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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 proinflamatory 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 class 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, 67). 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,

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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 $239_{(\Delta 31–37)}$, $239_{(\Delta 64–67)}$, $239_{(\Delta 23–74)}$, $239_{(LM194AA)}$, $239_{(LL20AA)}$, $239_{(P104A,P107A)}$, $239_{(RR137AA)}$, $239_{(DD88NN,E*DDD91NQNNN)}$ (Aci1), $239_{(Y223F)}$, $239_{(Y28A,Y39A)}$ and HIV-1 NA7 and SF2 *nef* genes were described previously (28, 40, 42, 66, 68). Bicistronic pCGCG vectors coexpressing GFP and the HIV-2 ROD, HIV-2 BEN, HIV-1 Priso-k1, 93BRO29k1, and 13127K2 *nef* alleles were described previously (38, 59).

The HIV-2 CBL23 (60) *nef* was PCR amplified using primers HIV2Xba1, 5′-CGGTCTAGATGCAATATGGGTGCGAG-3′, and pHIV-2MluI, 5′-CCACGCGTTAACTAAATGGTATTCCT-3′. The remaining HIV-2 *nef* genes were amplified from the CDC 310319 and 60415K strains (22, 48) obtained from Mark Rayfield, Stephen Wiktor, Feng Gao, and Beatrice Hahn through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institutes of Health (46). The primers were pHIV2Xba10319, 5′-CGGTCTAGATGGGATCAGCTGGTTC-3′, or pHIV260415KXba, 5′-CGGTCTAGATGGGTGCGAGTGGAT-3′, paired with either p310319Mlu, 5′-GTACGCGTCTAATCTGTTGGTATTCCCCTT-3′, or p60415KMlu, 5′-GT*ACGCGT*TTAATTAAATGGTATTCCT-3′.

The SIVsm FWr *nef* allele was amplified by nested PCR with the inner primers pSIVsmXba, 5′-ACCTATCTAGAGCCCTTATGGGTGGCGTTACC-3′, and SIVsm(MluI), 5′-GTCCCTACGCGTCAGCTTGTTTCCTTCTTG-3′, from the blood of a naturally infected sooty mangabey, kindly provided by Francis Novembre. Five to six individual *nef* clones per SIVsm or HIV-2 sample were sequenced and one representative allele was selected for functional analyses. SIVcne5 and SIVblu31 *nef* clones were kindly provided by Beatrice Hahn and Frederic Bibollet-Ruche. The *nef* genes were amplified with pSIVcne5XbaI, 5′-TCTGGGTCTAGAG-TCAATATGGGTGGAAAG-3′, paired with pSHIVcne5Mlu, 5′-CGGTTAACGCGTCTACTTCCTCTTAGGGAACAT-3′, and pSIVblu3.1XbaI, 5′-TCTGTGTCTAGATCGGCTATGGGTTCCACG-3′, together with pSHIVblu3.1MluI, 5′-CTAGGAACGCGTCTACTTAAGTGCTGACTTTGA-3′, respectively.

The full-length pSAB-1 (31) and pSIVagmTAN-1 (63) molecular clones were obtained from Mojun Jin, Marcelo Soares, and Beatrice Hahn through the AIDS Research and Reference Reagent Program, Division of AIDS. The *nef* alleles were PCR amplified using SIVagmsabXbaI, 5′-TCCTCTAGATACAATATGGGTGGCAAGAGCTCAA-3′, SIVagmsabMluI, 5′-TGCACGCGTTCAGGCAGACACTGACGGCAC-3′, and SIVagmtanXbaI, 5′-GCCTCTAGATTCAGTATGGGTGGCAGCAATTCAA-3′, together with SIVagmtanMluI, 5′-GCGACGCGTCTACTTCCTCTTAACAAGACCATGC-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 (28). Aliquots of 10^7 Jurkat T cells from exponentially growing cultures were electroporated at 200 V and 960 μF with 20 μg of an appropriate expression vector as described (28). HEK 293T cells and CV1 cells were maintained and transfected as described previously (29, 66).

Proviruses and infections. SIVmac239 nef^+ IRES-GFP and SIVmac239 nef^- IRES-GFP each contain the IRES-EGFP cassette immediately downstream of the *nef* gene and were provided by Jan Münch. *env*-defective variants of the above proviruses were generated by filling in a unique ClaI 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, CD4, CD3, CD28, and class I MHC on GFP-expressing cells was analyzed 3 days later by flow cytometry.

Flow cytometry. Aliquots of 3×10^5 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 (CD28.2, BD Pharmingen), class I MHC (W6/32, BD Pharmingen), or CD3ε (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 and CD4 from the cell surface were measured by flow cytometry as previously described (26, 66, 68). Briefly, 12 to 16 h after transfection 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 10^5 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 1.5×10^7 per ml, incubated for 1 h in a CO_2 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-fluorescence 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 downmodulated 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 chemokine 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 CD4. 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

