HIV-1-Induced Cell-Cell Fusion: Host Regulation And Consequences For Viral Spread

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HIV-1-INDUCED CELL-CELL FUSION: HOST REGULATION AND CONSEQUENCES FOR VIRAL SPREAD

A Dissertation Presented

by

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ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) is a human retrovirus of the lentivirus subgroup which primarily infects T cells and macrophages, and causes acquired immune deficiency syndrome (AIDS). Since its emergence in the early 1980s, HIV-1 has caused a global pandemic which is still responsible for over one million deaths per year, primarily in sub-Saharan Africa.

HIV-1 has been the subject of intense study for over three decades, which has resulted not only in major advances in cell biology, but also in numerous drug treatments that effectively control the infection. However, cessation of treatment always results in reemergence of the infection due to the ability of HIV-1 (and other lentiviruses) to establish a persistent quiescent infection known as latency. The elimination of latently-infected cells is the primary goal of current research towards a cure for HIV-1, alongside efforts to develop vaccines, which have thus far been fruitless.

The spread of HIV-1 to susceptible target cells (which express the receptor CD4 and a co-receptor; CXCR4 or CCR5) can take place when antigen-presenting cells, such as dendritic cells, capture virus particles and then pass them on to target cells, without themselves becoming infected. Alternatively, productively infected T cells or macrophages can spread HIV-1 either by shedding virus particles to the milieu, which are then stochastically acquired by target cells, or through transient contacts between infected and uninfected cells known as virological synapses (VSs). VS-mediated cell-to-cell transmission is thought to be highly efficient due to the release of virus directly onto (or very near to) a target cell, and some evidence suggests that the VS is a privileged site which allows the virus to evade neutralizing antibodies and drugs. However, and most importantly, it is of central interest to us because the same transient cell adhesions that facilitate virus transfer can also result in the fusion of the two cells to form a syncytium, due to the presence of the viral fusogen Env and its receptor and co-receptor on either side of the VS. While T cell syncytia can be found in vivo, they remain small, and it appears that the majority of VSs resolve without fusion.

The regulation of HIV-1-induced cell-cell fusion and the fate of those syncytia are the focus of the work presented here. A family of host transmembrane proteins, the tetraspanins, which regulate cell-cell fusion in other contexts (e.g. the fusion of myoblasts to form and maintain myotubes), were found to inhibit HIV-1-induced cell-cell fusion. Our investigations have further characterized this regulation, concluding that tetraspanins allow cells to reach the fusion intermediate known as hemifusion before their ability to repress fusion takes effect. In parallel, because syncytia are nevertheless found both in infected individuals and in a humanized mouse model for HIV-1, we also became interested in whether small T cell-based syncytia were able to participate in HIV-1 spread by transmitting virus to target cells. Using a simple three dimensional in vitro culture system which closely recapitulates those in situ observations, we found that small syncytia can contact target cells and transmit virus without fusing with them. Overall, these studies further our understanding of HIV-1-induced syncytia and reveal a previously unrecognized role for these entities as active participants in HIV-1 spread.
CITATIONS

Material from this dissertation has been published in the following form:


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CHAPTER 1: INTRODUCTION

1.1. Natural history of HIV-1

HIV-1 was first isolated in 1983 by the Montagnier group, and soon after by the Gallo group, and by 1984 was recognized as the causative agent of acquired immune deficiency syndrome (reviewed in [1]). The virus was initially named lymphadenopathy-associated virus (LAV) and human T-lymphotropic virus type 3 (HTLV-III), before it was given the consensus name human immunodeficiency virus type 1 (HIV-1). Due to the speed with which the virus was spreading (initially in men who have sex with men and drug users, but quickly expanding to the broader population), and with credit due to activists and healthcare professionals who pushed for its recognition, HIV-1 research quickly rose to the forefront of the biomedical sciences and has remained a major focus since. This intense research has resulted in the elucidation of key biological paradigms, but also the development of a number of drug treatments that, when used in combination, effectively control the infection (but do not cure it).

HIV-1 is a zoonotic virus (reviewed in [2]) derived from the primate lentivirus simian immunodeficiency virus (SIV), specifically the SIV which uses chimpanzees as its host (SIVcpz). SIVcpz has undergone several transmission events into humans that have established spreading HIV-1 groups. The major HIV-1 group (group M) consists of a number of subtypes which have spread through human populations to different degrees, of which HIV-1 group M subtypes A and C are most widely spread in the Eastern hemisphere and in sub-Saharan Africa, and subtype B is prevalent in the Western hemisphere. The first cross-species transmission events of SIVcpz into humans are
thought to have occurred amongst hunters in the Democratic Republic of Congo (then named Zaire) in the early 1920s [3], likely when SIVcpz-infected chimpanzees were butchered for meat by individuals who had open wounds on their hands (resulting in blood to blood contact). However, the virus did not begin spreading more widely in human populations and into the Western hemisphere until much later, when group M HIV-1 circulating in Haiti spread to the United States to establish the now prevalent subtype B [4]. The nascent pandemic was first detected in the early 1980s (likely over a decade after its initial introduction to the United States from Haiti [4]) when many high-risk patients (i.e. men who have sex with men) presented with unusual infections that indicated immunodeficiency of unknown cause.

The disease progression for untreated HIV-1 infection begins with flu-like symptoms, which soon subside and lead into a lengthy refractory period lasting two to five years. The immune system responds to episodes of resurgence of the virus with hyperactivation, which in turn facilitates further spread of the virus (due to its “preference” for infecting activated CD4+ T cells) and establishment of a systemic infection. Through a combination of CD4+ T cell exhaustion, and active killing of immune cells by HIV-1, a profound immune deficiency is established, which in turn allows numerous pathogens (otherwise well-controlled in immunocompetent individuals) to establish aggressive infections that ultimately result in death.

HIV-1 spreads from person to person through sexual contact, direct blood to blood contact (including across mucosal tissues), and from mother to child either during birth or breast feeding. The skin forms an effective barrier against transmission, and there
is no evidence of transmission of the virus mediated by aerosol, feces, urine, or other bodily fluids. When HIV-1-infected individuals are being treated with the triple antiretroviral therapy (ART) regimen, they exhibit high CD4$^+$ cell counts and no viremia for the majority of the time, and are not at risk for transmission through sexual contact or from mother to child. Although this treatment is not curative of an established HIV-1 infection, treatment with ART in the first few days post exposure (e.g. in the case of an accidental exposure in a healthcare or research setting) can prevent the establishment of the infection altogether. Additionally, pre-exposure prophylaxis is now available for HIV-1-uninfected individuals who are at some risk of transmission, e.g. because they are in a relationship with someone who is HIV-1-infected.
1.2. Taxonomy and genome structure

HIV-1 is a retrovirus of the genus *Lentivirus*, which in turn consists of groups of viruses with bovine, equine, feline, ovine, and primate hosts. HIV-1 is an enveloped virus with two copies (i.e. alleles) of a positive sense single stranded RNA genome (refer to [5]). The ~9.7 kb genome consists of the major genes *gag*, *pol*, and *env*, and the accessory/regulatory genes *tat*, *rev*, *nef*, *vpu*, *vif*, and *vpr* (Figure 1). The *gag* gene encodes for the structural assembly polyprotein Gag, while the *pol* gene encodes for the viral protease (PR), reverse transcriptase (RT), and integrase (IN) proteins. The product of the *env* gene is the envelope glycoprotein Env, which is the viral adhesion and fusion protein that determines the ability of virus particles to attach to and enter target cells which bear the receptor CD4 and a chemokine co-receptor (CCR5 or CXCR4). Alternate reading frames and splicing variants of the *env* gene and its surrounding region encode for the transactivator protein Tat, and the RNA-shuttling protein Rev. While the functions of the remaining accessory proteins are still the subject of extensive research, in a broad sense they operate in concert with host proteins to create a cellular environment that favors virus spread and overcome specific restriction factors, e.g. by directing degradation of the hypermutagenic APOBEC3 proteins that become incorporated into virions (in the case of Vif [6-8]), or through downregulation of BST-2/tetherin (by Vpu [9,10]) and SERINC3/5 (by Nef [11-13]) from the surface of producer cells to allow virus particles to be released and to then infect target cells, respectively.
Figure 1: Genome structure of HIV-1, NL4-3 strain (reproduced with permission from Watts et al., 2009 [14]). Note that all three reading frames are utilized to minimize genome size. MA, matrix; CA, capsid; NC, nucleocapsid; PR, protease; RT, reverse transcriptase; IN, integrase; SP, signal peptide.
Figure 2: The HIV-1 replication cycle (reproduced with permission from Ganser-Pornillos et al., 2008 [15]).
1.3. Replication cycle

The HIV-1 replication cycle (Figure 2) can be divided into the entry, uncoating, reverse transcription, integration, transcription, translation, assembly, release, and maturation stages (though some of them can overlap with adjacent stages). Entry (reviewed in [16] and [17]) is primarily mediated by virion-decorating trimers of Env, the viral fusion protein which consists of the ectodomain gp120 and the transmembrane domain gp41. HIV-1 Env variants are broadly characterized according to their tropism, determined by the co-receptor which they can utilize for fusion. CCR5-tropic (or R5) Envs are thought to be the most relevant as they can target both T cells and macrophages in vivo and prevail in early infection, while CXCR4-tropic (or X4) Envs are thought to be characteristic of late stage infections, but have been extensively used in research because many transformed T cell lines do not express CCR5.

Although many viral fusogens are only active at low pH, thus allowing them to only trigger membrane fusion in an appropriate compartment such as the acidified endosome, HIV-1 Env (and other lentiviral glycoproteins) are active at neutral pH. An important consequence of this is that Env can induce the formation of syncytia, i.e. multinucleated cells, when an infected cell with Env on its surface and an uninfected cell with the receptor CD4 and the appropriate co-receptor come into close contact. The various ways in which syncytium formation is prevented will be discussed throughout this section.

Fusion of the virion with the target cell membrane begins when gp120 on the surface of a mature virus particle ligates with CD4 on the surface of a target cell. A
cascade of conformational changes is triggered when the co-receptor is then contacted, and gp41 becomes exposed. The external region of gp41 extends and the fusion peptide is inserted into the target cell membrane, which is followed by zippering of the heptad repeat regions to bring the apposed membranes close enough to favor the initiation of membrane merger. The outer leaflets of the virion envelope and the target cell membrane initially merge, in an intermediate known as hemifusion, before the inner leaflets also merge and a fusion pore is formed. All the steps of fusion up to this point are driven by folding energy within gp41, and do not require any energy expenditure, and they can be slowed or arrested entirely when the temperature is reduced from 37°C to below 23°C [18]. Pore expansion, which requires energy expenditure within the target cell e.g. by dynamin [19], is followed by release of the viral core into the cytoplasm, which completes the fusion process (see also Chapter 2, Figure 1).

Disassembly of the viral core, reverse transcription of the genome by RT, nuclear import of the resulting proviral DNA, and integration into the host genome mediated by IN are reviewed in [20]. One notable consequence of the reverse transcription stage is the introduction of mutations into the proviral genome, which result from the fact that RT is very error-prone. These mutations are the reason that HIV-1 sequences recovered from single infected individuals can be grouped into variants known as quasispecies (reviewed in [21]). The mutagenic nature of RT drives the fast evolution of the virus, and underlies its ability to develop resistance to drug,

Following integration, the expression of the early genes tat and rev establishes an environment favorable for HIV-1 transcription and RNA export (reviewed in [22]). Tat
stimulates transcription of the provirus through the long terminal repeat (LTR) region, which acts as the viral promoter (after its earlier role of facilitating integration). Rev mediates nuclear export of the resulting RNA species, which consist of a complex mixture of partially and fully spliced mRNAs, as well as the full-length viral genomic RNA (vRNA). The mRNAs are then translated into the various viral proteins. Notably, the GagPol mRNA is translated into either Gag or GagPol, based on a programmed frameshift that allows for read-through and full GagPol translation ~1 out of 20 times (reviewed in [23]). Maintenance of this ratio is crucial for the infectivity of the resulting virions [24], and some evidence suggests that eukaryotic release factor 1 (eRF1), which facilitates stop codon recognition and translation termination, can modulate GagPol frameshift read-through efficiency [25,26].

The matrix (MA) domain of Gag contains a patch of basic residues which favors its interaction with acidic plasma membrane lipids, in particular phosphatidylinositol 4,5-bisphosphate (PIP$_2$) [27-29]. Once MA binds PIP$_2$, an N-terminal myristyl moiety becomes exposed and strongly anchors the polyprotein to the plasma membrane [28]. Additional targeting of Gag to specific sites of the plasma membrane, namely the uropod of polarized T cells, is mediated by the nucleocapsid (NC) domain [30,31]. Multimerization of Gag into a lattice is facilitated by interactions between the capsid (CA) domains of adjacent Gag monomers, and further enhanced by the interaction between NC and the viral genomic RNA (reviewed in [32]).

