Review

From Tango to Quadrilla

Current Views of the Immunological Synapse

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Original manuscript submitted: 01/16/07

Manuscript accepted: 02/07/07

Previously published online as a *Cell Adhesion & Migration* E-publication:
<http://www.landesbioscience.com/journals/celladhesion/abstract.php?id=3982>

KEY WORDS

T lymphocyte, immunological synapse, signaling, microcluster, TCR, lipid raft, costimulation

ABBREVIATIONS

APC	antigen presenting cell
CD	cluster designation
cSMAC	central supramolecular activation clusters
CTL	cytotoxic T lymphocyte
CTLA 4	cytotoxic T lymphocyte antigen 4
DC	dendritic cell
dSMAC	distal supramolecular activation cluster
IS	immunological synapse
LBPA	lysobisphosphatidic acid
LFA 1	leukocyte function associated molecule 1
MHC	major histocompatibility complex
MTOC	microtubule organizing centre
pSMAC	peripheral supramolecular activation cluster
TCR	T cell receptor

ACKNOWLEDGEMENTS

We thank Marco Necci for graphics and Adelaida Sarukhan for critical reading of the manuscript. A.V. is supported by grants from Italian Association for Cancer Research (AIRC) and MIUR PRIN.

ABSTRACT

All T cell functions require establishing contacts with other cells. In the last ten years, the immunological synapse, the contact-site between T cells and their partners, has been the object of numerous investigations and recent advances in imaging technologies have provided significant insights into the mechanism of immunological synapse formation and its functional outcomes. Considering all the available data, the immunological synapse can be defined as a dynamic structure, formed between a T cell and one or more antigen-presenting cells, showing lipid and protein segregation, signaling compartmentalization, and bidirectional information exchange through soluble and membrane-bound transmitters. In this review, we present the current views on the immunological synapse and discuss about some interesting unresolved questions.

INTRODUCTION

T lymphocytes are activated when their T-cell receptors (TCRs) recognize and interact with specific antigenic complexes formed by antigen-derived peptides bound to integral membrane proteins encoded by the class I or class II genes of the major histocompatibility complex (MHC). TCR ligands are therefore not soluble but presented on the surface of an antigen-presenting cell (APC). Since a single APC presents on its membrane many different combinations of peptide-MHC (pMHC) molecules, the number of specific antigenic complexes for a T cell can be very low (10–100).^{1–3} Thus, the elaborate process of T-cell activation, resulting in a variety of cellular responses, including proliferation, secretion of cytokines and cytotoxic mediators, can be initiated by the interaction of TCRs with very few ligands. Moreover, T cells must be able to discriminate precisely between an infectious stimulus and a non-infectious one, and tune their response in accordance with the molecular context in which the antigen is presented. Thus, whereas the interaction between an individual TCR and its ligand occurs over a time-frame of a few seconds, the interaction between a single T cell-APC pair can be sustained for several hours.⁴ During the prolonged interaction with APCs, the T lymphocyte can scan the surface of its partner, organize a specialized junction to compartmentalize signaling molecules and integrate signals continually delivered by its TCRs and costimulatory receptors. This long-lasting contact is necessary to ensure the sustained signaling that initiates and maintains gene transcription and promotes T-cell cycle progression.^{5,6}

The fact that T cells are activated through an interaction with other cells has three intriguing consequences. First, several receptors are simultaneously triggered, allowing T cells to interpret the context in which antigen is presented. Second, signaling compartmentalization allows spatiotemporal segregation of signaling messengers, this segregation being necessary to activate specific downstream responses. Last, effector functions are localized toward the specific T-cell target.

During physiological T-cell stimulation, the organization of an “immunological synapse”—a complex specialized junction between the T cell and the APC—is pivotal to sustain and amplify signaling, and costimulatory molecules actively participate in this process by reorganizing actin cytoskeleton and recruiting lipid rafts into the T-APC junction.

