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In Vivo Imaging-Driven Approaches to Study Virus Dissemination and Pathogenesis

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Keywords

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Abstract

Viruses are causative agents for many diseases and infect all living organisms on the planet. Development of effective therapies has relied on our ability to isolate and culture viruses in vitro, allowing mechanistic studies and strategic interventions. While this reductionist approach is necessary, testing the relevance of in vitro findings often takes a very long time. New developments in imaging technologies are transforming our experimental approach where viral pathogenesis can be studied in vivo at multiple spatial and temporal resolutions. Here, we outline a vision of a top-down approach using noninvasive whole-body imaging as a guide for in-depth characterization of key tissues, physiologically relevant cell types, and pathways of spread to elucidate mechanisms of virus spread and pathogenesis. Tool development toward imaging of infectious diseases is expected to transform clinical diagnosis and treatment.

1. INTRODUCTION

Viruses are intracellular obligate parasites that depend on cellular machines of other organisms for their replication. As capsid-encoding organisms, viruses can self-assemble and are composed of proteins and nucleic acids that parasitize a ribosome-containing organism for the completion of their life cycle (1). Viruses can be further categorized broadly as nonenveloped and enveloped based on the absence or presence of a lipid bilayer that encloses the capsid. Metagenomic studies from randomly sequenced environmental samples have revealed that viral genes constitute the largest part of the genosphere (2). This incredible abundance means that viruses constantly probe the available biological space on our planet for a niche that allows their proliferation and hence survival. Once the niche is identified, viruses evolve proficiently to use their environment, thus becoming the ultimate insiders of host biology. They manipulate cellular pathways, cell-cell communication, and adhesion biology to spread successfully within and between cells. They exploit the circulatory blood and lymphatic systems for their dissemination through body fluids as well as use tissue architecture and cell migratory patterns to disseminate within hosts. Viruses also adeptly exploit the host mucosal biology to facilitate horizontal and vertical transmission to successfully infect the next host, ensuring their continued survival. Owing to millions of years of coevolution with their hosts, viruses often develop a host-pathogen relationship that leans toward coexistence. Disease then mainly arises under situations of immune deficiency, lack of resistance, or exuberant host immune response (3). Pathogenic interactions also arise when a virus-carrying species makes a first contact with an uninfected permissive species or when the tropism broadens. Most devastating viral diseases originate from such cross-species transmissions—for example, influenza (birds to humans or via pigs as intermediates) (4), human immunodeficiency virus-1 (HIV-1) (chimpanzees to humans) (5), severe acute respiratory syndrome (civet cats to humans) (6), rabies (animals to humans) (7), and Ebola virus (EBOV) (bats to humans, chimps, and gorillas) (8). When humans, livestock animals, or plant crops become diseased, viral infections demand our attention to find treatments and cures.

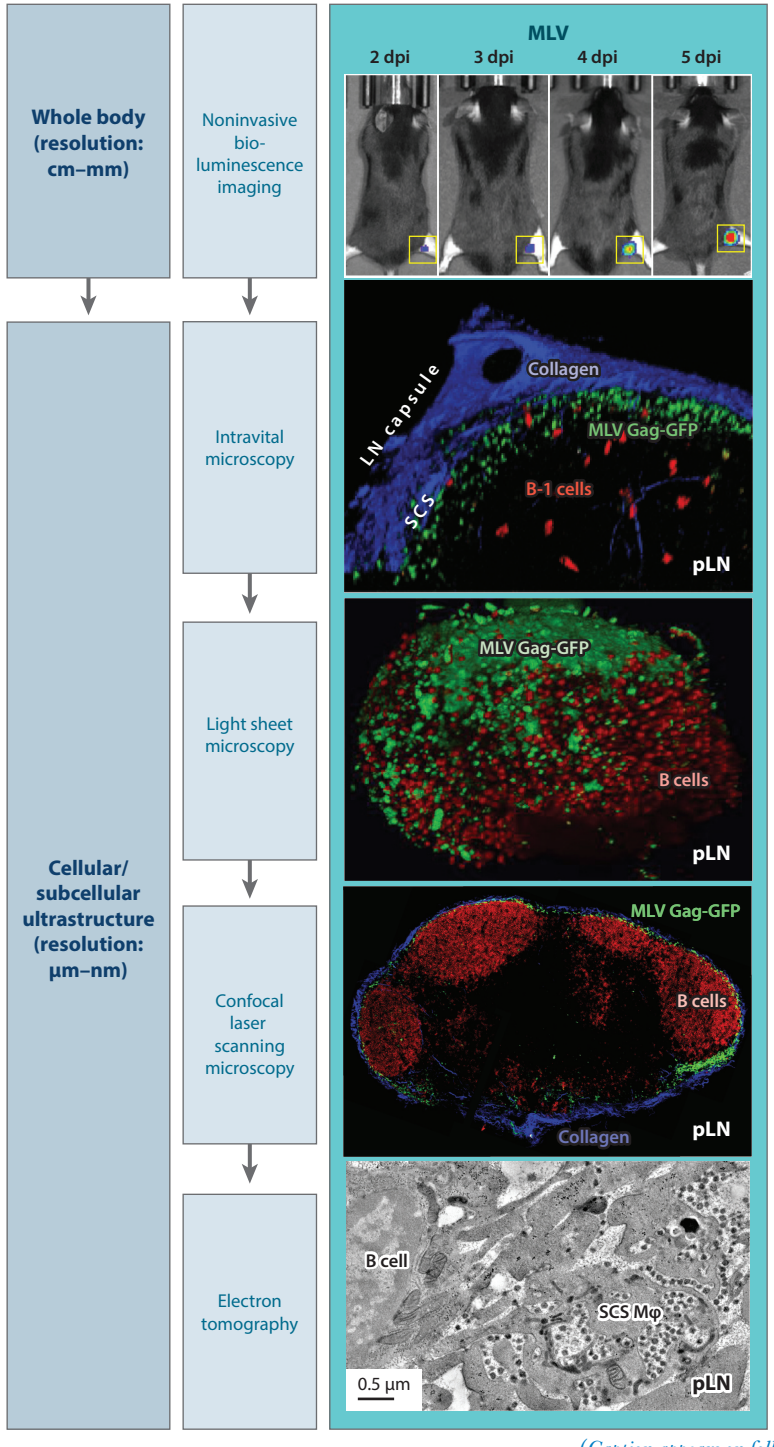
For finding therapeutic strategies, scientific explorations have traditionally followed a reductionist approach, starting with simple in vitro systems and subsequently extending information to complex in vivo scenarios. This process, while very successful, has its own risks, as the translation of in vitro findings to in vivo context is often difficult. Therefore, an early complementary in vivo approach that identifies physiologically relevant routes of virus transmission, spread, relevant target tissues, and cell types can greatly enhance the translatability of in vitro studies. In this review, we delineate a top-down approach using noninvasive whole-body imaging as a guide for subsequent in-depth characterizations at multiple spatial and temporal resolutions.

2. THE NEED FOR WHOLE-BODY GUIDED IMAGING APPROACHES FOR STUDYING VIRUS INFECTIONS

The classical reductionist approach has been vastly successful in advancing our understanding of viruses and combating viral diseases, especially for viruses such as hepatitis C virus (HCV), which lacks appropriate small animal models. On the other hand, although we know the molecular basis for replication in target cells and have generated drugs effective against viruses such as HIV-1, the lack of good animal models results in a poor understanding of the nature of the viral reservoir from which the virus rebounds in vivo and remains a main barrier for a cure (9, 10). This aspect underscores the need to change our approach and answer basic questions

to tackle these challenging viruses through in vivo lenses. One reason for this in vitro to in vivo disparity is that the concepts developed in vitro often rely on either immortalized tissue culture cell lines or easily accessible blood cells from patients, which often do not reflect the biology of infected cells in tissues. In vivo, tissue-resident cells interact with other cell types and receive specific cues in the form of cell-cell contacts and cytokines, which shape cell differentiation, activation, and their functional responses. Consequently, physiologically relevant cell types exhibit expression profiles that are often distinct from cultured cells. Here, we propose a complementary research program driven by noninvasive imaging-based in vivo studies to direct in-depth in vitro studies of physiologically relevant aspects of virus replication to increase the pace of achieving therapeutically viable advances. We outline this vision, discuss available imaging modalities and necessary viral tools, discuss the need for suitable animal models, and then describe emerging insights into in vivo mechanisms of virus dissemination and pathogenesis.

