What is the importance of the immunological synapse?

Daniel M. Davis[1] and Michael L. Dustin[2]

[1]Department of Biological Sciences, Sir Alexander Fleming Building, Imperial College London, South Kensington Campus, London, UK, SW7 2AZ
[2]Skirball Institute of Biomolecular Medicine and Department of Pathology, New York University School of Medicine, New York, NY 10016, USA

The immunological synapse (IS) has proved to be a stimulating concept, particularly in provoking discussion on the similarity of intercellular communication controlling disparate biological processes. Recent studies have clarified some of the underlying molecular mechanisms and functions of the IS. For both T cells and natural killer (NK) cells, assembly of the IS can be described in stages with distinct cytoskeletal requirements. Functions of the IS vary with circumstance and include directing secretion and integrating positive and negative signals to determine the extent of response.

Although the immunological synapse (IS) was first described in terms of directed secretion of cytokines between T cells and antigen-presenting cells (APCs) [1,2], it was the landmark discovery of micrometer-sized segregated clusters of proteins at the T cell–APC intercellular contact [3–5] that led to the common use of the concept, and triggered an ongoing major research effort in imaging immune responses. Initial excitement stemmed from the strong correlation of specific patterns of protein clustering with T-cell proliferation. This suggested, but did not prove, a causal relationship. Observations of segregated protein clusters at contacts between targets and NK cells [6] or B cells [7] required that the concept of the IS was expanded to include other immunological effectors. Indeed, segregated clusters of proteins could form even when an effector response was not elicited, such as when ligation of inhibitory killer immunoglobulin-like receptors (KIRs) prevented NK-cell activation at a so-called inhibitory NK-cell IS ([6,8–11] and references therein). Variation in the organization of different types of IS is illustrated in Box 1 and Figure I). Most recently, a supramolecular structure resembling the IS was also observed during intercellular viral transmission [12,13]. Thus, assembly of an immune cell synapse can occur in different circumstances for a variety of functions (Table 1).

Stages in assembly of the IS

From early studies using T cells interacting with a supported lipid bilayer containing agonist peptide–MHC and intercellular cell adhesion molecule-1 (ICAM-1), T-cell receptor (TCR)–peptide–MHC interactions were first seen to accumulate in a ring surrounding a central cluster of leukocyte function-associated molecule-1 (LFA-1)–ICAM-1 interactions, creating an immature T-cell synapse, which later inverts such that a ring of integrin, the peripheral supramolecular activation cluster (p-SMAC) [3], surrounds a central cluster of TCR–peptide–MHC, the c-SMAC [3], at a mature IS [4,5]. The central accumulation of TCR–peptide–MHC is clearly dependent on actin cytoskeletal processes [14] and might be regulated in part by ERM protein phosphorylation controlling the link between membrane proteins and the cytoskeleton [15]. NK-cell cytotoxicity is controlled by a balance between activating and inhibitory signals [16–18] and uses the same cytolytic mechanisms as cytotoxic T lymphocytes (CTLs). Recent evidence that NK-cell synapses also form in stages is that, at an activating (i.e. cytolytic) NK-cell IS, CD2, f-actin, CD11a and CD11b rapidly accumulate in the peripheral ring of an NK-cell IS before recruitment of perforin towards the center of the synapse [19]. Importantly, colchicine, an inhibitor of microtubule formation, prevented accumulation of perforin but not CD2, CD11a or CD11b, at the IS.

NK cells and CTLs might use the IS differently to integrate signals. For example, polarization of the microtubule organizing center (MTOC), which occurred before accumulation of f-actin at the NK-cell IS, could also occur in some non-cytolytic NK–target conjugates [20]. Thus, an additional process after reorientation of the MTOC seems necessary for NK cell-mediated lysis. Strikingly, moderate interference of actin polymerization dramatically impaired NK-cell, but not CTL, cytotoxicity. Thus, NK cells might use cytoskeletal rearrangements as 'checkpoints' on the way to being committed to deliver a lytic hit, whereas CTLs commit to lysis more rapidly and robustly. These differences might relate to the strategy each cell uses for detection of disease: activation of CTLs is kept stringent by requiring TCR recognition of agonist peptide–MHC, whereas NK-cell cytotoxicity is regulated by a balance between activating and inhibitory signals triggered by invariant epitopes. Evidence that activating and inhibitory NK-cell signals integrate by control of cytoskeletal processes is that signaling by inhibitory KIR dephosphorylates Vav1, directly blocking actin cytoskeletal processes [21].

