CHAPTER 13

Visualization of Cell-Cell Interaction Contacts—Synapses and Kinapses

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Abstract

T-cell activation requires interactions of T-cell antigen receptors (TCR) and peptides presented by major histocompatibility complex molecules (MHCp) in an adhesive junction between the T-cell and antigen-presenting cell (APC). Stable junctions with bull's eye supramolecular activation clusters (SMACs) have been defined as immunological synapses. The term synapse works in this case because it joins roots for "same" and "fasten," which could be translated as "fasten in the same place." These structures maintain T-cell-APC interaction and allow directed secretion. We have proposed that SMACs are not really clusters, but are analogous to higher order membrane-cytoskeleton zones involved in amoeboid locomotion including a substrate testing lamellipodium, an adhesive lamella and anti-adhesive uropod. Since T-cells can also integrate signaling during locomotion over antigen presenting cells, it is important to consider adhesive junctions maintained as cells move past each other. This combination of movement (kine-) and fastening (-apse) can be described as a kinapse or moving junction. Synapses and kinapses operate in different stages of T-cell priming. Optimal effector functions may also depend upon cyclical use of synapses and kinapses. Visualization of these structures in vitro and in vivo presents many distinct challenges that will be discussed in this chapter.

Introduction

The partnership between dendritic cells (DC) and T-lymphocytes (T-cells) defends the body against microbes, parasites, abnormal cells and environmental toxins that breach the barrier function of skin and epithelial surfaces.[1,2] Diverse tools including those of biochemistry, cell biology, genetics and imaging have been employed to understand the mechanistic basis of this partnership. In recent years, imaging approaches have become increasingly useful as molecular technologies for labeling cells and proteins and imaging hardware and software have improved. In vitro imaging led to the initial definition of the immunological synapse (IS, or synapse) based on the organization of polarity and adhesion molecules to fasten (-apse) the T-cells to the same (syn-) antigen presenting cells (APCs) or place.[3-8] Advances in near-field in vitro imaging have led to the description of TCR microclusters that sustain signaling in the periphery of synapses.[9-11] Introduction of two-photon laser-scanning microscopy and methods for long-term in vivo observation have led to a basic understanding of the dynamics of T-cell-APC interactions in the living lymph node and the affects of antigen, which leads to signal integration via both short and long-lived T-cell-APC contacts.[12-14] The long-lived interactions can be defined as synapses since they fasten the T-cell to

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the same APC. The short-lived interactions both early and late in the process appear to be the product of combining motility and cell-cell communication as a continuous kinetic process.[15] The T-cells move while maintaining extensive contact with the APC. This dynamic interaction, for which there is no convenient descriptor, could be described as a kinapse—combining roots indicating movement (kine-) and fastening (-apse) at the same time. A challenge for in vitro molecular imaging is to provide insight into how T-cells integrate signals from synapses and kinapses. It is likely that TCR microclusters will be common structures in this process.

One of the basic biological questions in immunology is what distinguishes T-cell responses to DC that lead to tolerance or priming. One concept is that the outcome of antigen presentation depends upon the activation status of the DC.[2] Immature DCs patrol the tissue spaces and boundaries of the body and gather antigenic structures, both self and foreign. Induced or spontaneous maturation of DC triggers their migration to the lymph node and concurrent processing of antigens to generate peptides that bind to major histocompatibility complex molecules (MHCp) that are then presented at the cell surface. DC migrate to the lymph node via the lymphatics and then migrate in the parenchyma and join DC networks in the T-cell zones where they encounter many T-cells.[16,17] The level of costimulatory molecules expressed by the DC is determined by the level of cytokines-like TNF produced in response to various endogenous or exogenous activators of innate immunity.[18] This level of innate stimulation appears to control whether the antigen-dependent T-DC interactions lead to tolerance or priming of an immune response over a period of 5–7 days.[19] While some have argued that tolerance induction does not involve synapses in vitro or in vivo,[20,21] we have found that the TCR-MHCp interactions alone control in vitro synapse formation[22] and that T-cells do synapse with DC during tolerance induction in vivo.[14] T-cells also synapse with DC during induction of oral tolerance.[23]

Once T-cells are primed they may take on a number of fates. They may become memory cells that continue to recirculate,[24] exit the secondary lymphoid tissues altogether to sites of inflammation,[25-27] remain in T-cells zones to help $CD8^+$ T-cell responses,[28] or move to follicles within the lymph node to help B-cells.[29] It has been demonstrated that effector $CD8^+$ T-cells are active in killing targets within lymph nodes.[30] The manner in which these fates are established is poorly understood, but may involve processes such as asymmetric cell division set up by synapses or different cytokine milieus encountered by daughter cells as the migrate.[31] Memory T-cells have been shown to accumulate in the bone marrow and to interact with bone marrow DC during secondary stimulation.[32]

Peripheral tissue scanning by DC is only one mode of innate immune surveillance of tissues. Two striking examples are the surveillance of the brain by the dynamic processes of microglial cells[33] and the active patrolling of liver sinusoides by natural killer T-cells, an innate-like T-cell.[34]

In this review we will summarize a new view of sustained T-cells activation through the synapses and kinapses. Then, how the synapse and kinapse work together in T-cell tolerance and immune surveillance will be discussed. Throughout the chapter the various visualization methods that are employed will be described and critiqued with respect to potential and limitations.

New Model for Sustained Signaling through the Synapse

Studies on the synapse bring together three parallel lines of experimentation in immunology through high-resolution fluorescence microscopy: TCR signal transduction, T-cell adhesion and polarity mechanisms. TCR signaling is based on a tyrosine kinase cascades that leads to rapid activation of phospholipase C γ.[35] The key tyrosine kinases are Lck, which initiates phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic domain of the TCR, ZAP-70, which is recruited to phosphorylated ITAMs and phosphorylates LAT and ITK, which phosphorylates phospholipase C γ that is recruited to phosphorylated LAT. Phospholipase C γ activation leads to generation of inositol-1,4,5-triphosphate, leading to Ca^{2+} mobilization and diacylglycerol leading to activation of protein kinase C and Ras exchange factors.[31] The triggering of the cascade is based on recruitment of Lck-associated coreceptors to the TCR and on TCR oligomer formation (see also Chapters 6 and 11).

