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From optical bench to cageside: intravital microscopy on the long road to rational vaccine design

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Summary

No anti-viral vaccine is perfect. For some important pathogens, there are no effective vaccines. Many current vaccines are based on the working principles of Jenner and Pasteur, i.e. empiric administration of attenuated or inactivated forms of the pathogen. Tapping the full potential of vaccination requires a thorough understanding of the mechanism of immune activation by pathogens and their individual components. Though the rate of discovery continues to accelerate, the complexity of the immune system is daunting, particularly when integrated into the overall physiology of the host. Here, we review the application of multiphoton microscopy to examine host-pathogen interactions, focusing on our recent efforts to understand mouse CD8⁺ T-cell responses to viruses at the level of cellular interactions in lymph nodes draining the infection site. We also discuss our recent efforts to understand the influence of the sympathetic nervous system on antiviral immunity, with the ultimate goal of appreciating the traditional elements of immunity as just one facet of the total organismal response to infection and immunization.

Keywords

intravital microscopy; sympathetic nervous system; T cell; vaccine; virus

Intravital microscopy: back to the future

One of the principal roots of biological research extends 300+ years to Hooke's and van Leeuwenhoek's discoveries, respectively, of cells and 'animalcules' using improved versions of early microscopes. Cellular immunology has its own roots in Metchnikoff's 1882 visualization of macrophage phagocytosis made possible by Abbe's newly advanced objectives which greatly increased image resolution (and remarkably, essentially remain state-of-the-art). With the introduction of lasers and electronics to microscopy a century later, light microscopy entered a new golden age of direct observation-based biological discovery. The pace of technological advances has only accelerated in the past decade, expanding the limits of the possible at a dizzying pace.

Tellingly, Metchnikoff discovered phagocytosis as it occurred in starfish larvae that he had pierced with a tiny thorn. He chose starfish larvae for their availability (his laboratory was in Sicily) and, above all, transparency. Though cellular immunology began thus with intravital microscopy (IVM), a century would lapse before IVM returned to the forefront of immunology research, largely through the development of the multiphoton microscope, which enables the visualization of fluorescent molecules in tissues at a depth sufficient to image immune cells in relevant contexts of activation and effector function.

Though like any technique, multiphoton microscopy (MPM) is subject to limitations and artifacts, it has become a vital technique for translating indirect knowledge from functional *in vitro* and *in vivo* experiments into direct knowledge of immune function, and for making original discoveries on immune mechanisms. Here we review and contextualize the application of MPM to our overall goal of understanding the activation of CD8⁺ T cells by viruses in a true physiological setting, which includes contributions from all vertebrate control systems, including the nervous system. Aside from its basic interest, we view this information as crucial in moving CD8⁺ T-cell vaccine development from an empiric to a rational exercise. For readers interested in practical aspects of applying MPM to infectious disease research in mouse model systems, we point them our recent review (1).

Lymphatics: where immunogenicity begins

The immune system is organized into two basic physical entities (with an abundance of gray areas): immune tissues, sites where immune responses are orchestrated (including lymphocyte activation), and everywhere else, i.e. non-immune organs, where lymphocytes (or the antibodies they secrete) act to provide immunity. For immunogens introduced by standard vaccination routes into skin or muscle, lymphocyte activation occurs in lymph nodes (LNs) draining the vaccination site. Antigens are delivered to LNs by the lymphatics, a remarkable array of fluid collecting vessels that are present in every organ and have the dual role of immune function and maintaining the proper volume of extracellular fluid (2). Fluid initially collects in lymphatic capillaries, small bore (10 - 80 μ m diameter), blind-ended, highly permeable vessels that form a mesh-like network in the interstitia (3-5). Fluctuation in tissue hydrostatic pressure opens the lumen of lymphatic capillaries, drawing in water, solutes, and small particles for a one-way trip to the LN (6, 7). Increased hydrostatic pressure dramatically fills lymph capillaries, but it probably does not greatly increase the rate of lymph flow to the LN (8). This has important practical ramifications, since relatively large volumes are typically used to introduce immunogens in mouse studies (e.g. we typically introduce virus subcutaneously to a mouse in a volume of 25 μ l). As lymphatic capillaries fill, overlapping cell junctions serving as flap-valves close, preventing backflow and propelling lymph into collecting, or afferent lymph vessels (9).

Collecting lymphatics are more highly structured than lymph capillaries, being surrounded by a sheath of contractile smooth muscle cells that propel lymph (the intrinsic lymphatic pump) in a coordinated fashion by electrical stimulation, controlled in part by the sympathetic nervous system (10, 11). Valves divide these larger collecting vessels into units, termed lymphangions, which are $\sim$ 100 μ m in diameter and up to a mm in length (12). As in the venous system, the valves generate directional flow toward DLNs. Lymph can be transported against a pressure gradient in a wavelike manner between lymphangions, probably via gap-junction mediated communication between lymphatic endothelial cells which activate the smooth muscles cells they contact (12). Lymph percolates through the receiving node (described in detail below) before exiting via efferent lymphatic vessels. Eventually, lymph empties into the blood through the thoracic duct.

