Immunity, Vol. 21, 331–339, September, 2004, Copyright ©2004 by Cell Press

Review

Illuminating the Landscape of In Vivo Immunity: Insights from Dynamic In Situ Imaging of Secondary Lymphoid Tissues

Alex Y.C. Huang, Hai Qi, and Ronald N. Germain
Lymphocyte Biology Section, Laboratory of Immunology
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland 20892

A central feature of the immune system is the migratory behavior of its cellular components. Thus, fully understanding the generation and maintenance of immune responses must include consideration of how hematopoietic cells home to, interact within, and exit from secondary lymphoid organs as well as peripheral tissues. Recent advances in in situ imaging techniques now permit direct observation of these events in their physiologic settings with high spatiotemporal resolution. This review summarizes progress in this area of investigation from a lymphocentric perspective. We highlight controversies, point out key unanswered questions, and briefly outline what we believe are some of the near-term directions that in situ microscopic analysis of the immune system will take.

Introduction

The normal functions of the nervous system critically depend on information storage and data transmission involving the network of synaptic connections among neurons. Similarly, operation of the immune system also requires sophisticated cellular communications that rely to a great extent on intimate membrane contacts (Dustin and Colman, 2002). While neuronal connections are frequently long-lived and, at least late in development, can often be considered "hard-wired," the immune system is uniquely "soft-wired." Its cells constantly traffic through different parts of the body (Butcher et al., 1999; von Andrian and Mackay, 2000), establishing or relinquishing key physical contacts with various partner cells at different times and in diverse locations. For example, the induction of a productive T cell response requires dendritic cells (DCs) that have captured antigens (Ags) at peripheral sites of infection to migrate into secondary lymphoid organs such as lymph nodes (LNs), spleen, and Peyer's patches. Here DCs come in contact with naive T cells that repeatedly circulate between the blood compartment and these organized secondary lymphoid sites. After Ag-specific T cells are activated by Ag-bearing DCs through a series of contact-dependent signaling events, they terminate this pairing and reenter the circulation, often emigrating and accumulating at peripheral sites of inflammation and Ag deposition (Banchereau and Steinman, 1998). In these latter locations, the differentiated T cells carry out their effector functions, a process that itself typically involves direct cell contact. Immunologists seeking a deeper understanding of host defense and of autoimmunity must dissect critical cellular interactions like these by observing and analyzing them in detail at the right time and in the proper location.

*Correspondence: rgermain@niaid.nih.gov

Until recently, collecting the data needed to understand the complex migratory behavior and transitory cell associations of lymphocytes has been limited by available techniques. Our knowledge of immune cell interactions has come primarily from in vitro studies of various coculture systems, ex vivo examination of consequences of cellular interactions in situ, and static histological analyses of lymphoid tissues, all of which have been indispensable to our current mechanistic understanding of such interactions (Germain and Jenkins, 2004; Jenkins et al., 2001). However, these methods do not permit direct examination of inherently dynamic processes at the single-cell level in both a temporally and spatially resolved fashion within the physiologic environment of lymphoid tissues or created by active inflammation at peripheral sites. In the past few years, however, advances in imaging instruments, software, fluorescent reagents, and animal manipulation tools have begun to address these limitations.

Epifluorescence-based video intravital microscopy was the first of these dynamic imaging methods to be employed (MacPhee et al., 1992; Mempel et al., 2003). Its use provided new insights into cell adherence to and penetration of microvasculature endothelium but did not permit analysis of cells within dense organized lymphoid tissues or the extravascular spaces of inflamed tissues. The recent advent of new micro-PET (Herschman, 2003), luminescence (Hardy et al., 2001), and micro-MRI (Kircher et al., 2003) methods has permitted the repetitive analysis of lymphocyte distribution in animals, but only with limited resolution that does not reveal individual cell behavior or have the capacity to track signaling events and molecular distribution patterns (Choy et al., 2003). These limitations have been overcome in mouse models by the application of conventional confocal microscopy and, most recently, by the powerful approach of two-photon laser scanning microscopy. The technical aspects of each microscopic method have been well described in a recent review (Cahalan et al., 2002). In brief, standard epifluorescence microscopy yields an image that represents emitted light from both well above and well below the focal plane within a specimen. In contrast, both confocal and two-photon imaging limits the collected light to a small axial (z) distance, thereby improving the resolution of objects in three-dimensional space by limiting superposition of signals. The rapid sequential collection of several colors of x-y data at multiple z levels in a thick specimen and the repetition of this volume imaging over time allows the shape, movement, molecular patterning, and even gene activation responses of multiple cell types to be simultaneously tracked in a dynamic manner with high resolution (Bousso et al., 2002; Bousso and Robey, 2003; Cahalan et al., 2002; Mempel et al., 2004; Stoll et al., 2002). In the case of two-photon methods, this type of data collection can extend hundreds of microns into native tissues. With the proper specimen handling, imaging can be extended over many hours, and when combined with sequential

sample analysis, these methods permit the visualization of different stages of immune responses. This is an exciting development that has already begun to provide new understanding of the dynamic nature of the immune system and promises to revolutionize our concepts of immune cell behavior.

In this review, we summarize and discuss what has been learned from live tissue-imaging studies in the context of the still limited literature on this topic and, wherever possible, point out what we believe can or should be studied with this new approach in the future. Because most of the published studies have been focused on DC-T cell interactions, we will also center our discussion on this topic.

Dynamic Tissue-Imaging Techniques

Currently, two methods of tissue preparation exist for in situ confocal and two-photon laser scanning microscopy: tissue explant microscopy and intravital microscopy. By using two-photon imaging of explanted LNs bathed in medium perfused with 95% O_2 (a procedure chosen based on prior neurobiological studies of brain slices [Yuste et al., 1999] whose relevance to the physiological condition within LNs is uncertain [Caldwell et al., 2001]), Miller et al. (2002) provided the first detailed, quantitative analysis of the movement of adoptively transferred, ex vivo fluorescent-dye-labeled naive B and $CD4^+$ T cells in a lymphoid tissue. Both the T and B cells were extremely mobile, moving at 11 and 6 μm/min, respectively. A similar high motility was also subsequently observed for naive $CD8^+$ T cells by Bousso and Robey (2003) by using an O_2-perfused LN explant method similar to that of Miller et al. Extrapolation from the imaging data involving $CD8^+$ T cells suggested that up to 500 naive cells probed a single DC every hour in the absence of antigen.

