Differential Ability of Primary HIV-1 Nef Isolates To Downregulate HIV-1 Entry Receptors

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ABSTRACT

HIV-1 Nef downregulates the viral entry receptor CD4 as well as the coreceptors CCR5 and CXCR4 from the surface of HIV-infected cells, and this leads to promotion of viral replication through superinfection resistance and other mechanisms. Nef sequence motifs that modulate these functions have been identified via in vitro mutagenesis with laboratory HIV-1 strains. However, it remains unclear whether the same motifs contribute to Nef activity in patient-derived sequences and whether these motifs may differ in Nef sequences isolated at different infection stages and/or from patients with different disease phenotypes. Here, nef clones from 45 elite controllers (EC), 46 chronic progressors (CP), and 43 acute progressors (AP) were examined for their CD4, CCR5, and CXCR4 downregulation functions. Nef clones from EC exhibited statistically significantly impaired CD4 and CCR5 downregulation ability and modestly impaired CXCR4 downregulation activity compared to those from CP and AP. Nef’s ability to downregulate CD4 and CCR5 correlated positively in all cohorts, suggesting that they are functionally linked in vivo. Moreover, impairments in Nef’s receptor downregulation functions increased the susceptibility of Nef-expressing cells to HIV-1 infection. Mutagenesis studies on three functionally impaired EC Nef clones revealed that multiple residues, including those at novel sites, were involved in the alteration of Nef functions and steady-state protein levels. Specifically, polymorphisms at highly conserved tryptophan residues (e.g., Trp-57 and Trp-183) and immune escape-associated sites were responsible for reduced Nef functions in these clones. Our results suggest that the functional modulation of primary Nef sequences is mediated by complex polymorphism networks.

IMPORTANCE

HIV-1 Nef, a key factor for viral pathogenesis, downregulates functionally important molecules from the surface of infected cells, including the viral entry receptor CD4 and coreceptors CCR5 and CXCR4. This activity enhances viral replication by protecting infected cells from cytotoxicity associated with superinfection and may also serve as an immune evasion strategy. However, how these activities are maintained under selective pressure remains elusive. We addressed this question by analyzing functions of primary Nef clones isolated from patients at various infection stages and with different disease phenotypes, including elite controllers, who spontaneously control HIV-1 viremia to undetectable levels. The results indicated that downregulation of HIV-1 entry receptors, particularly CCR5, is impaired in Nef clones from elite controllers. These functional impairments were driven by rare Nef polymorphisms and adaptations associated with cellular immune responses, underscoring the complex molecular pathways responsible for maintaining and attenuating viral protein function in vivo.

A number of viruses, including HIV-1 (1), other retroviruses (2), measles virus (3), influenza virus (4), and hepatitis B virus (5), have evolved ways to prevent superinfection of cells in which viral replication has been initiated. In HIV-1, the ~27-kDa accessory protein Nef plays an important role in the downregulation of the viral entry receptor CD4 (6) and coreceptors CCR5 (1) and CXCR4 (7) on the surfaces of infected cells. Downregulation of entry receptors and coreceptors may protect infected cells from superinfection-associated cytotoxicity due to overaccumulation of integrated viral genomes (8, 9), thus enhancing viral replication (1, 7). Receptor downregulation may also reduce signaling that could otherwise induce apoptosis, modulate intracellular viral transcription, and affect cellular chemotaxis (10, 11). In addition, recent reports have indicated that Nef-mediated CD4 downmodulation helps to protect infected cells from antibody-dependent cell-mediated cytotoxicity, thereby promoting viral persis-
tence (12, 13). The importance of Nef-mediated viral entry receptor downmodulation in HIV-1 pathogenesis is further demonstrated by natural variation in the ability of Nef clones isolated from infected individuals over the disease course to downregulate CD4 (14–17). Similarly, the impairment of these Nef activities in long-term nonprogressors (NP) (18), acute controllers (AC) (19), and elite controllers (EC) (20), who spontaneously suppress plasma viral loads (pVL) to undetectable levels in the absence of antiviral therapy, also supports their importance. However, it remains elusive whether Nef-mediated downregulation of CCR5 and CXCR4 also exhibits functional variation among primary clones and whether these functions are attenuated in HIV-1 controllers.

Despite being one of HIV-1’s most variable proteins, Nef nevertheless possesses several functionally important, highly conserved motifs. Motifs responsible for each of Nef’s functions have been identified in mutagenesis studies of laboratory-adapted HIV-1 strains (1, 6, 7, 21–25). For instance, CD4 and HLA class I downregulation activities are genetically separable. Loss of CD4 downregulation function can be achieved via disruption of the highly conserved motifs LL163,164 and DD174,175 (6, 22, 24), whereas alanine substitutions at M30 within the acidic cluster E62EEE65 and in the polyproline motif P72xxP78, affect HLA class I downregulation (21, 23, 25). The latter two motifs are additionally important for downregulation of both CCR5 and CXCR4 (1, 7).

In contrast, the locations and sequences of functionally important Nef motifs in naturally occurring (patient-derived) sequences remain poorly characterized. This is due in part to the extremely high sequence conservation of known motifs important for downregulation of CD4, HLA class I, and other receptors (14, 15, 17, 20, 26–30). Given that natural Nef sequences exhibit substantial functional heterogeneity (16, 17, 20, 31), it is reasonable to hypothesize that various secondary polymorphisms in Nef modulate these differences. Indeed, a recent report implicating a set of previously undescribed polymorphisms in modulating Nef-mediated HLA class I downregulation supports this hypothesis (26).

