Comprehensive Analysis of Nef Functions Selected in Simian Immunodeficiency Virus-Infected Macaques

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A variety of simian immunodeficiency virus (SIVmac) nef mutants have been investigated to clarify which in vitro Nef functions contribute to efficient viral replication and pathogenicity in rhesus macaques. Most of these nef alleles, however, were only functionally characterized for their ability to down-modulate CD4 and class I major histocompatibility complex (MHC-I) cell surface expression and to enhance SIV replication and infectivity. To obtain information on the in vivo relevance of more recently established Nef functions, we examined the ability of a large panel of constructed SIVmac Nef mutants and of variants that emerged in infected macaques to down-regulate CD3, CD28, and MHC-II and to up-regulate the MHC-II-associated invariant chain (II). We found that all these four Nef functions were restored in SIV-infected macaques. In most cases, however, the initial mutations and the changes selected in vivo affected several in vitro Nef functions. For example, truncated Nef proteins that emerged in animals infected with SIVmac239 containing a 152-bp deletion in nef efficiently modulated both CD3 and II surface expression. Overall, our results suggest that the effect of Nef on each of the six cellular receptors investigated contributes to viral fitness in the infected host but also indicate that modulation of CD3, MHC-I, MHC-II, or II surface expression alone is insufficient for SIV virulence.

Studies with the simian immunodeficiency virus (SIV) mac239 molecular clone demonstrated that large deletions in the accessory nef gene attenuate viral replication and pathogenicity in infected rhesus macaques (25). Premature stop codons in nef revert and come to predominate in infected animals within 1 or 2 weeks, thus confirming a strong selective pressure for Nef function in vivo (25). Subsequent studies suggest that intact nef genes are of similar relevance for the pathogenicity of human immunodeficiency virus type 1 (HIV-1) in infected human individuals (15, 27). It has been established that the SIVmac239 Nef performs a variety of in vitro functions that are likely relevant for efficient viral persistence and disease progression in vivo, including down-regulation of CD4 (7, 17), CD3 (5, 20, 45, 46, 53), CD28 (52), and class I major histocompatibility complex (MHC-I) (51) cell surface expression and enhancement of viral replication and infectivity (2, 29). More recently, it has been demonstrated that SIVmac and HIV-1 Nef also affect MHC-II antigen presentation by down-modulation of surface expression of mature MHC-II and up-regulation of the invariant chain (II) associated with immature MHC-II molecules (47, 50).

It is conceivable that these Nef activities increase viral spread both directly by enhancing virion infectivity and virus production and more indirectly by helping SIV and HIV to evade the immune response (reviewed in reference 4). However, we have only begun to understand their contribution to the pathogenesis of AIDS. Analysis of naturally occurring SIV Nef variants has demonstrated that substitution of R17Q18 to Y17E18 (YE-Nef) is associated with extensive T-cell activation and an acute disease in infected rhesus monkeys (16). In contrast, deletion of amino acids 143 to 146 (DMYL) in Nef (CS-Nef) leads to an attenuated phenotype in vivo (41), although the deletion is “repaired” and virulence is restored in some infected monkeys (55). Studies with specific SIVmac239 mutants demonstrated that the conserved acidic element (AcI-Nef) or potential protein kinase C phosphorylation site (PKC-Nef) are relevant for Nef function both in vitro and in vivo (9). A major drawback of most of these early studies, however, was that the results did not allow definitive conclusions about the relevance of specific Nef activities for viral pathogenicity because the mutations impaired most or all aspects of Nef function. Over the last years, however, it has become clear that different Nef functions are genetically separable and require distinct elements located throughout the Nef molecule (1, 19, 21, 22, 30, 33, 42). This knowledge allowed the generation of SIVmac mutants that are selectively impaired in one or a few in vitro Nef functions but not in others. Subsequent studies in the SIV macaque model provide evidence that multiple in vitro Nef activities, such as lymphocyte activation (16) and down-modulation of CD4 (23), MHC-I (35), or CD3 (36) surface expression, confer a selective advantage for viral replication in infected monkeys.

