

# Intravital Microscopy: Visualizing Immunity in Context

## Review

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*The most pervasive fallacy of philosophic thinking goes back to neglect of context.*

John Dewey (1859–1952)

Recent advances in photonics, particularly multi-photon microscopy (MPM) and new molecular and genetic tools are empowering immunologists to answer longstanding unresolved questions in living animals. Using intravital microscopy (IVM) investigators are dissecting the cellular and molecular underpinnings controlling immune cell motility and interactions in tissues. Recent IVM work showed that T cell responses to antigen in lymph nodes are different from those observed *in vitro* and appear dictated by factors uniquely relevant to intact organs. Other IVM models, particularly in the bone marrow, reveal how different anatomic contexts regulate leukocyte development, immunity, and inflammation. This article will discuss the current state of the field and outline how IVM can generate new discoveries and serve as a “reality check” for areas of research that were formerly the exclusive domain of *in vitro* experimentation.

### Introduction

Regardless of the value of the diverse array of *in vitro* experimental systems to probe immunological cellular and molecular functions and notwithstanding its unique challenges, the most relevant experimental setting to measure such attributes remains the live animal. Intravital microscopy (IVM) affords a view into the lives and fates of diverse immune cell populations in lymphoid organs and peripheral tissues. The practice of IVM was first described in the nineteenth century (Cohnheim, 1889; Wagner, 1839) and has led to numerous discoveries, especially regarding the molecular and biophysical mechanisms of leukocyte adhesion to endothelial cells (Mempel et al., 2004b). However, advances in molecular and genetic tools and optical equipment as well as immunological understanding during the past few years have dramatically enhanced microscopists' ability to observe, quantify, and probe the complex behaviors of units such as T cells, B cells, granulocytes, and dendritic cells (DC) in their native anatomical context. Traditional IVM approaches to observe immune cell adhesion and migration *in situ* have employed two-dimensional imaging methods such as brightfield transillumination or

epifluorescence videomicroscopy (Mempel et al., 2004b). These microscopy technologies and molecular tools for IVM, intact organ studies, and live cell interactions have been extensively reviewed elsewhere (Cahalan et al., 2002; Huppa and Davis, 2003; Mempel et al., 2004b). A relatively recent development is multiphoton microscopy (MPM), featuring infrared pulsed laser excitation to generate optical sections of fluorescent signals hundreds of micrometers below the surface of solid tissues (Denk et al., 1990). The combination of MPM technology with IVM (MP-IVM) enables the analysis of cell migration in time-lapse recordings of 3D tissue reconstructions (Cahalan et al., 2002).

Traditional IVM approaches can be used to study practically any cell in numerous physiological models (Table 1). This article will focus on the current state and likely future of IVM technology and related fields to study T cells and dendritic cells (DC). What do we currently know about T cell and DC migration to and within different tissues? What are the surface receptors and cytoskeletal as well as intracellular signaling mechanisms that control their interactions with other cells and with each other? Given that most current answers to these questions are based on non-IVM experimentation, often from reductionist *in vitro* assays, how can future IVM studies provide much-needed validation, refinement (or rejection) of prevailing immunological and cell biological concepts? What are the available hardware, computational, and biological tools? What are their current limitations? Which technological improvements are needed to further accelerate the emerging renaissance of IVM as a unique tool for immunologists?

### Focus on T Cells

Hematopoietic progenitor cells (HPC) that are slated to ultimately give rise to antigen-sensitive, functional T cells need to traverse many anatomical barriers in a steady progression from the BM through lymphoid organs and eventually into peripheral tissues (Figure 1A). Common lymphoid progenitors that express IL-7R arise in BM and migrate to the thymus, where Notch1 and pre-TCR signaling control differentiation pathways that generate weakly self-recognizing naive T cells that journey to secondary lymphoid organs (SLO) via the systemic circulation (von Boehmer et al., 2003). Extrathymic development of T cells, while the object of less intense scrutiny, has nonetheless been proposed to contribute to specialized T cell subsets with roles in guiding innate immunity and appropriate responses to damage and altered self, such as natural killer-T (NKT) cells in the liver and  $\gamma\delta$  TCR<sup>+</sup> intraepithelial lymphocytes in the gut (Abo et al., 2000; Barrett and Bluestone, 1995). Naive T cells recirculate continuously between the blood and SLO such as the spleen, lymph nodes (LN), and Peyer's patches (PP). This process is tightly regulated, with egress controlled by sphingosine-1-phosphate (Matloubian et al., 2004) and homing to SLO (other than the spleen) by multistep adhesion cascades involving L-selectin (CD62L), the integrins LFA-1 and/or  $\alpha 4\beta 7$ , and

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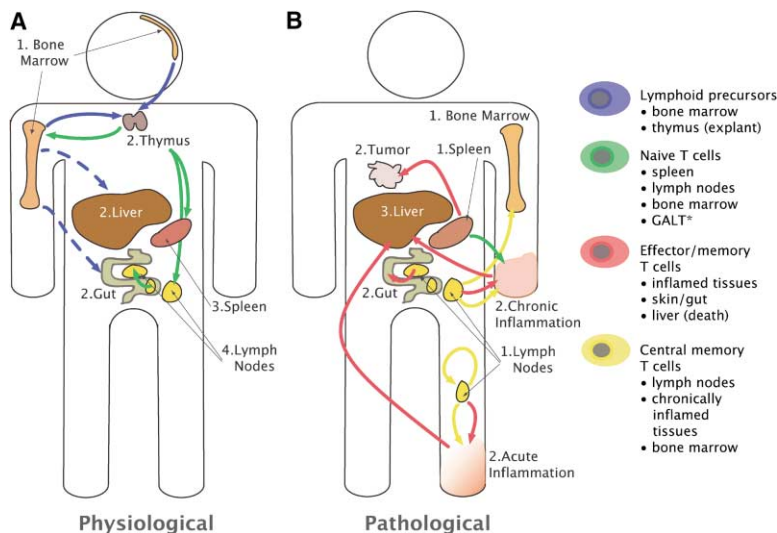


Figure 1. Schematic Representation of Different Stages in T Cell Development and Migration between Primary and Secondary Lymphoid Organs and Other Tissues in Physiological and Pathological Settings.

Colored arrows represent migration pathways of common lymphoid progenitors from the BM (blue), naive T cells to SLO (green), and antigen-experienced effector/memory (red) or central memory (yellow) T cells. Existing IVM models that allow in situ visualization of distinct migration and/or differentiation events in rodents are listed on the right (see also Table 1). For inflamed tissues, these include cremaster muscle, skin (ear, footpad), small intestine, and others. Abbreviations: \*GALT, gut-associated lymphoid tissues, including PP and mesenteric LN.

the CCR7 binding chemokines CCL21 and CCL19 (Butcher and Picker, 1996; von Andrian and Mackay, 2000).

Upon activation, antigen-specific naive T cells clonally expand and acquire effector functions and the ability to home to sites of inflammation (Figure 1B). After antigen clearance, most of these effectors die in peripheral tissues or central sites such as the liver (John and Crispe, 2004), but a small fraction enter the long-lived memory pool. Memory cells are further subdivided into central memory and effector memory subsets; whereas the former continue to survey lymphoid organs, the latter patrol peripheral tissues (Sallusto et al., 1999; Weninger et al., 2001). While many intra- and extravascular migration events that mark the life of a T cell are yet to be visualized in situ, there is now a plethora of IVM models and in vitro systems that could conceivably be useful to access virtually every stage in a T cell's career (Figure 1; Table 1).

