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Imaging the choreography of lymphocyte trafficking and the immune response

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The functioning of the immune system depends upon exquisitely choreographed interactions between its cellular constituents. Two-photon microscopy now enables us to visualize cell motility and cell–cell interactions deep within intact tissues and organs, both in explanted preparations and *in vivo*. Real-time immunoimaging techniques have illuminated the roles of random and chemokine-driven motility for cellular search strategies, the complex dynamics of cellular interactions, and the micro-anatomical localization and control of lymphocyte trafficking. Recently, advances have been made in these areas of research, as exemplified by studies investigating T cell–dendritic cell interactions, T cell–B cell interactions, and the regulation of lymphocyte egress from the lymph node.

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Introduction

The immune system is the most disseminated and mobile system in our bodies. Its component parts (largely individual cells) continuously circulate, endlessly surveying peripheral tissues for possible infection, transiting through lymphoid organs and returning to mount defenses. Key aspects of the immune response thus involve the intrinsic motility of the immune cells themselves, external factors that regulate their trafficking, and cell–cell interactions such as the presentation of antigens by dendritic cells (DCs) to T cells within lymph nodes.

Until recently, studies of these processes had been constrained by methodological limitations, and two distinct lines of investigation have dominated the field: first, *in vivo* experiments that examine the behavior of populations of cells in living animals during an immune response; and, second, *in vitro* experiments that utilize individual cells in artificial environments. However, the immune response is the sum of many complex and dynamic individual cellular behaviors that are shaped by a host of environmental factors. Whereas *in vivo* experiments maintain this natural environment, histological studies of fixed preparations ('snapshot immunology') cannot resolve the behaviors of individual cells. Conversely, *in vitro* experiments provide real-time, single-cell information, but fail to replicate the complexity and dynamics of intact tissue environments.

This situation changed dramatically in 2002 with the application of laser-scanning microscopy to image the real-time behavior of individual cells within intact lymphoid organs and tissue slices. In particular, two-photon (2-photon) microscopy [1] has enabled non-injurious visualization of motility and of interactions of fluorescently labelled cells at depths of hundreds of μm into tissues and organs including lymph node [2–9,10••,11], thymus [12,13•,14,15•], spleen [16], intestinal tissue [17] and brain slices [18]. The method relies upon scanning a pulsed laser beam such that fluorescence is excited by the nearly simultaneous absorption of two low-energy (long-wavelength) photons only at the plane of focus. To date, most studies have employed 'explanted' preparations, surgically removed from the animal and maintained in warmed, oxygenated, artificial medium, but 2-photon imaging has also been extended to surgically exposed 'intravital' preparations in an anesthetized animal, permitting visualization within lymph nodes [3,7]. Immunoimaging studies have employed both explants (an organ or tissue surgically removed from the animal and maintained in a warmed, oxygenated artificial medium) and intravital preparations where an organ or tissue is imaged following minimal surgery to expose it within the living, anesthetized animal [3,7]. Explant and intravital imaging preparations each offer differing advantages for specific experiments but, whenever possible, it is advisable that results obtained using explants be confirmed by intravital imaging (Table 1).

During the past two years there has been explosive growth in this new field of 'immunoimaging' as well as a growing appreciation of the importance of single-cell dynamics and cell–cell contacts. Several major themes have emerged. An example is the question of random versus directed cell motility. Initial reports have demonstrated that T cells in the lymph node parenchyma follow a random walk [2,3], but examples of chemokine-directed and structurally regulated motility have subsequently

Table 1

Explant versus intravital preparations.

	Pro	Con
Explant/slice	Technical ease; higher throughput (e.g. image multiple nodes from a single mouse). Greater stability (no pulse or respiratory movements). Imaging access from all surfaces. Defined superfusion medium; relatively rapid addition and washout of drugs. Applicable to biopsied human tissue.	Vasculature and lymphatics present, but no flow. Innervation lost. Concerns regarding physiological oxygenation within the tissue. (Most studies employ medium bubbled with 95% O_2/5% CO_2.)
Intravital	Truly *in vivo* environment in which vascular and lymphatic circulation are maintained. Physiological oxygenation. Possible to image homing and egress processes. Intact innervation.	Technically difficult; lower success rate and throughput. Requirement for rigid mounting to minimize pulse and respiratory movement artefacts while allowing unimpeded blood and lymph flow. Possible effects of anaesthetics. Potential inflammation and trauma from surgery. Restricted access for imaging. Temperature control less precise, leading to variation in lymphocyte velocities. Pharmacological studies hindered by whole-animal pharmacokinetics and by possible effects on other organ systems.

