HIV-Captured DCs Regulate T Cell Migration and Cell-Cell Contact Dynamics to Enhance Viral Spread

1. Mature DCs capture and compartmentalize HIV particles near the uropodia via Siglec-1 receptor

2. HIV-captured DCs migrate towards lymph node-homing chemokinetants

3. Prolonged interactions between HIV-captured DCs and CD4+ T cells are facilitated by Env:CD4 mediated signaling transduction

HIGHLIGHTS
Mature DCs compartmentalize HIV particles near the uropodia via Siglec-1 receptor

HIV-captured DCs respond to lymph node-homing chemokines and access lymphatics

Prolonged contacts between HIV-captured DCs and CD4 T cells facilitate virus transfer

Surface-accessible HIV particles can induce T cell signaling via Env:CD4 engagement
HIV-Captured DCs Regulate T Cell Migration and Cell-Cell Contact Dynamics to Enhance Viral Spread

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SUMMARY

Trafficking of cell-associated HIV-1 from the genital mucosa to lymphoid organs represents a critical first step toward systemic infection. Mature DCs capture and transmit HIV-1 to T cells, but insights into DC-to-T cell viral spread dynamics within a 3-dimensional environment is lacking. Using live-cell imaging, we show that mature DCs rapidly compartmentalize HIV-1 within surface-accessible invaginations near the uropod. HIV-1 capture did not interfere with DC migration toward lymph node homing chemo-attractants and their ability to enter lymphatic vessels. However, HIV-captured DCs engaged in prolonged contacts with autologous CD4+ T cells, which led to high T cell infection. Interestingly, we show that surface bound, virion-associated Env induced signal transduction in motile T cells that facilitated prolonged DC:T cell interactions, partially through high-affinity LFA-1 expression. Together, we describe a mechanism by which surface bound HIV-1 particles function as signaling receptors that regulate T cell motility, cell-cell contact dynamics, and productive infection.

INTRODUCTION

The majority of new human immunodeficiency virus-1 (HIV-1, referred to as HIV hereafter) infections worldwide occur through sexual mucosal transmission, of which young women are most vulnerable (Hladik et al., 2007; Piot et al., 2015; World Health Organization, 2018). During sexual transmission, HIV gains entry at mucosal sites (vaginal, penile, and anal mucosae) through breaks in the mucosal epithelial barrier to infect a founder population of susceptible targets (Baggaley et al., 2010; Haase, 2010, 2011; Tebit et al., 2012). During the eclipse phase of infection, focal clusters of infected cells replicate sufficient virus in mucosal T cells and macrophages that then leads to viral spread to draining and distant lymphoid organs (Haase, 2010, 2011; Rodriguez-Garcia et al., 2017). Our current understanding of viral dissemination kinetics following vaginal HIV infection come from studies with SIV-infected macaques (Miller et al., 2005), where locally infected cells in cervical vaginal tissues (CVTs) is followed by rapid dissemination into the draining lymph nodes (LNs), before spreading to distant lymphoid tissues and detection in blood (Deruaz et al., 2017; Haase, 2011; Hu et al., 2000; Masurier et al., 1998). Similar viral dissemination kinetics were also observed after vaginal challenge of BLT humanized mice (Deruaz et al., 2017) supporting the paradigm that HIV spread from the CVT to distant tissues is a stepwise progression of events that, once systemic dissemination is achieved, becomes difficult to eradicate with current drug regimens owing to the establishment of the latent reservoir (Whitney et al., 2014, 2018).

HIV reaches the genital-draining LN by two plausible mechanisms: (1) passive transport of infectious HIV particles released by infected CVT cells that enter the LNs via the lymphatics and (2) transport by infected or virus-carrying leukocytes via their natural trafficking into and within LNs, followed by their delivery to susceptible CD4+ T cells through cell-cell contact (Cunningham et al., 2010; Hladik et al., 2007; Jolly et al., 2004; Sigal et al., 2011). Although these mechanisms are not mutually exclusive, we previously showed that blocking leukocyte trafficking in and out of tissues after vaginal or subcutaneous HIV challenge in BLT humanized mice led to significant inhibition or delay in viremia and tissue viral loads (Deruaz et al., 2017; Murooka et al., 2012). Interfering with leukocyte recirculation in chronically infected animals did not impact viremia (Murooka et al., 2012), suggesting that HIV is actively carried into peripheral tissues and blood by migratory cells predominantly during the early stages of infection. More recently, we showed...
Cervical dendritic cells (DCs) can promote systemic viral spread through capture of infectious HIV particles and their transport to the draining lymph nodes, where virus is spread to susceptible T cells via cell-cell contact, termed DC trans-infection (Cameron et al., 1992; Geijtenbeek et al., 2000; Hu et al., 2004; Trifonova et al., 2018; Wu and KewalRamani, 2006). Although the cellular aspects of cell-cell HIV transmission are well described, most studies were done exclusively using culture systems that do not consider the highly motile nature and cell-cell contact dynamics between DCs and T cells observed in the lymph node. Another outstanding question is how DCs, in the absence of productive infection, restrain motile T cells to promote stable DC:T cell conjugates to facilitate viral transfer. We and others have utilized 3D collagen model systems to better characterize the dynamic on-off kinetics between infected and uninfected cells and have described key aspects of cell-cell contact behaviors that impact viral dissemination kinetics and pathogenesis (Lopez et al., 2019; Symeonides et al., 2015; Usmani et al., 2019). Herein, we addressed whether HIV-captured DCs retain their ability to exit the genital mucosa, enter the lymphatic vessels, and migrate toward LN-homing chemo-attractants and whether they can restrain motile T cells for long enough to permit viral transfer in the absence of cognate antigen recognition. We show that mature monocyte-derived DCs were highly efficient at capturing cell-free HIV particles which, over time, became condensed within compartments close to the uropodia of motile DCs. Consistent with previous studies, HIV particle capture was dependent on Siglec-1 and required actin cytoskeletal mobilization to compartmentalize virus for at least 24 h. HIV-captured DCs retain their ability to respond to tissue exit chemo-attractants CCL19/21 and S1P by facilitating entry into and within lymphatic vessels. Using our 3D collagen imaging model, we show that CD4 T cells engaged in prolonged contacts with HIV-captured DCs that were dependent on gp120:CD4 interactions and further strengthened through LFA-1:ICAM-1 binding. Stable DC:T cell contacts resulted in increased T cell infection, highlighting an unexpected mechanism by which surface-bound HIV particles on DCs can function as adhesive receptors to contact and restrain motile T cells to facilitate HIV spread.

RESULTS

Mature Dendritic Cells Readily Capture and Compartmentalize HIV into Clusters near the Uropodia