#### T Cell Activation: Dynamic Topography Marks the Interface with APC

Immune responses rely on the exchange of information between cells. This crosstalk is thought to involve, at least in part, soluble factors such as chemokines diffusing through and being retained by the extracellular matrix or being transcytosed across cellular barriers (Rot and von Andrian, 2004). Immune cells can achieve additional discriminatory potential by creating tight interfaces with other cells to bring complementary membrane receptors into apposition. One paradigm that has emerged from in vitro modeling of such interactions is the formation of the immunological synapse or supramolecular activation clusters, a highly ordered yet dynamic macromolecular organization between lymphocytes and APC that is thought to aid in signal modulation and propagation (Dustin and Chan, 2000; Grakoui et al., 1999; Monks et al., 1998). T cells as well as NK cells can form synapses in vitro, and there is some evidence that B cells also organize their antigen receptors and integrins in a configuration resembling the T cell synapse (Batista et al., 2001; Carrasco et al., 2004). Consistent with the concept that synapse formation may also occur

in situ, exclusion from the contact zone of CD43, a large, anti-adhesive T cell-expressed mucin, has been observed during T cell stimulation by DC in intact excised LN (Stoll et al., 2002). However, IVM studies must provide the ultimate test whether synapse-like cell-cell interfaces form in lymphoid organs or elsewhere in vivo.

In order for a synapse to form, T cells must specifically adhere to an APC. Leukocytes upregulate their adhesiveness primarily through integrins upon activation by antigen, chemoattractants, and other signals (Dustin and Springer, 1989; Giagulli et al., 2004; Jones et al., 1998). Provided the target cell expresses a sufficient ligand density, this allows for much stronger adhesive contacts and hence resistance to dispersal forces. Although best established for the case of leukocyte-endothelial cell interactions in blood vessels (Springer, 1994), adhesive strength is also thought to play a role for stable synapse formation and during transendothelial and extravascular migration by establishing transient, mechanically stable contacts. It is unclear whether tissue culture-based assays to study these events can fully describe the more complex in vivo situation. For example, cells in 3D matrices rarely exhibit the integrin-dependent focal adhesions commonly seen with cells cultured on a 2D extracellular matrix (ECM) substrate. Instead, they form transient adhesions and have an elongated morphology, which may optimize their navigation through crowded spaces (DeMali et al., 2003; Wolf et al., 2003).

#### T Cell Activation: Adhesion and Cytoskeletal Remodeling

Naive T cells are remarkably nonmotile and poorly adhesive when placed in a Petri dish, which is a reason why most in vitro studies have been performed with preactivated primary T cells or effector-like clones that are much more motile and sticky. By contrast, naive T cells and DC display remarkably high spontaneous motility and undergo continuous rapid shape changes in intact LN (Bousso and Robey, 2003; Mempel et al., 2004a; Miller et al., 2002). Neither the signals that prompt their incessant migration nor the molecular machinery

Table 1. Mouse Imaging Models for Tracking Lymphocyte Function in Tissues

Process	Organ Model	In Vivo	In Vitro	References	
Hematopoiesis, lymphopoiesis	BM		LTBMC	Dexter et al., 1977	
	Thymus	EPF	TOC	Mazo et al., 1998 Mandel and Kennedy, 1978	
Homing to and migration within SLO	Peripheral LN HEV		TOC, MPM	Bousso et al., 2002	
			PPFC	Campbell et al., 1998	
	Inguinal LN	EPF		PPFC	Lawrence et al., 1995
		MPM			von Andrian, 1996
	Popliteal LN			WOC, MPM	Miller et al., 2003
		MPM			Miller et al., 2002
	Mesenteric LN	EPF		WOC, LSCM	Mempel et al., 2004a
	Spleen	EPF			Stoll et al., 2002
	Peyer's Patch	EPF			Grayson et al., 2003
	Peyer's Patch HEV			PPFC	Grayson et al., 2003
Inflamed venules			PPFC	Bargatze et al., 1995 Berg et al., 1993	
Inflammation, immunosurveillance by memory cells	Pancreas	EPF		Lawrence et al., 1987	
	Intestine	EPF		Enghofer et al., 1995	
		EPF		Fujimori et al., 2002	
	Cremaster muscle	BRF		Massberg et al., 1998	
	Mesentery	BRF		Baez, 1973	
	Skin (DSFC)	EPF		Atherton and Born, 1972	
	Skin (ear)	EPF		Falkvoll et al., 1984	
	Skin (allograft)	EPF		Reus et al., 1984	
	BM	MPM		Jones et al., 2003	
				I.B.M. and U.H.v.A., unpublished data	
	CNS (spinal cord)	EPF		Vajkoczy et al., 2001	
	CNS (brain)	EPF		Carvalho-Tavares et al., 2000	
	Synovial tissue	EPF		Veihelmann et al., 1998	
	Liver	LSCM		Hoffmeister et al., 2003	
		EPF		Nakagawa et al., 1996	
Tumor	Bladder	EPF, MPM		Unpublished, U.H.v.A.	
	Angiogenesis	EPF		Leunig et al., 1992	
		EPF, MPM		Jain et al., 2002	
Transplanted organs	Metastasis	MPM		Condeelis and Segall, 2003	
	Pancreatic islets	EPF		Vajkoczy and Menger, 1994	
	Lung	EPF		Sikora et al., 2003	

Abbreviations: LTBMC, long-term BM cultures; PPFC, parallel plate flow chamber; TOC, thymic organ culture; WOE, whole organ extraction; DSFC, dorsal skin fold chamber; EPF, epifluorescence; BRF, bright field; MPM, multiphoton microscopy; and LSCM, laser-scanning fluorescent microscopy.

for their propulsion are known. One possible scenario is that migrating cells use certain integrins to establish transient footholds on extracellular matrix molecules and/or integrin ligands on other cells. For example, IVM studies have shown that neutrophils use several  $\beta 1$  integrins to migrate in inflamed tissues (Werr et al., 1998). However, the extent and importance of integrin activity have not been evaluated on cells migrating within SLO. One technical consideration is that some integrins (particularly the  $\alpha 4$  integrins and LFA-1) are required for lymphocyte homing (von Andrian and Mackay, 2000). Thus, experimental approaches relying on inhibition of such integrins to address their function must be timed to target cells after they have already entered an SLO of interest. IVM methods that can unobtrusively track and selectively modulate integrin distribution and activity during T cell migration and interaction with APC could reveal whether and how T cell behavior is linked to integrin function.

In most experimental settings, T cells form stable adhesive contacts with APC that present their cognate

antigen. However, whether such interactions are formed in vitro depends on the assay conditions (Dustin et al., 1997; Gunzer et al., 2000). As will be discussed below, in vivo interactions of naive T cells with DC in LN occurs in three distinct phases, whereby transient (<5 min) interactions predominate early on and stable contacts are primarily formed several hours after T cells and DC have first encountered each other (Mempel et al., 2004a). Whether stable and/or transient contacts require LFA-1 has not yet been determined, but a key role appears likely, since TCR signaling triggers LFA-1-mediated synapse formation (Grakoui et al., 1999). Moreover, LFA-1 engagement with its ligand, ICAM-1, is necessary for optimal T cell response to cognate peptide-MHC (pMHC) complexes presented by APC (Davignon et al., 1981; Scharffetter-Kochanek et al., 1998; Schmits et al., 1996; Sligh et al., 1993). The transition from rapid migration to stationary interaction probably requires cytoskeletal remodeling from a configuration geared toward rapid myosin tracking to a more stable and polarized distribution, which can stabilize membrane remodeling

events that accompany receptor triggering driven in part by PI3K signaling and the Rho/Rac family of GTPases (Dustin and Chan, 2000).

