

DC-SIGN-Mediated Internalization of HIV Is Required for *Trans*-Enhancement of T Cell Infection

Douglas S. Kwon,² Glenn Gregorio,³ Natacha Bitton,² Wayne A. Hendrickson,³ and Dan R. Littman^{1,2,4}

¹Howard Hughes Medical Institute and

²Molecular Pathogenesis Program

Skirball Institute of Biomolecular Medicine

New York University School of Medicine

New York, New York 10016

³Howard Hughes Medical Institute

Department of Biochemistry and Molecular Biophysics

College of Physicians and Surgeons

Columbia University

New York, New York 10032

Summary

Fusion of the human immunodeficiency virus (HIV) to the plasma membrane of target cells is mediated by interaction of its envelope glycoprotein, gp120, with CD4 and appropriate chemokine receptors. gp120 additionally binds to DC-SIGN, a C-type lectin expressed on immature dendritic cells. This interaction does not result in viral fusion, but instead contributes to enhanced infection in *trans* of target cells that express CD4 and chemokine receptors. Here we show that DC-SIGN mediates rapid internalization of intact HIV into a low pH nonlysosomal compartment. Internalized virus retains competence to infect target cells. Removal of the DC-SIGN cytoplasmic tail reduced viral uptake and abrogated the *trans*-enhancement of T cell infection. We propose that HIV binds to DC-SIGN to gain access to an intracellular compartment that contributes to augmentation or retention of viral infectivity.

Introduction

The human immunodeficiency virus (HIV) infects cells that express CD4 and a variety of chemokine receptors, particularly CCR5, at their surface (reviewed in Simmons et al., 2000). Infection is initiated by binding of the surface subunit of envelope glycoprotein (Env), gp120, to CD4 on the target cell. This interaction results in a conformational change in gp120 that allows it to bind to a member of the family of seven transmembrane domain G protein-coupled chemokine receptors (Lapham et al., 1996; Trkola et al., 1996; Wu et al., 1996). Interaction with the chemokine receptor releases the fusogenic potential of the transmembrane subunit of Env, gp41, whose N-terminal fusion peptide is inserted into the target cell lipid bilayer, initiating fusion of the viral and host membranes. These contacts between envelope glycoprotein and CD4 plus chemokine receptors are believed to be sufficient for viral entry. Isolated strains of HIV utilize distinct chemokine receptors for infection (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Dragic et al., 1996; Feng et al., 1996). Those that use

the chemokine receptor CCR5 (R5 strains) have been found to be preferentially transmitted following initial exposure to virus (Cornelissen et al., 1995; Huang et al., 1996; Samson et al., 1996; van't Wout et al., 1994). Viruses that use the chemokine receptor CXCR4 (X4 strains) often prevail during progression to immunodeficiency (Connor et al., 1997; Schuitemaker et al., 1992).

Although the mechanism of HIV entry has been studied extensively in cultured cells, the factors that contribute to infection in vivo remain poorly understood. It has been proposed that dendritic cells (DC), which are prevalent at mucosal surfaces and function as antigen-presenting cells in draining lymphatic tissues, have an important role in HIV infection (reviewed in Rowland-Jones, 1999; Steinman et al., 1999). These cells have been shown to facilitate infection with HIV when they are cocultured with T cells (Cameron et al., 1992; Pope et al., 1995). HIV replicates poorly in immature DC in vitro (Cameron et al., 1992; Graneli-Piperno et al., 1999), and it has been difficult to study infection of DC in vivo. A novel function for DC was proposed after it was found that HIV binds to DC-SIGN, a cell surface C-type lectin on these cells (Geijtenbeek et al., 2000a). DC-SIGN had been previously identified as a gp120 binding protein (Curtis et al., 1992) and was subsequently isolated from monocyte-derived DC as a binding partner for ICAM-3 (Geijtenbeek et al., 2000b). We showed that, in contrast to CD4 and chemokine receptors, DC-SIGN does not mediate the entry of HIV bound to it. However, DC-SIGN-expressing cells pulsed with HIV transmit virus to appropriate target cells and retain infectious particles for several days in culture. In addition, at low viral multiplicity of infection (MOI), activated T lymphocytes cocultured with DC-SIGN-expressing cells are much more readily infected than lymphocytes exposed to control cells. Similar results were observed with several laboratory-adapted and primary R5 strains of HIV. Because DC-SIGN is expressed at high levels on immature dendritic cells, we proposed that it may have an important role in DC-mediated transport of virus from mucosal sites to secondary lymphoid tissues, where it could efficiently transfer virus to T cells (Geijtenbeek et al., 2000a).

Dendritic cells are specialized sentinel cells that take up antigen, process it, and present it to T lymphocytes following migration to lymphoid organs (reviewed in Banachereau et al., 2000; Thery and Amigorena, 2001). Because antigens are engulfed and digested by DC, it is puzzling that HIV infectivity increases and even persists for days after virions bind to the surface of these cells. Electron micrographic examination of HIV-exposed DC has shown the presence of intact viral particles in intracellular vesicular compartments (Hladik et al., 1999). Because DC-SIGN has putative tyrosine and dileucine internalization motifs in its cytoplasmic tail, we reasoned that HIV particles may undergo endocytosis after binding to the cell surface lectin. In the current study, we have examined the role of HIV endocytosis in the process of DC-SIGN-mediated augmentation of viral infectivity. We show that HIVs of variable tropism, but not murine retroviruses, are captured and display enhanced infectivity.

⁴Correspondence: littman@saturn.med.nyu.edu

ity following interaction with cells that express DC-SIGN. Following binding to DC-SIGN, HIV is internalized into a low pH nonlysosomal compartment, which allows retention of infectivity. The DC-SIGN-mediated internalization enhances the amount of virus captured by cells, and truncation of the cytoplasmic domain of DC-SIGN results in decreased viral uptake and loss of enhancement of infectivity. Together, these findings suggest that HIV has evolved to exploit the cellular machinery and the physiological functions of dendritic cells to its advantage for optimizing transmissibility between individuals.

Results

DC-SIGN Binds Numerous HIV and SIV Strains but Not MLV-Pseudotyped Virus

To examine the specificity of the interaction between DC-SIGN and viral envelope glycoproteins, a human monocytic cell line, THP, was transduced with a retrovirus encoding the cDNA for DC-SIGN. This THP DC-SIGN cell does not express CD4 or CCR5 and cannot be infected by HIV (data not shown). Binding of purified fluorescently labeled envelope glycoproteins to these cells demonstrated that DC-SIGN could interact with gp120 from HIV strains with R5, X4, and R5X4 tropism and with envelope glycoprotein from simian immunodeficiency virus (SIV) (Figure 1A). This was consistent with assays in which binding of HIV particles was measured by utilizing a replication defective HIV-1 encoding the firefly luciferase reporter gene (Figure 1B). Expression of the reporter provides a quantitative measure of a single round of HIV infection. THP DC-SIGN cells were pulsed with HIV-luciferase pseudotyped with envelope glycoproteins from primary or laboratory-adapted R5, X4, and R5X4 HIV strains or from SIV. The cells were then washed extensively and added to HIV-permissive 293T target cells expressing human CD4, CXCR4, and CCR5. Cells were harvested 48 hr later and assayed for luciferase activity. DC-SIGN was able to capture and transmit all virus strains tested; thus, DC-SIGN is capable of binding to a broad range of primate lentiviruses.

DC-SIGN was previously shown to enhance HIV infection of T cells at low virus titer (Geijtenbeek et al., 2000a). To test the specificity of this enhancement, we used HIV-luciferase pseudotyped with Env from multiple strains of HIV and SIV to infect T cells. When low amounts of virus were used, infection was greatly enhanced by coculture with THP DC-SIGN cells when compared to the same amount of virus added to a coculture with the parental cell line or T cells alone (Figure 1C). Enhancement was obtained with all HIV and SIV strains tested and also with replication-competent virus when activated T cells were cocultured with THP DC-SIGN cells or human DC (Figure 1D, and data not shown). Enhancement with replication-competent virus was not observed upon coculture with THP cells or DC pretreated with an anti-DC-SIGN antibody.

