On the whereabouts of HIV-1 **cellular** entry and its fusion ports

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Abstract

HIV-1 disseminates to diverse tissues through different cell types and establishes long-lived reservoirs. The exact cellular compartment where fusion occurs differs depending on the cell type and mode of viral transmission. This implies that HIV-1 may modulate a number of common host cell factors in different cell types. In this review, we evaluate recent advances on the host cell factors playing an important role in HIV-1 entry and fusion. New insights from **restriction factors** that inhibit virus-cell fusion in vitro may contribute the development of future therapeutic interventions. Collectively, novel findings underline the need for potent, host-directed therapies that disrupt the earliest stages of the virus life cycle and preclude the emergence of resistant viral variants.

HIV-1 infects different cell types

Tens of millions of people are infected with the human immunodeficiency virus type-1 (HIV-1) worldwide, and a similar number have died from the spectrum of acquired immune deficiency syndrome (AIDS) related diseases (Gallo, 2016). Despite the ability to administer anti-retroviral therapy (ART) to prevent HIV-1 morbidity and mortality in AIDS patients, this therapeutic approach does not eradicate the virus and remains inaccessible in many countries burdened with the majority of global infections (Medlock et al., 2017). Basic research focusing on the interaction of HIV-1 with numerous cell types is important to understand the mechanisms of virus persistence, transmission, and pathogenesis. Existing ART treatment lowers viremia to near undetectable levels but fails to eliminate HIV-1 from infected individuals, presumably due to the presence of **latent viral reservoirs** in CD4⁺ T cells, and other cell types (Boxes 1 and 2)(Gandhi et al., 2010). Indeed, other cell types are susceptible to HIV-1 infection, such as macrophages and other hematopoietic cells of the myeloid lineage (Simmons et al., 1997), and can also contribute to the establishment of chronic infection. While a well-established non-human primate model using Simian Immunodeficiency Virus (SIV) has revealed the importance of myeloid cell infection (Honeycutt et al., 2017; Laguette et al., 2012), a better understanding of how human tissue macrophages respond to HIV-1 infection is required to more effectively engineer strategies for virus eradication. The role of **dendritic cells** (DCs) in HIV-1 infection is clearly of interest, as they facilitate virus transmission to T cells despite being refractory to HIV-1 fusion and productive infection themselves (Geijtenbeek et al., 2000b). It is possible that HIV-1 circumvents direct infection of DCs to prevent activation of an innate immune response. In this review, we consider recent advances in the study of HIV-1 entry in different cellular contexts. Indeed, the application of new imaging techniques (like super-resolution (Chojnacki et al., 2012), spectral imaging (Jones et al., 2017; Russell et al., 2017; Zaitseva et al., 2017) or Cryo-EM (Lee et al., 2016; Wu et al., 2010)) have led to recent discoveries that highlight the importance of the point of HIV-1 fusion or how HIV-1 hijacks host proteins for entry (also termed **host restriction factors**). Here, we describe important advances on the molecular mechanisms leading to HIV-1 fusion in different cellular contexts: macrophages, dendritic cells (DCs) and T cells and the role of different host restriction factors during entry. Interestingly, the point of fusion (i.e. plasma membrane versus endocytic compartments) differs depending on the cell line.

Entry of HIV-1 in macrophages

Endocytosis has surfaced as a potentially important route of HIV-1 entry (de la Vega et al., 2011; Miyauchi et al., 2009a). Several studies have dealt with this hypothesis in different biological contexts. In particular, evidence in macrophages provides a strong case for virus-cell fusion occurring within endosomal compartments (Li et al., 2017; Marechal et al., 2001; Russell et al., 2017). Macrophages exhibit high endocytic activity relative to T cells (Mellman, 1996) and HIV-1 uptake following endocytosis could be a route to productive infection in this cell lines (Russell et al., 2017). In an initial study documenting the observation that HIV-1 could enter macrophages via endocytosis Carter and co-workers (Carter et al., 2011); employed a pharmacological approach utilizing inhibitors for caveolin-1-related endocytosis, micropinocytosis, actin rearrangements and Rho GTPases, finding that most likely HIV-1 entry into macrophages proceeds via an endocytic pathway related to micropinocytosis. Furthermore, genetic approaches have been used to demonstrate that the overexpression of CD4 at the surface of induced pluripotent stem cell (iPSC)-derived human macrophages (PSC-macrophages) can increase HIV-1 fusion but not HIV-1 infection efficiency, as evidenced from viral DNA expression assays (van Wilgenburg et al., 2014). Indeed, HIV-1 was demonstrated to fuse within endosomes in PSC-macrophages via a Dynamin-2 (DNM2), Ras-related C3 botulinum toxin substrate 1 (RAC1 GTPase) and Pak-dependent (kinase) pathway. The authors, measured fusion and reverse transcription in PSC-macrophages expressing LCK tyrosine protein kinase, -which tethered CD4 in the cell surface altering its normal endocytic rate increasing CD4 expression 3-fold. This led to a significant increase in HIV-1 fusion and reverse transcription but strikingly, productive HIV-1 infection was unaffected, implying that productive infection by HIV-1 in macrophages happens via an endocytic uptake pathway.

Recent evidence utilizing real-time single virus tracking and imaging of double labeled HIV-1 virions demonstrates that HIV-1 can enter primary derived macrophages and fuse from endosomal compartments within these cells (Russell et al., 2017) (Li et al., 2017). Moreover, the data seem to indicate that HIV-1 can enter the cell via clathrin-mediated endocytosis as HIV-1 fusion was inhibited when using dynasore (Kirchhausen et al., 2008), a small molecule DNM2 inhibitor, and is guided toward Rab5a-positive early endosomal compartments where fusion can then occur as visualized by advanced optical microscopy (Russell et al., 2017) (Li et al., 2017). One plausible hypothesis for this would be that, in macrophages, the fusion reaction might be initiated by HIV-1 Env upon engagement of CD4 and CCR5, but yet, cannot be completed in the plasma membrane because the increased endocytic rate relative to T cells.

