Down-Modulation of CD8αβ Is a Fundamental Activity of Primate Lentiviral Nef Proteins

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It is well established that the Nef proteins of human and simian immunodeficiency viruses (HIV and SIV) modulate major histocompatibility complex class I (MHC-I) cell surface expression to protect infected cells against lysis by cytotoxic T lymphocytes (CTLs). Recent data supported the observation that Nef also manipulates CTLs directly by down-modulating CD8αβ (J. A. Leonard, T. Filzen, C. C. Carter, M. Schaefer, and K. L. Collins, J. Virol. 85:6867–6881, 2011), but it remained unknown whether this Nef activity is conserved between different lineages of HIV and SIV. In this study, we examined a total of 42 *nef* alleles from 16 different primate lentiviruses representing most major lineages of primate lentiviruses, as well as nonpandemic HIV-1 strains and the direct precursors of HIV-1 (SIVcpz and SIVgor). We found that the vast majority of these *nef* alleles strongly down-modulate CD8β in human T cells. Primate lentiviral Nefs generally interacted specifically with the cytoplasmic tail of CD8β, and down-modulation of this receptor was dependent on the conserved dileucine-based motif and two adjacent acidic residues (DD/E) in the C-terminal flexible loop of SIV Nef proteins. Both of these motifs are known to be important for the interaction of HIV-1 Nef with AP-2, and they were also shown to be critical for down-modulation of CD4 and CD28, but not MHC-I, by SIV Nefs. Our results show that down-modulation of CD4, CD8β, and CD28 involves largely overlapping (but not identical) domains and is most likely dependent on conserved interactions of primate lentiviral Nefs with cellular adaptor proteins. Furthermore, our data demonstrate that Nef-mediated down-modulation of CD8αβ is a fundamental property of primate lentiviruses and suggest that direct manipulation of CD8+ T cells plays a relevant role in viral immune evasion.

Cellular immune responses, mediated by CD8+ cytotoxic T lymphocytes (CTLs), play a significant role in the control of viral replication (9, 39). However, human and simian immunodeficiency viruses (HIV and SIV) can evade CTL control by the acquisition of escape mutations in major histocompatibility complex class I (MHC-I)-restricted epitopes and have evolved elaborate mechanisms to counteract the host immune response (8, 29). As a consequence, CTLs are usually unable to suppress viral replication to harmless levels and, ultimately, almost invariably fail to prevent progression to AIDS. In particular, the multifunctional accessory Nef protein of HIV and SIV is well known for its capability to manipulate multiple cellular functions to support viral immune evasion, replication, and pathogenesis (4, 30). Nef undermines the efficiency of the CTL response by several mechanisms. First, it down-modulates MHC-I molecules from the cell surface (2, 32, 33, 59). This Nef function reduces CTL lysis of HIV-1-infected cells (13) and is associated with a strong selective advantage in SIV-infected rhesus macaques (42, 66). Notably, Nef affects MHC-I cell surface expression in a selective manner, i.e., it down-modulates HLA-A and -B but not HLA-C or -E alleles, most likely to balance escape from CTL lysis with protection from attack by natural killer (NK) cells (11, 15, 62). Second, Nef impairs the function of CD4+ T cells and antigen-presenting cells (APCs) that are required to maintain proper CD8+ T cell responses. In virally infected CD4+ T cells, Nef reduces the levels of CD4, MHC-I, and (less efficiently) CD28 and CXCR4 (CXCL12) cell surface expression and alters signal transduction and trafficking pathways (4, 30). In APCs, Nef perturbs MHC-II-restricted antigen presentation by up-modulation of Ii cell surface expression (56, 64). Third, it has been proposed that Nef-mediated up-modulation of FAS-L induces apoptosis of "attacking" CTLs (47, 71).

Accumulating evidence suggests that HIV-1 may also infect CD8+ T cells and manipulate them directly. During development in the thymus, a significant proportion of T cells express both CD4 and CD8 (17, 21), and low levels of CD4 expression are induced on activated memory CD8+ T cells, thereby rendering them susceptible to HIV and SIV infection (10, 18, 31). Finally, it has been reported that some rare HIV-1 strains can use CD8 as a receptor for viral entry (49, 50, 72). In support of a relevant role *in vivo*, HIV-1-infected CD8+ T cells have been detected in infected individuals, particularly in late-stage AIDS patients (50). Thus, although CD4+ T cells are the main targets of HIV-1 infection, it may also be advantageous for the virus to manipulate infected CD8+ T cells. In fact, it has been reported that some Nef proteins interact specifically with the cytoplasmic tail (CT) of the β-chain of CD8αβ to down-modulate it from the cell surface (35, 63) and that a CD8+ T cell subpopulation with reduced CD8β chain expression emerges in HIV-1-infected individuals (57). CD8β cell surface expression is dependent on coexpression of the CD8α chain, and both form covalently linked CD8αβ heterodimers. Furthermore, CD8 also exists as an αα homodimer that is expressed mainly by intestinal γδ T cells and thymic T cell precursors, whereas the CD8αβ receptor is found mostly on thymocytes and mature cytotoxic/suppressor T cells (19). Since the latter T cell population is the one that attacks and eliminates HIV-infected

cells, it is conceivable that the virus specifically targets the β-chain of the CD8 receptor.

The elegant study of Stove and coworkers established that some HIV-1 group M (major) Nef proteins, as well as the HIV-2 Rod and SIVmac239 Nef proteins, down-modulate CD8$\alpha\beta$ heterodimers (63). More recent work by the Collins laboratory suggested that AP-1 is relevant for down-modulation of CD8β by the HIV-1 Nef protein (35). It remained largely elusive, however, whether Nef-mediated down-modulation of CD8β is functionally and mechanistically conserved between different lineages of primate lentiviruses and whether this Nef function changed after zoonotic transmission of SIVs from apes and monkeys to humans, during adaptation of HIV-1 and HIV-2 to the new host (60). To address this, we functionally analyzed a large panel of highly divergent Nef proteins. Our data demonstrate that the vast majority of primate lentiviral Nef proteins efficiently down-modulate human CD8$\alpha\beta$ from the cell surface by specific interaction with the cytoplasmic tail of CD8β. We also show that CD8β down-modulation is generally dependent on the dileucine (E/DXXXLL/M) motif and on two adjacent acidic residues (D174D175 in HIV-1 NL4-3 Nef and D/ED/E in other Nef proteins), referred to herein as the "DD" motif, in the C-terminal flexible loop of primate lentiviral Nef proteins and generally involves domains highly similar to those employed by Nef to down-regulate CD4 and CD28.

MATERIALS AND METHODS

Nef expression vectors. The generation of pCGCG vectors (22) coexpressing enhanced green fluorescent protein (EGFP) and AU-1-tagged *nef* genes of HIV-1 NL4-3, NA7, JRCSF, YBF30, CK1.62, 8161K9, 13127K2, HJ162, and HJ736, SIVcpz MT145, MB897, GAB1, EK505, TAN1, TAN2, and TAN3, HIV-2 BEN, and SIVgor, -gsn, -mus, -mon, and -mac239 from a bicistronic RNA via an internal ribosome entry site (IRES) has been described previously (52, 56). Additional *nef* alleles from HIV-1 YBF116 and DJ131, SIVcpz US, GAB2, CAM5.1, Nok5, and Nik4, HIV-2 Cbl23, 60415K, and 310319, SIVsmm Fmm1, Fyr1, and Fwr1, and SIVrcm, -deb40, -syk51, -blue31, -sun, -tan1, and -sab1 were PCR amplified using primers introducing XbaI and MluI sites flanking the *nef* reading frame and were cloned into the pCGCG vector (56). An overview of these *nef* alleles is provided in Table S1 in the supplemental material. Site-directed mutagenesis was performed by splice overlap extension PCR. The pCGCG (*nef⁻*) control vector expressing only EGFP contains a *nef* gene with a mutation in the ATG initiation codon and two premature stop codons, at positions 3 and 40 of the reading frame (56). All constructs were verified by sequence analysis.

Proviral constructs. Generation of HIV-1 (NL4-3-based) proviral constructs carrying functional *nef* genes followed by an IRES element and the *eGFP* gene has been described previously (1, 55, 56). Splice overlap extension PCR was used to replace the HIV-1 NL4-3 *nef* allele with the *nef* genes listed in Table S1 in the supplemental material, via the single restriction sites for HpaI and MluI. Further splice overlap extension PCR was used to introduce alanine substitutions in SIVagmSab Nef and to mutate the LL/M residues in the C-terminal flexible loop of various primate lentiviral Nef proteins as described elsewhere (55, 56). All constructs were verified by sequence analysis.

Cell culture. 293T and SupT1 cells were cultured as described elsewhere (35, 56). CD4⁺ and CD8⁺ T cells from healthy human donors were isolated using human CD4⁺ or CD8⁺ T cell RosetteSep kits (Stemcell Technologies) or CD4 or CD8 MicroBeads (MACS; Miltenyi Biotec) following the protocols provided by the manufacturers. The cells were stimulated for 3 days with human CD3/CD28 T-Activator Dynabeads (Invitrogen) at a cell-to-bead ratio of 1:1 and with 10 ng/ml interleukin-2 (IL-2) prior to transduction.

