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Nef Proteins from Diverse Groups of Primate Lentiviruses Downmodulate CXCR4 To Inhibit Migration to the Chemokine Stromal Derived Factor 1

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Nef proteins of primate lentiviruses promote viral replication, virion infectivity, and evasion of antiviral immune responses by modulating signal transduction pathways and downregulating expression of receptors at the cell surface that are important for efficient antigen-specific responses, such as CD4, CD28, T-cell antigen receptor, and class I and class II major histocompatibility complex. Here we show that Nef proteins from diverse groups of primate lentiviruses which do not require the chemokine receptor CXCR4 for entry into target cells strongly downmodulate the cell surface expression of CXCR4. In contrast, all human immunodeficiency virus type 1 (HIV-1) and the majority of HIV-2 Nef proteins tested did not have such strong effects. SIVmac239 Nef strongly inhibited lymphocyte migration to CXCR4 ligand, the chemokine stromal derived factor 1 (SDF-1). SIVmac239 Nef downregulated CXCR4 by accelerating the rate of its endocytosis. Downmodulation of CXCR4 was abolished by mutations that disrupt the constitutively strong AP-2 clathrin adaptor binding element located in the N-terminal region of the Nef molecule, suggesting that Nef accelerates CXCR4 endocytosis via an AP-2-dependent pathway. Together, these results point to CXCR4 as playing an important role in simian immunodeficiency virus and possibly also HIV-2 persistence in vivo that is unrelated to viral entry into target cells. We speculate that Nef targets CXCR4 to disrupt ordered trafficking of infected leukocytes between local microenvironments in order to facilitate their dissemination and/or impair the antiviral immune response.

Chemokine receptors mediate cell responses to chemokines, a family of polypeptides that regulate leukocyte migration and activation (30, 44). Many chemokines are proinflammatory cytokines induced to attract leukocytes mediating inflammatory reactions. Some chemokines, such as stromal derived factor 1 (SDF-1), also perform other basic functions in the immune system, which are not directly related to the inflammatory functions, such as regulating the trafficking of immature blood cells and naïve lymphocytes (2, 34). Ordered movement of leukocytes between specific sites within lymphoid tissue and eventually to sites of inflammation is required for normal development of the lymphoid compartment as well as for the development and maturation of the immune response (45).

Chemokine receptors, in conjunction with CD4, are also essential for the entry of primate lentiviruses into their target cells (18, 20). Simian immunodeficiency virus (SIV), human immunodeficiency virus type 1 (HIV-1), and human immunodeficiency virus type 2 (HIV-2) use overlapping yet distinct sets of chemokine receptors as entry cofactors (8). HIV-1 strains that prevail during the early course of infection usually use CCR5 chemokine receptor (6) while those isolated from AIDS patients frequently use CXCR4 or are dual tropic for CCR5 and CXCR4 (70). HIV-2 strains are relatively promiscuous in their use of chemokine coreceptors. They frequently use CXCR4, but other receptors such as CCR5, STRL33 (Bonzo), and GPR-15 (Bob) often support viral infection (55). SIVmac239 and SIVsm strains, which are closely related to HIV-2, use monkey and human CCR5 (13, 37, 41). The role of STRL33 for infection of primary cells by these strains is unclear (53). Notably, SIVmac239 and SIVsm strains do not use CXCR4 as an entry cofactor (13, 43, 69).