In the present study, we examined the downregulation of the HIV-1 entry receptor CD4 and coreceptors CCR5 and CXCR4 by Nef proteins derived from patients of diverse infection stages and phenotypes, including 45 EC, 46 CP, and 43 AP. We demonstrate that Nef clones from EC are significantly impaired in CD4 and CCR5 downregulation activity and modestly impaired in CXCR4 downregulation compared to those from CP and AP. In addition, by using EC Nef sequences with severely impaired function, we have identified various polymorphisms, including novel ones, that modulate Nef activity in natural sequences.

**MATERIALS AND METHODS**

**Study subjects.** A total of 45 EC (median pVL of 2 RNA copies/ml with an interquartile range [IQR] of 0.2 to 14 RNA copies/ml; median CD4 count of 811 [IQR, 612 to 1,022] cells/mm<sup>3</sup>) (20, 32) and 46 CP (median pVL, 80,500 [IQR, 25,121 to 221,250] RNA copies/ml; median CD4 count, 292.5 [IQR, 72.5 to 440] cells/mm<sup>3</sup>) (17) were studied as described previously. A total of 43 AP were identified during acute/early HIV-1 infection as defined by the Acute Infection Early Disease Research Program (AIEDRP) criteria (33) from cohorts in Boston, New York, and Berlin, Germany, as described previously (19, 34, 35). For each AP, the earliest available plasma sample was studied; these were collected a median of 54 [IQR, 36 to 72] estimated days postinfection. The median pVL among AP was 380,000 [IQR, 33,300 to 750,100] RNA copies/ml. All EC, CP, and AP were untreated at the time of sample collection and infected with HIV-1 subtype B. This study was approved by the institutional review board of Massachusetts General Hospital, Boston, MA, USA, and all participants provided written informed consent.

**Cloning and plasmid construction.** Control and patient-derived nef genes were amplified from plasma HIV-1 RNA by nested reverse transcription-PCR as described earlier (36) and cloned into the pIRE2-EGFP vector (Clontech), which coexpresses Nef and enhanced green fluorescent protein (EGFP), the latter via an internal ribosome entry site (IRES). A median of 3 nef clones was sequenced per patient, and a single clone with an intact Nef reading frame closely resembling the original bulk sequence was selected for analysis (17, 19, 20).

Nef-GFP fusion constructs were also generated for control and patient-derived Nef sequences. To do this, DNA fragments encoding Nef from the HIV-1 reference strain SF2, fused to GFP, were cloned into pCDNA3.1 (Invitrogen) as described previously (37). Chimeric domain constructs between Nef from primary isolates and SF2 cells were generated by overlay extension PCR. Defined mutations of interest were introduced into Nef-GFP fusion constructs by site-directed mutagenesis. All control, patient, and site-directed mutant Nef clones were reconfirmed by DNA sequencing of the entire nef region.

Naturally occurring Nef sequences exhibit length polymorphisms. To facilitate a consistent codon numbering scheme (based on the Nef<sub>HXB2</sub> reference strain), all clonal Nef sequences were aligned pairwise to Nef<sub>HXB2</sub> by using an in-house algorithm based on the HyPhy platform (38), and insertions were stripped out.

**Western blot analysis.** TZM-bl cells (39) were transfected for 48 h with plasmid DNAs carrying genes for GFP alone or Nef-GFP fusion proteins, after which the cells were lysed in a buffer composed of 50 mM Tris-HCl (pH 8.0) containing 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS (20, 27). The lysates (10 μg of protein each) were then subjected to SDS-PAGE in triplicate and transferred to nitrocellulose membranes. The blots were separately probed using anti-GFP polyclonal antibody (Medical & Biological Laboratories, Nagoya, Japan), anti-Nef polyclonal antiserum (NIH AIDS Research and Reference Reagent Program), and anti-β-actin monoclonal antibody (MAB; Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Nef clones of interest were transferred into a pNL4.3ΔNef plasmid as described previously (20, 30) and confirmed by DNA sequencing. Recombinant viruses harboring nef from HIV-1 strain SF2 (NL43-Nef<sub>SF2</sub>) or lacking nef (NL43-ΔNef) were used as positive and negative controls, respectively. HEK-293T cells were transfected with each proviral clone, and viruses were harvested from the supernatant 48 h later. Cell lysates (10 μg protein each) were prepared, subjected to SDS-PAGE, and transferred to nitrocellulose membranes as described above. The blots were separately probed using anti-HIV-1 Gag p24 polyclonal antibody (BioAcademia, Osaka, Japan) and the same anti-Nef polyclonal antisera and anti-β-actin monoclonal antibody described above. In both cases, band intensities were quantified by using ImageQuant LAS 600 (GE Healthcare Life Sciences).

**Receptor downregulation analysis.** TZM-bl cells (39) were transfected for 48 h with plasmid DNAs harboring genes for GFP alone, Nef-RES-GFP, or Nef-GFP fusion proteins. The resultant cells were stained with allophycocyanin-Cy7-conjugated anti-human CCR5 MAb (BD Pharmingen), brilliant violet-conjugated anti-human CD4 MAb (Biolegend), allophycocyanin-conjugated anti-human CXCR4 MAb (Biolegend), and 7-amino-actinomycin D (7-AAD).

Cells of the human T cell line CEM, as well as primary CD4<sup>+</sup> T lymphocytes, were electroporated with plasmid DNAs encoding GFP alone or Nef-GFP fusion proteins as previously described (16). Primary CD4<sup>+</sup> T lymphocytes were prepared from PBMC of two HIV-negative donors followed by activation with phytohemagglutinin for 5 days and then separation of the CD4<sup>+</sup> subset by magnetic cell sorting (Miltenyi Biotech). For the CCR5 downregulation assay, the plasmid encoding human ccr5 (kindly provided by Kei Sato, Kyoto University, Kyoto, Japan) was electroporated together with the plasmid DNAs encoding GFP alone or Nef-
GP fusion proteins. The resultant cells were stained with either anti-human CCR5 MAb or anti-human CD4 MAb and 7-aminoactinomycin D (7-AAD).

