

Stop and Go Traffic to Tune T Cell Responses

Review

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Adaptive immune responses are initiated by interactions of T cells with antigen-presenting cells, but the basic nature of these interactions during an immune response in vivo has been a matter of speculation. While some in vitro systems provide evidence for stable interactions, referred to as immunological synapses, compelling evidence supports T cell activation through serial transient interactions. Deep tissue intravital and organ culture microscopy studies suggest that both modes of interaction are employed, but new issues have emerged. This review will discuss in vitro results that framed the hypotheses that are currently being tested in vivo. I present a model in which TCR stop signals compete with chemokine-mediated go signals to adjust the duration of immunological synapse formation and tune the immune response between tolerance and full activation.

Introduction

The interaction of T cells with antigen-presenting cells is the determinative event in the initiation of an adaptive immune response. These interactions can turn T cells on or off depending upon the context. Antigen under steady-state conditions leads to T cell deletion or tolerance (Hawiger et al., 2001), while antigen in the context of infection or antigen-presenting cell activation with adjuvants induces generation of effector T cells, immunity, and memory (Ahmed and Gray, 1996; Bonifaz et al., 2004). The distinctions between tolerogenic and immunogenic interactions are central to our understanding of the immune response. One potentially important parameter is the duration and molecular organization of the T cell-antigen presenting cell interaction and the molecular organization of the cytoskeleton, adhesion molecules, costimulatory molecules, and antigen receptors in the stable or transient contact areas. Results from in vitro studies have established a number of hypotheses that are being tested in vivo and in situ by advanced imaging methods.

The earliest studies on the kinetics of the immune response suggested a process in which immune cells surveyed the body for antigen by “recirculation” between blood and secondary lymphoid tissues (Gowans and Knight, 1964). When specific antigen reached the lymph nodes, this triggered a period of antigen specific lymphocyte retention in the secondary lymphoid tissues

and proliferation of the antigen specific clones, followed by release of effector cells to the blood and peripheral tissues in a process spanning 3–5 days (Sprent et al., 1971). The details of the in vivo search process have been examined only recently, but some insights into antigen-specific T cell-APC interactions have been generated by in vitro studies.

In vitro antigen-specific T cell-APC interactions were observed as early as the 1970s when in vitro antigen-specific T cell systems were first emerging and in early studies of T cells interacting with dendritic cells (Inaba and Steinman, 1986; Lipsky and Rosenthal, 1975). The duration of these interactions varies with the function. Effector cells can execute functions rapidly and may only require a few minutes, whereas naive T cells require several hours of interaction with antigen-presenting cells to induce proliferation and cytokine production (Iezzi et al., 1998; van Stipdonk et al., 2003). It has been demonstrated that sustained TCR signaling is needed to induce rearrangement of chromatin required for cytokine gene expression, at least in the case of induction of Th2 effector T cells from naive T cells (Iezzi et al., 1999). These studies were performed under conditions where the T cells were in continuous contact with antigen but did not examine the nature of this interaction, whether the T cells stayed in place or migrated rapidly over the surface, or if they interacted in phases that mixed these modes of interaction over the critical time period. In this review I will summarize the in vitro results that framed the question for recent in vivo observations, discuss the initial results from in vivo imaging of T cell-APC interactions, and then propose a simple model of how T cells make decisions between forming transient and stable cell-cell interactions that lead to tolerance or immunity.

The Stop Signal, the Immunological Synapse, and Serial Encounters

The molecular architecture of antigen-specific interactions between T cells and antigen-presenting cells was first worked out for cytotoxic T cells, which engaged in a short cycle of conjugate formation and killing, taking only several minutes. The stability of these interactions was related to polarization of the T cell (Geiger et al., 1982) and the activation of adhesion molecules (Sung et al., 1986). Polarization was visualized through tracking of the microtubule organizing center and Golgi apparatus of cytotoxic cells and, later, helper T cells (Kupfer et al., 1987). TCR signaling was initiated very rapidly in a time frame faster than these cytoskeletal rearrangements (Poenie et al., 1987). The magnitude of the initial and sustained cytoplasmic Ca^{2+} increase regulates migration of the T cells, with high levels stopping migration and thus allowing a prolonged interaction with the APC (Negulescu et al., 1996). The combination of the adhesion molecule ICAM-1 and agonist MHC-peptide complexes was sufficient to induce T cell stopping and polarization in a two-dimensional system with planar bilayers (Dustin et al., 1996, 1997). Studies on T cell-APC interac-