Therefore, the transition towards **full fusion** might occur once HIV-1 virions are harboured within early endosomes as previously shown in TZM-bl cells in (de la Vega et al., 2011) and primary macrophages in (Li et al., 2017; Russell et al., 2017). However, the finding that HIV-1 fusion could be arrested in macrophages using dynasore, a small molecule DNM2 inhibitor, does not necessarily imply that HIV-1 necessarily enters the cell through clathrin-mediated endocytosis because the target of the drug, GTPase dynamin-2 (DNM2), might also be implicated in other aspects of virus fusion (Jones et al., 2017; Wen et al., 2016).

In brief, recent studies show evidence for endosomal HIV-1 fusion in primary derived macrophages and PSC-macrophages. The occurrence of HIV-1 fusion in the plasma membrane of macrophages, however, cannot be totally discarded. The potential endocytic route for productive HIV-1 entry and fusion is not fully elucidated yet; as there is some discrepancy in the literature about this (micropinocytosis (Marechal et al., 2001; van Wilgenburg et al., 2014) versus clathrin-mediated endocytosis (Li et al., 2017)).

Interactions between HIV-1 and dendritic cells

Because of their physiological role as professional antigen presenting cells, and their localization at mucosal surfaces, it has been proposed that DCs are among the first cells to encounter HIV-1 during transmission (Piguet and Steinman, 2007). DCs express relatively low levels of the CD4 receptor and CCR5/CXCR4 co-receptors, which is thought to be one reason why they are not regularly infected by HIV-1 (Parrish et al., 2012). Nonetheless, HIV-1 interacts with DCs in a number of ways to promote virus spread (Geijtenbeek et al., 2000b; Sol-Foulon et al., 2002) (Figure 1A). For example, DCs express a number of attachment molecules which can mediate virus internalisation (Turville et al., 2002) as well as virus transfer to other cell types, such as CD4⁺ T lymphocytes (Lambert et al., 2008). For instance The c-type lectin dendritic cell-specific intercellular adhesion molecular 3-grabbing non-integrin (DC-SIGN) is thought to play an important role in the entry of HIV-1 into cells (Feinberg et al., 2001). In DCs expressing both CD4 and DC-SIGN, the HIV-1 Env preferentially binds to DC-SIGN tetramers, which have been described as a “docking site” for HIV-1 (Cambi et al., 2004; Geijtenbeek et al., 2000b; Izquierdo-Useros et al., 2014). Moreover, the cytoplasmic region of DC-SIGN contains an **internalization motif**, and upon binding, the virus can be endocytosed into clathrin-coated pits (Cambi et al., 2009; Engering et al., 2002; Kwon et al., 2002). This process is thought to be dependent on clathrin, DNM2, and membrane cholesterol (Cambi et al., 2009; Ménager and Littman, 2016). HIV-1 may also

be internalised by DCs through interactions with caveolin or processes involving **bleb retraction** and **macropinocytosis**, with the latter thought to be the primary method of virus entry (Ménager and Littman, 2016).

The fate of HIV-1 following internalization into DCs may depend on the mode of entry into the cell and has not been fully elucidated. **Ratio fluorescence imaging** has revealed that, in mature DCs, antigen-bound DC-SIGN localises to compartments with a neutral pH located close to the cell surface which may represent early endosomes (Cambi et al., 2004). In contrast, in immature DCs, internalised DC-SIGN localises to large compartments close to the nucleus with a low pH, characteristic of lysosomes (Engering et al., 2002). Within lysosomes, HIV-1 might be targeted for degradation and processed for presentation by the major histocompatibility complex (MHC) in T cells (Leonard et al., 2011). **However, HIV-1 internalised by mature DCs is presumably protected from this pathway, observed by fluorescence confocal microscopy (Kwon et al., 2002); in these experiments DC cells were exposed to HIV-1 viruses at low temperature and then labelled with anti-HIV serum and fluorescently labelled transferrin, after shifting to 37 °C, most of the HIV fluorescence was colocalized with the fluorescent transferrin in a sac-like nonlysosomal (Figure 2).** Indeed, HIV-1 has been observed to remain stable and infectious in mature DCs for many days following entry into the cell (Kwon et al., 2002).

Although the frequency of productive infection of DCs is low, it has been proposed that they may serve as virus reservoirs mediating infection of **CD4⁺ T cells**, and hence facilitate the spread of HIV-1 to lymph nodes (Wu and KewalRamani, 2006). HIV-1 can be transmitted from DCs to CD4⁺ T cells in a number of ways, collectively referred to as trans-infection or trans-enhancement (Figure 1B) (Ménager and Littman, 2016). DC-SIGN-bound HIV-1 on the surface of DCs may be transported to lymph nodes by these DCs, where it can be brought into close proximity with CD4 and the CCR5/CXCR4 co-receptors on T cells (Hijazi et al., 2011; Wilen et al., 2012). HIV-1 can also be transferred between DCs and T cells by exocytosis of captured virus particles or via transmission of HIV-1 through the **virological synapse**, which forms upon direct contact between DCs and T cells (Ellwanger et al., 2016). Of note, this has been observed following extension of CD4⁺-rich **filopodia** from the T cell into HIV-1-containing compartments located on DCs; the DC-T cell contact is thought to be mediated by the binding of DC-SIGN to ICAM-3 on the CD4⁺ T cell (Felts et al., 2010; Geijtenbeek et al., 2000a).