Preparation of CEM A2-CD8β fusion cell line. A CEM T cell line stably expressing the HLA-A2-CD8β fusion was generated as previously described (35). Briefly, the construct expressing the HLA-A2-CD8β fusion molecule was introduced into CEM-SS cells by using a murine stem cell virus (MSCV) retroviral vector pseudotyped with vesicular stomatitis virus G protein (VSV-G). A uniform population was selected by culturing the cells in neomycin.

Virus stocks and transduction. To generate viral stocks, 293T cells were cotransfected with the proviral HIV-1 constructs and a plasmid (pHIT-G) expressing VSV-G (1, 55). The latter was used to achieve comparably high initial infection levels for flow cytometric analysis. The medium was changed after overnight incubation, and virus was harvested 24 h later. All cell types were transduced with NL4-3-based proviral constructs coexpressing the various Nef proteins and EGFP as described previously (55). These constructs have the advantage that *nef* alleles are expressed from the wild-type HIV-1 long terminal repeat (LTR) promoter and via the regular splicing sites. For the detection of CD4 down-modulation, proviral HIV-1 constructs defective in *vpu* and *env* were used, since both gene products also reduce CD4 cell surface expression and thus mask the effect of Nef. Flow cytometric analysis was performed at 3 days posttransduction.

Electroporation of CEM and SupT1 cells. Electroporation of CEM and SupT1 cells with pCGCG vectors expressing different Nefs was performed with a Gene Pulser Xcell apparatus (Bio-Rad) as recommended by the manufacturer. Briefly, 10 μg of vector DNA coexpressing EGFP and Nef was electroporated using 0.4-cm cuvettes (Bio-Rad) at 200 V and 950 μF. At 2 days posttransfection, receptor expression was determined by fluorescence-activated cell sorter (FACS) analysis.

Flow cytometric analysis. CD4 (Invitrogen), CD28 (BD Biosciences), and MHC-I (Dako) expression in SupT1 or purified human CD4⁺ T cells transduced with HIV-1 (NL4-3) constructs coexpressing Nef and EGFP was measured as described previously (55). Flow cytometric analyses of A2-CD8β fusions in CEM cells or CD8β in SupT1 and primary CD8⁺ T cells were performed using the primary antibodies BB7.2 (Abcam) and CD8β (clone 2ST8.5H7; Acris), which recognize CD8$\alpha\beta$ but not CD8$\alpha\alpha$, and a secondary allophycocyanin-conjugated goat anti-mouse antibody (Invitrogen). For quantification of Nef-mediated modulation of the specific surface molecules, the levels of receptor expression were determined for cells expressing EGFP. The extent of down-modulation (*n*-fold) was calculated by dividing the mean fluorescence intensity (MFI) obtained for cells transduced with the *nef*-negative NL4-3 control viruses by the corresponding values obtained for cells transduced with viruses coexpressing Nef and EGFP.

Western blot analysis. To examine the expression of most Nef alleles, 293T cells were transfected as described above with 5 μg DNA of expression vectors coexpressing EGFP and AU-1-tagged Nefs. At 2 days posttransfection, cells were lysed with RIPA buffer (1% NP-40, 0.5% sodium desoxycholate, 3% SDS, 150 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA, protease inhibitor cocktail). Lysates were boiled at 95°C for 10 min, separated in 4 to 12% Bis-Tris gradient acrylamide gels (Invitrogen), blotted onto nitrocellulose filters, and probed with anti-AU-1 (MMS-130P; Covance), anti-GFP (ab290; Abcam), and anti-β-actin (ab8227; Abcam). Subsequently, blots were probed with anti-mouse or anti-rabbit IRDye Odyssey antibodies, and proteins were revealed using a Li-Cor Odyssey scanner.

Statistical analysis. All statistical calculations were performed with two-tailed Student's *t* test, using Graph Pad Prism, version 5.0. *P* values of <0.05 were considered significant. Correlations were calculated with the linear regression module of the software.

RESULTS

To determine whether down-modulation of CD8β is a conserved property of primate lentiviruses, we examined a total of 42 HIV and SIV *nef* alleles representing the majority of primate lentiviral lineages known to date (see Table S1 in the supplemental material

for details). Western blot analysis of 293T cells with pCGCG vectors expressing AU-1-tagged versions of these Nef proteins showed that all of them were expressed at detectable, albeit variable, levels (see Fig. S1). To determine the reproducibility, specificity, and possible significance of the effect of Nef on CD8β, we used three different target cell lines. (i) The first cell line was a CEM T cell line stably expressing the ectodomain of HLA-A2 fused to the cytoplasmic tail (CT) of the CD8β chain (A2-CD8β fusion) (35). The A2-CD8β fusion helped to address whether the CT, which consists of only 17 amino acids (CCRRRRARLRFMK QMYK), is sufficient for Nef-mediated modulation. (ii) The human T cell lymphoblastic lymphoma cell line Sup T1, which expresses full-length CD8αβ, CD4, CD28, and MHC-I, allowed measurement of Nef-mediated modulation of unmodified Nef target proteins from the same HIV-1-infected cell culture. (iii) To exclude the possibility of *in vitro* artifacts, we verified all effects of Nef in primary CD8+ or CD4+ T cells.

All cell types were transduced with HIV-1 NL4-3-based proviral constructs coexpressing the various Nef proteins and EGFP as described previously (55, 56). We found that HIV-1-infected (EGFP+) cells showed substantially lower levels of A2-CD8β fusion or CD8αβ surface expression than uninfected cells (examples are shown in Fig. 1A). This effect was not observed after infection with an otherwise isogenic HIV-1 reporter construct containing a disrupted *nef* gene and was thus Nef specific (Fig. 1A). Notably, some Nef proteins, e.g., those of SIVcpz, SIVmac, and SIVsmm, were highly effective at preventing CD8β surface expression in HIV-infected primary T cells at very low levels of EGFP (and thus Nef) expression (Fig. 1A). This is noteworthy because only CD4 and T cell receptor (TCR)-CD3 (in the case of HIV-2 and most SIVs) are down-modulated by Nef with similar efficacies, whereas significant modulation of MHC-I, CD28, and CXCR4 requires substantially higher levels of Nef expression (55). To quantify the efficiency of Nef-mediated receptor modulation, we divided the MFI for cells infected with the *nef*-defective control virus by the MFI for cells infected with viral constructs coexpressing Nef and EGFP. Statistical analyses showed that the effects of the various *nef* alleles on the surface expression of the A2-CD8β fusion on CEM cells correlated well with down-modulation of CD8αβ in SupT1 cells ($R^2 = 0.5153$; $P < 0.0001$) and primary CD8+ T cells ($R^2 = 0.4952$; $P < 0.0001$) (Fig. 1B). Thus, the CT of the human CD8β chain is sufficient for down-modulation by the vast majority of primate lentiviral Nefs, and down-modulation of the A2-CD8β fusion recapitulates the effect of Nef on unaltered CD8αβ molecules.

It has been suggested previously that down-modulation of the human CD8β chain and CD4 involves common molecular interactions, i.e., Nef-mediated recruitment of AP-2 to the cytoplasmic domain of CD8β at the plasma membrane to promote clathrin-dependent endocytosis (63). In agreement with this assumption, we found that the potency of the 42 primate lentiviral Nef proteins in down-modulation of CD8αβ correlated with the effects on CD4 ($R^2 = 0.1305$; $P = 0.0187$) and CD28 ($R^2 = 0.2672$; $P = 0.0004$) in primary T cells but not with the modulation of MHC-I (Fig. 1C). However, the correlation between Nef-mediated modulation of CD4 and CD8αβ was imperfect, suggesting that both activities are mediated by overlapping domains in Nef but are functionally separable.