In parallel, Env trimers assemble in the endoplasmic reticulum and traffic to the plasma membrane (reviewed in [33]). The cytoplasmic tail of Env (EnvCT) contains two
independent recycling motifs: a YxxL motif which recruits AP-2 [34-36], and a dileucine motif which recruits AP-1 [37]. These adaptor proteins associate with clathrin to mediate endocytosis of Env, which likely serves to both prevent unintended syncytium formation, and to reduce the immunogenicity of the protein by sequestering it intracellularly. However, such recycling is prevented when Env trimers are trapped by the Gag lattice [38], likely through modification of the membrane microenvironment by Gag, as well as a (possibly direct) interaction between MA and EnvCT [39]. This interaction also serves to repress the fusogenicity of Env while on the surface of producer cells, again reducing the chances of syncytium formation [40]. Furthermore, Gag recruits host factors to the assembly site which are then incorporated into virus particles. Of particular relevance to our work are the tetraspanins (further discussed in Section 1.6), which accumulate at HIV-1 budding sites [41,42], are trapped by Gag [43], and are incorporated into virus particles (more specifically, the tetraspanins CD9, CD53, CD63, CD81, CD82, and Tetraspanin-14) [44,45]. The Gag assembly platform therefore serves to organize the membrane and recruit viral and host proteins, but it also ultimately orchestrates virus release.

Like many enveloped viruses, the C-terminal-most domain of HIV-1 Gag, p6, contains two “late domain” signals which recruit the proteins Tsg101 (the PTAP signal), and AIP1/ALIX (the LYPx(n)L motif). This results in assembly of the cellular ESCRT machinery, which is co-opted by HIV-1 to drive membrane scission, i.e. virus particle release (reviewed in [46] and [32]). Within virus particles, either concomitant with or shortly after release, the viral protease (PR) becomes active and follows a well
characterized sequence of cleavage events, a process known as maturation. The result of maturation is the formation of the viral core, which has an outer shell made of the CA domain of Gag, and contains the two copies of the viral genomic RNA and the associated NC, RT, and IN proteins. Another result of maturation is the relief of the interaction between MA and EnvCT, which in turn renders Env more fusogenic [47-49], and allows Env trimers to become more mobile in the virion envelope and accumulate at one pole of the virion [50], likely facilitating stronger CD4 binding and entry into target cells. Maturation also alters the biophysical properties of the virion, reducing its stiffness and enhancing its propensity for fusion [51,52].

1.4. Latency and potential cures

HIV-1 establishes a productive infection most efficiently in activated target cells. However, when an infected T cell later reverts to a resting memory state (rather than dying off as the immune reaction declines), HIV-1 can establish a quiescent infection state known as latency (reviewed in [53] and [54]). The integration site preference for HIV-1 is thought to contribute to its ability to establish latency, due to several host factors which guide integration of the provirus to specific regions within the highly organized nucleus in which the state of chromatin could affect viral transcription [55]. An alternative model for latency centers around the idea that HIV-1 can stochastically establish either a productive or a latent infection in a relatively homogeneous cell population, rather than requiring infected cells to transition from an activated to a resting
state (reviewed in [56]). In either model, the state of chromatin is considered to be of key importance for allowing the virus to remain latent.

When a patient is treated with ART, the lack of viremia and productively infected cells prevents the immune hyperactivation which characterizes the untreated infection. It is this situation which most favors the establishment of latency, when a subset of infected T cells fall back to a resting state and can persist for months to years, even undergoing homeostatic proliferation without activation of the provirus. Upon cessation of ART, small reactivation events can result in reemergence of the virus from this latent reservoir, and return of the full-blown infection state. Therefore, ART is not curative of the infection, and in recent years researchers have pursued elimination of the latent reservoir as a potential true therapeutic, i.e. a cure for HIV-1 infection in people.

One strategy for combating the latent reservoir is the “shock-and-kill” approach [57], where ART treatment is ceased, and patients are treated with drugs that stimulate reemergence of latent viruses, allowing immune clearance of those infected memory T cells. Subsequent ART treatment would then prevent new infections, and the virus would be cleared altogether. Drugs which alter the chromatin state of latently infected cells have been shown to increase transcription of the virus in latently infected T cells, and clinical trials are underway to determine whether this approach could eliminate the latent reservoir and cure HIV-1 infection (reviewed in [55]).

In parallel, genome editing approaches are being investigated for the excision of the HIV-1 provirus from latently infected cells in order to eliminate the viral reservoir (reviewed in [58]). However, this strategy has been to some extent set aside in favor of
pharmacological chromatin modification, due to the availability of already approved drugs with such function (e.g. the histone deacetylase inhibitor vorinostat) that could be relatively easily applied to cure HIV-1 infection, and because genome editing technology has not yet reached sufficient levels of accuracy to allow their use in people without off-target effects.

Another potential application for genome editing towards achieving a cure is to mutate the CCR5 co-receptor in immune cells, which would render them non-permissive to HIV-1. This approach is based on the discovery of a rare allele of CCR5 which harbors a 32 base pair deletion (termed CCR5-Δ32) and codes for a non-functional protein that does not support HIV-1 infection [59] (for both R5- and X4-tropic viruses, as the mutated protein also has a secondary scavenging effect on CXCR4 [60]). CCR5-Δ32 was found to be capable of functionally curing an individual who was previously infected with HIV-1 (the so-called Berlin patient), who was treated for a leukemia with a bone marrow transplant from a donor who was homozygous for the CCR5-Δ32 allele [61]. The Berlin patient has since tested negative for HIV-1 without being treated with ART, making this the only known functional cure for the infection, though of course the patient needs to remain on anti-rejection drugs to retain the transplant. If an HIV-1-infected individual could have their own bone marrow stem cells rendered CCR5-mutant by ex vivo gene therapy (e.g. by excising a portion of the gene using zinc finger nucleases), and then having those cells returned to their body, they could achieve the same functional cure without the risk of transplant rejection as they would be receiving an autologous transplant (reviewed in [62]). This approach would also eventually lead to the elimination
of latently-infected cells, as ART would be ceased in those patients and the re-emerging productive infections from the latent reservoir would be cleared by the immune system, as seems to have occurred in the Berlin patient [63], though the chemotherapy and treatment for graft-versus-host disease that this patient received may have helped clear the reservoir.

1.5. Routes for infection of target cells

Target cells of HIV-1 can be infected through three main routes: when they are contacted by an antigen-presenting cell (APC), such as a dendritic cell, which harbors virus particles; when they stochastically encounter free virus particles in the milieu; or when they are contacted by a productively infected cell, such as a T cell or a macrophage, which then releases virus particles directly towards or near to the target cell.

APC-mediated transmission of HIV-1 is termed trans-infection, which reflects the fact that the cell delivering the virus to the target cell is not itself productively infected, but is merely carrying virus particles that are then shed towards target cells. Such events take place when transient adhesions known as infectious synapses [64] form between a virus-carrying APC and a target cell, and they have been observed to occur in a humanized mouse model for HIV-1 infection [65] (with the donor cells being CD169+ macrophages, which act as APCs, in that case), lending credence to the idea that trans-infection is a mode of spread that HIV-1 utilizes in vivo.

Transmission of HIV-1 to target cells upon encounter of virus particles in the milieu is also named cell-free infection, primarily to differentiate it from cell-to-cell
transmission (accordingly, the terms “cell-free virus” and “cell-associated virus” are also in use). Cell-free infection is the most extensively studied mode of transmission of the virus, modeled in vitro by simply incubating target cells with virus-containing cell culture supernatant, or concentrated and/or purified preparations of virus derived from supernatant. It remains unclear whether such experiments bear any resemblance to any transmission events that could take place in vivo (see also Section 3.3.4.).

Cell-to-cell transmission of HIV-1 (reviewed in [66]) takes place through transient adhesions, known as virological synapses (VSs) [67], which form between an infected cell and an uninfected target cell, and lead to the transfer of large amounts of virus. VS-mediated cell-to-cell transmission of HIV-1 between susceptible cells has been extensively documented in vitro (e.g. [68-70]). Live cell imaging has been of paramount importance for the understanding of cell-to-cell transmission, especially with the use of fluorescently-tagged virus strains that allow not only the tracking of infected cells, but the visualization of assembly platforms and released virus particles [69] (see also Chapter 3). Cell-to-cell transmission has also been found by various groups to shield transmitted viruses from drugs [71] and neutralizing antibodies (reviewed in [72]), which would otherwise prevent infection e.g. in the case of cell-free virus transmission. This shielding may be due to the simple fact that this mode of transmission allows for many more viruses to be taken up by the same target cell, so that there is a greater probability that a virus particle that happened by chance to be unaffected by the inhibitory agent could be transferred to the target cell [71], though this idea has been challenged as overly simplistic [73]. Alternatively, it has been proposed that VS-mediated transmission could
favor the uptake of viruses into endosomal compartments within the target cell, where they are protected from the inhibitor-containing extracellular milieu [70].

Although for other retroviruses, such as murine leukemia virus (MLV) [74] and HTLV-1/2 (reviewed in [75]), cell-to-cell transmission is widely accepted as the primary mode of transmission, this remains an open question in the case of HIV-1. The evidence for cell-to-cell transmission of HIV-1 in vivo or in an animal model is limited mainly to a study by the Mempel group, in which a humanized mouse model for HIV-1 infection (further discussed in Chapter 3), was utilized to determine that the motility of infected T cells is crucial for the spread of HIV-1 between cells in lymph nodes and to distal tissues, suggesting that shed extracellular (i.e. cell-free) virus is not sufficient to sustain spread of the virus, with the implication that cell-to-cell transmission is instead required [76]. Importantly, that report documents that a substantial fraction of infected T cells are small syncytia, meaning that infected cells formed Env-dependent stable contacts with target cells (i.e. VSs) that in some cases resulted in cell-cell fusion, but in other cases could have resulted in cell-to-cell transmission [76]. No evidence of cell-to-cell transmission was reported in the earlier mentioned study in which trans-infection of HIV-1 was documented in humanized mice [65].

The relative contribution of each of the above outlined modes of HIV-1 transmission during an in vivo infection is unknown. Recent computational modeling studies and inferences from in vitro experiments suggest that cell-free and cell-to-cell transmission may contribute roughly equally to overall HIV-1 spread [77-79]. However, no long-term population-level in vitro experiments addressing this question have been
carried out to date, and none of the above mentioned studies included or specifically considered APCs to also measure or model the contribution of trans-infection to overall spread.

1.6. Formation of syncytia

As introduced in Section 1.3, HIV-1 can induce cell-cell fusion between infected and uninfected cells to form multinucleated cells, or syncytia. HIV-1-induced cell-cell fusion was observed in T cell cultures very early on, and quickly became a convenient way to monitor the progression of infection, as a cytopathic effect that was likely an artifact of cell culture. Syncytium formation was also utilized to monitor whether mutant Envs were functional and to therefore elucidate the function of specific domains and residues in Env, and to develop neutralizing antibodies and fusion inhibitors whose intended function was not to prevent cell-cell fusion, but to prevent virus-cell fusion, i.e. infection of target cells, which was much more difficult to monitor. Furthermore, it seemed unlikely that HIV-1 induced the formation of T cell syncytia *in vivo* because of the numerous mechanisms (mentioned in Section 1.3) that are in place to prevent cell-cell fusion.

An additional mechanism for cell-cell fusion prevention in the context of HIV-1 involves tetraspanins (earlier mentioned in Section 1.3), a family of small transmembrane proteins which act as scaffolds, recruiting numerous partner proteins, and forming signaling platforms (reviewed in [80] and [81]). Tetraspanins have been known to play a role in membrane fusion processes for some time, e.g. by promoting sperm-egg fusion,
or by inhibiting macrophage fusion (reviewed in [82]). Because (as mentioned in Section 1.3) tetraspanins are incorporated into virus particles, the Koyanagi group tested their (specifically CD9, CD63, CD81, CD82, and CD231) effect on virus infectivity and found that it was strongly inhibited, specifically at the membrane fusion stage [83]. When considering the idea that infected cells likely frequently align with target cells (i.e. a “risky” setting where cell-cell fusion could, in theory, easily take place), and the fact that tetraspanins are enriched at virus assembly sites [41] and polarized at the VS along with Gag [42], the Thali group tested whether tetraspanins (CD9, CD63, and CD81) could inhibit cell-cell fusion as well as virus-cell fusion, and found that this was indeed the case [84]. Further, it was determined that the presence of Gag was required to allow tetraspanins to inhibit Env’s ability to fuse cells, which falls in line with the fact that Gag actively recruits and traps CD9 at assembly sites [43], along with Env [40].

Another aspect of the involvement of tetraspanins in HIV-1 replication is that tetraspanins are downregulated from the surface of infected T cells upon infection [85]. Tetraspanin downregulation upon HIV-1 infection is determined primarily by Vpu, which affects both surface and intracellular levels of tetraspanins, along with Nef, which only affects surface levels of tetraspanins [86,87]. Despite this overall downregulation, tetraspanins are still enriched at virus assembly sites, likely because they are trapped there by Gag [43], though it appears that Vpu and Nef are nevertheless able to affect tetraspanin levels within budding sites, as viruses deficient in one or both of those accessory proteins were shown to incorporate increased levels of CD81 into virus particles with a concomitant decrease in infectivity [87]. In summary, HIV-1 infection
reconfigures the tetraspanin profile of the cell, likely to achieve a balance between the negative effect of tetraspanins on virus infectivity, and the positive effect of tetraspanins in preventing cell-cell fusion and therefore allowing VS-mediated transmission.