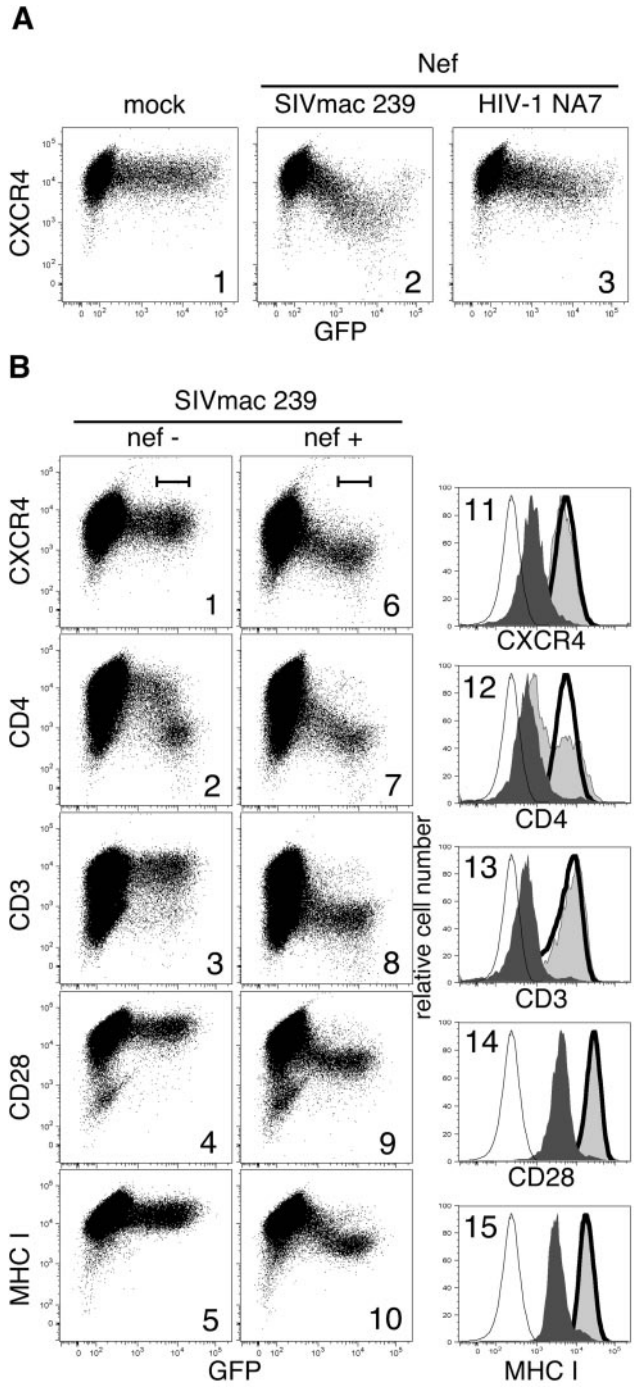

FIG. 1. SIVmac239 Nef downregulates CXCR4 cell surface expression. (A) Flow cytometry analysis of CXCR4 and GFP expression in Jurkat T cells transiently coexpressing SIVmac239 or HIV-1 NA7 Nef and GFP reporter from bicistronic transcription units (panels 2 and 3). Cells expressing GFP alone were used 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.

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 (Δ23–74) (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 (Δ31–37) 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 (Δ64–67) 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