At the same time that Miller et al. described naive cell behavior in the absence of antigen, Stoll et al. (2002) reported results of tissue explant imaging of $CD4^+$ T cell interactions with antigen-bearing DC using a confocal instrument and without super-oxygenation of LNs. In contrast to the findings first reported by Miller et al. (2002), they found that naive $CD4^+$ T cells were relatively immobile within the region of observation ($<$80 μm below the capsule) when Ag-bearing DC were absent, but after 36 hr of DC interaction and antigen-dependent activation, these lymphocytes moved rapidly in a manner similar to that seen by the other groups using naive T cells.

Given the known constant recirculation of naive lymphocytes (Ford and Gowans, 1969), Stoll et al. pointed out in their initial report that there must be a technical reason for their failure to observe the expected motility of the $CD4^+$ T cells prior to TCR-dependent activation. Several possible explanations were considered, among them tissue oxygenation, laser phototoxicity, and the specific region of the LN imaged. Phototoxicity seemed an unlikely source of artifact, because T cells recovered after prolonged imaging of a LN with foreign antigen-pulsed DC showed activation markers such as CD69 and those imaged divided and moved vigorously after more than 8 hr of intermittent illumination. Nonetheless, little naive cell movement was seen under the same conditions. Although many investigators have ascribed differences in T cell motility observations between the Stoll report and others to oxygenation effects, more recent work has pointed in another direction with intriguing physiologic implications, namely a striking degree of variation in lymphocyte migratory behaviors in distinct subregions of the LN.

The limit for high-resolution confocal work is typically less than 80–100 μm, whereas two-photon studies can extend 200–300 μm into the LN under optimal conditions. A variety of experiments have been conducted in our laboratory to explore whether this imaging depth difference or oxygenation is the principle factor responsible for the differing data on naive cell motility in the various published studies. These experiments have revealed that there are substantial variations in the migration of naive T cells in distinct areas of the LN and especially in shallow versus deep segments relative to the capsule. The mobility of naive $CD4^+$ and $CD8^+$ T cells in explants imaged using the room air conditions of Stoll et al. but employing two-photon methods is very similar to that reported by Miller et al. and Bousso and Robey, provided that the tracking involves depths not typically visualized with confocal instrumentation. This can be seen in Movie 1 (available online at http://www.immunity.com/cgi/content/full/21/3/331/DC1), in which most of the lymphocytes show very dynamic movements deep under the capsule ($>$100 μm), whereas cells in the "shallower" regions of the same LN ($<$80 μm) show either slow movement or are immobile, as originally seen with confocal microscopy (Stoll et al., 2002). Despite the slow moving or immobile T cells in the shallower region, however, we are able to observe T cells in clusters with antigen-bearing DCs that are present in these as well as deep T-zone regions (A.Y.C.H, unpublished data).

Could these regional differences themselves still be an experimental artifact due to the use of explanted LNs, which lack blood flow, lymph flow, and innervation, or because the surfaces of exposed tissues are prone to hypothermia? Several observations suggest not. First, although the confocal explant experiments of Stoll et al. (2002) did not show naive T cell movement near the capsule, antigen-activated $CD4^+$ T cells migrated rapidly within the same region. Second, a new method of imaging intact lymphoid organs in anesthetized animals (intravital microscopy) has recently been developed to provide for more physiologic conditions of observation. In our hands, when applied to a range of subcapsular depths, such intravital imaging has again reproducibly demonstrated significant heterogeneity in lymphocyte migration pattern and speed, with naive T cells in deeper regions moving rapidly and those in shallow regions showing limited movement or static behaviors. The data on T cell mobility that we have obtained deep in the T cell zone agree with those first reported using this technique by Miller et al. (2002), who described comparable naive $CD4^+$ T cell motility in the LN of living animals and in LN explants (9 μm/min and 11 μm/min, respectively) (Miller et al., 2003). We have also imaged a single inguinal LN sequentially in two opposing directions, from the capsule toward the hilus and then from the hilus out toward the capsule (A.Y.C.H., unpublished data). In each data set, the zone of lymphocyte immobility corresponded to the shallow subcapsular and interfollicular

areas of the LN (Movies 2 and 3, available online at http://www.immunity.com/cgi/content/full/21/3/331/DC1), rather than the region in which observation began and in which the potential for phototoxicity was greatest. Finally, tissue-draining DCs also showed robust migration in the subcapsular region (Movie 4, available online at http://www.immunity.com/cgi/content/full/21/3/331/DC1), adding to the evidence against a temperature, phototoxicity, or oxygenation artifact as the origin of the limited lymphocyte mobility in this area of the LN. Taken as a whole, these findings suggest a striking difference in T cell migration patterns corresponding to different anatomical locations within the LN. Such differences may have been under-appreciated in published studies that typically excluded the interfollicular and subcapsular zones from analysis (Bousso and Robey, 2003; Mempel et al., 2004; Miller et al., 2002, 2003, 2004). Both the potential immunological significance and the mechanistic origins of these differences in lymphocyte mobility are discussed more fully below.

Such evidence for regional differences in lymphocyte migratory behavior also suggests that intravital imaging has certain limitations despite being a more physiologic method of analysis. Live animal surgical approaches to certain tissues of interest may prevent effective data collection under some conditions due to anatomical constraints they place on the available field of view within the target lymphoid structure. Explant methods circumvent this problem by allowing multiple imaging angles in an isolated tissue and the collection of more representative data throughout the LN structure, while also minimizing motion artifacts.

Respecting Anatomy

Regional intranodal variations in T cell migration highlight an essential parameter critical to proper interpretation of dynamic imaging data, namely an appreciation of the intricate underlying architecture of an organized lymphoid organ. While certain unstained elements such as collagen-rich fibers can be visualized due to a unique feature of two-photon illumination ("second harmonic emission" [Zoumi et al., 2002]), many key nonlymphoid structures (e.g., high endothelial venules [HEV], the draining sinus, conduits, stromal cells, and many components of the extracellular matrix) along with endogenous, unlabeled hematopoietic cells are represented as a dark background. When viewing movies made from such data sets, it is tempting to interpret these dark zones as "empty space" akin to the open areas between cells in a culture dish. Of course, nothing could be further from the truth, and the dense packing of cells along with the complex distribution of stromal elements seen in conventionally stained fixed or frozen sections of lymphoid tissues makes this perception error readily apparent. These unseen structures and cells undoubtedly influence the observed behavior of labeled cells, but the true impact of this "missing data" on proper interpretation of imaging studies will remain unknown until better landmarks and multicolor detection methods can be developed to delineate the contributions of these structures to the pattern of cellular behavior in complex lymphoid tissues.

