

The Immunological Synapse

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Abstract

The molecular interactions underlying regulation of the immune response take place in a nanoscale gap between T cells and antigen-presenting cells, termed the immunological synapse. If these interactions are regulated appropriately, the host is defended against a wide range of pathogens and deranged host cells. If these interactions are dysregulated, the host is susceptible to pathogens or tumor escape at one extreme and autoimmunity at the other. Strategies targeting the synapse have helped to establish immunotherapy as a mainstream element in cancer treatment. This Masters' primer will cover the basics of the immunological synapse and some of the applications to tumor immunology. *Cancer Immunol Res*; 2(11); 1023–33. ©2014 AACR.

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Learning Objectives

The T-cell immunological synapse is the junction formed between a T cell and an antigen-presenting cell, comprising interactions for antigen recognition regulating host immunity. If these interactions are dysregulated, the host is susceptible to pathogens or tumor escape at one extreme and autoimmunity at the other. Immunotherapeutic strategies targeting the synapse have shown promise for cancer treatment. Upon completion of this activity, the participant should gain a basic knowledge of the organization and function of the immunological synapse that modulate immune responses.

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Introduction

T-cell-dependent immune responses protect the host from cancer (1, 2), but also participate in destructive autoimmunity (3, 4). T-cell receptor (TCR) engagement leads to suppression of T-cell locomotion, formation of a specialized junction, and T-cell polarization (5–7). This combination of a specialized junction, cell polarization, and positional stability bears a striking similarity to the classical synapse of the nervous system (8, 9). T cells can also integrate signals through the antigen receptor during migration, and we have referred to this type of mobile, antigen recognition structure as a kinapse (10). The immunological synapse or kinapse integrates three broad categories of receptors: antigen (TCR), adhesion, and costimulatory/checkpoint. The orga-

nization of these receptors in the interface affects how they function in a way that cannot be predicted without this spatial and temporal information. The textbook picture of antigen recognition across an approximately 15-nm gap between a T cell and an antigen-presenting cell (APC) provides the justification for why the immune system needs a synapse or kinapse (11). Adhesion molecules, identified first by function with blocking antibodies and then by expression cloning, provide the energy needed to pull cells together, allowing sustained antigen recognition and precise execution of effector functions (12). Finally, costimulatory and checkpoint receptors alter the functional outcome of immunological synapse formation substantially and can also influence the synapse-kinapse balance (13, 14). To date, immunotherapies targeting checkpoint receptors have provided the most promise (15).

Immunological Synapse Models

Determining how antigen recognition, adhesion, and costimulation/checkpoint receptors come together in the immunological synapse or kinapse required visualization of the close interface between the T cell and the APC. Two major approaches dominated the efforts at molecular analysis.

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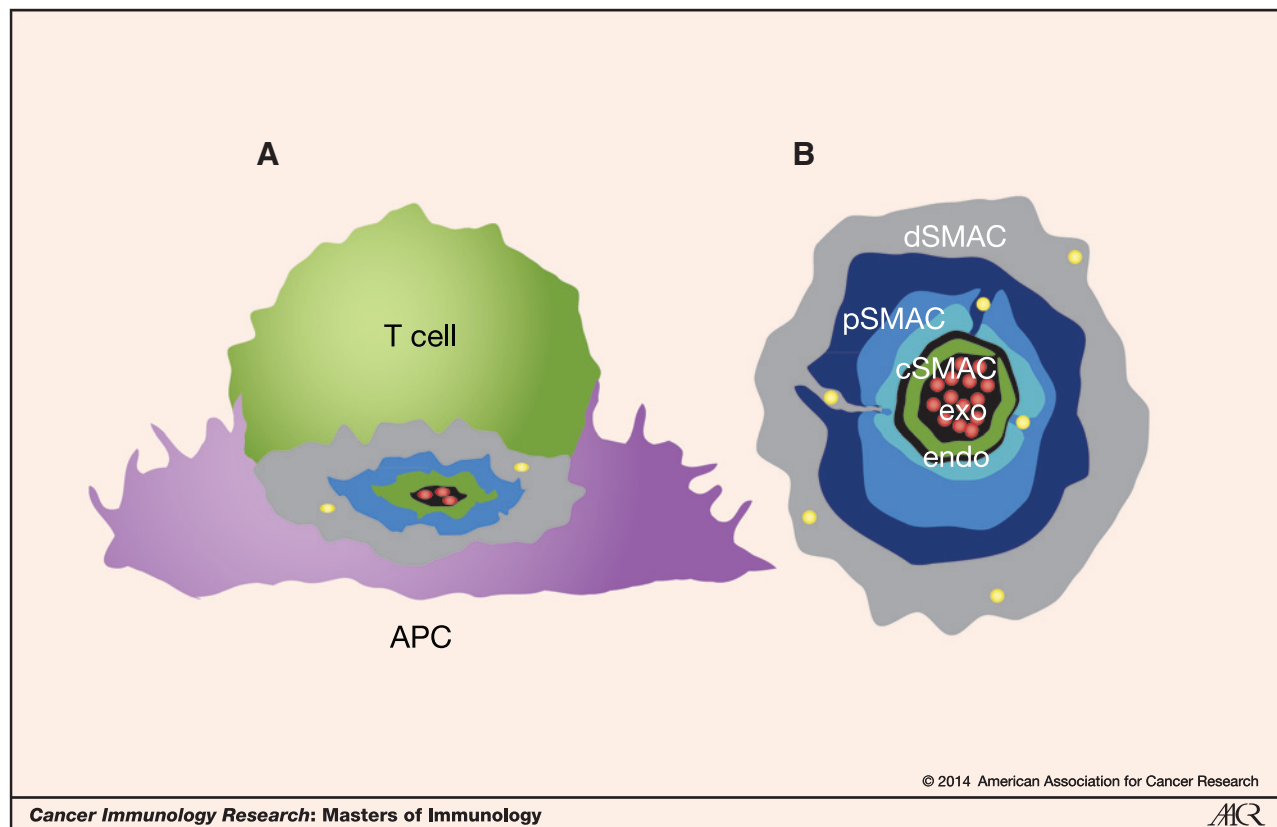


