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Inhibition of Human Immunodeficiency Virus Type 1 Env-Mediated Fusion by DC-SIGN

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DC-SIGN, a lectin expressed on dendritic cell and macrophage subsets, binds to human immunodeficiency virus Env glycoproteins, allowing capture of viral particles. Captured virions either infect target cells or are efficiently transmitted to lymphocytes. Cellular mechanisms underlying the effects of DC-SIGN remain poorly understood. Here we have analyzed the effects of DC-SIGN on viral entry and on syncytium formation induced by Env glycoproteins. The lectin enhanced susceptibility to viral infection and dramatically increased virion internalization. Captured virions accumulated in the vesicular pathway, and their access to the cytosol was altered. Strikingly, the presence of DC-SIGN on target cells inhibited their ability to form syncytia with Env-expressing cells. However, increasing CD4 surface levels on target cells alleviated this inhibitory effect of DC-SIGN. Moreover, the potency of the viral fusion inhibitor T-20 was not affected in DC-SIGN-expressing cells. Altogether, our results indicate that DC-SIGN exerts subtle and complex effects during early steps of HIV type 1 replication. DC-SIGN facilitates capture and accumulation of viral particles in a vesicular compartment and inhibits viral fusion. Competition between CD4 and DC-SIGN for Env binding likely affects virus access to the cytosol and syncytium formation.

Human immunodeficiency virus type 1 (HIV-1) infects human $CD4^+$ T cells, macrophages, and dendritic cells (DCs) via interactions between the viral envelope glycoprotein gp120/gp41, the CD4 molecule, and a chemokine receptor. Other surface molecules are involved in viral capture and transmission. In DCs, a variety of receptors and lectins can efficiently bind HIV-1 virions (34). Among them, DC-SIGN, a recently identified C-type lectin, is believed to play a peculiar role during virus transmission (12, 13). HIV-1 dissemination within an individual necessitates the transfer of virus from mucosal surfaces of entry to lymph nodes, where the virus actively replicates in $CD4^+$ T lymphocytes. Immature DCs residing in the skin and mucosa are thought to be the first cells targeted by HIV-1 during sexual transmission. DC migration from the periphery and recruitment of T cells within lymphoid tissues is likely exploited by HIV-1 to ensure its propagation (4, 9). HIV-1 replicates rather inefficiently in DC cultures. DCs express low levels of CD4 and coreceptor CCR5 or CXCR4. R5-tropic, but not X4-tropic, HIV-1 strains replicate in immature DCs (15, 27). However, both R5- and X4-tropic HIV-1 readily bind and enter DCs (9, 16).

DC-SIGN (or CD209) is a type II transmembrane protein with an external C-type (Ca^{2+} dependent) mannose-binding domain, expressed at the surface of DC and macrophage subsets (8, 13, 21, 30). High surface levels of the lectin are detected on immature monocyte-derived DCs, with more than 10^5 copies per cell (3). The known cellular ligands of DC-SIGN are intercellular adhesion molecule 2 (ICAM-2) and ICAM-3. DC-SIGN–ICAM-2 interaction regulates DC transmigration across the vascular and lymphoid endothelium (11). DC-SIGN binding to ICAM-3 mediates loose adhesion between DCs and T cells (13). This early contact may enable the T-cell receptor to scan for processed antigens, allowing the initiation of primary immune responses. Besides ICAM ligands, DC-SIGN interacts with various pathogens. The lectin efficiently binds glycoproteins of leishmania and envelope glycoproteins of Ebola virus and cytomegalovirus and of the lentiviruses HIV and simian immunodeficiency virus (1, 5, 12, 17, 28). DC-SIGN-expressing cells retain attached lentiviruses in an infectious state for several days and transmit them to lymphocytes, with a striking enhancement of infection efficiency (13). These observations led to a model in which virus is captured by DCs through DC-SIGN binding, allowing efficient *trans* infection of T lymphocytes. Furthermore, we recently reported that DC-SIGN surface levels are upregulated in HIV-infected cells (31). This process is caused by the viral protein Nef, which acts by inhibiting DC-SIGN endocytosis. Upregulation of DC-SIGN at the cell surface dramatically increases clustering of DCs with T lymphocytes and HIV-1 transmission, providing a mean for the virus to facilitate its spread in the organism.

However, the scenario is more complex than initially thought. gp120 has the potential to bind to various receptors, including CD4 and several types of lectin receptors, not exclusively DC-SIGN (33–35). Other lectins include the mannose receptor and langerin, a molecule expressed in Langerhans cells, which may also play a role in virus transmission, especially in DC subsets that do not express DC-SIGN (34). Accordingly, incubation with cocktails of anti-DC-SIGN monoclonal antibodies (MAbs) and with mannan, which both efficiently block virus transmission from $DC\text{-}SIGN^+$ cell lines, is much less potent in primary DCs (3). Furthermore, though rhesus macaque DCs express a homolog of human DC-SIGN, they transmit primate lentivirus independently of this lectin (37). Therefore, although DC-SIGN is important for viral cap-

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ture and transmission from DCs to lymphocytes, there may be additional mechanisms used by the virus in this process.

Regarding DC-SIGN, the cellular mechanisms involved in HIV-1 capture and transmission are only partly understood. DC-SIGN mediates rapid internalization of virions into an acidic compartment, where they retain and even enhance their competence to infect target cells in *trans* (20). Removal of the cytoplasmic tail of the lectin abolishes *trans* enhancement of infection (20). A dileucine (LL) sequence and a tyrosine-based sequence present in this cytoplasmic tail are potential recognition motifs for the cell-sorting machinery. Removal of the LL sequence inhibits antibody-induced DC-SIGN endocytosis and induces accumulation of the lectin at the cell surface (31). However, an LL mutant of DC-SIGN remains capable of transmitting infection (31). This suggests that distinct mechanisms govern the process of virus transmission and of *trans* enhancement of infection: an anchor of viral particles at the cell surface may be sufficient for transmission, whereas enhancement of infection would require endocytosis. In addition to mediating virus transfer to lymphocytes, DC-SIGN also exerts a *cis*-enhancing effect on HIV-1 infection (21). Expression of DC-SIGN on target cells does not alleviate the requirement for CD4 and a coreceptor(s) but increases their susceptibility to infection. Furthermore, DC-SIGN allows infection of cells with otherwise limiting concentrations of these receptors. It has been suggested that the high affinity of DC-SIGN for the viral envelope glycoproteins ($K_d \approx 1.5$ nM) may serve to anchor the virions and thus to increase their local concentrations in proximity to the cognate receptors (21). However, as for its effect on virus transmission, the precise mechanism by which DC-SIGN enhances infection in *cis* remains poorly characterized.

In this study, our aim was to characterize how DC-SIGN impacts the replicative cycle of HIV-1. We have analyzed the fate of incoming virions in target cells in the presence and in the absence of DC-SIGN. We have designed a variety of HeLa cell derivatives expressing wild-type (WT) or mutant DC-SIGN molecules, with or without CD4 and HIV coreceptor(s). We have performed single-cycle assays of HIV-1 replication, subcellular fractionation analysis of target cells early after viral exposure, and HIV-1 Env-mediated cell-cell fusion experiments. We demonstrate that the effects of DC-SIGN are multiple. The lectin facilitates capture and accumulation of viral particles in an intracellular vesicular compartment but inhibits HIV-1 Env-mediated fusion. Our results provide new insight into how the lectin facilitates *cis* and *trans* infection of HIV-1.