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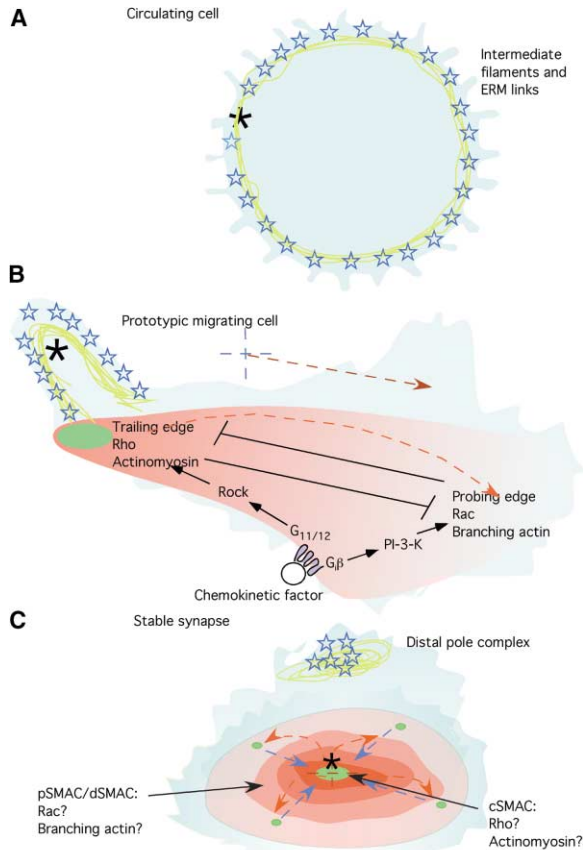


Figure 1. Cytoskeletal Reorganization in T Cells

(A) Model for circulating T cells. Circulating T cells are relatively rigid because both intermediate filament (yellow lines) and actin cytoskeletal linkages mediated by ERM family members (stars) support the microvilli studded surface. While they have an MTOC (*) it does not clearly indicate a probing of trailing edge.

(B) Model for generic migrating cell. Adhesion molecules form a gradient from front to rear with formation of interactions in the probing edge and de-adhesion at the trailing edge (red gradient). The trailing edge is characterized by a uropod with the MTOC, intermediate filaments, and ERM family proteins. A model for leukocyte chemokinesis is represented where spatially uniform engagement of GPCR triggers phosphatidylinositol-3-kinase (PI-3-K) to activate Rac and branched actin networks characteristic of probing edge. The same GPCR activates $G_{11/12}$ to activate Rock, Rho, and actinomyosin contractility to mediate contraction and de-adhesion. The mutual inhibition of these signaling systems promotes formation of these structures to reinforce cell polarity and allows the cells to migrate without a gradient of the GPCR ligand. The green patch near the trailing edge is a region where small adhesion molecules like CD2-CD58 and TCR-MHC-peptide interaction accumulate in a migrating cell until cell-cell contact is broken. The blue crosshair reflects the stable position of the substrate and the brown arrows reflect the movement of the cell.

(C) Model for immunological synapse. The surface has freely mobile MHC-peptide complexes (green) and ICAM-1 (red). In the mature synapse the central TCR cluster is focused in the cSMAC and LFA-1-ICAM-1 interactions are concentrated in the pSMAC after initiation in the distal or dSMAC (blue area at periphery of contact). Additional TCR-MHC-peptide interactions may also be initiated in the pSMAC and transported toward the cSMAC to sustain signaling (small green dots). The dSMAC/pSMAC appears similar to a probing edge of a fibroblast with a branched actin networks at the leading edge and an actinomyosin contractile structure to promote deadhesion at the cSMAC/pSMAC boundary. The bent red arrows reflect recycling of LFA-1 to the outer edge of the pSMAC. The dashed brown crosshair

is the position of the cell, which is stable, while the blue arrows reflect the sliding of molecules in the cell membrane and bilayer toward the cSMAC. The distal pole complex is represented as an accumulation of ERM family proteins and intermediate filaments (not demonstrated experimentally) at the pole of the cell furthest from the pSMAC.

tions in vitro suggested that antigen receptor and adhesion signaling was sufficient to induce rapid T cell polarization and a stop signal for migration in vitro. In addition to movement of the MTOC and Golgi apparatus T cells were also shown in vitro to undergo an extensive membrane-cytoskeletal reorganization at the interface resulting in a central cluster of antigen receptors, the central supramolecular activation cluster (cSMAC), and a ring of adhesion molecules, the peripheral SMAC (pSMAC) in the mature immunological synapse (Grakoui et al., 1999; Monks et al., 1998). The pSMAC is formed by interaction of the integrin LFA-1 with ligands including ICAM-1. The mature immunological synapse is the dominant mode of interaction of T cells with B cells, mature dendritic cells in suspension and with reconstituted systems with MHC-peptide complexes, and ICAM-1 in a supported planar bilayer (Benvenuti et al., 2004; Grakoui et al., 1999; Krummel et al., 2000; Monks et al., 1998). Stable immunological synapses are formed by $CD4^+$ and $CD8^+$ naive, memory, and effector T cells (Stinchcombe et al., 2001; Watson and Lee, 2004).

