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The c-SMAC: sorting it all out (or in)

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T cells integrate and transduce the key signals necessary to mount an appropriate immune response. To do this, they rely on both secreted factors as well as physical cell–cell contact. Much attention has focused on the organization of proteins at the contact area between a T cell and an antigen-presenting cell, known as the immunological synapse. It has been shown in vitro that proteins segregate into two distinct regions within this contact area, a central area referred to as the c-SMAC, where the T cell receptor and associated signaling molecules are enriched, and a peripheral region called the p-SMAC containing LFA-1 and the scaffolding protein talin. Whether or not these structures form in vivo and how they function in T cell activation remain issues of great interest. Here, we review recently published work and propose several possible functions for the role of the c-SMAC in T cell activation.

During the course of an immune response, many different cell types work together to respond to microbial infections and maintain resistance against pathogens. T cells play a key role in controlling the adaptive immune response via the production of cytokines and in some cases may act directly as cytotoxic T lymphocytes to kill infected host cells. T cell activation in response to foreign antigens is the pivotal event in developing immunity; hence, this process must be tightly regulated to prevent inappropriate and potentially harmful responses to self-antigens. How the molecular interactions at the immunological synapse ensure the fidelity of T cell signaling is an important issue.

T cell activation is initiated by the engagement of the T cell receptor (TCR) with peptide-bound MHC (pMHC) on the surface of an antigen-presenting cell (APC). During cell–cell contact, the TCR and other coreceptors cause the T cell to polarize, leading to remodeling of the actin cytoskeleton and repositioning of the Golgi apparatus and microtubule-organizing center (MTOC) between the nucleus and the contact area (Kupfer et al., 1983). Although it has been known for years that physical cell–cell contact between T cell and APC is required for T cell activation, only recently have studies examined the molecular organization of the contact area (Monks et al., 1998; Dustin and Shaw, 1999; Grakoui et al., 1999). These imaging studies have shown that proteins within the contact area are organized into distinct compartments, suggesting that the specific organization of proteins in the contact area may be critical in modulating T cell activation.

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Abbreviations used in this paper: APC, antigen-presenting cell; c-SMAC, central-supramolecular activation complex; DC, dendritic cell; LFA-1, leukocyte function-associated molecule-1; MTOC, microtubule-organizing center; p-SMAC, peripheral-SMAC; TCR, T cell receptor.

Defining the contact site

Using deconvolution microscopy, Monks et al. (1998) first described the spatial segregation of proteins at the contact site between a CD4$^+$ T cell and an antigen-loaded B cell. They reported that TCR clustered at the center of the contact, an area termed the central-supramolecular activation complex (c-SMAC), whereas the adhesion molecule leukocyte function-associated molecule-1 (LFA-1) localized in a ring-shaped structure surrounding the c-SMAC, referred to as the peripheral-SMAC (p-SMAC). Others have confirmed the formation of a c-SMAC and p-SMAC in a variety of different cell types, including primary cells (for review see Bromley et al., 2001). These studies have also helped identify additional constituents of the c-SMAC, including CD2, CD28, PKC-θ, Lck, Fyn, CD4, and CD8 (for review see Huppa and Davis, 2003). To date, only talin and LFA-1 are known to reside in the p-SMAC. Other molecules such as CD43 are excluded from the contact area entirely (Allenspach et al., 2001; Delon et al., 2001).

Mechanisms of protein segregation in the immunological synapse

The precise mechanism by which c-SMACs and p-SMACs are formed is unknown, but it is believed to be dependent on signals generated by the TCR. However, this may not be strictly true as TCR-independent c-SMAC formation mediated by NKG2D has been reported for certain types of T cells (CD8$^+$ and γδ T cells; Favier et al., 2003; Somersalo et al., 2004). Surprisingly, little is known regarding the required signals that trigger c-SMAC formation in these systems.