Lymph transports soluble antigens, viruses and unicellular pathogens, and cells. For tissue resident sentinel lymphocytes and antigen-presenting cells (APCs) [such as dendritic cells (DCs) and Langerhans cells (LCs)], the distance to lymph nodes is substantial. Even in a mouse, cells may have to travel several cm to reach the DLN (in whales, the distance can be meters). Even at several μ ms per minute (a high speed for cells in dense tissue), this would take days. Rather than arduously crawling through tissues, cells enter lymphatics and flow to the DLN within hours (13, 14).

DC migration is an area of intense research, owing to its central contribution to immunogenicity and self-tolerance. Under steady-state conditions, DCs are a major cellular component of the afferent lymphatics but a minor component of efferent lymph (15-17). This finding, along with the short lifespan of DCs and the observation that severing lymphatic vessels leads to the near absence of nodal DCs, led to the prevalent view that at steady state, myeloid DCs populate the LNs via lymphatic trafficking (14, 18-21). Recently, however, this conclusion was challenged by more direct findings that only a small fraction of DCs in the node originate from peripheral tissue, as determined using transgenic mice whose tissue DCs express enhanced green fluorescence protein (eGFP) (22). Inflammation enhances (up to 10-fold) the migration of DCs via the lymphatics (23). Under these conditions, lung-derived DCs can represent a substantial proportion (25% compared to 4%) of LN DCs (22, 23), with the bulk of DCs still derived from blood.

Tissue DCs enter lymphatics following various inflammatory stimuli, including infection, contact sensitization, and tissue trauma. Release of pro-inflammatory cytokines such as interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) promote DC maturation, a key event leading to their emigration via lymphatics (24). Along with increased expression of costimulatory and major histocompatibility complex (MHC) class II molecules, mature DCs modulate chemokine receptor expression to those favoring lymphatic entry (25-28). The best characterized of these LN-tropic chemokine receptors is CCR7. CCR7's ligands, CCL19 and CCL21, are expressed by both lymphatic endothelial cells and LN cells, and lymphatic endothelial expression is greatly increased by inflammation (29). Studies in *CCR7*^{-/-} mice, as well as in *plt/plt* mice (deficient in CCL21), confirm the role of CCR7-CCL21 as the predominant guiding interaction in lymph-borne emigration of DCs to DLN (24, 29-32).

In contrast to cell egress from blood vessels, under non-inflammatory conditions, cell entry into lymphatics does not entail integrin-based attachment and diapedesis (33). The porous nature of lymphatic structures facilitates DC entry. Entry does not require proteolytic digestion of the extracellular matrix, since the basement membrane leaves bare 5% of secondary lymphatic vessels and 30% of the surface lymphatic capillaries (34). Rather, DCs squeeze through the overlapping, oak-leaf shaped flap-valves into the fenestrated lymphatics. *In vivo*, mobilized DCs penetrate the lymphatic vessels in a manner of minutes (34). Inflammation induces lymphatic integrin expression and alters the requirements for cell emigration, in keeping with the complications entailed with maintaining effector cells at the inflammation site (7, 35).

Lymph drainage and cellular movement within the node

Afferent lymphatics empty into LNs, highly organized collagen- and fat-encapsulated (36, 37) immune organs. Lymph is first collected into the subcapsular sinus (SCS) located immediately below the capsule. The SCS is lined by specialized macrophages and separated from the LN parenchyma by a basement membrane, although this membrane is likely porous (at least in some areas) with gaps as large as 1 μ m detectable by electron microscopy (38-40). Deeper in the LN, naive lymphocytes enter through high endothelial venules (HEVs) in the T-cell zone of the inner paracortex. Lymphocytes traffic along stromal cell 'highways' post-entry (41, 42), before B lymphocytes diverge to populate more superficially located follicles, utilizing follicular DCs to guide their movement (41). Naive CD4⁺ and CD8⁺ T cells remain in the T-cell zone near sites of HEV entry, along with a variety of DCs and macrophage subtypes.

Lymph entering the DLN is sampled by SCS macrophages, which can be distinguished from other nodal macrophages by their expression of CD169 and lack of F4/80 (43, 44). Lymph-borne antigens escaping filtration by SCS macrophages enter the node parenchyma through

a system of conduits, although diffusion through SCS-floor gaps may also occur (45-47). While small molecular weight molecules freely enter the conduit system and transit into the T-cell zone, molecules with a molecular radius of >5 nm (typically proteins of > 70 kDa) are largely excluded and are retained in the SCS (48, 49). A substantial fraction of substances size-excluded from conduits pass from the SCS directly to the medullary sinus, a large cavity near the LN hilum (50) and egress with efferent lymph, thereby completely bypassing the LN cortex (13, 48). Size- included molecules channel through the conduits to LN capillaries and venules, enabling chemokines from the periphery to rapidly access HEVs and recruit naive lymphocytes to the LN to enhance immune surveillance (51).