Members of the integrin and immunoglobulin families mediate T-cell adhesion to APCs. These interactions greatly extend the sensitivity of TCR to small numbers of MHCp-bearing agonist peptides.[36] Costimulatory molecules also are configured as adhesion molecules and the line between adhesion and costimulation molecules if often blurry.[22,37] By definition, adhesion enhances the physical interaction of T-cells with APC and the interaction of TCR and MHCp, while costimulation enhances TCR signaling or produces independent signals that integrate with the TCR signal to influence T-cell activation. However, the major T-cell adhesion molecules have some costimulatory activity. For example, LFA-1 contributes to the adhesion of T-cells in many contexts, contributes to TCR-MHCp interactions and provides signals that enhance Ca^{2+}, phosphatidylinositol-3-kinase and MAPK pathway activation.[38] There are also negative costimulators. For example, CTLA-4 and PD-1 negatively regulate T-cell expansion at intermediate and late periods of activation.[39,40]

Polarity of secretion is a hallmark of the neural synapses and is one of the most compelling parallels between the IS and neural synapse.[7] Early studies on the mechanism of T-cell-mediated killing suggested that killing worked by exocytosis of preformed granules containing lytic molecules with activity-like complement.[41] It was later found that the primary role of perforin was to induce the target cell to take a "poison pill" by introducing granzyme A or B into the cytoplasm, which initiates a pro-apoptotic caspase cascade.[42,43] Evidence that cell T-cell polarity was related to directed secretion was provided by seminal studies of Geiger and Kupfer showing that the microtubule organizing center and Golgi apparatus reorients toward the target cell for killing.[44,45] Kupfer published a series of studies on molecular makeup of the T-cell-B-cell interface with the first demonstration of CD4, LFA-1, IL-4, Talin and protein kinase C-θ polarization to the interface.[46-50] All of these studies were performed with fixed cells so temporal information was deduced from populations of images for cells fixed at different times.

In 1998, Kupfer published a paper on the organization of LFA-1, Talin, TCR and protein kinase C-θ in the interface between antigen-specific T-cells and antigen-presenting B-cells.[4] LFA-1 and Talin were shown to form a ring in the interface and TCR and protein kinase C-θ were shown to cluster in the middle. These structures were defined as supramolecular activation clusters (SMAC). The TCR cluster marked the central SMAC (cSMAC), while the LFA-1 ring marked the peripheral SMAC (pSMAC). It was implied that TCR signaling was initiated and sustained by the cSMAC. My collaborators and I published a paper in parallel in which live T-cells interacting with supported planar bilayers were imaged in real time to visualize segregated adhesive domains composed of LFA-1-ICAM-1 and CD2-CD58 interactions.[3] It was posited that the segregation of the adhesion molecules was driven by the different topology of the LFA-1-ICAM-1 (40 nm domain) and CD2-CD58 (15 nm domain) interactions.[3,51] The antigen-dependent organization of these domains into a bull's eye pattern, similar to that reported in several international meetings by Kupfer, was an active process.[3] We proposed the definition of "immunological synapse" for the bull's eye pattern described by Kupfer and colleagues and our studies with adhesion molecules, linking a specific molecular pattern to the widely discussed concept.[52,53] Taking these two studies together, the synapse was defined as a specialized cell-cell junction composed of a cSMAC and a pSMAC. The term immunological synapse has subsequently been applied to a more diverse array of structures, but we will focus on the positional stability and polarity and how these are generated by the cell, which are the functionally important aspects.

The formation of the synapse was first evaluated in live T-cell-supported planar bilayer models.[5] Supported planar bilayers are formed from phospholipid liposomes on clean glass coverslips and can contain physiological densities of purified adhesion molecules that are anchored to the upper leaflet of the bilayer such that they are laterally mobile.[54,55] When ICAM-1 and MHC-peptide complexes are included, T-cells are fully activated by the substrates and organize SMACs similarly to T-cell-B-cell synapses. These systems can be imaged by wide-field or confocal fluorescence microscopy and near-field methods as will be discussed below. It was shown that TCR are engaged first in the periphery within 30 seconds and then these TCR clusters translocate to the center of the synapse to form the cSMAC by five minutes. Examination of cell-cell systems showed a

similar pattern with peripheral TCR clusters merging in the center to form the cSMAC.[56,57] This process could take up to 30 minutes with naive T-cells. IS formation is enhanced by CD28-CD80 mediated costimulation,[58] but CD28-CD80 interactions are dependent upon TCR-MHCp interactions,[22,59,60] perhaps due to local rearrangements of actin that are compatible with enhanced CD28-CD80 interaction. Thus, costimulation mediated by CD28 interaction with CD80/86 is a positive feedback loop in IS formation and function.

The IS pattern was highly correlated with full T-cell activation in vitro in multiple studies using both T-cell-B-cell, T-cell-MHCp and ICAM-1 bearing planar bilayer and NK cell-targeT-cell IS.[4,5,61] While it was recognized early on that TCR signaling was initiated well before the cSMAC was formed, it was still posited that the cSMAC might be involved in sustained signaling. While kinases can be localized to the cSMAC at 1–5 minutes, there is a consensus that the cSMAC has relatively low levels of phosphotyrosine, activated phospho-Lck, or activated phospho-ZAP-70 at later times.[57,62,63] Phosphotyrosine staining was retained in the cSMAC at one hour in CD2AP deficient T-cells. Since CD2AP regulates TCR degradation, it was argued that the cSMAC is engaged in continuous signaling, which is made occult by TCR degradation processes.[63]

A striking property of the cSMAC is that TCR-MHCp interactions in the cSMAC are stable as measured by fluorescence photobleaching recovery.[5] Since each TCR-agonist MHCp interaction has a half-life of 5–30 seconds it would be expected that half of the TCR-MHCp interactions in the interface would exchange with free MHCp over a period of several minutes. In fact, the TCR-MHCp interactions in the cSMAC do not exchange with MHCp in the bilayer over a period of one hour. The basis of this stabilization is not clear, but may be as simple as the very high local density of TCR and MHCp creating diffusion barriers that exclude free MHCp or favor rebinding of the same MHCp to the TCR following spontaneous dissociation.[63] In contrast, sustained signaling by T-cells appears to be maintained by new TCR-MHCp interactions since it can be acutely inhibited by antibodies to MHCp that compete for TCR binding.[64] This acute inhibition by anti-MHCp appears to be inconsistent with a central role of stable TCR-MHCp interactions in the cSMAC with TCR-MHCp interactions; however, in the absence of other TCR-containing structures after 5–30 minutes, attention has continued to focus on the cSMAC as a signaling structure, even though the formation of a cSMAC is not required for T-cell activation.[63,65]

To search for other TCR-containing structures in the IS, my lab has employed total internal reflection fluorescence microscopy (TIRFM) with the live T-cell-supported planar bilayer system. This is a uniquely advantageous combination for increasing sensitivity to small, low-contrast structures. Through the lens, total internal reflection fluorescence microscopy is based on using very high-resolution oil immersion objectives with a laser focused at the outer edge of the back aperture to steer the shaft of illumination to the sample at an angle that exceeds the "critical angle."[66] Under these conditions, all the light is reflected off of the interface between the coverglass and the cell, but an evanescent wave is generated that can excite fluorescence in the "near-field" (within 200 nm of the interface) (Fig. 1A,B). This method is generally not useful in cell-cell interfaces because these structures are many microns away from the interface, but it can be very effective for examination of the interface with cells and the supported planar bilayer, which is only 2 nm off the surface of the coverglass. Thus, the entire IS can be illuminated with lateral and axial resolution of ~ 200 nm, a uniquely optimal situation in light microscopy. TIRFM is used for single-fluorophore imaging, so as a long as contrast exists, a small fluorescent structure can be detected.[67,68]

