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Adding new dimensions: Towards an integrative understanding of HIV-1 spread

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Abstract

In vitro studies in primary or immortalized cells continue to identify essential principles governing interactions of HIV-1 with isolated target cells. Until recently, significant technical barriers prevented this wealth of information to be efficiently translated to the more complex scenario of HIV-1 spread inside the host. Our current understanding of the impact of physiological parameters on HIV-1 spread thus remains very limited. In this Review, we discuss the recent development of imaging approaches for the visualization of HIV-1 spread and their adaptation to organotypic *ex vivo* as well as animal models. We will focus on new concepts such as mechanisms and *in vivo* relevance of cell-cell transmission for HIV-1 spread and the pathobiological principles of the HIV pathogenesis factor Nef that emerge from the application of such integrative approaches using complex cell systems.

The molecular biology of HIV-1, the causative agent of AIDS, has been intensively studied since its discovery 30 years ago. These efforts identified many essential aspects of the virus-host interactions that govern HIV-1 infection of individual cells. Such studies have for example identified the host receptor and co-receptors (CD4, CCR5 and CXCR4, respectively) and main target cells (CD4⁺ T cells and macrophages) of HIV-1¹. Yet, other key aspects of virus propagation, in particular of virus spread between cells and tissues inside the host, remain poorly characterized. Such knowledge is essential to fully understand HIV-1 pathogenesis and progression to AIDS.

The majority of studies on HIV-1 replication to date have been conducted in simple two-dimensional (2D) cell culture systems, often relying on monotypic cultures of immortalized cell lines at non-physiologically high cell densities. These systems ignore important parameters that govern viral replication and spread in the infected host such as tissue architecture and composition, biophysical parameters such as shear flow of body fluids, as

well as cell communication and motility. Transmission of the virus typically occurs at genital or rectal mucosal surfaces and later, the bulk of virus replication will occur within lymphoid tissues. HIV-1 thus needs to surmount substantial physical, mucosal and endothelial barriers to gain access to its target cells and, in the course of infection, encounters remarkably different physiological environments in the infected host (see FIG. 1 for an overview of vaginal HIV-1 transmission and spread inside the host). Mucosal dendritic cells (DCs) constitute one of the first cells that take up virus particles and store them without becoming productively infected². It is widely assumed that virus uptake triggers a migratory program in DCs that allows these cells to home to draining lymph nodes, where they efficiently transmit the virus to T cells, which results in substantial virus replication and dissemination². HIV-1 also infects macrophages, which have a considerably longer life span than T cells and could constitute an important viral reservoir¹. Once inside cells, HIV-1 can either establish productive or latent infection, depending on the type of target cell and their activation status¹. We are beginning to understand which specific molecular mechanisms govern HIV-1 replication in different cells when studied in isolation. It can, however, be anticipated that even subtle alterations in physiological parameters, as recently illustrated for body temperature³, profoundly affect the efficacy of replication and that the specific strategies employed by the virus to optimize its spread depend on the nature of target cells.

To gain a more integrative view on the mechanisms that control HIV-1 replication and spread in infected individuals, significant efforts are made to visualize and study HIV-1 replication in physiologically relevant complex systems. Important recent advances include (i) novel virus labeling strategies, (ii) the implementation of organotypic cell culture models and the generation of tunable three dimensional tissue culture models, (iii) the development of suitable small animal models, and (iv) and the application of intravital imaging techniques. In this Review, we will summarize these recent developments and address how such integrative approaches have already shaped our understanding of HIV-1 pathogenesis by providing insights into the mechanisms and *in vivo* relevance of HIV-1 cell-cell transmission and the physiological role of the viral pathogenesis factor Nef. Finally, we will discuss critical technical and conceptual challenges that need to be overcome in order to achieve a fully integrated understanding of HIV-1 replication in the infected host.

Model systems for integrative analyses of HIV-1 spread

Novel labeling approaches (Box 1) facilitate the visualization of HIV-1 spread in *in vivo* and complex *ex vivo* systems. Model systems that are currently being used and further developed as surrogates for the physiological events during HIV-1 infection include organotypic *ex vivo* cultures, synthetic 3D model systems and small animal models.

Ex vivo complex cell systems

An important development of the last decade has been the use of human tissue (mainly lymphoid and mucosal tissues) as organotypic cultures to study HIV-1 infection *ex vivo* (FIG. 2a). Although such models cannot simultaneously integrate all parameters that govern HIV-1 pathogenesis, they allow studying HIV-1 replication and select aspects of

pathogenesis in the context of physiological tissue architecture, which involves relevant communication between different cell types. Reflecting the origin of the tissue used for these organotypic cultures, select aspects can be studied in individual model systems.

Mucosal transmission models—Early events of HIV-1 transmission can be studied in mucosal explants of the reproductive tract, mainly from the cervix and vagina ⁴⁻⁶, although penile, placental and colorectal tissue also have been used ^{7,8}. Initial techniques ⁹ were improved to better sustain tissue polarization and integrity ¹⁰. Briefly, the tissue is retrieved directly after surgery, dissected into small blocks and cultured on collagen sponges or membranes at an air-liquid interface for about two weeks (FIG. 2a). At various time points post infection cells or tissue can be retrieved for analysis of viral spread by flow cytometry and/or immunohistochemistry ¹⁰.

These models were very helpful for studying early steps in HIV-1 infection, such as defining the main target cells ¹¹ and viral kinetics and factors that influence it ^{11,12}. For instance, skin-resident DCs (such as Langerhans cells) and mucosal DCs were reported to emigrate from the tissue and to trans-infect CD4⁺ T cells without being themselves productively infected ^{13,14}, which suggests a role of these cells in spreading the infection to lymphoid organs. Furthermore, explants of the female reproductive tract are valuable tools to test the efficacy of microbicides in the prevention of HIV-1 transmission ^{15,16}.