LN conduits are tube-like structures ranging in diameter from 0.2 to $3\ \mu\text{m}$ that course through the LN T-cell zone, frequently terminating near HEVs (13). Lymph travels through an inner core composed of loosely packed collagen bundles with microfibrils filling non-collagenous voids (52, 53). Fibroblastic reticular cells (FRCs) form an outer sheath of basement membrane covering the core; however, small gaps in the sheath are covered by DCs (54, 55). FRC-associated-DCs extend dendrites into the conduit lumen, allowing DC access to transported material (56). Mature DCs localize adjacent to conduits (56), potentially enabling their acquisition of soluble antigen from peripheral infection sites and participation in T-cell cross-priming. This would impose a size limit on immunogens cross-priming (see below) via this route, which to our knowledge has yet to be examined experimentally.

Conduits were believed to play little role in B-cell activation, due to their apparent paucity in B-cell follicles (46, 57). The abundance of follicular conduits is controversial, however, and may more closely resemble T-cell-zone follicles (58). Regardless, Roozendaal *et al.* (57) reported that follicular conduits transport small soluble proteins to naive B cells. In place of FRCs, follicular conduits are sheathed by follicular dendritic cells (FDCs), enabling FDC-acquisition of conduit-transported antigen for B-cell priming (59).

Following the initiation of lymph-borne transport of particles and soluble material to the DLN (which persists as long as peripheral antigen is present to drain), a second wave of compartmentalized antigen delivery occurs as APCs arrive from peripheral inflammation sites (60). DCs are typically the first arrive, beginning 12-18 h after infection/immunization, with LCs arriving 3-4 days later (61). Entering DLNs from the SCS entails traversing the SCS basement membrane and a layer of marginal reticular cells. How emigrating APCs manage this is uncertain, though the pores in the SCS floor may simplify their task (13). Regardless of the mechanism, within a day, migrant DCs establish residence near the HEVs, where they are poised to interact with naive T cells entering from HEVs (62, 63). It is likely that DCs follow a CCL21 gradient to the paracortex, since migratory DCs accumulate in the SCS of mice lacking CCL21 (*plt/plt*) (64). Likewise, CCR7-deficient mice have few migratory DCs in the LN paracortex (31). Migrating DCs may traverse FRC networks (41, 65), akin to lymphocytes, with FRC expression of CCL19/21 (64) providing the requisite chemotactic signals.

Activating naive CD8⁺ T cells: direct- versus cross-priming

By killing or reprogramming cells bearing antigenic peptides complexed with MHC class I molecules, CD8⁺ T cells exert immune effector functions against viruses, other intracellular pathogens, and tumors. For pathogens resistant to antibody-based vaccines (e.g. HIV, *Plasmodium falciparum*) and tumors, there is great hope for the effectiveness of vaccines that induce a robust CD8⁺ T-cell response.

It has been known for decades that protein and inactivated virus-based vaccines only poorly activate naive CD8⁺ T cells (but never say never), fueling interest in the field of antigen

processing and presentation. The rationale being that detailed knowledge of CD8⁺ T-cell activation (and tolerization) will enable rational vaccine design. It is becoming clear that the exact circumstances of CD8⁺ T-cell priming can have a significant influence on their effectiveness in both primary and recall responses, due to differences in immunodominance hierarchies elicited, homing capabilities, and effector functions, providing added impetus for detailed understanding (66).

There are two basic routes of priming naive CD8⁺ T cells: direct priming, where CD8⁺ T cells are activated by APCs synthesizing the cognate antigen, and cross-priming, where APCs acquire antigen from exogenous sources. In the case of viruses, which can infect both professional APCs (i.e. DCs and other immune cells capable of priming) and non-immune cells, dissecting the contributions of direct-priming and cross-priming to the overall antiviral CD8⁺ T-cell responses is a challenging task. Rising to the task, a number of new approaches have been described to better define priming of anti-viral CD8⁺ T cells (reviewed in 67).

These approaches focus on ablating or inactivating CD8 α ⁺ DCs, LN/spleen-resident cells highly efficient *ex vivo* at phagocytosing dead cells and processing acquired exogenous antigen (68-70), potentially allowing them to acquire antigen from moribund LN-resident virus-infected cells. Prior studies with diverse viruses (including herpes simplex, influenza, vaccinia, respiratory syncytial, and lymphocytic choriomeningitis) demonstrated that mouse CD8 α ⁺ DCs that have acquired viral antigen *in vivo* from infected cells, potently activate naive CD8⁺ T cells *ex vivo* (reviewed in 71). Three strategies now are available for characterizing the contribution of CD8 α ⁺ DCs *in vivo*: (i) Batf3^{-/-} mice, which lack CD8 α ⁺ (as well as CD103⁺ langerin⁺) DCs (72); (ii) injecting cytochrome C into mice, which induces apoptosis when delivered the cytosol thereby selectively ablating the CD8 α ⁺ DC subset with highest cross-priming capacity (71); and (iii) injecting of TLR ligands into mice, which induces CD8 α ⁺ DC maturation, greatly reducing their cross-presentation capacity (73, 74).

