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Cytotoxic immunological synapses

Michael L. Dustin¹ and Eric O. Long²

¹ Helen and Martin Kimmel Center for Biology and Medicine, Skirball Institute of Biomolecular Medicine, New York University School of Medicine, New York, NY, USA

² Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD, USA

Summary

One of the most fundamental activities of the adaptive immune system is to kill infected cells and tumor cells. Two distinct pathways mediate this process, both of which are facilitated by a cytotoxic immunological synapse. While traditionally thought of as innate immune cells, natural killer (NK) cells are now appreciated to have the capacity for long-term in reactions to chemical and viral insults. These cells integrate multiple positive and negative signals through NK cell cytotoxic or inhibitory synapses. The traditional CD8⁺αβ T-cell receptor-positive cells are among the best models for the concept of an immunological synapse, in which vectorial signaling is linked to directed secretion in a stable interface to induce apoptotic cell death in an infected cell. Large-scale molecular organization in synapses generated a number of hypotheses. Studies in the past five years have started to provide clear answers regarding the validity of these models. *In vivo* imaging approaches have provided some hints as to the physiological relevance of these processes with great promise for the future. This review provides an overview of work on cytotoxic immunological synapses and suggests pathways forward in applying this information to the development of therapeutic agents.

Keywords

activation; cytotoxicity; synapse; actin; microscopy; adhesion

Introduction to the immunological synapse

Immunological synapses are antigen specific cell-cell junctions with a synaptic cleft stabilized by *bona fide* adhesion molecules for vectorial cell-cell communication between an immune cell and an antigen-presenting cell (APC) (1). We use the term synapse to describe junctions that meet these criteria. The cytotoxic synapse is one of the earliest and best defined of immunological synapse types based on a number of key findings in immunology in the 1970s and early 1980s that exploited this system, and clear functional importance that sustained interest even as other T-cell subsets were described. Zinkernagel and Doherty (2) defined the role of the major histocompatibility complex (MHC) in killing of virally infected cells by sensitized T lymphocytes, which are described as cytotoxic T lymphocytes (CTL) to distinguish them from helper T lymphocytes. The description of adhesion molecules like leukocyte function-associated antigen-1 (LFA-1) by Springer (3), the polarization of cytotoxic T cells (4,5), and directed secretion of perforins and granzymes triggered by

Address for correspondence: Michael L. Dustin, Helen and Martin Kimmel Center for Biology and Medicine, Skirball Institute of Biomolecular Medicine, New York University School of Medicine, 550 First Avenue, New York, NY 10016, USA, Tel.: +1 212 263 3207, Fax: +1 212 263 5711, michael.dustin@med.nyu.edu.

cytoplasmic Ca^{2+} elevation (6,7) led to the proposal of a synaptic basis for T-cell killing (8). The cloning of the T-cell receptor (TCR) (9) and the definition of peptides bound to the groove of MHC molecules as TCR ligands (10,11) allowed the generation of monoclonal T-cell mice and the biochemical preparation of defined TCR ligands that set the stage for further molecular dissection of the immunological synapse.

The study of natural killer (NK) cell synapses was on a parallel track to CTLs. Natural killing was described in the mid 1970s (12). Early studies on the cell biology of NK mediated killing noted dramatic secretory and cytoskeletal polarization that accompanied the cytotoxic process (13–16). The inverse relationship between natural killing and MHC class I expression was noted in 1986 by Kärre (17). Yokoyama described MHC class I binding inhibitory receptor Ly49 as a prototype molecular basis for ‘missing self’ recognition (18). Identification of the structurally unrelated but functionally equivalent MHC class I inhibitory receptors in human NK cells, the killer-cell Ig-like receptors (KIRs), led to the definition of the immunotyrosine inhibition motif (ITIM) sequence as V/IxYxxL, and the finding that tyrosine phosphatase SHP-1 is recruited by phosphorylated ITIMs to turn off activation signals (19). The description of many activating NK cell receptors and associated signal transduction modules suggested additional modes of positive signaling that are integrated with the negative signals in the NK synapse (20,21). NK cells could also link into adaptive immunity via FcRs but were initially thought of as innate effector cells. Recently, NK cell ‘memory’ responses were described, which blur the line between adaptive CTLs and innate NK cells (22,23). While both the CTL and NK synapses can be cytotoxic in nature, the distinct triggering mechanisms and checkpoints make the two cells synapse with potential targets quite differently. The analysis of the NK cell synapse includes both a cytotoxic synapse and an inhibitory synapse in which the negative regulatory receptors are dominant.