Next, we examined the effects of *nef* alleles on CD8αβ based on their species of origin and phylogenetic relationships. First, we examined 28 *nef* alleles from HIV-1 and its SIV precursors from apes and monkeys. This set encompassed HIV-1 M (major), the main causative agent of AIDS, and the nonpandemic or rare HIV-1 O (outlier) and N (non-M, non-O) groups, which arose from independent zoonotic transmissions of SIVcpz from the *Pan troglodytes troglodytes* subspecies of chimpanzees; SIVcpz from both *P. t. troglodytes* and *Pan troglodytes schweinfurthii*, which has not been found in humans; SIVgor from gorillas, which may be the original host of HIV-1 O; and SIVrcm, SIVgsn, SIVmus, and SIVmon, from red-capped mangabeys and greater spot-nosed, mustached, and mona monkeys, respectively (*Cercocebus torquatus*, *Cercopithecus nictitans*, *Cercopithecus cephus*, and *Cercopithecus mona*, respectively) (60). The latter are relevant for the emergence of HIV-1 because current data indicate that the precursor of SIVrcm recombined with the common ancestor of SIVgsn/mus/mon in chimpanzees to give rise to SIVcpz (6). Notably, most SIV *nef* alleles were obtained directly from uncultured ape or monkey material and thus did not contain adaptive changes in response to exposure to human cells (52, 55). For detailed quantitative analyses, we used the results obtained with CEM cells expressing A2-CD8β fusions because the effects of Nef showed the greatest dynamic range (Fig. 1A). Although all *nef* alleles that modulated A2-CD8β fusions also down-modulated unmodified CD8αβ molecules (Fig. 1B), the effects were often saturated in primary CD8+ T cells (Fig. 1A) and were (to some extent) dependent on the peripheral blood mononuclear cell (PBMC) donor (data not shown). We found that HIV-1, SIVcpz, and SIVgor *nef* alleles down-modulated A2-CD8β fusions with efficiencies ranging from 1.3- to 10.6-fold (Fig. 2A). On average, *nef* alleles from SIVcpz (7.8 ± 2.3; $n = 12$ [numbers give mean *n*-fold down-modulation ± standard deviation]) were significantly more active ($P = 0.036$) in modulating A2-CD8β fusions than HIV-1 *nef* alleles (5.6 ± 2.5; $n = 11$) (Fig. 2B). Our finding that SIVcpz Nefs are capable of targeting the CT of human CD8β is not surprising, because the amino acid sequence of this domain is identical in humans and chimpanzees (16). On average, *nef* alleles from the *P. t. troglodytes* subspecies of chimpanzees (which have transmitted the virus to humans) were more active in down-modulating A2-CD8β fusions than *nef* genes derived from the *P. t. schweinfurthii* subspecies (Fig. 2B). Furthermore, as a group, *nef* alleles from pandemic HIV-1 M strains reduced cell surface expression of A2-CD8β fusions more effectively than those from the nonpandemic N and O groups of HIV-1 (Fig. 2B). However, due to the limited sample size and relatively high variation of Nef function within each group, these differences failed to reach significance. In comparison to HIV-1 and SIVcpz Nefs, those of SIVgsn, SIVmus, and SIVmon showed only modest activity in down-modulating A2-CD8β fusions, although they potently kept MHC-I molecules away from the cell surface (Fig. 2B). The sequences of the CT domains of the CD8β molecules of greater spot-nosed, mustached, and mona monkeys are not available. Thus, it remains to be elucidated whether the SIVgsn, SIVmus, and SIVmon Nefs are more effective in down-modulating the CD8β variants found in their cognate host species. Most likely, SIVcpz obtained its *nef* gene from the precursor of SIVrcm, not from a possible common ancestor of SIVgsn/mus/mon (6, 55). Thus, it is noteworthy that the single SIVrcm Nef available for analysis was more active in modulating A2-CD8β fusions and CD8αβ than the SIVgsn, SIVmus, and SIVmon Nefs but was less effective than the majority of HIV-1 and SIVcpz Nefs (Fig. 2). Altogether, our results demon-
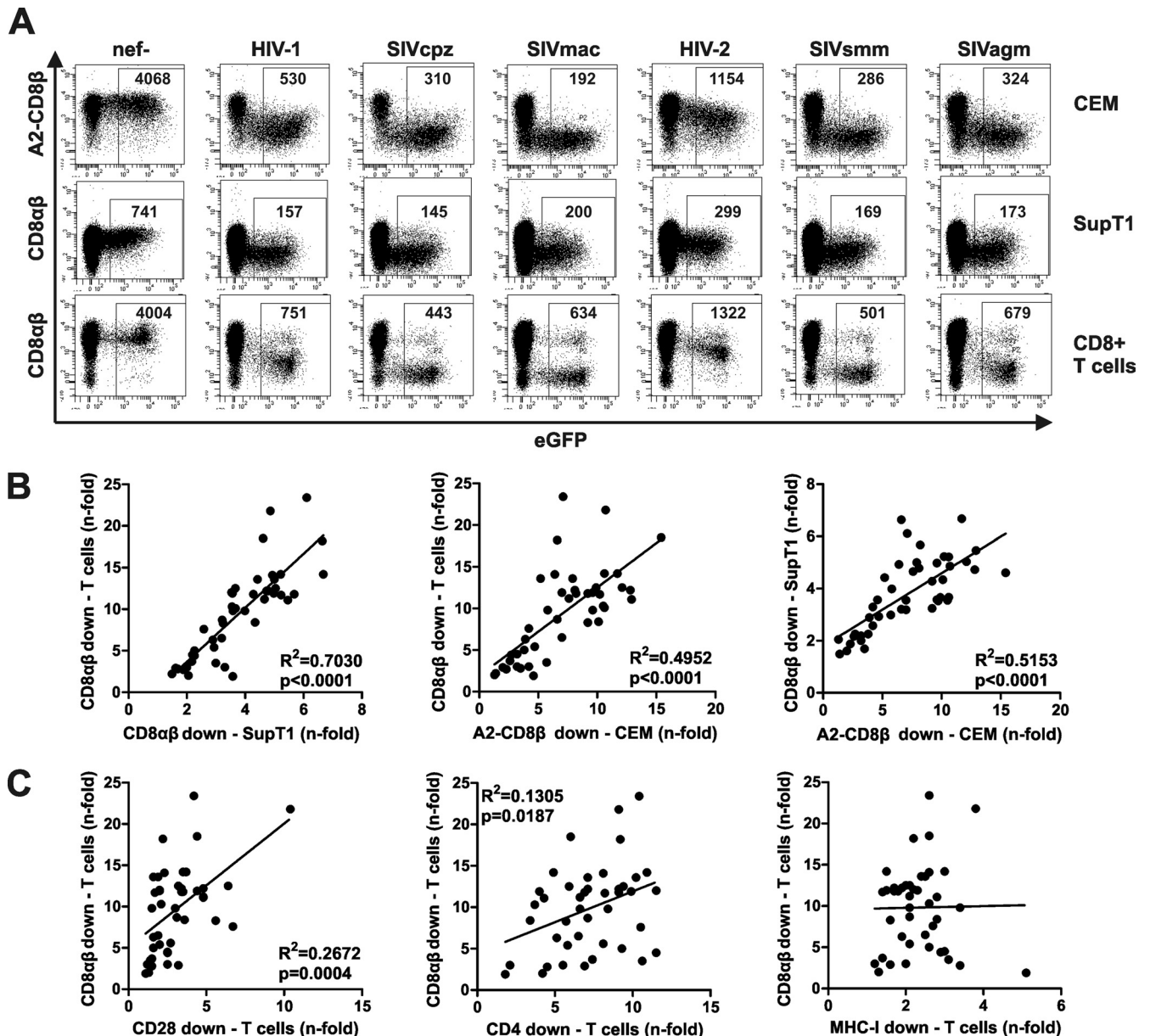
FIG 1 Nef-mediated down-modulation of human CD8$\alpha\beta$ is conserved between primate lentiviruses. (A) Flow cytometric analysis of CEM cells expressing A2-CD8β fusions, SupT1 cells, and primary CD8+ T cells transduced with HIV-1 recombinants expressing EGFP alone or together with the indicated *nef* alleles. The ranges of EGFP expression used to calculate the MFI of A2-CD8β fusion or CD8$\alpha\beta$ expression are indicated. (B) Correlations between the efficiencies of Nef-mediated down-modulation of CD8$\alpha\beta$ in primary CD8+ T cells and of A2-CD8β fusions in CEM cells or CD8$\alpha\beta$ in SupT1 cells. (C) Correlations between the efficiencies of Nef-mediated down-modulation of CD8$\alpha\beta$ in primary CD8+ T cells and of CD28, CD4, and MHC-I in primary CD4+ T cells. Each symbol represents average *n*-fold down-modulation ($n = 3$) of the indicated receptor molecule by one individual *nef* allele analyzed. An overview of the HIV and SIV *nef* alleles used is provided in Table S1 in the supplemental material.

strate that down-modulation of CD8$\alpha\beta$ is a conserved property of the M, N, and O groups of HIV-1 and their simian counterparts, SIVcpz and SIVgor. Since the CT domains of CD8β are identical between chimpanzees and humans, SIVcpz Nefs were already capable of modulating human CD8$\alpha\beta$ after zoonotic transmission, and this function was largely maintained in the new human host. Further studies are necessary to clarify whether more common infection of CD8+ T cells in SIVcpz-infected chimpanzees than in HIV-1-infected humans may explain why SIVcpz *nef* alleles are, on average, more active in modulating A2-CD8β fusions than those of HIV-1.

