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DC-SIGN, a Dendritic Cell–Specific HIV-1-Binding Protein that Enhances *trans*-Infection of T Cells

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Summary

Dendritic cells (DC) capture microorganisms that enter peripheral mucosal tissues and then migrate to secondary lymphoid organs, where they present these in antigenic form to resting T cells and thus initiate adaptive immune responses. Here, we describe the properties of a DC-specific C-type lectin, DC-SIGN, that is highly expressed on DC present in mucosal tissues and binds to the HIV-1 envelope glycoprotein gp120. DC-SIGN does not function as a receptor for viral entry into DC but instead promotes efficient infection in *trans* of cells that express CD4 and chemokine receptors. We propose that DC-SIGN efficiently captures HIV-1 in the periphery and facilitates its transport to secondary lymphoid organs rich in T cells, to enhance infection in *trans* of these target cells.

Introduction

Transmission of human immunodeficiency virus type 1 (HIV-1) infection in humans requires the dissemination of virus from sites of infection at mucosal surfaces to T cell zones in secondary lymphoid organs, where extensive viral replication occurs in $CD4^+$ T-helper cells (Fauci, 1996). These cells express both CD4 and the chemokine receptor CCR5, which together form the receptor complex required for entry by the R5 viral isolates that are prevalent early after infection (Dragic et al., 1996; Lu et al., 1997; Littman, 1998). Viruses with tropism for other chemokine receptors, particularly CXCR4, are rarely transmitted and generally appear only late in infection.

The mechanism of early viral dissemination remains vague, but based on anatomical distribution of different hematopoietic lineage cells and on in vitro infectivity studies it has been inferred that immature dendritic cells (DC) residing in the skin and at mucosal surfaces are the first cells targeted by HIV-1. DC are the most potent antigen-presenting cells in vivo (Valitutti et al., 1995; Banchereau and Steinman, 1998). Immature DC in peripheral tissues capture antigens efficiently and have the unique capacity to subsequently migrate to the T cell areas of secondary lymphoid organs. As the cells travel, they mature and alter their expression profile of cell surface molecules, including chemokine receptors, lose their ability to take up antigen, and acquire competence to attract and activate resting T cells in the lymph nodes (Adema et al., 1997; Banchereau and Steinman, 1998). HIV-1 is thought to subvert the trafficking capacity of DC to gain access to the $CD4^+$ T cell compartment in the lymphoid tissues (Grouard and Clark, 1997; Rowland-Jones, 1999; Steinman and Inaba, 1999).

Immature DC express CD4 and CCR5, albeit at levels that are considerably lower than on T cells (Granelli-Piperno et al., 1996; Rubbert et al., 1998), and they have been reported to be infectable with R5 strains of HIV-1. In contrast, immature DC do not express CXCR4 and are resistant to infection with X4 isolates of HIV-1 (Weissman et al., 1995; Blauvelt et al., 1997; Granelli-Piperno et al., 1998). Entry of HIV-1 into immature DC has also been reported to proceed through a CD4-independent mechanism (Blauvelt et al., 1997), suggesting that receptors other than CD4 could be involved. There have been conflicting reports regarding the significance of HIV-1 replication within DC (Cameron et al., 1994; Ayehunie et al., 1997; Canque et al., 1999). Although replication can be observed in some circumstances, it has also been reported that, in immature DC, replication is incomplete and that only early HIV-1 genes are transcribed.

It has been proposed that virus-infected immature DC migrate to the draining lymph nodes where they initiate both a primary antiviral immune response and a vigorous productive infection of T cells, allowing systemic distribution of HIV-1 (Cameron et al., 1992; Weissman et al., 1995; Granelli-Piperno et al., 1999). However, in a nonhuman primate model of mucosal infection with the simian immunodeficiency virus, it has been difficult to demonstrate productive infection of DC despite rapid dissemination of virus (Stahl-Hennig et al., 1999). Other efforts to model primary HIV-1 infection in vitro by exposing DC derived from skin or blood to HIV-1 have indicated that these cells are poorly infected. Nevertheless, only DC and not other leukocytes, including monocytes, macrophages, B cells, and T cells, were able to induce high levels of infection upon coculture with mitogen-activated $CD4^+$ T cells after being pulsed with HIV-1 (Cameron et al., 1992, 1992b, 1996; Weissman et al., 1995; Blauvelt et al., 1997; Granelli-Piperno et al., 1999). In an early study, Cameron et al. (1992) proposed that DC have a unique ability to "catalyze" infection of T cells with HIV but do not become infected themselves.

The mechanism by which DC capture HIV-1 and promote infection of $CD4^+$ T cells has not been elucidated,

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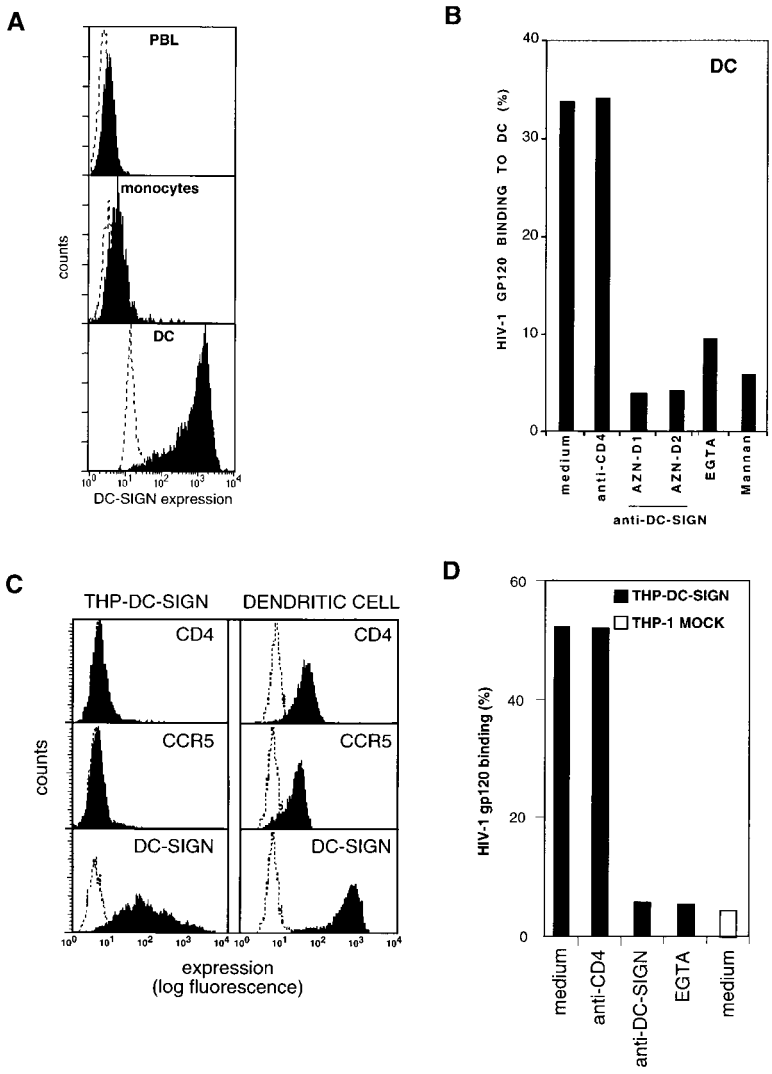

Figure 1. DC-SIGN Is a DC-Specific Receptor for HIV-1 gp120

(A) DC-SIGN is expressed specifically by DC. Immature DC, cultured from monocytes in the presence of GM-CSF and IL-4, express high levels of DC-SIGN, whereas resting peripheral blood lymphocytes and monocytes do not express DC-SIGN. Expression of DC-SIGN (AZN-D1) was determined by FACScan analysis. One representative experiment out of three is shown.