Mature DCs capture and transmit infectious HIV particles to susceptible T cells more efficiently than immature DCs, whereas the latter are more susceptible to HIV infection (Turville et al., 2004). LPS-stimulated DCs upregulated HLA-DR, CD80/86, and CD83 and were more migratory compared with immature DCs in 3D collagen matrices (Figure S2). To better characterize HIV capture dynamics by motile DCs, we generated a dual-fluorescent HIV reporter that incorporates both the Gag-iGFP gene and nef-IRES-dTomato cassette (Gag-iGFP/dTomato). The resulting viral particles are brightly labeled by incorporation of the Gag-iGFP fusion protein, whereas infected cells express high levels of dTomato, allowing us to discriminate between productively infected (GFP + dTomato +) and HIV-captured but uninfected (GFP + dTomato -) DCs and T cells (Figures 1A and S1). Cell surface CD4 downregulation was observed only in dTomato + cells, confirming functional Nef expression (Figure S1). Immature and LPS-stimulated MDDCs were incubated with virus for up to 28 h, and viral capture kinetics was evaluated by flow cytometry. As expected, mature DCs efficiently captured and retained HIV particles for up to 28 h, whereas immature DCs rapidly degraded HIV particles shortly after capture and only 2.6% of cells contained measurable HIV-iGFP after 28 h (Figures 1B and 1C). We did not observe infected, dTomato + only DCs during this time course experiment (Figure 1B). Next, we sought to visualize viral capture dynamics by DCs within a 3D fibrillar space that allowed cells to migrate and retain morphological features observed at mucosal surfaces in vivo. Celltracker blue-labeled DCs were co-embedded with HIV-iGFP particles in a 3D collagen matrix and visualized for up to 24 h (Figure 1D and Video S1). Although immature DCs remained immotile and weakly retained HIV clusters, mature DCs exhibited large dendritic extension, were migratory, and contained HIV particles. In the first hour, HIV particles were found dispersed throughout the cell, but over time, large viral clusters became visible near the trailing edge of migratory DCs, followed by distinct HIV compartmentalization by 5 h post-viral exposure (Figures 1D and 1E). This was confirmed by line profile analysis, showing that all DCs analyzed have compartmentalized virus by 24 h (Figure 1E). These studies confirm that mature DCs capture
Reduction of both wild-type and HIV further confirmed that Siglec-1 was the predominant receptor that facilitated HIV capture, as blockade showed that Siglec-1 (CD169) was largely responsible for HIV capture by DCs in a gp120-independent manner. Indeed, wild-type and HIV-iGag experiments are shown (n = 303 total cells).

Confined within the training edge of polarized DCs. Relative proportion of HIV found compartmentalized over time is shown. Data from two independent "HIV capture" and "HIV compartmentalization" line profiles are shown. HIV compartmentalization was defined as >70% of total GFP fluorescence that is confined within the training edge of polarized DCs. Relative proportion of HIV found compartmentalized over time is shown. Data from two independent experiments are shown (n = 303 total cells).

and retain HIV within clusters that seem to polarize toward the trailing edge, or uropodia, of motile DCs within a 3D environment.

HIV Clusters within CD81-Rich Compartments near the Uropodia of Motile DCs
To better define the HIV-containing compartment, HIV-containing DCs (1 h virus pulse) were labeled with fluorophore-conjugated antibodies recognizing the uropodal marker P-selectin glycoprotein-1 (PSGL-1) or CD81 prior to collagen embedding for live-cell imaging. We observed strong co-localization between HIV clusters and both PSGL-1 and CD81, but not with dextran-containing endosomes, confirming that virus-containing compartments (VCCs) are distinct from endosomes (Figures 2A and 2B). Ultrastructural analysis using electron microscopy of HIV-captured DCs in collagen confirmed that mature viral particles were contained close to the cell surface membrane within invaginated pockets (Figures 2E and S3). These data confirm that the observed GFP + clusters in DCs were indeed mature HIV particles and not endocytosed free Gag-iGFP proteins. We observed some DCs that had more than one irregularly shaped VCCs near the uropodia containing mostly mature HIV, and no viral budding events in this compartment was observed in our analysis. Menanger and Littman (2016) showed that dynamin-2, a regulator of actin polymerization, helped facilitate the formation of HIV-containing compartments within DCs. Indeed, pre-treatment of DCs with Dynasore, a reversible inhibitor of dynamin, did not interfere with HIV capture but resulted in a complete loss of viral cluster polarization and migration (Figure 2C and Video S2). Phalloidin staining of formalin-fixed, Dynasore-treated DCs in collagen, which preserved the dendritic features of motile DCs, confirmed lower F-actin content and increased endocytosis of HIV particles (Figure 2D). When Dynasore was removed from the cell suspension by media wash after 2 h of drug treatment, DC motility and virus compartmentalization was restored, supporting the notion that actin networks are crucial for VCC maintenance in motile DCs.

Siglec-1 Facilitates HIV Compartmentalization in Motile DCs
Previous studies (Akiyama et al., 2015; Izquierdo-Useros et al., 2012a, 2012b, 2014; Perez-Zsolt et al., 2019) showed that Siglec-1 (CD169) was largely responsible for HIV capture by DCs in a gp120-independent manner. Indeed, wild-type and HIV-iGagEnv were captured with equal efficiencies and compartmentalized by DCs near the uropodia (Figure 3A). As Siglec-1 is upregulated in mature MDDCs (Figure 3B), we further confirmed that Siglec-1 was the predominant receptor that facilitated HIV capture, as blockade with anti-Siglec-1 antibody (at a titrated dose that yielded ~90% receptor blockade) led to near-complete reduction of both wild-type and HIVEnv capture and retention (Figures 3C and 3D). This was also confirmed by immunostaining in collagen gels, where wild-type and HIVEnv both co-localized with Siglec-1 at the uropodia of migrating DCs (Figures 3E and 3F). Conversely, blockade with anti-DC-SIGN did not lead to significant reduction in HIV capture (Figure S4B), although DCs did express the receptor. DC-SIGN expression did not co-localize with viral clusters near the uropodia of polarized DCs in collagen (Figures S4B and S4C), indicating that DC-SIGN played a minimal role in viral capture in our model. Notably, Siglec-1 expression in control DCs were not localized exclusively in the uropodia, suggesting that HIV-binding caused HIV-Siglec-1 clusters to relocate toward the uropodia.

HIV-Captured DCs Retain Migration toward Lymph Node Homing Chemokines
Mucosal DCs capture antigens and actively migrate to the draining lymph node via the afferent lymphatics (Martin-Fontecha et al., 2009; Permanyer et al., 2018; Platt and Randolph, 2013; Randolph et al., 2005). Although HIV-captured DCs appear to retain their baseline motility, albeit at a lower velocity (Figure S1),
we addressed whether virus capture altered their migration toward tissue exit chemo-attractants CCL19/21 and S1P. No changes in their respective receptors CCR7 or S1PR1 on DCs before and after HIV exposure was observed, and comparable chemotactic responses toward CCL19/21 and S1P gradients were noted between the two DC populations (Figures 4A and 4B). Chemotactic responses of HIV-captured DCs were significantly reduced by anti-CCR7 antibody and FTY720 treatment that blocks CCR7- and S1PR1-mediated chemotaxis, respectively. We next addressed whether HIV-captured DCs were able to enter and migrate within lymphatic vessels, a critical step for active dissemination into the lymph node. We utilized an ex vivo explant model of the murine ear dermis, taking advantage of the fact that human immune cells responded robustly to murine CCL19/21 (Deruaz et al., 2017). Lymphatic vessels from mouse explants were labeled using anti-LYVE-1 antibody, then a 1:1 mixture of control and HIV-captured DCs were overlaid for 15 min to allow for cells to enter the dermal layers of the skin for microscopy analysis (Figure 4C). We were not productively infected and did not express de novo gp120 on the cell surface, we interpreted these data as surface-accessible Siglec-1:HIV complexes functioning as adhesive molecules to retain motile

Figure 2. HIV Capture Kinetics and Localization by Mature DCs in 3D Collagen
(A) Micrograph of mature DCs stained for PSGL-1, CD81 or pulsed with fluorescently labeled dextran after pulsing with HIV particles for 1 h. DCs were embedded into collagen matrix for live-cell imaging.
(B) Representative life profile analysis of GFP and PSGL-1, dextran or CD81 fluorescent intensity in a polarized, HIV-captured DC (n = 33 total cells). Fluorescence intensity is expressed as arbitrary units (a.u.).
(C) Time-lapse micrograph of HIV-captured DCs that were either left alone or treated with Dynasore and prepared for live-cell imaging in collagen matrix. HIV-iGFP signal intensity is depicted using LUT union Jack analysis. Dynasore “wash out” condition involved washing DCs with media after 2 h of drug treatment.
(D) HIV-captured DCs were either left alone or treated with Dynasore, then embedded in collagen and fixed with PFA in situ at 37°C. DCs were stained with phallloidin-Texas red. Representative micrograph of HIV-captured DCs are shown.
(E) Transmission electron micrograph of a polarized DC in collagen matrix. HIV particles are observed within surface-accessible compartments (yellow arrowheads in enlarged insets). The leading and training edges are indicated, based on the location of the nucleus (N).