Two recent studies emphasize the importance of conducting imaging studies in the context of appropriate anatomic landmarks. Bajenoff and Guerder used sequential static imaging methods to show that T cells first accumulate in contact with Ag-bearing DCs just outside the HEV through which the lymphocytes entered the LN (Bajenoff et al., 2003). Likewise, another group has recently reported that T- and B-lymphocytes, as well as DCs, congregate in the interfollicular region or "cortical ridge" and that T-DC interactions in the presence of antigen preferentially occur here rather than in the deep T zone (Katakai et al., 2004). In our own live imaging studies, the use of fluorescent dextrans injected intravenously allowed blood vessels (including HEV) to be visualized and demonstrated that many of the poorly motile lymphocytes in a LN are arrayed along the vessel walls (Movie 5, available online at http://www.immunity.com/cgi/content/full/21/3/331/DC1). Without this marking of the vessels, these immobile cells would just be a subpopulation of the total pool of tracked lymphocytes whose "aberrant" behavior could not be related to any particular feature of lymph node organization.

T Cell Interactions with Antigen-Bearing DCs: Long-Lasting or Short-Lived Clusters

Early studies using dynamic lymphocyte-imaging methods were not focused solely on naive cell migration; they also examined the nature and duration of T cell contact with DC bearing cognate Ag. By employing adoptive transfer of T cells from TCR transgenic mice with a known specificity and DC bearing the corresponding pMHC ligands (Ingulli et al., 1997; Kearney et al., 1994), it was possible to examine how agonist recognition influenced this critical cell-cell interaction. A key issue was the duration of such associations in relation to the resulting functional state of the T cell. Prior to the emergence of live tissue-imaging tools, two widely cited in vitro studies explored this question and reached drastically differing conclusions. By using pMHC-coated plastic to simulate the cell surface of antigen-presenting cells (APC) and soluble anti-CD28 antibody to mimic costimulatory signals, Iezzi et al. (1998) found that naive T cells needed approximately 10 hr of costimulation and TCR signaling or more than 20 hr TCR signaling alone before they committed to cell proliferation (Iezzi et al., 1998). Although this initial study did not directly address the issue of whether required period of stimulation needed to be continuous, a more recent report suggests that this is the case (Huppa et al., 2003). Gunzer et al. (2000) examined this issue with a different approach. Employing DCs as APCs and a collagen-gel matrix to mimic a three-dimensional tissue environment, these authors found that a large fraction of T cells engaged in short (10–15 min) and sequential contacts with the same or different DCs, eventually becoming activated as measured by changes in surface marker expression and cell proliferation. Based on these results, these authors proposed a "digital" counter mechanism inside T cells that determined when the accumulated signals from these short encounters exceeded a threshold necessary for cellular activation (Friedl and Gunzer, 2001; Gunzer et al., 2000).

Each of these in vitro systems has serious drawbacks. That employed by Iezzi et al. (1998) lacked living Ag-

bearing DCs and involved a two-dimensional environment. In contrast, while Gunzer et al. employed live DCs as APCs and tried to mimic the three-dimensional tissue environment, the collage gel matrix differs from the lymphoid tissue at least in one major respect: T cells do not seem to be exposed to collagen fibers anywhere in LNs (Gretz et al., 1997). In addition, the assessment of T cell activation by using a combination of cell-surface phenotype and bulk proliferation, as done in this study, may have been misleading with respect to whether the majority of the cells that showed transient interactions with DC actually became fully activated and synthesized cytokines. It is possible that only the small fraction of T cells showing long-lived associations with APC did so, as was found in another in vitro imaging study (Hurez et al., 2003). Clearly, direct observation of T-DC interactions in their native tissue environment was needed to resolve the contact duration-activation issues.

Two groups initially approached this subject by using explant imaging. Cahalan and colleagues visualized stable (>50 min) clusters of DO11.10 $CD4^+$ TCR transgenic cells in draining LNs 24 hr after a subcutaneous ovalbumin immunization (Miller et al., 2002). Although antigen-bearing DCs were not directly visualized in this particular study, previous experiments demonstrated that with the same combination of Ag and transgenic T cells, Ag-specific T cells formed clusters exclusively around DCs 24 hr after Ag immunization or direct injection of Ag-pulsed DCs (Ingulli et al., 1997, 2002). Therefore, the T cell clusters observed by Miller et al. (2002) most likely contained Ag-bearing DCs, and these data would thus be consistent with a long-lasting T-DC conjugation model. This latter group did, however, also observe "swarming" behaviors by some Ag-specific T cells that did not form clusters but were nonetheless confined to a relatively small area with much reduced motility. This behavior is superficially consistent with the second model, that individually short-lived but repetitive T-DC contacts drive T cell activation.

Data from an independent live-imaging study reported simultaneously with the results of Miller et al. and involving LN explants with dual color analysis of both T cells and DC, however, provided clear evidence for a long-lived interaction model. Stoll et al. (2002) reported that the bulk of transferred $CD4^+$ T cells present in the subcapsular region of a LN explant formed highly stable antigen-dependent associations with DC, with individual unbroken associations tracked for up to 15 hr in some cases. Only after more than 36 hr of potential T-DC contact and the onset of cell division in the T cell population were many of the lymphocytes seen to move rapidly and make only transient contacts with the DC. In a subsequent study the physical contacts between Ag-bearing DCs and $CD8^+$ T cells were also estimated to last for several hours (Bousso and Robey, 2003). Therefore, for both $CD4^+$ and $CD8^+$ T cells, long-lasting physical conjugation with DCs appears to be the predominant mode of interactions that preceded robust clonal expansion in explanted LNs. Interestingly, these data, obtained by using the latest technical methods, are very similar to observations made more than 25 years ago involving guinea pigs' lymphocytes and antigen-presenting cells analyzed in vitro (Stingl et al., 1978; Yamashita and Shevach, 1978). These studies showed prolonged antigen-specific interactions that preceded the onset of cell division.