The relationship of cytoskeletal polarity to motility and stopping can be understood in terms of recent molecular models of mammalian cell crawling (Figure 1). Circulating lymphocytes are relatively rigid and are studded with many microvilli that facilitate interactions with endothelial cells (Brown et al., 2001). The basis of this rigidity is cage-like cortical cytoskeleton formed by intermediate filaments in circulating cells. Upon activation of circulating T cells by chemokines, this structure “collapses” to the side of the cell nearest the MTOC. Another important process is the coordinate “relaxation” of the cortical actin cytoskeleton, which is mediated by Rac activation, leading to dephosphorylation of Ezrin, Radixin, and Moesin (ERM) family proteins (Faure et al., 2004). These processes set up the morphology of a migrating T cells. In T cells migrating over a planar surface coated with ICAM-1, the LFA-1-ICAM-1 interactions form an asymmetric wedge-shaped pattern due to the initiation of LFA-1-ICAM-1 interactions in the forward-probing cell edge and disrupted near the trailing edge (uropod), where the MTOC and intermediate filaments are located (Brown et al., 2001; Dustin et al., 1997; Grakoui et al., 1999). CD43, ICAM-3, and ERM proteins are also concentrated in the uropod of migrating cells (Sanchez-Madrid and del Pozo, 1999; Dustin and Cooper, 2000). This pattern is fully consistent with general models for amoeboid cell motility where adhesive interactions are formed in a forward-probing edge, move backward in the cell based on actin treadmill and actinomyosin contractions, and then are disengaged nearer the back of the cell by actinomyosin-induced contraction (Laukaitis et al., 2001; Lawson and Maxfield, 1995; Xu et al., 2003). This process can be driven externally by G

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protein-coupled receptors that couple to two different heterotrimeric G proteins: one that preferentially triggers phosphatidylinositol-3-kinase, Rac, and branched actin polymers consistent with formation of forward-probing membrane structures and one that preferentially couples to Rho kinase (Rock), Rho, and activation of actin-myosin-based contractility consistent with retraction and de-adhesion (Figure 1B). These structures are mutually antagonistic, so once polarity is established it is stable, even in the absence of chemotactic gradients. This mode of chemically induced migration is known as chemokinesis. This model is fully consistent with the LFA-1-ICAM-1 interaction pattern formed by T cell migration on planar surfaces. It has been shown that LFA-1 detachment in migrating lymphocytes is dependent on Rock (Smith et al., 2003). Furthermore, nonmuscle myosin II (MyH9) is required for T cell migration, but not for adhesion, again suggesting a role in traction and detachment (Jacobelli et al., 2004). Chemokinetic factors for naive T cells are not known, but factors from dendritic cells appear to be effective (Gunzer et al., 2000). Effector T cells do not appear to require external signals for motility *in vitro* or LFA-1 interactions alone are sufficient to deliver these signals (Bromley et al., 2000; Dustin et al., 1997).

How does this model for motility apply to the sessile immunological synapse? The pSMAC appears to be a radially symmetrical probing structure that is induced during spreading and can be maintained for a long period (Grakoui et al., 1999) (Figure 1C). This interpretation is also consistent with studies from Samelson on actin organization in spreading Jurkat T cells (Bunnell et al., 2001). The cSMAC then may be the surrogate of the trailing edge where receptors are normally transported and disengaged in migrating cells (Figure 1C). I speculate that LFA-1 is disengaged and recycles from this point much like recycling integrins in motile leukocytes (Lawson and Maxfield, 1995). The TCR that are also moved to the cSMAC cannot disengage and so accumulate in the center as has been directly observed (Grakoui et al., 1999). A prediction of this model is that Rac activation will be observed in the periphery of the pSMAC, which may transition to Rho activation as one moves toward the cSMAC. Studies of this resolution have not been performed, but it is known that Vav1, a Rac activator, and WASP, a Cdc42 effector, play an important role in the formation and function of the immunological synapse (Ardouin et al., 2003; Cannon and Burkhardt, 2004). This model does not explain two important aspects of immunological synapse function. First, the cSMAC or cSMAC/pSMAC boundary is a target for directed secretion in the immunological synapse (Stinchcombe et al., 2001), a feature not explained by this model that must involve the recruitment to exocytic vesicle-targeting receptors, Snares, specifically to the cSMAC/pSMAC boundary (Das et al., 2004). Second, the pole of the T cell away from the cSMAC is called the "distal pole complex" and has unique features consistent with a role in sequestration of negative regulators (Cullinan et al., 2002). The distal pole complex contains CD43 and ERM family members, which are excluded from the interface.

The behavior of the pSMAC in cells with stable synapses versus cells that begin to migrate is consistent

with provisional function of the pSMAC as a probing membrane in that initiation of migration to break the immunological synapse is preceded by a loss of symmetry of the pSMAC with higher LFA-1/ICAM-1 interaction in the direction of impending cell movement. This behavior is readily observed in anergic T cells and T cells reacting to mixtures of agonist and antagonist MHC-peptide complexes (Heissmeyer et al., 2004; Sumen et al., 2004). These studies demonstrate a continuum from the asymmetric probing edge of a migrating cell to the axisymmetric pSMAC of an immunological synapse-forming cell.