The location of APC subsets in the LN dictates their access to incoming lymph or lymphatic-conduit filtered lymph and must therefore play a critical role in their potential participation in CD8⁺ T-cell activation. Under steady-state conditions, CD8 α ⁺ DCs are located in the LN paracortex in frozen sections (75). However, their behavior remains to be examined *in vivo*, particularly their access to lymph conduits or the SCS. This latter property is critical for direct priming, since even the smallest virions exceed the conduits size-exclusion limit by many orders of magnitude. Intact viral particles would therefore only infect those cells residing in or accessing the SCS, unless of course, a mechanism for transporting virions into deeper areas of the T cell exists. Likewise, direct priming could occur deeper in the node, if DCs infected in the periphery migrate centripetally.

Back to Metchnikoff

Ex vivo approaches are inherently limited by their uncertain relationship to what actually occurs *in vivo*. The distinguishing feature of higher multicellular organisms is the spatial organization of their tissues. Obviously, the remarkably intricate structures of LN and other immune organs (with some structural elements still to be discerned, no doubt) evolved to optimize immune functions. Immune cells incessantly interact with other cells and structures based on their location in the three-dimensional tissue matrix. Equally important, the spatial constraints imposed by lymphoid structures must influence the extracellular environment of immune cells in highly specific ways that modulate the concentrations of gases, nutrients, cytokines, etc. Simply culturing cells in synthetic media containing 5 to 10 times more oxygen than encountered *in vivo* (i.e. standard tissue culture conditions) must alter the behavior of cells in important ways that are nearly impossible to establish experimentally.

Ex vivo observations can provide information about potential functions and interactions of immune cells, but function must be established *in vivo*.

Understanding immune function in the context of tissue architecture ultimately relies on microscope-based visualization of immune cells in tissues. Light and electron microscopy have played important historic roles in immunology research in defining physical structures visualized by various staining methodologies, and increasingly by the use of immunofluorescence to detect cellular subsets identified by their interaction with antibodies, or by their expression of fluorescent proteins controlled by lineage-specific promoters. The introduction of laser scanning confocal microscopy (LSCM) and associated hardware and software innovations has enabled the three-dimensional visualization of fluorescent cells and structures. Confocal microscopy is inherently limited in its ability, however, to penetrate tissue to depths sufficient to visualize cell interactions in critical anatomic regions of immune tissues. So while LSCM can be used on living tissue sectioned *ex vivo* (though laser-mediated tissue damage is a serious problem), its use in IVM is limited to near surface interactions in skin or exposed tissues (10-20 μm). While even standard epifluorescence microscopy has been used in important studies on immune cell trafficking in blood vessels, returning cellular immunology to its IVM roots in Metchnikoff's microscopy required the development of multiphoton microscopy (MPM). MPM was first applied to biology in 1990 and more than a decade later to imaging immunity principally by the Cahalan and Von Andrian groups using home-made machines (76-79). With the introduction of turn-key systems by a number of manufacturers, MPM has become a mainstream, though costly, technology.

MPM is enabled by intense lasers that deliver infrared ultra short (femto- to picosecond) pulses of photons to biological specimens (reviewed in 1, 80). Power is essential, since two-photon excitation, predicted by quantum mechanics theory in Maria Goppert-Mayer's 1931 Ph.D. dissertation (and confirmed experimentally only 30 years later), occurs when a fluorophore absorbs two-photons within 10^{-18} sec (an impressively small number: the universe itself is less than 10^{18} sec old!), resulting in excitation approximating the summed energy of the photons (i.e. half the wave length of the merging photons). The longer wavelength photons generated by the MP laser scatter less, penetrate deeper into the tissue, and are less phototoxic than shorter wavelength photons used for standard fluorescence excitation. Since there is sufficient photon flux only at the focal plane to create the two-photon effect, excitation exclusively occurs at the focal plane, obviating the need for a confocal pinhole to obtain high resolution, greatly increasing the sensitivity of the method.

MPM images are acquired in a manner similar to standard confocal images. After selecting a field, serial scans are performed in the z-dimension by incrementally adjusting the focal plane with a computer-controlled mechanical stage or objective. Reconstructing these images with software creates a virtual three-dimensional image of the region that, depending on circumstances, ranges in depth to nearly 500 μm . Since phototoxicity is minimized (though still an important consideration) by MPM, images can be repetitively collected over time to generate four-dimensional movies of fluorescent-labeled cells/viral proteins/structures in their native environment. With sophisticated tracking software, it is now possible to quantitate the movements and interactions of cells during the course of an immune response.

