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New insights into antiviral immunity gained through intravital imaging

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Viral infections pose an ongoing challenge for mankind. Much of our knowledge of the immune response to viral infections comes from *ex vivo* analyses of infected animals, which provide important yet static information about events occurring within the host. Recently, a relatively new technique known as intravital microscopy (IVM) has been applied to the study of antiviral immunity. Intravital imaging affords a unique, real-time view of both viral dynamics and the ensuing immune response (along with their interplay) in the living animal. This review details some of the newest observations about the antiviral immune response gained using IVM.

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Introduction

After viral infection, the precise nature of the immune response and the myriad factors that shape it determine whether a virus will be cleared and at what cost to the host. Accordingly, the antiviral immune response has been the subject of intense study, often aimed at informing rational vaccine design for human pathogens. Key methodological advances have expanded our knowledge of the behavior of both the virus and antiviral effectors, however most of the technologies thus far employed only yield a snapshot of infection. The recent application of intravital microscopy (IVM) to visualize the dynamics of viral infection as it occurs in real time *in vivo* has generated important insights into both the spread and containment of infection.

The primary advance allowing widespread adoption of IVM was the creation of turn-key multiphoton (MP) lasers ([1], reviewed in [2]). Instead of conventional excitation of fluorescent molecules that results from the absorption of a single photon of the appropriate wavelength, powerful MP lasers excite fluorophores with two (or more) simultaneously arriving photons of longer wavelengths. These longer wavelengths scatter less in the tissue, affording greater tissue penetration with less photodamage. Using MP lasers, the immune response can be imaged over large time periods in living animals (with the aid of different fluorescent reporter viruses and fluorescently labeled cell populations). As past immunological advances gained through the use of IVM have been summarized in other manuscripts [3–6], this review will focus only on recent advances in antiviral immunity.

Virus spread within the animal

At the earliest phase of infection, a virus often must breach barrier tissues to gain entry into cells permissive for replication. Several recent IVM studies have examined viral dissemination in mice, primarily focusing on the contribution of cellular motility to viral spread. To analyze the dissemination of human immunodeficiency virus (HIV) within the host, Murooka *et al.* infected $CD4^+$ T cells with an HIV-reporter virus expressing GFP and then transferred these cells into humanized mice [7]. Intriguingly, naïve, infected T cells retain mobility and migration patterns within the LN, although they move at slower speeds than their uninfected counterparts. Imaging revealed that some of the infected T cells have cellular extensions up to 20× larger than the cell soma that contact numerable nodal cells. This increased cellular surface area, combined with retained cellular mobility, suggested that infected T cells could serve as disseminatory vehicles for HIV. Indeed, chemical inhibition of T cells' LN egress prevents HIV from establishing viremia in this model.

Looking at another retrovirus, Seewald *et al.* infected B and T cells *in vivo* by subcutaneously injecting Friend murine leukemia-virus (F-MuLV)-GFP [8]. In the draining LN, GFP^+ T cells remain mobile, however, B cells (with normal rapid intrafollicular movement) become sessile when infected. This restricted B cell movement allows the formation of virological synapses with uninfected cells *in vivo*, putatively for virus spread. Thus, at least two viruses can use cellular migration or alterations thereof to infect new cells.

Innate antiviral immunity

In comparison to T and B cells that can be readily removed from the LN for viral infection or for labeling with fluorescent dyes, innate lymphocytes are typically harder to isolate and less amenable to adoptive transfer. Thus, most IVM studies of the innate antiviral immune

response have utilized reporter mice to illuminate innate cellular populations, and these studies have generally lagged behind examination of adaptive immunity. Nonetheless, the important role of innate lymphocytes during viral infection can clearly be captured by IVM. After infection with lymphocytic choriomeningitis virus (LCMV), myeloid cells in the brain rapidly respond to infection with type I IFN-dependent morphological changes and increased vascular patrolling [9]. Innate lymphocytes also robustly respond to systemic delivery of myxoma virus, with neutrophils quickly accumulating in the liver [10•]. Once at the site of infection, these neutrophils release extracellular traps (NETs), which protect host cells from viral infection. Intriguingly, inducing NET formation prior to injecting myxoma results in a marked decrease in infected cells in the liver, suggesting neutrophil NET formation prevents further virus spread.