Figure 1. Model immunological synapse—elevated (A) and front-on view (B) of model for synapse focusing on interface. Color code: gray, F-actin lamellipodium (dSMAC); blue, LFA-1-ICAM-1 adhesion (pSMAC); green, CD28-CD80 costimulatory molecules (Endo-cSMAC); and red, TCR-MHC antigen recognition (Exo-cSMAC). The yellow dots are TCR microclusters that include both CD28 (green) and TCR (red). This is where signals are generated.

Top-down analysis of T cell-APC pairs using immunofluorescence (16) and bottom-up approaches of applying purified molecules to substrates have both contributed to our understanding of the immunological synapse. Reconstitution methods based on fluid-supported lipid bilayers (SLB), in which the purified molecules are combined in a laterally mobile form, have been effective in recreating the organization of the immunological synapse formed with live T cells (16, 17). The canonical organization of the immune synapse is a bull's eye structure with a central TCR-MHC interaction cluster surrounded by a ring of LFA-1-ICAM-1 adhesion and a distal ring that includes the transmembrane tyrosine phosphatase CD45 (18, 19). Kupfer referred to these radially symmetric compartments as supramolecular activation clusters (SMAC; Fig. 1; ref. 16). The segregation of TCR-MHC interactions from LFA-1-ICAM-1 interactions was a confirmation of the working model formulated by Springer in 1990 that, based on their sizes, the TCR-MHC interactions would need to segregate laterally from the LFA-1-ICAM-1 interactions (12). This is a stereotypical response of helper, cytotoxic, and regulatory T cells (20-23). The SMACs of a stable synapse correspond to polarized compartments in a kinapse (24). T cells and dendritic cells (DC) have a more complex organization of similar compartments, perhaps due to the intricate topology of DCs (25, 26). We first consider the three categories of receptors

that are organized in the synapse and then how they are coalesced into a functional synapse or kinapse.

T-cell antigen receptor

TCR interaction with MHC-peptide complexes controls the specificity of the immune response and the source of antigens in both cellular and humoral responses. TCR genes undergo rearrangement similar to that of antibody genes (27). T cells with subunits encoded by the α and β genes make up the classical diverse repertoire of naïve T cells that can respond to pathogens and tumors. T cells with subunits encoded by the γ and δ genes include specialized cells that are not MHC restricted. The complete TCR on the cell surface is composed of two highly diversified antigen recognition subunits that are essentially unique for each T cell, either $\alpha\beta$ or $\gamma\delta$, in a noncovalent complex with nonpolymorphic CD3 ϵ -CD3 δ , CD3 ϵ -CD3 γ , and CD3 ζ -CD3 ζ dimers (28). The entire complex is critical for surface expression and signal transduction, but it lacks any intrinsic catalytic activity. Instead, the TCR complex has 10 immunotyrosine activation motifs (ITAM) with paired tyrosine residues that, when phosphorylated, can recruit the nonreceptor tyrosine kinase zeta-associated protein of 70 kDa (ZAP-70; Fig. 2; refs. 29, 30).

Although it was recognized by 1975 that cell surface-expressed gene products of the highly polymorphic MHC were