Application of TIRFM to the IS led to a striking discovery. While the field had seen the IS formation process as a single wave of TCR-MHCp movement from the periphery to the cSMAC, TIRFM revealed continued formation of TCR-MHCp microclusters in the periphery of the IS.[9-11] TCR clustering had been recognized as an important concept on theoretical grounds based on work with growth factor receptors[69,70] and the FcεRI receptor[71] (see also Chapter 6), but the minimal clusters sufficient to sustain TCR signaling had been assumed to be too small for direct visualization.[72,73] Initially, TCR microcluster can contain up to ~150 TCR each at which point they are readily detectable by conventional methods, but by 60 minutes of sustained signaling the TCR clusters contain only ~10 TCR per cluster and could only be detected by TIRFM.[9]

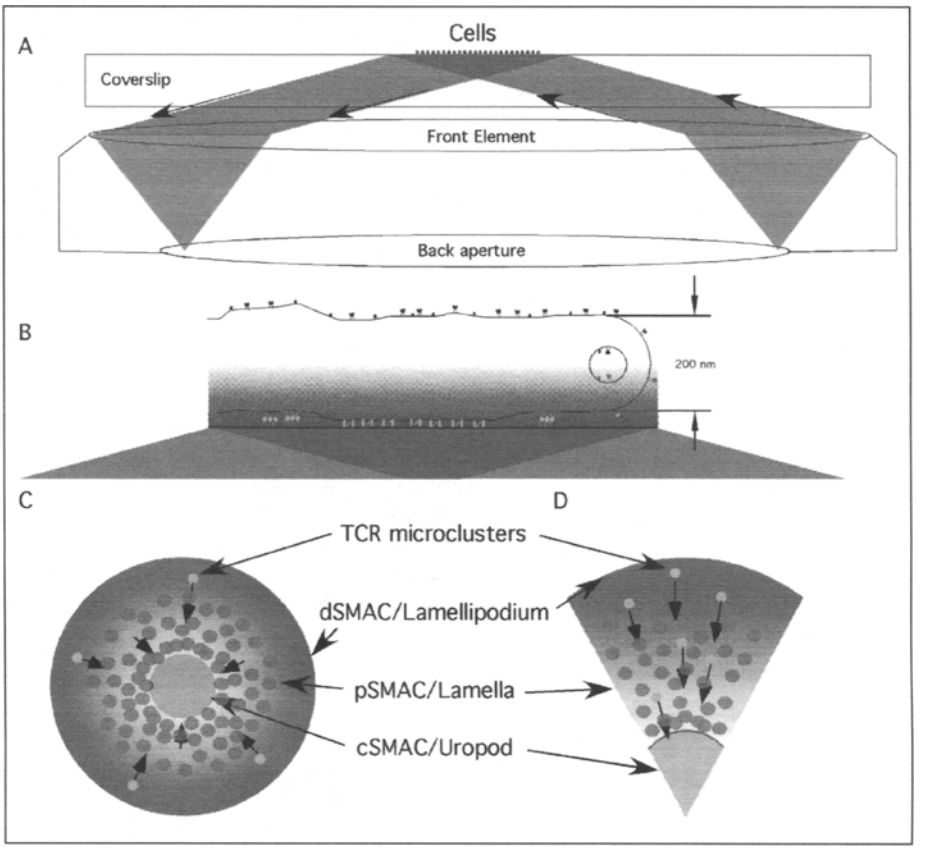

Figure 1. Total internal reflection fluorescence microscopy and schematics of synapse and kinapse. A) Schematic of TIRFM objective (NA > 1.45) with laser light path indicated and incidence on supported planar bilayer with attached cells. B) Expanded view of evanescent wave (gradient region above the coverslip) in which two sides of a thin cellular structure can be selectively imaged. Molecules in contact area with the bilayer are visualized whereas objected on the upper side or weakly or not visualized. This greatly increases contrast. However, it should be pointed out that some cytoplasmic membrane systems such as exocytic vesicles can be detected in the TIRFM field. C) En face view of the synapse with cSMAC, pSMAC and dSMAC as defined by Kupfer.[4,62] The grayscale gradient approximates the pattern of f-actin, which is highest in dSMAC, lower in the pSMAC and lowest in the cSMAC. TCR (fine speckling) and LFA-1 (coarse speckling) microclusters are indicated. The SMACs are not homogeneous clusters, but at high resolution are collections of different types of micron scale molecular clusters that function in microcluster initiation (dSMAC), microcluster translocation (pSMAC) and degradation (cSMAC) with respect to the TCR. Arrows on schematic indicate direction of TCR microcluster movement. D) En face view of a kinapse. In migrating cells the leading edge is defined as a lamellipodium, followed by the lamella and trailed by the uropod. The kinapse has not been extensively studied, so this model is based on the hypothesis that the structures in the synapse and kinapse can be paired as indicated.

TCR microclusters are detected over the entire range of MHCp densities leading to cSMAC formation.[9] Each microcluster lasts about two minutes prior to capture by the cSMAC. TCR microclusters are stained with anti-phosphotyrosine antibodies; they recruit ZAP-70-GFP and anti-MHCp Ab that block Ca^{2+} signaling within two minutes by blocking formation of

new TCR microclusters.[9-11] Interestingly, TCR-MHCp interactions in the cSMAC persisted for many minutes after anti-MHCp addition, demonstrating that stable interactions in the cSMAC are not sufficient to sustain TCR proximal signaling. Therefore, contrary to initial perceptions we could demonstrate with near-field imaging methods that TCR microclusters are newly formed in the periphery of the IS to sustain signaling. This finding redirects our attention from the cSMAC to the more dynamic T TCR structures in the periphery of the IS. It also emphasizes the essential heterogeneity of SMACs. These are not homogeneous clusters but complex membrane-cytoskeletal zones composed of distinct microclusters (Fig. 1C).

The TCR microclusters are formed in the periphery of the IS–a structure that Kupfer described as a distal SMAC (dSMAC).[62] The dynamics of the dSMAC are very similar to the lamellipodium of a spreading or migrating fibroblasts in that it displays cycles of extension and retraction referred to as contractile oscillations.[74,75] Consistent with this, the dSMAC is also highly enriched in dynamic f-actin. Based on studies with Jurkat T-cells on anti-CD3 coated surfaces, the dynamic f-actin structures are dependent upon the Rac1 effector WAVE2 and the lymphocyte cortactin homology HS-1[76,77] (Fig. 1C). The dSMAC is also rich in CD45, the transmembrane tyrosine phosphatase that primes activation of Src family kinases including Lck and Fyn.[62,78] The near-field imaging clearly demonstrated that CD45 is excluded from peripheral TCR microclusters (see also Chapter 7), but is included in the cSMAC.[9] A model for CD45 function could be stated as Lck is activated by CD45 outside the TCR microclusters and then active Lck diffuses a short distance to capture in the TCR microcluster where it operates until rephosphorylated on the inhibitory site by Csk.[67]

The TCR microclusters traverse the pSMAC, a domain rich in the larger LFA-1-ICAM-1 interaction. The pSMAC is not a solid ring, but a meshwork of micron-scale, LFA-1 rich clusters with interspersed spaces lacking LFA-1-ICAM-1 interaction.[5] The TCR microclusters appear to navigate these holes in a tortuous fashion.[9,10] Thus, the TCR microclusters do not take a straight-line path to the cSMAC, but zigzag through the pSMAC, perhaps because of the obstacles formed by the dynamic LFA-1 clusters (Fig. 1C). It is notable that ZAP-70 recruitment and activation appears to be maximal as the TCR microclusters traverse the pSMAC.[9-11] Thus, the close juxtaposition of TCR microclusters and surrounding integrin microclusters appears to be an optimal condition for TCR signaling, but signaling turns off as, or shortly after, the TCR clusters join the cSMAC, which is typically free of LFA-1-ICAM-1 interactions.

