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Immunity, Vol. 16, 145–155, January, 2002, Copyright ©2002 by Cell Press

HIV-1 Nef-Induced Upregulation of DC-SIGN in Dendritic Cells Promotes Lymphocyte Clustering and Viral Spread

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Summary

DC-SIGN, a dendritic cell (DC)-specific lectin, mediates clustering of DCs with T lymphocytes, a crucial event in the initiation of immune responses. DC-SIGN also binds HIV envelope glycoproteins, allowing efficient virus capture by DCs. We show here that DC-SIGN surface levels are upregulated in HIV-1-infected DCs. 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. These results provide new insights into how HIV-1 spreads from DCs to T lymphocytes and manipulates immune responses. They help explain how Nef may act as a virulence factor in vivo.

Introduction

HIV-1 primary infection is characterized by a stage of intense viral replication. Sexual transmission is mainly restricted to R5-tropic viral strains. 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 dendritic cells (DCs) residing in the skin and mucosa are thought to be the first cells targeted by HIV-1 (Cameron et al., 1996; Klagge and Schneider-Schaulies, 1999). DCs perform a crucial role in the generation and regulation of immunity (Banchereau and Steinman, 1998). They are the only antigen-presenting cells that can stimulate resting naive T lymphocytes and initiate CTL responses. Immature DCs residing in peripheral tissues capture antigens from various sources, including microbes, infected cells, cell debris, proteins, and immune complexes. Antigen-loaded DCs travel toward secondary lymphoid organs and mature, processing antigens for presentation and acquiring the capacity to attract and activate resting T cells during their journey. DC's migration from the periphery and recruitment of T cells within lymphoid tissues is likely exploited by HIV-1 to ensure its propagation (Cameron et al., 1996; Sewell and Price, 2001; Steinman, 2000). HIV-1 replicates rather inefficiently in DC cultures. DCs express low levels of the HIV receptor CD4 and coreceptors CCR5 or CXCR4. R5-tropic, but not X4-tropic, HIV-1 strains induce chemotaxis and replicate in immature DCs (Granelli-Piperno et al., 1998; Lin et al., 2000). However, both R5- and X4-tropic HIV-1 readily bind and enter DCs (Granelli-Piperno et al., 1999; Klagge and Schneider-Schaulies, 1999). How this happens has been unravelled recently by the identification of DC-SIGN, a DC-specific protein that interacts with HIV-1 (Geijtenbeek et al., 2000b).

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 both mature and immature DCs (Geijtenbeek et al., 2000c). The known cellular ligands of DC-SIGN are ICAM-2 and ICAM-3. DC-SIGN-ICAM-2 interaction regulates DC transmigration across vascular and lymphoid endothelium (Geijtenbeek et al., 2000a). DC-SIGN binding to ICAM-3, a molecule constitutively expressed at the T cell surface, mediates loose adhesion between DCs and T cells (Geijtenbeek et al., 2000c). This early contact may enable the TCR to scan for processed antigens, allowing the initiation of primary immune responses (Geijtenbeek et al., 2000c). Besides ICAM ligands, DC-SIGN efficiently binds viral envelope glycoproteins from R5 and X4 HIV-1 isolates and from HIV-2 and SIV (Geijtenbeek et al., 2000b; Pohlmann et al., 2001). DC-SIGN-expressing cells retain attached virions in an infectious state for several days and transmit them to lymphocytes, thus enhancing infection efficiency (Geijtenbeek et al., 2000b). Binding and transmission of HIV-1 from DCs to T cells is blocked by DC-SIGN antibodies. These observations led to a model in which virus is captured by DCs through DC-SIGN binding, allowing efficient trans-infection of T lymphocytes. They account for the apparent discrepancies between the ability of DCs to capture virus and their susceptibility to productive infection (Blauvelt et al., 1997). Thus, although DC-SIGN neither triggers virus fusion nor circumvents the requirement for CD4 and coreceptors for infection, it contributes significantly to the efficiency of these processes.

Whereas the role of DC-SIGN in HIV capture by DCs is demonstrated, how virions are then conveyed to lymphoid organs is not fully understood. It is not clear whether infectious virions remain associated with the cell surface or, more likely, are internalized into intracellular vesicles (Blauvelt et al., 1997; Geijtenbeek et al., 2000b; Granelli-Piperno et al., 1999; Pohlmann et al., 2001). Of note, the form under which HIV-1 is transported likely depends on virus tropism, since R5 HIV-1 strains replicate in DCs, whereas captured X4 strains may be

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only "passively" ferried. Information about the next step of viral spread, which is virus transmission to lymphocytes, is scarce. In primary cell culture experiments, DCs exposed to HIV readily form clusters with unstimulated T cells. Interestingly, a vigorous HIV-1 replication was observed in DC-T cell clusters, but not in separate preparations of DCs or T cells (Granelli-Piperno et al., 1998, 1999; Pope et al., 1994, 1995). Efficient viral replication in this system requires a direct contact between DCs and T cells (Tsunetsugu-Yokota et al., 1997), and virus production mainly originates from syncytia involving DCs and T cells (Pope et al., 1994, 1995). This observation is relevant to the in vivo situation. In lymphoid tissues of HIV-1-infected individuals, DC-derived syncytia are actively formed and become the sites of intense viral replication (Frankel et al., 1997, 1996). It has thus been proposed that DC-T cell clusters are first generated, leading to the formation of syncytia, which further enhance viral replication (Pope et al., 1995). How these clusters are generated and the role of DC-SIGN in this process remain obscure.

The Nef protein of HIV and SIV is required for efficient in vivo viral replication and pathogenicity (Cullen, 1998). Experimental infection of macaques with SIVmacΔnef is characterized by a low level of viral replication (Kestler et al., 1991). In humans, nef-deleted proviruses were detected in several long-term nonprogressors (Deacon et al., 1995). Mechanisms accounting for the role of Nef remain unclear. Nef is dispensable for viral replication in most culture settings. In macrophages, Nef mediates lymphocyte chemotaxis and activation (Swingler et al., 1999). In immature DCs, Nef exerts a marginal role on the replication of R5 HIV strains, but it is required for optimal virus production in DC-T cell mixtures (Petit et al., 2001). Identified functions of Nef include the activation of cellular signal transduction pathways and the downregulation of cell surface expression of CD4 and MHC-I. The latter effects may facilitate viral replication and immune evasion and are mediated by interactions of Nef with components of the cell sorting machinery (Piguet et al., 1999).

We have examined here the effects of HIV-1 infection on DC-SIGN intracellular trafficking, with a particular focus on the role of Nef. We show that DC-SIGN surface levels are significantly upregulated in HIV-infected cells. This phenomenon is induced by Nef, which inhibits DC-SIGN endocytosis. Nef activity requires a dileucine-based sorting motif located in the cytoplasmic tail of DC-SIGN. Stabilization of DC-SIGN at the cell surface dramatically enhances the ability of DCs to form clusters with lymphocytes and increases virus transmission.