We recently visualized another poxvirus (vaccinia (VACV)) after inoculation into the skin of mice with the same needle employed for human vaccination [11]. Using a blue-fluorescent-protein (BFP)-expressing virus, we visualized large areas of viral replication in epidermal keratinocytes. While the adaptive immune response is necessary for clearance of a subset of dermal VACV-infected leukocytes, monocytes rapidly accumulate near VACV-infected keratinocytes. These monocytes facilely penetrate replicative foci and are necessary for clearance of infectious virus from the skin.

While IVM analyses of innate immunity to viral infection are relatively sparse at this point, the studies available all detail the rapid recruitment and mobility changes of innate lymphocytes in response to infection. No doubt as more reporter mice become available for imaging, numerous additional aspects of the innate response to viruses will be visualized.

Activation of adaptive immunity

Adaptive immunity to viral infections begins in the LN draining the site of infection. Because of its central role in the immune response, the first immunologic studies utilizing IVM visualized reactive LNs; these organs remain the subject of intense investigation. After a large number of labs visualized LNs after protein or peptide immunization, a consensus emerged of both the complex organization and cellular migration speeds and patterns of nodal cellular populations. Most basically, the LN can be subdivided into several key areas: the subcapsular sinus (SCS) into which lymph first drains, the B cell follicles containing naïve B cells, and the T cell area of the LN containing the sites of T cell entry (the high endothelial venules) and high numbers of DCs.

To enter the LN, cell-free virions can travel in lymph fluid deposited into the SCS, where these viruses encounter a specialized layer of macrophages (termed SCS macrophages). Exquisitely adept at filtering incoming particulates, SCS macrophages capture large quantities of free virus and remove it from further lymphatic circulation [12–14]. Additionally, SCS macrophages can pass acquired antigen along to subjacent B cells for development of humoral immunity [13,15]. Perhaps as expected, SCS macrophages are often infected after filtering virus from the lymph, which leads to their death. However, even when SCS macrophages are not productively infected, inflammatory stimulation through abortive infection or the infection of neighboring cells leads to attrition of this important cellular layer [16••]. Using IVM, Gaya *et al.* demonstrated that incoming DCs disrupt this macrophage barrier as they move through the floor of the SCS *en route* to the T cell zone. Accordingly, this disruption of the SCS macrophage layer impairs B cell responses to subsequent viral infections [16••].

What could be the purpose of infection and attrition of SCS macrophages? Sagoo *et al.* recently examined LNs infected with modified vaccinia Ankara (MVA, a poxvirus that does not replicate in mammalian cells) [17]. After subcutaneous injection of MVA, SCS macrophages in the draining LN activate inflammasomes, which can be visualized by oligomerization of ASC-GFP. As SCS macrophages die after inflammasome activation, they release ASC specks, previously shown to propagate inflammatory signals. Thus, SCS macrophages provide a unique inflammatory stimulus after virus infection that further incites the immune response [18]. Intriguingly, it has recently been shown that cellular debris from pyroptotic SCS macrophages sequester bacteria, allowing for clearance by other cells [19]. Perhaps this mechanism also confines the spread of viral infection.

Macrophages do not appear to play a predominant role in priming the T cell response to virus infection, although they can prime tumor-specific T cells [20]. Naive, virus-specific $CD8^+$ T cells contact virus-infected macrophages, however, $CD8^+$ T cells preferentially interact with $CD11c^+$ DCs for priming [12,21,22•]. After subcutaneous injection of VACV, naïve $CD8^+$ T cells relocate from the central T cell zone of the LN to the peripheral interfollicular regions and cortical ridge areas to interact with infected DCs [12,23]. Fascinatingly, $CD8^+$ and $CD4^+$ T cells interact with distinct DC subsets for priming, clustering around antigen presenting cells (APCs) in different areas of the LN [22•,24•]. While $XCR1^+$ DCs are dispensable for $CD8^+$ T cell priming after subcutaneous MVA injection, they are required for $CD8^+$ T cell priming after cutaneous herpes simplex virus (HSV) infection. Additionally, $CD8^+$ and $CD4^+$ T cells interact with APCs at different times following infection: with HSV, $CD4^+$ T cells cluster early with migratory DCs while $CD8^+$ T cells interact later with $XCR1^+$ DCS. After MVA injection, $CD8^+$ T cells cluster first with DCs followed by $CD4^+$ T cells. With both viruses, however, $XCR1^+$ DCs are