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Nef-Mediated Suppression of T Cell Activation Was Lost in a Lentiviral Lineage that Gave Rise to HIV-1

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SUMMARY

High-level immune activation and T cell apoptosis represent a hallmark of HIV-1 infection that is absent from nonpathogenic SIV infections in natural primate hosts. The mechanisms causing these varying levels of immune activation are not understood. Here, we report that *nef* alleles from the great majority of primate lentiviruses, including HIV-2, downmodulate TCR-CD3 from infected T cells, thereby blocking their responsiveness to activation. In contrast, *nef* alleles from HIV-1 and a subset of closely related SIVs fail to downregulate TCR-CD3 and to inhibit cell death. Thus, Nef-mediated suppression of T cell activation is a fundamental property of primate lentiviruses that likely evolved to maintain viral persistence in the context of an intact host immune system. This function was lost during viral evolution in a lineage that gave rise to HIV-1 and may have predisposed the simian precursor of HIV-1 for greater pathogenicity in humans.

INTRODUCTION

Progression to AIDS in HIV-1-infected humans is characterized by high levels of immune activation associated with accelerated T cell turnover rates and apoptotic death (reviewed in Stevenson, 2003). In contrast, markedly lower levels of T cell activation, proliferation, and apoptosis are found in naturally SIV-infected primates, e.g., sooty mangabeys and African green monkeys, which do not develop immunodeficiency despite high systemic viral loads (reviewed in Hirsch, 2004; Silvestri, 2005). Consistent with a deleterious role in primate lentiviral infection, T cell activation is the strongest predictor of progression to AIDS in HIV-1-infected humans (Giorgi et al., 1999; Sousa et al., 2002) and the only consistent difference between pathogenic and nonpathogenic HIV/SIV infections (reviewed in Silvestri, 2005); however, the mechanisms responsible for the observed differences in infection-associated activation levels remain unknown.

One viral gene product that has been implicated in immune activation and viral pathogenicity is the accessory protein Nef (reviewed in Renkema and Saksela, 2000; Wei et al., 2003). Humans infected with *nef*-defective HIV-1 exhibit a slow/nonprogressor phenotype (Deacon et al., 1995; Kirchhoff et al., 1995), and macaques inoculated with *nef*-deleted SIVmac have extremely low viral loads, resulting in either no pathogenicity or a markedly protracted disease course (Kestler et al., 1991). Several Nef functions have been identified that may contribute to this phenotype, such as its ability to downregulate CD4, CD28, and class I MHC (MHC-I) (reviewed in Wei et al., 2003), which is believed to facilitate virus immune evasion (reviewed in Johnson and Desrosiers, 2002; Skowronski et al., 1999). HIV-1 Nef has also been reported to directly enhance the responsiveness of T cells to activation (Fenard et al., 2005; Fortin et al., 2004; Wang et al., 2000), but this effect was not uniformly observed among primate lentiviruses (Bell et al., 1998; Howe et al., 1998; Münch et al., 2005). Taken together, Nef has been characterized as a potent, multifunctional virulence factor of primate lentiviruses.

SIVs infect a wide variety of nonhuman primate species in sub-Saharan Africa (reviewed in Hahn et al., 2000; Sharp et al., 2005). In contrast to HIV-1 and HIV-2 infections of humans, natural SIV infections are usually not pathogenic for their primary hosts (reviewed in Hirsch, 2004; Silvestri, 2005). Currently, over 30 different SIVs have been molecularly characterized. Although all of them encode a *nef* gene (Leitner et al., 2005), functional data are almost exclusively derived from the HIV-1 Nef protein. To examine the function of Nef from an evolutionary perspective, we report here a comprehensive analysis of *nef* alleles from a variety of divergent SIV lineages. Using an experimental system allowing us to investigate Nef function in the context of replicating virus, we found that all primate lentiviral Nefs downmodulate CD4, CD28, and MHC-I. However, the ability to modulate the surface expression of the T cell receptor (TCR) complex differed markedly depending on the particular lentiviral lineage analyzed. Nef proteins from the great majority of primate lentiviruses efficiently downmodulated TCR-CD3, thereby suppressing the responsiveness of infected T cells to activation and activation-induced cell death (AICD). By contrast, the Nef proteins of HIV-1 and its closest simian relatives failed to downmodulate TCR-CD3 and to prevent AICD. Our data thus show that Nef exerts an important protective function that was lost during lentiviral evolution in a lineage that gave rise to HIV-1. We posit that this Nef activity is needed to prevent the chronic generalized T cell activation typical of pathogenic HIV infection and thus contributes to the nonpathogenic phenotype of natural SIV infections.

RESULTS

The Great Majority of Primate Lentiviral Nef Proteins Downmodulate TCR-CD3

Nef alleles (n = 30) from different primate lentiviruses (see Table S1 in the Supplemental Data available with this article online) were cloned into a replication-competent HIV-1 (NL4-3-based) proviral vector designed to coexpress Nef and eGFP from a bicistronic RNA (Schindler et al., 2003). In these constructs, *nef* expression is mediated by the wild-type HIV-1 LTR promoter and naturally occurring splice sites; however, cells infected with these reporter viruses coexpress Nef and eGFP at correlating levels (Schindler et al., 2005). Thus, in this system, the effect of Nef on the surface expression of cellular receptors can be examined directly in virally infected cells. Figure 1 shows such an analysis for CD4, MHC-1, CD3, and CD28 molecules on Jurkat T cells transduced with viral constructs expressing highly divergent HIV/SIV *nef* genes (as well as a *nef*-minus control). All *nef*-containing viruses downregulated CD4 and MHC-I, indicating that they expressed biologically active Nef proteins. However, the effect on TCR-CD3 surface expression was highly variable. The majority of viral *nef* constructs, including those of SIVsmm, SIVmac, and HIV-2 but also those of SIVrcm, SIVdeb, SIVsyk, SIVblu, SIVsun, SIVtan, and SIVsab, downmodulated TCR-CD3 efficiently; however, no such effect was observed for *nef* alleles of HIV-1, SIVcpz, SIVgsn, SIVmus, and SIVmon (Figure 1; Table S1).

To quantify the extent of receptor downmodulation, the mean fluorescence intensity (MFI) of cells infected with *nef*-defective virus (e.g., 48.7 for CD4) was divided by the MFI of cells infected with constructs coexpressing Nef and eGFP (e.g., 7.4 for NL4-3 Nef-mediated downregulation of CD4; Figure 1A). Results were tabulated separately for viral constructs that failed (group 1; n = 12) versus those that were capable of TCR-CD3 downmodulation (group 2; n = 18). For clarity, both groups were further subdivided to distinguish Nef proteins from the two human AIDS viruses and their immediate SIV counterparts (HIV-1/CPZ and HIV-2/SMM/MAC) from those of the remaining SIVs (GSN/MUS/MON and all other SIVs). The extent of TCR-CD3 downmodulation of group 2 *nef* alleles ranged from 5.7- to 18.0-fold, whereas group 1 Nefs completely failed to diminish TCR-CD3 surface expression (Figure 1B). Many group 2 *nef* alleles were also highly active in downregulating CD28, an important costimulatory molecule of T cell activation (reviewed in Riley and June, 2005), but this phenotype was more variable. The functional differences between the two groups were statistically highly significant ($p < 0.004$ for both CD28 and TCR-CD3 downmodulation) and were not biased by different transduction efficiencies because CD4, MHC-I, TCR-CD3, and CD28 expression levels were all measured simultaneously in the same cultures.