While the ‘synaptic basis of T-cell killing’ was first noted in 1984 (8) with a prominent review adopting the term in 1994 (24), this concept remained mostly latent until work by Kupfer on organization of molecules in the helper T cell-B cell interface and work from our group on the dynamics of pattern formation provided a molecular signature for an immunological synapse (25,26). Studies by Kupfer revealed a striking segregation of adhesion receptors in the interface between T cells and antigen-presenting cells. These reconstructed images were presented at meetings in 1996, and they were so convincing of important underlying mechanism that Janeway introduced them into his *Immunobiology* textbook (27) as early as 1997, a year prior to peer-reviewed publication. The original publication in 1998 introduced the term supramolecular activation cluster (SMAC) into the immunology vocabulary to describe two distinct micron scale domains formed in a bull’s eye pattern: a central (c)SMAC rich in TCR and a peripheral (p)SMAC configured as a ring of LFA-1 adhesion receptors (25). Lck and protein kinase C- θ (PKC θ), a novel PKC isoform that is uniquely recruited to the T-B interface (28), were co-localized with TCR in the cSMAC. The widely expressed integrin-cytoskeletal linking protein talin was co-localized with LFA-1. This structure was observed under conditions of T-cell activation, and the organized SMACs were not observed when antagonistic MHC-peptide ligands were presented on the B-cell tumors used as APCs. In the same time frame, we were utilizing the supported planar bilayer system to investigate the organization of adhesive contacts formed by LFA-1 and CD2, a second important adhesion receptor utilized by human CTLs. Using a supported planar bilayer model, we demonstrated segregation of LFA-1 from CD2 and further demonstrated active concentration of CD2 through its interaction with the adapter CD2AP (26). In this paper and without knowledge of the SMAC nomenclature, we proposed the use of the term immunological synapse to describe the bull’s eye pattern of integrins around TCR and isometric adhesion systems like CD2 and CD28. That summer we succeeded in reconstituting T-cell activation by supported planar bilayers presenting

ICAM-1 and MHC-peptide complexes. We again found the same end point as Kupfer but could watch the evolution of the patterns from inverted nascent structures in which TCR were engaged in peripheral clusters that translocated to the center of the interface to generate the SMACs (29). These results suggested that the immunological synapse functioned as a molecular machine to convert early TCR signals into a stable structure that would sustain signaling to achieve full activation (29). Thus, the mature immunological synapse was provisionally defined as a stable and antigen specific T cell-APC junction composed of SMACs.

The concept that actinomyosin-mediated transport plays an important role in forming the SMACs has been supported by studies in primary helper T cells and the Jurkat T-lymphoma model system. Supramolecular topology and membrane fluctuations can drive segregation of receptor-ligand interactions into microclusters, and this process may be particularly important in defining adhesion domains with the large integrin and small immunoglobulin superfamily receptors (26,30). These domains are typically sub-micron and can be organized into larger domains when coupled to cortical actin (31–33). Comparative studies demonstrate that the immunological synapse has parallels to integrin-mediated tissue cell spreading on planar substrates (34). One of the signatures of this process is membrane extension and retraction cycles (contractile oscillations) driven by actin polymerization and myosin II mediated contraction in the lamellipodium. This is considered a sensory process related to the ability of tissue cells to measure and eventually influence mechanical properties of the three dimensional (3D) tissue environment. These experiments demonstrated that the CD45-rich immunological synapse compartment defined by Kupfer as a distal (d)SMAC (35) is a radial lamellipodium that bestows the immune cells with the ability to sense both chemical and mechanical properties of the antigen-presenting cell (36). The predicted retrograde F-actin flow has been imaged directly in Jurkat cells (32) (Fig. 1A). Submicron TCR and LFA-1 microclusters that form in the dSMAC are transported through the pSMAC at ~40% of the rate of the actin flow. The actin flow dissipates at the inside edge of the cSMAC. Incorporation of TCR into the core of the cSMAC is dependent upon expression of Tsg101 (37), a protein that recognizes ubiquitinated cargo and mediates transport into small vesicle into the interior of endosomes (multivesicular bodies) or into the extracellular space in the context of retroviral budding (38). Small, dynamic microclusters have been observed exclusively with the planar bilayer system using total internal reflection fluorescence microscopy due to sensitivity and contrast issues. Methods to improve imaging at cell-cell interfaces to resolve such faint structures do not currently exist, but some promising ideas are in development (39).

The adhesion ring of the immunological synapse is established based on centripetal actin flow. This radial symmetry allows the T cell to dramatically slow or stop its motility without losing the sensory advantages of the lamellipodium in detection of MHC peptide complexes (40,41) and the interpretation of mechanical cues (42). This symmetrical actin flow creates the pSMAC. Breaking the symmetry of the synapse restores motility and this process has been directly observed during T-cell priming on supported planar bilayers (36). This asymmetrical retrograde actin flow creates an LFA-1 focal zone that drive motility (43). It was surprising that even during antigen recognition T cells use the PKC θ signaling pathway to induce symmetry breaking and bursts of migration followed by Wiskott Aldrich syndrome protein (WASp)-dependent reestablishment of the symmetric synapse. We have proposed that the motile phases be referred to as a kinapse (Fig. 1B), with the distinct, but etymologically related name to reflect the functional implications of signal integration and effector functions executed while migrating (44). Similar symmetry breaking and kinaptic behavior have been observed in NK cells receiving a combination of activating and inhibitory signals (45).

