Imaging immune cell interactions and functions: SMACs and the Immunological Synapse
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1. Introduction
A key role of peripheral lymphocytes is the immune surveillance against foreign pathogens. The initiation and the specificity of immune responses strictly depend on clonotypic antigen (Ag) receptors, such as the TCR and BCR that are expressed on T and B lymphocytes, respectively. The biologic outcome of Ag recognition by T lymphocytes is determined by a large number of monomorphic receptors, such as CD4, CD28, and LFA-1, which, together with the TCR, regulate lymphocyte maturation, differentiation as well as effector functions. The roles of these receptors have been studied extensively. In most cases, their biochemical signaling potential was determined using anti receptor antibodies or purified recombinant soluble ligands. The physiological ligands recognized by the monomorphic receptors and by the TCR are themselves membrane proteins expressed on the surfaces of the various Antigen Presenting Cells (APCs). Consequently, Ag recognition by developing and by mature T cells depends on the ability of the T cells to interact with APCs and to form stable T–APC cell conjugates.

Although a great deal is known about the biochemical basis of TCR signaling, much less is known about the coordinate signaling of the TCR and the other receptors under physiological conditions. Over the last several years, a new approach has been successfully applied to address this issue. It involves the microscopic visualization of the T cells during their interaction with APC. These studies employ a variety of imaging techniques including live cell imaging and multi-dimensional imaging of fixed cell conjugates.

The most exciting fundamental finding of these studies is the fact that receptors are not randomly engaged or clustered at the interface between the T cell and its bound APC. Instead, the engaged receptors form higher order clusters that are highly organized both spatially and temporally. Moreover, intracellular signaling, adaptor, and cytoskeletal proteins appear to be associated with these molecular structures. These highly organized molecular assemblies have been termed Supra-Molecular Activation Clusters (SMACs). The cell contact containing these SMACs has been generically termed the “Immunological Synapse” (IS). Although the SMACs and the IS were initially discovered in cell conjugates between CD4 T cells and B cell lines, they have since been observed in many other cell conjugates involving almost all cell types of the immune system.

The kinetic molecular composition and the functional role of the SMACs and the IS are now under intense investigation. The reviews in this issue present the diversity of the current knowledge of this emerging new field. These reviews describe the structure and diverse functions of the IS in T helper, CTL, NK cells, and B cells. The emerging theme from all of these studies is that the SMACs and the IS serve as a means of transferring information between the interacting cells. Although the precise modes of information transfer, that are presented in these reviews, are different depending on the interacting cell types, the SMACs and IS provide a means to restrict the transfer of signals only to the bound cells thus ensuring the Ag specificity of the responses.

2. The directed secretion of effector molecules and cytokines at the T–APC cell contact (IS)
The hallmark of lymphocyte function is the requirement for strict Ag specificity in the recognition of foreign Ags and the effective specific response to eliminate that Ag. Remarkably, while the recognition phase is mediated by Ag-specific receptors like the TCR and BCR, the T cell responses are mediated by secretion of effector molecules that lack Ag specificity. In the case of CTL, killing of target cells can be achieved by secretion of perforins (for a complete discussion, see the review in this issue by G. M. Griffiths). In the case of T helper cells, the activation, proliferation, and differentiation of B cells depends of cytokines produced by the
T cells (for a complete discussion, see the review in this issue by J. Cambier). Since neither the secreted perforins nor the cytokines are Ag specific, it was unclear how their secretion would result in the selective killing of target cells, or the induction of Ag-specific B cells without similar effects on bystander cells.

To address these questions conjugates of CTL–TC and T–B cells were imaged at the single cell level by immunofluorescence microscopy [1–4]. Microscopic observations of the CTL–TC and NK–TC couples demonstrated that the intracellular secretory machinery, namely the Golgi Apparatus (GA) and the microtuble organizing center (MTOC), of the CTL and NK cells were rapidly reoriented to face the contact site with the lysable target cells. Sequential repositioning of the GA/MTOC towards multiple TCs by a single CTL resulted in the sequential killing of these TCs. Based on these studies it was proposed that the secretion of perforins is directed and limited to the contact site between the CTL and TC, therefore avoiding the killing of bystander cells.

A similar rapid reorientation of the GA/MTOC was seen in Ag-specific T helper cells bound to B cells. When the T–B conjugates were labeled intracellularly, after several hours of interaction, with antibodies specific for IL-2, IL-4, IL-5, and IFN-gamma the newly synthesized cytokines were heavily localized in the T cell at the contact site with the bound B cell [5]. Longer term (24–48 h) microscopic observations of Ag-specific conjugates between T cells and multiple bound small resting B cells demonstrated that only the B cells that were facing the site of cytokines production in the cells underwent proliferation [6] (Fig. 1). Thus, T helper cells, like CTL, direct the secretion of their effector molecules towards the contact site with the APC. Moreover, in T–B conjugates this interaction persists for many hours.