(B) DC-SIGN, but not CD4, mediates binding of HIV-1 gp120 to DC. DC were allowed to bind HIV-1 gp120-coated fluorescent beads. Adhesion was blocked by anti-DC-SIGN antibodies (20 μg/ml), mannan (20 μg/ml), and EGTA (5 mM), and not by neutralizing anti-CD4 antibodies (20 μg/ml). One representative experiment out of three is shown.

(C) Immature DC express low levels of CD4 (RPA-T4) and CCR5 (2D7/CCR5) and high levels of DC-SIGN (AZN-D1). THP-1 cells stably transfected with DC-SIGN (THP-DC-SIGN) express high levels of DC-SIGN (AZN-D1) while CD4 and CCR5 are not expressed (filled histograms). Antibodies against CD4 and DC-SIGN were isotype matched, and the appropriate isotype controls are represented by dotted lines.

(D) DC-SIGN transfectants (THP-DC-SIGN) bind HIV-1 gp120. THP-DC-SIGN and mock transfectants were allowed to bind HIV-1gp120-coated fluorescent beads. Adhesion was blocked by anti-DC-SIGN antibodies (20 μg/ml) and EGTA (5mM) and not by neutralizing anti-CD4 (RPA-T4) antibodies (20 μg/ml). One representative experiment out of three is shown.

and it has been unclear whether there is specificity in the interaction of DC with virus. In the accompanying paper, we describe the identification of a DC-specific C-type lectin, designated DC-SIGN, that binds with high affinity to ICAM-3 present on resting T cells (Geijtenbeek et al., 2000 [this issue of *Cell*]). Nucleotide sequence analysis of the cDNA indicated that this molecule is identical to a previously described HIV-1 gp120-binding C-type lectin (Curtis et al., 1992) isolated from a placental cDNA library. Here, we demonstrate that this HIV-1-binding protein, which is highly expressed on DC present at mucosal sites, specifically captures HIV-1 and promotes infection in *trans* of target cells that express CD4 and appropriate chemokine receptors. Our findings suggest that, during transmission of HIV-1, the virus initially binds to mucosal DC through DC-SIGN, allowing subsequent transport to secondary lymphoid organs and highly efficient infection of $CD4^+$ T cells by a novel *trans* infection mechanism.

Results

DC-SIGN Is a DC-Specific HIV-1-Binding Protein

DC-SIGN was recently identified as a DC-specific ICAM-3 adhesion receptor that mediates DC-T cell interactions (Geijtenbeek et al., 2000). Flow cytometric analysis of an extensive panel of hematopoietic cells with anti-DC-SIGN antibodies demonstrated that DC-SIGN is preferentially expressed on in vitro cultured DC but not on other leukocytes, such as monocytes and peripheral blood lymphocytes (PBL) (Figure 1A). Identification of DC-SIGN by peptide amino acid sequencing of the 44 kDa immunoprecipitated protein revealed it to be 100% identical in its amino acid sequence to the HIV-1 envelope glycoprotein gp120-binding C-type lectin previously isolated from a placental cDNA library (Curtis et al., 1992). To determine whether this molecule has a role in binding of HIV to DC, we used a flow cytometric adhesion assay (Geijtenbeek et al., 1999) to examine the ability of HIV-1 gp120-coated fluorescent beads to bind to immature DC (Figure 1B). The gp120-coated beads bound efficiently to the DC, and the binding was completely blocked by the anti-DC-SIGN antibodies AZN-D1 and AZN-D2. In contrast, neutralizing anti-CD4 antibodies had no effect on gp120 binding to DC. This result indicates that, although the primary HIV-1 receptor CD4 is expressed on DC (Figure 1C), HIV-1 gp120 preferentially binds to DC-SIGN. Similarly, the monocytic cell line THP-1, which lacks expression of both CD4 and CCR5, bound the gp120-coated beads after

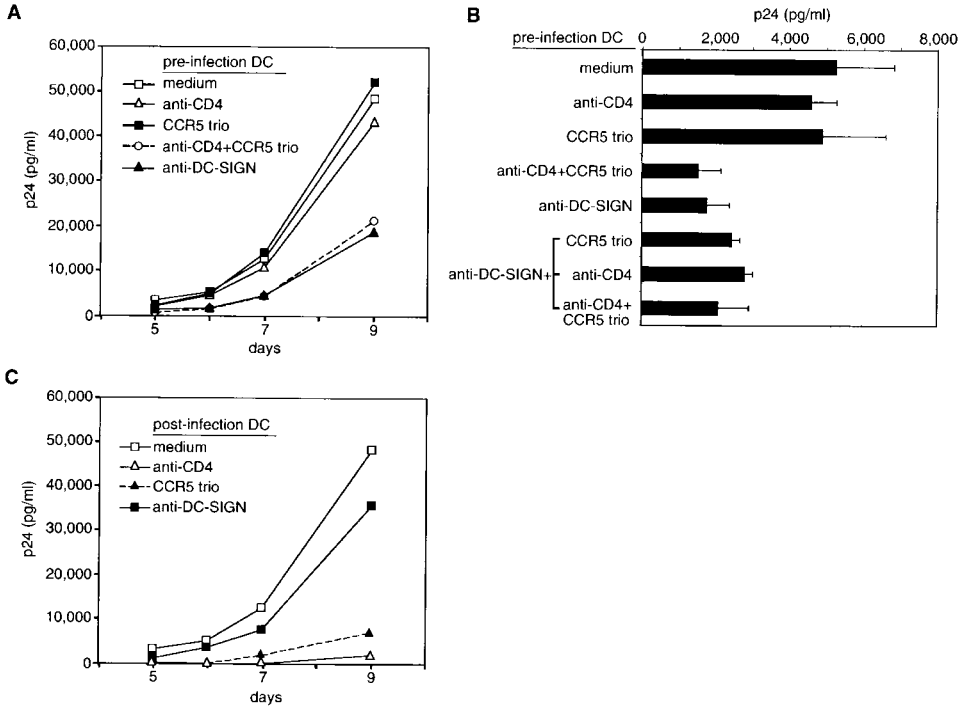

Figure 2. DC-SIGN Mediates HIV-1 Infection in a DC-T Cell Coculture

(A) Antibodies against DC-SIGN inhibit HIV-1 infection as measured in a DC-T cell coculture. DC (50×10^3) were preincubated for 20 min at room temperature with blocking mAb against CD4 (RPA-T4) or DC-SIGN (AZN-D1 and AZN-D2) (20 μg/ml) or with a combination of CCR5-specific chemokines (CCR5 trio: RANTES, MIP-1α, and MIP1β; 500 ng/ml). Preincubated immature DC were pulsed for 2 hr with HIV-1 (M-tropic HIV-$1_{Ba\text{-}L}$ strain), and unbound virus particles and mAb were washed away. Subsequently, DC were cocultured with activated PBMC (50×10^3) for 9 days. Coculture supernatants were collected, and p24 antigen levels were measured by ELISA. One representative experiment out of two is shown.

(B) Inhibition of HIV-1 infection in a DC-T cell coculture by blocking DC-SIGN, CD4, and CCR5. HIV-1 replication in the DC-T cell coculture at day 5 of the experiment is described in Figure 5A. The results of day 5 are representative for days 6, 7, and 9 of DC-T cell coculture. DC were also preincubated with mAb against DC-SIGN together with anti-CD4 and CCR5-specific chemokines. p24 values represent mean ± SD of triplicate cultures. One representative experiment out of two is shown.

