

Blocking of integrins inhibits HIV-1 infection of human cervical mucosa immune cells with free and complement-opsonized virions

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The initial interaction between HIV-1 and the host occurs at the mucosa during sexual intercourse. In cervical mucosa, HIV-1 exists both as free and opsonized virions and this might influence initial infection. We used cervical explants to study HIV-1 transmission, the effects of opsonization on infectivity, and how infection can be prevented. Complement opsonization enhanced HIV-1 infection of dendritic cells (DCs) compared with that by free HIV-1, but this increased infection was not observed with CD4⁺ T cells. Blockage of the α 4-, β 7-, and β 1-integrins significantly inhibited HIV-1 infection of both DCs and CD4⁺ T cells. We found a greater impairment of HIV-1 infection in DCs for complement-opsonized virions compared with that of free virions when α M/ β 2- and α 4-integrins were blocked. Blocking the C-type lectin receptor macrophage mannose receptor (MMR) inhibited infection of emigrating DCs but had no effect on CD4⁺ T-cell infection. We show that blocking of integrins decreases the HIV-1 infection of both mucosal DCs and CD4⁺ T cells emigrating from the cervical tissues. These findings may provide the basis of novel microbicidal strategies that may help limit or prevent initial infection of the cervical mucosa, thereby reducing or averting systemic HIV-1 infection.

Keywords: CD4⁺ T cells · Complement system · DCs · HIV · Integrins



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Introduction

The most common route of HIV-1 transmission is by hetero-sexual intercourse, with women accounting for more than half of the newly HIV-1-infected individuals [1]. During sexual transmission, the first interactions between HIV-1 and the host occur at

mucosal sites, such as genital and rectal mucosa. Within hours of initial exposure, the virions can reach the submucosa and local events that contribute to the establishment of a systemic infection are set in motion [2], leading to high levels of infection and destruction of CD4⁺ T effector memory cells, particularly in the gastrointestinal tract [3]. While immune cells can be found at

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sites of inflammation throughout the female genital tract, in the absence of such inflammation, immune cells are found most frequently in the cervical transformation zone and surrounding tissues and are likely sites for HIV infection [4, 5].

Studies in macaques inoculated with SIV, to model vaginal transmission of HIV, have shown that SIV virions cross the single layer mucosal barrier of the endocervix, however, virus has also been shown to enter through the stratified epithelium [6], especially at sites of preexisting inflammation. Sexually transmitted diseases and genital infections increase the risk of acquiring HIV-1 infection due to disturbed epithelial layer and increased availability of target cells in the mucosa; moreover, the production of proinflammatory cytokines, such as TNF- α , is associated with an enhanced HIV-1 replication [7].

Another potential mechanism available to the virus to penetrate the mucosa is by exploiting dendritic cells (DCs) and Langerhans cells (LCs) localized in the stratified epithelium, where these cells can pick up HIV-1 and transfer the virus to the submucosa [8]. When HIV-1 has reached the submucosa, the primary target cells for the virions are CD4⁺ T cells, macrophages, and DCs. In addition to binding to CD4 and the coreceptor CCR5/CXCR4 [9], HIV-1 can be captured by DCs via an array of cell surface receptors, such as heparin sulfate proteoglycans, C-type lectins, and integrins [9–11]. As soon as 24 h postexposure, DCs with captured virions can reach draining LNs where they can transfer infectious HIV-1 to surrounding cells [2, 12]. In macaques challenged vaginally with SIV, small founder populations of productively infected cells are demonstrable in the cervical mucosa 3 to 4 days postexposure. The founder populations normally consist of resting CD4⁺ T cells with a receptor pattern and functionality that support a productive infection [13, 14], i.e. CD4⁺ T cells with high expression of CCR5 and α 4 β 7-integrins [15]. After local amplification, the infection spreads to draining and then distant lymphoid tissues, resulting in a disseminated infection [6, 13].

The mucosal epithelium is not a simple passive barrier; it also mediates host defenses by secreting mucus, defensins, and complement proteins [16]. HIV-1 can activate the different complement cascade pathways, which all converge at the cleavage of C3, but HIV-1 can escape complement-mediated lysis as the virions incorporate host proteins that will cleave activated C3 into inactivated iC3b [17]. The iC3b fragments are covalently attached to the viral protein gp120 and can interact with complement receptor 3 (CR3: CD11b/CD18, α M β 2 integrin). After seroconversion, virions are covered with both specific antibodies and complement [17]. In addition, seroconversion enhances complement activation and increases the amount of C3 cleavage products on the surface of HIV-1 [18].

Complement-opsonized virions already exist at the mucosal surface during the initial phase of infection [18]. We have shown earlier that complement-opsonized HIV-1 is internalized more efficiently by DCs than free HIV-1 [9]. Complement opsonization of HIV-1 has also been shown to enhance *in vitro* infectivity for cultured monocytes [19] and immature DCs [20–22]. Given the potential impact of opsonization of HIV-1 on both the acute and chronic phases of infection, it is important to understand the

effects of opsonization on the ability of HIV-1 to interact with cells and to infect them. The aim of this study was to investigate the initial HIV-1 infection of cervical mucosa, using an *ex vivo* cervical tissue explant model, comparing free and opsonized virions. The degree of infection of mucosal DCs and CD4⁺ T cells, and the receptors involved in the initial infection were analyzed.

Complement opsonization of virions resulted in a higher level of infection in DCs emigrating from explant tissues in culture compared with that of free HIV-1, whereas the effect on infection of emigrating CD4⁺ T cells was the opposite with decreased infection for opsonized HIV-1. Blocking of CD4, and integrins β 1, β 2, α 4, and β 7 all resulted in significantly decreased infection of DCs and CD4⁺ T cells. We found a higher impairment in HIV-1 infection in emigrating DCs for complement-opsonized virions when interactions with α M/ β 2- and α 4-integrins were blocked than for free virions. Blockade of integrins decreased both the infection of DCs and CD4⁺ T cells emigrating from the cervical explant tissues, suggesting novel approaches for microbicidal strategies to limit or prevent vaginal transmission of HIV.

Results

Complement opsonization of HIV-1 enhances infection of DCs emigrating from cervical mucosa tissue

We studied the initial phase of HIV-1 infection in cervical mucosa. Cervical tissues were challenged with HIV-1_{BaL} in different forms: free HIV-1 (F-HIV); complement opsonized HIV-1 (C-HIV), immune complexed HIV-1 with a mixture of unspecific, and HIV-1 specific neutralizing IgG (IgG-HIV); or HIV-1 opsonized with both complement and a mixture of unspecific and HIV-1 specific neutralizing IgG (C-IgG-HIV) and infection was measured on day 3–6. The level of HIV-1 infection of emigrating DCs was highest for C-HIV and C-IgG-HIV (Fig. 1A), whereas F-HIV gave the highest level of infection in CD4⁺ T cells (Fig. 1B). Compared with F-HIV, normalized as 100% value for infection, both C-HIV (151%; $p < 0.005$) and C-IgG-HIV (141%) showed enhanced infection of DCs emigrating from cervical explants (Fig. 1C), while infection using IgG-HIV was significantly decreased (60%; $p < 0.005$) (Fig. 1C). However, HIV-1 infection of mucosal CD4⁺ T cells emigrating from explant cultures was significantly decreased when exposed to opsonized forms of virions (C-HIV (80%, $p = 0.01$), IgG-HIV (70%, $p = 0.025$), or C-IgG-HIV (64%, $p = 0.026$) compared with F-HIV (normalized to 100%) (Fig. 1D). The infection profile was the same for endocervix and ectocervix (Supporting Information Fig. 1A and B).