Prolonged DC:T Cell Engagement Is Facilitated through gp120:CD4 Interaction
Mature DCs migrate into and within the T cell zone of the lymph node to maximize access to naive T cells (Mempel et al., 2004; Miller et al., 2003). Virological synapse (VS) formation between HIV-infected and uninfected cells are well described and serve as a direct conduit for efficient HIV spread to susceptible T cells (Jolly et al., 2004; Jolly and Sattentau, 2004). We next sought to visually characterize the cellular dynamics during DC:T cell trans-infection within a fibrillar 3D environment that allows cells to migrate, locate, and engage other cells in a manner that are reflective of interactions observed in the LN. Activated CD4+ T cells were co-cultured with either HIV-captured DCs or cell-free HIV particles in collagen matrices for 4 h, after which T cells were isolated and incubated in fresh media for an additional 48 h to measure productive, dTomato expression (Figure 5A). The presence of DCs significantly increased T cell infection compared with cell-free virus alone, and raltegravir pre-treatment completely inhibited infection in both experimental conditions. Next, we addressed how physiological DC:T cell contact dynamics were impacted upon HIV exposure using live-cell microscopy. As expected, DC:T cell contacts in the absence of infection remained brief, with mean durations lasting under 5 min, whereas extended DC:T cell contacts (mean duration of ~17 min) were observed in the presence of HIV particles (Figures 5B and 5C and Video S4). When DC:T cell contact durations were further characterized as brief (<5 min), scanning (5–17 min), or stable (>17 min), we noted that more than 70% of all DC:T cell conjugates were either scanning or stable contacts in the presence of virus (Figure 5D), indicating a substantial increase in DC:T cell dwell times. Surprisingly, stable DC:T cell contacts were completely abrogated in the presence of HIV-iGagΔEnv, suggesting that gp120:CD4 interactions facilitate durable DC:T cell contacts. Similar reductions in DC:T cell contact duration was observed after CD4 antibody blockade (Figures 5C and 5D). Since HIV-captured DCs were not productively infected and did not express de novo gp120 on the cell surface, we interpreted these data as surface-accessible Siglec-1:HIV complexes functioning as adhesive molecules to retain motile
Figure 3. Siglec-1 Mediates HIV Capture and Uropodial Compartmentalization in Motile DCs
(A) Micrograph of mature DCs pulsed with either wild-type or ΔEnv HIV-iGFP, washed, and embedded in collagen for live-cell imaging. Time stamp: min:sec.
(B) Expression of cell surface Siglec-1 on mature DCs.
T cells as they scan the DC surface. Indeed, T cells contacting control DCs or Env-deficient virus-containing DCs did not decelerate during contacts, whereas a substantial reduction in scanning speeds was observed in T cells contacting HIV-captured DCs (Figures 5E–5G). These data suggest that surface-accessible HIV particles may function as adhesive molecules to “slow” T cell migration, prolonging DC:T cell contact times to enhance cell-cell HIV spread.

**gp120:CD4 Binding Induces Signaling that Further Stabilizes DC:T Cell Interactions**

We postulated that repeated gp120:CD4 interactions during T cell scanning of the DC surface may invoke signals that promote stable DC:T cell conjugates. Previous studies have demonstrated that gp120:CD4 engagement induces phosphorylation of Lck, TCRβ chain, and AKT and induces T cell stop (Hioe et al., 2011; Vasiliver-Shamis et al., 2008, 2009). Since LFA-1:ICAM-1 adhesive contacts are also an essential facilitator of DC trans-infection (Hioe et al., 2011; Wang et al., 2009), we tested whether Siglec-bound HIV can regulate LFA-1 activation in scanning T cells. Using a monoclonal antibody that binds to high affinity confirmation LFA-1 (clone 24) (Laufer et al., 2018), a measurable increase in high affinity LFA-1 was observed in T cells after co-culture with wild-type HIV-pulsed DCs, but not with control or HIV-iGag:Env-pulsed DCs, suggesting that gp120-mediated modulation of T cell migration was at least partially dependent on LFA-1 activation (Figure 6A). Blocking LFA-1:ICAM-1 interactions resulted in a substantial reduction in stable DC:T cell contacts and lower T cell infection, supporting their role in cell-cell stabilization and viral transmission (Figures 6B–6D).

To further identify signaling networks activated by gp120:CD4 interactions, T cells were co-cultured with DCs pulsed with either wild-type or HIV-iGag:Env, harvested after 15 min and analyzed for signaling networks using a custom human peptide kinome array (Nickol et al., 2019). Ingenuity pathway analysis (IPA) identified upregulation of the PI3K pathway, calcium signaling, and ERK/MAPK pathways in T cells, among others, during contacts with wild-type, but not HIV-iGag:Env-captured DCs (Figure 6E and Table S1). InnateDB analysis also predicted upregulation of “TCR signaling,” “MAPK pathways,” and “Focal adhesions” (Table S2), identifying signaling pathways that are initiated through Env:CD4 engagement.

**DISCUSSION**

During vaginal transmission, HIV establishes a focal infection within the CVT, which disseminates to draining and distant lymphoid organs prior to viral detection in blood. In order for HIV to establish a persistent infection, it needs to replicate quickly and find new target cells in the face of increasing adaptive immune pressure. It is now well documented that HIV takes advantage of the physiological trafficking properties of immune cells to gain access to large numbers of target T cells and that these mechanisms are particularly important during the early stages of transmission (Deruaz et al., 2017; Fackler et al., 2014; Murooka and Mempel, 2012; Pena-Cruz et al., 2018; Usmani et al., 2019). DCs can enhance viral spread in cultures (Cameron et al., 1992), but insights into cellular dynamics that orchestrate DC-to-T cell viral dissemination using experimental models that allow cells to migrate, locate, and engage each other are still lacking. The main question we addressed in this study is how HIV-captured DCs, which do not express de novo Env on the cell surface, regulate T cell behaviors in the absence of cognate antigen recognition. Herein, we have mapped key mechanisms of how migratory DCs capture, transport, and transmit infectious HIV to susceptible T cells and demonstrate that surface accessible Siglec:HIV clusters act as adhesivesignaling receptors to form a crucial contact point with scanning T cells. This in turn leads to gp120-mediated activation of LFA-1 and signal transduction that induces T cell stop and activation to promote high viral spread. Thus, we describe a mechanism by which a relatively small number of HIV-captured DCs, in the absence of infection, can ignite exponential T cell infection using a previously unknown mechanism of restraining motile T cells to maximize viral spread efficiency.

DCs act as tissue sentinels throughout the body, including the female genital tract (FGT) (Lindquist et al., 2004; Satpathy et al., 2012). The presence of microbial products and inflammatory stimuli leads to their
mature DCs (Gummuluru et al., 2014). It is likely that endocytosed HIV particles are rapidly degraded by from endosomes, consistent with previous characterizations of these non-lysosomal compartments in vaginal HIV exposure in humanized mice, suggesting that S1PR1 may play a dominant role in regulating DC Notably, FTY720 treatment was more effective in suppressing viremia compared with CCR7 blockade afterported with the lymph once they reach collecting lymphatics (Girard and Springer, 1995; Tal et al., 2011).

lymphatic entry, DCs crawl on endothelial cells in the direction of lymph flow, but they get passively trans-
migration from the skin to the lymph nodes is driven by S1PR1 and CCR7 expression (Bromley et al., 2005; Czeloth et al., 2005; Debes et al., 2005; Forster et al., 1999; Gollmann et al., 2008; Idzko et al., 2002; Lan et al., 2005, 2008; Russo et al., 2016; Tal et al., 2011; Weber et al., 2013) and during inflammation (Brown et al., 2010). Following lymphatic entry, DCs crawl on endothelial cells in the direction of lymph flow, but they get passively transported with the lymph once they reach collecting lymphatics (Girard and Springer, 1995; Tal et al., 2011). Notably, FTY720 treatment was more effective in suppressing viremia compared with CCR7 blockade after vaginal HIV exposure in humanized mice, suggesting that S1PR1 may play a dominant role in regulating DC egress. However, we cannot completely rule out the possibility that the CCR7 pathway might contribute to egress of other infected or HIV-containing cell types into peripheral tissues.