(C) DC-SIGN interactions with ICAM-3 are not involved in the transmission of DC-bound-HIV-1 to T cells. DC (50×10^3) were pulsed for 2 hr with HIV-1 (M-tropic HIV-$1_{Ba\text{-}L}$ strain), washed, and cocultured with activated PBMC (50×10^3) for 9 days in the presence of the CCR5-specific chemokines (CCR5 trio: RANTES, MIP-1α, and MIP1β; 500 ng/ml) or mAb against CD4 (RPA-T4) and DC-SIGN (AZN-D1 and AZN-D2) (20 μg/ml). Antibodies were added post-HIV-1 infection of DC, prior to the addition of PBMC. One representative experiment out of two is shown.

it was transfected with a DC-SIGN expression vector (Figure 1C). HIV-1 gp120 binding to this cell line, THP-DC-SIGN, was also blocked by anti-DC-SIGN antibodies, but not by anti-CD4 (Figure 1D). Binding of HIV-1 gp120 to DC-SIGN expressed on DC or THP-DC-SIGN was also inhibited by the carbohydrate mannan or EGTA, consistent with previous findings (Curtis et al., 1992) and with the observation that DC-SIGN is homologous to other members of the Ca^{2+}-binding mannose-type lectins (Weis et al., 1998). Together, these results demonstrate that DC-SIGN is a specific dendritic cell surface receptor for the HIV-1 envelope glycoprotein.

DC-SIGN Is Required for Efficient HIV-1 Infection in DC-T Cell Cocultures

Because DC-SIGN is exclusively expressed on DC and has a high affinity for HIV-1 gp120, we reasoned that it might play an important role in HIV-1 infection of DC or of T cells that make contact with DC. Immature DC, which express low levels of CD4 as well as CCR5 and abundant DC-SIGN (Figure 1C), were pulsed with the R5 isolate HIV-$1_{BA\text{-}L}$ for 2 hr, washed, and cultured in the presence of activated T cells (Figures 2A and 2B). To determine the contribution of each of these receptors in this assay system, we examined the effects of antibodies against CD4 and DC-SIGN and of a combination of three CCR5-specific chemokines (RANTES, MIP-1α, and MIP-1β). Preincubation of the immature DC with antibodies against DC-SIGN prior to infection resulted in significant inhibition of HIV-1 replication (Figure 2A). Neither anti-CD4 nor the CCR5-specific chemokines inhibited on their own, although a combination of these did block infection of DC (Figure 2A), which is probably due to efficient inhibition of the T cell infection by (un)bound anti-CD4/chemokines. Activated T cells challenged with the same viral load exhibited a weaker infection than those cultured with virus-pulsed DC (data not shown).

Since DC-SIGN binds to ICAM-3 on T cells, it is possible that antibodies against DC-SIGN could interfere with the DC-T cell interaction and thereby prevent HIV-1 transmission. To examine this possibility, antibodies against DC-SIGN were added after exposure of DC to HIV-1 but prior to the addition of activated T cells. In

this setting, only CCR5-specific chemokines and anti-CD4 antibody strongly inhibited HIV-1 infection of activated T cells, while antibodies against DC-SIGN had no effect (Figure 2C). These results thus suggest that DC-SIGN has an important function in propagation of HIV-1 in DC-T cell cocultures and that this function is related to the ability of DC-SIGN to bind to gp120 and not to its interaction with ICAM-3.

DC-SIGN Does Not Mediate HIV-1 Entry

To investigate whether DC-SIGN acts as a receptor that permits HIV-1 entry, similar to CD4 plus CCR5, we studied HIV-1 entry into 293T cells that expressed either DC-SIGN (293T-DC-SIGN) or CD4 and CCR5 (293T-CD4-CCR5). Cells were pulsed overnight with HIV_{BA-L} and washed the next day, and p24 levels were determined. There was no detectable p24 protein in the culture supernatants harvested from 293-DC-SIGN cells several days after the HIV-1 pulse, whereas the 293T-CD4-CCR5 cells were readily infected (Figure 3A).

To examine the possibility that DC-SIGN may work in conjunction with either CD4 or CCR5 to permit viral entry, we extended the studies by using HIV-1 pseudotyped with the envelope glycoprotein of the R5 isolate $HIV\text{-}1_{ADA}$. We employed a replication-defective HIV-1 genome that encoded a luciferase reporter gene, which allows a quantitative measure of the levels of single-round infection (Figure 3B) (Deng et al., 1996). Transiently transfected 293T cells expressing either CCR5 (293T-CCR5), CD4 (293T-CD4), or both (293T-CD4-CCR5), in the presence or absence of DC-SIGN, were infected with the reporter virus, and luciferase levels were determined after 2 days. As observed with replicating virus, HIV-1 entry was not detected in 293T cells that expressed only DC-SIGN (Figure 3B). No infection was observed if DC-SIGN was expressed with either CD4 or CCR5, indicating that DC-SIGN does not form a complex with these molecules to permit viral entry. In contrast, high luciferase activity was obtained after infection of 293T cells expressing both CD4 and CCR5, and expression of DC-SIGN did not contribute further to viral entry into these cells (Figure 3B). Therefore, DC-SIGN cannot substitute for CD4 or CCR5 in the process of HIV-1 entry.

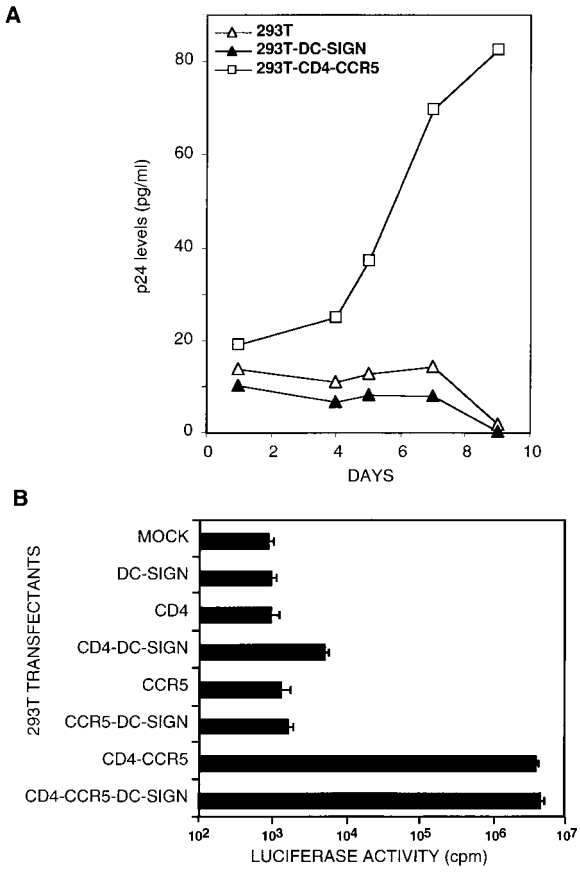

Figure 3. DC-SIGN Expressed on Target Cells Does Not Mediate HIV-1 Entry

(A) 293T cells were transfected with DC-SIGN or CD4 and CCR5 and pulsed for 2 hr with HIV-1 (CCR5-tropic $HIV\text{-}1_{Ba\text{-}L}$ strain). Subsequently, cells were cultured for 9 days. Supernatants were collected, and p24 antigen levels were measured by ELISA. One representative experiment of two is shown.

(B) 293T cells and 293T cells stably expressing either CD4, CCR5, or CD4 and CCR5 were transiently transfected with DC-SIGN and subsequently infected with pseudotyped CCR5-tropic $HIV\text{-}1_{ADA}$ virus in the presence of polybrene (20 μg/ml). Luciferase activity was evaluated after 2 days. One representative experiment out of three is shown.