CD8⁺ T cells in prime time

Our initial foray into visualizing CD8⁺ T-cell priming was largely based on examining frozen sections of popliteal lymph nodes (PLNs) after injecting recombinant vaccinia viruses (rVV) in the foot pad (though buried in the paper, is an early MPM image of immune cells, in this case, imaged in a LN explant). We visualized VV-infected cells by expressing NP-

SIINFEKL-eGFP (NP-S-GFP), a chimeric protein targeted to the nucleus, and T cells by adoptively transferring TCR transgenic T cells specific for SIINFEKL (OT-I T cells), or a NP-encoded peptide (F5 T cells) that were dye-labeled prior to transfer. We found that VV proteins were expressed in thousands of LN cells within 6 h post-injection, declined sharply in the next 18 h, and completely over the next day.

We extended these studies by using a Leica MP system to perform IVM (81). Since our microscope is in an inverted configuration, we imaged inguinal LNs (ILNs) rather than PLNs, which due to anatomical constraints are imaged using an upright microscope (each configuration is optimal for some organs). Imaging the ILN is tricky, due to its baroque lymphshed, requiring experience (and often multiple injections per node) to consistently inject into a relevant drainage area (A few days practice with India ink will speed the learning curve, and provide a naked eye demonstration that spatial separation is maintained as different injection sites may drain into distinct locations within a single LN).

Using rVV or recombinant vesicular stomatitis virus (VSV) also expressing NP-S-GFP, we found that ILN cells produce NP-S-GFP fluorescence at least as rapidly (within 2-3 h) as cells do *in vitro* after simple virus addition. Using brighter fluorescent proteins, we initially detect infection within an hour of injection (Hickman *et al.*, unpublished data), strongly implying that virions are delivered to the LN within minutes of injection. Indeed, the von Andrian and Carroll laboratories (82, 83) have reported that fluorescent VV, VSV or influenza A virions can be visualized in the PLN within minutes of subcutaneous footpad injection.

How do these virions, ranging from 100 nm (influenza) to 400 nm (vaccinia), enter the lymphatics and travel to LN? Although virion entry into the lymphatics remains to be directly visualized, a likely scenario is easy to conjure. Virions should easily pass into lymphoid capillaries, given their rather porous structure, with portals of $> 4 \mu\text{m}^2$ (34). The second barrier to free virion drainage, the flap-valves of the lymphatic endothelium, could also be easily traversed through 2-3 μm 'buttons' forming the hinges of the valves through which DCs pass (84). Indeed, it is likely that the lymphatic system evolved to facilitate delivery of particulate pathogens, including viruses and bacteria.

While the fluid dynamics of virion transport to the DLN are easy to understand, it is perhaps surprising that viruses are not adsorbed onto lymphoid endothelial cell surfaces *en route* to the DLN. VSV and VV bind ubiquitously to cells, attaching to virtually all cell types, and VSV has been reported to bind to lymphatic endothelial cells in the LN medulla (82). Could the collagen-based core of lymphatic vessels represent an adaptation to shield flowing virions from endothelial cells?

There is a potential artifact in these studies: virion transport could be artificially enhanced due to high numbers of viral particles needed for imaging or increased hydrostatic pressure resulting from subcutaneous injection. As discussed above, however, evidence suggests that tissue hydrostatic pressure does not greatly increase the rate of lymphatic flow. Moreover, using more natural routes of infection, CD8⁺ T-cell responses can initiate fully during the first 24 h post-infection, a period believed to be required for peripheral APC emigration to the DLN, supporting the physiological relevance of rapid pathogen transport into DLN (85). Still, the details of virion transport to DLN and infection of DLN cells needs to be compared under different infection conditions, including scarification, intramuscular injection, insect bite, and various mucosal routes.

The first cells virions encounter in the DLN are CD169⁺ (F4/80⁻) macrophages that line the SCS or project into the SCS from a caudal position, actively endocytosing viruses (82, 83), resulting in their preferential infection (43, 81, 86). Indeed, in our studies with VV and

VSV, this is the predominant infected cell population (43, 81). The filtration function of macrophages can contribute to antiviral immunity: in the case of VSV, viremia increases nearly 100-fold when SCS macrophages are depleted by clodronate liposome prior to infection (82). This function also limits the ability of VSV to access the CNS via sympathetic nerves innervating the DLN, at least in part due to type I IFN secretion by infected SCS macrophages (86).

Just how SCS macrophages capture incoming virions is controversial. Junt *et al.* (82) all reported that the cells are poorly phagocytic for 200 nm-diameter latex beads, suggesting a specific mechanism for virion phagocytosis. In contrast, Carrasco and Batista (87) visualized accumulation of similar beads by LN macrophages, and most other particulates [including fluorescently-labeled killed bacteria and fluorescent-protein encoding live parasites (88)] accumulate on or in these cells. CD169 itself binds many different sialylated glycoconjugates, preferring O-linked oligosaccharides terminating in Neu5Aca2,3Gal β 1,3GalNAc (89). While CD169's lectin activity is thought to primarily mediate cell-cell contacts (89), it might also function as a pattern recognition receptor for sialylated enveloped viruses (89). Given the strong evidence for their importance in immunity to viruses and other pathogens, it is of clear interest to study the function of SCS macrophages in detail and to determine the contribution of CD169 (90) and other surface proteins that could function (directly or indirectly) in pathogen binding/internalization (58).