HIV-1 entry in T cells

A recent paper published by Herold and colleagues (Herold et al., 2014) established that HIV-1 fuses in the plasma membrane of primary T cells. The authors concluded that endocytosed viruses do not lead to productive HIV-1 fusion. This was shown using a dominant-negative mutant that could block HIV-1 uptake by endocytosis but not HIV-1 fusion. The authors also used an impermeable HIV-1 fusion inhibitor (T20) employed in primary CD4 T cells preincubated at 22 °C (which allowed for HIV-1 endocytosis and not HIV-1 fusion). T20 turned out to fully block HIV-1 fusion when raising the temperature at 37 °C, indicating that endocytosed virus were unable to fuse. In contrast, Miyauchi and co-workers (Miyauchi et al., 2009a) suggested that HIV-1 virus fuses within endosomes in TZM-bl and CEM cells as it can engage CD4 and co-receptors at low temperatures but the rate of escape from T20 as compared to temperature rise was delayed when performing population-based bulk HIV-1 fusion experiments (Marin and Melikyan, 2015). The same group also showed that DNM2 inhibitor dynasore (Kirchhausen et al., 2008) can block fusion (de la Vega et al., 2011; Miyauchi et al., 2009a; Miyauchi et al., 2009b). Moreover they visualized the process of fusion in endosomes in TZM-bl reporter cells using double color real-time single particle tracking; however the authors did not use primary CD4 T cells in their imaging experiments and therefore both studies do not necessarily contradict each other. In a recent paper, we (Jones et al., 2017) and others (Aggarwal et al., 2017) have shown that DNM2 is crucial for HIV-1 fusion in primary CD4 T cells and it is not involved in endocytosis. Using advanced microscopy and functional-based assays for HIV-1 fusion, we described the role of DNM2 during HIV-1 entry in TZM-bl cells and primary CD4 T cells showing that DNM2 played a crucial role, establishing hemi-fusion and in a later stage stabilizing the fusion pore, most likely at the plasma membrane. These data, were further supported by Aggarwal and co-workers (Aggarwal et al., 2017). Combining dynamin inhibition with functional HIV-1 fusion assays (Jones and Padilla-Parra, 2016); real-time single HIV-1 tracking (Padilla-Parra et al., 2013); detection of HIV DNA products (van Wilgenburg et al., 2014) the authors of this recent study identified that dynamin in CD4 T cells directly regulates the fusion reaction at the plasma membrane. In conclusion, in recent years it has been established by us and two other groups that HIV-1 most likely fuses in the plasma membrane of primary CD4 T cells, and also, that the role of dynamin in these cells is strictly related to the HIV-1 fusion reaction and not endocytosis.

Cell-to-cell transmission of HIV-1

Contacts between infected cells and neighbouring uninfected target cells can enhance HIV-1 fusion and productive infection through the formation of the virological synapse (VS) (Groot et al., 2008; Jolly et al., 2004; Jolly and Sattentau, 2007). During the establishment of the VS, host and viral proteins are recruited to the site of intercellular contact, such as viral Gag and Env from the donor cell, and CD4 in the target cell (Murooka et al., 2012). Initial Env-CD4 interactions can then lead to a second stage of VS stabilization in which other molecules interact at contact sites: e.g. Lymphocyte function-associated antigen-1 (LFA-1) and Intercellular Adhesion Molecule-1 (ICAM-1) (Li et al., 2015), which enhance adhesion between donor and target cell. Subsequently, Gag is recruited to the contact site of the infected cell and virion budding occurs into the interstitial space of the synapse (Hubner et al., 2009). How this process is regulated and the exact mechanism by which cell-cell fusion is avoided during the establishment of the VS is unknown but a subject of intense debate (Compton and Schwartz, 2017).

One report showed that HIV-1 could be taken up by DNM2-dependent endocytosis using both CXCR4- and CCR5-tropic viruses during cell-to-cell transmission between CD4⁺ T cells (Sloan et al., 2013). The authors employed both RNA interference and small molecule inhibition in primary CD4 T cells to describe the role of DNM2 in productive cell to cell HIV-1 transmission. In the same study, the authors observed, using a pharmacological and RNAi approach in primary CD4 + T cells, that certain transmission events were DNM2-dependent but not clathrin-dependent, suggesting that DNM2 might regulate HIV-1 cell-cell transmission independently of endocytosis (Sloan et al., 2013); this suggests that in HIV-1 cell to cell transmission, DNM2 might also be implicated in the fusion reaction, --as is the case in single virus entry in CD4 T cells (Jones et al., 2017), -- and not necessarily in endocytosis.

The role of endocytosis in cell-to-cell transmission is also a matter of debate. One study reported that HIV-1 virus transfer, fusion, and productive infection in target primary CD4⁺ T cells were not blocked by dynasore administration at sub-toxic concentrations (Kirchhausen et al., 2008), implying that endocytosis does not appear to constitute a major entry pathway in this setting (Permanyer et al., 2012). These results, however, contradict single virus tracking data in which HIV-1 was found to cross the VS and accumulate in endosomal compartments in target primary CD4 T cells (Dale et al., 2011). Furthermore, they proposed, based on data visualizing double labelled HIV-1 virions with rapid confocal microscopy, that HIV-1 could

undergo maturation and posterior fusion in these compartments, meaning that immature HIV-1 viruses could undergo maturation in endosomal compartments of the target cell (Dale et al., 2011). More data coming from tracking single double labelled HIV-1 virions with confocal microscopy also suggested that membrane fusion within endosomes might present delayed kinetics (several hours) to allow the proteolytic maturation of Gag to occur (Dale et al., 2011). This is important because endosomal delivery of virus following cell-to-cell transmission could serve to evade neutralization by circulating HIV-1-specific antibodies. To summarize, there is also a certain degree of controversy on the role of endocytosis during the establishment of the VS for HIV-1 cell to cell transmission (Permanyer et al., 2012) (Dale et al., 2011). In this context, DNM2 seems also to be crucial (Sloan et al., 2013), but its role could not be strictly related to endocytosis but perhaps in the HIV-1 fusion reaction; as is the case in single HIV-1 entry in CD4 T cells (Jones et al., 2017).

Roles of DNM2 in HIV-1 fusion during infection

DNM2 is known to contribute to cellular endocytosis by pinching the clathrin-coated pit upon forming an octameric, ring-like structure that is important for its GTPase activity (Ferguson and De Camilli, 2012). The finding that DNM2 contributes to HIV-1 entry was inferred to suggest that HIV-1 might enter cells via endocytosis (Miyauchi et al., 2009a). However, as explained above, recent reports have shown that DNM2 is directly involved in the virus-cell fusion process (Jones et al., 2017) (Aggarwal et al., 2017). The transition to full fusion or fission of two lipid bilayers depends on the competition between fusion and a calcium/ DNM2-dependent fission mechanism (Artalejo et al., 2002; Chiang et al., 2014; Jaiswal et al., 2009; Zhao et al., 2016). This transition is very slow (seconds to minutes) and stresses the importance of the **hemi-fusion** state to regulate the transition towards full fusion (Zhao et al., 2016). A recent study demonstrated the importance of actin dynamics in this process, where actin must provide enough tension to merge fusing vesicles into the plasma membrane (Wen et al., 2016). This was shown by using confocal and super-resolution stimulated emission depletion (STED) microscopy, force measurements and gene knockouts. Indeed, filamentous actin, in conjunction with **actin regulators** **N-WASP** and Formin, seems to mediate the curvature between membranes favouring fusion by providing enough tension to transition toward full fusion of exosomes in **chromaffin cells** (Wen et al., 2016). In CD4⁺ T cells (Jones et al., 2017), hemi-fusion between HIV-1 and the plasma membrane of the host cell might evolve toward full fusion if two conditions are met: i) DNM2 forms a low oligomeric state, and ii) actin is selectively modulated to accommodate formation of the fusion **pore** (Yoder et al., 2008).