The infection profiles were similar, independently of whether cultures were harvested at day 3, 5, or 6 (Fig. 1E and F) with the greatest difference in the level of infection in DCs using F-HIV versus C-HIV seen at day 5 (Fig. 1E). Impairment of T-cell infection by virion opsonization was more pronounced at day 3 than at day 6 (Fig. 1F). To assess the infection at a later time point than day 3–6, it was necessary to add exogenous GM-CSF and IL-2 to the culture to maintain cell viability. We

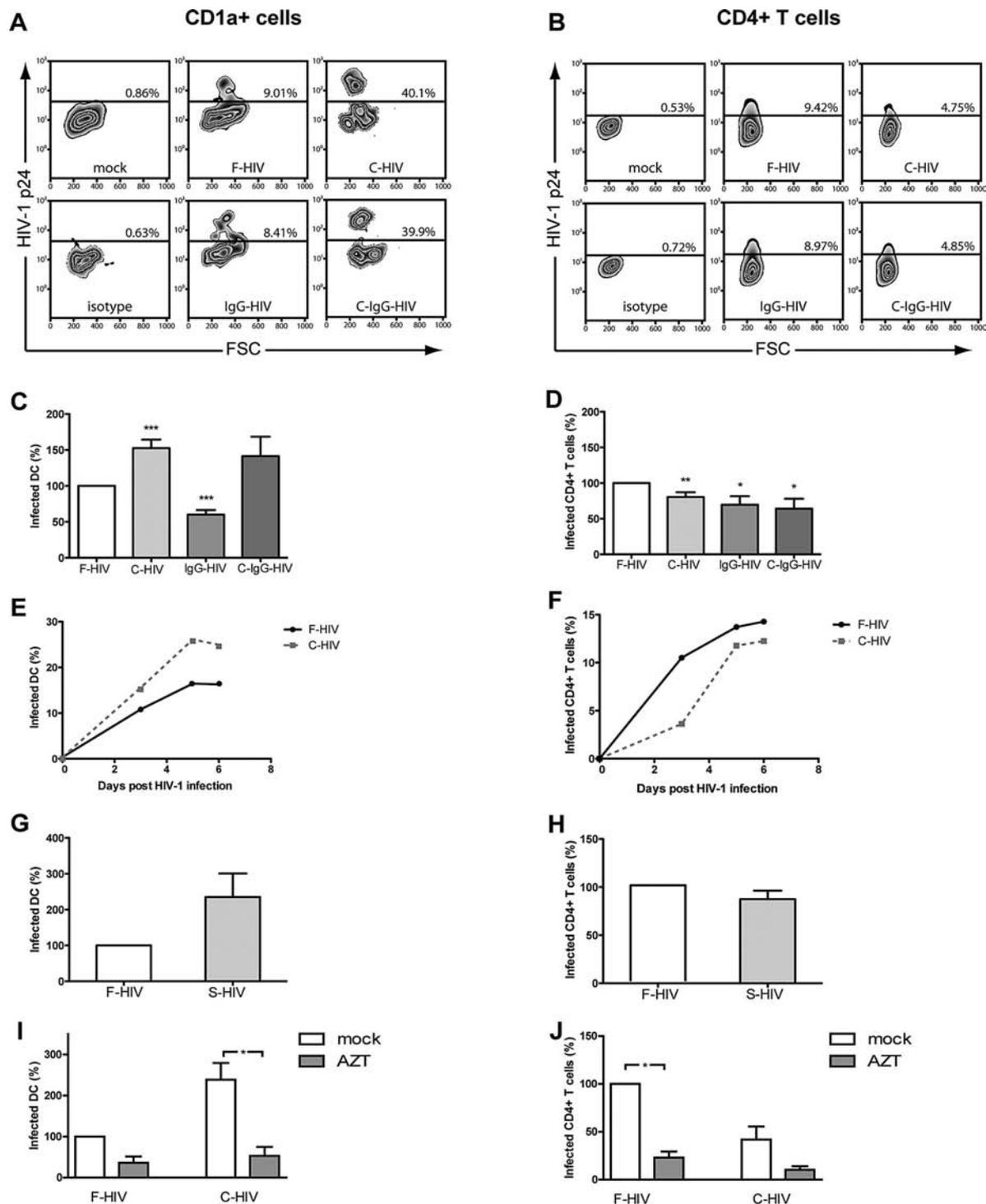


Figure 1. Complement opsonization of HIV-1 enhances infection of DCs but decreases infection of CD4⁺ T cells. The cervical tissue biopsies were infected with different forms of HIV-1_{BaL}, either free (F-HIV), complement opsonized (C-HIV), antibody opsonized (IgG-HIV), virions opsonized by a cocktail of complement and antibodies (C-Ig-HIV) or mock-treated, by spinning the cultures for 2 h at 37°C. The tissues were washed and transferred to six-well plates and cultured for 3–6 days. (A, B) The emigrating cells were harvested and stained with (A) anti-CD1a and anti-HIV-1 mAbs for DCs, and (B) anti-CD3, anti-CD4, and anti-HIV-1 mAbs for CD4⁺ T cells and the level of infection was assessed by flow cytometry. (C, D) The level of HIV-1 infection in the different (C) DC (N = 10–32) or (D) T-cell (N = 11–32) experiments was normalized with F-HIV set as 100%. (E, F) The kinetics of HIV-1 infection in the emigrating (E) DCs and (F) CD4⁺ T cells were measured day 3–6. (G, H) The level of HIV-1 infection of emigrating (G) DCs and (H) T cells (N = 4–5) exposed to F-HIV or HIV-1 opsonized with seminal fluid was also assessed. (I, J) The level of HIV-1 infection of emigrating (I) DCs and (J) T cells from cervical tissue biopsies (N = 3) pre-exposed to mock or AZT followed by infection with F-HIV or C-HIV was measured on day 4. Mock or AZT was present throughout the whole 6-day culture. Statistical significance was tested using a two-sided paired t-test. *p < 0.05, **p < 0.001, ***p < 0.0001. Data are shown as mean + SEM of the indicated number of samples, each assessed in its own experiment.

found the same profile but enhanced infection at day 8 in both DCs and T cells compared with day 3–6 (Supporting Information Fig. 1C).