Classical studies of in vivo virus infection and pathogenesis have relied on sampling tissues predicted to be infected at investigator-defined time points. Such approaches remain limited by spatial and temporal predictions, thus presenting opportunities to miss important events and introduce investigator bias. In contrast, methods that provide temporal and whole-body perspectives of virus infection permit unbiased insights over time. Noninvasive imaging techniques enable expansive views of the host body, with capabilities to monitor ongoing virus infection and ensuing immune responses in parallel. With the right tools in hand, noninvasive imaging can allow observation of the entire gamut of infection events, including virus entry into hosts, virus flow through tissues, virus replication in target tissues, dissemination, and even transmission. Importantly, noninvasive imaging can guide subsequent in-depth single-cell studies as well as ultrastructural imaging analyses to shed light on cellular mechanisms that govern viral spread within the native tissue architecture. Medical diagnostic imaging in combination with tissue biopsies even makes these technologies available in patients (11). In animal models, noninvasive bioluminescence imaging (NBLI) can identify exact time points at which infections reach critical stages in specific tissues, many of which can then be surgically exposed for subsequent multiphoton intravital microscopy (MP-IVM) to provide dynamic and single-cell-resolved information. Additionally, bioluminescence imaging (BLI) after necropsy can identify tissue samples and areas of specific disease states that can be examined using large-volume three-dimensional (3D) light sheet fluorescence imaging with sub-single-cell resolution, as well as electron tomography (ET) for ultrastructural characterization (12). This is particularly important for long and extended organs such as the gastrointestinal tract, where identification of infected areas can tremendously simplify downstream higher-resolution analyses. As such, noninvasive imaging can direct a pipeline of analyses from the macroscopic to ultrastructural levels, spanning 8–9 orders of increasing resolution (**Figure 1**). In addition to reducing subject numbers through longitudinal studies, the prospect of imaging the same subject over a course of disease progression adds a correlative dimension to interpretations of disease outcome. The unbiased nature of noninvasive imaging allows visualization of animal to animal variation during infection spread. Such readouts are also bound to reveal unexpected facets of virus infection and dissemination, such as natural barriers and kinetic bottlenecks that can be exploited therapeutically. Having identified the physiologically relevant tissues/areas and specific cell types that play roles in host-pathogen interactions in vivo, researchers using subsequent mechanistic in vitro studies and high-throughput screening in identified cell types have increased probabilities for human applications. Thus, a noninvasive, imaging-assisted approach can help us counter the growing threat of emerging viruses as well as chronic infections that are difficult to cure.



(*Caption appears on following page*)

Figure 1 (*Figure appears on preceding page*)

Illustration of a technological pipeline for a whole-body imaging-driven multiscale investigative approach to study virus infection. MLV is used as an example. (*Top right*) Longitudinal NBLI reveals infection of NLuc-expressing MLV at the pLN after subcutaneous challenge and informs subsequent focused studies at the pLN using various imaging modalities (*middle column*) at increasing scales of resolution (*left column*). (*Right column*) Intravital microscopy shows the arrival of fluorescent Gag-GFP-labeled MLV at the pLN (*blue* collagen capsule) and reveals *trans*-infectious synapses between MLV-laden SCS macrophages (*green*) and B-1 cells (*red*). Light sheet fluorescence microscopy shows the entire LN with MLV (*green*) captured at the pLN. Confocal immunohistochemistry of pLN sections shows MLV Gag-GFP (*green*) accumulating below collagen capsule (*blue*) next to B cell-rich follicles (*red*). Electron tomography reveals cell-cell contacts between B cells and MLV-laden SCS macrophages in LNs. Abbreviations: GFP, green fluorescent protein; LN, lymph node; MLV, murine leukemia virus; NBLI, noninvasive bioluminescence imaging; NLuc, Nanoluciferase; pLN, popliteal lymph node; SCS, subcapsular sinus. The images for intravital, confocal, and electron microscopy are reproduced from Reference 72 with permission from AAAS.

3. IMAGING TECHNIQUES

3.1. Noninvasive Imaging

Recent years have seen a significant improvement in noninvasive biomedical imaging technologies originally developed for patients, such as magnetic resonance imaging (MRI), positron-emission tomography (PET), single-photon emission computerized tomography (SPECT), and computed tomography (CT), which have subsequently been applied for small animal imaging (13) (see **Table 1** for details). BLI and fluorescence imaging, developed for cellular imaging, have been applied to small animal studies (**Table 1**). For patients and nonhuman primates (NHPs), PET imaging, which monitors the distribution of introduced radioactive tracers, is the method of choice (14). PET and SPECT have been used to identify sites of virus binding, fusion, and infection through the use of radiolabeled viral molecules as probes (15–17). Virus and infected-cell localization can be studied using radiolabeled viruses as well as through administration of radiotracer-conjugated antibodies to viral proteins (immuno-PET) (18). The tracers used for PET scans emit positrons, which react with electrons in the body to produce two photons that are detected by the PET scanner to generate images (19). In contrast, NBLI is currently a dominant method for small animal imaging. NBLI can employ various luciferases such as firefly (FLuc), Renilla (RLuc), Gaussia (GLuc), and Nanoluciferase (NLuc), each with unique emission characteristics and specific substrates, enabling multiparameter imaging studies (20). NBLI relies on detection of photons emitted from the oxygen-mediated conversion of injected substrates and does not require an external light source, unlike fluorescence. As substrate diffuses throughout the body, photons are generated only at the site of luciferase activity. A high signal-to-noise ratio provides superior detection sensitivity of as few as 30–50 cells under true infection conditions. The wide availability of bacterial/viral luciferase expression systems and luciferase-expressing transgenic mice under the control of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), interferon (IFN) or IFN-induced genes for visualizing resulting host responses, in conjunction with expansion of available instruments, has resulted in the wide application of NBLI to study host-pathogen interactions. Detection sensitivities of bioluminescent signals, especially from deeper tissues, are strongly hampered by several factors, such as light scattering and absorption properties of different tissue types (21). Light absorption by hemoglobin and melanin occurs strongly for light in the visible spectrum but is reduced at wavelengths longer than 600 nm, making red light–emitting luciferases the preferred choice for deep tissue imaging (22).