The implications of the immunological synapse and SMACs for cytotoxic cells were immediately evident. It was already known that cytolytic granules of CTLs move to the interface with the microtubule-organizing center (MTOC) prior to lethal hit delivery by the CTL. The bull's eye-like pattern suggested a central secretory target with a ring of adhesion molecule to prevent leakage of cytolytic cargo and spare bystander cells. However, the next synapse to be described was not the cytotoxic synapse but the inhibitory NK cell synapse. We review the data on NK and CTL synapses and particularly focus on the role of synapse organization in function where there are mechanistic insights.

Inhibitory NK cell synapses

NK cells look similar to T lymphocytes but lack antigen receptors and instead express an array of activating and inhibitory receptors and the Fc receptor CD16. Most host cells are protected from NK cells by expressing MHC-peptide complexes on their surface. In mice, these molecules are recognized by members of the Ly49 family, a group of dimeric type 2 transmembrane proteins with C-type lectin domains, which nonetheless recognize protein determinants of MHC class I (46). Just how rapidly this system is evolving is underscored by the fact that humans have a completely different family of immunoglobulin superfamily receptors, the KIRs, to serve the same function (47). In both cases, the inhibitory receptors have cytoplasmic domains with ITIMs. Mice and men express also the more conserved lectin-like inhibitory receptor CD94-NKG2A, which binds to the non-classical MHC class I molecule Qa1 and HLA-E, respectively. ITIMs are phosphorylated by Src family kinases, recruit Src homology (SH) domain-containing phosphate 1 (SHP-1), and terminate signaling (48) at a proximal step through dephosphorylation of Vav1 (49,50). There are activating members of all three NK cell inhibitory receptor families. The ability of the activating receptors to bind host MHC class I is crippled by mutations in the binding sites, but it seems that they are evolved for recognition of MHC class I like viral antigens that may have initially evolved to engage inhibitory receptors (51). It is likely that such 'arms races' with viruses drive the rapid evolution of NK cell receptor families (52).

sModel systems with NK cell lines and target cells transfected to express green fluorescence protein (GFP)-tagged forms of human leukocyte antigen C (HLA-C) and KIR were developed to visualize NK cell inhibitory synapses (53). When NK cell lines contact cells expressing the MHC class I (HLA-C) recognized by a KIR, these molecules undergo dramatic accumulation in the contact area, and the NK cell migrates past the putative target (53). Typically, KIR and HLA-C molecules accumulate in a central area surrounded by LFA-1 and ICAM-1 (54,55). Interestingly, the interaction of KIR with HLA-C and their central accumulation was F-actin, temperature, and energy independent, in striking contrast to the F-actin, temperature, and energy-dependent interactions in the helper T-cell immunological synapse. Although F-actin can accelerate KIR recruitment to the synapse, it is not absolutely required (56). Furthermore, inhibitory KIR does not block the actin-dependent accumulation of activation receptors but promotes an actin-independent accumulation of activation receptors at inhibitory synapses, where it prevents their phosphorylation (55). This observation suggested that the interactions driving inhibition would operate under a broader range of physical conditions than activating systems like the TCR and thus would dominantly inhibit NK killing under any condition where the NK cells contact another cell expressing the appropriate ligand. Rather than targeting activation receptors and their associated signaling subunits for dephosphorylation, ITIM-containing receptors appear to block activation by different types of receptors and signaling pathways through actin-independent inactivation of the Vav1-Rac1 pathway (20). This actin-independence is a unique situation in immune synapse formation. Surprisingly, phosphorylated KIR is not evenly distributed at inhibitory synapses but is concentrated in a few microclusters (57). Live imaging of the dynamics of NK cell inhibitory synapses should

provide insights into the unique and unusual mechanism of inhibition by ITIM-containing receptors. It remains to be determined if inhibitory receptors expressed on T cells, such as programmed death-1 (PD-1), and other cells behave similarly or have distinct biophysical mechanisms.