MATERIALS AND METHODS

Cells, reagents, viruses, and infections. HeLa $CD4^+$ DC-$SIGN^+$ cells were derived from HeLa $CD4^+$ long terminal repeat (LTR)-*lacZ* cells (clones P4 and P4C5, which also express CCR5) (22) by infection with flap-derived lentiviral vectors coding for either the WT or LL/AA mutant of DC-SIGN under the control of the cytomegalovirus promoter (31). Individual clones were screened and bulk populations were sorted for DC-SIGN expression. High CD4 surface levels in P4 derivatives were obtained by transient infection with a lentiviral vector coding for CD4 (a kind gift of Y. Percherancier, Institut Pasteur, Paris, France) and sorting with a cell sorter. HeLa243Env cells stably express HIV-1_{LAI} gp120/gp41, Tat, Rev, and Vpu (29). Immature DCs were derived from monocytes as described earlier (31) and were a kind gift of J. P. Abastado (IDM, Paris, France). Production of HIV_{NL43}, $HIV_{YU\text{-}2B}$, HIV_{NLAD8}, and HIV(VSV) strains has been described elsewhere (22, 27). Bafilomycin A1 was from Sigma, and T-20 was from American Peptide. Single-cycle infections of P4 derivatives were performed as described earlier (22), and viral infectivity was measured 24 h after viral exposure. Immature DCs (2.5×10^5 cells per well) were infected with HIV_{NLAD8} (25 ng of p24), and viral replication was monitored as described earlier (27).

Flow cytometry analysis. Cells were stained with anti-DC-SIGN (MAb 161-PE; R&D Systems) and anti-CD4 MAbs (SK3-PE; Becton Dickinson) and were processed for flow cytometry as described earlier (31).

Entry assay. The entry assay was performed by using a cell fractionation protocol modified one described in reference 23. Briefly, subconfluent cultures of HeLa or P4 derivatives ($\sim 6 \times 10^5$ cells in six-well plates) were exposed to the indicated HIV-1 preparations (200 ng of p24/ml) in culture medium containing 20 mM HEPES, pH 7.4, for 2 h at 37°C. Cells were then washed three times in ice-cold phosphate-buffered saline. To remove virus adsorbed at the cell surface, cells were then treated for 10 min with 7 mg of pronase (Boehringer Mannheim)/ml in ice-cold RPMI medium–20 mM HEPES. Cells were washed three times in RPMI medium containing 10% fetal calf serum in order to eliminate pronase. To selectively permeabilize plasma membranes, cells were then treated with an ice-cold digitonin buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 1 mM EDTA, and 100 μg of digitonin [RBI-Sigma]/ml) during 10 min at 4°C. Cells were then centrifuged at 3,000 rpm in a Biofuge Pico centrifuge (Heraeus) for 4 min at 4°C. Supernatants and pellets, corresponding to cytosolic and vesicular fractions, respectively, were adjusted to 0.5% Triton. Samples were then briefly centrifuged at 10,000 rpm to remove debris before measurement of p24 concentrations by enzyme-linked immunosorbent assay (NEN; Perkin-Elmer Life Sciences). Total internalized p24 content was calculated by addition of cytosolic and vesicular p24 contents.

Fusion assay. Syncytium formation assays between P4 derivatives and HeLa243Env cells were essentially performed as described earlier (29). Briefly, cells were mixed at the indicated cell ratio in 24-well plates. HeLa243Env cells also express Tat, which activates production of β-galactosidase (β-Gal) after fusion with P4 cells. After 6-h or overnight incubation, cells were fixed and analyzed for in situ β-Gal detection. When stated, syncytial assays were performed in 96-well plates for quantitative measurement of β-Gal production.

RESULTS

Enhancement of viral infectivity by DC-SIGN. We examined the effects of DC-SIGN on HIV-1 entry and infection of target cells. To this end, we designed HeLa $CD4^+$ cells (P4 clone) stably expressing DC-SIGN. P4 cells were transduced with a lentiviral vector coding for DC-SIGN. Two independent clones, P4 DC3 and P4 DC4, were selected for further analysis. They express DC-SIGN surface levels in the range of those observed in immature DCs (Fig. 1A), whereas CD4 and CXCR4 surface expression was similar to that of parental P4 cells (not shown). P4 cells carry an integrated HIV LTR-*lacZ* cassette that is activated by Tat upon HIV-1 infection. β-Gal expression levels correlate with infection efficiency in a single-cycle viral replication assay (22). We compared the susceptibility of P4, P4 DC3, and P4 DC4 to infection with the X4-tropic HIV-1 strain NL43. We observed a three- to sixfold increase of viral infectivity in the presence of DC-SIGN (Fig. 1B). In contrast, the infectivity of HIV-1 particles coated with the VSV-G envelope protein, which does not bind DC-SIGN (21), was not enhanced but instead slightly decreased in P4 DC3 and P4 DC4 cells (Fig. 1B). Thus, the enhancement of HIV-1 infectivity in these clones was mediated by interactions between gp120/gp41 complexes and DC-SIGN.

We extended these observations to various HIV-1 isolates. We derived P4C5 (HeLa $CD4^+$ $CXCR4^+$ $CCR5^+$) cell populations expressing DC-SIGN (P4C5 DCWT cells [Fig. 1A]). The infectivity of the X4-tropic strain NL43 and R5-tropic strains NLAD8 and YU-2 was increased two- to fourfold in these cells (Fig. 1C). It was previously reported that DC-SIGN was rapidly internalized from the cell surface (31). An LL sequence located in the cytoplasmic tail of DC-SIGN functions