#### **T Cell Activation: What IVM Might Reveal**

It is likely that a major effort in future IVM work (primarily conducted by MPM) will be geared toward tracking cytoskeletal, signaling, and cell surface molecules in migrating and interacting lymphocyte and DC subsets to relate these processes to the regulation of T cell responses in the context of different physiological settings. This will require the use of advanced fluorescent probes that must meet several requirements, such as a high signal-to-noise ratio; selectivity for specific molecular or subcellular targets; and functional inertia, i.e., their presence must not interfere with the normal distribution or activity of the imaged object. The most straightforward approach to image intracellular events in living cells is arguably the use of genetically encoded fluorescent proteins that may be incorporated into chimeric signaling and/or cytoskeletal molecules or inducibly expressed to indicate gene activation, such as for IL-2 in T cells (Naramura et al., 1998). However, it should be cautioned that the latter approach will only allow detection once a T cell has synthesized a sufficient amount of fluorescent protein, which is likely to occur several hours after it has received an activating stimulus. By contrast, tracking of chimeric fluorescent signaling molecules that become rapidly redistributed upon T cell stimulation might afford real-time detection of activation events *in situ*. This strategy will push the limits of spatial resolution and detection sensitivity of current state-of-the-art IVM equipment, but the goal seems attainable with current technology.

#### **Using IVM to Listen to the T Cell-APC Dialog**

Even though the immunological synapse model and its relevance to T cell biology has not been demonstrated in intact lymphoid organs, several groups have recently utilized confocal microscopy or MPM to visualize the formation of T cell-DC contacts in LN and have verified the appearance of stable contacts that can last several hours in the presence of antigen (Bouso and Robey, 2003; Mempel et al., 2004a; Miller et al., 2004; Stoll et al., 2002). MP-IVM studies can take advantage of excellent tissue penetration afforded by the infrared excitation, which allows much deeper imaging within solid organs than with conventional single-photon excitation for confocal imaging (in LN up to 450  $\mu\text{m}$  versus less than 100  $\mu\text{m}$ , respectively [Cahalan et al., 2002]). Modern laser scanheads allow the rapid acquisition of stacks of 2D optical tissue sections, which are then digitally reassembled into 3D renderings of the original sample. Iterative generation of multiple image stacks at defined time intervals can then be used to produce 3D time-lapse movies, which can be analyzed by various means (Figure 2; Table 2). For migrating cells in solid organs it is not optimal to measure velocities from maximum intensity projections of image stacks (since the z velocity is not accounted for); 3D velocity along the migration track should ideally be quantified. In order to achieve optimal automated measurements with 3D tracking software, fluorescent cell densities, scanning volumes, the spac-

ing between z sections, and the rate of z stack acquisition must be carefully controlled for accurate tracking and to minimize loss of cell identity between z sections or consecutive image stacks (Figure 2B).

The quantitative analysis of cell-cell interactions is similarly complex. Typically, two (or more) cell types, such as purified T cells and DC, are tagged with distinct fluorophores that can be detected in separate fluorescence channels (refer to Movies for examples; Movies are available online at <http://www.immunity.com/cgi/content/full/21/3/315/DC1>). A distinct advantage of MPM excitation over conventional single-photon techniques is that several fluorescent dyes or proteins with distinct emission and excitation characteristics can be visualized simultaneously to facilitate side-by-side analysis of different cell subsets. However, this analysis is only possible after extensive processing of the raw data and merging of image stacks from different fluorescence channels. In our hands, current software for automated image analysis is useful to track large numbers of individual cells and to measure a variety of parameters to describe migratory behavior at both the single-cell and population level. However, automated programs are still limited in their ability to extract reliable data that describe complex cell-cell interactions, thus necessitating time-consuming image analysis by trained human observers. For example, this approach was taken to examine whether, when, and for how long T cells engage in transient and stable interactions with mature antigen-presenting DC (Mempel et al., 2004a).

While this and other studies have clearly shown that T cells in intact LN engage in stable contacts with DC that may last several hours (Bouso and Robey, 2003; Mempel et al., 2004a; Stoll et al., 2002), the question whether such contacts are required to achieve full activation and effector commitment has been the subject of debate. *In vitro* data have generally suggested that long contacts are necessary for optimal proliferation and cytokine secretion of CD4 and CD8 T cells (Huppa et al., 2003; Lee et al., 2002; van Stipdonk et al., 2003), but not for CTL killing (Faroudi et al., 2003a; Purbhoo et al., 2004). However, CD4 T cell activation has also been achieved in experimental settings where interactions with DC were frequent but limited to short duration (Faroudi et al., 2003b; Gunzer et al., 2000).

To address these conflicting observations *in vivo*, we devised a strategy using MP-IVM in popliteal LN to follow naive TCR transgenic CD8 T cells over a 48 hr period after their entry into LN (Mempel et al., 2004a). Recipient animals were first injected into a footpad with immature peptide-pulsed or control DC plus 10 ng LPS (to induce DC maturation and migration into the draining popliteal LN) and 18 hr later with an intravenous bolus of TCR transgenic naive T cells. To ensure synchronicity of intranodal T cell dwell times, further homing to PLN was blocked 2 hr after T cell injection by MAb inhibition of L-selectin, an essential adhesion receptor for lymphocyte homing via high endothelial venules (HEV). After thus restricting T cell homing to PLN to the first 2 hr after injection, we observed that during the initial  $\sim 8$  hr after entering the popliteal LN, T cells rarely stopped for more than a few minutes, even when presented with antigen by mature DCs. Instead, they scanned many different DCs rapidly (phase one). Subsequently, their

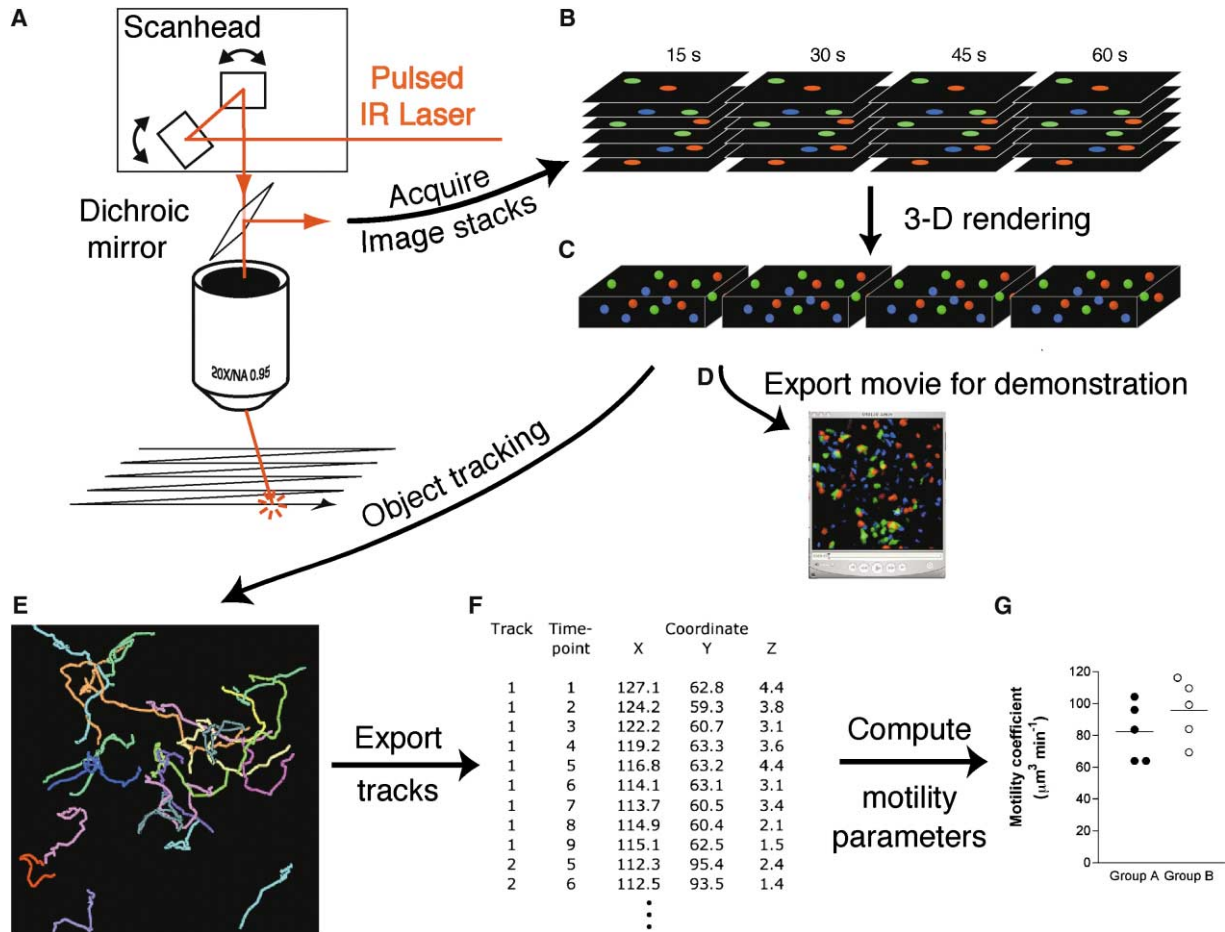


Figure 2. Steps Involved in the Analysis of In Vivo Cell Motility by MP-IVM

(A) A pulsed beam of infrared (IR) laser light is raster scanned via a microscope objective with high numerical aperture onto a thick specimen by rapid, synchronized movement of a pair of steering mirrors. Fluorescent signals are generated by the multiphoton effect in a  $\sim 1 \mu\text{m}$  deep section in the objective's focal plane (Denk et al., 1990).