Indeed, Yoder and co-workers (Yoder et al., 2008) showed by flow cytometry, western blot and kinase assays in resting CD4 T cells that cortical actin is a restriction factor overcome by HIV-1 by Gai-dependent signalling from the chemokine receptor (CXCR4) to activate the actin-depolymerizing factor **cofilin** (Yoder et al., 2008)

In DCs, however, the role of DNM2 is less clear and does not appear to be related to fusion. Moreover, DNM2 and tetraspanin (TRPM7) have been found to play an important role in promoting **actin nucleation** and stabilization allowing HIV-1 accumulation in **dendrites**, concomitantly avoiding HIV-1 macropinocytosis (Ménager and Littman, 2016). The importance of macropinocytosis during **transenhancement** was shown by employing several drug inhibitors selective for this particular endocytic process. The authors (Ménager and Littman, 2016) rescued HIV-1 trans-infection by DCs lacking DNM2; also reviewed in (Padilla-Parra and Dustin, 2016). In DCs the cortical actin cytoskeleton was shown to play a crucial role as opposed to endocytosis.

In addition to its role in early events of HIV-1 entry and fusion, DNM2 also regulates the fusogenicity of HIV-1 particles via its interaction with viral accessory protein Nef, in infected cells in Jurkat Tag lymphoid cells (Pizzatto et al., 2007). Furthermore, the incorporation of Serine Incorporator 3 and 5 (**SERINC3** and **SERINC5**, known to be involved in serine incorporation into lipids and promote phosphatidylserine (PS) and sphingomyelin (SM) biosynthesis) into HIV-1 virions has been found to decrease virus infectivity in **Jurkat cells** and is antagonized by Nef (Rosa et al., 2015; Usami et al., 2015). Indeed, Usami and co-workers found that the infectivity of nef-deficient virions increased 100-fold in double-knockout primary CD4 T cells missing both **SERINC3** and **SERINC5** (Usami et al., 2015). In addition, in the presence of Nef, **SERINC3** and **SERINC5** can be sequestered from the cell surface to an endosomal compartment, thereby preventing incorporation into budding virions (Figure 3) (Usami et al., 2015). The mechanism by which **SERINC** proteins decrease fusogenicity is not understood, but recent evidence indicates that these proteins act immediately following fusion pore formation (Sood et al., 2017); this was shown using functional virus-cell and cell-cell fusion assays combined with single virus imaging using TZM-bl reporter cells, CV-1 cells stably expressing CD4 and CSCR4 and NSP/CD4/CXCR4/DSP-2 cells. A potential mechanistic clue comes from the observation that **SERINC5** promotes the inactivation of Env glycoproteins by enhancing the exposure of gp41 domains (Sood et al., 2017). It now remains to be determined whether **SERINC** proteins or other inhibitors of lentiviral fusogenicity (Compton et al. 2014) can have an impact on **Env clustering and motility**, processes which

are crucial for HIV-1 engagement and productive fusion with an infected cell (Chojnacki et al., 2012) (Figure 4).

It is therefore clear that DNM2 has a multifaceted role during HIV-1 cell cycle: i) regulation of the fusion reaction in CD4 T cells (Aggarwal et al., 2017; Jones et al., 2017), ii) regulation of actin dynamics during trans-enhancement from DCs to CD4 T cells (Ménager and Littman, 2016) and iii) its role in downregulating SERINC3 and SERINC5 during egress and budding in primary CD4 T cells (Usami et al., 2015).

Restriction by cell-intrinsic innate immunity influences the route of HIV-1 cellular entry

Another potential explanation for the heterogeneity of sites available for HIV-1 fusion comes from studies of cell-intrinsic innate immune factors, such as the interferon-induced transmembrane (IFITM) proteins. IFITM proteins can inhibit numerous virus-cell and cell-cell fusions at the entry step prior to fusion pore formation; mechanisms involved may include membrane deformation and altered lipid trafficking (Amini-Bavil-Olyaei et al., 2013; Desai et al., 2014; Li et al., 2013). A wide array of viruses are sensitive to IFITM-mediated antiviral activities, including HIV-1 (Compton et al., 2014; Compton et al., 2016; Lu et al., 2011) (Yu et al., 2015). In a recent study, the sensitivity of HIV-1 entry to IFITM-mediated restriction was reported to depend on co-receptor usage and the subcellular localisation of IFITM in the host cell (Foster et al., 2016). For example, X4 tropic HIV-1 strains were shown to exhibit sensitivity to IFITM2 and IFITM3, which are predominantly localized to endosomes and lysosomes, while R5 tropic strains were sensitive to IFITM1 at the plasma membrane of U87 neuroblastoma cells which do not express detectable IFITMs without IFN induction (Foster et al., 2016) (Figure 5). This differential susceptibility to different IFITM family members suggests that HIV-1 fusion might occur in endosomes or at the plasma membrane depending on which virus co-receptor is engaged at the cell surface. Moreover, Using transmitted/founder (TF) clones, which represent viruses that establish early infection following HIV-1 transmission, the authors showed that TF strains were resistant to IFITM-mediated restriction of virus entry (Foster et al., 2016). In addition, matched viral clones derived 6 months following initial infection exhibited a gain of sensitivity to endosomal IFITM2 and IFITM3. Further, When IFITM2 and IFITM3 were mutated to enforce stable plasma membrane localization, the ‘6 month’ viruses remained resistant to their antiviral activities (Foster et al., 2016). Therefore, the progressive sensitization of primary virus isolates to IFITM2 and IFITM3 suggest that founder viruses enter cells at the plasma membrane while viruses isolated at later stages of