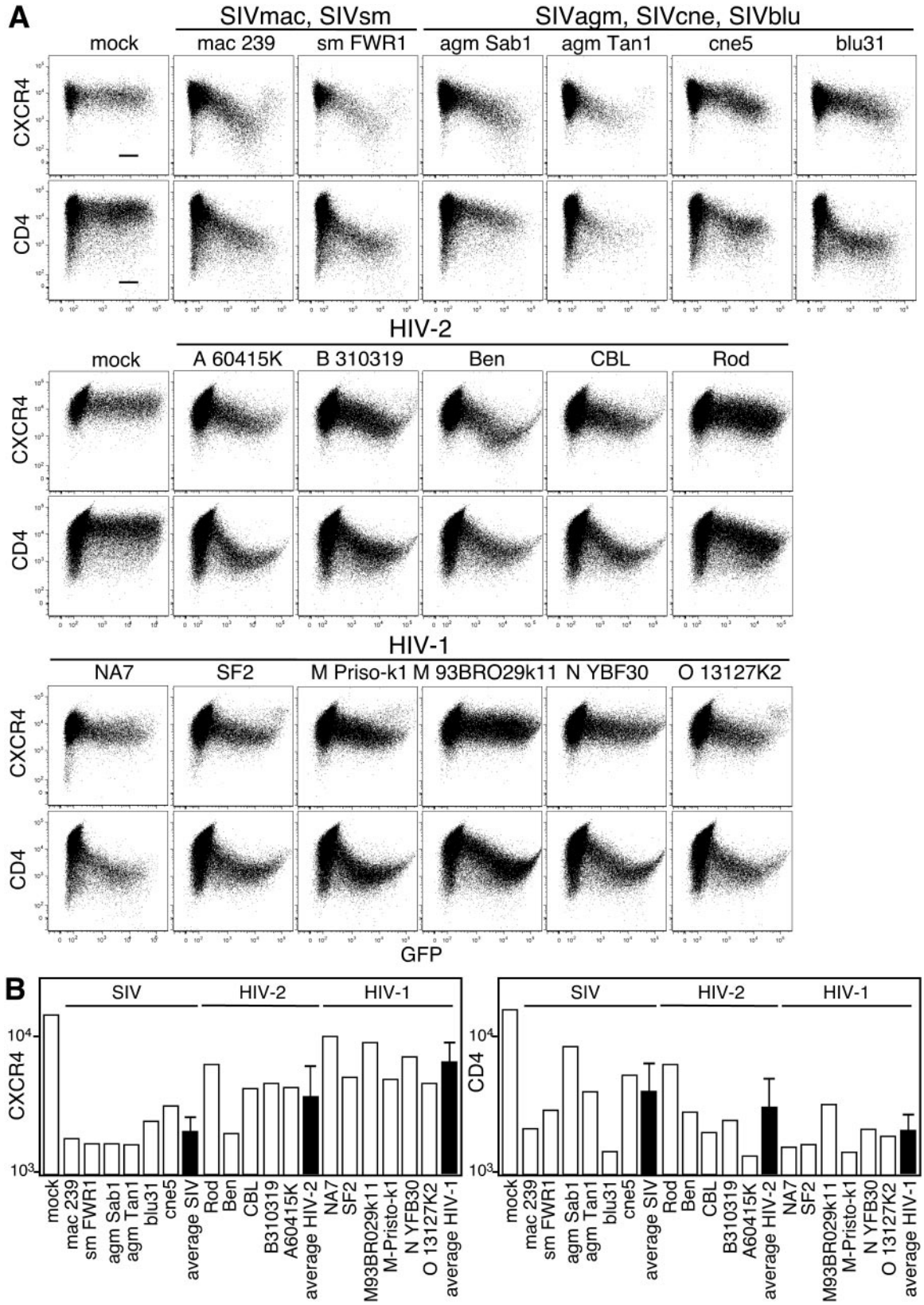

FIG. 2. Effects of SIV, HIV-1 and HIV-2 Nef proteins on CXCR4 and CD4 cell surface expression. (A) Flow cytometry analysis of CXCR4 and CD4 expression on the surface of Jurkat T cells transiently transfected to coexpress the indicated Nef proteins and GFP marker from bicistronic expression vectors. Cells 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.

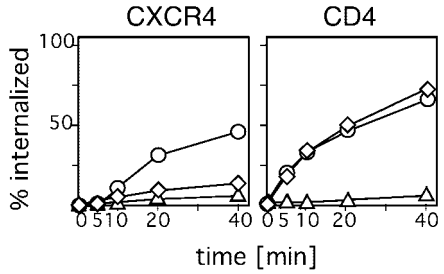

FIG. 3. SIVmac239 Nef accelerates CXCR4 endocytosis rate. The rates of CXCR4 endocytosis (left panel) and CD4 endocytosis in Jurkat T cells expressing SIVmac239 Nef (○), HIV-1 NA7 Nef (◇), and control cells (△) were measured by a flow cytometry-based endocytosis assay. The percentage of CXCR4 internalized from the cell surface is shown as a function of time.

MHC (LL20AA and Y223A) (67) had little detectable effect on CXCR4 downregulation (Fig. 4, panels 21 and 41).

Finally, alanine substitutions for prolines P104 and P107, which in HIV-1 Nef are required for association with PAK2 kinase (58), did not have a noticeable effect on the downregulation of any of the four receptors under study (Fig. 4, panel 33 to 36). Together, these data are consistent with the possibility that the ability of SIVmac239 Nef to bind AP-2 via its N-distal AP-2 binding element is crucial for CXCR4 downregulation and 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 $r = 0.93$, $P < 0.0001$).

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. Notably, CXCR4 and CD4 contain in their cytoplasmic domains similar phorbol ester-activated endocytosis signals that are reactive with the AP-2 clathrin adaptor. These signals contain either a dileucine or leucine-isoleucine motif and are activated by phosphorylation of a serine residue located in its vicinity (62). Furthermore, CXCR4 undergoes slow constitutive internalization through coated pits and coated vesicles into the endosomal compartment from which it can recycle back to the cell surface, similar to CD4 (50, 62). Finally, both constitutive and Nef-induced CD4 and/or CD28 internalization occurs via the AP-2/coated-pits pathway (24, 49, 51, 67). Thus, the possibility that Nef induces CXCR4 endocytosis via the AP-2 clathrin adaptors is consistent with the general model wherein Nef downregulates target receptors by exploiting the same mechanisms that mediate their internalization under physiologic conditions.

The observation that Nef proteins of diverse SIV and HIV-2 strains specifically downregulate CXCR4 indicates that this function is important for effective viral persistence in the host. Notably, the ability of Nef to decrease CXCR4 cell surface expression does not appear to correlate with the ability of these strains to use CXCR4 as coreceptor for entry. For example, SIVmac and SIVsm do not use CXCR4 and strongly diminish CXCR4 cell surface expression (13). Conversely, HIV-2 Ben virus uses CXCR4 (35) (data not shown), yet the Nef protein it encodes strongly downregulates CXCR4. Additionally, Nef proteins specified by HIV-1 strains, which frequently utilize