Among the many unknowns, the role of SCS macrophages in the initiation of CD8⁺ T-cell responses, particularly as direct-priming APCs, is unknown. *Ex vivo* approaches have reported that LN macrophages do not present antigen after viral infection; however, these results are complicated by difficulty in isolating CD169⁺ macrophages from the LN, as well as the possible incapacitation of infected cells due to virus-induced cytopathogenicity (91-93). Using either confocal microscopy or MPM, we found that despite accounting for 85% of infected cells, SCS macrophages do not form prolonged contacts with CD8⁺ T cells, which interact instead with DCs (43, 81). The factors governing CD8⁺ T-cell APC selection remain to be investigated, but our recent findings point to a central role for chemokines in T-cell discrimination of APCs (Hickman *et al.*, manuscript in preparation).

Most LN cells imaged by MPM movies are motile, either moving rapidly [i.e. T cells and neutrophils (77, 88)] or at a more relaxed pace [steady-state DCs within the LN paracortex (94)] SCS macrophages, however, are nearly immobile, perhaps due to physical constraints associated with their SCS location. Likewise, virus-infected LN cells move very slowly (around 1.5 μ m/min) (81), though the locomotion of macrophages versus DCs has not been specifically characterized. The immobility of infected APCs at the node periphery may facilitate their detection by rapidly moving LN lymphocytes, similar to the localization of LN immigrant DCs near the HEVs.

How do virus-infected LN DCs interact with CD8⁺ T cells? LN T-cell activation was the focus of many of the initial MPM studies (77, 79, 95, 96) and has been reviewed in detail (97-100). Using peptide-pulsed, LPS-activated adoptively transferred DCs as APCs, Mempel *et al.* (96) proposed a three step model for CD8⁺ T-cell priming. Phase one occupies the first 8 h post T-cell transfer (performed 18 h after APC injection) and is characterized by transient T-cell contacts with DCs and upregulation of T-cell early activation markers. During the 12 h phase two period, T cells slow and engage in prolonged interactions with DCs. In phase three, T cell-DC interactions return to phase one-type interactions. Hugues *et al.* (101) confirmed and refined this model also using peptide-pulsed DCs.

MPM of CD8⁺ T cells interacting with DCs presenting antigen targeted by conjugation to a monoclonal antibody specific for a DC surface protein (Dec-205) revealed a different picture, however (102). CD8⁺ T cells bypassed phase one, forming stable interactions from the onset of imaging. Complications ensued, as Henrickson *et al.* (103) reported that following peptide-pulse DC transfer, T-cell behavior varies with TCR avidity and density of antigen-presenting DC, with stronger activation again resulting in bypassing phase one interactions.

These situations, while relevant for specific vaccine scenarios, are a far cry from natural circumstances, such as viral infections. What happens when APCs are nearly exclusively located in or about the LN SCS, e.g. after subcutaneous virus injection? Using MPM, we found that naive CD8⁺ T cells centrally located in the LN pre-infection, move centrifugally to interact with VV- or VSV-infected DCs in the peripheral interfollicular region (PIR), the areas between B-cell follicles at the outer edge of the LN (81). Even with the artificially high numbers of adoptively transferred, fluorescent-labeled TCR-transgenic cells used in this study, relocation to the PIR was nearly complete by 6 h post infection.

Although it is generally accepted that LNs are designed to optimize interactions between naive T cells exiting HEVs with vicinal DCs, this does not preclude trafficking of immigrating T cells to the PIR. This observation suggests the existence of a mechanism to maximize interactions of PIR APCs with the substantial population of T cells that are already present in the naive LN (~ 10% of LN cells at any given time). Since Seedhom *et al.* (104) reported that the frequency of anti-VV CD8⁺ T cells is ~ 1/1500, there would be ~200 VV-specific CD8⁺ T cell in a small (e.g. popliteal) naive mouse LN. Accessing this pool of immediately available T cells could accelerate the CD8⁺ T-cell response by hours, with every minute potentially important in the race to generate sufficient numbers of CD8⁺ T cells to mount an effective antiviral response. To this end, we found that preventing naive CD8⁺ T cells from entering DLN after initiating VV-infection reduced the number of effector cells in the periphery only by half (81), consistent with the idea that ~1/2 of naive anti-VV precursors reside in the DLN at the time of infection. The extent to which these findings extend to other viruses and other routes of infection is an important question in future experiments.