infection might increasingly rely on endosomal entry (Foster et al., 2016) (Figure 5). Alternatively, since a pool of IFITM2 and IFITM3 is present at the plasma membrane prior to endosomal targeting via endocytosis, the differential sensitivities of HIV-1 strains to IFITM proteins might indicate that they could potentially fuse at different portions of the plasma membrane (Foster et al., 2016). The authors also show by infectivity assays that HIV-1 entry site choice, as inferred by sensitivity to IFITM, depends on the relative amounts of CD4 expressed in these cells (U87 neuroblastoma cells and primary CD4 T cells), thus linking receptor and co-receptor usage with viral entry route (Foster et al., 2016). Subsequent work further reinforced the link between IFITM and the port of entry used by HIV-1. A recent report demonstrated that X4 tropic HIV-1, but not R5 tropic, was hypersensitive to a splice variant of IFITM2 lacking the N-terminus (Wu et al., 2017). Jurkat E6-1 R5 cells expressing IFITM proteins were incubated with p24 antigen X4 tropic NL4-3 or R5-Tropic AD8 and virus titers measured as a function of time. R5 virus infection was unaffected (Wu et al., 2017). Since this IFITM variant was especially abundant in primary human cells (CD4 T cells and monocytes) susceptible to HIV-1 infection in vivo, the authors postulated that IFITM might limit X4 tropic infection, and thus, that it might contribute to the tendency of TF viruses to exhibit CCR5-dependency during primary infection.

In brief, IFITM proteins might play an important role in the point of HIV-1 fusion. Understanding the different molecular mechanisms for the family of innate immunity of IFN-induced transmembrane (IFITM) proteins is very important to develop novel approaches to treat HIV-1 diseases. In particular the work of Wu and co-workers describing the specific restriction of Delat20 IFITM2 for CXCR4 (X4) tropism but not CCR5 (R5) may help to stop transmission of X4 viruses (acute phase).

Concluding remarks

In order to understand the restrictions imposed by different cell types on virus entry and fusion, it is crucial to explore the role of HIV-1 endocytosis and fusion globally in different tissues (CD4+ T cells, macrophages, and DCs) using different strategies. In fact, the heterogeneity of virus entry has to be addressed by studying each of the models mentioned above at molecular resolution, as the cell type and/or activation state of the target cell may impose different cytoskeletal and endocytic requirements. Describing these mechanisms might help us devise strategies to keep HIV-1 and other enveloped viruses at bay, and perhaps enable us to improve the efficiency of antiviral drugs and **neutralizing antibodies**. Here, we have stressed the

problem of dissecting the port of HIV-1 entry in different cell lines and contexts and how cellular host proteins, such as DNM2, SERINC3, SERINC5 and IFITMs, might modulate this process together with the HIV-1 fusion reaction. We have discussed recent advances in the determination of the point of fusion of HIV-1 in primary T cells, the plasma membrane (Aggarwal et al., 2017; Herold et al., 2014; Jones et al., 2017)) and primary macrophages (Li et al., 2017; Russell et al., 2017). We have reviewed the multi-dimensional role of DNM2: regulation during HIV-1 entry, fusion, and budding in different cellular contexts. These new insights constitute a challenging and promising field of research to potentially modulate host restriction factors and stop HIV-1 infection in the early stages. Clearly, many questions regarding HIV-1 cellular entry remain to be addressed (see Outstanding Questions and Box 3). Furthermore, In order to design alternative and novel therapeutic interventions that focus on the host cell rather than the virus itself, the current virus-centric approach must be reimagined. The problem of heterogeneity in HIV-1 entry and fusion is therefore very complex and we need to address it with an integrative approach using technologies from different disciplines: from advanced imaging approaches and tissue engineering to mathematical modelling.

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368 **Glossary**

369 **Actin**

370 Family of multifunctional proteins that form microfilaments. Actin participates in cell
371 motility, cell division, cytokinesis, vesical and organelle movement, cell signalling and cell
372 shape.

373 **Actin nucleation**

374 Actin filaments are generated by actin polymerization. The first step of this process is known
375 as nucleation, and it's the formation of three actin monomers from which filaments may
376 elongate.

377 **Actin remodelling**

378 Cellular process organized by actin monomers polymerization in response to signalling
379 cascades that initiated by environment cues. The remodelling of actin filaments is mediated
380 by actin-binding proteins and leads to change of the cell organization and shape.

381 **ART**

382 Anti-retroviral therapy. The gold standard in treatment of HIV-1, ART usually involves the
383 combination of three antiviral drugs which act in different ways to limit spread of HIV-1.
384 These may include fusion inhibitors, non-nucleoside reverse-transcriptase inhibitors
385 (NNRTIs), nucleoside reverse-transcriptase inhibitors (NRTIs), protease inhibitors (PIs),
386 entry inhibitors, or integrase strand transfer inhibitors (INSTIs). ART manages but does not
387 cure HIV-1 infection.

388 **BET family inhibitors**

389 Protein inhibitors that reversibly bind proteins of the bromodomain and extra terminal motif
390 family and prevent protein protein interactions between them and acetylated histones and
391 transcription factors.

392 **Bispecific antibodies**

393 An artificial protein composed of two fragments of two different monoclonal antibodies or a
394 monoclonal antibody and a neutralizing antibody.

395 **Bleb retraction**

396 Blebs are spherical cellular protrusions, not supported by an actin cytoskeleton, in
397 mammalian cells observed during cytokinesis, apoptosis and cell migration. Retraction
398 happens when cell's membrane lacks the stability afforded by cell-substrate contacts.

399 **CCR5**

400 7- transmembrane G protein coupled chemokine receptor for inflammatory chemokines.
401 Upon binding it transduces a signal for increase of intracellular calcium ion level. It acts like
402 a co-receptor (CD4 is the primary) for HIV R5 strains.

403 **CD4**

404 Membrane protein involved in the MHC- T cell interaction. It is the primary receptor for
405 HIV-1.

406 **CD4⁺ T cells**

407 Mature T cells with CD4 protein on their cell surface. They are the main targets of HIV-1.

408 **CEM cells**

409 T lymphoblastoid human cell line.

410 **Chromaffin cells**

411 Neuroendocrine cells part of the medulla of the adrenal glands in mammals. They are related
412 to the sympathetic nervous system and they can migrate to sympathetic ganglia and to adrenal

medulla. They secrete adrenaline, dopamine, enkephalin and other hormones into the blood stream.

Cofilin

Protein which binds and disassembles actin filaments.

Cortical actin network

The richest area of actin filaments are found in a narrow zone underneath the plasma membrane (cortex). Most filaments there are organized into a network that excludes most organelles from the cortical cytoplasm.