The accessory protein Nef is an important virulence factor of primate lentiviruses (17, 33, 36). Nef proteins modify cellular environments so that they become more conducive for viral replication. They also facilitate immune evasion of the infected cells by modulating signal transduction pathways and downregulating cell surface receptors that are important for antigen presentation and for antigen-specific responses. SIV and HIV-1 Nef proteins share many functions, including the abilities to downmodulate cell surface CD4, CD28, and class I and II major histocompatibility complex (MHC) by accelerating their endocytosis rates and possibly by blocking their transit to the plasma membrane (1, 4, 5, 23, 24, 32, 59, 61, 64, 66). They also share the abilities to associate with the regulators and effectors of the small GTPase Rac, such as DOCK2-ELMO1 complex and p21-activated kinase 2 (PAK-2) (29, 38, 56). Despite these similarities, important differences exist between the functions of SIV and HIV-1 Nef proteins. For example, SIVmac239 and HIV-2 Nef proteins strongly downmodulate the T-cell antigen receptor-CD3 complex while HIV-1 Nef does not have such an effect (3, 27). Nef proteins downmodulate cell surface expression of CD4,
CD28, and T-cell receptor-CD3 by recruiting these receptors to sites of the AP-2 clathrin-adaptor-dependent endocytosis (16, 24, 40, 51, 67, 68). Observations from structural and biochemical studies indicate that this involves formation of trimERIC complexes where Nef, the target receptor, and AP-2 bind each other cooperatively (11, 16, 26, 40, 54, 68). SIVmac239 Nef possesses two constitutively strong AP-2 binding determinants in its N-terminal region (40). It uses the N-distal AP-2 binding determinant to bind AP-2 in the context of CD4 and CD28 (40, 67), in contrast, another Nef surface is required for cooperative binding of the SIV Nef-CD3 complex to AP-2 (68). The use of distinct AP-2 interaction surfaces of Nef to recruit different membrane receptors provides a mechanism for independent and specific selection of distinct types of target receptors and their recruitment to AP-2 for endocytosis. Class I MHC complexes are downregulated by an AP-2 independent mechanism(s) (9, 25, 39, 52).

Previous studies revealed that an HIV-1 nef allele inhibited SDF-1-induced migration of Jurkat T cells and of primary human CD4+ T lymphocytes without affecting CXCR4 expression at the cell surface (14). Additionally, we reported that a different, patient-derived HIV-1 nef allele (NA7) disrupts migration to SDF-1 by hyperactivating a Rho-family GTPase Rac through the DOCK2-ELMO1 guanine exchange factor, which signals downstream of chemokine receptors (21) and is bound by Nef (29). Importantly, though we found that NA7 Nef slightly lowers the cell surface expression of the SDF-1 chemokine receptor CXCR4, this effect in and of itself cannot account for the observed perturbations of HIV-1 Nef-induced lymphocyte migration (29).

Here we show that lymphocyte migration to SDF-1 is also strongly inhibited by SIVmac239 Nef. Furthermore, Nef proteins encoded by diverse SIV strains strongly downregulate the cell surface expression of CXCR4 by accelerating its endocytosis. In contrast, HIV-1 and the majority of HIV-2 Nef proteins tested did not have such strong effects. Since SIVmac239 and other SIV strains usually do not use CXCR4 as an entry cofactor, our observations suggest that Nef targets CXCR4-controlled processes to inhibit the physiological role(s) of this chemokine receptor.

MATERIALS AND METHODS

Plasmids. HIV-1, HIV-2, and SIV nef alleles and mutants were subcloned downstream of the cytomegalovirus promoter into the pCGCG bicistronic vector (40). The same transcription unit also contains the green fluorescent protein (GFP) gene under the translational control of the encephalomyocarditis virus internal ribosome entry site (IRES). The SIVmac239 nef mutants 239(LA177), 239(LA6-07), 239(LA7-4), 239(LA8-06), 239(LA20-02), 239(LA30-01), 239(LA3-01), 239(LA5-01), 239(LA7-01), 239(LA8-02), 239(LA9-01), 239(LA10-01), and 239(LA11-01) were amplified with pSIVcne5XbaI, 5'-CACCCGCTTACATATGGGCGTCC-3' and SIVagmsabXbaI, 5'-TCCTCTAGATCAATATGGGTGCGAG-3'. All primers introduced XbaI and MluI restriction sites flanking the nef gene for cloning into the bicistronic pCGCG vector. Rhesus CXCR4 cDNA was cloned between XbaI and MluI restriction sites in pCGCG bicistronic vector.

