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Dynamic Behavior of T Cells and Thymocytes in Lymphoid Organs as Revealed by Two-Photon Microscopy

Review

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The initial application of two-photon imaging to the study of lymphoid tissues has provided a dramatic glimpse into how cells of the immune system move and interact in their native environments. In this review we summarize what we have learned so far and point out areas for future investigation using this approach.

Introduction

Lymphocytes exist within highly organized cellular environments. These tissue environments serve to segregate cells into different compartments, provide extracellular matrix for lymphocytes to crawl on, and help to present guidance and signaling molecules. Studies of lymphocytes removed from their normal environments provide an essential foundation for understanding the cellular responses to different stimuli. A full understanding of lymphocyte biology, however, will require integrating the information obtained from studies of isolated lymphocytes with studies of how the cells behave in their normal tissue environments. Analysis of the behavior of cells within tissues should help to address a number of important questions. How is organization of lymphoid tissues maintained? How do cells know where they are supposed to go and how do they get there? How do rare antigen specific lymphocytes find antigen-presenting cells bearing their cognate antigens? How is cell motility related to antigen recognition and signaling events? How does activation and differentiation effect a cell's location and migratory behavior?

These are challenging questions, and solving them will undoubtedly require many independent approaches (Table 1). In the past few years, the application of multiphoton imaging to analysis of lymphoid tissue has made it possible for the first time to perform real-time analysis of cells within their normal tissue environments. This review will focus on the application of this important new tool to understanding how T cells and thymocytes function in their native environments.

Two-Photon Microscopy: Advantages and Limitations

For questions that require imaging live cells for extended time periods deep within tissues, two-photon microscopy is the current method of choice. Like confocal microscopy, two-photon microscopy uses a laser to excite a fluorescent tag within a sample and detectors to measure the emitted light. However, unlike the lasers used for confocal microscopy, which provide single-photon excitation, the lasers used in two-photon microscopy excite by using near simultaneous absorption of two long wavelength (~800 nm) photons. This leads to several distinct benefits. The long wavelengths used for two-photon microscopy are less damaging and penetrate more deeply into tissues than those used in confocal microscopy. In addition, the requirement for near simultaneous absorption of two photons means that excitation is only achieved near the focal plane where the laser light is most concentrated. This has two distinct advantages. One is that there is little tissue damage to the regions above and below the focal plane that are not being imaged. In addition, there is no out-of-focus light that could make the image blurry and difficult to interpret. For a detailed explanation of two-photon microscopy, we refer the reader to the excellent review by Cahalan and colleagues (Cahalan et al., 2002).

While two-photon microscopy represents a powerful advance in our ability to probe the behavior of lymphocytes, it also has some significant limitations. One drawback is the cost of the lasers, which are many times more expensive that those used for confocal microscopy. In addition, the difficulties of imaging deep into tissues are only partially overcome with existing two-photon technology. Perhaps the major challenge is light scattering by tissues, which reduces the signal and limits the depth one can image into tissues to a few hundred microns. In some cases, the actual limit may be closer to ~100 microns when using less intense labels or when imaging into denser tissues that scatter light more strongly. Finally, there is the challenge of introducing informative fluorescent labels into tissues. Thus far, several approaches have been used. These include labeling cells with vital dyes prior to introducing them back into mice or organ culture (Figure 1) (Bousso et al., 2002; Bousso and Robey, 2003; Mempel et al., 2004; Miller et al., 2002, 2003), injecting vital dyes in situ (Miller et al., 2004), and expressing GFP derivatives as retroviruses or transgenes (Movie 1 [originally published online in Robey and Bousso, 2003, Visualizing Thymocyte Motility Using 2-Photon Microscopy, Immunol. Rev, Blackwell Publishing] and C. Witt and E.A.R., unpublished data). In the future, the use of fluorescent fusion proteins and other indicators of signaling and differentiation events should expand the range of questions that can be addressed by using this technique. However, the challenge of obtaining a sufficiently bright signal to allow detection deep within scattering tissues is likely to continue to pose limits on the types of questions that can be addressed with this technique.

