Enhanced CD4 Down-modulation by Late Stage HIV-1 nef Alleles Is Associated with Increased Env Incorporation and Viral Replication*

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Three viral proteins participate in the down-modulation of CD4 in human immunodeficiency virus type 1 (HIV-1)-infected cells. The underlying mechanisms have been extensively investigated. However, the physiological relevance of this phenomenon remains poorly understood. To address the role of CD4 down-modulation in HIV-1 pathogenesis in vivo, we have characterized the functional properties of nef alleles isolated from seven HIV-1-infected patients at either the stage of AIDS (late alleles) or during the asymptomatic phase of infection (early alleles). HIV-1 variants carrying these nef alleles showed striking differences in CD4 down-modulation, virus infectivity, and replication properties. Infection of T cells with late strains resulted in production of viral particles with enhanced infectivity, as compared with variants carrying early nef alleles. These differences in infectivity were observed only when viruses were produced in cells with high levels of the viral receptor, suggesting a functional link between CD4 levels and the ability of Nef to down-modulate CD4 and to enhance viral infectivity. Similarly, late nef alleles were substantially more active than early nef genes in stimulating HIV-1 replication in high CD4-positive cells, including primary lymphocytes, but not in cells expressing low levels of the CD4 receptor. Single-round assays showed that differences in infectivity between late and early strains are largely reduced when evaluated in target cells with high levels of CD4, suggesting that the inhibitory effect occurs at the entry step. Supporting this, enhanced CD4 down-modulation by late nef alleles was associated with higher levels of envelope incorporation into viral particles, a phenomenon that likely accounted for the augmented infectivity. Our data suggest a mechanistic link between the Nef-mediated CD4 down-modulation and the enhancement of replication in CD4-positive lymphocytes. As progression to disease occurs, HIV-1 Nef variants with enhanced ability to down-modulate CD4 are selected. These strains efficiently overcome the deleterious effects of CD4 and replicate more aggressively in CD4-positive primary lymphocytes. These results highlight the importance of the virus-induced CD4 down-modulation in HIV-1 pathogenesis.

Down-modulation of the CD4 receptor is one of the hallmarks of HIV infection. Three viral proteins participate in this process: Nef, Env, and Vpu. The mechanisms of action of these proteins have been extensively characterized. Nef enhances internalization of CD4 from the cell surface and targets the receptor for degradation into lysosomes (1–6). Env binds CD4 in the endoplasmic reticulum and blocks its transport to the cell surface (7–11), whereas CD4 molecules retained in the endoplasmic reticulum are targeted by Vpu for degradation in the cytosolic proteasome (12, 13). Nef is the only CD4 down-modulator expressed early during infection. Nef transcripts constitute the most abundantly expressed early viral gene, and overall Nef plays the major role in down-modulation of the receptor (14, 15). Furthermore, Nef is the only gene product capable of down-regulating CD4 molecules already present on the cell surface prior to infection.

Early studies with SIV-infected monkeys highlighted the importance of Nef in viral pathogenesis. SIV viruses carrying deletions in nef replicate poorly in rhesus macaques and usually do not cause AIDS (16). Other studies have shown that some of the pathologies associated with HIV infection, including immunodeficiency and loss of CD4-positive cells, can be recapitated in transgenic mice expressing HIV-1 Nef, emphasizing the importance of this protein in HIV pathogenesis (17, 18). However, it has been difficult to determine which of the in vitro functions of Nef contribute to the pathogenesis of HIV in vivo (reviewed in Ref. 19). In addition to down-modulating the viral receptor, HIV-1 Nef reduces cell surface levels of class I MHC and CD28 (20–22), increases infectivity of virions in a CD4-independent manner (23–26), increases viral replication in primary lymphocytes (27, 28), and modulates T cell activation, cytokine production, and lymphocyte chemotaxis (29–31). Studies with monkeys have specifically evaluated the role of CD4 down-modulation in SIV pathogenesis in vivo. Mutations in SIV Nef that disrupt the ability to down-modulate CD4 strongly reduce viremia in infected monkeys and revert effi-

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The abbreviations used are: SIV, simian immunodeficiency virus; HIV-1, human immunodeficiency virus; MHC, major histocompatibility complex; LTNPs, long-term nonprogressors; PBMCs, peripheral blood mononuclear primary cells; FCS, fetal calf serum; PHA, phytohemagglutinin; RT, reverse transcriptase; PSL, photo-stimulated luminescence; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium.