Table 1 Examples of imaging modalities used for studies of viral infection in animal models

	Imaging modalities for studies of viral infection					
	Imaging modality	**Functional principle**	**Resolution**	**Sensitivity**	**Depth in vivo**	**Example applications to infection**
Nuclear	positron-emission tomography	γ-ray-emitting radioisotopes	1–2 mm (110, 111)	10^{-11}–10^{-12} mol/L (110, 111), few hundred to 10^5 cells (112, 113)	unlimited (110, 111)	sites of inflammation (114), sites of virus binding, fusion (16), infection (114), release (radiolabeled viral molecules as probes) (16, 115)
	single-photon emission computerized tomography	low-energy γ-ray emission from radioisotope decay	1–2 mm (110, 111)	10^{-10}–10^{-11} mol/L (110, 111)	unlimited (110, 111)	virus-induced brain encephalopathy (116), sites of inflammation, infection-induced apoptosis (115), sites of virus binding, fusion, infection, release (radiolabeled viral molecules as probes) (15)
	computed tomography	2D X-ray scans complexed with contrast agents and digital geometry processing for 3D image reconstruction	50 μm (110, 111)	not well characterized	unlimited (110, 111)	imaging of infection-induced apoptosis, infection-induced tissue changes (117)
Magnetic/radio	magnetic resonance imaging	nuclear magnetic resonance	10–100 μm (110, 111)	10^{-3}–10^{-5} mol/L (110, 111), 1–10^5 cells, depending on tracking method (118)	limited by magnet bore (110)	sites of inflammation, infection-induced tissue changes (115)
Sonic	ultrasound	>20,000 Hz sound wave pulse/echo transduction	50–500 μm (111)	excellent with use of microbubbles (111)	mm to cm (110, 111)	imaging of congenital infection (119, 120), infection-induced tissue changes (120)
Optical	noninvasive bioluminescence/ fluorescence imaging	bioluminescence emission via luciferases, fluorescent proteins	3–5 mm (110, 111)	10^{-15}–10^{-17} mol/L (110, 111), 3 cells (121)	1–2 cm (110, 111)	identification of infected organs infection dissemination (122), interferon-response induction (115, 123)
	multi-photon intravital microscopy	near-infrared excitation of fluorescent probes	29 μm (lateral), 97 μm (axial) (124)	10^{-9}–10^{-12} mol/L (110, 111), single cells	1 mm (125)	virus dissemination, spatiotemporal dynamics of infected cells (125)
	light sheet fluorescence microscopy	excitation of fluorescent probes	250–500 nm (126)	10^{-9}–10^{-12} mol/L (110, 111), single cells	N/A	3D identification of infected cells within context of tissue milieu, localization of incoming virus particles, identification of virus-capturing cells
	confocal laser scanning microscopy	excitation of fluorescent probes	180 nm (lateral), 500 nm (axial) (127)	10^{-9}–10^{-12} mol/L (110, 111), single cells	N/A	identification of infected cells within context of tissue milieu, localization of incoming virus particles, identification of virus-capturing cells

Functional principles, resolutions, and sensitivities of each imaging modality are listed for consideration before use.
Abbreviation: N/A, not applicable.

3.2. Intraoperative Imaging

MP-IVM is the method of choice for investigating dynamic behaviors of infected cells, as well as responding immune cells, in tissues of interest identified by noninvasive imaging. MP-IVM allows imaging of live cells for extended time periods within deep tissues but often requires surgical procedures (23). Two-photon IVM uses high-pulsed longwave laser light, which can penetrate tissues to excite traditional fluorophores in the visible range using near-simultaneous absorption of two long wavelength photons. High photon density allowing simultaneous absorption of two photons is achieved only near the focal plane, resulting in confocal imaging and limiting tissue damage.

3.3. Cellular and Subcellular Imaging

Confocal laser scanning microscopy and light sheet fluorescence microscopy (LSFM) are the standard imaging techniques for 2D and 3D histology of tissues, respectively. The increasingly applied LSFM uses one or several sheets of laser light that illuminate an entire focal plane within the tissue. However, tissue needs to be optically cleared prior to imaging with detergents or organic solvents to maximize penetration of the illuminating laser as well as minimize the scattering and absorption of emitted light (24). LSFM allows large portions of entire organs such as the brain, spleen, liver, and lymph nodes (LNs) to be imaged without physically sectioning them to obtain information on infected cells within their native 3D tissue architecture. Transmission electron microscopy and ET provide the resolution and ultrastructural detail required to visualize and characterize viruses, their replication stages, and associated host structures (12).

3.4. RNAscope and DNAscope In Situ Hybridization

Next-generation in situ hybridization techniques use two double-Z target probes that bind in tandem to their respective complementary RNA (RNAscope) or DNA (DNAscope) sequences. The prerequisite tandem binding of probes to target nucleic acids to initiate a signal amplification cascade via sequential hybridization steps ensures very low to zero background. As a result, a single RNA or single-copy DNA molecule can be visualized in individual cells and tissue sections (25, 26). Importantly, results can be obtained in just 1 day (<8 h) compared to radioactive probe-dependent in situ hybridization, which takes weeks. This technology is rapidly transforming our ability to visualize rare virus-infected reservoirs as well as host immune responses (27).

4. ANIMAL MODELS FOR IN VIVO IMAGING

The choice of in vivo imaging modality is largely determined by the animal model in use. Large animals such as primates require PET, SPECT, and MRI, all capable of deep tissue imaging, while BLI and micro-CT play important roles in small animal models. It is, however, important to note that PET, SPECT, and MRI were developed for clinical diagnoses in human patients (28). The noninvasive nature of these techniques is predicted to allow their application in clinics. Indeed, it appears feasible that whole-body imaging can inform subsequent biopsies and permit multiscale imaging in patients sooner than thought.

Rigorous mechanistic and preclinical translational studies require prudent choice of animal models. The strengths and limitations of each animal model in their ability to recapitulate human disease should be carefully assessed for the research question under study. Small animal models such as mice are tractable and cost-effective, as well as inbred, allowing researchers to generate reproducible data. They are particularly well-suited for implementation of in vivo imaging

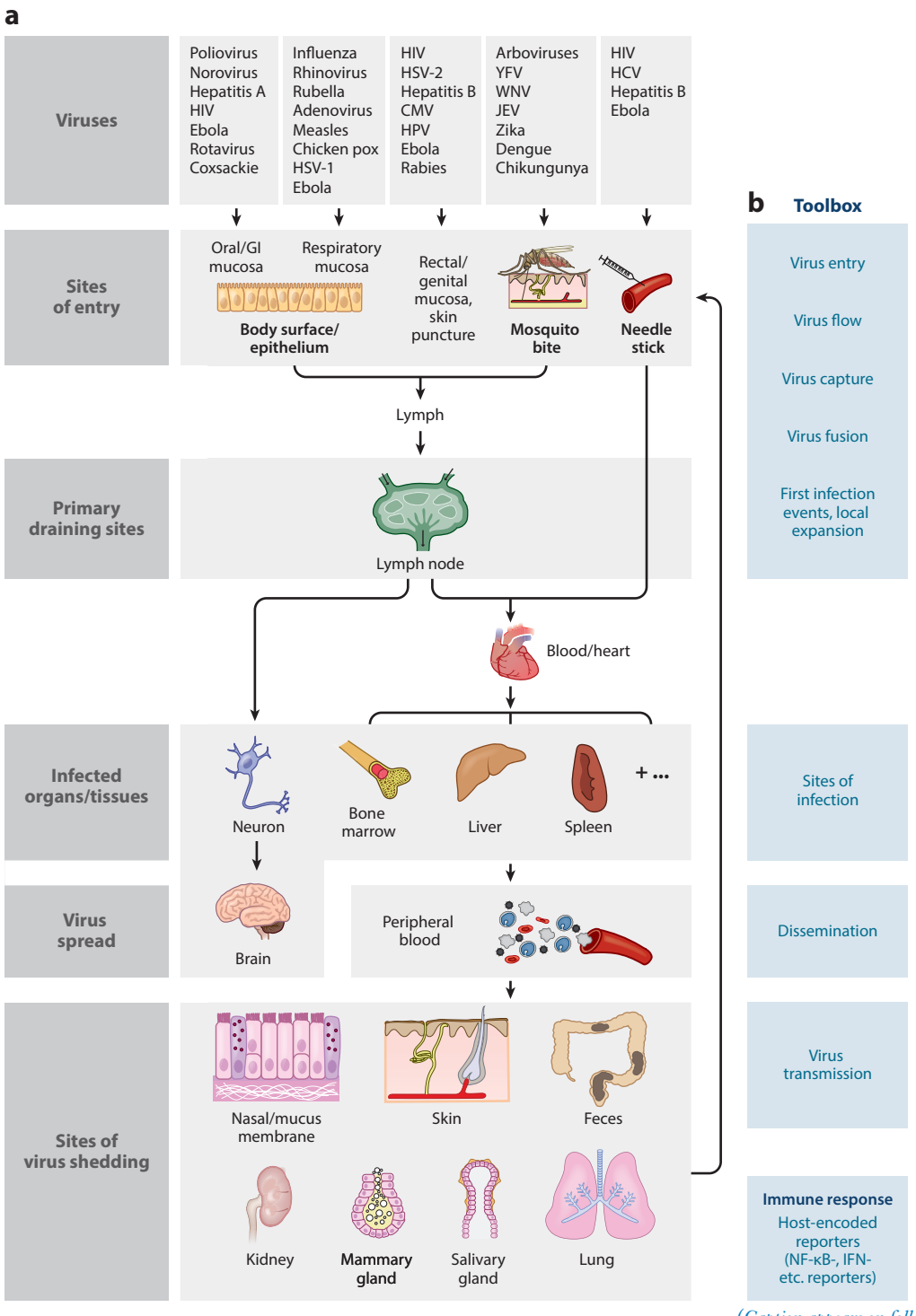
approaches. They remain workhorses for modeling the immune system and provide genetic tools in the forms of transgenic and knockout mouse models. However, many clinically relevant viruses such as HIV, herpes, HCV, and EBV do not infect mice (29, 30). In such cases, NHPs have become essential models for simulating infection caused by HIV and zoonotic viruses, such as flaviviruses and togaviruses, and for testing efficacies of emerging EBOV vaccines (29, 31). However, NHP models also have several disadvantages compared to mice, as they are large, expensive, and less tractable, in addition to raising ethical considerations. In this regard, next-generation mice with human immune systems—humanized mice (hu-mice)—have become important tools to bridge this gap. For generation of hu-mice, immunodeficient strains are engrafted with human immune cells, including stem cells, to regenerate a wide range of lymphocyte and monocyte populations in mice (32, 33). Recent advances to improve humanization through the use of immunodeficient mice transgenic for human cytokines and growth factors have significantly increased the translational potential of murine small animal models (34, 35).