Next, we examined the capability of *nef* alleles from the SIVsmm/SIVmac/HIV-2 lineage to modulate A2-CD8β fusions in more detail. HIV-2 arose from multiple independent transmissions of SIVsmm from sooty mangabeys (*Cercocebus atys*) to humans (23, 60). The SIVsmm/SIVmac/HIV-2 lineage is of particular interest for studies on determinants of the pathogenesis of AIDS because SIVsmm is usually nonpathogenic in its natural monkey host but is highly pathogenic in experimentally infected rhesus macaques (SIVmac). The phenotype of HIV-2 in humans is intermediate: it can cause AIDS but is substantially less virulent than HIV-1, and many HIV-2-infected individuals show low viral
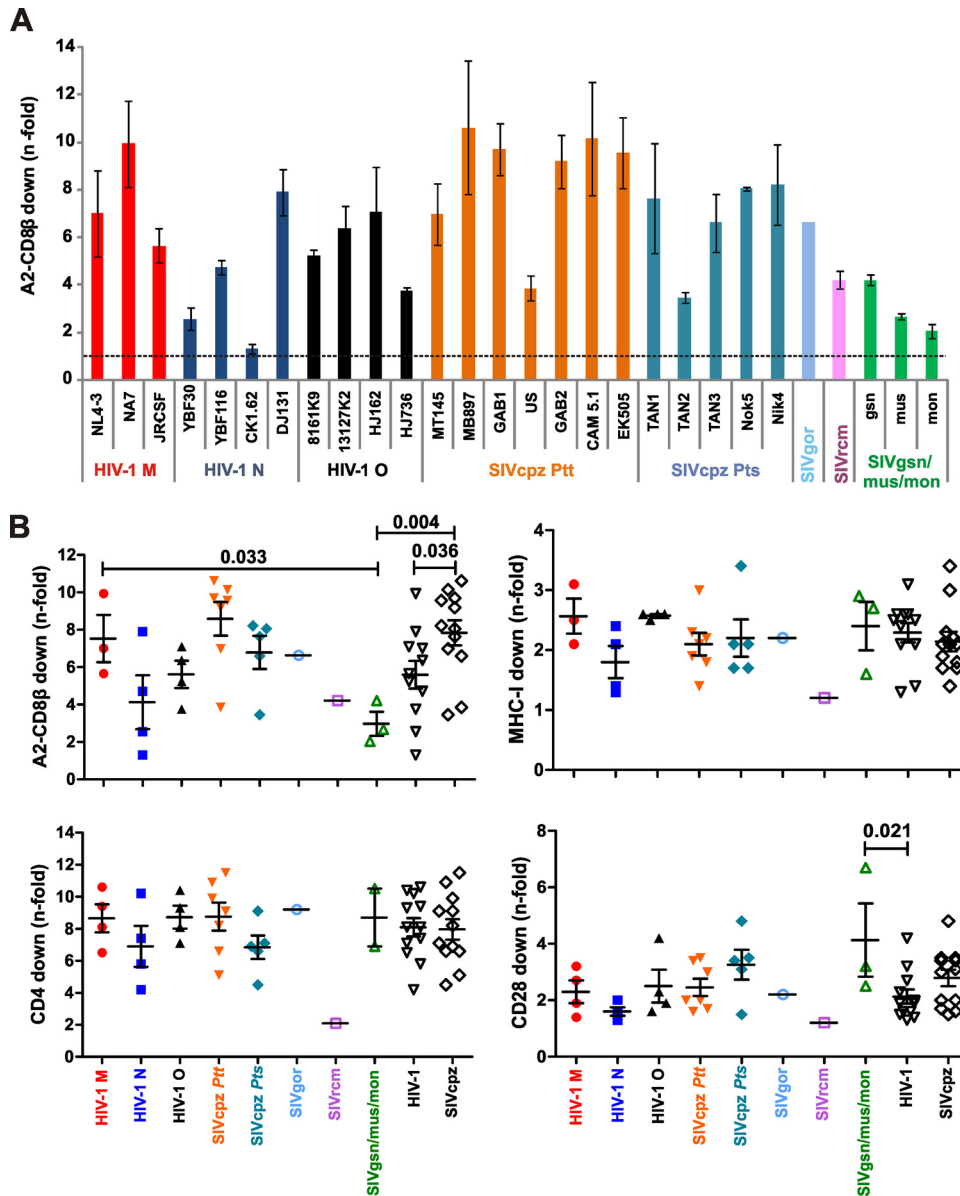
FIG 2 Modulation of A2-CD8β fusions and other receptors by *nef* alleles from HIV-1 and its closest SIV counterparts. (A) Efficiencies of A2-CD8β fusion down-modulation by *nef* alleles from the indicated HIV-1 and SIV strains. Data shown are average values ± standard deviations (SD) derived from triplicate experiments. (B) Comparison of A2-CD8β fusion, MHC-I, CD4, and CD28 down-modulation by *nef* alleles from the indicated groups of primate lentiviruses. A2-CD8β fusion modulation was measured in CEM cells, and down-modulation of the remaining receptors was measured in primary CD4+ T cells. Each symbol represents the average for three independent measurements, and similar results were obtained in SupT1 cells.

loads and become long-term nonprogressors (24, 38, 43). All seven HIV-2 and SIVsmm *nef* alleles tested significantly reduced the levels of A2-CD8β fusions and CD8αβ cell surface expression, albeit with differential efficiencies, ranging from 3.2- to 12.8-fold in CEM cells and from 1.9- to 21.8-fold in primary T cells (Fig. 3A and data not shown). Unexpectedly, the SIVsmm Nefs were significantly more effective in down-modulating A2-CD8β fusions than those of HIV-2 (11.8- ± 1.2-fold versus 6.1- ± 3.2-fold; *P* = 0.0360), although HIV-2 Nefs were more effective at removing MHC-I molecules from the cell surface (3.8- ± 0.9-fold versus 2.2- ± 0.4-fold; *P* = 0.0332) (Fig. 3B). In agreement with published data (63), the *nef* allele of the molecular SIVmac239 clone,

which is commonly used for studies on viral pathogenesis in the SIV-macaque model, was highly effective at down-modulating A2-CD8β fusions and CD8αβ from the cell surface (Fig. 3 and data not shown). Notably, it has been reported that activated intestinal double-positive CD4+ CD8+ memory T cells are a primary target of SIVmac infection in neonatal rhesus macaques (69) and are rapidly depleted in adult macaques with acute SIV infection (68). Our observation that *nef* alleles from SIVsmm and the macaque-adapted SIVmac derivative down-modulate CD8αβ with a high efficiency suggests a relevant role of this Nef function in viral immune evasion in both nonpathogenic natural and pathogenic experimental SIV infections.
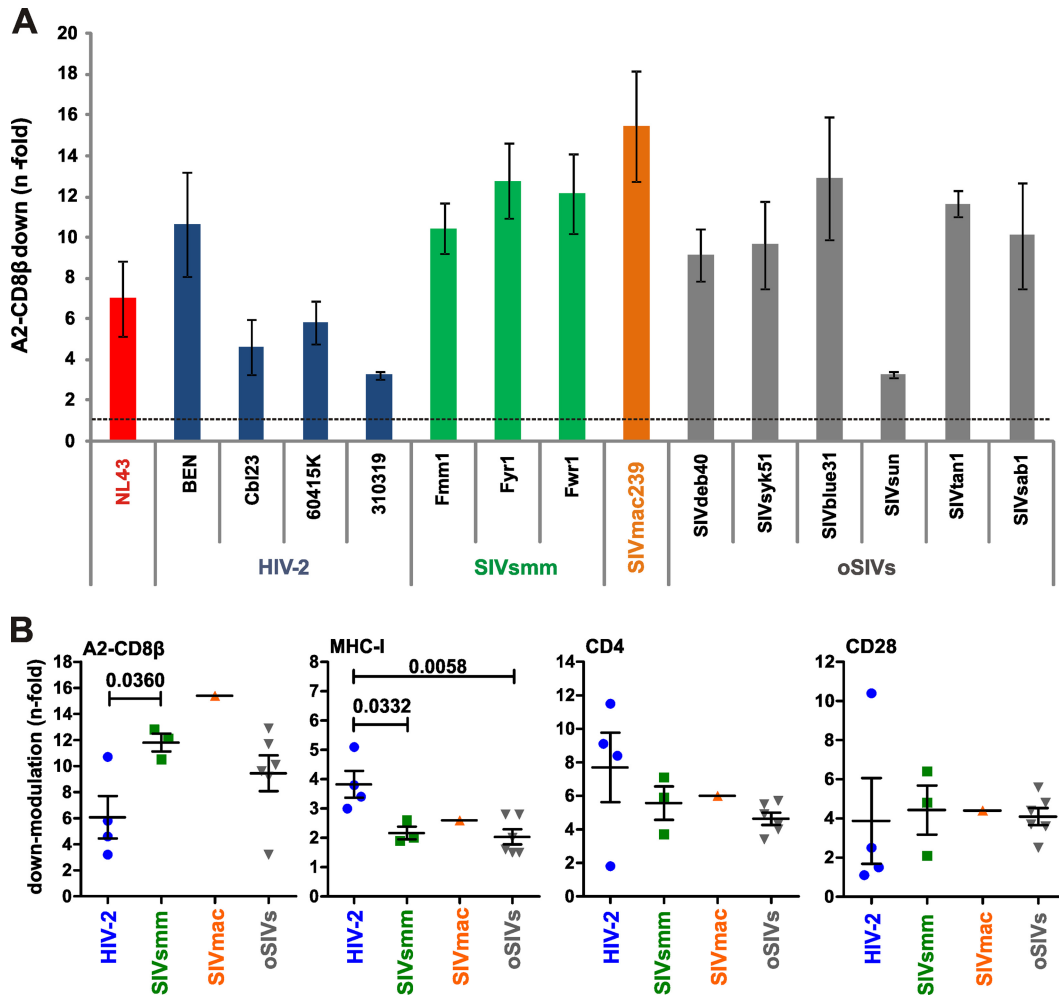
FIG 3 Modulation of A2-CD8β fusions and other receptors by *nef* alleles from HIV-2, their SIVsmm and SIVmac counterparts, and other highly divergent primate lentiviruses. (A) Efficiencies of A2-CD8β fusion down-modulation by *nef* alleles from the indicated HIV-2 and SIV strains. (B) Comparison of A2-CD8β fusion, MHC-I, CD4, and CD28 down-modulation by *nef* alleles from the indicated groups of primate lentiviruses. oSIVs, SIVs that were not involved in the evolution of HIV-1 and HIV-2. A2-CD8β fusion modulation was measured in CEM cells, and down-modulation of the remaining receptors was measured in primary CD4⁺ T cells. Refer to the legend to Fig. 2 for further details.