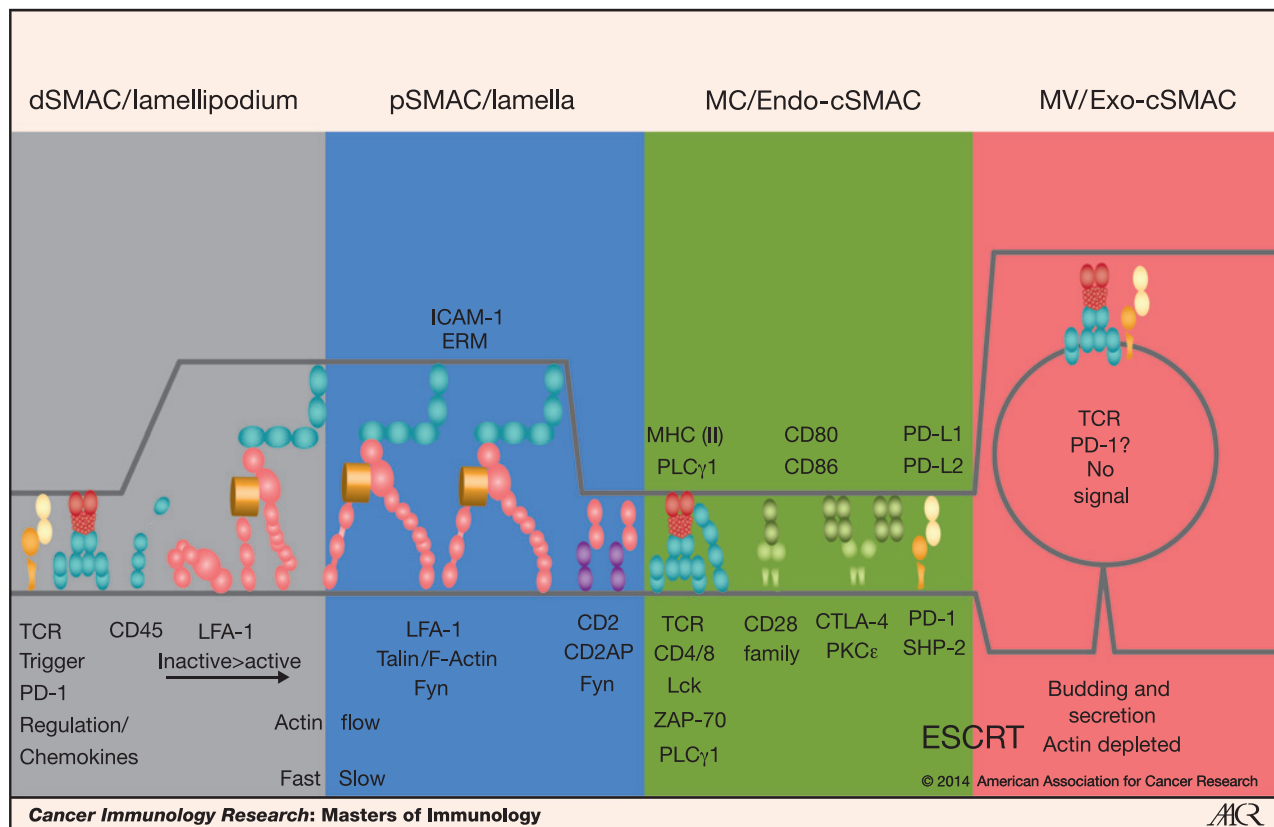


Figure 2. SMACs and representative receptor classes in immunological synapse. See text for description and citations.

required to "restrict" T-cell recognition of antigen (31), the full molecular picture of how antigenic peptides bound to MHC was not clear until the crystal structure of an MHC molecule was solved in 1987 (32). There are two distinct types of MHC that control antigen recognition—one set of genes for cytotoxic T cells (CTL) that bind endogenously synthesized cytoplasmic peptides generated by the proteasome (class I), and a distinct set of genes for helper T (Th) cells that bind exogenous antigens taken into the cell by endocytosis (class II). The process of cross-presentation or cross-dressing allows specialized APCs needed for T-cell priming to present exogenous antigens through the class I pathway, which is likely critical for anticancer cellular immunity (33). MHC class II molecules can also engage in signaling (Fig. 2; ref. 34). Other MHC-like molecules present nonpeptide antigenic structures. These include CD1d presentation of glycolipids to invariant natural killer (NK) T cells and MR1 presentation of bacterial metabolites to mucosa-associated invariant T cells (MAIT). These innate-like T cells may have utility in immune response to tumors (35).

Conventional $\alpha\beta$ T cells are marked by different coreceptors, CD8 and CD4 (36). CD8 binds to nonpolymorphic elements of MHC class I, and CD4 to that of MHC class II. In T-cell development, thymocytes rearrange TCR genes and test receptors for interaction with self-peptides on MHC. Strong interactions lead to apoptosis or, for a limited number of clones, the generation of actively suppressive thymic regulatory T cells

(Treg), which are important in tumor immunology as their formation of immunological synapses can suppress antitumor responses in part by destabilizing T-effector immunological synapses (37–40). Differences in signaling between conventional $\alpha\beta$ T cells and Tregs offer therapeutic opportunities to push the balance toward regulation in autoimmunity and toward responsiveness in cancer therapy (23, 41). A weaker recognition process is required for thymocyte survival, and this is thought to bias the TCR repertoire toward recognition of MHC with either CD4 or CD8 (42). This weak self-recognition is thought to set the stage for foreign peptide recognition by providing the T cell with weak recognition of the peptide flanking, polymorphic α -helices of the MHC proteins, and also provides survival signals to mature T cells. It is still debated whether the germline-encoded TCRs are evolutionarily biased to recognize MHC molecules in a certain preferred orientation, or if the germline TCR repertoire has an antibody-like lack of bias and entirely learns to recognize MHC molecules in the thymus (43, 44).