Lymphoid organ culture infection models—Most lymphoid organs such as the spleen, lymph nodes and the thymus are not readily available for *ex vivo* studies, therefore studies of acute HIV-1 replication in lymphoid organs most often rely on *ex vivo* tonsil cultures obtained through routine tonsillectomy. Tonsils support HIV-1 replication either as tissue blocks (human lymphoid histocultures (HLH)) (FIG. 2a) or as aggregates of isolated cells (human lymphoid aggregate culture (HLAC)) ^{17,18}. HLH and HLAC have been widely used to study key pathogenic parameters ¹⁹⁻²³. In contrast to peripheral blood mononuclear cells and probably owing to their tissue composition and cytokine milieu, lymphoid tissue cultures support HIV-1 replication without exogenous stimulation ^{18,20} and even show HIV-1 infection of resting, non-activated target cells ²⁴. Although macroscopically non-inflamed parts of tonsils are used, one aspect sometimes criticized about HLH and HLAC is that the inflammation that provokes the clinical manifestation and need for tonsillectomy may influence experimental outcomes. However, HIV-1 replication is equally independent from exogenous stimulation in *ex vivo* cultures of non-inflamed spleen tissue ²⁵.

Whereas tonsil histocultures allow studying acute HIV-1 infection and depletion of mature CD4⁺ T cells, fetal thymus organ cultures (FTOC) shed light on the influence of infection on T cell development in the thymus, a process that significantly contributes to CD4⁺ T cell depletion in AIDS patients. Thymic tissue is retrieved from children undergoing cardiac surgery and cells are either cultured in aggregates ²⁶ or as tissue blocks ²⁷ (FIG. 2a). FTOC studies showed that HIV-1 depletes various progenitor cells and thus reduces thymic output ^{28,29}, a finding that mirrors the thymic atrophy seen in HIV-1 positive children ³⁰. In line with results from transgenic mice ³¹, the viral pathogenicity factor Nef emerged as a key determinant of this process ^{32,33}.

Synthetic 3D cultures models—Although organotypic *ex vivo* tissue cultures recapitulate important aspects of HIV-1 pathogenesis in a close-to-physiological setting, alterations of experimental parameters and thus mechanistic studies are difficult. To complement organotypic cultures, synthetic support matrices are used for the development of complex 3D cultures (FIG. 2b). In the standard 2D cultures, lymphoid cells grown in suspension will sediment at the bottom of the culture dish in a densely packed monolayer (FIG. 2c). This can be avoided by using an extracellular matrix (ECM) of either natural or synthetic origin for the embedding of single cells. As there are in general many different cell types and respective environments, there are also various 3D models. Typically cells are suspended in a liquid matrix precursor, which then solidifies without compromising cell viability. Materials consist either of a single component such as purified collagen I, fibrin or hyaluronic acid, or comprise a complex mixture of reconstituted basement membrane (for example Matrigel) or fibroblast-derived stromal ECM^{34,35}. The resulting porous matrix provides a scaffold structure, allows nutrient and waste exchange, and can contain adhesion sites for cellular functionality. Furthermore, 3D cultures are less heterogeneous and more transparent than tissue cultures, which makes them ideal for live-cell imaging (Box 1).

Whereas collagen systems contributed immensely to our current knowledge of differentiation and cell migration, including motility modes of T cells, macrophages, and DCs³⁶⁻³⁸, their application in virology is still very limited³⁹ and only recently individual studies started to use 3D matrices to reconstitute select aspects of HIV-1 pathogenesis *ex vivo*. Focusing on effects of the viral protein Nef on target cell motility, 3D T lymphocyte cultures allowed recapitulating the inhibitory effect that Nef has on the motility of CD4⁺ T lymphocytes *in vivo* and revealed that this effect is specific to migration modes employed at high matrix density⁴⁰. Similarly, the use of 3D cultures allowed identifying that Nef differentially affects the two migration modes used by macrophages to infiltrate 3D matrices with distinct architectures (Verollet and Maridonneau-Parini, personal communication. As such effects cannot be observed in classic 2D cultures, the use of matrices will likely emerge as a powerful approach to address differences of HIV-1 susceptibility between suspension cultures and organ explants.

Small animal models—The development of suitable small animal models has been a continuous focus of HIV research. These efforts were hampered by the fact that rodent cells are intrinsically resistant to HIV-1 infection. Many steps in the viral life cycle depend on factors that are present in human target cells but not in rodents. Therefore integration of human transgenes, such as CD4 and CCR5⁴¹⁻⁴³, into rodents was an obvious strategy to enhance their permissivity for HIV-1 infection. Although this is sufficient to support HIV-1 entry, viral replication halts at post entry steps and additional transgenes, such as human Cyclin T1^{44,45,46} and other, yet to be identified host factors^{47,48}, are needed. In addition, species-specific cellular restriction factors such as TRIM5a, APOBEC3G, CD317/tetherin, or SAMHD1 also limit HIV-1 replication in rodents⁴⁹. The viral evasion mechanisms used to overcome restriction are highly adapted to the human host and often ineffective in rodent cells. Despite all these difficulties, current transgenic small animal models are sufficiently robust to test the *in vivo* efficacy of potential antivirals^{44,46,50}.

A parallel effort has been to ‘humanize’ immune-compromised mice through transplantation of human tissues such as fetal thymus, liver, and hematopoietic stem cells and thus render them susceptible to HIV-1 infection^{49,51-53}. These models have their own limitations. The problem of xenograft-versus-host disease⁵⁴ may have serendipitously been resolved through the use of recipients deficient for the phagocytosis modulator CD47⁵⁵, whose macrophages appear to tolerate the human graft much better than CD47-sufficient animals. However, the molecular interactions between the human graft and the murine host are likely still suboptimal at many levels and require validation of these models for each particular experimental use and caution in the interpretation of the results. These models are continually being improved, for example by developing recipient mouse strains that express human MHC molecules as well as human cytokines or their receptors (reviewed in⁵⁶).