(B) Incremental vertical motion of the objective relative to the specimen yields stacks of optical sections, which are serially reacquired at defined time intervals (in this example, 15 s are required to acquire one z stack composed of six optical sections).

(C and D) In (C), each z stack is rendered as a three-dimensional volume, and in (D) two-dimensional projections of image stacks are exported as movie files for demonstration and further off-line analysis.

(E) Determination of cell centroids to represent cell position allows automated tracking of migration paths of three or more cell populations recorded in separate color channels. This is achieved by assigning track identities to serial images of individual cells.

(F and G) In (F), tracks consisting of serial sets of xyz coordinates of single cell centroids are exported as numerical databases, and in (G) are used to compute parameters of cell motility (see also Table 2). Specialized software for automated 2D or 3D cell tracking and computing of the acquired tracks is essential for in-depth, large-scale analysis of migratory behavior at the single-cell level.

motility decreased at 6–8 hr after entry, and the majority of T cells then formed contacts lasting longer than 1 hr (phase two). Tight, long-lasting contacts continued throughout the first day after T cell entry, but completely disappeared thereafter when T cells began to proliferate rapidly (phase three). These results reconcile the seemingly conflicting in vitro observations that antigen encounter by T cells provides an acute stop signal in some settings (Dustin et al., 1997) but does not prolong T cell-APC contacts in others (Gunzer et al., 2000); both interactive modes are relevant in that they each recapitulate distinct phases of T cell behavior during activation.

Which factors determine the transition between different phases of interactive behavior? One possibility is that after their entry into the LN from peripheral tissues, DC must continue to mature before they can form long-

lasting conjugates with T cells. However, the adoptively transferred DC that were recovered from popliteal LN during early phase 1 (i.e.,  $\sim 20$  hr after footpad injection) expressed high levels of MHC class II and costimulatory molecules, which is typically interpreted as a sign of full maturity (Mempel et al., 2004a). Nevertheless, it is likely that DC continue to undergo functional changes after entering a LN. For example, we observed that their motility was high between 20 hr and 38 hr after footpad injection (corresponding to phases one and two), but declined thereafter. DC also changed their intranodal distribution after entering the T cell area; while they were initially concentrated in close vicinity to HEV, their distribution was more uniform throughout the T cell area during the second day (Mempel et al., 2004a). However, these changes in motility and distribution did not coin-

Table 2. List of Parameters Used in Defining Cell Migration

Parameter	Definition/Comments	Units
Mean displacement	Average distance, $x$ , from arbitrary points of origin after time interval, $t$ . Plotting $x$ versus $t^{1/2}$ yields the MDP and allows determination of the motility coefficient.	$\mu\text{m}$
Instantaneous velocity	$v$ , velocity between two measurement time points.	$\mu\text{m}/\text{min}$
Mean velocity	$v$ , average velocity over an extended imaging period.	$\mu\text{m}/\text{min}$
Shape index	Long axis/short axis, measure of polarization.	dimensionless
Motility coefficient	$M = x^2/4t$ (for 2D measurements) or $M = x^2/6t$ (for 3D), analogous to the diffusion coefficient of Brownian particles.	$\mu\text{m}^2/\text{min}$
Chemotactic index	Ratio of a cell's net displacement to the total path length.	dimensionless
Turning angle	Angle of the two velocity vectors before and after a measurement time point.	degrees

cide with the transition between phase 1 and 2. Furthermore, we noted recently that long-lasting interactions tend to occur somewhat earlier in superficial regions of the T cell area, such as directly below and between the B cell follicles, than in the deep paracortex (T.R.M. and U.H.v.A., unpublished data). T cells migrating in superficial LN regions have a priori a lower motility than in deeper areas (Stoll et al., 2002 and Movies 4 and 5, available online at <http://www.immunity.com/cgi/content/full/21/3/315/DC1>), which might explain the differential timing of stable conjugate formation. Nevertheless, these observations argue against the notion that immaturity of DC could explain the prevalence of short contacts during phase 1 (i.e., 18 hr to ~26 hr after footpad injection).

A related question is whether DC or T cells determine the duration of contacts during phase two. When T cells were synchronized, the vast majority of contacts that formed between 8 hr to 12 hr after adoptive transfer were long lasting (Mempel et al., 2004a). However, in experiments in which T cells were not synchronized with anti-L-selectin new T cells continuously enter LN from the blood. In the latter setting, interactions were much more heterogeneous during this time interval, with stable and dynamic interactions occurring side-by-side. The most likely explanation for these findings is that the newcomer T cells could only engage in phase one-like short interactions, whereas T cells with longer dwell times had already accumulated sufficient activation signals to transition to long-lasting, phase two-like interactions.

We therefore favor the hypothesis that changes within the T cells after their entry from the bloodstream or during their sequential encounters with DC are at least in part responsible for this phased sequence of interactive behavior, although this does not exclude the possibility that DC may over time modulate their receptivity for different interactive modes. It can be argued that signal buildup has occurred in T cells in the first phase, as indicated by increased expression of the early activation markers CD44 and CD69 (Mempel et al., 2004a), and that T cells settle down once a certain number of DC bearing antigen have been scanned. During the stationary period in phase two, T cells reach maximal expression levels of activation markers and begin to produce effector cytokines, such as IL-2 and IFN- $\gamma$ . However, it is also possible that shortly after T cells have left the bloodstream to enter a LN, they may possess a reduced responsiveness to pMHC ligands (Stefanova et al., 2002). Thus, phase one could reflect a potential require-

ment for T cells for several hours of LN residency before optimal TCR function is attained. However, to date IVM has not yet resolved the timing, duration, nature, or amount of T cell signaling with respect to the behavioral changes outlined herein. This remains an important goal for future studies.

Published MPM work to date has concentrated on analyzing T cell interactions with DC that have carried antigen to draining LN from a peripheral location. It must be kept in mind that LN contain a priori a considerable number of DC, which contribute to T cell activation. For example, in the mouse both the induction of self-tolerance and crosspriming of CD8 T cells are preferentially mediated by CD8 $\alpha^+$  DC (Allan et al., 2003; Belz et al., 2002; den Haan et al., 2000), which migrate very poorly to LN upon deposition in the skin (Mempel et al., 2004a; Smith and Fazekas de St Groth, 1999). Thus, it is likely that LN-resident members of this DC subset acquire antigen from the lymph or possibly from other DC to contribute to T cell stimulation (Itano et al., 2003). The geometry and kinetics of T cell interactions with the resident DC network in SLO has not yet been visualized in vivo. IVM measurement of signaling in T cell populations interacting with different DC subsets after different LN dwell times will provide critical clues in this regard.