In general, cellular behaviors observed in appropriately oxygenated explants closely replicated those in *in vivo* preparations; for example, velocities and motility patterns of naïve T cells are comparable in both preparations [2,3].

emerged [10••,15•]. Other topics of discussion surround the exquisite and varied choreography of various cell–cell interactions during activation of the immune response, and the importance of local tissue organization and structural elements. In 2-photon images, fluorescently labelled cells appear to 'swim' within a black void, but in reality they are, of course, surrounded by numerous unlabeled cells and stromal elements.

In this article, we review progress in elucidating these topics during the past two years, as exemplified by studies investigating T cell–DC and T cell–B cell interactions, as well as the regulation of lymphocyte egress from the node (Figure 1).

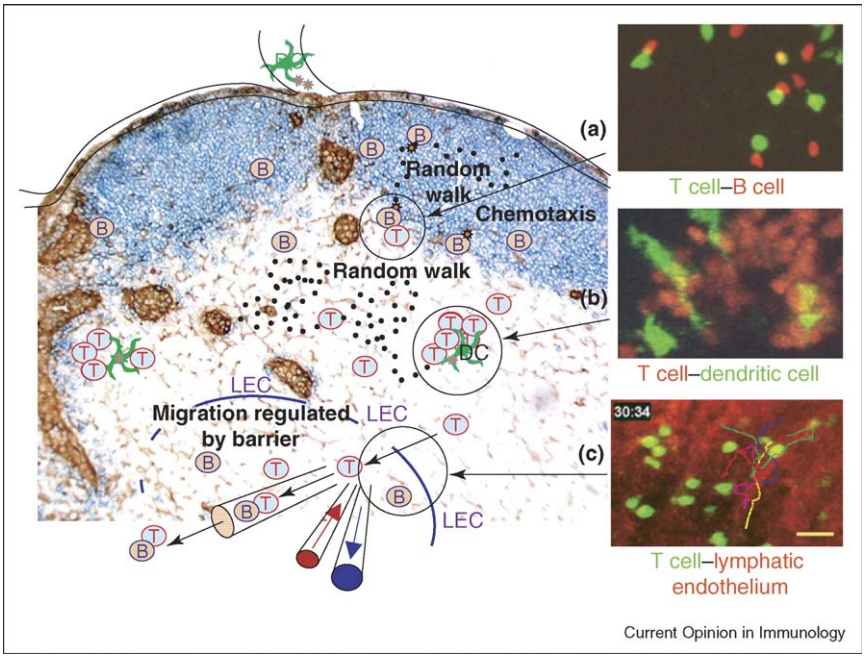
T cell interactions with dendritic cells

DCs ingest antigens in peripheral tissues and, in response to inflammatory signals, migrate to lymph nodes where they present MHC-bound peptide antigens to re-circulating T cells, thereby initiating the adaptive immune response. The cell dynamics of the immune response had long been hidden within the 'black box' of the lymph node, but *in vivo* imaging studies are now beginning to answer two key questions: how do T cells and DCs find one another; and what is the nature of their mutual interactions that lead to T cell activation?

It now appears that random encounters driven by T cell motility and DC probing play a vital role in repertoire scanning to detect the presence of antigen. According to this theory, the frequency of T cell contacts with DC processes in the lymph node, estimated to be 500 per DC per hour for $CD8^+$ T cell contacts by Bousso and Robey [6] and 5000 per DC per hour for $CD4^+$ contacts by Miller *et al.* [4], is sufficient to allow rare antigen to be detected by the pool of T cells in a lymph node within a reasonable timeframe. But, in addition, there are probably other structural factors involved, including the network of DCs [9] through which T cells must 'run the gauntlet' immediately after entering the node at sites of homing within high endothelial venules. Thus, T cells that are entering the lymph node would have a high probability of encountering antigen soon after their arrival, enhancing the likelihood of antigen detection over and above a purely stochastic mechanism. In addition, DCs might change their properties in response to inflammation or to local signaling to recruit T cells, either by emitting chemokines to exert localized chemotaxis or perhaps even by directly contacting additional T cells in the vicinity through submicroscopic processes termed 'tunneling nanotubes' [19].