5. TOOLBOX FOR VISUALIZING VIRUS-HOST INTERACTIONS

Viral infection and dissemination occur through several sequential steps (**Figure 2**) traversing an array of tissues and organs to reach the final shedding point for transmission. The host mounts immune responses at these sites to control the infection. To study these aspects through imaging-guided approaches, it is necessary for researchers to generate tools that allow monitoring each step of infection. In the following sections, we discuss tools that will have to be developed to elucidate different aspects of virus infection.

5.1. Virus Entry, Fusion, and Capture

Viruses must overcome physical barriers maintained by organisms and their tissues. Although most viruses enter organisms through mucosal surfaces, they can also enter through direct injection into the bloodstream or subcutaneously. Following entry, viruses can be captured locally or disseminate through the lymphatic vascular system, which drains into LNs (36) (**Figure 2**). In order to examine spatiotemporal progression of viral infection, it is necessary for researchers to employ systems that allow monitoring of the viral life cycle in the context of the whole organism as well as at the tissue level. Visualizing each step requires distinct tools and various levels of complexity. Noninvasive studies to monitor these early processes require (*a*) the labeling of virus particles for PET, SPECT, or BLI to allow the observation of viral flow and particle capture at specific tissues as well as cells; (*b*) reporters that specifically measure fusion into cells; and (*c*) expression of reporters in infected cells to monitor first infection events. Labeling of virus particles can be achieved through incorporating luciferases, fluorescent proteins, or radiotracers for use in appropriate imaging techniques. Using fluorescently tagged virus particles, researchers can identify virus-capturing cells with subsequent higher-resolution studies using intraoperative, cellular, and subcellular imaging. Reporters to measure fusion into cells may involve the use of Cre recombinase (37) or split reporter systems, such as split NLuc or green fluorescence protein (GFP), in which specific reporter subunits are incorporated into viruses and cells (38). Finally, infection by reporter-encoding viruses can be monitored at early time points for observation of initial infection events. As with other stages of infection, these reporters can be applied in several imaging techniques to gain insight into these early infection events at multiple resolutions, allowing spatiotemporal and molecular characterization of early infection determinants. Finally, studies of first infected cells can be carried out with single-round reporter viruses, which can be easily generated for different viruses.



(*Caption appears on following page*)

Figure 2 (*Figure appears on preceding page*)

General illustration showing spatiotemporal progression of virus infection in a mammalian host and the toolbox required to monitor virus-host interactions. (*a*) This illustration shows the various stages of virus infection from initial replication to systemic dissemination and its transit through various organs in the host after entering through indicated routes until its transmission from various shedding sites. (*b*) Shown are the indicated phases of virus infection and ensuing host immune responses that need to be monitored using the toolbox of viral as well as host-encoded reporters for in vivo imaging-guided studies of infection. Abbreviations: CMV, cytomegalovirus; GI, gastrointestinal; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HPV, human papillomavirus; HSV, herpes simplex virus; IFN, interferon; JEV, Japanese encephalitis virus; WNV, West Nile virus; YFV, yellow fever virus.

5.2. Virus Spread

Viruses can spread as cell-free virions (picornaviruses, togaviruses) or in cell-associated forms in vivo [mumps, measles, rubella, HIV, influenza virus, cytomegalovirus (CMV), Epstein-Barr virus (EBV), Colorado tick fever virus]. Transmission modes are also predetermined by exit mechanisms from infected cells (39). Lytic nonenveloped viruses such as picorna-, adeno-, and reoviruses, as well as bacteriophages, can lyse infected cells, most clearly initiating transmission in their cell-free forms. Nonlytic release of nonenveloped viruses has also been observed, particularly when cells contact each other (40). Enveloped viruses, which bud from host cell plasma membranes, can in principle use both cell-free and cell-mediated contact mechanisms to spread. Viruses can exploit existing cell-cell contacts or induce new ones to rapidly spread between cells. Various cellular structures and processes co-opted by viruses to spread include filopodial bridges, cytonemes, nanotubes, virological synapses, infectious synapses (ISs) that mediate *trans*-infection, uropods, extracellular matrix biofilms, phagocytosis, and cell-cell fusion; these have been described in great detail in excellent reviews (39, 41–43). Monitoring virus spread requires replication-competent reporters or the application of label-conjugated antibodies, which is possible for both PET and BLI (38, 44) (**Figure 2**). An elegant way to identify sequentially infected cells is to use floxed color-switching viruses, which permit fate mapping (45). Following noninvasive studies, higher-resolution approaches can help precisely define characteristics of viral spread. Specific modes of spread used by viruses within tissues can be studied at higher resolutions through the observation of fluorescently labeled viral components, such as capsids, with MP-IVM.

5.3. Virus Transmission

Following establishment of infection and systemic spread, viruses shed from specific sites to infect new hosts. Viruses can shed through blood, stool, mucus, semen, and/or milk. Virus transmission to new hosts can occur days or weeks following initial infection of the original host. Thus, long-term transmission studies require replication-competent reporter viruses that can stably retain reporters from the initial point of infection to the time of transmission (**Figure 2**). DNA virus genomes are generally more stable and insertion-tolerant than those of RNA viruses, engineering of which can be technically challenging. Small reporters such as NLuc can be helpful in maintaining viral replication competence and genomic reporter stability. To overcome challenges associated with long-term reporter virus stability, researchers may administer viruses to known sites of shedding to study transmission to new hosts. This approach can prevent reporter loss that may otherwise occur during the multiple rounds of virus replication required for accumulation of virus to the site(s) of transmission.

5.4. Host Immune Response

A large number of transgenic mice express luciferases (usually FLuc) under promoters that permit the detection of host signaling responses to incoming viral infections (**Figure 2**). The inflammatory and IFN signaling pathways are most useful for the study of host-pathogen interactions.