In a tumor context, antigen recognition can enhance or inhibit responses. The classical tumor-infiltrating lymphocyte (TIL) is a CD8 CTL that can directly kill tumor cells, and the infiltration of tumors by CD8 T cells correlates with better outcome for some tumors (2). APCs in tumors can include CD11c⁺ cells with characteristics of DCs (45). Cancer vaccines can promote expansion and differentiation of T cells with antitumor specificity in a therapeutic setting (46).

T-cell specificity can also be engineered through introduction of chimeric antigen receptors into patient cells in adoptive immunotherapy (47).

Adhesion receptors

Adhesion molecules are critical for sensitive antigen recognition required for tumor rejection (48). Strategies based on immunizing mice with CTL lines followed by selection for mAbs that block killing led to the discovery of lymphocyte function-associated antigen-1 (now CD11a/CD18, but often referred to as LFA-1), LFA-2 (now CD2), and LFA-3 (now CD58; Fig. 2; ref. 49).

CD2 and CD58 define a heterophilic adhesion receptor pair (50). CD2 and CD58 are members of the immunoglobulin (Ig) superfamily and are similar in size to the TCR and MHC-peptide complex, suggesting that CD2 may cooperate very closely with the TCR (12, 51). CD2 and CD58 are members of the signaling lymphocyte activation molecule (SLAM) family. Most other SLAM family members are homophilic (they mediate adhesion by binding between products of the same gene) that interact through their cytoplasmic domain with SLAM-associated protein (SAP), which links to the tyrosine kinase Fyn (52). SLAM family members are required for particular interactions between thymocytes in the development of certain specialized T-cell lineages and for early T-cell-B-cell interactions required for germinal center formation (53).

LFA-1 is a heterodimer with a unique α subunit that shares a β subunit with three other cell-surface heterodimers, each of which has an α subunit with a distinct expression pattern. Functional screening for mAbs that block LFA-1-dependent aggregation after immunization with B cells from LFA-1-deficient patients identified the first LFA-1 ligand, intercellular adhesion molecule-1 (ICAM-1 or CD54; ref. 54). LFA-1 is a member of the integrin family, and ICAM-1 is a member of the Ig superfamily. The intercellular link formed by the interaction of LFA-1 with ICAM-1 is much longer than that with the TCR, and it was suggested that the TCR-MHC and LFA-1-ICAM-1 interactions would need to segregate laterally in the interface, the first prediction on the structure of the immunological synapse (12). ICAM-2, a second ligand for LFA-1 that was cloned from an endothelial cell library, is also an Ig superfamily member (55). ICAM-3 was defined by functional blocking in a screen in which ICAM-1 and ICAM-2 were blocked (56). T cells express other adhesion molecules including additional integrins (57). Integrin ligands are expressed on all nucleated cells and can be further induced, in the case of ICAM-1, by exposure of stromal cells to inflammatory cytokines, including TNF, IL1 β , or IFN γ (58). A complication of the therapeutic use of anti-LFA-1 mAbs is that LFA-1 is important for interactions with endothelial cells (59), which are important for optimal localization of cells, in addition to immunological synapse formation. Anti-LFA-1 mAbs were approved for treatment of psoriasis, but the therapeutic window between reduction of disease and increased vulnerability to infection was too small (60). There are activating Abs to LFA-1, but the affinity of LFA-1 for ICAMs is optimized to allow rapid leukocyte migration, and constitutively active LFA-1 is defective in mediating migration,

contraindicating LFA-1 affinity enhancement as a therapeutic strategy (61).

Although adhesion molecules are diverse in structure, both integrins and the SLAM family signal through the Src family kinase Fyn rather than Lck, which is the primary partner for antigen and costimulatory receptors (62). Another common feature is that both LFA-1 and CD2 engage ligands in the periphery of the immunological synapse, even though the CD2-CD58 interactions are the correct size to colocalize with TCR in the center (Fig. 2; see Supplementary Information in ref. 17).

Costimulation

Costimulatory receptors have minimal signaling or adhesive activity on their own, but can enhance adhesion and signaling locally when combined with other stimuli, primarily through the TCR (63, 64). Costimulatory receptors were predicted as a corollary of the clonal selection model, in which T cells must be able to attenuate their responses to harmless foreign proteins from the environment that are not present in the thymus and could drive inappropriate immune responses if not checked by some mechanism for extrathymic tolerance (65). Pathogens or tumors would need to upregulate costimulation to drive a T cell to respond, and incorporation of costimulatory ligands directly into tumor cells has been implemented in tumor vaccines (66). Activation of mature T cells in the absence of costimulation leads to a second round of clonal deletion that protects the host against immune responses to harmless environmental antigens. Jenkins and Schwartz (67) discovered that antigen recognition with suboptimal costimulation could induce a state of nonresponsiveness that they named anergy.