In both cases, the live cells (negative for 7-aminoactinomycin D) were gated, and the mean fluorescence intensity (MFI) of CD4, CCR5, or CXCR4 in GFP+ (Nef-expressing) and GFP- subsets was analyzed by flow cytometry (FACSCanto II or FACSVerse; BD Biosciences, San Jose, CA). Results were expressed as the means of triplicate experiments, normalized to control plasmid expressing NefSF2, such that values of >100% or <100% indicated increased or decreased activity, respectively.

**Superinfection protection assay.** The CCR5-tropic molecular clone pJRFL (kindly provided by Yoshio Koyanagi, Kyoto University, Japan) was digested with DraIII and Xhol, and the fragment encompassing the envelope region was subcloned into similarly digested pNL43 (40), giving rise to pNL43-ENVJRFL. Also, a part of the envelope region of pNL43 (between two BglII sites) was removed by digestion with BglII followed by religation of the resultant fragment (41), giving rise to pNL43-ΔENV. HEK-293T cells were transfected with NL43 (harboring intact ENV_{NL43}, HIV-1 ENV_{ JRFL}, or NL43-ENV_{ JRFL}, or were cotransfected with NL43-ΔENV and DNA encoding vesicular stomatitis virus glycoprotein (VSVg). Virus-containing culture supernatants were collected 48 h later as previously described (28). TZM-bl cells, seeded at 8 × 10^4 cells/well in a 24-well plate, were first transfected for 24 h with plasmid DNAs carrying genes for GFP alone or Nef-GFP fusion proteins containing various mutations of interest, collected, and reseded at 8 × 10^4 cells/well in a 24-well plate. At 24 h after transfection, the resultant cells were then exposed to HIV-1 ENVNL43, HIV-1 ENV_{ JRFL}, or VSVg-pseudotyped virus for 4 h. In addition, cultures treated with the following inhibitors were used as additional controls: 1 μg/ml anti-CD4 MAb (clone SK3; Biologic), 100 nM AMD3100 (CXCR4 inhibitor; NIH AIDS Research and Reference Reagent Program), or 100 nM Maraviroc (MVC; CCR5 inhibitor; NIH AIDS Research and Reference Reagent Program). The resultant cells were collected, stained with 7-AAD followed by intracellular staining with phycoerythrin-labeled anti-p24 Gag MAb (KC-57; Beckman Coulter, CA), and analyzed by flow cytometry. The live (negative for 7-AAD) GFP+ subsets were gated and analyzed for the frequency of p24 Gag-expressing cells.

**Statistical analysis.** Unless otherwise indicated, nonparametric statistics were employed throughout: the Mann-Whitney U test was used to test for differences between two groups, while correlations were analyzed using Spearman’s test. In univariate analyses, a two-tailed value analogues of the false-discovery rate (FDR), which denotes the expected proportion of false positives among results deemed significant at a given P value threshold (42). For example, at a q level of ≤0.2, we expect 20% of identified associations to represent false positives. For this analysis, statistical significance was defined as a P value of <0.05 and a q level of <0.2.

**Nucleotide sequence accession numbers.** The GenBank accession numbers assigned to the clonal nef sequences are JX71199 to JX71243 (EC) (20), JX440926 to JX440971 (CP) (17), and LC018135 to LC018177 (AP).

**RESULTS**

**Downregulation of viral receptors CD4, CCR5, and CXCR4 by patient-derived Nef clones.** Transfection of TZM-bl cells with the NefSF2-ires-GFP control strain resulted in a marked reduction in the cell surface expression of CD4, CCR5, and CXCR4 receptors (Fig. 1A presents a representative set of flow cytometry plots). Residual mean fluorescence intensities (calculated as the MFI of the GFP+ [Nef-expressing] subset divided by the GFP- subset) were 15.1 ± 2.8% (CD4), 42.7 ± 5.2% (CCR5), and 31.0 ± 6.5% (CXCR4) (means ± standard deviations [SD]), confirming previous observations (1, 6, 7, 28).

We then analyzed 45 EC, 46 CP, and 43 AP Nef clones for their ability to downregulate these viral entry receptors. Receptor downregulation activities of the patient-derived Nef clones was normalized to activity of the control strain NefSF2, such that values of >100% or <100% indicated increased or decreased activity compared to NefSF2, respectively. EC Nef clones exhibited median CD4 downregulation activities of 80.2% (IQR, 68.9% to 90.3%) of that of NefSF2, values that were significantly lower than those of CP Nef clones (median, 95.3% [IQR, 80.2% to 99.8%]) and AP Nef clones (median, 99.4% [IQR, 92.9% to 102%]) (Fig. 1B). Importantly, the present data obtained for CD4 downregulation in TZM-bl cells by Nef clones from EC and CP were highly consistent with those previously derived from testing the same set of Nef clones in CEM T cells (17, 20) (Spearman’s R = 0.9, P < 0.001).

Moreover, EC Nef clones exhibited median CCR5 downregulation activities of 60.0% (IQR, 49.8% to 71.3%) relative to control strain NefSF2, values that were significantly lower than those of CP Nef clones (median, 78.3% [IQR, 67.8% to 86.6%]) and AP Nef clones (median, 81.3% [IQR, 67.8% to 86.2%]) (Fig. 1C). In contrast, somewhat, EC Nef clones exhibited only modestly lower CXCR4 downregulation activity (median, 83.2% [IQR, 79.0% to 88.3%]) than CP Nef clones (median, 88.7% [IQR, 79.4% to 97.5%]) and AP Nef clones (median, 89.5% [IQR, 83.4% to 95.7%]), with only the latter comparison achieving statistical significance (Fig. 1D). CP and AP Nef clones differed only with respect to CD4 downregulation, with CP exhibiting modest yet significantly lower function than AP (Fig. 1B).