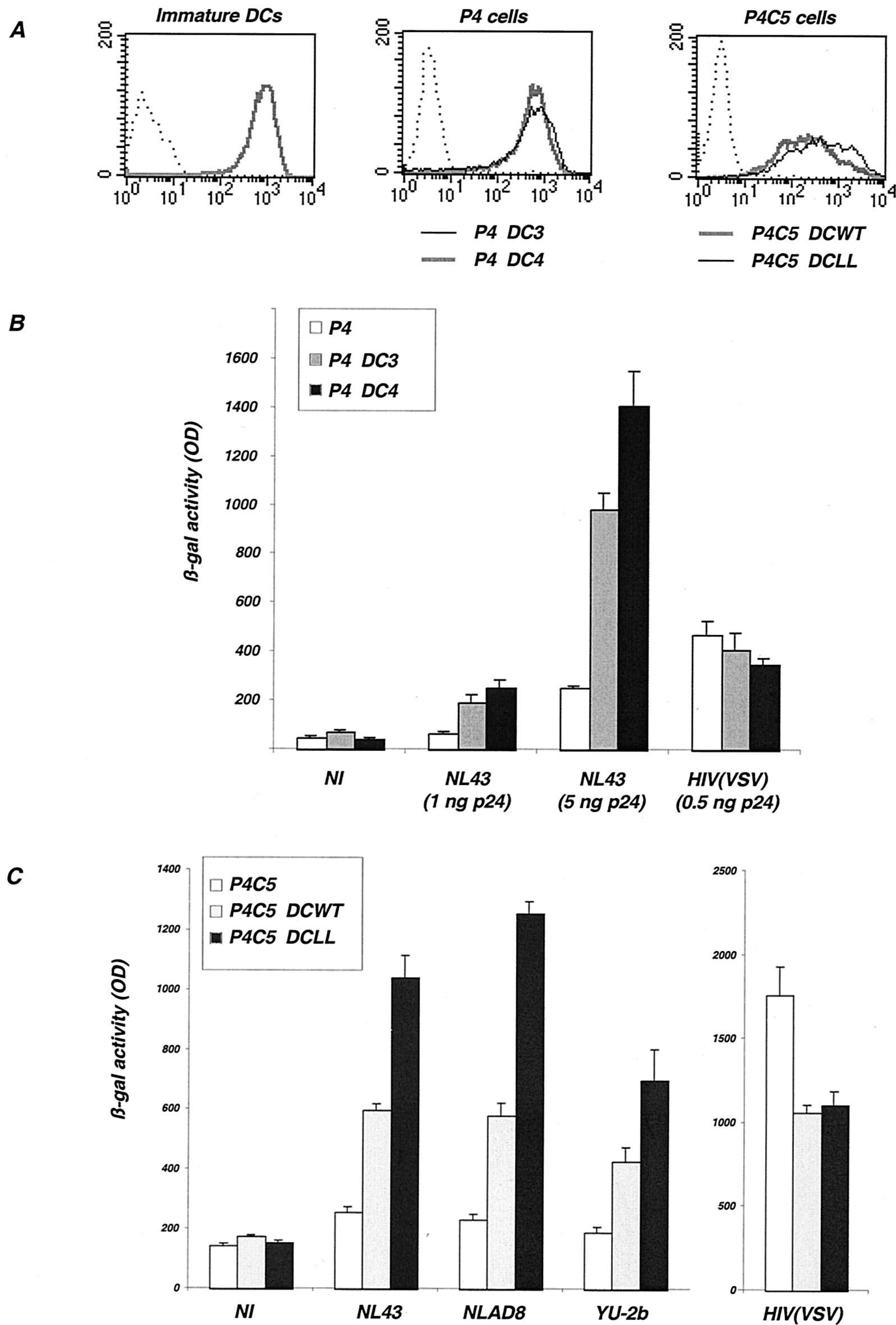

FIG. 1. DC-SIGN enhances viral infection. (A) Surface expression of DC-SIGN in immature DCs, in HeLa $CD4^+$ DC-$SIGN^+$ (P4 DC3 and P4 DC4 clones) cells, and in P4C5 (HeLa $CD4^+$ $CCR5^+$) cells expressing WT (DCWT) or an LL mutant (DCLL) of DC-SIGN. Cells were stained with anti-DC-SIGN antibodies and were analyzed with flow cytometry. An isotypic MAb was used as a negative control (dotted lines). (B) Enhancement of HIV-1 infection by DC-SIGN. HeLa $CD4^+$ (P4 clone) and P4 DC3 and P4 DC4 cells, which both carry an integrated HIV LTR-*lacZ* cassette, were infected with the indicated doses of HIV_{NL43} or HIV(VSV), an HIV-1 variant with a deletion of Env pseudotyped with the VSV-G envelope. Similar results were obtained with higher viral inputs of HIV(VSV) (not shown). (C) Enhancement of infection of various X4 and R5 HIV strains. P4C5 cell derivatives were infected with the indicated viral strain (5 ng of p24). Infection was assessed by measuring β-Gal activity in cell extracts 24 h later. Data are means plus or minus standard deviations of triplicates and are representative of at least three independent experiments. OD, optical density.

as a sorting signal regulating the intracellular trafficking and endocytosis of the lectin. DC-SIGN molecules mutated in this motif (DC-SIGN LL/AA) accumulate at the cell surface and are not prone to spontaneous or antibody-induced internalization (31). We examined whether *cis* enhancement of viral infectivity required the ability of DC-SIGN to undergo endocytosis. As expected, in P4C5 cell populations expressing DC-SIGN LL/AA (P4C5 DCLL cells), steady-state surface levels of the lectin were about fourfold higher than in P4C5 DC WT cells (Fig. 1A). Interestingly, the increase of HIV-1 infectivity was more pronounced in P4C5 DC LL than in P4C5 DCWT cells (Fig. 1C). Again, infection of HIV(VSV) pseudotypes was increased neither by the WT nor by the LL/AA mutant of DC-SIGN (Fig. 1C).

Altogether, these results confirm that expression of DC-SIGN in target cells allows for a more efficient infection of HIV-1. Enhancement of viral infectivity was observed in P4 DC3 and P4 DC4 clones, as well as in P4C5 populations expressing DC-SIGN. The LL sorting signal mediating internalization of the lectin is not required for the increase of viral infectivity.

Dual effect of DC-SIGN during viral entry: increased virion uptake and reduction of cytosolic access. We next compared viral uptake in P4 and in P4 DC3 cells (Fig. 2A). Cells were exposed to HIV_{NL43} or, as a control, to viral particles devoid of viral envelope (HIVΔenv). After 2 h at 37°C, cells were treated with pronase to remove extracellular viral material and the intracellular p24 content was measured (Materials and Methods). When virus binding was performed at 4°C, virtually no p24 was detected in target cells, indicating that pronase treatment efficiently removed all extracellular particles (not shown). With HIVΔenv, after 2 h at 37°C, similar amounts of p24 were measured in P4 and P4 DC3 cells (Fig. 2A). Thus, DC-SIGN does not influence the nonspecific uptake of virions. In contrast, the internalization of HIV-1 particles carrying competent gp120/gp41 complexes was dramatically increased in the presence of the lectin. About 8- to 10-fold more intracellular p24 was measured in P4 DC3 cells (Fig. 2A). Similar results were obtained in P4C5 cells expressing DC-SIGN, with both X4 and R5 strains (Fig. 2E and not shown), indicating that the increased ability to capture HIV-1 virions is not a special feature of the P4 DC3 clone.

As P4 and P4 DC3 cells express the CD4 molecule, we asked whether CD4 is involved in DC-SIGN-mediated enhancement of viral capture. We derived HeLa cells (which lack CD4) stably expressing DC-SIGN (HeLa DC cells) and performed a similar analysis of viral entry. HeLa DC cells internalized ~10- to 15-fold more HIV-1 particles than HeLa cells, in a process requiring the viral envelope (Fig. 2B). Therefore, DC-SIGN allows an efficient capture and subsequent internalization of HIV-1, irrespective of CD4 expression.

A subcellular fractionation assay that measures cytosolic and vesicular HIV-1 Gag p24 content early after viral exposure was previously described (22, 23). In the absence of envelope glycoprotein on virions or of viral receptors, p24 was incorporated in intracellular vesicles but was not detected in the cytosolic fraction. In contrast, when appropriate envelope-receptor interactions could occur, the cytosolic fraction represented 5 to 20% of intracellular p24, depending on the cell type analyzed. Thus, cytosolic p24 is a reliable indicator of virus internalization leading to authentic infection. However, it has been reported that infectious virus undergoes nonspecific vesicular uptake similarly as nonenveloped virions and that the majority of internalized particles end up being degraded in lysosomes (22, 23).

There is an apparent quantitative discrepancy between the ability of DC-SIGN to mediate viral capture (8- to 15-fold more particles internalized) and the increase of viral infectivity in single-cycle assays (three- to sixfold enhancement). To investigate this discrepancy, we compared in P4 and P4 DC3 cells the subcellular repartition (in cytosolic and vesicular fractions) of incoming viral particles (Fig. 2C). With HIVΔenv, the cytosolic content was close to background levels in both cell types, confirming that it is a reliable marker of productive entry events. Exposure of P4 cells to infectious HIV-1 was associated with the detection of p24 in the cytosolic fraction (Fig. 2C). Interestingly, cytosolic p24 content was increased only two- to threefold in P4 DC3 cells when compared to that in P4 cells. This increase is within the range of the improvement of viral infectivity observed in $DC\text{-}SIGN^+$ cells. Therefore, even if there is a dramatic enhancement in the capture of virions in $DC\text{-}SIGN^+$ cells, most particles are restricted to the vesicular pathway and do not gain access to the cytoplasm. This is particularly obvious when results of the entry assay are presented as the ratio of cytosolic to total internalized p24 (Fig. 2D). With HIVΔenv, more than 99% of captured particles are detected in vesicles; the remaining 1% found in the cytosol likely corresponds to contamination during the fractionation procedure (22). With infectious HIV-1, about 5% of the internalized material was detected in the cytosol and 95% in the vesicular fraction of P4 cells (Fig. 2D). In $DC\text{-}SIGN^+$ cells, the cytosolic ratio significantly dropped to 1% of the internalized particles.