Nef-Mediated TCR-CD3 Downmodulation Blocks Activation of Virally Infected T Cells

HIV-1 Nef is known to enhance the responsiveness of T cells to activation (Fenard et al., 2005; Fortin et al., 2004; Wang et al., 2000). Since most primate lentiviral Nefs tested downmodulated TCR-CD3 and CD28 (Table S1), we hypothesized that they might inhibit, rather than enhance, T cell activation. Nuclear factor of activated T cells (NFAT) represents an important regulator of IL-2 gene expression, one of the hallmarks of T cell activation. We thus infected Jurkat T cells stably transduced with an NFAT-dependent luciferase gene (Fortin et al., 2004) with the HIV-1 Nef/eGFP constructs and determined their responsiveness to activation. Compared to uninfected control cultures, T cells infected with *nef*-defective HIV-1 exhibited an average of 4.6-fold increase of NFAT activity after stimulation with phytohemagglutinin (PHA) (Figure 2A). This increase in NFAT activation was even greater in cells expressing HIV-1/CPZ (7.7 ± 1.2, n = 9) and GSN/MUS/MON *nef* alleles (5.8 ± 0.7, n = 3; numbers indicate average values ± SD). However, virtually no NFAT activity was detected in cell cultures expressing HIV-2/SMM/MAC (0.7 ± 0.1, n = 8) or other SIV *nef* alleles (0.6 ± 0.1, n = 10). Remarkably, in these latter cases, NFAT activities were lower than those detected in uninfected cells, indicating active suppression of early T cell activation by these *nef* alleles. Similar results were observed for a second activation marker, CD69, which was only induced in

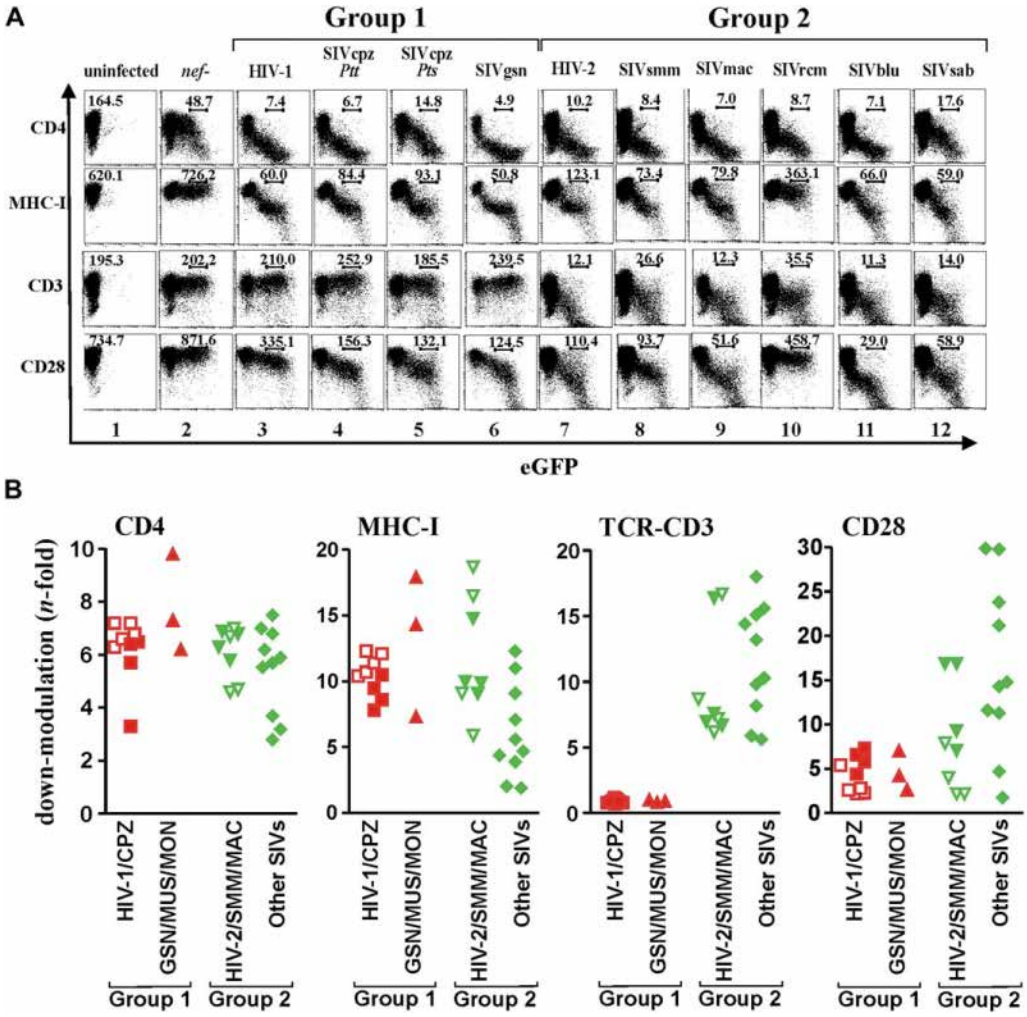

Figure 1. Differential Regulation of Surface-Receptor Expression by Primate Lentiviral Nef Proteins

(A) Flow cytometric analysis of CD4, MHC-I, CD3, and CD28 expression in Jurkat cells infected with HIV-1 recombinants expressing eGFP alone (*nef*−) or together with *nef* alleles derived from the primate lentiviruses indicated. eGFP expression levels used to calculate receptor downmodulation and the MFIs are indicated.

(B) Quantitative assessment of Nef-mediated downmodulation of the indicated cellular receptors. Each symbol represents n-fold downmodulation of the indicated receptor molecule by one of the 30 individual *nef* alleles analyzed. Group 1 and 2 *nef* alleles are color coded red and green, respectively. Open symbols indicate values obtained for Nef proteins of human viruses (HIV-1 and HIV-2).

cells expressing group 1 *nef* alleles (Figure S1A). Notably, Jurkat cells infected with group 1 and group 2 HIV-1 Nef/eGFP constructs responded equally well to stimulation with PMA/ionomycin, which bypasses surface receptors and directly activates intracellular signaling pathways (data not shown).

To mimic stimulation by APCs, we next examined the effect of the different *nef* alleles on the activation status of infected Jurkat T cells treated with anti-CD3/CD28 beads which crosslink the TCR with CD28 (Trickett and Kwan, 2003). Again, HIV-1/CPZ and GSN/MUS/MON *nef* alleles enhanced and the remaining *nef* alleles inhibited T cell activation (Figure 2B), and these results correlated with NFAT induction data obtained following PHA stimulation (Figure S1B). Thus, various stimuli, including PHA, anti-CD3/CD28 beads, and anti-CD3 antibody (data not shown), all yielded the same results: *nef* genes derived from HIV-1 and a subset of closely related simian relatives caused hyper-responsiveness of virally infected T cells, whereas the *nef* alleles of HIV-2 and all other SIVs inhibited T cell activation.

Nef-Mediated TCR-CD3 Downmodulation Protects Infected PBMCs against AICD

In acute primate lentiviral infection, the primary target cells are memory $CD4^+CCR5^+$ T cells that are phenotypically resting but are believed to represent a recently activated cell population (Li et al., 2005; Mattapallil et al., 2005).