It has been observed in many studies that when T-cells migrate on ICAM-1 containing surfaces they produce "focal zones" of LFA-1-ICAM-1 interaction that accumulate in the lamella, the force generating structure in amoeboid cell locomotion.[5,37,79-83] These focal zones can vary in shape from crescents to wedges that are analogous to a half or quarter synapses. We would posit that the focal zone is similar or identical to the "lamella" zone of a migrating tissue cell.[82,83] In a synapse, the inward-directed forces generated in the pSMAC/lamella are balanced and the cell moves slowly or not at all.[84] If the symmetry of the synapse is broken, then the asymmetric lamella mediates rapid cell movement, but this does not necessarily terminate signaling. Thus, we propose that the contact structure used by a migrating T-cell to integrate signal from an APC is not a synapse, but can be defined as a "kinapse" translated as a moving junction. Kinapses signal by forming TCR microclusters in the leading lamellipodia that signal as they translocate through the lamella and are inactivated in the uropod, the trailing structure that is analgous to the cSMAC (Fig. 1D). This model suggests a way to resolve the controversy regarding synapse formation and signaling between groups that typically observe synapse formation during T-cell activation and those who observe migration only.[85] Since TCR signaling is initiated and sustained in leading lamellipodium by TCR microclusters, the formation of these structures by migrating T-cells is fully compatible with integration of signals. The uropod and cSMAC may differ in that a cSMAC receives and can preserve, to some degree, TCR-MHCp complexes and associated molecules, although there is no evidence that the molecules accumulated in the cSMAC continue to signal in normal T-cells, while the uropod may only maintain long-term connections using membrane nanotubes, which may prolong signaling connections between

immune cells, but are likely to break beyond a few 10s of micrometers.[86,87] The major differences between synapse and kinapse include that a synapse maintains contact with the same APC over hours, whereas a kinapse may maintain communication with one APC for only a few minutes. Synapses polarize some secretion toward the APC, whereas kinapses would not appear to have the same possibility.

The current challenge in the field is to image TCR microclusters in T-DC or other T-APC interfaces. This is a challenge because the near-field imaging technologies that are available cannot access the cell-cell junction. Closer examination of a study from Kupfer using wide-field microscopy and computational deconvolution suggests that these methods can detect early microclusters, but its not clear if the later-sustained microclusters, which are smaller, would be visible.[62] T-DC synapses appear to have multiple foci of TCR-MHCp interaction,[88] but other studies observe classical synapses with rings of pSMACs and cSMACs formed with mature DC.[20] The observation that the classical synapses have multiple signaling TCR microclusters may reconcile these observations, but dynamic imaging of T-DC microclusters would be required to validate this position.[89] T-cell synapses with CHO cells as model APC appeared multifocal and demonstrated a surprising segregation of TCR clusters from CD28 clusters,[90] which have been observed to colocalize in other model systems.[22,59,60] Davis and colleagues have visualized single MHCp complexes in the interface between T-cells and APC by labeling peptides with phycoerythrin, but the limitation of this approach is that its not clear how many TCR are involved in recognition of these peptides[61,91,92] (see also Chapter 6). In fact, the evidence that these peptides are recognized at all is circumstantial rather than direct. When a single peptide-agonist-peptide complex is seen in the interface, the T-cell fluxes Ca^{2+} transiently, but the limitation that the single peptide can only be imaged once due to fluorophore bleaching makes it impossible to follow dynamic processes that could provide more direct evidence for interaction. Technologies with the potential to collect such dynamic interaction include spinning disc confocal and line-scan technologies that have recently been commercialized.[82] The problem is not single molecule sensitivity, but contrast and the ability to distinguish small clusters from random fluctuations.

In Vivo Functions of Synapse and Kinapse

TCR signal integration through microclusters formed in synapses or kinapses provides a framework for thinking about results from recent in vivo studies. Since we can now understand the ability of T-cells to signal while migrating, we can consider regulation of T-cell migration not in terms of signaling changes, but in terms of controlling the network of spatiotemporal cell-cell interactions that recruit differenT-cells into the immune response or allow them to execute effector functions. Another consideration is directed secretion, which is likely best delivered through a synapse, whereas a kinapse may be much less efficient at delivering secreted molecules to specific APC.

Naive T-cell priming is a central process in initiation of immune responses. Priming of naive T-cells requires interactions with DCs in vivo.[93] However, not all antigen-specific T-DC interactions will lead to priming. When an antigen is presented by DCs in the steady state (absence of inflammation), the result is induction of peripheral tolerance.[94,95] Presentation under steady-state conditions leads to T-cell proliferation followed by induction of antigen-specific nonresponsiveness in the effector cells or their deletion.[96] A second mechanism of peripheral tolerance is the induction of antigen-specific regulatory T-cells.[97] The steady-state process of inducing tolerance preconditions the active peripheral T-cell repertoire to react selectively with pathogen-associated foreign antigens under conditions of infection or tissue damage since T-cells specific for self antigens and benign foreign antigens are deleted or anergic.[2] The dynamics of priming or priming versus tolerance has been the subject of several papers.

Stand-alone studies on priming of naive T-cells have been carried out using three different TCR transgenic models, two different adjuvant systems and both explanted intact lymph node and intravital imaging approaches. Consistent themes are emerging and hypotheses can be developed regarding the basis for differences between studies.

In Vivo Analysis of CD4+ T-Cell Priming and Tolerance Induction

Stoll et al[98] examined dynamics of T-cell priming by transferred mature DC. CFSE labeled 5C.C7 TCR Tg T-cells were introduced into the lymph nodes by intravenous adoptive transfer and DiI- or DiD-labeled DC were introduced to specific draining lymph nodes by sub-cutaneous injection. 5C.C7 is an I-E^k restricted TCR that binds moth cytochrome C (MCC) peptide 91-103 as an agonist, so DC were either pulsed with this peptide or a control peptide. The lymph nodes were then explanted, embedded and imaged with a conventional confocal microscope. This is the only study of lymph node T-cell dynamics by conventional confocal microscopy, which limits depth of penetration to ~50 μm. T-cells displayed low basal motility in the absence of antigen, which may be attributed to low perfusion (media movement over the surface of the lymph node). Nonetheless, many more 5C.C7 T-cells interacted with antigen-pulsed DC compared to unpulsed DC. The antigen-specific interactions appeared to be stable in that T-cells and DC formed extensive interfaces that excluded CD43, a characteristic of in vitro synapse. Naive 5C.C7 T-cells expressing CD43-GFP were prepared by retroviral transduction of bone marrow stem cells followed by reconstitution of irradiate mice. A remarkable transformation took place at 36 hours when the activated antigen-specific blasts initiated rapid migration. This 36-hour period took place in vivo, not in the organ culture; nonetheless, the imaging conditions were the same so it is apparent that recently activated blasts "break" synapse-like interactions after 36 hours when they initiate rapid migration under conditions where naive T-cells were incapable of migration. Subsequent encounters with antigen-positive DC did not lead to synapses. This study suggested that CD4 T-cells remain in synapse until they start to proliferate such that all communication prior to the first division is through synapses and all communication after the first division takes place through kinapses.