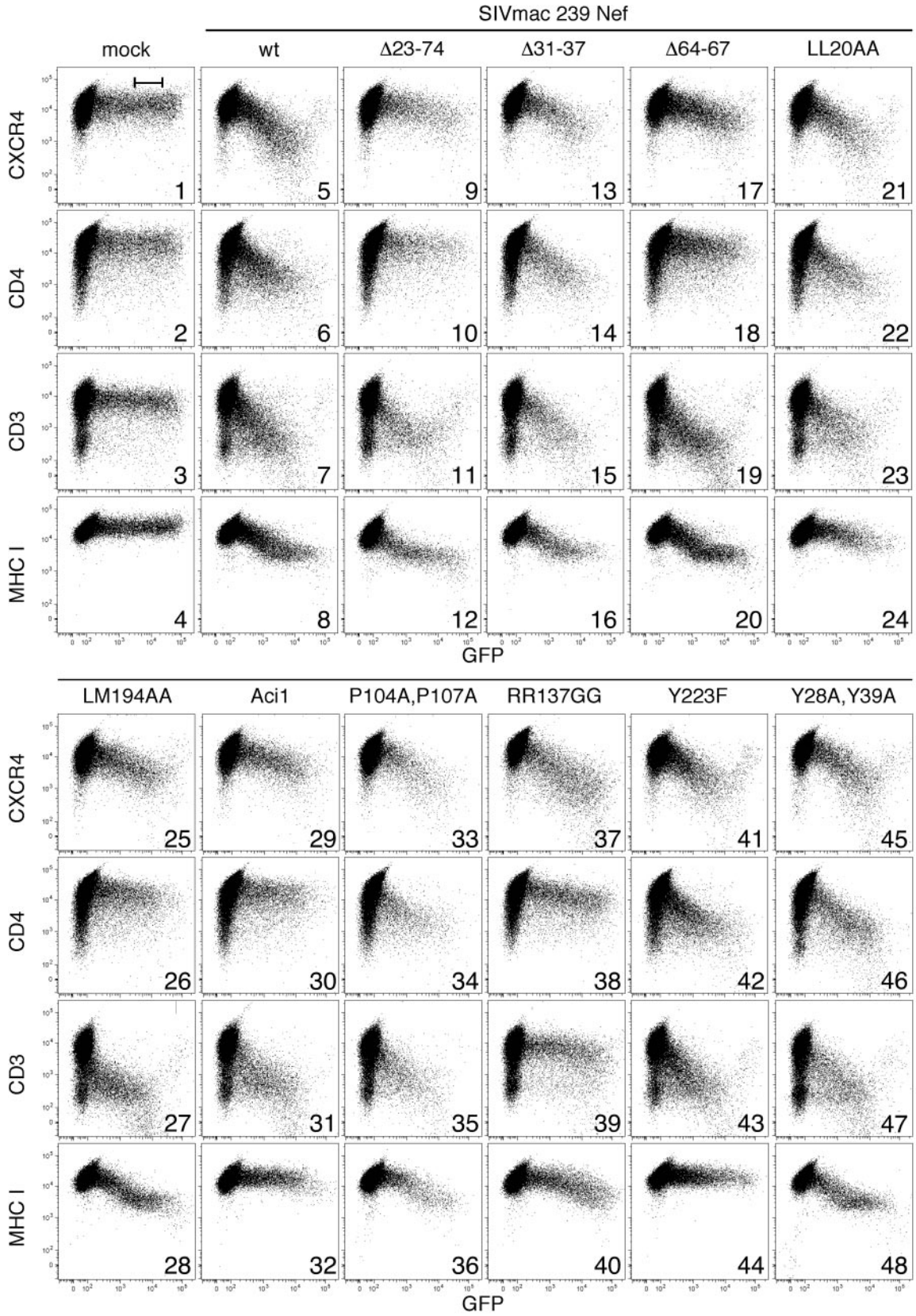

FIG. 4. Effects of mutations in SIVmac239 Nef on downregulation of CXCR4 and other cell surface receptors. Flow cytometry analysis of CXCR4, CD4, CD3, and class I MHC expression on the surface of Jurkat T cells transiently expressing the indicated Nef proteins and GFP marker from bicistronic expression vectors. Cells expressing GFP alone were used as a negative control (mock).

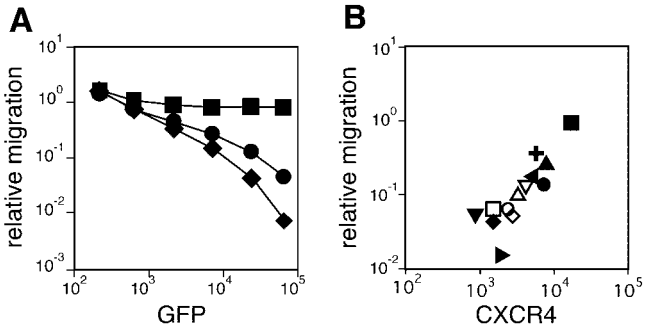

FIG. 5. SIVmac239 Nef inhibits lymphocyte migration to SDF-1. (A) SIV Nef strongly inhibits migration to SDF-1. Jurkat T cells transiently expressing SIVmac239 Nef (◆) or HIV-1 NA7 Nef (●) and GFP or GFP alone (■) were used in transwell migration assays with SDF-1. A relative fraction of cells that migrated to SDF-1 as a function of GFP fluorescence are shown. (B) The ability of SIV Nef to strongly inhibit lymphocyte migration to SDF-1 correlates with CXCR4 downmodulation. The effects of mutations in SIVmac239 Nef on migration to SDF-1 (abscissa) and on CXCR4 downmodulation (ordinate) were measured for populations of cells showing identical levels of GFP fluorescence. The following mutations in *nef* were tested: Δ31–37 (Δ), Δ64–67 (◀), Δ23–74 (▲), Aci1 (+), LL20AA (○), Y28A,Y39A (◇), P104A,P107A (□), RR137GG (▼), LM194AA (▽), Y223F (▶). Wild-type SIVmac239 Nef (◆),HIV-1 NA7 Nef (●), and GFP alone (■) were used as controls.