Altogether, these results indicate that DC-SIGN exerts a dual effect during the early steps of the viral cycle. The lectin binds and captures viral particles, which are efficiently internalized in intracellular vesicles. However, the fraction of viral material reaching the cytosol, when compared to the overall amount of internalized virions, is reduced in $DC\text{-}SIGN^+$ cells.

Of note, P4C5 cells expressing DC-SIGN LL/AA mediated the internalization of HIV-1 virions in a pronase-resistant compartment as efficiently as those expressing the WT molecule (Fig. 2E). Moreover, the ratio of cytosolic to total internalized p24 was similarly reduced in cells expressing DC-SIGN WT or LL/AA, as determined by cell fractionation assays (Fig. 2E). This indicates that the LL motif of DC-SIGN, previously shown to be involved in the spontaneous or antibody-mediated internalization of DC-SIGN, does not influence the endocytosis of HIV-1 virions or their subsequent access to the cytosol.

pH-independent productive viral entry in $DC\text{-}SIGN^+$ cells. HIV-1 infection is a pH-independent process (24, 32) that is not inhibited by weak bases or by inhibitors of vesicular acidification, such as bafilomycin A1 (22). Since DC-SIGN induces strong endocytosis of incoming viral particles, we asked whether the lectin influences the pH sensitivity of viral entry. The effect of bafilomycin A1 on HIV_{NL43} and HIV(VSV) was examined in a single-cycle replication assay. As expected, in P4 cells, bafilomycin A1 did not alter HIV-1 replication, whereas it inhibited infection of virions coated with the VSV-G envelope, which requires an acidic environment to become fusogenic (Fig. 3). The same phenomenon was observed in P4 DC4

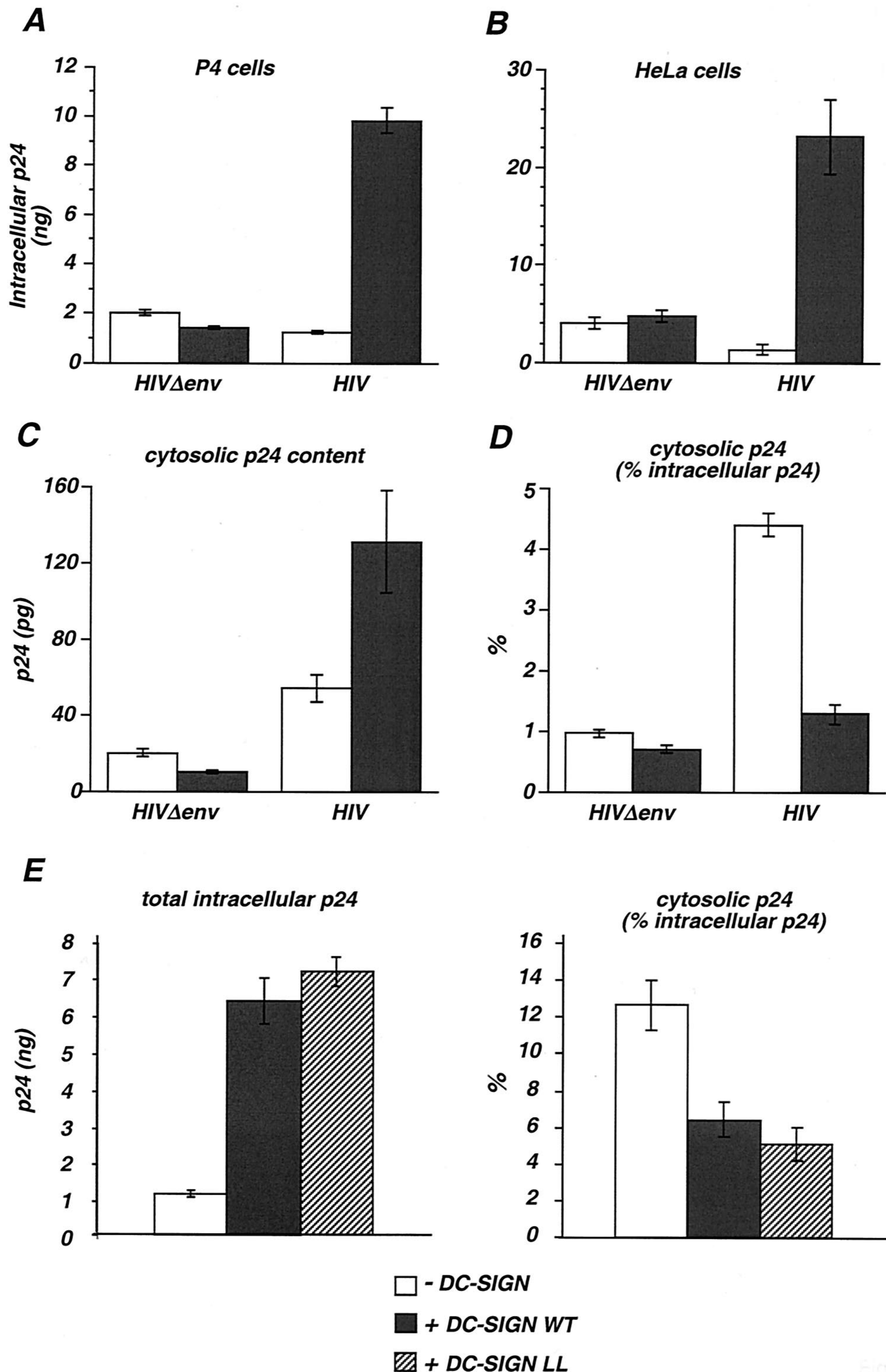

FIG. 2. Effect of DC-SIGN on viral entry. (A and B) Total intracellular p24 levels. P4 and P4 DC3 cells (A) and HeLa and HeLa–DC-SIGN cells (B) were exposed to HIV_{NL43} or to noninfectious HIV-1 particles devoid of envelope protein (HIVΔenv) as a control. Viral input corresponded to 200 ng of p24 for 2 h at 37°C. After viral exposure, cells were treated by pronase to eliminate virus adsorbed at the surface and total intracellular p24 contents were measured. (C and D) Cytosolic intracellular p24 levels. P4 and P4 DC3 cells were treated as for panel A, except that p24 contents were measured in the cytosolic and vesicular (pellet) fractions. (C) Cytosolic p24 levels (in picograms) are shown. (D) The ratios of cytosolic p24 to total intracellular (vesicular plus cytosolic) p24 are shown. (E) Role of the LL motif of DC-SIGN during viral entry. Parental P4C5 cells and P4C5 cells expressing WT (DCWT) or expressing an LL mutant (DCLL) of DC-SIGN were exposed to HIV_{NL43} as described for panels A and B. Left panel, total intracellular p24 levels. Right panel, ratios of cytosolic p24 to total intracellular (vesicular plus cytosolic) p24 (after subtraction of background levels obtained with HIVΔenv). Data are means plus or minus standard deviations of triplicates and are representative of at least three independent experiments.