**Relationship between ccr5 genotype and Nef's ability to downregulate CCR5.** Heterozygosity for the ccr5Δ32 mutation is associated with durable control of HIV-1 infection (43, 44). We postulated that Nef-mediated CCR5 downregulation activity may be reduced in ccr5Δ32 subjects compared to ccr5+/+ subjects, as lower cell surface CCR5 expression in the former could reduce in vivo pressure on Nef to maintain robust CCR5 downregulation function. Seven out of 38 genotyped subjects were ccr5Δ32/− in this EC cohort (32). However, no significant difference in Nef-mediated CCR5 downregulation between ccr5Δ32/− and ccr5+/− EC was observed (Mann-Whitney, P = 0.3).
Amino acids associated with Nef-mediated downregulation of CD4, CCR5, and CXCR4. To investigate the contribution of naturally occurring polymorphisms at Nef’s variable sites on the function of patient-derived Nef sequences, we performed an exploratory sequence-function analysis restricted to amino acids observed at a minimum frequency of $n = 5$ in our data set. At the predefined $P$ threshold of $<0.05$ and a $q$ of $<0.2$, only one polymorphism (Arg-8) in EC Nef appeared to be associated with higher CCR5 downregulation function, although introduction of this mutation into NefSF2 resulted in no obvious functional difference (data not shown). No Nef codons were identified as significantly associated with CD4 or CXCR4 downregulation in EC. Among CP and AP, no Nef codons were identified as being significantly associated with any of the three Nef functions evaluated.

Distinct regions responsible for functional impairment in EC Nef clones. We next sought to identify naturally occurring polymorphisms responsible for impaired Nef-mediated viral receptor downregulation in individual Nef clones. We focused on three EC Nef clones, EC12, EC19, and EC51, that exhibited substantially diminished CD4 and CCR5 downregulation activities (Fig. 2A). These three patient-derived Nef clones encoded a large number of amino acid differences distributed throughout the Nef
protein, so we first tried to broadly identify the Nef domain(s) responsible for reduced function. Chimeric constructs between EC clones and the SF2 control strain were constructed in the Nef-GFP fusion backbone (Fig. 3A). Consistent with the results obtained with the IRES system (Fig. 1A), the NefSF2-GFP fusion protein reduced cell surface CD4, CCR5, and CXCR4 expression (Fig. 3B), exhibiting residual MFIs of receptor staining in the GFP/(Nef-expressing) subset of 18.2%, 54.7%, and 49.7%, respectively, relative to the GFP subset.

For clone EC12, substituting the N- and C-terminal halves of EC12 with the corresponding part of NefSF2 (yielding chimeric constructs EC12-N and EC12-C, respectively) did not appreciably rescue CD4 downregulation function (Fig. 3C). This indicated that both the N- and C-terminal halves of Nef are responsible for the poor CD4 downregulation activity of this sequence. In contrast, CD4 downregulation activity in EC19 was rescued from 24.4% to 94.6% of the level of NefSF2 in chimera EC19-C (Fig. 3C), suggesting that the major determinants of poor CD4 downregulation function in this sequence map to its native N-terminal domain. Furthermore, in EC51, CD4 downregulation activity was rescued from 0.5% to 54.8% of the level of NefSF2 in chimera EC51-N (Fig. 3C), suggesting that important determinants of poor CD4 downregulation function in this sequence mapped to its native C-terminal domain. Taken together, results suggest that different Nef

![Diagram of receptor downregulation](https://example.com/diagram.png)
motifs are responsible for impaired CD4 downregulation activity in EC12, EC19 and EC31 Nef clones.

We also evaluated these chimeric constructs with respect to their CCR5 downregulation activity. In contrast, somewhat, to results for CD4 downregulation, EC12-N rescued CCR5 downregulation activity from 50.8 to 81.2% (Fig. 3D), indicating that different motifs modulate CD4 and CCR5 downregulation activities in this clone. EC19 was generally functional for CCR5 downregulation; nevertheless, exchanging the N terminus of this clone with the NefSF2 sequence (EC19-C) modestly improved CCR5 downregulation activity from 75.4 to 88.2% (Fig. 3D). Similar to results for CD4, CCR5 downregulation activity was rescued from 10.8 to 63.5% in EC51-N (Fig. 3D), suggesting that key determinants of poor CCR5 downregulation function in this sequence also map to its native C-terminal domain. Taken together, results suggest that different motifs were responsible for impaired downregulation of CCR5 in EC12, EC19, and EC51 Nef. Moreover, key determinants of CXCR4 downregulation function were similarly mapped for these clones (Fig. 3E). Whereas EC12 and EC19 were generally functional for CXCR4 downregulation, exchanging the C terminus of EC51 with the NefSF2 sequence (EC51-N) improved CXCR4 activity from 56.2 to 93.0%.