Intravital microscopy

New HIV-labeling approaches (Box 1) and small animal models described above now permit the visualization of HIV-1 infection *in vivo*. Whereas conventional brightfield and widefield fluorescence microscopy are suitable to examine the intravascular interactions of leukocytes with vascular endothelium that control their targeted recruitment to tissues⁵⁷, multiphoton fluorescence microscopy⁵⁸ allows for dynamic imaging of cellular behavior in extravascular tissue compartments⁵⁹. Over the past 15 years, multiphoton intravital microscopy (MP-IVM) has been embraced by researchers in many fields, including immunologists, who have developed a variety of approaches to use this technology to study dynamic cellular functions of immune cells *in situ* (Box 2). Their investigations of viral pathogens in this context was, at least initially, mostly geared towards understanding how and where these elicit immune responses^{60,61}, and how the effector functions of the immune system are coordinated to control viral infection^{62,63}. Only recently, the mechanisms of viral spread in the host began to receive attention.

MP-IVM is technically challenging, and one important obstacle in the development of new and the adoption of existing IVM models is to preserve tissue physiology (for example, blood perfusion, lymph flow, innervation and temperature) to the largest extent possible during microsurgical exposure, while at the same time immobilizing the specimen sufficiently to permit high-resolution time-lapse imaging. Another challenge is to render tissue structures, cells, and molecules of interest visible. In conventional mice this is routinely achieved through the use of endogenous sources of contrast (tissue autofluorescence, second harmonic generation), organic fluorescent dyes used for the labeling of adoptively transferred cell populations or direct injection as intravital dyes, and through the use of transgenic and gene-targeted mice expressing fluorescent proteins. The latter approach is not readily available for the study of human tissues engrafted in immune-deficient animals, and in order to study the interaction of HIV-1 with the immune system alternative approaches will need to be employed. In this regard, the use of viral vectors or of emerging gene-editing techniques, such as CRISPR/Cas to achieve stable expression of reporter genes in engrafted hematopoietic stem cells currently appear as the most promising approaches to visualize immune cells without prior purification and/or *ex vivo* labeling in humanized animals in complementation of strategies to label virions and infected cells (Box 1).

It can be expected that with the rapid development of new imaging technology and more and more sophisticated fluorescent reporters, the potential of imaging studies in the immune system will continue to increase, and that some of these advances will also translate into novel approaches to study HIV-1 replication and its interaction with the immune system *in vivo* in humanized mice.

Recent lessons from integrative HIV-1 spread analyses

The integration of some of the above described model systems with appropriate labeling and imaging approaches for the visualization of HIV-1 spread recently provided fundamentally new insights into important aspects of HIV-1 spread in the host that would not have been gained by more traditional approaches. These include (i) mechanisms⁶⁴⁻⁶⁷ and (ii) *in vivo* relevance⁶⁸ of HIV-1 cell-cell transmission for virus spread and (iii) the pathobiological principles of the HIV-1 pathogenesis factor Nef^{40,69}, on which we will focus to illustrate the power of such integrative analyses.

Virus spread across the virological synapse

One area in which imaging-based integrative approaches contributed important new insights is the mechanism by which the virus is transmitted from an infected donor to a new target cell. Over the past years it was established that HIV-1, in addition to cell-free infection, can spread between target cells via cell-associated modes⁶⁴⁻⁶⁷. Cell-cell transmission can occur directly from an infected donor T cell or macrophage to a target cell and involves formation of cell contacts referred to as virological synapses. Virological synapses are enriched in adhesion molecules as well as viral budding structures and HIV-1 entry receptors that specifically polarize towards cell-cell contacts in donor and target cells, respectively⁷⁰⁻⁷². A variation of this process is trans-infection, in which DCs capture and store virus particles without becoming productively infected themselves and then efficiently pass on virus to adjacent T cells. At least in *in vitro* 2D T cell cultures, cell-associated modes predominate over cell-free infection to efficiently spread virus from infected donor to uninfected target cells⁷⁰⁻⁷².

Although the efficiency of cell-cell transmission is easily revealed by bulk measurements, the magnitude and dynamics of the transmission process as well as the profound impact it has on cell organization and polarity can only be appreciated by live imaging^{66,67,73,74}. Visualization of individual transmission events of fluorescently labeled particles by live and high resolution microscopy techniques provided an initial characterization of the cellular and viral machinery involved. These studies also revealed that retroviral cell-cell transmission can occur via several mechanistically distinct processes such as transport of virions along or in the lumen of cell protrusions (cytonemes or nanotubes) or crossing of the short distance of the synaptic cleft at the virological synapse as cell-free particle^{66,67,73,74}. Irrespective of the exact modes of transmission, cell-cell transmission ensures targeted delivery of high concentrations of infectious virions to target cells. This effect may even be enhanced by the formation of so called polysynapses, in which one infected donor cell simultaneously engages multiple target cells for efficient virus dissemination⁷⁵. Live-cell imaging of GFP-tagged HIV-1 Gag established that following virological synapse formation, efficient cell-

cell transmission occurs in less than 90 mins⁷⁴. These analyses further suggested that cell-cell transmission may result in endocytic uptake of HIV-1 virions in target cells⁷⁴. Fusion of HIV-1 with target cell membranes was for many years considered to occur at the plasma membrane, despite several reports of productive infection following endocytic uptake^{76,77}. The introduction of high resolution imaging with increasing temporal resolution now provides several lines of direct evidence for productive HIV-1 infection following escape from target cell endosomes^{73,78,79}.