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The release of the new progeny occurs. Alternatively, down-modulation signals may also induce apoptosis and cytopathic effects before transcription through the HIV long terminal repeat (43). These studies have failed to explain why CD4 down-modulation might diminish in late progressors or asymptomatic carriers (40). Another study has analyzed the modulation of HIV-1, Nef functions during progression to disease. Interestingly, nef alleles isolated at early stages of infection efficiently down-modulate class I MHC. In comparison, nef alleles isolated after progression to AIDS showed enhanced ability to down-modulate CD4 and to stimulate HIV-1 replication in PBMCs, whereas class I MHC down-modulation activity is diminished in late nef alleles (41). These findings suggest that down-modulation of class I MHC plays an important role at early stages of infection, in which selective pressure to evade the action of CD8-positive cytotoxic lymphocytes is high.

The above studies also suggest that down-modulation of CD4 may influence HIV pathogenesis in vivo. However, these studies have failed to explain why CD4 down-modulation might confer a replicative advantage to HIV-1. Several hypotheses have been presented to address this issue (reviewed in Ref. 42). Binding to, or cross-linking of CD4 on the surface of infected cells may result in the transduction of signals that inhibit transcription through the HIV long terminal repeat (43). These signals may also induce apoptosis and cytopathic effects before release of the new progeny occurs. Alternatively, down-modulation of the CD4 receptor may impede superinfection, a phenomenon that may also jeopardize particle production (44). More recent findings suggest that elevated levels of CD4 may interfere with infectivity and release of viral particles (45–48).

To understand the role of CD4 down-modulation in HIV pathogenesis we have functionally characterized nef alleles isolated from seven HIV patients at different stages of infection. To evaluate specifically the contribution of CD4 down-modulation to viral pathogenesis we have utilized an experimental approach where the infectivity of HIV particles is analyzed in producer and target cells expressing varying degrees of CD4. Our results suggest that HIV variants with strong CD4 down-modulation activity, emerging as progression to disease occurs, overcome more efficiently the inhibitory effects of CD4 and, thus, replicate more aggressively in CD4-positive T cells.

METHODS AND MATERIALS

Cell Lines—Construction of Jurkat T cells expressing different amounts of surface-CD4 has been described before (48). Jurkat T cells were grown in RPMI medium containing penicillin, streptomycin, glutamine, Hepes, and 10% fetal calf serum. High CD4 cells were maintained in the presence of 20 U of IL-2 and 30 μg/mL of NaHCO3 (pH 9.0) (G418 (0.2 mg/mL), whereas low CD4 cells were grown with G418 alone (0.2 mg/mL) to avoid selection of high CD4 expressing cells. The enrichment/purification procedure was optimized to engineer high CD4 cells with receptor levels about 2-fold higher than those found in human CD4-positive primary lymphocytes, as estimated by flow cytometry. Jurkat-Low-CD4 cells expressed surface-CD4 amounts 25-fold lower than high CD4 cells. Infectivity assays were performed in either MAGIC-5 or MAGIC-5B cells (kindly provided by Dr. M. Matsuda and Dr. M. Tatsumi, Osaka University). These cell lines are derivatives of HeLa CD4-positive cells and express the β-galactosidase gene under the control of the HIV-1 long terminal repeat promoter (49). MAGIC-5B cells (referred to herein as MAGIC-High-CD4) have CD4 receptor levels 12-fold higher than MAGIC-5 (MAGIC-Low-CD4), as estimated by flow cytometry after staining with CD4-specific antibodies.

Viruses—The HIV-1 nef alleles analyzed in this study have been described before (41). These samples were obtained from seven individuals from the New England Hemophilia Center at the University of Massachusetts. For each patient, samples were taken at time points when CD4-positive T cells were within the normal range (median, early alleles: 763 ± 125 CD4+/μl and after declining to less than 200 cells/μl (median, late alleles: 84 ± 31 CD4+/μl). Primary nef alleles were PCR-amplified and used to replace the nef gene in HIV-1 NL4.3. Except in the case of SP7–88, SP7–91, P2–87, and P2–93, which were isolated at time point representative individual clones, the nef alleles used in these experiments were cloned as pools.