Because all primate lentiviruses that we tested displayed interaction with DC-SIGN, we wished to determine whether other retroviruses containing glycosylated envelope protein could similarly bind to DC-SIGN. We therefore pseudotyped an envelope-deficient HIV parti-

cle that encoded the *Renilla* luciferase reporter gene with the glycosylated ecotropic Moloney murine leukemia virus (MLV) envelope glycoprotein. The MLV *Renilla* luciferase-pseudotyped virus was mixed with HIV firefly luciferase particles pseudotyped with the R5 tropic envelope JRFL. The virus particles encoding two different forms of luciferase allowed us to simultaneously quantify infectivity of each particle within the same culture. The viruses were mixed to give comparable levels of infection of murine 3T3 target cells expressing human CD4, CCR5, and Cyclin T1 (Figure 1E). Human cyclin T1 is required for efficient transcription of LTR-driven HIV genes in murine cells. When this same mixture was added to human 293T targets expressing the necessary HIV entry receptors, only the HIV JRFL firefly luciferase virus could infect, thus verifying that the *Renilla* luciferase virus was in fact pseudotyped with the ecotropic MLV envelope. THP or THP DC-SIGN cells were incubated with this mixture of virus, washed, and then added to 3T3 target cells (Figure 1F). Only the virus pseudotyped with the HIV envelope could be captured and transferred to the 3T3 target cells. Together, these data demonstrate that although DC-SIGN can capture and enhance a wide variety of HIV and SIV strains, this broad specificity among primate lentiviruses does not extend to other retroviruses containing glycosylated envelopes.

HIV Is Internalized into a Low pH Endosomal Compartment following Binding to DC-SIGN

The cytoplasmic tail of DC-SIGN contains two classically defined putative internalization motifs, a dileucine-based motif and a tyrosine-based motif (Mellman, 1996; Trowbridge et al., 1993). This observation led to the question of whether virus remains on the cell surface or is internalized following interaction with DC-SIGN. To answer this question, we incubated THP DC-SIGN cells on ice with purified gp120 that was directly conjugated to the fluorescent marker FITC (Figure 2A); the integrity of the gp120 was confirmed by binding to THP cells expressing CD4 (data not shown). After washing, cells were shifted to 37°C for the times indicated. They were then treated with trypsin or mock treated and then stained for remaining surface expression of DC-SIGN as a control for proteolysis. Without shifting to 37°C, trypsin treatment resulted in the loss of both gp120-FITC and surface DC-SIGN. After incubation for 10 min at 37°C, however, a portion of the gp120-FITC signal was protected from proteolysis despite complete loss of surface DC-SIGN expression following treatment. THP DC-SIGN cells that were first fixed with paraformaldehyde or that were continually maintained on ice did not show gp120-FITC protease protection (Figure 2B, and data not shown). A time course showed that DC-SIGN mediated rapid internalization of HIV envelope (Figure 2B).

Following internalization by DC-SIGN, the intensity of the gp120-FITC signal was found to increase with time (Figures 2C and 2D). Since the fluorescence of FITC has been reported to be pH sensitive (Geisow, 1984), we sought to determine if the increase in gp120-FITC fluorescence intensity was due to internalization into a low pH compartment. When the intracellular pH of THP DC-SIGN cells was neutralized by pretreatment of cells with weak bases such as NH₄Cl or chloroquine, the increase

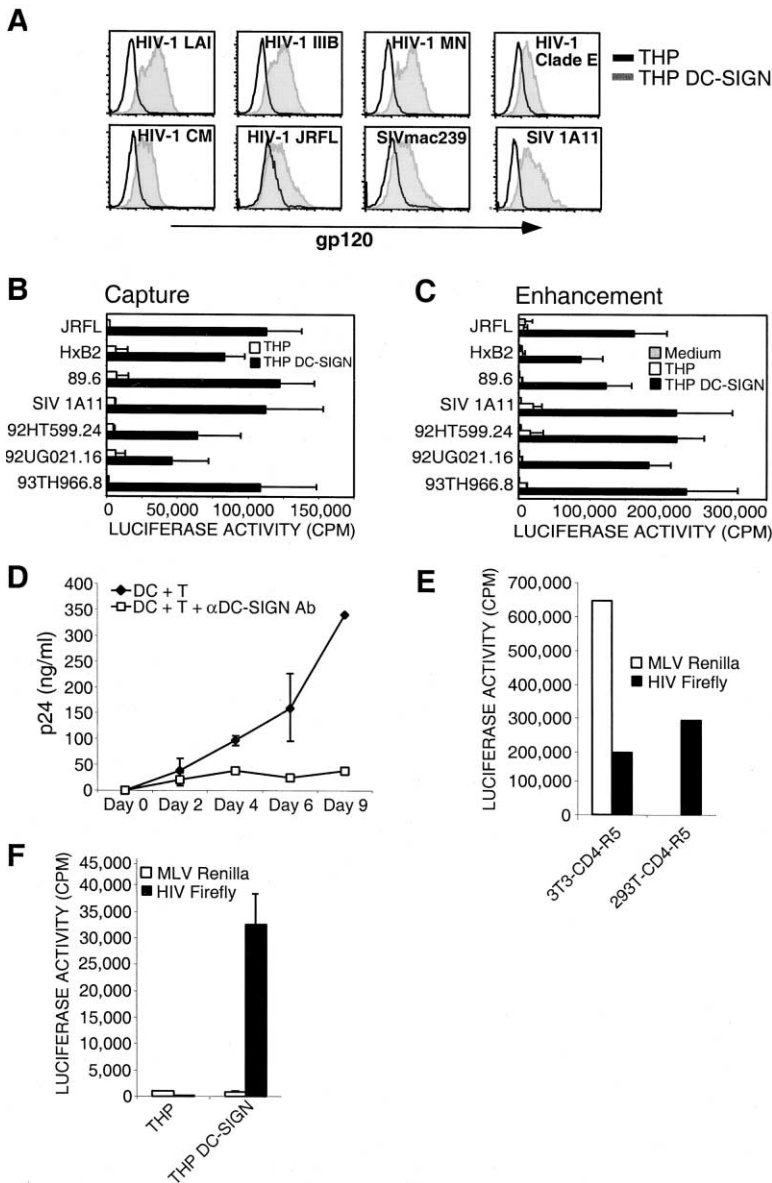


Figure 1. Interaction of Primate Lentivirus but Not MLV Envelope Glycoproteins with DC-SIGN

(A) Binding of recombinant envelope from several strains of HIV and SIV to DC-SIGN. THP or THP DC-SIGN cells were incubated with purified HIV gp120 or SIV gp140 and subsequently stained with pooled human anti-HIV serum or monkey anti-SIV serum, and a fluorescently labeled secondary antibody. Cells were then washed and analyzed for envelope binding by FACS. LAI, IIB, MN, and CM are CXCR4 tropic envelopes, while JRFL and Clade E are CCR5 tropic envelopes.

(B) DC-SIGN can capture R5, X4, and R5X4 strains of HIV and SIV. THP or THP DC-SIGN cells were pulsed with pseudotyped virus encoding luciferase at high multiplicity of infection, washed, and then added to 293T cells expressing the HIV entry receptors CD4, CCR5, and CXCR4. Infection was assayed 2 days later by measuring luciferase activity in the 293T cells. JRFL and 93TH966.8 are R5 strains; HxB2, 92HT599.24, and 92UG021.16 are X4 strains; 89.6 is an R5X4 strain.

(C) DC-SIGN can enhance infection with R5, X4, and R5X4 strains of HIV and SIV at low virus multiplicity of infection. HIV-luciferase-pseudotyped virus was incubated with THP cells, THP DC-SIGN cells, or in medium alone for 2 hr at 37°C. Activated T cells were then added and infection was assayed 2 days later. (D) DC-SIGN expressed on dendritic cells is required for enhancement of infection with replication-competent HIV. Monocyte-derived DC were incubated with replication-competent HIV-1 LAI and then added to activated T cells. For antibody blocking experiments, DC were preincubated with anti-DC-SIGN antibody (25 μ g/ml) for 1 hr prior to addition of virus. Aliquots of the coculture supernatants were taken at the indicated time points and assayed for HIV p24 antigen by ELISA.