Siglec-1 is expressed in cervical CD14^+CD11c^+HLA-DR^+DCs and MDDCs and has been implicated in HIV capture and retention that is dependent on ganglioside GM3 binding and not HIV gp120 (Crocker et al., 2007; Gummuluru et al., 2014; Izquierdo-Useros et al., 2012a, 2012b, 2014; Perez-Zsolt et al., 2019). This was confirmed in our study, as dense clusters of wild-type and Env-deficient HIV both co-localized with Siglec-1 near the DC uropodia. Ultrastructural analysis by TEM confirmed the presence of mature HIV particles within surface-accessible virus containing compartments (VCCs) and membrane invaginations near the rear end of polarized DCs. The observed HIV clusters also co-localized with CD81^+ but were distinct from endosomes, consistent with previous characterizations of these non-lysosomal compartments in mature DCs (Gummuluru et al., 2014). It is likely that endocytosed HIV particles are rapidly degraded by
phagolysosomes for antigen presentation, whereas those that are contained within Siglec-1^CD81^ compartments remain intact for up to 24 h, based on high HIV-GFP retention in our studies. Retained HIV particles can then access target CD4^+^ T cells through DC:T cell interactions in a way that is analogous to the capture of ganglioside-rich exosomes by mDCs and amplification of the immune response without antigen reprocessing (Izquierdo-Useros et al., 2014; Nasr et al., 2014). Subcapsular sinus (SCS) macrophages also capture and transport to DCs or the B cell follicles (Grabowska et al., 2018; Phan et al., 2009; Veninga et al., 2015). HIV and MLV (murine leukemia virus) have been shown to exploit SCS macrophages to access target CD4 T cells and B cells, respectively, and facilitate efficient viral spread in vivo (Sewald et al., 2015). In our study, Siglec-1 was rarely found near the uropodia in control DCs, whereas dense HIV:Siglec-1 clusters localized near the trailing edge shortly after HIV exposure. How HIV:Siglec-1 relocates near the uropodia is not clear, as Siglec-1 lacks tyrosine-based signaling motifs and the cytoplasmic tail is poorly conserved compared with others in the Siglec family (Crocker et al., 2007). However, the uropodia of migrating cells is a specialized platform for organelles, adhesion receptors, and key regulators of the actin polymerization machinery, suggesting that HIV:Siglec-1 clusters may be actively transported to the uropodia rather than passive accumulation. The trailing edge is also enriched with tetraspanin-containing microdomains that are crucial for the formation of VCCs (Barreiro et al., 2008; Suárez et al., 2018). Interestingly, the uropodia of polarized T cells has been shown as the preferred site for membrane-associated Gag accumulation and VS formation (Hatch et al., 2013). Together, Siglec-1-mediated HIV capture and retention occur highly localized within the uropodia of migrating DCs, but within compartments that are accessible by T cells during DC:T cell interactions.

Two-photon microscopy analyses have revealed that naive T cells crawl along the surface of the FRC network in an apparently random manner and continually scan dendritic cells that are also situated on the same FRC networks, in search for cognate antigen (Mempel et al., 2004; Miller et al., 2003). These studies have forced us to re-evaluate the interplay between cell motility, cell-cell contact dynamics, and trans-infection mechanisms, where dynamic DC:T cell interactions occur continuously. We performed live-cell imaging studies in collagen matrices to visually characterize how HIV-containing DCs alter the speed and duration of T cell scanning behaviors in a fibrillar 3D setting that better reflect their physiological interactions in vivo. High T cell infection rates were observed in the presence of DCs in collagen compared with free-virus alone, and this corresponded with a high proportion of stable DC:T cell contacts. Interestingly, we observed that stable DC:T cell conjugates were abrogated in the presence of Env-deficient HIV, or after CD4 antibody blockade, despite similar viral capture kinetics and localization to wild-type HIV. These data seemed to suggest that the presence of virus-containing invaginations modulated T cell scanning and migration and that this involved Env:CD4 interactions. Elegant ultrastructural analysis of the DC:T cell conjugate site has revealed T cell-derived filopodial protrusions that penetrate into the virion-rich folds of dendritic cells and attachment of viral particles along these actin-rich extensions (Felts et al., 2010). Addition of blocking antibodies against gp120 resulted in retention of virions by dendritic cells, whereas CD4 antibody blockade prevented DC-to-T cell viral transmission. Together, these data illustrate a scenario where actively scanning T cells probe the DC surface with CD4-rich filopodial protrusions, which upon contact with HIV clusters embedded within membrane invaginations, leads to generation of signals that culminate in T cell arrest and VS formation in a gp120:CD4-dependent manner. Our observations that T cells appear either arrested...
Figure 6. LFA-1:ICAM-1 Binding Stabilizes Stable DC:T Cell Contacts and Facilitates Viral Transmission

(A) Flow cytometry analysis of high-affinity LFA-1 expression (mAb clone 24) on T cells after co-culture with HIV-captured DCs. Data are normalized to no virus DC:T cell controls. Data from two healthy donors. Mean ± SEM are shown. **p < 0.01, Unpaired Student’s t test.

(B) DC:T cell contact duration in the presence or absence of LFA-1:ICAM-1 interactions. ICAM-1, anti-ICAM-1 antibody; aLFA-1, anti-LFA-1 antibody. Red lines indicate median values. **p < 0.01, ***p < 0.001, Mann-Whitney U test. Data from eight independent experiments are shown (n = 957 total DC:T cell contact events).

(C) Percent distribution of DC:T cell contact times defined as brief (>7 min; black), prolonged (7–17 min; blue), or stable (>17 min; red) from data in (B) are shown.

(D) Percent normalized T cell infection with the indicated antibody blockade. Data are from two independent experiments. Mean ± SEM are shown. n.s., not significant. **p < 0.01, Unpaired Student’s t test.

