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Importance of the N-Distal AP-2 Binding Element in Nef for Simian Immunodeficiency Virus Replication and Pathogenicity in Rhesus Macaques

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Point mutations in SIVmac239 Nef disrupting CD4 downmodulation and enhancement of virion infectivity attenuate viral replication in acutely infected rhesus macaques, but changes selected later in infection fully restore Nef function (A. J. Iafrate et al., J. Virol. 74:9836–9844, 2000). To further evaluate the relevance of these Nef functions for viral persistence and disease progression, we analyzed an SIVmac239 Nef mutant containing a deletion of amino acids Q64 to N67 (Δ64-67Nef). This mutation inactivates the N-distal AP-2 clathrin adaptor binding element and disrupts the abilities of Nef to downregulate CD4, CD28 and CXCR4 and to stimulate viral replication in vitro. However, it does not impair the downmodulation of CD3 and class I major histocompatibility complex (MHC-I) or MHC-II and the upregulation of the MHC-II-associated invariant chain, and it has only a moderate effect on the enhancement of virion infectivity. Replication of the Δ64-67Nef variant in acutely infected macaques was intermediate between grossly *nef*-deleted and wild-type SIVmac239. Subsequently, three of six macaques developed moderate to high viral loads and developed disease, whereas the remaining animals efficiently controlled SIV replication and showed a more attenuated clinical course of infection. Sequence analysis revealed that the deletion in *nef* was not repaired in any of these animals. However, some changes that slightly enhanced the ability of Nef to downmodulate CD4 and moderately increased Nef-mediated enhancement of viral replication and infectivity in vitro were observed in macaques developing high viral loads. Our results imply that both the Nef functions that were disrupted by the Δ64-67 mutation and the activities that remained intact contribute to viral pathogenicity.

The *nef* gene is conserved among all human and simian immunodeficiency viruses (HIV and SIV, respectively). Nef is dispensable for efficient viral replication in many cell culture systems but critical for the full pathogenic potential of primate lentiviruses (4, 20, 36, 37). Several Nef activities that likely contribute to efficient viral spread and disease progression are conserved among HIV type 1 (HIV-1) and SIVmac. These include downmodulation of CD4, CD28, and class I and II major histocompatibility complexes (MHC-I and -II, respectively); upregulation of the invariant chain (Ii) associated with immature MHC-II molecules; enhancement of virion infectivity; and stimulatory effects on virus replication in primary T cells (1–3, 5, 8, 14, 18, 24–26, 28, 30, 40, 41, 44, 46, 55, 56, 59, 63). In contrast to HIV-1, the SIVmac Nef also efficiently downmodulates CD3 surface expression (7, 31). Studies with SIVmac Nef mutants selectively altered in some functional aspects indicate that most or all of these activities mediate some selective advantage in vivo and might contribute to viral pathogenesis (22, 34, 47, 48, 54, 61). However, their relative

contributions to the virulence of SIVmac and HIV-1 remained largely elusive.

Downmodulation of CD4, the primary receptor of HIV and SIV, is one of the best established Nef functions. The viral Env protein and the accessory HIV-1 Vpu protein also participate in CD4 downmodulation, suggesting that this function is critical for efficient viral spread in the infected host (reviewed in references 15, 21, 39, and 66). The mechanism of Nef-mediated CD4 downmodulation has been extensively studied. Nef induces CD4 endocytosis via the AP-2 clathrin adaptor pathway, and this probably involves the formation of ternary complexes comprising Nef, CD4, and AP-2 (29, 50), analogous to the previously reported CD3-Nef-AP-2 complex (62). The internalized CD4 is degraded in an acidic compartment (23, 29, 50). The physiological relevance of CD4 downmodulation is less clear, but it has been suggested that this activity might prevent superinfection, facilitate virion release, increase Env incorporation into virions, and impair T-cell receptor function (6, 9, 17, 38, 52).

Several lines of evidence indicate that CD4 downmodulation is important for viral pathogenesis. Nef alleles derived from some HIV-1-infected long-term nonprogressors are defective in this function (12, 45, 65). In contrast, HIV-1 *nef* alleles obtained during late stages of disease progression show enhanced activity (13). It has also been shown that CD4 down-

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modulation by HIV-1 Nef correlates with the efficiency of HIV-1 replication in primary T lymphocytes (43) and with viral replication and CD4$^+$ T-cell depletion in human lymphoid tissue ex vivo (27). We have previously analyzed an SIVmac239 variant containing three amino acid substitutions in Nef disrupting the downregulation of CD4 and enhancement of viral infectivity and replication but not the downmodulation of CD3, MHC-I, and MHC-II and the upregulation of Ii (34). These mutations attenuated SIV replication during acute infection. Thereafter, however, viruses containing reversions and compensatory changes in Nef emerged. Notably, restoration of the disrupted Nef functions correlated with increasing viral loads and disease progression. Thus, these results showed that the ability of Nef to downmodulate CD4 and/or to enhance viral infectivity is critical for efficient replication during acute infection but did not provide information on the relevance of these Nef activities to viral persistence and the clinical outcome of infection.

To further evaluate the relevance of CD4 downmodulation to viral replication and pathogenesis, we investigated an SIVmac239 Nef variant containing a difficult-to-revert deletion of amino acids Q64 to N67 (Δ64-67Nef). It has been previously shown that these residues are involved in the interaction of Nef with AP-2 clathrin adaptor proteins and critical for the efficient downregulation of CD4, CD28, and CXCR4 and the stimulation of viral replication but not for other Nef functions (32, 42, 63). Here we demonstrate that the Δ64-67Nef virus shows pathogenic properties intermediate between wild-type SIVmac239 and forms containing grossly defective *nef* genes. In conclusion, Nef-mediated CD4 downregulation and enhanced replication are important in vivo, but other functions also contribute to the virulence of SIVmac.

MATERIALS AND METHODS

Plasmids and proviral constructs. Generation of bicistronic pCGCG expression vectors coexpressing the green fluorescent protein (GFP) alone or in conjunction with the wild-type SIVmac239 (239wt) or Δ64-67Nef has been described previously (42, 63). The proviral 239wt clone, the 239$_{(EDR)}$-Nef mutant, and ΔNU, containing deletions of 515 bp in the *nef*-long terminal repeat region, have been previously described (34, 36).

Cells, virus stocks, and infectivity assays. HeLa CIITA, P4-CCR5, 293T, and Jurkat cells were cultured as described previously (47, 49, 53). For virus production, 293T cells were transfected by the calcium phosphate method (19) with 10 μg of the proviral constructs. The medium was changed after overnight incubation, and virus was harvested 24 h later. The p27 antigen concentrations were quantified by using an SIV enzyme-linked immunosorbent assay provided by the NIH AIDS Reagent Program. Cells were infected with virus stocks containing normalized quantities of p27 antigen, and virus production was measured by using a reverse transcriptase assay at 2- to 3-day intervals (51). Rhesus blood mononuclear cells (rPBMC) were isolated as described previously (40), and immediately infected with aliquots of the virus stocks containing 1 ng of p27 and kept in RPMI 1640 with 10% fetal calf serum (FCS). Residual virus was removed by washing the cells 16 to 18 h after infection. At 3 days after infection cells were stimulated with phytohemagglutinin (2 μg/ml; Sigma) for 3 days, washed, and maintained in RPMI 1640 with 20% FCS and 100 U of interleukin-2 (IL-2)/ml. The rhesus T-cell line 221-89 (3) was maintained in the presence of 100 U of IL-2/ml (Boehringer, Heidelberg, Germany) and 20% FCS. Infections were performed in the absence of exogenous IL-2 or in the presence of 50 U of IL-2/ml and 5% FCS. In the Nef *trans*-complementation assay 293T cells were cotransfected with 4 μg of a *nef*-defective SIVmac239 proviral construct and 3 μg of a bicistronic pCGCG vector expressing Nef. Virus was harvested 3 days posttransduction and used to infect P4-CCR5 or TZM-bl indicator cells as described previously (49).