Transfections, Virus Preparations, and Infectivity Assays—Viruses were produced by calcium phosphate chemical transfection of human kidney fibroblastic CD4-negative 293T cells with HIV-1 NL4.3 plasmids or by electroporation of CD4-positive Jurkat-T cells. When indicated, a CD4 expression plasmid (pCMX-CD4) was included in 293T cell transfection mixtures. Jurkat-T cells were electroporated in 4-mm gap cuvettes with a Bio-Rad Gene Pulser II electroporation system set at 240 V and 950 microfarads. Unless specified, infection of T cells was performed following a spinoculation method. Briefly, 0.5 × 10^6 cells were incubated with viral supernatants (2 μg of p24 protein) in 24-well plates for the presence of 4 μg/ml polybrene and 10 μM Hepes (pH 7.4) and centrifuged at room temperature for 90 min (2,500 rpm) in a table-top centrifuge (Sorvall RT6000B). After centrifugation, cells were washed three times with RPMI medium supplemented with 10% fetal calf serum and then incubated in the same medium for 1–2 days at 37 °C. The amount of p24 antigen in supernatant fluids was estimated with an enzyme-linked immunosorbent assay kit (Beckman Coulter) and used to normalize samples containing virus. Infection of MAGIC cells was performed for 48 h in the presence of 20 μg/ml DEAR-dextran. Routinely, after 8 h of infection of target cells 10 μM 3'-azido-3-deoxythymidine was added to block new cycles of virus replication. Infected cells were scored after staining with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal).

Viral Replication Assays—The impact of Nef in viral replication was evaluated in transformed T cell lines (Jurkat, H9, or SupT1) and peripheral blood mononuclear primary cells (PBMCs). Infection of T cell lines was initiated by electroporation with 10 μg of proviral DNA, as described above. Production of p24 in culture supernatants was estimated at regular intervals. Human PBMCs were isolated with a lymphocyte separation medium (Organon Teknika). Immediately afterward, cells were infected with virus stocks containing 2 ng of p24 core antigen and kept in RPMI 1640 with 10% FCS. Three days postinfection, cells were stimulated with PHA (4 μg/ml, Sigma). Supernatants were collected at regular intervals, and virus production was measured by reverse transcriptase (RT) assay. The level of p24 present in each dot was quantitated as photo-stimulated luminescence (PSL) by using a Fuji BAS1000 phosphorimaging device as described elsewhere (50).

Flow Cytometry—Surface staining of CD4 was performed with either OKT4 mAb, followed by addition of goat anti-mouse Cy-5-conjugated antibody, or with CD4-V4 mAb (R-phycocerythrin-conjugated, BD Biosciences). The epitopes recognized by these mAbs do not overlap with the gp120-binding domain (51). Intracellular staining of p24 was performed with a p24-specific mAb (fluorescein isothiocyanate-conjugated KC57 clone, Coulter) using a cell permeabilization kit following the recommendations of the manufacturer (Caltag Laboratory). After staining, cells were fixed with 1% paraformaldehyde and analyzed in a FACS Calibur system (BD Biosciences) running with CellQuest software.

Detection of Virion-bound Env and CD4 Proteins—Incorporation of viral and cellular proteins into viral membranes was determined with a quantitative p24 virion-binding ELISA assay. Briefly, 96-well plates were coated overnight at 4 °C with 100 μl of carbonate buffer (15 mM Na2CO3, 35 mM NaHCO3, pH 9.0) containing 7.5 μg/mL of either goat anti-mouse IgG or goat anti-human IgG (Jackson ImmunoResearch). Wells were washed five times with PBS and then blocked for 1 h at 37 °C with 3% bovine serum albumin in PBS. After one wash with PBS, plates were incubated with 100 μl of either 20 μg/ml anti-CD4 mAb (OKT4) in Dulbecco's modified Eagle's medium containing 5% fetal calf serum (DMEM-FCS) or 7.5 μg/mL of anti-CD8 mAb (b12, National Institutes of Health AIDS Research and Reference Program) in DMEM medium containing 5% fetal calf serum (DMEM-FCS). After 150 min at 37 °C, wells were washed five times with bovine serum albumin in PBS, and then 100 μl of virus-containing supernatants were