(E and F) DC-SIGN cannot bind HIV pseudotyped with MLV envelope glycoprotein. HIV expressing the reporter *Renilla* luciferase was pseudotyped with the MLV envelope and mixed with HIV firefly luciferase pseudotyped with the HIV envelope JRFL. The viral mixture was used to directly infect murine 3T3 cells expressing human CD4, CCR5, and cyclin T1 or 293T-CD4-CCR5 cells (E) or to infect 3T3 cells following capture with THP or THP DC-SIGN cells (F).

in gp120-FITC fluorescence intensity was abolished, even though the kinetics of internalization, as judged by protease protection, was unchanged from untreated cells (Figures 2C and 2D, and data not shown). This suggests that internalization by DC-SIGN results in entry into a low pH compartment.

To verify that DC-SIGN could mediate internalization of whole virus particles in addition to gp120, we examined HIV-pulsed human dendritic cells by fluorescence confocal microscopy. The cells were pulsed with HIV particles on ice and then fluorescently labeled with an anti-HIV serum. The cells were simultaneously pulsed with fluorescently labeled transferrin, which is selectively internalized by the transferrin receptor into early endosomal and perinuclear recycling compartments

(Hopkins, 1983; Hopkins and Trowbridge, 1983; Yamashiro et al., 1984). After binding on ice, the cells were shifted to 37°C for various times and then fixed and permeabilized so that they could be stained for the late endosomal/lysosomal marker LAMP (Chen et al., 1988). Without the shift to 37°C, both transferrin and HIV were at the cell surface (Figure 3A). After a short incubation of 5–15 min at 37°C, most of the HIV fluorescence was colocalized with transferrin in intracellular nonlysosomal compartments. After 45 min, however, much of the HIV was segregated from transferrin and localized in a distinct compartment that is nonlysosomal, as evidenced by the failure to colocalize with LAMP. Even after a 2 hr incubation at 37°C, the HIV did not enter LAMP-positive compartments (data not shown). THP DC-SIGN cells

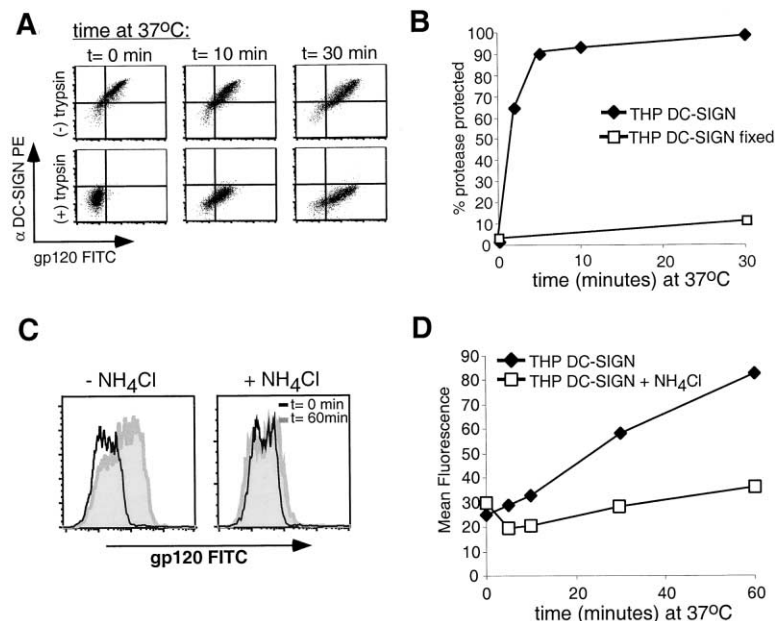


Figure 2. HIV gp120 Is Internalized by DC-SIGN into a Low pH Compartment

(A) Binding to DC-SIGN results in protection of gp120-FITC from trypsin treatment. THP DC-SIGN cells were stained with gp120-FITC on ice, washed, and then shifted to 37°C for the times indicated. Cells were then treated with trypsin or mock treated, fixed, and stained with an anti-DC-SIGN antibody and a PE-labeled secondary antibody for the remaining surface expression of DC-SIGN to verify proteolysis. Cells were then washed thoroughly and analyzed by FACS.

(B) Kinetic analysis of gp120-FITC proteolysis protection. THP DC-SIGN cells were treated as above or were first fixed with 2% paraformaldehyde to assay for gp120-FITC internalization. The percent gp120-FITC protection was calculated by determining the ratio of % FITC-positive cells with trypsin treatment versus % FITC-positive cells upon mock treatment.

(C and D) pH-dependent change in fluorescence of internalized gp120-FITC. gp120-FITC was bound to THP DC-SIGN cells on ice. After washing, the cells were incubated at 37°C for the times indicated in the presence

or absence of NH_4Cl to neutralize intracellular pH. Cells were then proteolyzed to remove surface gp120-FITC and analyzed by FACS. FACS profile (C) and kinetic analysis (D) of change in gp120-FITC fluorescence following shift to 37°C are shown.

were also found to internalize HIV with similar kinetics (data not shown).

Internalized HIV Remains Competent for Infection of a Second Target Cell

We next sought to confirm that internalized virus was still infectious and could be transferred to a second infectable target cell. We therefore pulsed either THP DC-SIGN cells or DC on ice with replication-defective HIV-luciferase pseudotyped with the HIV JRFL envelope glycoprotein (Figure 3B). We then incubated the cells for the indicated times at 37°C, returned them to ice, and pulsed them with an HIV JRFL-pseudotyped virus expressing the reporter GFP. The cells were then treated with trypsin or mock treated, washed, and mixed with 293T target cells expressing human CD4 and CCR5. Without the incubation at 37°C, both the HIV-luciferase virus and the HIV-GFP virus were sensitive to proteolysis. After incubation for 20 min at 37°C, however, the HIV-luciferase virus was protected from proteolysis and could be transmitted to the target cell, while the HIV-GFP remained protease sensitive, thus verifying that the trypsin treatment was effective. A time course of protease protection was also performed (Figure 3C). THP DC-SIGN cells were incubated on ice with an HIV reporter virus expressing firefly luciferase. THP cells expressing a truncated form of DC-SIGN ($\Delta 35$) lacking the cytoplasmic internalization motifs were incubated on ice with HIV expressing the reporter *Renilla* luciferase. Following incubation with virus, both cell lines were washed thoroughly to remove unbound virus and the THP DC-SIGN and THP DC-SIGN $\Delta 35$ cells were mixed. The cells were then incubated at 37°C for the indicated times and mock treated or protease treated, and then added to infectable 293T target cells. After incubation at 37°C, the HIV bound to the THP DC-SIGN cells was

protected from protease, whereas the HIV-*Renilla* luciferase bound to the THP DC-SIGN $\Delta 35$ cells was eliminated (Figure 3C). These results indicate that even after internalization of HIV by DC-SIGN, infectivity is preserved and internalized virus remains competent for transmission to a second target cell.

Soluble DC-SIGN Does Not Mediate Enhancement of Infection

Contact with DC-SIGN alone may be sufficient to mediate conformational changes in envelope glycoprotein that result in enhanced HIV infection. Alternatively, there may be requirements that necessitate that DC-SIGN be expressed on a cell. To test the former possibility, we generated a soluble form of DC-SIGN (sDC-SIGN) by expressing and purifying the entire extracellular domain of the protein. The sDC-SIGN blocked capture of virus by THP DC-SIGN cells (Figure 4A), but did not enhance infectivity when mixed with virus and target cells (Figure 4B). This suggests that the interaction between envelope glycoprotein and DC-SIGN alone is not sufficient for enhancement of HIV infection of T cells, and it is consistent with the notion that DC-SIGN must be expressed on a cell to enhance viral entry.