DC-SIGN Captures HIV-1 and Facilitates Infection of HIV-1 Permissive Cells In *trans*

Because DC-SIGN did not appear to mediate virus entry into target cells, we hypothesized that in a DC-T cell coculture (Figure 2) DC-SIGN might facilitate both capture of HIV-1 on DC, independent from CD4 and CCR5, and subsequent transmission of HIV-1 to the CD4/CCR5-positive T cells. To test this, THP-DC-SIGN transfectants, which do not express CD4 or CCR5 (Figure 1C) and which cannot be infected by HIV-1 (data not shown), were pulsed with single-round HIV-luciferase virus pseudotyped with the $HIV\text{-}1_{ADA}$ envelope glycoprotein. After washing to remove unbound virus, the cells were cocultured with CD4/CCR5-expressing 293T cells, which are permissive for HIV-1 infection, or activated T lymphocytes. THP-DC-SIGN cells were able to capture the pseudotyped virus and transmit it to the target cells that expressed the receptors required for viral entry (Figure 4A). HIV-1 capture was completely DC-SIGN dependent, as antibodies against DC-SIGN inhibited HIV-1 infection (Figure 4A), and DC-SIGN-negative parental THP-1 cells were unable to capture and transmit HIV-1 (Figures 4A and 4B). Similar to our previous findings, the DC-SIGN-mediated infection of the target cells was not due to DC-SIGN binding to ICAM-3, since 293T cells are ICAM-3 negative. These findings indicate that DC-SIGN expressed at the surface of heterologous cells can capture HIV-1 in a form that retains its capacity to subsequently infect HIV-1-permissive cells. The ability of DC-SIGN to capture and transmit HIV-1 was also observed with HIV-luciferase viruses pseudotyped with envelope glycoproteins from an additional five R5 isolates, including three primary isolates (Figure 4B), and from the X4 isolate HXB2 (data not shown).

Analysis of luciferase activity in both adherent (293T-CD4-CCR5) and nonadherent (THP-DC-SIGN) cell frac-

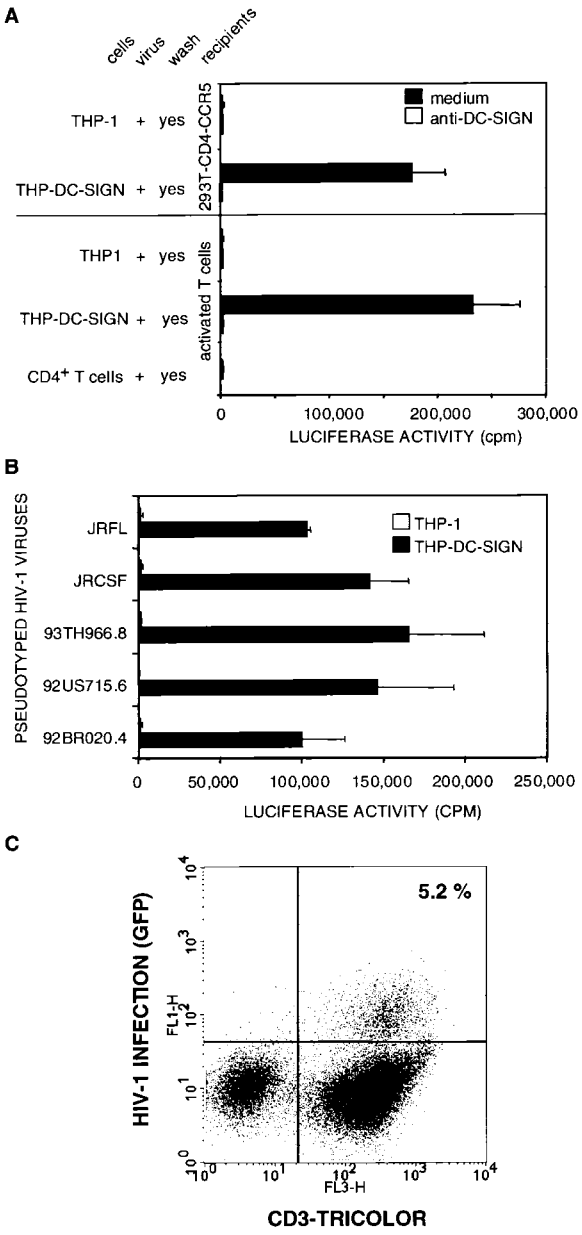

Figure 4. DC-SIGN Captures HIV-1 that Retains Infectivity for $CD4^+$ T Cells

(A) DC-SIGN captures HIV-1 and facilitates infection of HIV-1 permissive cells in *trans*. DC-SIGN transfectants (100×10^3) were preincubated for 20 min at room temperature with blocking mAb against DC-SIGN (AZN-D1 and AZN-D2; 20 μg/ml). The THP-DC-SIGN cells were infected with HIV-luciferase virus pseudotyped with R5 strain $HIV\text{-}1_{ADA}$ Env. Alternatively activated T cells were infected with pseudotyped $HIV\text{-}1_{ADA}$ virus. After 2 hr at 37°C, the infected cells were extensively washed and added to either 293T-CD4-CCR5 cells or activated primary T cells (100×10^3). HIV-1 infection was determined after 2 days by measuring the luciferase activity. One representative experiment out of three is shown.

(B) DC-SIGN is able to mediate capture of HIV-1 viruses pseudotyped with M-tropic HIV-1 envelopes from different primary isolates. DC-SIGN-mediated capture was performed as described in (A) on 293T-CD4-CCR5 with HIV-luciferase viruses pseudotyped with the CCR5-specific HIV-1 envelopes from JRFL and JRCSF and from primary viruses 92US715.6, 92BR020.4, and 93TH966.8. One representative experiment out of two is shown.

tions after 2 days of coculture demonstrated that productive HIV-1 infection occured only in the HIV-1 permissive 293T-CD4-CCR5 cells (data not shown). Similarly, by using a pseudotyped HIV-1 vector with the green fluorescent protein gene in place of Nef (HIV-eGFP), we demonstrated that cells expressing CD4/CCR5 and not those expressing DC-SIGN were infected in cocultures. Thus, after coculture of virus-pulsed THP-DC-SIGN cells with T cells, only the $CD3^+$ T cells expressed virus-encoded GFP (Figure 4C).

Sexual transmission of HIV-1 is likely to require a means for small amounts of virus to gain access to cells that are permissive for viral replication. This may be achieved because of the ability of virus to interact with DC, which can capture HIV-1 and present it to the permissive cells. To mimic in vivo conditions in which HIV-1 levels are likely to be limiting, we challenged THP-1 transfectants with low titers of pseudotyped HIV-1 and subsequently cocultured these cells with HIV-1 permissive cells, without washing away unbound virus (Figure 5A). As expected, neither 293T-CD4-CCR5 cells nor activated T cells were efficiently infected with the low titers of pseudotyped HIV-1 (Figure 5A). Strikingly, when these permissive cells were challenged with an identical amount of HIV-1 in the presence of THP-DC-SIGN, but not of the parental THP-1 cells, efficient HIV-1 infection was observed in *trans* (Figure 5A). The enhancement of HIV-1 infection of primary T cells by DC-SIGN was also observed with HIV-luciferase viruses pseudotyped with five other R5 envelopes, including three from primary virus isolates (Figure 5B). These results indicate that DC-SIGN not only sequesters HIV-1 but also enhances CD4-CCR5-mediated HIV-1 entry by presentation in *trans* to the HIV-1 receptor complex. Antibodies against DC-SIGN completely inhibited infection (Figure 5A), demonstrating that the efficient enhancement of HIV-1 entry into CD4/CCR5-positive cells is DC-SIGN dependent.