Results

Upregulation of DC-SIGN in HIV-1-Infected DCs

We first analyzed the effects of HIV-1 infection on DC-SIGN expression. DCs were prepared from PBMCs of seronegative individuals. They expressed DC lineage markers, including DC-SIGN and the HIV receptors CD4 and CCR5, and were phenotypically immature (see Experimental Procedures). As expected (Granelli-Piperno et al., 1998; Petit et al., 2001), R5-tropic HIV_{NLAD8} and HIV_{YU-2} strains replicated at low levels in immature DCs, whereas the X4-tropic laboratory-adapted HIV_{NL43} strain was unable to grow (data not shown). Productive infection of DCs by HIV_{NLAD8} and HIV_{YU-2} strains was evidenced by p24 release into culture supernatants (Petit et al., 2001) (data not shown) and by immunofluorescence (IF) analysis revealing HIV-1 Gag-positive cells (Figure 1). Depending on donors, 10%–80% of the cells were Gag positive 4–6 days postinfection (pi). To study DC-SIGN expression in the fraction of productively infected cells, samples were doubly stained with anti-Gag and anti-DC-SIGN antibodies. Numerous DC-SIGN-positive dots were distributed in the cytoplasm and at the periphery of DCs not exposed to virus (Figure 1). In samples infected with R5-tropic HIV-1, intense DC-SIGN surface staining and weak intracellular signals were detected in DCs positive for Gag (Figure 1), suggesting that HIV-1 infection induces the accumulation of DC-SIGN at the cell surface. This observation was made with both HIV_{NLAD8} and HIV_{YU-2} isolates (Figure 1). In contrast, in cells exposed to HIV_{NL43}, Gag-expressing cells were not detected, and DC-SIGN localization was not affected (data not shown). Therefore, the accumulation of DC-SIGN at the surface of infected cells required de novo synthesis of HIV-1 proteins.

We examined the role of Nef in this phenomenon. DCs were infected with nef-deleted viruses (Δnef HIV_{NLAD8} and HIV_{YU-2}). We previously reported that WT and Δnef HIV-1 replicate with similar kinetics in DCs (Petit et al., 2001). The fraction of Gag-positive DCs, as well as the levels of p24 released into supernatants, was similar after infection with WT or Δnef isogenic viruses (data not shown). Nef expression was detected by IF in cells infected with WT, but not with Δnef viruses (data not shown). Interestingly, DC-SIGN staining patterns in Δnef-infected DCs positive for Gag expression resembled those in noninfected cells (Figure 1). Thus, accumulation of DC-SIGN at the surface of HIV-1-infected DCs was associated with Nef expression. Maturation of DCs in the presence of cytokines does not enhance DC-SIGN surface expression (Geijtenbeek et al., 2000c). This suggests that HIV-1-induced DC-SIGN upregulation was not due to modifications of the maturation state of DCs.

Immature DCs display various morphologies, potentially affecting DC-SIGN localization. Thus, we examined the effects of HIV-1 infection in HeLa $CD4^+$ cells stably expressing DC-SIGN (P4-DC3 cells). Cells were infected with WT and Δnef HIV_{NL43}, doubly stained 2 days later with anti-CD4 and anti-DC-SIGN antibodies and analyzed by flow cytometry (Figure 1B). We observed in cells infected with WT virus a CD4-low cell population in which DC-SIGN surface expression was increased. With Δnef virus, a CD4-low cell population was detected, which likely corresponded to cells expressing HIV-1 proteins known to downregulate CD4 (Vpu or Env). However, the DC-SIGN surface level was unaffected in these cells (Figure 1B). P4-DC3 cells were then infected with env-deleted HIV-1 pseudotyped with VSV-G (HIV(VSV) virions). Similarly, a CD4-low, DC-SIGN-high cell population was detected with WT, and not with Δnef HIV(VSV). Thus, a concurrent cell surface upregulation of DC-SIGN and downmodulation of CD4 occurs in HIV-infected cells. Moreover, HIV-1 Env is not involved in the upregulation of DC-SIGN.

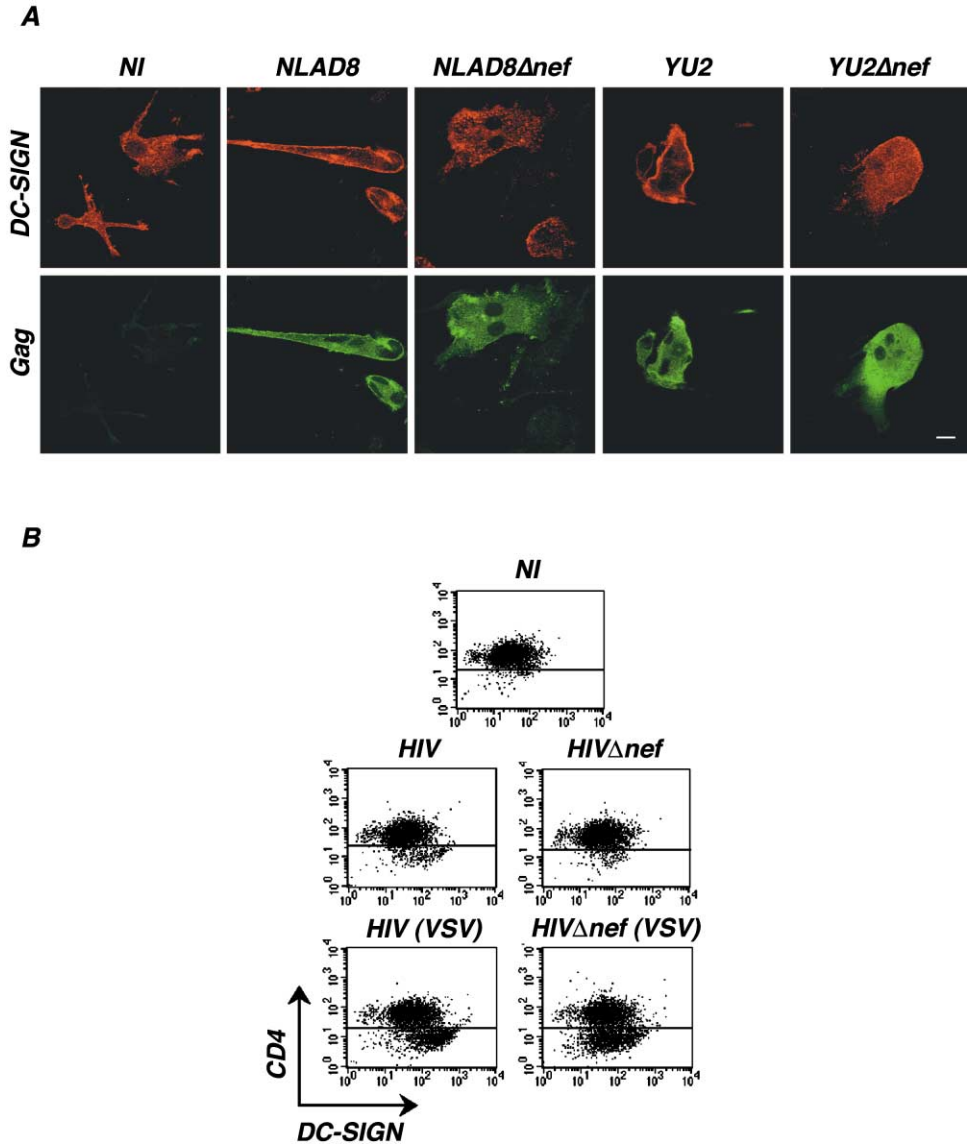

Figure 1. Upregulation of DC-SIGN in HIV-1-Infected Cells

(A) Immature DCs were exposed to the indicated HIV-1 strains. Cells were doubly stained 4 days later with anti-DC-SIGN and anti-HIV-1 Gag antibodies (Abs) and analyzed by IF and confocal microscopy. A representative medial section is shown. (NI, noninfected cells; scale bar, 10 μm.)