The goal of these studies was to assess the relevance of different in vitro Nef functions for viral pathogenesis. However, several Nef activities that could play an important role for SIV or HIV-1 virulence, such as modulation of CD28, MHC-II, and II surface expression, have only recently been established (47, 50, 52). Therefore, we investigated the effect of a large panel of mutant SIV nef alleles and of variants that emerged in infected macaques on CD4, MHC-II, and II as well as CD3, CD28, and MHC-I surface expression. Taken together, our results indicate that a selective pressure for all
these Nef functions exist in vivo. A combination of most or all of these in vitro Nef activities seems important for the maintenance of high viral loads and development of disease in infected macaques.

MATERIALS AND METHODS

Plasmids. For most SIV nef alleles used in the present study, generation, sequence analysis, and functional activity in down-modulation of CD4 and MHC-I cell surface expression and enhancement of viral infectivity and replication has been described previously (summarized in Table 1). The G,A Nef was generated by PCR mutagenesis of the SIVmac239 wild-type (239wt) nef using primer p239nefXba-G2A (5'-ATATGGGTGGAGC-3') and p239nefMlu and the inner primers p239RR5 (5'-CAGTGCAGCAGCACATAGAATC-3') and p239RR3 (5'-GATTTCTAGTGGCTGCAGT-3'). Mutated positions are underlined. All nef alleles were cloned into the bicistronic cytomegalovirus-based pcG expression vector coexpressing the green fluorescent protein (GFP) and Nef (19) using the unique XbaI and MluI restriction sites (shown in bold) flanking the nef open reading frame. All PCR-derived inserts were sequenced on both strands to confirm that the constructs expressed the correct Nef variants.

Transfections and cell culture. HeLa CIITA and Jurkat T cells were cultured as described previously (12, 50). Transfection of Jurkat T cells was performed using the DMRIE-C reagent (Gibco-BRL, Karlsruhe, Germany) following the manufacturer’s instructions. HeLa CIITA cells were transfected with Metfectene (Biontech, Munich, Germany). Briefly, 25 μg of DNA in 100 μl of OMEM (Invitrogen, Karlsruhe, Germany) was mixed with 12 μl of Metfectene in 100 μl of Dulbecco’s modified Eagle medium (DMEM) and incubated for 30 min at room temperature. Subsequently, the mixture was added to 2 × 105 cells and incubated for 6 h at 37°C. Thereafter, the medium was changed and cells were analyzed by a fluorescence-activated cell sorter (FACS) on the following day.

Western blotting. 293T cells were transfected with 5 μg of pcG expression vectors coexpressing GFP and Nef using the calcium phosphate method as described previously (10, 12). Two days posttransfection, cells were pelleted and lysates were generated and separated through sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis as described previously (10). Expression of Nef proteins in whole cellular lysates was analyzed by immunoblots. Proteins were detected with a 1:2,500 dilution of an alkaline phosphatase-conjugated secondary anti-human IgG antibody (Jackson Immuno Research) as described by the manufacturer.

Flow cytometry. CD4, CD3, MHC-I, CD28, and GFP reporter molecules in Jurkat T cells and down-modulation of MHC-II and up-regulation of CD8 in HeLa CIITA cells transfected with bicistronic vectors coexpressing Nef and GFP were measured as described previously (12, 47). The following phycoerythrin-conjugated antibodies were used: anti-CD4 (RPA-T4), anti-CD3 (UCHT1), anti-LeuM-28 (L293) (BD Biosciences Pharmingen), anti-CD74/R/PE (M-B741) (Anzelli), anti-HLA-ABC antigen (W6/32)/RPE (DAKO), mouse anti-human HLA-DR TUB3 (Calltag Laboratories), and L243 (BD Biosciences Pharmingen). The levels of CD4, CD3, MHC-I, and CD28 on Jurkat cells or of MHC-II and CD8 on HeLa CIITA cells, respectively (red fluorescence), were measured from aliquots of the same transfection as a function of GFP fluorescence. For the quantification of Nef-mediated down- or up-regulation of cellular receptors, the mean channel numbers of red fluorescence were determined for cells expressing no, low, medium, or high levels of GFP as described previously (12, 47). The mean channel numbers of red fluorescence obtained for cells transfected with a control construct expressing GFP only were divided by the corresponding numbers obtained for cells coexpressing Nef and GFP to calculate the values for n-fold regulation with respect to nontransfected control. The flow cytometry data were acquired on a FACScan from Becton Dickinson and analyzed using CellQuest software. The gate settings were determined by the use of isotype controls and corresponded to the levels of expression observed in untransfected cells.
down- or up-modulation, respectively (12, 47). The same ranges of green fluorescence were used in all calculations.