THE IMMUNOLOGICAL SYNAPSE: STRUCTURE AND FUNCTIONS

The immunological synapse (IS) is a high-order, dynamic structure formed at the T-cell plasma membrane, as a consequence of the interaction between TCRs and pMHC molecules displayed at the APC surface.⁷

TCR triggering leads to a general relocation of T-cell molecules and organelles—a process termed polarization⁸—which involves complex asymmetric redistribution of membrane receptors and signaling molecules, reorganization of the actin cytoskeleton, and repositioning of the Golgi apparatus and the microtubule-organizing centre (MTOC). In particular, at the contact-region between T cell and its partner, major rearrangements are known to occur, leading to the organization of ‘supramolecular activation clusters’.⁹ Several *in vitro* imaging studies have shown that the formation of the mature IS requires the coordinated organization of signaling and cytoskeletal components into two distinct compartments within the T cell-APC contact site^{9,10}: an inner ring termed central supramolecular activation cluster (cSMAC), highly enriched in TCR and pMHC complexes and signaling molecules (CD2, CD28, PKC- θ , Lck, Fyn, CD4 and CD8), which is formed within minutes of T cell-APC contact, and an outer ring termed peripheral supramolecular activation cluster (pSMAC), which contains the adhesion molecule leukocyte function-associated molecule-1 (LFA-1). In addition, there is a more peripheral region than pSMAC, which is called distal (d)SMAC, enriched in CD43 and CD45 molecules.^{11,12} The molecular segregation process begins as inverse of the pattern of the mature synapse, with a central cluster of adhesion molecules surrounded by a peripheral ring of engaged TCR¹³ and is thought to be dependent on actin-cytoskeleton rearrangements¹⁴: T-cell treatment with cytochalasin D leads to free diffusion of TCRs within the cell surface and lack of molecular segregation at the contact region.¹⁵

The expression “immunological synapse” was firstly associated to the specialized junction between a T lymphocyte and an APC, but it is now widely used to describe any effector-target cell contact site; its most important feature is the molecular organization of transmembrane proteins into activating clusters which results in integration of key signals delivered through several receptor systems.¹⁶

Whether the formation of a mature IS, with a well-defined cSMAC surrounded by a pSMAC, represents an absolute physiological requirement in T cell activation is still a controversial issue. Although cSMAC and pSMAC formation has been demonstrated in several experimental systems, such as CD4⁺ T cells stimulated by antigen-loaded B cells¹⁷ and in lipid planar bilayers containing pMHC complexes as artificial models of APC,¹⁸ there are indeed productive interactions that do not result in cSMAC formation. Thus, multi-focal synapses, characterized by a pSMAC and multiple cSMACs, have been described during interactions between thymocytes and thymic epithelial cells^{19–20} or CD8⁺ lymphocytes and their targets.^{21–22} When dendritic cells (DCs) were used as APCs, both “classical” and multi-focal synapses have been observed,^{23–24} even though T cell-DC interactions are in general less stable than the T-B cell ones, and more dependent on APC cytoskeleton.²⁵ Considering the great diversity of possible structures, current terminology refers to an IS as the contact region between T cells and their partners during prolonged interactions.

cSMAC formation observed in productively activated T cells has been initially reported as involved in early T-cell signaling events by driving receptor aggregation.¹³ However, it has been proved later that TCR signaling can be initiated before the organization of detectable activation clusters²⁶ suggesting that, rather than being a prerequisite for TCR signaling, IS represents a complex structure consequent to TCR stimulation. The idea that the cSMAC is not involved in the primary signaling for T-cell activation is supported by the correlation between the inability to form a mature IS observed in CD2-associated

protein (CD2AP) deficient mice and T-cell hyper-responsiveness.²⁷ Binding of CD2AP to the cytoplasmic domain of CD2 is induced by T-cell activation and is required for receptor clustering at the T cell-APC contact region.²⁸ In the absence of CD2AP, T cells show impaired TCR recycling and/or degradation,²⁹ suggesting a role for cSMAC in TCR downregulation and in signal exhaustion. This theory is supported by the recent observation that lysobisphosphatidic acid (LBPA), a lipid generated in multi-vesicular bodies in late lysosomal pathway, is found in proximity of most cSMACs but not in the peripheral T cell-APC contact region.³⁰