(E) IPA identified biological pathways that were significantly (p < 0.01) upregulated (Z score > 1) in T cells co-cultured with wild-type HIV-pulsed DCs (T cells co-cultured with HIV-iGagEnv-pulsed DCs set as background controls). The p value (red line), calculated with the Fischer’s exact test, reflects the likelihood that the association between a set of activated kinases and a biological function is significant (p value ≤ 0.05).
or display low motility when contacting HIV-captured DCs, but not Env-deficient or control DCs, are in line with this model. Soluble and virion-associated gp120 can induce Ca\textsuperscript{2+} flux downstream of the chemokine receptors (Melar et al., 2007; Weissman et al., 1997), and calcium signaling mediates T cell arrest upon cognate antigen recognition (Bromley et al., 2000, 2001). Whether calcium signaling is required for DC:T cell conjugate formation is unclear, since gp120 molecules embedded into planar lipid bilayers arrested T cells without detectible calcium release (Vasiliver-Shamis et al., 2009). Other surface molecules can play a role in stabilizing the VS, as CD81 tetraspanin (Gousset et al., 2008), GM1 ganglioside (Jolly and Sattentau, 2005), and integrins (Jolly et al., 2004, 2007) have all been shown to accumulate at the site of cell-cell contact. Of particular importance are LFA-1:ICAM-1 adhesive interactions that facilitate VS formation (Jolly et al., 2004, 2007; Rodriguez-Plata et al., 2013; Vasiliver-Shamis et al., 2010). Importantly, we found that gp120:CD4 binding was required for activation of the high-affinity conformation of LFA-1 in T cells during co-cultures with HIV\textsuperscript{+} DCs, suggesting that HIV:CD4 binding represents an initial, critical step in facilitating prolonged DC:T cell contacts. Our data are consistent with previous studies where HIV gp120 triggered LFA-1 activation in resting CD4 T cells in a CD4-dependent manner (Hioe et al., 2011). Additionally, LFA-1 activation can also function beyond their adhesive properties to promote VS formation, as T cell polarization and MTOC reorganization toward the cell-cell contact site upon LFA-1 ligation has been reported (Starling and Jolly, 2016). Thus, our data describe a model where surface accessible HIV on DCs modulates T cell scanning behaviors by activating a gp120-dependent signaling cascade that favors stable DC:T cell contacts and viral transmission. It is unlikely that DC:T cell conjugates observed in our studies are the result of cognate antigen recognition, since T cells were isolated from healthy donors and the number of HIV-specific T cells are expected to be low. However, given that HIV-specific T cells are preferentially infected during acute infection (Douek et al., 2002; Lore et al., 2005), an intriguing question is whether antigen recognition further facilitates DC:T contacts that can simultaneously prime and transmit HIV particles to T cells, and is a focus of our ongoing studies.

HIV Env induces calcium flux, MEK/ERK activation, Lck phosphorylation, and partial ZAP-70 activation, among others, in CD4 T cells, but their role in viral entry or infection has been debated (Cicala et al., 2006; Goldman et al., 1994; Hioe et al., 2011; Juszczak et al., 1991; Melar et al., 2007; Popik et al., 1998; Vasiliver-Shamis et al., 2008, 2009). Recent studies by Lens et al. showed that Env:CD4 clustering at the site of T cell-T cell contact initiated signaling downstream of the T cell receptor in infected cells, leading to enhanced cell-cell HIV transmission (Len et al., 2017). In their study, robust signaling in target T cells were also observed, suggesting that de novo Env expression on infected T cells delivered activation signals during cell-cell contacts. Here, we addressed whether surface-bound HIV clusters induce similar T cell signaling pathways that modulate migratory behaviors in a way that favors viral spread. Our kinase array and IPA analysis confirmed activation of calcium signaling, TCR signaling, and ERK/MAPK pathways, among others, through Env:CD4 binding, which can collectively modulate T cell migration behaviors. Tec family of tyrosine kinases (Z score 2.1, p value 3.4x10^-6) plays an indispensable role in T cell maturation, activation, and differentiation (Andreotti et al., 2010; Schwartzberg et al., 2005), where Itk phosphorylation downstream of the T cell receptor regulates Ca\textsuperscript{2+} mobilization, actin mobilization, and cytoskeletal reorganization. Previous studies have demonstrated that Itk is phosphorylated in T cells shortly after contact with gp120- and ICAM-1-embedded lipid bilayers (Vasiliver-Shamis et al., 2009) and that Itk deficiency disrupts F-actin assembly and reduces intracellular p24 levels upon HIV infection (Francois and Klotman, 2003), suggesting that this pathway may regulate both T cell motility and virus transcription. Similarly, activation of the PI3K pathway (Z score 2.1, p value 1.3x10^-5) through gp120:CD4 interactions may regulate T cell migration behaviors and help promote HIV infection post-viral entry (Francois and Klotman, 2003; Nabel and Baltimore, 1987). Finally, the canonical MAPK/ERK pathway (Z score 1.9, p value 1.1x10^-4) regulates cell adherence, metabolism, and cell proliferation, and inhibiting ERK signaling is known to restrain ECM-induced cell motility (Cuevas et al., 2007; Klemke et al., 1997; Krueger et al., 2001). Earlier work showed that ERK activation regulated HIV infectivity and was required for efficient organization of the reverse transcription complex (Jacque et al., 1998; Yang and Gabuzda, 1999). Activation of the Th17 signaling pathway may increase virus infection, as Th17-lineage CCR6\textsuperscript{+} CD4\textsuperscript{+} T cells are preferentially infected early during infection (Planas et al., 2017; Stieh et al., 2016). Together, kinome analyses suggest that DC-bound HIV can activate signaling pathways that regulate both T cell motility and viral susceptibility. Signaling activation by surface-bound HIV is not surprising, given that as little as two HIV particles were sufficient to stimulate calcium influx in primary T cells (Melar et al., 2007). Although there are a relatively low number of Env per virion...
(~14 trimers) (Zhu et al., 2006), their access to CD4 molecules is augmented by the large numbers of HIV particles found within VCCs and the high frequency of serial DC:T cell interactions that occur in lymphoid organs. It is important to note that, although gp120-dependent signaling is initiated downstream of either the CD4 molecule or the CXC4/CCR5 co-receptor, gp120:CD4 engagement is a requirement for both (Melar et al., 2007). Lck phosphorylation occurs downstream of the CD4 molecule, whereas calcium signaling is exclusively downstream of chemokine receptors, but these signals converge to regulate T cell migration behavior and function. Prior engagement of gp120 by the CD4 molecule can induce a conformational change that supports subsequent binding with the co-receptor, indicating that CD4 and co-receptor do not have to be present at the same time (Popik et al., 1998; Weissman et al., 1997). This raises an intriguing possibility that serial T cell scanning behaviors can “prime” virion-associated Env within VCCs, potentiating subsequent T cell encounters that induce a unique signaling cascade to modulate T cell motility, cell-cell adhesion, and HIV susceptibility.

In summary, we undertook a visualization-based approach using 3D collagen to better characterize the dynamics of DC-HIV capture, retention, and transmission of virus to susceptible T cells. We describe a mechanism by which surface-bound HIV particles on motile DCs function as adhesive receptors to contact and restrain migrating T cells to facilitate DC-to-T cell viral spread. Subsequent activation of signaling cascade facilitated by Env:CD4 binding further modulates T cell migration behaviors to enhance HIV susceptibility, providing additional insights into cellular mechanisms that contribute to the exponential viral replication observed in lymphoid organs during acute infection.

Limitations of the Study
The present study examined DC:T cell interaction dynamics and activation of signaling pathways in the presence of HIV particles within a 3D collagen model to more closely recapitulate cell-cell contacts in lymph nodes. These studies are yet to be verified in vivo using multiphoton intravital microscopy (MP-IVM) (Murooka et al., 2012; Murooka and Mempel, 2013; Usmani et al., 2019), where interactions with stromal and other immune lymph node cells in the presence of physiological blood and lymph flow can further modulate DC:T cell conjugate formations. The current study was also limited to monocyte-derived dendritic cells to model HIV-capture dynamics in collagen, and thus further validation using primary DC subsets isolated from mucosal tissues is warranted.

Lead Contact
Further information and requests for resources and reagents should be directed to the Lead Contact, Dr. Thomas Murooka.

Materials Availability
All materials developed and used in this study will be made available upon request.

Data and Code Availability
Data and code used in this study will be made available upon request.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101427.