DC Present in Mucosal Tissues at Sites of HIV-1 Exposure Express DC-SIGN and Are CCR5 Negative

Demonstration that cells that express DC-SIGN can capture HIV-1 and efficiently transmit the virus to other cells in *trans* suggested that DC that express this C-type lectin have a key role in viral infection in vivo. To determine whether such cells are indeed present in vivo, we performed immunohistochemical analyses of mucosal tissues that are the sites of first exposure during sexual transmission of HIV-1 (Figure 6A). DC-SIGN was expressed on DC-like cells with large and very irregular morphology that were present in the mucosal tissues, such as cervix, rectum, and uterus (Figures 6Aa, 6Ab, and 6Ac, respectively), in regions beneath the stratified

(C) Activated T cells are infected by HIV-1 in the T cell/THP-DC-SIGN coculture. THP-DC-SIGN cells were incubated with HIV-eGFP viruses pseudotyed with M-tropic $HIV\text{-}1_{ADA}$ and subsequently cocultured with activated T cells. The CD3-negative THP-DC-SIGN cells were not infected by HIV-1, whereas the CD3-positive T cells were infected. T cells, gated by staining for CD3 (tricolor), were positive for eGFP, whereas CD3-negative THP-DC-SIGN that initially captured HIV-eGFP did not express eGFP. One representative experiment out of two is shown.

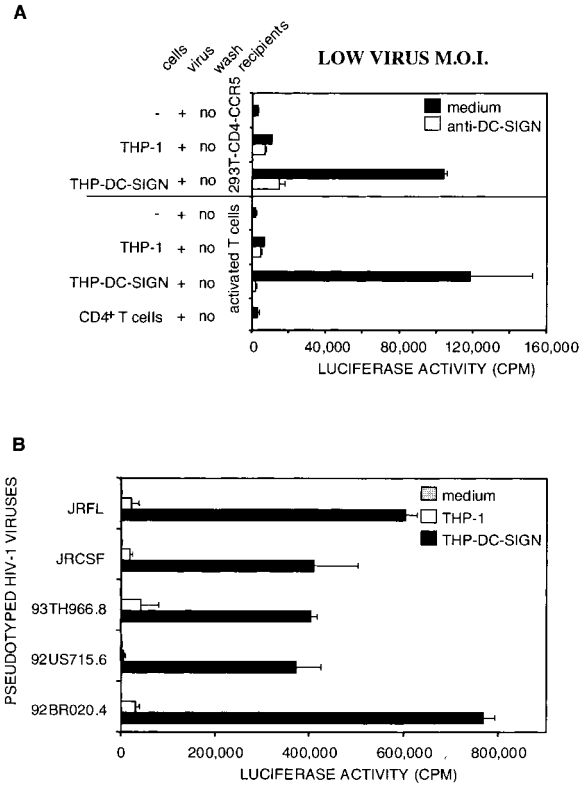

Figure 5. DC-SIGN Enhances HIV-1 Infection of T Cells by Acting In *trans*

At a low virus load, DC-SIGN in *trans* is crucial for the infection of HIV-1 permissive cells. THP-1 transfectants (100×10^3) were preincubated for 20 min at room temperature with blocking mAb against DC-SIGN (AZN-D1 and AZN-D2; 20 μg/ml). The cells were infected by low amounts of pseudotyped HIV-1_{ADA} virus (A) or other R5 isolates of HIV-1 (B), i.e., at the threshold of detection in a single round infection assay. After 1 hr at 37°C, the cell/virus suspension was directly added to either 293T-CD4-CCR5 or activated T cells (100×10^3). The infectivity was determined after 2 days by measuring the luciferase activity. One representative experiment out of two is shown.

squamous epithelium in the lamina propria. Analyses of serial sections stained for CD3, CD20, CD14, and CD68 confirmed that DC-SIGN-expressing cells were distinct from T cells, B cells, monocytes, and macrophages (data not shown). Similarly, in the accompanying paper (Geijtenbeek et al., 2000), staining of lymph nodes and skin has shown DC-restricted expression of DC-SIGN. We have also compared expression of DC-SIGN, CD4, and CCR5 on DC in the mucosa of the uterus and rectum and found in serial sections that the majority of DC-SIGN-positive DC in these tissues coexpressed CD4 but lacked CCR5 (Figure 6B). This suggests that DC present at mucosal sites, that have first contact with HIV-1 during sexual transmission, are not infected with HIV-1 through usage of CD4/CCR5. This observation is consistent with the recent demonstration that DC at sites of mucosal infection of nonhuman primates do not become infected (Stahl-Hennig et al., 1999).

DC-SIGN-Bound HIV-1 Retains Infectivity after Long-Term Culture

If HIV-1 gains access to secondary lymphoid organs by way of binding to DC, then virus would have to retain infectivity during the transport from the mucosal tissues to the T cell zones in draining lymph nodes. To determine if virus bound to DC-SIGN retains infectivity for a prolonged period of time, we first conducted a time-course experiment to determine the length of time that HIV-1 gp120 remains bound to DC-SIGN expressed on transfected THP-1 cells. We observed that gp120-coated beads remained bound to DC-SIGN for more than 60 hr (Figure 7A). We next investigated the length of time during which HIV-1-pulsed THP-DC-SIGN cells could retain infectious virus. The DC-SIGN-expressing transfectants were pulsed with pseudotyped HIV-1 for 4 hr and then washed extensively. The pulsed cells were subsequently placed in culture and were removed at defined intervals and cocultured with activated T cells (Figure 7B). Remarkably, after 4 days the HIV-1-pulsed cells were still able to efficiently infect target cells. In contrast, virus in the absence of DC-SIGN-positive cells lost its infectivity after 1 day. These findings support the hypothesis that limiting numbers of HIV-1 particles, captured by mucosal DC that express DC-SIGN and CD4 but not CCR5, retain infectivity during and after migration to regional lymphoid tissues. T cells, which express CD4 and CCR5, would then be productively infected due to DC-SIGN-mediated enhanced *trans* infectivity of the small numbers of HIV-1 particles (Figure 7C).

Discussion

We have identified a novel DC-specific adhesion receptor, DC-SIGN, that is identical to the high-affinity HIV-1 gp120-binding C-type lectin cloned from a human placental cDNA library (Geijtenbeek et al., 2000). We have demonstrated that DC that express both DC-SIGN and CD4 preferentially use DC-SIGN to capture HIV-1 via its high affinity for HIV-1 gp120. DC-SIGN not only efficiently recruits HIV-1 but also facilitates HIV-1 infection of $CD4^+$ T cells by a novel in *trans* mechanism. Our findings thus indicate that HIV-1 utilizes a novel receptor strategy that has not been previously described in other viral systems, and suggest that the virus exploits multiple cell surface receptor systems to ensure that it can establish a productive infection in its host organism.

