereas CD2

and LFA-1 are engaged in different subregions of the pSMAC (5) (Figure 1). Therefore, CD28 is perfectly positioned to directly manipulate sustained TCR signals.

Coligation of TCR and CD28 or LFA-1 activates the actin-myosin-dependent transport (7) of receptors and lipid domains to IS. The coengagement of TCR and CD28 by antibodies on beads also triggers enhanced accumulation of GM1 at the interface, compared to engaging CD3 alone (87). This enhanced raft accumulation correlates with increased lck activation and degradation and increases in immediate and sustained tyrosine phosphorylation (87, 88). In comparison to TCR or CD28 engagement alone, coligation of TCR and CD28 results in the synergistic and sustained phosphorylation and membrane localization of Vav (88). CD28 may influence immediate and sustained TCR signaling by enhancing activation of PI-3K or lck or some combination of these effects (89). Thus, CD28 effects the earliest processes associated with TCR signaling and formation of the IS and is an ideal candidate for enhancing sustained engagement processes in the cSMAC.

Natural Killer Cell Synapse

Natural killer (NK) cells represent a newer frontier in the study of IS formation and extend the model beyond helper T cell interactions. Natural killer cells have large granular lymphocyte (LGL) morphology and play a role in the early protection against microbial infection and tumor cell invasion prior to development of adaptive immunity. They have the ability to lyse targets and also form part of both innate and adaptive immune responses (90). Most NK cells express the low-affinity (Fcγ) receptor type IIIA (CD16) that recognizes IgG-coated targets that can then be killed by antibody-dependent cell cytotoxicity (ADCC). In contrast, tumor- and virus-infected cells induce an innate immune response that integrates multiple signals from the target cells and utilizes invariant receptors. A central concept for NK cell killing is that they detect the absence of self-MHC molecules, the missing-self hypothesis (91). While this area is developing rapidly, the initial gestalt of the field provides a vivid example of how IS formation may play a central role in the integration of complex receptor information.

NK cells express two main types of innate recognition receptors (Table 1): activating receptors associate with ITAM-bearing polypeptide chains and inhibitory receptors with immunotyrosine base inhibitory motifs (ITIM). Activating NK receptors signal in much the same way as TCR, through a kinase cascade involving lck and Syk or Zap-70. These activating receptors are counteracted by the inhibitory receptors that recruit SHP-1 following phosphorylation of the ITIMs by src family kinases (92). The activation of the src family kinases is initiated by the activating receptors such that there is a sequential aspect to this process. SHP-1 extinguishes signaling through neighboring ITAMs. The key to this model is that the activating and inhibitory receptors are thoroughly intermixed in the IS.

TABLE 1 Human natural killer (NK) cell receptors involved in triggering or inhibition of cytotoxicity

	Ligand	Expression	Signaling	Components
Non-MHC specific activating receptors				
NCR				
NKp46	?	all	CD3ζ + FcR1γ	3 + 1 ITAM
NKp30	?	all	CD3ζ	3 ITAM
Nkp44	?	all IL2 act.	DAP12	
CD16	?/IgG Fc	all	CD3ζ FcR1γ	3 + 1 ITAM
NKRP-1 (= NK1.1)	?		FcR1γ	
Various adhesion receptors	variable	all	variable	
MHC specific activating receptors				
Immunoglobulins:				
KIR-S = NKR				
2DS1	HLA-Cw4	clonally	DAP12	1 ITAM
2DS2	HLA-Cw3	clonally	DAP12	1 ITAM
2DS3	HLA-Cw3 & Cw4	clonally	DAP12	1 ITAM
2DS4	?	clonally	DAP12	1 ITAM
2DS5	?	clonally	DAP12	1 ITAM
3DS1	HLA-Bw4	clonally	?	
ILT1 (= LIR7)		clonally	FcR1γ	1 ITAM
Lectin like receptors				
NKG2D	MICA	clonally	DAP10	PI3K docking site
NKG2C/CD94	HLA-E	clonally	DAP12	1 ITAM
MHC specific inhibitory receptors				
Immunoglobulins				
KIR-L				
2DL1 (= p58.1)	HLA-Cw4 group*	clonally	2 ITIM	
2DL2 (= p58.2)	HLA-Cw3 group*	clonally	2 ITIM	
2DL3	HLA-Cw3& Cw4	clonally	2 ITIM	
2DL4	HLA-G	all	1 ITIM	
3DL1 (= p70 & NKB1)	HLA-Bw4	clonally	2 ITIM	
3DL2 (= p140)	HLA-A3	clonally	4 ITIM	
ILT2 (= LIR-1& MIR-7 & CD85) (E, C) UL-18	HLA-A, -B, G	clonally	4 ITIM	
Lectin like				
NKG2A/CD94	HLA-E	clonally	1 ITIM	
Non-MHC_specific inhibitory receptors				
LAIR-1 (= p40)	?		2 ITIM	
AIRM-1 (= p75)	Sialic acid		2 ITIM	
Irp60	?		2 ITIM	