More recently, Mempel et al. reported a more detailed analysis of Ag-specific interactions between $CD8^+$ T cells and Ag-bearing DCs by using intravital rather than explant methods (Mempel et al., 2004). Two hours after transfer of dye-labeled T lymphocytes, a monoclonal antibody specific for CD62L was administered to interfere with the tethering of circulating lymphocytes to HEV (Butcher et al., 1999; von Andrian and Mackay, 2000; Warnock et al., 1998) and prevent T cells that had not already entered the LN from doing so. This produced a labeled T cell cohort within the lymphoid organ that was reasonably well synchronized in terms of its residence time in the tissue. The characteristics of the interactions of these lymphocytes with Ag-bearing DCs could then be related to how long after LN entry the observed behavior took place. T cells were found to initially engage in brief and multiple contacts with antigen-bearing DCs whose motility decreased over time. This "phase one" was followed by a 12 hr period ("phase two") during which Ag-specific T cells established and maintained stable conjugates with DCs. After this period of prolonged interactions with DCs, T cells were found to dissociate and regain rapid motility, accompanied by cellular proliferation ("phase three"). These latter two phases seen with $CD8^+$ T cells analyzed by using intravital conditions correspond closely to the results previously obtained by Stoll et al. with $CD4^+$ T cells and explant methods. Although the two studies involved different DC populations, T cell subsets, and different imaging depths, they both demonstrated that very long-lived antigen-driven T-DC associations characteristically precede activation for cell division and rapid migration of the activated cells within the LN parenchyma. Very importantly, T cells in phase one as defined by Mempel et al. did not upregulate CD25 or produce IL-2, despite their transient and multiple contacts with DCs. In fact, these authors did not observe increased CD25 expression or IL-2 and IFN-γ production until after the stable-contact phase (phase two), suggesting the necessity of prolonged T-DC conjugation for full-fledged T cell activation (Huppa et al., 2003; Iezzi et al., 1998). The Mempel report also provided a potential explanation for the "swarming" behavior as originally described by Miller et al. (Cahalan et al., 2002), suggesting that the T cells involved may represent recent immigrants displaying the phase one behavior. It has been proposed that this period of transient DC contacts may be needed for "resensitization" of the cells after they emerge from blood where they have been deprived of the self-recognition necessary for optimal foreign antigen reactivity (Stefanova et al., 2002), but this is presently only an intriguing speculation. An alternative is that the change in contact duration is not the result of alterations in T cell physiology but, rather, reflects the further differentiation/maturation of the cotransferred DCs over the observation period. Indeed, various studies have shown that more mature or activated DCs express increased levels of adhesion molecules (Teunissen et al., 1994) that are important in establishing long-lived contacts with T cells (Benvenuti et al., 2004).

Taken together, these live-imaging studies conducted by different research groups using distinct methods

have indicated that stable and long-lasting association is a major mode of T cell interaction with foreign Ag-bearing DC in situ and is required for T cells to proliferate and subsequently develop effector functions. On the other hand, a shared deficiency in all of above live-imaging studies is the lack of real-time functional readouts in vivo. As a result, we are still unable to accurately estimate how long a particular T cell needs to contact an Ag-bearing DC in situ in order to turn on a relevant gene, e.g., IL-2. Indeed, in vitro studies are still more advanced in this regard. For example, GFP expressed under the control of a large IL-2 promoter fragment was used to assess in a dispersed cell culture environment the relationship between the longevity of a T cell-DC conjugate and the capacity of a given T cell to activate the IL-2 reporter (Hurez et al., 2003). In this study, several hours of stable contact were necessary before a cell would show reporter GFP activity; brief encounters allowed CD69 upregulation but never IL-2 gene activity.

Going forward, a key technical issue in the field will thus be the development and use of fluorescent reporter technology (Zhang et al., 2002) to assess gene activity (Hu-Li et al., 2001; Mohrs et al., 2001; Naramura et al., 1998), to observe molecular redistributions such as those occurring during immune synapse formation (Egen and Allison, 2002; Krummel et al., 2000; Wetzel et al., 2002), and/or to follow intracellular signaling events (Miyawaki et al., 1999; Siegel et al., 2000; Ting et al., 2001). Endogenous expression of fluorescent reporters is also essential for moving beyond the current methods of adoptive transfer of ex vivo manipulated, dye-labeled cells that are particularly problematic with respect to DC biology (Banchereau and Steinman, 1998). The use of CD43-GFP to reveal T-DC immunological synapse formation in vivo shows the feasibility of this approach (Delon et al., 2001; Stoll et al., 2002), but many hurdles remain. Current microscope detectors require rather high levels of fluorescence from cells for adequate imaging deep in tissues, but for many molecules, this demands gross overexpression of the protein of interest (Tsien, 1998), with the likely outcome that the normal physiology of the cell will be disturbed. Fluorescent protein expression by all of a particular population of cells present in a transgenic animal can make the image too complex for analysis. Only a few color variants of GFP with substantially distinct emission spectra are available (Tsien, 1998; Zhang et al., 2002), limiting the extent to which several cells or molecular parameters can be followed simultaneously. Retroviral transduction of activated T cells results in poor homing of these cells to LN, although culture in certain cytokines can minimize this problem (Weninger et al., 2001).

Ways around these limitations are emerging. Infection of bone marrow precursors followed by generation of chimeric animals can be used to obtain fluorescent naive cells that localize to the LN upon further transfer (Stoll et al., 2002). Cells purified from animals with global expression of a fluorescent protein (Okabe et al., 1997) can also be employed for this purpose, an approach that prevents the loss of signal that accompanies cell division of dye-labeled cells. Some of the other problems will disappear as new generations of detectors appear with enhanced sensitivity, as spectral separation hardware and software makes distinguishing minor color variants of fluorescent proteins more practical (Dickinson et al., 2003), as new fluorescent proteins with enhanced quantum efficiencies and more distinct emission spectra are developed (Kalb et al., 2004; Zhang et al., 2002), and as 64-bit computers allow more channels of data collection and analysis. As just one example of the potential of active reporter technology in the context of two-photon imaging, we have been able to readily visualize two different fluorescent proteins simultaneously in the LN of immunized mice carrying IL-2 promoter/enhancer driven fluorescent protein transgenes (D. Bruniquel, R.H. Schwartz, R.N.G., and A.Y.C.H., unpublished data). Other studies have shown the feasibility of imaging sites such as Peyer's patches and of combining analysis of lymphoid cells with infectious agents that express endogenous fluorescent proteins (M. Chieppa, unpublished data).

Finding Ag-Bearing DCs: Chance Encounter or Planned Date?

How do rare antigen-specific T cells find the only slightly more abundant antigen-bearing DCs that are present in LNs draining a peripheral site of infection or presenting a tissue-specific antigen in the "steady-state" (Scheinecker et al., 2002), thus generating the long-lived cell clusters described above? One intriguing feature of T-DC interactions that has been reported and that our own imaging has confirmed is the role the dendrites of DCs play in promoting antigen-dependent cell interactions (Miller et al., 2004). Both in vitro (K. Eichelberg, unpublished data) and in situ (Movie 6, available online at http://www.immunity.com/cgi/content/full/21/3/331/DC1), T cells often first touch the tip of a rapidly moving dendrite when they are in the vicinity of a mature DC (Miller et al., 2004). When the latter has cognate foreign pMHC ligands on its surface, this brief and physically limited contact frequently results in a rapid change in local T cell membrane morphology and the movement of the T cell into close contact with the body of the presenting cell. Cahalan and colleagues have measured the volume swept by DC processes in situ and estimated that they increase the effective zone of potential T cell contact by at least 3-fold (Miller et al., 2004). These data provide a dramatic example of how dynamic imaging can link morphology with function.