NK cell effector functions are controlled by a balance –or rather an integration– of multiple activating and inhibitory signals. A recent study examined in detail how different signals control NK cell motility and shape (45). NKL cells stimulated by glass slides coated with the NKG2D ligand MICA spread and contracted, and a symmetrical ring of F-actin formed. In contrast, NKL cells spread asymmetrically and moved over the LFA-1 ligand ICAM-1. In the presence of both ligands, NKG2D engagement imposed a stop signal and a symmetrical synapse with peripheral F-actin formed. Interestingly, addition of HLA-E, the ligand of inhibitory receptor CD94-NKG2A, reversed the stop signal. This migratory behavior of NK cells under conditions where inhibitory signals dominate, which is reminiscent of the T-cell kinapses described above (44), may facilitate disengagement from cells that have to be spared, thus allowing NK cells to sample target cells more rapidly.

Cytotoxic NK cell synapse

NK cell-mediated killing can be triggered by a number of pathways, and in most cases, activation actually requires integration of multiple signals (58). The most potent mechanism for triggering degranulation is linked to antibody recognition through the Fc receptor CD16, but this mechanism does not induce polarity of granule release on its own. The transmembrane isoform of CD16 that mediates killing is a classical immunoreceptor in which signal transduction is accomplished by forming a complex between the ligand binding transmembrane receptor with an ITAM-containing signal transduction module on a separate transmembrane protein, most significantly FcR- γ , which is non-covalently associated through a process requiring charged residues in the transmembrane domain (59). CD16 signals can trigger killing by human NK cells without other signals and have the potential to overcome inhibition by KIRs despite the advantages of KIRs noted above. Other NK cell-activating receptors must be engaged in combinations in order to trigger cytotoxicity and the most synergistic combinations are activating receptors with different types of motifs (60). For example, several activating receptors associate with DAP12, which has an ITAM, while NKG2D associates with DAP10, which has a YINM motif shared with costimulatory receptors like CD28. Activating receptors in the SLAM family possess phosphotyrosine motifs that link to the small adapter SAP [SLAM (signaling lymphocytic activating molecule)-associated protein] to deliver activating signals through recruitment of Fyn. Several of the activating NK cell receptors have unknown but widely expressed ligands, such that not all of the receptors engaged in an activating NK synapses can be known at present.

Recruitment of signal transduction molecules to cytotoxic and inhibitory synapses were compared by Vyas and Dupont using NK cell lines, NK clones, and primary NK cells (61–64). NK cells readily formed cSMAC and pSMAC-like compartments in 1–10 min, regardless of whether the synapses would lead to cytotoxicity or inhibition. Thus, in these studies, the LFA-1-talin system seemed to be uniformly activated in this time frame to allow for sampling of signals present on apposed cells. The significant difference between cytotoxic and inhibitory synapses was the ratio of activating tyrosine kinases, like Syk, ZAP70, and Lck, to the tyrosine phosphatase SHP-1 in the cSMAC. This ratio was high in cytotoxic synapses and low in inhibitory synapses. This finding is consistent with the model that SHP-1 recruitment by inhibitory receptors must act locally on key phosphorylated tyrosines generated by activating receptors to prevent the tyrosine kinase cascade from propagating. Cytotoxic NK synapses are similar in many respects to T-cell synapses in that

the active signaling molecules are recruited to the synapse and a well-defined pSMAC is formed. Delivery of lytic granules to the cytotoxic synapse requires actin cytoskeleton remodeling and microtubule-dependent transport. While actin rearrangement is required for polarization of NK cells, cortical F-actin forms a barrier that lytic granules must traverse in order to reach and fuse with the plasma membrane. Recent studies have reported that myosin IIA is not required for the formation of an organized and polarized NK cell synapse but is essential for the final step of lytic granule exocytosis (65,66). Myosin IIA is associated with lytic granules and promotes their transport through the final layer of F-actin at the cytotoxic synapse (66).

Several groups working on NK cell synapses have converged on the conclusion that classical NK-mediated killing results from a multistage process with respect to patterning, cytoskeletal polarization and killing (67,68). Early studies suggested that NK cells sustained nascent immunological synapses over longer periods compared to T cells (53). This prolonged nascent synapse, although not observed by all, suggested that NK cells might use the nascent synapse over time to test the ratio of activating to inhibitory inputs prior to commitment. The relationship between receptor accumulation, actin polarization, and killing suggested that NK cells use formation of a mature synapse as a checkpoint, the passage of which is dependent upon the ratio of activating and inhibitory signals (69,70). While a few NK cells rapidly committed to the mature synapse and killed the target cell, as many as half of the cells took many minutes after contact to form a synapse and kill the target, if the target would be killed at all (54). This finding suggests that with a population of normal NK cells and nominally susceptible targets, there is a probability that the balance of activating and inhibitory synapses will fail to pass checkpoints for synapse formation and cytotoxic triggering or may be delayed in passing these checkpoints. Thus, compared to CTL synapses described below, the NK cytotoxicity commitment process is prolonged.