In addition to tetraspanins, another host factor involved in preventing HIV-1-induced cell-cell fusion is ezrin, a protein of the ezrin-radixin-moesin family which is also enriched at virus assembly sites and polarized at the VS [88]. Ezrin serves to link transmembrane proteins (such as CD81) to actin, thus connecting the cytoskeleton to outside-in signaling platforms and enabling the cell to respond to its environment.

Taken together, numerous viral and host proteins and protein interactions prevent cell-cell fusion between HIV-1-infected and uninfected cells. Consequently, the finding that a significant minority of HIV-1-infected T cells in humanized mice were small syncytia [76] was very much unexpected. Although large syncytia derived from macrophage-T cell or dendritic cell-T cell fusions were noticed in infected individuals relatively early on [89,90], purely T cell-derived syncytia were considered to be solely occurring in culture and never in vivo. However, small T cell-derived syncytia were in fact reported in a widely ignored study [91], falling in line with the above mentioned finding in humanized mice [76]. The morphology of T cell syncytia observed in the lymph node of humanized mice, which is characterized by long and thin processes with nuclei located very distally from each other, would have made it very difficult for such entities to be found in two to three micron-thick tissue slices that are routinely used in pathologic examinations [92]. It therefore seems likely that the number of small T cell syncytia in HIV-1-infected individuals has been vastly underestimated, though the fact
that these syncytia stay small suggests that the mechanisms that prevent cell-cell fusion are nevertheless still operating.

1.7. Summary of results and conclusions

The work presented in Chapters 2 and 3 addresses questions relating to the formation and fate of syncytia upon HIV-1 infection. More specifically, Chapter 2 further explores the role of tetraspanins in the prevention of cell-cell fusion, and Chapter 3 asks whether small T cell syncytia can transfer virus to target cells through contact without cell-cell fusion.

Although tetraspanins have been recognized as modulators of cell-cell fusion for some time [82], in none of the known paradigms was the specific action of tetraspanins on the membrane fusion process elucidated. The cascade of events leading up to and following membrane merger (see also Section 1.3) can be separated into distinguishable intermediates through methods such as temperature shifts, drug treatments, or monitoring the transfer of lipophilic dyes between cells [18]. Therefore, in Chapter 2 we employed a small panel of fusion inhibitors which targeted specific stages of HIV-1-induced fusion, and asked whether the inhibition profile of any of those inhibitors could be altered by overexpressing CD9 in producer cells. We found that sensitivity to dynasore, an inhibitor of dynamin which was found to affect the pore expansion stage (a very late stage of fusion), was significantly reduced in CD9-overexpressing cells, meaning that the fusion-inhibitory action of CD9 operated prior to the pore expansion stage, but after all the other stages targeted with other fusion inhibitors. Furthermore, we observed the transfer of a
lipid dye from target cells to producer cells, while simultaneously measuring full fusion using a cytosolic dye, to examine whether the hemifusion intermediate (where only the outer leaflets of the membranes merge, without an open fusion pore) was affected by CD9 or CD63 overexpression. Strikingly, we found that tetraspanin overexpression allowed normal progression to the hemifusion intermediate, but prevented the completion of fusion beyond that stage, corroborating the results from the drug treatment approach.

In Chapter 3, in collaboration with the Mempel group, we investigated the prevalence and behavior of small T cell syncytia induced by HIV-1 infection. Because culturing HIV-1-infected T cells in classical culture, i.e. in liquid medium-containing vessels where the cells settle to the bottom and essentially experience a two-dimensional (2D) environment, usually results in the formation of large syncytia which do not resemble what was observed in the lymph node of HIV-1-infected humanized mice [76], we introduced extracellular matrix and developed a simple three-dimensional (3D) culture system. Upon doing this, we found that we could recapitulate those in situ observations, finding that T cell syncytia stayed small and exhibited unusual elongated morphologies as they do in the lymph node. We then performed live cell time lapse imaging of infected T cells in these 3D cultures, and strikingly found that small T cell syncytia could contact target cells, transfer virus to them, and finally separate from them without cell-cell fusion, much like uninucleated infected cells had already been shown to do [69]. Furthermore, we found that migrating infected cells could deposit dense
accumulations of virus particles into the milieu, and can envision that target cells migrating through such virus “clouds” could become infected.
1.8. References


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CHAPTER 2: EVIDENCE SHOWING THAT TETRASPININS INHIBIT HIV-1-INDUCED CELL-CELL FUSION AT A POST-HEMIFUSION STAGE

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2.1. Abstract

Human immunodeficiency virus type 1 (HIV-1) transmission takes place primarily through cell-cell contacts known as virological synapses. Formation of these transient adhesions between infected and uninfected cells can lead to transmission of viral particles followed by separation of the cells. Alternatively, the cells can fuse, thus forming a syncytium. Tetraspanins, small scaffolding proteins that are enriched in HIV-1 virions and actively recruited to viral assembly sites, have been found to negatively regulate HIV-1 Env-induced cell-cell fusion. How these transmembrane proteins inhibit membrane fusion, however, is currently not known. As a first step towards elucidating the mechanism of fusion repression by tetraspanins, e.g., CD9 and CD63, we sought to identify the stage of the fusion process during which they operate. Using a chemical epistasis approach, four fusion inhibitors were employed in tandem with CD9 overexpression. Cells overexpressing CD9 were found to be sensitized to inhibitors targeting the pre-hairpin and hemifusion intermediates, while they were desensitized to an inhibitor of the pore expansion stage. Together with the results of a microscopy-based dye transfer assay, which revealed CD9- and CD63-induced hemifusion arrest, our investigations strongly suggest that tetraspanins block HIV-1-induced cell-cell fusion at the transition from hemifusion to pore opening.
2.2. Introduction

HIV-1 enters target cells by fusing the viral lipid envelope with the cell membrane, allowing the release of the viral core into the cell (reviewed in [1]). The viral envelope glycoprotein Env, however, not only mediates fusion of viral and cellular membranes, but can also drive the fusion of the plasma membranes of infected and uninfected cells, thus giving rise to the formation of multinucleated entities, i.e., syncytia. HIV-1-induced syncytia are readily observable not only in cell culture systems but have also been documented in infected individuals [2-4] as well as in a humanized mouse model [5], though a quantification of how many infected cells exist as syncytia in vivo is not feasible. However, in vitro analyses as well as certain in vivo observations [6,7] suggest that, in the majority of cases, contacts between infected and uninfected cells, which can lead to particle transmission via the virological synapse ([8], and for a recent review, see [9]), dissolve without resulting in cell-cell fusion.

While, theoretically, virus dissemination through a succession of syncytia is possible because syncytia produce large amounts of progeny viruses [10], it has been established that syncytia tend to undergo apoptosis (reviewed in [11,12]). Also, establishment of latency (for a review, see [13]) is likely not possible in these short-lived syncytia. Thus, fusion regulation, beyond simply controlling proper timing of the viral entry process, may have evolved to ensure continued virus spread through particle transmission without cell-cell fusion. Indeed, by now, several ways by which HIV-1 regulates the fusogenicity of Env have been identified. These include: (a) the rapid internalization of newly synthesized Env from the surface of the infected cell (reviewed
in [14]); (b) an interaction between the cytoplasmic tail of the gp41 transmembrane domain of Env and the matrix domain of immature Gag, which strongly represses the fusogenicity of Env not only within the virion, but also already at the virological presynapse [15-19]; and (c) the active recruitment of tetraspanins to viral assembly sites [20,21], where they repress cell-cell fusion [22] and, upon their acquisition by newly formed particles, virus-cell fusion [23,24]. An involvement of tetraspanins in the regulation of Env-induced membrane fusion should not be surprising, as these proteins have been shown to regulate numerous membrane fusion processes, including mammalian spermatocyte-oocyte fusion (reviewed in [25]), macrophage fusion [26,27], and myoblast fusion [28,29]. Indeed, a very recent report also implicates a tetraspanin in yet another virus-triggered membrane fusion event [30].

How tetraspanins regulate membrane fusion, in any context, is currently unknown. In order to pave the way towards understanding the mechanism of fusion regulation by these proteins as well as the involvement of potential cofactors, we sought to determine which stage of Env-induced fusion is affected by tetraspanins (see [31,32] for detailed descriptions of the HIV-1 Env-induced fusion reaction, and Figure 1 for a schematic of the steps involved). To achieve this, we monitored Env-induced cell-cell fusion while applying a panel of fusion inhibitors that operate at different stages of fusion in tandem with tetraspanin overexpression (i.e., a chemical epistasis approach), as well as by using an imaging-based fluorescent dye transfer assay.
2.3. Results and Discussion

2.3.1. Temporal Delineation of CD9-Mediated Fusion Repression Using Chemical Epistasis

Previously, we and others reported that overexpression of the tetraspanins CD9 and CD63 (as well as other members of this family of membrane scaffolds) in HIV-1-producing cells represses virus-cell and cell-cell fusion induced by Env [22-24]. In contrast, L6, a host transmembrane tetraspanin-like protein (also known as TM4SF1) has no such effect, despite the fact that it colocalizes with tetraspanins at the plasma membrane and is incorporated into HIV-1 particles [23,24,33]. Applying a chemical epistasis approach in order to identify the step in the fusion process affected by CD9 overexpression, we used four fusion inhibitors with known action (the steps of the fusion process they affected are delineated in Figure 1). The four inhibitors target: (1) the co-receptor engagement stage (the small molecule AMD3100, which blocks CXCR4); (2) the prehairpin intermediate (the C-peptide C34); (3) the hemifusion intermediate (the Abl kinase inhibitor Imatinib [34]); and (4) pore expansion (the small molecule dynamin inhibitor dynasore [34-36]). It should be noted that the fusion inhibitory action of Imatinib has only been reported once so far, and the underlying mechanism is not yet clear. Similarly, that dynasore affects pore expansion is a relatively recent finding. Consequently, the action of both inhibitors will thus likely be the subject of future scrutiny. Given, however, the lack of more established inhibitors of these stages of the fusion process, we decided nevertheless to use them here. Inhibitor concentrations were empirically determined in order to span a range of fusion inhibition (data not shown) and
five different concentrations of inhibitor in addition to a vehicle control were employed in each case.

Cell-cell fusion assays were carried out using the dual split protein (DSP) system developed by the Matsuda group [37,38], whereby fusion can be measured by the development of GFP fluorescence in syncytia upon bimolecular complementation. HeLa cells were co-transfected with HIV-1 pNL4-3 and L6 or CD9, and later co-cultured with TZM-bl cells, while fusion inhibitors were titrated in so that an inhibition profile could be obtained by fitting an inhibition model (as described in the Experimental section; Figure 2). The basal effect of CD9 overexpression on the level of fusion (typically a ~50% reduction in this assay, as shown in Figure 3B, inset) was eliminated by normalizing to the level of fusion when vehicle alone was used. The inhibitory concentrations leading to 25% or 50% inhibition (IC$_{25}$ or IC$_{50}$) for each inhibitor could then be compared between the L6 and CD9 overexpression treatment. Possible outcomes of such experiments are depicted in Figure 2 and described in the following. If there was no difference in IC$_{25}$ or IC$_{50}$ between L6 and CD9, we would conclude that the inhibitor acted before CD9, or that the inhibitor was epistatic over CD9 (Figure 2, left panel). However, if the CD9 treatment exhibited a desensitization to the inhibitor (an increased IC$_{25}$ or IC$_{50}$), we would conclude that the effect of CD9 was epistatic over the inhibitor (Figure 2, middle panel). Furthermore, if sensitization was observed (a reduced IC$_{25}$ or IC$_{50}$), we interpreted that as the inhibitor again being epistatic over CD9, but the action of CD9 is in some way affecting the inhibitor’s action (Figure 2, right panel).
The results of our analyses are shown in Figure 3. The inhibition profile of AMD3100 within the L6 and CD9 treatments was not significantly different (Figure 3A), and we can thus conclude that CD9 regulates a stage that follows co-receptor engagement. Interestingly, CD9 overexpression led to significant sensitization to the C-peptide C34, indicating that a process which closely follows the formation of the pre-hairpin intermediate was affected by CD9 overexpression (Figure 3B). Similarly, significant sensitization to the Abl kinase inhibitor Imatinib, which has been shown to arrest fusion at the hemifusion intermediate [34], was observed upon CD9 overexpression (Figure 3C), strongly suggesting that CD9 inhibits the fusion process after the hemifusion intermediate has formed. Furthermore, and strikingly, when the dynamin inhibitor dynasore (which impedes pore expansion [36]) was titrated, CD9 overexpression led to significant desensitization to this inhibitor (Figure 3D), showing epistasis of CD9 over dynasore in fusion repression. Together, these results allowed us to conclude that CD9 regulates a stage of fusion concurrently with or after lipid mixing and prior to pore expansion.