The NF-κB reporter mouse is based on the HIV-1 LTR and permits the visualization of inflammatory signaling in response to infection and tissue damage (46). Luciferase expressed under the control of the IFN-β promoter allows visualization of type-I IFN signaling (46). Since these transgenic mice are based on FLuc, they can be elegantly combined with virus-expressed NLuc, GLuc, or RLuc to simultaneously visualize the spreading infection and host immune response. Using Cre-lox technology, it is possible to express IFN-induced luciferase reporters in cell types of choice to visualize the activation and recruitment of specific leukocytes (47). Alternatively, immune cell types such as dendritic cells, macrophages, and B and T cells can be purified from mice ubiquitously expressing FLuc luciferase under the β-actin promoter. Adoptive transfer of purified cell types into virus-challenged mice can allow observation of the recruitment of luciferase-expressing lymphocytes or monocytes to the infected tissue, organ, or draining sites (46).

6. IN VIVO IMAGING-BASED STUDIES OF VIRUS INFECTIONS

In the following sections, using different viruses as examples, we discuss studies that elegantly implement several imaging-based tools for studying various aspects of viral infection as well as the host immune response. They illustrate how imaging-based approaches have clarified our models, revealed crucial new mechanistic insights, or identified new aspects of host biology.

6.1. Simian Immunodeficiency Virus

Simian immunodeficiency virus (SIV) and SIV carrying HIV-1 Env infection of rhesus macaques are important in vivo model systems to understand the highly related HIV-1. The Villinger laboratory (44) has carried out noninvasive imaging to study SIV infection using a novel multimodal immuno-PET/CT-based imaging technique. They used radioactive ^{65}Cu-labeled antibodies to viral antigens (7D3, SIV envelope; non-neutralizing) or immune cells (CD4) as probes. This technique allowed whole-body visualization of wild-type SIV-infected sites and T cell reconstitution in viremic, antiretroviral drug–treated as well as elite controller monkeys (44, 48). The immuno-PET/CT technique was subsequently used to gain mechanistic insights for sustained SIV control in rhesus macaques after a combination of antiretroviral and α4β7 antibody therapy (49). The integrin α4β7 regulates trafficking of lymphocytes into and between gut inductive and effector sites. The gut-resident α4β7$^+$ CD4$^+$ CCR5$^+$ T cells are highly permissive to SIV infection and are characteristically depleted during the early phase of infection. Immuno-PET/CT revealed that the α4β7 mAb reduced viral antigen loads not only in the colon, as expected, but also in several other tissues, including the lungs, spleen, and axillary and inguinal LNs. Surprisingly, imaging also showed that the α4β7 mAb treatment did not prevent the characteristic depletion of CD4$^+$ T cells in gut. Rather, the treatment facilitated the restoration of CD4$^+$ T cells in gut tissues at later stages of infection, potentially reviving the gut immune system for better prognosis (49, 50). Thus, it is easy to see why investment in improving the noninvasive immuno-PET/CT approach will pay rich dividends as it can be rapidly translated for use in humans, particularly with the available array of antibodies against the HIV-1 envelope to uncover unexpected facets of ongoing infection and implement course correction strategies. Such a strategy is already in use in the cancer field, where imaging modalities such as PET, SPECT, MRI, and ultrasonography form the cornerstone in early cancer detection and treatment (51).

The Hope laboratory (52) applied various imaging techniques to monitor early transmission events during vaginal transmission in rhesus macaque. Single-round non-replicating SIV expressing FLuc allowed a macroscopic survey of the infected regions throughout the female reproductive tract (FRT). The global distribution of infection sites showed that virions disseminated rapidly

and gained access to target cells throughout the FRT from the labia to the distant ovaries, as well as draining LNs. BLI-aided detection of infected regions guided the investigators to the earliest infected cells, which were revealed as Th17 $CD4^+$ T cells, in agreement with previous studies (53–55). Th17 $CD4^+$ T cells play a critical role in maintaining gut integrity and regulating mucosal immune responses (56). Therefore, these data clarify the preferential early loss of Th17 $CD4^+$ T cells from the gut, leading to microbial translocation and chronic immune activation, which exacerbate SIV pathogenesis. Another set of studies carried out by the Haase laboratory (57–59) used a combination of immunohistochemistry and in situ hybridization for sensitive detection of infected cells and viral RNA. They elucidated the role of the chemokine immune response in establishment of SIV infection. Their study showed that epithelial cells at the ecto- and endocervix junction (also called the transition zone), when exposed to SIV, initiate a chemokine relay system by secreting CCL3, CCL20, and CXCL8. These chemokines attract plasmacytoid dendritic cells (pDCs) and macrophages to accumulate beneath the epithelium. The innate sensing of viruses in macrophages and pDCs stimulates production of CCL3, CCL5, CXCL8, and CXCL10, which attract beta-chemokine-producing $CD4^+$ T cells along with more macrophages and pDCs. This recruitment generates a positive feed-forward loop that sustains further increases in $CD4^+$ T cell targets, resulting in local expansion and establishment of self-propagating infection. Their imaging analyses further revealed that in SIVmac239Δnef-vaccinated macaques, the inflammatory chemokine relay is inhibited at the source in epithelial cells, and that infection does not progress beyond the initial foci, preventing mucosal transmission. These data revealed a window of opportunity between early infection and local expansion that was exploited through use of glycerol monolaurate, an anti-inflammatory agent and inhibitor of T cell signaling, to prevent SIV transmission (58).

6.2. Human Immunodeficiency Virus-1

HIV-1 can exploit cell-cell contacts that cells use to communicate with each other or induce them de novo in the form of virological synapses for efficient spread. In vitro studies have shown that cell-to-cell transmission of HIV-1 is more efficient than free virus infection of cells (39, 60, 61). In addition, lymphotropic retroviruses such as HIV-1 can use the trafficking of infected T cells or migratory DCs to spread to distant tissues. Imaging-based studies by the Chen (62) and Mempel (63, 64) laboratories have therefore centered around elucidating the in vivo mode of HIV-1 spread at the popliteal LN (pLN) and spleen of HIV-1-infected hu-mice, respectively, using MP-IVM. Unexpectedly, their studies first revealed that CCR5-tropic HIV-1-infected T cells readily form small, multinucleated syncytia containing up to 5 nuclei (62, 64). Moreover, syncytia were motile and formed transient adhesive contacts that were dependent on viral envelope glycoprotein expression, suggesting that they form virological synapses (64). The ability of HIV-1-induced syncytia to spread virus by cell-cell contact was confirmed in vitro in 3D matrix gel cultures (65). HIV-1 infection was observed to spread as foci within the splenic lymphoid tissue of hu-mice (62). In addition, one hallmark of in vitro cell-to-cell spread, namely the simultaneous transfer of many viral particles through the cell-cell interface resulting in a high local multiplicity of infection, was also observed in the spleen (62, 66). Both findings suggested that HIV-1 spread was confined to regions around infected cells. The Bjorkman laboratory (12, 67) examined HIV-1 infection in the gut tissues and spleens of hu-mice by ET and LSFM. In addition to finding evidence for virological synapses, they found pools of immature and mature virions possibly released in semisynchronous waves accumulating next to HIV-1-infected cells. These data implied that there could be a contribution of cell-free HIV-1 to spread even within tightly packed tissues. Ultimately, true mechanistic insights can be revealed only by carefully monitoring the dynamics of cell-cell contacts and possibly the flux of cell-free virus particles coupled with infection outcomes.