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AUTHOR CONTRIBUTIONS
Conceptualization and methodology, W.H.K. and T.T.M.; Investigation, W.H.K., P.L., O.A., U.M., and R.H.; Resources, J.K. and R.P.; Writing W.H.K. and T.T.M. Funding Acquisition, W.H.K., J.K., and T.T.M.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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Supplemental Information

HIV-Captured DCs Regulate T Cell Migration and Cell-Cell Contact Dynamics to Enhance Viral Spread

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### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Human CD3           | Biolegend | clone: HIT3a |
| Human CD4           | Biolegend | clone: RPA-T4 |
| Human CD11c         | Biolegend | clone: Bu15 |
| Human CD14          | Biolegend | clone: 63D3 |
| Human CD80          | Biolegend | clone: 2D10 |
| Human CD81          | Biolegend | clone 5A6 |
| Human CD83          | Biolegend | clone: HB15E |
| Human CD86          | Biolegend | clone: IT2.2 |
| Human CD169         | Biolegend | clone: 7-239 |
| Human CD209         | Biolegend | clone: 9E9A8 |
| Human CCR7          | Biolegend | clone: G043H7 |
| Human CD11+CD18 (high affinity conformation) | Abcam | clone: 24 |
| PSGL-1              | Biolegend | clone KPL-1 |
| goat anti-mouse IgG antibody conjugated with AF568 | Thermo Scientific | Cat #A11004 |
| Neutralizing Siglec-1 antibody | R&D | Cat #AF5197-SP |
| Human LFA-1         | BD Biosciences | clone L130 |
| Human ICAM-1        | BD Biosciences | clone LB-2 |
| Neutralizing CCR7 antibody | R&D | MAB197R-SP |
| Mouse LYVE-1        | R&D | Clone: 223322 |
| anti-human CD3ε/CD28 antibody coated dynabeads | Life Technologies | Cat #11131D |
| LPS from Salmonella Minnesota | Invivo Gen | Cat #tlrl-smlps |
| Fetal calf serum    | VWR Seradigm | Cat #1500-500 |
| Nuncclon Spera flasks | Thermo Scientific | Cat #174951 |
| Bovine collagen     | PureCol | Cat #5005-100ML |
| Dynasore hydrate    | Sigma | Cat #D7693 |
| Texas Red conjugated dextran (70kDa) | Thermo Scientific | Cat #D-1830 |
| Texas Red conjugated phalloidin | Thermo Scientific | Cat #T7471 |
| Collagenase D       | Roche | Cat #11088866001 |

### Bacterial and Virus Strains

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| HIV-iGag/dTomato (BaL envelope) | This paper | N/A |
| HIV-iGag/dTomato Δenv (BaL envelope) | This paper | N/A |

### Biological Samples

### Chemicals, Peptides, and Recombinant Proteins

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| human CCL19         | Biolegend | Cat #582102 |
| Chemical Name                  | Company          | Catalog Number |
|-------------------------------|------------------|---------------|
| human CCL21                   | Biolegend        | Cat #582202   |
| human S1P                     | Sigma            | Cat #73914    |
| Human IL-4                    | Biolegend        | Cat #574008   |
| Human IL-2                    | Peprotech        | Cat #200-02   |
| Human GM-CSF                  | Biolegend        | Cat #578606   |
| FTY720-phosphate              | Caymen           | Cat #10008639 |
| Raltegravir                   | NIH ARRRP        | Cat# 11680    |

**Critical Commercial Assays**

| Assay Name                    | Company          | Catalog Number |
|-------------------------------|------------------|---------------|
| GenElute HP Plasmid Maxiprep Kit | Sigma           | NA0310-1KT    |
| Human CD14+ monocyte isolation kit | Stemcell Technologies | Cat #17858   |
| Human CD4+ T cell isolation kit | Stemcell Technologies | Cat #17952   |

**Deposited Data**

**Experimental Models: Cell Lines**

| Cell Line                      | Company          | Catalog Number |
|--------------------------------|------------------|---------------|
| HEK 293T cells                 | ATCC             | ATCC CRL-11268|
| MAGI CCR5 cells                | NIH ARRRP        | Cat #3580     |

**Experimental Models: Organisms/Strains**

**Oligonucleotides**

**Recombinant DNA**

**Software and Algorithms**

| Software/Algorithm             | Company          |
|--------------------------------|------------------|
| Imaris 8.2                     | Bitplane         |
| ImageJ                         | NIH/freeware     |
| Prism 6                        | GraphPad         |
| FlowJo                         | Treestar         |
| Matlab                         | Mathworks        |
| Ingenuity pathway analysis (IPA)| Ingenuity Systems|
| InnateDB                       | www.innatedb.com  |

**Other**
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Thomas Murooka (thomas.murooka@umanitoba.ca).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cells

Human CD14+ monocytes and CD4+ T cells (both from Stem cell Technologies) were isolated from PBMCs of healthy donors (NetCAD, Canadian Blood Services). All cells derived from donors are anonymized and contain no information regarding the gender, race or health status. Studies using human blood products were approved by the University of Manitoba Biomedical Research Ethics Board (B2015:030).

MAGI.CCR5 cells were obtained from the NIH ARRRP (Cat #3522) and grown in DMEM supplemented with 10% fetal calf serum (VWR Seradigm), 2 mM GlutaMAX (Gibco), 1mM sodium pyruvate (Corning) and 10mM HEPES (Sigma-Aldrich) under 37°C/5% CO2 conditions. The parental cell line of MAGI is a HeLa cell clone, which is a female cell line. This cell line has not been authenticated.

Viral Constructs and Preparation of Viral Stocks

The R5-tropic HIV Gag-iGFP/dTomato (HIV-iGFP for short) reporter encodes for two fluorescent proteins: (1) a fusion protein between GFP and the structural polypeptide Gag (Gag-iGFP) to visualize GFP-containing HIV particles (Muller et al., 2004), and (2) a nef-ires-dTomato gene cassette that express soluble dTomato (Usmani et al., 2019) after productive infection. The env-deleted plasmid was constructed by digesting the HIV Gag-iGFP plasmid
with PsiI to generate a frameshift mutation in the env gene (HIV-iGFPΔenv), as described previously (Murooka et al., 2012). All plasmids were confirmed by Sanger sequencing. All HIV-1 stocks were generated by transient transfection of HEK 293T cells using calcium phosphate. To generate HIV-iGag virus, cells were co-transfected with the pGagPol plasmid (NIH ARRRP). Viral supernatants were collected 48 hours after transfection and layered over a 20% sucrose solution before ultra-centrifugation at 35,000 rpm for 90 min using an SW70Ti rotor (Beckman Coulter). Virus pellets were resuspended in PBS, aliquoted and stored at -80°C until use. Each HIV stock was titrated using MAGI.CCR5 cells and expressed as infectious blue focus units (bfu) per ml.

**METHOD DETAILS**

**Cell culture**

Human CD14+ monocytes and CD4+ T cells (Stem cell Technologies) were isolated from PBMCs of healthy donors (NetCAD, Canadian Blood Services). Cell purity for both populations were routinely >95%. Monocytes were differentiated into immature dendritic cells by seeding into Nunclon™ Sphera™ flasks (Thermo Scientific) with 50ng/mL each of human granulocyte-macrophage colony-stimulating factor (hGM-CSF; Biolegend) and interleukin 4 (IL-4; Biolegend) in complete RPMI1640 media supplemented with 10% FBS (VWR Seradigm), 2 mM GlutaMAX (Gibco), 1mM sodium pyruvate (Corning Cat #25-000-CI) and 10mM HEPES (Sigma-Aldrich), for five days. On day 5, immature monocyte-derived dendritic cells (MDDCs) were stimulated with 100ng/mL purified lipopolysaccharide (LPS) from *Salmonella minnesota* (Invivo Gen) for 24-48 hours. Naïve CD4+ T cells were activated with anti-human CD3ε/CD28 antibody coated dynabeads (1:1 bead:cell ratio, Life Technologies) in complete RPMI 1640
media. After two days, beads were magnetically removed and T cells were cultured for another 4-6 days in complete medium containing 50 IU/mL human rIL-2 (Peprotech), keeping cell densities at 2 x 10^5 cells/mL. Day 7 expanded T cells were used for all experiments. Studies using human blood products were approved by the University of Manitoba Biomedical Research Ethics Board (B2015:030).