The Dynamics of Thymocyte-Stromal Cell Interactions

Until recently, many of us suspected that thymocytes were relatively nonmotile cells based on their rounded

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Table 1. Comparison of Methods Used to Study T Cell-APC Interactions

Technique	Advantages	Limitations
Two-photon imaging of tissues (Bousso et al., 2002; Bousso and Robey, 2003; Mempel et al., 2004; Miller et al., 2002, 2003, 2004)	Provides real-time information about behavior of cells in tissue environments.	Difficulty in detecting signal deep in tissues limits the use of certain markers. Cannot simultaneously examine marker expression of cells being imaged.
Immunofluorescence analysis of fixed tissue sections (Ingulli et al., 1997; Lind et al., 2001; Saiki et al., 2001; Schaefer et al., 2001).	Can use antibodies to identify different cell types. Provides information about location of specific cells within tissue environments.	Static information only.
Isolation of T cell and DC clusters from lymph nodes (Hommel and Kyewski, 2003)	Can use immunofluorescence and flow cytometric analyses to characterize cells within clusters.	Static information only.
Time-lapse imaging of T cell-APC interactions in culture (Gunzer et al., 2000; Hurez et al., 2003; Krummel et al., 2000)	Real-time information. Can follow individual cells for long periods. More sensitive detection compared to two-photon microscopy, allowing for a broader range of markers.	Culture conditions do not recapitulate normal cellular environment.
Imaging of fixed T cell-APC conjugates (Freiberg et al., 2002; Monks et al., 1998)	High-resolutions analysis of proteins at T cell-ACP interface.	Static information only. Does not recapitulate normal cellular environment.
Imaging of T cells interacting with ligand in planar bilayers (Grakoui et al., 1999; Hailman et al., 2002)	High resolution, dynamic and quantitative information about movement of proteins.	Effects of APC and cellular environment cannot be studied. T cell itself not imaged.

appearance within fixed tissue sections and their inert behavior in a tissue culture dish. However, our first glimpse of the behavior of thymocytes within 3D tissues showed that this image of the passive thymocyte was incorrect (Bousso et al., 2002). Instead we found that thymocytes within a 3D stromal cell network were highly motile and were capable of actively exploring their local environment and traveling thousands of microns in a matter of hours. Thymocytes displaying high motility have now been seen in several different systems including reaggregate thymic organ cultures, fetal thymic organ cultures seeded with retrovirally transduced thymocytes, and intact adult thymic lobes (Movies 1–4 [originally published online in Robey and Bousso, 2003, Visualizing Thymocyte Motility Using 2-Photon Microscopy, Immunol. Rev, Blackwell Publishing] and C. Witt and E.A.R., unpublished data). Interestingly, thymocyte motility was highly dependent on contact with stromal cells, since thymocytes that reached the edge of the tissue immediately rounded up and stopped crawling (Bousso et al., 2002). This suggested that substances produced by stromal cells, such as chemokines and extracellular matrix, might promote thymocyte motility.

The high motility of thymocytes has implications for the formation of the T cell repertoire. During thymic development, thymocytes rearrange and express their α and β TCR genes and test out the specificity of their newly formed receptor for recognition of peptide-MHC

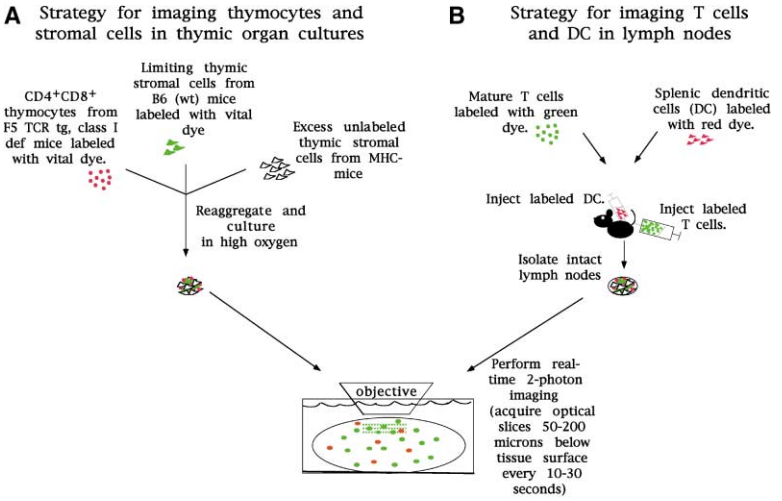

Figure 1. Experimental Strategies for Two-Photon Analysis of Thymocytes and T Cells