RESULTS

We performed a thorough functional analysis of mutant nef alleles that have been previously investigated in the SIV macaque model (summarized in Table 1) to obtain further information on the relevance of different in vitro Nef functions for efficient viral replication in vivo. The location of most mutations investigated in the Nef molecule is shown in Fig. 1. The major focus was on Nef-mediated modulation of MHC-II and Ii cell surface expression because both activities were described only very recently (47, 50) and were not investigated in previous in vivo studies. Additionally, in many cases the effects of mutations on CD3 and CD28 cell surface expression have also not been reported. Furthermore, utilization of different methods and/or Nef expression vectors complicates the assessment of previous data. Therefore, to extend the previous studies, we subcloned all nef alleles into a bicistronic vector that allows a quantitative FACS analysis and thereby permits direct functional comparison of various nef alleles (12, 19, 47). Notably, CD4, CD3, CD28, and MHC-I cell surface expression on Jurkat T cells and MHC-II and Ii surface expression on HeLa CIITA cells, respectively, were always measured simultaneously from the same transfection to avoid any bias that could result from variation in transfection efficiencies.

Western blot analysis revealed that the 239wt and most mutant Nef proteins were efficiently synthesized in transfected 293T cells, whereas no Nef-specific signal could be detected in cells transfected with the nef-deleted construct expressing only GFP (Fig. 2). Consistent with a previous study (10), only very low amounts of Nef could be detected in cells transfected with the expression vector containing the C8 and 1823ΔC8 nef alleles carrying a 4-amino-acid deletion in the highly conserved core of the Nef protein (Fig. 2).

As expected, the 239wt Nef efficiently modulated cell surface expression of all six receptors investigated (Fig. 3, lane 1). In contrast, no significant changes were observed in cells transfected with a plasmid containing an inactivating point mutation in the ATG initiation codon and premature in-frame stop codons in the nef gene (Fig. 3, lane 2). Consistent with previous findings on HIV-1 Nef function (13), disruption of the N-
terminal myristoylation signal by a G2A mutation impaired all SIVmac Nef functions. Our sensitive assay revealed, however, that the G2A-Nef showed some residual activity in down-modulation of CD4, CD28, and CD3 surface expression (Fig. 3, lane 3). This is not due to overexpression because the G2A-Nef also had some weak effects in SIVmac-infected cells (data not shown) and was expressed at levels comparable to the 239wt Nef (Fig. 2).

The YE-Nef contains a change of R17Q18 to Y17E18, which generates a potential SH2-domain binding site (31) and is associated with extensive T-lymphocyte activation and acute disease in infected macaques (16). The YE-Nef modulated CD4, CD3, CD28, MHC-II, and Ii surface expression as well as 239wt Nef. Unexpectedly, the activity of the YE-Nef in MHC-I down-modulation was moderately reduced (Fig. 3, lane 4). These observations support the previous conclusion that the dramatic enhancement in virulence of the SIVmac YE-Nef variant is due to enhanced T-cell activation (16) rather than an increased activity in other aspects of Nef function.

Mutations in N-proximal tyrosine residues (Y28A and Y39A; YYAA-Nef) and in the central PxxP motif (P104A and P107A; AxxA-Nef) were previously shown not to disrupt major aspects of SIVmac239 Nef function in vivo in infected macaques (11, 29), even though a weak selective pressure for A104Po rA107P reversions was observed in chronically infected animals (26) and the alanine substitution for proline P107 impaired the interaction of Nef with PAK (26, 29). As shown in Fig. 3 (lane 5), the AxxA-Nef efficiently down-modulated CD3, CD28, MHC-I, and MHC-II and up-regulated Ii cell surface expression. The Y28F and Y39F mutations had little disruptive effect on Nef function, except for a moderately reduced activity in MHC-II down-modulation (Fig. 3, lane 6). Concordant with these observations, we found previously that the Y28F and Y39F mutations do not revert in infected rhesus macaques (11). Thus, the N-terminal tyrosine and the central proline residues are not critical for the ability of SIVmac Nef to modulate the surface expression of these six cellular receptors nor for 239Nef function in vivo.