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 SIVsm 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 of 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 paracortex. In contrast, in animals infected with the Δ*nef* 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 inhibition 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 periphery and thus facilitate their dissemination throughout the immune 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 lentiviruses that do not use CXCR4 as an entry cofactor with a selectable advantage in the infected host.

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REFERENCES

1. **Aiken, C., J. Konner, N. R. Landau, M. E. Lenburg, and D. Trono.** 1994. Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain. Cell **76:**853–864.
2. **Ansel, K. M., and J. G. Cyster.** 2001. Chemokines in lymphopoiesis and lymphoid organ development. Curr. Opin. Immunol. **13:**172–179.
3. **Bell, I., C. Ashman, J. Maughan, E. Hooker, F. Cook, and T. A. Reinhart.** 1998. Association of simian immunodeficiency virus Nef with the T-cell receptor (TCR) zeta chain leads to TCR down-modulation. J. Gen. Virol. **79:**2717–2727.

4. **Bell, I., T. M. Schaefer, R. P. Trible, A. Amedee, and T. A. Reinhart.** 2001. Down-modulation of the costimulatory molecule, CD28, is a conserved activity of multiple SIV Nefs and is dependent on histidine 196 of Nef. Virology **283:**148–158.
5. **Benson, R. E., A. Sanfridson, J. S. Ottinger, C. Doyle, and B. R. Cullen.** 1993. Downregulation of cell-surface CD4 expression by simian immunodeficiency virus Nef prevents viral super infection. J. Exp. Med. **177:**1561–1566.
6. **Berger, E. A., P. M. Murphy, and J. M. Farber.** 1999. Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. Annu. Rev. Immunol. **17:**657–700.
7. **Bibollet-Ruche, F., E. Bailes, F. Gao, X. Pourrut, K. L. Barlow, J. P. Clewley, J. M. Mwenda, D. K. Langat, G. K. Chege, H. M. McClure, E. Mpoudi-Ngole, E. Delaporte, M. Peeters, G. M. Shaw, P. M. Sharp, and B. H. Hahn.** 2004. New simian immunodeficiency virus infecting De Brazza's monkeys (*Cercopithecus neglectus*): evidence for a *Cercopithecus* monkey virus clade. J. Virol. **78:**7748–7762.
8. **Bieniasz, P. D., and B. R. Cullen.** 1998. Chemokine receptors and human immunodeficiency virus infection. Front. Biosci. **3:**d44–58.
9. **Blagoveshchenskaya, A. D., L. Thomas, S. F. Feliciangeli, C. H. Hung, and G. Thomas.** 2002. HIV-1 Nef downregulates MHC-I by a PACS-1- and PI3K-regulated ARF6 endocytic pathway. Cell **111:**853–866.
10. **Bleul, C. C., M. Farzan, H. Choe, C. Parolin, I. Clark-Lewis, J. Sodroski, and T. A. Springer.** 1996. The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. Nature **382:**829–833.
11. **Bresnahan, P. A., W. Yonemoto, S. Ferrell, D. Williams-Herman, R. Geleziunas, and W. C. Greene.** 1998. A dileucine motif in HIV-1 Nef acts as an internalization signal for CD4 downregulation and binds the AP-1 clathrin adaptor. Curr. Biol. **8:**1235–1238.
12. **Carl, S., A. J. Iafrate, S. M. Lang, C. Stahl-Hennig, E. M. Kuhn, D. Fuchs, K. Matz-Rensing, P. ten Haaft, J. L. Heeney, J. Skowronski, and F. Kirchhoff.** 1999. The acidic region and conserved putative protein kinase C phosphorylation site in Nef are important for SIV replication in rhesus macaques. Virology **257:**138–155.
13. **Chen, Z., P. Zhou, D. D. Ho, N. R. Landau, and P. A. Marx.** 1997. Genetically divergent strains of simian immunodeficiency virus use CCR5 as a coreceptor for entry. J. Virol. **71:**2705–2714.
14. **Choe, E. Y., E. S. Schoenberger, J. E. Groopman, and J. W. Park.** 2002. HIV Nef inhibits T-cell migration. J. Biol. Chem. **277:**46079–46084.
15. **Cinamon, G., V. Grabovsky, E. Winter, S. Franitza, S. Feigelson, R. Shamri, O. Dwir, and R. Alon.** 2001. Novel chemokine functions in lymphocyte migration through vascular endothelium under shear flow. J. Leukoc. Biol. **69:**860–866.