We also tested CD4, CCR5, and CXCR4 downregulation activity of these constructs in the human CEM T cell line as well as primary CD4+ T cells. CD4, CCR5, and CXCR4 downregulation activities in CEM cells for NefSF2 were all comparable with those obtained in TZM-bl cells (data not shown). In contrast, receptor downregulation activities of NefSF2 were relatively weak in primary CD4+ T cells: residual MFIs (of the GFP subset divided by the GFP” subset) were 32.0% ± 2.8% (CD4), 70.6% ± 2.7% (CCR5), and 80.3% ± 0.6% (CXCR4). Importantly however, the relative differences in CD4, CCR5, and CXCR4 downregulation activities in all three EC Nef clones and their chimeric constructs measured in primary CD4+ T cells were consistent with those measured in TZM-bl cells and CEM cells (Pearson correlation, all
Distinct regions responsible for steady-state protein expression levels in EC-Nef clones. The steady-state expression level of Nef-GFP fusion proteins in TZM-bl cells was examined with an anti-GFP antibody (Fig. 4A). EC12 Nef exhibited protein expression of 25.2% of the level of NefSF2, and EC12-C rescued it to 41.8%, whereas the protein expression level of EC12-N (25.1%) remained compromised (Fig. 4B). All EC19 and EC19 chimeras showed protein expression levels broadly comparable to NefSF2 (Fig. 4B). In contrast, EC51 Nef exhibited protein expression that was 21.6% of the NefSF2 level, and EC51-N rescued it to 57.3%, whereas the protein expression level of EC51-C (25.1%) remained compromised (Fig. 4B). We also analyzed the Nef-GFP protein expression level via staining with an anti-Nef antibody (Fig. 4A) and confirmed that both results were in good agreement.

To characterize Nef expression in the context of a whole-virus construct, EC-derived Nef clones and their respective chimeras were cloned into NL43-based proviral plasmids, and the expression level of Nef and Gag proteins was analyzed in HEK-293T cells 48 h after transfection. Consistent with the Nef-GFP transfection experiments, a substantially reduced steady-state Nef expression level was observed for EC12 and EC12-N, as well as EC51 and EC51-C, though the expression levels of Gag protein and β-actin in the same samples was not much affected (Fig. 4C). In contrast, EC19 and their chimeric constructs showed expression levels of Nef and Gag comparable to the control, NL43-NefSF2 (Fig. 4C). Taken together, these data indicate that different Nef motifs affected the steady-state protein expression levels among the EC-Nef clones.

FIG 4 Western blot analysis of Nef chimeric constructs. (A) Western blot analysis of total cell lysates (10 μg of protein each) from TZM-bl cells transfected with DNA constructs encoding GFP alone or NefSF2-GFP, as well as EC-derived clones and GFP chimeras. The membranes with transferred proteins were stained with antibodies to GFP (top), Nef (middle), and β-actin (bottom). (B) Quantification of band intensities obtained from the anti-GFP staining. Steady-state expression was normalized to NefSF2, which was arbitrarily set at 100%, and each data point reflects the mean ± SD of triplicate determinations. (C) Western blot analysis of total cell lysates (10 μg of protein each) from HEK-293T cells transfected with full-length proviral HIV-1 genomes expressing the various indicated Nef proteins. The membranes were stained with antibodies to Nef (top), Gag, and β-actin (bottom). Representative data (of two independent assays) are shown.

Fine-mapping sequence motifs responsible for impaired functions in individual EC-Nef clones. Our initial chimeric experiments indicated that impairment of CD4, CCR5, and/or CXCR4 downregulation activities in different EC Nef clones was mediated by different Nef genetic regions. Specifically, CD4 downregulation and steady-state Nef protein levels in clone EC12 appeared to be modulated by components located throughout the Nef coding region (while CCR5 and CXCR4 downregulation in EC12 appeared to be modulated in part by motifs in its C-terminal domain). Due to the complexity of mapping such widespread sites, and the observation that CXCR4 downregulation activity of EC12 and its chimeras varied to a lesser extent than for other EC Nef clones (Fig. 3E), this clone was not followed further. In contrast, for clones EC19 and EC51, our initial chimeric experiments clearly implicated specific regions for follow-up. For EC19, determinants of both CD4 and CCR5 appeared to map to its native N terminus, whereas for EC51, key determinants of both function and steady-state protein levels appeared to map to its native C terminus. As such, these two clones were examined in fine-mapping experiments to identify specific Nef sequence determinants of CD4 and CCR5 downregulation activity in these clones.

First, we further minimized the N-terminal part of EC19 on the NefSF2 backbone by constructing and functionally evaluating chimeras expressing EC19 Nef codons 1 to 35 (EC19-N1) and 36 to 91 (EC19-N2) in the NefSF2 backbone. In chimera EC19-N1, CD4 downregulation function was rescued to 82.0% of NefSF2 levels, while the function of EC19-N2 remained substantially compromised (27.7%). This result indicated that the major determinants of the CD4 downregulation defect of EC19 map to Nef positions 36 to 91, as function was largely rescued when this region was

R values > 0.9, P < 0.01), suggesting that the observed impairments in EC-derived Nef clones are independent of cell type.

To characterize Nef expression in the context of a whole-virus construct, EC-derived Nef clones and their respective chimeras were cloned into NL43-based proviral plasmids, and the expression level of Nef and Gag proteins was analyzed in HEK-293T cells 48 h after transfection. Consistent with the Nef-GFP transfection experiments, a substantially reduced steady-state Nef expression level was observed for EC12 and EC12-N, as well as EC51 and EC51-C, though the expression levels of Gag protein and β-actin in the same samples was not much affected (Fig. 4C). In contrast, EC19 and their chimeric constructs showed expression levels of Nef and Gag comparable to the control, NL43-NefSF2 (Fig. 4C). Taken together, these data indicate that different Nef motifs affected the steady-state protein expression levels among the EC-Nef clones.
replaced by that of NefSF2 (Fig. 5A). In this region, EC19-Nef and NefSF2 differ at 9 residues: 38, 43, 50, 51, 54, 55, 57, 83, and 87 (Fig. 5A). It is notable that EC19 harbored exceedingly rare substitutions at some of these sites (e.g., 55C and 57R).