2.3.2. Tetraspanin Overexpression Leads to Accumulation of the Hemifusion Intermediate

Based on the results shown in Figure 3, which indicate that tetraspanins act at a post-hemifusion stage, we sought to further test this hypothesis using a different, more direct approach. We employed an imaging-based fluorescent dye transfer assay [39,40], whereby target Jurkat T cells labeled with the cytosolic dye CMAC and the fixable lipophilic dye Vybrant CM-DiI were co-cultured with producer HeLa cells transfected
with pNL4-3Gag-iGFP and a tetraspanin or the L6 control. After 5 h of co-culture, excess unbound target cells were washed off, and the cultures were imaged after fixation. Gag-iGFP-positive cells were counted, and the proportion of those which were positive for CM-DiI alone was taken as indicative of reactions that had reached the hemifusion stage, while the proportion which was positive for both CM-DiI and CMAC (visible as an additional nucleus in the syncytium and corresponding to a gap in the GFP channel) was taken as quantification of full fusion or syncytium formation. Example images of each of these cases are shown in Figure 4A.

As expected, reduced levels of full fusion (measured as the number of CMAC-positive nuclei in infected syncytia divided by the number of infected cells) were consistently observed when CD9 or CD63 were overexpressed compared to the L6 or empty vector controls (Figure 4B). However, taking into account total fusion activity (hemifusions plus syncytia), we found a striking increase in the hemifusion proportion upon tetraspanin overexpression (Figure 4C). This indicates that tetraspanin overexpression allowed fusing cells to progress to the hemifusion intermediate, but arrested the process at a later stage, leading to a build-up of this intermediate. Notably, the total fusion activity upon CD9 or CD63 overexpression was no different than that in the L6 control (Figure 4D), meaning that the reduction in full fusion could be fully recovered at the hemifusion stage. This indicates that there is likely no pre-hemifusion repression effect of tetraspanin overexpression.

Taken together, the results of these experiments render strong support for the hypothesis that CD9 and CD63 regulate HIV-1-induced cell-cell fusion after the outer
membrane leaflets of the fusing cells have merged and prior to pore expansion. Considering potential mechanisms, we can envision two scenarios: tetraspanins may interact directly with viral or host factors which regulate pore stabilization and/or expansion; alternatively (or additionally), tetraspanins may modulate the mechanical properties of membranes in a way that renders fusion pore stabilization and/or expansion less energetically favorable. The former scenario may involve, for example, cytoskeletal and associated proteins (in unpublished data we find that depletion of ezrin in HIV-1 producing cells leads to enhanced Env-induced cell-cell fusion [41]). Support for the latter possibility comes from studies on the effect of virion stiffness on virus-cell fusion [42,43]; tetraspanins at sites of fusion may similarly increase the local stiffness of membrane (we are currently testing this hypothesis). The sensitization to C34 observed in our experiments (Figure 3B) would also be in line with this potential mechanism; that is, the action of C34 comes at a stage where the apposed membranes of the fusing cells are being brought closer using folding energy, which acts against not only the water molecules found in the intercellular space, but also against the stiffness of the apposed membranes. A stiffer membrane would conceivably slow down the zippering of the heptad repeat regions, thus allowing better opportunity for C34 to bind, leading to sensitization.

Irrespective of the precise mechanism of tetraspanin-mediated fusion repression, the hemifusion intermediate is an important facet of HIV-1 pathogenesis. Bystander cell killing, one of the mechanisms thought to contribute to T cell depletion, can be triggered by transient hemifusion between infected and uninfected cells, resulting in apoptotic
death of the uninfected cell (an event sometimes referred to as “kiss of death”, first described in [44] and reviewed in [45,46]). Hence, although tetraspanins are actively recruited by the virus [20,21] to prevent excessive syncytium formation and thus likely promoting efficient virus spread, by arresting Env-induced fusion at the hemifusion stage, these proteins may also be co-responsible for one of the key pathogenic features of HIV-1.
2.4. Experimental Section

2.4.1. Cells, Plasmids, Antibodies, and Reagents

The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: TZM-bl cells from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc, Jurkat clone E6-1 cells from Dr. Arthur Weiss. HeLa cells were obtained from Dr. Eric Cohen. HeLa and TZM-bl cells were maintained in DMEM supplemented with 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin. Jurkat T cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin. All experiments were conducted in media supplemented with 10% FBS and without antibiotics.

The following proviral plasmids were used: pNL4-3 and pNL4-3-ΔEnv (KFS) from Dr. Eric Freed (National Cancer Institute, Frederick, MD, USA); pNL4-3Gag-iGFP and pNL4-3-ΔEnvGag-iGFP from Dr. Benjamin Chen (Mount Sinai School of Medicine, New York, NY, USA). pCMV-Sport6 expression plasmids containing L6, CD9, or CD63 cDNA were provided by Dr. Yoshio Koyanagi (Kyoto University, Kyoto, Japan) and modified with a C-terminal HA tag by standard PCR-based cloning. Expression plasmids Rluc8-DSP\textsuperscript{1−7} and Rluc8-DSP\textsuperscript{8−11} used in the dual split protein assay were provided by Dr. Zene Matsuda (The University of Tokyo, Japan).

2.4.2. Dual Split Protein (DSP)-Based Cell-Cell Fusion Assay

HeLa cells were co-transfected with the HIV-1 pNL4-3 provirus, an overexpression plasmid (L6 or CD9), and a construct encoding DSP-1 (the N-terminal
half of GFP fused to the N-terminal half of Renilla luciferase 8; Rluc8). In parallel, TZM-bl cells, serving as target cells, were transfected with a construct encoding DSP-2 (the C-terminal half of the aforementioned split-Rluc8-GFP protein). 48 h later, the two cell types were co-cultured for 5 h in order for cell-cell fusion to take place. Upon fusion between virus-producing HeLa and target TZM-bl cells and mixing of their contents, the two parts of the DSP assemble through bimolecular complementation and the resulting syncytium develops green fluorescence, as well as the ability to cleave luciferase substrates (not utilized here).

Cell co-cultures were detached using Trypsin-EDTA and fixed using PBS/4% PFA before resuspension in PBS. Flow cytometry was performed on an LSR II cytometer (BD Biosciences) using a 488 nm excitation laser, with 100,000 events recorded per sample. Flow cytometry datasets were analyzed using FlowJo v.X.0.6 software [47]. The proportion of cells which were GFP-positive compared to an Env-deleted control (pNL4-3-ΔEnv) was taken as the rate of cell-cell fusion. This was multiplied with the mean GFP fluorescence intensity within GFP-positive cells (in order to account for multiple fusions within each syncytium) and normalized to the vehicle control within each treatment (L6 or CD9) at 100% fusion. Three to seven biological replicates were performed for each experiment, consisting of two technical replicates at each inhibitor concentration for each treatment.

2.4.3. Dye Transfer Cell-Cell Fusion Assay

A classical three-color dye transfer cell-cell fusion assay [39,40] was adapted for our purposes. HeLa cells were transfected with a fluorescently tagged HIV-1 provirus
(pNL4-3<sup>Gag−iGFP</sup> or pNL4-3-ΔEnv<sup>Gag−iGFP</sup>) and an overexpression plasmid (empty, L6, CD9, or CD63). 48 h later, transfected HeLa cells were co-cultured with Jurkat cells labeled with CellTracker Blue CMAC and Vybrant CM-DiI (Molecular Probes). After 5 h of co-culture, unattached Jurkat cells were washed off using PBS, and cells were fixed using PBS/4% PFA, before imaging on a DeltaVision deconvolution microscope (Applied Precision, Issaquah, WA, USA) using a 10× objective. Randomly selected fields were imaged, and later manually scored for the presence of GFP, CMAC, and CM-DiI within each cell. At least 900 infected cells were scored in each treatment across three biological replicates, each consisting of two technical replicates. Syncytium formation was measured as the proportion of GFP-positive cells which were also positive for CMAC and CM-DiI, normalized to the background rate of fusion in the ΔEnv condition. The full fusion index was measured by dividing the number of CMAC-positive nuclei found within GFP-positive syncytia by the total number of GFP-positive cells scored. Hemifusion was measured as the proportion of GFP-positive cells that also exhibited CM-DiI membrane labeling but no internalized CMAC, background-subtracted using the non-specific CM-DiI transfer observed in the ΔEnv control. Finally, hemifusion was also measured as a proportion of total fusion activity by dividing the number of hemifusions by the sum of hemifusions and syncytia.

2.4.4. Imaging

Fluorescence images shown in Figure 4A were acquired from cells prepared and co-cultured as described in the dye transfer assay, using the 60× objective on a DeltaVision deconvolution microscope (Applied Precision, Issaquah, WA, USA).
Images were deconvolved, Z-stacks were projected, contrast and gamma adjustments were made, and the same adjustment was used for all images displayed.

2.4.5. Statistical Analysis

Statistical tests and curve fitting were performed using GraphPad Prism version 6.01 for Windows (GraphPad Software, San Diego, CA, USA). Cell-cell fusion data using the dual split protein assay were analyzed by nonlinear regression using a standard variable slope inhibitor response model, and comparing the IC50 and IC25 values between conditions using an extra sum-of-squares F test. Three-color dye transfer fusion assays were analyzed by two-way analysis of variance and differences between conditions were evaluated using Fisher’s LSD Test. Correction for multiple comparisons was applied where appropriate. All error bars shown represent the standard error of the mean. Significance is indicated as follows: *: $p \leq 0.05$ **: $p \leq 0.01$; ***: $p \leq 0.001$. 
2.5. Conclusions

Tetraspanins regulate a wide variety of cell-cell fusion processes, including syncytium formation induced by HIV-1 Env. We found that overexpression of tetraspanins blocks HIV-1-driven fusion after hemifusion but before pore expansion. To the best of our knowledge, this is the first description of a virus-associated host cell factor which regulates cell-cell fusion at a post-hemifusion stage. As such, this finding also provides a basis for further studies aimed at elucidating how tetraspanins can negatively regulate membrane fusion processes, both viral and non-viral.

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2.7. Author Contributions

Menelaos Symeonides, Nathan H. Roy, and Markus Thali conceived and designed the experiments; Menelaos Symeonides performed the experiments and analyzed the data; Marie Lambelé cloned the HA-tagged tetraspanin plasmids; Menelaos Symeonides and Markus Thali wrote and revised the manuscript; All authors helped edit the manuscript. The authors declare no conflict of interest.
2.8. Figure Legends

Figure 1. Schematic of fusion steps, and stage of action of fusion inhibitors used, denoted as numbered red circles: (1) AMD3100; (2) C34; (3) Imatinib; and (4) dynasore. Schematic key: Outer membrane leaflets—blue; Inner membrane leaflets—green; HIV-1 Env gp120—yellow, gp41—brown (cytoplasmic tail not shown); CD4—orange; CXCR4—grey; Tetraspanins—red.

Figure 2. Chemical epistasis approach; possible outcomes of tandem tetraspanin overexpression with fusion inhibitor titration.

Figure 3. Chemical epistasis experiments using fusion inhibitors AMD3100 (A); C34 (B); Imatinib (C); and dynasore (D) in virus-producing HeLa cells overexpressing L6 or CD9 co-cultured with TZM-bl target cells. Fusion levels were quantified using the DSP fusion assay. The basal effect of L6 and CD9 overexpression on fusion (shown here as an inset in B; data taken from the same experiment) was equalized by normalizing to vehicle. The data points were fitted to a classical inhibition response model and the inhibitor concentrations leading to 25% inhibition (IC$_{25}$) or 50% inhibition (IC$_{50}$) were compared between L6 and CD9 for each inhibitor. Shown are the only comparisons which exhibited a significant difference.

Figure 4. Accumulation of the hemifusion intermediate caused by tetraspanin overexpression. (A) HeLa cells transfected with pNL4-3$^{Gag−iGFP}$ and the indicated
plasmids were co-cultured with CMAC and CM-DiI-labeled Jurkat cells, fixed, and imaged. The three possible outcomes of cell-cell contact are shown as examples. Inset in Hemifusion/CM-DiI panel is contrast-enhanced. Bar = 10 μm; (B) Quantification of full fusion. The number of CMAC-positive nuclei found within GFP-positive syncytia was divided by the number of GFP-positive cells and normalized to the empty vector control; (C) Quantification of hemifusion (cells positive for GFP and CM-DiI, but not CMAC) as a percentage of the sum of hemifusions and syncytia; (D) Total fusion activity measured as the sum of hemifusions and syncytia, normalized to the empty vector control. Statistical comparisons made against L6, except where denoted by a horizontal bar.
2.9. Figures

Figure 1: Schematic of fusion steps, and stage of action of fusion inhibitors used.
Figure 2: Chemical epistasis approach.
Figure 3: Chemical epistasis experiments using fusion inhibitors.
Figure 4: Accumulation of the hemifusion intermediate caused by tetraspanin overexpression.
2.10. References


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CHAPTER 3: HIV-1-INDUCED SMALL T CELL SYNCYTIA CAN TRANSFER VIRUS PARTICLES TO TARGET CELLS THROUGH TRANSIENT CONTACTS

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3.1. Abstract

HIV-1 Env mediates fusion of viral and target cell membranes, but it can also mediate fusion of infected (producer) and target cells, thus triggering the formation of multinucleated cells, so-called syncytia. Large, round, immobile syncytia are readily observable in cultures of HIV-1-infected T cells, but these fast growing “fusion sinks” are largely regarded as cell culture artifacts. In contrast, small HIV-1-induced syncytia were seen in the paracortex of peripheral lymph nodes and other secondary lymphoid tissue of HIV-1-positive individuals. Further, recent intravital imaging of lymph nodes in humanized mice early after their infection with HIV-1 demonstrated that a significant fraction of infected cells were highly mobile, small syncytia, suggesting that these entities contribute to virus dissemination.