CRISPR/cas9 gene editing system

Gene editing system based on the delivery of Cas9 endonuclease complexed with a synthetic guided RNA into a cell. Based on specific nucleotide repeats can alter the cell's genome at a desired location by allowing existing genes to be removed and new ones to be added.

CXCR4

Chemokine receptor with 7-transmembrane regions and part of the G protein coupled receptors. It acts as a co-receptor (CD4 is the primary) for HIV X4 strain.

Dendrites

Branched projections of a neuron that act to propagate the electrochemical stimulation received from other neural cells to the cell body of the neuron from which they project.

Dendritic cells

Professional antigen presenting cells located in numerous tissues (eg. skin, mucosal, lymphoid) throughout the body. They process antigens and then they present them on their cell surface to cytotoxic T cells while they express molecules as cytokines, chemokines and proteases to initiate immune response.

Dendritic cell maturation

Their ability to regulate immunity depends on their maturation. The process of maturation is based on phenotypical and functional changes and involves redistribution of MHC complex from intracellular compartments to their surface, down regulation of antigen internalization, cytoskeleton re-organization, secretion of cytokines, chemokines, proteases and surface expression of adhesion molecules and chemokine receptors.

Dynamin-2

Protein of the dynamin family, subfamily of the GTP-binding proteins. Dynamin-2 is found in all cell types. They are implicated in endocytosis, cell motility and in membrane alterations.

Dynasore

Small GTPase inhibitor that targets dynamin-1 and dynamin-2.

Endocytosis

The cell process of uptake of solid particles, small molecules or fluid droplets in small vesicles from the surrounding medium. It is divided in Clathrin mediated and non-clathrin mediated endocytosis.

Env

Viral envelope proteins encoded by the *env* gene. Env proteins are type I fusion machines thus bind to receptors at the cell membrane. This action triggers conformational changes allowing for binding of the fusion peptide. The *env* gene encodes for the precursor gp160 protein that is then cleaved to gp120 and gp41.

459 **Env clustering and motility**
 460 Trimeric Env proteins are distributed in the viral envelope. Studies have correlated the
 461 maturation stage of Gag protein with the Env mobility and proposed that Env proteins can be
 462 polarized towards CD4 receptors.

463 **Filopodia**
 464 Slender bundled actin containing plasma membrane protrusions. They are important for cell
 465 adhesions, migration and cell-to-cell contact.

466 **Follicular CD8⁺ T cells**
 467 HIV-1 specific cytolytic T cells usually found in the B cell follicles.

468 **Founder Virus Clone**
 469 A single viral variant that initiates the HIV-1 infection.

470 **Fusion Assays**
 471 Experimental procedures to test membrane fusion of enveloped viruses onto target cells, or
 472 cells to other cells.

473 **HIV-1**
 474 Human immunodeficiency virus type 1. An envelope lentivirus of the *retroviridae* family.
 475 HIV-1 infection in humans leads to acquired immunodeficiency syndrome (AIDS).

476 **HIV-1 R5**
 477 Viral strain that can infect cells expressing CD4 and CCR5 on their cell surface.

478 **HIV-1 X4**
 479 Viral strain that can infect cells expressing CD4 and CXCR4 on their cell surface.

480 **Histone Deacetylase inhibitors**
 481 Drugs with important role in epigenetic and non-epigenetic transcriptional regulation. In
 482 HIV-1 reactivation they induce transcription at the HIV-1's long terminal repeat.

483 **Histone methyltransferase inhibitors**
 484 Drugs for inhibiting known methyltransferases from methylating specific lysines of histones
 485 and thus repressing HIV-1 transcription.

486 **iPSCs**
 487 Induced pluripotent stem cells.

488 **IFITMs**
 489 Interferone induced transmembrane proteins that inhibit virus entry and fusion in cells.

490 **Internalization motif**
 491 Short linear motifs in the cytosolic tails of membrane proteins, that bind transiently to
 492 proteins and protein complexes.

493 **Interferones**
 494 Signalling proteins/cytokines released by cells of the immune system in response to presence
 495 of pathogens.

496 **Latent viral reservoirs**
 497 A group of long-lived cells that are infected with HIV but are not actively expressing it, as
 498 patients undergo ART.

499 **Latency reversing agents**
 500 Drugs used to induce HIV-1 expression in cells of patients under ART or *in vitro*.

501 **Lentivirus**
 502 Genus of retroviruses that cause chronic and deadly diseases.
 503
 504

505 **Lipid raft**
 506 Different lipids present in plasma membrane have different biophysical propensities to
 507 associate with each other and form distinct and stable domains in membranes. Their existence
 508 it is still an area of debate.

509 **Macrophages**
 510 White blood cells of the myeloid lineage that arise from the differentiation of monocytes.
 511 Macrophages engulf and destroy cells and materials that are recognised as foreign or
 512 damaged.

513 **Major Histocompatibility Complex**
 514 Host proteins that are part of the adaptive immune system in all jawed vertebrates. They
 515 present antigens from intracellular proteins of infected or malignant cells to the cell's surface,
 516 where they can be surveyed by cytotoxic T lymphocytes.

517 **Macropinocytosis**
 518 Is a form of endocytosis and it depends on actin cytoskeleton. It is the uptake of fluid droplets
 519 and it occurs in many cell types.

520 **Membrane Fission**
 521 The process of splitting of a single lipid bilayer into two parts.

522 **Membrane Full Fusion**
 523 The process where two distinct lipid bilayers merge to become one, also called closed fusion.

524 **Membrane Hemi-Fusion**
 525 The intermediate state during membrane fusion when only the outer bilayer leaflets are
 526 mixed.

527 **Neutralizing antibodies**
 528 Antibodies that are capable of blocking the virus by neutralization. This process can be
 529 mediated by blocking of the virion binding on cell receptors, block virus uptake from cells,
 530 prevent uncoating of viral genomes in endosomes or promote aggregation of virions.

531 **Nucleocapsid**
 532 The viral genome and the protein capsid that surrounds it.

533 **N-WASP**
 534 Neural Wiskott-Aldrich syndrome protein. It is a member of the WAS proteins which
 535 mediate signal transduction and interact with actin-binding proteins.