Cells and transfections. Jurkat T cells (JJK) expressing human CD4 at a high level were maintained in RPMI 1640 medium supplemented with 2 mM glutamine and 15 mM HEPES, pH 7.4, and cultures were diluted 1:10 every 3 to 4 days. LTR-driven luciferase reporter plasmids containing 103 Jurkat T cells expressing the luciferase reporter gene and were provided by Jan Münch. env-defective variants of the above proviruses were generated by filling in with a unique Clal site located in env coding sequence immediately downstream of arginine codon R491 and thereby creating a frameshift mutation at this site. Vesicular stomatitis virus G pseudotyped viral stocks were produced from HEK 293T cells cotransfected with SIVmac proviral constructs carrying either nef open or nef-defective reading frames, followed by IRES-GFP, and a plasmid expressing Env protein of the vesicular stomatitis virus. Viral stocks were stored frozen at −86°C. Jurkat T cells were transduced with the pseudotyped virus particles, and cell surface expression of CXCR4 and CD4, CD3, and class I MHC on GFP-expressing cells was analyzed 3 days later by flow cytometry.

Flow cytometry. Aliquots of 3 × 107 cells were washed once with phosphate-buffered saline (PBS) containing 1% fetal bovine serum and 0.1% sodium azide (PBS-FA). Cells were reacted at 4°C with 50 µl aliquots of PBS-FA containing saturating amounts of phycoerythrin-conjugated monoclonal antibodies specific for CXCR4 (12G5, BD Pharmingen), CD4 (SK3, BD Pharmingen), CD28 (BD28.2, BD Pharmingen), class I MHC (W6/32, BD Pharmingen), or CD3e (HIT3A, BD Pharmingen), washed twice with PBS-FA, and suspended in 200 µl PBS-FA, and receptor expression and GFP expression were analyzed on an LSR II flow cytometer (Becton Dickinson). Cells infected with SIV-derived vectors were fixed with 3% paraformaldehyde following staining and analyzed by flow cytometry.

Endocytosis assays. Jurkat T cells coexpressing Nef and GFP from bicistronic pCGCG vectors or expressing GFP alone as a negative control were used. Internalization rates of CXCR4 were measured in cells transduced with flow cytometry as previously described (26, 66, 68). Briefly, 12 to 16 h after transduction cells were reacted with saturating amounts of phycoerythrin-conjugated monoclonal antibodies specific for CXCR4, or CD4, in RPMI 1640 medium containing 0.2% bovine serum albumin and 10 mM HEPES (pH 7.4) on ice for 20 min. Cells were then washed to remove the unbound antibody, and aliquots of 106 cells were incubated at 37°C to allow receptor internalization. The incubations were terminated on ice and each sample was divided into two aliquots. One aliquot was diluted fivefold with PBS to measure total cell surface receptor. The other aliquot was also diluted fivefold with RPMI 1640 adjusted to pH 2 to remove monoclonal antibody that had not been internalized. This permitted us to determine specifically the internalized receptor. Total and internalized receptors were determined by flow cytometry for cells showing iden-
tical levels of GFP expression. The fraction of receptor internalized was calculated as described previously (67, 68).

**Migration assays.** Jurkat T cells were transfected by electroporation with plasmids coexpressing Nef and GFP marker protein from a single bicistronic transcription unit (pCGCG) (40); 24 to 48 h later the transfected cells were washed once in RPMI 1640 supplemented with 1% bovine serum albumin (RPMI/BSA), resuspended in RPMI/BSA at 1.5 × 10^6 per ml, incubated for 1 h in a CO₂ incubator and 100-μl aliquots of cell suspensions were applied to upper chambers of 24-well Trans-Well plates (Costar 3421), and 450 μl of RPMI/BSA supplemented with 10 ng/ml SDF-1 (R&D Systems) was loaded into the lower chambers. Cells were allowed to migrate for 2 h, and the relative frequencies of GFP-positive cells in the initial and migrated populations were determined by using an LSR-II flow cytometer (Becton Dickinson). For each GFP-ssuorescence gate the fraction of migrated cells was calculated by dividing the fraction of cells found in that gate in the migrated population by that in the initial population of cells (29).