The archetypal costimulator is CD28, which is an Ig superfamily member with a homodimeric structure and a cytoplasmic domain lacking enzymatic activity but containing motifs that recruit and activate Lck and indirectly, protein kinase C (PKC)- θ , an important PKC isoform in T cells that contributes to the activation of NF- κ B transcription factors and promotes IL2 production (Fig. 2; ref. 68). The activity of CD28 is dependent upon the upregulation of B7-1 (CD80) and B7-2 (CD86) on APCs and the interaction in an immunological synapse (26). Both CD80 and CD86 are upregulated on DCs by stimulation through maturation signals delivered by Toll-like receptors and inflammatory cytokines (69, 70). This is a critical link between innate and adaptive immunity, and the potential of some tumors to grow without inducing expression of costimulatory ligands may allow them to tolerize tumor-specific T cells.

The inducible T-cell costimulator (ICOS, CD278) is a second Ig superfamily costimulatory system. In contrast with CD28, it is not expressed on naïve T cells, but is upregulated following T-cell activation and binds ligand of ICOS (LICOS, CD275; ref. 71). LICOS is expressed prominently on DCs and B cells, but can be induced on stromal cells by inflammatory cytokines. ICOS is critical for germinal center reactions, in which its ability to activate phosphatidylinositol-3 kinase is particularly important (72). In the context of cancer immunotherapy, ICOS costimulation promotes Th17 differentiation in CD4 T cells, which is an effective response mode for some solid tumors (71).

The other large costimulatory receptors are members of the TNF receptor (TNFR) superfamily, including CD27, GITR (CD357), 4-1BB (CD137), and OX40 (CD134). These receptors signal through TNFR-associated factors (TRAF) using K63 ubiquitination to signal through activation of NF- κ B2 transcription factors (73, 74). With the exception of CD27, which is constitutively expressed, these costimulatory receptors are induced on conventional T cells in hours to days following initial activation. Therefore, they are involved in effector and memory functions of T cells following the initial expansion phase. These receptors are also constitutively expressed on Tregs, and this perhaps offers an opportunity to modulate negative regulation in the tumor microenvironment (TME). The last set of costimulatory receptors are members of the lectin-like receptor superfamily that pair noncovalently with DAP10, a disulfide-linked heterodimer containing a sequence motif that allows recruitment of phosphatidylinositol-3-kinase, which contributes to activation of the growth-promoting kinase AKT (75). These receptors, with NKG2D as a prime example, are expressed on CTLs as well as NK cells and promote killing. The counter-receptors for NKG2D are induced by cellular stress and are potentially important in recognition of deranged host cells (69, 76). However, tumor cells proteolytically shed one of the human counter-receptors, MICA, leading to jamming of this system as an escape mechanism (77).

There is evidence for combinations of adhesion and costimulatory ligands generating partial signals that can lead to transient formation of ligand-independent immunological synapses. This has been observed with NKG2D in human (22) and mouse (78) CTLs and recently for CD28 in Th cells (79). In this context, the upregulation of ICAM-1, CD80, and NKG2D ligands such as MICA (human) and Rae-1 (mice) can induce a state of heightened surveillance in inflamed or stressed tissues.

Checkpoint blockade

Coinhibitory or checkpoint receptors are a natural complement to the costimulators—they use various negative signaling pathways, often recruiting tyrosine phosphatases, such as SHP-1 and SHP-2, to attenuate tyrosine kinase cascades or other signaling pathways.

One of the best-defined checkpoint inhibitors is cytotoxic T-lymphocyte antigen-4 (CTLA-4, CD152), which is induced rapidly following T-cell activation (80, 81). CTLA-4 has a constitutively active internalization motif in its cytoplasmic domain such that it does not accumulate on the cell surface except when ligated in the synapse or when the Y-based internalization motif is phosphorylated (82). CTLA-4 competes with CD28 for binding to CD80 more potently than for CD86 (83). In Tregs, CTLA-4 is constitutively expressed, and coupling to the endocytic pathway leads to the removal of CD80 and CD86 from APCs, which impairs CD28-dependent responses of other T cells (84). CTLA-4 associates with PKC ϵ in Tregs (Fig. 2; ref. 85). Anti-CTLA-4 mAbs are approved for treatment of melanoma (86). Although CTLA-4-deficient mice suffer from fatal immunopathology (87), there is a significant therapeutic window in which antitumor effects can be achieved with limited toxicity.