To further fine-map the residues responsible for the functional rescue, the following amino acid changes were introduced into EC19-N2: insertion of A and T residues at positions 50 and 51 (EC19-N2ins), introduction of an S-to-C substitution at codon 55 (SF2-57R).
(EC19-N2C), and a substitution of the rare R with the highly conserved W at codon 57 (EC19-N2W) (Fig. 5A). These changes resulted in increases in CD4 downregulation activity from 27.7% (EC19-N2) to 36.1% (EC19-N2ins), 37.5% (EC19-N2C), and 54.2% (EC19-N2W) of that of NefSF2, respectively (Fig. 5A). Moreover, introductions of both the S-to-C and R-to-W substitutions at codons 55 and 57, respectively (EC19-N2CW) further increased CD4 downregulation activity to 66.4% of that of NefSF2, suggesting additive effects of these amino acid residues on modulation of CD4 downregulation activity. Conversely, changing Nef codon 57 from the highly conserved W to the rare R in NefSF2 (SF2-57R) decreased its CD4 downregulation activity to 76.9%, while changing both codons 55 and 57, respectively, by introducing C-to-S and W-to-R substitutions (SF2-SR) further decreased NefSF2’s CD4 downregulation activity to 42.7% (Fig. 5A). In general, CCR5 downregulation activities of these mutant constructs were similarly affected by these changes, albeit to lesser extents (Fig. 5A).

Taken together, these results indicated that the presence of a rare residue at Nef codon 57 (57R), and to a lesser extent the presence of an uncommon residue at codon 55 (55S) and deletions at codons 50 and 51, contributed to the impaired CD4 downregulation activity of EC19’s Nef sequence. Of interest, the 55S substitution has been identified as a noncanonical HLA-B*57-associated polymorphism in EC (20). Elite controller EC19 expressed HLA-B*57, suggesting that Nef-55S could have arisen via HLA-B*57-mediated immune escape in this individual.

We then moved on to EC51. For this clone, we further minimized its C-terminal part on the NefSF2 backbone by constructing and functionally evaluating chimeras expressing EC51 Nef codons 150 to 170 (EC51-C1) and EC51 Nef codons 171 to 206 (EC51-C2) on the NefSF2 backbone (Fig. 5B). In chimera EC51-C1, CD4 downregulation function was rescued to 100% of NefSF2 levels, while the function of EC51-C2 remained substantially compromised (0.4%) (Fig. 5B). This result indicated that the major determinants of the CD4 downregulation defect of EC51 map to Nef positions 171 to 206 (as function was 100% in all clones harboring the NefSF2 sequence in this region, but essentially 0% in all clones harboring EC51’s sequence in this region). In this region, EC51-, Nef and NefSF2 differ at 8 residues: 171, 173, 176, 183, 184, 188, and 192 (Fig. 5B). Again, it is notable that EC51 harbored exceedingly rare substitutions at some of these sites (e.g., 171Q and 183R).

To further fine-map the residues responsible for the functional rescue, the following amino acid changes were introduced into EC51-C2: introduction of a Q-to-H substitution at 171 (EC51-C2H), and a double substitution from R to the highly conserved W at codon 183 and Q to R at codon 184 (EC51-C2WR). Whereas the function of EC51-C2H remained substantially compromised (1.1%), introduction of highly conserved 183W and 184R into EC51 (EC51-C2WR) resulted in its functional rescue to 90.5% of that of NefSF2 (Fig. 5B). Conversely, changing Nef codon 183 from the highly conserved W to R in NefSF2 (SF2-183R) decreased its CD4 downregulation activity to 17.7%, while changing Nef codon 184 from R to Q in NefSF2 (SF2-184Q) remained functional (98.1%) (Fig. 5B). Introducing both W183R and R184Q substitutions into NefSF2 decreased its CD4 downregulation activity to 13.5% (Fig. 5B). Taken together, these results indicated that the presence of a rare residue at Nef codon 183 (183R) nearly fully explains the impaired CD4 downregulation function in EC51’s Nef sequence.

CCR5 downregulation activities and steady-state protein expression levels of these mutant constructs were similarly affected by these changes (Fig. 5B). Moreover, in these constructs, significant positive associations were observed between CD4 downregulation function and protein expression level (Pearson \( R = 0.96, P = 0.0021 \)) as well as CCR5 downregulation function and protein expression level (Pearson \( R = 0.94, P = 0.005 \)). Taken together, these results indicate that the presence of a highly uncommon residue at Nef codon 183 (183R) not only contributed to the impaired CD4 downregulation function, but also impaired CCR5 downregulation activity in patient EC51’s Nef sequence. Moreover, the Nef-183R-mediated functional impairment was also associated with the reduced steady-state protein expression level of this elite controller-derived Nef clone.

**Effects of EC-Nef clones on susceptibility to HIV-1 superinfection.** We postulated that the differential ability to down-modulate viral entry receptors by patient-derived Nef clones would have consequences for the protection of HIV-infected cells from deleterious HIV-1 superinfection (1, 7, 28). Before testing this directly, we first undertook the following control experiments. We transfected GFP alone into TZM-bl cells and then exposed them to HIV-1 expressing various envelopes: ENVJRFL, a CCR5-using strain; NL43-ENVNL43, a CXCR4-using strain; and VSVg-pseudotyped HIV-1, which does not require CD4 or CCR5/CXCR4 coreceptors for entry. As expected, infection by HIV-ENVJRFL was nearly completely blocked by CD4 MAb or the CCR5 antagonist Maraviroc (MVC), while infection by HIV-ENVNL43 was nearly completely blocked by CD4 MAb or the CXCR4 antagonist AMD3100 (Fig. 6A). Also as expected, infection by VSVg-pseudotyped virus was not blocked by any of these reagents (Fig. 6A).