**RESULTS**

A transient expression assay in human Jurkat T cells was used to study the effect of SIV Nef and HIV Nef on CXCR4 cell surface expression. Cells were transfected with plasmids expressing Nef and a GFP reporter protein from the same bicistronic transcription unit and CXCR4 expression and GFP expression were detected by flow cytometry. As shown in Fig. 1A, expression of SIVmac239 Nef (SIVmac239 Nef) resulted in a dose-dependent decrease in CXCR4 expression on the cell surface. In contrast, expression of HIV-1 Nef protein encoded by the NA7 allele had a diminished effect on CXCR4 cell surface levels, in agreement with previous observations (29).

To confirm that CXCR4 is downregulated in virally infected cells, we characterized its expression on the surface of cells expressing the SIVmac239 provirus. As controls, we also measured cell surface expression of CD4, CD3, CD28 and class I MHC, which are all downregulated by SIVmac239 Nef (3, 4, 5, 39, 67). To directly visualize the productively infected cells we used viral constructs expressing GFP from an IRES element placed immediately downstream of the nef gene. The viruses also carried a frameshift mutation in the env gene to permit only a single infection cycle. Viral stocks pseudotyped with Env protein of the vesicular stomatitis virus (vesicular stomatitis virus G) were produced from HEK 293T cells cotransfected with proviral constructs carrying either the nef open or nef-defective reading frame and a plasmid expressing vesicular stomatitis virus G and were then used to infect CD4-positive Jurkat T cells. As shown in Fig. 1B, CXCR4 was strongly downregulated from the surface of cells infected with viruses containing only the intact nef gene. We also found that SIVmac239 Nef also downregulated rhesus CXCR4 in transient expression assays in monkey CV1 cells (data not shown). We concluded that SIVmac239 Nef strongly downregulates cell surface expression of CXCR4 receptor for SDF-1 chemo- kine in infected cells.

The observation that SIVmac239 Nef strongly downregulates CXCR4 but HIV-1 NA7 Nef does not suggested that the NA7 allele may be an exceptional loss-of-function allele. Alternatively, it was possible that the ability to downregulate CXCR4 is conserved only in Nef proteins from a certain subset of primate lentiviruses. To distinguish between these possibilities we tested additional SIV and HIV-1 Nef proteins for their ability to downregulate CXCR4 from the cell surface. We also characterized Nef proteins from several HIV-2 strains. HIV-2 is closely related to SIVmac yet it frequently uses CXCR4 as an entry coreceptor, while SIVmac does not (13, 43, 69). We also characterized the abilities of all Nef proteins tested to downmodulate CD4, since this function is known to be widespread among lentiviral Nef and therefore this assay could identify generally inactive proteins.

As shown in Fig. 2, all SIV nef alleles tested downmodulated CXCR4. Nef protein encoded by SIV naturally infecting sooty mangabeys (SIVsm, FWR1), which is closely related to SIVmac239, caused a strong decrease in CXCR4 surface expression. This decrease was comparable to that seen with the SIVmac239 protein. Nef proteins of two SIV strains isolated from African green monkeys (Cercopithecus aethiops, SIVagm, Sab1, and Tan1) had similar effects even though SIVagm is only distantly related to SIVmac and SIVsm. Nef proteins of SIV isolated from De Brazza’s monkey (Cercopithecus neglectus, SIVcne5) (7) and from blue monkeys (Cercopithecus mitis, SIVblu31) also downmodulated CXCR4, but their effects were not as strong as those seen with SIVmac-, SIVsm-, and SIVagm-derived alleles. Additional SIVcne and SIVblu Nef proteins need to be characterized to determine whether the alleles tested are representative or rather unusual weak alleles.