A series of studies by Miller et al using two-photon laser-scanning microscopy (TPLSM) from 2002 to 2004[13,99-101] shed light on repertoire scanning and priming in a similar hybrid in vivo/explant system with some modifications compared to Stoll et al.[98] For information on TPLSM, which allows imaging of cells at depths of up to 500 μm in tissues, I refer the reader to recent reviews.[102-104] Briefly, TPLSM uses pulsed infrared light to excite fluorophores with two photons of red light, a nonlinear process that can be tuned to provide excitation in a tightly defined focal point of a laser within the tissues followed by wide-field detection of all photons emitted by the tissue. The advantage of infrared light is that it penetrates tissues better than visible light due to lower scattering, except in the wavelength range around 950 nm were infrared light is absorbed strongly by water. Since fluorescence is excited only in the focal volume bleaching above and below the focal plane in which the laser beam is scanned, photobleaching is minimized and no pin-hole is needed for detection. In fact, the photomultiplier should be very close to the objective to collect scattered photons in the appropriate wavelength range. Two-photon excitation spectra tend to be broad, so a single laser wavelength can be used to excite three fluorescent proteins and all quantum dots colors simultaneously. The limitation then becomes the number of photomultiplier tubes (PMTs), which is limited to two or three on most commercial systems. The ideal geometry of having the PMTs very close to the objective means that tradeoffs in depth are required to gain more colors. Figure 2 shows a spectral unmixing strategy to use two PMTs to identify cells expressing CFP, GFP and YFP, with a second channel for a quantum dot or red organic dye (e.g., Texas red™). Despite these advantages, great care in optimizing excitation power and collection is essential since deep-tissue imaging with brightly labeled cells is typically performed near the damage threshold for the tissue and the line between optimal imaging and tissue damage is easily crossed in a 2–3 fold range.[104] Adoptively transferred DO11.10 TCR Tg T-cells were activated with ovalbumin in alum.[13,99] Alum is an adjuvant that primes Th2 responses perhaps due to the activation of IL-4 producing APC.[105] Unlike the Stoll et al system where the node was immobilized by implanting it in a drop of agarose and imaging it from the immobilized side,[98] Miller et al attached the lymph node to the bottom surface of a dish via the hillus and then imaged from above with a continuous perfusion of highly oxygenated media.[99] These conditions and perfusion may be the key difference, resulted in the first observations of dramatic motility of naive T-cells in the lymph node, which is similar to that observed by intravital microscopy,[100] and established the

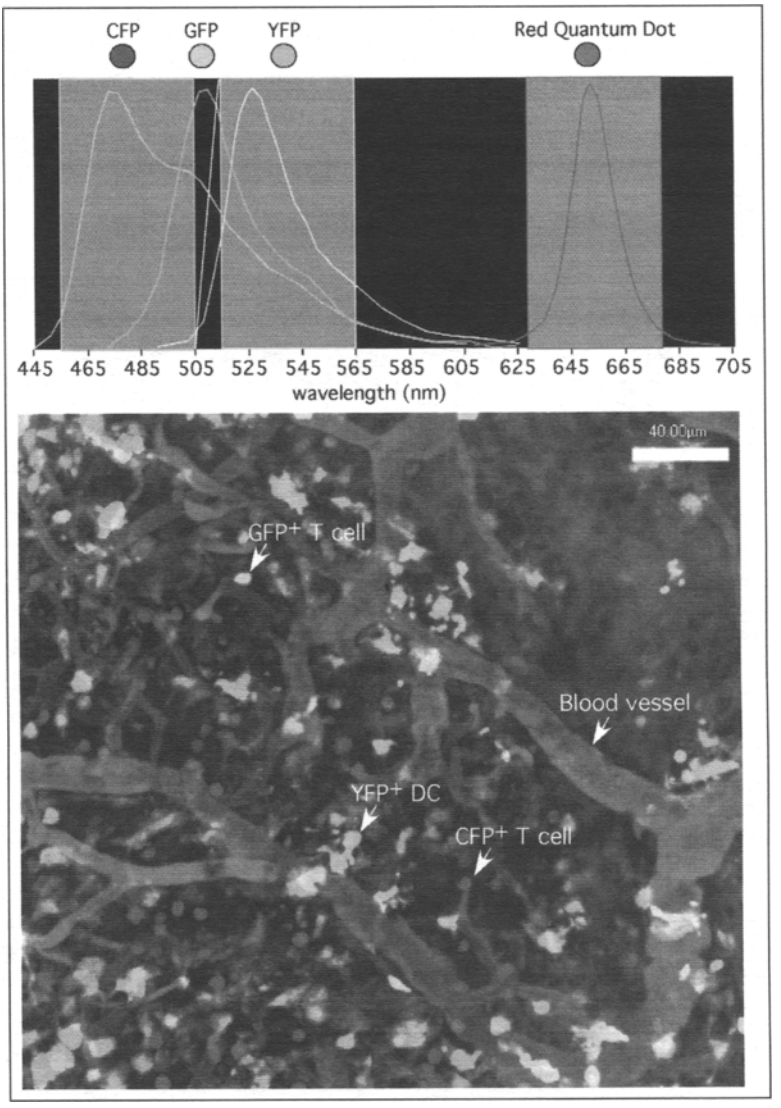

Figure 2. Two-photon excitation of three fluorescent proteins and intravascular quantum dots in the inguinal lymph node. The emission spectra of enhanced cyan fluorescent protein (ECFP), enhanced green fluorescent protein (EGFP), enhanced yellow fluorescent protein (EYFP) and red quantum dots, and the band-pass limits of the filters used for emitted light collection (grey). Images utilizing this approach can be found in Shakhar et al.[14]

current paradigm for stochastic repertoire scanning,[101] In studies on priming purified DO11.10, T-cells were labeled with CMTMR and adoptively transferred i.v., while DC were labeled in situ by subcutaneous injection of CFSE with the alum/antigen mixture. Therefore, all DC that were present at the injection site and them migrated to the lymph node would be labeled. Resident DC in the lymph node that were exposed to antigen draining via the lymph or immigrant DC that entered the tissue hours after the injection would not be labeled because the reactive dye would be hydrolyzed in a few minutes. It is not clear how effective the alum is at preventing drainage of

soluble antigen to the lymph node ahead of immigrant DC, which may have an impact on early priming.[106] Nonetheless, this was a highly effective method for examining T-cell interactions with DC that migrated from the injection site.