It will also be of great interest to examine the degree to which the time window of activation post-infection influences CD8⁺ T-cell differentiation with regard to effector function and memory pool generation. For even acute viral infections, viral clearance from peripheral tissues can take weeks, providing a steady delivery of antigen to DLN in the form of free antigens or immigrant antigen-bearing APCs (or much longer, since viral antigens and genes from classically acute viruses can persist for months or years following mouse infection) (105-107). Catron *et al.* (108) reported that CD4⁺T-cell function was influenced by their time frame of activation in the DLN post-antigen administration. If nothing else, alterations in CD4⁺ T-cell function can influence CD8⁺ T-cell interactions, particularly once the adaptive immune response kicks into gear, and the increased numbers of responding cells (CD4⁺ and CD8⁺ T cells and B cells) have a much greater likelihood of interacting with each other.

This latter point raises the issue of the contribution of CD4⁺ cells to CD8⁺ T-cell responses. We previously reported that the absence of CD4⁺ T cells has only minor effects on the primary CD8⁺ T-cell response to influenza A virus (IAV) (109), echoing many other reports of CD4⁺ independent antiviral CD8⁺ viral responses (110). Still, even 'minor' effects imply a potential interaction between CD4⁺ and CD8⁺ T cells, consistent with our finding that ablating CD25⁺ regulatory T cells enhances CD8⁺ T-cell responses to VV and IAV and alters the immunodominance hierarchies (111). Although CD4⁺ T cells are not critical for

the initial generation of memory CD8⁺ T-cell generation, they are required for maintenance of memory cells and their recruitment to secondary challenge sites (112, 113). Clearly, the interactions between T-cell subsets in the DLN is well worth a look by MPM, as are the potential contributions to T-cell activation of NK cells and even less likely suspects such as neutrophils (114).

B cells: the next frontier

Our laboratory recently returned to a topic that we had not studied in decades, antibody driven evolution of the IAV hemagglutinin (HA)(115). The HA has a discrete number of antigenic sites (5 or so) that induce neutralizing antibodies, and it has always been puzzling why these sites are immunodominant and to what extent an immunodominance hierarchy exists among individual B cell responses to HA (or other antigens for that matter). We've spent much of the last 15 years studying immunodominance in CD8⁺ T-cell responses (66, 116-118) (we'd still like to visualize the competitive interactions between T cells occupying different rungs of the immunodominance hierarchy), and are intrigued to examine the possible parallels with B-cell responses. While there are many functional approaches to explore, eventually all roads lead to MPM (particularly when the instruments are sitting in your lab), which will be an important element in understanding IAV activation of B cells.

B-cell responses have been the topic of a number of MPM studies (reviewed in 58, 119). Three studies visualized SCS macrophages capturing and relaying Ag to follicular B cells (82, 87, 120). Junt *et al.* imaged fluorescent dye-labeled VSV virions accumulating on SCS macrophages, while Carrasco and colleagues visualized SCS macrophage acquisition of antigen-coated fluorescent virion sized-beads. More recently, Phan *et al.* (44) reported 'relay' of antigen (immune complexes) is needed for affinity maturation of antibodies. Interestingly, we have not observed that follicular B cells are infected with VV or VSV (81, 86). Since splenic B cells can be infected with these viruses *in vitro*, this suggests that either follicular B cells differ in their infectability or that infectious virus is not trafficked into the follicle. Interestingly, Catron *et al.* (121) recently reported bead-coupled antigen can be proteolytic liberated before antigen capture by follicular B cells, suggesting this might also be an important pathway for viral antigen transport into the node.

SCS macrophages are not essential for B-cell activation by some viruses. Purtha *et al.* (122) used clodronate-liposome-depletion experiments to show that B-cell activation after West Nile virus infection is unaffected by ablation of SCS macrophages. Similarly, macrophage depletion does not impair the humoral immune response to subcutaneously injected IAV (83), although this challenge route is more relevant for vaccination rather than infection.

For imaging in tissue sections, B cells offer a significant advantage over T cells, in that fluorescent antigens (rather than MHC-peptide complexes, which are notoriously finicky for this application) can be used to identify cells expressing cognate antibodies. This will enable many interesting experiments correlating specific B-cell activation with various APCs, whose presented viral antigens can be characterized using antibodies specific for different viral proteins and distinct forms of viral proteins (e.g. antibodies specific for HA trimers vs. monomers vs. denatured HA).

The larger context

Antiviral immune responses in vertebrates typically unfold in a starkly different physiological state than exists in the uninfected healthy animal. Viral infections trigger rapid changes in cytokines that induce global alterations in vertebrate physiology, resulting in the acute phase response, fever, and alterations in the nervous and endocrine systems. Sorting

out the contributions of these changes to the antiviral immune response is a daunting challenge, not the least, because they are all intimately interweaved.

We have begun to tackle this issue by characterizing the role of sympathetic nervous system (SNS) in antiviral immunity. The SNS is the Yin of the autonomic nervous system (with the cholinergic nervous system as its Yang). SNS neurons act by releasing granules containing norepinephrine, neuropeptide Y, and adenosine triphosphate (ATP). As mentioned above, lymph flow is controlled by the SNS, and immune organs are innervated, possibly exclusively, by the SNS. SNS involvement in immunity has long been studied, but the focus has favored phenomenology over mechanism.