Spatiotemporal segregation, integration and balancing of signaling are likely the main IS functions. A single signaling pathway can often modulate a wide variety of cellular events, indicating that spatiotemporal segregation of messengers plays a key role in activating specific downstream responses. The polarized and compartmentalized nature of the T-cell IS may thus facilitate local signaling, leading to specific cellular responses at specific location and times. Establishing checkpoints for signaling is also a very important aspect in T-cell activation. The activating and inhibitory molecular interactions have half-lives in the order of seconds,^{31,32} while the duration of signaling that is required to achieve T-cell priming ranges from few to several hours. In addition, physiological T-cell activation is generally achieved by very few antigenic complexes on the APC, suggesting that signaling amplification is an important requirement for T-cell priming. Recent studies demonstrated recruitment of low-affinity self-pMHC complexes into the IS of the APC, where they may contribute to T cell activation by providing low levels of additive signaling.^{33,34}

Another possible role for the IS is favoring polarized secretion of cytokines or cytotoxic granules toward the T-cell partner/target. In a polarized, IS-forming T cell trafficking of vesicles is directed to the T cell-APC contact region and this may be important to facilitate T-cell functions. However, recent evidence indicated that polarized secretion is not a general rule. In CD4⁺ T cells, while IL-2 and IFN γ are indeed secreted in a polarized fashion toward the APC, other cytokines, such as IL-4 and TNF, or chemokines are secreted in a multidirectional manner.³⁵ In addition, the release of lytic granules by cytotoxic T lymphocytes (CTLs) does not require formation of a mature IS,^{36–38} can simultaneously occur at distinct sites of the T cell and, at least *in vitro*, can be uncoupled, in terms of time and space, with the stable, stimulatory synapse.³⁹

CD28 COSTIMULATION: BUILDING AN IS

Several receptors are stimulated by their ligands during the sustained T cell-APC interaction. Although the classical “two-signals” model of T-cell activation—proposing that T-cell priming requires “signal one” delivered by the TCR and “signal two” by CD28, in whose absence TCR stimulation result in T-cell anergy—can be considered obsolete, it is however clear that TCR-signaling amplification by CD28 plays a key role under physiological conditions of T-cell activation.⁴⁰

Ligand-dependent CD28 recruitment into the cSMAC has been described,⁴¹ although CD28 signaling starts prior to cSMAC formation.⁴²

CD28 is required for induction of gene transcription and is responsible of lowering T-cell threshold of activation, allowing T-cell priming by few antigenic complexes,⁴³ suggesting that the molecule acts as an enhancer of early TCR signaling.⁴⁴ By reorganizing lipid rafts and regulating the actin-cytoskeleton rearrangements,^{45–47} CD28 recruits signaling molecules such as Lck and PKC- θ into the

IS, thus generating an environment where signal transduction is organized and amplified.⁴⁸⁻⁵⁰ In support of this role of CD28, it has been recently shown that CD28 in-trans costimulation fails to induce IL-2 transcription.⁵¹

The C-terminal proline motif of CD28 seems to be required for the costimulatory function of this molecule^{52,53} and is responsible for CD28-mediated lipid rafts recruitment into the IS.⁴⁹ The same domain mediates association of CD28 with filamin-A (FLNa), an actin-binding protein with signaling-scaffold properties that accumulates at the IS in response to CD28 coligation.⁴⁷ Interestingly, FLNa knockdown in human T lymphocytes results in loss of both lipid-raft recruitment into the IS and CD28 costimulation, whereas anti-CD3 induced response, as measured by IFN γ production, is not affected.⁴⁷