To further determine whether down-modulation of CD8β is a general property of primate lentiviral Nef proteins, we analyzed *nef* alleles from several additional highly divergent primate lentiviruses: SIVdeb from De Brazza monkeys (*Cercopithecus neglectus*), SIVsyk from Sykes monkeys (*Cercopithecus albogularis*), SIVblu from blue monkeys (*Cercopithecus mitis*), SIVsun from sun-tailed monkeys (*Cercopithecus solatus*), SIVtan from tantalus monkeys (*Chlorocebus tantalus*), and SIVsab from Sabaeus monkeys (*Chlorocebus sabaeus*). Together with the HIV and SIV *nef* alleles described above, these *nef* genes represented 5 of the 6 major primate lentiviral lineages known to date (23, 60). We found that all but the SIVsun sol-36 *nef* allele efficiently down-modulated A2-CD8β fusions and CD8αβ (Fig. 3 and data not shown). On average, these SIV *nef* alleles were more active in modulating A2-CD8β fusions than *nef* alleles derived from HIV-2 (9.5- ± 3.3-fold versus 6.1- ± 3.2-fold; P = 0.15), although the latter were more effective at modulating MHC-I (3.8- ± 0.9-fold versus 2.0- ± 0.6-fold; P = 0.0058) and CD4 (7.7- ± 4.1-fold versus 4.6- ± 0.9-fold; P = 0.11) cell surface expression (Fig. 3B).

The effects of Nef on MHC-I, CD4, and CD28 correlated well in SupT1 and primary T cells, and these functional differences were observed in both cell types (data not shown).

To further assess possible differences in Nef-mediated receptor modulation dependent on phylogenetic relationships, species of origin, and biological properties, we next compared the efficiencies of all *nef* alleles derived from HIV-1 and HIV-2, their direct SIV precursors, SIVs that (to our current knowledge) have not been transmitted to humans, viruses of human and nonhuman primate (NHP) origin, and viruses that do or do not down-modulate TCR-CD3 (55) in down-modulating A2-CD8β fusions, MHC-I, CD4, and CD28. HIV-1 Nefs were significantly less active than those of HIV-2 in down-modulating MHC-I (2.3- ± 0.6-fold versus 3.8- ± 0.9-fold; P = 0.001) but not in modulating A2-CD8β fusions or CD4 (Fig. 4). As a group, *nef* alleles derived from HIVs (Hu-Ori) were significantly (P = 0.022) less potent in modulating A2-CD8β fusions but more active in modulating MHC-I and CD4 than those derived from SIVs (NHP-Ori) (Fig. 4). Finally, we found that *nef* alleles from viruses that are unable to
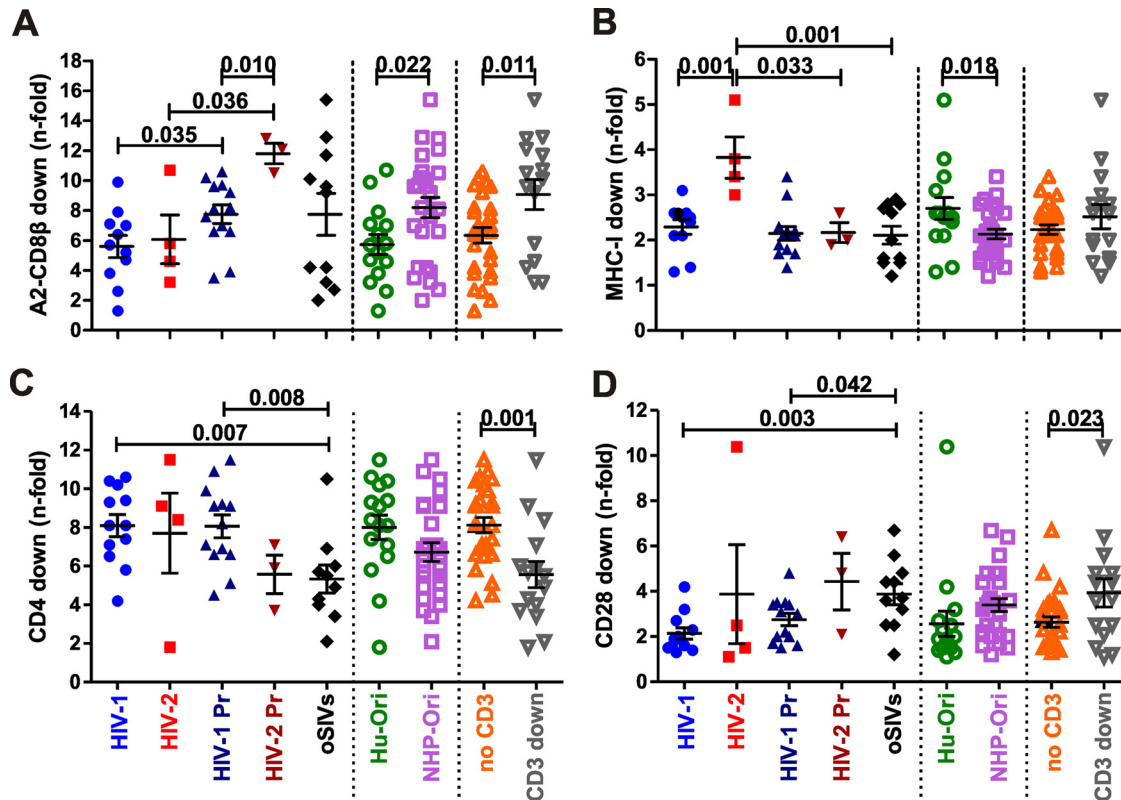
FIG 4 Comparison of Nef-mediated receptor modulation by human and nonhuman primate lentiviruses. Quantitative assessment was performed to measure down-modulation of the A2-CD8β fusion (A), MHC-I (B), CD4 (C), and CD28 (D) by *nef* alleles, based on the species of origin (humans versus nonhuman primates) and biological properties (unable or able to down-modulate TCR-CD3) of the *nef* alleles examined. A2-CD8β fusion modulation was measured in CEM cells, and down-modulation of the remaining receptors was measured in primary CD4+ T cells. Abbreviations: Pr, precursor; Hu, human; NHP, nonhuman primate.

down-modulate TCR-CD3 (i.e., HIV-1 and its *vpu*-containing SIV precursors) were, on average, significantly less effective in down-modulating A2-CD8β fusions (6.3- ± 2.7-fold versus 9.1- ± 3.9-fold; $P = 0.011$) and CD28 (2.6- ± 1.2-fold versus 3.9- ± 2.4-fold; $P = 0.023$), but more active in down-modulating CD4 (8.1- ± 2.0-fold versus 5.6- ± 2.6-fold; $P = 0.001$), than *nef* genes from primate lentiviruses that down-modulate TCR-CD3 (Fig. 4).

The data described above show that down-modulation of CD8β is a conserved property of the vast majority of primate lentiviral Nef proteins. Previous studies suggested that some conserved Nef functions, such as down-modulation of MHC-I, may involve different domains in different primate lentiviruses and thus may have evolved independently during primate lentiviral evolution (22). Thus, we next examined whether or not down-modulation of CD8β involves similar or distinct domains in highly divergent primate lentiviral Nef proteins. First, we focused on the E/DXXXLL motif in the C-terminal flexible loop of Nef because previous studies have shown that this motif is important for the ability of HIV-1 Nef to interact with different adaptor proteins (i.e., AP-1, AP-2, and AP-3) and to down-modulate CD4 and CD8β (12, 35, 63). The acidic residue and the first leucine are preserved in the great majority of HIV and SIV strains (12). In contrast, the second leucine is conserved in HIV-1 Nefs but is frequently replaced by a valine, threonine, or methionine residue in SIV and HIV-2 Nef proteins (Fig. 5A and data not shown). To