Another checkpoint receptor is programmed cell death-1 (PD-1, CD279). PD-1 binds PD-1 ligands 1 and 2 (PD-L1, CD274 and PD-L2, CD273; refs. 88, 89) and is recruited to the immunological synapse in a manner related to MHC-peptide strength and abundance (90, 91). PD-1 recruits SHP-2 tyrosine phosphatase and attenuates early TCR signaling upon ligand binding (Fig. 2; ref. 92). The impact of PD-1 on the stability of the immunological synapse depends upon the context, with destabilization in autoimmune and delayed-type hypersensitivity (93, 94), but stabilization in antiviral responses (95). Stabilization of the immunological synapse in this context results in an immune paralysis in which the T cell cannot respond nor be released to engage other targets. PD-1 is associated with T-cell exhaustion in chronic viral infection, and this mechanism can protect the host from immunopathology related to toxicity of chronic immune responses (96). However, it can be exploited by pathogens and tumors as an evasion mechanism (97). Recently, mAbs to PD-1 have been approved for treatment of melanoma in Japan and the United States; so far, results in melanoma and lung cancer are promising (98). The ability to predict patient response based on information about the tumor is a significant goal of personalized medicine. Trials are in progress to determine if PD-L1 expression on the tumor predicts patient response. PD-1 is expressed on Tregs and suppresses its activity. Thus, blockade of PD-1 may increase Treg function, suggesting a rationale for combining anti-PD-1 with anti-CTLA-4 (99), as the latter suppresses Treg function. Other checkpoint inhibitors include Vista and SIRP α (CD172A; refs. 100, 101).

Hierarchy of interactions

Antigen, adhesion, and costimulatory/checkpoint receptors are highly interdependent. LFA-1 activity on T cells is increased by TCR signaling in a process referred to as inside-out signaling (102). This observation leads to a "chicken and egg" problem for initiating TCR signaling in which LFA-1 clearly is important for T-cell sensitivity to antigen (103), yet TCR signaling is necessary for activation of LFA-1. A likely solution involves chemokines in the tissue microenvironment, which stimulate the activity of LFA-1 at the leading edge of T cells and establish a low level of transient LFA-1-dependent adhesion, thus increasing the chances that TCR can detect rare MHC-peptide complexes (104). Once the TCR is triggered, this can further enhance LFA-1 activity and promote formation of an immunologic synapse (105). Increases in LFA-1 affinity are driven by large conformational changes in the heterodimers (Fig. 2; ref. 106). Costimulatory receptor CD28 interacts with ligands in a TCR-dependent fashion, with the critical regulatory step being its low expression on quiescent cells (64), indicating that CD28 only engages its ligand at sites "seeded" by TCR microclusters (MC; ref. 13). One way to consider this is that in the steady state, LFA-1-ICAM-1 interactions allow rapid scanning of APC surfaces for TCR-MHC complexes, whose signaling increases LFA-1-dependent adhesion and generates local MCs, which favors the participation of CD2, CD28, and other small adhesion molecules. Some adhesion molecules and costimulatory receptors cooperate in a process called superagonism. Antibody combinations to CD2 monomer (107) or

single antibodies to CD28 homodimer (108) can induce full T-cell activation in a TCR-dependent manner. The structural basis of these effects is not known, but it appears that in some extreme conditions induced by antibodies, the normal process of TCR induction of adhesion/costimulation can operate backwards. It is not clear if this happens physiologically, but the phenomenon may be useful in a clinical setting if it can be controlled and directed (109).

TCR microclusters

When TCRs are ligated by MHC-peptide complexes presented on a cell, e.g., anti-CD3 antibodies on a solid substrate or MHC-peptide complexes and ICAM-1 on a supported planar bilayer, the first structures that can be visualized at the interface are sub-micron TCR clusters that form in an F-actin-dependent manner (110–113). It has been argued that TCRs are preclustered on T cells, but the ligand-induced clusters have a number of distinct characteristics (79, 114). Ligand-induced MCs recruit the src family kinase Lck to the TCR, leading to the phosphorylation of ITAMs and the recruitment and activation of ZAP-70. ZAP-70 phosphorylates the membrane-anchored linker of activated T cells (LAT) that recruits phospholipase C γ (PLC γ) and facilitates its activation by the inducible T-cell kinase (ITK; ref. 115). PLC γ -mediated generation of diacylglycerol and inositol-1,3,5-trisphosphate leads to the activation of Ras, PKC, and calcium ion effector pathways that can account for transcriptional activation of the T-cell growth and regulatory cytokines such as IL2 (116, 117). TCR can use a module, including the guanine nucleotide exchange factor Vav that activates Notch, to directly control Myc transcription and T-cell proliferation independent of IL2 (118).

Src family kinases Fyn (mentioned in adhesion context) and Lck (dominant kinase downstream of TCR) are regulated by intramolecular interactions. All Src family kinases have a C-terminal tyrosine (Y505 in Lck) that serves as an intramolecular ligand for its own Src homology 2 (SH2) domain. When the SH2 domain is bound to the C-terminal tyrosine in parallel with a second intramolecular interaction of the Src homology 3 (SH3) domain with the polyproline motif between the kinase domain and the SH2 domain, the kinase is locked in an inhibited state. The C-terminal Src kinase (Csk) mediates this phosphorylation when recruited to membrane-anchored Src substrates in the vicinity of the active Src kinase. This negative feedback is counteracted by the transmembrane tyrosine phosphatase CD45. Selective Csk inhibition results in ligand-independent TCR signaling that synergizes with CD28 signals (119). CD45 deficiency results in inhibition of TCR signaling (120). However, CD45 also can reverse activating phosphorylations mediated by Lck and ZAP-70 (121). Interestingly, CD45 is excluded from TCR MCs, and this reduction in CD45 density, based on the large size of its extracellular domain, appears to be important in setting the appropriate local level of phosphatase activity for TCR triggering (113, 122, 123; Fig. 2). Any small adhesion system will generate interactions that exclude CD45, so it is unlikely the CD45 exclusion is sufficient for full TCR signaling, but some level of exclusion appears to be necessary (79, 124).