While selective accumulation of lipid microdomains at the IS has been clearly established, the role of rafts in T-cell activation is still under investigation. A prevailing model suggests that raft redistribution influences the segregation of signaling proteins required in T-cell activation, thus favoring spatiotemporal compartmentalization of signaling.^{48,54,55} Recently, this hypothesis was confirmed by a study on the transmembrane tyrosine-phosphatase CD45, showing that CD45 partitioning into rafts dictates whether the phosphatase functions as a negative or positive regulator of T-cell activation.⁵⁶

During lymphocyte activation, lipid rafts may function as platforms for the formation of multi-component transduction complexes. Indeed, these microdomains are constitutively enriched in proteins involved in the early phases of TCR signaling, such as the Src-family kinases Lck and Fyn, the adapter protein LAT, phosphoprotein associated with glycosphingolipid-enriched domains (PAG) or Csk-activating protein (Cbp) and Lck-interacting molecule (LIME).⁵⁷ In addition, the composition of raft-associated proteins changes after T-cell stimulation, suggesting that rafts are dynamic platforms for T-cell signaling. Finally, lipids raft may be involved in regulating membrane elasticity that, in turn, controls the fitting of the T cell-APC at the IS.⁵⁷

THE NEW CONCEPTS

Microclusters: Signaling units at the IS. As already mentioned, the organization of a mature IS, with defined c- and pSMACs, is not required for TCR signaling. Indeed, recent evidence indicate that microclusters of signaling molecules, at the periphery of the IS, are responsible for initiating and maintaining T-cell activation.^{58,59} TCR microclusters contain key signaling molecules, such as Lck, Zap70, LAT and SLP-76 and are responsible of tyrosine phosphorylation and calcium fluxing in an actin polymerization-dependent manner. The generation of these early signaling events occurs within seconds after T-cell contact with a planar bilayer used as artificial model of APC^{60,61} and with B cells,⁵⁹ suggesting a non-uniform distribution of TCR and MHC molecules on the cell surface.^{62,63} In agreement, clustered pMHC molecules inserted in planar lipid bilayers exert enhanced T-cell activation as compared with uniformly-distributed MHC ligands.⁶⁴

A single-molecule-imaging analysis revealed that microclusters are relatively static, although their components are in a highly-dynamic state, moving in and out of the cluster.⁶⁵ TCR microclusters move independently toward the center of the IS and fuse to form large clusters: the interconnected large clusters become then immobilized at the center of the IS, and form the TCR-cSMAC.⁶¹ Migration towards the centre to form cSMAC correlates with loss of their

association with adaptors molecules and loss of phosphotyrosine activity,⁵⁹ while retention of microclusters in the periphery is associated with sustained calcium influx and phosphotyrosine activity.^{58,61} TCR microclusters continue to form after cSMAC organization and are sites of TCR signaling, based on the recruitment of key signaling molecules.⁶² This model suggests that early-signaling events provide a highly-dynamic microenvironment enabling large-scale molecular reorganization processes observed in the formation of a mature synapse. The long-lasting signaling required for T cell activation seems thus to be provided by newly formed microclusters at the cell-cell contact site.

Whereas cSMAC size is directly correlated with antigen density, the size of microclusters appears to be independent of the antigen concentration.⁶¹ The mechanisms of TCR-microcluster formation are still unknown, although the actin cytoskeleton seems to be involved. Latrunculin A inhibits movement of preexisting microclusters and formation of new ones, without disturbing already-formed microclusters and cSMAC. This correlates with a reduction in the ongoing calcium elevation.⁶¹