Flow cytometry. Jurkat T cells were either transfected by using the DMRIE-C reagent (Gibco-BRL) for subsequent detection of CD3, CD4, MHC-I, and
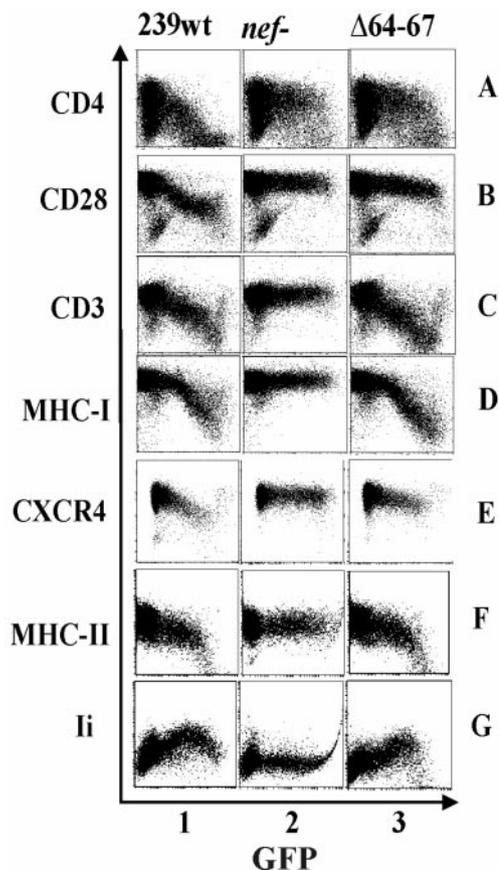

FIG. 1. Modulation of human cell surface receptors by the SIVmac239 Δ64-67 Nef. Human CD4$^+$ Jurkat T cells (A to E) or HeLa CIITA cells (F and G) were transiently transfected with plasmids expressing GFP alone (*nef−*) or together with the 239wt and Δ64-67 Nef alleles. The surface expression of CD4 (A), MHC-I (B), CD3 (C), CD28 (D), CXCR4 (E), MHC-II (F), and Ii (G) was measured as described in Materials and Methods. The results were confirmed in independent experiments.

CD28 or electroporated as described previously (32, 33) to analyze the effect of Nef on CXCR4 surface expression. Efficient transfection with the DMRIE-C reagent involves stimulation with phytohemagglutinin, which leads to CXCR4 downmodulation. Therefore, Jurkat cells were transfected by using two different methods. HeLa CIITA cells were transfected with Metafectene (Biontex) according to manufacturer's instructions. Expression of CD3, CD4, MHC-I, CD28, and CXCR4 on Jurkat T cells and MHC-II or Ii surface expression on HeLa CIITA cells transfected with bicistronic vector coexpressing Nef and GFP were measured as described previously (32, 53, 54). Quantification of Nef-mediated down- or upregulation of the respective surface markers was performed as described elsewhere (13, 53, 54). Briefly, the mean channel numbers of red fluorescence were determined for cells expressing no (N), low (L), medium (M), or high (H) levels of GFP. The mean channel numbers of red fluorescence obtained for cells transfected with the *nef* construct expressing GFP only were divided by the corresponding numbers obtained for cells coexpressing Nef and GFP to calculate the values for X-fold down- or upmodulation, respectively.

Infection of rhesus macaques. Six juvenile rhesus macaques of Indian origin were infected by intravenous inoculation of SIVmac239 Δ64-67Nef containing 10 ng of p27 produced by transfected 293T cells. Animals were housed at the German Primate Center in Gottingen in accordance with the institutional guidelines. The macaques were healthy and seronegative for SIV, D-type retroviruses, and simian T-cell leukemia virus type 1 at the time of infection. Sera and cells were collected at regular intervals, and serological, virological, and immunological analysis was performed as described previously (57, 58, 64).
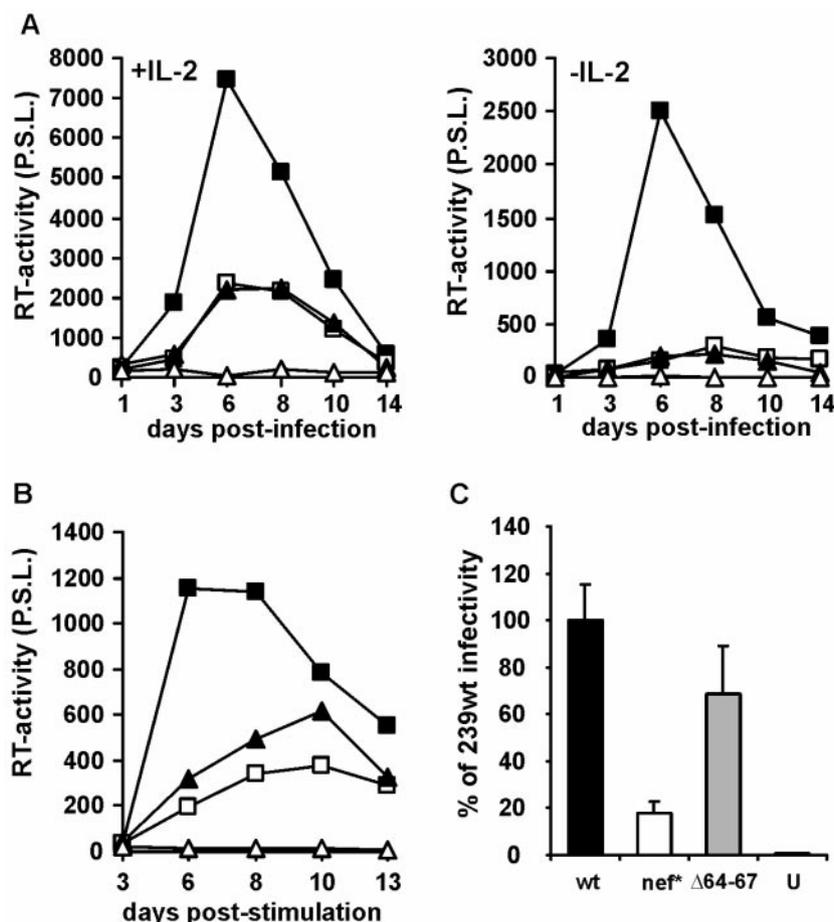
FIG. 2. Deletion of residues 64 to 67 impairs the ability of Nef to stimulate SIV replication but not to enhance the infectivity of progeny virions. (A) Replication of SIVmac239 containing wild type (■), Δ64-67 (▲), or a disrupted *nef* gene (□) in 221-89 cells in the presence (left) or absence (right) of exogenous IL-2 (100 U/ml). (B) Replication of SIVmac239 *nef* variants in rhesus PBMC infected immediately after isolation and stimulated 3 days later. PSL, photon-stimulated light emission. Similar results were obtained in two independent experiments using different virus stocks. △, Uninfected cells. (C) P4-CCR5 cells were infected in triplicate with aliquots of three different 293T cell-derived virus stocks containing 100 ng of p27 core antigen. Infectivity is shown relative to the 239wt virus. U, uninfected cells.

Nef amplification and sequence analysis. Viral plasma RNA was isolated with the QIAamp RNA Kit (Diagen, Basel, Switzerland) and reverse transcribed with superscript reverse transcriptase (Gibco-BRL, Eggenstein, Germany). SIV sequences spanning the entire *nef* gene were amplified from cDNA with a nested PCR approach essentially as described previously (34). All *nef* alleles were first cloned into the bicistronic CMV-based pCGCG vector coexpressing GFP and Nef using XbaI and MluI restriction sites flanking *nef*. Subsequently, some *nef* alleles were introduced into the proviral SIVmac239 genome by splice overlap extension PCR as described previously (34, 48). All PCR-derived inserts were sequenced on both strands to confirm that the constructs expressed the correct Nef variants.

Western blot. 293T cells were transfected with 5 μg of pCGCG expression vectors (42) coexpressing GFP and Nef using the calcium phosphate method as described previously (11, 54). At two days posttransfection cells were pelleted, and lysates were generated and separated by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis as described previously (11, 54). Expression of Nef and p27 in whole cellular lysates was analyzed by immunoblotting. Proteins were detected with a 1:2,500 dilution of pooled sera from SIVmac239-infected rhesus macaques and a 1:5,000 dilution of an alkaline phosphatase-conjugated secondary anti-human immunoglobulin G antibody (Jackson Immunoresearch) as described by the manufacturer.