*HLA-Cw3 group contains HLA-Cw1, -Cw3, -Cw7, Cw8 etc.
*HLA-Cw4 group contains HLA-Cw2, -Cw4, -Cw6, Cw15.

Non-MHC-Specific Receptors

Many adhesion and costimulatory molecules contribute to NK killing (e. g. LFA-1, CD2, 2B4) (Table 1). Antibodies to these molecules profoundly inhibit killing of target cells by NK cells (93). Some of these receptors would appear to be adhesion or costimulation structures; they may serve these roles in NK cells or alternatively may directly trigger killing. The ability of adhesion molecules to trigger killing may depend on the distribution of ligands. For example, ICAM-2, a ligand for LFA-1, appears to trigger NK cells when it is clustered on the uropod of target cells (94).

Natural cytotoxicity receptors (NCR) are recently described non-MHC-specific triggering receptors and include NKp46, NKp30, and NKp44. All resting human NK cells express NKp46 and NKp30; IL-2-activated cells also express NKp44 (95). The ligands for these molecules have not been identified.

MHC-Specific Receptors

There are two groups of activating receptors that bind specifically to HLA class I. Triggering killer inhibitory receptor-short (KIR-S) receptors are type I glycoproteins with Ig-like domains. Different NK-cell clones express various KIR-S receptors. One clone may express one to five KIR-S (96). The ligands of four KIR-S are known (97) (Table 1).

Another group of specific activating receptors are dimeric type II membrane proteins with C-type lectin domains. NKG2 is a receptor for stress-inducible MHC I–like molecule MICA (98). CD95/NKG2C is a receptor for HLA-E (99). In rodents, there are no KIRs. Instead, a family of C-type lectins of the Ly49 family fill this role (100).

Most HLA-specific activating receptors have inhibitory counterparts with the same specificity. Inhibitory receptors form three groups: six Ig-family KIR-L receptors with different specificities, one Ig family member ILT2, and the lectin-like NKG2A/CD95 (90). These molecules have cytoplasmic ITIMs (101). The crystal structure of KIR/MHC I complex shows that KIR and TCR footprints are different and recognition by KIR is also less allele-specific and less dependent on peptide than TCR/MHC class I recognition (102). The distance spanned by KIR/MHC class I is similar to that of the TCR/MHC interactions (<15 nm). Therefore, the topology of NK inhibitory interactions is consistent with interactions like that in the cSMAC of the helper T cell IS.

Interactions Between Receptors and Ligands

The affinity of the HLA-specific receptors is dependent on a peptide bound in the HLA groove. The Kd values of inhibitory KIR-Ls and inhibitory NKG2A/CD94 are approximately 10 μM with fast on and off rates, whereas the Kd for activating NKG2C/CD94 to the HLA-E ligand is more than 74 μM (103). The kinetics of the interactions of KIR and ligand are fast, faster than activating TCR/MHC

interactions. From the crystal structure of KIR2DL2 in a complex with HLA-C3w, it can be concluded that there may be another binding site that can be connected to ligand-induced receptor aggregation (102).

Recently Davis et al described an inhibitory IS between an NK-cell line expressing KIR2D and a class I–transfected B cell line (104). In the NK cell IS, a ring of KIR/MHC interactions formed around the central LFA-1/ICAM-1 cluster, the opposite of the mature T cell IS (Figure 2). Zn^{2+} modulates the negative signal of KIR2D when it binds to HLA-Cw4 or -Cw8. It was also demonstrated that MHC I clustering by KIR2DL may require Zn^{2+}, possibly to stabilize the clusters. It is not known what form the NK synapse takes when the activating signals outweigh the inhibitory signals. However, it is likely that the KIR/MHC ring of the inhibitory NK IS also contains engaged activating KIR, each surrounded by inhibitory receptors that recruit SHP-1 on demand.