Is the entry of a T cell into the contact range of a DC a random or directed event? Innate receptor (e.g., TLR) signals that accompany pathogen invasion induce activated DC to produce chemokines recognized by receptors on memory T cells (Tang and Cyster, 1999). This implies that directional T cell migration toward an antigen-bearing DC should be seen under such conditions. More limited evidence suggests that such activated DC also make at least one chemokine (CCL19) (Ngo et al., 1998) that interacts with a receptor (CCR7) found on naive T cells (Butcher et al., 1999). Naive T cells entering the LN through HEV might thus be expected to chemotax toward mature DCs that had trafficked through the afferent lymphatic and entered the paracortical region through the floor of the subcapsular sinus (Weinlich et al., 1998). These Ag-specific T cells would then be retained in this region following TCR engagement by the "stop" signal arising from this engagement that is partly

the consequence of increased integrin-mediated adhesion (Dustin et al., 1997). T cells that did not find a cognate ligand would presumably show only transient contacts with these DCs. In the case of a noninfected host lacking inflammatory signals that promote DC activation, it is less clear whether chemotactic signals would be available to guide T cells to these key antigen-presenting cells, and thus, one might expect a different pattern of T cell migration within the LN under such conditions.

Cahalan and colleagues were the first to conduct the extensive quantitative analyses necessary to evaluate whether T cells show any particular pattern in their migratory behavior. Their in situ analyses of T cell motility have challenged the notion that DCs (activated or not) provide specific location cues to T cells and modify the their directionality within lymphoid tissues (Cahalan et al., 2002; Wei et al., 2003). These investigators reported that the mean displacement of a migrating naïve $CD4^+$ T cell population was not linearly proportional to the amount of time they spend in locomotion, as would be expected for directional movement; rather, the displacement was linearly proportional to the square root of time, a characteristic feature of random diffusion that has most often been used to describe the behavior of molecules rather than cells. This feature of T cell migration was also reported to be shared by B lymphocytes in the primary follicle (Cahalan et al., 2002) and similar data have been presented for $CD8^+$ T cells by Mempel et al. (2004). Furthermore, in a separate study Cahalan's group specifically analyzed T cell motion in the immediate vicinity of endogenous antigen-bearing DCs that were presumed to be mature and activated, once again failing to find a directional pattern (Miller et al., 2004). Clearly, these results are not consistent with a chemotaxis-driven T-DC encounter for either the steady-state condition or in the presence of inflammatory activation of DC. These investigators thus proposed that the initial meeting between Ag-specific T cells and Ag-bearing DCs is a stochastic process in which T cells and antigen-bearing DCs mutually scan for potential partners through autonomous "random walks" (Miller et al., 2003).

While there is little direct evidence at present for T cell chemotaxis in vivo, it is also necessary to be cautious about concluding from these data that lymphocytes truly engage only in "random walks." First, the proper use of this term in physics requires the observations made of a system to meet a number of rather stringent conditions, of which the displacement-time relationship as used in the above-mentioned studies is a necessary, but not sufficient, one. A more comprehensive way of conducting such an analysis involves simulating a pure random walk in silico as a control and testing experimental observations against the results of such simulations, similar to the practice employed in studies of molecular dynamics involving membranes (Saxton and Jacobson, 1997). Second, due to the technical limitations already discussed, the physical complexity of lymph node structure is not visible to an observer with the current two-photon methods. Therefore, certain seemingly autonomous features of lymphocyte motility may actually be imposed by structural constrains and not by the lack of an underlying chemotactic drive in the system.

One possible illustration of this process of guided T cell mobility involves the observation that naive T cells appear to rapidly reverse direction upon reaching the border of a B cell follicle (Miller et al., 2003) (Movies 7 and 8, available online at http://www.immunity.com/cgi/content/full/21/3/331/DC1). This is quite difficult to explain by the current random walk interpretation of T cell mobility. However, structural analyses of the fibroblastic reticular cell (FRC) network of lymph nodes have revealed a striking difference between primary follicles and the adjacent T cell cortex: collagen fibers and the fibroblastoid reticular cells (FRCs) that wrap around them are abundant in the T zone but sparse in the follicle (Kaldjian et al., 2001). Thus, an intriguing explanation for the behavior of naive T cells at this border zone is that they preferentially utilize the FRC network as the substrate for migration and will not continue moving beyond the limits of this "track" system. Such confinement of T cell movement to extracellular matrix elements has been observed in the collagen gel matrix model (Friedl et al., 1998). If this notion of physical constraints is correct, then activated T cells that have acquired CXCR5 expression and are attracted to forming germinal centers within B cell follicles (Ansel et al., 1999) must either lose this limitation to permit their migration through the follicle or the follicle must undergo some remodeling to create the necessary "tracks" for T cell invasion.

A related point is that because the FRC network has been shown to divide the cortex into interconnected "labyrinthine cavities" (Kaldjian et al., 2001), the distribution of any potential chemokine gradients in the T zone is unlikely to be linear in space. Thus, even when T cells are indeed committed to chemokine-directed migration, they would have to change directions as often as required by the dimensions of individual cavities and the convoluted distribution of chemokines. This would certainly increase the apparent randomness as measured in the displacement-versus-time analysis, blurring the distinction between chemotaxis and true random motion. Finally, in the case of local behaviors near DCs, at least for mature activated DCs that are known to produce T cell-attracting chemokines (Morelli et al., 2001; Nagorsen et al., 2004; Ngo et al., 1998; Tang and Cyster, 1999), one wonders if chemokine production by nonfluorescent DCs in the vicinity complicates the analysis.

As evident from the above discussion, the few live tissue-imaging studies performed to date have yet to define the precise dynamics by which the initial encounter of Ag-specific T cells and DCs occurs. On the other hand, a conceptual framework for this line of research may be extracted from static imaging analyses of tissue sections. By using DO11.10 $CD4^+$ T cells and peptide-pulsed splenic DCs in a adoptive transfer model, Ingulli et al. (1997) observed T-DC clusters in draining lymph node as early as 8 hr after subcutaneous DC injection. While the size and number of these clusters increased substantially within the next 16 hr, their general location appeared to remain the same: the paracortical T cell region close to B cell follicles (Ingulli et al., 1997). Because at least for peripheral lymph nodes, this region is supplied with abundant HEV (Gretz et al., 1997), it is interesting to consider the possibility that DCs coming from peripheral tissues and T cells coming from the circulation would concentrate their scanning around

HEV areas in seeking their cognate partners, with the result that many such interactions would occur shortly after T cell diapedesis from the blood rather than after a prolonged period of wandering deep in the LN parenchyma. As noted above, tissue-derived DCs are preferentially localized to the vicinity of HEVs 24 hr after immunization and these DCs form clusters with Ag-specific T cells in this location shortly after the adoptive transfer of the latter (Bajenoff et al., 2003). Similarly, a high concentration of interacting T cells and DCs was also observed in the interfollicular ("cortical ridge") region near the HEV in a separate study (Katakai et al., 2004). Such a preferential distribution of incoming DCs to HEV regions has been confirmed by two published live imaging analyses, although neither study revealed directional migration of DCs based on displacement-time analysis (Mempel et al., 2004; Miller et al., 2004). The concentration/retention of DC and T cells in this region might be driven by the locally high level of CCL21 acting on CCR7 expressed by both the incoming DC and lymphocytes (Cyster, 2000).