DC-SIGN Internalization Results in Increased Viral Capture and Is Necessary for *Trans*-Enhancement of HIV Infection

To determine whether DC-SIGN-mediated internalization is required for enhancement of HIV infection, we generated THP cells expressing mutant forms of DC-SIGN in which the cytoplasmic domain of the molecule was either partially ($\Delta 20$) or fully ($\Delta 35$) truncated. The partial truncation removed the 20 amino acids from the N terminus that include the putative dileucine-based internalization motif, while the full truncation removed 35 amino acids that include both the dileucine motif and

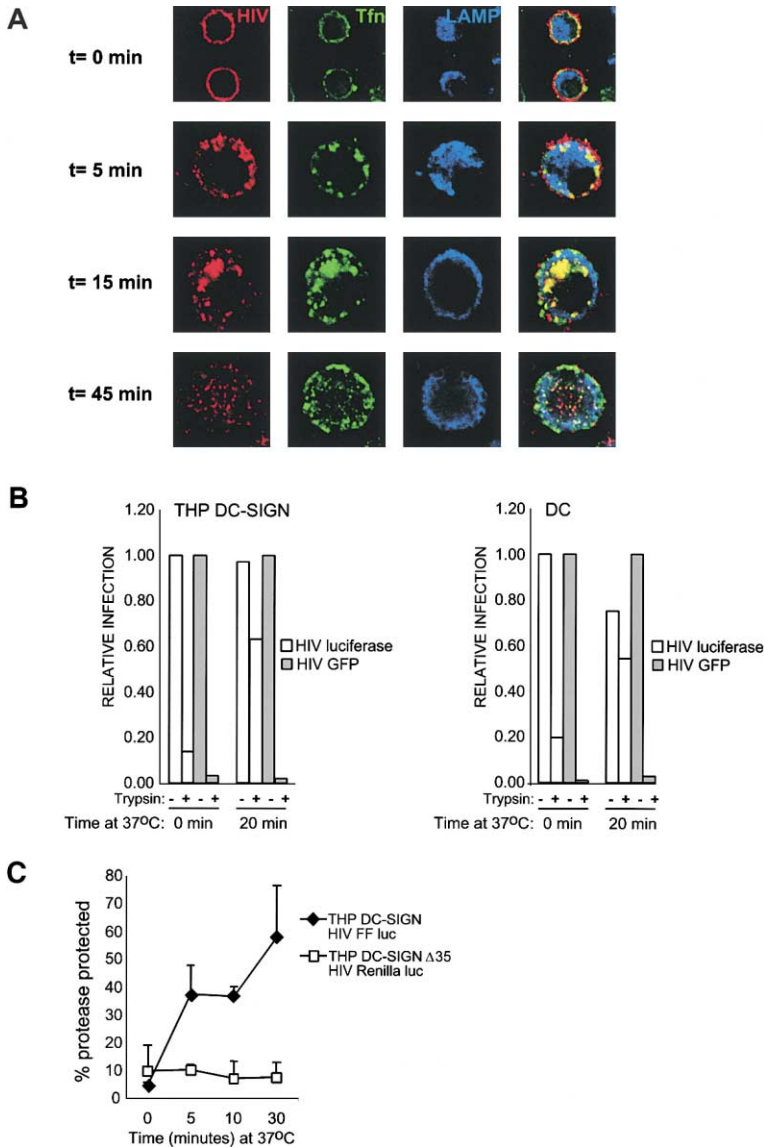


Figure 3. DC-SIGN-Mediated HIV Internalization and Its Effect on Viral Infectivity

(A) Colocalization of internalized HIV with transferrin receptor. Monocyte-derived DC were incubated on ice with Cy3-labeled inactivated HIV and Alexa 633-conjugated transferrin. After incubation at 37°C for the indicated times, the cells were stained intracellularly with an anti-LAMP antibody conjugated to FITC. Cells were visualized using confocal microscopy. Alexa 633 and FITC color assignment were exchanged to allow better visualization.

(B and C) Internalized HIV remains competent for infection of a second target cell. (B) HIV-luciferase (JRFL) was incubated with THP DC-SIGN cells or DC on ice. Cells were then washed and shifted to 37°C for the times indicated, placed back on ice, and incubated with a second pseudotyped virus, HIV-GFP (JRFL). The cells were then treated with trypsin or mock treated and added to 293T-CD4-CCR5 target cells. HIV infection was determined by measuring luciferase activity with a luminometer or GFP expression by FACS. (C) THP cells expressing wild-type or a truncated form of DC-SIGN ($\Delta 35$) were incubated on ice with pseudotyped particles encoding firefly luciferase or *Renilla* luciferase, respectively. The cells were then washed, mixed, and incubated at 37°C for the indicated times. They were then mock treated or treated with protease, and mixed with 293T-CD4-CCR5 target cells. Infection was read out 2 days later by measuring firefly and *Renilla* luciferase signals in cell lysates.

the tyrosine-based motif. THP cells transduced with the mutant and wild-type (wt) DC-SIGN were sorted by flow cytometry to obtain comparable levels of expression (Figure 5A). THP DC-SIGN wt cells were also sorted for high expression.

These cells were used in capture experiments with incubations performed on ice to block internalization and receptor recycling. When cells were pulsed with virus at high MOI, washed extensively with cold buffer, and then added to target cells, the mutant and wild-type cells showed approximately equal levels of viral capture and transmission (Figure 5B). This was expected since the extracellular domain of DC-SIGN was intact on all cells, and at high virus doses, a sufficient number of particles would likely be retained on the surface and could then be transmitted to infectable target cells. When incubation with virus was performed at 37°C, cells expressing wild-type DC-SIGN showed a significant increase in captured infectious virus. Cells expressing DC-SIGN with deleted internalization motifs, how-

ever, showed little increase when incubation was carried out at 37°C. The THP DC-SIGN Hi cell line captured more virus under both conditions relative to the wild-type cell line with lower expression. This suggests that internalization is a mechanism by which DC-SIGN-expressing cells increase viral capture, which was also confirmed by incubating THP DC-SIGN cells with virus, washing, and then lysing the cells and directly measuring the captured p24 HIV antigen by ELISA (Figure 5C). We also found that gp120 internalization by THP cells expressing cytoplasmic truncated DC-SIGN was significantly impaired (data not shown). Despite being able to bind to gp120 with high affinity, THP cells expressing CD4 captured only modest amounts of virus (Figure 5B, and data not shown).

Although THP cells expressing DC-SIGN with cytoplasmic domain truncations could capture virus, they failed to mediate enhancement of T cell infection at low multiplicity of infection (Figure 5D). Even when the wild-type THP DC-SIGN cells were incubated with 5-fold less

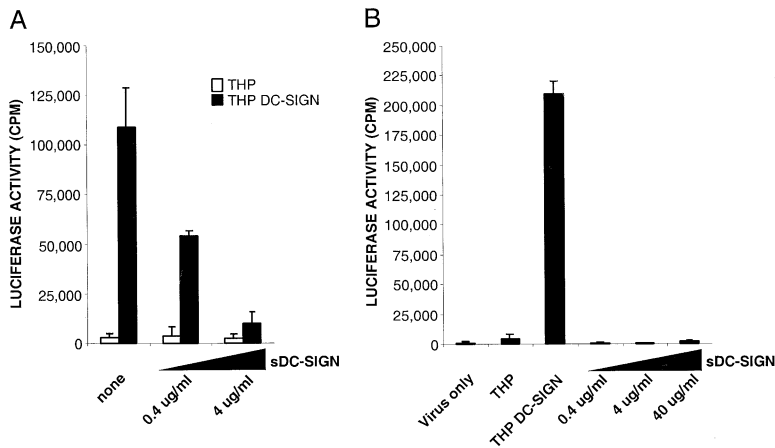


Figure 4. Soluble DC-SIGN Does Not Enhance HIV Infection

(A) sDC-SIGN inhibits capture of HIV by THP-DC-SIGN cells. sDC-SIGN was preincubated with HIV for 30 min before mixing with THP DC-SIGN cells in a capture assay.

(B) Absence of sDC-SIGN enhancement of HIV infection of T cells. Pseudotyped HIV was incubated at 37°C at low MOI with THP DC-SIGN cells or sDC-SIGN alone at the concentrations indicated for 2 hr and then added to activated T cells.

virus, the enhancement of target T cell infection was considerably greater than with cell lines expressing truncated DC-SIGN (data not shown). Thus, internalization appears to be critical for mediating *trans*-enhancement of HIV infection.