In vivo imaging: From stable to dynamic IS. The fact that TCR-signaling microclusters are organized within seconds after T-cell interaction with APCs suggests to review the previous definition of IS, in favor of a new dynamic model. Recent sophisticated imaging techniques, permitting direct observation of T cell-DC interaction in lymph nodes,⁶⁶⁻⁶⁹ provided new insights in understanding the complex dynamics of in vivo T-cell activation. Two-photon microscopy analysis revealed that in vivo T cell-APC pairing occurs only after a long series of short encounters of rapidly moving T cells with numerous APCs. In lymph nodes, both CD4⁺ and CD8⁺ T cells may have transient, dynamic interactions with DCs^{69,70} before they choose the partner and form stable conjugates. However, it's only during this prolonged interaction that T cells are induced to proliferate and produce cytokines, indicating that productive T-cell activation corresponds to the formation of a stable IS.⁶⁹ After this period, T cell-DC return to short-lived contacts. Thus, in contrast to what observed in vitro, T cell-APC interactions are highly dynamic during a first phase, corresponding to T-cell entrance into lymph node, and this may be strategic to find the right partner. Moreover, these data indicate that, even when in contact with antigen-specific DCs, T cells can be distracted by signals present in the environment, which prevent formation of stable pairs. This prolonged period of transient interactions is not observed when antigen is presented by many resident DCs,⁷¹ suggesting that antigen concentration regulates T cell-DC dynamics in vivo. As illustrated in Figure 1, when antigen is not limiting or when the TCR/pMHC interactions are characterized by high avidity, encounters between T cells and APCs likely result in quick formation of stable conjugates, followed by organization of classical IS. In contrast, when pMHC complexes are few and do not engage the TCR with high avidity, T cells may require several short-lived interactions with APCs before forming stable pairs.

The concept that IS formation is a highly-dynamic process is confirmed by the observation that a single T cell can form ISs with different APCs at the same time, and finally polarize their secretory machinery toward the APC that is providing the strongest stimulus.⁷² This dynamic aspect may be extremely important in the case of CTLs, which can serially kill numerous target cells⁷³ and may need rapid pairing with and detach from the target cells.^{74,75}

Chemokines and their receptors: Soluble transmitters at the IS. The in vivo data, showing T cells unable to form stable conjugates with APCs after entry in lymph nodes and hesitating between

several potential partners, may be explained by the presence in the environment of chemoattractive signals competing with TCR stop signals.^{61,76} However, chemokines may have a different role during IS formation. Although the contribution of costimulatory molecules to IS formation has long been recognized, only recently chemokine receptors have been described as new T-cell costimulatory molecules.⁷⁶ During T-cell activation, recruitment of CCR5 and CXCR4 chemokine receptors into the IS, by a mechanism requiring chemokine secretion by APCs, results in prolonged T cell-APC interaction, and facilitates T-cell activation by reinforcing T cell-APC pair attraction and delivering costimulatory signals.⁷⁷ TCR activation leads to a major change in the signaling of chemokine receptors that, in this condition, couple preferentially to G_q/G_{11} instead of G_i ,⁷⁷ thus inducing cell adhesion rather than chemotaxis,^{78,79} either by enhancing LFA-1 affinity^{80,81} or by overriding G_i -mediated chemotactic signaling. In addition, G_q -mediated signaling also triggers the translocation of nuclear factor of activated T cells (NFAT) to the nucleus,⁸² providing another additional mechanism by which chemokine-receptor engagement at the IS may enhance T-cell activation.

A dual role for chemokines in T-cell activation has been therefore proposed⁷⁶: while the presence of chemoattractant forces when T cells are searching for the right partner may indeed prevent T cell-APC pairing, production of chemokines by the APCs, and subsequent accumulation and trapping of G_q -coupled chemokine receptors at the IS, may represent a strategy to reinforce T cell-APC interaction and facilitate T-cell activation.

At the IS, chemokines may act as soluble transmitters allowing information exchange during contact between several types of immune cells, thus representing the functional equivalent of neurotransmitters at the neurological synapse.⁸³

FUTURE RESEARCH ON THE IS

Understanding the dynamic synapse. Although the emerging imaging techniques are providing new insights into the dynamics involved in IS formation, further studies are necessary to elucidate several unresolved questions concerning events occurring at this level *in vivo*.