Two recent concepts in NK cell development have not been extensively studied with respect to synapse formation. At an early developmental stage, NK cells must be 'licensed' by interactions with MHC class I (71). This process, which generates mature NK cells, appears to be akin to positive selection in T cells. An alternative hypothesis to explain this phenomenon of NK cell 'tolerance' is that NK cells that do not receive inhibitory signals through MHC class I receptors become desensitized, or 'disarmed', through persistent activation signals (72). Epigenetic factors that are not well understood define the array of activating and inhibitory receptors that are expressed in any particular NK cell. Once these cells develop, only the NK cells that express sufficient levels of inhibitory receptors that recognize host MHC class I gain functional competence and are considered licensed, self-tolerant effector cells. Mouse models and human systems in which inhibitory receptor expression can be linked to specific MHC class I alleles demonstrate that licensed and unlicensed cells both express perforin and granzymes, but only licensed cells can engage in missing self recognition. The epigenetic mechanisms that control expression of inhibitory receptor also generate NK cells (10–15%) that lack MHC class I-binding inhibitory receptors, and these cells are hyporesponsive (73–76). The licensing interactions and cytotoxic synapse formation have not been examined in models where licensing can be controlled, and the signaling differences between licensed and unlicensed cells are not well defined. For example, it is not known if unlicensed cells form a nascent synapse similar to the bulk of primary NK cells, which are licensed.

A second process that is of great interest in NK cells is based on recent observations that virus-specific NK cells can engage in adaptive responses with primary expansion, contraction, memory, and recall phases (23). While populations of mouse cytomegalovirus (mCMV)-specific NK cells expressing the Ly49H activating receptor are much more abundant than mCMV-specific naïve T cells, the Ly49H⁺ NK cells nonetheless undergo a

100-fold expansion during infection. These cells then contract back to near pre-infection levels to initiate a memory phase. Recall responses are more efficient, as memory Ly49H⁺ NK cells elaborate a 10-fold enhanced ability to protect NK cell-deficient mice from mCMV infection compared to naive Ly49H⁺ NK cells. The characteristics of synapse formation by memory and naive NK cells are not known, but this could certainly be addressed in the mouse models. While most mice are raised in specific pathogen-free conditions, humans experience many viral infections, and it is likely that peripheral blood NK populations contain both naive and memory NK cells. It remains to be determined how much of the heterogeneity in human NK cell behavior is related to differences in these subsets.

The cytotoxic NK cell synapse has been modeled using supported planar bilayers (77). Bilayers containing CD48 and ULBP1 trigger synergistic granule release in primary human NK cells. In human NK cells, the receptor for CD48 is 2B4, a member of the SLAM family that associates with signaling adapter SAP, and ULBP1 is a ligand of NKG2D. Whereas both CD48 and ULBP-1 are needed to trigger degranulation, CD16 engagement with immunoglobulin G (IgG) alone is sufficient for degranulation. Lysosomal membrane glycoprotein-1 (LAMP-1) (CD107a) molecules that have been delivered to the cell surface upon degranulation are not allowed to diffuse at the plasma membrane but are retrieved into a stable and central endocytic compartment. Formation of an organized cytotoxic synapse and retrieval of LAMP-1 at the center were absolutely dependent on LFA-1 interaction with ICAM-1 on the bilayer. Thus, an unanticipated function of an LFA-1 pSMAC in the cytotoxic NK synapse may be for the efficient recycling of granule membrane to form new cytolytic granules—a process that may be critical for serial killing. Another surprise in this study was that although both 2B4-CD48 and NKG2D-ULBP interactions span less than 15 nm between membranes and might be expected to co-cluster based on size rules for microcluster formation (78), these molecules were dramatically segregated. The 2B4-CD48 interactions were positioned at the center, perhaps as expected, whereas the NKG2D-ULBP-1 interactions were co-localized with the LFA-1-ICAM-1 interactions in a peripheral region. Thus, NK cells integrate NKG2D and 2B4 signals from different compartments of the synapse to trigger degranulation into the cSMAC.

Cytotoxic T cells

Cajal described the neural synapse as a ‘The protoplasmic osculation (kiss) ... the final saga in an epic love story.’ Following on this precedent, the cytotoxic synapse of CTL has been aptly described as a kiss of death (79), certainly the final saga for the target. There are two modes of CTL-mediated killing: Ca²⁺-dependent killing by perforin and granzymes and Ca²⁺-independent killing mediated by Fas ligand (FasL) binding to Fas (CD95) on target cells. Perforin-mediated killing can also be diagnosed with the vacuolar acidification inhibitor concanamycin A (80). Both pathways trigger death by apoptosis, but the perforin pathway is typically faster. Perforin-mediated killing is more general, since it is based on a highly conserved membrane injury response and endosomal lysis leading to introduction of granzymes into the target cell cytoplasm (81), and thus, no specific receptor is needed. Since perforin and granzymes are released from the cell, the synaptic cleft has been assumed to enhance function through high local concentration and also to prevent bystander exposure to active perforin. Fas must also function in a cell-cell contact, since FasL and Fas are membrane proteins with a limited reach (~15 nm). FasL has been reported to be present in the granules of CTLs that co-express perforin and granzymes and to be exocytosed into the synaptic cleft in response to TCR triggering (82). This is in part explained by the sorting of FasL into multivesicular body core vesicles that are released from cells during degranulation (83). It has been observed that in transplant rejection, early CTL express perforin and FasL, while during the retraction phase the CTL lose the perforin pathway, but continue to be active killers using FasL. In CTLs utilizing only FasL, the pathway is not dependent upon