Neutralization of Intracellular pH Results in Loss of DC-SIGN-Mediated *Trans*-Enhancement of HIV Infection

Since HIV internalization appears to be critical for enhancement of infection, we surmised that internalization

of HIV into a low pH compartment might be important. We therefore used concanamycin A, a specific inhibitor of vacuolar proton pumps, to neutralize intracellular pH. THP DC-SIGN cells that were pretreated with concanamycin A for 2 hr and for an additional 2 hr during incubation with HIV at high MOI were still able to capture and transmit virus to target cells after washing to remove free viral particles (Figure 6A). In contrast, concanamycin A treatment completely abolished the ability of THP DC-SIGN cells to enhance viral infection of T cells at low viral titer (Figure 6B). Similar results were obtained when

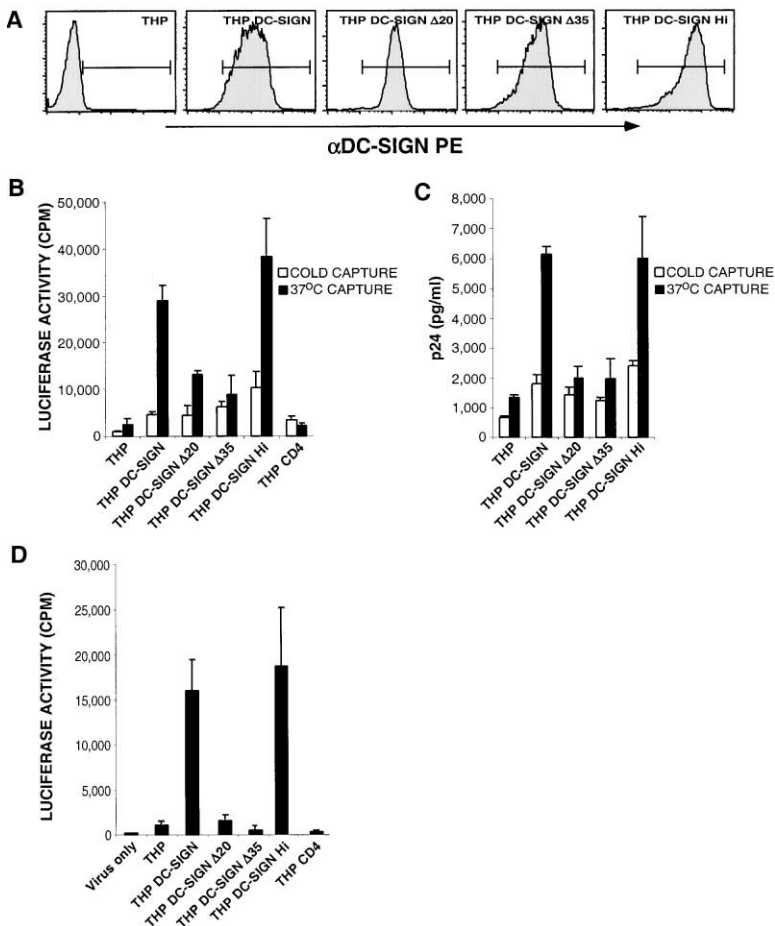


Figure 5. Mutant DC-SIGN Molecules that Lack the Cytoplasmic Domain Have Reduced HIV Internalization and Are Deficient for Enhancement of HIV Infection

(A) FACS analysis of THP cells transduced with wild-type or mutant DC-SIGN molecules lacking the first 20 aa (Δ20) or 35 aa (Δ35) of the cytoplasmic domain.

(B) Reduced capture of HIV at 37°C by cells expressing truncated DC-SIGN. THP cells expressing wt or mutant forms of DC-SIGN were incubated with HIV-luciferase (JRFL) on ice or at 37°C. The cells were then washed extensively and mixed with 293T-CD4-CCR5 target cells and luciferase activity was measured 2 days later.

(C) Reduced HIV particle binding to truncated DC-SIGN at 37°C. THP cells expressing wt or mutant forms of DC-SIGN were incubated with HIV-luciferase (JRFL) on ice or at 37°C. The cells were then washed extensively, lysed, and assayed for HIV p24 antigen by ELISA.

(D) Requirement for DC-SIGN cytoplasmic domain in *trans*-enhancement of infection. Wild-type or mutant THP DC-SIGN cells were incubated with pseudotyped HIV-luciferase (JRFL) at 37°C at low virus concentration and then added to activated T cell targets.

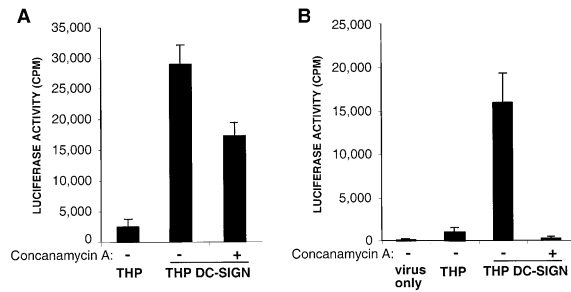


Figure 6. Neutralization of Intracellular pH Results in Loss of DC-SIGN-Mediated Infection Enhancement

(A) Capture of HIV after treatment with concanamycin A. THP DC-SIGN cells were pretreated with 10 nM concanamycin A for 2 hr or mock treated, and then pulsed with HIV-luciferase (JRFL) at high MOI for 2 hr in the continued presence of drug. Cells were then washed extensively and treated cells were resuspended in medium-containing drug. Concanamycin was diluted out upon addition to 293T-CD4-CCR5 cells. Luciferase was measured 2 days later. (B) Abrogation of *trans*-enhancement of HIV infection by treatment with concanamycin A. THP DC-SIGN cells were pretreated with 10 nM concanamycin A for 2 hr before the addition of HIV-luciferase (JRFL) at low MOI. After 2 additional hr, cell/virus was added to activated T cells in a large volume to dilute the concanamycin, and infection was read 2 days later.

intracellular pH was neutralized by the weak bases NH_4Cl or chloroquine (data not shown).

Discussion

We have described a novel mechanism by which DC-SIGN mediates internalization of HIV particles into a nonlysosomal intracellular compartment that appears to be critical for DC-SIGN enhancement of HIV infection. This activity occurs with a broad range of primate lentiviruses. Previous studies have proposed that DC may be responsible for the preferential transmission of R5 tropic viruses following initial exposure of the host. The observation that infection by R5, X4, and R5X4 viruses can all be enhanced by DC-SIGN suggests that this molecule is not responsible for the observed bottleneck in transmission. Although DC-SIGN shows broad specificity among primate lentiviruses, it cannot bind to HIV particles pseudotyped with the retroviral ecotropic MLV envelope. The inability to bind to MLV may be due to the reduced level of MLV gp70 glycosylation compared to that of HIV gp120, different orientation of the sugars, or lack of amino acid sequences that may contribute to specificity. Overall, however, it suggests that primate lentiviruses have uniquely adapted to coopt DC-SIGN on dendritic cells.

Following the interaction of HIV with DC-SIGN, the virus is rapidly internalized. Virions remain infectious even after internalization into a low pH compartment. Previous studies have demonstrated that even early endosomal/recycling compartments are mildly acidic, with a pH estimated to be between 6.0 and 6.8 (reviewed in Mellman, 1996). The pH of late endosomal/lysosomal compartments is significantly lower at 5.0–5.5. The vast majority of internalized HIV does not enter late endosomal or lysosomal compartments, as demonstrated by the failure to colocalize with LAMP, even at late time points. Additionally, the low pH of these degradative

compartments has been shown to result in rapid loss of viral infectivity (Kempf et al., 1991). Exposure to the moderate pH of early endosomal/recycling compartments, however, does not significantly reduce viral infectivity (data not shown).

Internalization appears to be critical for the enhancement of viral infectivity since mutant DC-SIGN molecules that lack internalization signals can no longer enhance. However, cells expressing the cytoplasmic truncated forms of DC-SIGN are still capable of binding and transmitting virus to a second target cell at high viral MOI. This suggests that the observed enhancement is not simply due to effective increases in the local concentration of virus due to adsorption of virus on DC-SIGN-expressing cells. In addition, the observation that neither soluble DC-SIGN nor the THP DC-SIGN $\Delta 35$ mutant can mediate infection enhancement argues that contact between DC-SIGN and HIV alone is unlikely to result in changes (e.g., conformational) in viral proteins that lead to increased infectivity or stability.