Corresponding authors: Daniel M. Davis (d.davis@imperial.ac.uk), Michael L. Dustin (dustin@saturn.med.nyu.edu).

Box 1. Variety in the structure of immunological synapses (ISs) for different types of immune cell interactions

Supramolecular organization of proteins has been observed in synapses involving various types of immune cells, including T cells, natural killer (NK) cells, B cells and dendritic cells. There is evidence that the underlying molecular mechanisms by which proteins are arranged at the IS involves both spontaneous processes and cytoskeleton-driven mechanisms. However, between different cell types, it has recently emerged that there are many intriguing differences in the specific organization of proteins at the IS. Thus, as is perhaps commonplace after an initial burst of research in a new area, complexities now suggest things are not quite as simple as perhaps once assumed.

In Figure I, two cells are shown to interact and below, one cell has been removed to reveal the organization of proteins at the intercellular contact for various types of immune cell interaction. In the helper T-cell IS, LFA-1 (leukocyte function-associated molecule-1) and talin initially cluster in the central supramolecular activation cluster (c-SMAC) with T-cell receptor (TCR) segregated into a peripheral ring. After some time, in the order of minutes, this arrangement inverts so that TCR clusters in the c-SMAC with LFA-1 and talin in the peripheral SMAC (p-SMAC) [3,5]. TCR clusters in small areas surrounded by LFA-1 in the early cytotoxic T-cell IS [25]. After some time, a peripheral ring of LFA-1 forms to enclose two segregated domains in the c-SMAC, one containing the TCR and the other containing the lytic granules [40,41]. Studies of rare genetic diseases have recently revealed some of the molecular determinants underlying the movement of lytic granules [42]. At the cytolytic NK-cell IS, SH2-domain-containing

phosphotyrosine phosphatase-1 (SHP-1) initially clusters in small areas surrounded by LFA-1 [43]. At a later time, lytic granules cluster in the c-SMAC with LFA-1 in the p-SMAC [9,43,44]. MHC class I clustering in the target cell at the non-cytolytic NK-cell IS has several different patterns [8]. It has been speculated that this inhibitory NK-cell IS represents a stage towards assembly of a mature activating synapse from which progression has been prevented [11]. Although not depicted, γδ T cells also form an IS that is just beginning to be studied [45].