There is compelling evidence that the actin cytoskeleton plays an important role in c-SMAC formation (for review see Dustin and Cooper, 2000). The strongest evidence is that treatment with cytochalasin D, an actin polymerization inhibitor, can block c-SMAC formation. Moreover, substantial actin polymerization occurs at the APC contact site, implicating many of the proteins that regulate actin polymerization in T cells, such as the Rho family of GTPases (Rac, Rho, and

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Cdc42), their GEFs and GAPs, and their downstream targets in the establishment of a c-SMAC (for review see Miletic et al., 2003). Furthermore, recent work suggests that intracellular protein trafficking may also play a role in c-SMAC formation, with newly synthesized and recycled proteins transported directly to the c-SMAC by intracellular vesicles (Das et al., 2004). Because vesicular trafficking events are generally dependent on the actin cytoskeleton, these data further highlight the importance of cytoskeletal rearrangement during c-SMAC formation.

Because the formation of the immunological synapse occurs concomitantly with MTOC polarization, microtubules and microtubule motors may also be important. Dominant negative forms of Cdc42 block MTOC polarization in T cells, implying that actin and microtubule-based cytoskeletal rearrangements are interconnected (Stowers et al., 1995). Other studies, examining T cell spreading in response to TCR signaling demonstrated that early T cell spreading is actin dependent; however, microtubules stabilize the spread T cell at later time points (Bunnell et al., 2001). It is interesting to speculate that proteins involved in the regulation of apical/basolateral polarity may also be involved in MTOC polarization in T cells. One family of proteins known as the partitioning defective (Par) family, has been shown to regulate asymmetric protein localization and MTOC positioning in several different systems (for review see Macara, 2004). Interestingly, Par6 contains a CRIB domain that interacts with active Cdc42 and Rac1 (Lin et al., 2000), and Par1 has been demonstrated to phosphorylate microtubule-associated proteins (Drewes et al., 1998). Further examination of the proteins that regulate microtubule assembly and disassembly may shed some light on whether MTOC polarization plays a role in c-SMAC formation.

Immunological synapse versus c-SMAC formation

Although it was originally coined to describe the contact area between a helper T cell and B cell, the term immunological synapse is today generically used to describe the contact surface between any lymphoid effector cell (T, B, or NK cell) and APC or target cell. Thus, the term does not necessarily imply any segregation of proteins in the contact area into c-SMAC and p-SMAC regions but simply the contact site (Fig. 1).

Although the formation of immunological synapses is self-evident, whether c-SMACs and p-SMACs form at every immunological synapse is not clear. C-SMAC formation is well documented using lipid bilayer systems (Grakoui et al., 1999; Somersalo et al., 2004; Krogsgaard et al., 2005) and experiments with in vitro maintained T cell lines. The degree of protein enrichment is reported to range from less than twofold for the TCR to approximately four- to fivefold for LFA-1 (Monks et al., 1998). Even under the most favorable conditions, however, many contacts do not exhibit obvious c-SMAC and p-SMAC regions. This raises the possibility that c-SMAC and p-SMAC formation is not a physiological requirement for T cell signaling; however, given the low levels of protein enrichment, the failure to observe c-SMACs in these experiments may be due to limits in imaging sensitivity. Moreover, the detection of organized synapses in cell–cell conjugates is highly dependent on the orientation of the conjugates and complicated by the fact that not all antibodies are able to penetrate the contact area between a T cell and APC. The use of GFP fusion proteins may circumvent some of the problems associated with antibody staining, but the relatively high expression levels required to detect these GFP fusion proteins with conventional imaging systems raises another set of issues. Because of the technical limitations associated with imaging studies, there is no definitive answer yet regarding the absolute requirement of c-SMAC and p-SMAC formation for T cell signaling.

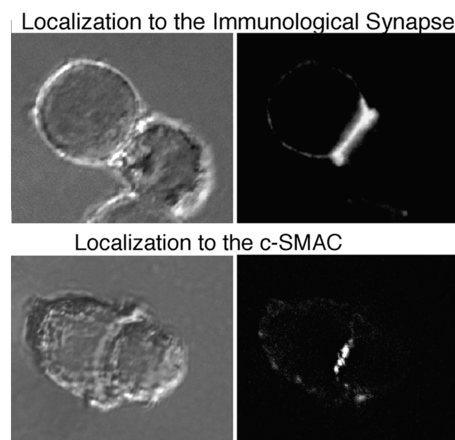

Figure 1. **Immunological synapse versus c-SMAC formation.** T cell–B cell conjugates depicting immunological synapse localization (top) and c-SMAC localization (bottom).