Next, we studied the Y223F Nef mutant, which was previously shown to be defective specifically in MHC-I down-modulation (35) but whose effect on MHC-II expression has not been defined. Notably, the Y223F mutation consistently reverted, and MHC-I down-regulation was fully restored within 4 weeks following experimental infection of rhesus macaques (35). As shown in Fig. 3 (lane 7), we found that the Y223F mutation also does not affect down-modulation of MHC-II or up-regulation of Ii. This evidence indicates that reversions of the Y223F mutation indeed reflect a selective pressure for MHC-I down-modulation in vivo.

In contrast to the Y223F substitution, combined changes of P73E, A74D, and D204R in Nef (EDR-Nef) were reported to disrupt CD4 down-modulation and enhancement of viral replication in vitro but had no significant effect on MHC-I down-modulation (23). The SIVmac239 EDR-Nef variant was attenuated early during infection of rhesus macaques, and subsequent increases in viral load coincided with restorative changes in Nef (23). As shown in Fig. 3 (lane 8), the EDR mutation disrupted up-regulation of Ii and impaired CD28 down-modulation. In contrast, the EDR-Nef was fully active in down-modulating MHC-II and CD3 surface expression. Interestingly, only the D204R mutation reduced the ability of Nef to up-regulate Ii, although both the P73A74/E73D74 and D204R mutations disrupted CD4 and CD28 down-modulation (Fig. 3, lane 8). Concordant with these observations, we found previously that the Y28F and Y39F mutations do not revert in infected rhesus macaques (11). Thus, the N-terminal tyrosine and the central proline residues are not critical for the ability of SIVmac Nef to modulate the surface expression of these six cellular receptors nor for 239Nef function in vivo.
lanes 9 and 10). The peak levels of viral load during acute infection of macaques with the SIVmac EDR-Nef variant were indistinguishable from those seen in animals infected with a nef-deleted virus and about 100-fold lower than those in animals infected with SIVmac239 containing a functional nef (23). Together with the present data, these findings indicate that Nef-mediated modulations of MHC-II, Ii, CD3, and MHC-I surface expression are insufficient for effective SIV replication at least during the early stages of infection and possibly throughout the span of infection.

In contrast to CD4 down-modulation (18, 23, 49), the relevance of the interaction of HIV-1 and SIVmac Nef with PAK-2 for viral pathogenesis is less clear. Mutation of a diarginine motif (R136R137 to L136L137) in SIV Nef was shown to disrupt its association with PAK-2 and to revert in infected macaques (43). Therefore, it was suggested that the interaction of Nef with PAK might be relevant for viral pathogenicity (43). However, an intact RR sequence in Nef was also reported to be critical for down-modulation of CD4 and CD3 (22). We found that substitution of R136R137 to A136A137 (AA-Nef) impairs all in vitro Nef activities investigated, except up-regulation of Ii (Fig. 4). Notably, the tNefs also down-modulated MHC-II, albeit less efficiently than the 239wt Nef (Fig. 4). The Δ153Nef missing amino acid residues 59 to 110 behaved similar to the tNefs (Fig. 4A, lane 3), whereas the Δ183Nef lacking residues 59 to 120 (36) was generally inactive (Fig. 4A, lane 4). The Δ183Nef differs from the Δ153Nef by the additional deletion of amino acid residues 111 to 120. Western blot analysis revealed that both tNefs were expressed at similar levels (Fig. 2; also data not shown), indicating that residues 111 to 120 might be required for both down-modulation of CD3 and up-regulation of Ii. These results show that large regions in the conserved core of the Nef protein are dispensable for modulation of CD3 and Ii cell surface expression and again indicate that these activities are insufficient for a virulent phenotype in infected macaques (36).