In vitro observations using cell culture systems have provided a wealth of molecular detail regarding the functional contact (immunological synapse) between lymphocytes and antigen-presenting cells, yet the cellular dynamics of this central process remained poorly understood. In particular, contrasting studies pointed to requirements for either sustained (many hours) [20,21] or sequential brief (approximately 15 min) contacts [22]. Two independent 2-photon imaging studies of $CD4^+$ T cells [4,5] and $CD8^+$ T cells [7] in the lymph node have now revealed an elaborately choreographed sequence of interactions during antigen activation that lead to a robust T cell response. These groups utilized different experimental approaches (explant versus *in vivo* preparations, and subcutaneous labeling of DC versus transfer of *in*

Figure 1



Localization of different cell–cell interactions within a lymph node. The schematic shows T cells (T), B cells (B), dendritic cells (DC), lymphatic endothelial cells (LEC) and antigen. **(a)** Chemotaxis of B cells to the follicular border and interaction with helper T cells to form motile conjugate pairs. **(b)** Interaction of antigen-specific T cells with antigen-bearing dendritic cells within the T cell zone, leading to initial transient interactions and subsequent clusters, as illustrated here. **(c)** Regulation of T cell egress into medullary sinuses under basal conditions without antigen by stromal endothelial cells that constitute a barrier that can be closed by $S1P_1$ receptor agonists.

vitro labeled DCs, respectively), but reached the broadly similar conclusion that T cell priming progresses through distinct phases that involve sequential T cell–DC contacts of varying duration.

Mempel *et al.* [7] described three distinct phases of $CD8^+$ T cell priming: first, multiple brief encounters with DCs; second, long-lasting stable DC–T cell conjugates; and a third phase, coincident with T cell proliferation, which involves short DC contacts. T cell motility was reduced during the first phase when events have been initiated, including upregulation of CD69, but high motility resumed on day two as cells began to proliferate. Surprisingly, this three-phase trafficking program was also observed in the absence of antigen, although the second phase was shorter. Mempel *et al.* thus proposed that recirculating lymphocytes pass through the same three phases during their transit across the lymph node independent of cognate antigen. Although sharing many similarities, the subsequent findings of Miller *et al.* [5] for $CD4^+$ cells revealed several key differences. Most importantly, Miller *et al.* did not observe antigen-independent changes in naïve T cell behavior; the vast majority of $CD4^+$ T cells maintained robust motility and made only brief contacts with DCs, regardless of the time after adoptive transfer. It is presently unclear whether this might reflect fundamental differences in trafficking behaviors between $CD4^+$ and $CD8^+$ T cells or if it results from methodological differences. A further difference was that Miller *et al.* observed contacts between $CD4^+$ T cells and DCs primarily at 'arms length' on dendrites, whereas DCs visualized by Mempel *et al.* did not show an elaborate dendritic structure and $CD8^+$ T cells primarily contacted the body of the DC. There is general agreement that both $CD4^+$ and $CD8^+$ T cells form stable contacts with antigen-bearing DCs that last several hours following an initial phase of transient interactions. In addition, Miller *et al.* distinguished a 'swarming' phase when enlarged T cell blasts dissociate from DCs following the period of stable interactions, but remain in the local vicinity and make contacts with additional DCs [5]. Starting 24 hours after the initial encounter with antigen-bearing DCs, T cell blasts were observed to round up and divide, leading to several rounds of division in the lymph node during the next few days.

Extending the theme of serial encounters between T cells and DCs, the ability of successive waves of antigen-bearing DCs to influence subsequent T cell behavior and activation has been evaluated [23•]. $CD4^+$ T cells that were previously activated by contact with a first wave of DCs can encounter and respond to a second wave of antigen-bearing DCs. During the swarming phase described previously [5], these secondary prolonged interactions resulted in increased cytokine production over and above that which

was elicited by the first wave alone [23•], demonstrating that T cells can integrate signals during successive encounters with DCs, leading to a more vigorous response.

Taken together, these three imaging studies paint a consistent picture in which the immunological synapse in native tissues is remarkably fluid during the first hours of an immune response, and that stable synapses form only at specific stages of antigen presentation to T cells. Moreover, the progressive nature of these interactions implies that T cells activate by way of multiple antigen recognition events. The initial period of scanning for specific antigen interactions might be optimized by sequential interactions with several DCs during the early phase of T cell activation, and be further modulated by additional interactions with newly arrived DCs at later times.