**Flow cytometry and antibodies**

Phenotypic characterization of MDDCs and CD4+ T cells were performed using the BD FACSCanto-II and analyzed with FlowJo software (Tree Star). Human Fc receptors (FcR) blockade was performed using the TruStain FcX™ (Biolegend) solution. MDDC and T cells were phenotyped with a panel of directly conjugated anti-human mAb (Biolegends) as indicated in the Key Resources Table. HIV-captured DCs and HIV infected T cells were fixed with 2.5% paraformaldehyde prior to flow cytometry analysis.

**Virus capture and retention assays**

3-6 million DCs were pulsed with HIV-iGFP (1,550 – 3,300 bfu) and their capture efficiency measured by flow cytometry. Because HIV-iGFPΔenv titers were not attainable using MAGI.CCR5 indicator cell lines, we pulsed DCs with increasing amounts of either parental or HIV-iGFPΔenv stocks to achieve equivalent numbers of HIV-captured DCs by flow cytometry (data not shown). To evaluate the role of Siglec-1 on HIV capture efficiency, DCs were pre-treated with anti-Siglec-1 neutralizing antibody (2ug; R&D) for 15 mins at room temperature prior to virus incubation and imaging studies.
**Live-cell imaging in 3D collagen chambers**

Collagen type I was used to recapitulate the three-dimensional (3D) fibrillar networks found in the lymph node T cell zone (Wolf et al., 2009) and were assembled as previously described (Sixt and Lammermann, 2011). Bovine collagen (PureCol) was used to achieve a final concentration of 1.7mg/mL in each chamber. To characterize the HIV capture dynamics by DCs, 1-2 million DCs were labeled with Celltracker Blue (CMAC; 30µM) or Celltracker Red (CMTPX; 7.5μM), washed and embedded into collagen along with HIV particles. Chambers were allowed to solidify for 45 minutes at 37°C / 5% CO₂ and placed onto a custom-made heating platform attached to a temperature controller apparatus (Werner Instruments). A thermocouple device was used to continuously monitor and maintain the chamber temperature at 37°C. A multiphoton microscope with two Ti:sapphire lasers (Coherent) was tuned to between 780 and 920 nm for optimized excitation of the fluorescent probes used. For four-dimensional recordings of cell migration, stacks of 13 (or 26) optical sections (512 x 512 pixels) with 4 µm z-spacing were acquired every 15 (or 30) seconds to provide imaging volumes of 48 (or 96) µm in depth. Emitted light was detected through 460/50 nm, 525/70 nm and 595/50 nm dichroic filters with non-descanned detectors. All images were acquired using the 20X 1.0 N.A. Olympus objective lens (XLUMPLFLN; 2.0mm WD).

To perform live imaging studies of antibody-labeled DCs, DCs were pulsed with HIV particles for 4 hours, washed extensively with warm complete media to remove free virus, and then incubated with primary antibodies against Siglec-1, PSGL-1 and CD81. Secondary goat anti-mouse IgG antibody conjugated with Alexa Fluor 568 or Alexa Fluor 594 (Thermo Fisher Scientific) were added at 1:100 dilution for an additional 15 minutes, then cells were washed and embedded into collagen chambers for imaging studies. In some experiments, cells were
pretreated with either isotype or blocking antibodies for LFA-1 (BD Biosciences), CD4 (Biolegend) or ICAM-1 (BD Biosciences) prior to embedding into collagen chambers. To study the role of actin, CMAC-labeled cells were pre-treated with Dynasore (100µM) for 20 mins at room temperature and embedded into collagen chambers with HIV particles. As Dynasore is a reversible drug, for some experiments, Dynasore were washed out after 2 hours of incubation. These washed cells were then embedded with HIV-1 particles in the collagen chamber. In some studies, HIV-captured DCs were pulsed with Texas Red-conjugated dextran (70kDa; Thermo Fisher Scientific) for 15 mins at 37°C prior to embedding into collagen chambers. A 1:4 or 1:8 ratio of DCs and CD4 T cells, respectively, was used for visualizing cell-cell contact dynamics in collagen chambers.

**Immunofluorescent staining in collagen chambers**

To preserve the migratory DC morphology in collagen, chambers were fixed with 4% paraformaldehyde/5% sucrose overnight at 37°C / 5% CO₂, as described previously (Lopez et al., 2019). Glycine buffer (0.15M) was used to quench residual PFA. Next, chambers were permeabilized using a 0.5% triton-X solution for 48 hours at 4°C. Collagen gels were blocked using 1% bovine albumin serum (BSA) and labeled with Texas Red™-conjugated phalloidin (ThermoFisher), as per manufacturer’s protocol.

**Transmission electron microscopy**

Transmission electron microscopy was performed to reveal the ultrastructure of the HIV-containing compartments in migratory DCs in collagen. DC-embedded chambers were fixed, quenched with glycine buffer and incubated in 0.05% Tween in PBS. Gels were fixed with 1%
osmium tetroxide for 2 hours, then dehydrated using the following solutions: 30% ethanol, 50% ethanol, 70% ethanol, 90% ethanol, 100% ethanol, 100% methanol, then 100% propylene oxide. Gels were placed in embedding solution (mixture of EMbed 812, DDSA, NMA and DMP-30) for 24 hours prior to ultra-sectioning with the Reichert ultrathin microtome at a thickness of 70-90nm. The EM grids were then stained with uranyl acetate for 30 minutes and lead citrate for 10 minutes. Electron micrographs were taken with a Philips CM10 transmission electron microscope.

**Transwell® assay**

To investigate the chemotactic migration of HIV-bearing MDDCs towards lymph node chemokines, 0.5-1 million DCs were placed into the top insert at a final volume of 100µl of a Transwell plate (5µm pore size; Corning). 600µl of media containing human CCL19 (2µg; Biolegend) and CCL21 (2µg; Biolegend) or S1P (10nM) was added to the bottom chamber. Transwell plates were incubated for 3 hours at 37°C/5% CO₂, after which migrated DCs in the bottom well were collected and counted using counting beads by flow cytometry (Biolegend). For receptor blockade studies, DCs were pre-treated with anti-CCR7 antibody (2µg; R&D) or FTY720-P (10 µM) prior to analysis in transwell assays.

**Ex vivo skin crawl-in assay**

A crawl-in assay using explanted mouse ears was used to assess chemokine-dependent DC migration in the dermal layer in real time, as previously described, with some modifications (Weber and Sixt, 2013). Briefly, the ears of female/male 6-8-week-old Balb/c mice were excised, and ear sheets composed of the epidermis and dermis was obtained using forceps. After FcR
blockade, the lymphatic vessels were labeled with Alexa Fluor-594 conjugated mouse anti-LYVE-1 antibody (R&D) for 30 minutes at room temperature. Ear sheets were overlaid with 0.25 million DCs with or without HIV for 20 minutes at 37°C to allow them to crawl into the dermal layer. After washing away unbound DCs, skin sheets were mounted onto a glass slide with complete media and prepared for live-cell microscopy. In some studies, DCs were pre-treated with anti-CCR7 and/or FTY720 prior to overlay onto dermal tissue layers.

**Image analysis**

Time lapse micrograph images were transformed using Imaris 8.3 (Bitplane) to generate maximum intensity projections (MIPs) and exported as Quicktime movies. Automated 3D tracking of DC centroids was performed for all motility analyses, and subsequent cell track parameters (arrest coefficient, mean displacement) were analyzed using a custom script in Matlab (Mathworks). To characterize the spatial distribution of HIV-containing compartments in motile DCs, line profile analyses were performed to determine fluorescent intensity profiles on the z-axis, where 0 represents the uropodia and 1 represents the leading edge. Cell surface area and cell perimeter measurements were performed using ImageJ using the wand (tracing) tool after color thresholding. Circularity measurements were also performed using ImageJ, where a measure of 0 indicated a straight line and 1 indicated a perfect circle, as previously described (Usmani et al., 2019). Contact duration and cell migration speeds during contacts were analyzed using ImageJ.