The fundamental findings from this series of experiments were: (1) T-cells move rapidly and, to a first approximation, randomly in the T-cell zones,[99] (2) similar movement of T-cells is observed in vivo using intravital microscopy of the inguinal lymph node,[100] (3) DC move slowly, but contact many T-cells by probing with many veil-like processes such that each DC can contact up to 5000 T-cell per hour,[101] and (4) antigen-specific interactions can be more dynamic that those observed by Stoll et al[98] with stable interaction between 3-16 hours, with resumption of antigen-specific swarming and rapid migration by 16-24 hours and beyond.[13] Even at the most stable phases of interaction, the apparent contact area size and the changes over time suggested a more dynamic situation than in vitro synapse; however, no molecular imaging was performed. The same model was also used to study oral tolerance in mesenteric and peripheral lymph nodes.[23] In conditions of priming and tolerance, T-cell clusters were formed that were similar to those in Miller et al. 2004.[13] Under conditions of priming, the clusters were larger and longer-lived, but synapses were observed in both priming and tolerance induction. The prevalence of a spectrum of interaction types from rapid migration to swarming in addition to stable interactions suggest that kinapses also have a role in in vivo priming and tolerance.

Steady-state DC have been visualized in vivo using CD11c promoter YFP transgenic (CD11c-YFP) mice.[17] Lymph node DCs include immigrants from the tissues and resident cells that may enter directly from the blood. While tissue inflammation stimulates synchronous migration of many DC to the draining lymph node, there is a poorly understood steady-state migration of DC from the tissues to the lymph nodes that is likely to be critical for peripheral tolerance to tissue antigens.[107] These immigrant DC are mature in that they express high levels of MHC class II on their surfaces, but they are not activated since they only express low to intermediate levels of CD80 and CD86. The CD11c promoter was used to generate a number of transgenic strains that were tested for bright enough fluorescence to be useful in two-photon intravital microscopy in up to 300 μm depth. One founder of several tested had high expression in $CD11c^{hi}$ myeloid DC. In the T-cell zones of inguinal lymph nodes of live mice, steady-state DC form extensive sessile networks.[17] Distinct DC behaviors were described in the subcapular sinus (migrating DC), superficial surface of B-cell follicles (layer of dim stationary DC), interfollicular zones (clusters of DC trapping T and B-cells) and T-cell zones (DC networks). Immigrant DC migrated from the subcapular sinus to the T-cell zones rapidly and join the T-cell zone networks.[17] Thus, rapid T-cell migration through the DC networks and DC outreach to passing T-cells through formation of long membrane processes are the mechanisms that drive repertoire scanning.

$CD4^+$ T-cell priming and tolerance was studied in vivo using the CD11c-YFP Tg mice and a strategy for targeting antigen to DC by attaching antigenic peptides to mAb specific for the scavenger receptor DEC-205.[14,94] Under these conditions all of the DEC-205 positive DC, which include CD11c high and low DC types, will present antigen. In the absence of innate immune stimulation, presentation of antigen to three different MHC class II-restricted TCR tested thus far (3A9/HEL, 2D2/MOG, OTII/OVA) leads to tolerance.[14,94,95] Tolerance involves initial activation and expansion of T-cells followed by death of the expanded cells between days three and seven after initial activation. Induction of tolerance requires both the early exposure to antigen, which induces activation and proliferation and late exposure to antigen after day three to induce deletion (M. Nussenzweig, personal communication).

To perform these experiments, OTII TCR Tg/chicken β actin promoter-GFP (GFP) Tg T-cells (specific) and non-TCR Tg/CFP Tg T-cells (nonspecific) were transferred into CD11c-YFP Tg hosts that had been injected four hours prior with DEC-205-Ova peptide such that the specific and nonspecific T-cells enter lymph nodes that already contain antigen-presenting DC. Blood flow in the intravital inguinal lymph node preparation was followed using red quantum dots, which excite well at the same TPLSM wavelength, 900-920 nm. Tolerance was induced with the DEC-205-Ova alone and priming was induced by addition of anti-CD40 mAb to induce activation of DC. This study is

distinguished from other studies on CD4+ T-cells and other studies on tolerance by using intravital microscopy in which blood and lymph flow are intact. Imaging was initiated in three time frames from 0-6 hours, 6-12 hours and 12-18 hours after T-cell transfer. Specific T-cells showed rapid arrest near high endothelial venules (HEV) within an hour of injection, regardless of whether the conditions favored tolerance or priming. Over the next 18 hours, the specific T-cell regained rapid motility in the DC networks and did not systemically form stable interactions at later times. Some statistically significant differences were detected between tolerance and priming in that under conditions of priming, the rate of return to control migration velocity was slower than for tolerizing conditions. Since activation and proliferation are induced under both tolerizing and priming conditions, there was a positive correlation between synapses and proliferation. It appeared that critical signals for tolerance were integrated during later, T-DC kinapse formation. In fact, continued interaction of T-cells with antigen-positive DC appears to be critical for full expansion of CD4+ T-cells.[108] Since all three studies on CD4+ T-cells, Stoll et al.[98], Miller et al[13] and Shakhar et al[14] concur that T-DC interactions are dynamic after proliferation is initiated, it seems very likely that both synapses and kinapses play a key role in CD4+ T-cells priming and tolerance.

In Vivo Analysis of CD8+ T-Cell Priming and Tolerance Induction

The priming of CD8 T-cells has been shown to require a short time of interaction with antigen-presenting cells followed by a long antigen-independent expansion process.[109-111] As mentioned in the previous section, this is very different from the current understanding of antigen requirements for CD4+ T-cell expansion.[108,112] However, induction of antigen-dependent tolerance of CD8+ T-cells appears to require sustained contact with antigen-positive DC after 72 hours.[19] Thus, while priming of CD8+ T-cell responses may be imprinted by early interactions with mature, activated DC, the peripheral deletion of auto-reactive CD8+ T-cells appears not to be imprinted, but to require sustained interactions with DC for longer than three days. These biological issues need to be considered in interpretations of the imaging data.

Bousso and Robey studied the priming of LCMV gp33 specific P14 TCR Tg T-cells using transferred DCs[113] and a similar explanted lymph node imaging approach as Miller et al.[99] They focused on one time point that was 24 hours after transfer. It is assumed that DC migrated rapidly to the lymph node since they were injected into very proximal subcutaneous sites. Bousso et al[113] provided the first estimate of DC repertoire scanning rate at 500 T-cells per DC per hour. This is 10-fold lower than the subsequent estimate by Miller et al of 5000 T-cells per DC per hour and the major difference may be in the visualization of fine dendritic processes that greatly increase the effective target size of the DC in the Miller/Cahalan study.[101] This may be an imaging issue or an issue related to handling of the DC altering their morphology. Like Stoll et al,[98] Bousso et al[113] emphasize the formation of synapses, but only at one (24 hour) time point. A unique aspect of this study is that it is the only intact lymph node study where antigen dose was varied. They found that synapses were formed over the entire range that would lead to priming.