(B) HeLa $CD4^+DC\text{-}SIGN^+$ cells (P4-DC3 clone) were infected with HIV_{NL43} or with HIV(VSV), an env-deleted HIV-1 pseudotyped with the VSV-G envelope. Cells were doubly stained 2 days later with anti-CD4 and anti-DC-SIGN Abs and analyzed by flow cytometry. Data are representative of two independent experiments.

A Dileucine-Based Sorting Motif Regulates DC-SIGN Trafficking

We investigated the intracellular trafficking of DC-SIGN, which is poorly characterized, and the mechanisms of the action of Nef on this lectin. Experiments were performed using a transient transfection assay, which we had previously designed for studying the effect of Nef on MHC-I (Le Gall et al., 1998). HeLa cells, which are DC-SIGN negative, were cotransfected with a GFP vector along with plasmids expressing DC-SIGN or Nef. About 30%–50% of the cells were transfected, as measured by detecting GFP at 24 hr posttransfection (data not shown). DC-SIGN expression was examined by flow cytometry and IF confocal microscopy. Analysis was focused on the fraction of transfected (GFP^+) cells. We established previously that under these experimental conditions, the amount of Nef protein expressed in transfected cells was similar to that produced in HIV-infected cells (Le Gall et al., 1998).

A variety of leucine-based and tyrosine-based motifs are located in the cytoplasmic tail of integral membrane proteins, where they function as sorting signals for the endocytic pathway (Bonifacino and Dell'Angelica, 1999). These motifs are recognized by adaptor protein (AP) complexes (Hirst and Robinson, 1998). Association of AP complexes with cytosolic clathrin induces formation

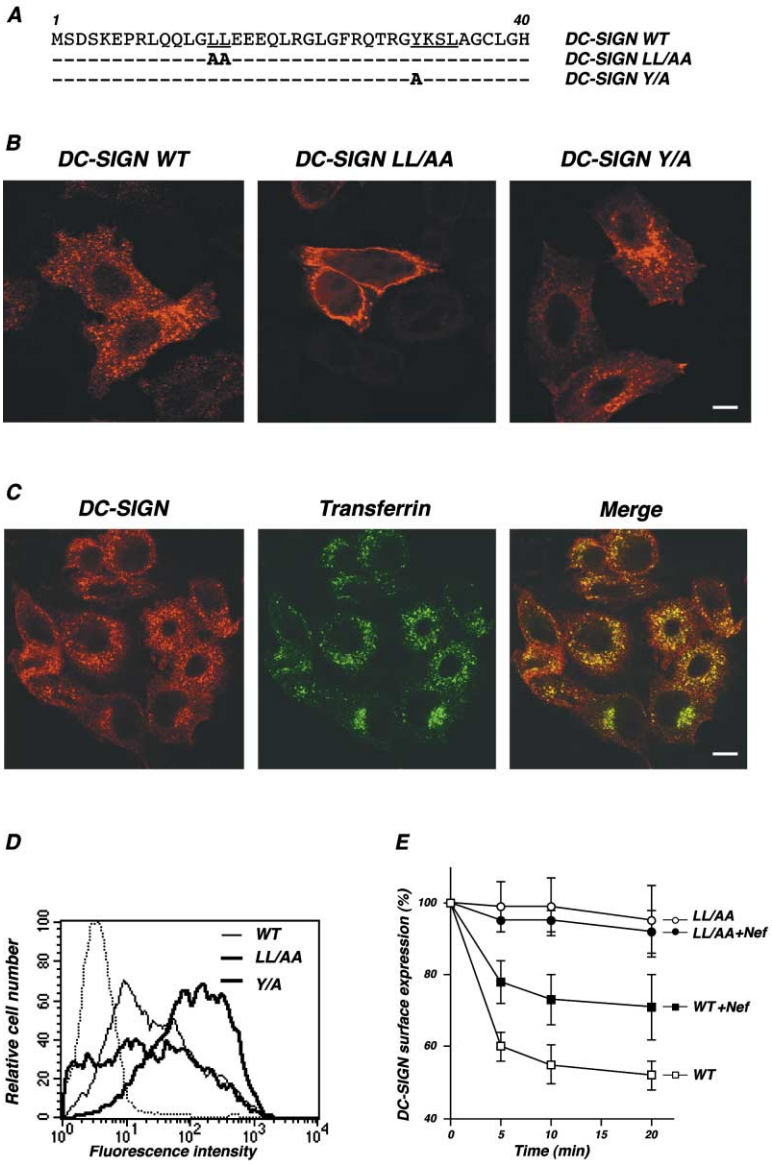

Figure 2. Cellular Localization of Wild-Type and Mutant DC-SIGN Proteins

(A) Amino acid (aa) sequence alignment of the cytoplasmic domain of WT and mutant DC-SIGN proteins. Bold letters identify aa substitutions. The two putative sorting signals are underlined.

(B) Subcellular localization of WT and mutant DC-SIGN proteins. HeLa cells were transfected with the indicated DC-SIGN vectors along with a GFP reporter plasmid. Cells were stained with anti-DC-SIGN mAb 24 hr later. Localization of DC-SIGN was examined by confocal microscopy in GFP^+ cells. (Scale bar, 10 μm.)

(C) Colocalization of DC-SIGN with transferrin-FITC, a marker of early endosomes. HeLa cells were transfected with DC-SIGN WT vector. After 24 hr, cells were incubated with transferrin-FITC, stained with AZN-D1 mAb, and analyzed by confocal microscopy. The right panel is a superposition of the two stainings, in which costained regions appear in yellow.

(D) Surface levels of WT and mutant DC-SIGN. Transfected HeLa cells were stained with AZN-D1. DC-SIGN surface levels were analyzed in GFP^+ cells by flow cytometry.

(E) Kinetics of internalization of WT and mutant DC-SIGN proteins. HeLa cells were transfected with the indicated DC-SIGN vectors along with a GFP reporter vector and a HIV_{LAI} Nef expression plasmid. After 24 hr, cells were labeled at 4°C with the anti-DC-SIGN mAb AZN-D2, washed, and incubated at 37°C for the indicated periods of time. Cells were then cooled at 4°C and stained with fluorescent anti-mouse IgG antibodies. Data are the ratios of the fraction of positive cells at different time points to the fraction of positive cells at time zero. Results from three independent experiments (mean ± SD) are shown.

of clathrin-coated vesicles and routing of proteins toward endosomal compartments. Two putative sorting signals (LL and YSQL sequences; Figure 2A) are located in the cytoplasmic tail of DC-SIGN (Soilleux et al., 2000). We examined whether these sequences play a role in DC-SIGN trafficking. Mutants were constructed in which the tyrosine and leucine residues were replaced by alanine (DC-SIGN Y/A and LL/AA, respectively). We compared the intracellular localization of the mutant and wild-type proteins (DC-SIGN WT). Confocal microscopy indicated that DC-SIGN WT surface staining was low, with numerous cytoplasmic dots visible in the perinuclear region and at the cell margins (Figure 2B). DC-SIGN WT significantly colocalized with transferrin, a marker of early endosomes (Figure 2C), strongly suggesting that the receptor is mostly distributed within the endocytic compartment. Mutation of the tyrosine-based sequence did not modify the localization of DC-SIGN, except that perinuclear staining was slightly brighter (Figure 2B). In striking contrast, the LL/AA mutant was primarily located at the plasma membrane, with weak intracellular staining (Figure 2B). Flow cytometry analysis confirmed that surface levels of the LL/AA mutant were 4-fold higher than those of DC-SIGN WT or Y/A (Figure 2D). We then compared the rate of endocytosis of WT and LL/AA DC-SIGN in a flow cytometry-based assay (Le Gall et al., 2000). Surface molecules were stained with a DC-SIGN mAb (AZN-D2) at 4°C, and then cells were incubated at 37°C. At different times, AZN-D2-bound DC-SIGN surface molecules were revealed with a secondary Ab. DC-SIGN WT proteins present at the surface were rapidly endocytosed, with a half-life of about 15 min (Figure 2E). In contrast, the LL/AA mutant was steadily expressed at the plasma membrane, with more than 90% of the molecules remaining at the surface after 20 min (Figure 2E).