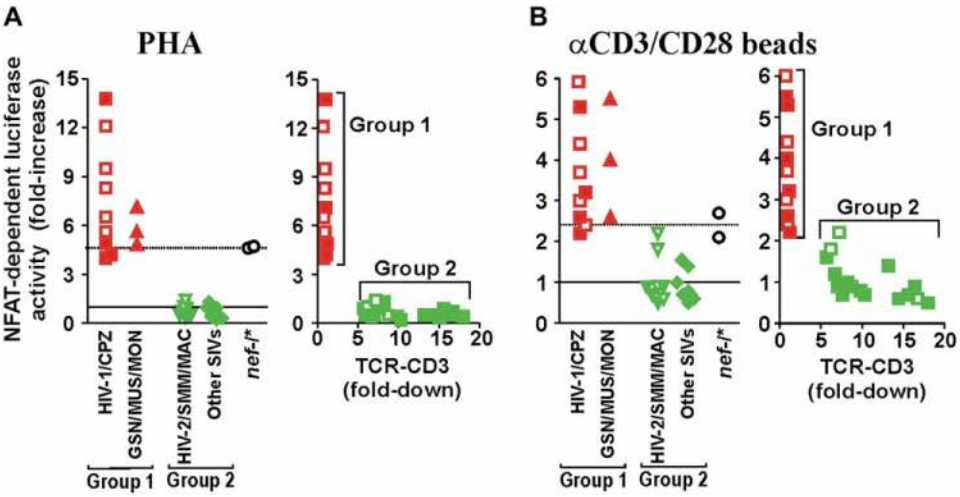

Figure 2. Nef-Mediated Differences in Responsiveness to T Cell Stimulation

Jurkat cells stably transfected with an NFAT-dependent reporter gene were transduced with HIV-1 Nef/eGFP constructs expressing either no *nef* (*nef*−/*) or *nef* alleles derived from the various groups of primate lentiviruses. Cultures were stimulated with PHA (A) or anti-CD3/CD28 beads (B). The left panels indicate NFAT-dependent luciferase reporter activities in Jurkat T cells infected with the respective HIV-1 Nef variants relative to those measured in uninfected cells. The average levels of NFAT induction measured in uninfected cells and in *nef*-defective HIV-1 infection are indicated by solid and broken lines, respectively. The right panels show that inhibition of NFAT induction correlates with Nef-mediated downmodulation of TCR-CD3. Group 1 and 2 *nef* alleles are color coded as in Figure 1.

To mimic this phenotype in vitro, we transduced PHA-stimulated peripheral blood mononuclear cells (PBMCs) with the HIV-1 Nef/eGFP constructs and incubated them in the absence of exogenous stimuli until they expressed low levels of activation markers and resembled resting cells (Figure 3A). Thereafter, the cells were exposed to a second PHA stimulus and examined for the expression of activation markers and signs of apoptosis. Flow cytometric analysis showed that all *nef* alleles that downmodulated CD28 and TCR-CD3 on Jurkat T cells also did so on human PBMCs (Figures S1C and S1D). Interestingly, downmodulation of TCR-CD3 in infected primary cells required much lower levels of *nef* expression than downregulation of CD28 (Figure 2B). Induction of CD69 as an early marker, and the IL-2 receptor (IL-2R) as a late marker, for T cell activation was enhanced by group 1 Nefs but inhibited by all other Nef proteins. Most importantly, PHA-treated PBMC cultures infected with HIV-1 constructs expressing group 1 Nefs exhibited substantially higher levels of AICD compared to those expressing group 2 Nef proteins. For example, the frequency of annexin V^+ apoptotic cells infected with the HIV-1 NA7 Nef construct increased from 8.5% to 33.9% over a period of 2 days, while only about 10% of cells expressing HIV-2, SIVmac, or SIVsab Nefs became apoptotic in this same time period (Figure 3C).

Quantitative assessment of the effects of all 30 *nef* alleles demonstrated that PBMCs infected with viruses containing group 1 Nefs expressed about 4-fold higher levels of CD69 (Figure 3D) and 2-fold higher levels of IL-2R (Figure 3E) compared to cells expressing group 2 Nefs ($p < 0.0001$ in each case). CD69 and IL-2R expression levels in these cultures correlated significantly (Figure S1E). Moreover, group 2 Nefs (14.2% ± 0.9%) prevented AICD much more efficiently than group 1 Nefs (28.0% ± 1.7%; $p < 0.0001$; numbers indicate mean percentages ± SEM of apoptotic cells) (Figure 3F). Again, the ability of Nef to inhibit the induction of CD69, IL-2R, and apoptosis was dependent on TCR-CD3 downmodulation (Figures 3D–3F, right panels), and a higher state of activation correlated with enhanced apoptosis (Figure S1F). In contrast to PHA activation, treatment with an anti-Fas antibody (CD95 mAb) resulted in virtually identical levels of programmed death in PBMCs infected with group 1 and group 2 Nef-containing HIV-1 (Figure S2). This result shows that cells infected with viruses expressing group 1 Nef exhibit higher levels of TCR-CD3-mediated, but not FAS-mediated, activation-induced apoptosis. Interestingly, the caspase inhibitor zVAD-FMK abolished the higher levels of apoptosis in PHA-activated PBMC cultures expressing group 1 Nefs, pointing to enhanced caspase activity as a potential underlying mechanism (Figure S2).

To demonstrate a causal relationship between Nef-induced TCR-CD3 downmodulation and reduction of T cell activation and apoptosis, we generated *nef* mutants that were selectively active or defective in this function. A truncated form of the SIVmac239 *nef* gene (tNef) efficiently downmodulated TCR-CD3 but was defective in other Nef functions (Figure 4A, column 6) (Münch et al., 2002). Conversely, deletion of amino acids 23–30 in the SIVdeb *nef* gene (Δ23–30) specifically abrogated this protein's ability to downmodulate TCR-CD3 but did not disrupt its other activities (Figure 4A, column 8). Functional analysis demonstrated that the SIVmac tNef, but not the

SIVdeb Δ23–30 Nef, blocked the induction of NFAT and upregulation of CD69 in HIV-1-infected Jurkat T cells (Figures 4B and 4C). Moreover, the SIVmac tNef, but not the SIVdeb Δ23–30 Nef, inhibited activation and AICD of HIV-1-infected primary PBMCs (Figures 4D–4F). Thus, Nef-mediated TCR-CD3 downmodulation is required and sufficient to inhibit T cell activation and AICD.

Nef-Mediated TCR-CD3 Downmodulation Inhibits Activation of Sooty Mangabey T Cells

To assess whether Nef function was dependent on the proviral context or the species origin of the target cell, we used HIV-1, SIVmac, and SIVagm Nef/eGFP constructs to transduce primary PBMCs from sooty mangabeys (SMs), a natural host of SIVsmm (Silvestri, 2005). As shown in Figure 5A, SIVmac and SIVagm Nefs, but not the HIV-1 Nef, downmodulated TCR-CD3 and inhibited IL-2R activation in SM PBMCs (note that although infected SM cells expressed overall reduced levels of CD4, downmodulation was more efficient in the presence of Nef). Remarkably, proviral constructs expressing different *nef* alleles exhibited the same phenotype in human and SM PBMCs (Figures 5B and 5C). For example, group 2 SIVsmm, SIVmac, and SIVdeb *nef* alleles as well as the SIVmac tNef mutant all downmodulated TCR-CD3 and inhibited activation of SM PBMCs (Figure 5C), and there was an overall highly significant correlation between the extent of Nef-mediated modulation of TCR-CD3, CD28, and IL-2R surface expression in human- and SM-derived PBMCs (Figures 5D–5F). Thus, the effect of Nef on T cell activation is independent of the proviral context and is conserved in target cells from divergent primate species.