The effects of HIV-2 Nef proteins on CXCR4 cell surface expression were somewhat less pronounced. Of five alleles tested only the Ben allele decreased CXCR4 expression to levels seen with SIVmac- and SIVsm-derived nef genes. The remaining four HIV-2 nef alleles also diminished cell surface CXCR4 expression but to lesser extents. Finally, all six HIV-1 nef alleles tested affected CXCR4 cell surface expression but only marginally. We concluded that Nef proteins from diverse SIV strains strongly downregulate CXCR4, those from HIV-1 do so weakly, and those from HIV-2 have intermediate effects. In contrast, all Nef proteins tested strongly downregulated cell surface expression of CXCR4. Clearly, there was no correlation between the ability of various Nef proteins to downregulate CXCR4 and CD4.

Nef proteins downmodulate cell surface levels of target receptors by recruiting them to sites of endocytosis (9, 24, 38, 51, 61, 67). Therefore we asked whether SIVmac239 Nef enhances CXCR4 endocytosis rate. Jurkat T cells were transfected to transiently express SIVmac239 Nef or HIV-1 NA7 Nef and GFP or GFP alone as controls. Cells were reacted with anti- CXCR4 monoclonal antibody labeled with phycoerythrin. Subsequently, the rates of CXCR4 internalization in Nef-expressing and in control cells were determined for populations showing identical levels of GFP fluorescence using a flow cytometry-based endocytosis assay (67, 68). As a control, we determined the rates of CD4 endocytosis in the same transfected cell populations.

As shown in Fig. 3, expression of SIVmac239 Nef resulted in an approximately 10-fold increase in the rate of CXCR4 internalization over that seen in cells expressing GFP protein alone. The magnitude of this increase was almost as high as that seen for CD4 in cells expressing SIVmac239 and HIV-1 NA7 Nef proteins. Thus, the accelerated endocytosis of CXCR4 was probably responsible for its downregulation from the surface of SIVmac239 Nef-expressing cells. Notably, HIV-1 Nef also increased the CXCR4 endocytosis rate compared to that in control cells, but to a much lesser extent than SIVmac239 Nef. This is consistent with the observations that the NA7 Nef
modestly decreased CXCR4 cell surface expression and likely explains this phenomenon (Fig. 1A and 2A) (29).

To elucidate how SIVmac239 Nef induces CXCR4 endocytosis, we studied which surfaces and elements of the protein are required for this effect. Previous studies revealed that SIV Nef induces endocytosis of CD4, CD28, and CD3 via the AP-2 clathrin adaptor pathway and class I MHC via an AP-2-independent pathway (40, 66–68). Mutations in SIVmac239 Nef that selectively disrupt molecular interactions required for these different functions were previously identified (40, 51, 67, 68). Therefore we tested the effects of these mutations on the ability of SIVmac239 Nef to downregulate CXCR4 cell surface expression. Figure 4 shows the results of these experiments.

We first tested a mutation that disrupts the interaction of SIVmac239 Nef with the AP-2 clathrin adaptor by deleting two constitutively strong AP-2 binding elements located in the N-terminal region of this Nef molecule (7/H900423–74) (40). As shown in Fig. 4, this mutation severely disrupted CXCR4 downregulation (Fig. 4, panel 9). Next we tested two smaller deletions in the same region that each removes a different constitutively strong AP-2 binding element in Nef (40). Deletion of amino acid residues including lysine L31 through aspartic acid D37 (7/H900431–37) removes the N-proximal AP-2 binding element that is dispensable for known functions of Nef (40, 68). This mutation had a weak disruptive effect on the ability of Nef to downregulate CXCR4 (Fig. 4, panel 13). A more limited disruption of this region by alanine substitutions for tyrosines Y28 and Y39, which were implicated as important for the interaction with the μ2 subunit of the AP-2 complex (51), had no detectable effect on downregulation of CXCR4 or any of the other receptors tested (Fig. 4, panels 45 to 48). Thus, another aspect of the N-proximal element likely contributes to CXCR4 downregulation.