These results indicated that Ab-bound DC-SIGN molecules are rapidly internalized from the cell surface. Re-

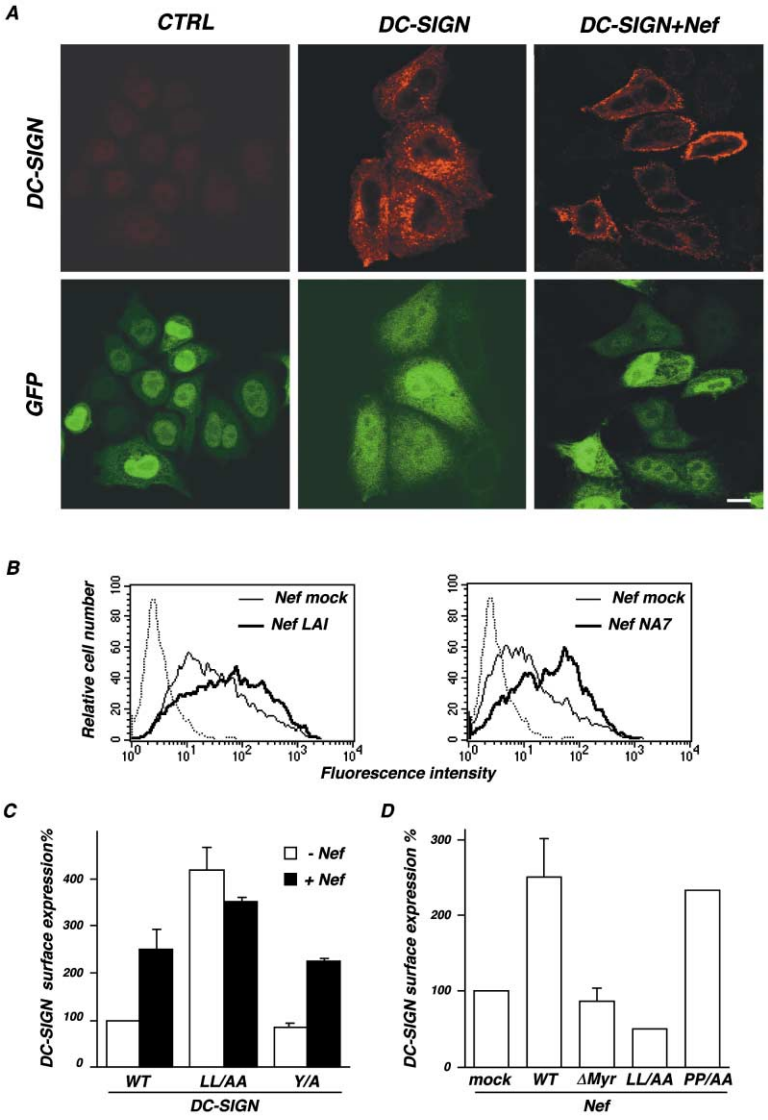

Figure 3. Effect of Nef on DC-SIGN Surface Expression

(A) Subcellular localization of DC-SIGN with or without Nef. HeLa cells were transfected in order to express DC-SIGN, GFP, and, when indicated, the Nef protein (NA7 strain). After 24 hr, cells were stained with anti-DC-SIGN mAb. Localization of DC-SIGN in GFP^+ cells was examined by confocal microscopy. Cells transfected with the Nef NA7-GFP plasmid only (CTRL) were stained as a negative control. (Scale bar, 10 μm.)

(B) Surface levels of DC-SIGN with or without Nef. HeLa cells were transfected in order to express DC-SIGN, GFP, and the Nef proteins from HIV_{LAI} (Nef LAI, left panel) or HIV_{NA7} (Nef NA7, right panel). A plasmid encoding the *nef* gene in an antisense orientation was used as a negative control (Nef mock curves). After 24 hr, DC-SIGN surface expression was measured in GFP^+ cells by flow cytometry. Data are representative of three independent experiments.

(C) Surface levels of WT and mutant DC-SIGN with or without Nef. HeLa cells were transfected in order to express WT or mutant DC-SIGN, GFP, and, when indicated, the Nef protein (NA7 strain). After 24 hr, surface expression of DC-SIGN was measured in GFP^+ cells by flow cytometry. Steady-state surface levels (mean fluorescence intensity, or MFI) of DC-SIGN WT measured without Nef were defined as 100%. Results from three independent experiments (mean ± SD) are shown.

(D) Effect of Nef mutants on DC-SIGN surface expression. HeLa cells were transfected in order to express DC-SIGN WT, GFP, and, when stated, Nef WT, NefΔmyr (G_2A mutant), NefLL/AA (Nef $L_{165}L_{166}AA$), or NefPP/AA (Nef $P_{72}P_{75}AA$) proteins. After 24 hr, surface expression of DC-SIGN was measured in GFP^+ cells by flow cytometry. DC-SIGN steady-state surface levels (MFI) measured without Nef were defined as 100%. Results from three independent experiments (mean ± SD) are shown.

cruitment by the cell sorting machinery is mediated by the recognition of a dileucine motif located in the cytoplasmic tail of the molecule.

Nef Upregulates DC-SIGN Surface Expression

We further examined the effect of Nef on DC-SIGN trafficking. HeLa cells were transiently transfected with a DC-SIGN vector, with or without a plasmid encoding Nef. Expression of the viral protein was verified by Western blotting and by IF analysis (data not shown). Expression levels were comparable to those detected in HIV-infected cells (Le Gall et al., 1998). Confocal microscopy confirmed that in the absence of Nef, DC-SIGN was mostly located in intracellular vesicles (Figure 3A). Nef induced significant changes in the localization of DC-SIGN, which accumulated at the plasma membrane (Figure 3A). Of note, the intracellular localization of DC-SIGN in Nef-expressing cells was reminiscent of that of DC-SIGN LL/AA (compare Figures 2B and 3A). Flow cytometry analysis revealed that DC-SIGN surface levels were increased 2.5-fold in the presence of Nef protein from the prototypic HIV_{LAI} strain (Figures 3B and 3C). Upregulation was also induced by NA7 Nef, a protein derived from a primary HIV-1 isolate (Greenberg et al., 1998) (Figure 3B). These results indicated that among HIV-1 proteins, Nef is necessary and sufficient for inducing DC-SIGN upregulation. This property of Nef is not restricted to laboratory-adapted HIV-1 strains.