Inefficient Nef-Mediated Downmodulation of TCR-CD3 Is Associated with $CD4^+$ T Cell Loss in SIVsmm-Infected Sooty Mangabeys In Vivo

Although the great majority of SMs do not develop immunodeficiency or $CD4^+$ T cell depletion as a consequence of SIVsmm infection (Silvestri, 2005), recent studies indicate that approximately 10%–15% of animals experience considerable $CD4^+$ T cell loss (B. Sumpter, R. Dunham, S. Gordon, J. Engram, M. Paiardini, M. Cervasi, H. McClure, S. Staprans, D.L.S., and G.S., unpublished data). To examine whether this in vivo phenotype is associated with differences in Nef function, we analyzed SIVsmm *nef* alleles from 8 SMs with low (<450/mm^3) and 11 SMs with normal $CD4^+$ T cell counts (>450/mm^3). Nef alleles were amplified directly from the plasma of infected animals and cloned into a vector coexpressing *nef* and GFP from a bicistronic RNA (Greenberg et al., 1998). To ensure representation of the *nef* quasispecies, all 19 amplicons were cloned and tested in bulk (for quality control, two clones per amplicon were also sequenced and phylogenetically analyzed; Figure S3). These analyses revealed that *nef* genes from SIVsmm-infected SMs with low $CD4^+$ T cells counts were significantly less active in downmodulating TCR-CD3 than *nef* alleles from animals with normal $CD4^+$ counts, while these two groups of *nef* alleles did not differ significantly in their ability to downmodulate CD4 (Figure 6A). It should be noted that the *nef* allele from a single SM with very low $CD4^+$ T cell counts (3/mm^3) downregulated all four surface receptors only poorly and may thus be generally functionally impaired. However, exclusion of this animal from the analyses did not change the results: The difference in TCR-CD3 downmodulation between "CD4 high" and "CD4 low" animals remained statistically significant ($p = 0.0056$). A linear regression analysis illustrating this correlation is shown in Figure 6B. Finally, *nef* genes from SMs with low $CD4^+$ T cell counts were frequently also less active in downmodulating MHC-I and CD28, but these differences were less pronounced. In summary, Nef-mediated downmodulation of TCR-CD3, and to a lesser extent of CD28 and MHC-I, is associated with stable $CD4^+$ T cell counts in SIVsmm-infected SMs. Since in most of these animals there is also a significant correlation between decreased $CD4^+$ T cell counts and increased immune activation (B. Sumpter, R. Dunham, S. Gordon, J. Engram, M. Paiardini, M. Cervasi, H. McClure, S. Staprans, D.L.S., and G.S., unpublished data), these results suggest that Nef plays an important protective role in maintaining low levels of T cell activation in naturally infected primates in vivo.

DISCUSSION

Nef-Mediated TCR-CD3 Downmodulation Is Highly Conserved throughout Primate Lentiviral Evolution

Previous studies of HIV-1 Nef have identified this accessory protein as an enhancer of T cell activation (Fenard et al., 2005; Fortin et al., 2004; Wang et al., 2000). In this paper, we confirm this finding for all three groups of HIV-1 (M, N, and O) but also show that the Nef proteins of most other primate lentiviruses exhibit a remarkably different phenotype. Essentially all SIV Nefs, except for a small subset, downmodulate TCR-CD3, in addition to CD4 and MHC-I. The exceptions include the chimpanzee precursor of HIV-1, SIVcpz, as well as three *Cercopithecus* viruses, SIVgsn, SIVmus, and SIVmon. The latter three form a tight clade in phylogenetic trees (Figure 7A) and are closely related to SIVcpz (and HIV-1) in the *env* gene (Bailes et al., 2003). However, in the *nef* gene (as well as *gag* and *pol* regions), SIVcpz is most closely related to SIVrcm, reflecting its recombinant ancestry (Bailes et al., 2003). Indeed, the entire SIVcpz *nef* gene is SIVrcm derived, since the recombination event that generated SIVcpz also introduced a duplication of the 3′ terminus of *env*/5′ terminus of *nef*, thereby eliminating the *nef*/*env* overlap that is typical of all other primate lentiviruses (Figure 7B). Since the SIVrcm Nef protein downmodulates TCR-CD3 but the (SIVrcm-related) SIVcpz Nef protein does not (Table S1), this function must have been lost twice during primate lentiviral evolution: once on the branch leading to the SIVgsn/mus/mon clade, and a second time after the recombination event that generated SIVcpz. In the context of primate lentivirus evolution, these two events are comparatively recent, whereas the Nef-mediated TCR-CD3 downmodulation

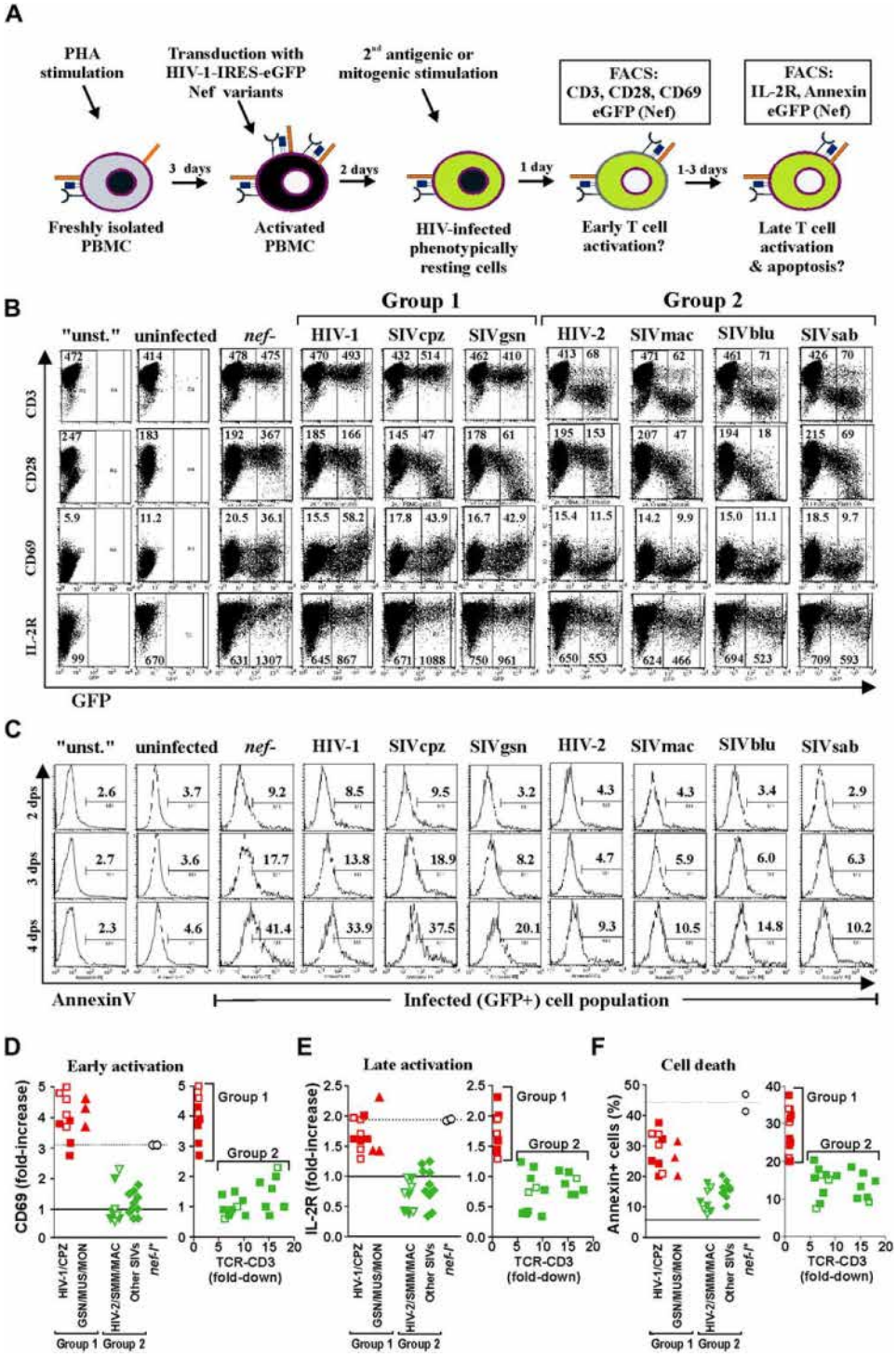

Figure 3. Activation and AICD of Virally Infected PBMC Cultures

(A) Schematic representation of the experimental procedure. Human PBMCs were transduced with HIV-1 eGFP/Nef constructs 3 days after PHA stimulation and cultured until they expressed low levels of CD69 and IL-2R, i.e., exhibited a resting phenotype. Thereafter, the PBMCs were exposed to a second PHA stimulus and examined for the expression of T cell activation markers and apoptotic features.