Intracellular pH appears to be critical for DC-SIGN-mediated enhancement since treatment of DC-SIGN-expressing cells with lysosomotropic agents results in a loss of *trans*-enhancement. HIV is known to rapidly lose infectivity at 37°C, and we have shown that HIV bound to THP DC-SIGN cells can retain the infectivity of virus for several days (Geijtenbeek et al., 2000a). Virus bound to THP cells expressing a cytoplasmic truncated form of DC-SIGN, however, cannot retain viral infectivity (data not shown). Therefore, it may be that internalization into a mildly acidic compartment results in stabilization of virus. It has been reported that follicular dendritic cells (FDC) can protect immune complexes from degradation and retain antigen in native conformation for several months (Mandel et al., 1980; Tew et al., 1980). In addition, FDC have recently been described to retain infectious HIV (Smith et al., 2001). This was demonstrated by injecting mice with HIV in the footpad and recovering infectious virus 9 months later in draining lymph nodes. Since HIV cannot replicate in the mouse, it suggests that the infectivity of the original virus was maintained. The retention of HIV infectivity by FDC may involve internalization (Tacchetti et al., 1997). One possibility is that HIV is internalized by immature DC into an intracellular compartment that has similar functions, such as maintaining the native conformation of HIV envelope glycoprotein and protecting the virus from degradation. Preservation of viral infectivity may require low pH exposure, although this alone does not appear to be sufficient for enhanced infectivity (data not shown). Other enveloped animal viruses such as influenza and Semliki Forest virus have been demonstrated to have pH-induced changes that significantly increase viral stability (Bullough et al., 1994; Fuller et al., 1995; Hernandez et al., 1996; Ruigrok et al., 1988). Although low pH exposure is not required for HIV infection, it is possible that such exposure in an intracellular compartment within the DC results in conformational changes that preserve or increase the infectivity of virus. It has been reported that low pH exposure may prime gp41 for fusion (Fackler and Peterlin, 2000). This is consistent with the observation that use of lysosomotropic agents that neutralize intracellular pH abrogate DC-SIGN-mediated enhancement of T cell infection.

We propose a model in which DC located at mucosal

sites bind and internalize intact HIV particles, which can then be transported to secondary lymphoid tissue by migrating DC. This internalization increases the amount of virus that can be retained by DC-SIGN-expressing cells, which may be critical *in vivo* since it would be expected to lead to a commensurate increase in the amount of virus that can be transported by DC from peripheral mucosal sites, where virus amounts are likely to be low, to secondary lymphoid tissue. Internalization may also result in changes in the viral particles that are dependent upon low pH exposure and that may result in the retention and/or augmentation of viral infectivity.

Although internalization of HIV may be important for the observed increase in viral infectivity, several important questions remain. It is still unclear whether HIV virions continue to associate with DC-SIGN after internalization and whether DC-SIGN mediates the return of virus to the surface. Since DC-SIGN-mediated enhancement of infection is observed with nonreplication-competent pseudotyped virus, it is unlikely that the viral membrane fuses with the DC-SIGN-expressing cell membrane. This means that the virus must recycle back to the surface so that it can contact entry receptors on the target cell. Whether this recycling of virus is constitutive or regulated remains to be determined. The exact nature of the changes that occur upon internalization is also unclear. Internalization may function simply to concentrate virus for more efficient transfer to the target, or the virus may undergo covalent modifications or conformational changes that result in higher viral fusogenicity or increased stability. Additionally, the importance of potential contacts made between DC-SIGN and molecules present on the T cell remains to be determined. These contacts may potentially increase the efficiency of viral transfer from the DC-SIGN-expressing cell to the target cell, or they may alter the intrinsic susceptibility of the T cell to infection. The novel mechanism of specific receptor-mediated HIV endocytosis described here may be critical to our understanding of *in vivo* HIV transmission and pathogenesis.

Experimental Procedures

Cells

Immature DC were generated and cultured as described previously (Geijtenbeek et al., 2000b). Mononuclear cells were purified from buffy coats using Ficoll (Amersham Pharmacia, Piscataway, NJ) and then adhered to plastic for 2 hr. Adherent cells were washed extensively and then cultured in RPMI (Life Technologies, Rockville, MD), 2% autologous serum, penicillin (50 U/ml), streptomycin (50 U/ml), and stimulated with GM-CSF (800 U/ml; Immunex, Seattle, WA) and IL-4 (500 U/ml; R&D Systems, Minneapolis, MN) for 7 days. Mutant and wild-type THP DC-SIGN cells were generated by packaging pMX DC-SIGN into a retrovirus pseudotyped with the VSV-G envelope protein (Deng et al., 1997; Emi et al., 1991). THP cells were then infected, stained with the anti-DC-SIGN antibody 16d5 (a kind gift of Y. van Kooyk) (Geijtenbeek et al., 2000b), and sorted for expression of DC-SIGN. THP cells were grown in RPMI supplemented with 10% fetal calf serum (FCS), penicillin (50 U/ml), streptomycin (50 U/ml), and L-glutamine (2 mM).

Pseudotyped Virus Production

Pseudotyped viruses were produced as described previously (Laudau et al., 1991). Briefly, 293T cells were cotransfected with viral envelope expression vectors and envelope-deleted HIV NL4-3 encoding the reporter firefly luciferase, *Renilla* luciferase, or GFP cloned into the Nef open reading frame. Cell supernatants were

harvested 48 hr posttransfection, filtered, and frozen in aliquots at -120°C . Virus was titered by infecting 293T-CD4-CCR5 cells and measuring luciferase activity in cell lysates 48 hr postinfection using a luciferase reporter assay kit (Promega, Madison, WI). Low titer infections were performed with virus dilutions that were at the low limit of detection in purified activated T cell targets. High titer infections utilized virus at concentrations 10- to 100-fold higher.

gp120 Binding Assay

THP DC-SIGN cells were incubated on ice with 50 nM of purified HIV gp120 or SIV gp140 (obtained from the NIH AIDS Research and Reference Reagent Program) for 1 hr in staining buffer (phosphate buffered saline [PBS] with 2% FCS, 1 mM CaCl_2 , and 1 mM MgCl_2). All subsequent washes and antibody dilutions were performed with cold staining buffer. Cells were washed and incubated with pooled serum from HIV-positive patients (for gp120 stains; a kind gift of J. Moore) or monkey anti-SIV serum (for gp140 stains; a kind gift of J. Moore) on ice, washed, and labeled with goat anti-human IgG secondary antibody conjugated to phycoerythrin (PE) (R&D Systems). After a final wash, cells were analyzed by FACS.

DC-SIGN Capture, Particle Binding, and Infection Enhancement

Capture and enhancement of infection by DC-SIGN-expressing cells has been described previously (Geijtenbeek et al., 2000a). Briefly, 5×10^4 THP or THP DC-SIGN cells were incubated with HIV for 2 hr at 37°C or on ice as indicated. For capture experiments, cells were washed thoroughly in medium and then added to an equal number of 293T cells expressing CD4 and CCR5 or 3T3 cells expressing CD4, CCR5, and human Cyclin T1 in a 24-well plate. To assay HIV particle binding by DC-SIGN, THP or THP DC-SIGN cells were incubated with HIV for 2 hr at 37°C or on ice. Cells were then washed thoroughly, pelleted, lysed, and assayed for HIV p24 antigen by ELISA (Coulter, Miami, FL). Enhancement experiments were performed by incubating 5×10^4 THP, THP DC-SIGN cells, or DC with HIV for 2 hr at 37°C . HIV/cells were then directly added to an equal number of T cells that were stimulated with IL-2 (10 U/ml; R&D Systems) and PHA (10 $\mu\text{g}/\text{ml}$; Sigma, St. Louis, MO) for 2 days. For experiments performed with HIV-luciferase-pseudotyped virus, cells were harvested 48 hr postinfection and luciferase activity was measured using a luciferase reporter assay kit (for reading of firefly luciferase; Promega, Madison, WI) or a dual luciferase reporter assay kit (for reading of firefly and *Renilla* luciferase; Promega) and an Anthos Lucy1 microplate luminometer (Labtech International, Salzburg, Austria). For enhancement experiments performed with replication-competent HIV, aliquots of coculture supernatants were taken at the indicated time points and assayed for p24 antigen by ELISA (Coulter). In experiments using concanamycin A, THP DC-SIGN cells were pretreated with medium containing 10 nM concanamycin A (Sigma) for 2 hr at 37°C . Cells were then incubated with virus in medium containing drug for 2 hr at 37°C and then added to target cells in a large volume to dilute concanamycin A at least 10-fold.