DC localized in the skin and mucosal tissues such as the rectum, uterus, and cervix have been proposed to play a role in initial HIV-1 infection. DC constitute a heterogeneous population of cells that are present in minute numbers in various tissues just beneath the dermis or mucosal layer and form a first-line defense against viruses and other pathogens. DC have previously been shown to sequester HIV-1 and efficiently transmit the virus to $CD4^+$ T cells. We have demonstrated here that this property of DC can be ascribed to the ability of HIV-1 to bind specifically to these cells through the interaction of gp120 with DC-SIGN. DC thus efficiently capture HIV-1 through a specific interaction that is independent from binding of virus to CD4 and CCR5. DC-SIGN cannot mediate HIV-1 entry but rather functions as a unique HIV-1 *trans* receptor facilitating HIV-1 infection of CD4/CCR5-positive T cells (Figures 4 and 5). At low virus titer, CD4/CCR5-expressing cells are not detectably infected without the help of DC-SIGN in *trans* (Figure 5A). Conditions in which the number of HIV-1 particles is limiting are likely to resemble those

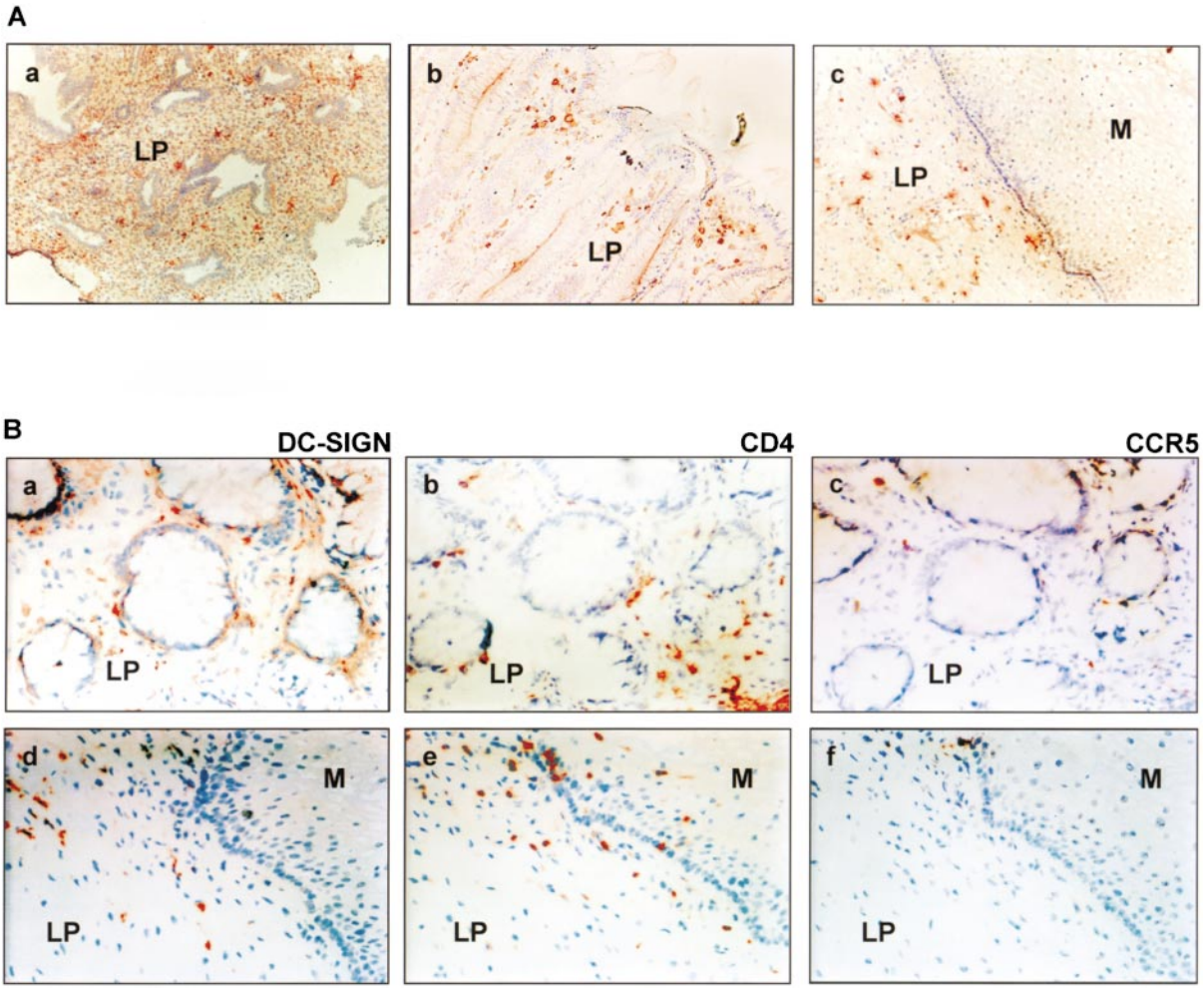

Figure 6. DC-SIGN Is Expressed on DC Present in Mucosal Tissue that Do Not Express CCR5

Immunohistochemical analysis of DC-SIGN expression on mucosal tissue sections.

(A) Different tissue sections were stained with anti-DC-SIGN mAb: cervix (a), rectum (b), and uterus (c) (original magnification, 200×) All mucosal tissues contain DC-SIGN-positive cells in the lamina propria. Staining of serial sections demonstrate that these DC-SIGN-positive cells do not express CD3, CD20, CD14, and CD68 (data not shown).

(B) Immunohistochemical staining of serial sections of rectum (a–c) and uterus (d–f) with antibodies against DC-SIGN (a and d), CD4 (b and e), or CCR5 (c and f).

found in vivo, and the results thus suggest that DC-SIGN may be required for viruses to be transmitted from mucosa to T cells that express CD4 and chemokine receptors. In addition, our studies demonstrate that virus bound to DC-SIGN is remarkably stable and can thus retain infectivity for the prolonged periods of time required for DC to traffic via lymphatics from mucosa to regional lymph nodes (Figures 7A and 7B).

Mechanism of DC-SIGN-Mediated Enhancement of HIV-1 Infectivity

The mechanisms by which HIV-1 exploits the machinery of DC and the properties of DC-SIGN to achieve efficient infection of cells that are competent for viral replication remain unclear. The process through which DC-SIGN promotes efficient infection in *trans* of cells through their CD4/chemokine receptor complex is of particular interest. Binding of the viral envelope glycoprotein to DC-SIGN may induce a conformational change that enables a more efficient interaction with CD4 and/or the chemokine receptor. As multiple conformational transitions are required before the envelope glycoprotein initiates fusion with target membranes, the binding of DC-SIGN to gp120 may facilitate or stabilize one of these transitions. Anti-gp120 antibodies that increase infectivity of viral particles have been described (Lee et al., 1997), and it is possible that DC-SIGN has a similar effect upon binding to the envelope glycoprotein. Alternatively, binding of viral particles to DC-SIGN may focus or concentrate them at the surface of the DC and may thus increase the probability that entry will occur after they bind to the receptor complex on target cells. Although the molecular mechanism has to be investigated in more detail, it is clear that DC-SIGN enhances the infection of T cells, since at low multiplicity of infection T cells are not infected in the absence of DC-SIGN.