Deletion of amino acids Q64 to N67 (7/H900464–67) removes the N-distal AP-2 binding element in SIVmac239 Nef, which is required for downregulation of CD4 and CD28 through their recruitment to AP-2. Notably, the Δ64–67 mutation severely disrupted the ability of Nef to downregulate cell surface CXCR4 (Fig. 4, panel 17). This observation further suggested that Nef uses the N-distal element to downregulate CXCR4 through the AP-2 mechanism, similar to how it downregulates CD4 and CD28 (40, 67) (data not shown).

Then we analyzed additional mutations in Nef that compromise CD4 downregulation and/or other Nef functions. Initially we focused on mutations that do not affect constitutively strong Nef binding to AP-2 but rather disrupt other molecular interactions required for CD4 downregulation (40). Alanine substitutions for leucine L194 and methionine M195 (LM194AA) selectively diminished CXCR4 and CD4 downregulation without affecting T-cell receptor-CD3 and class I MHC cell surface expression (Fig. 4, panels 25, 27, and 28). Mutation in acidic element (Aci1, residues D88 to D95) (12) disrupted in part the effect of Nef on CXCR4 (Fig. 4, panel 29). This mutation was shown previously (28, 67) to have a pleiotropic effect on several Nef functions including CD4 and class I MHC downregulation (Fig. 4, panels 30 and 32). The mutation of the arginine residues R137 and R138 (RR137GG) also had a pleiotropic effect on receptor expression but had little detectable effect on CXCR4 downregulation (Fig. 4, panel 37). Similarly, two mutations that selectively disrupted downregulation of class I

FIG. 1. SIVmac239 Nef downregulates CXCR4 cell surface expression. (A) Flow cytometry analysis of CXCR4 and GFP expression in Jurkat T cells transiently coexpressing SIVmac239 or HIV-1 NA7 Nef and GFP reporter from bicistronic transcription units (panels 2 and 3). Cells expressing GFP alone were used as a negative control (panel 1, mock). (B) Flow cytometry analysis of CXCR4, CD4, CD3, CD28 and MHC I cell surface expression in Jurkat T cells infected with vesicular stomatitis virus G pseudotyped SIVmac239 containing a wild type (nef+, panels 6 to 10) or inactive (nef- , panels 1 to 5) nef gene, and a GFP reporter. Histograms of cell surface expression of the receptors for populations of cells infected with nef+ SIV (light gray), nef- SIV (dark gray), and showing identical levels of GFP fluorescence gated as indicated in panels 1 and 6, are also shown (panels 11 to 15). Histograms for uninfected cells (thick line) and unstained cells (thin line) are included as positive and negative controls for receptor expression.
FIG. 2. Effects of SIV, HIV-1 and HIV-2 Nef proteins on CXCR4 and CD4 cell surface expression. (A) Flow cytometry analysis of CXCR4 and CD4 expression on the surface of Jurkat T cells transiently transfected to coexpress the indicated Nef proteins and GFP marker from bicistronic expression vectors. Cells expressing GFP alone were used as a negative control (mock). (B) CXCR4 and CD4 downregulation by Nef proteins. For each Nef protein tested in A, a mean level of CXCR4 (left) and CD4 (right) on the surface of positively transfected cells is shown. The analysis was performed for positively transfected cell populations gated for a narrow range of GFP expression as indicated in the upper leftmost panels in A.
Mutations such as LL20AA, Y223A, LL20AA, and Y223A disrupted CXCR4 downmodulation by SIVmac239 Nef to inhibit lymphocyte migration to SDF-1. Thus, generally there was a strong positive correlation between the ability of mutant Nef proteins to downmodulate CXCR4 and to inhibit SDF-1-induced lymphocyte migration (Pearson correlation coefficient r = 0.93, P < 0.0001).