16. **Craig, H. M., W. M. Pandori, and J. C. Guatelli.** 1998. Interaction of HIV-1 Nef with the cellular dileucine-based sorting pathway is required for CD4 down-regulation and optimal viral infectivity. Proc. Natl. Acad. Sci. USA **95:**11229–11234.
17. **Deacon, N. J., A. Tsykin, A. Solomon, K. Smith, M. Ludford-Menting, D. J. Hooker, D. A. McPhee, A. L. Greenway, A. Ellett, C. Chatfield, V. A. Lawson, S. Crowe, A. Maerz, S. Sonza, J. Learmont, J. S. Sullivan, A. Cunningham, D. Dwyer, D. Dowton, and J. Mills.** 1995. Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. Science **270:**988–991.
18. **Deng, H., R. Liu, W. Ellmeier, S. Choe, D. Unutmaz, M. Burkhart, P. Di Marzio, S. Marmon, R. E. Sutton, C. M. Hill, C. B. Davis, S. C. Peiper, T. J. Schall, D. R. Littman, and N. R. Landau.** 1996. Identification of a major co-receptor for primary isolates of HIV-1. Nature **381:**661–666.
19. **Douek, D. C., J. M. Brenchley, M. R. Betts, D. R. Ambrozak, B. J. Hill, Y. Okamoto, J. P. Casazza, J. Kuruppu, K. Kunstman, S. Wolinsky, Z. Grossman, M. Dybul, A. Oxenius, D. A. Price, M. Connors, and R. A. Koup.** 2002. HIV preferentially infects HIV-specific CD4+ T cells. Nature **417:**95–98.
20. **Feng, Y., C. C. Broder, P. E. Kennedy, and E. A. Berger.** 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science **272:**872–877.
21. **Fukui, Y., O. Hashimoto, T. Sanui, T. Oono, H. Koga, M. Abe, A. Inayoshi, M. Noda, M. Oike, T. Shirai, and T. Sasazuki.** 2001. Haematopoietic cell-specific CDM family protein DOCK2 is essential for lymphocyte migration. Nature **412:**826–831.
22. **Gao, F., L. Yue, D. L. Robertson, S. C. Hill, H. Hui, R. J. Biggar, A. E. Neequaye, T. M. Whelan, D. D. Ho, and G. M. Shaw.** 1994. Genetic diversity of human immunodeficiency virus type 2: evidence for distinct sequence subtypes with differences in virus biology. J. Virol. **68:**7433 7447.
23. **Garcia, J. V., and A. D. Miller.** 1991. Serine phosphorylation-independent downregulation of cell-surface CD4 by nef. Nature **350:**508–511.
24. **Greenberg, M. E., S. Bronson, M. Lock, M. Neumann, G. N. Pavlakis, and J. Skowronski.** 1997. Colocalization of HIV-1 Nef with the AP-2 adaptor protein complex correlates with Nef-induced CD4 down-regulation. EMBO J. **16:**6964–6976.
25. **Greenberg, M. E., J. I. Iafrate, and J. Skowronski.** 1998. The SH3 domain-binding surface and an acidic motif in HIV-1 Nef regulate trafficking of class I MHC complexes. EMBO J. **17:**2777–2789.
26. **Greenberg, M., L. DeTulleo, I. Rapoport, J. Skowronski, and T. Kirchhausen.** 1998. A dileucine motif in HIV-1 Nef is essential for sorting into clathrin-coated pits and for downregulation of CD4. Curr. Biol. **8:**1239–1242.
27. **Howe, A. Y., J. U. Jung, and R. C. Desrosiers.** 1998. Zeta chain of the T-cell receptor interacts with *nef* of simian immunodeficiency virus and human immunodeficiency virus type 2. J. Virol. **72:**9827–9834.
28. **Iafrate, A. J., S. Bronson, and J. Skowronski.** 1997. Separable functions of Nef disrupt two aspects of T-cell receptor machinery: CD4 expression and CD3 signaling. EMBO J. **16:**673–684.
29. **Janardhan, A., T. Swigut, B. Hill, M. P. Myers, and J. Skowronski.** 2004. HIV-1 Nef activates Rac and disrupts T-cell chemotaxis by binding DOCK2: ELMO1:Rac complex. PloS Biol. **2:**65–76.
30. **Jason, G., and J. G. Cyster.** 2005. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. Annu. Rev. Immunol. **23:**127–159.
31. **Jin, M. J., H. Hui, D. L. Robertson, M. C. Mueller, F. Barre-Sinoussi, V. M. Hirsch, J. S. Allan, G. M. Shaw, P. M. Sharp, and B. H. Hahn.** 1994. Mosaic genome structure of SIV from west African green monkeys. EMBO J. **13:**2935–2947.
32. **Kasper, M. R., and K. L. Collins.** 2003. Nef-mediated disruption of HLA-A2 transport to the cell surface in T cells. J. Virol. **77:**3041–3049.
33. **Kestler, H. W. 3rd, D. J. Ringler, K. Mori, D. L. Panicali, P. K. Sehgal, M. D. Daniel, and R. C. Desrosiers.** 1991. Importance of the *nef* gene for maintenance of high virus loads and for development of AIDS. Cell **65:**651–662.