Studies in a great number of macaques demonstrated that the SIVmac C8-Nef variant containing a deletion of amino acids 143 to 146 (DMYL) is attenuated in vivo (41). In some animals, however, the deletion was repaired by a 12-bp duplication, and subsequent changes evolved until this region closely resembled the wild-type Nef sequence (4-amino-acid deletion, EKIL→EYIL→DILY) (55). We have shown that the
FIG. 5. Modulation of human cell surface receptors by repaired nef alleles that evolved in macaques infected with the SIVmac C8-Nef variant. (A) Primary FACS data and (B) quantitative analysis of the modulation of cell surface expression levels by the indicated Nef mutant proteins. The experiments were performed and the values were calculated as described in the legend to Fig. 3. The amino acid sequences of the indicated Nef variants and their functional activity in CD4 and MHC-I down-modulation have been previously reported (10). The results were confirmed in an independent experiment.

C8-Nef is unstable and that the initial duplication and subsequent mutations gradually restored CD4 and MHC-I down-modulation as well as enhancement of viral infectivity and replication (10). As shown in Fig. 5A (lanes 1 and 6), the original C8-Nef and a mutant 239Nef (239ΔC8) containing the corresponding deletion were also unable to down-modulate CD3, CD28, and MHC-II. However, the C8- and 239ΔC8-Nefs caused up to 5.8-fold increases in II surface expression (Fig. 5B). One possible explanation is that this function requires only low Nef expression levels (47, 50). The initial 12-bp duplication (4-amino-acid deletion, EKIL) enhanced Nef activity mainly restored Nef functions that likely allow efficient down-modulation of CD28 (Fig. 5B). This is most likely due to a glutamine substitution for histidine H196 (H196Q) (10), since H196 is known to be required for the effect of Nef on CD28 (6). The changes selected in vivo also increased the ability of Nef to down-modulate CD3 (Fig. 5B). However, unlike the 239wt, the EKIL-, EIYL-, and DMYL-Nef variants down-modulated CD3 most effectively at medium expression levels and became less efficient at higher levels (Fig. 5A, lanes 2 to 4). A similar phenotype was reported for some 239Nef deletion mutants (53). Notably, the initial 4-amino-acid deletion EKIL→EIYL changes selected in Mm45R (55) mainly enhanced Nef’s ability to down-modulate MHC-I and -II and to enhance virion infectivity (Fig. 6, upper panel). In contrast, the EIYL→DIYL changes selected later during infection also restored Nef-mediated down-regulation of CD4 and stimulation of viral replication. Together, these findings confirm that a selective pressure for down-modulation of MHC-I (35), CD3 (36), and CD4 (23) exists and further suggest that down-regulation of MHC-II also is advantageous for the virus in vivo.

In addition to the constructed C8-Nef variants containing changes only in the DMYL sequence, we also analyzed nef alleles that emerged in two macaques infected with the SIVmac C8-Nef (10). A representative nef allele obtained from Mm1820 at 42 weeks postinfection (1820EKFL10) carried a 30-bp deletion close to the 3’ end of nef together with a 12-bp duplication (10). The 1820EKFL10 Nef modulated MHC-I and -II and II surface expression but was largely inactive in down-regulating CD4, CD3, or CD28 (Fig. 5A, lane 5). A nef allele derived from Mm1823 at 15 weeks postinfection contained the original deletion and did not show restored function (Fig. 5A, lane 6). In contrast, the 1823DIYL20 Nef obtained 5 weeks later was active in all in vitro assays investigated, except for down-modulation of CD28 (Fig. 5A, lane 7). Notably, restoration of Nef function in Mm1823 was associated with declining CD4+ T-cell counts and progression to simian AIDS (14). In agreement with the observation that only some Nef activities were restored in Mm1820 (Table 1), this animal did not develop severe clinical alterations (14). Interestingly, a representative nef allele obtained from Mm1823 at a later time point (DMYL42-Nef) efficiently down-modulated CD28 (Fig. 5A, lane 8). It was less active than the 1823DMYL20 Nef, however, in down-modulating CD3 and MHC-I (Fig. 5A, lanes 7 and 8). Restoration of CD28 down-modulation coincided with a Q196H reversion, which became predominant in Mm1823 between 20 and 42 weeks postinfection (10). Notably, the initial sequence alterations that emerged in Mm1820 at 42 weeks postinfection contained a selective pressure for down-modulation of MHC-I (35), CD3 (36), and CD4 (23) exists and further suggest that down-regulation of MHC-II also is advantageous for the virus in vivo.