protein synthesis, suggesting the FasL is stored in an intracellular compartment such as cytotoxic granules, but FasL-mediated killing is selectively inhibited by brefeldin A (84,85). Brefeldin A inhibits both endoplasmic reticulum to Golgi and some regulated trans-Golgi to plasma membrane secretion pathways, but not the release of granules (secretory lysosome) or recycling endosomes (86). Thus, FasL-mediated killing can be diagnosed by anti-FasL antibodies and by acute treatment with brefeldin A (long-term treatment will eventually limit the availability of MHC-peptide complexes on the target).

The CTL immunological synapse

Studies on the CTL synapse have focused primarily on understanding of the perforin and granzyme-mediated killing. CTLs are known to engage in a killing cycle that involves tenacious adhesion, release of cytotoxic agents, and detachment from the target (79). CTLs undergo dramatic polarization changes with movement of the CTLs' microtubule organizing center (MTOC) and linked cytotoxic granules to the synapse (4). Electron microscopy demonstrated that the MTOC is moved directly to the membrane, much like the basal body of a primary cilium (87). Some of the mechanisms mediating granule movement in the CTL synapse have been illuminated by study of patients and mouse models selected based on defects in pigmentation arising from melanocyte granule movement defects, which in some cases predict CTL granule movement defects (88–90). Molecules involved in this process include Rab27a, Munc13–4, and myosin V. Separating CTLs from target cells during the peak of adhesion is difficult and traumatic to both cells suggesting strong cell adhesion (91). LFA-1 and CD2 adhesion pathways mediate human CTL adhesion to an array of targets (92). It is likely that other pathways contribute in different contexts. How CTLs know to let go after targets are programmed for lysis is not clear. It is possible that some chemical or mechanical feedback is involved, as membranes of cells undergoing apoptosis undergo loss of phospholipid asymmetry and dramatic blebbing based on changes in the cortical cytoskeleton (93).

Molecular patterns in the immunological synapse were first described for CD4⁺ helper T cells as described above. Naive CD8⁺ T cells were subsequently found to form similar bull's eye synapses during priming *in vitro* (94) and in mature CTLs during target lysis (95). The cytotoxic synapses were characterized by a well-defined pSMAC (LFA-1 ring) and cSMAC (TCR, CD8 and Lck). This process could be reconstituted with ICAM-1 and MHC class I peptide complexes in supported planar bilayers (96). Surprisingly, CTLs were very efficient at forming pSMAC-like adhesion rings and would do so transiently even in the absence of specific MHC-peptide complexes (96). Griffiths (95) described the cSMAC as being divided into signaling and secretory domains. Such functional compartmentalization of the cSMAC is likely to be important in integration of costimulatory signals with TCR signal and receptor degradation in helper cells (97). While both LFA-1 and CD2 adhesion pathways contribute to conjugate formation, only the LFA-1 pathway leads to formation of organized SMACs (98). Recently, the secretion of granules directly into the model cytotoxic synapse with a planar bilayer has been observed by TIRFM microscopy based on the detection of CD107a⁺ foci at the cSMAC (99,100). These studies confirm that there is a secretory domain in the cSMAC. It has been reported that target lysis can take place without an immunological synapse (101). The quantitative advantages of forming a synapse versus other modes of interaction are only beginning to be understood.

The competitive edge offered by the synapse

The model that secretion of granule contents into the immunological synapse is important for efficiency of killing has recently been tested and an unexpected new parameter governing efficiency has been revealed. Sykulev and colleagues (99,100) initiated studies

comparing human cytotoxic T-cell clones expressing CD8 and CD4. The CD8⁺ T cells are 100-fold more efficient than the CD4⁺ CTLs in terms of the time and granule investment needed to kill targets. Both types of CTLs kill primarily using the perforin/granzyme pathway, and both cell types are equivalently armed at the level of granule activity. Comparison of synapse formation by both cell types revealed that the CD8⁺ CTLs form more stable synapses (80% vs 40% of total conjugates), whereas CD4⁺ CTLs have more prevalent kinapses (the asymmetric junctions that lead to migration and absence of a protected secretory domain). As described above, the generation of kinapses in CD4⁺ T cells is promoted by activation of PKC θ , such that inhibition of PKC θ stabilized the synapses of CD4⁺ CTLs. Synapse stabilization by this mechanism resulted in a threefold increase in killing efficiency. Since ~40% of CD4⁺ CTLs did form synapses without the inhibitor, it is suggested that stable synapses are sixfold more efficient than kinapses (100). The remaining 30-fold difference in efficiency between CD4⁺ and CD8⁺ T cells was due to a different, unexpected mechanism (Fig. 1C).