We then examined whether DC-SIGN mutants were susceptible to Nef regulation. The DC-SIGN Y/A mutant was upregulated by Nef (Figure 3C), indicating that the effect of Nef is not mediated by the YSQL sequence of the lectin. In contrast, DC-SIGN LL/AA surface levels, which were already high, were not increased by Nef (Figure 3C). Thus, the effect of Nef and that induced by the removal of the dileucine signal were not additive. We also compared the rates of endocytosis of DC-SIGN WT and LL/AA molecules in the absence and in the presence of Nef. Stability of DC-SIGN at the cell surface was enhanced by Nef, showing 70% of WT molecules at the plasma membrane after 20 min (Figure 2E). Thus, Nef increased DC-SIGN surface levels mostly by pre-

venting its internalization. The effect of Nef was less pronounced than that of the dileucine sorting signal. Moreover, the high surface stability of DC-SIGN LL/AA was not modified by Nef (Figure 2E).

All together, these data indicated that Nef upmodulates DC-SIGN expression by modifying the intracellular trafficking of the lectin. Nef inhibits the endocytosis of surface DC-SIGN molecules. This effect is reminiscent of that induced by the destruction of the dileucine sequence located in the cytoplasmic tail of the lectin, strongly suggesting that Nef antagonizes the activity of this sorting motif.

We next investigated which determinants of Nef contribute to modify DC-SIGN trafficking. We analyzed three well-characterized Nef mutants, whose expression levels are equivalent to those of the WT viral protein. This point was verified by Western blot analysis (data not shown). A myristoylation-negative Nef mutant (NefΔmyr) was defective in upregulating DC-SIGN surface expression (Figure 3D), strongly suggesting that attachment of Nef to cellular membranes is required for its activity. A conserved dileucine motif located in an exposed carboxy-terminal loop of Nef is responsible for interaction with AP complexes (Bresnahan et al., 1998; Craig et al., 1998; Greenberg et al., 1998). This motif is crucial for CD4 downregulation, but not for MHC-I downregulation. Mutating the dileucine motif (Nef LL/AA) abolished the effect of Nef on DC-SIGN (Figure 3D) and on CD4 (data not shown). Nef also bears a proline-rich region, which is part of an SH3 binding motif and is required for MHC-I downregulation (Mangasarian et al., 1999). A Nef mutant of two of these proline residues (Nef PP/AA), which is defective for downregulating MHC-I, was still active on DC-SIGN (Figure 3D) and on CD4 (data not shown). We conclude that Nef attachment to cellular membranes and binding to the clathrin AP complexes are required for its activity on DC-SIGN. In contrast, interaction of the polyproline region of Nef with proteins carrying an SH3 domain appears dispensable.

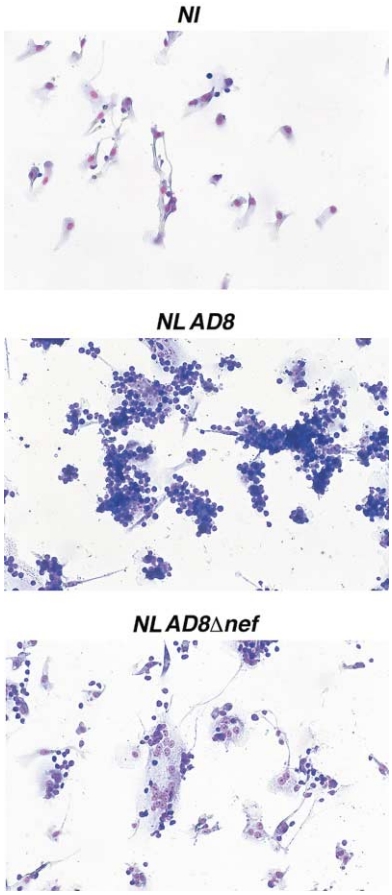

Figure 4. Clustering of DCs with Lymphocytes

Immature DCs were exposed to R5-tropic NLAD8 and NLAD8Δnef HIV-1 strains. Six days after infection, cells were incubated with PBMCs for 45 min at 37°C. Cells were then stained with May-Grüenwald-Giemsa (MGG). DCs appeared as large cells with light pink nucleus. Lymphocytes were smaller and dark blue stained. (NI, noninfected cells.) Representative fields are shown.

Clustering of HIV-Infected DCs with Lymphocytes

We examined the functional consequences of the upregulation of DC-SIGN. A crucial role of DC-SIGN is to mediate contact between DCs and T cells through binding to ICAM-3 (Geijtenbeek et al., 2000c). We asked whether Nef-induced DC-SIGN upregulation impacts the ability of DCs to form clusters with lymphocytes. To this aim, DCs were infected with WT or Δnef NLAD8 viruses. After 6 days of culture, numerous syncytia were visible, confirming virus replication. Activated PBLs were added to DCs for 45 min. Cells were fixed and stained with May-Grüenwald-Giemsa. DCs appeared as large cells with light pink nuclei. Lymphocytes were much smaller and stained dark blue, and thus were easily distinguishable from DCs. In DCs not exposed to virus, only a few DC-T cell clusters were visible, each containing a few lymphocytes (Figure 4). After infection with NLAD8 WT virus, the capacity of DCs to form clusters was dramatically increased (Figure 4). This phenomenon was observed with both isolated DCs and syncytia, indicating that it was not due to special features associated with syncytia. Remarkably, DC-T cell clusters were much less abundant when DCs had been infected with NLAD8Δnef. Similar results were observed when DCs were infected with another R5 strain (YU-2; data not shown).

We wondered whether the increase of lymphocyte clustering upon HIV infection of DCs was related to the effect of Nef on DC-SIGN surface expression. To study this, we used two potent inhibitors of DC-SIGN-ICAM-3 interactions, the carbohydrate mannan and a mixture of two anti-DC-SIGN antibodies (AZN-D1 and AZN-D2) (Geijtenbeek et al., 2000c). In the experiment depicted in Figure 5, about 70% of the cells expressed Gag after infection with NLAD8 (data not shown). Syncytia, whose abundance varied depending on the DC source, were barely visible with the donor shown. Numerous clusters were formed upon addition of lymphocytes to HIV-infected DCs. Both mannan and anti-DC-SIGN antibodies strongly inhibited the formation of lymphocyte clusters with HIV-1-infected DCs (Figure 5A), whereas an unrelated isotypic antibody was ineffective (data not shown).