(B) Flow cytometric analysis of CD3, CD28, and CD69 expression by PBMCs infected with HIV-1 constructs expressing the indicated *nef* alleles at 1 day poststimulation (dps) and of IL-2R expression at 4 dps. "Unst." specifies control cells that did not receive the second PHA stimulus.

(C) Kinetics of apoptosis in a representative experiment. Percentages of annexin V^+ apoptotic cells in uninfected cultures or in HIV-1-infected cells expressing eGFP alone (*nef*−) or together with the indicated *nef* alleles are indicated.

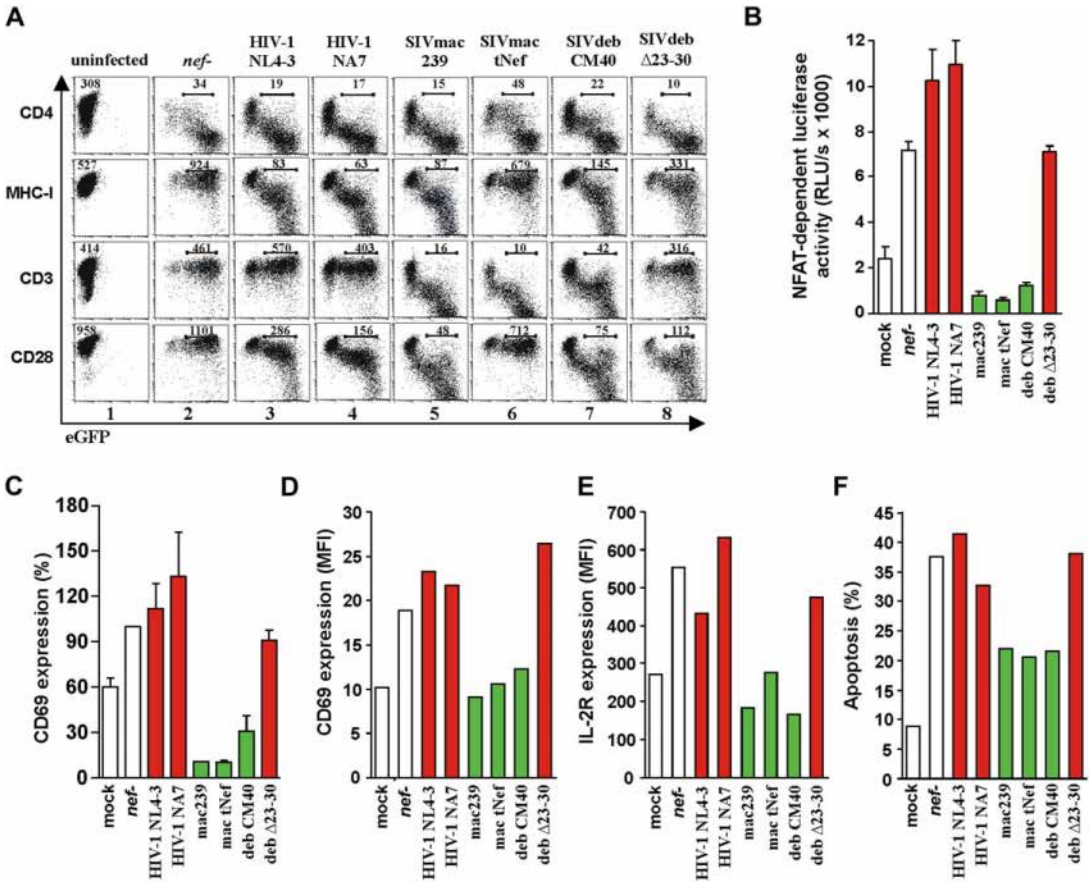

Figure 4. Modulation of Receptor Expression, T Cell Activation, and Apoptosis by Selective *nef* Mutants

(A) Flow cytometric analysis of Jurkat cells infected with HIV-1 constructs expressing eGFP alone (*nef*–) or with the *nef* alleles indicated.

(B and C) Analysis of Jurkat cells stably transfected with an NFAT-dependent reporter gene following transduction with the indicated HIV-1 Nef/eGFP constructs indicated. Levels of NFAT-dependent luciferase reporter activity (B) and CD69 expression (C) are the average (±SEM) of four experiments. Mock specifies uninfected control cells. *Nef* alleles capable or defective of TCR-CD3 downmodulation are shown in green and red, respectively.

(D–F) Analysis of human PBMCs following transduction with the indicated HIV-1 IRES/eGFP constructs. CD69 (D) was measured 1 day following a second PHA stimulus; IL-2R expression levels (E) and the frequencies of apoptotic cells (F) were measured 4 dps. Results are representative of three to six experiments.

function represents a characteristic feature of longstanding virus/host relationships.

Nef-Mediated TCR-CD3 Downmodulation: A Key Contributor to the Nonpathogenic Phenotype of Natural SIV Infections?

$CD4^+$ T cell activation requires the interaction of the TCR-CD3 complex with the MHC class II complex presenting antigen specific peptides as well as a CD28/B7-mediated costimulation. Primate lentiviral Nefs that effectively block T cell activation by downmodulating TCR-CD3 do so in human as well as sooty mangabey PBMCs (Figure 5), indicating that this function is independent of the species origin of the infected host cell. Moreover, SMs infected with SIVsmm strains whose Nefs were capable of downmodulating TCR-CD3 maintained normal $CD4^+$ T cell counts, while animals infected with SIVsmm strains whose Nefs were less active in this function experienced progressive $CD4^+$ T cell loss (Figure 6; note that Nef-mediated TCR-CD3 downmodulation is generally much more pronounced in primary PBMCs than in immortalized T cells). Since the latter animals also exhibited signs of increased in vivo immune activation (B. Sumpter, R. Dunham, S. Gordon, J. Engram, M. Paiardini, M. Cervasi, H. McClure, S. Staprans, D.L.S., and G.S., unpublished data), it is tempting to speculate that, in most natural SIV infections, there

(D–F) CD69 (D) was measured 1 day after the second stimulus, and IL-2R expression levels (E) and the frequencies of apoptotic cells (F) were measured by FACS at 4 dps. The average levels of CD69, IL-2R, and apoptotic cells in uninfected or *nef*– HIV-1 infected cells are indicated by solid or broken lines, respectively. The right panels show that inhibition of CD69 and IL-2R induction and apoptosis correlate with Nef-mediated downmodulation of TCR-CD3. Group 1 and 2 *nef* alleles are color coded as in Figure 1.