Assuming c-SMACs do form in vivo, what is the function of the c-SMAC? This has been a controversial area with many different hypotheses proposed. In the following sections, we discuss recent findings related to the function of the c-SMAC. In the last section, we review evidence for whether c-SMACs form in vivo.

Signaling and the c-SMAC

One of the most remarkable properties of the TCR is its astonishing sensitivity and specificity for a specific pMHC complex. Because a single APC displays a vast array of different peptides, a T cell must sift through countless numbers of nonantigenic peptides to find enough antigenic peptides to become activated. Most data suggest that the minimum number of specific antigenic peptides required to activate a T cell ranges from about ten to a few hundred (Demotz et al., 1990; Harding and Unanue, 1990; Irvine et al., 2002).

Early models of c-SMAC function proposed that the c-SMAC might help to initiate T cell activation by driving receptor aggregation (Grakoui et al., 1999). However, it is now known that c-SMAC formation is not required to initiate signaling by the TCR. TCR signaling can be initiated by small numbers of antigenic pMHC complexes immediately after contact, before reorganization of the contact area can occur (Sykulev et al., 1996; Irvine et al., 2002; Lee et al., 2002; Wulfing et al., 2002). Also, many proximal readouts of TCR stimulation, such as Ca^{2+} mobilization and the peak of tyrosine phosphorylation, occur well before c-SMACs can form (Lee et al., 2002; Zaru et al., 2002).

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Because c-SMACs can be long-lived structures, it was then suggested that c-SMACs enhance T cell activation by concentrating the TCR and pMHC in one area of the plasma membrane and holding them there for long periods of time (Dustin and Shaw, 1999). This model reasoned that the increased local concentration of both TCR and its ligand would facilitate engagement of the relatively low affinity TCR and potentially slow its disengagement due to molecular crowding. Computer modeling experiments support the idea that c-SMAC formation can enhance TCR signaling by facilitating rebinding events due to the high concentration of TCR and ligand (serial triggering; Lee et al., 2003).

The c-SMAC and its role in TCR down-regulation

The c-SMAC may also function to facilitate TCR down-regulation. Receptor down-regulation as a means of tuning down responses has been demonstrated in other receptor-induced signaling cascades (Stoscheck and Carpenter, 1984). In resting T cells, the TCR constitutively recycles from the surface to an intracellular compartment and back (Liu et al., 2000). After TCR engagement, internalized tyrosine-phosphorylated receptors are ubiquitinated and then targeted to lysosomes (Alcover and Alarcon, 2000; Liu et al., 2000). T cells with a defect in c-SMAC formation, such as T cells from mice deficient in CD2AP, have defects in TCR down-regulation (Lee et al., 2003). The inability to down-regulate activated receptors results in sustained signaling, suggesting that the c-SMAC enhances TCR down-regulation and signal attenuation. A computer simulation of c-SMAC formation suggests that phosphorylation is more efficient in the c-SMAC because of the higher TCR and pMHC concentrations (Lee et al., 2003). Although this should augment TCR signaling, greater levels of TCR phosphorylation result in enhanced down-regulation of the TCR. The model suggests that the higher levels of phosphorylation mediated by c-SMAC formation facilitate the recruitment of ubiquitin ligases leading to increased TCR degradation (Naramura et al., 2002).

The c-SMAC and the maintenance of signaling

T cell proliferation and cytokine production require contact between a naive T cell and an APC for many hours before the T cell is committed to an activation program (Iezzi et al., 1998; Lee et al., 2002). The exact amount of time required, however, is still a matter of some debate and may vary between $CD4^+$ and $CD8^+$ cells (van Stipdonk et al., 2003). Most estimates for the duration of contact range from 4 to 10 h to achieve at least one round of cell division (Lee et al., 2002). Longer contact times may allow for secondary rounds of division and maximal cytokine production (Gett et al., 2003).