Because direct contact with APC is the *modus operandi* within the immune system for activation of T cells, the ability of rare antigen-specific cells to find APC can be likened to a 'needle in the haystack' problem. We have postulated that a stochastic scanning mechanism within the lymph node can provide a sufficiently large number of contacts to solve this problem for the first encounter between CD4 and DCs [3,4]. The problem is severely compounded in the case of antigen-specific $CD8^+$ cells, which must receive help in order to lyse targets more effectively and to persist as memory $CD8^+$ cells. Is T cell help delivered by sequential interactions first with DC and then with $CD8^+$ T cells, or must these cells come together as a 'threesome'? A recent study from Ron Germain's group provides some answers to this dilemma [24••]. In contrast to the random walk observed by Miller *et al.* in the absence of antigen [2–4], $CD8^+$ cells were frequently seen to move directly toward $CD4^+$–DC clusters during a specific antigen response. This 'smart' behavior would recruit $CD8^+$ cells to the cluster and enhance the likelihood of forming a threesome. The chemokines CCL3 and CCL4, ligands of CCR5, were shown to be produced by $CD4^+$ T cells and by DCs; moreover, antibody neutralization inhibited $CD8^+$ memory recall responses. Under inflammatory conditions, CCR5 upregulation in $CD8^+$ T cells would facilitate chemotaxis to $CD4^+$ T cells clustering around DCs, greatly increasing the likelihood that highly motile antigen-specific $CD4^+$ and $CD8^+$ cells would find each other in order to produce more lethal killers and promote a $CD8^+$ memory recall response.

T cell interactions with antigen-engaged B cells

A second crucial antigen-specific interaction within the lymph node occurs when helper T cells interact with antigen-engaged B cells of the same specificity; this provides 'help' for these B cells to differentiate into antibody-producing plasma cells. Earlier imaging studies had shown that B cells and T cells are constantly migrating in a random walk pattern within their compartments (B cell follicles and T cell zone of the paracortex, respectively); however, in the absence of antigen, they appear to respect the invisible boundary at the follicle edge [25]. How then do the T cells and B cells locate one another, and what is the nature of their interaction? A recent study [10••] addressed these questions by use of 2-photon imaging and, in addition to outlining the cellular dynamics associated with T cell-dependent antibody responses, provided the first evidence for lymphocyte chemotaxis *in vivo*. Moreover, this study illuminates the importance of microanatomical localization within the lymph node for the immune response to function correctly. In this instance the compartmentalization of the T cells and B cells themselves (each labeled with fluorescent dyes of differing emission wavelength) provided a ready means to identify B cell follicles and T cell zones in 2-photon images, and localization was further facilitated by second-harmonic imaging of collagen fibers in the capsule — a technique based on non-linear frequency-doubling of light by organized structures.

In the presence of soluble antigen, the behavior of B cells near the follicle edge changed dramatically: cells moved along relatively straight paths toward the follicle edge, where they then moved more randomly without deviating far from the T cell zone. Simultaneous imaging of antigen-engaged B cells together with control, unactivated, B cells of differing antigen specificity revealed that the antigen-engaged cells were selectively depleted within a band extending 20–100 μm from the follicle boundary. This directional migration is believed to be driven by a concentration gradient of CCL21, and, consistent with this, immunofluorescence staining in fixed node preparations revealed a gradient of CCL21 tapering from the T zone ~100 μm into the follicle.

Once they arrived at the follicle edge, B cells were observed to interact with antigen-specific T cells in a different manner to T cell–DC contacts. Highly motile T cell–B cell conjugates moved at a velocity (~9 μm/min) characteristic of the activated B cells (versus ~12 μm/min for T cells), and B cells invariably led the way, dragging behind rounded T cells as they 'waltzed' together. Antigen-engaged B cells remained paired with antigen-specific helper T cells for as long as 1 hr, whereas non-specific interactions typically lasted <10 min. B cells occasionally contacted more than one T cell leading to serial monogamy, but T cells remained strictly monogamous. The authors proposed that motility and partner exchange might be vital to optimize T cell–B cell cooperation during a mixed antigen response and to colonize fresh territory for formation of germinal centers [10••].