Whether a transient quaternary complex is formed between DC-SIGN, HIV-1 Env, CD4, and CCR5 remains

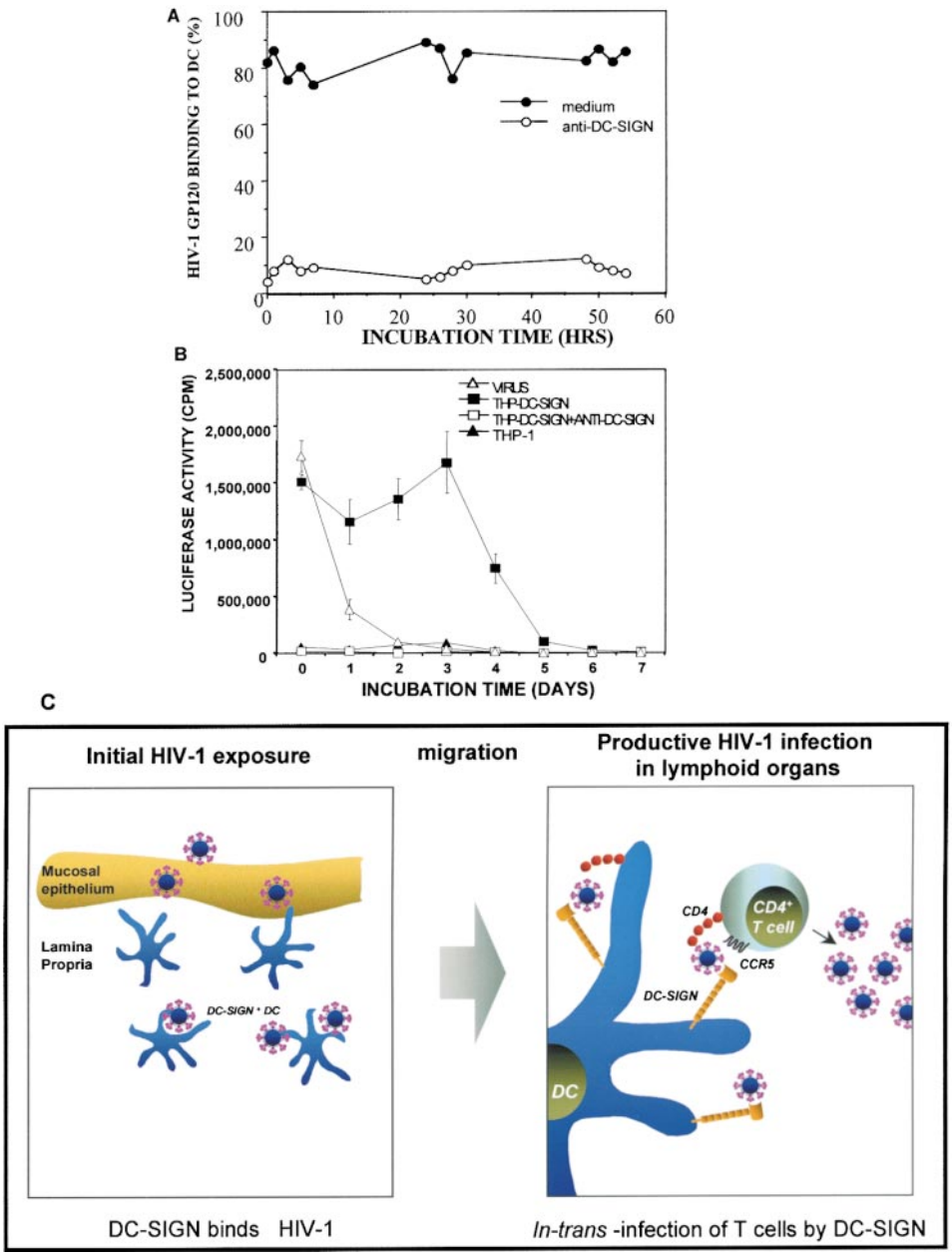

Figure 7. DC-SIGN Captures HIV-1 and Retains Long-Term Infectivity

(A) Time course of HIV-1_{MN} gp120 binding to THP-DC-SIGN. DC-SIGN-positive cells were incubated with gp120-coated beads. Beads bound for more than 60 hr as determined by FACScan analysis.

(B) DC-SIGN binds HIV-1 and retains for more than 4 days virus that infects T cells in *trans*. THP/THP-SIGN cells were pulsed for 4 hr with HIV-1 pseudotyped virus in the presence or absence of anti-DC-SIGN antibodies (AZN-D1 and AZN-D2, 20 μg/ml). After washing, the HIV-1 pulsed cells were cultured at 37°C for several days. As a control, identical amounts of virus were incubated at 37°C in medium without cells. Every day, aliquots of the HIV-1 pulsed cells were added to HIV-1 permissive 293T-CD4-CCR5 cells in order to measure infectivity. Lysates to examine luciferase activity were obtained after 2 days of coculture.

(C) Model of HIV-1 coopting DC-SIGN as a *trans* receptor after initial exposure. DC are the primary cells targeted by HIV-1 during mucosal exposure and are DC-SIGN positive. HIV-1 adheres to DC-SIGN via a high-affinity interaction, and the immature DC carrying HIV-1 migrates to the lymphoid tissues. Upon arrival, DC will cluster with T cells, and DC-SIGN enhances HIV-1 infection of T cells in *trans* leading to a productive and sustained infection.

to be determined. Elucidation of the crystal structure of a gp120-CD4 complex has revealed that most glycosylation sites within gp120 reside in a ridge that flanks the CD4-binding pocket (Kwong et al., 1998). Since mannan blocks the binding of gp120 to DC-SIGN, it is likely that this C-type lectin binds to one or more carbohydrate moieties in gp120. It remains possible, however, that the lectin domain of DC-SIGN interacts with the polypeptide

backbone of gp120. Further studies with mutant forms of gp120 and with soluble DC-SIGN may be informative in efforts to elucidate the mechanism of enhanced infectivity in *trans*.

In a separate study, we have shown that DC-SIGN binds to ICAM-3, which is expressed constitutively on the surface of T lymphocytes (Geijtenbeek et al., 2000). Enhancement of target cell infectivity by DC-SIGN-bound HIV-1 was not dependent on the presence of ICAM-3 on target cells. However, we observed that enhancement of infectivity was consistently better when target cells were T cells rather than 293-CD4-CCR5 cells. It remains possible that the efficiency of viral transmission from carrier DC to target T cells may also be enhanced by specific adhesive interactions other than DC-SIGN-ICAM-3, such as LFA-1-ICAM-1, which predominates the adhesion between DC and activated T cells (Geijtenbeek et al., 2000). Therefore, antibodies against DC-SIGN do not inhibit the DC-T cell transmission of HIV-1 postinfection (Figure 2C).

Role of DC in HIV Infection In Vivo

The only HIV-1 receptors previously known to have a role in HIV-1 entry were CD4 and a subset of the G protein–coupled chemokine receptors, including CCR5 and CXCR4. CCR5 functions as the major receptor for strains of virus previously classified as "macrophage-tropic," and only those strains that can utilize this chemokine receptor can be efficiently transmitted between individuals (Littman, 1998). Other gp120-binding receptors had been previously identified, including DC-SIGN and galactosyl ceramide (Harouse et al., 1991), but these had not been shown to be involved in viral entry. This study shows that DC-SIGN not only binds HIV-1 but can also sequester it and catalyze its entry into cells that express CD4 and chemokine receptors. Although it remains to be determined whether DC-SIGN has a significant role in HIV-1 pathogenesis in vivo, our in vitro results and the pattern of expression of the different receptors in mucosal tissues are consistent with its having a key function in the early stages of viral infection. Remarkably, our immunohistochemical analyses clearly demonstrate that CCR5 is not expressed in the lamina propria of HIV-1-related mucosal tissue (Figure 6), whereas DC-SIGN is abundantly expressed. This observation confirms and extends the findings of Hladik et al. (1999), who showed that DC present in the genital tract also lack CCR5, and strongly suggest that HIV-1 cannot infect DC present at mucosal sites.