The notion that once cytokines are produced they are secreted directly and locally towards the area of membrane–membrane contact, which is similar to the localized secretion of neuro-transmitters in neuronal synapses, led William Paul to term the T–APC contact the “Immunological Synapse” [7].

3. Ag-dependent rearrangement of receptors and cytoskeleton at the T–APC contact site

The generation of cytokines by activated T cells is dependent on early activation signals that may precede the cytokines production by many hours. These signals require the recognition of a specific Ag by the TCR and the involvement of additional receptors.

These receptors can be studied using either activating or blocking anti-receptor antibodies. Clearly, such high-affinity antibodies can activate or inhibit the TCR and the T cells, but they may over-ride some important regulatory mechanisms that may be in place in real T–APC interactions. If such mechanisms exist they must be studied under more physiological conditions during T cell interactions with their APCs. We proposed that it would be possible to specifically identify and study, at the single cell level, the roles of engaged receptors and their associated cytoplasmic proteins by visualizing their translocations and clustering at the T–APC contact area. Indeed, as predicted, Ag-specific clustering of the TCR in the contact was seen in T–B couples [8].
Localization studies of two monomorphic receptors CD4 and LFA-1, which bind class II MHC and ICAM-1, respectively, on the APC yielded surprising results [8,9]. Both receptors clearly clustered in the cell contacts of Ag-specific conjugates but failed to cluster in Ag-non-specific conjugates, in spite of the fact that their ligands on the APC were the same as in the Ag-specific conjugates. These findings demonstrated that the engagement of the TCR can modulate the properties of multiple monomorphic receptors, even those that are not associated directly with the TCR. Moreover, different mechanisms may be used with each class of receptors.

Inside the T cell, the Ag-specific interaction with APC resulted in the rapid recruitment of the cytoskeletal protein talin to the cell contact and the reorientation of the MTOC [10]. Using imaging of T–APC conjugates and antibody-induced capping in resting and activated T cells it was concluded that talin associates with activated/lipid-enriched LFA-1 [11]. It was proposed that the clustering of talin at the contact serves to stabilize the integrin mediated cellular interaction between the T cell and the APC. The reorientation of the MTOC and its associated microtubules plays a role in directed secretion and membrane insertion as discussed above.

Although both cytoskeleton reorientations depend on the engagement of the TCR, they are not mechanistically tightly coupled [9]. Interestingly these reorientations were correlative with the biologic outcome of the activation process. The engagement of the TCR with suboptimal levels of Ag that failed to cause T cell proliferation or cytokine production did not result in MTOC reorientation but caused the clustering of talin at the cell contact. In contrast, MTOC re-orientation was observed only when the T cells were optimally activated and were able to proliferate. Thus, different TCR mediated signals can trigger different early molecular rearrangements and subsequent later biologic outcomes.

4. Imaging as a tool to identify proteins involved in T cell activation

The finding that molecular events that are imaged within minutes of T–APC conjugation can predict the long-term outcome of the activation can be a very useful and rapid measure of activation. In addition the rapid recruitment of intracellular proteins to the T–APC contact site, under full or partial activation conditions, may serve as a new tool to identify and assign new functions to specific proteins. In particular, it is important to understand how the many signals that are delivered in vivo by multiple receptors are integrated into a decision making process that affects the outcome of the response. The imaging approach provides a means to study complex signal integration rather than signal dissection during the interaction of T cells with live APCs.

Traditionally, understanding the mechanisms of such complex biological systems depended on the ability to molecularly dissect these pathways using reductionist experimental approaches. Such studies were instrumental in identifying multiple intracellular biochemical pathways that can link the surface receptors to downstream responses. As the number of these pathways continues to expand, it is increasingly obvious that they do not function independently of each other. Instead, it appears that these biochemical pathways may be interconnected in multiple branching points. Unfortunately, the experimental methods that were optimized to dissect these activation pathways are inappropriate for the task of complex signal integration.

The nearing completion of the human and mouse genome project is expected to further compound this task. It is expected that computational analysis of the new ORF would identify many more receptors, enzymes, and adapter proteins that may be involved in the signaling cascades. New experimental approaches would be required to study the roles of specific proteins in cell activation and signaling, because of the presence of many other closely related proteins.

T cells express multiple genes of related protein kinases. Among these, the protein kinase C (PKC) family of serine/threonine kinases has long been known to be essential for T cell activation. It was unknown which of the 10 different PKC isoforms that are expressed in T cells are directly responsible for TCR mediated activation. Sequence analysis failed to detect any specific traits that would link a particular isoform to the TCR. To address this issue Ag-specific T–APC conjugates were labeled with antibodies specific for each of the expressed PKC isoforms [12]. Surprisingly, of all the PKCs, only PKC-θ responded to the activation by translocating to the contact area, the site of TCR activation. Interaction of T cells with APCs that were pulsed with suboptimal concentrations of Ag or with antagonist peptides failed to cause the recruitment of PKC-θ to the cell contact. Additional microscopic and functional studies suggested that activation and translocation of PKC-θ was required for TCR-dependent proliferation. This notion was confirmed by analysis of PKC-θ knockout mice [13]. The T cells from these mice express all the other PKCs, yet the mature T cells fail to respond to TCR activation.