Agonist regulation of lymphocyte egress from the lymph node

FTY-720 is a newly discovered immunosuppressant that acts on sphingosine 1-phosphate (S1P1) receptors to

inhibit the egress of lymphocytes from thymus, lymph node and Peyer's patch (but not from the spleen), thereby producing a depletion of lymphocytes from the circulating blood and inhibition of effector function [26]. Two contrasting hypotheses have been proposed to account for this action [27,28]. One envisages that immunosuppressants such as FTY720 operate by way of functional antagonism, whereby binding to $S1P_1$ receptors on lymphocytes promotes receptor down-regulation, which in turn prevents their chemotaxis from the lymph node towards endogenous S1P within lymph [27]. A second hypothesis proposes instead that $S1P_1$ agonists act on endothelial cells to close 'gates' in the sinus endothelium and thereby block lymphocyte egress [28]. A recent imaging study has now provided the first visualization of transendothelial migration of T cells into lymphatic sinuses, and lends strong support to the stromal gate model [29••].

Imaging was achieved using an explanted node preparation. Although this did not fully replicate *in vivo* processes of lymphocyte egress, it offered advantages of unrestricted access for the microscope to the medullary region of the node, together with ready application and washout of drugs without the pharmacokinetic hurdle of long plasma half-lives in intact animals (Table 1). Despite the disruption of blood and lymphatic circulation, T cells were observed to exit from medullary cords into lymphatic sinuses, indicating that the explanted node provides a readily accessible preparation in which to study transendothelial migration of lymphocytes. Moreover, these studies hinged on structural identification of the stromal tissue that surrounds medullary sinuses, which was achieved by incubating nodes with fluorescently labeled wheat germ agglutinin. On a final technical note, the use of a reversible $S1P_1$ agonist and antagonists greatly strengthened the pharmacological interpretation of the results and enabled kinetic changes in lymphocyte migration and motility to be monitored within the typical timeframe ($\sim$1 hr) of imaging experiments.

Nodes treated with the specific $S1P_1$ receptor agonist SEW2871 showed tightly packed T lymphocytes 'log jammed' along the borders of medullary sinuses that were void of T cells. This confirmed previous findings in fixed tissue sections but, more importantly, live-cell imaging revealed that log-jamming is characterized by a discrete directional alteration in lymphocyte behavior and not merely by complete arrest. Specifically, T cells moved within medullary cords and along sinus boundaries but were unable to traverse into lymphatic sinuses, which suggests that $S1P_1$ agonists inhibit the physical crossing of the sinus boundary into efferent lymphatics. Although T cell motility was slowed by SEW2871, this was specific to cells in the medulla because T cell velocities in cortical areas were unaffected. The effects of SEW2871 were rapidly reversed on washout, resulting in recovery of T cell motility and the crossing of the endothelial boundary at particular 'portals' to cause refilling of sinus spaces. Addition of $S1P_1$ antagonists in the continued presence of the agonist SEW2871 closely replicated the effects of washout of SEW2871, whereas application of antagonist alone had no effect.

These results appear inconsistent with a model of 'functional antagonism' for several reasons: the effects on T cell motility migration following application and washout of SEW2871 were much faster than expected for catabolism and subsequent re-synthesis of $S1P_1$ receptors; effective concentrations of agonists are within the steep agonist binding range of $S1P_1$ receptors; and, most strikingly, the actions of SEW2871 were reversed by high-affinity selective antagonists, rather than mimicking the action of SEW2871 as expected if lymphopenia resulted from receptor desensitization or downregulation. Instead, the authors proposed a mechanism whereby agonist actions on $S1P_1$ receptors expressed in endothelial cells (rather than on lymphocytes) inhibit lymphocyte egress from the node by closing constitutively open stromal gates. The combination of live-cell imaging studies with reversible chemical probes thus opens a window on one of the basic mechanisms that regulates lymphocyte trafficking through the lymph node, and has important biomedical implications because $S1P_1$ receptor agonists might serve as useful immunosuppressant drugs.

Conclusions

In this article, we review three specific instances in which 2-photon microscopy has revealed new insights into cellular trafficking and interactions within the immune system, and such immunoimaging techniques are now being applied to a diverse array of problems. Recent examples include: comparisons of cell interactions during priming and tolerizing immune responses [8,11,30] (for review, see Amigorena *et al.* in this issue of *Current Opinion in Immunology*); visualization of effector memory T cells specific for myelin basic protein interacting with central neurons as they cause demyelination in a rat model of multiple sclerosis [31]; and the first imaging studies of how regulatory T cells modulate T cell–DC interactions [32,33]. It is clear that the next few years will bring a new level of complexity to our understanding of the cellular choreography between visitors and permanent residents within the lymph node and numerous other tissues.

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