(A) Mixed reaggregate thymic organ culture (RTOC) system for real-time analysis of MHC-driven contacts (Bousso et al., 2002). Isolated DP thymocytes from TCR transgenic mice were labeled with a red vital dye. Isolated thymic stromal cells from wild-type embryonic mice were labeled with a green vital dye. Labeled cells were mixed with an excess of unlabeled thymic stromal cells from MHC-deficient mice. Cells were allowed to reaggregate in high oxygen submersion cultures. Thymocytes differentiate to CD8 SP thymocytes after 3 days of culture. Two-photon imaging of RTOC cultures was performed after 1 day of culture, at which point thymocytes had begun the process of positive selection, but remained CD4+CD8+. The use of excess unlabeled, nonselecting, MHC-deficient stromal cells allowed us to demonstrate preferential interactions of thymocytes with MHC-bearing stromal cells and to monitor MHC-driven interactions in real-time (see Movies 2–4). Figure was adapted from Robey and Bousso, 2003, Visualizing Thymocyte Motility Using 2-Photon Microscopy, Immunol. Rev, Blackwell Publishing.

(B) An in vivo cell transfer system for real-time analysis of T cell-DC interactions (Bousso and Robey, 2003). T cells from wild-type or TCR transgenic mice were labeled with a green vital dye and were intravenously injected into mice. Splenic dendritic cells were labeled with a red vital dye prior to injection. After 20 hr, the draining lymph node was removed and imaged by using two-photon microscopy while perfusing with oxygenated media at 37°C. (See Movie 5–7, available online at http://www.immunity.com/cgi/content/full/21/3/349/DC1).

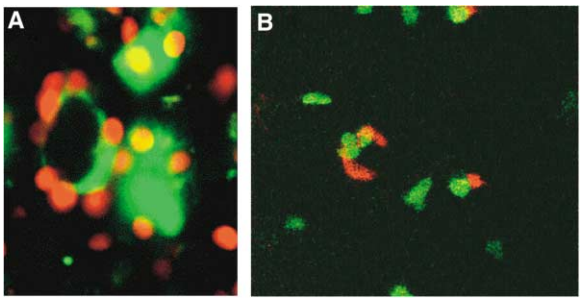

Figure 2. Visualization of T Cell-APC Interactions in Tissue via Two-Photon Imaging

(A) Thymocyte: thymic stromal cell interactions during MHC recognition in RTOC. RTOCs were formed with F5 thymocytes (red), selecting wild-type stromal cells (green), and excess unlabeled MHC negative stromal cells. MHC recognition results in the selective accumulation of thymocytes around MHC-bearing stromal cells.

(B) T cell-DC contacts during priming in the lymph node. Imaging of intact lymph nodes containing P14 TCR CD8 T cells (green) and DC bearing their cognate antigen (red).

complexes expressed on thymic stromal cells. Weak reactivity to self peptide-MHC on thymic epithelial cells leads to positive selection and differentiation to the CD4 or CD8 lineage. Strong reactivity to self-peptide MHC leads to negative selection. The active crawling of thymocytes within the thymic tissue environment suggests that thymocytes might initially form relatively short and frequent encounters with multiple stromal cells and raises the question of how these encounters might change during MHC recognition.

We developed a mixed thymic reaggreagate system to investigate this question (Figure 1A) (Bousso et al., 2002). In this system fluorescently labeled naive CD4+CD8+ thymocytes bearing a fixed TCR transgene were cocultured with unlabeled MHC-deficient thymic stromal cells, spiked with a limited number of labeled wild-type thymic stromal cells that express peptide-MHC complexes that can positively select thymocytes bearing the transgenic TCR. Under these conditions, thymocytes and stromal cells form a three-dimensional organo-typic culture that supports the positive selection of thymocytes from DP to mature CD8 SP cells (Anderson et al., 1994; Bousso et al., 2002). Time-lapse two-photon imaging of these cultures allowed us to observe the contacts between thymocytes and thymic stromal cells and to examine the impact of MHC recognition on these contacts.

In vitro studies of T cell-DC interactions show that under some circumstances, peptide-MHC recognition can occur without prolonged cellular interactions (Gunzer et al., 2000). It was therefore important to ask whether recognition of positive-selecting ligands prolonged contacts between thymocyte and thymic stromal cells. We found that DP thymocytes tend to form clusters around thymic stromal cells expressing selecting MHC, but not around MHC-deficient stromal cells (Figure 2A; Bousso et al., 2002). This implies that MHC recognition leads to prolonged contacts between thymocytes and positive-selecting stromal cells.