The model where stable synapses are always correlated with T cell responses has been challenged by observations of T cell-dendritic cell interactions in 3D collagen. Time-lapse studies in 3D collagen gels with mature DC demonstrate rapid migration of antigen-specific T cells over the time period required for commitment to proliferation (Gunzer et al., 2000). This process where T cells appear to integrate a series of transient interactions to become activated has been referred to as "serial encounters," and the frequency of these encounters has been evoked as the key variable that would lead to tolerance or immunity. The importance of the collagen gel in this system is not known, but collagen fibers in lymph nodes are sequestered in cellular sheaths formed by reticular fibroblasts (Gretz et al., 2000). Thus, a 3D collagen gel is not a good model for the lymph node parenchyma (Dustin et al., 2001). In another study in which suspension culture was performed without collagen it was noted that long-lived T-DC interactions were necessary, but not sufficient, for IL-2 production, but T cells that formed a series of transient interactions were able to proliferate (Hurez et al., 2003). Additional insight was gained by comparing immunological synapse formation with mature and immature dendritic cells in suspension culture (Benvenuti et al., 2004). Interactions with mature dendritic cells led to more long-lived interactions with well-organized immunological synapses, whereas interaction with immature dendritic cells led to only short-lived interactions with poorly formed immunological synapses. Presentation by immature dendritic cells leads to tolerance *in vivo* leading these authors to suggest that transient interactions may be tolerogenic on balance. It's important to note that proliferation as measured by Gunzer et al. is not always an indication of an effective response but can also be a component of tolerance-induction processes (Hawiger et al., 2001). Mature and immature dendritic cells differ in MHC levels, costimulation, and cytoskeletal organization. All of these factors may influence immunological synapse stability.

Another factor that may influence immunological synapse stability is competing go signals. It will be very important to understand how chemotactic and chemokinetic go signals compete with TCR stop signals, since this type of competition may determine the duration of T cell-APC interactions in some *in vivo* microenvironments. An analysis of chemokine go signals versus the TCR stop signal revealed that there is a hierarchy (Bromley et al., 2000). In this study ICAM-1 was immobilized on filters with and without agonist MHC-peptide complexes and spontaneously motile, antigen-specific effector T cells were allowed to interact with the filters. The T cells transmigrate on ICAM-1 alone based on sponta-

neous motility in the absence of a chemokine gradient but do not transmigrate when agonist MHC-peptide complexes are included. This system of stopped cells on filters with ICAM-1 and MHC-peptide complexes is then exposed to a chemokine gradient across the filter and the resistance of the T cells to dislodgement is determined. Half of the chemokines tested did not dislodge the stopped T cells (e.g., CCL3 and CXCL12), but the other half potently induced migration (e.g., CCL21, CCL19, CXCL10), thus overriding the antigen stop signal. The dominant effect of ligands for CCR7 (CCL21 and CCL19) and CXCR3 (CXCL10) was chemotactic because it was only detected when the chemokine was in the chamber opposite the stopped cells, not when it was in the same chamber with the stopped cells (fugitaxis) or when it was in both chambers (chemokinesis). However, it is possible that the chemokinetic factors produced by dendritic cells could also override the MHC-peptide stop signal and allow T cells to migrate in the presence of antigen stop signals. While the ability of T cell to maintain a synapse in the presence of CCL3 or CXCL12 gradients was intact over a wide range of antigen doses, the ability of CCL21 to dislodge T cells was most prominent at low antigen dose and could be overcome at high antigen dose. Thus, TCR stop signals and CCL21 go signals engage in quantitative competition where the relative strengths of the stop and go signals determine the outcome. This model argues that chemotactic and chemokinetic factors in the local environment will have a profound influence on the duration of stable interactions.

The significance of stable or serial transient interactions for signal integration by the T cell is not well understood. Proliferation and cytokine production require signaling over a period of 6–20 hr in naive T cells and for about 2 hr for rested effector T cells (Huppa et al., 2003; Iezzi et al., 1998; van Stipdonk et al., 2003). While Huppa et al. emphasized the stability of T cell-APC interactions in those systems over many hours, the other studies did not differentiate between stable and transient interactions. Faroudi et al. (2003) performed an analysis of whether continuous stimulation was required for a human T cell line to produce IFN- γ . They found that signaling in a cycle with 20 min on and 10 min off for five cycles was equivalent to continuous stimulation for 150 min. A potent but reversible Src family tyrosine kinase inhibitor was used to turn off signaling during the 10 min rest periods. These conditions were intended to simulate serial encounter models where T cell signaling is interrupted by migration away from APCs (Gunzer et al., 2000), but Src kinase signaling would likely continue due to engagement of other receptors during intervening periods in serial encounter models, which may limit to the applications of approaches with general inhibitors to simulate natural disengagement. Similar experiments have not been performed for IL-2 production by naive T cells, but these experiments are only meaningful if the cells produce the cytokine—a negative result would not be informative. Therefore, *in vivo* systems that allow examination of molecular aspects of signal integration under conditions of stable and transient interactions are needed to address these issues more completely. Translating these systems to *in vivo* imaging, as discussed below, would be a further challenge.