GenBank accession numbers. SIVmac239 *nef* sequences derived from macaques infected with the Δ64-67Nef variant were submitted to the GenBank sequence database and have been assigned the accession numbers DQ411004 to DQ411025.

RESULTS

Functional characterization of the SIVmac239 Δ64-67Nef. Prior to animal studies, we confirmed and extended the in vitro characterization of the Δ64-67Nef. In agreement with previous reports (32, 42, 63), we found it to be defective in downregulation of CD4, CD28, and CXCR4 cell surface expression (Fig. 1A, B, and E). Notably, deletion of residues 64 to 67 did not affect the activity of Nef in downmodulating CD3 and class I or II MHC (Fig. 1C, D, and F). The Δ64-67Nef also upregulated Ii surface expression albeit with moderately reduced efficiency compared to 239wt Nef (Fig. 1G). Thus, the Δ64-67 mutation, which removes the N-distal AP-2 binding element (42, 63), disrupted the effect of SIVmac239 Nef on three of the seven cell surface receptors under study.

Next, we introduced the Δ64-67Nef into the full-length SIVmac239 provirus and investigated its effect on viral replication and infectivity. Infection of the rhesus macaque T-cell line 221-89 presents a useful system for investigating the ability of Nef to cause lymphocyte activation (3). As shown in Fig. 2A, the Δ64-67Nef was unable to enhance SIVmac239 replication
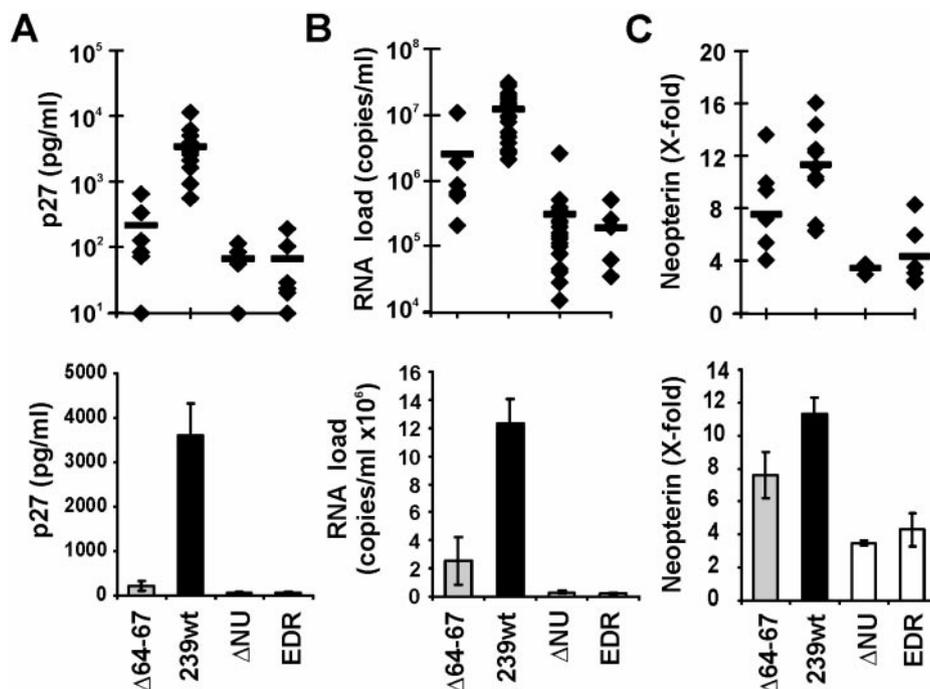
FIG. 3. Replication of SIVmac239 containing the Δ64-67 Nef variant early during infection. The peak plasma levels of p27 and viral RNA in six acutely infected rhesus macaques, which were observed at 2 weeks postinfection, are shown. (A) Levels of p27 plasma antigenemia. The limit of detection is approximately 10 pg/ml. (B) Viral RNA load. (C) Levels of neopterin, a marker for nonspecific immune activation (58). For comparison, values obtained from animals infected with 239wt, the grossly *nef*-defective ΔNU virus, and from six macaques inoculated with the $239_{(EDR)}$Nef mutant (34) are also indicated. The upper panels give individual values, and the lower panels indicate the average values ± the standard deviation. U, uninfected cells.

in 221-89 cells both in the absence and in the presence of exogenous IL-2. Compared to 239wt the Δ64-67Nef mutant virus also replicated with delayed kinetics and reduced efficiency in rPBMC that were infected immediately after isolation and stimulated 3 days later (Fig. 2B). In most experiments, however, the Δ64-67Nef variant replicated slightly better than the 239*nef** containing a disrupted *nef* open reading frame (ORF), indicating that it maintains some residual activity in this assay. Consistent with the published data (42), the Δ64-67Nef enhanced virion infectivity, albeit with moderately reduced efficiency (Fig. 2C). These results are in agreement with previous observations with HIV-1 *nef*, suggesting that Nef-mediated CD4 downregulation usually correlates with the efficiency of viral replication in vitro but not with enhancement of virion infectivity (27, 40, 43). Together, our in vitro data demonstrate that the Δ64-67Nef does not efficiently down-modulate CD4, CD28, and CXCR4 or stimulate viral replication in vitro but is active in modulating MHC-I, MHC-II, Ii, and CD3 surface expression and enhancing virion infectivity.

The Δ64-67Nef attenuates SIV replication during acute infection. Six rhesus macaques were intravenously infected with SIVmac239 containing the Δ64-67Nef to evaluate the effect of this deletion on viral replication and pathogenesis in vivo. As expected from previous studies (34, 36, 40), peak levels of plasma viremia and of viral RNA were observed at 2 weeks postinfection (wpi). During acute infection, the average levels of p27 plasma antigenemia in the animals that received the Δ64-67Nef mutant (219 ± 101 pg/ml, $n = 6$) were ~16-fold lower than those detected in 239wt-infected macaques (3,612 ±

708, $n = 11$) (Fig. 3A). However, they were threefold higher than those observed in grossly *nef*-deleted 239ΔNU-infected macaques (68 ± 23, $n = 4$) and in six animals that received the $239_{(EDR)}$-Nef variant (65 ± 30, $n = 6$) (34). In comparison, the peak levels of viral RNA ($2.5 \times 10^6 \pm 4.2 \times 10^6$) in Δ64-67Nef infection were only 4.8-fold lower than those observed in the 239wt infection ($1.2 \times 10^7 \pm 1.8 \times 10^6$, $n = 22$; $P = 0.01$) but 8.5-fold higher than those observed in macaques inoculated with the ΔNU variant ($2.9 \times 10^5 \pm 1.2 \times 10^5$, $n = 22$; $P = 0.02$) (Fig. 3B). On average, the levels of RNA were also 13.3-fold higher than those previously observed in macaques infected with the $239_{(EDR)}$-Nef variant ($1.9 \times 10^5 \pm 7.7 \times 10^4$, $n = 6$). Consistent with the intermediate levels of SIVmac239 Δ64-67 replication, the urinary neopterin levels, a marker for nonspecific immune stimulation (58), were also higher than in $239_{(EDR)}$-Nef and ΔNU-infected animals but lower than in 239wt infection (Fig. 3C). These results demonstrate that the deletion of residues 64 to 67 in Nef attenuates SIVmac239 replication early in infection. The disruptive effects were less severe, however, than those of large deletions in ΔNU Nef or the point mutations present in the $239_{(EDR)}$-Nef variant (Fig. 3) (34).