Although these live cell imaging analyses significantly enhanced our understanding of the mechanisms by which efficient HIV-1 cell-cell transmission is achieved in 2D cell culture, they cannot predict the relative contribution of cell-cell over cell-free transmission modes for HIV-1 spread *in vivo*. Estimations of relative efficiencies of cell-free and cell-cell transmission modes so far have been deduced from experiments in which isolated cell populations were incubated in low volume and thus under conditions that largely favor formation of cell-cell contacts and do not reflect physiological cell densities of diverse tissues. In addition, such experimental conditions do not account for the intrinsic motility of donor and target cells on the frequency as well as stability of virological synapse formation and thus virus spread. As a consequence, biophysical parameters such as cell density, pore size and stiffness of the surrounding tissue and matrix, and adhesive properties of the cell environment are likely to represent critical determinants of HIV-1 replication in target organs that may dictate which transmission route is more effective. First answers to these questions were recently provided by studies that pioneered the visualization of HIV-1 spread in humanized mice.

Visualization of HIV-1 spread *in vivo*

Several groups have started to employ MP-IVM to study different aspects of retroviral infection *in vivo*. Murooka *et al.* first validated the bone marrow-liver-thymus (BLT) humanized mouse model for studies on T cell migration by showing that recruitment of naïve or central memory-like human CD4⁺ T cells to murine lymph nodes via skin-draining lymphatics or from the bloodstream, respectively, is largely intact and that their migratory behavior within the lymph node cortex is indistinguishable from that of their murine counterparts⁶⁸. They then infected these animals through subcutaneous injection of a HIV-1 reporter strain that expresses GFP in productively infected cells. Within the first few days after infection, the vast majority of cells in the draining lymph node that expressed detectable quantities of GFP were T cells. Using MP-IVM they demonstrated that these infected cells migrate at reduced speeds compared to their uninfected counterparts, but nevertheless efficiently roam the lymph node cortex (FIG. 3a), which suggests that the continued motility of infected cells might contribute to local viral dissemination, at least within lymphoid tissues. As T cells physiologically recirculate through lymphoid tissues, it was also tested whether infected T cells can contribute to systemic viral dissemination. Indeed, using the functional sphingosine-1-phosphate receptor 1 (S1P1)-antagonist FTY720 to block lymphocyte egress from lymphoid tissue at the onset, but not in established systemic infection, limits plasma viremia to background levels in BLT mice. This suggests that the migration of infected T cells (or of another leukocyte population, such as B lymphocytes, that is capable of transporting infectious virus and requires S1P1 to

recirculate) out of the lymph node, which drains the site of infection, and into remote lymphoid and non-lymphoid tissues facilitates systemic infection of the host.

This study also observed that in early infection ~10-20% of infected T cells in lymph nodes form unusually long membranous extensions at their uropods (FIG. 3b). These depend on the ability of the viral Env protein to interact with CD4 and primarily reflected the formation of multinucleated syncytia since they connected multiple cells that intermittently migrated in opposite directions. However, some of the elongations also ended blindly, and might have resulted from tethering interactions with non-visualized CD4⁺ lymph node cells. The authors speculated that these tethering interactions could reflect the formation of virological synapses that mediate cell-cell transfer of HIV-1, and that in some instances synapse formation results in cell fusion and formation of syncytia owing to imperfectly controlled fusogenicity of Env during viral budding. Interestingly, T cells infected with HIV expressing non-functional Env still migrate at reduced speeds⁶⁸, indicating that HIV reduces T cell motility through additional mechanisms, possibly through the expression of Nef, as discussed below.

Murine leukemia viruses (MLVs) are natural mouse pathogens that can be used to investigate general properties of retroviral Env and Gag proteins relevant to HIV infection *in vivo* without prior humanization of the animals analyzed. Sewald *et al.* recently examined the spread of Friend MLV (F-MLV)-infection *in vivo* through MP-IVM⁸⁰. F-MLV uses the almost ubiquitously expressed murine cationic amino acid transporter (mCAT)-1 as entry receptor and, in addition to B cells, infects CD4⁺ and CD8⁺ T cells with comparable efficiency *in vitro*. *In vivo*, however, F-MLV preferentially infected CD4⁺ T cells, while CD8⁺ T cells are largely spared⁸⁰, an yet unexplained observation that highlights the fact that tissue environmental conditions critically determine retroviral spread. Since B cells are absolutely required for infection of mice with F-MLV⁸⁰, one possible interpretation is that interactions of infected B cells with CD4⁺, but not with CD8⁺ T cells, can mediate contact-dependent virus transfer *in vivo*. Interestingly, infected B cells showed a 3 to 5-fold higher propensity *in vitro* to form virological synapses with CD4⁺ T cells than with CD8⁺ T cells. Using MP-IVM and fluorescent reporter virus, Sewald *et al.* further demonstrated that, dependent on intact Env-mCAT-1 interactions, some infected B cells transiently interrupt their migration in lymph nodes and polarize GFP-tagged Gag proteins in ways suggestive of the formation of virological synapses (FIG. 3d, e). Syncytia formation was not apparent as for HIV infection⁶⁸, but some motile infected B cells developed elongated uropods and some arrested B cells formed thin membrane extensions resembling filopodia⁸⁰. Potential factors contributing to the transmission of F-MLV from infected B cells to CD4⁺, but not CD8⁺ T cells *in vivo* include differences in the microenvironmental positioning of CD4⁺ and CD8⁺ T cells, and specific molecular interactions that take place between B cells and CD4⁺ but not CD8⁺ T cells. It will be exciting to explore whether HIV spread exhibits similar unique features *in vivo* that are not predicted from *in vitro* studies.