T cell activation occurs upon TCR contact with pMHC ligands that are of sufficiently high affinity to trigger activation signals above a certain threshold. Whether a full signal is delivered is determined by additional factors, such as the stability (Holler and Kranz, 2003) and changes in heat capacity (which implies conformational change) of the pMHC complex (Krogsgaard et al., 2003). T cells can also respond to signals through interactions with weaker peripheral ligands that may be kinetically similar to positively selecting ligands in the thymus. Indeed, freshly isolated naive T cells from LN show evidence of partial TCR activation, presumably triggered by self-ligands, whereas peripheral blood T cells are essentially quiescent (Stefanova et al., 2002). Recent MP-IVM observations suggest that exposure to self-ligands may also influence T cell behavior in LN possibly through signal integration after T cells enter from the blood (Mempel et al., 2004a); phase 1-like T cell conjugates are formed with mature DC whether antigen is present or not, and these transient interactions become prolonged after ~8 hr, even in the absence of activating antigen. However, this phase two-like (possibly self-antigen-driven) interactive behavior is abbreviated and rarely induces the hour-long conjugates that are seen

with antigen-pulsed DC. The overall impact of these putative signaling noise effects on T cell fate and function are just beginning to emerge. In normal nonlymphopenic hosts, CD4 T cells do not seem to depend on encounters with self-antigens, whereas CD8 T cells die rapidly when denied the opportunity to interact with self (Germain et al., 2002). Hence, periodic TCR stimulation with self-antigens during passage through SLO may constitute a requirement for T cell survival, at least for CD8 T cells.

#### **Random Walk versus Chemoattractant-Directed Migration**

Numerous experiments have demonstrated a role for chemokines in the establishment and maintenance of lymphoid tissue microenvironments (reviewed in (Ansel and Cyster, 2001; Mackay, 2001; Rot and von Andrian, 2004)). It has also been suggested that chemokines orchestrate cellular interactions in SLO, such as those between T cells and DC during T cell priming in LN (Tang and Cyster, 1999). The prevailing view has been that migrating cells sense a gradient of soluble or surface-immobilized chemokine(s) released from a distant source, which provides a chemotactic cue for directed migration. However, MPM studies in explanted LN as well as in live mice found no convincing evidence for directional trafficking of naive T cells under steady-state conditions (Bousso and Robey, 2003; Mempel et al., 2004a; Miller et al., 2002, 2003, 2004). Rather, the migration pattern followed by T cells during their search for cognate antigen seems best described as a random walk (Miller et al., 2004; Wei et al., 2003). However, no data have been presented so far that assess the impact of functional inactivation of chemokines or their receptors on the migratory behavior of lymphocytes or DC in the interstitium of SLO.

#### **Beware of the Unseen**

While *in situ* imaging has clearly shown that earlier concepts of chemokine action in SLO must be revised, it should be noted that genetic deficiencies in several chemokine pathways result in severe disorganization of SLO architecture and lymphocyte and DC distribution (Ansel and Cyster, 2001). Thus, chemokines must act on migrating cells in more subtle ways but with nonetheless dramatic consequences. Chemotaxis *in vivo* may not necessarily follow the simple principles established by *in vitro* experiments where groups of cells follow uniform, long-range, soluble gradients. More complex traffic guidelines are likely to apply, potentially involving hierarchical systems of multiple competing short-range chemoattractant gradients (Foxman et al., 1997), rapid receptor de- and resensitization, patterned deposition of chemoattractants on ECM and cell surfaces (reviewed in Rot and von Andrian, 2004), and physical barriers imposed by other cells and stroma elements that impact on both migrating cells and the transport, diffusion, convection and turnover of chemotactic mediators. Direct experimental evidence for or against such hidden traffic signals awaits further *in situ* imaging but might only become apparent with more sensitive analytical tools than are currently employed. For this, it is also important to consider which aspects of cell migration can be accu-

rately measured and how to model and mathematically describe complex migratory behavior (for a list of commonly used parameters to describe cell migration see Table 2). It will also take some time to accumulate the prerequisite experience to understand the biological meaning and limitations of such measurement parameters.

A large body of experimental data and theoretical framework have been generated describing the migratory behavior of cells on two-dimensional substrates (Maheshwari and Lauffenburger, 1998). Typically, these studies employed systems in which both cells and chemoattractants were essentially uninhibited in their two- or three-dimensional migration and diffusion, respectively. However, cellular movement in SLO is restricted by structural scaffolds composed of blood vessels; lymphatic sinuses; ECM molecules such as the reticular network of collagen fibers, which are mostly ensheathed by fibroblastic reticular cells (Gretz et al., 1997); as well as densely packed motile and sessile lymphoid and non-lymphoid cells. Recent studies, employing MPM or confocal microscopy to track T cell movement in SLO have only visualized T cells and sometimes DC, while the ubiquitous multitude of nonfluorescent obstacles remains unseen (Bousso and Robey, 2003; Mempel et al., 2004a; Miller et al., 2004; Stoll et al., 2002). Hence, the impact of the putative tug of war between physical barriers and biochemical cues on a particular cell's migration path remains to be assessed. Indeed, although anecdotal, MPM time-lapse videos suggest that trabecular collagen fibers may serve as footholds and possibly guidance structures for some migrating T cells and DC in intact LN (Mempel et al., 2004a; Miller et al., 2002). By acting as impediments or preferred points of contact to a migrating cell, such hardwired microenvironmental features will inevitably alter migratory paths and may thereby introduce the appearance of randomness. This greatly complicates the final determination of whether migrating leukocytes are guided by directional cues or act as truly autonomous agents.

The current acquisition speed of most laser-scanning microscopes sets stringent limits to the tissue volume that can be scanned per unit time and thereby to the duration over which a highly motile cell can be tracked, because of the cell's tendency to migrate out of the imaged tissue volume (Figure 2). This can, on the one hand, create a bias when measuring average velocities of single cell tracks, particularly in the dimension in which the acquired dataset is most limited (usually the *z* direction), because highly motile cells may be represented more than once as they exit and reenter a region of interest. On the other hand, these technical limitations can potentially disallow the detection of long-range chemotactic guidance cues, which may only become detectable when a cell's path is followed over a sufficiently long distance and period of time.

Arguably the most accurate analysis vehicle currently available to detect migration patterns is the mean displacement (MD) plot (Miller et al., 2002). Here, the average 2D or 3D displacement of a population of cells over distinct time intervals (typically multiples of the acquisition cycle time for individual *z* stacks) is plotted against the square root of time (Figure 3). The slope of the resulting curve can be used to determine a cell popula-

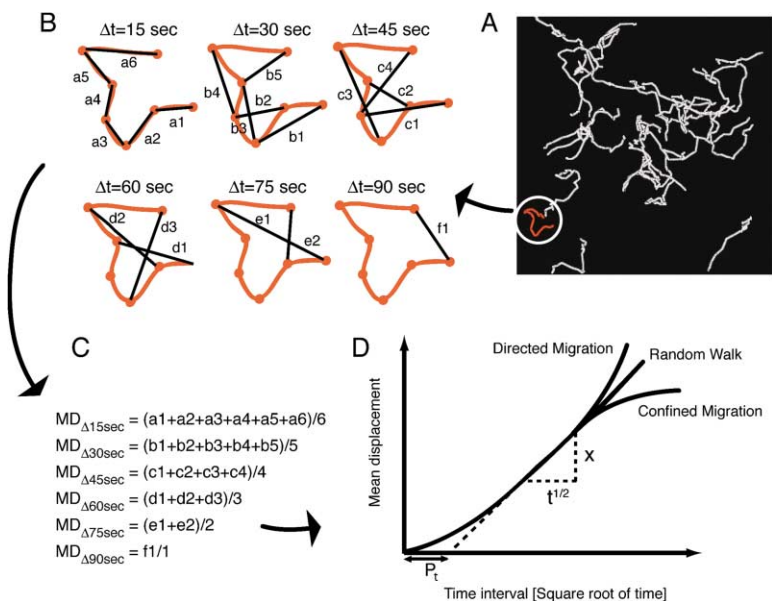


Figure 3. Mean Displacement Plots (MDP) Integrate Directionality and Speed of Cell Migration.