In order to quantify cluster formation, lymphocytes

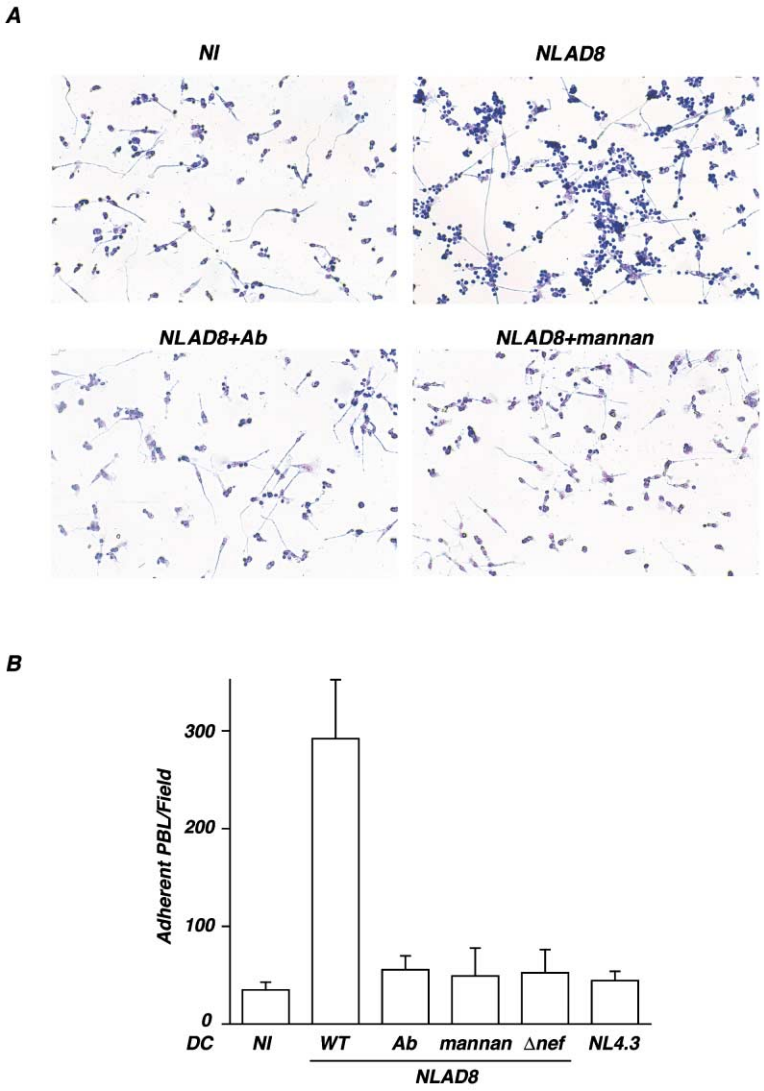

Figure 5. Clustering of HIV-Infected DCs with Lymphocytes Is Mediated by DC-SIGN

Immature DCs were exposed to the R5-tropic NLAD8 and NLAD8Δnef or to the X4-tropic NL43 HIV-1 strains.

(A) Effect of anti-DC-SIGN mAbs and of mannan on DC-T cell clustering. Six days after infection of DCs with NLAD8, cells were preincubated for 20 min with blocking antibodies against DC-SIGN (AZN-D1 and AZN-D2) or with mannan before addition of PBMCs for 45 min. Cells were then stained with MGG. Representative fields are shown.

(B) Quantitative analysis. PBMCs were labeled with a fluorescent dye before incubation with DCs for 45 min at 37°C. Cells were then fixed, and the number of fluorescent cells per field was scored. At least three fields were analyzed. Data are mean ± SD from two independent experiments. DCs and PBMCs used in Figure 5 are from a different donor than those used in Figure 4. (NI, noninfected cells.)

were labeled with the fluorescent dye Calcein-A and added to DCs for 45 min at 37°C. Clustering was revealed by microscopy, and the number of adherent PBLs per field was scored. Infection with HIV NLAD8 induced a significant increase in the ability of DCs to cluster with lymphocytes (Figure 5B). This increase was not observed with NLAD8Δnef. Mannan and anti-DC-SIGN antibodies reduced cluster numbers to levels observed in noninfected DCs (Figure 5B). Furthermore, low levels of clustering in DCs exposed to the X4 strain NL43 indicated that the process required de novo synthesis of viral proteins. Similar results were observed when resting T cells or purified $CD8^+$ cells were added to DCs instead of activated lymphocytes (data not shown). Thus, HIV-1-infected DCs form clusters with lymphocytes, irrespective of their activation state.

All together, these results demonstrated that HIV-1 infection of DCs dramatically increases their ability to form clusters with lymphocytes . This phenomenon involves Nef-induced upregulation of DC-SIGN expression.

DC-SIGN Surface Stabilization Increases HIV-1 Transmission

We previously reported that Nef is required for efficient HIV-1 replication in cocultures of DCs and lymphocytes (Petit et al., 2001). We examined whether upregulation of DC-SIGN plays a role in this process. Experiments were performed using HeLa cells as donor cells. They are CD4 negative and thus not susceptible to HIV infection. DC-SIGN WT, with or without Nef, or DC-SIGN LL/AA was transiently expressed by transfection. Cells were then exposed to a low-HIV-1 inoculum (10 ng of p24 per 10^6 cells) for 2 hr and incubated with activated PBMCs after extensive washing. Viral replication was measured as p24 production into supernatants. A representative experiment is shown in Figure 6. When cells were transfected with the control vector, virus production was close to background levels. Therefore, HeLa cells do not efficiently transfer HIV-1 to lymphocytes. Expression of DC-SIGN was associated with viral replication in PBMCs, confirming that virion capture by this molecule promotes transmission to T cells (Geijtenbeek et al.,

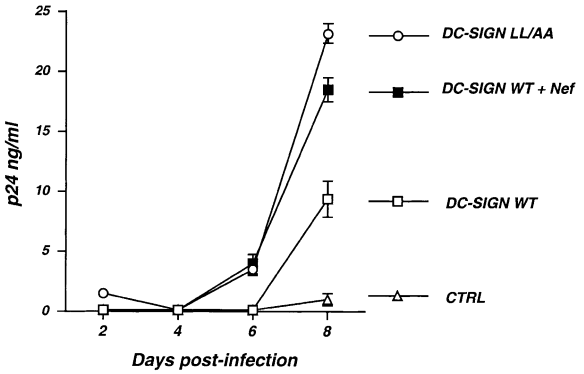

Figure 6. Upregulation of DC-SIGN Facilitates Trans-Infection of Lymphocytes

HeLa cells were transfected with the indicated DC-SIGN and Nef LAI vectors. After 24 hr, cells were incubated with HIV-1 (NL43 strain) for 2 hr at 37°C, washed to remove unbound virus, and cocultured with activated PBMCs. Viral replication was assessed by measuring p24 production in culture supernatants. Similar results were obtained with NLAD8 (data not shown). Data are expressed as mean ± SD of triplicates and are representative of two independent experiments.

2000b). Interestingly, virus transmission was significantly enhanced in the presence of Nef or when DC-SIGN LL/AA was expressed, as observed by higher levels of p24 production in PBMCs (Figure 6). Thus, DC-SIGN upregulation significantly increases HIV-1 transmission to lymphocytes.

Discussion

This work elucidates a mechanism by which lymphocytes efficiently adhere to HIV-infected DCs. Upon infection with R5-tropic HIV-1 strains, DC-SIGN is upregulated at the surface of DCs. This process is induced by Nef, which inhibits DC-SIGN endocytosis. Upregulation of DC-SIGN increases the ability of DCs to form clusters with lymphocytes, thus facilitating transmission of infectious viral particles. This novel mechanism potentially contributes to HIV-1 spread in vivo.