Although tyrosine phosphorylation peaks within a few minutes after TCR engagement, signaling by the TCR appears to persist for many hours after stimulation. Using a fluorescent probe for PIP_3, several groups demonstrated that PIP_3 is continuously generated throughout the entire time the T cell remains in contact with an APC (Costello et al., 2002; Harriague and Bismuth, 2002; Huppa et al., 2003). Signaling appeared to depend on continual TCR engagement because the addition of antibodies to disrupt TCR-pMHC engagement interrupted PIP_3 production (Huppa et al., 2003). This supported previous data showing that premature termination of signaling affected IL-2 production and proliferation proportionally to the duration of stimulation (Weiss et al., 1987; Iezzi et al., 1998; Lee et al., 2002).

Whether TCR signaling by antigenic pMHC complexes can be maintained in the c-SMAC for long periods of time is not clear given evidence suggesting that T cells can internalize pMHCs from the APC (Huang et al., 1999; Hwang et al., 2000). But it is now clear that a fraction of endogenous self-pMHC complexes are recruited to the c-SMAC (Wulfing et al., 2002; Li et al., 2004; Krogsgaard et al., 2005). It is interesting to speculate that endogenous self-peptides are responsible for sustained TCR signaling events. In this model, antigenic peptides initiate c-SMAC formation, but the c-SMAC may allow the usually nonstimulatory self-peptides to induce a signal sufficient to sustain T cell activation. This could explain why endogenous self-peptides alone cannot initiate T cell activation but still play a role in augmenting T cell activation in the presence of low antigenic pMHC numbers (Wulfing et al., 2002; Krogsgaard et al., 2005). Thus, in conditions where the presence of antigenic peptides is limiting, c-SMAC formation may be critical to sustain signaling for many hours.

The c-SMAC, polarity, secretion, and T cell cytolysis

T cell engagement with an APC initiates a complex intracellular reorganization of the T cell toward the APC (Kupfer and Dennert, 1984; Kupfer et al., 1986). A hallmark of this "polarization" is the movement of the Golgi and the MTOC to a position just underneath the contact surface. Although it was initially thought that the c-SMAC was formed exclusively by the reorganization of transmembrane proteins on the surface of the T cell, the trafficking of molecules from intracellular compartments to the cell surface and back may also play an important role (Das et al., 2004). Because most proteins are sorted through the Golgi, T cell polarization could participate in c-SMAC formation by regulating the inward and outward trafficking of membrane-targeted proteins.

Polarized trafficking may also allow for the directional secretion of cytokines or cytolytic granules to the c-SMAC (Kupfer et al., 1983; Poo et al., 1988). In addition, work with cytolytic T cells suggests that the p-SMAC may function as a kind of "gasket" to prevent the leakage of cytolytic granules into the extracellular space (Stinchcombe et al., 2001). This would enhance the efficacy of killing by cytolytic enzymes and prevent their actions on unintended targets. However, a recent study demonstrated that T cell killing does not require the formation of a stable c-SMAC. It should be noted that the efficiency of killing, the potential for bystander killing, or the minimal versus the optimal number of peptides required to kill were not addressed in this study (Purbhoo et al., 2004). These issues may be important during killing in vivo where target cells are in very close proximity to other cells.