(A) As an example, a single cell's 3D migration track (red line) generated as described in Figure 2E is chosen. This track was generated from 3D centroid positions in 6 consecutive image stacks.

(B) The cell's trajectory is broken down into displacements over all multiples of the acquisition time interval for individual z stacks. In this example,  $1 \times 15 \text{ s}$  for the first migration interval (a1),  $2 \times 15 \text{ s}$  for the second (a2), and so on. Displacements are determined for all positions along the trajectory as starting points and for all measurable acquisition intervals (e.g., b1 to b5 for 30s cycle time).

(C) Mean displacements (MD) for each acquisition interval are then calculated for all cell trajectories from a recording.

(D) Plotting the mean displacement of all cells within a population against the square root of the corresponding time interval yields the MDP. The slope of the linear segment of the curve is a measure for a cell population's propensity to move away from an arbitrary point

of origin and thereby of the territory that cells traverse per unit time. The initial exponential segment of the curve reflects the fact that cells initially migrate for some time in one preferred direction (the mean free path) before making a significant turn. The time interval to the transition from the exponential to the linear segment is the persistence time,  $P_t$ . The slope of the linear segment of the curve represents the motility coefficient  $M$ , calculated for 3D measurements as  $M = x^2/6t$  ( $M = x^2/4t$  for 2D measurements) in analogy to the diffusion coefficient used to describe the motion of Brownian particles. If the migration of a cell population follows a random walk model, the curve remains linear over a time interval that is determined by the time during which the average cell can be tracked (more motile cells tend to leave the scanned volume more quickly than slow moving cells). Transition of the linear segment to a plateau indicates that the cell population is confined due to physical or biological restriction. An increasing slope is expected when cells migrate in a biased fashion, e.g., due to the influence of long-range chemoattractants.

tion's motility coefficient (analogous to the diffusion coefficient used to describe Brownian particles) and gives a measure of the area or volume an average cell scans per unit time. Moreover, the shape of the MD-time curve yields additional information (Saxton, 1997): a plateau of the curve after an initial linear segment indicates confinement of a cell population; for instance, by a physical barrier or a biochemical retention signal. We have directly observed such confined motion of  $CD8^+$  T lymphocytes in the presence of LPS-activated dendritic cells in mouse lymph nodes in vivo (Mempel et al., 2004a). An increase in the slope of the MD curve, on the other hand, would indicate directed motion, as one would expect when cells fall under the influence of a distant chemotactic signal. Either of these behavioral characteristics may become overt only when one tracks individual T cell migration for longer than the  $\sim 20 \text{ min}$  time interval that has been achieved so far.

### Focus on DC

DC are the most potent APCs involved in priming and shaping the T cell responses and in regulating many aspects of immunity and tolerance (Banchereau et al., 2000; Steinman et al., 2003). While many cell types can elicit recall responses by antigen-experienced T cell, DC are exceptionally capable of processing and presenting antigenic pMHC complexes in the context of the costimulation needed to activate naive T cells. Mammals possess numerous DC subsets that are defined by distinct surface markers and the type of immune response they induce. DC can enter LN either through blood or lymph

(Cavanagh and von Andrian, 2002) and can be functionally subdivided based on their ability to process and present certain types of antigens, the kinetics of antigen presentation, and their differential access to various anatomical compartments (Itano and Jenkins, 2003).

A good example in this context is the recent finding that subcutaneous injection of a soluble, nonreplicating antigen elicits two waves of antigen presentation in draining LNs (Itano et al., 2003). The first wave is generated by lymph-borne transport of free antigen to the LN, followed by local processing and presentation by LN-resident DC. These APCs then present the processed peptides to  $CD4$  T cells, which proliferate, but are nonetheless unable to mediate a delayed type hypersensitivity (DTH) response. The second wave of antigen presentation is mediated by newly immigrated dermal DC, that carry processed antigen from the skin and elicit full-fledged effector T cells capable of initiating DTH responses (Itano et al., 2003). Dynamic visualization of these events in situ will likely provide additional information regarding the dynamics of T cell-APC encounters.

Additional complexity is added to this scenario by the finding that upon infection of mice with herpes simplex virus via the skin, Langerhans cells (LC) are not directly involved in the priming of  $CD8^+$  T cells in draining LNs (Allan et al., 2003). Rather, it was suggested that the role of LC may be restricted to initial uptake and transport of antigen to the lymph node where it is presented to T cells by a distinct  $CD8\alpha^+$  DC population. Whether, where and how antigen gets transferred from one DC to another could conceivably be observed using existing IVM tech-



nology. However, the ability of dynamic *in vivo*-imaging approaches to erect a conceptual framework reconciling these challenges to existing paradigms of antigen presentation will rely on the generation of suitable reporters; e.g., fluorescent protein-based genetic markers enabling the identification of distinct DC sub-populations in tissues. Additionally, it will be necessary to visualize and track DC-associated antigen. In this context, the specialization of different DC subtypes to preferentially acquire certain classes of antigen may be exploitable. For example, one convenient way to target specific DC types without altering DC function or state of maturation is to conjugate antigen or fluorochrome to a monoclonal antibody to the scavenger receptor DEC-205, whose expression is restricted to CD8 $\alpha$ <sup>+</sup> and epithelium-derived DCs (Bonifaz et al., 2004). Beyond the labeling of specific functional compartments, ultimately the use of reliable genetic markers for tagging putative DC lineages for IVM imaging will allow further insight into the development and differentiation of DC subsets.

### Emerging Models

#### *Popliteal Lymph Node*

Due to its large size and ease of surgical exposure, the inguinal (or subiliac) LN has long been the primary model to conduct IVM studies on peripheral LN in the mouse (Miller et al., 2003; Okada et al., 2002; Stein et al., 2000; von Andrian, 1996; Warnock et al., 1998). However, while this preparation is well-suited for epifluorescence IVM at video-rate acquisition speeds, it is less suitable for MP-IVM (Mempel et al., 2004b). First, and most importantly, it is difficult to collect high-resolution time series image stacks of fluorescent cells from inguinal LN, due to respiration-induced tissue movement that must be mechanically constrained (Miller et al., 2003). In our hands, application of mechanical force to the soft tissue surrounding the LN affects afferent lymph flow (Mempel et al., 2004b). The acute impact of altered lymph flow on lymphocyte or DC behavior has not been examined, but earlier studies have shown that interruption of afferent lymphatics virtually abolishes lymphocyte traffic and HEV function (Hendriks et al., 1987; Mebius et al., 1991). Second, the inguinal LN is relatively large (>1 mm diameter), and given the current limit in penetration depth for MPM imaging (up to ~450  $\mu$ m), the deep T cell area is often visually inaccessible to *in situ* imaging (T.R.M. and U.H.v.A., unpublished data). Third, the lymphatics that drain lymph fluid to the inguinal LN typically have anastomoses to several other LN (Palframan et al., 2001). Thus, when cells or other biological material are deposited into the skin in an animal's flank, the amount that reaches the inguinal LN can be somewhat variable.

These confounding factors can be largely avoided by using the popliteal LN, which makes this preparation more suitable for MP-IVM (Mempel et al., 2004a). A small skin incision behind the knee allows easy access to this LN, and several bone pivots along the mouse skeleton can be immobilized without compromising the surrounding soft tissue, thus maintaining virtually motionless stability as well as physiological blood and lymph flow, which can be directly visualized upon injection of fluorescent lymph markers in the animal's footpad (von Andrian and Mempel, 2003). Cells or reagents that

are injected into the footpad and enter the afferent lymph are bound to appear in the upstream popliteal LN, and the LN in the opposite knee serves as a convenient matched control. Furthermore, the small size of the popliteal LN facilitates imaging of the deep cortex and medulla region. This model has already allowed high-resolution cell tracking and 3D mean displacement measurements for T cell and DC migration *in vivo* (Videos; Mempel et al., 2004b). The reader may refer to the Movies accompanying this article, which are available online; further examples of video footage may be viewed at <http://cbr.med.harvard.edu/uva>.