This mechanism could make T cell detection of Ag-bearing DC very efficient, as all the incoming T cells from the blood would face a dense gauntlet of antigen-bearing DCs shortly after entering the LN. To precisely estimate the efficiency of such strategy, one would need knowledge of the life span and antigen-presentation competency of individual DCs around HEVs and the frequency of T cell egressing from HEVs, all of which can be analyzed by live-tissue imaging. Furthermore, while the static imaging analysis cannot formally differentiate recent immigrant T cells from the blood and those cells relocated from the deep cortex (Bajenoff et al., 2003), live-tissue imaging provides an excellent opportunity to test whether Ag-specific T cells recently homing from HEVs indeed dominate during the early interactions with Ag-bearing DCs.

Concluding Remarks

For many tissues and organs whose cells in the adult have a fixed relationship to one another, a great deal of understanding can be obtained by classical static histological examination. While the details of the underlying stromal architecture of lymphoid tissues can be usefully determined by the same techniques, the hematopoietic elements of the immune system are constantly changing partners and positions while moving between the blood circulation, lymphatic vessels, secondary lymphoid elements, and inflamed peripheral tissues. Only through dynamic imaging modalities can these features of immune cell behavior be appreciated, quantified, and related to function. Furthermore, only microscopic-based methods are currently able to provide enough resolution to allow examination of the membrane shape changes and protein redistributions that play major roles in guiding cellular immune responses.

Many of the results obtained to date by these new dynamic optical imaging methods merely confirm preexisting views derived from less direct approaches, which is understandable given the early stage of development and application of this technology. Some potential new insights have emerged, especially concerning the rates of lymphocyte migration within the LN parenchyma and the duration as well as frequency of T-DC contacts, but even in these areas, limitations in both the methods and our ability to interpret the images are evident. This is particularly true with respect to the complexity of lymph node structure and the substantial variation in lymphocyte and DC behavior in distinct subregions of these organs. It is also important to keep in mind that as beautiful as such images can be, they complement rather than replace more traditional methods of immunological investigation that provide a richer assessment of function as opposed to just form.

Despite these current constraints, the future is bright (no pun intended). With improvements in the tool kit for microscopic imaging studies, there will surely be an increasingly sophisticated set of observations emerging in the near future on the dynamic aspects of immune cell behavior in situ. With careful and cautious interpretation, together with the right cohort of accompanying studies, we can look forward to a deepening understanding of how the intricate dances of immune cells contribute to the overall behavior of the system.

Acknowledgments

The authors wish to thank Ina Ifrim for her valuable help in establishing two-photon microscopy in the LBS. They also thank Katrin Eichelberg, Marcello Chieppa, Denis Bruniquel, Ronald Schwartz, and Parizad Torabi-Parizi for allowing them to mention some of their unpublished observations, along with Jackson Egen for his helpful comments on the manuscript. A.Y.C.H. and H.Q. are supported by postdoctoral fellowship grants from Cancer Research Institute.

Received: July 16, 2004
Revised: August 10, 2004
Accepted: August 11, 2004
Published: September 14, 2004

References

Ansel, K.M., McHeyzer-Williams, L.J., Ngo, V.N., McHeyzer-Williams, M.G., and Cyster, J.G. (1999). In vivo-activated CD4 T cells upregulate CXC chemokine receptor 5 and reprogram their response to lymphoid chemokines. J. Exp. Med. *190*, 1123–1134.

Bajenoff, M., Granjeaud, S., and Guerder, S. (2003). The strategy of T cell antigen-presenting cell encounter in antigen-draining lymph nodes revealed by imaging of initial T cell activation. J. Exp. Med. *198*, 715–724.

Banchereau, J., and Steinman, R.M. (1998). Dendritic cells and the control of immunity. Nature *392*, 245–252.

Benvenuti, F., Lagaudriere-Gesbert, C., Grandjean, I., Jancic, C., Hivroz, C., Trautmann, A., Lantz, O., and Amigorena, S. (2004). Dendritic cell maturation controls adhesion, synapse formation, and the duration of the interactions with naive T lymphocytes. J. Immunol. *172*, 292–301.

Bousso, P., Bhakta, N.R., Lewis, R.S., and Robey, E. (2002). Dynamics of thymocyte-stromal cell interactions visualized by two-photon microscopy. Science *296*, 1876–1880.

Bousso, P., and Robey, E. (2003). Dynamics of $CD8^+$ T cell priming by dendritic cells in intact lymph nodes. Nat. Immunol. *4*, 579–585.

Butcher, E.C., Williams, M., Youngman, K., Rott, L., and Briskin, M. (1999). Lymphocyte trafficking and regional immunity. Adv. Immunol. *72*, 209–253.

Cahalan, M.D., Parker, I., Wei, S.H., and Miller, M.J. (2002). Two-photon tissue imaging: seeing the immune system in a fresh light. Nat. Rev. Immunol. *2*, 872–880.

Caldwell, C.C., Kojima, H., Lukashev, D., Armstrong, J., Farber, M., Apasov, S.G., and Sitkovsky, M.V. (2001). Differential effects of phys-

iologically relevant hypoxic conditions on T lymphocyte development and effector functions. J. Immunol. *167*, 6140–6149.

Choy, G., Choyke, P., and Libutti, S.K. (2003). Current advances in molecular imaging: noninvasive in vivo bioluminescent and fluorescent optical imaging in cancer research. Mol. Imaging *2*, 303–312.

Cyster, J.G. (2000). Leukocyte migration: scent of the T zone. Curr. Biol. *10*, R30–R33.

Delon, J., Kaibuchi, K., and Germain, R.N. (2001). Exclusion of CD43 from the immunological synapse Is mediated by phosphorylation-regulated relocation of the cytoskeletal adaptor moesin. Immunity *15*, 691–701.

Dickinson, M.E., Simbuerger, E., Zimmermann, B., Waters, C.W., and Fraser, S.E. (2003). Multiphoton excitation spectra in biological samples. J. Biomed. Opt. *8*, 329–338.