The preferential association of thymocytes with MHC-bearing thymocytes allowed us to use this system to identify and monitor MHC driven contacts. Examination of these MHC-driven contacts in mixed RTOC cultures revealed two distinct modes of interaction (Movies 2–4). Approximately half of the contacts were stable and long lasting, with thymocytes adopting a rounded, nonmotile morphology. The remaining one-half of contacts were dynamic and relatively short-lived (mean duration of approximately 20 min), with thymocyte remaining elongated and actively crawling over the surface of the stromal cell. Positive selection is thought to require prolonged or repeated recognition of peptide-MHC and is accompanied by gradual changes in the differentiation stage of the thymocytes. Thus it is tempting to speculate that the different modes of MHC-driven interactions seen in mixed RTOC culture correspond to distinct temporal stages of the positive section process.

The Thymus: What's Next?

Clearly the application of two-photon imaging to the study of thymic development is in its infancy, and there are many important questions that need to be addressed. Prominent among these are the nature of the contact regions between thymocytes and selecting stromal cells, and the organization of signaling molecules at the site of contact. For mature T cells there is evidence that the region of contact with an APC can be highly ordered, with a ring of adhesion molecules surrounding a core of signaling molecules, structures that have been called super molecular activation complexes or SMACs (Monks et al., 1998) or immunological synapses (Grakoui et al., 1999). In considering the potential of thymocytes to form synapse-like structures, the high motility of thymocytes, and what is known about thymocyte selection, many questions arise. To what extent do ordered contact regions form between thymocytes and stromal cells during peptide-MHC recognition? Do distinct types of contact regions form during negative versus positive selection, or during class I versus class II MHC recognition? What types of contacts form during stable versus dynamic contacts? Do thymocytes receive signals from sequential encounters with multiple stromal cells, or are contacts monogamous?

Regarding the ability of thymocytes to form immunological synapses: initial studies of dissociated thymocytes have provided some key information. In one study (Richie et al., 2002), thymocytes expressing fluorescent fusion proteins consisting of GFP fused to the TCR signaling molecules (lck-GFP, and CD3zeta-GFP) were observed interacting with thymic stromal cells. In another study (Hailman et al., 2002), synapse formation was monitored by using fluorescent versions of MHC and ICAM-1 embedded in a planar lipid bilayer as a surrogate APC. These studies indicated that, rather than forming "mature synapses" with central accumulations of signaling molecules, the contact regions between thymocytes and their APC were more disordered, with multiple concentrations of signaling molecules at the edge of contacts or spread throughout the contact area. In the future, it will be important to monitor the location of signaling molecules during MHC recognition in thymic organ cultures or intact thymic lobes, in which thymocytes are motile and undergo normal positive and negative selection. By combining two-photon imaging with

the use of fluorescent fusion proteins, it should be possible to obtain spatial and temporal information about the movement of signaling molecules as thymocytes form dynamic interactions with stromal cells.

Another set of questions concerns the long-range migrations of thymocytes and how these relate to selection and differentiation events in the thymus. Immunofluorescence analysis of fixed tissue sections of the thymus has revealed a remarkable compartmentalization of the thymus, in which thymocytes representing different developmental stages are found in discrete anatomical locations (Lind et al., 2001; Robey and Bousso, 2003). By putting these static pictures together with what we know about the developmental stages of thymocytes and the signals that drive them, we can piece together the outlines of the journey of a developing T cell through the thymus. The earliest thymic progenitors enter through blood vessels located deep in the thymus at the cortical/medullary junction and then migrate outward to the capsule where they begin to undergo TCR gene rearrangements. After TCRβ selection, thymocytes migrate inward to fill the cortex and eventually complete rearrangement of TCRα, leading to the formation of the αβTCR. A fraction of cortical thymocytes then undergo positive selection thymocytes and migrate further inward to fill the medulla.

The analysis of fixed tissue sections provides essential information about the migration patterns of thymocytes but does not reveal the dynamics of these migrations. For example, do selection signals alter the speed of migration and/or directionality of thymocytes? Do thymocytes arrive at their appropriate location in a highly directed fashion, or do they get there by "trial and error"? To what extent do thymocytes respond to short-range versus long-range directional cues? Do these cues affect migration speed, directionality, or both? These questions can best be addressed by tracking the movement of individual thymocytes over time deep within the thymus. Thus two-photon microscopy is likely to be an important tool in future studies of thymocyte migration.