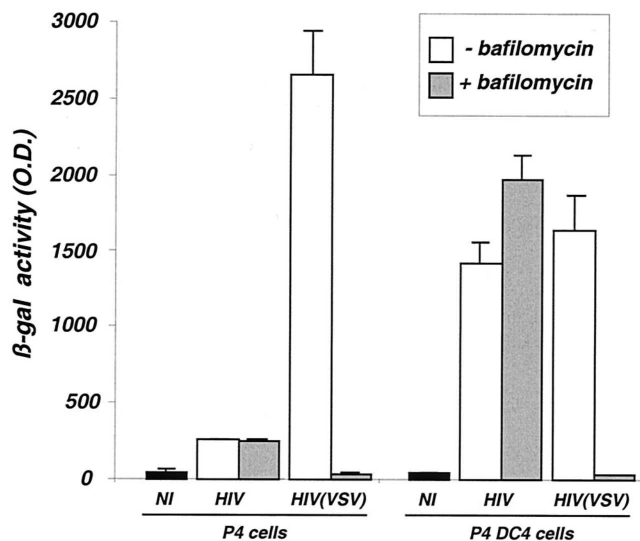

FIG. 3. Effect of bafilomycin A1 on HIV-1 infection in DC-SIGN$^+$ cells. P4 and P4 DC4 cells were preincubated with or without bafilomycin A1 (0.25 μM) and were exposed either to HIV_{NL43} or HIV(VSV) (5 ng of p24). Viral infection was monitored as described in the Fig. 1 legend. Data are means plus or minus standard deviations of triplicates and are representative of three independent experiments. O.D., optical density.

(Fig. 3) and P4 DC3 (not shown) cells. In summary, HIV-1 infectivity was increased by DC-SIGN and this enhancement was not affected by bafilomycin A1. Thus, vesicular capture of incoming virions by DC-SIGN does not lead to a pH-dependent productive entry of HIV-1.

Inhibition of viral fusion by DC-SIGN. HIV-1 infection leads to the formation of multinucleated giant cells or syncytia, which are produced by fusion between Env-expressing cells and CD4$^+$ cells. We examined whether DC-SIGN affected Env-mediated syncytium formation. To this end, we used HeLa243Env cells, which express functional gp120/gp41 complexes at the surface, as well as the HIV-1 Tat transactivator (29). Coculture of HeLa243Env with P4 cells leads to cell-cell fusion and β-Gal production, thus providing a convenient means of monitoring syncytium formation. P4, P4 DC3, and P4 DC4 cells were mixed with HeLa243Env cells for 6 h at 37°C, and cells were then stained for in situ β-Gal activity (Fig. 4A). Numerous blue syncytia were detected in P4-HeLa243Env mixtures. Interestingly, the number of syncytia was significantly reduced when P4 DC3 or P4 DC4 cells were used. In order to quantify this inhibition, we measured β-Gal activity in a colorimetric assay (Fig. 4B). In the absence of HeLa243Env cells, background β-Gal levels were measured. With P4 cells, β-Gal activity was detected after 6 h of coculture with HeLa243Env. The levels of enzymatic activity correlated with the amount of Env-expressing cells present in the cultures (Fig. 4B). About three- to fivefold less β-Gal activity was detected with P4 DC3 or P4 DC4 cells than with P4 cells. Moreover, about 10-fold more Env-expressing cells were required to achieve similar levels of fusion with P4 DC3 and -4 cells than with P4 cells. We also used in the assay two P4 clones expressing the DC-SIGN LL/AA mutant. We observed a similar inhibition of viral fusion with these clones (Fig. 4B), indicating that the endocytic ability of DC-SIGN is not involved in this inhibition. Of note, similar results were observed after overnight incubation of Env$^+$ and target cells (not shown).

Thus, expression of DC-SIGN in target cells significantly inhibits the ability of gp120/gp41 complexes to mediate syncytium formation.

Fusion inhibitor T-20 is efficient in DC-SIGN$^+$ cells. We next examined the potency of the fusion inhibitor T-20 in the presence of DC-SIGN. Binding of the HIV-1 Env complex with CD4 and coreceptor initiates a series of conformational changes that leads to exposure of the fusogenic structure of gp41 (7, 25). T-20, a synthetic peptide corresponding to a region of gp41, is a potent inhibitor of HIV-1 entry and cell-cell fusion (18, 36). T-20 binds to a conserved hydrophobic groove of gp41 that is transiently exposed after CD4-gp120 interactions and blocks formation of the hairpin structure necessary for fusion. We examined whether the sensitivity of gp120/gp41 complexes to T-20 was affected by DC-SIGN. We first compared the inhibitory activity of T-20 on syncytium formation between HeLa243Env and P4 or P4 DC4 cells. T-20 efficiently inhibited fusion between P4 and Env-expressing cells (50% inhibitory concentration [IC_{50}] and IC_{90} of ~8 and 50 ng/ml, respectively [Fig. 5A]). With P4 DC4 cells, which are much less prone to Env-mediated fusion, we used a 10-fold excess of HeLa243Env cells, in order to achieve similar levels of syncytium formation in the absence of inhibitor. However, T-20 efficiently inhibited syncytium formation between P4 DC4 and HeLa243Env cells (IC_{50} and IC_{90} of ~6 and 50 ng/ml, respectively [Fig. 5A]). Of note, similar results were obtained at other HeLa243Env/P4 DC4 ratios or when P4 DC3 cells were used (not shown). The effect of T-20 was then examined in single-cycle replication assays in P4 and P4 DC4 cells. The peptide potently inhibited infection by cell-free HIV-1. In both cell types, the inhibition reached 30 to 50% at 200 ng of T-20/ml and was over 90% at 1,000 ng/ml (Fig. 5B). We then verified that T-20 was active in cells that naturally express DC-SIGN. To this end, we monitored replication of the R5-tropic strain NLAD8 in primary immature DCs. T-20 (1 μg/ml) efficiently abolished viral replication, as measured by p24 production in the culture medium (Fig. 5C).

Altogether, these results indicate that T-20 is efficient in blocking cell-cell fusion and cell-free virus replication in DC-SIGN-positive cells. Therefore, DC-SIGN–Env interactions do not inhibit the conformational changes of the gp120/gp41 complex leading to the exposure of the fusogenic structure targeted by T-20. The inhibitory effects of T-20 and DC-SIGN on the fusion process are governed by distinct mechanisms.

CD4 and DC-SIGN compete for HIV-1 interaction. We then examined whether the inhibition of viral fusion by DC-SIGN was due to a competition between CD4 and the lectin for binding to gp120/gp41. To address this point, we overexpressed CD4 in P4 and P4 DC3 cells and studied their ability to fuse with HeLa243Env cells. P4 cells express rather low levels of CD4, a situation reminiscent of that observed in human DCs. After transient transduction with a lentiviral vector encoding CD4, we observed a 6- to 14-fold increase of the surface levels of this receptor, yielding P4-CD4high and P4 DC3-CD4high cells, respectively (Fig. 6A). P4-CD4high cells formed syncytia with HeLa243Env cells more potently than P4 cells, with an approximately twofold increase in β-Gal activity (Fig. 6B).