FTY720 treatment, which blocks lymphocyte egress, reduced systemic dissemination of HIV-1, indicating that migration of infected cells was required for virus spread to other organs (64). Additional support for a major role of lymphocyte migration in HIV-1 dissemination came from studies in the Luster laboratory (68). Blocking chemoattractant receptors and lymphocyte egress through Pertussis toxin and FTY720 treatment, respectively, prevented both viremia and infection of the gut following an intravaginal HIV-1 challenge in hu-mice (68). The HIV-1 accessory protein Nef disrupts host cell signaling, downregulates cell surface proteins, and perturbs the actin cytoskeleton (69, 70). In vitro, these activities impact cell polarity, duration, and stability of cell-cell contacts, both within virological synapses and of immunological synapses, in addition to curtailing migration of infected $CD4^+$ T cells. The Fackler laboratory (71) examined the effect of HIV-1 Nef expression in primary mouse $CD4^+$ T cells using MP-IVM. They found that Nef expression impaired actin remodeling and led to their accumulation inside high endothelial venules (HEVs) due to impaired extravasation. As a result, T cell homing into LNs was significantly blocked. The Mempel laboratory (63) extended these observations in hu-mice to find that disrupting Nef function restored T cell motility and accelerated systemic viral dissemination after intravaginal HIV-1 challenge. However, the actin-remodeling function was required to counter immune pressure imposed by $CD8^+$ T cells and enhance HIV-1 persistence during later stages of infection. The manipulation of lymphocyte trafficking and homing by HIV-1 accessory proteins like Nef is important, as it can not only affect ensuing immune responses but also shape formation of distinct long-term HIV-1 reservoir populations in lymphoid tissues compared to blood.

Finally, the detailed characterization of the latent HIV-1 reservoir requires the identification of cells that carry integrated HIV-1 DNA as well as transcribed RNA. This has been accomplished using highly sensitive in situ hybridization technologies such as DNAscope and RNAscope (27). The addition of these imaging approaches will be vital to understand the nature of the HIV-1 reservoir that remains the last barrier to an HIV-1 cure.

6.3. Murine Leukemia Virus

The murine retrovirus murine leukemia virus (MLV) infects mice as its natural host. As a result, mouse genetics can be exploited to elucidate virus dissemination, establishment, and ensuing host immune responses in great detail. Sewald et al. (72, 73) used MP-IVM, immunofluorescence, and ET to monitor the FrMLV (ecotropic Friend MLV strain) infection in vivo and elucidated several interesting aspects of host-retrovirus interactions. They sought to understand how free retroviruses disseminate when they gain access to the host circulatory system via the lymph or blood. They used subcutaneous infection (intrafootpad) of mice to model dissemination of lymph-borne retroviruses using colored MLV and HIV-1 (capsid fused to GFP). MP-IVM revealed that retroviruses can access lymph flow immediately and arrive at the draining pLN through the afferent lymphatic vessel. They observed dynamic accumulation of cell-free MLV and HIV-1 at the floor of the subcapsular sinus (SCS) (**Figure 3**). This was due to specific interaction of sialic acid gangliosides present on retroviral membranes with the I-type lectin Siglec-1/CD169 expressed on sentinel macrophages (**Figure 3**). Retroviruses such as MLV and HIV-1 likely evolved to incorporate sialic acid ligands for targeting the filtering function of CD169 (74, 75). In contrast, HIV-2 fails to efficiently use this pathway, as its membranes have diminished levels of sialic acid ligands, reflecting its evolutionary choice (76). Retrovirus binding by SCS macrophages allowed virus dissemination to transition from a cell-free to the more efficient cell-associated mode. MLV-laden SCS macrophages promoted infection of captured viruses by delivering them to susceptible B lymphocytes, also known as *trans*-infection, through formation of viral Env glycoprotein-dependent infectious synapses s (**Figure 3**). Such a process for HIV-1 handover was shown ex vivo from DCs to $CD4^+$ T cells by the Hope (77) and Cunningham (78) laboratories. However, under in

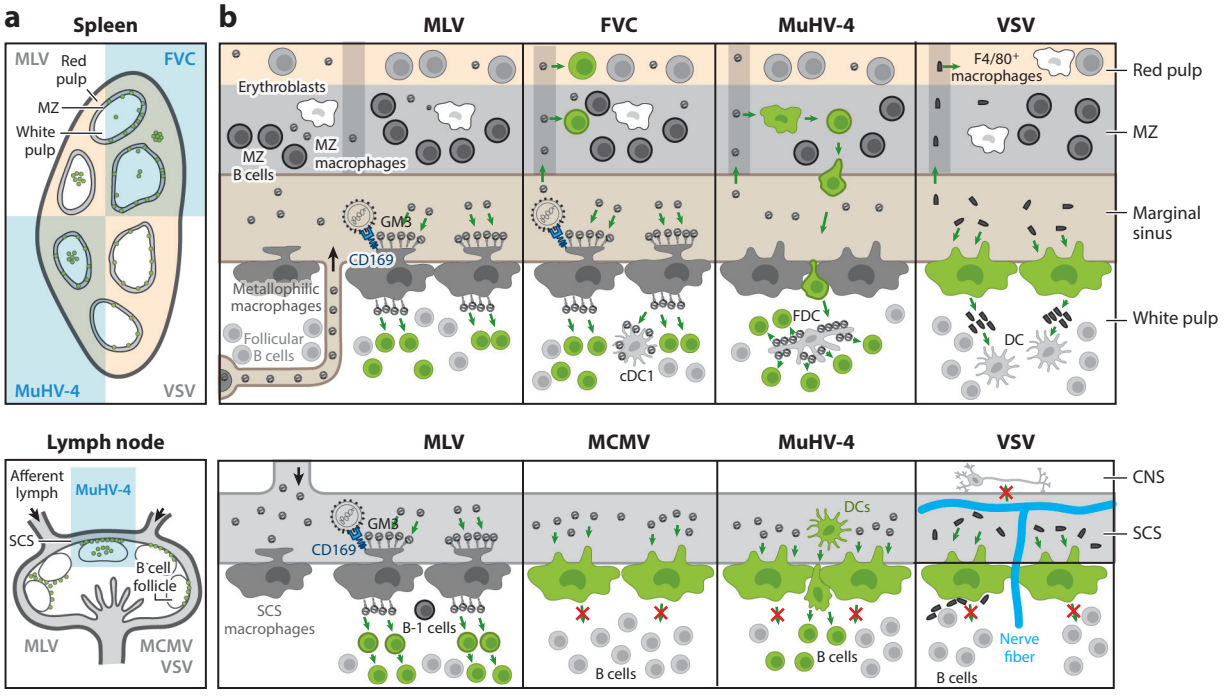


Figure 3

Illustration showing the fate of blood- and lymph-borne viruses after capture by CD169-expressing MMMs in the splenic MZs and SCS-lining macrophages of the popliteal LN. MMMs and SCS macrophages play an infection-promoting role by capturing MLV particles from blood and lymph before *trans*-infecting permissive lymphocytes, such as B-1 cells. In the case of the pathogenic FVC, MMMs in the spleen limit virus dissemination to the red-pulp resident erythroblasts and orchestrate protective $CD8^+$ T cell responses by collaborating with cDC1. MuHV-4 infects splenic MZ macrophages, followed by MZ B cells. The infected MZ B cells migrate to the white pulp, where they transfer virus to FDCs, which spread the infection to interacting B cells. In the LN, SCS macrophages restrict MuHV-4 spread by sequestering viruses from lymph. However, migratory DCs carry MuHV-4 from the upper respiratory tract to the LN and promote infection by spreading virus to B cells. MCMV infects SCS macrophages in the LN, which do not support robust virus infection, thus restricting viral dissemination to potential targets. Blood-borne VSV infects MMMs and red-pulp resident $F4/80^+$ macrophages in the spleen. Unlike $F4/80^+$ macrophages, MMMs are productively infected by VSV and serve as vessels for antigen and IFN production. The DC activation that follows helps in eliciting protective humoral and cell-mediated responses against VSV. Lymph-borne VSV infects SCS macrophages, which produce IFN to protect peripheral nerves from lethal VSV infection. Individual viruses are demarcated in the overview (*left*) using blue and white quadrants. Black arrows denote blood or lymph flow, and green arrows show the flow of viral particles. Virus-infected cells are shown in green, and red crosses depict restricted viral spread. Abbreviations: cDC1, conventional dendritic cells 1; CNS, central nervous system; DC, dendritic cell; FDC, follicular dendritic cell; FVC, Friend virus complex; GM3, monosialodihexosylganglioside; IFN, interferon; LN, lymph node; MCMV, murine cytomegalovirus; MLV, murine leukemia virus; MMM, marginal zone metallophilic macrophage; MuHV-4, murid herpesvirus 4; MZ, marginal zone; SCS, subcapsular sinus; VSV, vesicular stomatitis virus.