Here, we report that the formation of small, migratory syncytia, for which we provide further quantification in humanized mice, can be recapitulated \textit{in vitro} if HIV-1-infected T cells are placed into 3D extracellular matrix (ECM) hydrogels rather than being kept in traditional suspension culture systems. Intriguingly, live-cell imaging in hydrogels revealed that these syncytia, similarly to individual infected cells, can transiently interact with uninfected cells, leading to rapid virus transfer without cell-cell fusion. Infected cells were also observed to deposit large amounts of viral particles into the extracellular space. Altogether, these observations suggest the need to further evaluate the biological significance of small, T cell-based syncytia and to consider the possibility that these entities do indeed contribute to virus spread and pathogenesis.
3.2. Introduction

Human immunodeficiency virus type 1 (HIV-1) primarily infects CD4+ T lymphocytes and macrophages. Acute infection is characterized by flu-like symptoms, and is followed by a long asymptomatic period, until finally developing into profound immune deficiency resulting primarily from CD4+ T cell depletion. The spread of virus between infected and susceptible target cells can take place via several pathways, including: release of cell-free virus particles which are then stochastically encountered by a target cell; sequestration of virus particles by dendritic cells and subsequent delivery of these particles to a target cell (a process termed trans-infection [1]); and the process of cell-to-cell transmission, whereby an infected cell directly interacts with a target cell, thus forming a transient adhesion structure known as the virological synapse (VS; [2,3]), which facilitates transfer of newly released viral particles. Such interactions are thought to occur most frequently in secondary lymphoid tissue, such as in lymph nodes and the gut-associated lymphoid tissue (GALT), where high cell density and migratory scanning behavior of T cells, facilitated by the architecture of the stromal environment, provide conditions conducive to controlled cell-cell interactions, including antigen presentation through the immunological synapse [4-7].

Cell-to-cell transmission of HIV-1 has been extensively studied for more than two decades (for recent reviews: [8,9]). While in vitro studies have long suggested that this mode is more efficient than cell-free virus transmission [10], it remained unclear why producer cells (which express the viral envelope glycoprotein, Env) would not automatically fuse with target cells (which express the viral receptor/coreceptors) once a
VS forms. However, various viral and cellular mechanisms/factors, including retrieval of Env from the surface of infected cells [11,12] and Env's interaction with immature Gag, which is known to repress Env's fusion activity in particles [13-17] and at the virological presynapse [18], have since been shown to help preserve the integrity of the VS by preventing producer-target cell fusion (for a discussion, see also [19]). Syncytia, which are multinucleated entities that form when Env-expressing (infected) cells fuse with target cells, were thus considered to be artifacts of cell culture and/or were thought to occur in infected individuals only if HIV-1-infected dendritic cells or macrophages occasionally fuse with target T cells. As will be described in the following, however, observations made in lymph nodes of HIV-1-infected humanized mice [20], together with two (largely ignored) earlier reports that documented lymphocyte-based small syncytia in secondary lymphoid tissue of infected individuals [21,22], forced us to reconsider the significance of HIV-1-induced T lymphocyte-based syncytia.
3.3. Results and Discussion

3.3.1. Quantification of HIV-1-induced small syncytia in lymph nodes of humanized mice

A considerable proportion of HIV-1-infected cells in the lymph node of humanized bone marrow/liver/thymus (BLT) mice exhibit elongated morphologies and reduced migration speed. Further, multiphoton intravital microscopy (MP-IVM) revealed that, surprisingly, a large fraction of these cells were syncytia [20]. To document this finding with more granularity, the number of discernible nuclei (revealed using an HIV-1 reporter strain that expresses EGFP fused to a nuclear localization signal, referred to as HIV-nGFP; see Figure 1A and [20]) and the instantaneous skeletal length of all infected cells in the lymph node were measured. As shown in Figure 1B, ~20% of infected cells are multinucleated with two, three, or four discernible nuclei (in decreasing frequency), and we did not observe any cells with five or more discernible nuclei during our imaging studies. However, it is possible that visualizing syncytia using HIV-nGFP may underestimate the number of nuclei in syncytia, since overlapping nuclei may appear as a single nucleus in some instances. Alternatively, larger syncytia may be more susceptible to apoptosis. Nevertheless, we conclude that HIV-1-induced syncytia are numerous in the lymph node, but remain small two days post-infection despite having demonstrated fusion competence. At a later time-point large syncytia develop occasionally [23], though they likely involve non-lymphoid cells and thus may not be purely T cell-derived.
We also sought to further quantify the unusual morphologies and behaviors adopted by HIV-1-induced (small) syncytia. As documented in Figure 1A and Movie S1, the nuclei within syncytia can be arranged either in a tightly clustered (coordinated) or in a scattered (uncoordinated) fashion, and dynamically switch between these two modes (see also [24]). This is reflected in the measurements of instantaneous skeletal length (Figure 1C), where the morphologies of syncytia with three or four nuclei appear to stratify into two groups: one of syncytia with short skeletal length (corresponding to coordinated forms, and resembling uninucleated cells), and one of syncytia with three to four times larger skeletal length (corresponding to an uncoordinated form). Syncytia with two nuclei exhibited much more continuous range of sizes and shapes, suggesting that nuclei in these entities were better able to decouple from each other.

Despite their varied morphologies, small syncytia have one thing in common: their nuclei are often not situated in close proximity (within a few microns) to each other (see also Figure 1D-E for further quantification). Consequently, if such entities were to form in the lymph nodes of HIV-1-infected individuals, most of them would not be detected using methods typically used to visualize infected cells in lymphoid tissues, such as immunocytochemistry of thin (three to five micron) sections. Under these conditions, only small syncytia that happen to be in the plane of the tissue slice and in a small compact shape when the tissue is fixed would become discernable as syncytia. Thus, it is possible that small HIV-1-induced syncytia are much more frequent within infected lymphoid tissues than previously thought [21,22].
Due to the high prevalence of small syncytia (two to four nuclei, but not larger) in the lymph node, we asked whether they continue to undergo fusion with target cells, or rather, if they can transiently contact uninfected T cells (i.e. without fusing with them). To test this, we imaged the interactions between HIV-1-induced syncytia and adoptively transferred CellTracker Orange-labeled uninfected central memory-like T (T\textsubscript{CM}) cells in the lymph node of humanized mice using MP-IVM. We found that syncytia typically formed transient contacts (no longer than two minutes) with uninfected T cells that did not lead to fusion (Figure 1F and Movie S2). These transient contacts show that the observed HIV-1-induced T cell syncytia are not merely “fusion sinks” [25], as is typically observed when T cells are grown in 2D culture.

3.3.2. \textit{In vitro} recapitulation of HIV-1-induced small T cell syncytia

Given the surprisingly high proportion of syncytia in the lymph node of HIV-1-infected humanized mice and, as discussed above, possibly also in HIV-1-infected individuals, we began to further investigate the formation, morphology, and behavior of HIV-1-induced syncytia within reductionist cell culture systems that permit live cell imaging at (relatively) high spatial resolution. However, syncytia grown in traditional cell culture systems, such as suspension culture, do not display the elongated morphologies that we observed in the lymph node of humanized mice ([20] and Figure 1), but rather remain rounded and can consist of dozens to hundreds of nuclei. Further, while a small series of \textit{in vitro} studies published two decades ago showed that large T cell syncytia can display behavior that is reminiscent of that of infected individual cells [26-29], those syncytia contained thousands of nuclei and thus obviously are not
representative of the small syncytia observed in lymph nodes either. As documented, however, in recent studies and as discussed in commentaries [30-32], T cells grown in three-dimensional (3D) cell culture systems, such as hydrogels composed of ECM, better reflect their *in situ* behaviors [6,33] compared to those grown in suspension culture, likely because distinct ECM tracks, structurally similar to the fibroreticular networks in lymph nodes, provide guidance structures for migrating lymphocytes in those *in vitro* systems [34]. We therefore started using 3D ECM hydrogels for our experiments and, as will be described in the following, immediately noticed profound differences in the appearance of syncytia, compared to syncytia observed in 2D culture.

When HIV-1-infected primary CD4+ T cells or CEM-SS T cells were cultured in 3D ECM hydrogels composed of either Matrigel or human collagen type I, the syncytia that formed were typically small (2-5 nuclei), and exhibited dynamically changing morphologies, including very elongated forms that have not been observed in 2D cultures (Figure 2A-B, Movies S3-S4 and S6-S9). The migratory behavior of infected T cells, whether uninucleated or multinucleated, in 3D cultures was also different, with cells showing directed amoeboid (non-adhesive) migration (Figure 2C and Movie S5), as already documented by others in detail for uninfected lymphocytes [31,35,36]. When quantifying the rate of syncytium formation, we also found that the number and size of syncytia tended to be reduced in 3D compared to 2D culture (data not shown), but these analyses will need to remain preliminary while we further investigate the contribution of factors that likely impact fusion, such as cell density or ECM composition. Still, during the development of 3D ECM hydrogel cultures, we already made several observations
(which we do not document here, however) that we think should guide further development of this *in vitro* system. First, collagen better facilitated fast and directed amoeboid migration compared to gels composed of Matrigel, whereas cells projected large pseudopodia more frequently in Matrigel than in collagen. Second, virus release, seen as large accumulations of extracellular Gag-iGFP puncta (to be discussed later, in 3.3.4) was enhanced in Matrigel compared to collagen. Third, primary CD4⁺ T cell syncytia were more elongated than CEM-SS syncytia, but syncytia of both cell types exhibited switching behavior.

Importantly, while the current *in vitro* studies of small HIV-1-induced syncytia need to remain qualitative in nature, for the reasons outlined above, the experiments shown in Figure 2 and Movies S3-S5 nevertheless already provide additional relevant new information about HIV-1-induced syncytia: First, due to the presence in humanized BLT mice of multiple human immune cell lineages, including macrophages and dendritic cells (which can undergo fusion with HIV-1-infected T cells), we could not yet state with confidence that the observed small syncytia (in humanized mice) are resulting purely from homotypic T cell-T cell fusions, and whether this might explain differences noted between this model and the classical T cell culture system. Now, using only T cells in 3D culture, we observe very similar phenotypes, which strongly suggests that the small syncytia observed in situ are indeed also purely T cell-based. Second, in the 3D culture system, cells were infected with an X4-tropic HIV-1 (NL4-3Gag-iGFP), whereas R5-tropic virus was used in the humanized mouse experiments, demonstrating that the phenotype of these syncytia is not determined by virus tropism, but by the environment the cells
experience. Third, whereas a fully replicative virus was used in the humanized mouse experiments, with or without the inclusion of nGFP to mark nuclei, in the 3D culture experiments we utilized the Gag-iGFP-tagged virus which is not fully replicative and where the nuclei are instead marked by the absence of GFP signal due to the exclusion of the marker from the nucleus. We can therefore corroborate the observations made using nGFP-tagged virus with the use of another reporter system to mark the nuclei, and show that these syncytia form during the first round of infection and likely are not a consequence of genetic drift or adaptation to the particular cells utilized in either study.

Overall, these in vitro experiments demonstrate that the migratory behavior and multinucleated cells closely resemble those that are found in the lymph node of humanized mice (Figure 1). Although additional modifications to this model, for instance through addition of stromal and dendritic cell populations, would further recapitulate the complex intercellular interactions observed in lymphoid tissues [37], we conclude that even a very basic 3D experimental system much better reflects the consequences of viral infections than traditional (2D) cell culture systems (see also [32]).

3.3.3. Small T cell syncytia can transfer viral particles to target cells through transient contacts

Thus far, syncytia have been thought of as merely cytopathic features of HIV-1 infection, exaggerated in the artificial environment of 2D cell cultures, where syncytia continue to fuse with uninfected cells [29] until they die, likely due to excess karyogamy (nuclear fusion within the syncytium) [38]. Given our data showing that syncytia actually stay small in lymphoid tissues of humanized mice (and likely in infected individuals)
shortly after infection, we reasoned that they may contribute to virus dissemination by transmitting virus.