**HIV infection in collagen matrix**
0.5 million DCs and 2-4 million CD4+ T cells were embedded into collagen gel with HIV particles and placed into a 48-well plate. Solidified gels were incubated for 4 hours at 37°C, then cells were recovered after digestion with collagenase D (Roche), washed and subsequently placed in complete media containing 50IU/mL hrIL-2 in a 24 well plate. T cells in the culture supernatant were gently removed, whereas DCs remained attached to the plastic culture vessel (which removed >95% of DCs). Recovered T cells were incubated for another 48 hours and productive infection was assessed by flow cytometry. Cell-free infection in collagen matrix was performed in parallel but in the absence of DCs. In some experiments, cells were pretreated with either isotype or neutralizing antibodies prior and during DC co-cultures in collagen. Raltegravir (20µM) was used to prevent productive infection and served as a negative control.

**Phenotypic analysis of high affinity LFA-1**

One million DCs were pulsed with HIV (WT and Δenv) for 3 hours and washed with RPMI medium to remove unbound virus. DCs were co-cultured with 4 million resting autologous CD4+ T cells in collagen gel for 3.5 hours. T cells were recovered using collagenase D and were immediately stained for high affinity LFA-1 expression (Human CD11+CD18, clone 24) for flow cytometry analysis.

**Kinome analysis**

Design, construction and application of the human peptide arrays were based on a previously reported protocol (Kindrachuk et al., 2014). DCs were pulsed with either wildtype or HIV-iGFPΔEnv, washed extensively and co-cultured with autologous T cells for 15 minutes at 37°C. Cells were placed on ice and T cells were separated using a negative selection magnetic column
to remove CD11c+ DCs (purity >98%). T cells were pelleted, cell lysates prepared and incubated with human kinome arrays (JPT Technologies). Briefly, cell pellets were lysed with 100uL buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM Na3VO4, 1 mM NaF, 1 g/ml leupeptin, 1 g/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride) and incubated on ice for 10 min. Cell lysates were clarified by centrifugation at 14,000 rpm. Cell lysates were transferred to fresh microcentrifuge tubes, and the total protein concentrations were measured using the Pierce BCA Protein Assay Kit. Activation mix (50% glycerol, 50 µM ATP, 60 mM MgCl2, 0.05% Brij 35, 0.25 mg/mL bovine serum albumin) was added to the equivalent amounts of total protein (100 µg) for each sample, and total sample volumes were matched by the addition of kinome lysis buffer. Samples were spotted onto kinome peptide arrays (JPT Peptide Technologies GmbH, Berlin, Germany) and incubated for 2 h at 37 °C and 5% CO2. Following incubation, arrays were washed once with PBS containing 1% Triton X-100, followed by a single wash in deionized H2O. Arrays were stained with PRO-Q Diamond phosphoprotein stain (Invitrogen, Carlsbad, CA, USA) for 1 h with gentle agitation. Arrays were subsequently destained (20% acetonitrile, 50 mM sodium acetate, pH 4.0) 3 times × 10 min each with the addition of fresh destain each time. A final 10 min wash was performed with deionized H2O. Arrays were dried by gentle centrifugation. Array images were acquired using a PowerScanner microarray scanner (Tecan, Morrisville, NC, USA) with a 580-nm filter to detect dye fluorescence. Signal intensity values were collected using Array-Pro Analyzer version 6.3 software (Media Cybernetics, Rockville, MD, USA).

**Kinome data analysis**
The specific responses of each peptide were calculated by subtracting background intensity from foreground intensity. Signal intensities induced by ΔEnv HIV-iGFP were subtracted from the intensities from the time-matched, wildtype HIV-iGFP biological conditions, and test statistics were calculated. Average intensities were then taken over the three replicate intensities, and these values were subjected to hierarchical clustering analysis using InnateDb (www.innatedb.com) and ingenuity pathway analysis (IPA) software (Ingenuity Systems, Redwood City, CA). For our investigations, input data were limited to peptides that showed consistent responses across the biological replicates (p<0.05) as well as statistically significant changes between the wildtype and ΔEnv HIV conditions to identify biological pathways that are specifically induced through Env:CD4 interactions. In Figure 6E, we used the IPA regulation z-score algorithm to identify biological functions that were above a z-score >1 (black bars), and p-values (red line), calculated with the Fischer’s exact test, reflects the likelihood that the association between a set of active kinases and a biological function is significant [p-value < 0.05].

MATERIAL AVAILABILITY

HIV reporter plasmids generated in this study will be available to researchers upon request.

QUANTIFICATION AND STATISTICAL ANALYSIS

Unpaired Student’s t test and Mann-Whitney U test were used for comparisons of datasets with normal and non-normal distribution, respectively, using Prism 5 (GraphPad). Median and p
values from statistical analyses are indicated in each graph. When p values were higher than 0.05, differences were considered as not significant.

**DATA AND SOFTWARE AVAILABILITY**

MATLAB cell motility analysis scripts will be made available upon request. Motility parameters were calculated as described by Beltman et al. (2009). Briefly, mean velocities were defined as the average of the distance a cell traveled over the time period between the two consecutive frames. Confinement ratio is calculated based as the ratio of the displacement a cell has traveled over the total length of the path that cell has travelled. Figures and illustrations were prepared using Adobe® Illustrator and BioRender.com.
Supplementary Figure 1: Schematic and validation of the HIV Gag-iGFP/dTomato reporter, Related to Figure 1 and 4. (A) Schematic illustration of the reporter proviral vector. PR = protease cleavage sites. (B) T cells were infected with HIV-iGFP and characterized by flow cytometry and confocal microscopy. (C) T cells were infected with HIV-iGFP in the presence or absence of DCs, and assessed for infection after 48 hours. Raltegravir was added at the time of infection to serve as control. The presence of DCs enhanced T cell infection, and productively infected dTomato+ T cells (red dots) downregulate CD4 expression, as expected. Representative data from 3 independent experiments are shown.
Supplementary Figure 2: 3D migration behavior of mature DCs after HIV exposure, Related to Figure 1. 

(A) Phenotypic analysis of monocyte-derived dendritic cells (MDDCs) before and after stimulation with LPS for 24 hours. 

(B) Immature or mature DCs were either left alone or exposed to HIV-iGFP for 4 hours, washed, and embedded into collagen chamber for live-cell microscopy studies. Each data point represents a single cell. Bar graph indicate median values. Cumulative data from 3 independent experiments are shown. n.s. = not significant. **** p ≤ 0.001.
Supplementary Figure 3: Transmission electron micrograph of polarized HIV-captured DCs in collagen matrix, Related to Figure 2. Mature HIV particles are found in membrane invaginations close to the cell surface (yellow arrowheads in enlarged insets). The leading and trailing edge are indicated, based on the location of the nucleus (N).
Supplementary Figure 4: DC-SIGN does not facilitate HIV capture by motile DCs in 3D collagen, Related to Figure 3. (A) Phenotypic expression of DC-SIGN between immature and mature MDDCs. (B) % HIV capture by DCs, relative to DCs treated with vehicle prior to incubation with virus. Representative data from two independent experiments is shown. Mean +/- SEM. n.s. = not significant. (C) Representative micrograph of a HIV-captured DC in collagen matrix stained for DC-SIGN. (D) Representative line profile analysis of HIV-Gag-iGFP and DC-SIGN fluorescence intensity in a polarized HIV-captured DC shown in (B) (n=13).