Dustin, M.L., Bromley, S.K., Kan, Z., Peterson, D.A., and Unanue, E.R. (1997). Antigen receptor engagement delivers a stop signal to migrating T lymphocytes. Proc. Natl. Acad. Sci. USA *94*, 3909–3913.

Dustin, M.L., and Colman, D.R. (2002). Neural and immunological synaptic relations. Science *298*, 785–789.

Egen, J.G., and Allison, J.P. (2002). Cytotoxic T lymphocyte antigen-4 accumulation in the immunological synapse is regulated by TCR signal strength. Immunity *16*, 23–35.

Ford, W.L., and Gowans, J.L. (1969). The traffic of lymphocytes. Semin. Hematol. *6*, 67–83.

Friedl, P., and Gunzer, M. (2001). Interaction of T cells with APCs: the serial encounter model. Trends Immunol. *22*, 187–191.

Friedl, P., Zanker, K.S., and Brocker, E.B. (1998). Cell migration strategies in 3-D extracellular matrix: differences in morphology, cell matrix interactions, and integrin function. Microsc. Res. Tech. *43*, 369–378.

Germain, R.N., and Jenkins, M.K. (2004). In vivo antigen presentation. Curr. Opin. Immunol. *16*, 120–125.

Gretz, J.E., Anderson, A.O., and Shaw, S. (1997). Cords, channels, corridors and conduits: critical architectural elements facilitating cell interactions in the lymph node cortex. Immunol. Rev. *156*, 11–24.

Gunzer, M., Schafer, A., Borgmann, S., Grabbe, S., Zanker, K.S., Brocker, E.B., Kampgen, E., and Friedl, P. (2000). Antigen presentation in extracellular matrix: interactions of T cells with dendritic cells are dynamic, short lived, and sequential. Immunity *13*, 323–332.

Hardy, J., Edinger, M., Bachmann, M.H., Negrin, R.S., Fathman, C.G., and Contag, C.H. (2001). Bioluminescence imaging of lymphocyte trafficking *in vivo*. Exp. Hematol. *29*, 1353–1360.

Herschman, H.R. (2003). Micro-PET imaging and small animal models of disease. Curr. Opin. Immunol. *15*, 378–384.

Hu-Li, J., Pannetier, C., Guo, L., Lohning, M., Gu, H., Watson, C., Assenmacher, M., Radbruch, A., and Paul, W.E. (2001). Regulation of expression of IL-4 alleles: analysis using a chimeric GFP/IL-4 gene. Immunity *14*, 1–11.

Huppa, J.B., Gleimer, M., Sumen, C., and Davis, M.M. (2003). Continuous T cell receptor signaling required for synapse maintenance and full effector potential. Nat. Immunol. *4*, 749–755.

Hurez, V., Saparov, A., Tousson, A., Fuller, M.J., Kubo, T., Oliver, J., Weaver, B.T., and Weaver, C.T. (2003). Restricted clonal expression of IL-2 by naive T cells reflects differential dynamic interactions with dendritic cells. J. Exp. Med. *198*, 123–132.

Iezzi, G., Karjalainen, K., and Lanzavecchia, A. (1998). The duration of antigenic stimulation determines the fate of naive and effector T cells. Immunity *8*, 89–95.

Ingulli, E., Mondino, A., Khoruts, A., and Jenkins, M.K. (1997). *In vivo* detection of dendritic cell antigen presentation to $CD4^+$ T cells. J. Exp. Med. *185*, 2133–2141.

Ingulli, E., Ulman, D.R., Lucido, M.M., and Jenkins, M.K. (2002). In situ analysis reveals physical interactions between $CD11b^+$ dendritic cells and antigen-specific CD4 T cells after subcutaneous injection of antigen. J. Immunol. *169*, 2247–2252.

Jenkins, M.K., Khoruts, A., Ingulli, E., Mueller, D.L., McSorley, S.J., Reinhardt, R.L., Itano, A., and Pape, K.A. (2001). *In vivo* activation of antigen-specific CD4 T cells. Annu. Rev. Immunol. *19*, 23–45.

Kalb, J., Nielsen, T., Fricke, M., Egelhaaf, M., and Kurtz, R. (2004). In vivo two-photon laser-scanning microscopy of $Ca2^+$ dynamics in visual motion-sensitive neurons. Biochem. Biophys. Res. Commun. *316*, 341–347.

Kaldjian, E.P., Gretz, J.E., Anderson, A.O., Shi, Y., and Shaw, S. (2001). Spatial and molecular organization of lymph node T cell cortex: a labyrinthine cavity bounded by an epithelium-like monolayer of fibroblastic reticular cells anchored to basement membrane-like extracellular matrix. Int. Immunol. *13*, 1243–1253.

Katakai, T., Hara, T., Lee, J.H., Gonda, H., Sugai, M., and Shimizu, A. (2004). A novel reticular stromal structure in lymph node cortex: an immuno-platform for interactions among dendritic cells, T cells and B cells. Int. Immunol. *16*, 1133–1142. Published online July 5, 2004. 10.1093/intimm/dxh113.

Kearney, E.R., Pape, K.A., Loh, D.Y., and Jenkins, M.K. (1994). Visualization of peptide-specific T cell immunity and peripheral tolerance induction *in vivo*. Immunity *1*, 327–339.

Kircher, M.F., Allport, J.R., Graves, E.E., Love, V., Josephson, L., Lichtman, A.H., and Weissleder, R. (2003). *In vivo* high resolution three-dimensional imaging of antigen-specific cytotoxic T-lymphocyte trafficking to tumors. Cancer Res. *63*, 6838–6846.

Krummel, M.F., Sjaastad, M.D., Wulfing, C., and Davis, M.M. (2000). Differential clustering of CD4 and CD3ζ during T cell recognition. Science *289*, 1349–1352.

MacPhee, P.J., Schmidt, E.E., and Groom, A.C. (1992). Evidence for Kupffer cell migration along liver sinusoids, from high-resolution in vivo microscopy. Am. J. Physiol. *263*, G17–G23.

Mempel, T.R., Henrickson, S.E., and Von Andrian, U.H. (2004). T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. Nature *427*, 154–159.

Mempel, T.R., Moser, C., Hutter, J., Kuebler, W.M., and Krombach, F. (2003). Visualization of leukocyte transendothelial and interstitial migration using reflected light oblique transillumination in intravital video microscopy. J. Vasc. Res. *40*, 435–441.

Miller, M.J., Wei, S.H., Parker, I., and Cahalan, M.D. (2002). Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. Science *296*, 1869–1873.