In summary, two qualitatively different behaviors have

been reported for T cells encountering antigen on antigen-presenting cells: stopping of movement with formation of a stable immunological synapse or continued rapid movement from APC to APC. The finding that appears to link these two types of observations is that some chemokines can inhibit stable interaction in favor of motility. Thus, rapid migration of T cells over antigen-positive APCs could be explained by dominant chemotactic or chemokinetic signals that prevent T cell stopping while still allowing TCR signaling. These environmental signals could then separate TCR-mediated polarization from TCR-mediated transcriptional regulation. Overall, many data are consistent with the model that long periods (hours) of stable immunology synapse formation are correlated with IL-2 production, while transient interactions fail to induce IL-2 production but can produce proliferation and production of effector cytokines.

Live In Situ and In Vivo Observations

The picture of the lymph node up to 2002 was based on static light and electron microscopy (Ebnet et al., 1996). The concept was that the reticular fiber network connected the afferent lymph in the subcapsular sinus to the high endothelial venules (HEV) and DCs lining the reticular fibers in the T cell zone. T cells were then proposed to move down corridors lined with DCs like guests in a receiving line. The movement of the T cells would be driven by chemotactic gradients, and they would then exit through the medullary lymphatics. The clearest picture of the impact of antigen on T cell-antigen presenting cell interactions was based on studies from Jenkins and colleagues that showed cluster formation with several antigen specific T cells clustering around each antigen positive immigrant DC around 24–48 hr after introduction of the DCs (Ingulli et al., 1997). When soluble antigen was injected subcutaneously there were two waves of antigen delivery, one of soluble antigen to lymph node-resident DC and a second of tissue DC migration to the lymph node, which led to distinct proliferative responses (Itano et al., 2003). A recent study by Bajénoff et al. (2003) using similar histological methods demonstrated complete Freund's adjuvant-stimulated positioning of newly immigrant DCs around the high endothelial venules, where they appeared to retain antigen-specific T cells for over 24 hr after entry into the lymph node. Two histological studies also found evidence for TCR polarization in T cell-APC interactions within the T cell zones and in the meninges during viral infection (McGavern et al., 2002; Reichert et al., 2001). These static views were informative but could not address issues of stability of interactions or synapse formation because even molecular polarization takes place both in the context of synapse formation and cell migration (Figure 1).

Organ Culture and Intravital Imaging of Immune Responses

Two approaches emerged to study T cell-APC interactions *in situ*. Intravital microscopy has been practiced for over 40 years in physiology research (Arfors et al., 1969). This approach typically involves surgical exposure of a tissue of an anesthetized animal so that high-resolution imaging can be performed. The advantage of

this approach is that blood flow, lymph flow, natural tissue oxygenation and neural innervation are maintained. Caveats include effects of surgical trauma, motion artifacts in imaging due to breathing and blood flow, and the need for anesthesia that must be carefully titrated. The other approach is to remove the tissue of interest from a euthenized mouse and prepare an organ culture in which the tissue cells will remain alive and functional for several hours or even days. This approach is technically easier because the challenges of immobilizing the tissue are greatly reduced. Caveats include setting the ambient conditions for the culture, which are often determined by the requirements to keep the tissue cells alive, and can involve extreme conditions like use of 95% oxygen, the standard in neural slice cultures. The first images of T cell interactions with APC in intact lymph nodes were based on the organ culture approach.

A study by Germain and colleagues employed the organ culture approach to the study of T cell-APC interactions in intact explanted lymph nodes partially embedded in agarose and imaged with a conventional laser scanning confocal microscope at depths up to 50 μm s (Stoll et al., 2002). Antigen-pulsed, fluorophore-labeled mature DCs were introduced via lymph and different fluorescent-labeled T cells from a TCR transgenic mouse were introduced by intravenous injection to a syngeneic recipient mouse. The lymph nodes were then removed at different times and imaged for a period of hours. In this situation most of the action takes place in the live, awake animal, and the lymph node is isolated and imaged *ex vivo* to obtain time window of a few hours in a longer process. The conditions used in these studies did not reveal any migration of naive T cells in the absence of antigen. It has been noted in deep tissue-imaging studies that naive T cell migration varies with depth in the tissue (Mempel et al., 2004), and it's possible that the limited T cell movement is related to this, although no systematic study on this issue has been published. Stable, specific interactions were observed out to 36 hr after the introduction of DCs. After 36 hr the T cells dissociated from the DCs and moved vigorously. By 48 hr all the T cells were dividing and moving rapidly. This study supported the formation of a stable immunological synapse and actually showed that CD43-GFP was excluded from the interface in the stable contact areas *in situ*. The absence of migration of naive T cells in the lymph node in the absence of antigen, however, meant that the stable interactions with APC could be related to the conditions selected for the organ culture preparation and not a true antigen stop signal. An exciting and unequivocal result was the rapid migration of the activated T cell blasts in the lymph nodes that revealed a second phase of T cell movement after a prolonged stable synapse.