further determine the functional relevance of the EXXXLL motif, we introduced alanine substitutions into the LL/M motif in eight highly divergent SIV Nefs and the control HIV-1 NA7 Nef. Notably, two SIV strains examined contained changes, to EXXXLM (SIVmac) and PXXXLL (SIVsun), in the otherwise conserved EXXXLL motif (Fig. 5A, inset). Mutations in the LL/M residues generally disrupted the capability of primate lentiviral Nefs to down-modulate CD4, CD28, and CD8β but had no significant effect on protein expression levels and MHC-I modulation (Fig. 5A to D and data not shown). Correlation analyses showed that the effects of the various wild-type and mutant *nef* alleles on the surface expression of A2-CD8β fusions on CEM cells correlated well with down-modulation of CD8αβ in CD8+ T cells ($R^2 = 0.6254$; $P < 0.0001$) and with down-modulation of CD4 ($R^2 = 0.3622$; $P = 0.0082$) and CD28 ($R^2 = 0.7448$; $P < 0.0001$) but not MHC-I (Fig. 5E). The wild-type SIVsun Nef protein, which contains PXXXLL instead of EXXXLL, had little effect on CD8β but effectively down-modulated CD4 and CD28 from the cell surface (Fig. 5A to C). The latter is in agreement with the previous finding that the acidic residue plays a differential role in the modulation of dileucine-dependent Nef targets and is not generally required for Nef-mediated receptor modulation (12). Altogether, our results support the hypothesis that the E/DXXXLL motif is of general importance for the capability of primate lentiviral Nef proteins to down-modulate CD4, CD28, and CD8 from the cell surface. Our data also imply that some Nef proteins that contain changes in the
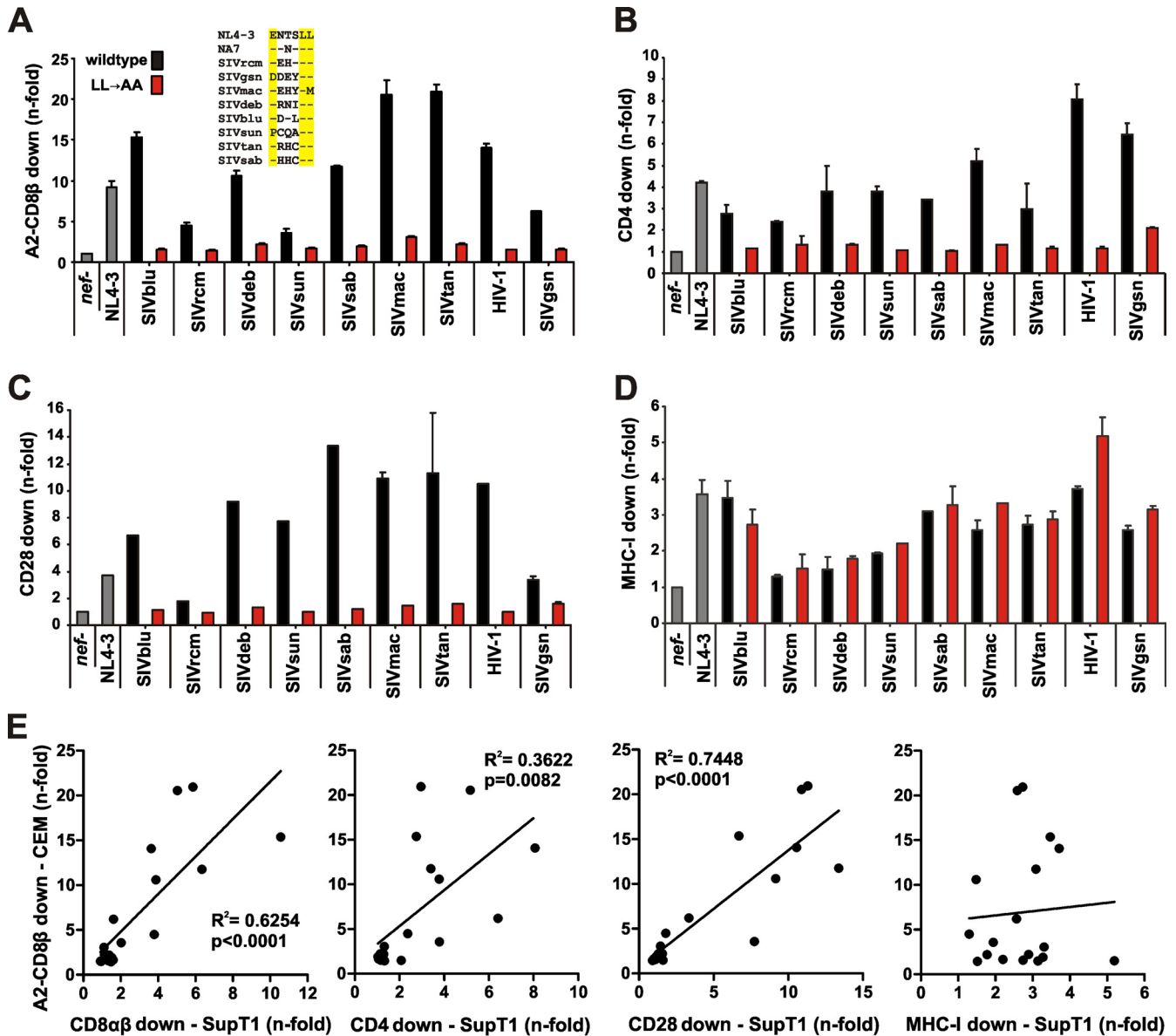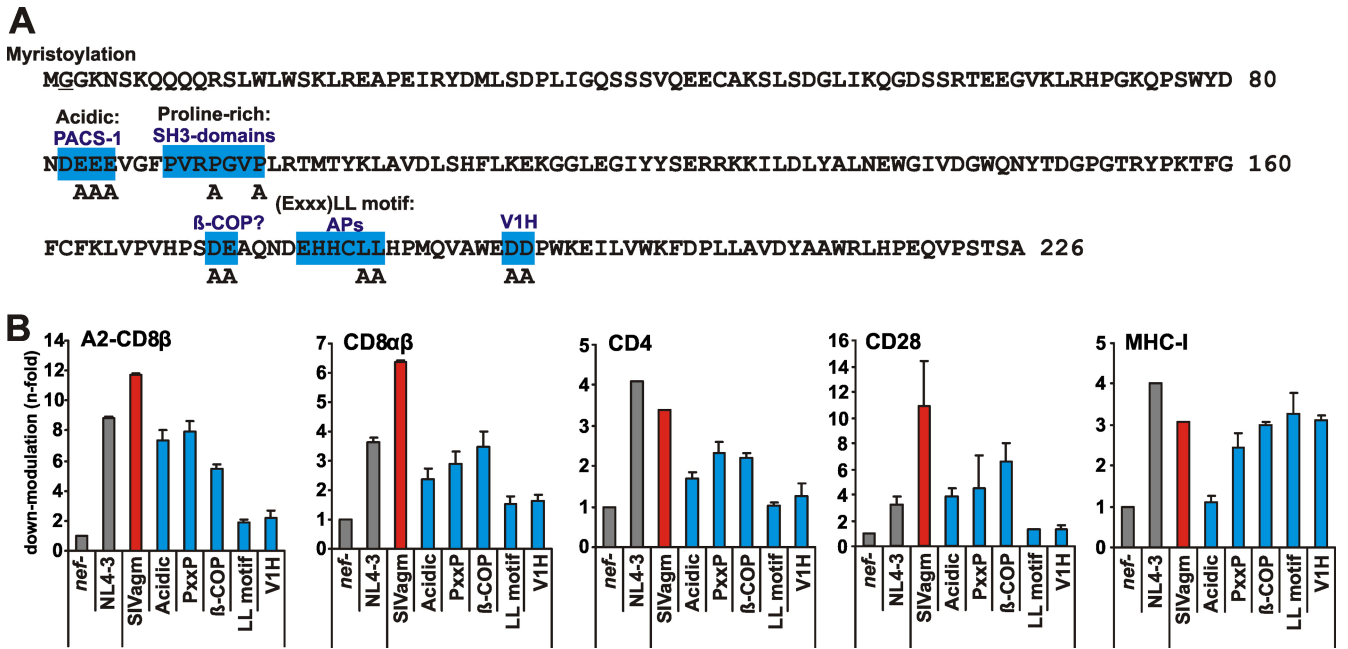
FIG 5 Importance of the EXXXLL motif in the C-terminal flexible loop for the modulation of cellular receptors by primate lentiviral Nef proteins. (A to D) CEM cells expressing the A2-CD8β fusion (A) or SupT1 cells (B to D) were transduced with VSV-G-pseudotyped *vpu-* and *env*-defective NL4-3-based constructs expressing the indicated *nef* alleles and assayed for surface expression of the A2-CD8β fusion (A), CD4 (B), CD28 (C), and MHC-I (D). Quantification was performed as described in Materials and Methods. Data shown are average values $\pm$ SD derived from two independent experiments. The inset in panel A shows an amino acid sequence comparison of the various EXXXLL-corresponding regions analyzed. The NL4-3 motif is shown as a reference at the top. Dots indicate amino acid identity. (E) Correlations between efficiencies of down-modulation of the A2-CD8β fusion on CEM cells and modulation of CD8$\alpha\beta$, CD4, CD28, and MHC-I on SupT1 cells.

acidic residue of the EXXXLL motif are still able to connect CD4 and CD28 to adaptor proteins.