Reduced virulence of SIVmac239 Δ64-67Nef during later stages of infection. After the acute phase of infection, the viral loads decrease in the great majority of animals inoculated with *nef*-deleted SIVmac239 but usually remain high in animals infected with 239wt (36). In contrast, the outcome of infection in the animals infected with the Δ64-67Nef variant varied. Three of the six animals—Mm9024, Mm10026, and Mm10027—maintained intermediate to high levels of viral RNA and cell-associ-
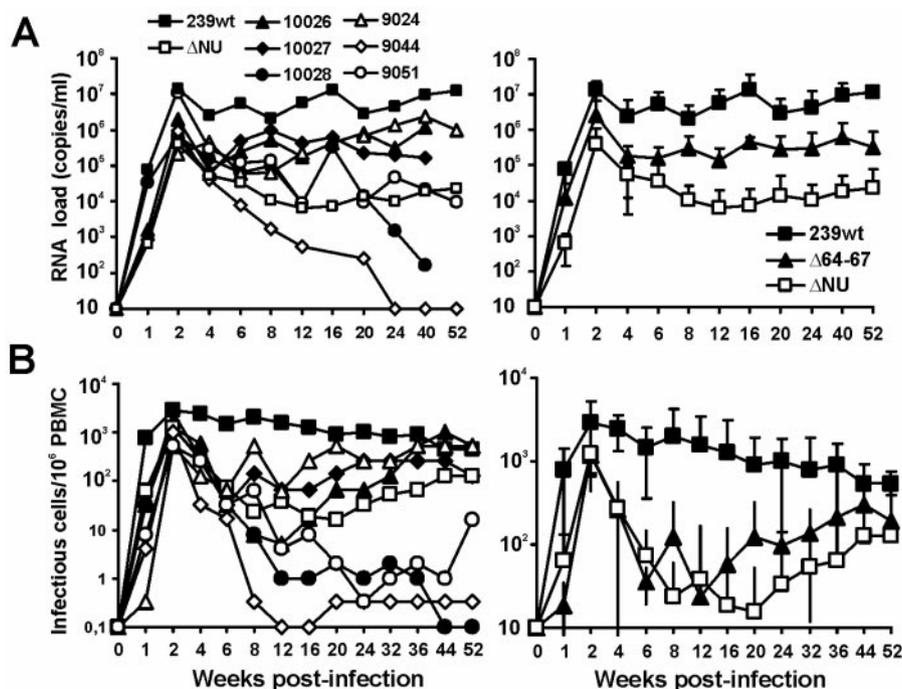
FIG. 4. Persistence of the SIVmac239 Δ64-67 Nef variant in rhesus macaques. (A) Viral RNA load and (B) number of infectious cells per 1 million PBMC. The right panels show average values ± the standard deviations. For comparison, values obtained from macaques infected with SIVmac239 ΔNU (□) or wild-type SIVmac239 (■) are indicated. Parameters were determined as described in Materials and Methods.

ated viral loads (Fig. 4, left panels). The remaining macaques—Mm9044, Mm9051, and Mm10028—developed low RNA loads, similarly to attenuated ΔNU infection (Fig. 4A, left panel). Furthermore, the cell-associated viral loads were 2 to 3 orders of magnitude lower than those usually observed in 239wt infection (Fig. 4B, left panel). Between 12 and 52 wpi, the average RNA loads in the six animals that were inoculated with the Δ64-67Nef mutant ($3.5 \times 10^5 \pm 1.6 \times 10^5$) were 27-fold higher compared to ΔNU infection ($1.3 \times 10^4 \pm 6.7 \times 10^3$; $P = 0.0004$) but 22-fold lower than those in 239wt-infected macaques ($7.9 \times 10^6 \pm 4.2 \times 10^6$; $P = 0.0015$) (Fig. 4A, right panel). Typically, the cell-associated viral loads declined only marginally after the acute phase of 239wt infection but dropped dramatically in animals that received the ΔNU or Δ64-67Nef variants (Fig. 4B, left panel). However, 3 of 6 Δ64-67Nef and 3 of 15 ΔNU-infected macaques showed increasing cell-associated viral loads after 12 and 20 wpi, respectively (Fig. 4B and data not shown). In contrast, the average cell-associated virus loads in 239wt-infected animals declined slowly during later stages of 239wt infection due to the death of several rapid progressors showing high levels of replication during the investigation period. On average, the number of infectious cells increased earlier in Δ64-67Nef-infected animals compared to ΔNU infection (Fig. 4B, right panel). It is noteworthy that the cell-associated viral loads in Δ64-67Nef-infected macaques were reduced more severely than the viral RNA loads. Consequently, the plasma RNA copy numbers per infectious cell detected in the periphery of both Δ64-67Nef (3,273 ± 693)- and 239wt (5,899 ± 2,111)-infected macaques were significantly higher than those detected in ΔNU infection (352 ± 70; $P = 0.0006$ and 0.013, respectively; the numbers indicate the average number of RNA

copies per infectious cell ± the standard error of the mean [SEM]).

Consistent with the variable efficiency of viral replication, the six animals infected with the Δ64-67Nef variant showed differential clinical courses of infection. The two animals with the highest viral loads, Mm9024 and Mm10026, showed declining CD4^{+} T-cell numbers after 16 wpi and developed a moderate lymphadenopathy by 4 wpi. Mm9024 also developed increasing thrombocytopenia after 6 wpi and persistent splenomegaly by 16 wpi and had to be euthanized by 69 wpi because of diarrhea and severe immunodeficiency. Histopathological examination revealed moderate to severe follicular hyperplasia with progression to depletion of multiple lymphatic organs and a chronically active gastroenteritis induced by opportunistic infections (*Giardia*). Mm10026 also became immunodeficient, and autopsy at 53 wpi revealed a moderate chronically active gastroenteritis partially induced by parasites, SIV-induced arteriopathy of the respiratory tract, and severe generalized lymphatic hyperplasia with the beginning of a pleomorphic malignant lymphoma in some lymph nodes and spleen. The third animal that showed relatively high viral loads after infection with the Δ64-67Nef variant, Mm10027, developed a persistent mild lymphadenopathy from 8 wpi and a mild anemia and thrombocytopenia after 28 wpi. Histological examination at 53 wpi revealed mild generalized follicular hyperplasia in lymphatic organs. Consistent with the low viral loads detected in Mm9044, this animal remained healthy with stable CD4 counts throughout the observation period and was euthanized at 75 wpi. Postmortem examination revealed no clinical abnormalities except a mild hyperplasia in lymphatic tissues.

TABLE 1. Viral load and changes in *nef* in macaques infected with the Δ64-67Nef variant

Animal[a]	wpi[b]	Viral load[c]		*nef* sequence analysis[d]			
		RNA copies	Cell associated	No. of *nef* alleles analyzed	No. of ns/*nef*	No. of aas/Nef	No. of non-synonymous changes (%)
Mm9024	24	1.3×10^6	256	5 (0)	8.0	7.6	95
	40	2.3×10^6	512	4 (0)	14.8	14.5	98
	Ln69		128	5 (1)	9.8	9.4	96
Mm9044	24	b.d.l.	0.33	4 (1)	1.8	1.3	71
	40	b.d.l.	<0.33	5 (1)	1.2	1.2	100
Mm9051	24	4.5×10^4	0.33	5 (0)	6.6	5.6	85
	40	2.1×10^4	1	4 (0)	1.3	1.3	100
Mm10026	24	3.1×10^5	67	5 (0)	12.6	11.2	89
	40	1.2×10^6	512	11 (0)	8.5	7.7	91
	Ln53		512	6 (1)	10.3	9.3	90
Mm10027	24	2.0×10^5	256	5 (0)	10.2	8.0	78
	40	1.6×10^5	256	6 (0)	6.2	5.5	89
	Ln53		128	4 (0)	4.8	4.3	89
Mm10028	24	1.5×10^3	1	3 (2)	5.0	4.3	87
	40	1.6×10^2	1	7 (8)	5.3	3.9	74
	Ln54		0.1	5 (0)	1.4	1.2	86
Mm-M		$9.1 \times 10^5 \pm 8.5 \times 10^5$	310 ± 173	36 (0)	10.1 ± 3.2	9.1 ± 3.2	91
Mm-L		$1.1 \times 10^4 \pm 1.8 \times 10^4$	0.7 ± 0.4	28 (12)	3.5 ± 2.4	2.9 ± 1.9	75

[a] Mm-M refers to the three animals with medium (Mm9024, Mm10026, and Mm10027), and Mm-L are animals with low viral loads (Mm9044, Mm9051, and Mm10028).
[b] Parameters were determined at the indicated weeks postinfection (wpi), except the cell-associated viral loads for Mm10026, Mm10027, and Mm10028, which were obtained at 36 wpi. Ln, lymph node.
[c] Viral loads were measured as described in the legend to Fig. 4. b.d.l., below detection limit (40 copies/ml).
[d] Numbers of intact and defective (in parentheses) *nef* genes per sample analyzed. The average frequency of nucleotide and amino acid changes (ns and aas, respectively) were only calculated for full-length *nef* ORFs.