To further enhance the potential in vivo relevance, we performed similar studies using fresh seminal fluid as the opsonizing agent. The seminal fluid gave similar results as the fresh blood serum with an increased infection of DCs (211%) and a decreased infection of T cells (74%) for opsonized versus nonopsonized virions (Fig. 1G and H). Infection of cervical tissues was also assessed with two additional HIV-1 strains, the CXCR4 tropic HIV-MN and CCR5 tropic HIV-ADA, but these viruses gave very low or no infection (Supporting Information Fig. 1D) and this is in accordance with findings by Greenhead et al. [23]. To distinguish between productive infection of the DCs and CD4⁺ T cells and p24 immunostaining of internalized virions without productive infection, experiments were performed where the reverse transcriptase inhibitor azidothymidine (AZT) was present throughout the whole course of culture. Tissues exposed to AZT had decreased levels p24 gag positive cells compared to untreated HIV-1-infected cells (Fig. 1I and J) confirming that most of the p24 signal was attributable to productive infection. To further characterize HIV infection of cervical mucosa, we assessed the levels of HIV-1 p24 in the supernatants at day 4 and we found that C-HIV gave a higher infection compared with the level obtained with F-HIV (Supporting Information Fig. 1E).

Characterization of C-type lectin and integrin expression on cervical mucosa DCs and T cells

To better understand the establishment of HIV-1 infection in the cervical mucosa, we characterized the expression of an array of receptors, and the location of DCs and T cells. The expression and both cellular and anatomic localization of the C-type lectin receptors MMR (CD206), DC-SIGN (CD209), and Langerin (CD207), and integrins β 1, β 2, β 7, α 4, and α M were assessed by flow cytometry (for emigrating cell populations) and fluorescence microscopy (for cervical tissues). Expression of Langerin was detected almost exclusively on LCs located in the epithelium (Fig. 2A). The vast majority of CD3⁺ T cells were located within the lamina propria (LP), but a few T cells could be found in the epithelium (Fig. 2B). CD1a expression was detected both on the LCs in the epithelial layer and on submucosal DCs in the LP (Fig. 2C). MMR expression was only detected in cells in the LP, presumably on submucosal DCs and macrophages (Fig. 2D). Cells expressing α M/ β 2-integrins were located both in the epithelium and LP, and likely consist of LCs in the epithelial layer and submucosal DCs and macrophages in LP (Fig. 2E). β 1-integrin expression was found on cells in LP and on the basement membrane (Fig. 2F), while cells expressing the β 7-integrin were detected in the LP (Fig. 2G). The specificity of all positive staining was verified by use of isotype control antibodies (Fig. 2H and I). Emigrating DCs and T cells were assessed for expression of C-type lectin receptors MMR, DC-SIGN, and Langerin, and integrins β 1, β 2, β 7, α 4, and α M. Expression of C-type lectin receptors MMR, DC-SIGN, or Langerin was detected

on DCs (Fig. 2J) but not on T cells (our unpublished observation). β 1, β 2, β 7, α 4, and α M integrins were expressed by DCs (Fig. 2J), whereas CD4⁺ T cells only expressed β 1, β 7, and α 4 (our unpublished observation).

Blockage of CD4 binding abolishes infection of DCs and CD4⁺ T cells by free and opsonized HIV-1

We investigated the infection in DCs and CD4⁺ T cells after blocking CD4 with the mAb b12, a neutralizing antibody that specifically targets the CD4 binding site on gp120, and CCR5 with the inhibitor TAK779. We mostly used the b12 experiment to establish the level of inhibition reached in this model by a known blocker of HIV-1 infection [24]. b12 significantly decreased the infection of migrating DCs to 45% of unblocked control for F-HIV ($p = 0.019$) and to 62% of control for C-HIV ($p = 0.028$) (Fig. 3A). When b12 was used, infection of CD4⁺ T cells emigrating from cervical explants was decreased by 85% ($p < 0.0001$) for F-HIV and by 74% ($p < 0.0001$) for C-HIV (Fig. 3B). The CCR5 inhibitor TAK779 decreased the HIV-1 infection of emigrating DCs by 41% ($p = 0.037$) for F-HIV and 66% for C-HIV ($p = 0.06$) and of CD4⁺ T cells by 48% ($p = 0.02$) for F-HIV and 68% ($p = 0.01$) for C-HIV (Fig. 3C and D).

Preventing HIV-1 binding to MMR inhibits infection in emigrating cervical DCs

To study the impact of C-type lectins on infection in DCs and CD4⁺ T cells, we exposed cervical mucosa to the mannose polymer mannan, a competing ligand for C-type lectins, before challenging the tissues with HIV-1. Infection was decreased for both F-HIV (46%, $p = 0.005$) and C-HIV (40%, $p = 0.023$) in emigrating DCs after blocking C-type lectins located in the cervical mucosa (Fig. 4A). In contrast, no effect on the infection of emigrating CD4⁺ T cells was observed (Fig. 4B). DCs emigrating from cervical tissues have been shown to express MMR [24]. Blocking this C-type lectin using anti-MMR mAb gave similar results as when C-type lectins were blocked using mannan with a 51% ($p = 0.006$) decrease in F-HIV infection in DCs (Fig. 4C). When the cervical tissue was challenged with C-HIV, the corresponding decrease in DC infection was 43% ($p = 0.029$) (Fig. 4C). As expected, infection of CD4⁺ T cells was not affected by blocking MMR in the cervical mucosa (Fig. 4D). Blocking of MMR had a slightly higher effect on DC infection with F-HIV than with C-HIV.

Blockade of integrins decreases HIV-1 infection in emigrating DCs and CD4⁺ T cells

Integrins are used by different viruses to attach or infect host cells [25]. Many integrins are expressed by DCs and T cells and we investigated the role of the α M (CD11b) and β 2 (CD18) integrins, i.e. CR3, expressed on both DCs and LCs, in the establishment