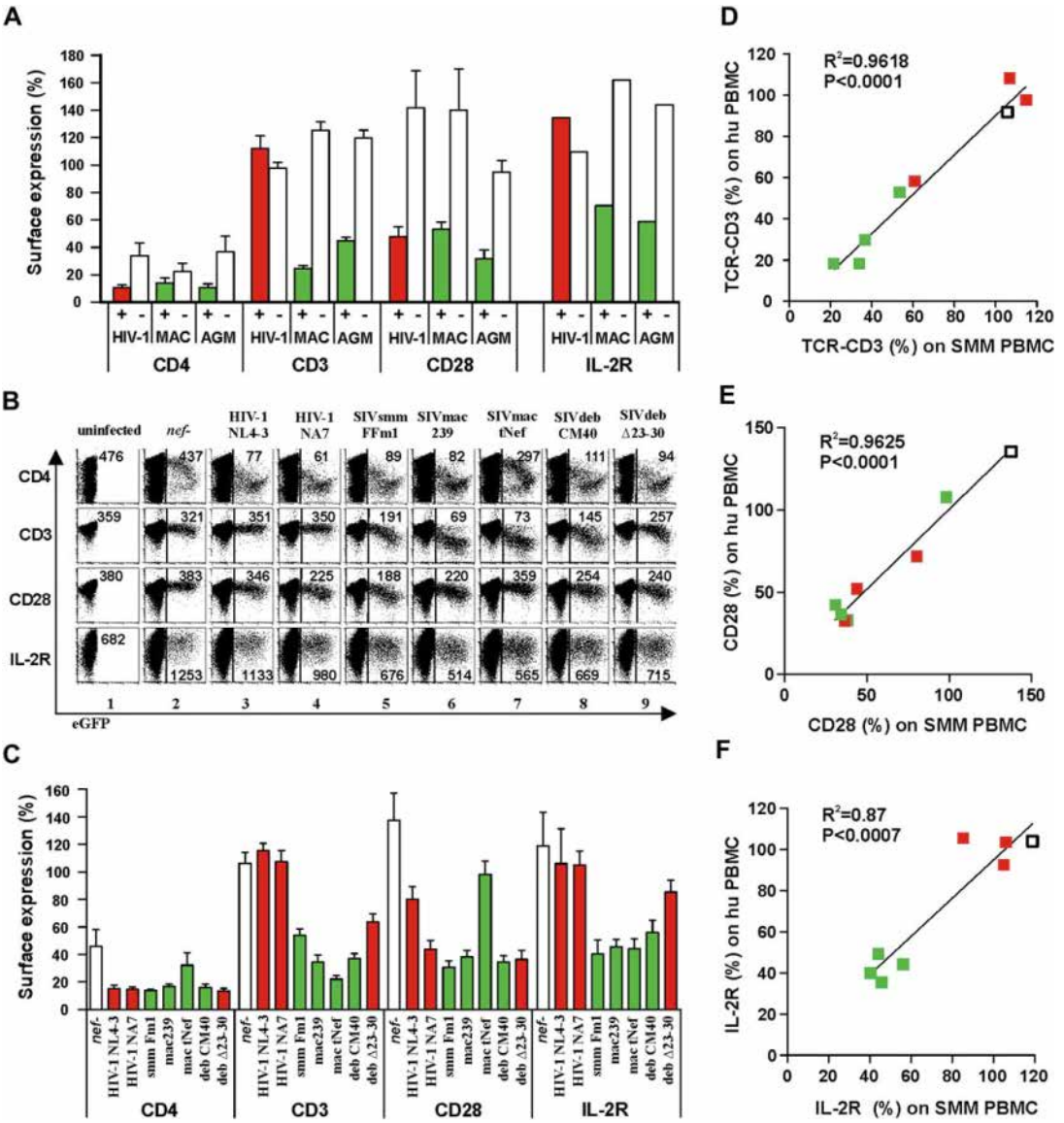

Figure 5. Nef-Mediated Receptor Modulation in Primary Sooty Mangabey PBMC

(A) Surface expression of CD4, CD3, CD28, and IL-2R receptors on SM PBMCs infected with HIV-1, SIVmac, or SIVagm Nef/eGFP constructs containing intact (+) or disrupted (−) *nef* genes. Receptor expression levels on uninfected cells were set to 100% in (A) and (C)–(F), and values in (A) and (C) are means ±SEM derived from four or five transductions.

(B) Flow cytometric analysis of SM PBMCs infected with HIV-1 Nef/eGFP constructs expressing HIV-1, SIVsmm, SIVmac, and SIVdeb wild-type (NL4-3, NA7, FFm1, 239, and CM40), mutant (tNef and Δ23–30), or no (−) *nef* genes. The range of eGFP expression used to calculate the percentages of receptor expression and MFIs is indicated.

(C) Relative CD4, CD3, CD28, and IL-2R cell-surface expression levels on SM PBMCs infected with the indicated HIV-1 Nef/eGFP construct. *Nef* alleles are color coded as in Figure 4.

(D and E) Nef-mediated modulation of TCR-CD3, CD28, and IL-2R in human and SM PBMCs. Open symbols indicate values obtained for the *nef*− control.

is a direct link between Nef's ability to downmodulate TCR-CD3 and the maintenance of an intact immune system.

In HIV-1-infected humans, most $CD4^+$ T cell killing occurs in the acute phase of infection when the majority of mucosal $CD4^+$ memory T cells are destroyed by direct, virally mediated mechanisms (Brenchley et al., 2004; Li et al., 2005; Mattapallil et al., 2005; Mehandru et al., 2004). It has been proposed that acute HIV-1 infection should be viewed as a "selective memory T cell lymphopenia" and that the high levels of immune activation observed during chronic infection are secondary to this

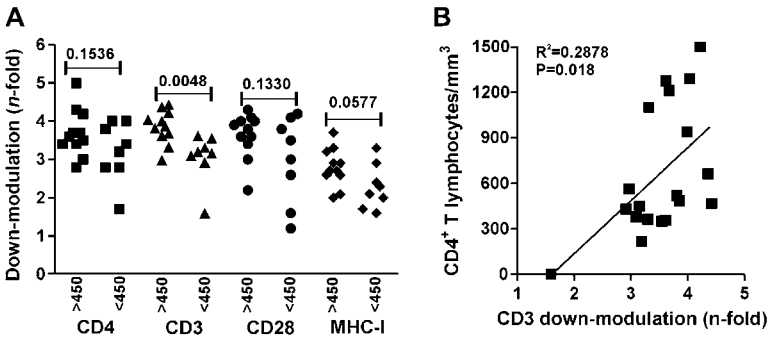

Figure 6. Nef Protein Function in SIVsmm-Infected SMs with and without Progressive CD4$^+$ T Cell Loss

(A) SIVsmm *nef* genes were grouped by CD4$^+$ T cell counts of the animals from which they were derived. Values for downregulation of CD4, CD3, CD28, and MHC-I at a medium range of eGFP expression levels are indicated and are representative of two experiments (p values are shown).

(B) Correlation between Nef-mediated TCR-CD3 downmodulation and CD4$^+$ T cell counts.

depletion, due to a breakdown of gut immune responses and/or compensatory mechanisms aimed at replenishing CD4$^+$ T cells in lymphatic organs (Brenchley et al., 2006). Interestingly, SIV infection of SMs is also associated with substantial depletion of mucosal memory CD4$^+$ T cells (S. Gordon, J. Engram, J. Milush, R. Dunham, N. Klatt, E. Strobert, I. Pandrea, S. Staprans, D.L.S., and G.S., unpublished data); however, in these infections, CD4$^+$ T cell loss is not followed by generalized immune hyperactivation. The reasons for this are not understood, but the results reported here strongly suggest a protective role of the SIV *nef* gene product. Nef-mediated downmodulation of TCR-CD3 reduces T cell activation and activation-induced cell death and may possibly also decrease bystander apoptosis through reduced expression of proapoptotic proteins or inflammatory cytokines. Moreover, SIV Nef is likely to protect infected cells against killing by cytotoxic T lymphocytes via MHC-I downregulation (Collins et al., 1998). Finally, SIV Nef may further inhibit immune activation and apoptosis by efficient downmodulation of CD28 and possibly other functions, such as inhibition of T cell trafficking (Hrecka et al., 2005). Thus,