Mm9051 also did not show signs of immunodeficiency, except for a mild lymphadenopathy occurring between 4 and 48 wpi. Autopsy was performed at 75 wpi and revealed a moderate generalized follicular hyperplasia in lymphatic tissues and generalized lymphatic activation but no opportunistic infections. Despite low levels of viral replication (Fig. 4) Mm10028 showed a decline in the number of CD4+ T cells after 36 wpi and developed a persistent anemia by 20 wpi, which was severe at necropsy at 54 wpi. Histopathological examination revealed a moderate to severe chronic active gastroenteritis and a moderate interstitial pneumonia.

In summary, all six animals infected with the Δ64-67Nef variant became slow progressors and survived at least 1 year after infection, whereas ca. 70% of the SIVmac 239-infected rhesus macaques developed severe immunodeficiency and died within this time period. However, two animals (Mm9024 and Mm10026) with high viral loads developed AIDS. In contrast, Mm9044 and Mm9051 with low viral loads remained clinically healthy during the 75-week observation period. The remaining two macaques, Mm10027 with intermediate viral loads and Mm10028 with low viral loads, showed signs of progressive infection but did not develop AIDS for about 1 year of follow-up. Thus, both the replicative capacity and the pathogenetic properties of the Δ64-67Nef variant were intermediate between 239wt and ΔNU.

Nef sequence alterations selected in vivo. Three of the six animals infected with the Δ64-67Nef variant (Mm9024, Mm10026, and Mm10027, [designated Mm-M for moderate]) developed viral loads that were moderately reduced compared to the pathogenic SIVmac 239wt, whereas the remaining three macaques (Mm9044, Mm9051, and Mm10028 [designated Mm-L for low]) showed more attenuated levels of viral replication resembling grossly *nef*-deleted ΔNU infection (Table 1 and Fig. 4). It is known that small deletions in *nef* can be "repaired" and function can be restored during the course of SIV infection (11, 67). Therefore, we next investigated whether the different levels of viral replication observed in the six animals infected with the Δ64-67Nef variant correlate with reversions or second-site compensatory changes in Nef. Sequence analysis of PCR fragments amplified from plasma viral RNA at sequential time points revealed that the Δ64-67 deletion in *nef* was not repaired in any of the six animals (Fig. 5). However, as summarized in Table 1, the frequency of nucleotide changes and deduced amino acid substitutions was ~3-fold higher in the three animals with high viral loads. Although this may be the consequence rather than the cause of efficient replication, it is noteworthy that 91% of the nucleotide changes in Mm-M but only 75% in Mm-L were nonsynonymous. Moreover, all 36 *nef* ORFs derived from Mm-M at 24 or 40 wpi predicted full-length proteins, whereas 12 of the 40 sequences derived from
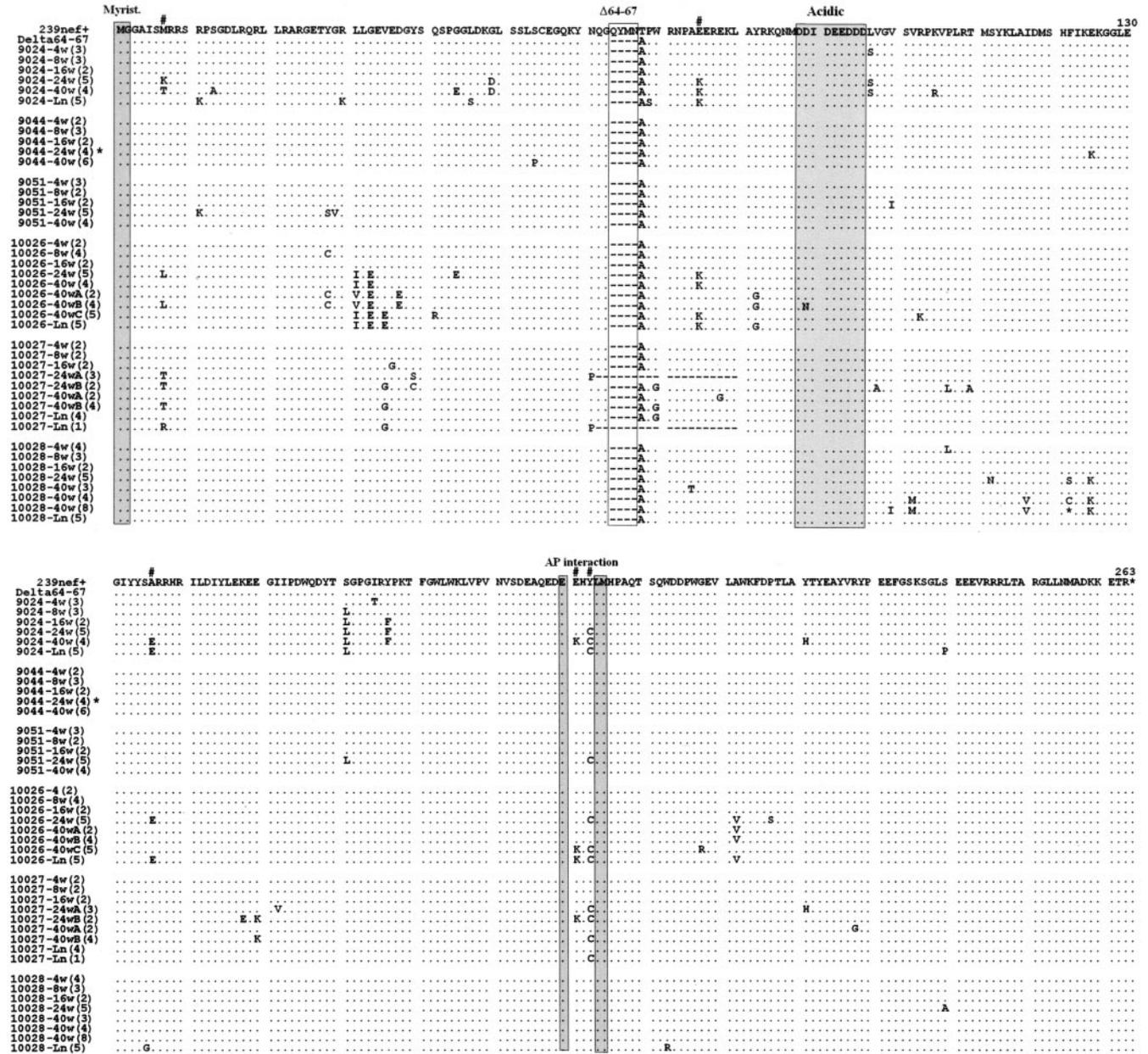
FIG. 5. Alignment of Nef sequences derived from macaques infected with the SIVmac239 Δ64-67 Nef variant. The SIVmac239 sequence is shown in the upper panel for comparison. Dots indicate amino acid identity, and dashes indicate gaps introduced to optimize the alignment. Some sequence motifs in Nef, including the N-terminal myristoylation signal, the acidic region, a C-proximal adaptor-protein (AP) interaction site, and the position of the Δ64-67 deletion are indicated. The "#" symbol denotes positions where changes in Nef were selected in several animals. The numbers in parenthesis give the number of clones analyzed. w, wpi; Ln, lymph node.

Mm-L were truncated (Table 1). These results indicate a high selective pressure for amino acid changes and full-length Nef expression in animals that developed relatively high viral loads. To further assess the Nef sequence alterations selected in vivo, we also analyzed genomic DNA samples derived from lymph nodes obtained at necropsy. *env-nef* fragments could be readily amplified from the three animals with moderate to high viral loads (Mm9024, Mm10026, and Mm10027) but only from one animal with low viral loads (Mm10028). The results confirmed that the Δ64-67 deletion was stable and that a larger number of changes was observed in the *nef* sequences derived from the

three animals with the relatively high viral loads compared to Mm10028 showing low viral loads (Table 1).