34. **Kim, C. H., and H. E. Broxmeyer.** 1999. Chemokines: signal lamps for trafficking of T and B cells for development and effector function. J. Leukoc. Biol. **65:**6–15.
35. **Kirchhoff, F., K. D. Jentsch,, B. Bachmann,, et al.** 1990. A novel proviral clone of HIV 2: biological and phylogenetic relationship to other primate immunodeficiency viruses. Virology **177:**305–311.
36. **Kirchhoff, F., T. C. Greenough, D. B. Brettler, J. L. Sullivan, and R. C. Desrosiers.** 1995. Absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. N. Engl. J. Med. **332:**228–232.
37. **Kirchhoff, F., S. Pohlmann, M. Hamacher, R. E. Means, T. Kraus, K. Uberla, and P. Di Marzio.** 1997. Simian immunodeficiency virus variants with differential T-cell and macrophage tropism use CCR5 and an unidentified cofactor expressed in CEMx174 cells for efficient entry. J. Virol. **71:**6509–6516.
38. **Kirchhoff, F., M. Schindler, N. Bailer, G. H. Renkema, K. Saksela, V. Knoop, M. C. Muller-Trutwin, M. L. Santiago, F. Bibollet-Ruche, M. T. Dittmar, J. L. Heeney, B. H. Hahn, and J. Munch.** 2004. Nef proteins from simian immunodeficiency virus-infected chimpanzees interact with p21-activated kinase 2 and modulate cell surface expression of various human receptors. J. Virol. **78:**6864–6874.
39. **Le Gall, S., F. Buseyne, A. Trocha, B. D. Walker, J. M. Heard, and O. Schwartz.** 2000. Distinct trafficking pathways mediate Nef-induced and clathrin-dependent major histocompatibility complex class I down-regulation. J. Virol. **74:**9256–9266.
40. **Lock, M., M. E. Greenberg, A. J. Iafrate, T. Swigut, J. Muench, F. Kirchhoff, N. Shohdy, and J. Skowronski.** 1999. Two elements target SIV Nef to the AP-2 clathrin adaptor complex, but only one is required for the induction of CD4 endocytosis. EMBO J. **18:**2722–2733.
41. **Marcon, L., H. Choe, K. Martin, M. Farzan, P. D. Ponath, L. Wu, W. Newman, N. Gerard, C. Gerard, and J. Sodroski.** 1997. Utilization of C-C chemokine receptor 5 by the envelope glycoproteins of a pathogenic simian immunodeficiency virus, SIVmac239. J. Virol. **71:**2522–2537.
42. **Mariani, R., and J. Skowronski.** 1993. CD4 down-regulation by nef alleles isolated from human immunodeficiency virus type 1-infected individuals. Proc. Natl. Acad. Sci. USA **90:**5549–5553.
43. **Marx, P. A., and Z. Chen.** 1998. The function of simian chemokine receptors in the replication of SIV. Semin. Immunol. **10:**215–223.
44. **Moser, B., and P. Loetscher.** 2001. Lymphocyte traffic control by chemokines. Nat. Immunol. **2:**123–128.
45. **Moser, B., M. Wolf, A. Walz, and P. Loetscher.** 2004. Chemokines: multiple levels of leukocyte migration control. Trends Immunol. **25:**75–84.
46. **Münch, J., M. Schindler, S. Wildum, E. Rücker, N. Bailer, V. Knoop, F. J. Novembre, and F. Kirchhoff.** Primary sooty mangabey simian immunodeficiency virus and human immunodeficiency virus type 2 *nef* alleles modulate cell surface expression of various human receptors and enhance viral infectivity and replication. J. Virol.. **79:**10547–10560.
47. **Oberlin, E., A. Amara, F. Bachelerie, C. Bessia, J. L. Virelizier, F. Arenzana-Seisdedos, O. Schwartz, J. M. Heard, I. Clark-Lewis, D. F. Legler, M. Loetscher, M. Baggiolini, and B. Moser.** 1996. The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. Nature **382:**833–835.
48. **Owen, S. M., D. Ellenberger, M. Rayfield, S. Wiktor, P. Michel, M. H. Grieco, F. Gao, B. H. Hahn, and R. B. Lal.** 1998. Genetically divergent strains of human immunodeficiency virus type 2 use multiple coreceptors for viral entry. J. Virol. **72:**5425–5432.
49. **Pelchen-Matthews, A., I. Boulet, D. R. Littman, R. Fagard, and M. Marsh.** 1992. The protein tyrosine kinase p56lck inhibits CD4 endocytosis by preventing entry of CD4 into coated pits. J. Cell Biol. **117:**279–290.
50. **Pelchen-Matthews, A., I. J. Parsons, and M. Marsh.** 1993. Phorbol ester-