Protease Protection Experiments

DC-SIGN-expressing cells were incubated with pseudotyped viruses or gp120-FITC in medium on ice for 1 hr. Cells were then washed extensively with serum-free medium, resuspended in serum-free medium, and shifted to 37°C for various times. Cells were mock treated with serum-free medium or treated with 0.05% trypsin (for cells pulsed with pseudotyped virus; Life Technologies) or 0.05% trypsin and 0.53 mM EDTA (for cells pulsed with gp120-FITC; Life Technologies) for 5 min. Proteolysis was stopped by adding an equal volume of medium containing 20% FCS. For gp120-FITC protection experiments, cells were then stained for surface expression of DC-SIGN using the mouse monoclonal antibody 16d5, followed by a secondary goat anti-mouse IgG antibody conjugated to PE (R&D Systems). In gp120-FITC protection experiments using NH_4Cl , cells were pretreated for 1 hr at 37°C in medium with 50 mM NH_4Cl and maintained in medium with drug during incubation with gp120. Following washing, cells were resuspended in medium with NH_4Cl until protease treatment. For protection experiments with HIV pseudotypes, cells were added to 293T targets and luciferase activity was assayed 48 hr postinfection.

Confocal Microscopy

DC-SIGN-expressing cells were adhered to polylysine (Sigma)-treated glass slides for 45 min in PBS at room temperature. Slides were then incubated with Aldrithiol-2-inactivated HIV particles (a kind gift of J. Lifson) for 1 hr on ice, washed, and then stained for bound HIV using pooled human serum from HIV-positive patients, followed by a donkey anti-human IgG secondary antibody conjugated to Cy3 (Molecular Probes, Eugene, OR). Cells were washed, placed in medium containing 10 μ g/ml transferrin conjugated to Alexa 633 (Molecular Probes), and shifted to 37°C for the indicated times. After incubation, cells were washed again, fixed with 4% paraformaldehyde, and permeabilized with PBS, 0.05% saponin, 0.2% BSA. Cells were then stained with a mouse anti-LAMP antibody directly conjugated to FITC (BD PharMingen, San Diego, CA). After a final wash, cells were imaged with a Zeiss LSM-510 laser scanning confocal microscope.

Soluble DC-SIGN

Soluble DC-SIGN (sDC-SIGN) was made by bacterial expression of the extracellular domain of DC-SIGN linked to an N-terminal His tag to allow rapid purification. Protein was purified to >95% purity using a nickel-charged Hi-Trap chelating column (Amersham Pharmacia), followed by a Hi-Trap Q-ion exchange column (Amersham Pharmacia), and a final Superdex 200 26/60 size exclusion column (Amersham Pharmacia). sDC-SIGN was incubated with luciferase-pseudotyped HIV at the concentrations indicated in 50 μ l DMEM supplemented with 10% FCS for 30 min at 37°C. For competition assays, virus and sDC-SIGN were then added to 5×10^4 THP or THP DC-SIGN cells in 50 μ l of medium and incubated for an additional 2 hr at 37°C. Cells were washed extensively with medium and added to 5×10^4 293T-CD4-CCR5. For enhancement assays, HIV was incubated with sDC-SIGN at the indicated concentrations for 2 hr at 37°C before the addition to 5×10^4 activated T cells. Luciferase activity was measured 48 hr postinfection.

Acknowledgments

The authors are grateful to Y.R. Zou, F. Geissmann, J.M. Binley, and S. Bolland for helpful discussions. We would also like to thank J. Lifson for providing inactivated HIV virions, J. P. Moore for providing pooled human and monkey serum, and J. Hirst for assistance with FACS sorting. This work is supported by grants from the National Institutes of Health to D.R.L. N.B. is supported by a fellowship from the Cancer Research Institute. D.R.L. and W.A.H. are investigators of the Howard Hughes Medical Institute.

Received September 9, 2001; revised November 30, 2001.

References

Alkhatib, G., Combadiere, C., Broder, C.C., Feng, Y., Kennedy, P.E., Murphy, P.M., and Berger, E.A. (1996). CC CKR5: a RANTES, MIP-1 α , MIP-1 β receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* 272, 1955–1958.

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y.J., Pulendran, B., and Palucka, K. (2000). Immunobiology of dendritic cells. *Annu. Rev. Immunol.* 18, 767–811.

Bullough, P.A., Hughson, F.M., Skehel, J.J., and Wiley, D.C. (1994). Structure of influenza haemagglutinin at the pH of membrane fusion. *Nature* 371, 37–43.

Cameron, P.U., Freudenthal, P.S., Barker, J.M., Gezelter, S., Inaba, K., and Steinman, R.M. (1992). Dendritic cells exposed to human immunodeficiency virus type-1 transmit a vigorous cytopathic infection to CD4 $^+$ T cells. *Science* 257, 383–387.

Chen, J.W., Cha, Y., Yuksel, K.U., Gracy, R.W., and August, J.T. (1988). Isolation and sequencing of a cDNA clone encoding lysosomal membrane glycoprotein mouse LAMP-1. Sequence similarity to proteins bearing onco-differentiation antigens. *J. Biol. Chem.* 263, 8754–8758.

Choe, H., Farzan, M., Sun, Y., Sullivan, N., Rollins, B., Ponath, P.D., Wu, L., Mackay, C.R., LaRosa, G., Newman, W., et al. (1996). The

beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* 85, 1135–1148.

Connor, R.I., Sheridan, K.E., Ceradini, D., Choe, S., and Landau, N.R. (1997). Change in coreceptor use correlates with disease progression in HIV-1-infected individuals. *J. Exp. Med.* 185, 621–628.

Cornelissen, M., Mulder-Kampinga, G., Veenstra, J., Zorgdrager, F., Kuiken, C., Hartman, S., Dekker, J., van der Hoek, L., Sol, C., Coutinho, R., et al. (1995). Syncytium-inducing (SI) phenotype suppression at seroconversion after intramuscular inoculation of a non-syncytium-inducing/SI phenotypically mixed human immunodeficiency virus population. *J. Virol.* 69, 1810–1818.

Curtis, B.M., Scharnowske, S., and Watson, A.J. (1992). Sequence and expression of a membrane-associated C-type lectin that exhibits CD4-independent binding of human immunodeficiency virus envelope glycoprotein gp120. *Proc. Natl. Acad. Sci. USA* 89, 8356–8360.

Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Di Marzio, P., Marmon, S., Sutton, R.E., Hill, C.M., et al. (1996). Identification of a major co-receptor for primary isolates of HIV-1. *Nature* 381, 661–666.

Deng, H.K., Unutmaz, D., KewalRamani, V.N., and Littman, D.R. (1997). Expression cloning of new receptors used by simian and human immunodeficiency viruses. *Nature* 388, 296–300.

Dragic, T., Litwin, V., Allaway, G.P., Martin, S.R., Huang, Y., Nagashima, K.A., Cayanan, C., Maddon, P.J., Koup, R.A., Moore, J.P., et al. (1996). HIV-1 entry into CD4 $^+$ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* 381, 667–673.

Emi, N., Friedmann, T., and Yee, J.K. (1991). Pseudotype formation of murine leukemia virus with the G protein of vesicular stomatitis virus. *J. Virol.* 65, 1202–1207.

Fackler, O.T., and Peterlin, B.M. (2000). Endocytic entry of HIV. *Curr. Biol.* 10, 1005–1008.

Feng, Y., Broder, C.C., Kennedy, P.E., and Berger, E.A. (1996). HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* 272, 872–877.