To then start addressing this question, i.e. whether syncytia, specifically the small, highly mobile ones that we now can recapitulate in vitro, can transfer viral particles without fusing with target cells, we performed live cell time-lapse microscopy studies in the 3D ECM hydrogel system, using T cells infected with HIV-1 NL4-3Gag-iGFP [39]. The GFP tag inserted between the MA and CA domains of Gag, flanked by protease sites, allows for the visualization of assembly and polarization of Gag in infected cells as well as the subsequent release of virus particles followed by transfer to uninfected target cells. Because this virus is not fully replicative, we did not seek to evaluate whether virus transfer events result in productive infection of target cells (though we have no reason to assume that they would not, if fully competent virus is utilized; see below for further discussion), but instead focused on whether we could observe contact-mediated virus transfer and subsequent separation of producer and target cell without the formation of a syncytium. Because interactions between HIV-1-infected and uninfected T cells have not previously been examined in 3D cultures per se, we also tracked uninnucleated infected cells to study how cell-to-cell transfer occurs under conditions that more closely recapitulate their in vivo behaviors.

Strikingly, very similar virus transfer events were observed when either uninnucleated or multinucleated infected cells were viewed (Figure 3A, left and middle, and especially Movie S6 where both types of events occur almost simultaneously in the same field). In both cases, transfer of virus to target cells, which can be discerned in time-
lapse movies (Movies S6-S8), is reflected in a relatively sudden increase in fluorescence intensity observed (Figure 3A, left and middle) and measured (Figure 3B, red trace) on the recipient cells. The sudden increase contrasts with a much more gradual increase that can be observed (Figure 3A, right) and measured (Figure 3B, green trace) in cells that became infected at an earlier, unseen time point, and eventually (likely 12-18 h later; [39]) started to newly synthesize Gag-iGFP as part of a productive infection at the time of imaging. Further, the two distinct types of events can be identified by the localization and nature of the Gag-iGFP signal: Firstly, upon virus transfer, material is deposited onto the surface of target cells (Figure 3A, left and middle), in contrast to newly synthesized Gag-iGFP, which appears only within the cytoplasm (Figure 3A, right). Secondly, the punctate nature of transferred Gag-iGFP (Figure 3C, red trace) is clearly distinct from the much more diffuse signal (likely corresponding to GFP that was intracellularly cleaved out of the tagged protein) seen when Gag-iGFP is newly synthesized within the cell (Figure 3C, green trace). These patterns closely match the original report of this tagged virus strain by the Chen group [39].

A defining feature of transfer events was that producer and target cells showed directed motility before or after (or even during) the moment of transfer (somewhat reminiscent of kinapses; [40]). Additionally, the Gag puncta (which we interpret to be released virus particles) transferred upon contact seemed to rapidly distribute to other uninfected cells that were already in contact with the uninfected cell closest to the infected cell (Movies S6–S7). In one case (Movie S8), the infected syncytium was seen to “drag” the previously immobile uninfected cell across the imaging field, and then
“drop” it while migrating away, leaving Gag puncta on its surface. Overall, such dynamic Transfer events have not previously been observed. They clearly differ from what appeared to be more static contacts between infected and uninfected cells during which virus transfer has been documented so far [39]. Most importantly though, syncytia have not previously been reported to transfer virus to target cells by contact, or even to merely be able to contact an uninfected cell without fusing with it.

3.3.4. Pools of cell-free virus particles are deposited by migrating HIV-1-infected T cells

Intriguingly, besides observing particle transfer upon cell-cell contact, we also found distinct accumulations of densely packed virus (released by infected cells) in what appeared to be pockets that had formed within the ECM hydrogel (Figure 4 and Movie S9; also see the beginning of Movie S7). We have also observed cells depositing a trail of released Gag puncta in their wake as they migrated through the ECM (from a uninucleated cell in Movie S6, and from a syncytium in Movie S9). Although the accumulations appeared to dissipate a few minutes after they were visible (data not shown), it was not clear whether this was due to photobleaching or whether the virus particles had diffused away.

The significance of these observations (the deposition of free virus by small syncytia) remains unclear, but it would seem quite likely that potential target T cells, when they encounter such deposits, could become infected. Notably, a recent electron tomography study of the GALT documented comparable dense pools of free virions between cells (but apparently not in clefts of VSs) in HIV-1-infected humanized mice.
Together, these observations suggest that free viral particles may contribute more to virus spread than expected based on numerous in vitro studies which concluded that cell-free virus is inefficiently transmitted to target cells. All those in vitro analyses, however, were performed in traditional systems, i.e. by incubating target cells in medium containing suspended virus particles, or even with experiments where cultures of cells are (gently) shaken [42]. Obviously, pools of virus, as they can be found in our 3D system and in stroma of lymphoid tissue in humanized mice [41], would not form under those conditions, and potential target cells would not be exposed to locally concentrated virus. This may have led us to underestimate the relative contribution of cell-free HIV-1 infection to virus spread (e.g. [43-45]).

Of note, though, we have thus far observed these cell-free virus accumulations only in Matrigel hydrogels, and not in collagen hydrogels. However, both ECM models have limitations: Matrigel is derived from murine sarcoma cells which secrete a very complex basement membrane-like ECM that does not closely model the lymph node stroma (though it includes collagen type I), whereas the collagen utilized here was pure recombinant human collagen type I alone, which is a much more simplistic ECM that also does not accurately represent the lymph node. Therefore, we cannot conclude that one or the other model is more appropriate, and some of the events (such as the cell-free virus accumulations) may be influenced by the composition of the ECM.
3.3.5. Perspectives: A role for small T cell syncytia in HIV-1 spread and/or pathogenesis?

This communication establishes that small HIV-1-induced T cell syncytia, as they occur to a significant extent in lymphoid tissue of humanized mice and likely in infected individuals, can be recapitulated in simple 3D cell culture systems. It also demonstrates that such small syncytia can transfer virus to uninfected cells upon transiently contacting them, and it reveals that HIV-1-infected T cells (uninucleated and multinucleated) deposit pools of particles that likely would be able to infect cells as they migrate through those accumulations.

Given their emergence early in the infection process (as documented in humanized mice; [20] and Figure 1), it seems likely that small HIV-1-induced syncytia significantly contribute to virus spread and, equally importantly, that they contribute to pathogenesis (e.g. by triggering bystander killing through pyroptosis [46,47]. Confirming (or refuting) these hypotheses, however, necessitates extensive additional studies in humanized mice and/or the implementation of imaging techniques that allow quantitative, long-term analyses at the cell population level in 3D cultures. In conclusion, we propose that our view of what constitutes a potentially propagative or pathogenic HIV-1-infected entity should include not only uninucleated cells but also small, T cell-based syncytia.
3.4. Experimental Section

3.4.1. Cells, Plasmids, and Reagents

The following reagent was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: CEM-SS cells (Cat. #776) from Dr. Peter L. Nara [48-50]. CEM-SS T cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin. HEK293T cells were maintained in DMEM supplemented with 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin. All experiments were conducted in media supplemented with 10% FBS and without antibiotics.

Human primary CD4+ T cells were isolated from whole blood of a healthy donor by ficoll separation and subsequent negative selection (Miltenyi Biotech Cat. #130-091-155). Cells were activated with 5 μg/mL phytohemagglutinin in the presence of 50 units/mL interleukin-2 in RPMI 1640 supplemented with 10% FBS and antibiotics. After 24 hours, cells were washed and cultured in the same medium containing interleukin-2 and without phytohemagglutinin. Cells were used for infections 24 to 48 hours later.

The proviral plasmid NL4-3Gag−iGFP [39] was obtained from Dr. Benjamin Chen (Mount Sinai School of Medicine, New York, NY, USA). An R5-tropic pLeG-nef+ reporter strain (on the NL4-3 proviral backbone) that expresses EGFP with an N-terminal nuclear localization signal (termed HIV-nGFP) was used to visualize the nuclei of syncytia in vivo, as previously described [20]. nGFP is enriched in all nuclei of HIV-1-induced syncytia [20], and their numbers were determined by discrete increases in GFP intensity, based on an 80% of maximum fluorescence intensity threshold.
Matrigel (Cat. #354234) was purchased from BD Biosciences (Sparks, MD, USA). Human collagen type I solution (VitroCol; Cat. #5007) was purchased from Advanced BioMatrix (San Diego, CA, USA)

3.4.2. Intravital multiphoton microscopy and image analysis

MP-IVM of HIV-1-infected cells within the popliteal lymph node of BLT mice [51] was performed as described previously [20]. A MaiTai Ti:sapphire laser (Newport/Spectra-Physics) was tuned to 920 nm for optimized excitation of the fluorescent probes used. Intravital movies (S1, S5) are maximum intensity projections of 11 optical sections (512 x 512 pixels) with 4 μm Z-spacing, taken every 15 seconds to provide imaging volumes of 40 μm in depth. Imaging depth was typically confined to 80-150 μm below the lymph node capsule. 2D skeletal lengths were measured in ImageJ as the longest path connecting front and end of unbranched cells or as the sum of path lengths of all branches of multi-branched syncytia at each time frame (“instantaneous skeletal length”). The instantaneous skeletal length data shown in Fig. 1C were stratified into “high” and “low” skeletal lengths using a cutoff at 30 μm based on the evident bimodal distribution of the data in order to display means, but these were not used for any statistical tests. Nuclei within infected cells (using the HIV-nGFP reporter) were identified based on a discrete increase in fluorescent intensity, rendered using an 80% of maximum fluorescent intensity threshold, and enumerated at each time frame. To visualize cell-to-cell interactions, T_{CM} cells were generated in vitro, labeled with CellTracker Orange (CMTMR: Molecular Probes Cat. #C2927), and adoptively transferred into the footpad of BLT mice, as previously described [20].
3.4.3. Virus production and infection

Viruses were produced in HEK293T cells by cotransfection of pNL4-3\textsuperscript{Gag-iGFP} and pVSV-G using calcium phosphate precipitation (Invitrogen, Carlsbad, CA, USA). Virus-containing supernatants collected 48 h post-transfection were cleared of cell debris by centrifugation at 2000 rcf for 10 min, filtered through a 0.4 μm filter, aliquoted, and stored at -80°C.

To infect CEM-SS or primary CD4+ T cells with HIV-1 NL4-3\textsuperscript{Gag-iGFP}, 2×10\textsuperscript{6} or 5×10\textsuperscript{6} cells (respectively) were pelleted and resuspended in 450 μL of CO2-independent medium (Gibco, Grand Island, NY, USA) supplemented with 10% FBS, and 10 μL of virus-containing supernatant was added (resulting in ~20% of cells being infected by the time of imaging). The cells were gently shaken in round-bottom 5 mL tubes for 2 h at 37°C, then 3 mL of prewarmed RPMI 1640/10% FBS were added, the cells were pelleted, resuspended in 8 mL RPMI 1640/10% FBS, and incubated at 37°C/5% CO2.

3.4.4. Embedding of T cells in 3D ECM hydrogels

Note that the data presented in this report were acquired over several imaging sessions utilizing evolving (though obviously recorded) variations of the method described below, e.g. using different glass-bottom vessels, different hydrogel volumes, or different cell numbers. For simplicity, the method described here is the one which we found to present the fewest technical problems (e.g. poor hydrogel adherence).

Glass-bottom dishes (MatTek Corporation, Ashland, MA, USA) were first functionalized to enhance adherence of the hydrogel. The glass microwell was first treated with 0.1 N sodium hydroxide for 30 min at room temperature (RT), and
subsequently aspirated and air-dried. (3-Aminopropyl)trimethoxysilane (APTMS; Sigma-Aldrich Cat. #281778) was then spread onto the glass microwell and incubated for 3 min at RT, and aspirated before five washes with water for five minutes each on a plate shaker. After aspirating the final wash, 0.5% glutaraldehyde in water was added to the glass microwell and incubated for 1 h at RT. The dish was then washed five times as before, and the microwell was coated with dilute VitroCol solution (500 μg/mL in 0.01 N hydrochloric acid) overnight at 37°C. After one wash with water and three washes in PBS, the dish was covered with PBS and stored at 4°C (for no more than 48 h) until use.

To embed T cells in ECM hydrogels, cells at 24 to 48 h post-infection were counted, pelleted, and resuspended in ice-cold serum-free RPMI 1640 at an appropriate cell density. For the experiment shown in Movie S8, uninfected CEM-SS cells were labeled with CellTracker Blue CMAC (Molecular Probes Cat. #C2110) according to manufacturer guidelines, and then mixed with infected CEM-SS cells at a 1:1 ratio prior to centrifugation.