The T cell zones of lymph nodes are greater than 50 μm deep in the tissue such that conventional confocal imaging has limitations. Fully addressing the controversies framed by the *in vitro* studies described above required deeper high-resolution imaging offered by two-photon laser scanning microscopy (Dustin et al., 2001). Two-photon microscopy uses femtosecond pulsed lasers with peak energies high enough to excite fluorophores with two photons of infrared light instead of one photon of visible light (Zipfel et al., 2003). The laser

power is adjusted so that two-photon excitation is only obtained at the focal point of the high numerical aperture objective and then power must be increased exponentially with depth to counteract the loss of power due to scattering (Theer et al., 2003). Emitted photons are collected efficiently by detectors positioned close to the objective as the laser beam is raster scanned through the tissue. Two-photon imaging with mode-locked (femtosecond pulse) Titanium-Sapphire lasers has been very useful for imaging T cells and DCs in lymph nodes in ways that would not be possible with conventional confocal imaging.

Cahalan and colleagues were the first to use two-photon microscopy on explanted lymph nodes with 95% oxygen (Miller et al., 2002). Miller et al. concurred with Germain that antigen induced stopping of T cells even in deeper parts of the lymph node, although DC were not visualized, but the most exciting aspect of the study was the observation of the baseline migration of T cells and antigen-dependent swarming of T cells. In the absence of antigen, naive T cells moved rapidly and in random directions within the T cell zones. Miller et al. concluded that the T cells move in a random walk in the deep paracortex based on the observation that the mean squared displacement increased linearly with time. This random behavior was restricted to the deep paracortex away from boundaries with B cell follicles. Nonetheless, this largely random and rapid movement within the core of the T cell zones was very different than that envisioned by the field and suggested an alternative model for the "needle in a haystack" search of rare, naive T cells for antigen-positive dendritic cells. This movement pattern was confirmed using the first intravital two-photon imaging of the inguinal lymph node (Miller et al., 2003). Concurrent imaging of T cells and DCs in cultured lymph nodes showed that each DC contacted in the 500–5000 T cell per hour (Bousoo et al., 2002; Miller et al., 2004). Thus, it was proposed that T cells and DC each carry out a random search in the T cell zone so that a small number of DC can efficiently scan the naive T cell repertoire. The view first exposed by Germain that T cells form stable interactions with DCs in the presence of antigen in intact lymph nodes was reinforced by studies from Robey on naive CD8 T cells (Bousoo et al., 2002). Miller et al. also reported swarming of antigen-specific T cells in which the T cells did not form stable synapses, but they also did not move away from specific locations, suggesting a tethering or attraction to a particular APC.

von Andrian and colleagues performed a more extensive time analysis of CD8 and CD4 T cell interactions with transferred DC by using intravital microscopy of the popliteal lymph node, a new preparation that provided convenient access to afferent lymphatics draining uniquely to this lymph node through subcutaneous injection into the footpad (Mempel et al., 2004). Another innovative aspect of this study was the use of anti-L-selectin antibodies to restrict T cell entry into the lymph node to a 2 hr window, effectively synchronizing the T cell interactions with DCs. Synchronizing the response provided a clear visualization of the T cell-DC interaction in three phases. Antigen-specific interactions of T cells and DC were transient between 2–8 hr, were stable between 8–24 hr, and then reverted to transient by 23–36 hr. Dendritic cell were only injected at one time with

respect to T cells such that it was not clear if the three phases were due to changes in the T cell or changes in the dendritic cells. Changing the phasing of dendritic cell injection would address this issue. Interestingly, IL-2 production took place during and after the stable interaction phase. Thus, all the *in vivo* imaging studies highlight stable antigen-dependent interactions, but they also reveal that T cells are highly dynamic in the process of reaching and then departing from these stable interactions. Mempel et al. (2004) also provided evidence that T cell receptor signaling was taking place in the early dynamic phase based on up-regulation of CD69—direct evidence for signaling through serial encounters *in vivo*.

Imaging Effector T Cells

After activated lymphocytes exit the lymph node they move through the blood to peripheral tissues where they interact with diverse cell types (watch the Movie online at <http://www.immunity.com/cgi/content/full/21/3/305/DC1>). These interactions have not been examined to date. In addition to conventional T cells, the peripheral tissues are inhabited at steady state by different populations of nonconventional T cells including $\alpha\beta$ TCR expressing natural killer T cells and $\gamma\delta$ TCR expressing T cells. Natural killer T cells are a small population in the spleen and lymph nodes, but a relatively large population in the liver (Emoto and Kaufmann, 2003). Selection of these cells requires CD1d in the thymus, and the $\alpha\beta$ TCR repertoire is highly restricted (Brigl and Brenner, 2004). In the periphery they have a partially activated phenotype with expression of NK cell markers and T cell activation markers such as chemokine receptor CXCR6. Visualization of NK T cells in the liver by intravital confocal microscopy was made possible by knockin mice in which CXCR6 was replaced by GFP (F. Geissmann, T.O. Cameron, S. Sidobre, N. Manlongat, M.J. Briskin, M. Kronenberg, M.L.D., and D.R. Littman, unpublished data). The heterozygous mice have normal NKT populations, and >70% of the liver GFP^{hi} cells are NKT cells. Imaging of the liver NKT cells revealed that they remain in the sinusoids and migrate rapidly ($\sim 15 \mu\text{m}/\text{min}$) regardless of the direction of blood flow in the sinusoids and do not extravasate (F. Geissmann, T.O. Cameron, S. Sidobre, N. Manlongat, M.J. Briskin, M. Kronenberg, M.L.D., and D.R. Littman, unpublished data). This remarkable behavior is likely an adaptation to the liver parenchyma where there is little extravascular space, but very effective access from the sinusoids to most cell types. Activation of the NKT cells with anti-CD3 or Concanavalin A results in immediate stopping of migration and formation of stable interactions with cells on the wall of the sinusoids for greater than 1 hr. NKT cells store message for IL-4 and IFN- γ and secrete the cytokines within 1 hr of activation (Matsuda et al., 2003). It appears that activated NKT cells by antigen in the liver will result in release of cytokines into the blood to achieve a system wide immunomodulation that has found diverse applications. The study of effector cells in the peripheral tissues promises to be as full of insights and surprises as imaging of primary activation in the lymph nodes.