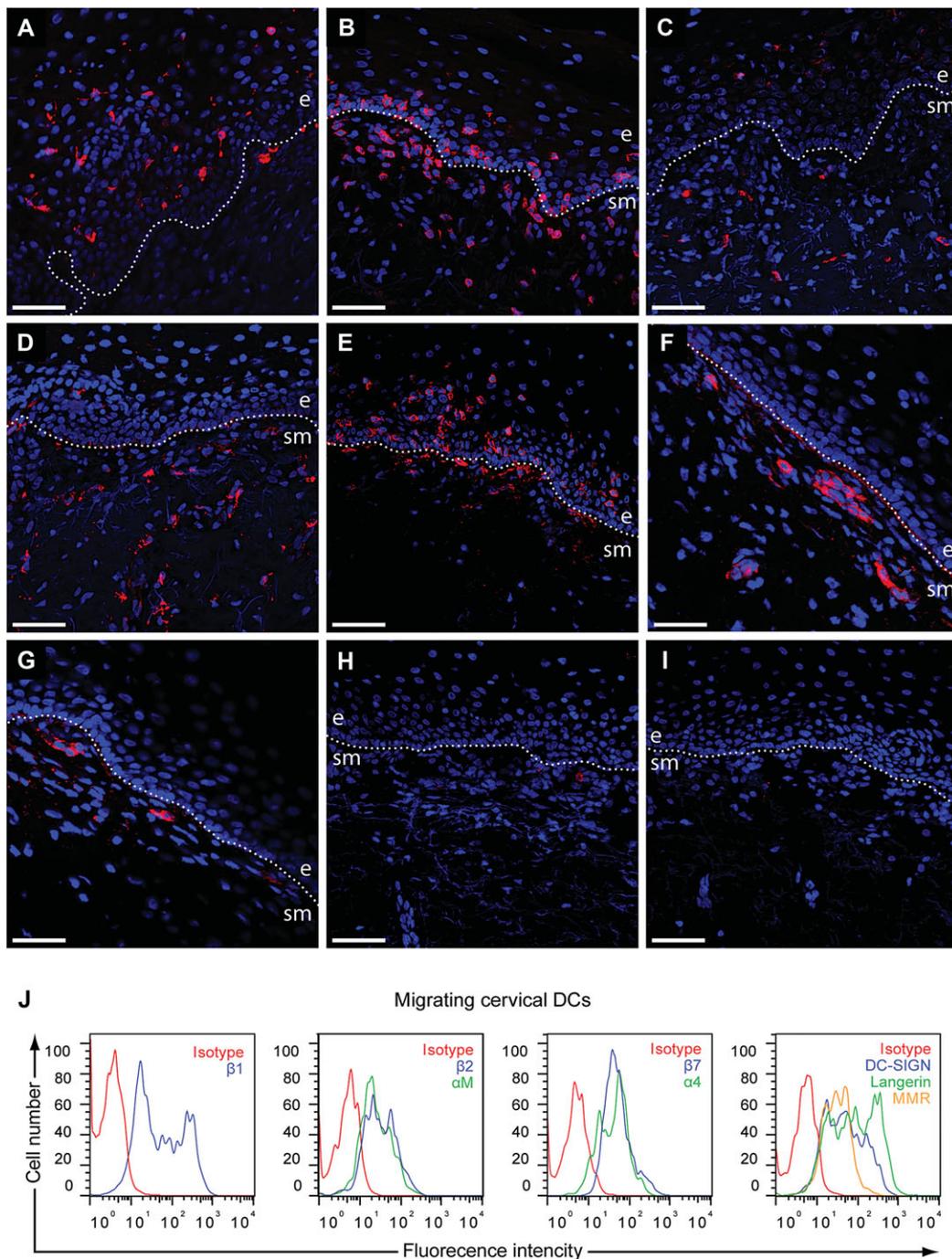


Figure 2. Expression of C-type lectins and integrins in cervical mucosa tissue and on emigrating immune cells. (A–I) Cryopreserved cervical mucosa tissue sections were stained with (A) anti-Langerin, (B) anti-CD3, (C) anti-CD1a, (D) anti-MMR, (E) anti-CD11b/CD18, (F) anti- β 1, (G) anti- β 7, or (H, I) isotype control. The primary antibody was visualized by an sAb conjugated to Rhodamin Red-X. The sections were mounted using mounting medium for fluorescence containing DAPI. Receptor expression is shown in red in the cervical mucosa, scale bar, 50 μ m. (J) DCs emigrating from cervical mucosa tissue were labeled with anti-Langerin, anti-CD3 anti-DC-SIGN anti-MMR, anti-CD11b, anti- β 1, anti- β 7, anti- α 4, or isotype control followed by goat-anti-mouse Alexa Fluor 488 and analyzed by flow cytometry. e = epithelium, sm = submucosa. Data shown are representative of three to five stainings performed on different tissue samples.

of HIV-1 infection. Cervical mucosa was incubated with anti- α M integrin and anti- β 2 integrin mAbs to block CR3 before adding F-HIV or C-HIV. Upon blockade of CR3, the infection of emigrating DCs was decreased by 54% for F-HIV and 82% for C-HIV ($p =$

0.023) (Fig. 5A). The infection in emigrating CD4⁺ cells was also decreased but to a lesser extent. Blocking CR3 inhibited infection in the CD4⁺ T-cell population for F-HIV with 38% ($p = 0.021$) and C-HIV with 54% ($p = 0.001$) (Fig. 5B).

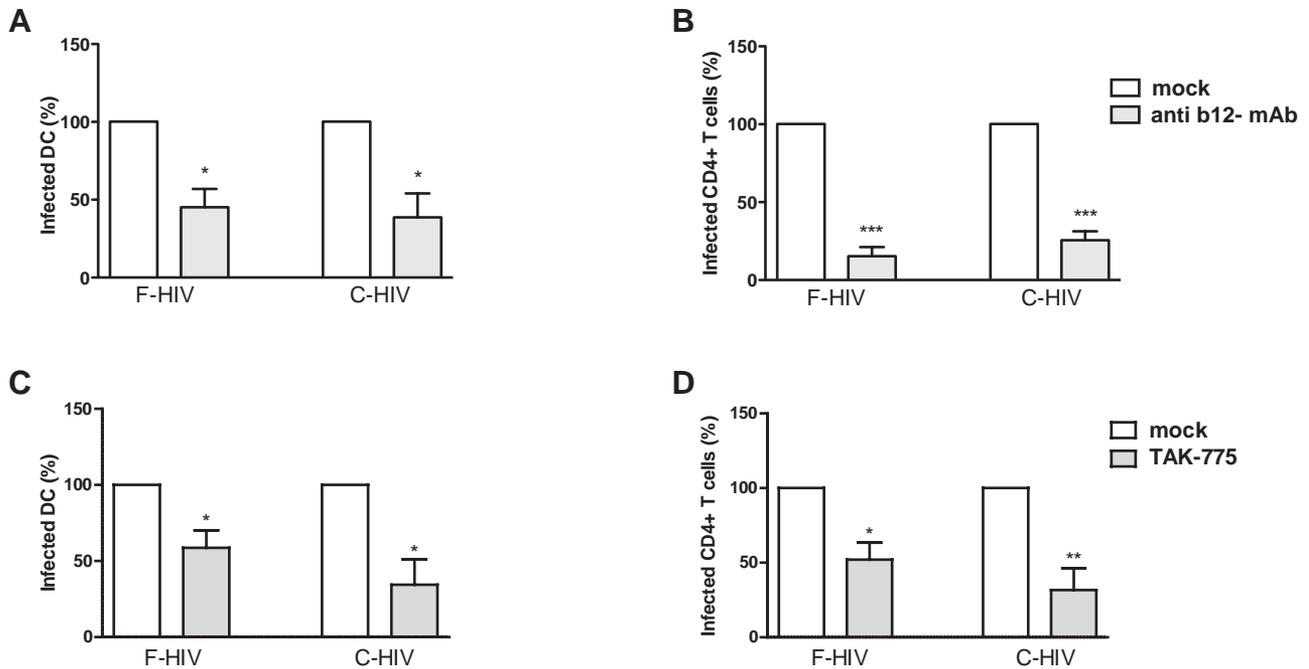


Figure 3. Blockade of CD4 and CCR5 decreases the infection of emigrating cervical mucosa DCs and T cells. Cervical tissue biopsies were infected with HIV-1_{BaL}, either as free virions (F-HIV) or complement opsonized virions (C-HIV) that had been mock treated or incubated with b12 mAb, for 2 h at 37°C. The cervical tissue biopsies were pretreated for 30 min at 37°C with TAK779 followed by infection with different forms of HIV-1_{BaL}, either free (F-HIV) or complement opsonized (C-HIV), for 2 h at 37°C. The tissues were washed and transferred to six-well plates and cultured for 3–6 days in the absence or continuous presence of b12 mAb or TAK779. The emigrating cells were harvested and stained with anti-CD1a, and anti-HIV-1 mAbs for DCs and anti-CD3, anti-CD4, and anti-HIV-1 mAbs for CD4⁺ T cells and level of infection was assessed by flow cytometry for HIV-1 p24 expression. (A, B) The level of HIV-1 infection after mock treatment or treatment with b12 in the different (A) DCs ($N = 4–6$) or (B) T cells ($N = 4–7$) was normalized with mock-treated F-HIV set as 100%. (C, D) The level of HIV-1 infection after mock or TAK779 treatment in the different (C) DC or (D) T-cell experiments was normalized with F-HIV set as 100% ($N = 3–4$). Statistical significance was tested using a two-sided paired t-test. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$. Data are shown as mean + SEM of the indicated number of samples, each assessed in its own experiment.