Supramolecular activation clusters

SMACs defined by Kupfer are divided into the central, peripheral, and distal regions, or cSMAC, pSMAC, and dSMAC (Fig. 1). On the basis of analysis of immunological synapse dynamics using the SLB system, dSMAC behaves like the leading lamellipodium of migrating cells in that it undergoes cycles of protrusion and retraction (24). In contrast, pSMAC is rich in LFA-1 and the integrin to cytoskeletal linker talin, defining it as a lamellum, based on an abrupt change in F-actin dynamics and filament decorations compared with the lamellipodium (24, 125). Higher-resolution imaging has forced a revision of Kupfer's SMAC definitions. First, the active TCR-MHC interactions that lead to signaling in the immunological synapse are concentrated in small MCs that are formed in the dSMAC and move centripetally through the pSMAC to the cSMAC (111, 113, 126). This traversal of the integrin-rich pSMAC by the smaller TCR is possible because the LFA-1-ICAM-1 interaction also begins in MCs in the dSMAC and consolidates into a reticular network with submicron holes in which the TCR-MHC MCs can be fully segregated (127). The LFA-1-ICAM-1 reticulum in the pSMAC is continually moving inward, which allows the central transport of the TCR-MHC MCs (128). Although the immunological synapse can be stable for hours, the dSMAC and pSMAC are highly dynamic structures that are completely renewed every few minutes (24). The second major revision is the structure of the cSMAC. The original cSMAC, as defined by Kupfer, is a TCR-MHC-rich central structure with accumulation of PKC- θ . We now know that the prominent accumulation of PKC- θ is a signature of CD28-dependent costimulation (68). Work with SLB containing only ICAM-1 and activating MHC-peptide complexes revealed a very different cSMAC in which TCR-MHC interactions were highly stabilized and signaling was terminated (17, 113). Further study of bilayers containing ICAM-1, MHC, and CD80 revealed that CD28 and PKC- θ are initially colocalized with TCR in MCs, but on convergence in the cSMAC segregate into a TCR-rich, CD28-deficient zone, in which signaling is terminated, and a TCR-poor, CD28-rich region, in which CD28-PKC- θ -enhanced TCR signaling continues (13, 26). Further analysis of the TCR-MHC-rich signal termination zone in the cSMAC revealed that it is composed of TCR-enriched extracellular microvesicles that bud from the plasma membrane and become trapped in a persistent interaction with the MHC in the SLB (129). Vesicles that bud from the plasma membrane are defined as ectosomes. The budding process is dependent on tumor-susceptibility gene 101 (TSG101), and the buds are arrested by the expression of dominant-negative vacuolar protein sorting 4 (VPS4), implicating the endosomal sorting complexes required for transport (ESCRT) pathway (Fig. 2; ref. 129). This is a property shared with exosomes. In T-cell-B-cell interactions, these TCR-enriched extracellular vesicles are collected by the B cell and moved away from the interface. CD28-CD80 interactions are efficiently sorted away from the bulk of the TCR that is sorted to the extracellular vesicles. Thus, the cSMAC can be divided into two components: the endo-cSMAC, in which TCR and CD28 continue to signal within the synapse, and the exo-cSMAC, composed of TCR-enriched extracellular vesicles. We

have found that the checkpoint receptor PD-1 is highly enriched in the exo-cSMAC. This would suggest that TCR and PD-1 are closely associated and co-sorted or that PD-1 is ubiquitinated. The TCR-enriched vesicles continue to activate signaling in the B cell through engaging surface MHC molecules, and in the case of MHC class II may be relevant to T-cell help delivery as an active form of exo-TCR (129, 130). Studies using the SLB system suggest that all T cells engage in this type of vesicle transfer, opening the possibility that helper, cytotoxic, and regulatory cells may generate exo-TCR in a carrier that can contain other information, including nucleic acids (131). Whether exo-TCR plays a role in the TME is an open question. The modular structure of the immunological synapse thus incorporates a lamellipodium (dSMAC), lamellum (pSMAC), TCR MCs, a sorting domain that can enrich CD28-enhanced TCR signaling complexes (endo-cSMAC), and an extracellular compartment of TCR-enriched extracellular vesicles (exo-cSMAC; Figs. 1 and 2). The exo-cSMAC is a transient compartment that is normally internalized by the APC. When projected into the more complex three-dimensional setting of a T-cell–DC interface, the lamellipodium, lamellum, and multiple endo-cSMACs remain clearly visible at the interface. It is likely that the multifocal close contacts observed in electron microscopy are not MCs, but are multiple, larger endo-cSMAC structures. It has also been proposed that the MCs can manifest as small projections that indent the APC surface, rather than moving laterally (132). There is also an LFA-1–dependent actin cloud or lamellum that imparts a more three-dimensional aspect to the actin cytoskeleton, and this structure incorporates a subset of signaling components (133, 134). This raises issues of subsynaptic vesicular components that may also play an important role in signaling (135, 136).