#### *Bone Marrow*

The bone marrow (BM) supports intense traffic of blood cells in the extravascular space and at the interface with the intravascular compartment, particularly in sinusoids and venules. The most primitive stem cells are in close contact to the endosteum in the subendosteal region of BM cavities (Calvi et al., 2003; Nilsson et al., 2001; Zhang et al., 2003). As these cells give rise to increasingly more lineage-committed progenitors, the more differentiated cells are thought to migrate toward the central region of the BM cavity (Hermans et al., 1989; Lord et al., 1975). Thus, it is believed that developing cells move actively between specific niches within the BM (Tokoyoda et al., 2004). Newborn fully differentiated blood cells must ultimately penetrate the vessel wall to reach the lumen. Conversely, circulating leukocytes, such as memory B cells (Paramithiotis and Cooper, 1997; Slifka et al., 1998), plasma cells (Hallek et al., 1998), HPC (Wright et al., 2001) and subsets of memory T cells (Di Rosa and Santoni, 2003) continuously leave the blood and home to the BM. Traffic of DC to the BM has found relatively little consideration so far. However, it is well known that DC can migrate from the blood into peripheral tissues (reviewed in (Cavanagh and von Andrian, 2002)), possibly including the BM. However, the traffic signals that control the migration of these diverse cell populations are still poorly understood. IVM will allow these events to be explored.

The thickness of bone surrounding BM cavities in most anatomical locations renders these tissues inaccessible for *in vivo* imaging (Pannarale et al., 1997). Thus, early attempts at IVM in BM of rabbits resorted to the use of surgically implanted chambers, which may cause considerable trauma and inflammation (Branemark, 1959; McCuskey et al., 1971). More recently, we have introduced an alternative IVM technique that maintains tissue integrity without requiring surgical manipulation, except a small skin incision. This model visualizes the BM in the flat bones of murine skull (Figures 4A and 4B). High-resolution epifluorescence and MP-IVM are facilitated by the ability to immobilize the skull in a stereotactic holder. The thin layer of bone that covers the BM cavities in the frontoparietal skull of adult mice is only a few tens of  $\mu$ m thick and sufficiently transparent to allow the observation of underlying BM cavities and microvessels as well as cell traffic in intra- and extravascular compartments (Mazo et al., 1998; Mazo et al., 2002). Representative footage from this model are provided with the Movies accompanying this article.

Topographical features of the skull BM can be depicted, and quantitative measurements can be performed by analyzing the differential distribution of free

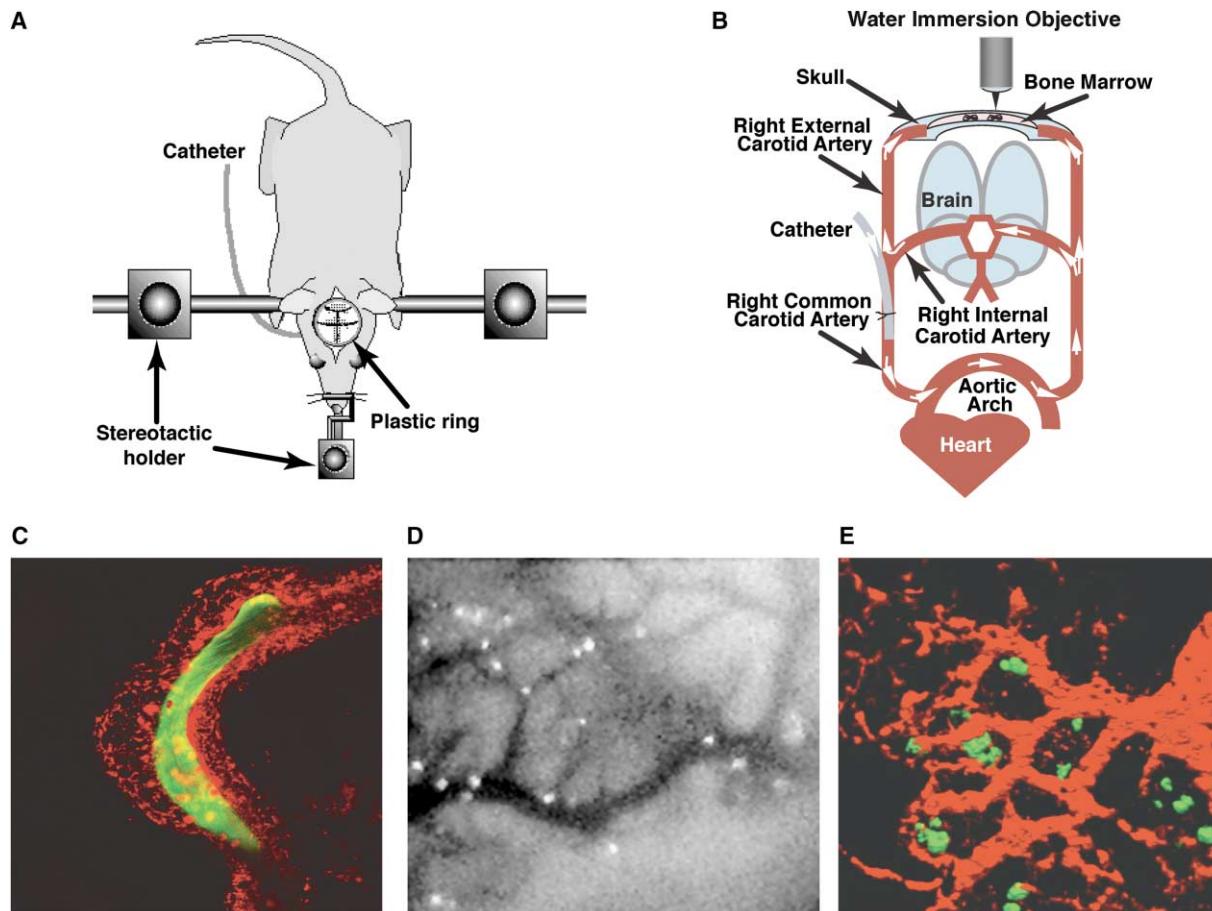


Figure 4. The Mouse BM IVM Model

(A) Schematic drawing of the preparation. Mice are anesthetized and the scalp is incised in the midline to expose the frontoparietal skull without inflicting damage to the bone. The head is fixed in a stereotactic holder on a Plexiglas stage (not shown) and a plastic ring is inserted to spread the skin and allow application of physiological buffer to avoid drying of the tissue.

(B) A catheter is inserted into the right common carotid artery to allow injection of cells and reagents upstream of the left common carotid artery, which feeds the skull BM. The schematic drawing shows the major arteries and a frontal section through the skull. Upon retrograde injection through the catheter, fluorescently labeled cells are carried with the arterial blood flow (direction indicated by arrows) and some are transported to the bones of the skullcap. Here, they can be visualized by epifluorescence imaging through the intact bone.

(C) Representative 3D projection generated by MP-IVM demonstrating accumulation of rhodamine 6G (red) and FITC-dextran (green) in skull BM. While low molecular weight rhodamine 6G (479D) extravasates and stains nucleated hematopoietic cells that fill the entire BM cavity, high molecular weight FITC-dextran (2 MD) stays confined to the intravascular compartment, delineating only the BM microvasculature. Several adherent intravascular leukocytes are also stained by rhodamine 6G. Refer to Movie 11.

(D) Epifluorescence micrograph of central memory CD8 T cells in BM microvessels. The image shows a segment of the parietal sinusoidal network. Fluorescently labeled T cells are injected through the carotid artery catheter and their adhesive interactions with BM venules and sinusoids can then be detected by epi-fluorescent video IVM (MP-IVM acquisition speeds are too slow to capture the fast kinetics of intravascular T cell adhesion). Refer to Movie 10.