Next, TZM-bl cells were transfected with the NefSF2-GFP fusion protein and exposed to infection by these viruses. The NefSF2 expression in TZM-bl cells substantially protected the infection of HIV-ENVJRFL and HIV-ENVNL43 (Fig. 6A), with the frequency of infected cells (calculated as the frequency of the p24 Gag subset within GFP+ [Nef-expressing] cells) reduced to 22.0% ± 4.2% and 26.3% ± 2.8%, respectively, compared to TZM-bl cells expressing GFP only (no treatment). As expected however, NefSF2 expression in TZM-bl cells did not protect cells against infection by VSVg-pseudotyped HIV-1 (Fig. 6A).

We then tested the following NefSF2 mutations known to specifically disrupt downregulation of CCR5/CXCR4 coreceptors and CD4, respectively: P27XXP29 to A27XXA27 (AxxA) and ED174,175 to AA174,175 (EDAA) (1, 7). As expected, the AxxA mutant exhibited substantial impairments in CCR5 and CXCR4 downregulation but no substantial change in CD4 downregulation function, whereas the EDAA mutations exhibited the opposite (Fig. 6B). We next examined the susceptibility of target cells expressing these Nef variants to infection by our panel of HIV-1 strains. The AxxA variant reduced the protection from infection by HIV-ENVJRFL and HIV-ENVNL43 only modestly (Fig. 6B). In contrast, the EDAA variant completely lost the protection from infection by both HIV-ENVJRFL and ENVNL43 (Fig. 6B). The results suggested that Nef-mediated CCR5 and CXCR4 downregulation modestly reduces the susceptibility of infected cells to HIV-1 infection, whereas Nef-mediated CD4 downregulation profoundly reduces their susceptibility to HIV-1 infection.

Next, we delivered the three EC-Nef clones (EC12, -19, and...
and their chimeras into TZM-bl cells and tested these cultures for susceptibility to HIV-ENVJRFNL infection. EC12 and its chimeras provided substantially decreased protection (<20%) against HIV-ENVJRFNL infection compared to NefSF2 (Fig. 6C). EC19 and its chimera EC19-N also provided substantially decreased (<25%) protection against HIV-1 ENVJRFNL infection. In contrast, EC19-C, which exhibited comparable CD4 and CCR5 downregulation activities to NefSF2 (Fig. 3C and D), also protected against HIV-ENVJRFNL infection comparably to NefSF2 (Fig. 6C). As expected given their diminished CD4 and CCR5 downregulation...
functions (Fig. 3C and D), EC51 and EC51-C provided very poor protection against HIV-ENVJRFL infection (Fig. 6C). In contrast, EC51-N, which exhibited >50% of the CD4 and CCR5 downregulation activities of NefSF2 (Fig. 3C and D) provided 45.9% protection against HIV-ENVJRFL infection relative to NefSF2 (Fig. 6C). Overall, the extent of Nef-mediated CD4 downregulation function correlated positively with protection against HIV-ENVJRFL infection (Pearson $R = 0.98$, $P = <0.001$). However, preservation of Nef-mediated CCR5 downregulation function in some clones (e.g., EC12, EC12-N, EC12-C, EC19, and EC19-N, which exhibit >50% activity compared to NefSF2) (Fig. 3D) plays a part in retaining at least some protection against HIV-ENVJRFL infection.

We also evaluated the same set of Nef clones with respect to protection against HIV-ENVNL43 infection (Fig. 6C). Again, protection against HIV-ENVNL43 infection in Nef-expressing cells correlated positively with Nef’s CD4 downregulation function (Pearson $R = 0.93$, $P < 0.001$). Furthermore, preservation of CXC4 downregulation functions in patient-derived Nef clones (Fig. 3E) may play a role in retaining some protection against HIV-ENVNL43 infection (Fig. 6C).

### DISCUSSION

In this study, we showed that the downregulation functions of the viral entry receptor CD4 as well as the coreceptors CCR5 and CXC4 differed markedly among Nef clones isolated from 45 EC compared to 46 CP and 43 AP. Specifically, the median CD4 and CCR5 downregulation activities of EC-derived Nef clones were significantly impaired compared to those of CP- and AP-derived Nef clones. Also, the median CXC4 downregulation activity of EC-derived Nef clones was modestly impaired compared to that of CP- and AP-derived Nef clones, with the latter significantly so. In addition, these reductions in Nef’s receptor downregulation functions increased the susceptibility of Nef-expressing cells to viral superinfection. By fine-mapping sequence determinants associated with decreased Nef-mediated CD4 and CCR5 downregulation activities in EC Nef clones, we revealed that combinations of amino acid variations unique to individual EC Nef clones (rather than particular Nef genetic determinants common to EC) were responsible, at least in part, for impairments in Nef function and steady-state protein expression levels in these patient sequences. Specifically, rare polymorphisms at highly conserved tryptophan residues (e.g., Trp-57 and Trp-183), deletions (e.g., AT50,51), and polymorphisms at HLA-associated sites (e.g., Cys-55) were responsible for reduced functions in the EC-derived Nef clones examined. These results indicated that unique sequence determinants, likely modulated by viral and host immune factors, contribute to the Nef functional attenuation observed in EC.

Certain cytotoxic T lymphocyte escape and HLA-associated polymorphisms in Nef have been shown to affect Nef functionality in chronic progressors (26, 27, 30), elite controllers (20), and patients at acute/early phases of infection who subsequently become controllers (19). Although previous studies investigated various Nef functions (including downregulation of CD4 and HLA class I, upregulation of CD74, enhancement of virion infectivity, and stimulation of viral replication), the effects of HLA-associated mutations on Nef’s ability to downregulate chemokine receptors CCR5 and CXC4 remain uncharacterized. As protective HLA class I alleles, most notably HLA-B*57, are overrepresented in EC (32, 44), it is possible that mutations associated with such alleles may impair Nef function in these individuals. Indeed, we previously identified noncanonical HLA-B*57-associated polymorphisms in the present EC cohort, including Nef substitutions 3G, 19R, E28D, C55X, V85L, 187L, Q105X, G178X, and M198X (20).