Directed Secretion: A Synaptic Advantage?

A central element of the immunological synapse concept is directed secretion of soluble components into the synaptic cleft (137–139). This is fundamental to models of T-cell help and cell-mediated killing of infected cells or tumor cells. A major assumption has been that orientation of the centrioles and Golgi toward the APC reflects directed secretion. This has been challenged by studies using antibody-mediated capture to determine the orientation of secretion (140). In this case, molecules that move through the highly oriented Golgi can be secreted randomly based on the manner in which secretory vesicles are transported after leaving the Golgi apparatus. For example, IFN γ was found to undergo directed secretion from helper T cells, but TNF and chemokines were not directed to the synapse. The TCR can be directly delivered to the synapse in vesicles (141). Cytolytic granules are directly delivered to a secretory domain near the endo-cSMAC compartment (20). Recently, it has been proposed that the centrosome docking at the immunological synapse sets up a *de facto* primary cilium that enables intraflagellar transport machinery and hedgehog pathway signaling to be implemented in the immunological synapse (142, 143). Despite this high level of specialization, it has been shown that CTLs may operate in a "quick draw" mode and release cytolytic granules at targets without reorienting

and docking the centrioles to the synapse (144). NK cells also use more dynamic kinapses in signal integration with tumors, with advantages in the ability to penetrate into the tumor over more stable synaptic interactions (145). However, even for NK cells, converting kinapses into synapses with tumor-targeting antibodies increases the efficiency of killing (146). The potential advantage of synapses is the ability to concentrate the cytolytic components on the target surface. Comparison of synaptic versus kinaptic killing in the context of CD8 versus CD4 cytotoxic T cells *in vitro* suggests that the advantage of forming a stable synapse is about 3-fold (147, 148). In a murine breast cancer model, anti-CTLA-4 mAbs stimulate motility of activated T cells in the tumor (149), consistent with other evidence that CTLA-4 engagement could stimulate T-cell motility (150). Stabilization of activated T-cell interactions through induction of Rae-1, which binds NKG2D on T cells, was required to decrease tumor growth (149). Antigen recognition processes that lead to stable synapses may be desirable in immunotherapy settings. It will be of great interest to determine the mode of interaction induced by chimeric receptors that combine ITAM and costimulatory signaling in one polypeptide (47), particularly as conventional synapses with B chronic lymphocytic leukemia cells are defective (3). Exocytic trafficking in the synapse is likely to be balanced by endocytic trafficking. One manifestation of endocytic processes is trogocytosis, in which one cell "gnaws" off pieces of another cell. In addition to ESCRT-dependent budding to generate TCR-enriched extracellular vesicles, some fraction of TCRs in the cSMAC regions undergoes internalization and can bring MHC from the APC with it (129, 151). CD8 T cells that capture MHC-peptide from target cells through trogocytosis actually become targets for killing by other CTLs in the culture (152). What controls the ratio of TCR that is transferred to the APCs in extracellular vesicles versus MHC that is captured by the T cell from the APCs is not known, but both processes are readily detectable across a single immunological synapse (129). The consequence of exo-TCR transfer for tumor cells is not known. CTLs have been shown to release TCR-positive vesicles at target cells (153, 154). It is not clear if these are similar to the exo-TCR reported by Choudhuri and colleagues (129) as it is not clear if these exosome-like structures contain recently engaged TCRs or were sorted into these structures without prior ligand engagement through a distinct mechanism of TCR sorting. It may be possible to isolate the vesicles budded from the plasma membrane versus those released from granules and subject these to proteomic analysis and functional testing.

Conclusions

The immunological synapse mode of stable engagement of targets benefits the attack on tumor. One of the challenges is that *bona fide* tumor antigens are likely to be weaker agonists that may be more dependent on costimulation for stabilization of synapses. Current therapies that target checkpoint receptors are directly manipulating the immunological synapse. Anti-CTLA-4 mAbs may both disinhibit conventional cytotoxic and helper T cells while taking away one mechanism by which Tregs function to reduce suppression in the draining lymphoid

tissues, among surrounding DCs and in the TME. The impact of PD-1 blockade on T-cell dynamics is determined by the context, including integration of other costimulatory signals. Potential autoimmune toxicity of anti-PD-1 may be offset by the enhancement of Treg function due to blockade of PD-1 on Tregs. Thus, anti-CTLA-4 and anti-PD-1 may be complementary in the TME by offsetting any enhancement of Treg function from PD-1 with blockade by anti-CTLA-4 (99). Antibodies can also have agonistic effects, particularly if Fc binding is intact, leading to additional parameters. TCR-enriched extracellular vesicles, unique structures that share properties with both ectosomes and exosomes, may also have multiple roles in the TME. Further study of the immu-

nological synapse in the TME is needed to appreciate other interactions within and between SMACs to achieve optimal results for immunotherapy.

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