It is important to note that the data represented in Figure I derive from many researchers using a variety of particular systems and methodologies. For example, representation of the early cytotoxic T-cell IS derives from data examining the interaction of the cytotoxic T cell with a supported lipid bilayer containing intercellular adhesion molecule-1 (ICAM-1) and peptide–MHC and remains to be seen in cytotoxic T cell–target cell interactions. Also, it should be noted that the organization of proteins at an IS will probably vary with local concentrations of cytokines, chemokines and other environmental stimuli. A detailed tabulation of the location of molecules at the T-cell and NK-cell IS has been presented in Ref. [46], although it is rapidly emerging that many other proteins, such as potassium channel proteins [47] and even transcription factor signaling intermediates [48], can also be organized at the IS. As such, Figure I illustrates that the current data suggest that there are differences in the organization of types of IS but it remains a major challenge to the field to clarify the importance of these differences in different immune cell interactions.
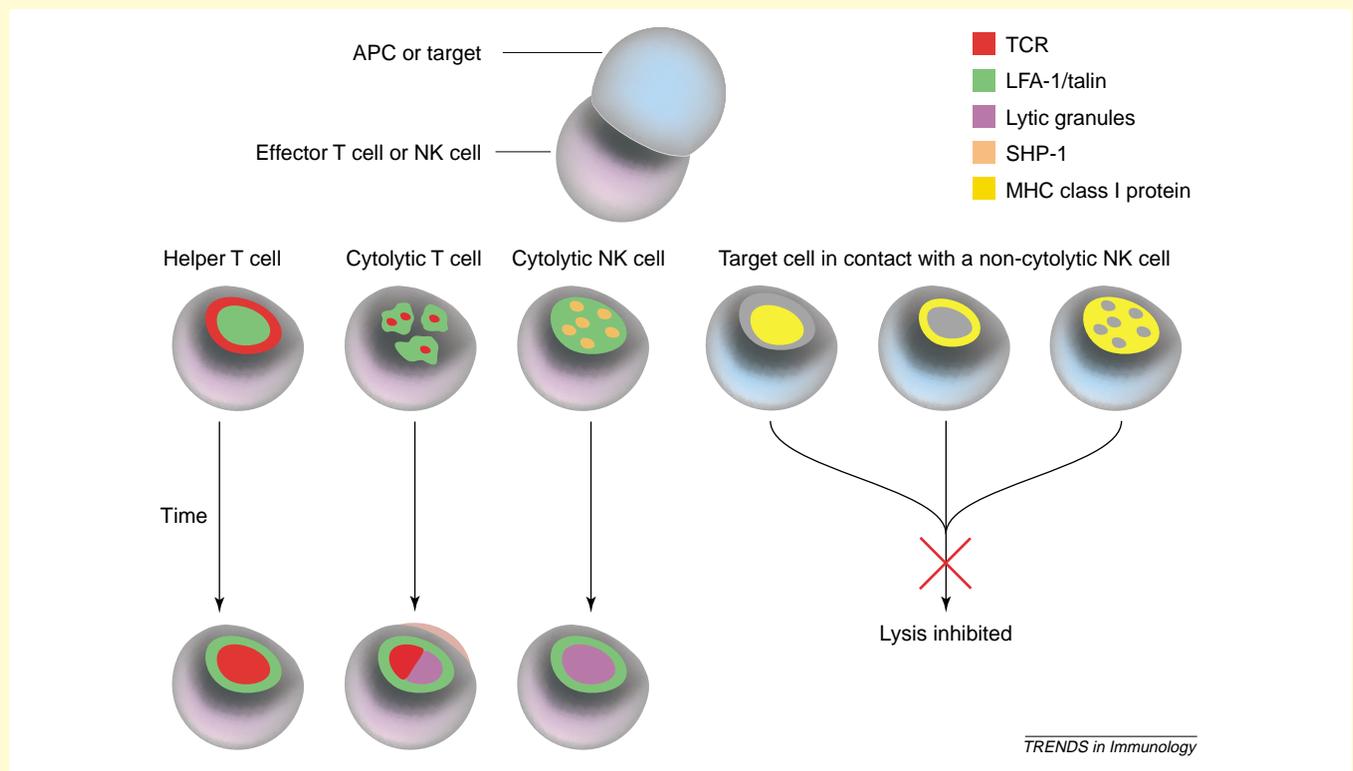

Figure I. Representation of the structure of the immunological synapse (IS) in different types of immune cell interactions. More research is needed to clarify if and how intercellular communication is influenced by such variations in protein organization at the IS. Abbreviations: APC, antigen-presenting cell; LFA-1, leukocyte function-associated molecule-1; NK, natural killer; SHP-1, SH2-domain-containing phosphotyrosine phosphatase-1; TCR, T-cell receptor.

Some of the checkpoints for a CTL might also be bypassed by activating signals associated with inflammation or stress, such as high expression of ICAM-1 or induction of NKG2D ligands. NKG2D is a costimulatory receptor for T cells and an activating receptor for NK cells [22,23] that recognizes stress-inducible MHC class I-related proteins expressed in specific membrane

microdomains [24]. Recently, NKG2D, along with ICAM-1, was found to contribute to formation of an antigen-independent p-SMAC-like structure, at least at the IS formed between T cells and a supported lipid bilayer containing ICAM-1 and agonist peptide–MHC [25]. The presence of an antigen-independent p-SMAC-like structure might accelerate later stages of CTL-mediated killing

Table 1. The immunological synapse (IS) can have several functions, with varying importance for particular cell–cell interactions