To investigate the relationship between coreceptor downregulation and the presence of these HLA-B*57-associated polymorphisms among the 17 EC expressing this allele in our cohort, we assigned each of these Nef sequences a score reflecting the total number of HLA-B*57-associated polymorphisms contained therein. This number correlated negatively (albeit not significantly) with CCR5 (Spearman $R = -0.45$, $P = 0.07$) and CXC4 (Spearman $R = -0.39$, $P = 0.12$) downregulation activities in these 17 HLA-B*57-expressing EC. These results suggest that Nef’s ability to downregulate CCR5 and CXC4 can be influenced at least to some extent by Nef polymorphisms selected under host cellular immune pressure.

Our study also illustrates that Nef motifs modulating function in natural Nef sequences may be different, and more complex, than those previously mapped in mutagenesis studies of reference strains. Nef-mediated CD4 downregulation has been reported to occur via two mechanisms: direct binding of Nef to the cytoplasmic tail of CD4 via Nef’s WL57,58 motif (45), and Nef-mediated hijacking of the clathrin sorting pathway of CD4, mediated in part by Nef’s LL163,164 motif (22, 46). In the former pathway, introduction of WL57,58 to AA57,58 mutations into laboratory-adapter Nef strains substantially impaired CD4 downregulation activity (47). In contrast, in our study, introduction of the natural yet rare R57 polymorphism into NefSF2 conferred only a 20% reduction in CD4 downregulation activity; additional natural neighboring mutations (e.g., S57) were necessary to further impair Nef function. Moreover, among three EC Nef clones carrying R57, in our study (no CP or AP Nef clone carried this residue), only EC19 Nef exhibited <50% CD4 downregulation activity relative to NefSF2 (the other two exhibited >80% activity). These results suggest that, though individual polymorphisms can underlie functional impairments in some cases, in other cases their individual contributions are more subtle, and multiple naturally arising polymorphisms are required in combination to reduce Nef-mediated CD4 downregulation ability in EC. Measurement of direct binding of Nef to CD4 may help in understanding the differential downregulation function of Nef variants harboring mutations around W57. The LL163,164 motif of Nef was conserved in all Nef clones from all three cohorts; therefore, this motif is less likely to mediate functional heterogeneity of patient-derived Nef clones.

Mechanistic pathways of Nef-mediated downregulation of CCR5 and CXC4 remain unclear. SIVmac239 Nef exhibits a more pronounced ability to downregulate CXC4 than HIV-1 Nef (48). Moreover, this activity of simian immunodeficiency virus (SIV) is abolished by mutations that disrupt the clathrin adaptor protein 2 binding element located in the N-terminal region, which is unique to SIV Nef (48), suggesting the involvement of clathrin adaptor protein 2 in Nef-mediated CXC4 downregulation. In HIV-1 Nef, introduction of alanine mutations in the acidic cluster E62EEE65 and the polyproline motif P72xxP78 disrupts both CCR5 and CXC4 downregulation (1, 7). Nef’s P72xxP78 motif overlaps those known to mediate HLA class I downregulation (21, 23, 25), which is mediated by clathrin adaptor protein 1 (49, 50). Furthermore, a recent report demonstrated that HIV-1 Nef downregulates C-C and C-X-C chemokine receptors, including CCR5 and CXC4, via ubiquitin-dependent and...
-independent mechanisms (51). In our study, Nef-mediated viral entry receptor downregulation functions correlated positively in patient-derived sequences (Fig. 2), suggesting that these Nef functions are mechanistically linked to each other through common functional motifs or interactions with host proteins in vivo. Alternatively, various amino acid combinations within or outside known motifs may allow individual Nef clones to simultaneously adapt to each host’s unique milieu in vivo. The observation that each EC-Nef clone harbored unique polymorphisms that contributed to its functional attenuation supports the latter hypothesis. Of note, we found no evidence of functional trade-offs or substitutions/domains that enhanced one function at the expense of another in any of the Nef clones examined here.

The HIV-1 Nef subtype B consensus sequence contains 7 tryptophan residues (at positions 5, 13, 57, 113, 124, 141, and 183); all of these are >95% conserved in the Los Alamos database. Conservation of Trp-183 is particularly high (1,466 out of 1,469 [99.8%] in the Los Alamos sequence database), suggesting its functional importance in vivo. Indeed, a recent report demonstrated that introduction of an unnatural W-to-A mutation at codon 183 alone impaired Nef’s ability to enhance virion infectivity (52). However, the role of this residue in the function of natural Nef sequences, especially in terms of viral coreceptor downregulation, remains incompletely characterized. We demonstrated that introduction of the natural, albeit rare, W-to-R mutation at codon 183 in NefW induced its steady-state protein expression level by 70% and reduced downregulation activities of CD4 and CCR5 also by 70%. The downregulation of HLA class I was similarly impaired to its functional attenuation supports the latter hypothesis. Of note, we found no evidence of functional trade-offs or substitutions/domains that enhanced one function at the expense of another in any of the Nef clones examined here.

In conclusion, our results suggest that Nef-mediated CD4, though not CCR5 or CXCR4, downregulation function differs across infection stages, with acute/early sequences exhibiting modestly higher function than those sampled at the chronic stage. More notably, Nef-mediated downregulation of primary (CD4) and secondary (CCR5 or CXCR4) viral entry receptors and resultant protection against HIV-1 superinfection were significantly impaired in Nef clones isolated from elite controllers. In the latter sequences, functional impairments were modulated by unique, host-specific combinations of rare polymorphisms (including some likely selected under in vivo cellular immune pressures), indicating that the functional modulation of primary Nef sequences is likely mediated by complex polymorphism networks.

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