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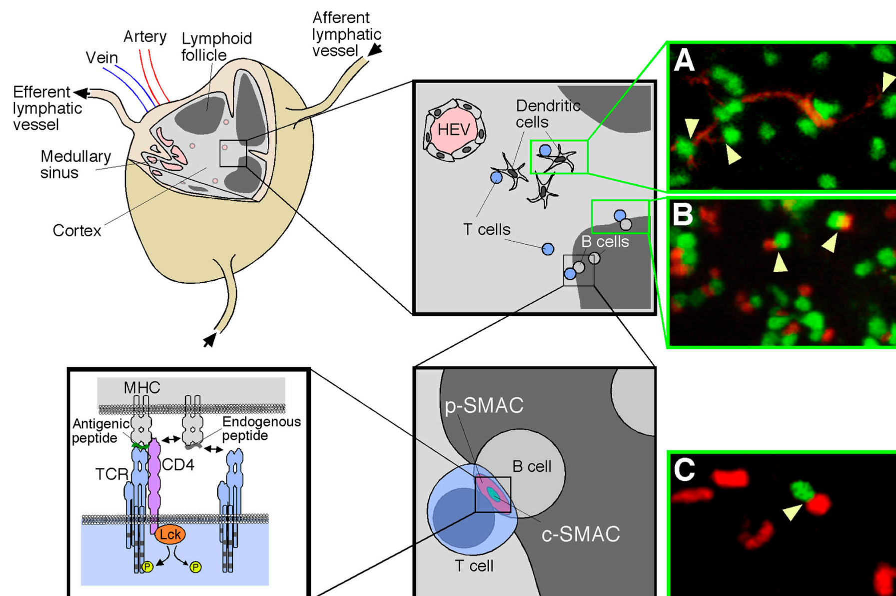

Figure 2. **During the course of an immune response, many different cell types work together to initiate an immune response.** Antigen-loaded DCs from the periphery first migrate to the cortex of the lymph node via the afferent lymphatic vessels. There, they display peptides to T cells, which have entered the lymph node via high endothelial venules (HEVs). T cells scan the DCs looking for reactive antigenic peptides. If a T cell encounters such a peptide, it will form a conjugate with the DC. (A) A two-photon image of an endogenous DC (red) presenting antigen to CD4$^+$ T cells (green) in a mouse lymph node. After the appropriate activation signals have been received from the DC, the T cell proliferates and then migrates to the edge of the follicle where it can interact with B cells. If the B cells are presenting the proper peptide, the T cell will form a stable conjugate. (B) Cognate interactions between T helper cells (green) and antigen-specific B cells (red). This interaction induces the T cell to produce cytokines and provide other signals to the B cell, allowing for the propagation of the adaptive immune response. During a T cell–B cell interaction, c-SMAC and p-SMAC regions form at the site of the synapse. Within the c-SMAC, the TCR then uses signals from both antigenic and reactive endogenous self-peptides loaded onto MHC to keep signaling pathways activated. These cascades are initiated when the TCR becomes phosphorylated by the tyrosine kinase Lck. Effector CD8$^+$ T cells also use a similar mechanism of T cell activation when encountering target cells. (C) A CD8$^+$ cytotoxic T lymphocyte engaging a peptide-pulsed target cell in a mouse spleen. Arrowheads denote T cell–APC contacts.

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Does the c-SMAC form in vivo?

Recent in vivo work raises questions about the significance of c-SMAC formation. Do T cells and APCs form c-SMACs and p-SMACs in vivo? Do c-SMACs form between T cells and all APCs?

Mature dendritic cells (DCs) are the most efficient and potent APC (Mellman and Steinman, 2001). DCs are usually the first to acquire antigen in the periphery. There, inflammatory signals induce the DC to mature and carry the antigen to the lymph node (Fig. 2). In the lymph node, naive CD4$^+$ T cells reactive to that particular antigen interact with the DC and become activated, leading to proliferation. After this initial stimulation, T cells then migrate to B cell–rich zones of the lymph node, where they encounter B cells displaying the same antigen. The function of this interaction is to induce the T cell to produce cytokines that allow the B cell to proliferate, differentiate, and produce high affinity antibodies.

Although c-SMACs form between T cells and antigen-loaded B cells, it is not clear whether T cells form clearly defined c-SMACs with other APCs such as DCs and macrophages. Only a single study has demonstrated c-SMAC formation between activated T cells and DCs (Benvenuti et al., 2004). In contrast, c-SMAC formation has been demonstrated between CD4$^+$ T cells and antigen-loaded B cells, thymic epithelium, and artificial lipid bilayers (Monks et al., 1998; Grakoui et al., 1999; Richie et al., 2002). It has also been shown that CD8$^+$ T cells can form c-SMACs with their target cells (Potter et al., 2001). Thus, it is possible that the formation of c-SMACs differs among different APCs.