T Cell-DC Interactions: A Not-So-Improbable Cellular Encounter

The lymph node has been viewed as a "black box" where those DC that bear antigen somewhat manage to interact with the rare T cells bearing an appropriate TCR. At first glance, such an event may appear relatively improbable. Typically, the frequency of naive T cells displaying a given peptide specificity do not exceed 1×10^{-5}. Recent studies have shed some light on mechanisms that facilitate this initial encounter. First, DC migrating from inflamed tissues and naive T cells express the LN homing chemokine receptor CCR7 and both cell types migrate to the paracortical region of the LN through the afferent lymph and high endothelial venules (HEV), respectively (Cyster, 2000). Second, tissue-derived DC home preferentially in the vicinity of HEVs, a positioning that might optimize the scanning of incoming T cells (Bajenoff et al., 2003). Importantly, two-photon imaging of intact LN has revealed that the cellular behaviors of DC and T cells also promote this encounter by allowing for a very efficient scanning of the T cell repertoire (Bousso and Robey, 2003; Miller et al., 2004) (Figure 3, Movie 5 available online at http://www.immunity.com/cgi/content/full/21/3/349/DC1). Naive T cells are highly motile (mean velocity of 10–12 μm/min) and follow relatively chaotic trajectories with frequent turns. As a result of this frantic motility, the pool of T cells that surrounds an individual DC is constantly changing over time. On the other hand, DC migrate more slowly (2–6 μm/min) but undergo rapid shape changes and dendrite deployments. These dynamic behaviors further increase the territory effectively surveyed by each DC and the overall efficiency of the scanning process. It has been estimated that in the absence of Ag, each DC interacts with 500–5000 different T cells per hour, with each contact lasting no more than a few minutes (Bousso and Robey, 2003; Miller et al., 2004). Thus, on a second look, it appears that even a few antigen-bearing APC should have the opportunity to encounter an appropriate T cells with a high probability. For example, only one to ten DC would be required to scan 1×10^5 different T cells in a day. Certainly, such a high scanning rate is a key feature of the immune system, allowing for the rapid detection of T cells of interest in a context of a high diversity of TCR receptors. It remains to be determined whether the distinct DC populations found in LNs (including dermal DC, Langerhans cells, or blood-borne DC subsets) have distinct migratory patterns and scanning ability reflecting for example differences in morphology and/or maturation stage. So far, both in vitro (Bousso and Robey, 2003; Mempel et al., 2004) and in vivo (Miller et al., 2004) vital dye labeling of DC have been described, and these different methods will almost certainly label distinct subsets of DCs. Future use of appropriate GFP reporter systems should help visualize particular endogenous DC subsets.

Finally, it is unclear whether DC themselves have the ability to attract naive T cells by chemotactic gradients. A recent report found no differences in the velocities of T cells approaching or departing dendritic cells, strongly suggesting that T cell-DC contacts occur mostly by chance (Miller et al., 2004). In another study, injection of DC plus LPS appeared to significantly restrict T cell trafficking but the underlying mechanism remains to be elucidated (Mempel et al., 2004).

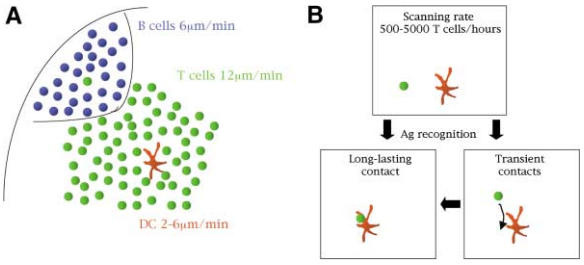

Figure 3. Cellular Dynamics in the Lymph Nodes

(A) Schematic representation of a B cell follicle and of the T cell zone of lymph node. The mean velocities of the different cell types are indicated.

(B) Dynamics of antigen-independent and antigen-dependent T cell-DC contacts. The various proposed modes of T cell activation are shown. Data are summarized from Bousso and Robey, 2003; Mempel et al., 2004; Miller et al., 2004; Miller et al., 2002.

Distinct Modes of T Cell-APC Interactions: How Do T Cells Get Activated?