Integrative understanding of HIV-1 Nef function

Investigating the pathophysiological role of HIV-1 Nef constitutes another aspect of HIV biology that has strongly benefited from an integrative approach. Nef has remained one of

the more enigmatic HIV-1 encoded proteins, as indicated by its name, an abbreviation for negative factor. This name was deduced from initial experiments in cell lines that indicated a negative effect of the protein on virus replication. By contrast, *in vivo* Nef strongly promotes virus spread and thus disease progression⁸¹⁻⁸³. The identification of Nef as key factor for HIV-1 pathogenesis spurred intense efforts to decipher the underlying molecular mechanisms. This revealed a complex array of physical interactions with host cell proteins by which Nef alters a large set of cellular functions^{84,85}. Nef most prominently affects vesicular transport and signal transduction^{84,85}, but Nef also protects infected cells from lysis by cytotoxic T or NK cells^{86,87}, prevents superinfection^{88,89} and optimizes host cell responses to exogenous stimulation to increase cell survival and production of infectious progeny virions⁹⁰⁻⁹³. The need for integrative approaches for studying Nef is illustrated best by the fact that the prominent growth advantage that Nef-positive viruses display *in vivo* is not reflected by any of the *ex vivo* models thus far employed to study Nef function^{84,85}. This difference can be explained in part by the lack of HIV-1 specific cytotoxic T cells in the *ex vivo* models but this also indicates that the true effector functions of Nef may only be revealed in complex models that better mimic the host environment than monotypic 2D cell cultures. Previous work showed that Nef inhibits T cell migration in standard transwell chemotaxis assays^{94,95}, mostly by disrupting host cell actin dynamics and reprogramming the substrate specificity of the cellular p21-activated kinase 2^{96,97}. Addressing how impaired motility of infected T cells is related to Nef's role as a pathogenesis factor, however, required *in vivo* studies. Following the fate of Nef-expressing T cells after adoptive transfer into recipient mice revealed that their homing to lymph nodes is potently reduced⁴⁰. Intravital imaging and 3D immunofluorescence reconstruction demonstrated that Nef blocks extravasation of T cells through high endothelial venules and, consistent with the study by Murooka and colleagues on the *in vivo* motility of infected T cells⁶⁸, reduces T cell motility inside the lymph node (FIG. 4a). These results could be recapitulated in *ex vivo* models of transendothelial migration and 3D collagen matrices⁴⁰, providing accessible experimental systems for future mechanistic dissection. Furthermore, histological analyses of lymph nodes of simian immunodeficiency virus infected rhesus macaques showed that Nef expression is associated with the accumulation of viral antigen at the subcapsular sinus and with a reduction of productively infected T cells in the paracortex⁹⁸. Together these results suggest that Nef may reduce the frequency and motility of HIV-1 infected cells inside lymph nodes, which could have profound impacts on virus dissemination as well as immune evasion. Given the high intrinsic motility of uninfected T cells, reduced motility of infected T cells may favor the formation of stable virological synapses and polysynapses to facilitate virus spread (FIG. 4a). At the same time, the probability of engagement with specific antigen presenting cells and thus their probability to activate B cells may significantly decline by such a reduction in motility (FIG. 4b). This would synergize with Nef's ability to modulate immune cell communication in several ways: First, Nef potently disrupts formation of immune synapses between infected T cells and antigen presenting cells^{92,99,100}, which might impair their specific T helper function. Second, Nef promotes the formation of long cell conduits from infected T cells and macrophages¹⁰¹⁻¹⁰³ that appear to deposit Nef protein inside B cells to prevent class switching. Third, Nef triggers the release of soluble factors including ferritin from infected cells which results in unspecific activation of bystander B cells. Since specific B cell activation is required for the generation of

protective antibodies that recognize their target antigen with high affinity, this unspecific activation results in hyperproduction of low affinity antibodies ⁶⁹. Finally, elevated levels of Nef expression correlate in an HIV-1 transgenic mouse model with the development of B cell lymphoma ¹⁰⁴. Together, these insights gained from complex *ex vivo* and *in vivo* studies suggest disruption of B cell function as a previously unrecognized pathogenic activity of Nef. As such, Nef may be a central viral determinant for the complex B cell dysfunction in AIDS patients ¹⁰⁵ and the experimental systems established will allow further testing of this hypothesis and dissecting of the underlying mechanisms.

Conclusions

Here we have described existing and emerging experimental models that aim to account for the influence of the tissue environment on the dynamic cellular and viral functions that determine HIV-1 replication and spread *in vivo*, but are largely neglected in conventional 2D cell-culture systems. In reviewing some of the findings facilitated through these models, which range from reconstituted 3D tissue systems and explanted organ cultures to small animal models, we tried to illustrate their potential and the unique features of the data they can yield, such as observations of altered migratory behavior of infected cells that result from the specific function of viral proteins. Further development of these experimental systems and of virus labeling techniques provides a fresh approach to investigate HIV-1 pathogenesis through the dynamic visualization of viral replication and spread in its physiological context. Based on the substantial research of the mechanisms of cell-to-cell spread *in vitro*, an important initial focus of emerging *in vivo* studies will be to dissect to which extend retroviral spread in the host occurs via this transmission mode (FIG. 1d). Considering that the loss of CD4⁺ T cells in HIV-infected individuals is thought to, at least in part, reflect abortive infection upon efficient cell-to-cell transfer of virus particles to uninfected target cells ^{23,106}, these studies will also shed light on mechanism and dynamics of HIV-1 mediated T cell depletion. Results may vary depending on the particular tissue examined, but there will likely be common principles of how HIV-1-infected or HIV-1-carrying cells interact with target cells and potentially transfer virions. As suggested recently ¹⁰⁷, it is possible that cell contact-dependent spread greatly facilitates viral dissemination, but that free virus can still transmit the infection between cells at a low efficiency. To determine the relevance of contact-dependent spread, it will be important to examine to what extent antagonizing it diminishes the ability of the virus to spread within and between tissues.