Importantly, at five positions (M7, E75, A136, E191, and Y193) amino acid substitutions were observed in at least two of the three macaques with relatively high viral loads but not in the remaining animals (Fig. 5). Of these alterations the E191K and Y193C changes were most interesting because they were consistently observed in Mm-M infection and became predominant in animals Mm9024 and Mm10026, which developed the most severe clinical symptoms. Furthermore, they are located in the ExxxLM sequence, which may be involved in the inter-
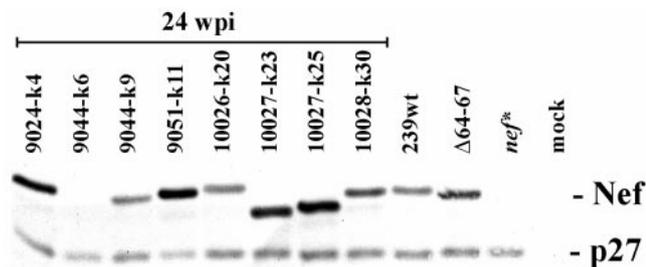
FIG. 6. Expression of *nef* alleles selected in SIVmac239 Δ64-67Nef-infected macaques. 293T cells were transfected with proviral SIVmac239 constructs encoding the indicated Nef proteins or containing a disrupted *nef* gene (*nef**). Pooled sera from SIVmac239-infected rhesus macaques were used for the detection of Nef and p27, respectively.

action of Nef with clathrin adaptor protein complexes (10, 18) and critical for downmodulation of CD4 and CD28 (63), as well as upregulation of Ii by SIVmac239 Nef (53, 59). Notably, HIV-1 Nef proteins do not contain the N-terminal AP-binding motif, and some HIV-1 Nef proteins contain a cysteine at the position corresponding to Y193 in SIV Nef. Thus, the Y193C change might partially compensate for the loss of the N-terminal AP-2 binding element in Δ64-67 SIV Nef by generating a more "HIV-1-like" C-terminal sequence. In addition to these point mutations, 45 nucleotides flanking the Δ64-67 mutation were deleted in three of five *nef* alleles derived from Mm10027 at 24 wpi, predicting the deletion of amino acid residues 61 to 80 (Fig. 5). The majority of *nef* alleles derived from Mm9044 at 24 wpi and from Mm10028 at both 24 and 40 wpi also contained a mutation of AAAAGAAAAGGGGGG to AAAAAAAAAGGGGGG in the polypurine tract, predicting the substitution of E125K (Fig. 5).

Functional activity of Nef variants selected in vivo. Our observation that some changes were consistently observed in *nef* alleles derived from animals with relatively high viral loads, suggested that they may functionally compensate for the Δ64-67Nef deletion. To evaluate this possibility, representative *nef* alleles PCR amplified from the six animals at 24 and 40 wpi were cloned into the bicistronic vector coexpressing Nef and GFP for flow cytometric analysis. Western blot analyses revealed that, with the exception of the 9,044-k6 allele containing a premature stop codon at position 122 of the *nef* ORF, all Nef variants were expressed in transfected 293T cells (Fig. 6 and data not shown). The electrophoretic mobility differed from that of the 239wt Nef due to the Δ64-67 and Δ61-80 deletions and the presence of up to 10 amino acid substitutions.

Flow cytometric analysis revealed that the ability of Nef to efficiently downmodulate CD4, CD28, and CXCR4 was not restored in any of the six animals infected with the Δ64-67Nef variant (examples shown in Fig. 7A). In contrast, all intact *nef* alleles derived from these animals efficiently modulated surface expression of CD3, MHC-I, MHC-II, and Ii. For quantitative fluorescence-activated cell sorting analysis, we divided the mean fluorescence intensity obtained on Jurkat or HeLa CIITA cells transiently transfected with the different *nef* alleles by the mean fluorescence obtained for cells transfected with a *nef*-defective control construct as described previously (13, 53). As shown in Fig. 7B, all *nef* alleles selected in vivo showed little

if any activity in downmodulating CD4 and CD28. They were functionally active, however, in modulating the remaining surface receptors investigated, albeit with different efficiencies.

Finally, we evaluated whether more subtle differences in Nef function are associated with the development of relatively high or low load viral loads in vivo. We compared the average activities of seven *nef* alleles derived from the three Mm-M animals with high viral loads (Mm9024, Mm10026, and Mm10027) at 24 or 40 wpi with those derived from the remaining Mm-L macaques. At these time points, the virals loads differed by 2 to 3 orders of magnitude between the two groups of animals (Table 1). Quantitative analysis revealed some minor restoration of CD4 but not CD28 or CXCR4 downmodulation in animals with high viral loads (Fig. 7C and data not shown). In contrast, the functional activity in CD3 downregulation was reduced at high levels of Nef expression. Notably, most *nef* alleles selected in vivo showed enhanced activity in Ii upregulation compared to the Δ64-67Nef (Fig. 7C). Finally, both groups of animal-derived *nef* alleles and the Δ64-67Nef showed similar activities in modulating MHC-I and MHC-II surface expression. Thus, the differences in viral loads in the animals that received the Δ64-67Nef mutant did not correlate with efficient restoration of CD4 and CD28 downmodulation. However, *nef* alleles derived from animals with low or high viral loads showed some subtle differences in their abilities to downregulate CD4 and CD3 surface expression.

To evaluate whether the changes selected in macaques infected with the Δ64-67Nef variants restored the ability of Nef to stimulate SIV replication, we introduced the animal-derived *nef* alleles obtained at 24 wpi into the 239wt provirus and assayed the effects of these *nef* variants on the replicative capacity of SIVmac 239 in 221-89 cells. In the absence of exogenous IL-2, only the parental 239wt virus replicated efficiently (Fig. 8A), demonstrating that none of the changes selected in vivo fully restored this Nef function. In the presence of IL-2, however, several *nef* alleles were more active in enhancing SIV replication than the original Δ64-67Nef (Fig. 8B). Those derived from Mm9024 and Mm10027 showed the highest activity and resulted in levels of replication intermediate between 239wt and the Δ64-67Nef variant. In comparison, SIVmac239 containing the 9044-k9 and 10026-k20 *nef* alleles replicated only slightly better than the original Δ64-67Nef mutant virus. Finally, introduction of the 9051-k11 and 10028-k30 *nef* alleles resulted in inefficient replication in 221-89 cells. In addition to the changes in *nef*, the G-A mutations in the polypurine tract of sequences derived from Mm9044 and Mm10028 described above might also affect the efficiency of viral replication. Furthermore, the Mm10028 *nef* alleles contained changes of TTTTAT to TTCTAT (24 wpi) and TTGTAT or TTGAAT in the T-rich region just upstream the polypurine tract, which is known to be important for efficient replication of SIVmac239 (32). On average, the reverse transcriptase production in 221-89 cells infected with SIVmac239 carrying *nef* alleles derived from the three animals with high viral loads was intermediate (78.3 ± 24.4, $n = 12$) between 239wt (100%) and Δ64-67Nef (47.2 ± 1.4, $n = 3$) infection (Fig. 8C). In contrast, intact *nef* alleles derived from animals with low viral loads did not enhance viral replication (44.1 ± 18.6, $n = 9$).