ANTIGEN-PRESENTING CELLS: The Other Half of the Synapse

The IS is formed only in the context of MHC-peptide complexes on the APC. The APC plays a crucial and active role in the generation of the MHC-peptide complexes, but it is not as clear that the APC plays an active role once the MHC-peptide complexes are on the surface. The B cell cytoskeleton is apparently dispensible for IS formation by primed T cells (7). Nevertheless, different types of APC are functionally distinct, and their unique characteristics may include active contributions to IS formation. For example, dendritic cells (DC) activate naïve T cells most efficiently among APC, whereas a wider variety of APC can activate effector or memory T cells. Variations in molecule expression, membrane organization, antigen processing, and active responses of the APC to IS formation may define unique properties of each APC.

The general topic of antigen processing and determinant selection has been reviewed in detail (105, 106). Generally, antigen is separated in two pools to be presented by MHC molecules; class I presents antigens of intracellular origin to CD8 T cells, and class II presents antigens of extracellular origin to CD4 T cells. These pools are loaded with peptide in the endoplasmic reticulum and MIIC compartments, respectively, but appear to move to the surface in a common transport vesicle, the CIIV.

Dendritic Cells

DC encounter antigen in the periphery and then migrate to lymphoid organs where they present antigen in class I and II in the T cell area of the lymph node (107). Immature DC retain pools of MHC molecules intracellularly but express few MHC molecules on the surface until they are exposed to maturation signals. DC internalize antigen by macropincytosis and by using their C-type lectin and Fc receptors.

Upon maturation (in response to endotoxin, TNFα, or IL-1) (108) and entry into the lymph node T cell area, antigen is processed and mature MHC molecules are transported to the surface via CIIV compartments (109). The CIIV are in some manner associated with the actin cytoskeleton since latrunculin A blocks transport to the surface (110). With maturation, DC rearrange their cytoskeleton and increase their motility (111). The signals for CIIV fusion with the plasma membrane are not known, but it is interesting to consider that T cell contact may influence this process.

MHC at the Cell Surface

Interaction of MHC molecules with the cytoskeleton might control the mobility of MHC molecules and regulate IS formation. Class II diffusion rates are dependent on the cytoskeleton in some cell lines and on the cytoplasmic domain in I-A^k in fibroblast L cells, with cytoplasmic-tail deleted class II moving faster than wild type (112).

Superstructures of stimulatory molecules may efficiently present antigen to T cells. Fixed T and B lymphoma cells display coclustered class I and II on the cell surface (113) and could facilitate interactions between cells and influence signal transduction within a cell. The presence of the costimulatory molecules and the tetraspanin family members in the cluster also indicates a potential for a preformed complex of importance for T cell interactions (110, 114). These protein oligomers may be formed in the CIIV where class I, class II, and CD86 (B7-2) colocalize before class II appears at the DC surface (110). A role for cholesterol in formation of these clusters and the presence of GPI anchored proteins implicates a relationship of these structures to rafts in the APC membranes (110, 115). The grouping of MHC molecules has implications for signaling in the APC and for the nature of the ligand for the TCR. MHC class II preclustering in rafts enhances T cell activation (40). These MHC clusters may also transduce signals to the APC cytoskeleton upon receptor engagement. Induced association of the engaged MHC-ligands with the cytoskeleton could alter the dynamics of IS formation and may enhance signaling by promoting stabilization of the central cluster of engaged MHC molecules in the IS. The APC may also then display a degree of polarization of CIIV fusion with the plasma membrane, possibly delivering new complexes to existing IS to maintain these structures.

CONCLUSIONS

The IS is a dynamic structure that builds on pre-existing membrane organization in the form of small dynamic rafts in the context of a polarized lymphocyte to create a machinery for sustained T cell signaling. The stabilization of the central cluster of engaged MHC-peptide complexes is correlated with full T cell activation. The recruitment of PKCθ appears to be a marker for attainment of the stable IS. Natural killer cells appear to use a similar principle to measure the relative levels of positive

and negative signals to identify infected or abnormal cells using a complex array of invariant receptors. Since APC differ in their ability to activate T cells at different stages of differentiation, it also is likely that at least some APC will engage in active processes to manipulate IS formation and stabilization. The potential for a role for rafts and cytoskeleton of the APC in the process of IS stabilization is particularly exciting. Future studies in this rich new area of research are likely to yield further insights as the high order organization of the IS is appreciated in greater detail.