DC-SIGN may therefore play a crucial role in initial HIV-1 exposure by mediating viral binding to DC present in mucosal tissues, rather than infection of these cells. The high level of expression of DC-SIGN on immature DC and its high affinity for gp120, which exceeds that of CD4 (Curtis et al., 1992), indicate that DC-SIGN is endowed with the ability to efficiently capture HIV-1, even when the virus is present in minute amounts. HIV-1 may subsequently exploit the migratory capacity of the DC to gain access to the T cell compartment in lymphoid tissues. DC must be activated to commence their migration, and it is hence possible that multimerization of DC-SIGN on the cell surface of DC by interaction with the multivalent virus particles may initiate this process. Interestingly, the time course experiment shows that DC-SIGN is able to capture and bind to HIV-1 for more than 4 days, after which the virus can still infect permissive cells. This long-term preservation of HIV-1 in an infectious state would appear to allow sufficient time for it to be transported by DC trafficking from mucosal surfaces to lymphoid compartments, where virus can be transmitted (Figure 7C) (Steinman et al., 1997). Several groups have reported that DC can migrate from the periphery to draining lymph nodes within 2 days after antigen exposure or HIV-1 challenge (Barratt-Boyes et al., 1997; Stahl-Hennig et al., 1999). Viral particles have been reported within endocytic vesicles of DC. This observation suggests that DC-SIGN-bound HIV-1 may be internalized and protected during the time required for the cells to complete their journey to the regional lymph nodes. Further studies will be required to determine if viral internalization is essential for maintenance of infectivity.

Our data suggest that, after HIV-1 has been ferried by DC to the lymphoid compartment, DC-SIGN presents the bound viral particles to the CD4/CCR5 complex present on T cells and greatly enhances their entry into these cells (Figure 7C). We showed that monoclonal antibodies directed against DC-SIGN blocked productive infection occurring in the T cell cocultures with CD4/CCR5-positive monocyte-derived DC. Therefore, even in the presence of obligatory HIV-1 receptors present in *cis* on target cells, DC-SIGN functions as a *trans* receptor for HIV-1 infection of T cells and is critical in the primary cocultures. This is an important example of how a receptor can work in *trans*. Interestingly, CD4 can facilitate HIV-1 infection of CD4-negative cells that express CCR5 by a *trans* receptor mechanism, although it remains unclear whether this is an important route of infection in vivo (Speck et al., 1999). In that case, interaction of envelope glycoprotein with CD4 results in a conformational change that permits binding of the virus to CCR5 on CD4-negative cells. Together with the results presented here, these studies indicate that HIV-1 can use receptors in *trans* to facilitate infection of cells that otherwise may be difficult to infect either because of lack of proper receptors or because of their anatomical distribution relative to the sites of HIV-1 exposure.

The discovery of the role of DC-SIGN in HIV-1 infection may have significant implications for understanding the mechanism of HIV-1 transmission and for developing strategies to prevent or block viral infection. The observation that transmission of infection is confined to R5 strains of HIV-1 has remained a major enigma. In preliminary studies, we found that DC-SIGN captures and enhances infection of both X4 and R5 strains, and it is thus unlikely that preferential interaction of DC-SIGN with CCR5 would account for the restriction in tropism during transmission. Nevertheless, the demonstration that uninfected DC contribute to the process of viral entry raises the possibility that the requirement for CCR5 utilization may reflect a requirement for interaction of multiple cell types. The inhibition of HIV-1 infection observed in the presence of anti-DC-SIGN antibodies suggests that interfering with the gp120-DC-SIGN interaction either during the capture phase of DC in the mucosa or during DC/T cell interactions in lymphoid organs could inhibit dissemination of the virus. Small molecule inhibitors, potentially carbohydrate-based, that block

the ability of gp120 to bind to DC-SIGN may be effective in prophylaxis or therapeutic intervention. Vaccine strategies aimed at eliciting mucosal antibodies that inhibit gp120 binding to DC-SIGN may also be efficacious in preventing early establishment of infection. The efficacy of gp120 vaccines has been measured as a function of the levels of neutralizing antibodies that inhibit HIV entry through CD4 and CCR5. Our results thus suggest that levels of antibodies that block virus binding to DC-SIGN or the DC-SIGN-mediated enhancement of infection may also be predictive of protection.

Experimental Procedures

Antibodies

The following mAb were used: 2D7 (anti-CCR5; Becton Dickinson and Co., Oxnard, CA) and CD4 (RPA-T4; PharMingen, San Diego, CA). Anti-DC-SIGN mAb AZN-D1 and AZN-D2 were obtained by screening hybridoma supernatants of human DC-immunized BALB/c mice for the ability to block adhesion of DC to ICAM-3, as measured by the fluorescent bead adhesion assay.

Cells

Immature DC were cultured as previously described (Geijtenbeek et al., 2000). Stable THP-1 transfectants expressing DC-SIGN were generated by transfection of THP-1 cells with pRc/CMV-DC-SIGN by electroporation similarly as described (Lub et al., 1997).

Fluorescent Bead Adhesion Assay

Carboxylate-modified TransFluorSpheres (488/645 nm, 1.0 μm; Molecular Probes, Eugene, OR) were coated with M-tropic HIV-1_{MN} envelope glycoprotein gp120 similarly as was described for ICAM-1 beads (Geijtenbeek et al., 1999). Streptavidin-coated beads were incubated with biotinylated F(ab′)2 fragment rabbit anti-sheep IgG (6 μg/ml; Jackson Immunoresearch) followed by an overnight incubation with sheep-anti-gp120 antibody D7324 (Aalto Bio Reagents Ltd., Dublin, Ireland) at 4°C. The beads were washed and incubated with 250 ng/ml purified HIV-1 gp120 (provided by Immunodiagnostics, Inc., through the NIH AIDS Research and Reference Reagent Program) overnight at 4°C. The fluorescent beads adhesion assay was performed as described by Geijtenbeek et al. (1999).

HIV-1 Infection of Both DC and DC-SIGN Transfectants

The M-tropic strain HIV-$1_{Ba\text{-}L}$ was grown to high titer in monocyte-derived macrophages (MDM). Seven days after titration of the virus stock on MDM, $TCID_{50}$ was determined with a p24 antigen ELISA (Diagnostics Pasteur, Marnes la Coquette, France) and estimated as 10^4/ml. DC (50×10^3) preincubated with mAb against DC-SIGN (AZN-D1 and AZN-D2) or CD4 (RPA-T4) (20 μg/ml) or a combination of CCR5-specific chemokines (RANTES, MIP-1α, MIP-1β; each 500 ng/ml) for 20 min at room temperature were pulsed for 2 hr with HIV-$1_{Ba\text{-}L}$ (at a multiplicity of infection of 10^3 infectious units per 10^5 cells), washed, and cocultured with activated PBMC (50×10^3). No DC-T cell syncytium formation was observed. The postinfection experiment was performed similarly except that the mAb or chemokines were added after the washing step of the HIV-1 pulse, together with the activated PBMC. Culture supernatants were collected at day 5, 6, 7, and 9 after DC-T cell coculture and p24 antigen levels, as a measure of HIV-1 production were determined by a p24 antigen ELISA. PBMC were activated by culturing them in the presence of IL-2 (10 U/ml) and PHA (10 μg/ml) for 2 days.

Pseudotyped viral stocks were generated by calcium-phosphate transfections of 293T cells with the proviral plasmid pNL-Luc-E^-R^- (containing a luciferase reporter gene) or the proviral pHIV-eGFP (containing a GFP reporter gene) and expression plasmids for ADA, JRFL, and JRCSF gp160 envelopes. The isolation, identification, and construction of the plasmids encoding the primary virus envelopes from 92US715.6, 92BR020.4, and 93TH966.8 has been previously described (Bjorndal et al., 1997). Viral stocks were evaluated by limiting dilution on 293T-CD4-CCR5 cells. HIV-1 pseudotyped with murine leukemia virus (MLV), amphotropic Env, and vesicular stomatitis virus glycoprotein (VSV-G) were used to ensure target cell viability.

Immunohistochemical analyses were performed as described previously (Geijtenbeek et al., 2000).

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