Finally, we examined the ability of *nef* variants to enhance the infectivity of SIVmac. To avoid a possible bias due to
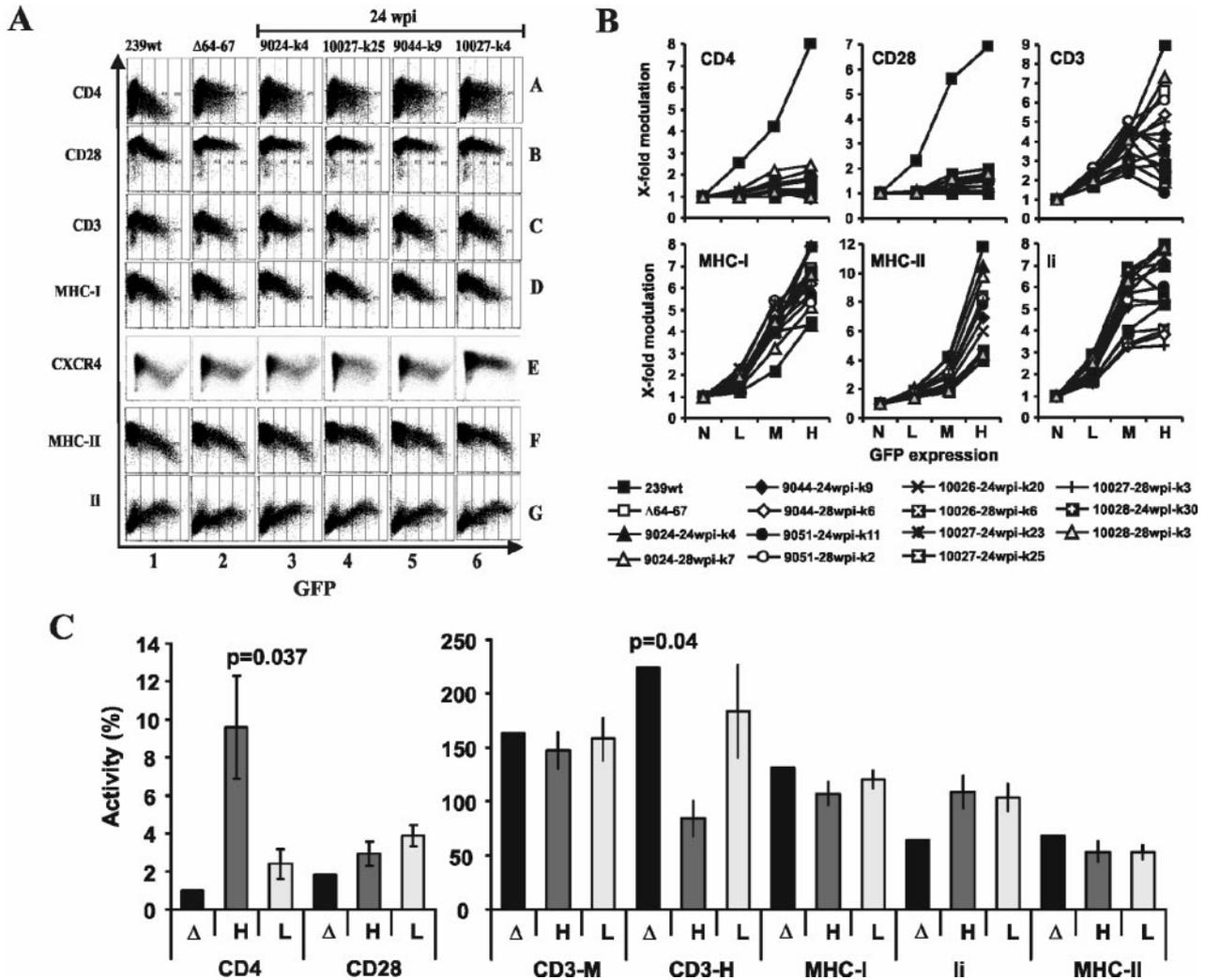
FIG. 7. Functional activity of *nef* alleles derived from SIVmac239 Δ64-67Nef-infected macaques. (A) Jurkat T (rows A to D) or HeLa-CIITA (rows E and F) cells were transfected with bicistronic vectors coexpressing the indicated *nef* alleles and GFP and assayed for surface expression of CD4, CD3, CD28, MHC-I, MHC-II, and Ii. (B) Quantitative analysis of the functional activity of individual *nef* alleles. Ranges for green fluorescence on cells defined as expressing no (N), low (L), medium (M), or high (H) levels of GFP are indicated in panel A. X-fold down- or upmodulation was calculated as described in Materials and Methods. (C) Average activities ± the standard deviations of *nef* alleles derived from macaques with relatively high (H) or low (L) virus loads. Values are shown relative to the activity, calculated as the X-fold modulation of the respective receptors at high expression levels, of the SIVmac 239wt Nef protein (100%). Δ, Activity of the original Δ64-67Nef. The effect of several Nef proteins on CD3 was saturated at medium expression levels. Therefore, CD3 downmodulation was calculated for both medium and high levels of GFP expression.

changes in overlapping *env* or LTR sequences, we expressed Nef in *trans* in the virus producer 293T cells. The 239wt Nef enhanced viral infectivity in TZM-bl cells ~20-fold (Fig. 9A). The Δ64-67Nef showed ~2-fold reduced activity (50.2% ± 8.8%, $n = 9$; numbers give mean infectivity ± the SEM as a percentage of that obtained for 239wt Nef). Unexpectedly, *nef* alleles derived from six animals infected with the 239Δ64-67Nef variant differed considerably in their ability to promote viral infectivity (Fig. 9A). These differences were highly reproducible and confirmed in two different indicator cell lines (Fig. 9B). Importantly, *nef* alleles derived from the three animals with relatively high viral loads were usually more active (59.4% ± 7.3%, $n = 45$) than those derived from macaques showing more attenuated levels of viral replication (24.3% ± 2.5%, $n = 36$; $P <$

0.0001) (Fig. 9A). Taken together, our results show that the second-site mutations in Nef selected in animals with moderate to high viral loads slightly increased the ability of the Δ64-67Nef to downmodulate CD4 and had more significant effects on its ability to enhance viral replication and infectivity in vitro.

DISCUSSION

Disrupting the N-distal AP-2 binding element in SIV Nef eliminates downregulation of CD4, CD28, and CXCR4 (32, 42, 63). We demonstrate that this mutation attenuates viral replication both in vitro and in vivo in infected rhesus macaques. Attenuation was clearly less severe, however, than that achieved by large deletions that disrupt all aspects of Nef function. There-
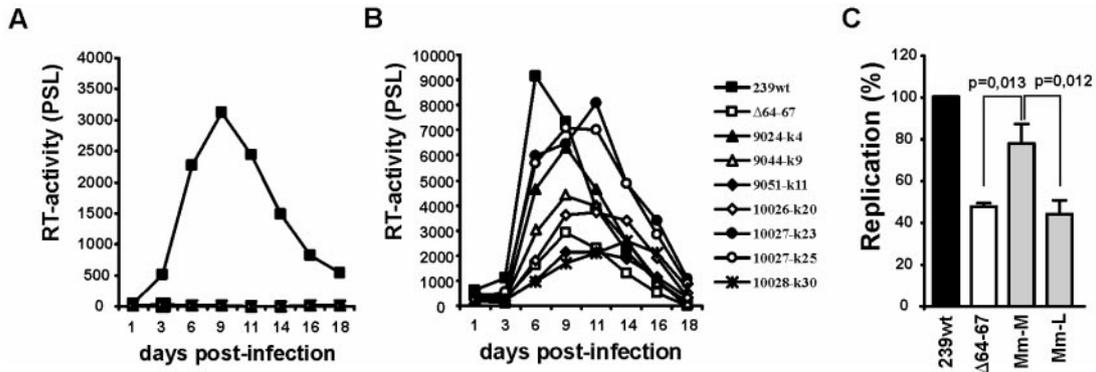
FIG. 8. Activity of *nef* alleles derived from SIVmac239 Δ64-67Nef-infected animals at 24 wpi in enhancing viral replication. Replication of the indicated SIVmac239 *nef* variants in 221-89 cells in the absence (A) or presence (B) of exogenous IL-2. The reverse transcriptase activity was determined by using a phosphorimager. PSL, photon-stimulated light emission. Similar results were obtained in three independent experiments. (C) Virus production by 221-89 cells infected in the presence of IL-2 with SIVmac239 variants expressing the 239wt and Δ64-67 Nefs or *nef* alleles derived from the three animals with moderate (Mm-M, Mm9024, Mm10026, and Mm10027) or low (Mm-L, Mm9044, Mm9051, and Mm10028) viral loads. For each virus variant, we determined the total reverse transcriptase activity in the culture supernatants over the 18-day period. The reverse transcriptase values obtained for 239wt were considered 100%. Means and SEMs derived from three experiments are shown.

fore, some or all of the functions that were not disrupted by the Δ64-67 deletion, such as downmodulation of class I MHC and CD3, upregulation of Ii cell surface expression, or the increased virion infectivity also contribute to viral replication in vivo. These findings are consistent with the accumulating evidence suggesting that a combination of multiple separable Nef functions enables both SIV and HIV to replicate efficiently and to persist at high levels in the infected host (11, 22, 34, 47–49, 54, 61).