When triggered by agonist MHC-peptide complexes, CD8⁺ CTLs release granules faster than CD4⁺ CTLs. Triggering using the same anti-CD3 antibody eliminated the kinetic difference, and eliminating CD8⁺ CTL interactions slowed granule release from CD8⁺ CTLs. Examination of the movement of the MTOC and the granules to the synapse revealed that strong TCR signals promoted by CD8 led to fast granule recruitment to the MTOC and movement of that MTOC-granule complex to the secretory domain, such that the granules were tightly localized in the cSMAC. Signals generated by weaker agonist MHC-peptide complex, CD8 blockade, or use of CD4 as the coreceptor led to movement of the MTOC to the secretory domain without the granules, which then needed to slowly make their way to the secretory domain via the pSMAC such that the granules appear loosely arrayed in the synapse or kinapse (99). Similar results were obtained by the Griffith laboratory utilizing altered peptide ligands in a CD8 system, suggesting that both the CD8 and TCR-MHC-peptide interaction strength can determine the early signaling kinetics and pathway for granule delivery (102). Granule exocytosis rates and the diffusion rate for perforin and granzymes from the synaptic cleft will determine the efficiency of granzyme delivery to the cytoplasm. Therefore, the formation of a cytotoxic synapse based on pSMAC integrity and polarized granule delivery to the secretory domain are two components that offer a significant competitive advantage in the race with pathogens.

Cytotoxic synapses *in vivo*

In vitro studies have provided insights into the function of the immunological synapse, but *in vivo* studies have the potential to put this system in a more physiological context. Histological analysis supports a role of organized immunological synapses, particularly in the central nervous system (CNS) (103,104). Two photon laser scanning microscopy particularly has enabled imaging of micron scale details hundreds of microns deep in live tissues to capture the dynamics of interactions (105). Priming of CD8⁺ T cells involves prolonged interactions with dendritic cells (106,107). The effector phase involves stable interactions, but CTLs could kill 2.4 specific B-cell targets per hour. Interestingly, the presence of regulatory T cells reduced the killing rate to 0.4 per hour, a sixfold decrease in the killing rate (108). The tumor microenvironment further decreases the rate of killing to <0.2 per hour (109). Less has been done at this point with CTL in the context of infection. Recently, we examined the dynamics of LCMV-specific CTLs in the meninges during fatal LCMV meningitis (110). While histological analysis suggested the presence of stable synapses in this model, dynamic imaging revealed that the CTLs are constantly in motion, suggesting that they are forming kinapses. It is difficult in fixed images to distinguish a synapse with a transient break in the pSMAC from kinapse (103). It is only through dynamic imaging that the stability of interactions can be assessed. The kinapse formation by CTLs in

the meninges precedes massive recruitment of myelomonocytic cells that is fatal to the host (110). It is not clear why more stable synapses leading to efficient killing of virally infected cells is not observed in this site. Future advances in intravital imaging should also provide more insight into cytotoxic synapse functioning *in vivo*. *In vivo* imaging methods with the required molecular resolution are already in use in the nervous system (111) and may soon illuminate immune cell function in physiological settings.

Conclusions

The immunological synapse defined by formation of an intact adhesion ring and the movement of cytolytic granules to a secretory domain provides a site for signal integration in NK cells and contributes to the efficiency of CTL-mediated killing. Studies of NK cells have revealed a central role of integrin LFA-1 in the organization and dynamics of NK cytotoxic synapses. Imaging of T-cell cytotoxic synapses has demonstrated physical differences between CD4⁺ and CD8⁺ cells that result in vastly superior cytotoxic activity of CD8⁺ cells. Outstanding problems in the field remain. For example, the permeability of the pSMAC ring is not known, and studies in CD4⁺ T cells suggest that it is surprisingly permeable to antibodies and Fab fragments (112). In fact, the anti-LAMP-1 CD107a Fab-based method used to rapidly detect granule exocytosis in the synapse with planar bilayers and TIRFM is dependent upon the ability of the labeled Fab to diffuse freely into the synaptic cleft for both NK cells and CTLs (77,99,100). Another issue is the minimal signal integration time for NK cells versus CTLs. It had been argued that CTLs were much more decisive than NK cells, but this apparently depends upon the ligand of the CTL. NK cells may make decisions in a more tentative process, but this seems to be followed by an efficient killing process. In contrast, the rate of commitment with CTLs depends upon ligand strength, with lower potency ligands leading to an inefficient killing process. This factor may be one that leads to slow killing of tumor cells.