For Matrigel hydrogels, the cell suspension was mixed with Matrigel solution on ice to a final concentration of ~8 mg/mL and final cell density of 2 to 5 million cells/mL (CEM-SS) or 5 to 15 million cells/mL (primary CD4+). For collagen hydrogels, ice-cold VitroCol solution was neutralized to pH 7.0 using 0.1 N sodium hydroxide, and 10× PBS was added to achieve a final 1× concentration. The cell suspension was then mixed with the neutralized gel solution to achieve a final collagen concentration of 1.6 mg/mL and cell densities as above. The cold gel (Matrigel or collagen) suspension was then pipetted onto the functionalized glass microwell (300 μL for a 14 mm microwell in a 35 mm dish)
and was quickly placed into a 37°C/5% CO2 incubator. After 30 min, 1.7 mL of prewarmed culture medium was carefully pipetted onto the hydrogel, and the dish was placed back in the incubator for 24 h.

3.4.5. Live cell time-lapse imaging of T cells embedded in 3D ECM hydrogels

The medium covering the 3D ECM hydrogel was aspirated and replaced with prewarmed CO2-independent medium supplemented with 10% FBS. The glass-bottom dish was then placed on the microscope stage surrounded by a temperature-controlled enclosure preheated to 37°C with a beaker of water for humidity. Fluorescence and brightfield images were acquired using 20× or 40× air objectives on a DeltaVision microscope (Applied Precision, Issaquah, WA, USA) on an Olympus IX70 base with a Xenon light source and automated Z-axis control, point visitation, and time-lapse acquisition. Using Fiji 2.0.0-rc-41 [52], Z-slices were projected (by maximum intensity for fluorescence images, and by minimum intensity for brightfield images), intensity levels were adjusted, and gamma correction was applied where indicated. Quantification of mean fluorescence intensity and skewness (Fig. 3B-C) was performed using Fiji 2.0.0-rc-41 [52] by manually circling each cell at each time point as a region of interest, which was then exported using the Analyze/Measure tool. Any frames where a cell of interest was obscured by another cell were omitted from the analyses. Note that the times shown in Fig. 3 do not necessarily correspond to the times in Movies S6-S7, due to the realignment of the traces as described in the legend for Fig. 3, though the same realignment was used for Figs. 3B and 3C. The in vitro data shown in Figs. 2-4 were taken from six 18-24 h imaging sessions and a total of 55 imaging fields.
3.5. Conclusions

HIV-1-induced T cell-based syncytia so far have been considered short-lived artifacts of *in vitro* cell culture. Results presented in this study, however, show that when cultivated in 3D ECM hydrogels, which more closely resemble the environment in lymphoid tissue of infected individuals, HIV-1-infected T lymphocytes can form small syncytia that closely mimic those observed in lymph nodes of humanized mice (and possibly in secondary lymphoid tissue of infected individuals). Using this 3D *in vitro* system allows us also to document that such small T cell-based syncytia can transfer virus to uninfected cells. Further investigations of potential contributions to virus spread and pathogenesis thus are clearly warranted.

3.6. Supplementary Materials

The following are available online at http://www.mdpi.com/1999-4915/7/12/2959/s1.

Movie S1: HIV-1-infected cells in the lymph node of humanized mice

Movie S2: Syncytia in the lymph node contact uninfected T cells without undergoing cell-cell fusion.

Movie S3: CD4+ T cells in 3D culture form small syncytia with elongated morphology.

Movie S4: Small CEM-SS syncytia in 3D culture can dynamically change their morphology.

Movie S5: CD4+ T cells in 3D culture exhibit in vivo-like migratory behavior.

Movie S6: Uninucleated infected cells and syncytia can transfer virus to target cells without fusion.
Movie S7: A virus transfer event between a syncytium and two uninfected target cells.

Movie S8: Cell-to-cell transfer of virus can take place while cells are migrating.

Movie S9: Migrating infected cells can deposit a trail of released virus particles.

3.7. Acknowledgments

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3.8. Author Contributions

Menelaos Symeonides, Thomas T. Murooka, Thorsten R. Mempel and Markus Thali conceived and designed the experiments; Menelaos Symeonides and Thomas T. Murooka performed the experiments and analyzed the data; Menelaos Symeonides, Lauren N. Bellfy and Nathan H. Roy developed the 3D ECM hydrogel system; Menelaos Symeonides and Markus Thali wrote the manuscript; All authors helped edit and revise the manuscript.
3.9. Figure Legends

Figure 1. Morphology, frequency, and cellular interactions of HIV-1-induced syncytia in the lymph node. (A) Intravital micrographs of lymph node cells infected *in situ* with HIV-nGFP (day 2) reveal both individual infected cells and multinucleated syncytia. In the bottom panels, the nuclei of infected cells, whose location is identified by a discrete increase in fluorescence intensity, are rendered white, based on an 80% of maximum fluorescence intensity threshold. (B) Frequency of HIV-1-infected cells with increasing numbers of discernable nuclei. (C) Instantaneous skeletal lengths of individual infected cells and multinucleated syncytia. Red lines indicate means. A threshold length of 30 microns (dotted line) was used to differentiate between coordinate versus uncoordinated movements. Data from 8 movies recorded in two independent experiments are shown. (D) Representative traces of HIV-1-infected lymph node cells showing instantaneous skeletal lengths over time. (E) Percent of time an individual infected cell or a multinucleated syncytium displayed coordinated and uncoordinated movements, based on a skeletal length threshold of 30 microns. Data from 8 movies recorded in two independent experiments are shown. (F) 3D time-lapse image sequence of a transient cellular interaction between an HIV-1-induced syncytium (HIV-nGFP; green) and an uninfected T cell (CMTMR-labeled; red) in the lymph node of BLT mice. In the bottom panel, 3D cellular surface rendering shows the transient nature of the contact. Elapsed time in min:s. Yellow arrowheads indicate the interacting uninfected T cell. Scale bar = 15 μm. (G) Interaction times between HIV-1-induced syncytia and uninfected T cells. Data from 2 movies (n = 9) is shown. Red line indicates the median.
Figure 2. Small T cell syncytia form in HIV-1 NL4-3Gag-iGFP-infected 3D ECM hydrogel cultures and closely recapitulate in situ observations. (A) A syncytium with two nuclei in a collagen hydrogel culture of infected human primary CD4+ T cells (stills from Movie S3). (B) A syncytium with two nuclei in a Matrigel hydrogel culture of infected CEM-SS T cells exhibiting switching from uncoordinated to coordinated migration (stills from Movie S4). (C) A uninucleated infected human primary CD4+ T cell exhibiting directed amoeboid migration in a collagen hydrogel (stills from Movie S5). Scale bars = 30 μm. Elapsed times in h:min:s for (A) and (B), and in min:s for (C). Gag-iGFP signal is pseudocolored in green. Brightfield images are in shades of gray. In (A) and (B), the syncytium is shown without the brightfield image for the first and last stills for clarity.

Figure 3. Transfer of virus particles from HIV-1 NL4-3Gag-iGFP-infected uninucleated CEM-SS cells and CEM-SS syncytia to uninfected CEM-SS cells in 3D ECM hydrogels. (A) (Left); A syncytium with 3 nuclei transfers virus to target cells, one of which then migrates away (see also Movie S7). (Middle); A uninucleated infected cell transfers virus to three target cells (yellow arrowheads; see also Movie S6). (Right); An uninfected cell with Gag-iGFP puncta on its surface (asterisk) begins developing newly synthesized Gag-iGFP signal ~12 h later, while a cell it is attached to throughout this time that did not harbor Gag-iGFP puncta is not seen to develop Gag-iGFP signal. Scale bars = 30 μm (left and middle) or 10 μm (right). Elapsed time in h:min. Gag-iGFP signal is pseudocolored in green. Brightfield images are in shades of gray. To show transferred
virus particles more clearly, the areas indicated by yellow boxes are shown in white-boxed enhanced insets with 1.5× enlargement and a 0.6 gamma correction applied. (B) Average of the mean fluorescence intensities of five target cells exhibiting either transferred Gag-iGFP (red), or five other target cells developing newly synthesized Gag-iGFP from a previously unseen infection event (green). An uninfected cell is shown in grey. The individual traces were aligned according to the time where Gag-iGFP signal began increasing (which was the time of contact with the producer cell in all cases for the transfer traces; red arrow), and the averaged curves were manually normalized to baseline and smoothed (2nd order and 2 nearest neighbors). (C) The average skewness of the signal across these same cells was plotted. Skewness close to zero indicates evenly distributed, or hazy signal, while higher positive skewness indicates punctate signal. Error boundaries in (B) and (C) represent the standard error of the mean.

Figure 4. Infected cells deposit virus particles into pools within their surrounding stroma. (A) A CEM-SS syncytium in Matrigel culture releases a very dense accumulation of Gag-iGFP puncta into a cell-free pocket (area bounded by yellow box; still from Movie S7). (B) A CEM-SS syncytium in Matrigel culture migrates across the field, leaving behind a trail of dense Gag-iGFP puncta (area bounded by yellow box), shown also at 2× magnification and with brightness enhanced in a white-boxed inset (see also Movie S9). Scale bars = 20 μm. Elapsed time in h:min. Gag-iGFP signal is pseudocolored in green. Brightfield images are in shades of gray.
3.10. Figures

Figure 1: Morphology, frequency, and cellular interactions of HIV-1-induced syncytia in the lymph node.
Figure 2: Small T cell syncytia form in HIV-1 NL4-3Gag-iGFP-infected 3D ECM hydrogel cultures and closely recapitulate in situ observations.
Figure 3: Transfer of virus particles from HIV-1 NL4-3Gag-iGFP-infected uninucleated CEM-SS cells and CEM-SS syncytia to uninfected CEM-SS cells in 3D ECM hydrogels.
Figure 4: Infected cells deposit virus particles into pools within their surrounding stroma.
3.11. Supplementary Movie Legends

Movie S1 – HIV-1-infected cells in the lymph node of humanized mice. Humanized BLT mice were injected in the footpad with HIV-nGFP, where GFP is highly enriched in cellular nuclei, and the draining popliteal lymph node prepared for MP-IVM at day 2. Representative infected cells (GFP+; green) that display one, two or three discernible nuclei are shown (top). In the bottom panels, green fluorescence signals above 80% of the intensity maximum were used to define cell nuclei, which are shown in white. The syncytium with two discernible nuclei remains elongated throughout the recording, while the syncytium with three discernible nuclei switches between coordinated and uncoordinated motility. Each individual frame is a maximum intensity projection of 11 z-stacks spaced 4 μm apart (for a total volume of 40 μm). Time is shown in minutes and seconds. Scale bar = 20 μm. See also Figure 1A.

Movie S2 – Syncytia in the lymph node contact uninfected T cells without undergoing cell-cell fusion. In vitro-generated central memory CD4+ T cells, either infected with HIV-GFP (GFP+; green) or uninfected (labeled with CellTracker Orange; red), were adoptively transferred by footpad injection into BLT mice pretreated with antiretroviral drugs (100 mg/kg FTC, 150 mg/kg TDF). After 12 hours, the draining popliteal lymph node was prepared for MP-IVM. Two representative movies of T cell migration prior to (yellow circle) and during (blue circle) transient interactions with syncytia are shown, demonstrating cellular interactions without fusion. Each individual frame is a maximum
intensity projection of 11 z-stacks spaced 4 μm apart (for a total volume of 40 μm). Time is shown in minutes and seconds. Scale bar = 40 μm. See also Figure 1F-G.

Movie S3 – CD4+ T cells in 3D culture form small syncytia with elongated morphology. Primary human CD4+ T cells isolated from a healthy donor were infected with VSV-G-pseudotyped NL4-3\textsuperscript{Gag-iGFP} virus. The next day, cells were embedded in a 3D collagen gel as described in the Experimental Section, and 12 h later imaged live at 37 °C using a 20× objective on a DeltaVision widefield microscope. Six 3 μm-spaced z-slices were taken every 20 s, and were subsequently projected into one image. The syncytium seen here in green has two nuclei located at opposite ends, and a central bulged region with high amounts of viral Gag. The diffuse fluorescence is a result of this syncytium being located at a higher part of the gel, where the limitations of widefield imaging become more prominent. See also Figure 2A.

Movie S4 – Small CEM-SS syncytia in 3D culture can dynamically change their morphology. CEM-SS cells were infected with VSV-G-pseudotyped NL4-3\textsuperscript{Gag-iGFP} virus. The next day, cells were embedded in a 3D Matrigel gel as described in the Experimental Section, and 24 h later imaged live at 37 °C using a 40× objective on a DeltaVision widefield microscope. Seven 2 μm-spaced z-slices were taken every 5 min, and were subsequently projected into one image. A syncytium with two nuclei (dark areas within the cell in the GFP channel) begins with two lobes, which merge into a coordinated round
morphology as the cell begins to migrate through the gel and leaves the plane of focus. See also Figure 2B.

Movie S5 – CD4+ T cells in 3D culture exhibit *in vivo*-like migratory behavior. Primary human CD4+ T cells were infected, embedded in collagen, and imaged as in Movie S3 (though with a 10 s time lapse). The uninucleated infected cell migrating across the field moves by 108 μm over 560 s, for a mean velocity of 11.58 μm/min. Such fast directed amoeboid motility is not typically observed in classical 2D culture and requires the presence of a 3D ECM. See also Figure 2C.