In lymph nodes, before conjugates turn stable, most T lymphocytes have dynamic interactions with DCs leading to upregulation of activation markers in a significant proportion of T cells, suggesting that productive TCR signaling is occurring. These results are compatible with a multiphasic, balanced model of activation, in which T cells integrate signals from dynamic short contacts with DCs in order to achieve polarization toward the “best” APC (Fig. 1). *In vitro* experiments, aimed at periodically blocking signal transduction in T cells interacting with APCs, have supported the idea that T cells can be activated by intermittent signals.^{84,85} However, the molecular changes that a T cell requires to choose one single APC, stop and organize a mature IS are still obscure.

In the absence of a stable interaction, transient TCR-signaling microclusters are probably organized at the membrane of T cell

Figure 1. A dynamic immunological synapse. *In vitro* and *in vivo* experiments suggest that, depending on antigen concentration and/or TCR avidity, the encounter between T cells and APCs may rapidly result in the formation of a stable IS, described by a central signaling-silent TCR cluster and peripheral TCR signaling microclusters (A), or may be characterized by short-lived interactions finally culminating in formation of a stable conjugate (B). We speculate that TCR-pMHC interactions occurring during short-lived encounters induce receptor clustering and allow T cell activation by APCs carrying few specific pMHC complexes.

during its short-lived encounters with several APCs. Whether these transient microclusters are different, in terms of signaling, compared with those organized during a stable T cell-APC interaction is an interesting question to be addressed. In addition, it is tempting to speculate about the possible relation between the TCR-signaling microclusters organized upon antigen recognition and the multivalent architecture of the TCR/CD3 complex recently described.⁸⁶ In particular, it would be interesting to evaluate whether TCR-signaling microclusters are preferentially assembled from mono- or multivalent TCR/CD3 complexes and what is the role of lipids and cytoskeletal proteins in stabilizing TCR nano- and microclusters.

The immunological synapse dissolution. Once the IS between a T cell and an APC is formed, the interaction may be stable in the absence of disturbing influences but can be destroyed by cell division, death of the APC or external influences, such as chemokines. Recent progress has greatly increased the understanding of the T-cell orchestrated signaling networks that regulate the process of synapse formation. Yet, a major question concerning the mechanisms regulating IS disassembly remains unresolved. The mechanism proposed, but not completely elucidated, involves downmodulation of TCRs and adhesion molecules,^{87,88} upregulation and recruitment into the IS of receptors delivering inhibitory signals,⁸⁹ and redistribution of molecules such as CD43, a cell-surface mucin that has been proposed to function as a negative regulator of T cell signaling, into the contact zone.⁹⁰

One of the best-studied negative regulators of T cell activation is the cytotoxic T lymphocyte antigen-4 (CTLA-4).⁹¹ Mice lacking CTLA-4 develop a severe autoimmune phenotype with organ destruction.⁹² How CTLA-4 suppresses excessive immune responses

and autoimmunity is still unclear. Proposed mechanisms include ligand competition with the costimulatory molecule CD28, activation of inhibitory signaling pathways⁹³ and effects on membrane lipid rafts.⁹⁴ Recently, it has been shown that, in the presence of antigen, T cells expressing CTLA-4 continue their random migration at unchanged velocities, whereas CTLA-4-null T cells slow down and form long-lasting conjugates with APCs.⁹⁵

CONCLUSIONS

The expression “immunological synapse” was used to underline the similarities between the junction formed by T cells with their partners and the specialized contact-region between neurons. Although the IS, in contrast to the neurological synapse, is not stable and is characterized by a bi-directional transmission of information between T cells and APCs, it is indeed a structurally and functionally compartmentalized area of the plasma membrane, in which communication between cells occurs through soluble and membrane-bound transmitters. During the last ten years, several laboratories have focused their studies on the IS, using imaging techniques often accompanied with classical biochemical or cell biology approaches. Importantly, in most of the cases, the IS study has not been a mere description of cell-cell interaction or molecular segregation, but it greatly contributed to our understanding of the T cell activation process. We believe that important insights may still stem from this area of study and further work is indeed needed to understand how analogic signals collected at the IS are converted in a digital-like signal determining the choice between T cell tolerance or activation.

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