Miller, M.J., Wei, S.H., Cahalan, M.D., and Parker, I. (2003). Autonomous T cell trafficking examined in vivo with intravital two-photon microscopy. Proc. Natl. Acad. Sci. USA *100*, 2604–2609.

Miller, M.J., Hejazi, A.S., Wei, S.H., Cahalan, M.D., and Parker, I. (2004). T cell repertoire scanning is promoted by dynamic dendritic cell behavior and random T cell motility in the lymph node. Proc. Natl. Acad. Sci. USA *101*, 998–1003.

Miyawaki, A., Griesbeck, O., Heim, R., and Tsien, R.Y. (1999). Dynamic and quantitative $Ca2^+$ measurements using improved cameleons. Proc. Natl. Acad. Sci. USA *96*, 2135–2140.

Mohrs, M., Shinkai, K., Mohrs, K., and Locksley, R.M. (2001). Analysis of type 2 immunity in vivo with a bicistronic IL-4 reporter. Immunity *15*, 303–311.

Morelli, A.E., Zahorchak, A.F., Larregina, A.T., Colvin, B.L., Logar, A.J., Takayama, T., Falo, L.D., and Thomson, A.W. (2001). Cytokine production by mouse myeloid dendritic cells in relation to differentiation and terminal maturation induced by lipopolysaccharide or CD40 ligation. Blood *98*, 1512–1523.

Nagorsen, D., Marincola, F.M., and Panelli, M.C. (2004). Cytokine and chemokine expression profiles of maturing dendritic cells using multiprotein platform arrays. Cytokine *25*, 31–35.

Naramura, M., Hu, R.J., and Gu, H. (1998). Mice with a fluorescent marker for interleukin 2 gene activation. Immunity *9*, 209–216.

Ngo, V.N., Tang, H.L., and Cyster, J.G. (1998). Epstein-Barr virus-induced molecule 1 ligand chemokine is expressed by dendritic cells in lymphoid tissues and strongly attracts naive T cells and activated B cells. J. Exp. Med. *188*, 181–191.

Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T., and Nishimune, Y. (1997). "Green mice" as a source of ubiquitous green cells. FEBS Lett. *407*, 313–319.

Saxton, M.J., and Jacobson, K. (1997). Single-particle tracking: ap-

plications to membrane dynamics. Annu. Rev. Biophys. Biomol. Struct. *26*, 373–399.

Scheinecker, C., McHugh, R., Shevach, E.M., and Germain, R.N. (2002). Constitutive presentation of a natural tissue autoantigen exclusively by dendritic cells in the draining lymph node. J. Exp. Med. *196*, 1079–1090.

Siegel, R.M., Chan, F.K., Zacharias, D.A., Swofford, R., Holmes, K.L., Tsien, R.Y., and Lenardo, M.J. (2000). Measurement of molecular interactions in living cells by fluorescence resonance energy transfer between variants of the green fluorescent protein. Science's STKE 2000: pl1 10.1126/stke.2000.38.pl1.

Stefanova, I., Dorfman, J.R., and Germain, R.N. (2002). Self-recognition promotes the foreign antigen sensitivity of naive T lymphocytes. Nature *420*, 429–434.

Stingl, G., Katz, S.I., Clement, L., Green, I., and Shevach, E.M. (1978). Immunologic functions of Ia-bearing epidermal Langerhans cells. J. Immunol. *121*, 2005–2013.

Stoll, S., Delon, J., Brotz, T.M., and Germain, R.N. (2002). Dynamic imaging of T cell-dendritic cell interactions in lymph nodes. Science *296*, 1873–1876.

Tang, H.L., and Cyster, J.G. (1999). Chemokine Up-regulation and activated T cell attraction by maturing dendritic cells. Science *284*, 819–822.

Teunissen, M.B., Rongen, H.A., and Bos, J.D. (1994). Function of adhesion molecules lymphocyte function-associated antigen-3 and intercellular adhesion molecule-1 on human epidermal Langerhans cells in antigen-specific T cell activation. J. Immunol. *152*, 3400–3409.

Ting, A.Y., Kain, K.H., Klemke, R.L., and Tsien, R.Y. (2001). Genetically encoded fluorescent reporters of protein tyrosine kinase activities in living cells. Proc. Natl. Acad. Sci. USA *98*, 15003–15008.

Tsien, R.Y. (1998). The green fluorescent protein. Annu. Rev. Biochem. *67*, 509–544.

von Andrian, U.H., and Mackay, C.R. (2000). T-cell function and migration. Two sides of the same coin. N. Engl. J. Med. *343*, 1020–1034.

Warnock, R.A., Askari, S., Butcher, E.C., and von Andrian, U.H. (1998). Molecular mechanisms of lymphocyte homing to peripheral lymph nodes. J. Exp. Med. *187*, 205–216.

Wei, S.H., Parker, I., Miller, M.J., and Cahalan, M.D. (2003). A stochastic view of lymphocyte motility and trafficking within the lymph node. Immunol. Rev. *195*, 136–159.

Weinlich, G., Heine, M., Stossel, H., Zanella, M., Stoitzner, P., Ortner, U., Smolle, J., Koch, F., Sepp, N.T., Schuler, G., and Romani, N. (1998). Entry into afferent lymphatics and maturation in situ of migrating murine cutaneous dendritic cells. J. Invest. Dermatol. *110*, 441–448.

Weninger, W., Crowley, M.A., Manjunath, N., and von Andrian, U.H. (2001). Migratory properties of naive, effector, and memory $CD8^+$ T cells. J. Exp. Med. *194*, 953–966.

Wetzel, S.A., McKeithan, T.W., and Parker, D.C. (2002). Live-cell dynamics and the role of costimulation in immunological synapse formation. J. Immunol. *169*, 6092–6101.

Yamashita, U., and Shevach, E.M. (1978). The histocompatibility restrictions on macrophage T-helper cell interaction determine the histocompatibility restrictions on T-helper cell B-cell interaction. J. Exp. Med. *148*, 1171–1185.

Yuste, R., Majewska, A., Cash, S.S., and Denk, W. (1999). Mechanisms of calcium influx into hippocampal spines: heterogeneity among spines, coincidence detection by NMDA receptors, and optical quantal analysis. J. Neurosci. *19*, 1976–1987.

Zhang, J., Campbell, R.E., Ting, A.Y., and Tsien, R.Y. (2002). Creating new fluorescent probes for cell biology. Nat. Rev. Mol. Cell Biol. *3*, 906–918.

Zoumi, A., Yeh, A., and Tromberg, B.J. (2002). Imaging cells and extracellular matrix in vivo by using second-harmonic generation and two-photon excited fluorescence. Proc. Natl. Acad. Sci. USA *99*, 11014–11019.