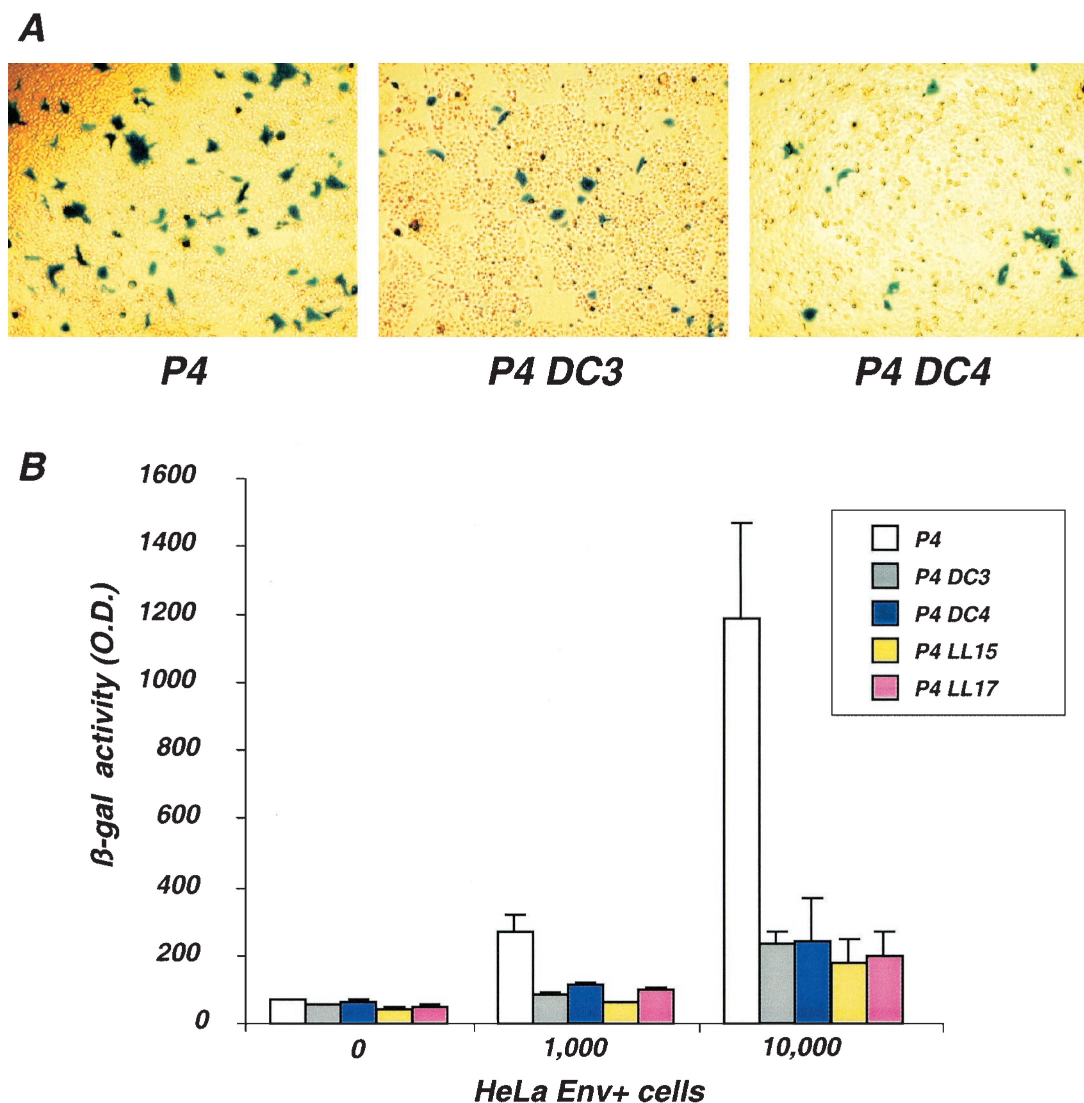

FIG. 4. DC-SIGN inhibits syncytium formation between HIV-1 Env^+ and $CD4^+$ cells. (A) Syncytium formation assay. HeLa243Env cells, which express gp120/gp41 complexes and Tat, were mixed (at a 1:5 ratio) with the indicated P4 cell clones. After a 6-h incubation at 37°C, cells were fixed and stained for β-Gal detection. Giant multinucleated cells express β-Gal after Tat-induced transactivation of the HIV LTR-*lacZ* cassette. Representative fields are shown. (B) Quantitative analysis of the inhibitory activity of DC-SIGN. The indicated P4 cell clones (10^4 cells per point) were cocultivated with 10^3 or 10^4 $HeLa243Env^+$ cells. After 6 h, β-Gal activity was measured in cell extracts. P4 LL15 and P4 LL17 are two independent clones expressing the DC-SIGN LL/AA mutant. Data are means plus or minus standard deviations of triplicates and are representative of three independent experiments. O.D., optical density.

Interestingly, in P4 DC3-CD4high cells, this increase was more pronounced (three- to fourfold increase) and syncytium levels reached those observed in P4-CD4high cells (Fig. 6B). Therefore, the inhibitory effect of DC-SIGN on HIV-1 fusion is no longer observed in the presence of high levels of CD4. These findings support a model in which CD4 and DC-SIGN compete for gp120 binding. Env interaction with CD4 leads to viral fusion, whereas Env binding to DC-SIGN prevents this process.

DISCUSSION

The mechanisms by which DCs capture and transmit HIV are poorly understood. HIV-1 attachment to target cells can occur via a large panel of cellular factors, which bind viral Env with rather low affinity (35). High-affinity binding is mediated by CD4, which leads to productive infection, as well as by lectins such as DC-SIGN (6, 12, 34), which efficiently captures viral particles but does not allow viral fusion by itself. Following interaction with DC-SIGN, the virus is kept in an infectious state and can be transmitted to lymphocytes several days after capture (8, 12). DC-SIGN also enhances the efficiency of virus infection in *cis*, when target cells express CD4 and a coreceptor (21). However, cellular mechanisms underlying the *cis* and *trans* effects of DC-SIGN remain incompletely unravelled.

We studied the role of DC-SIGN during early steps of the HIV-1 life cycle. We demonstrate here that the effects of DC-SIGN are complex and subtle. The lectin facilitates viral