In an earlier study we showed that mutations in the conserved acidic element (Aci-Nef) and in a putative PKC phosphorylation site (PKC-Nef) in 239Nef are important for down-modulation of CD4 and MHC-I and for SIVmac replication in vivo (9). A reexamination of these mutants in additional assays revealed that the Aci-Nef containing a total of eight amino acid substitutions (Fig. 1) was also inactive in CD28 down-regulation but efficiently modulated CD3 and II surface expression.
It was reported that the acidic element mediates HIV-1 Nef binding to the cellular protein PACS-1 and that this interaction is required for down-regulation of MHC-I (8, 38). Our data suggest that the acidic element in SIV Nef has a similar function in down-modulation of MHC-I. It remains to be elucidated, however, why mutations in the acidic element in SIV Nef disrupt CD4 down-modulation more dramatically than those in HIV-1 Nef. Reversion of three of the eight amino acid residues mutated in Aci-Nef (Aci\(\text{NNQQN}\)) enhanced Nef activity in down-modulating MHC-I and MHC-II but not CD4 or CD28 (Fig. 7, lane 2). These changes were observed in Mm7209 at 44 weeks postinfection and coincided with a pronounced increase in viral load (9). The AciQQ-Nef containing reversions at six of the eight mutated positions, which evolved after Mm7209 had developed immunodeficiency, was fully functional in CD4 and CD28 but only moderately active in MHC-I down-regulation (Fig. 7, lane 3). Notably, a high proportion of nef alleles derived from Mm7209 during a late stage of infection contained a deletion of amino acids 60 to 68 (9). The 7209LnAx1 Nef, which was derived from a lymph node biopsy obtained from animal 7209 during necropsy (9) and contains this deletion, was inactive in down-modulating CD4 and MHC-II but active in other assays (Fig. 7, lane 4). This result is in agreement with published data demonstrating that residues 60 to 68 are critical for the effect of SIV Nef on CD4 (30). In contrast to the changes in the acidic region, mutation of the putative PKC phosphorylation site impaired the ability of Nef to modulate all six receptors investigated (Fig. 7, lane 5). This was unexpected because the PKC-Nef was efficiently expressed and enhanced viral infectivity, albeit with reduced efficiency compared to the 239wt Nef (9). A nef allele selected in Mm7065 infected with the SIVmac239 PKC-Nef mutant (9) efficiently modulated CD3, MHC-I, and II surface expression but showed little activity in other in vitro functions, including CD4 down-regulation (Fig. 7, lane 6). These results are further evidence that Nef-mediated modulation of CD3, MHC-I, and II surface expression mediates a selective advantage in vivo but is usually not sufficient for progression to AIDS as Mm7065 remained asymptomatic with low viral loads during 1.5 years of follow-up (9).

**DISCUSSION**

Previous studies of SIVmac239 Nef variants revealed that down-modulation of CD4 (23), MHC-I (35), and CD3 (36) confers a selective advantage for viral replication and persistence in infected macaques. More recently, additional Nef functions were described that have the potential to impair the host immune response to HIV-1 and SIV. Nef down-modulates the CD28 costimulatory factor of T-cell activation (52). Furthermore, it down-regulates mature MHC-II molecules, while up-regulating II associated with immature MHC-II complexes (47, 50). Here we show that these Nef functions are selected for in SIVmac-infected animals. Most changes selected...
in vivo also affected other aspects of Nef function. Nonetheless, our findings are evidence that modulation of CD28, MHC-II, and Ii surface expression provides a selective advantage for primate lentiviruses in vivo. Our data also indicate, however, that Nef-mediated down-modulation of MHC-II and up-regulation of Ii is usually not sufficient for a fully virulent phenotype.