In vivo imaging

A crucial question remains: do descriptions of c-SMACs obtained in vitro reflect reality in vivo? Recent technological advances in fluorescence imaging have made it possible to directly observe antigen presentation within intact lymphoid tissues. Confocal microscopy has been used to study T cell–DC contacts in lymph node explants; however, imaging depth is restricted (<80 μm from the capsule) and the high degree of sample photodamage generated by this technique is problematic for live cell imaging. An approach that overcomes these limitations is two-photon microscopy (Fig. 2; for reviews see Cahalan et al., 2002; Huang et al., 2004; Sumen et al., 2004). Because two-photon excitation is achieved with a near IR laser, it is possible to image deep within the T cell regions of a lymph node (100–450 μm below the capsule) where the bulk of T cell–DC interactions are believed to take place. Furthermore, because excitation occurs only at the point of focus, the rest of the sample is exposed to relatively harmless long wavelength light, thus greatly reducing sample photodamage. Several groups have used two-photon imaging to record time-lapse images of living lymphocytes and APCs in explanted lymph node preparations (Bousso and Robey, 2003; Hugues et al., 2004; Miller et al., 2004a,b; Okada et al., 2005) and in surgically exposed lymph nodes in anesthetized mice (Miller et al., 2003; Lindquist et al., 2004; Mempel et al., 2004). The ability to image single cells in native tissues is an important step toward understanding the dynamics and topology of immunological synapse formation in vivo.

Tissue imaging studies reveal that T cell–DC interactions during the immune response are far more dynamic in situ than predicted by in vitro systems. For both CD8$^+$ and CD4$^+$ T cells (Mempel et al., 2004; Miller et al., 2004b), initial interactions with DCs are serial and transient, lasting only minutes. Using an intravital preparation, Mempel et al. (2004) showed that for CD8$^+$ T cells, interactions with DCs were remarkably dynamic for the first ~8 h before progressing to comparatively stable

contacts lasting >1 h. For $CD4^+$ T cells in lymph node explants, the change in behavior is more rapid and many cells formed stable interactions within 2 h of adoptive transfer. In both studies the period of stable interaction lasted for 13–16 h before T cell motility resumed and contacts with DCs returned to short-lived contacts. This suggests that T cell activation involves both stable and serial interactions in vivo.

Although two-photon microscopy has generated a picture of T cell behavior and T cell–DC contact morphology during an immune response, the question of whether c-SMACs and p-SMACs form in vivo is still unanswered. There is some evidence that protein segregation does occur in vivo. Reichert et al. (2001) showed that the TCR concentrates toward one side of a T cell in whole lymph node sections after antigen challenge. Also, using confocal microscopy to image intact lymph nodes, Stoll et al. (2002) demonstrated that CD43 was excluded from the contact site, implying that some molecular reorganization occurs between T cells and DCs in native tissues. Although these data are not definitive evidence that c-SMACs and p-SMACs form in vivo, they suggest that immunological synapses in vivo have some organizational characteristics that are common with synapses seen in vitro.

The major limitation with current in vivo microscopy is the low resolution of the images. For conclusive proof of c-SMAC and p-SMAC formation in vivo, it will be necessary to image the components of the c-SMACs and p-SMACs in single cells during antigen recognition. However, the main technical challenge is to create fluorescent probes bright enough for two-photon microscopy to detect physiological concentrations of these molecules on cells in native tissues.

Conclusions

Recent efforts have permitted single cell analyses of antigen presentation in vivo using sophisticated imaging techniques. Emerging techniques such as two-photon microscopy have given us an appreciation for the complex and dynamic nature of T cell interactions with APCs in lymphoid tissues. Although these approaches have yielded new insights into the process of T cell activation, there still remain many fundamental questions regarding the events that occur at the immunological synapse. It is still not clear whether T cells form c-SMAC and p-SMAC structures in vivo and what role these structures play in T cell activation. With advances in imaging technology and computer modeling, some of these long-standing questions may soon be answered.

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