$\alpha 4$ - (CD49d), $\beta 1$ - (CD29), and $\beta 7$ -integrins are expressed by DCs (Fig. 2) and T cells in the cervical mucosa in different heterodimeric complexes, and $\alpha 4\beta 7$ -, $\alpha E\beta 7$ -, and $\alpha 4\beta 1$ -integrins are expressed on T cells [26], while $\alpha E\beta 7$ - and $\alpha 4\beta 1$ -integrins are expressed on DCs. We investigated whether blocking $\beta 1$ -integrins would affect the infection of emigrating immune cells from cervical tissue after exposure to free or complement opsonized HIV-1. Pretreatment of the cervical mucosa with anti- $\beta 1$ integrin mAb inhibited infection in both emigrating DCs and CD4⁺ T cells. Infection of DCs was inhibited by 66% ($p = 0.008$) for F-HIV and by 57% for C-HIV ($p = 0.013$) (Fig. 5C). In CD4⁺ T cells, the blocking of $\beta 1$ -integrins gave a 49% decrease in infection for F-HIV ($p = 0.01$) and a 46% decrease for C-HIV ($p = 0.01$) (Fig. 5D). HIV-1 gp120 binds to $\alpha 4\beta 7$ -integrins expressed on T cells and facilitates the formation of virological synapses and spread of the virus via LFA-1 [26], whereas the effects of virion interaction with $\alpha 4$ and $\beta 7$ on DCs remain unknown. Targeting the integrins $\alpha 4$ or $\beta 7$ in the cervical mucosa with specific blocking antibodies [27–29] resulted in a decreased infection in both emigrating DCs and CD4⁺ T cells (Fig. 5E–H). Blocking the $\alpha 4$ integrin decreased the infection in DCs by F-HIV by 62% ($p = 0.006$) and by C-HIV by 80% ($p = 0.006$) (Fig. 5E). In the CD4⁺ T-cell population, infection was decreased by 38% ($p = 0.022$) for F-HIV and 55% ($p = 0.0025$) for C-HIV (Fig. 5F). The blocking of $\beta 7$ -integrins in the cervical

tissue also decreased infection in the emigrating DCs by 49% ($p = 0.012$) for F-HIV and 66% ($p = 0.001$) for C-HIV (Fig. 5G). The infection of CD4⁺ T cells was inhibited by 44% ($p = 0.004$) for F-HIV and by 58% ($p = 0.004$) for C-HIV (Fig. 5H). We found the same level of inhibition of infection when a lower concentration (5 $\mu\text{g}/\text{mL}$ compared with 20 $\mu\text{g}/\text{mL}$) of the anti- $\beta 7$ integrin or anti- $\alpha 4$ integrin antibodies was used (Supporting Information Fig. 2).

Of note, areas positive for HIV-1 p24 were detected in the submucosa tissues in all cervical tissues infected with F-HIV or C-HIV, whereas these areas were rarely or not found when the use of $\alpha 4$ and $\beta 7$ -integrins had been inhibited (our unpublished observation).

Discussion

It is important to gain a better understanding regarding the initial events in HIV-1 infection in the female genital tract in order to develop new strategies that prevent the initial infection or target the first week of local infection, with the ability to block the development of systemic infection. When studying the first steps of HIV-1 infection, there are many layers of interactions occurring within the mucosal microenvironment to take into account,