The first kinetic study of T-cell-DC interaction in vivo was Mempel et al[114] performed extensive studies with the P14 line also used before by Bousso et al.[113] Unlike Bousso, Mempel et al imaged the popliteal lymph node of live anesthetized mice with intact blood and lymph flow. While studies were performed with the DO11.10 CD4+ T-cells, this data set was very limited compared to the more complete study by Miller et al.[13] Mempel et al used LPS, treated, mature CMTMR-labeled DC that were injected into the foot pad, which then drained uniquely to the ipsilateral popliteal lymph node. They injected CFSE labeled P14 T-cells, allowed the cells an hour to enter the lymph node via the HEV and then injected mAb to L-selectin throughout the rest of the experiment to block further entry. This ensured that the one temporally defined cohort of T-cells is followed. Mempel et al[114] is the first study to clearly demonstrate three phases of interaction during priming. Antigen-specific T-cells began to encounter DC soon after entering the lymph node, but for the first eight hours the encounters were short-lived kinapses. Early kinapse formation activated the T-cells since CD69 was upregulated in this time frame, referred to as phase 1. In the 8-12 hour time period synapses formed. After this synapse period, referred to as phase 2, cytokine production was

initiated. At 24-26 hours there was a mixed picture of synapses and kinases and by 44-48 hours all interactions were kinapses. This period, referred to as phase 3, was characterized by proliferation. Thus, although it was found that $CD8^+$ T-cells only require a few hours of stimulation to fully commit to many rounds of cells division, the in vivo profile of T-DC interaction is very similar to what was reported subsequently for $CD4^+$ T-cells, which integrate signals over longer periods. It is possible that $CD8^+$ T-cells in vitro immediately form synapses with antigen-rich DC and integrate signals quickly to commit fully to an effector program. In contrast, the sparse antigen-positive DC in vivo may require a longer period of signal integration requiring phase 1 and 2 before becoming antigen independent in phase 3. More work would be required to determine if $CD8^+$ T-cells do or do not integrate meaningful signals through kinapses in phase 3.

Hugues et al[21] studied tolerance versus priming of $CD8^+$ OTI TCR Tg T-cells using an explant system very similar to that of Miller et al.[99] They used the DEC-205 antigen delivery approach after CFSE labeled OTI T-cells were transferred and labeled all DC in the explanted lymph nodes by injecting fluorescently labeled anti-CD11c IgG into the parenchyma prior to imaging. Thus, unlike Shakhar et al[14] Hugues et al[21] delivered antigen to DC after T-cells were equilibrated in the lymph nodes, rather than injecting T-cells into mice that had been equilibrated with the antigen and performed the imaging with explanted lymph nodes in which DC had CD11c and perhaps FcR engaged by anti-CD11c mAb, rather than performing intravital microscopy in a mouse in which DC express YFP. In this study, OTI T-cells were found to engage in a spectrum of interactions with DC that was biased toward synapses in priming conditions and kinapses in conditions of tolerance. It is not clear if the differences between Hugues et al[21] and Shakhar et al[14] are due to differences between $CD4^+$ and $CD8^+$ systems or due to technical differences in the way the experiments were performed. In Shakhar et al[14] the area around the HEV played a key role in early synapses and it might be argued this region may not function in the same way in the absence of blood flow. Zinselmeyer et al[23] used a totally different antigen-delivery route and also found that both priming and tolerance involved stable, IS-like T-cell migration patterns. It will be important to revisit the issue of T-DC dynamics during $CD8^+$ T-cell tolerance induction in light of these discrepancies.

Priming vs. Tolerance

Priming and tolerance require T-cell activation. Tolerance induction by deletion of effector cells appears to require prolonged TCR signaling beyond three days. Thus far, activation is associated with synapses in 9 of 10 data sets. Only one data set on tolerance induction is associated with activation without stable, IS-like interactions and this is contradicted by two other studies that readily detected these interactions under conditions of tolerance induction following primary activation. In most cases the formation of synapses is highly correlated to T-cell activation, but not to the generation of effector cells or memory, which is only correlated with more subtle changes in the dynamics of interaction. $CD4^+$ T-cells and $CD8^+$ T-cells undergoing tolerance induction all integrate signals at a later time (>24 hours) and all show rapid migration with short duration interactions with DC in this time frame. Thus, kinapse signal integration is likely to be critical for full activation of $CD4^+$ T-cells and tolerance induction and $CD4^+$ and $CD8^+$ T-cells.

Dynamics of $CD4^+$ T-Cell Help for $CD8^+$ T-Cell Responses

Germain and colleagues[28] have examined the dynamics of CD4 T-cell help under conditions where the delivery of help and direct stimulation via MHC class I peptide complexes was independently controlled. A key result from this study was that early $CD8^+$ T-cell stimulation by antigen or innate signals upregulates CCR5 and appears to render the baseline movement of $CD8^+$ T-cell dependent upon expression of CCR5. DC that have interacted with $CD4^+$ T-cells produce CCR5 ligands and attract these partially activated $CD8^+$ T-cells to make additional contacts with these licensed DCs. This increases the probability that $CD8^+$ T-cells will interact with the licensed DC and improves production of memory $CD8^+$ T-cells. Whether other inflammatory chemokine receptors are used by $CD8^+$ T-cells is not known, but is likely. This study suggests an explanation

for earlier observations of "swarming" in which particular DC appeared to act as local attractors for subsets of T-cells.

Effector Sites

At the time of writing of this chapter, there are only a few papers on intravital microscopy of T-cell-APC interactions at effector sites. While there are a number of other studies that report on various lymphoid cells in the intestine and in tumors, I have not included these because there was no concept of antigen-specific interaction; rather, the movement of cell types was documented often with only a limited understanding of the cell type involved. The primary studies that I will discuss have focused on natural killer T (NKT) cells in the liver,[34] helper T-cells in the lymph node,[29] memory T-cells in the bone marrow,[32] and activated effector cells in the central nervous system (CNS).[115] These studies all suggest that effector cells form synapses with antigen-positive APC.

Geissmann et al[34] took advantage of the expression pattern of the chemokine receptor CXCR6 in the liver to follow NKT-cells in vivo. CXCR6 is highly expressed on activated T-cells and NKT-cells. In the liver of healthy mice, NKT-cells represent 30% of the mononuclear cells and 70% of the $CXCR6^+$ cells. Unutmaz et al[116] replaced the major coding exon of CXCR6 with GFP by homologous recombination. The $CXCR6^{gfp/+}$ mice have a normal number of NKT-cells in the liver, which are GFP^h, whereas the $CXCR6^{gfp/gfp}$ mice have 3-5 fold reduced numbers of NKT-cells in the liver, which remain GFP^{hi}.[34] The reason for the reduced numbers of NKT-cells in the $CXCR6^{gfp/gfp}$ mice appears to be reduced NKT-cell survival in the absence of CXCR6. The high levels of GFP expressed in the NKT-cells afforded the opportunity to track these cells in the liver of mice by intravital microscopy and to determine the effect of antigen. It had been reported that a population of leukocytes identified as Kupffer cells migrated within the sinusoids of the liver.[117] Geissmann et al[34] discovered that Kupffer cells stained by high molecular weight rhodamine dextran are stationary cells, while NKT-cells migrate rapidly within the sinusoids with or against blood flow. This rapid migration within the fenestrated sinusoids allowed WT NKT-cells to visit each hepatocyte in the liver on average every 15 minutes. Geissmann et al[34] never observed GFP^+ cells extravasating. NKT-cells have a dominant $V\alpha14$ rearrangement that produces a high affinity TCR for CD1d with α-galactosylceramide (AGC), which corresponds structurally to a class of bacterial lipids.[118,119] When AGC was injected i.v., most of the NKT-cells become activated within two hours based on cytokine production and 60% of the GFP^+ cells stopped migrating and formed synapses within 20 minutes,[34] apparently with Kupffer cells. NKT-cells have a previously activated memory/effector phenotype in vivo, such that these observations provide the first evidence of in vivo synapses in the effector phase of an immune response. We have also found that conventional effector T-cells patrol liver sinusoids. This suggests that patrolling of sinusoids is typical of immune surveillance by activated T-cells in the liver. By reducing the dose of AGC we have recently found that NKT-cells can be induced to produce cytokines without reduction in patrolling migration (P. Velazquez and MLD, unpublished observations). This suggests that NKT-cells are flexible in using synapses or kinapses for signal integration, depending upon the strength of signal.