(E) Visualization of tumor formation inside BM. GFP-transfected murine multiple myeloma cells were injected into a recipient mouse 3 weeks prior to MP-IVM of skull BM. In this image, the BM microcirculation was delineated by i.v. injection of TRITC-dextran (red). The majority of the tumor cells (green) formed clusters in BM cavities. Refer to Movie 12.

and dextran-coupled fluorochromes (Figure 4C). Upon intravenous injection, low molecular weight intravital dyes, such as rhodamine 6G, extravasate and accumulate in nucleated BM cells, but not in solid bone, thus detecting hematopoietically active BM and delineating the space in which it is enclosed. By contrast, large dextran-conjugated fluorescent markers stay confined to the BM vasculature unless an insult, such as ionizing irradiation causes enhanced vascular permeability (Mazo et al., 2002). Fluorescently tagged cells of interest can be injected upstream of the feeding carotid artery

and detected by epifluorescence IVM during their passage through downstream BM microvessels (Figure 4B). Utilizing this approach, it has been shown that leukocyte homing to BM involves a series of adhesive interactions with EC (Figure 4D) that mediate tethering, rolling, and firm arrest, in accordance with the classic multistep paradigm for leukocyte-endothelial cell interactions in other tissues (Butcher, 1991; Springer, 1994; von Andrian and Mackay, 2000). Interestingly, different subsets of blood-borne cells utilize unique combinations of traffic molecules to lodge in the BM (Katayama et al., 2003; Mazo

et al., 1998, 2002; Mazo and von Andrian, 1999; Shohet et al., 1998).

MP-IVM may also be well suited to investigate medically relevant events in this environment, such as HPC maturation and differentiation, a process that requires precise positioning of precursor cells at different stages of their development (Tokoyoda et al., 2004). The conjectured migration of individual precursor cells from one BM niche to another on their way from stem cell to mature blood cell has not been studied in vivo. Such studies will depend on the availability of gene-targeted mice that express fluorescent reporters driven by stage-specific promoters, some of which have already been generated (Yu et al., 1999).

The BM can support primary and secondary T cell responses to antigen. Numerous memory and a sizable number of naive T cells reside in the BM in steady-state conditions (Di Rosa and Santoni, 2002) and both naive and memory T cells home to the BM after adoptive transfer (Berlin-Rufenach et al., 1999; Di Rosa and Santoni, 2003; Feuerer et al., 2003). The long-term persistence of antigen-specific memory T cells in BM has also been demonstrated after viral infections and exposure to environmental antigens (Kuroda et al., 2000; Marshall et al., 2001; Price and Cerny, 1999; Slifka et al., 1997). Additionally, in breast cancer patients tumor-specific memory T cells are more frequent in BM than in peripheral blood and possess higher antitumor activity (Feuerer et al., 2001a, 2001b). It has also been shown that the BM can function as a SLO; under certain experimental conditions the BM supports the development of effector T cells from naive precursors (Feuerer et al., 2003; Tripp et al., 1997). The BM also contains T cell-rich areas (Schirrmacher et al., 2003) and B cell follicles (Horny et al., 1989), architectural features that are shared by all SLO. On the other hand, splenectomized *aly/aly* mice, which are devoid of classical SLO but possess presumably functional BM, cannot mount T cell-dependent antibody responses (Shinkura et al., 1996) and remain ignorant to cardiac allografts (Lakkis et al., 2000). IVM analysis of immune cell dynamics may eventually reveal why the BM can promote some, but apparently not all, types of adaptive immune responses.

As the major site of B cell generation after birth, the BM functions also as a primary lymphoid organ (Osmond, 1986). Its well-established function as a harbor for IgG-producing plasma cells as well as some memory B cells highlights its important role in humoral immunity (Ellyard et al., 2004; Manz et al., 1997; Slifka et al., 1998). Under pathological conditions, plasma cell precursors may undergo transformation into malignant blasts with subsequent development of BM resident tumors, particularly multiple myelomas (MM) (O'Connor et al., 2003; Potter, 2003). Several other types of tumors from diverse tissues such as prostate, breast, lung, and thyroid also possess BM tropism; i.e., they metastasize early and frequently to the BM where destruction of hematopoietic tissue contributes to tumor-associated morbidity and lethality. A better understanding of the migration signals that enable malignant cells to enter the BM could lead to new metastasis-preventing therapies.

The BM IVM model can be used to study tumor formation. Since MM spreads diffusely throughout the entire BM, tumor cell migration between the blood and BM is

probably critical for disease pathogenesis. Only upon seeding the BM microenvironment can MM cells survive and grow. Moreover, adhesion of MM cells to BM stroma elements enhances their resistance to chemotherapy, thus making the treatment of this cancer a difficult medical task (Hideshima et al., 2003). BM IVM allows the visualization of individual migrating tumor cells and tumor mass formation in living, undisturbed BM (Figure 4E). In the future, this might lead to the identification of molecules that support or inhibit MM establishment or dissemination. Detailed knowledge of these events could be of great significance for the development of improved therapeutic approaches, since it might highlight potential targets that control tumor cell migration to and within the BM and ways to block access of tumor cells to niches that support their growth. Recently, DC-based strategies to generate cytotoxic T lymphocytes (CTL) with anti-MM specificity have been developed (Hideshima et al., 2003). For effective treatment, it is crucial to identify a CD8 T cell population with not only tumor-killing ability but also the capacity for avid homing to the BM, since this is the primary site where CTL must lyse MM cells. IVM of BM will provide a powerful tool for direct and high-resolution observations of the interactions between tumor cells and tumor-specific T cells.

#### **Other Emerging Models**

Moving beyond LN and BM, other organ models that are increasingly explored with IVM methods include CNS, liver, spleen, pancreas, joints, atherosclerotic arteries, and other tissues that can be the target of inflammatory diseases (reviewed in Mempel et al., 2004b). Some internal tissues, especially the intrathoracic organs still present a challenging environment for IVM apart from their anatomical inaccessibility, because of the large tissue displacement during breathing cycles and the need to prevent pulmonary collapse upon opening of the thoracic cavity. Nevertheless, several pulmonary IVM approaches have been described that can partially overcome these difficulties (Funakoshi et al., 2000; Lawler et al., 2003; Sikora et al., 2003).

#### **Conclusion and Future Directions**

Although microscopy has been applied to observe cell migration in living animals for more than a century and a half, the use of IVM for immunological studies has been traditionally restricted to a relatively small number of specialized laboratories. However, dramatic improvements in technology during the past decade, especially the introduction of MPM, have greatly strengthened the scientific power of this approach. Aside from the benefits afforded by our increasing capacity to deconstruct and manipulate the molecular structure and function of genes and proteins in cells and animals, advances in laser technology and piezoelectronics and the increasing availability of commercial MPM systems now afford immunologists with the opportunity to study immune responses at subcellular resolution in a truly physiological context. Modern MP-IVM systems output massive digital data streams that require powerful software and computing resources to extract information about the spatial and temporal relationships of migrating cells and allow the acquisition of additional parameters, such as the color spectra and intensities of multiple fluorescent

objects. To make full use of these enabling technological advances to address immunological questions requires creative interfacing of imaging and computational hardware with sophisticated software and fluorescence-based biological tools some of which already exist, while many others remain to be created.

We have discussed herein examples of areas of immunological inquiry that are particularly likely to benefit from IVM-based strategies. These include the nature of T cell-APC contacts and information exchange, the role of the cytoskeleton and its linkage to the outside world via integrins, the rules that govern chemoattractant-guided migration in solid tissues, the nature of DC maturation and subset-specific influences on antigen processing and delivery, and the dynamic cellular organization of unique tissue compartments, such as the bone marrow. Given this broad applicability and the growing dissemination of powerful microscopy systems, it can be expected that IVM will be transmuted from a fringe technology to a unique component in the standard toolkit of modern immunology.

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