Interestingly, a closer examination of the data revealed that the Nef variant carrying the Y223A mutation inhibited migration more strongly than the wild-type SIVmac239 Nef even though the effect of the two proteins on CXCR4 expression was comparable. Therefore the robust disruption of lymphocyte migration to SDF-1 cannot be explained only by the ability of SIV Nef to downmodulate CXCR4, and additional components that contribute to this effect likely exist.

**DISCUSSION**

We report that Nef proteins encoded by several SIV strains strongly decrease expression of the CXCR4 chemokine receptor from the cell surface and that the HIV-2 Ben Nef had a similar strong effect. This effect requires the N-distal constitutively strong AP-2 binding determinant in the amino-terminal region of SIVmac239 Nef. The N-distal element is also critical for the ability of SIVmac239 Nef to recruit CD4 and CD28 to the AP-2 clathrin adaptor for endocytosis (40, 67). The above observations as well as evidence discussed below support the possibility that Nef induces CXCR4 endocytosis via the AP-2 clathrin adaptor.

CXCR4 mediates migration of cells to SDF-1 chemokine (10, 47). Since CXCR4 levels on the surface of SIVmac239 Nef-expressing cells were at least 10-fold lower than those on control cells, we characterized the migration of these cells to SDF-1. Jurkat T cells were transiently transfected with a control plasmid expressing GFP marker protein alone or with a plasmid that coexpresses Nef and a GFP marker protein from the same bicistronic transcription unit. Migration of the cell populations to SDF-1 was measured using a Transwell migration assay. The relative frequency of Nef-expressing and control cells in the migrated populations was then determined by flow cytometric measurement of GFP fluorescence (29).

Consistent with previous observations, approximately 30% of cells in the control populations migrated regardless of the level of GFP expression (29, data not shown). In contrast, in cell populations expressing SIVmac239 Nef migration was inhibited with increasing Nef/GFP expression in a dose dependent manner up to 100-fold (Fig. 5). Notably, the SIV Nef was more potent in inhibiting SDF-1-induced migration than HIV-1 Nef. We showed previously that inhibition of lymphocyte migration by HIV-1 NA7 Nef is independent of a slight decrease in CXCR4 expression levels seen on the surface of cells expressing this Nef protein (29). These observations together suggested that strong downmodulation of CXCR4 cell surface expression is an important component to the SIVmac239 induced inhibition of migration to SDF-1.

To demonstrate this directly we determined effects of mutations in SIVmac239 Nef that disrupted CXCR4 downmodulation on the inhibition of migration to SDF-1. The results are shown in Fig. 5 in a scatter plot as a function of CXCR4 expression levels on the surface of the transfected cell populations. Mutations such as Δ64–67, Δ23–74, AcI1, and LM194AA severely diminished the ability of SIVmac239 Nef to downmodulate CXCR4. All these mutations also severely disrupted the ability of SIVmac239 Nef to inhibit cell migration to SDF-1. Mutations such as LL20AA, Δ31–37, RR137GG, and Y223A disrupted CXCR4 downmodulation by SIVmac239 Nef to a lesser extent. However, these mutations had only a marginally negative effect on the ability of SIVmac239 Nef to inhibit lymphocyte migration to SDF-1. Thus, generally there was a strong positive correlation between the ability of mutant Nef proteins to downmodulate CXCR4 and to inhibit SDF-1-induced lymphocyte migration (Pearson correlation coefficient r = 0.93, P < 0.0001).

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FIG. 4. Effects of mutations in SIVmac239 Nef on downregulation of CXCR4 and other cell surface receptors. Flow cytometry analysis of CXCR4, CD4, CD3, and class I MHC expression on the surface of Jurkat T cells transiently expressing the indicated Nef proteins and GFP marker from bicistronic expression vectors. Cells expressing GFP alone were used as a negative control (mock).
CXCR4, are generally unable to strongly decrease cell surface CXCR4. Together this evidence points to CXCR4 having an important role for SIV and possibly also HIV-2 replication in vivo and suggests that this function is unrelated to viral entry into the target cells. Notably, we observed that SIVmac Nef downregulates CXCR4 just as strongly as SIVmac Nef. Since SIVsm replicates to high titers in sooty mangabeys, its natural host, but the infection is generally apathogenic (57), CXCR4 downregulation by Nef and the resulting migration defects are probably insufficient for the pathogenesis of lentiviral infections and have no apparent global immunosuppressive effects.