DC-SIGN Endocytosis

We show here that a dileucine sequence located in the cytoplasmic tail of DC-SIGN functions as a sorting signal, regulating the intracellular trafficking and endocytosis of the lectin. DC-SIGN is likely internalized by the clathrin-dependent machinery, since dileucine signals directly interact with AP complexes (Hirst and Robinson, 1998). What is the role of DC-SIGN endocytosis? Many lectins mediate both pathogen recognition and cell-cell interactions (Weis et al., 1998). DC-SIGN internalization could play a role in both of these processes. Internalization signals are present in other carbohydrate binding proteins, including the macrophage lectin, the mannose receptor, the dendritic DEC-205 molecule, and DC-SIGNR (or L-SIGN), a DC-SIGN-related molecule expressed in endothelial cells (Soilleux et al., 2000). DEC-205 and the mannose receptor direct captured antigens to specialized processing compartments. A likely role of DC-SIGN endocytosis would be to mediate antigen capture by DCs. So far, the only antigens known to bind DC-SIGN are lentiviral envelope glycoproteins. It will be worth examining whether DC-SIGN captures other antigens and whether internalization rates of the lectin are regulated upon binding of ligands, such as antigens or ICAM molecules. Also, DC-SIGN endocytosis may provide a pathway for the uptake of incoming HIV-1 particles into DCs, thus protecting them from the extracellular milieu (Blauvelt et al., 1997). Although this possibility was not addressed here, the endocytosis-defective DC-SIGN mutant provides a useful tool for assessing the consequence of virion internalization on the preservation of infectivity.

We focused our study on the role of DC-SIGN surface expression on DC-T cell interactions. DC-SIGN stabilization at the surface, induced by Nef or by removing the dileucine motif, enhanced the ability of target cells to bind ICAM-3-coated beads (data not shown). Furthermore, we observed a dramatic increase of lymphocyte adhesion to Nef-expressing, HIV-infected DCs. This process involved DC-SIGN, since it was inhibited by mannan and by anti-DC-SIGN antibodies. It has been established that DC-SIGN binding to ICAM-3 mediates a transient adhesion between DCs and T cells (Geijtenbeek et al., 2000c). Early DC-SIGN-ICAM-3 interaction may enable the TCR to scan for epitopes presented by MHC molecules, initiating and regulating immune responses. This early contact drives the formation of the "immunological synapse" between DCs and T cells (Steinman, 2000). It is conceivable that DC-SIGN endocytosis influences the timed and spatially organized contact between DCs and T cells. We show here that stabilization of DC-SIGN-ICAM-3 interactions significantly increases lymphocyte clustering. This will likely affect the complex array of molecular events leading to proper T cell activation. Our experiments demonstrate that DC-SIGN endocytosis is a pivotal phenomenon regulating interactions between DCs and lymphocytes.

In addition, DC-SIGN-ICAM-2 interaction mediates DC transmigration across the endothelium (Geijtenbeek et al., 2000a). DC-SIGN upregulation might also impact the interaction of the lectin with ICAM-2, thus potentially affecting DC trafficking.

Mechanism of Nef-Induced DC-SIGN Upregulation

We show that Nef induces a 2- to 3-fold increase in DC-SIGN steady-state surface levels by significantly inhibiting DC-SIGN internalization. The effects of Nef on DC-SIGN could be mimicked by removing the dileucine sorting motif of the lectin. DC-SIGN LL/AA was not further upregulated by Nef, indicating that the effects of the sorting signal and of Nef are not additive. Analysis of a series of Nef mutants demonstrated that a dileucine motif present within an exposed C-terminal loop of the viral protein is required for the effect of Nef on DC-SIGN. This critical dileucine motif is known to mediate direct interactions of Nef with AP complexes. Thus, these observations lead to a model in which Nef, by interacting with AP complexes, affects the proper recognition of DC-SIGN by the cellular sorting machinery.

Nef-induced MHC-I and CD4 downregulation are mediated by distinct mechanisms. Both events require the presence of sorting signals in target molecules. Nef-

induced MHC-I downregulation reflects mainly a misrouting of the immune receptor from the Golgi (Le Gall et al., 1998, 2000). Direct binding of Nef to AP complexes is not required for MHC-I downregulation, which rather involves the TGN sorting protein PACS-1 (Piguet et al., 2000). Nef-induced CD4 downregulation probably requires the establishment of a connection between the cellular endocytic machinery and the cytoplasmic domain of CD4 (Piguet et al., 1999). In this model, the N-terminal domain of Nef is involved in binding to CD4, whereas the dileucine motif is responsible for recruiting AP complexes of clathrin-coated pits (Bresnahan et al., 1998; Craig et al., 1998; Greenberg et al., 1998). Other Nef binding proteins, such as NBP1, a subunit of a vacuolar ATPase, might consolidate Nef-AP interactions (Lu et al., 1998). Our analysis of Nef mutants demonstrated that the effects of Nef on DC-SIGN and CD4 share similarities, both being mediated by a modification of the turnover of surface receptors and both likely involving interaction of Nef with AP complexes. However, whereas DC-SIGN is upregulated, CD4 is downmodulated. There are additional surface molecules affected by Nef. MHC-I and CD28 are downmodulated, while surface expression of the invariant chain of MHC-II and of TNF and LIGHT cytokines is enhanced (Lama and Ware, 2000; Stumptner-Cuvelette et al., 2001; Swigut et al., 2001). Although a number of surface molecules (including the receptors for EGF, LDL, mannose-6 phosphate, and the CD8, CD20, CD25, CD69, and HLA-C molecules) are not affected by Nef, our results indicate that the effects of Nef on cellular trafficking are more extensive than initially thought.

Why some receptors are upmodulated whereas others are downregulated by Nef remains speculative. Upregulation of DC-SIGN is not a consequence of the effects of Nef on CD4, since it is observed in HeLa cells, which lack CD4 expression. Most of the effects of Nef on trafficking require the critical dileucine motif, highlighting the importance of the interaction of Nef with AP complexes. Nef distorts the sorting function of AP complexes, scrambling the trafficking of CD4 and CD28 in lymphoid cells and of DC-SIGN in DCs. One can speculate that the trafficking of as yet unidentified proteins will also be affected by Nef.

DC-SIGN Upregulation and HIV Infection

The upmodulation of DC-SIGN in HIV-1-infected DCs has important immunological and virological implications. Nef-induced upregulation of DC-SIGN was observed in primary cells and with various viral strains, including primary isolates. Thus, it is likely that upregulation of DC-SIGN occurs in infected DCs in vivo. HIV-1 infection is characterized by a state of immune hyperactivation, associated with a progressive impairement of $CD8^+$ and $CD4^+$ T cell functions . We show that upregulating DC-SIGN surface levels significantly increased DC-T cell cluster formation. It is tempting to speculate that this phenomenon affects the potency of DCs to activate lymphocytes. Inhibiting DC-SIGN endocytosis may thus represent a pivotal feature in the ability of HIV-1 to manipulate the immune response.

R5-tropic, but not X4-tropic, HIV-1 strains replicate in cultures of immature DCs (Granelli-Piperno et al., 1998; Petit et al., 2001). Although virus production is low, it is vigorously increased upon binding of DCs to $CD4^+$ T cells. Numerous DC-T cell conjugates are formed, which ultimately fuse to form syncytia. We show that this process is promoted by Nef, which acts on DC-SIGN surface levels. Nef is expressed early and abundantly during the viral cycle. DC-SIGN upregulation likely occurs before the synthesis of viral structural proteins. This would allow for a sustained contact between DCs and $CD4^+$ T cells, which might facilitate viral spread and/or syncytia formation when fusogenic Env glycoproteins are produced. Other phenomena, such as maturation of DCs or CD40- and CD80-mediated contacts (Pinchuk et al., 1994), may additionally play an important role in the interaction between HIV-infected DCs and T cells.