To further define the functional domains involved in the interaction of primate lentiviral Nefs with CD8β and other cellular receptors, we generated five mutant SIVagmSab *nef* constructs containing changes in the following sequence motifs known to be important for HIV-1 Nef function: (i) the acidic domain that interacts with the sorting protein PACS-2 to target Nef to the perinuclear region (5, 45) and with the CT of MHC-I molecules to support their interaction with AP-1 (70); (ii) the PXXP motif, involved in interactions with signaling molecules, such as the SH3

domains of various tyrosine kinases (51); (iii) two negatively charged residues (E154E155 in NL4-3 and D172E173 in SIVagmSab) that may interact with β-COP (27, 44, 53); (iv) the E/DXXXLL AP interaction site described above; and (v) the DD motif implicated in Nef binding to the V1H subunit of the vacuolar ATPase and AP-2 (36, 37) (Fig. 6A). SIVagmSab Nef was selected for detailed analysis because infection of African green monkeys with this virus represents one of the best-studied models of nonpathogenic natural SIV infection (61). We found that mutations in the dileucine-based and DD motifs in the C-terminal flexible loop disrupted the effects of SIVagm Nef on CD8β, CD4,

A

Myristoylation

MGGKNSKQQQQRSLWLWSKLREAPEIRYDMLSDPLIGQSSSVQEECAKSLSDGLIKQGDSSRTEEGVKLRHPGKQPSWYD 80

Acidic:
PACS-1   Proline-rich:
          SH3-domains

NDEEEVGFPVRPGVPLRTMTYKLAVDLSHFLKEKGGLEGIYYSERRKKILDLYALNEWGIVDGWQNYTDGPGTRYPKTFG 160
    AAA   A   A

                ß-COP?   (Exxx)LL motif:
                           APs   V1H

FCFKLVPVHPSDEAQNDEHHCLLHPMQVAWEDDPWKEILVWKFDPLLAVDYAAWRLHPEQVPSTSA 226
           AA   AA   AA

B

FIG 6 Functional analysis of SIVagmSab Nef mutants. (A) The SIVagmSab Nef amino acid sequence is given in single-letter code. Mutated sequence motifs are highlighted by blue boxes, and positions of alanines are indicated. (B) CEM cells expressing A2-CD8β fusions or SupT1 cells were transduced with HIV-1 recombinants expressing EGFP alone or together with the indicated *nef* alleles and were assayed for surface expression of the A2-CD8β fusion, CD8αβ, CD4, CD28, and MHC-I. Quantification was performed as described in Materials and Methods. Data shown are average values ± SD derived from two independent experiments.
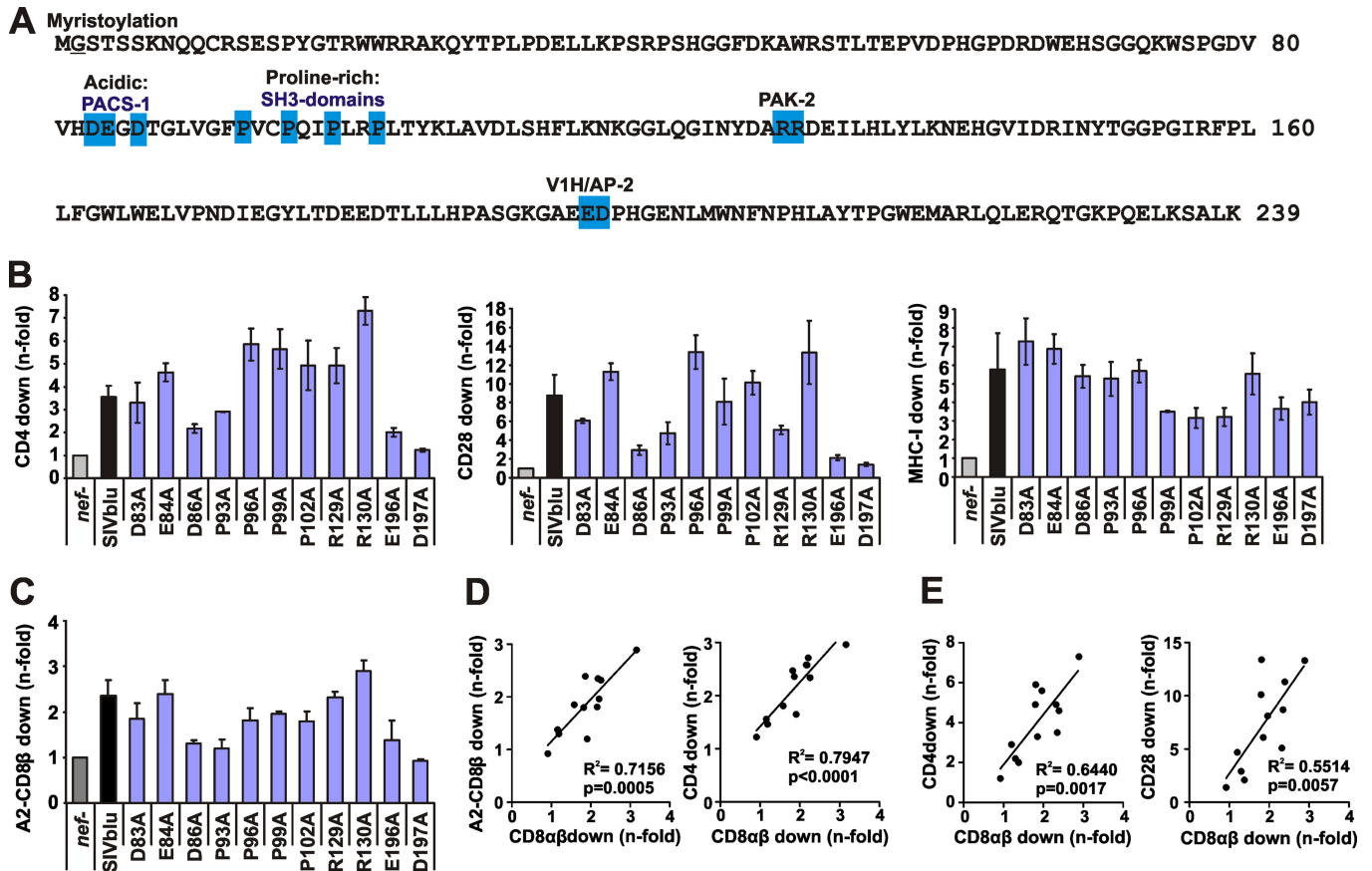
and CD28 but had no effect on MHC-I down-modulation (Fig. 6B). In contrast, changes in the acidic domain disrupted MHC-I modulation but only partially impaired the other Nef functions (Fig. 6B). Changes in the PXXP motif and the DE residues preceding the EXXXLL motif resulted in intermediate phenotypes and reduced the modulation of CD8β, CD4, and CD28 (Fig. 6B). Our results show that both the dileucine-based motif and the DD residues in the C-terminal flexible loop of SIVagm Nef are required for down-modulation of CD8β, CD4, and CD28. Previous data have shown that analogous changes in the HIV-1 Nef protein also disrupt the modulation of these receptors (63), supporting the observation that the EXXXLL and DD motifs play similar roles in SIVagm and HIV-1 Nef function.

The results described above and previously published data (35, 63) suggest that down-modulation of CD8β, CD4, and CD28 may involve similar domains in Nef. To further examine whether this may generally be the case, we generated a set of 11 SIVblu mutant Nefs containing alanine substitutions at single amino acid residues. As shown in Fig. 7A, these point mutations affected the acidic domain, the proline-rich region, a dibasic motif reported to be critical for PAK-2 binding (46, 48), and the diacidic putative V1H–AP-2 interaction site in the C-terminal flexible loop of Nef. The Nef protein from SIVblu, which infects blue monkeys (*Cercopithecus mitis*), was selected for analysis because it is highly divergent from other Nef proteins that have been examined functionally in some detail (i.e., HIV-1, SIVmac, and SIVagm Nef proteins) and is active in most aspects of Nef function (Fig. 7B and data not shown). The mutations had differential effects on SIVblu Nef function. As expected from previous results on HIV-1 (56, 63) and SIVagm (Fig. 6) Nef function, the ED residues (V1H–AP-2 interaction site) were critical for down-modulation of CD4,

CD8β, and CD28 (Fig. 7B and C). The D86A and P93A changes also markedly impaired these three Nef functions. This was unexpected, since the acidic domain and the PXXP motif are not critical for down-modulation of CD4 and CD28 by HIV-1 Nef (20). Notably, these changes did not reduce the efficiency of MHC-I down-modulation (Fig. 7B), and all SIVblu mutant Nefs were expressed at similar levels (see Fig. S2 in the supplemental material). The remaining mutations had little, if any, effect on modulation of CD4 and CD28, and in some cases (i.e., E84A, P96A, and R130A mutations) they even increased these Nef activities. The efficiencies of CD4 and CD28 down-modulation by this set of SIVblu Nefs correlated well with one another ($R^2 = 0.7718$; $P = 0.0002$) (data not shown) but not with down-modulation of MHC-I (data not shown). The effects of wild-type and mutant SIVblu Nefs on CD8β were highly reproducible in CEM cells expressing A2-CD8β fusions and in SupT1 cells ($R^2 = 0.7156$; $P = 0.0005$) (Fig. 7D). The efficiencies of the 12 SIVblu Nefs in down-modulating CD8αβ correlated very well with their potency in CD4 and CD28 modulation (Fig. 7D and E). These results thus further support the hypothesis that primate lentiviral Nefs use similar surfaces to modulate CD8β, CD4, and CD28 and further emphasize the important role of the DD motif in the C-terminal flexible loop of Nef in these activities. Our data also show, however, that residues in the acidic and proline-rich regions are more important for efficient modulation of CD8β, CD4, and CD28 by SIVblu Nef than by HIV-1 Nef proteins.