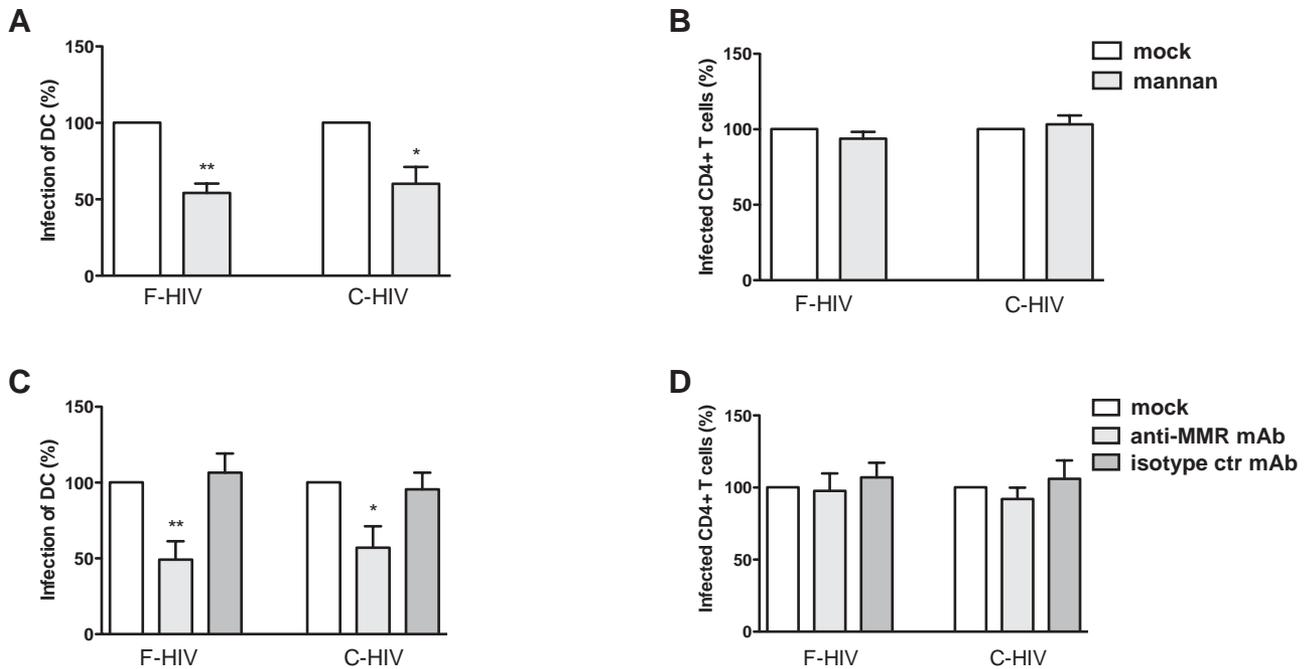


Figure 4. Blockade of the C-type lectin MMR decreases the infection of DCs emigrating out from cervical tissues. The cervical tissue biopsies were pretreated for 30 min at min 37°C with mock, mannan, anti-MMR mAb, or isotype control mAb followed by infection with different forms of HIV-1_{BaL}, either free (F-HIV) or complement opsonized (C-HIV), by spinning the cultures for 2 h at 37°C. The tissues were washed and transferred to six-well plates and cultured for 3–6 days with mock, mannan, anti-MMR mAb, or isotype control mAb. The emigrating cells were harvested and stained with anti-CD1a, and anti-HIV-1 mAbs for DCs and anti-CD3, anti-CD4, and anti-HIV-1 mAbs for CD4⁺ T cells and level of infection was assessed by flow cytometry. (A, B) The level of HIV-1 infection in (A) DCs (N = 4–5) or (B) T cells (N = 3) in the different experiments treated with mock or mannan was normalized with F-HIV set as 100%. (C, D) The level of HIV-1 infection in (C) DCs (N = 4–7) or (D) T cells (N = 4–9) in the different experiments treated with mock, anti-MMR mAb, or isotype control mAb was normalized with F-HIV set as 100%. Statistical significance was tested using a two-sided paired t-test. **p* < 0.05, ***p* < 0.001, ****p* < 0.0001. Data are shown as mean + SEM of the indicated number of samples, each assessed in its own experiment.

e.g. cervical secretions, complement components, epithelial cell barrier, hormones, and immune cells [16]. We used a tissue explant culture system to measure the HIV-1 infection of emigrating cervical DCs and CD4⁺ T cells after challenging the cervical explants with different forms of opsonized HIV-1 that are potentially relevant in vivo, i.e. virions covered in complement fragments or/and antibodies. Virions opsonized with complement produced higher levels of infection in the emigrating DCs than free HIV-1. In contrast, infection of emigrating CD4⁺ T cells was decreased when the virions were opsonized. The differential effects of complement-opsonized HIV could be due to the fact that only a few T cells express CR3 [30] and the coating of HIV with iC3b affects the ability of the viral protein gp120 to interact with the CD4 receptor on the cell surface. DCs on the other hand typically express CR3 and this receptor enhances the binding and uptake of C-HIV [9] and is involved in the enhanced HIV-1 infection [21]. Blockade of CD4, and the integrins $\beta 1$, $\beta 2$, αM , $\alpha 4$, and $\beta 7$, and the C-type lectin MMR resulted in significantly decreased infection of DCs. For emigrating CD4⁺ T cells, the highest impairment of infection was seen when HIV-1 ability to bind to the CD4 receptor was neutralized but blocking the integrins $\beta 1$, $\beta 2$, $\alpha 4$, and $\beta 7$ also decreased the infection. Blockade of the $\alpha M/\beta 2$ - and $\alpha 4$ -integrins inhibited infection of emigrating DCs by complement-

opsonized virions more than it inhibited infection by free virions.

Within the cervix, T cells and APCs are most frequently found in the cervical transformation zone and surrounding tissues [5]. Several different types of DCs exist in the cervical mucosa including myeloid DCs, with the LCs located in the epithelial layer and DCs in the submucosa, and a small number of plasmacytoid DCs [31]. In human explant cultures, HIV-1 can rapidly reach both LCs and CD4⁺ T cells located in the cervical epithelial layer [8], although DCs have been implicated in vaginal SIV transmission and dissemination to draining LNs in macaques [32].

We have previously demonstrated that complement opsonization of HIV-1 increased binding and uptake by both immature and mature DCs compared with free virions [9]. Furthermore, in other studies, infection of DCs, monocytes, and epithelial cells was enhanced when HIV-1 virions were complement opsonized [19, 20, 33]. This study shows for the first time that complement opsonization of HIV-1 significantly increases the infection of DCs/LCs emigrating from the cervical mucosa and decreases the infection of CD4⁺ T cells. This was observed when HIV-1 was opsonized not only with fresh human sera but also when HIV-1 was opsonized in seminal fluid, despite the tenfold lower amount of C3 fragments in semen [33].