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.
51. **Piguet, V., Y. L. Chen, A. Mangasarian, M. Foti, J. L. Carpentier, and D. Trono.** 1998. Mechanism of Nef-induced CD4 endocytosis: Nef connects CD4 with the mu chain of adaptor complexes. EMBO J. **17:**2472–2481.
52. **Piguet, V., C. Wan, L. Borel, A. Mangasarian, N. Demaurex, G. Thomas, and D. Trono.** 2000. HIV-1 Nef protein binds to the cellular protein PACS-1 to downregulate class I major histocompatibility complexes. Nat. Cell Biol. **2:**163–167.
53. **Pöhlmann, S., B. Lee, S. Meister, M. Krumbiegel, G. Leslie, R. W. Doms, and F. Kirchhoff.** 2000. Simian immunodeficiency virus utilizes human and sooty mangabey but not rhesus macaque STRL33 for efficient entry. J. Virol. **74:**5075–5082.
54. **Preusser, A., L. Briese, A. S. Baur, and D. Willbold.** 2001. Direct in vitro binding of full-length human immunodeficiency virus type 1 Nef protein to CD4 cytoplasmic domain. J. Virol. **75:**3960–3964.
55. **Reeves, J. D., and R. W. Doms.** 2002. Human immunodeficiency virus type 2. J. Gen. Virol. **83:**1253–1265.
56. **Renkema, G. H., A. Manninen, D. A. Mann, M. Harris, and K. Saksela.** 1999. Identification of the Nef-associated kinase as p21-activated kinase 2. Curr. Biol. **9:**1407–1410.
57. **Rey-Cuille, M. A., J. I. Berthier, M. C. Bomsel-Demontoy, Y. Chaduc, L. Montagnier, A. G. Hovanessian, and L. A. Chakrabarti.** 1998. Simian immunodeficiency virus replicates to high levels in sooty mangabeys without inducing disease. J. Virol. **72:**3872–3886.
58. **Sawai, E. T., A. Baur, H. Struble, B. M. Peterlin, J. A. Levy, and C. Cheng-Mayer.** 1994. Human immunodeficiency virus type 1 Nef associates with a cellular serine kinase in T lymphocytes. Proc. Natl. Acad. Sci. USA **91:**1539–1543.
59. **Schindler, M., S. Wurfl, P. Benaroch, T. C. Greenough, R. Daniels, P. Easterbrook, M. Brenner, J. Munch, and F. Kirchhoff.** 2003. Down-modulation of mature major histocompatibility complex class II and up-regulation of invariant chain cell surface expression are well-conserved functions of human and simian immunodeficiency virus *nef* alleles. J. Virol. **77:**10548–10556.
60. **Schulz, T. F., D. Whitby, J. G. Hoad, T. Corrah, H. Whittle, and R. A. Weiss.** 1990. Biological and molecular variability of human immunodeficiency virus type 2 isolates from the Gambia. J. Virol. **64:**5177 5182.
61. **Schwartz, O., V. Marechal, S. Le Gall, F. Lemonnier, and J. M. Heard.** 1996. Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. Nat. Med. **2:**338–342.
62. **Signoret, N., J. Oldridge, A. Pelchen-Matthews, P. J. Klasse, T. Tran, L. F. Brass, M. M. Rosenkilde, T. W. Schwartz, W. Holmes, W. Dallas, M. A. Luther, T. N. Wells, J. A. Hoxie, and M. Marsh.** 1997. Phorbol esters and SDF-1 induce rapid endocytosis and down modulation of the chemokine receptor CXCR4. J. Cell Biol. **139:**651–664.
63. **Soares, M. A., D. L. Robertson, H. Hui, J. S. Allan, G. M. Shaw, and B. H. Hahn.** 1997. A full-length and replication-competent proviral clone of SIVAGM from tantalus monkeys. Virology **228:**394–399.
64. **Stumptner-Cuvelette, P., S. Morchoisne, M. Dugast, S. Le Gall, G. Raposo, O. Schwartz, and P. Benaroch.** 2001. HIV-1 Nef impairs MHC class II antigen presentation and surface expression. Proc. Natl. Acad. Sci. USA **98:**12144–12149.
65. **Sugimoto, C., K. Tadakuma, I. Otani, T. Moritoyo, H. Akari, F. Ono, Y. Yoshikawa, T. Sata, S. Izumo, and K. Mori.** 2003. *nef* gene is required for robust productive infection by simian immunodeficiency virus of T-cell-rich paracortex in lymph nodes. J. Virol. **77:**4169–4180.
66. **Swigut, T., A. J. Iafrate, J. Muench, F. Kirchhoff, and J. Skowronski.** 2000. Simian and human immunodeficiency virus Nef proteins use different surfaces to downregulate class I major histocompatibility complex antigen expression. J. Virol. **74:**5691–5701.
67. **Swigut, T., N. Shohdy, and J. Skowronski.** 2001. Mechanism for down-regulation of CD28 by Nef. EMBO J. **20:**1593–1604.
68. **Swigut, T., M. Greenberg, and J. Skowronski.** 2003. Cooperative interactions of simian immunodeficiency virus Nef, AP-2, and CD3-zeta mediate the selective induction of T-cell receptor-CD3 endocytosis. J. Virol. **77:**8116–8126.
69. **Unutmaz, D., V. N. KewalRamani, and D. R. Littman.** 1998. G protein-coupled receptors in HIV and SIV entry: new perspectives on lentivirus-host interactions and on the utility of animal models. Semin. Immunol. **10:**225–236.
70. **Van't Wout, A. B., H. Blaak, L. J. Ran, M. Brouwer, C. Kuiken, and H. Schuitemaker.** 1998. Evolution of syncytium inducing and non syncytium inducing biological virus clones in relation to replication kinetics during the course of human immunodeficiency virus type 1 infection. J. Virol. **72:**5099–5107.