The successful identification of PKC-θ by imaging T–APC couples indicates that this approach can be used to identify the unique functional roles of individual members of other closely related gene products.

5. Visualizing the multi-dimensional complexity of T cell activation

Since quantitative difference in the extent of receptor engagement appear to have profound qualitatively different cellular responses it became important to quantify the extents of molecular translocations. To study quantitatively the participation of different receptors and intracellular proteins it is essential to take into account the fact that the cells are three-dimensional structures. We devel-
opened a computer-driven multi-dimensional digital imaging system that enables us to follow receptors and intracellular proteins during T–APC interactions. We studied the three-dimensional distribution of TCR and LFA-1, as well as PKC-θ and talin in T–APC conjugates. All of these proteins are specifically recruited to the cell contacts when the TCR is engaged. Surprisingly, when the three-dimensional data sets were deconvolved and rendered, we found that these proteins clustered into spatially segregated domains. We termed these segregated molecular clusters as SMACs [14]. The TCR and several protein kinases cluster in the central c-SMAC; LFA-1 and the cytoskeletal protein talin cluster in the peripheral p-SMAC (Fig. 2). It was demonstrated that the c-SMAC is the site of TCR engagement with its specific ligand on the APC. Moreover, it seems that the bound ligands on the APC and their receptors on the T cells form mirror image SMACs on both cells. We proposed that during physiological T–APC interactions the cells are productively activated by these spatially and temporally highly ordered SMACs. It should be noted that since the SMACs on the T cells and on the APCs contain different sets of receptors, it is highly likely that they would transmit different signals into the cells.

6. The functional roles of the SMACs

The observation that the tyrosine kinases Lck and Fyn are localized in the c-SMAC along with the engaged TCR suggested that the SMACs are important for T cell activation. This notion was supported by the observation that engagement of the TCR with altered peptides that did not induce productive T cell activation also failed to induce the formation of SMACs [14]. Interestingly, in these conjugates, the altered peptides were still able to trigger the clustering of talin to the cell contact. Thus, it appeared that SMAC formation was not necessary for the induction of some early signals, including the activation of cell adhesion, but was important for complete activation of the T cells. This notion was supported by a large number of other studies [15–17] (for a more detailed discussion, see the review in this issue by Mark Davis).

A recent microscopic study using antibodies directed against phosphotyrosine (p-Y) and tyrosine phosphorylated forms of Lck and Zap-70 questioned the role of SMACs in signaling [18]. The authors of that study proposed instead that T cell signaling precedes IS formation and no significant signaling happened in the SMACs. A unique feature of that study was the use of ex vivo splenic non-T non-B cells as the APCs, but contained also DCs. It was unclear if these APCs express normal levels of MHC and of most costimulatory receptors.

Another study that used a B cell line, which expresses high levels MHC, B7.2 and ICAM-1 yielded different conclusions about the roles of SMACs in signaling [19]. In this study too, signaling was detected before the formation of SMACs. The p-Y labeling and talin were clustered at the cell contact immediately after cell conjugation and before the formation of organized SMACs. By 3 min, SMACs were already formed (Fig. 3). Surprisingly, although Lck, a TCR-dependent tyrosine kinase, was present in the c-SMAC, there was no p-Y labeling there. Yet, several minutes later...
Fig. 3. The receptor–ligand interactions cause the formation of SMACs on both the T cell and the APC. Although the SMACs on both cells present an identical mirror image, their molecular contents are very different.

Fig. 4. A model staged T cell activation before and after SMAC formation.
did p-Y labeling reappear in the c-SMAC. To determine the cause of the temporary absence of p-Y from the c-SMAC, the location of the tyrosine-phosphatase CD45 was studied. Remarkably, CD45 was recruited to the early c-SMAC and rapidly thereafter was clustered in a newly discovered d-SMAC. The recruitment of CD45 to the early c-SMAC is triggered by the TCR and involves a transient interaction between CD3 and CD45[19]. Labeling with antibodies specific for phospho-Zap-70 indicated that Zap-70 was phosphorylated in two distinct stages: the first preceded SMAC formation and the second followed the clearing of CD45 from the c-SMAC. Based on these observation a new model of T cell activation was proposed (Fig. 4). This staged model of T cell activation includes at least two spatial-temporal distinct stages. The first occurs immediately upon initial antigen recognition, before the formation of SMACs. Signaling at this stage activates the β2 integrins, and signals the subsequent formation of SMACs, but is not sufficient to trigger productive T cell activation. The latter requires additional orchestrated signaling in the SMACs. This model addresses several key unresolved issues of T cell activation, namely the remarkable high sensitivity to low concentrations of Ag, the specific direct interaction of helper T cells and antigen-presenting B cells. Proc Natl Acad Sci USA 1998;85(16):6080–3.