Down-modulation of MHC-II likely reduces the stimulation of CD4+ helper T cells by antigen-presenting cells and might play an important role in the development of disease (50). This Nef function was disrupted by the C8 and Aci mutations in Nef and subsequently was restored in animals infected with the C8- and Aci-Nef variants (summarized in Table 1). Furthermore, the tNefs selected in macaques infected with SIVmac variants containing a 152-bp deletion in nef also showed some activity in MHC-II down-modulation. However, the changes selected in vivo in tNefs also increased MHC-I down-modulation and/or enhancement of viral infectivity. Furthermore, N-terminal Y28Y39 mutations did not revert in infected macaques (9), although they reduced the ability of Nef to down-modulate MHC-II (Fig. 3, lane 6). The effects of Nef on MHC-II surface levels on SIV- or HIV-1-infected cells are relatively weak (47, 50). Thus, the exact contribution of this Nef function to the efficiency of viral persistence in vivo remains to be clarified.

Efficient up-regulation of Ii surface expression is readily detectable even at low levels of Nef expression (47, 50). Stable surface expression of Ii prevents peptide presentation (39) and might contribute to the impaired helper T-cell responses observed in AIDS patients (40). We found that tNefs missing parts of the conserved core region efficiently up-regulated Ii (Fig. 4). However, they were inactive in all other aspects of Nef function, except CD3 down-regulation and a weak effect on MHC-II surface expression (Table 1). Our results constitute evidence that Ii up-regulation is advantageous for viral replication in infected macaques. Notably, this is consistent with the observation that nef alleles derived from some nonprogressors of HIV-1 infection were found not to up-regulate Ii (47). On the other hand, the attenuated phenotype of the SIVmac tNef.1 variant in infected macaques (36) implies that modulation of CD3 and Ii surface expression alone is insufficient for efficient viral replication in vivo. This conclusion is supported by the evidence that the EDR-Nef, which modulates CD3, MHC-I, MHC-II, and Ii surface expression, did not enhance SIV replication early during infection (23). It should be noted, however, that the tNefs were derived from animals with progressive disease (44). Thus, Nef-mediated modulation of CD3 and Ii surface expression might be sufficient for a virulent phenotype if additional changes elsewhere in the viral genome are present or if these Nef variants emerge in lymphatic tissues at the sites of robust viral replication.

Down-modulation of CD4 and CD28 require similar regions in Nef (52), and changes selected in animals infected with the SIVmac EDR-Nef variant restored both activities (23). Residue H196 in SIVmac Nef, however, is required for down-modulation of CD28 but not for other Nef functions (Fig. 5A, lane 4) (6). Changes of Q196H that restored CD28 down-modulation were observed in several monkeys infected with the SIVmac C8-Nef variant (10, 55), suggesting that this function provides a selective advantage in vivo. It remains, however, why the H196Q substitution consistently reverted between 15 and 25 weeks postinfection and thus more slowly than other inactivating point mutations in nef. One possible explanation is that the selective advantage of CD28 down-modulation in infected macaques is moderate because several other Nef functions also affect T-cell receptor-initiated activation of MHC-II restricted T cells.

Our analysis considerably extends the results of previous studies investigating the roles of specific Nef functions for viral pathogenicity. Of the constructed Nef mutants investigated, only the YE-Nef showed 239wt-like activity in all assays, except for a moderately decreased ability to down-regulate MHC-I (Table 1). These results confirm that the acute pathogenicity of the SIVmac YE-Nef variant (16) is associated with its ability to cause extensive T-lymphocyte activation and does not involve increased activity in other Nef functions. Our data also suggest that reversion of the Y17R mutation in Nef observed in animals that survive the acute phase of infection (48) could reflect selective pressure for efficient down-modulation of MHC-I to escape antiviral cytotoxic T-lymphocyte responses. It was also confirmed (35) that the F223Y reversion in Nef exclusively restores down-modulation of MHC-I. Consistent with findings in HIV-1-infected individuals (12), these results suggest that MHC-I down-modulation is relevant in vivo. Studies with SIVmac Nef mutants containing difficult-to-revert mutations that exclusively disrupt MHC-I down-modulation (51) will be of great importance to elucidate the relevance of this Nef function for viral replication and AIDS pathogenesis.