A further area of interest is how the motility and trafficking of HIV-1-infected and HIV-1-carrying cells contributes to virus spread in the infected host, for instance from mucosal transmission sites to draining lymph nodes (FIG. 1b), and then from there to remote tissues (FIG. 1c). This also includes the relevance of HIV-1-induced changes in the motility of infected cells, possibly through favoring stabilization of cell contacts that facilitate viral transfer.

It will likely also be highly instructive to visualize the spread of infection in different tissues, including the mucosae of the genital and intestinal tracts, the thymus and the central nervous system. A key unanswered question in HIV-1 pathogenesis remains how the virus is able to

overcome the epithelial barriers of the genital and rectal mucosae during sexual transmission (FIG. 1a). Microscopic imaging *in vivo* and in complex models will allow us to address whether luminal entry into the epithelium through diffusive percolation¹⁰⁸ is an efficient mechanism for HIV-1 to reach its initial target cells, or if transepithelial migration of HIV-1-carrying or infected cells, either donor or host-derived, predominate¹⁰⁹. Observing initial viral entry events, the pathways of spread and the growth of clusters of infected cells may also provide a better understanding of viral factors that influence transmission.

Beyond studies that predominately focus on the intrinsic replicative potential of HIV-1, the next step will be to couple such analyses with the visualization of antiviral innate, cellular and humoral immune responses, which will add to our understanding of the immunological activities that are effective against the virus at different stages of its life cycle (FIG. 1e). Similarly, *in situ*-visualization of HIV-1 replication will provide important insights into the activities of antiretroviral drugs or antibody-treatments by revealing their immediate impact on cellular and viral behavior *in vivo* in ways that static tissue analyses or conventional tissue culture systems cannot.

Only integration of experimental 2D, 3D and *in vivo* approaches, with their complementary characteristics regarding the balance of physiological relevance versus experimental control, will allow us to achieve a full understanding of the principles that govern HIV-1 spread in the infected host and thus AIDS pathogenesis. Beyond the improvement of basic mechanistic understanding, knowledge gained by the application of integrative approaches to study HIV-1 infection will facilitate the development of specific and targeted preventive and therapeutic antiviral strategies.

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Glossary

Productive and latent HIV infection

Productive infection comprises all steps of the viral life cycle, including viral protein and particle production. In latent HIV infection the life cycle halts as an integrated provirus without transcription of viral genes

Organotypic cultures

Ex vivo culture of tissue or tissue sections obtained from HIV-negative donors in which the physiological scenario of HIV-1 can be simulated. When tissue architecture of HIV target organs is preserved, such cultures are referred to as “organotypic”

2D/3D cell cultures

In classical culture systems cells settle at a two-dimensional (2D) plastic or glass surface. In contrast to this three-dimensional (3D) cell culture implements an extracellular matrix to provide spacing and a complex architecture

Lentiviral animal models

There are two main groups of lentiviral animal models: non-primate monkeys such as rhesus macaques that are naturally susceptible to SIV infection and small animal models, e.g. mice or rat which are rendered permissive to HIV infection by genetic manipulation or engraftment of human tissue

Bone marrow-liver-thymus (BLT) humanized mouse model

Immuno-deficient mice engrafted with fetal human thymus, fetal liver, and hematopoietic stems cells to recreate a functional human immune system in a small animal model

Lymph

Excess tissue fluid that enters afferent lymph vessels, which open into the subcapsular sinus of draining lymph nodes. Lymph can transport solutes, particles, or cells. Part of it reaches the medulla and from there enters efferent lymph vessels that eventually open into the bloodstream at the aortic arch

Lymph nodes

Lymph nodes are organized stromal environments where tissue lymph is filtered and where most T and B cells initially encounter their cognate antigen for the induction of adaptive immune responses. DCs and antigen enter lymph nodes through the afferent lymphatics, whereas T cells enter from the bloodstream

Virological synapse, cytonemes and nanotubes

The virological synapse represents an ordered assembly of viral and host proteins at the interface between infected (donor) and uninfected (target) cells that facilitates the transfer of infectious HIV-1. Virus transfer can occur via cell-free particles across the synaptic cleft but may also involve transport along cellular protrusions. This can occur via cytonemes that protrude from one cell towards its neighbor and allow transport of surface-bound virus particles. Alternatively, transport may occur in the lumen of tunneling nanotubes that form membrane bridges between donor and target cells allowing for exchange of cytoplasmic content

Immunological synapse

The contact interface between antigen presenting cells and T lymphocytes that supports adhesion, polarized signal transduction, and T cell activation

Uropod

The narrow trailing edge of a polarized, migrating leukocyte, which differs from the cell front not only in shape and position, but also in its content of organelles, cytoskeletal proteins, and membrane molecules involved in motility, such as adhesion molecules and signaling receptors

Biographies

Oliver T. Fackler is a Biologist by training and received his Ph.D. in 1997 from the University of the Saarland, Germany. Following a post doctorate with Matija Peterlin at UCSF, he started his own laboratory at the Department of Infectious Diseases at the University Hospital Heidelberg, where he obtained a tenured professorship in 2007 and is currently head of the section Integrative Virology. His main research interests are the molecular mechanisms of HIV accessory proteins, innate immune recognition of HIV infection by the host, and the analysis of HIV spread in complex cell culture systems.