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added (10 ng/ml p24 antigen). After 1 h at 37 °C, unbound HIV virions were washed six times with RPMI 1640 containing 1% FCS. Bound virions were lysed by 1 h at room temperature with 250 μl of PBS containing 1% Triton X-100. At this step lysates were frozen at −80 °C until quantification of bound p24 was performed with an enzyme-linked immunosassay kit (BD Biosciences). Nonspecific binding was estimated by coating wells with either mouse IgG or human IgG (SouthernBioTech). Additional controls for nonspecific binding were performed by estimating the capture of Env-defective virions to anti-gp120 mAb-coated plates.

Statistical Analysis—Mean values from early and late strains were compared with the paired T-Student test with software available in Microsoft Excel 98.

RESULTS

In Vivo Selection of HIV Viruses with Enhanced CD4 Down-modulation Activity—A previous study (41) has evaluated a set of nef alleles isolated from PBMC samples taken from seven HIV-infected patients either early after infection when CD4+ counts were normal and no symptoms were present (early alleles), or after declining to less than 200 counts/μl at the stage of AIDS (late alleles). Primary nef alleles were PCR-amplified and cloned into HIV-1 NL4.3 proviral constructs. To address whether subtle variations in the CD4 down-modulation activity of Nef may influence the ability of the virus to eliminate the receptor in the context of an HIV infection, we evaluated the extent of CD4 down-modulation in Jurkat T cells infected with HIV-1 NL4.3 strains. Isogenic NL4.3 proviral constructs differing solely in their nef genes were used to transfect 293T cells. The viral progeny was harvested and used to infect Jurkat T cells. The extent of CD4 down-modulation in p24-positive infected cells (region R4) was evaluated after staining with CD4-specific antibodies (Fig. 1, A and B). For comparison, Jurkat cells infected with a Nef-defective NL4.3 virus were also analyzed (Fig. 1C). In a representative experiment the mean CD4 levels in cells infected with the NL4.3 P10–96 late strain were half of those found on the surface of cells infected with the strain carrying the corresponding early nef allele P10–84 (mean CD4 levels: 109 and 205, respectively). Analysis of cells infected with the different alleles revealed that all the strains maintained the ability to reduce surface-CD4 beyond the levels achieved by Nef-defective viruses (Fig. 2A). Nevertheless, CD4 receptor levels were significantly lower in cells infected with HIV-1 late strains (130 ± 9), as compared with their early versions (190 ± 25, p = 0.04). These findings suggest that subtle variations in the CD4 down-modulation activity of Nef that are selected during disease progression may impact the ability of the virus to eliminate the receptor from the surface of infected cells.