Mutations in the SIVmac Nef PxxP motif only impaired its ability to stimulate viral replication in vitro and to interact with PAK (26, 29) (Table 1), whereas the analogous mutation in HIV-1 Nef impairs down-modulation of MHC-I, MHC-II, and CD28 (19) (data not shown). Thus, the PxxP element in SIV Nef clearly is less important than that in HIV-1 Nef. Previous studies suggested that reversion of A104P or A140P confers some selective advantage in chronically infected but not in
rapidly progressing animals (26, 29). The reduced replication efficiency of the SIVmac AxxA-Nef variant in rhesus peripheral blood mononuclear cell (29) cultures suggested that the AxxA-Nef might be less active than 239wt Nef only in causing T-cell activation. Revertants might have some selective advantage in chronically infected animals, in which most target cells are in a resting stage, but not in rapid progressors showing high levels of T-cell activation. Notably, a weak selective pressure exists for both P<0.07 which restores PAK association, and P<0.10 which does not (26, 29). Therefore, it remains to be clarified whether the interaction of Nef with PAK is important for its ability to stimulate viral replication.

An important question is whether the observations made in the SIV macaque model can be extended to humans infected with HIV-1. Notably, most in vitro Nef activities are conserved among HIV-1 and SIV Nef. Studies in HIV-1-infected individuals also indicated that multiple Nef functions, including down-modulation of CD4 and MHC-I as well as enhancement of viral infectivity and replication, all contribute to the pathogenesis of AIDS (12, 34, 54). Furthermore, expression of the HIV-1 Nef increases the pathogenicity of SIVmac in infected rhesus macaques, albeit with lower efficiency than the original 239wt Nef (3, 28, 32). However, in contrast to SIVmac Nef, the HIV-1 Nef does not down-modulate CD3 (5, 22), and the effects on CD28 surface expression are weak (6, 52). These functional differences suggest that SIVmac might affect T-cell activation through a T-cell receptor-dependent mechanism more severely than HIV-1.

Previous studies provided evidence that different Nef functions are preferentially selected for depending on the particular clinical stage of infection. In immunocompetent hosts, Nef activities were selected that allow the virus to efficiently escape the cytotoxic T-lymphocyte response, whereas the changes that predominated later in infection enhanced Nef activities, enhancing viral spread more directly (12, 37). The results of this study support these findings. For example, changes initially selected in monkeys infected with the SIVmac C8- or Aci-Nef variants predominantly enhanced Nef functions likely allowing immune evasion, such as modulation of MHC-I, MHC-II, and Ii surface expression (Fig. 6; Table 1). In comparison, CD4 down-modulation and enhancement of viral replication was restored later during infection.

Different in vitro Nef functions were restored in infected animals in various combinations, for example, CD3 and Ii (Δ152Nef versus tNefs); CD4 and CD28 (AciNNQQN versus AciQQ; EDR versus 239wt); CD3, MHC-I, and Ii (PKC versus PKC-7065); CD4, CD3, and MHC-II (EKIL versus EYIL); and MHC-I, MHC-II, and Ii (C8-Nef versus 1820EKKFLA10). Other changes emerging in vivo were more selective for individual Nef functions: MHC-I (Y223F versus 239wt); MHC-II (Acic versus AciNNQQN). Taken together, these results imply that a combination of different Nef functions contribute to efficient viral replication in vivo. However, our findings also indicate that several combinations of in vitro Nef functions (e.g., CD3 and Ii [tNefs]; CD3, MHC-I, and Ii [PKC-7065]; or CD3, MHC-I, MHC-II, and Ii [EDR-Nef]) are insufficient for efficient viral replication in infected macaques (10, 23, 36). Dissecting the relative contribution of these different Nef functions for the virulence of SIV and HIV-1 will be a major challenge because such an analysis requires mutations in Nef that are both highly selective and difficult to revert. Furthermore, the relative importance of specific Nef functions might differ between individual hosts. Nevertheless, such studies will provide important new insights into AIDS pathogenesis and into the complex mechanisms that HIV-1 and SIV have evolved to manipulate both helper T cells and antigen-presenting cells.

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