536 **Pore expansion**
 537 During membrane fusion between virus and cell membranes a pore is created and that is the
 538 spot that the viral genome gains access to the host cell's cytoplasm. This process requires
 539 time and energy until the pore achieves the optimal width.

540 **Pre-integration complex (PIC)**
 541 Nucleoprotein complex of viral genetic material associated with viral and host proteins
 542 capable of inserting the viral genome into the host genome.

543 **Ratio Fluorescence imaging**
 544 Spectroscopic technique expressing the relationship of signals at two or more wavelengths.

545 **Recombination (DNA)**
 546 Exchange of DNA strands to produce new nucleotide arrangements.

547 **Reporter cells**
 548 Genetically modified cell line that stably express a reporter gene of interest expressed at the
 549 endogenous level for rapid monitoring

550 **Restriction factors**

Cellular proteins of the host that can serve as powerful blockades to HIV entry or replication.

Reverse Transcription

Synthesis of a DNA molecule from an RNA template.

Secretory pathway

It is a set of organelles of a cell like endoplasmic reticulum, Golgi apparatus and the vesicles that travel between them and to the cell membrane or to the lysosomes. It is the pathway that includes the synthesis and delivery of proteins to cell membrane, lysosomes and the secretory organelles itself.

SERINC 3 and SERINC5

Serine incorporator 3 and 5. They enhance the incorporation of serine into phosphatidylserine and sphingolipids. They are also restriction factors of **lentiviruses** acting on an early step of infection.

SIV

Simian immunodeficiency virus. SIV is a member of the *retroviridae* family that infects non-human primates.

Tetraspasins

Protein transmembrane family involved in cell membrane compartmentalization, trafficking regulation.

Trans Enhancement (HIV-1)

Infection of CD4⁺ T cells with HIV-1 from infected Dendritic Cells when co-cultured. The viral particles have been internalized in a DC compartment and the transmission has been proposed to occur in the vicinity of a DC-T-cell contact known as the infectious synapse.

Trans infection (HIV-1)

The infection of a host cell, a T cell for example by a different type of cell, like a Dendritic Cell.

Viral DNA expression assays

Experimental procedures that identify if there is expression of the viral DNA in a host cell or organism.

Viral Tropism

Virus specificity for particular cell types determined by the interaction of viral surface proteins with host cell surface receptors.

Virological synapse

A tight adhesive junction between an HIV-infected cell and an uninfected target cell, across which virus can be efficiently transferred without cell-cell fusion.

Box 1. HIV-1 Latent Reservoirs

Individuals infected with HIV-1 are now able to live longer than ever before, and their health remains stable as HIV-1 infection is kept under control by anti-retroviral therapy (ART). However, HIV-1 infection remains a chronic illness due to the presence of latent viral reservoirs which persist within cells. The nature of these reservoirs has been best characterised in CD4⁺ T cells, however, the presence of viral reservoirs has also been observed in various other cells and tissues, such as in the lymph nodes, gut-associated lymphoid tissue, the spleen, the liver, and the central nervous system (Henrich et al., 2017). HIV-1 is thought to accumulate in these reservoirs in the first few days of viremia, however, several studies have shown that replication of HIV-1 within lymphoid tissues is ongoing, even during ART treatment (Lorenzo-Redondo et al., 2016). A possible explanation for this observation has been attributed to unfavourable pharmacokinetics of commonly used anti-retroviral drugs, which might only reach the lymph nodes at low concentrations (Boritz and Douek, 2017). In an attempt to eradicate these latent HIV-1 reservoirs, the so-called “shock and kill” strategy has been developed (Box 2).

A number of methods are being considered to address the problems associated with reactivating latent reservoirs, such as using improved **latency reversing agents (LRAs)**, **neutralizing antibodies**, **bispecific antibodies**, and by engineering T cells using the **CRISPR/Cas9 gene editing** system (Shan and Siliciano, 2013). Most LRAs target epigenetic factors; however, because viral reservoirs are present in a diverse range of cell types, the development of new small molecules or signalling molecules that act by altering the metabolism of cells may be required. Three to twelve months after the initiation of HIV-1 infection, broadly neutralising antibodies (bNAbs) are produced by the host to target parts of the virus. The identification of successful bNAbs produced by patients is currently the subject of extensive and promising research. The hope is that these bNAbs can be reproduced and combined to control viremia during ART interruption, engaging the host immune system to fight HIV-1 (Burton and Hangartner, 2016; Caskey et al., 2016). It was recently shown that a bispecific antibody acting as a bNAb recognising CD3 was able to increase the percentage of **follicular CD8⁺ T cells**, which killed HIV-1 infected cells in vitro (Petrovas et al., 2017). Another putative therapeutic approach involves the use of the CRISPR/Cas9 gene editing system, which has been used to eliminate the HIV-1 genome from human T lymphoid cells (Kaminski et al., 2016). However, it has also been suggested that CRISPR/Cas9 derived mutations may accelerate viral escape (Wang et al., 2016a; Wang et al., 2016b).

A main obstacle to the eradication of HIV-1 remains the presence of latent viral reservoirs. These reservoirs are hidden inside a diverse range of cell types which have not been equally represented in the literature. In order to address this, further research is required to identify the relevant cell types, their locations, and the overall amount of latent viral DNA in HIV-1 infected patients.

Box 2. The “Shock and Kill” Approach to HIV-1 Eradication

The “shock and kill” approach involves activating HIV-1 transcription using agents that reverse latency, such as **deacetylase inhibitors, histone methyltransferase inhibitors, or BET family inhibitors**, thus transforming these cells into targets for the innate HIV-1 specific immune system (Margolis and Archin, 2017; Siliciano, 2009).

Interrupting ART in this way presents a number of risks. The viral genome is carried by a pool of different cell types and remains functional in some cells while being inactivated in others. Only a fraction of the cells carrying the functional viral genome will be reactivated by the “shock and kill” procedure, leaving behind the cells containing non-functional viral genomes, which present possible sites of viral rebound, following **recombination**. The “shock and kill” procedure may also pose a risk to healthy cells, leaving them liable for infection. Studies of SIV have shown that following the “shock and kill” approach, variants of the SIV genome that evade the immune system may reach the brain of non-human primates and might infect neighbouring cells (Gama et al., 2017). Furthermore, the immune cell response is not broad enough to tackle all reactivated cells.

Box 3. Clinician’s corner.