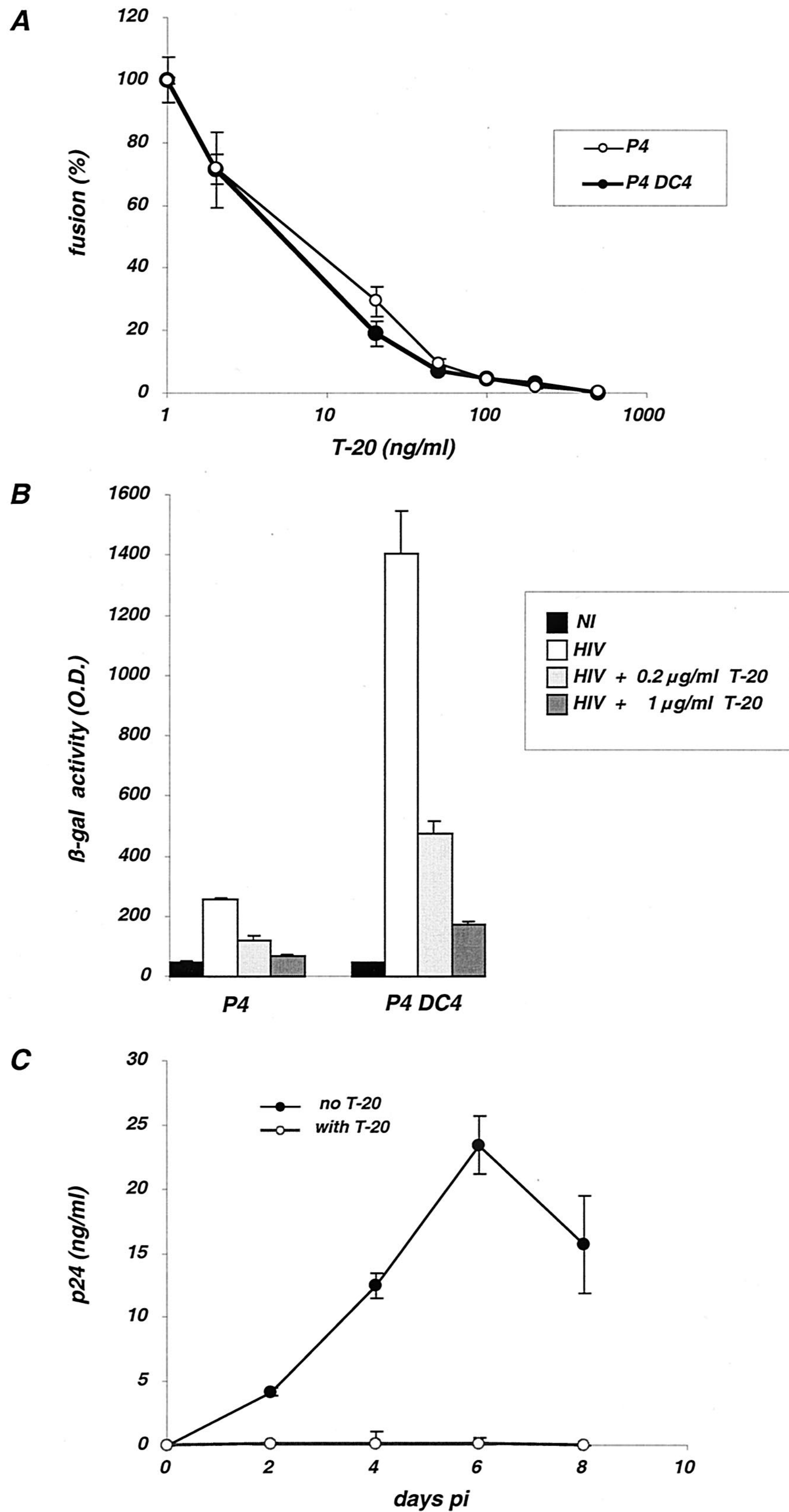

FIG. 5. Effect of the HIV-1 fusion inhibitor T-20 in DC-SIGN$^+$ cells. (A) Inhibition of syncytium formation by T-20. HeLa243Env$^+$ cells were mixed with P4 or P4 DC4 cells and with the indicated concentrations of T-20, and syncytia were quantified as described in the Fig. 4B legend. Similar results were obtained in P4 DC3 cells (not shown). (B) Inhibition of virus infection by T-20. P4 and P4 DC4 cells were exposed to HIV_{NL43} with the indicated concentrations of T-20. Viral infection was assessed as described in the Fig. 1B legend. Data are means plus or minus standard

capture and enhances infectivity. DC-SIGN induces accumulation of virions in an intracellular vesicular compartment but inhibits HIV-1 Env-mediated fusion. In DC-SIGN$^+$ cells, HIV-1 productive entry remains pH independent and the fusion inhibitor T-20 potently abolishes viral fusion, suggesting that contact between the lectin and the virus does not result in major conformational changes in the envelope. More likely, DC-SIGN competes with CD4 for attachment to gp120/gp41 complexes, leading to a significant decrease in viral fusion.

DC-SIGN and virus capture. The first step of entry of incoming HIV-1 is attachment to target cells. DC-SIGN strongly enhances virus adsorption (12). Binding of HIV-1 Env is dependent on the C-ter lectin domain of DC-SIGN and on carbohydrate and peptidic structures on the viral envelope (14, 28). Following interaction with DC-SIGN, HIV-1 is rapidly internalized into a low-pH endosomal compartment (20). We have documented further the fate of virions after internalization. To this end, we derived a variety of P4 (HeLa CD4$^+$) reporter cell clones and populations stably expressing the lectin. It was previously reported that, in HeLa cells, DC-SIGN is primarily located at the cell surface and in the endosomal compartment (31). This intracellular localization pattern is reminiscent of that observed in primary DCs. HIV-1 infection with various X4 and R5 isolates was three- to sixfold more efficient in the presence of DC-SIGN, confirming the *cis* enhancement activity of the lectin (21). We measured the Gag p24 content in these cells early after viral exposure. We observed a dramatic increase of viral uptake, with 10- to 15-fold more cell-associated p24, in DC-SIGN$^+$ cells. This increase also occurred in HeLa cells and thus does not require CD4. In contrast, it was observed neither with virons that have a deletion of Env nor with VSV-G-coated virions and therefore involves a direct interaction between gp120/gp41 complexes and the lectin. Moreover, cell-associated p24 was resistant to treatment with protease, confirming that incoming virions are rapidly internalized in DC-SIGN$^+$ cells.

Are DC-SIGN and virions internalized together? We previously reported that the LL sequence located in the cytoplasmic tail of DC-SIGN acts as a sorting signal and regulates trafficking of the protein. A DC-SIGN mutant (LL/AA) accumulates at the cell surface at the steady state and is no longer endocytosed by anti-DC-SIGN antibodies (31). We therefore examined how DC-SIGN LL/AA impacts HIV-1 replication. Interestingly, this mutant enhances infection in *cis*. It was even more efficient than the WT molecule in single-cycle infectivity assays. Moreover, fractionation assays demontrated that virions were rapidly internalized in cells expressing DC-SIGN LL/AA. Thus, the LL sequence of DC-SIGN is not involved in the endocytosis of HIV particles. How then may virions be internalized after binding to DC-SIGN LL/AA? One can postulate that other motifs in the lectin operate during virus-induced DC-SIGN endocytosis. This would imply that various pathways regulate DC-SIGN trafficking. The clathrin-dependent sorting pathway likely mediates DC-SIGN endocytosis and recycling through recognition of the LL motif. Clathrin-independent pathways may additionally be used during virus-induced DC-SIGN internalization. Alternatively, although less likely, virions might first be captured by DC-SIGN and then be internalized independently of the lectin. It will be important to determine if internalization and recycling of DC-SIGN are influenced by virus binding.

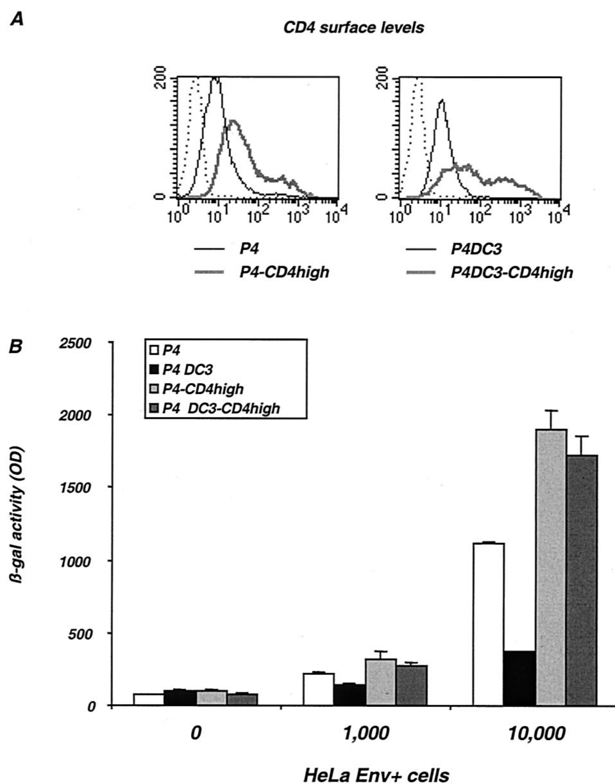

FIG. 6. High CD4 levels balance the antifusogenic activity of DC-SIGN. (A) Surface expression of CD4 in P4 and P4 DC3 cells. Cells were transiently transduced with a lentiviral vector encoding CD4, yielding P4-CD4high and P4 DC3-CD4high cells. Cells were then analyzed for CD4 expression by flow cytometry. An isotypic MAb was used as a negative control (dotted lines). (B) Syncytium formation assay. The indicated P4 derivatives (10^4 cells) were cocultivated with 10^3 or 10^4 HeLa243Env$^+$ cells. After 6 h, β-Gal activity was measured in cell extracts. Data are means plus or minus standard deviations of triplicates and are representative of two independent experiments. OD, optical density.