Nef-mediated CD4 Down-modulation Enhances Infectivity of HIV-1—We have previously reported that expression of CD4 in virus producer cells leads to decreased HIV-1 infectivity (47, 48). To address whether enhanced CD4 down-modulation by late nef alleles may lead to augmented HIV-1 infectivity, we utilized an in vitro system in which viral particles are produced from T cells expressing varying amounts of CD4 (Fig. 2). HIV-1 NL4.3 particles produced in CD4-negative 293T cells were used to infect Jurkat-High-CD4 cells. These cells express surface-CD4 levels 2-fold higher than those observed in CD4-positive lymphocytes, as estimated by flow cytometry (48). After 24 h of infection of Jurkat cells viral supernatants were harvested and the amount of p24 antigen protein estimated by ELISA. The infectivity of these particles was determined by infecting MAG-IC-Low-CD4 cells with equal amounts of p24 protein and scoring the number of blue foci (Fig. 2B). All the HIV-1 strains carrying late nef alleles showed significantly enhanced infectivities, as compared with their early variants (3- to 7-fold, average increase 4.2-fold, p = 0.0002). Interestingly, differences in infectivity were largely reduced when the same viruses were analyzed in the target cells MAGIC-High-CD4, expressing surface-CD4 levels 12-fold higher than MAGIC-Low-CD4 (Fig. 2, C and D) (early: 136 ± 7; late: 177 ± 13 blue foci/ng of p24; p = 0.015). These findings suggest that high levels of receptor expression in target cells may overcome the deleterious effects of CD4 in producer cells. A second set of experiments analyzed the infectivity of HIV-1 produced in 293T cells and then grown in Jurkat-Low-CD4 cells for 24 h (Fig. 2, E and F). These cells express 25 times less CD4 receptor than Jurkat-High, as estimated by flow cytometry with anti-CD4 mAbs (48). Infection of these cells with NL4.3 strains further lowered the levels of surface-CD4, as compared with those in mock infected cells or in cells infected with Nef-defective viruses (Fig. 2E). However, no significant difference was observed in the ability of early and late strains to down-modulate the receptor (average mean levels of surface-CD4 in early strains: 8.4 ± 1.6; late strains: 7.4 ± 0.9; p = 0.6). Interestingly, when the infectivity of viruses grown in Jurkat-Low-CD4 cells was analyzed, unlike in previous experiments with Jurkat-High-CD4, late viruses were just slightly more infectious than their early variants (early: 606 ± 51; late 818 ± 124; p = 0.13). In some cases, results were significantly different from those observed in Jurkat-High-CD4 cells. For instance, late viruses from pa-
patients P5 and P10 resulted in infectivities 3- and 5-fold higher, respectively, than their corresponding early strains when grown in high CD4 producer cells (Fig. 2B), whereas early strains displayed significantly higher infectivities than their late counterparts after replication in low CD4 cells (Fig. 2F). These results indicate a direct link between the ability of Nef to down-modulate the receptor and to enhance virus infectivity and suggest that enhanced CD4 down-modulation may confer a replicative advantage to HIV-1 replicating in T cells with physiological levels of surface-CD4.

**Nef Modulates Env Incorporation in Vivo**—Previous findings from our laboratory have shown that HIV-induced receptor down-modulation in T cells allows efficient incorporation of Env into nascent particles while preventing incorporation of CD4. Both phenomena may help to explain the mechanism by which overexpression of CD4 in producer cells inhibits HIV infectivity (47, 48). To investigate how variations in the ability of Nef to down-modulate CD4 may affect HIV-1 infectivity, we analyzed incorporation of Env and CD4 proteins into the viral membranes of NL4.3 virions carrying different nef alleles. For this purpose we used an ELISA-based assay. HIV-1 NL4.3 virions were produced by transfection of 293T cells with proviral constructs in the presence or absence of a CD4 expression plasmid. For comparison, wild-type and Nef-defective particles were also analyzed. The infectivity of the released particles was estimated in MAGIC-Low-CD4 cells as shown above, and it is given as percentage of the corresponding early strain produced in the absence of CD4 (Fig. 3A). Under these conditions, expression of CD4 in producer cells reduced HIV-1 infectivity by 70–95%. Interestingly, NL4.3 strains carrying late nef alleles were more resistant to the deleterious effects of CD4. With the exception of the pair P2–87/P2–93, in which no significant difference in infectivity was observed, the other late strains showed enhanced infectivities when compared with their respective early variants. On average, late HIV-1 strains were 2-fold more infectious than early strains carrying nef alleles from asymptomatic patients (infectivity values: early, 10.9 ± 1.2; late, 21.7 ± 3.0; p = 0.006). Viruses produced in the presence of CD4 were compared for their Env and CD4 protein content. Plates coated with the CD4-specific OKT4 mAb were
used to capture HIV-1 particles that had incorporated the receptor in their membranes (Fig. 3B). Plates coated with isotypic IgG were used to estimate nonspecific binding. Incorporation of CD4 was Env-independent, because binding to OKT4-coated plates was observed with Env-defective particles. All viral membranes from strains carrying early nef alleles showed higher levels of CD4 incorporation (early: 269 ± 52; late: 182 ± 5; p = 0.3). To determine the extent of Env incorporation into viral membranes we utilized plates coated with b12, a human mAb specific for gp120. Because b12 binds to the CD4 binding site in gp120 (52), this capture assay recognizes envelope protein that has no CD4 bound to it and is available for fusion to target cells. As previously reported, expression of CD4 led to reduced levels of Env incorporation in Nef-defective particles, as compared with wild-type viruses (48). As expected, no incorporation was detected in Env-defective NL4.3 produced in CD4-transfected 293T cells (ΔEnv), and for binding of p8–89 particles produced in High-CD4 cells to IgG-coated plates (IgG), are also included. Dark columns represent values in late strains; lighter columns denote viruses carrying early nef alleles.