vivo conditions, $CD169^+$ macrophages, and not DCs, engaged in *trans*-infection. Furthermore, the interrogating target lymphocyte was identified as innate-like B-1a cells that then formed Env-dependent virological synapses with susceptible cells for cell-to-cell transmission of viruses. Parallel ET of tissue sections revealed that uropods of MLV-infected cells formed virological synapses, recalling earlier in vitro work (79, 80). Importantly, labeling of virus particles allowed the monitoring of virus transfer via both the infectious and virological synapses. These data thus provided in vivo evidence that retroviruses use *trans*-infection and virological synapses to efficiently spread in real time during active infection. In a follow-up study, the Mothes laboratory (81)

examined the impact of CD169-mediated capture at the pLN on retrovirus dissemination and pathogenesis by comparing nonpathogenic FrMLV to the pathogenic splenomegaly-inducing MLV strain, Friend virus complex (FVC). In addition to lymphocytes, FVC can infect erythroblasts, where it causes uncontrolled proliferation and cancer. Both MLV strains were able to exploit CD169 to efficiently *trans*-infect permissive lymphocytes. However, CD169 played a protective role against the pathogenic virus by preventing systemic dissemination of cell-free virus both from the pLN and within the spleen. Immunostaining revealed that CD169 sequestered incoming viruses at the splenic marginal zones (MZs) and thereby protected the erythroblasts residing in the red pulp from fatal FVC infection (81) (**Figure 3**). Once the infection was established, FTY720 treatment revealed that FVC, like HIV-1, exploited the migratory behavior of infected lymphocytes to disseminate within the host. Together, these data indicate that retroviruses are adept at exploiting both cell-free and cell-dependent modes of transmission to facilitate their spread within the host. This study also highlighted how seemingly detrimental infection-promoting roles of host factors such as CD169 can be surmounted by its immune protective function upon encountering pathogenic viruses.

6.4. Vesicular Stomatitis Virus

CD169-expressing sentinel macrophages at the SCS of LNs and MZs of the spleen are strategically positioned at the fluid-tissue interface to filter out blood/lymph-borne pathogens from circulation and present antigens (82–87). Many of the seminal discoveries on dissemination-limiting and immune orchestration properties of SCS macrophages were made in von Andrian's laboratory (84) in collaboration with Sean Whelan (82, 86), through MP-IVM via use of fluorescently labeled ultraviolet (UV)-inactivated and replication-competent GFP-expressing vesicular stomatitis virus (VSV). $CD169^+$ SCS and MZ metallophilic macrophages (MMMs) in the splenic MZs capture lymph/blood-borne VSV (82, 88). Enforced VSV replication in these sentinel macrophages is critical for host survival. Before VSV replication kills these macrophages, they secrete large quantities of IFN as well as inflammatory cytokines that establish a local antiviral state. At the pLN, IFN produced by SCS macrophages prevents neurotropic VSV from invading the peripheral nerves within LNs and using it as a conduit to invade the CNS, thus protecting the host from paralysis and death (86). At the spleen, the paradoxical amplification of a potentially lethal virus in the MMM jump-starts a protective IFN response, increases viral antigen availability, and promotes adaptive immunity through DC activation (88).

6.5. Herpesviruses

The variety of applicable reporter systems and relevant in vivo herpesvirus infection models make this a fertile field for multiscale imaging approaches. Below we describe how imaging studies of three important herpesvirus models have unveiled numerous invaluable insights into both viral and host-driven infection mechanisms.

6.5.1. Rhadinoviruses. EBV and Kaposi's sarcoma-associated herpesvirus (KSHV) are two oncogenic human herpesviruses. EBV is a ubiquitous virus associated with multiple cancers, while KSHV is the causative agent of acquired immunodeficiency syndrome–associated Kaposi's sarcoma (89). Although EBV and KSHV lack tractable in vivo infection models, valuable insights can be gained through studies of murine herpesviruses in their natural hosts, mice. Murine gammaherpesvirus-68 (MHV-68) and murid herpesvirus 4 (MuHV-4) are two strains of rhadinovirus that are tractable in vivo infection models. Using these viruses, the Stevenson laboratory

(90, 91) has implemented an in vivo imaging-guided approach to study virus spread through different routes of transmission. These studies identified the intranasal route as a new portal for virus entry that is 100 times more efficient than oral inoculation. Visualization of virus spread of luciferase-expressing MuHV-4 and fluid markers by BLI demonstrated that incoming MuHV-4 viruses specifically localized to the nasal septum and turbinates at first, from where they gained access to the neuroepithelium and trafficked along neuronal cilia and infected neurons or proximate glial cells (92). Through an elegant series of immunostained tissue sections, MuHV-4 was found to spread via normal immune communication routes (e.g., from the olfactory epithelium via DCs to the draining cervical LN), where the virus spread to B cells, ultimately traveling to the spleen (**Figure 3**). In the spleen, it first infected MZ macrophages and spread to adjacent MZ B cells. Infected MZ B cells then relocated to the white pulp, transferring virus to follicular DCs. Finally, follicular DCs *trans*-infected B cells (93) (**Figure 3**). Luciferase-expressing MHV-68 combined with NBLI also revealed insights into female-to-male sexual transmission in mice (94). Intranasal infections resulted in vaginal viral shedding, and sexual transmission to male mice initiated in penis epithelium and *corpus cavernosum*. MHV-68 then spread to the draining LN and spleen.

Finally, SCS macrophages restricted MuHV-4 spread, not unlike as observed for MLV, by sequestering circulating virus particles from the lymph (95). Using Mx-1-cre mice in which floxed viral genomes were specifically tagged in type-I IFN-responding cells, Tan et al. (47) found a cell type–dependent IFN-induction in response to MuHV-4 infection. While IFN induction in macrophages suppressed infection, MuHV-4 evaded IFN responses in B cells, allowing the establishment of chronically latent infection (47). Through the sampling and imaging of different stages of infection guided by noninvasive perspectives, these studies illustrate the potential of imaging-based approaches to reveal pathways of viral dissemination within and between organisms.

6.5.2. Murine cytomegalovirus. Murine cytomegalovirus (MCMV) infection of mice is studied as a model for human CMV infection. Work in the Stevenson laboratory (45, 96, 97) has delineated and characterized specific MCMV dissemination pathways. For example, MCMV was believed to spread from its entry point to perivascular sites and finally to the salivary glands, from which it sheds. However, through an integrated approach comprising BLI and fate mapping using floxed reporter viruses, the Stevenson laboratory (45) defined intermediate steps in the dissemination pathway. Following subcutaneous challenge, MCMV first arrived at the pLN, where it directly infected SCS macrophages, which functioned to slow dissemination (**Figure 3**). When challenged intranasally, MCMV used DCs as vehicles to spread to distant infection sites. Through sequential tracking of luciferase-expressing MCMV, followed by floxed color-switching MCMV, Stevenson and colleagues (96) demonstrated a sequential migration of virus-carrying DCs from the lung to the mediastinal LN and then to salivary glands. Surprisingly, DCs traversed through LN HEVs to enter the blood, thus revealing an entirely new pathway for virus spreading. This was unexpected, as virus-laden DCs were thought to die following LN entry. Thus, study of MCMV revealed that HEVs may commonly allow DCs to enter blood from the LN against the direction of blood flow. Such findings demonstrate how imaging studies of viral infection can generate important insights into host physiology.