DC-SIGN-mediated internalization of HIV particles leads to an enhancement of infection in *trans* (20). This process is observed at low virus titers, when CD4$^+$ cells are not detectably infected without the assistance of DC-SIGN$^+$ cells (12). Internalization into a low intracellular pH is critical in this process (20). In this study, we have pointed out mechanistic

deviations of triplicates and are representative of three independent experiments. (C) Effect of T-20 on HIV-1 replication in DCs. Immature DCs (2.5×10^5) were infected with the R5 strain HIV_{NLAD8} (25 ng of p24) with or without T-20 (1 μg/ml). Viral replication was assessed by measuring p24 production in the culture supernatants. T-20 was maintained in the medium throughout the experiment. pi, postinfection. Data are means plus or minus standard deviations of duplicates and are representative of two independent experiments. No toxicity was observed with T-20 at the concentrations used.

differences between *cis* and *trans* enhancement of infection. DC-SIGN molecules lacking the LL signal are still capable of binding and enhancing infection in *cis* (this study), whereas a 35-amino-acid deletion of the cytoplasmic tail abolishes *trans* enhancement (20). It will be of interest to determine precisely which sequences of DC-SIGN are required for *cis* and for *trans* enhancement. Moreover, although the lectin induces a massive and rapid internalization of virions, we show that *cis* enhancement of infection remains pH independent, since it is not inhibited by bafilomycin A1. Entry by endocytosis does not necessarily mean that a pH-dependent step is required for fusion. For instance, pH-independent entry via endosomal vesicles has been described for poliovirus (26), and it was recently reported that pH-independent endocytosis of HIV-1 by macropinocytosis occurs in primary macrophages (23).

During early steps of viral replication, the majority of incoming virions are internalized in a vesicular compartment and end up being degraded in lysosomes (22, 23). However, a small fraction of endocytosed virions escape degradation and gain access to the cytosol, where they lead to productive infection. Therefore, measuring cytosolic p24 content provides a reliable marker of authentic infection (22). To document further the role of DC-SIGN during entry, we performed subcellular fractionation analysis of target cells. In the absence of the lectin, 90 to 95% of internalized virions were found in the vesicular fraction and 5 to 10% gained access to the cytosol. Interestingly, the presence of DC-SIGN significantly affected this subcellular repartition. Only 1 to 5% of incoming viral material was detected in the cytosolic fraction. This finding provides an explanation for the apparent quantitative discrepancy between the abilities of DC-SIGN to mediate viral capture (10- to 15-fold more particles internalized) and to increase viral infection in *cis* (three- to sixfold enhancement). Therefore, although DC-SIGN potently binds virions and induces their uptake, internalized particles accumulate in the vesicular compartment and their access to the cytoplasm is reduced.

DC-SIGN and virus fusion. That viral access to the cytoplasm is affected after binding to DC-SIGN suggests that the lectin may affect Env-induced membrane fusion. We compared the abilities of P4 cells expressing or not expressing DC-SIGN to fuse with HIV-1 Env^+ cells. The lectin profoundly inhibited syncytium formation between $CD4^+$ and Env^+ cells. It has been proposed that DC-SIGN binding may alter Env conformation in a manner that enables Env to bind to its receptors more readily or that makes Env more fusogenic (2). However, our results demonstrate that DC-SIGN-bound Env is less prone to fusion with its receptors. To document further this phenomenon, we studied the antiviral effect of T-20, a fusion inhibitor that targets a structural intermediate of the fusion process (18, 36). T-20 potently inhibited cell-free virus infection and cell-cell fusion, irrespective of DC-SIGN expression. Therefore, the inhibitory effects of T-20 and DC-SIGN on the fusion process are governed by distinct mechanisms. The lectin does not prevent access of T-20 to its structural target on gp41 and thus probably does not induce major conformational changes in gp120/gp41 complexes.

On the other hand, that T-20 blocks viral replication in $DC\text{-}SIGN^+$ cells as well as in primary DCs is of importance, since this compound is presently tested in clinical trials in HIV-infected patients. Our observation suggests that, in vivo, T-20 may be active not only in lymphocytes but also in DCs and in macrophages, in which virus infection can occur after the occurrence of macropinocytosis or other internalization pathways (9, 23).

Mechanisms other than conformational changes of Env should then account for our observation that DC-SIGN reduces both virion access to the cytoplasm and syncytium formation. It is possible that the capacity of DC-SIGN to capture virions surpasses the ability of the cell to mediate subsequent CD4-dependent entry events. DC-SIGN-bound virions might also accumulate in selective membrane microdomains or be routed toward an intracellular compartment(s), in which CD4 and the viral coreceptor are insufficiently or inadequately expressed. It will be interesting to determine if DC-SIGN accumulates in lipid raft membrane domains, which are known to be enriched in virus receptors. An additional, and not exclusive, possibility is that DC-SIGN and CD4 directly compete for Env binding. The affinity of DC-SIGN for gp120 slightly exceeds that of CD4 ($K_d \approx 1.5$ and ≈ 5 nM, respectively) (6). The relative expression levels of DC-SIGN and CD4 will thus determine which receptor will be preferentially used for binding. Interestingly, DCs express high surface levels of the lectin (1×10^5 to 2.5×10^5 copies per cell), whereas those of CD4 are much lower (3, 9, 19). One can speculate that, in DCs, incoming HIV virions will bind more efficiently to DC-SIGN than to CD4, which may significantly alter the efficiency of fusion. P4 derivatives used in this study express low CD4 and high DC-SIGN surface levels, a situation reminiscent of that of primary DCs. To examine if DC-SIGN competes with CD4 for attachment to gp120/gp41 complexes, we have transiently overexpressed CD4 in P4 $DC\text{-}SIGN^+$ cells. Their ability to form syncytia with Env^+ cells was readily increased, balancing the inhibitory effect of the lectin. We conclude that DC-SIGN inhibits Env fusion by competing with the viral receptor for gp120 attachment. We believe that this competition occurs not only at the cell surface but also in endosomes or macropinosomes, in which CD4 and DC-SIGN may colocalize. This competition will likely reduce access of internalized virions to the cytoplasm, as reported in this study.

Inhibition of HIV-1 fusion in DCs and viral spread. Our study provides new insight into how DC-SIGN contributes to HIV-1 dissemination by DCs. Inhibition of viral fusion may preserve internalized virions and maintain their replicative potential for prolonged periods of time. Once virus-loaded DCs encounter susceptible cells, a still poorly characterized mechanism may allow virions to recycle back to the surface and to be exocytosed, so that they can reach viral receptors expressed by target cells. Conversely, the presence of DC-SIGN may also protect DCs from massive infection by incoming virions and from fusion with Env-expressing cells. In cell cultures, DCs are much less prone to HIV-1 infection than are lymphocytes (9, 15, 27). In HIV-1-infected individuals, syncytia involving DCs and T cells are observed (10). One can speculate that the antifusogenic effect of DC-SIGN allows DCs to maintain their immunological functions after encountering viral antigens. Besides DC-SIGN, an array of other lectins expressed on different DC subsets are involved in HIV-1 binding (34). The inhibition of HIV-1 fusion by DC-SIGN described here may also apply to these other attachment factors and may be of critical importance for viral dissemination and immunopathogenesis.

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