We started with a standard neurobiology approach: ablating the mouse peripheral SNS by intraperitoneal injection of 6-hydroxydopamine (6-OHDA) (123). 6-OHDA is endocytosed by recycling synaptic vesicles where it is oxidized to generate lethal amounts of free radicals. The blood-brain barrier spares the CNS from nerve ablation. Peripheral nerves take several months to regenerate, providing a window to study chemically sympathectomized mice. We found that surprisingly, 6-OHDA ablation increases anti-IAV CD8⁺ T-cell responses by fourfold in a typical experiment. 6-OHDA also increased CD4⁺ T-cell responses, but this was not required for the increase in CD8⁺ T-cell responses. Antibody responses were not affected. The enhancing effect on CD8⁺ T-cell responses was also found for antigens presented by defined routes of cross-priming (antigen-expressing, non-histocompatible cells) and direct priming (VV-encoded, rapidly degraded cytosolic peptides).

We confirmed that sympathetic nerve-based activity is responsible for suppressing CD8⁺ T-cell responses by treating mice with SNS blockers. The β -antagonist naldolol enhanced responses to IAV, while the α -antagonist phentolamine had no significant effect. We further showed that a β_1 - but not a β -adrenergic antagonist enhanced responses. Adoptively transferring CD8⁺ T cells established that 6-OHDA treatment altered the response at the level of the recipient and not the donor, indicating that persistent alterations in CD8⁺ T cells do not explain the phenomenon. Consistent with this finding, 6-OHDA treatment enhanced ability of splenic APCs, including sorted CD11c⁺CD8 α ⁺ and CD11c⁺CD11b⁺ DCs, to activate antiviral CD8⁺ T cells. This could not be attributed to enhanced antigen presentation or expression of standard co-stimulatory molecules, and the molecular mechanism remains to be established.

We extended these findings to the mouse influenza pneumonia model, where we found that 6-OHDA-mediated sympathectomy decreased mortality (124). Perversely, this effect could not be attributed to the enhanced CD8⁺ T-cell response, since mice lacking functional K^b and D^b showed a similar effect. This is consistent with a distinct pharmacological profile: now naldolol had no effect, but phentolamine enhanced survival. Diminished mortality was not due to decreased viral replication, which was not affected by SNS ablation. Rather, it is attributed to a diminished inflammatory infiltrate, which likely resulted from remarkable effects on the early (2 d p.i.) innate cytokine response. IL-1 and IL-6 are the two most important pro-inflammatory cytokines, playing key roles in triggering the acute phase response and fever. Sympathectomy caused a 10-fold decrease in IAV-induced IL-6 and a more than 30-fold decrease in IL-1 β , and decreased IL-12p40, granulocyte-macrophage colony-stimulating factor (GM-CSF), and macrophage inflammatory protein-1 α (MIP-1 α). Since we showed that SNS innervation in the mouse lung is limited to large vessels and bronchi, we proposed the SNS exacerbates pathology by releasing neurotransmitters that diffuse into the lung and enhance release of cytokines by innate immune cells, many of which are known to express adrenergic receptors.

Taken together, these findings demonstrate that the SNS influences the innate and adaptive immune response to viruses and can play a deciding role in the survival of IAV-infected animals. We are anxious to use MPM to further explore effect of autonomic innervation on immune function.

The bottom line

A criticism frequently leveled at MP-based IVM, at least in private, is that it is a fancy technique that only confirms what is 'known' from simpler methods. Even if true, MPM would still be of great use, since an essential part of the scientific method is to test and increasingly refine hypotheses by observing nature with independent approaches. But the criticism is unwarranted. Only IVM can reveal the dynamics of antigen and cell movement at cellular scale. Only IVM can reveal the duration of cellular interactions. Only IVM is free from artifacts associated with tissue sectioning and from culturing cells in alien environments.

Technological advances will continue to increase the power of IVM. As reporter methodologies improve and expand, it will be increasingly possible to judge the outcome of cellular interactions based on functional criteria, e.g. expression of fluorescent reporter genes or binding/activation of fluorescent dyes. Photoactivatable fluorescent probes will enable observation of trafficking of selected cells. Events at immune synapses will be imaged *in vivo*. The application of fiber optics to MPM will facilitate imaging of internal organs, particularly the airways and GI tract, and will expand the use of MPM to larger animals, even humans at some point.

The knowledge generated from present and future IVM studies will impact vaccine development, but it is impossible to precisely predict just how. One of the most attractive features of basic research is its unpredictability; unknowns must be discovered, they cannot be predicted. From the practical standpoint, IVM studies have focused on activation of naive T cells and B with little attention to development of memory responses, which is the basis of vaccination. It is important therefore, to develop experimental strategies and tools that enable distinguishing activation events that lead to memory T-cell development. It will also be of great interest to visualize the activation of memory T cells upon challenge and their function at the sites of infection.

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