HIV-1 Nef proteins do not downregulate CXCR4 yet also inhibit lymphocyte migration to SDF-1 (14, 29). This effect is mediated by activation of Rac2 and/or Rac1 by the DOCK2-ELMO1 guanine exchange factor to which Nef binds (29). Notably, SIVmac239 Nef appeared to be a more potent inhibitor of lymphocyte migration to SDF-1 than HIV-1 Nef variants tested so far. Our data revealed that the robust effect of SIVmac Nef on chemotaxis correlated with its ability to downregulate CXCR4. SIVmac239 Nef also binds DOCK2-ELMO1 and activates Rac (data not shown). Thus, this mechanism may also contribute to the blocking of lymphocyte migration by SIVmac239 Nef.

SDF-1, through CXCR4, regulates multiple aspects of lymphocyte trafficking during the development and generation of the immune response (2, 15, 34). Experiments with SDF-1 and CXCR4 knockout mice revealed that SDF-1 promotes lymphocyte retention in lymphoid tissue, thereby preventing exit to the vasculature. Induction of transendothelial migration by diverse types of lymphocytes to lymph nodes and Peyer’s patches is another important function of SDF-1, which also directs lymphocyte migration in extravascular tissues. Through such actions, SDF-1 and other chemokines recruit lymphocytes to specific compartments in the lymphoid tissue. These events are crucial for the proper coordination of the immune response.

The possibility that Nef indeed disrupts the trafficking of the infected T cells in vivo is consistent with the observations from recent studies localizing T cells containing actively replicating SIV in lymph nodes from rhesus monkeys inoculated with either wild type or delta-nef SIVmac239 virus (65). In animals infected with wild-type SIVmac239, most of the infected T cells were located in the T-cell-rich cortex. In contrast, in animals infected with the Δnef virus, most were localized in B-cell-rich follicles and in the border region between the paracortex and the follicles. These differences were previously taken as evidence for the ability of Nef to activate T cells in the paracortex (65). However, they are also consistent with the possibility that the ability of the infected Nef-expressing T cells to respond to chemotactic cues that would normally direct them to B-cell follicles is suppressed. It will be important to understand whether this mislocalization results from Nef-disrupted migration of the infected T lymphocytes and to determine the scenarios in which Nef-mediated infection of infected leukocytes to SDF-1 occurs.

How CXCR4 downregulation in infected cells provides a selectable advantage for the virus is presently not known. Nonetheless, possible explanations are suggested by the known functions of SDF-1. One likely scenario is based on the fact that the fine tuning of leukocyte traffic through secondary lymphoid tissues by chemokines is essential for efficient immune surveillance and for the development of the immune response. Notably, in HIV-1 infection, a large fraction of HIV-1-infected CD4+ T cells are specific for HIV-1 antigens (19). Thus, disrupting lymphocyte traffic could provide yet another means to interfere with the development of the immune response to SIV antigens. Another scenario is suggested by the observation that SDF-1 can promote lymphocyte retention at local sites. Disruption of such cues could promote the exit of the infected T cells from the secondary lymphoid organs to the peripheral lymphoid system. Finally, it is conceivable that under some conditions, SDF-1-induced CXCR4 signaling could negatively affect SIV replication by means that are not related to lymphocyte migration. Future studies should focus on how CXCR4 downregulation by Nef provides those primate lentivirus that do not use CXCR4 as an entry cofactor with a selectable advantage in the infected host.

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REFERENCES

induced downregulation of CD4 is a multistep process involving dissociation from p56lck, increased association with clathrin-coated pits, and altered endosomal sorting. J. Exp. Med. 178:1209–1222.