New insights from restriction factors that inhibit virus-cell fusion may contribute to help in the potential development of future therapeutic interventions. Collectively, novel findings underline the need for potent, host-directed therapies that disrupt the earliest stages of the virus life cycle and preclude the emergence of resistant viral variants.

- A deep understanding of the HIV-1 fusion ports in different cell lines will impact the development of anti-HIV-1 agents (i.e. chemotherapeutic attack). With novel insights on the molecular mechanisms of HIV-1 entry three different type of inhibitors can be improved: i) attachment inhibitors, ii) co-receptor binding inhibitors and iii) fusion inhibitors.

- A number of strategies have been tried to block interactions between Env gp120 and CD4. For example, soluble CD4 that induces a non-fusogenic conformation of gp120, small molecule inhibitors targeting the CD4-gp120 binding site or CD4 antibodies. All these approaches will benefit from knowing the exact point of fusion in different cellular contexts.
- It has been reported that in 1% of the Caucasian population naturally occurs a homozygous deletion in the CCR5 allele not associated with immunological dysfunction. This led to the development of Maraviroc, which was approved by the US Food and Drug Administration (FDA) and the European Medicine Agency to treat patients with multi-resistant HIV-1 strains. This is, so far, the only example of a drug targeting a HIV-1 cellular factor in the clinic, that in this case is related to entry. A deep understanding of the molecular mechanisms that lead to HIV-1 fusion and the exact point of entry could help to develop other drugs targeting host restriction factors.

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Figure legends

Figure 1. HIV-1 cellular entry and dissemination in dendritic cells. **A.** HIV-1 can enter dendritic cells (DCs) in different ways, the most prolific ones being endocytosis and macropinocytosis. Upon binding, the HIV-1-DC-SIGN complex may be internalised into clathrin coated pits, pinched off through the action of dynamin. In macropinocytosis, sheet-like projections from the membrane engulf the virus and other materials present in the extracellular space, encapsulating them in a surface accessible compartment. Once internalised, virus-containing vesicles can be targeted for lysosomal degradation and processing for antigen presentation, or, may become early endosomes, protecting the virus. **B.** HIV-1 can be transferred from DCs to its main target, CD4⁺ T cells. Internalized viruses can be released extracellularly through exocytosis, infecting neighbouring cells. CD4⁺ T cells can also be infected by HIV-1 bound to DC-SIGN on DCs. CD4-rich filopodia projecting from the T cell can penetrate virus-containing compartments close to the surface of DCs, binding HIV-1 virions which then "surf" along filopodia to the T cell body and infect it. HIV-1 can also be transferred across the virological synapse between the DC and the CD4⁺ T cell, protected by sheet-like projections from the DC, (trans-infection). **C.** In the virological synapse, cell surface receptors and viral glycoproteins are recruited to the contact site between the DC and the T cell, stabilised by interactions between LFA-1-ICAM-1, and DC-SIGN-ICAM-3.

Figure 2. Formation of the HIV-1 fusion pore. The formation of the HIV-1 fusion pore involves a number of well-defined intermediate conformational states. HIV-1 Env binds to the CD4 receptor and then engages with the CCR5 co-receptor, forming the "early prebundle". This state progresses to that of the "binding prebundle", which gives rise to the "stalk-pore" model of membrane fusion. First, the outer leaflets of the lipid bilayer connect, forming a stalk. This leads to the "hemifusion" state, where the outer leaflets have fused but the inner halves of the two lipid bilayers remain uncorrupted, but in close contact, in a structure known as the "fusogenic prebundle". Finally, the inner membranes fuse, creating the "fusion pore", which forms due to the arrangement of proteins into the "6-helix bundle". Dynamin is thought to play a role at various stages of fusion pore formation, such as facilitating the binding of co-receptors, inducing hemifusion, and stabilising the fusion pore (2). Dynamin inhibitors, such as dynasore and TAK779, can inhibit the binding of the CCR5 co-receptor. Dynasore also interferes with

the stabilisation of the fusion pore by dynamin. The formation of the fusion pore can be inhibited by synthetic peptides T20 (HIV-1 fusion inhibitor) and temperature block (TB).

Figure 3. HIV-1 Cellular Egress. **A.** (1) Nef interacts with dynamin-2 (DNM2). (2) Activation of DNM2 by Nef facilitates clathrin mediated endocytosis of the CD4 and CCR5 receptors into clathrin coated pits. (3) Activation of DNM2 by Nef also results in (4) sequestration of SERINC3 and SERINC5 from the plasma membrane. DNM2 is involved in the scission of these endocytic vesicles from the membrane. The decrease in SERINC3/SERINC5 present at the plasma membrane results in the egress of fusion competent SERINC3/5 deficient virions in CD4 T cells. **B.** Inhibition (with mutants or RNA interference) of the (1) Nef-dynamin interaction results in the (5) release of fusion non -competent viruses with SERINC3/5 embedded in the viral envelope. CME: Clathrin mediated endocytosis

Figure 4. Structure and formation of HIV-1 particles. In mature HIV-1 particles the Env glycoproteins are able to move around in the viral membrane and can arrange themselves in trimers. In immature HIV-1 particles, Env motility is reduced due to the immatureGag lattice around RNA. This inhibits the ability of immature HIV-1 to undergo fusion with a cell membrane.

Figure 5. Role of IFITMs in HIV-1 restriction of entry and fusion. HIV-1 entry (left hand side of the cartoon, dashed line) occurs in CD4 T cells via R5 tropic viruses (R5 Env, blue) via interaction with CD4 and CCR5 and fusion occurs in the plasma membrane. HIV-1 X4 tropic viruses (X4 Env, green) occurs via interaction with CD4 and CXCR4 and internalization via endocytosis occurs. The virus might fuse within endosomes in the absence of IFITM2/3. IFITM2/3 proteins (red) present in endosomes might restrict HIV-1 fusion. The process of HIV-1 egress (right hand side of the cartoon, dashed line) is also subject to IFITM2/3 restriction. In this case, only the X4 tropic viruses with IFITM2/3 incorporated will be fusion defective. On the contrary, the R5 tropic HIV-1 virus with IFITM2/3 incorporated in its envelope will be fusion competent. In the particular case of 293T cells IFITM2/3 will also restrict X4 tropic HIV-1 viruses and therefore these viruses will be fusion defective.