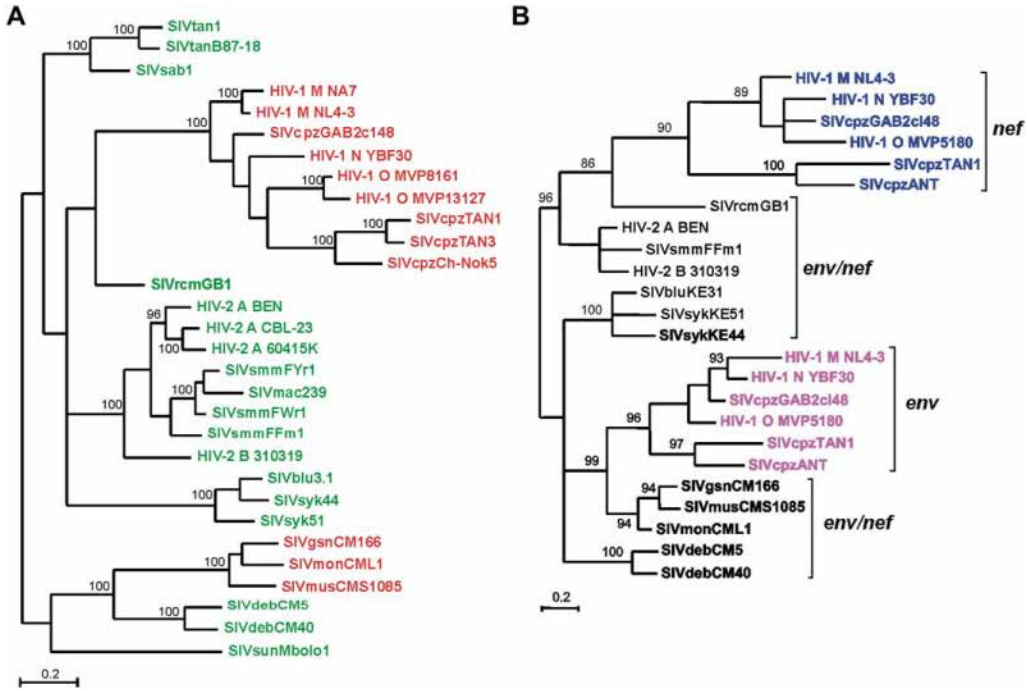

Figure 7. Evolutionary Relationship of Lentiviral Nef Proteins and Phylogenetic Evidence for a Sequence Duplication in the Genomes of SIVcpz and HIV-1

(A) Phylogenetic analysis of Nef proteins listed in Table S1. Group 1 and 2 sequences are color coded as in Figure 1. The numbers on branches are percentage posterior probabilities; only values of 95% and above are shown in (A), and only probabilities above 80% are shown in (B). The scale bars indicate 0.2 substitutions per site.

(B) Duplication in the SIVcpz/HIV-1 lineage. The 3′ end of *env* (magenta) and the 5′ end of *nef* (blue) sequences from the indicated HIV-1 and SIVcpz strains were aligned with the overlapping region of *env* and *nef* from various SIV and HIV-2 strains. Except for MVP5180 (L20571) and SIVcpzANT (U42720), all GenBank accession numbers are listed in Table S1. The gap-stripped alignment was 135 nucleotides. The tree was made by the Bayesian method using the general reversible (GTR) model of evolution, with γ distributed rates at sites run for 10 million generations.

Nef-mediated reduction of T cell activation may represent a key determinant in ensuring that natural SIV infections are nonpathogenic in their natural hosts.

Vpu Appears to Diminish the Selective Advantage of Nef-Mediated TCR-CD3 Downmodulation

If TCR-CD3 downmodulation serves to maintain virus replication and persistence in the context of an intact host immune system, how do primate species cope with SIVs that have lost this Nef function? While there is virtually no information about the natural history of SIVgsn, SIVmus, and SIVmon infections in their primate hosts, there is evidence that these viruses are much less prevalent than "regular" SIV infections. For example, only 3% and 4% of mustached and greater spot-nosed monkeys are infected with SIVmus and SIVgsn, respectively, compared to 50% to 90% of wild-living SMs, African green monkeys, and mandrills (Aghokeng et al., 2006; Bibollet-Ruche et al., 2004). SIVcpz infection infects up to 35% of wild-living chimpanzees but is unevenly distributed among wild populations and is much less transmissible than "regular" SIV infections (Sharp et al., 2005; Keele et al., 2006). Finally, SIVgsn/mus/mon and SIVcpz strains are quite unusual in that they encode a *vpu* gene, which is absent from other primate lentiviruses. Vpu itself does not downregulate TCR-CD3. However, there may be other (as yet unidentified) Vpu functions that may diminish the selective advantage of maintaining Nef-mediated TCR-CD3 downmodulation. This would explain the apparently coincidental loss of Nef function in two distinct lineages, both of which encode a *vpu* gene.

Nef-Mediated Inhibition of T Cell Activation Attenuates Even Pathogenic SIVmac Infection

A protective role of Nef-mediated TCR-CD3 downmodulation is consistent with the nonpathogenic phenotype of naturally occurring SIV infections; however, two models of experimental SIV infection appear to be at odds with this hypothesis. First, SIVmac (a virus inadvertently transmitted from naturally infected SMs to macaques in captivity; Daniel et al., 1985) causes immunodeficiency and AIDS in macaques (Kestler et al., 1991) despite the fact that its Nef protein efficiently downmodulates TCR-CD3. Second, SIVmac viruses containing HIV-1 *nef* genes (the so-called Nef-SHIVs) do not exert greater pathogenicity than wild-type SIVmac239 in experimentally infected macaques (Alexander et al., 1999; Kirchhoff et al., 1999). The Nef-SHIV chimeras are highly artificial viruses that express Nef from a different genomic location and at markedly reduced levels and are thus not really informative as to the role of TCR-CD3 downregulation in natural SIV infection. The in vivo phenotype of SIVmac is somewhat more difficult to explain, since the SIVmac Nef downmodulates TCR-CD3 not only in infected human but also in macaque PBMCs (unpublished data). SIVmac-infected macaques develop immunodeficiency and AIDS, indicating that even efficient Nef-mediated downmodulation of TCR-CD3 is not sufficient to prevent immunodeficiency and disease. However, SIVmac is not pathogenic when introduced back into SMs by experimental infection (Kaur et al., 1998). Thus, the pathogenic phenotype of SIVmac in macaques seems to be more a function of the host species than an inherent viral phenotype. Moreover, changes in SIVmac Nef that increase T cell activation also enhance its virulence (Du et al., 1995), whereas changes that selectively retain the TCR-CD3 downmodulation (but abrogate other Nef functions) result in an even more attenuated in vivo phenotype than an SIVmac strain that is lacking *nef* entirely (Münch et al., 2002). Thus, even in the pathogenic SIVmac/macaque model, the ability of Nef to inhibit T cell activation has a mitigating effect on viral virulence.

Conclusions

In summary, our data indicate that Nef-mediated downmodulation of TCR-CD3 is an evolutionarily highly conserved function of primate lentiviruses and a potential contributor to their nonpathogenic phenotype in most natural hosts. Thus, SIV Nef not only facilitates SIV persistence by downmodulation of CD4 and MHC-I but may also act as a "rheostat," allowing high enough levels of T cell activation to ensure sufficient viral replication and transmission, while at the same time preventing escalation of immune activation to levels that may be harmful to the host. Interestingly, this function was lost during primate lentiviral evolution in a subset of SIVs, including SIVcpz, the immediate precursor of HIV-1. While the presence of a *vpu* gene and other changes may have compensated for (and even precipitated) this loss of Nef function in the chimpanzee hosts, the inability to reduce T cell activation may have predisposed the simian precursor of HIV-1 to greater virulence upon transmission to humans. The fact that a second SIV (SIVsmm) with an intact TCR-CD3 downmodulation function resulted in an overall less pathogenic human (HIV-2) infection is consistent with this hypothesis (Marlink et al., 1988; Michel et al., 2000; Pepin et al., 1991). The present report provides the framework for future investigations aimed at exploring directly the contribution of Nef-mediated TCR-CD3 downmodulation to primate lentiviral pathogenesis (or lack thereof) in appropriate animal models.