Thomas T. Murooka obtained his Ph.D. in Immunology in 2008 at the University of Toronto, working in Eleanor Fish's lab on signaling through the chemokine receptor CCR5 and its role in vaccinia virus infection. Since 2009 he is a research fellow in Thorsten Mempel's laboratory at Massachusetts General Hospital and since 2013 an Instructor in Medicine at Harvard Medical School. His interest is to understand how HIV-1 parasitizes the migratory

activities of infected T cells and their interactions with antigen-presenting cells to spread in the host.

Andrea Imle studied Biomedicine at the University of Würzburg, Germany. She is currently working as a Ph.D. student in Oliver Fackler's laboratory on HIV spread in complex cell culture systems.

Thorsten R. Mempel received his M.D. from the University of Munich in 2000 and trained as a research fellow from 2002 to 2007 in the laboratory of Ulrich von Andrian at the Center for Blood Research in Boston, where he developed novel intravital microscopy approaches to study immune cell migration in vivo. He then started his own laboratory at the Center for Immunology and Inflammatory Diseases at Massachusetts General Hospital, and is currently an Associate Professor in Medicine at Harvard Medical School. His work focuses on cell migration and cell-cell interactions that facilitate intercellular communication in the adaptive immune system. He is also interested in how viruses have learned to interact with the immune system to their own benefit.

Box 1**Labeling approaches for the visualization of HIV-1 spread**

Analyses of the dynamics of HIV-1 spread involve the visualization both of virus particles and of productively infected cells. Classic approaches rely on the detection of viral antigen or genomes in tissue sections, for example from HIV-1 infected patients. Although this provides important information on the identity, location and frequency of infected cells, such static endpoint analyses by design fail to recapitulate the dynamic nature of infection processes. Studies of HIV-1 spread thus increasingly benefit from live cell imaging approaches that visualize defined aspects of virus replication, reveal individual virus components or identify productively infected cells. Several approaches were developed to visualize individual HIV-1 particles or subviral structures, including virion incorporation of fluorescent dyes by coupling to the virion associated accessory gene product Vpr, introduction of fluorescent dyes into the lipid envelope of the virus, or visualization of the viral genome ^{79,110,111} (see ^{112,113} for review), however all these approaches are limited to a single round of virus replication owing to subsequent loss of the dye. To overcome this limitation, virus variants were generated that encode fluorescent proteins or stainable tags fused to structural proteins of the virus, in particular to Gag ¹¹². These strategies generate particles well suited for live cell imaging, however tagging often compromises the biological properties of the virus, which can be partially rescued by production of particles that contain a mixture of tagged and untagged proteins from separate genomes ¹¹⁴. Furthermore, tag-encoding sequences are rapidly lost over a few rounds of virus replication. Alternatively, coding sequences for fluorescent reporters or cell surface receptors are placed under control of an internal ribosomal entry site (IRES) element to preserve expression of all viral genes in their natural form. The replication potential of such viruses is only slightly diminished relative to unmodified HIV-1 but, here too, reporter coding sequences are frequently lost. The generation of a fully replication competent HIV-1 variant that maintains a fluorescent label over multiple rounds of infection thus remains a major bottleneck for integrative studies of HIV-1 spread. Alternatively, for some applications, it might be sufficient to use cellular reporters that indicate productive infection of new target cells. Such constructs express luciferase or fluorescent reporters under the control of the viral promoter (LTR) and are commonly used in standard cell lines, for example TZM-bl. Introducing such reporters into transgenic animal models or complex cell systems might be a worthwhile endeavor.

Box 2

Multiphoton intravital microscopy (MP-IVM) studies of the immune system

MP-IVM is a versatile technique to directly visualize dynamic biological events in a variety of tissues of live, anaesthetized animals (see panel a). Some tissues are directly accessible, such as skin or the eye, but generally, MP-IVM studies require tissue exposure through installation of chronic imaging chambers, or through acute microsurgical preparations, as for instance for studies in lymph nodes (see panel b). MP-IVM studies of the immune system have established a range of read-outs of cellular behaviors that will likely be useful in the investigation of host-pathogen interactions, including HIV-1 spread (see panel c). Motility is the most widely analyzed immune cell behavior, including speed and directionality of cell migration through tissues, as well as stability and duration of cell-cell contacts (reviewed in ¹¹⁵). Cytotoxic killing of fluorescently tagged target cells, monitored through signs of apoptosis, such as loss of cell motility and structural integrity, or caspase activation, was the first immunological effector function to be visualized in vivo ^{116,117}. A prelude to examinations of host-live pathogen interactions has been to visualize the entry of sterile antigens, including inactivated viruses, into lymph nodes and their uptake by different types of antigen-presenting cells ¹¹⁸⁻¹²¹. Studies in explanted lymphoid tissues have demonstrated the utility of fluorescent protein-based reporters to monitor expression of host genes, for example of cytokines ¹²² or of viral genes in infected cells ⁶¹, or to track changes in subcellular protein localization as read-outs for the activation of signaling pathways in individual cells in tissues ¹²³⁻¹²⁵. The latter approach has further been advanced to study T cell receptor signaling in vivo by monitoring nuclear factor of activated T cells (NFAT) nucleocytoplasmic shuttling in T cells by MP-IVM ¹²⁶⁻¹²⁸. With the ongoing development of imaging technology and increasingly sophisticated fluorescent reporters, the possibilities of intravital microscopy studies will continue to grow, including their application in HIV-1 research.