Late nef Alleles Enhance Viral Replication in a CD4-dependent Manner—The single-round infectivity experiments de-
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Fig. 4. Nef enhances HIV-1 replication in a CD4-dependent manner in transformed and primary T cells. Viral replication assays in Jurkat-Low-CD4, Jurkat-High-CD4, H9, SupT1, and human PBMCs. Representative experiments with isolates from two different patients are shown (upper row: early P5–83 and late P5–95 alleles; bottom row: early P8–83 and late P8–89). Viral replication in transformed T cell lines was initiated by electroporation with proviral constructs. The extent of virus production was assessed by measuring the accumulation of p24 protein in cultures (values given in nanograms of p24/ml). Infection of human PBMC was initiated by incubation with equal amounts of p24 protein (see “Materials and Methods”). The extent of replication in primary cells was estimated by determining RT activity in culture supernatants. Values are given as photo-stimulated luminescence (PSL). ○, viruses carrying early nef alleles; ◊, late nef alleles; □, nef-defective viruses.

scribed above suggest that variations in nef occurring during progression to disease may enhance HIV infectivity in a CD4-dependent manner. It is predicted that HIV strains unable to efficiently down-modulate CD4 will replicate poorly in high CD4 cells. Conversely, elevated levels of surface-CD4 in target cells may enhance HIV entry and thus accelerate viral replication, minimizing the negative impact of CD4 down-modulation at late steps of the viral life cycle. To evaluate which one of these CD4 effects plays a major role during HIV replication, we infected transformed T cells and primary CD4-lymphocytes with NL4.3 recombinant viruses carrying different primary nef alleles (Fig. 4 and Table I). Viral replication in transformed T cells was initiated by electroporation of proviral DNA constructs. This procedure bypasses the entry step in the first round of replication and delivers HIV genomes into T cells to the same extent, regardless of their levels of surface-CD4. We first compared the Jurkat T cells utilized in the above experiments. The replication profiles of two pairs of representative early and late strains are shown in Fig. 4. Despite reduced viral entry observed in Jurkat-Low-CD4 (48), HIV replication in these cells occurred to higher levels (p24 accumulation in the range of 3–4 µg p24/ml) than in cells with higher amounts of surface-CD4. However, in Jurkat-Low-CD4 cells, no significant difference was observed in the rates of viral replication of viruses carrying either early or late nef alleles. The average time to accumulate 1 µg of p24/ml in culture supernatants was 19.4 ± 0.9 days for early strains and 18.9 ± 0.8 for late strains (p = 0.6) (Table I). Similarly, no significant differences were observed in the highest values of p24 accumulation in these cultures (early: 2772 ± 315; late: 2866 ± 286 ng of p24/ml; p = 0.83). In contrast, replication of the same alleles in Jurkat-High-CD4 cells was strikingly different. In these cells p24 values did not reach more than 10 ng of p24/ml after 3 weeks of infection, levels 100-fold lower than those observed in Jurkat-Low-CD4 cells. Interestingly, even though viral replication occurred to a much lower extent than in Jurkat-Low-CD4 cells, HIV strains carrying late nef alleles started to accumulate p24 earlier than their early counterparts and reached levels 10-fold higher than the early alleles (p < 0.001). We then decided to evaluate viral replication in two more cell lines highly permissive for HIV infection: H9, expressing low levels of surface-CD4, and SupT1, which expresses 12 times more receptor than H9 cells, as estimated by flow cytometry after staining with CD4-specific antibodies (data not shown). H9 cells showed a viral replication profile similar to that observed in Jurkat-Low-CD4 cells (Fig. 4). In most cases, the kinetics of HIV-1 strains carrying either early or late nef alleles were similar. No significant differences were observed in either the average time needed to reach 1 µg/ml p24 in cultures or in the highest p24 value (Table I). In contrast, replication profiles of early and late strains were significantly different in SupT1 cells. Cultures of SupT1 cells infected with HIV-1 strains carrying late nef alleles down-modulated the viral receptor more efficiently (data not shown), accumulated p24 protein faster, and to higher levels than their early variants (Table I, early: 3380 ± 182 ng of p24/ml; late: 5256 ± 496 ng of p24/ml, p = 0.004). We then compared viral replication profiles in PBMCs. CD4-positive primary lymphocytes expressed levels of CD4 receptor comparable to SupT1 (data not shown). Replication of Nef-defective viruses was largely impaired in PBMCs. As expected, late strains carrying nef alleles with stronger CD4 down-modulation activity replicated more efficiently than their early versions. RT activity in PBMC cultures reached values 2-fold higher in infections with late strains, as compared with infections with viruses carrying early alleles (early: 618 ± 118 PSL; late: 1248 ± 238 PSL; p = 0.03). The above results suggest that down-modulation of the CD4 receptor plays an important role in HIV-1 pathogenesis in vivo. Amino acid substitutions in Nef
conferring enhanced CD4 down-modulation activity enhance replication in CD4-positive primary lymphocytes and may contribute to the increased viral load observed in AIDS patients.