Fuller, S.D., Berriman, J.A., Butcher, S.J., and Gowen, B.E. (1995). Low pH induces swiveling of the glycoprotein heterodimers in the Semliki Forest virus spike complex. *Cell* 81, 715–725.

Geijtenbeek, T.B., Kwon, D.S., Torensma, R., van Vliet, S.J., van Duinhoven, G.C., Middel, J., Cornelissen, I.L., Nottet, H.S., Kewal-Ramani, V.N., Littman, D.R., et al. (2000a). DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell* 100, 587–97.

Geijtenbeek, T.B., Torensma, R., van Vliet, S.J., van Duinhoven, G.C., Adema, G.J., van Kooyk, Y., and Figdor, C.G. (2000b). Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell* 100, 575–585.

Geisow, M.J. (1984). Fluorescein conjugates as indicators of subcellular pH. A critical evaluation. *Exp. Cell Res.* 150, 29–35.

Granelli-Piperno, A., Finkel, V., Delgado, E., and Steinman, R.M. (1999). Virus replication begins in dendritic cells during the transmission of HIV-1 from mature dendritic cells to T cells. *Curr. Biol.* 9, 21–29.

Hernandez, L.D., Hoffman, L.R., Wolfsberg, T.G., and White, J.M. (1996). Virus-cell and cell-cell fusion. *Annu. Rev. Cell Dev. Biol.* 12, 627–661.

Hladik, F., Lentz, G., Akridge, R.E., Peterson, G., Kelley, H., McElroy, A., and McElrath, M.J. (1999). Dendritic cell-T-cell interactions support coreceptor-independent human immunodeficiency virus type 1 transmission in the human genital tract. *J. Virol.* 73, 5833–5842.

Hopkins, C.R. (1983). Intracellular routing of transferrin and transferrin receptors in epidermoid carcinoma A431 cells. *Cell* 35, 321–330.

Hopkins, C.R., and Trowbridge, I.S. (1983). Internalization and processing of transferrin and the transferrin receptor in human carcinoma A431 cells. *J. Cell Biol.* 97, 508–521.

Huang, Y., Paxton, W.A., Wolinsky, S.M., Neumann, A.U., Zhang, L., He, T., Kang, S., Ceradini, D., Jin, Z., Yazdanbakhsh, K., et al. (1996).

- The role of a mutant CCR5 allele in HIV-1 transmission and disease progression. *Nat. Med.* 2, 1240–1243.
- Kempf, C., Jentsch, P., Barre-Sinoussi, F.B., Poirier, B., Morgenthaler, J.J., Morell, A., and Germann, D. (1991). Inactivation of human immunodeficiency virus (HIV) by low pH and pepsin. *J. Acquir. Immune Defic. Syndr.* 4, 828–830.
- Landau, N.R., Page, K.A., and Littman, D.R. (1991). Pseudotyping with human T-cell leukemia virus type I broadens the human immunodeficiency virus host range. *J. Virol.* 65, 162–169.
- Lapham, C.K., Ouyang, J., Chandrasekhar, B., Nguyen, N.Y., Dimitrov, D.S., and Golding, H. (1996). Evidence for cell-surface association between fusin and the CD4-gp120 complex in human cell lines. *Science* 274, 602–605.
- Mandel, T.E., Phipps, R.P., Abbot, A., and Tew, J.G. (1980). The follicular dendritic cell: long term antigen retention during immunity. *Immunol. Rev.* 53, 29–59.
- Mellman, I. (1996). Endocytosis and molecular sorting. *Annu. Rev. Cell Dev. Biol.* 12, 575–625.
- Pope, M., Gezelter, S., Gallo, N., Hoffman, L., and Steinman, R.M. (1995). Low levels of HIV-1 infection in cutaneous dendritic cells promote extensive viral replication upon binding to memory CD4+ T cells. *J. Exp. Med.* 182, 2045–2056.
- Rowland-Jones, S.L. (1999). HIV: the deadly passenger in dendritic cells. *Curr. Biol.* 9, R248–R250.
- Ruigrok, R.W., Aitken, A., Calder, L.J., Martin, S.R., Skehel, J.J., Wharton, S.A., Weis, W., and Wiley, D.C. (1988). Studies on the structure of the influenza virus haemagglutinin at the pH of membrane fusion. *J. Gen. Virol.* 69, 2785–2795.
- Samson, M., Libert, F., Doranz, B.J., Rucker, J., Liesnard, C., Farber, C.M., Saragosti, S., Lapoumeroulie, C., Cognaux, J., Forcille, C., et al. (1996). Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature* 382, 722–725.
- Schuitemaker, H., Koot, M., Kootstra, N.A., Dercksen, M.W., de Goede, R.E., van Steenwijk, R.P., Lange, J.M., Schattenkerk, J.K., Miedema, F., and Tersmette, M. (1992). Biological phenotype of human immunodeficiency virus type 1 clones at different stages of infection: progression of disease is associated with a shift from monocytotropic to T-cell-tropic virus population. *J. Virol.* 66, 1354–1360.
- Simmons, G., Reeves, J.D., Hibbits, S., Stine, J.T., Gray, P.W., Proudfoot, A.E., and Clapham, P.R. (2000). Co-receptor use by HIV and inhibition of HIV infection by chemokine receptor ligands. *Immunol. Rev.* 177, 112–126.
- Smith, B.A., Gartner, S., Liu, Y., Perelson, A.S., Stilianakis, N.I., Keele, B.F., Kerkering, T.M., Ferreira-Gonzalez, A., Szakal, A.K., Tew, J.G., et al. (2001). Persistence of infectious HIV on follicular dendritic cells. *J. Immunol.* 166, 690–696.
- Steinman, R.M., Grenelli-Piperno, A., Tenner-Racz, K., Racz, P., Frankel, S., Delgado, E., Ignatius, R., and Pope, M. (1999). *Dendritic Cells: Biology and Clinical Applications* (New York: Academic Press).
- Tacchetti, C., Favre, A., Moresco, L., Meszaros, P., Luzzi, P., Truini, M., Rizzo, F., Grossi, C.E., and Ciccone, E. (1997). HIV is trapped and masked in the cytoplasm of lymph node follicular dendritic cells. *Am. J. Pathol.* 150, 533–542.
- Tew, J.G., Phipps, R.P., and Mandel, T.E. (1980). The maintenance and regulation of the humoral immune response: persisting antigen and the role of follicular antigen-binding dendritic cells as accessory cells. *Immunol. Rev.* 53, 175–201.
- Thery, C., and Amigorena, S. (2001). The cell biology of antigen presentation in dendritic cells. *Curr. Opin. Immunol.* 13, 45–51.
- Trkola, A., Dragic, T., Arthos, J., Binley, J.M., Olson, W.C., Allaway, G.P., Cheng-Mayer, C., Robinson, J., Maddon, P.J., and Moore, J.P. (1996). CD4-dependent, antibody-sensitive interactions between HIV-1 and its co-receptor CCR-5. *Nature* 384, 184–187.
- Trowbridge, I.S., Collawn, J.F., and Hopkins, C.R. (1993). Signal-dependent membrane protein trafficking in the endocytic pathway. *Annu. Rev. Cell Biol.* 9, 129–161.
- van't Wout, A.B., Kootstra, N.A., Mulder-Kampinga, G.A., Albrecht-van Lent, N., Scherpbier, H.J., Veenstra, J., Boer, K., Coutinho, R.A., Miedema, F., and Schuitemaker, H. (1994). Macrophage-tropic variants initiate human immunodeficiency virus type 1 infection after sexual, parenteral, and vertical transmission. *J. Clin. Invest.* 94, 2060–2067.
- Wu, L., Gerard, N.P., Wyatt, R., Choe, H., Parolin, C., Ruffing, N., Borsetti, A., Cardoso, A.A., Desjardin, E., Newman, W., et al. (1996). CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5. *Nature* 384, 179–183.
- Yamashiro, D.J., Tycko, B., Fluss, S.R., and Maxfield, F.R. (1984). Segregation of transferrin to a mildly acidic (pH 6.5) para-Golgi compartment in the recycling pathway. *Cell* 37, 789–800.