Okada et al[29] studied the T-B interactions during recognition of hen egg white lyozyme (HEL) or $I\text{-}A^b$-HEL 74-88 complexes by MD4 IgG Tg B-cells and TCR7 TCR Tg T-cells, respectively. The mice were immunized with HEL in alum and explanted lymph nodes were imaged exactly as in Miller et al 2004.[13] T-cell proliferation was extensive by day two, whereas B-cells did not proliferate extensively until day three. The B-cells slowed down in the first 1-3 hours and then again regained speed and migrated toward the junction between the T zone-B zone interface. This migration was guided by CCL21 gradients in the follicle and required CCR7 expression on the B-cells. This was the first demonstration of chemokine directed migration in an intact lymph node. By 30 hours the B and T-cells are activated and long-lived antigen specific T-B conjugates begin to form at the T zone-B zone interface. The interactions are strikingly different than priming T-DC interactions in that the B-cells continue to migrate rapidly and drag the T-cells behind them. This could be explained by the T-cell forming a synapse with the B-cell via the B-cell uropod, but the B-cell continuing to migrate under the influence of chemokinetic and chemotactic factors in the

environment. This is also different from an earlier study in which activated T-cells interacted with naive B-cells as viewed by conventional confocal microscopy in the inguinal lymph node.[120] In this case, the activated T-cells pushed the B-cells, which rounded up and became sessile after T-cell contact. While Gunzer et al[120] opens an interesting physical possibility that the lateral surfaces of a T-cell can function in migration while the front part forms an IS, it is less physiological than Okada et al[29] which studied a classical T-cell dependent antibody response. Again, effector cells form synapses rapidly after encountering APC.

von Andrian and colleagues[32] studied memory T-cells in the bone marrow and their rapid antigen-specific interactions with DC. They found that DC travel to the bone marrow and interact with central memory T-cells in an unexpected migration route for both of these cell types. This helps explain the well-known challenges of mature T-cells in bone marrow transplantation. Central memory T-cells and DC migrate randomly in the bone marrow cavity in the steady state. Introduction of antigenic peptide pulsed mature DCs and P14 central memory T-cells followed by imaging in the bone marrow cavity revealed rapid formation of synapses between the central memory T-cells and DC. This mechanism may help protect the bone marrow from infection and neoplastic events in the highly transformation sensitive hematopoetic system.

Kawakami et al[115] studied the dynamics of T-cells in acute spinal cord sliced from rats with early experimental allergic encephalitis (EAE) lesions. The EAE lesions were induced by injecting 5 million GFP^+ myelin basic protein (MBP) specific cloned T-cells i.v. Acute spinal cord slices were prepared four days after T-cell transfer, at which time antigen-specific and non-antigen-specific ovalbumin-specific cloned T-cells were also found in the lesions. This is characteristic of inflamed sites, which are equally attractive for extravasation of antigen-specific and nonspecific cells. Tracking of MBP-specific T-cells revealed that 40% were immobile over long periods, whereas only 5% of ovalbumin specific cells were similarly stationary. The remaining cells were migrating in the living brain tissue. Staining of the live sections in real time with antibodies to LFA-1, TCR and MHC class II revealed polarization of these molecules toward the shared interface between MBP specific T-cells and class II positive APC, but rarely with Ova-specific T-cells. These studies provide evidence from positional stability and molecular composition for in vivo synapses. Prior histological analysis in lymph nodes,[121] the meninges during the $CD8^+$ T-cell response to lymphocytic chriomeningitis virus,[122] and recent studies in adenovirus infection with thick section confocal imaging[123] have detected molecular signatures of synapses in fixed tissue with increasing acuity, but Kawakami et al[115] was the first study to be able to reference this staining to the dynamics of interaction in ex vivo live tissue. While the use of intact antibodies could be criticized—for example, Fab fragments with nonblocking Ab would be a better choice—this study provided a strong step toward imaging of SMACs in vivo.

Innate immune surveillance of the CNS was studied by Davalos et al[33] and Nimmerjahn et al[124] Microglial cells are the innate immune cells of the central nervous system in the steady state. These cells can be visualized in live mice in which the CX3CR1 chemokine receptor's major coding exon is replaced by eGFP. Microglial cells are the only cells in the CNS that express GFP in $CX3CR1^{+/gfp}$ mice. Both of these studies used the thinned skull technology developed by Gan and colleagues[125] to image microglial cells in the intact brain of anesthetized mice. Parenchymal microglial cells are referred to as "ramified" because each cell projects many long processes in three dimensions to cover a territory of 65,000 μm^3. The processes extend and retract at a velocity of 1-2 μm/min. Focal injury in the CNS results in a rapid response by the ramified microglial cells in which the cell bodies remain in place, but all the processes within a radius of 75 μm from the site of injury, dozens of individual processes, converge on the site while maintaining tethers to the cell bodies. While the biological function of this response is not known with certainty, the release ATP is necessary and sufficient to trigger the response and a reasonable biological hypothesis is that the cells are sealing off local injuries within 30 minutes. This does not appear to be a classical phagocytic response and in fact, classical phagocytes like neutrophils and monocytes appear to be excluded from small injuries that are completely surrounded by microglial foot processes.[126]

Whether microglial cells that respond to focal injuries are competent to present antigen to $CD4^+$ or $CD8^+$ T-cells is not known.

Summary

Recent evidence suggests that the basic unit of sustained T-cell receptor signaling are TCR microclusters dynamically generated near the leading edge of migrating T-cells forming kinapses or in the dSMAC of T-cells forming synapses. Retention of many TCR in the cSMAC is only possible when the T-cell stays with one APC, which requires a stop signal, but even then the continuous formation of peripheral TCR microclusters is required to sustain signaling. The specific function of the cSMAC is unknown, but it is not sufficient to sustain Ca^{2+} signals in T-cells. In vivo, activation of T-cells associated with activation and tolerance requires both synapses and kinapses. Synapses are a common feature of effector phase and these interactions are initiated rapidly after antigen recognition.

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