While it is firmly established that T cell activation relies on engagement of T cells by antigen-bearing APC, the dynamics of these cellular contacts as they happen in vivo are only beginning to be identified. In vitro imaging studies of T cell-APC interactions have suggested that T cells could achieve activation through distinct modes of cellular contact. Initial experiments aimed at visualizing these interactions were performed in suspension culture. In such experimental settings, T cells tend to form a tight and long-lasting (several hours) contact with antigen-bearing APCs (Delon et al., 1998; Monks et al., 1998; Negulescu et al., 1996). Shorter contacts have occasionally been observed in suspension culture, for example between T cells and immature DCs, but these resulted in abortive proliferation (Benvenuti et al., 2004). In one study, a long-lived contact appeared to be an absolute requirement for T cell commitment to IL-2 production (Hurez et al., 2003). In sharp contrast, T cells embedded in a collagen matrix formed multiple, transient contacts with DC lasting only 2–5 min (Gunzer et al., 2000). Interestingly, the presence of antigen on the DC did not influence the duration of contacts in this system, although it did promote T cell activation. Relatively similar cellular dynamics have been observed during the in vitro activation of a T cell clone interacting with macrophages in monolayer culture (Underhill et al., 1999). These observations suggest that T cells may sum signals received from multiple, short cell-cell contacts in order to achieve activation. In line with these observations, in vitro experiments aimed at periodically blocking signal transduction in T cells interacting with an APC have supported the idea that T cells can be activated by intermittent signals (Faroudi et al., 2003).

So how does T cell activation occur in vivo? This is an essential question since characteristics of T cell-DC interaction such as the duration of contact are likely to have a profound impact on the extent of T cell differentiation (Gett et al., 2003; van Stipdonk et al., 2003). The first direct observations of T cell-DC interactions in vivo comes from analysis of frozen lymph node sections. Selective accumulation of antigen-specific T cells around antigen-bearing DC has been visualized in several studies (Ingulli et al., 1997; Norbury et al., 2002; Saiki et al., 2001; Schaefer et al., 2001). Typically, clustering is maximal by day one and rapidly decreases over the next few days. As an alternative to in situ imaging approaches, T cell-DC clusters have also been physically isolated from lymph nodes and their composition and kinetics of formation extensively studied (Hommel and Kyewski, 2003).

Overall, the detection of antigen-driven T cell clusters surrounding DC has offered valuable information on when and how cell-cell contacts occur. It has also indicated that T cell contacts with antigen-bearing DC last significantly longer than nonspecific interactions. However, it does not provide information regarding the dynamics of these contacts. Two-photon imaging of intact lymph nodes has made it possible to fill this gap by tracking individual cell contacts over time. Miller and colleagues were able visualize CD4 T cells forming both stable and dynamic clusters upon immunization (Miller et al., 2002). While APCs were not visualized in this studies, it is likely that these behaviors were the result of T cell contacting antigen-bearing APC, raising the possibility that distinct modes of activation coexist in vivo. In another study, CD4 T cells and DC were visualized in the superficial area of the lymph node by confocal microscopy and were found to be stably interacting (Stoll et al., 2002). By using two-photon imaging, we observed CD8 T cells and DC bearing their cognate peptide one day after adoptive transfer and found that the duration of T cell-DC contact was in the range of hours (Bousso and Robey, 2003) (Figure 2B, Movies 6 and 7, available online at http://www.immunity.com/cgi/content/full/21/3/349/DC1). Similarly, in another study, long-lived ($>$1 hr) CD8 T cell-DC contacts dominated in lymph nodes analyzed between 8 and 24 hr after adoptive transfer (Mempel et al., 2004). Interestingly, at earlier time points ($<$8 hr), most T lymphocytes formed short-lived and dynamic interactions with DC. Although these short-lived interactions also occurred in the absence of antigen, the observation that a significant proportion of T cells had started to upregulate activation markers by 8 hr suggests that productive TCR signaling may occur during these short-lived interactions. These results suggested a multiphasic model of activation in which the T cells collect signals from multiple short contacts with DC prior to forming a long-lasting interaction. Figure 3 summarizes the cellular dynamics in lymph nodes as identified by two-photon microscopy.