Online summary

- Standard 2D cell cultures systems ignore critical parameters such as tissue architecture and composition, shear flow of body fluids, or communication and motility that determine the efficiency of HIV-1 spread and thus disease progression. New developments in the field increasingly allow for integrative *ex vivo* and *in vivo* analysis that take these aspects into account.
- Organotypic or synthetic 3D culture systems mimic the physiology of individual target organs to enable studies of select aspects of HIV pathogenesis *ex vivo*. Parallel developments in the visualization of productively infected cells or individual virus particles facilitate the tracking of dynamic infection processes in real time.
- Advanced life cell imaging approaches to follow the transmission of fluorescent HIV support the idea of cell-cell transmission as highly effective mechanism of HIV-1 infection.
- Small animal models such as BLT humanized mice provide new opportunities to study and visualize HIV-1 spread and pathogenesis *in vivo*. Intravital microscopy was recently applied for the first time to study retrovirus replication *in vivo* and provided support for an important role of cell-cell transmission in HIV-1 spread. This approach opens new avenues for gaining important insight into the mechanisms and dynamic of retroviral spread in the infected host.
- Integrative approaches helped to define the pathogenic principles of the HIV-1 accessory protein Nef and suggest Nef as a modulator of HIV target cell motility that may undermine host immune responses by altering immune cell communication.

Figure 1. Overview of crucial, but poorly understood steps in the biology of HIV-1 infection

(a) Under what conditions can HIV-1 cross the stratified epithelium of the vagina or the columnar epithelium of the uterine cervix during transmission? Can infectious virions penetrate mucosal tissues far enough to reach epithelial or subepithelial target cells? Which cells are initially infected? Is there a role of HIV trans-presentation by non-infected dendritic cells? **(b)** In what form does virus reach the draining lymph node? **(c)** How does the virus disseminate via the bloodstream to generate a systemic infection? **(d)** For all affected tissues, what is the role of (clockwise from top): free virus, *trans*-presentation by antigen-presenting cells, interactions with infected antigen-presenting cells, and direct T cell-T cell interactions for cell to cell spread? **(e)** What is the role for different immunological anti-HIV effector functions, such as (clockwise from top) direct T cell-mediated cytotoxic lysis of infected cells, antibody-mediated neutralization of free virus, or antibody-mediated cellular effector functions, such as cytotoxicity or phagocytosis, at different time-points of infection and in different tissues?

Figure 2. Complex *ex vivo* cell systems for studies of HIV-1 spread

Schematic depiction and characteristics of complex *ex vivo* cells systems for studies of HIV-1 replication and spread. The top panel depicts the schematic organization of the model system, the lower panel present histology of a tissue section (**a**) (reproduced from ⁶ with permission) or wide field images of cell organization (**b**, **c**). Scale bars = 40µm. (**a**) Organotypic histocultures of HIV-1 target organs. Tissue explants from cervix, vagina, tonsil or thymus are cultivated on support at the air-medium interface to maintain tissue organization and composition. (**b**) In synthetic 3D cultures systems, cells are embedded into

matrices for cultivation, allowing for tightly controlled experimental variation of cell and matrix composition. **(c)** Classical cultures, even for suspension cells, result in unphysiologically dense cell packing at the bottom of the tissue culture plate and thus a 2D scenario. **(d)** Table summarizing key characteristics of the culture systems shown above.

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Figure 3. Intravital microscopy analyses of retroviral spread *in vivo*

(a, b) MP-IVM of popliteal lymph nodes of HIV-1-infected BLT humanized mice has shown that HIV-1-infected T cells migrate half as fast as their uninfected counterparts (a) and that (b) they interact with CD4⁺ cells via gp120, which can lead to either tethering interactions or to formation of motile multinucleated syncytia. (c) Efficient viral dissemination from lymph nodes draining an HIV-1-inoculation site to distant tissues requires either HIV-1-infected or HIV-1-carrying cells as migratory vehicles (d) MLV-infection of C57BL/6 mice showed either unaltered migration or arrest of infected B cells in lymph nodes. (e) Visualizing Gag proteins revealed envelop-dependent polarization of virions in arrested B cells *in vivo*, suggesting formation of virological synapses during cellular interactions. Fluorescence micrograph in (e) is taken from the paper by Sewald *et al.*⁸⁰ and shown with permission from Dr. Walther Mothes.

Figure 4. Model for the effects of HIV-1 Nef on T lymphocyte trafficking and induction of humoral immunity

(a) Schematic depiction of effects of Nef observed on *in vivo* T lymphocyte trafficking. The scheme summarizes observations made in lymph node sections of SIV-infected macaques⁹⁸, infected humanized mice⁶⁸ and adoptive transfer of Nef-expression T lymphocytes into recipient mice⁴⁰. In the absence of Nef (left), T lymphocytes transmigrate from the HEV into the lymph node parenchyma where they remain fully motile. Infected cells spread virus but new infection may also occur by virus delivered via the afferent lymphatics. Exit of

infected cells via the medulla may support dissemination of the virus. The presence of Nef (right) significantly reduces the ability of infected T lymphocytes to transmigrate to the lymph node lumen and may also reduce their ability to exit. Successfully transmigrated cells preferentially remain in the proximity of the HEV and display reduced intra lymph node motility. These properties may favor the formation of stable virological synapses or even polysynapses and thus optimize virus transmission. As indicated by the enrichment of viral antigen at the subcapsular sinus, infection with virus via the afferent lymphatics may be particularly efficient with *nef*-positive virus. **(b)** Hypothetical model of effects of Nef on humoral immunity. In the absence of Nef, T lymphocytes are readily engaged in productive immunological synapses with antigen presenting cells. In the case of B cells, CD4⁺ help induces B cell activation driving germinal centre reactions and ultimately mounting of a potent humoral immune response (left). Nef expressed in HIV-infected CD4⁺ T lymphocytes disrupts immunological synapse organization to reduce signal transmission and might thereby limit B cell activation. Moreover, infected, Nef expressing macrophages can trigger unspecific B cell activation by a paracrine mechanism involving ferritin and deposit the viral protein in B cells from via nanotubes to interfere with Ig class switching. Together these effects likely inhibit humoral immune responses to HIV infection and suggest Nef as a key determinant for B cell dysfunction in AIDS patients.