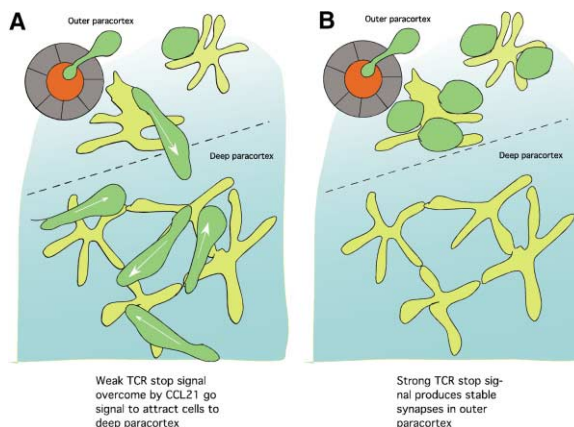


Figure 2. Model for Effect of Constant Chemokine Gradient on Weak and Strong TCR Stop Signals

The key feature of this model is that the outer paracortex is a site rich in HEV where chemokines from the T and B cell zones form overlapping gradients (blue is hypothetical CCL21 gradient). T cells that encounter weak stop signals will form synapses of short duration or simply migrate to the deep paracortex without forming a stable synapse at all (A). Cells encountering stronger stop signals (optimal agonist MHC-peptide amount and quality plus costimulation/adhesion) can resist the CCL21 gradient and form stable synapses in the outer-paracortex (B). Only antigen-specific T cells are represented. This model emphasizes the role of the stop and go signals in making decisions about stable interactions that are important for T cell fitness and differentiation.

Model for Regulation of Antigen-Dependent T-DC Interactions

Steady State

Little is known about physical interactions during steady-state presentation in the lymph node. However, there is enough information about *in vivo* dynamics and tolerance induction to begin to design informative experiments. About half of DCs in lymph nodes at the steady state have high expression of class II and express costimulatory molecules like CD86 and ICAM-1 (Wilson et al., 2003). These appear to be cells that have emigrated from the periphery. The MHC class II^{hi} cells process antigen poorly, but their MHC are likely to be loaded with peptides from self antigens acquired in the tissue. Under these conditions self-reactive recent thymic emigrants entering the lymph nodes for the first time will encounter antigen-positive DC immediately after extravasation. Since these DC have emigrated from healthy tissues, they are likely to have poor costimulation, and the lymph node itself does not react to inflammation so the environment is not adapted to retain lymphocytes for long periods (see below).

The go signals in the steady-state lymph node are likely to be a combination of chemotactic signals that maintain the T and B cell zones and chemokinetic signals that drive movement within these zones. The dominant chemokine CCL21 identifies T cell areas (Ohl et al., 2003). CCL21 is likely to be at its highest concentration in the deep paracortex and at a lower concentration in the outer paracortex, although this has not been demonstrated and is very important to test. Nonetheless, I hypothesize that the CCL21 concentration increases from the outer to deep paracortex and the gradient may

be well positioned to override weak TCR stop signals in the outer paracortex (Figure 2A). This may result in poor synapse formation in the outer paracortex when TCR and costimulatory signals are weak and direct entry of antigen-specific T cells into the inner paracortex under steady-state conditions. It is expected that weak stimulation of T cells by steady-state DC will leave these cells “unfit” and that most will die before exiting and the remaining cells will be anergic (Hawiger et al., 2001; Lanzavecchia and Sallusto, 2002). Therefore, we hypothesize that the steady-state T-DC interactions will be of shorter duration than under immunizing conditions, but the specific manner in which this will be achieved has to be determined experimentally. The concept of fitness, or the lack of fitness, in terms of survival signals provided by a variety of costimulatory systems seems very appropriate as a model for induction of tolerance in this context (Lanzavecchia and Sallusto, 2002). This steady-state process may lead to production of very little cytokine and relatively little disturbance of surrounding T cells or lymph node function. Direct data on this issue will be critical to understand this important process for maintaining self-tolerance and should provide insight into breaking self-tolerance under conditions where this is beneficial to the host, such as in tumor immunity, or where it must be restored, as in autoimmunity.