Immune responses to MCMV were similarly delineated through a multiscale optical imaging approach. SCS macrophages were found to capture incoming MCMV, and became infected, but restricted viral spread in a type-I IFN-dependent manner (97). Without type-I IFN signaling, MCMV disseminated to the spleen. Infection of CD11c- and LysM- (SCS macrophage markers) cre mice with floxed color-switching MCMV confirmed that SCS macrophages produced type-I IFN and induced an antiviral state to limit further dissemination. In addition, natural killer cells also contributed by eliminating MCMV-infected fibroblastic reticular cells. Thus, these studies

elucidated a two-pronged mechanism in LNs restricting MCMV dissemination but also inducing immune responses.

6.5.3. Herpes simplex virus. Herpes simplex viruses (HSVs) persist in nerve cells and can cause severe disease. Two groups used imaging approaches to better understand early infection events for HSV-1, which typically infects neurons following olfaction or sexual transmission. The Stevenson laboratory (91) illustrated the spreading pathway from the olfactory epithelium to trigeminal ganglia neurons, from where it could re-emerge in facial skin, without causing significant neurological disease. Using color-switching reporter viruses, they demonstrated that HSV-1 first infected both $LysM^+$ and $CD11c^+$ sub-epithelial myeloid cells near the site of entry. Following spread of HSV-1 through lymph to LNs, IFN responses were found to restrict HSV-1 infection of SCS macrophages and other LN myeloid cells. In the absence of IFN responses, infection spread became systemic (98).

The Iwasaki laboratory (99) illustrated the chain of infection events from vaginal acquisition to gastrointestinal pathogenesis during HSV-1 infection. After intravaginal transmission, HSV-1 specifically spread from the vagina to the enteric nervous system (ENS) through vaginal nociceptors. Immunofluorescence revealed that the virus then traveled through the dorsal root ganglia and infected the autonomic ganglia surrounding smooth muscle of the ENS. HSV-1 replication in the ENS led to neutrophil-mediated intestinal neuronal damage in infected mice, resulting in virus-induced megacolon. Thus, mechanisms of peristalsis loss and toxic megacolon were clarified by directly observing virus infection events in affected tissues in situ. These imaging-based studies illustrated the intricate mechanisms of virus spread through specific routes, leading to mechanistic insight into pathogenic or protective outcomes.

6.6. Influenza Virus

The ever-present risk of an influenza pandemic necessitates a better understanding of influenza virus infection as well as treatment regimens including vaccination. Realizing the potential of noninvasive imaging studies, three groups generated replication-competent NLuc- and GLuc-tagged influenza A viruses (100–103). Through a series of longitudinal imaging experiments, they demonstrated the utility of reporter viruses to track virus replication and dissemination in mice after intranasal challenge. In addition, they were able to evaluate preclinical efficacies of candidate vaccines, immunotherapies, and antiviral drugs by monitoring spatiotemporal kinetics of virus clearance in groups of passively or actively immunized mice. NLuc-encoding influenza virus has also been used to visualize virus transmission dynamics in real time using ferrets (104). The ferret model is extensively used to assess the pandemic potential of emerging influenza viruses (105). These studies illustrated the potential of NBLI in monitoring interhost transmission through direct contact and respiratory droplets followed by dissemination within the infected animal. In addition to highlighting individual differences in infection between animals, imaging also identified transmitted infection events that would have otherwise been missed by traditional methods.

Pulverer et al. (106) used BLI to monitor the dynamics and extent of the IFN response during influenza virus infection using mice expressing luciferase under the IFN-inducible Mx2 promoter. This is important given that an exuberant host immune response contributes to a diseased state (3). They showed that a wild-type influenza virus elicited a higher and more rapid IFN response than an NS1-deleted nonpathogenic counterpart. Moreover, the IFN response was not limited to the lungs, the primary site of infection, but extended to other organs such as the liver, kidney, spleen, and salivary glands. Raising an effective universal vaccine is challenging due to the antigenic drift and shift of influenza virus. To gain insight into the chain of events required for

effective immune response by vaccine candidates, the Carroll and Gonzalez laboratories (107, 108) used MP-IVM. They visualized the dynamic interplay between immune cells at the pLN in response to UV-inactivated PR8 influenza virus. As was true for lymph-borne particulate antigens such as VSV, the flu antigen was also captured by SCS and medullary macrophages (82). But unlike VSV, they were rapidly internalized, which led to an MyD88 Toll-like receptor 7-dependent necroptic death of SCS and medullary macrophages. In addition, the innate sensing induced IFN-β that promoted autocrine synthesis of interleukin-1α and chemoattractant MCP-1 by the medullary macrophages (108). This chemokine relay instigated a relocation of $CD11b^+$ DCs from the LN paracortex to antigen collection sites in the medullary regions next to dying macrophages (109). Antigen capture by $CD11b^+$ DCs was, however, dependent on the C-type lectin SIGN-R1 (107). The flu antigen–carrying DCs were then observed to directionally migrate toward the follicular DC network within the B cell follicle for an antigen hand off. Thus, visualization of intricate immune cell interplay revealed the essential steps required for germinal center formation, B cell memory, and durable humoral immunity to viral pathogens such as influenza through vaccination.

7. SUMMARY AND FUTURE PERSPECTIVES

Technological advances in imaging across temporal and spatial scales, from noninvasive whole-body imaging and LSFM for entire organs to ET for ultrastructural analyses, are currently transforming our ability to monitor viral infections. They have improved our understanding of the pathways forged by viruses, often in ways unpredictable by traditional tissue-sampling methods. Several lessons have emerged from early in vivo imaging applications in animal virus research: (*a*) A whole-body view can identify novel tissue sites relevant for virus dissemination and pathogenesis; (*b*) the sequential events of virus dissemination and spread within the host are influenced by routes of transmission and host circulatory system; (*c*) in vivo, viruses exploit the advantages of cell-free and cell-to-cell transmission, as well as the trafficking of immune cells for virus dissemination; (*d*) implicated cell types are often only sustained in vivo within the tissue context where they receive cues from neighboring cells; and (*e*) viral infections in vivo are fundamentally shaped by the innate and adaptive immune responses that are orchestrated within the specific tissue architecture.

Multiple viruses can infect the same host. For successful infection, viruses must find their unique solution for adapting to host tissue architecture and a mounting immune response. This host-pathogen relationship results in recurring themes that are best illustrated for the spleen and peripheral LN. Studies of several viruses have now confirmed the flypaper-like activity of SCS macrophages and MMMs in filtering out pathogens, tumor-derived vesicles, and other exosomes from circulation. In addition, they are capable of activating all arms of the immune response—innate, humoral, and cell mediated. The exact mechanism by which dissemination and immune response are orchestrated by SCS macrophages and MMMs is different among viruses. For example, viruses such as VSV and MCMV replicate in SCS macrophages, whereas MLV and FVC are captured and handed off to permissive cells. SCS macrophages are killed by VSV but trap MCMV in a non-amplifying pathway. Thus, despite differences, in each case, the sentinel macrophages limit systemic dissemination of pathogens and protect the host by producing IFNs and inflammatory cytokines. These cytokines activate DCs, $CD8^+$ T cells, follicular memory T cells, and memory B cells that are either present nearby or rapidly recruited to the subcapsular niche following infection and inflammation. Sentinel macrophages or interacting permissive cells become vessels for local virus amplification and antigen production. This is critical for generation and maintenance of protective immune responses. The emerging common themes can be implemented to design better vaccines to promote lifelong immunity.

Going forward, we can expect an increase in the sensitivity of the various imaging techniques and development of better reporter viruses as well as a seamless integration of all techniques across scales. The ability to detect a single infected cell at the whole-body level will be particularly transformative. PET/CT imaging of human infections for identifying tissues and informing treatment strategies will likely move into the clinic at a faster pace than thought before. We envision a future where treatment regimens and responses for various viral infections are determined by visual outputs. Until then, in vivo imaging of viral dissemination and pathogenesis in animal models will accelerate our scientific understanding of viral infections. These studies will improve the relevance and translatability of our classic reductionist in vitro approaches by providing anatomical context and identifying physiologically relevant cell types.

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