EXPERIMENTAL PROCEDURES

Proviral Constructs

Generation of HIV-1 (NL4-3-based) proviral constructs carrying functional *nef* genes followed by an IRES and the eGFP gene has been described (Schindler et al., 2003, 2005). Splice overlap extension PCR was used to replace the HIV-1 NL4-3 *nef* allele with the *nef* genes listed in Table S1. The proviral HIV-1 constructs are replication competent and express all viral genes, including *nef*, via the regular LTR promoter and splice sites. The integrity of all PCR-derived inserts was confirmed by sequence analysis. *nef*-defective control constructs contained a premature stop codon at position 40 of the HIV-1 NL4-3 *nef* (*nef**), or this same stop codon combined with a second stop codon at position 3 and a mutation of the initiation codon (*nef*−). Similar approaches were used to generate replication-competent SIVmac (strain 239) and SIVagm (strain TAN1) based IRES/eGFP constructs.

Amplification of SIVsmm *nef* Alleles from Plasma of Infected SMs

Blood samples were obtained from 19 naturally SIVsmm-infected SMs housed at the Yerkes National Primate Research Center of Emory University and maintained in accordance with NIH guidelines. Plasma viral RNA was extracted using the QIAamp Viral RNA Kit (QIAGEN), and *nef* genes were amplified by RT-PCR using primers SM-Nef-F1 (5′-GACA GATAGAATATATTCATTTCC-3′) and SM-Nef-R1 (5′-TCTGCCAGCCT CTCCGCAGAG-3′). For expression in Jurkat T cells, *nef* genes were PCR amplified using primers containing the XbaI and MluI restriction sites and cloned in bulk into a bicistronic expression vector (Greenberg et al., 1998). Sequence analysis of two clones per amplicon (two per animal) confirmed the integrity of these *nef* alleles. Transfection of Jurkat T cells and flow cytometric analyses were performed as described (Münch et al., 2005).

Cell Culture and Transfection

Jurkat and 293T cells were cultured as described (Münch et al., 2005; Kirchhoff et al., 2004). PBMCs from healthy human donors were isolated using lymphocyte separation medium (Biocoll Separating Solution, Biochrom), stimulated for 3 days with PHA (1 μg/ml), and cultured in RPMI1640 medium with 10% FCS and 1 ng/ml IL-2 prior to infection. SM PBMCs were isolated from 40 ml of anticoagulated peripheral blood using two sequential Ficoll gradients (Pharmacia). $CD8^+$ cells and $CD19^+$ cells were removed by antibody-coupled magnetic beads (Miltenyi Biotec). Cells were stimulated with PHA (0.3 μg/ml) and infected with HIV/SIV Nef/eGFP constructs 4 days following stimulation.

Virus Stocks and Transductions

To generate viral stocks, 293T cells were cotransfected with the HIV/SIV Nef/eGFP constructs and a plasmid (pHIT-G) expressing the vesicular stomatitis virus G protein (Schindler et al., 2003, 2005). The latter was used to achieve high initial infection levels for functional analysis. However, all HIV-1 constructs contained intact *env* genes and were thus replication competent following the first round of infection. VSV-G pseudotyping did not affect Nef-mediated surface-receptor modulation. Virus stocks were quantified using a p24 antigen capture assay provided by the NIH AIDS Research and Reference Reagent Program.

Flow Cytometric Analysis

CD4, TCR-CD3, MHC-I, CD28, and eGFP reporter expression in Jurkat T cells or human PBMCs transduced with HIV-1 (NL4-3) constructs coexpressing Nef and eGFP were measured as described (Schindler et al., 2003; Kirchhoff et al., 2004). CD69 and IL-2R expression was measured by standard FACS staining using CD69 (BD Pharmingen, Clone FN50) and CD25 (BD Pharmingen, Clone M-A251) mAbs. Flow cytometric analysis of SM PBMCs was performed on a LSRII (Becton & Dickinson) using FACSAria software, using anti-CD3 (SP-34-2), anti-CD4 (L200), anti-CD8 (RPA-T8), anti-CD25 (2A3), and anti-CD28 (CD28.2) antibodies (Becton & Dickinson). SM cells that stained positive for CD8 were excluded from the analysis. For quantification of Nef-mediated modulation, the levels of receptor expression (red fluorescence) were determined for cells expressing a specific range of eGFP (Schindler et al., 2003; Kirchhoff et al., 2004). The extent of downmodulation or induction (n-fold) was calculated by dividing the MFI obtained for cells infected with the *nef*-minus NL4-3 control viruses by the corresponding values obtained for cells infected with viruses coexpressing *nef* and eGFP.

NFAT Induction

Jurkat cells stably transfected with an NFAT-dependent reporter gene vector (Fortin et al., 2004) were either left uninfected or transduced with HIV-1 Nef/eGFP constructs expressing various *nef* alleles. Except for those cells used as controls, cultures were treated with PHA (1 μg/ml; Murex), anti-CD3 AB (1 μg/ml; BD Pharmingen, Clone HIT3a), or anti-CD3/CD28 beads (Trickett and Kwan, 2003) at a cell:bead ratio of 1:1. Luciferase activity was measured and n-fold induction determined by calculating the ratio between measured relative light units of treated samples over untreated samples as described previously (Fortin et al., 2004).

Induction of PBMC Activation and Apoptosis

Human PBMCs were first stimulated with PHA (1 μg/ml) for 3 days. Subsequently, the cells were cultured in RPMI1640 (10% FCS, 1 ng/ml IL-2), infected with various HIV-1 eGFP/Nef constructs, and cultured for another 2 days. At this time, the PBMCs expressed very low levels of CD69 and IL-2R and hence had a resting phenotype. Thereafter, the PBMCs were treated a second time with PHA, and CD69 and IL-2R expression levels were measured by FACS analysis 1 and 4 days later. The frequency of virally infected apoptotic cells was determined using the Annexin V (AnV) Apoptosis Detection Kit (BD Bioscience) as recommended by the manufacturer. To compare PHA- and FAS-induced apoptosis, PBMCs were stimulated with 1 μg/ml PHA or treated with 10 μl/ml CD95Ab (Apo-I hybridoma supernatant, kindly provided by K.M. Debatin) at 2 days posttransduction. Subsequently, aliquots of the cultures were treated with 20 μM zVAD-FMK (Sigma). CD95Ab-induced cell death was measured 12 hr postinduction, whereas PHA-induced apoptosis was determined 3 days poststimulation (dps) as described (Schindler et al., 2005).

Phylogenetic Analyses

Sequences were aligned using CLUSTAL W (Thompson et al., 1994). Sites with a gap in any sequence were discarded. Trees were made by the Bayesian method implemented in MrBayes v3.0 (Huelsenbeck and Ronquist, 2001) using 1 million or 10 million generations and burn-in of 10%. Estimated sample sizes from Tracer (Rambaut and Drummond, 2003) were above 100 for all trees.

Statistical Analysis

The activities of group 1 (n = 12) and group 2 (n = 18) *nef* alleles and of *nef* alleles derived from SMs with low (n = 8) or high (n = 11) $CD4^+$ T cell counts were compared using a two-tailed Student's t test. The PRISM package version 4.0 (Abacus Concepts, Berkeley, CA, USA) was used for all calculations.

Supplemental Data

Supplemental Data include three figures and one table and can be found with this article online at http://www.cell.com/cgi/content/full/125/6/1055/DC1/.

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Accession Numbers

GenBank accession numbers for the newly derived SIVsmm *nef* sequences are DQ408682 to DQ408725.