Function	Comment	Refs
(I) Establishing checkpoints for lymphocyte activation	Stages in the assembly of the IS, having distinct cytoskeletal requirements, can provide a framework for establishing checkpoints for cellular activation. This might be particularly important for integrating positive and negative signals in natural killer (NK) cells in which an inhibitory NK-cell IS might represent a stage towards the assembly of an activating IS from which progression has been prevented. Spatio-temporal movements of CD45 can facilitate discrete stages in T-cell signaling.	[11,19,20,49]
(II) Enhancing signaling	By its very nature, the dense accumulation of protein at the IS can increase the rate of T-cell receptor (TCR) triggering, at least initially.	[32]
(III) Terminating signaling and/or effector function	Membrane-proximal signaling can be terminated by downregulation and degradation of TCR and also perhaps by removal of TCR ligation at the IS by intercellular transfer of peptide–MHC. Transfer of peptide–MHC from an antigen-presenting cell (APC) to a cytotoxic T lymphocyte (CTL) might also terminate effector functions by facilitating 'fratricide'. Intercellular transfer of proteins that commonly occurs at an IS might also be required for detachment of conjugated cells, although direct evidence for this is lacking.	[32,50,51]
(IV) Balancing signaling	Balancing the relative influence of enhancing (II) and terminating (III) signaling to maintain agonist-triggered signals in normal T cells. Overbalances toward terminating signaling in anergic T cells.	[32,33]
(V) Directing secretion	This was the first function proposed and there is now considerable evidence for this being a major function of the IS. Current research addresses exactly when secretion of cytokine or lytic granules necessitates a mature IS.	[41,52,53]

on detection of agonist peptide. Thus, the definitive nature of the peptide–MHC for target identification might enable CTLs to pass some checkpoints that are needed to promote specificity of NK cells.

Self-assembly of protein clusters at the IS
Specific patterns of MHC/KIR can assemble at an inhibitory NK-cell IS even in the presence of drugs that inhibit cytoskeletal or ATP-dependent processes [6,8]. Thus, supramolecular organization of some proteins can occur by mechanisms other than cytoskeletal or other ATP-dependent processes and perhaps micrometer-sized domains could be created by spontaneous segregation of receptors and ligands spanning similarly sized intercellular distances. The thermodynamics underlying this idea have been mathematically formulated by modelling the IS as consisting of apposing elastic membranes containing two differently sized receptor-ligand pairs [26,27]. It seems that the loss of entropy by segregation of proteins can be offset by the gain in energy from increased receptor–ligand interactions and minimising bending of the opposing membranes. Consistent with this idea, MHC protein was found to accumulate at an inhibitory NK-cell IS preferentially where the size of the synaptic cleft matched the size of the extracellular portions of KIR/MHC [10]. Indeed, there might be something fundamentally important about the relative size of the extracellular portions of MHC/KIR and MHC/TCR that allows ATP-independent self-assembly and exclusion of larger receptors and ligands, such as ICAM-1 or LFA-1 [28].

Functions of the IS
A mature T-cell IS forms only on recognition of agonist peptide–MHC [3,5], and direct comparison of the timing of protein phosphorylation with accumulation at the IS demonstrated that initial membrane proximal TCR signaling has largely abated before a mature IS forms [29]. Thus, a mature IS is not required to initiate T-cell activation [3,29] but appears to form as early TCR signaling is waning [29] as a consequence of initial signals.

However, on ligation of T-cell NKG2D a ring of LFA-1 assembles [25], at least at the IS formed between T cells and a supported lipid bilayer, and thus supramolecular organization of some proteins might occur in the absence of agonist peptide–MHC.

For some time it has been known that T-cell cytotoxicity could be triggered by agonist peptide–MHC at a concentration too low to trigger other responses, such as IFN-γ secretion and internalization of CD3 [30]. Images of the CTL IS in the presence of low and high concentrations of agonist peptide–MHC, revealed that CTL cytotoxicity can be triggered without significant accumulation of CD2 or phosphotyrosine at the CTL–target interface [31]. Polarization of perforin and tubulin, however, was almost maximal even at a low concentration of agonist peptide–MHC. Thus, at least some aspects of a mature IS are unnecessary for cytotoxicity. A major next goal would be to uncover molecular mechanisms underlying different thresholds for the polarization of perforin and CD2.