**DISCUSSION**

In this report we have analyzed in detail the CD4 down-modulation activity of primary nef alleles obtained during early asymptomatic infection or after progression to AIDS. Our findings indicate that variations in the nef gene that occur during progression to disease confer stronger CD4 down-modulation properties to the virus. This fact highlights the important role of Nef in *in vivo* down-modulation of the viral receptor. By comparing the properties of viral particles produced in cells with different amounts of surface-CD4 expression, we were able to characterize Nef-induced phenotypes that occur in a CD4-dependent manner and dissect these activities from other functions of Nef. The infectivity of HIV-1 particles produced in cells expressing levels of surface-CD4 comparable to those found in CD4-positive primary lymphocytes was analyzed. Interestingly, HIV-1 strains carrying late nef alleles showed infectivities and viral replication properties significantly enhanced when compared with their early versions, suggesting that mutations in the nef region selected during disease progression may play an important role in enhancing the replicative and pathogenic properties of the virus. Two findings suggest that the enhanced infectivities promoted by late nef alleles can be directly attributed to their augmented CD4 down-modulation activity. First, enhanced infectivities in late strains were only observed if viral particles were produced in cells with high levels of surface-CD4. T cells expressing lower amounts of surface-CD4 were efficiently receptor-down-modulated by HIV-1 viruses regardless of the source of the nef alleles, and the viral particles produced from these cells were equally infectious. Second, differences in infectivity could be almost completely eliminated if target cells expressing high levels of surface-CD4 were utilized to analyze virion infectivity. As has been shown, increased levels of CD4 in target cells partially overcome entry defects due to limiting amounts of Env in viral particles (48). Further experiments with 293T cells confirmed the direct role of CD4 in inhibiting viral infectivity and ruled out the possibility that other properties, rather than CD4 expression, that might have arisen during selection of Jurkat-HighCD4 cells could be responsible for the observed phenotypes. Experiments with 293T cells also demonstrated that inefficient CD4 down-modulation observed in viruses carrying early nef alleles resulted in lower levels of Env incorporation and higher amounts of receptor in viral membranes. These findings likely explain the mechanism of inhibition mediated by CD4 expression in producer cells.

It is important to note that in our infectivity assays we have utilized a reporter cell line expressing low levels of CD4. This experimental approach enhances inhibitory effects on infectivity when Env amounts are limiting (48, 49, 53) and allowed us to enhance a phenotype that otherwise might not be revealed in *in vitro* experiments. The majority of the target cells *in vivo* are CD4-positive lymphocytes expressing levels of CD4 high enough to make the virus susceptible to CD4-inhibitory effects. Our findings also suggest that the Nef-induced CD4 down-modulation activity may be responsible for the long known Nef-mediated enhancement of viral replication in primary lymphocytes, cells expressing high levels of CD4 (27, 28). In agreement with this, recent reports also suggest that these two functions of Nef are closely related and may be mechanistically linked (54–56).