Movie S6 – Uninucleated infected cells and syncytia can transfer virus to target cells without fusion. CEM-SS cells were infected, embedded in Matrigel, and imaged as in Movie S4. Virus transfer from a uninucleated infected cell (top) and a syncytium (bottom left) to a number of target cells (denoted by T) can be seen here. The uninucleated infected cell transfers virus to target cells T1-5, and the syncytium transfers virus to target cells T6-7. Images shown represent brightfield in gray (bottom right), or Gag-iGFP in green, shown either with normal scaling (bottom left, and merged with brightfield at top right), or with a 0.6 gamma correction applied and enhanced scaling to better show appearance of Gag-iGFP puncta on target cells (top left). Scale bar = 30 μm. A yellow arrow indicates the moment where the uninucleated infected cell begins transferring virus to target cells T1-3, and all of the other transfer events in this field are happening at roughly the same time. At this time, Gag-iGFP puncta appear to distribute between cells
T1-T3 in a progressive fashion, beginning from the point of contact with the infected cell (see also Figure 3A, middle panel). Cells T4 and T5 also receive virus particles from this infected cell, and T4 can be seen migrating away at the end. Also note a trail of released virus left behind by the uninucleated infected cell as it migrates from left to right from 08:00:00 to 09:10:00 (see also Movie S7, Movie S9, and Figure 4 for similar events in syncytia). The syncytium’s targets, T6 and T7, are already in intimate contact with it at the start of the movie, and are obscured by lobes of the syncytium. At the 10:45:00 mark, cell T6 breaks free from the syncytium, now harboring a large amount of virus particles on its surface, as the syncytium slowly migrates away, and cell T7 also appears to harbor virus particles on its surface by the final time point.

Movie S7 – A virus transfer event between a syncytium and two uninfected target cells. CEM-SS cells were infected, embedded in Matrigel, and imaged as in Movies S4 and S6. The syncytium shown here initially has two nuclei, but soon fuses with an uninfected cell and now has three clearly visible nuclei. It remains stationary for several hours, before beginning to migrate towards a pair of uninfected target cells (top). Very soon after contact, virus particles can be seen covering the surface of both cells, one of which eventually migrates away. See also Figure 3A (left panel). Note also another instance of a dense accumulation of cell-free virus particles in what appears to be a pocket within the hydrogel that the syncytium moves one of its lobes out of, revealing the deposited free virus, before it moves back into the pocket. See also Figure 4A.
Movie S8 – Cell-to-cell transfer of virus can take place while cells are migrating. CEM-SS cells were infected, later mixed with CMAC-labeled uninfected CEM-SS cells (shown in blue), embedded in collagen, and imaged as above. A syncytium with two nuclei (one of which bears CMAC signal, indicating that it formed recently and not before the infected culture was mixed with the labeled uninfected cells) migrates across the field. Its trailing edge contacts an uninfected CMAC-labeled cell, which is then dragged along with it, and finally dropped in the corner of the field. The target cell now bears virus particles on its surface, and is no longer in contact with the syncytium, which has moved into a different focal plane and stopped migrating (not shown). The image was refocused at the 10:30:00 mark to better show the target cell and the virus particles on its surface. Note that this movie also shows an instance of newly synthesized Gag-iGFP appearing in a previously uninfected cell (not the target cell contacted by the syncytium). This non-CMAC labeled uninfected cell appears in the bottom left of the field at 07:20:00 and exhibits steadily increasing diffuse intracellular signal, as documented in Figure 3A (right panel) and Figure 3B-C (green traces).

Movie S9 – Migrating infected cells can deposit a trail of released virus particles. CEM-SS cells were infected, embedded in Matrigel, and imaged as in Movies S4, S6, and S7. A syncytium with 3 nuclei switches into a coordinated morphology and begins migrating across the field. Released virus particles can be seen in its wake (also shown enlarged and with increased brightness as an inset). Shortly after the end of the movie, the cell-free virus accumulation appeared to dissipate (not shown), but it could not be determined
whether this was because of photobleaching or if they had in fact diffused away. See also Figure 4B.
3.12. References


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CHAPTER 4: PERSPECTIVES AND FUTURE WORK

4.1. Relative contribution of cell-to-cell transmission for HIV-1 spread

The predominant mode of transmission of HIV-1 has not yet been determined. For the deltaretroviruses HTLV-1/2, cell-to-cell transmission and APC-mediated trans-infection are thought to be essential for continued spread, while (in sharp contrast to HIV-1) cell-free HTLV-1/2 particles are poorly infectious and infected individuals do not exhibit significant viremia [1]. Beyond the retroviruses, a number of herpesviruses (such as herpes simplex virus, varicella zoster virus, and pseudorabies virus) are also thought to primarily rely on cell-to-cell transmission, as (amongst other lines of evidence) neutralizing antibodies do not impair spread, which again is not the case for HIV-1 [2].

Computational models and data from in vitro HIV-1 spreading infection assays indicate that cell-free infection and cell-to-cell transmission contribute about equally [3-5]. However, these experiments and the parameters used in those models should be repeated using a more relevant culture system, such as organotypic cell culture or even simple 3D cell culture. Furthermore, because the relative contribution of APC-mediated trans-infection is unknown (though it appears to play an important role in very early infection [6]), T cell monocultures will likely not suffice and other cell types will need to be included in such experiments in the future. Beyond those essential modifications to previous experiments, however, based on the findings presented in Chapter 3, the contribution of small T cell syncytia (and likely larger syncytia or syncytia derived from dendritic cells or macrophages) will need to be given consideration.
4.2. Syncytia as super-spreaders/super-killers

While, as documented in Chapter 3, Figure 3, transmission to target cells of virus from small syncytia is comparable to transmission from uninucleated cells, we could imagine that allowing the formation of small syncytia nevertheless is advantageous for the virus, for the reasons outlined in the following. Although cell-to-cell transmission ultimately creates a newly infected cell that can migrate independently and spread the infection with theoretically unlimited range, there is a long delay (12 to 18 hours; [7]) between the transfer event and the point at which the target cell becomes productively infected and can transfer virus to other cells. By contrast, because of the very rapid mixing of cell contents that occurs upon cell-cell fusion, and because of the ability of the lobes of syncytia to then migrate quite distally from each other, the formation of a syncytium could be a mechanism by which an infected cell can extend the range at which it can further spread virus very rapidly (on the order of minutes to hours). Simply by having a larger volume of cytoplasm (and therefore many more ribosomes) to foster increased viral protein synthesis from the same pool of viral mRNAs, as well as more plasma membrane to support Gag assembly platform formation, syncytia can increase virus production without the need of a complete virus replication cycle. However, there is a point beyond which too much cell-cell fusion is detrimental (apparently past four or five nuclei), where the syncytium likely becomes poorly mobile and experiences so much destabilization that it is at risk for apoptosis. Thus, cell-cell fusion regulation is likely in place to prevent the formation of large syncytia in vivo, which is in line with the fact that we can observe transient contacts between small syncytia and target cells.
Bystander cell killing is thought to be a major driver of CD4+ T cell depletion in HIV-1-infected individuals (reviewed e.g. in [8]). Such cytopathicity can occur either through abortive infection of target cells, resulting in caspase-1-mediated pyroptotic death [9,10], or by Env-dependent transient hemifusions between infected and uninfected cells (see also Section 4.3), which result in caspase-3-dependent but death receptor-independent apoptosis of the uninfected cell. Because of the ability of T cell syncytia to form multiple distal transient cell-cell contacts, these may not only be more efficient than uninucleate infected cells in transmitting virus but also in bystander cell killing.

### 4.3. Tetraspanins may enhance HIV-1-induced bystander killing

The fact that tetraspanins (and likely ezrin) repress fusion at a late (post-hemifusion) and not an early stage (e.g. attachment) could have important consequences for interactions between infected and uninfected target cells: Such cell conjugates can form hemifusions or small incomplete fusion pores which are then able to separate again (rather than becoming permanently “adhered” through a hemifusion diaphragm). The observations we and others have made (see [11] and Fig. 6 in [12]) suggest that transient hemifusions and even transient fusion pores do occur between T cells and do not result in productive virus transmission, but instead likely in bystander cell killing. These “kiss-and-run” events, which have been previously described [13-15], may be facilitated by tetraspanins (and possibly also ezrin). The aforementioned phenomena may then be a direct consequence of the ability of these fusion repressors to act at a post-hemifusion stage.
Although all the above mentioned studies were carried out using classical 2D culture, the fact that cells cultured in 3D ECM hydrogels show differences in cell-cell fusion leads us to ask how such phenomena manifest in more life-like conditions. Because hemifusion is an intermediate on the pathway to full fusion, and because full fusion is reduced in this culture system (and in vivo, as evidenced by the lack of large T cell syncytia in the lymph node of humanized mice), we hypothesize that transient hemifusions and reversible full fusions happen at an even higher frequency than was reported in those studies (which was already on the order of 10-20%). In unpublished experiments, we have observed the formation of open fusion pores and exchange of cytoplasm between producer and target cell, followed by migration of the former target cell lobe away from the site of fusion, resulting in complete separation of the two entities. Such directed migration (combined with the overall fusion repressive environment in 3D ECM hydrogels) would not be found in classical 2D cell culture (as discussed in Chapter 3), hence this phenomenon is unlikely to have been previously observed, and has to our knowledge not yet been documented. Further investigation into this phenomenon could potentially confirm the very intriguing (though largely ignored) basic cell biological finding that reversible hemifusion events take place with considerable frequency when cells expressing fusogens meet cells that express the cognate receptor [11].

4.4. Significance of extracellular virus accumulations

We observed that cells infected with HIV-1 NL4-3 Gag-iGFP release fluorescent material into the extracellular milieu while migrating (see Chapter 3, Figure 4). These
accumulations, seen as small Gag-iGFP punctae, can most simply be interpreted to represent virus particles, likely as a mixture of (diffraction-limited) single virions and larger clusters of several virions. Alternatively, this material could be made up of small membrane fragments shed from the infected cell that harbor Gag-iGFP, and would therefore not be infectious. These hypotheses can be tested using two strategies: firstly, these extracellular accumulations can be observed by live cell imaging, and if uninfected target cells happen to migrate through them, those cells can then be tracked to see if they “pick up” these putative virions, and some time later (without any other infectious events) become themselves productively infected. Secondly, infected cultures can be fixed, embedded, and sectioned for transmission electron microscopy, through which it can be determined whether these pools of Gag-iGFP have the appearance of HIV-1 virions, i.e. if vesicles ~100 nm in diameter containing pyramid-shaped electron-dense cores can be found in large numbers in pockets within the extracellular matrix.

4.5. Fusion enhancement as therapeutic strategy

HIV-1-induced cell-cell contacts can progress to the formation of a syncytium, a result which is mutually exclusive with both productive viral transmission and contact-mediated killing of bystander cells. Therefore, forcing the outcome of HIV-dependent contacts towards more cell-cell fusion (e.g. using drugs that specifically enhance HIV-1-induced cell-cell fusion) will reduce both virus transmission and bystander cell killing. Furthermore, larger syncytia may apoptose more readily, as well as stimulate innate and adaptive immune mechanisms that will ultimately help clear the infection. Interestingly,
it has been reported that syncytia induced by measles virus produce large amounts of Type 1 interferon [16] (possibly acting as a danger signal resulting from “unscheduled fusion” [17]) and become more prone to being killed by natural killer cells [18]. Also, syncytia formed by canine distemper virus are prone to apoptosis [19], and, as shown very recently, a glycoprotein mutant of varicella zoster virus which forms more syncytia (without affecting viral egress) has dramatically impaired viral spread in a xenograft model [20]. Importantly, in the case of HIV-1, we recently found (in collaboration with Reuben Harris) that some strains less able to fuse cells are better able to transmit from cell to cell (data not shown).

We will therefore test this paradigm in the context of HIV-1 infection using a panel of 12 compounds which enhance HIV-1 Env-mediated cell-cell fusion, identified by the Sodroski group in a screen aimed at isolating fusion inhibitors [21]. If our experiments confirm that, as for other viruses, viral spread is impaired by increased cell-cell fusion, this would lend merit to further advancing this therapeutic strategy in the future. Preliminary data shows that non-cytotoxic concentrations of one of those compounds significantly enhance syncytium formation while negatively affecting cell-to-cell transmission of HIV-1 in vitro.

Beyond affecting the outcome of VSs to reduce transmission events, enhancing cell-cell fusion could also combat the latent reservoir (see Section 1.4). Even the very low levels of Env expression in latently infected cells [22] (which were recently shown to be high enough to be useful as targets for antibodies that recruit cytotoxic T lymphocytes [23]) could in theory support cell-cell fusion in the presence of a fusion
enhancing drug. If a latently infected (i.e. resting) T cell fuses with a neighboring cell that happens to be activated, the newly formed syncytium would be overall more activated than the originally infected cell was, therefore bringing the virus out of latency. Alternatively, the cell-cell fusion event could be destabilizing enough to result in death for that syncytium, and therefore elimination of that latent virus.
4.6. References


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