Immunizing Conditions

Exposure of lymph nodes to adjuvants like LPS or complete Freund’s adjuvant will trigger dramatic changes in the molecular and even physical architecture of the lymph node that are likely to alter the nature of T cell interactions. Adhesion molecules like ICAM-1 are increased on stromal elements and DCs (Dustin et al., 1986), and chemokine production is also likely to be increased—elements that contribute to antigen independent interactions (Bromley and Dustin, 2002; Real et al., 2004). It is also likely that the sphingosine-1-phosphate system is modified to mediate “shutdown” of the lymph node, thus preventing T cell exit (Winton et al., 2000). An interesting aspect of this global response of the lymph node to strong inflammation is that the entire organ is dedicating itself to responding to the antigens that are encountered in this context.

A simple two-stage model for T cell activation can be proposed based on the histological study by Bejenoff et al. (2003) where antigen was administered in CFA, a prototypic strong adjuvant that induces effector function and memory. In this situation antigen-specific T cells were retained in the outer paracortex for 24 hr prior to entering the deep paracortex, whereas nonspecific T cells rapidly transited to the deep paracortex. As indicated above, T cells enter through HEV into the outer paracortex so the retention of antigen-specific cells in this region is consistent with a prolonged stop signal but could also be accounted for in other, more dynamic ways, so it will be critical to look at events in live tissues. My working model favors the ideal that this would be a stable synapse formed relatively rapidly after extravasation with DC that have migrated specifically to the outer paracortex to intercept the recently extravasated T cells. This hypothetical strategy is more similar to the “receiving line” model of Shaw and Anderson than the random search model of Cahalan and colleagues; further experi-

ments are needed to test if both operate physiologically in different compartments. Greater levels of costimulation from the mature, prepositioned DC may generate more stable immunological synapses better able to resist the pull of CCL21 toward the deep paracortex compared to steady-state conditions (Figure 2B). The maintenance of a stable synapse is likely to be important over a time frame of at least 20 hr, the time frame defined by Schoenberger and colleagues as required for full programming of CD8 T cells for in vivo survival and production of effector function (van Stipdonk et al., 2003) and the time frame for stable interactions observed in a number of in vivo and organ culture studies (Bousso and Robey, 2003; Mempel et al., 2004; Miller et al., 2002; Stoll et al., 2002). The stable interactions observed in Stoll et al. were logically in the outer paracortex, since this region is the only part of the T cell zone accessible to conventional confocal microscopy (Stoll et al., 2002). After formation of a prolonged immunological synapse the T cell and DC interaction could be terminated by tipping the balance in favor of go signals through increased T cell motility, upregulation of potential synapse inhibitors like CTLA-4, or mitosis (Stoll et al., 2002).

How would this model incorporate the observations of Mempel et al. that there can be a prolonged phase of short serial encounters prior to forming a stable synapse? I would suggest that serial encounters described by Mempel et al. in the first hours may be a fail-safe mechanism that allows responses to go forward efficiently even when antigen and DC are not in optimal position for synapse formation at the moment that rare antigen-specific T cells enter the lymph node (Mempel et al., 2004). If antigen-specific T cells first encounter antigen on DC that are not optimal for stable synapse formation, then phase I interactions can hold the T cell in the vicinity of the DCs until the DCs are ready for stable interactions or changes in the T cell allow them to form stable synapses even with DC that are not in optimal position for stable interactions but have high levels of antigen and costimulation. There is probably more than one path to effective T cell activation under conditions where mature, antigen-bearing DC are available for interaction with T cells, but a common denominator will be a stable immunological synapse. In the scenario where synapse formation takes place in the deep paracortex there may be no CCL21 gradients, so how would this interaction be regulated to prevent inappropriate activation? I would speculate that the competing go signal in this region is likely to be the unidentified chemokinetic factor(s) that drive the rapid, apparently random T cell motility in the deep paracortex.

Whether they take place in the outer paracortex rapidly after T cell entry into the lymph node or in the deep paracortex after a prolonged period of serial encounters, the duration of the immunological synapse will be determined by a competition between the TCR stop signal and chemotactic or chemokinetic go signals. This is a quantitative competition so the formation of stable interaction tests the quality of the antigen and costimulatory signals. The resulting duration of stable synapses with mature DCs may be determinative not only of tolerance versus immunity, but may also contribute to differentiation decisions in parallel with potent signals like IL-4 and IL-12. The exciting thing about these hypothe-

ses is that with a great deal of planning and work, they are testable with current technologies.

Conclusions

Intravital microscopy has generated a number of insights that could not be obtained in any other way. The current view of the search mechanism of T cells for antigen in lymph nodes has been strongly shaped based on relatively few time-lapse, two-photon intravital and organ culture microscopy studies. The situation with T cell encounters with antigen is complex and involves both stable and transient interactions in some combination, but biologically relevant permutations have just begun to be explored including the conditions for induction of tolerance and immunity. Clear principles are beginning to emerge like the dominance of rapid T cell migration in the steady-state and the commonality of stable interaction in induction of full T cell activation. In the future magnetic resonance imaging and other methods may evolve to enable noninvasive imaging of single cells to further aid in understanding the wealth of information in single cell dynamics in tolerance and immunity (Pautler and Fraser, 2003).

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