The *in vivo* situation may be more complicated. First, it is important to emphasize that our viral assays were performed within the genetic background of a T-cell tropic laboratory adapted (LA) strain (NL4.3). In this experimental system, the vpu and env NL4.3 genes contribute to the virus-induced CD4 down-modulation. Env and vpu genes from primary isolates (PR) may behave in a different manner than the NL4.3 versions. For instance, it is important to note that PR and LA isolates largely differ in their dependence to receptor density for viral entry. Mutations in env selected during laboratory adaptation allow infection of cells with low levels of CD4 and decrease CD4 dependence for viral entry (57, 58). It is therefore tempting to speculate that in more physiological situations, in which all the primary CD4 down-modulator genes were evaluated together, the replication of primary isolates would be even more susceptible to the deleterious effects of CD4 and more dependent on the virus machinery to down-modulate the receptor.

Coreceptor density may also play a role *in vivo*, even more important than CD4, modulating the susceptibility to HIV entry, especially when Env levels in viral membranes are scarce and surface CD4 amounts in target cells are low (59). Therefore, in the context of CD4-positive primary lymphocytes, it is expected that low coreceptor levels will likely magnify the viral requirements for a CD4-down-modulation machinery.

One recent study has questioned the role of CD4 down-modulation *in vivo* (60). This study reported that one LTNP infected with an HIV-1 Nef-deleted strain developed AIDS after 18 years of infection. Progression to AIDS occurred with the characteristic changes accompanying viral evolution during disease, namely selection of CXCR4-tropic viruses with higher cytopathic properties. However, these changes occurred without restoration of the Nef-induced CD4 down-modulation activity. Unfortunately, this study did not evaluate the ability of late cytopathic variants to down-modulate the CD4 receptor. Compensatory changes in Vpu or Env may have allowed maintenance of this function even in the absence of a functional Nef product. Interestingly, Nef-deleted SIVmac239 acquires increased replicative capacity and pathogenic potential upon serial passages in rhesus monkeys. Sequence analysis of these serially passaged derivatives shows no propensity to restore the nef ORF but has revealed amino acid changes in the cytoplasmic membrane.
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domain of gp41 that enhanced its fusogenic potential and might partially overcome defects on CD4 down-modulation.²

In summary, our findings indicate that CD4 down-modulation plays an important role in HIV-1 replication and may contribute to the increase viral load observed in AIDS patients. Variations in the virus’ ability to down-modulate CD4 may impact the outcome of disease. Nevertheless, important questions remain to be addressed, as to why and when these changes occur. We have suggested that the need for Nef to perform other functions may impose constraints in the ability of this protein to evolve during infection (42). Amino acid substitutions that enhance a given function (e.g. class I MHC down-modulation) may negatively affect the performance of other tasks (e.g. CD4 down-modulation). It is also important to note that T-cell tropic HIV-1 strains, which are often observed at late stages of infection when they can be recovered from about 50% of infected patients, bind soluble CD4 with higher affinity and are more sensitive to inhibition of infectivity by sCD4 (61, 62). It will be interesting to investigate whether T-tropic strains are also more sensitive to the inhibitory effects of CD4 described here and whether the appearance of these strains in vivo may impose higher requirements to down-modulate CD4. This would explain why nef alleles are selected at a time in which CXCR4 strains are common. However, it is important to note that our studies do not exclude that changes leading to enhanced CD4 down-modulation may accumulate progressively during the asymptomatic stage. More extensive in vivo longitudinal studies will provide important information in this regard. Furthermore, it will be interesting to address the role of CD4 down-modulation in other primary cells such as macrophages and dendritic cells, in which low levels of CD4 expression may preclude the need for receptor down-modulation to achieve production of fully infectious particles.

To date, the HIV-induced down-modulation of CD4 has not been targeted for intervention. Our findings suggest an important role for this function in pathogenesis in vivo, and imply that drug interfering with this function might delay progression to disease and result in clinical benefits.

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