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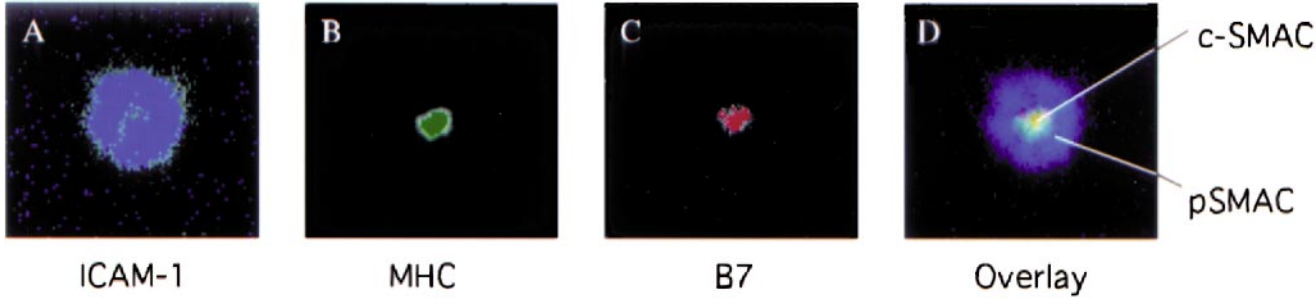

Figure 1 The mature immunological synapse. Patterns of LFA-1 (*A*), TCR (*B*), and CD28 (*C*) interaction in a functional synapse between a T cell and a supported planar bilayer containing Cy5-ICAM-1, Oregon Green-I-A^k, and Cy3-B7. All three markers are overlaid in panel D with the cSMAC and pSMAC labeled.

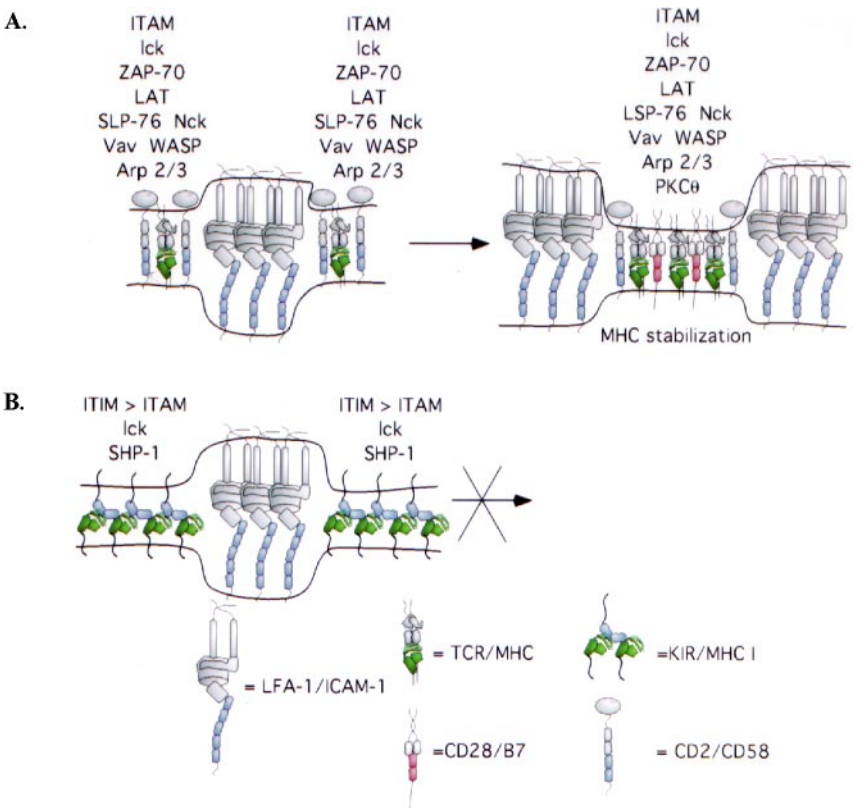

Figure 2 Model for signaling complex formation in the immunological synapse. (*A*) Formation of the T cell IS. Recruitment of PKC-θ is unique to the mature cSMAC. (*B*) Formation of the NK synapse arrested by inhibitory receptor signaling. The molecular pattern in an activating NK cell synapse is not known.