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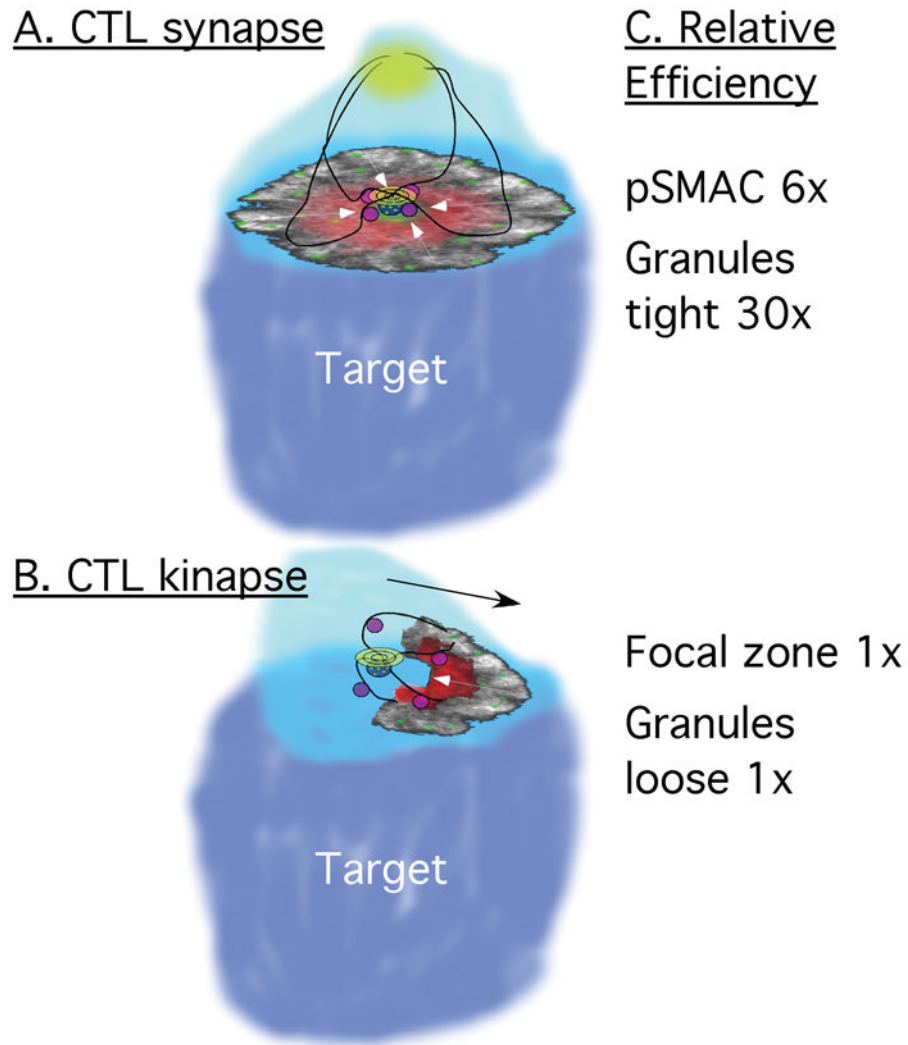


Fig. 1. Schematic of CTL synapse and kinapse

(A). CTL synapse. Strong TCR signal and CD8. The key feature is the symmetric actin pattern with centripetal flow (white arrows). This forms the pSMAC (red, ICAM-1) and positions the TCR for Tsg101 dependent movement in the cSMAC (green, TCR). In this efficient system the granules (purple) are targeted to the MTOC along microbutules (heavy black lines) prior to movement of the MTOC and Golgi to the cSMAC secretory domain. Some of the cSMAC-associated TCR is in multivesicular bodies (blue with green dots). (B). CTL kinapse. Weak TCR signal or CD4. The key feature is a asymmetric actin pattern with net retrograde actin flow (white arrow) inducing forward motion of the cell (black arrow). This forms the asymmetric focal zone (red, ICAM-1), whereas TCR microclusters do not accumulate in a cSMAC. The MTOC moves to the actin-depleted secretory domain, but the granules reach the domain more slowly. (C). Relative efficiency of the two configurations. The presence of an intact cSMAC gains $\sim 6\times$ increase in killing efficiency. The tight granule packing around the MTOC results in a $\sim 30\times$ increase in killing efficiency compared to the loose granule distribution.