DISCUSSION

In the present study, we show that the ability of Nef to down-modulate CD8αβ from the surfaces of virally infected primary T cells is conserved in the vast majority of primate lentiviruses and

A Myristoylation
MGSTSSKNQQCRSESPYGTRWWRRAKQYTPLPDELLKPSRPSHGGFDKAWRSTLTEPVDPHGPDRDWEHSGGQKWSPGDV 80

Acidic:
PACS-1

Proline-rich:
SH3-domains

PAK-2
VHDEGDTGLVGFPVCPQIPLRPLTYKLAVDLSHFLKNKGGLQGINYDARRDEILHLYLKNEHGVIDRINYTGGPGIRFPL 160

V1H/AP-2
LFGWLWELVPNDIEGYLTDEEDTLLLHPASGKGAEEDPHGENLMWNFNPHLAYTPGWEMARLQLERQTGKPQELKSALK 239

FIG 7 Functional analysis of SIVblu Nef mutants. (A) The SIVblu Nef amino acid sequence is given in single-letter code. The positions of single alanine substitutions are highlighted by blue boxes, and the domains affected by these mutations are indicated. (B and C) Jurkat T cells (B) or CEM cells (C) were transfected with pCGCG vectors expressing EGFP alone or together with the indicated *nef* alleles and were assayed for surface expression of CD4, CD28, MHC-I, and the A2-CD8β fusion. Quantification was performed as described in Materials and Methods. Data shown are average values $\pm$ SD derived from two independent experiments. (D) Correlations between efficiencies of down-modulation of the A2-CD8β fusion on CEM cells and of CD8$\alpha\beta$ (left) or CD4 (right) on SupT1 cells. (E) Correlations between Nef-mediated down-modulation of CD8$\alpha\beta$ on SupT1 cells and of CD4 (left) or CD28 (right) on Jurkat T cells.

that the 17-amino-acid CT sequence of CD8β is generally sufficient to confer sensitivity to Nef. Nef proteins of the SIV precursors of both human immunodeficiency viruses (i.e., SIVcpz, SIVgor, and SIVsmm) are highly effective in modulating human CD8$\alpha\beta$. Thus, like other Nef functions, such as modulation of CD4, CD3, CD28, and MHC-I and enhancement of viral infectivity and replication (3, 40, 41, 42, 55, 58, 62), and unlike Nef-mediated tetherin antagonism (28, 52, 74), the effect of Nef on CD8$\alpha\beta$ is species independent, and SIVs can manipulate human CD8$^+$ T cells without adaptive changes. This is not surprising, since the amino acid sequences of the cytoplasmic domains of human and macaque CD8β, encoded by the CY1 exon, which is present in all splice variants and targeted by Nef (63, 67), differ by only a single amino acid (CCRRRRARLRFMKQFYK to CCRRRR ARLRFIKQFYK). Our finding that the effect of Nef on CD8$\alpha\beta$ is conserved and usually highly effective supports a relevant role in direct HIV and SIV infections and manipulation of CD8$^+$ CTLs *in vivo*.

Previous studies have shown that some Nef activities, i.e., the ability to down-modulate CD4, CD28, and MHC-I and also to enhance viral infectivity and replication, are conserved across most or all primate lentiviral lineages (4, 55). Thus, although primate lentiviral Nef proteins are highly variable and often share

only about 30% amino acid identity (34), some functional interactions are obviously preserved. In some cases, however, the same activities may be mediated by different domains in Nef and thus evolved independently during primate lentiviral evolution (7, 25, 65). Our results support the hypothesis that the EXXXLL and (most likely) DD motifs in the C-terminal flexible loop of Nef are generally critical for Nef-mediated down-modulation of CD4, CD8β, and CD28. The finding that Nef proteins from several highly divergent primate lentiviruses use similar conserved motifs to perform these functions supports the idea that these motifs evolved early during primate lentiviral evolution.

Previous studies have demonstrated that the EXXXLL motif in the C-terminal flexible loop of Nef is critical for interaction with several clathrin adaptor proteins, i.e., AP-1, AP-2, and AP-3 (35, 36). In contrast, the adjacent DD motif is critical for AP-2 binding of HIV-1 Nef but dispensable for the interaction with AP-1 and AP-3 (36). Thus, our finding that changes in the DD motif disrupt the effects of HIV-1, SIVagm, and SIVblu Nefs on CD4, CD8β, and CD28 cell surface expression further supports an important role of AP-2 in the down-modulation of these receptors. Notably, recent data suggest that AP-1 is also important for down-modulation of CD8β and CD28 and (unlike the case with MHC-I) that this involves the EXXXLL motif in the HIV-1 Nef proteins

(35). These results are not mutually exclusive, because accumulating evidence suggests that Nef may interact with various adaptor proteins in order to remove them from the cell surface and to induce their redistribution to the *trans*-Golgi network and endosomes and/or lysosomal degradation. The relative contributions of the different adaptors and pathways (e.g., endocytosis versus intracellular retention) need further study and seem to vary not only for different Nef functions but also between different primate lentiviruses. In either case, it is becoming evident that the EXXXLL and DD motifs in the C-terminal flexible loop of Nef both seem to be generally critical for down-modulation of CD4, CD8β, and CD28 as well as for up-modulation of the invariant chain (Ii) by Nef (56). A recent study showed that the EXXXLL and DD motifs are also critical for tetherin antagonism (73), further supporting their important role in primate lentiviral Nef function.

We observed striking correlations between the disruptive effects of mutations in the SIVagm and SIVblu Nefs on down-modulation of CD4, CD8β, and CD28 (Fig. 6 and 7). In agreement with published data on HIV-1 Nef function (35, 63), these results suggest that primate lentiviral Nef proteins use similar domains and largely overlapping surfaces to reduce the cell surface expression levels of these receptors. Nonetheless, the potencies with which primary HIV and SIV *nef* alleles modulate these receptors vary considerably and do not often correlate with one another. Furthermore, it is known that most of these Nef activities are genetically separable by specific point mutations (2, 14, 22, 26, 54, 65). One straightforward explanation for these results is that down-modulation of CD4, CD8β, and CD28 by Nef involves conserved interactions with cellular adaptor proteins and more variable interactions with the cytoplasmic tails of the various cellular receptors. However, alternative possibilities cannot be dismissed entirely. For example, recent data suggest that substitutions in and near the E/DXXXLL and DD motifs can have differential effects on the interaction of Nef with various adaptor proteins (12, 36). Thus, it is possible that some mutations may disrupt the interactions with specific adaptor proteins that are critical for Nef-mediated modulation of particular cellular receptors.

While mutations in the EXXXLL motif in Nef generally disrupted modulation of CD4, CD8β, and CD28, the effects of alanine substitutions in other conserved domains of Nef were context dependent. For example, the D86A and P93A changes, which affect the acidic domain and the proline-rich region of SIVblu Nef, severely impaired down-modulation of these receptors but not modulation of MHC-I (Fig. 7). In contrast, we and others have found that alanine substitutions in the acidic domain of HIV-1 Nef impair down-modulation of MHC-I much more severely than that of CD4 or CD8β (45, 56; data not shown). Furthermore, mutations in the PXXP motif of HIV-1 Nef disrupt the interaction with the SH3 domains of cellular kinases but not with CD4 (51). These findings further illustrate the enormous versatility and plasticity of Nef activities and suggest that some conserved domains may be involved in distinct functions in Nef proteins from different primate lentiviruses. We found that even analogous mutations in various HIV-1 Nefs may have entirely different effects on their functional activity (data not shown). Thus, generalizations based on functional analyses of single *nef* alleles should be avoided, which is noteworthy because the vast majority of studies have used the *nef* allele from the T cell line-adapted HIV-1 NL4-3 strain.

Our observation that Nef-mediated down-modulation of CD8β is a well-conserved and effective Nef function, together with the recent finding that the CT of CD8β represents a major target of HIV-1 Nef (35), supports a relevant role in viral immune evasion *in vivo*. This may seem surprising, since CD8⁺ T cells are not known to be major target cells of HIV or SIV infection. In fact, when we infected CD8⁺ T cells purified from PBMCs with HIV-1, we observed infection rates of only about 0.4% (data not shown), similar to published results (35). However, this may be different *in vivo*, because T cells frequently express both CD4 and CD8 during thymic development (17), and activated CD8⁺ T cells may express low levels of CD4 (10, 18, 31). In fact, virally infected CD8⁺ T cells have been detected in AIDS patients (50). Thus, further studies, particularly in appropriate animal models, are warranted and will help to elucidate the possible relevance of Nef-mediated down-modulation of CD8β *in vivo*. Infections of macaques with SIVmac and of African green monkeys with SIVagm currently represent the most common nonhuman primate models for studying the pathogenesis of AIDS and the mechanisms of viral immune evasion. Since the *nef* alleles from both SIVmac and SIVagm effectively down-modulate CD8β, *in vivo* studies on the importance of this function for primate lentiviral infection and manipulation of CD8⁺ T cells *in vivo* seem feasible, and our ongoing studies aim to define mutations in Nef that selectively impair down-modulation of CD8β.

ACKNOWLEDGMENTS

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