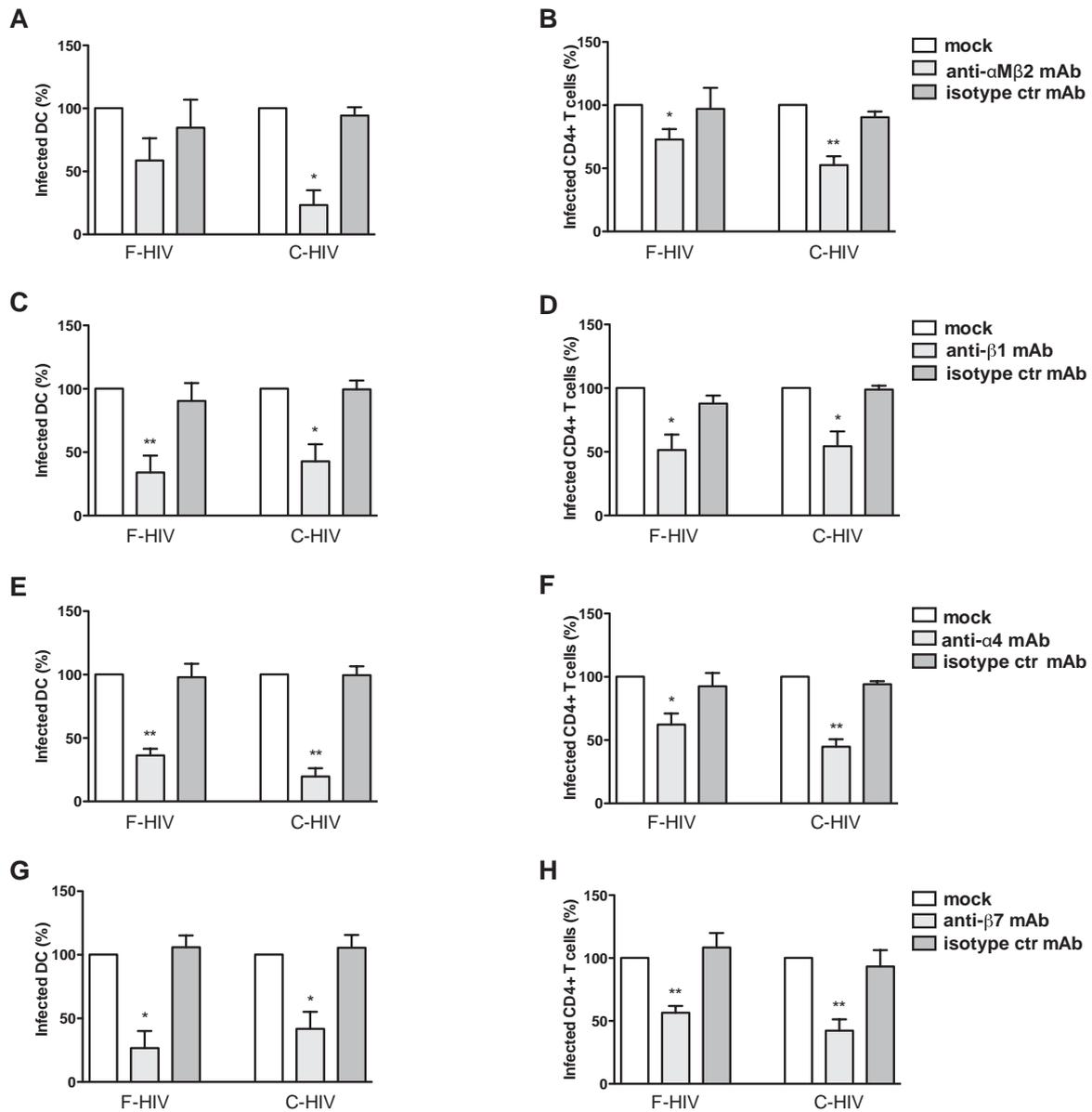


Figure 5. Blockade of integrins decreases the infection of DCs and CD4⁺ T cells emigrating from cervical mucosa tissues. Cervical tissue biopsies were pretreated for 30 min at 37°C with mock, anti-CD11b/CD18 mAbs, anti-β1 mAb, anti-α4 mAb, anti-β7 mAb, or isotype control mAbs followed by infection with different forms of HIV-1_{BaL}, either free (F-HIV) or complement opsonized (C-HIV), by spinning the cultures for 2 h at 37°C. The tissues were washed and transferred to six-well plates and cultured for 3–6 days with mock, anti-CD11b/CD18 mAbs, anti-β1 mAb, anti-α4 mAb, anti-β7 mAb, or isotype control mAbs. The emigrating cells were harvested and stained with anti-CD1a, and anti-HIV-1 mAbs for DCs and anti-CD3, anti-CD4, and anti-HIV-1 mAbs for CD4⁺ T cells and level of infection was assessed by flow cytometry (A, B) The level of HIV-1 infection in (A) DCs (N = 3) or (B) T cells (N = 6) in experiments treated with mock, anti-CD11b/CD18 mAbs, or isotype control mAbs was normalized with F-HIV set as 100%. (C, D) The level of HIV-1 infection in (C) DCs (N = 5) or (D) T cells (N = 6) in the different experiments treated with mock, anti-β1 mAb, or isotype control mAbs was normalized with F-HIV set as 100%. (E, F) The level of HIV-1 infection in (E) DCs (N = 3–4) or (F) T cells (N = 4) in the different experiments treated with mock, anti-α4 mAb, or isotype control mAbs was normalized with F-HIV set as 100%. (G, H) The level of HIV-1 infection in (G) DCs (N = 3–4) or (H) T cells (N = 4) in the different experiments treated with mock, anti-β7 mAb, or isotype control mAbs was normalized with F-HIV set as 100%. Statistical significance was tested using a two-sided paired t-test. **p* < 0.05, ***p* < 0.001, ****p* < 0.0001. Data are shown as mean + SEM of the indicated number of samples, each assessed in its own experiment.

Exposing the cervical tissue to the neutralizing mAb b12 decreased the infection of both F-HIV and C-HIV in emigrating DCs and CD4⁺ T cells, which is consistent with prior observations on the usage of the CD4 receptor for infection of cervical mucosal DCs and T cells [24]. Neutralization of HIV-1 with

b12 blocked both local infection of the cervical mucosa explants and viral dissemination of free HIV-1_{BaL} to emigrating immune cells [24], findings consistent with the observation that b12 inhibited HIV-1 replication in the vaginal mucosa in macaques when the antibody was given in high doses [34]. However, previous

studies have shown that HIV-1 can mutate to escape neutralization, potentially limiting the protective activity ability of b12 and other neutralizing antibodies. We clearly demonstrate that other receptors besides the CD4 receptor and coreceptor, i.e. integrins, can be of importance for the interactions between virus and potential target cells and for infection of CD4⁺ T cells in the cervical mucosa, possibly by aiding viral attachment to the cell surface and/or facilitating viral synapse formation [26] and dissemination of HIV-1.

MMR is expressed by DCs located in cervical tissues and on DCs emigrating from cervical explants [24]. HIV-1 uptake via MMR into macrophages and via Langerin into LCs can guide the virions away from the route leading to productive infection [35,36]. We established that C-type lectins were involved in events leading to infection of emigrating DCs as mannan and anti-MMR mAb decreased the infection. Although the effect of mannan on HIV-1 infection of cervical tissue explants and the ability of emigrating cells to cross-infect target cells has been shown previously [24], our data show direct infection of emigrating DCs from the cervical explants. However, no protection against SIV infection was seen for mannan in a macaque model [37]. Consequently, blocking of C-type lectins may not give the desired outcome as the viral interactions with these receptors are complex and may affect infection in different ways depending on the cell type.

Integrins bind to various cell surface and extracellular proteins to mediate cell-to-cell matrix adhesion and are involved in multiple important biological functions. Many viruses have been shown to use integrins to interact with host cells in order to facilitate or mediate infection [25]. For example, the integrin $\beta 1$ (CD29) is expressed by almost all cells and has been shown to be involved in rotavirus internalization and reovirus cell entry and infection [25]. In the cervical mucosa, $\beta 1$ -integrin is expressed on the basal membrane of epithelial cells and on immune cells, with strongest expression at the basal membrane layer [38]. A previous study showed that blocking $\beta 1$ -integrins on cervical mucosa epithelia diminished attachment of HIV-1 virions [38]. The blocking of HIV-1 usage of this receptor in the cervical mucosa decreased infection of both DCs and T cells emigrating out of the tissues and this could be an effect of direct interaction of HIV-1 with $\beta 1$ -integrins expressed on the immune cells or an indirect effect of viral binding to epithelial cells and the basal membrane expressing integrin $\beta 1$.

$\alpha 4\beta 7$ is the submucosal homing receptor expressed by T cells and DCs located in the gut and genital mucosa [26]. HIV-1 gp120 binds to an activated form of $\alpha 4\beta 7$, and on T cells this binding leads to activation of LFA-1, facilitating efficient spread of virus to other cells via virological synapses [26]. A variety of observations suggest that interactions between gp120 and $\alpha 4\beta 7$ may play a role in mucosal transmission and/or early mucosal replication of HIV-1 [15,39]. Of note, blocking $\alpha 4\beta 7$ -integrin by intravenous administration during acute SIV infection leads to decreased viral load in infected rhesus macaques [40]. Blocking of $\alpha 4$ - and $\beta 7$ -integrins significantly diminished HIV-1 infection of DCs and T cells emigrating from cervical tissue explants, possibly reflecting involvement of $\alpha 4\beta 7$ in complex with CD4 on the DCs and T cells

in direct infection and/or the formation of virological synapses and dissemination of virions between immune cells.