A number of questions remain unsolved. Perhaps the major limitation to these studies so far is the inability to follow an individual T cell from the time of its initial encounter with an APC to the time it begins to produce IL2 and proliferate. Rather, these studies so far have been limited to describing the kind of cellular contacts that dominate at a particular time point. This leads to ambiguities in interpreting of the consequences of a particular contacts on the eventual cellular outcome. For example, in the study of Mempel et al., 2004, it is unclear whether the T cells that upregulate activation markers at 8 hr are the same cells that made only transient contacts with DC. We also have a poor understanding of the immunological parameters that favor short versus long-term interactions. It will also be important to determine whether T cells that experience distinct patterns of APC encounter will end up with different functional capacities. Finally, it will be important to analyze the dynamics of T cell-DC interactions in physiologically relevant infectious models. Clearly, different immunization/infectious processes will differ in number, type, or phenotype of the APC involved. Such factors will undoubtedly influence both the way that T cells interact with antigen-bearing APC and the eventual consequences of the interactions.

To Explant or Not to Explant?

Our first glimpses into the dynamics of T cells, B cells, and dendritic cells in the lymph node came from imaging of intact, explanted lymph nodes (Bousso and Robey, 2003; Miller et al., 2002; Stoll et al., 2002). In some of these studies (Bousso and Robey, 2003; Miller et al., 2002), excised lymph nodes were imaged while being perfused with warmed, oxygenated media, conditions that are widely used by physiologists and neurobiolo-

gists for studies of thick tissue explants. More recently, two groups have described intravital imaging of lymph nodes (Mempel et al., 2004; Miller et al., 2003). In these studies, mice were anesthetized and lymph nodes were surgically prepared and submerged in warmed media while imaging. In theory the manipulations involved with either approach could alter the physiology of the tissue. Perfusing with oxygenated media may not achieve the same oxygen tension in the tissue that would normally be provided by the circulation, while the trauma associated with anesthesia and surgery could also introduce artifacts. Fortunately, the results obtained thus far via these two approaches have generally shown remarkable agreement, with the characteristic motility rates of the different cell types, the dynamics of cell movements, and antigen-driven contacts all appearing quite similar. The concordance between the results obtained with lymph node explants and intravital imaging suggests that both approaches can provide conditions that are physiologically appropriate. In one study of lymph node explants (Stoll et al., 2002), lower cell motility was observed, although the timing of stable T cell-DC interactions and the dissociation of T cell-DC conjugates was similar to those described in the other reports. The lower cell motility seen in this study could be due to the fact that relatively superficial areas of lymph node were examined. (Confocal, rather than two-photon microscopy was used for this study, limiting the depth of imaging to $<$50 μm from the surface.) In addition, the lack of perfusion in this study may also have contributed to lower cell motility.

Given that both tissue explants and intravital imaging can provide similar results, the choice of which approach to use will likely depend on which questions are being addressed. For example, when studying a process in which blood and lymph flow is likely to have an impact, such as cell migration into or out of a tissue, intravital imaging would clearly be a better choice. On the other hand for some tissues, such as thymus, intravital imaging poses particular challenges due to the relatively inaccessible location of the organ and the need to isolate tissue being imaged from movement caused by respiration. For such tissues, the explant approach may be advantageous. In either case, care must be taken to maintain the tissue under appropriate conditions. This is illustrated by the fact that lymphocyte motility drops rapidly when the perfusion is interrupted during imaging of explants (P.B., E.A.R., and C. Witt, unpublished data) or when cardiac arrest is induced during intravital imaging (Mempel et al., 2004).

Future Directions

Two-photon imaging has provided our first glimpse into how cells move and interact in their native tissue environments. As two-photon microscopes becomes accessible to more laboratories and as imaging and labeling methods become further refined, this technique will undoubtedly become a useful tool for addressing many different types of questions. Signaling events can be studied by examining the subcellular localization of fluorescent fusion proteins over time and by using calcium indicator dyes. Gene expression can be monitored with reporter transgenes such as GFP, whose expression is driven by interesting promoters. Such reporters could be used to identify particular cell types within tissues and to provide more information about changes in gene expression in individual cells over time. Another area in which further advances are likely is in the quantitation of two-photon data. The striking images generated by using this technique contain a wealth of information that is just beginning to be mined. Extracting this information will require improved software to identify objects, quantitate movement, and measure colocalization between different signals. Mathematical treatment of this information may help to confirm our visual impressions of the data and help to reveal patterns that are not obvious by simple inspection. With these advances, two-photon imaging will increasingly become a powerful tool not only to describe the behavior of immune cells in their in vivo context, but also to help dissect molecular and cellular interplay during immune responses at a mechanistic level.

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