Our results help explain the puzzling observation that R5-tropic strains are preferentially transmitted among humans, though this restriction process is probably multifactorial. It has been suggested to take place at the level of DC infection (Reece et al., 1998) and to involve the differential expression and signaling abilities of HIV-1 coreceptors (Lin et al., 2000; Zaitseva et al., 1997). Although DC-SIGN can transport both X4 and R5 isolates, only R5 strains will lead to productive infection and Nef expression in DCs. Nef-induced DC-SIGN upregulation could thus provide a decisive selective advantage for subsequent dissemination of R5 strains to T cells. It is currently unclear which DC subsets are the targets for HIV-1, and further work is required to address whether Nef functions in these subsets. HIV-1-infected DCs have been detected in adenoids and tonsils of seropositive individuals (Frankel et al., 1997, 1996). Infected cells appeared as multinucleated syncytia, potentially involving DCs and T cells, and were observed at all stages of disease. This suggests that Nef could promote viral spread during both acute and chronic infection. Moreover, DC-SIGN expression may be broader than initially thought, likely including certain types of macrophages in vivo (Mummidi et al., 2001). Our observations can be relevant to these cell types, which also support HIV infection.

In summary, our data emphasize the complex interactions between DCs and HIV-1. The viral protein Nef, by upregulating DC-SIGN surface expression, potentially manipulates both the immune response and viral dissemination.

Experimental Procedures

Generation of Mononuclear Subsets

DCs were prepared using a VacCell processor (Goxe et al., 1998). Briefly, PBMCs from leukapheresis were cultured 7 days in serum-free AIM-V medium (Gibco) supplemented with 500 U/ml GM-CSF (a kind gift from Novartis) and 50 ng/ml IL-13 (Sanofi), and DCs were isolated by elutriation. The isolation procedure yielded $CD1a^+MHC\text{-}I^+$, $MHC\text{-}II^+$, $CD64^-$, $CD83^-$, CD80-low, CD86-low cells, a phenotype corresponding to immature DCs. DC purity was >95%. When stated, PBMCs were activated with PHA and cultivated in the presence of IL-2 (50 U/ml; Chiron).

Cells, Viruses, and Infections

HeLa $CD4^+DC\text{-}SIGN^+$ cells (clone P4-DC3) were derived from HeLa-$CD4^+$ LTR-LacZ cells (clone P4) by infection with a lentiviral vector encoding for DC-SIGN. Individual clones were screened for DC-SIGN surface expression (C. Nobile et al., personal communication). The production and use of WT and Δnef HIV_{NL43}, $HIV_{YU\text{-}2}$, HIV_{NLAD8},

and HIV(VSV) strains have been described (Maréchal et al., 1998; Petit et al., 2001). Immature DCs (2.5×10^5 cells) were exposed to the indicated virus stocks (25 ng of p24). After overnight incubation, cells were washed and grown on glass coverslips. Transfected HeLa cells (5×10^5 cells) were exposed to the indicated virus preparations (10 ng of p24). After 2 hr at 37°C, cells were washed and PBMCs (10^6 cells) were added. P4-DC3 cells were infected (m.o.i., 0.1–0.4) as described (Maréchal et al., 1998).

Plasmid Construction and Transfection

The DC-SIGN WT vector contains the DC-SIGN gene in pRcCMV (Geijtenbeek et al., 2000c). The Nef-FT WT and Nef-mock CMV promoter-based plasmids carry the *nef LAI* gene in a sense and antisense orientation (Le Gall et al., 1998). Bicistronic plasmids coexpressing Nef NA7 (WT or mutant proteins) and GFP were kindly provided by J. Skowronsky (Greenberg et al., 1998). Nef LAI or DC-SIGN mutants were generated with the Quick Change kit (Stratagene). The sequence of DC-SIGN mutants was verified by sequencing. HeLa cells were cotransfected with Nef, DC-SIGN, and GFP expression vectors (2, 1, and 0.05 μg for 2.5×10^5 cells, respectively) as described (Le Gall et al., 1998). The GFP vector was omitted when the bicistronic Nef NA7-GFP vector was used. A vector encoding a defective (nonmyristoylated) Nef or the Nef-mock plasmid were transfected as negative controls, yielding similar results.

Flow Cytometry and Immunofluorescence Analysis

HeLa cells were processed for flow cytometry or immunofluorescence (IF) analysis 24 hr after transfection, as described (Le Gall et al., 1998). Cells were stained with anti-DC-SIGN mAbs AZN-D1 or AZN-D2 (Geijtenbeek et al., 2000c) or with an isotype IgG1 mAb as a control. Surface levels of DC-SIGN were measured in GFP^+ cells, which represented the fraction of the cell population (~30%–50%) that was transfected. Anti-CD4 mAb SK3-PE (Becton Dickinson) and rabbit polyclonal anti-DC-SIGN Abs (CSRD, raised against a C-terminal region of the molecule) were used for double stainings. When stated, transferrin-FITC (10 μg/ml; Molecular Probes) was added in serum-free medium for 5 min at 37°C before fixing the cells. For kinetics of DC-SIGN surface internalization (Le Gall et al., 2000), HeLa cells were stained for 45 min at 4°C with AZN-D2 mAb, washed, and incubated at 37°C. At different periods of time, AZN-D2-bound DC-SIGN surface molecules were revealed by a secondary Ab, and cells were analyzed by cytofluorometry. DCs were processed for IF as were HeLa cells, except that incubations were performed with 1% goat serum. Rabbit anti-Gag serum was obtained through the NIH AIDS Research and Reference reagent program. Confocal microscopy was performed on a Leica TCS4D instrument. Series of optical sections at ~0.5 μm intervals were recorded and mounted using Adobe Photoshop software.

DC-T Cell Clustering Assay

DCs (2.5×10^5 cells) were infected with the indicated viral strains (25 ng of p24) and incubated on glass coverslips. Four or six days after infection, PBMCs (5×10^5 cells/well) were added to DC, centrifuged at low speed (1000 × g for 1 min), and incubated for 45 min. Nonadherent cells were removed by extensive washing with PBS, and DC-T cell conjugates were fixed in methanol, stained with May-Grüenwald-Giemsa, and examined microscopically. When stated, mannan (20μg/ml), anti-DC-SIGN mAbs (AZN-D1 and -D2, 20μg/ml), or isotype control Ab (data not shown) was added 20 min before and during contact with PBMCs. For quantitative analysis, PBMCs were labeled with Calcein AM (1μg/ml; Molecular Probes) for 30 min at 37°C and incubated with DCs similarly to unlabeled PBMCs. The number of adherent fluorescent cells per field were scored. At least three fields per well were analyzed.

Acknowledgments

We thank S. Michelson and M. Alizon for critical reading of the manuscript, T. Geijtenbeek for helpful discussions, E. Perret for confocal microscopy analysis, and M. Feinberg, E. Freed, J. Skowronski, and the NIH AIDS Research and Reference reagent program for the kind gift of reagents. A.M. is a fellow of European Community funding (QLK2-CT 2000-01630). This work was supported by grants from the Agence Nationale de Recherche sur le SIDA (ANRS), SIDACTION, the European Community, the Dutch AIDS Foundation (5008), and the Dutch Heart Foundation (970701).

Received August 6, 2001; revised November 8, 2001.

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