Following vaginal SIV mucosal challenge of macaques, small populations of infected cells are established, which undergo a local expansion before disseminating to establish systemic infection [13]. These small founder populations must be sustained at a basic reproductive ratio (R_0) > 1 to give rise to systemic infection, otherwise the infection will be aborted [13,14]. The inhibition seen in infection of mucosal CD4⁺ T cells and DCs when blocking integrins may substantially decrease the founder populations in the cervical mucosa. These findings suggest that compounds targeting integrins might be successful in preventing the establishment of HIV-1 infection. However, there could be negative effects of blocking integrins on the mucosa and immune cells' ability to form immunological synapse and this needs to be assessed in animal models as these factors play important roles in modulating immune responses. Recent trials with microbicides and vaccines have shown some ability to prevent HIV-1 transmission but the efficacy has been low. A deeper understanding of how HIV-1 interacts with the immune cells in the cervicovaginal mucosa is necessary to prevent transmission and HIV infection in the future. New molecules to target for the use in new vaccines and microbicides are of great interest and our results suggest that integrins, such as $\alpha 4$ and $\beta 7$, may be promising microbicide candidates.

Materials and methods

Cervical tissue samples Ethics statement

This study was approved by Linköping Ethical Review Board (Ethical permit EPN M206-06) and informed consent of all participating subjects was obtained. Normal cervical tissue samples ($N = 42$) were obtained mainly from women who underwent prolapse surgery and from a few women who underwent hysterectomy for benign conditions comprising menstrual bleeding disorders and myomas at the Gynecology Clinic in Linköping, Sweden. All women had normal Pap smears prior to surgery and showed cytologically and clinically no signs of infection/inflammation at surgery.

Virus propagation

HIV-1_{Bal}/SUPT1-CCR5 CL.30 (Lot #P4143: Biological Products Core/AIDS and Cancer Virus Program, SAIC-Frederick, Inc., NCI Frederick) was produced using chronically infected cultures of ACVP/BPC cell line (No. 204). Virus was purified by continuous flow centrifugation for 30 min after sample loading. Virus containing fractions were pooled and diluted to <20% sucrose, and virus pelleted at approximately 100 000 $\times g$ for 1 h. The virus pellet was resuspended and aliquots were frozen in liquid N₂ vapor. All virus preparations were assayed for infectivity.

Opsonization of HIV-1

We used different preparations of HIV-1; F-HIV, HIV-1 opsonized with complement (C-HIV), HIV opsonized with a mixture of unspecific and HIV-1-specific IgG (IgG-HIV), or HIV-1 opsonized with a combination of both complement and IgG (C-IgG-HIV). C-HIV was obtained by incubation of HIV-1_{BaL} (30 µg/mL p24 equivalent) with an equal volume of human serum (HS) or seminal plasma containing 25% Veronal buffer [33]. To obtain C-IgG-HIV, 0.20 µg/mL neutralizing HIV-specific IgG (SMI, Stockholm, Sweden) and 20 µg/mL gamma globulin (Pharmacia, Stockholm, Sweden) were added besides the HS containing Veronal buffer, whereas IgG-HIV was obtained by adding only the antibodies. F-HIV was treated with media alone. All groups were incubated for 1 h at 37°C. As a negative control, human sera were heat-inactivated for 30 min at 56°C, incubated with HIV-1 and prepared under the same conditions as the other groups.

Cervical tissue samples and virus inoculations

The cervical tissues were kept on ice and processed within 30 min after resection. The epithelial layer and lamina propria were separated from underlying stroma and the tissue explants (8 mm²) were placed in cell culturing plates containing RPMI 1640, 20 µg/mL Gentamicin, 10 mM HEPES (Fisher Scientific, Gothenburg, Sweden), and 5% pooled HS (Fisher Scientific). Cervical explants were preincubated for 30 min at 37°C for mock treatment or with different inhibitors. The following inhibitors were used: 10 mg/mL mannan, 10 µM AZT (SIGMA-Aldrich), 5 mg/mL EDTA (SIGMA-Aldrich), mAb against the CD4 binding site on gp120 (b12, a kind gift from Professor D. Burton), anti-αM integrin mAb (CD11b: ICRF44, Ancell Corp., Bayport, NY, USA), anti-αM integrin mAb (KIM75), and anti-β2 integrin mAb (CD18: clone 6.5) (kind gifts from M. Robinson, Celltech, UCB group), anti-MMR mAb (CD206: clone 15-2, Biosite), anti-α4 integrin mAb (clone P1H4, Abcam), anti-β7 integrin mAb (clone F1B504, Biosite), and anti-β1 integrin (CD29) (clone JB1A, Millipore); matching isotype controls were used.

Tissue samples were challenged with HIV-1_{BaL} (250 ng/mL), spinoculated for 2 hours at 37°C, washed four times, transferred to six-well tissue culture plates, and cultured. After 3–6 days the emigrating cervical cells were collected and stained for flow cytometry. The infected cervical explants were fixed in 4% paraformaldehyde (PFA) and stored for later immunohistochemistry and/or immunofluorescence staining. To compare results obtained from separate experiments, using cervical explants derived from different donors, the F-HIV and C-HIV infection of emigrating DCs and CD4⁺ T cells in absence of inhibitors was normalized to 100% for each experiment.

Flow cytometry

Emigrated cells were harvested on day 3–6 from cervix cultures and stained with the following anti-human mAbs; CD4-

allophycocyanin (BD Biosciences), CD3-PerCP (BD Biosciences), and CD1a-PE (BD Biosciences). For detection of HIV-1, the cells were fixed in 4% PFA, permeabilized in 0.2% saponin, and incubated with the anti-HIV-1 mAb (KC57, clone FH190-1-1) and the corresponding isotype control (BD PharMingen, San Diego, CA, USA) for 45 min. The stained cells were assessed by flow cytometry (FACS Calibur) and acquired data were analyzed by FlowJo (Treestar, Ashland, OR, USA). In addition, the emigrating immune cells were stained with mAbs against Langerin (CloneD-CGM4/122D5 Biosite), MMR, DC-SIGN, α4, αM, β1, β7, α4, or β7 followed by sAb conjugated with FITC for 1 h followed by CD1a-PE or CD3-PerCP staining.

Immunofluorescence and confocal microscopy

Cryopreserved cervical tissue explants were sectioned and the sections were fixed in 4% PFA, quenched, and stained with the following mAbs: CD1a (HI149, BioLegend, San Diego, CA, USA), Langerin, MMR, α4, β7, α4β7, αVβ5, and β1. The slides were incubated with antibodies over night at 4°C, washed three times, and stained with a secondary mAb (Rhodamine Red-X conjugated donkey anti-mouse IgG, Jackson ImmunoResearch, Baltimore, PA, USA) for 1 h at room temperature. The sections were then washed and mounted using mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA). The samples were analyzed using an LSM 510 META confocal microscope (Carl Zeiss AB, Stockholm, Sweden) and LSM 510 software.

Statistical analysis

GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis. Results were tested for statistical significance using a two-sided paired *t*-test and *p*-values less than 0.05 were considered statistically significant. In all figures, *N* denotes the number of times each experiment was replicated, using cervical explants derived from different women.

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Abbreviations: AZT: azidothymidine · C-HIV: complement opsonized HIV-1 · F-HIV: free HIV-1 · HS: human serum · LC: Langerhans cell · LP: lamina propria · PFA: paraformaldehyde

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