Clustering of TCR in the central region of the IS might function to balance signaling. Recently, a Monte Carlo simulation of T-cell activation suggested that enhanced TCR signaling, caused by clustering in the IS, would be balanced by increased TCR downregulation [32]. In support, T cells lacking CD2AP are unable to downregulate TCR and are hypersensitive to antigen [32]. Also, phosphotyrosine was significantly increased in the central region of the IS formed by T cells lacking CD2AP, consistent with simulation of an IS in the absence of TCR downregulation. This collaboration of modeling, genetics and imaging allowed previously discussed concepts regarding adaptive signaling processes [33] to be integrated into the spatial framework of the IS with the conclusion that c-SMAC formation might have an important role in T-cell adaptation to different levels and strengths of antigen receptor signaling. Many biological systems display some degree of adaptive control in signaling processes, however, this might be a particularly crucial problem for the immune system given the diversity in amount and quality of antigenic structures, and

perhaps this lead to the evolution of a special structure, the c-SMAC, to assist this process. Signal-balancing properties of the synapse might dampen responses to the most abundant or high-quality peptide–MHC, allowing 'space' for other T cells to also proliferate. This might be important in allowing the immune system to establish a broad response to pathogens.

Counting of TCR-defining thresholds in the IS

The momentum of studies on the IS is fuelled by the development of innovative technology to meet the challenge of understanding the extreme sensitivity of T cells, able to detect even a single MHC–peptide complex [34]. Because T cells appear to have the ability of single molecule detection, there was clearly a need to adapt the emerging field of single molecule imaging to the study of T-cell activation. This was recently accomplished by tethering phycoerythin molecules to single MHC–peptide complexes on the surface of APCs [35]. The number of MHC–peptide complexes in the interface with a T cell could then be determined at the moment of T-cell interaction, and the consequences of this level of stimulation could be quantified through analysis of the Ca^{2+} signal. It was found that a single MHC–peptide complex triggers a transient Ca^{2+} signal and that 10 MHC–peptide interactions can trigger formation of a stable synapse. Similar ultrasensitive imaging methods will probably be required for understanding the early steps in signaling cascades, which rapidly drop below detectable levels despite the continued accumulation of downstream signaling intermediates [36]. It is not yet clear if or how the specific supramolecular structure of the IS has a role in defining thresholds at the IS, beyond the importance of clustering proteins at the intercellular contact.

There is still a lot to be learnt, just by watching

It is not the end of excitement about the IS, although the basis of the excitement has changed from the initial hype that these structures might explain T-cell activation decisions unilaterally. The picture is currently more complex in that protein clustering and localization can be observed in situations in which activation is blocked and activation can be observed in the absence of apparent supramolecular organization. However, the excitement remains because reactions at these interfaces are required for immune function and new tools to help understand the most fundamental aspects of this communication continue to move us forward rapidly. Particularly for understanding T-cell activation, we have identified the key receptors and ligands and the challenge now is to understand how they act in concert together to regulate T-cell responses. Similar challenges relate to understanding NK-cell activation, although some key receptor–ligand interactions, such as ligands for natural cytotoxicity receptors, also need to be identified.

As for so many biological processes, a lot can be learnt by simply imaging what goes where and seeing when known interactions happen. A caveat is that a deep understanding of the strengths and limitations of each imaging approach is necessary to attach reasonable interpretations to these images. New imaging methods,

for example to probe lipid phases [37], will also be needed to advance our understanding of the IS. Another major technical challenge is to be able to observe molecular rearrangements facilitating intercellular communication *in vivo*. An inspiring recent observation is that nano-tubular structures can allow intercellular transport of membrane vesicles [38]. About applying cutting edge microscopy to understand worm development in the mid-1970s, Nobel laureate John Sulston commented: 'Now to my amazement, I could watch the cells divide. Those Nomarski images of the worm are the most beautiful things imaginable… In one weekend I unraveled most of the postembryonic development of the ventral cord, just by watching' [39].

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