Early in infection the Δ64-67Nef variant replicated with significantly higher efficiency than the $239_{(EDR)}$Nef mutant analyzed in our previous study (34). Both mutant *nef* alleles were defective in downmodulating CD4, CD28, and CXCR4 and in stimulating viral replication. The major functional difference between them is that the Δ64-67Nef shows only moderately reduced activity in enhancing virion infectivity (Fig. 2), whereas the $239_{(EDR)}$Nef is entirely inactive (34). Although we compared

their functional activity in nine in vitro assays (CD4, MHC-I, CD3, CD28, CXCR4, MHC-II, Ii, infectivity, and replication), the possibility that both *nef* alleles also differ in other functional aspects cannot be dismissed. Nonetheless, our findings suggest that Nef-mediated infectivity enhancement, while insufficient for efficient stimulation of SIV replication in PBMC and in 221-89 cell cultures, may accelerate viral replication in infected macaques at early stages of infection. Moreover, our finding that *nef* alleles derived from macaques with progressing disease were significantly more active in enhancing viral infectivity than those obtained from macaques showing a more strongly attenuated course of infection suggests that this Nef function also contributes to viral spread and pathogenicity during later stages of infection.

Altogether, the SIVmac239 Δ64-67Nef variant showed pathogenic properties intermediate between 239wt and grossly *nef*-deleted SIVmac infection. The efficiencies of viral replication and
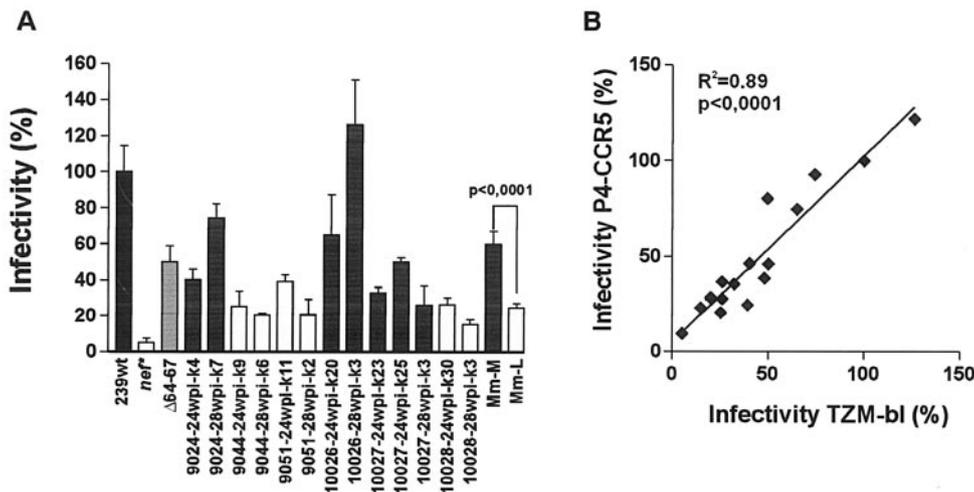

FIG. 9. Activity of *nef* alleles derived from SIVmac239 Δ64-67Nef-infected animals in enhancing viral infectivity. (A) TZM-bl cells were infected with virus stocks produced in 293T cells expressing no Nef (*nef**) or the indicated SIVmac239 *nef* alleles. The infectivities are shown relative to those obtained using 239wt Nef (100%). Average values (± the SEM) derived from triplicate infections (each) with three independent virus stocks are given. (B) Correlation between the infectivities of viral stocks quantified in TZM-bl or P4-CCR5 cells.

the clinical course of infection differed among the six Δ64-67Nef-infected animals. Half of the animals showed only moderately reduced viral loads compared to pathogenic 239wt infection and progressed slowly to immunodeficiency, whereas the remaining macaques remained asymptomatic and resembled grossly *nef*-deleted infection. Nef sequence analysis revealed that the deletion was not repaired in any of these animals during the course of infection. Furthermore, in contrast to the results obtained using the $239_{(EDR)}$Nef variant (34), the disrupted Nef functions were not efficiently restored in Δ64-67Nef-infected macaques. However, some second-site mutations in Nef were consistently selected in animals that developed relatively high viral loads. Functional analysis revealed that these changes had some minor restorative effects on CD4 downmodulation and moderately enhanced the ability of the Δ64-67Nef to enhance viral replication and infectivity in vitro. The fact that these alterations were only observed in animals developing relatively high viral loads and showing signs of disease progression suggests that the alterations contributed to efficient viral replication and pathogenesis in infected animals. However, given that Nef function was not fully restored, it is quite possible that changes elsewhere in the viral genomes contributed to this phenotype.

The Δ64-67Nef is impaired in two functions interfering with T-cell activation: the downmodulation of CD4 and CD28. However, it was even more active than 239wt Nef in downregulating CD3 from the cell surface. This function might be advantageous for the virus because it interferes with TCR-mediated T-cell activation by antigen-presenting cells and hence likely weakens the antiviral immune response. However, efficient CD3 downregulation is not sufficient to enhance SIV replication in infected macaques (48). We found that *nef* alleles derived from the two Δ64-67Nef-infected animals with the highest viral loads, Mm9024 and Mm10027, were less active than Δ64-67Nef in downmodulating CD3 cell surface expression. While the relevance of these subtle functional differences for viral replication in vivo remains elusive one could speculate that the reduced functional activity in CD3 downmodulation could lead to increased activation of infected T cells and hence increased virus production.

All *nef* alleles derived from Δ64-67Nef-infected macaques with relatively high viral loads predicted full-length reading frames and, as discussed above, contained amino acid changes that enhanced viral fitness. In contrast, *nef* ORFs were disrupted more frequently in animals with low viral loads, although fewer nucleotide changes were selected (Table 1). At 24 wpi, changes of AAAAGAAAAGGGGGG to AAAAAAAAAGGGGGG were consistently detected in Mm9044 and Mm10028, which both showed low viral loads. Unaltered polypurine tract sequences were detected later in Mm9044 infection. In Mm10028, however, additional changes in the PPT and the critical upstream T-rich region (35, TTTTATAAAAGAAAAGGGGGG to TTGAATAAAAAAAAAGGGGGG were also present at 40 wpi. Furthermore, the majority of *nef* genes were prematurely truncated (Table 1). Thus, it seems that in Mm10028 less fit viral variants predominated later during infection. Consistent with this, the viral RNA load in Mm10028 dropped by 2 orders of magnitude between 20 and 40 wpi, and no infectious cells could be detected after 36 wpi (Fig. 4). Less-fit viral variants have also been detected in some nonprogressors or slow progressors of HIV-1 infection (12, 45, 65).

It has been shown that Nef-mediated downregulation of class I MHC complexes from the cell surfaces protects infected cells from detection and lysis by cytotoxic T lymphocytes (CTL) (16). In agreement with an important role of this Nef function in vivo it has been demonstrated that MHC-I downmodulation is associated with a strong selective advantage (47, 61). It is thought that the emerging antiviral CTL response plays a key role in the control of viral replication after the acute phase of infection. Deletion of residues 64 to 67 did not reduce the ability of 239-Nef to downregulate surface expression of class I MHC complexes. Thus, it was unexpected that the cell-associated viral loads dropped as dramatically in the six animals that received the Δ64-67Nefs variant as in macaques infected with grossly *nef*-deleted ΔNU virus (Fig. 4B). Remarkably, the viral RNA levels in the plasma of Δ64-67Nef-infected animals dropped less strongly (Fig. 4A). Thus, cells infected with the Δ64-67Nef variant might produce more virus than those infected with the ΔNU virus. It is also possible, however, that our measurement of viral loads in the peripheral blood of the 239wt, ΔNU, and Δ64-67Nef-infected macaques may not accurately reflect the number of infected cells and virus production in lymphatic organs, the major site of HIV-1 and SIV replication. Nonetheless, our data show that efficient class I MHC downregulation is not sufficient to maintain high viral loads after the onset of the antiviral CTL response. However, the observations that the viral RNA load was intermediate and that the cell-associated viral load increased faster than in ΔNU infection provides some evidence that class I MHC downmodulation contributes to the development of high viral loads in vivo in infected rhesus macaques. Apparently a variety of complementary Nef activities allows the virus to replicate at high levels in the great majority of infected hosts.

Both the Δ64-67Nef and the previously analyzed $239_{(EDR)}$ Nef (34) are defective in at least four activities: downmodulation of CD4, CD28, and CXCR4 and stimulation of viral replication. Thus, it is not possible to discern the relative contributions of these functions to the attenuating effect of these mutations. Ideally, mutations that are both selective for one specific in vitro Nef function and difficult to revert should be analyzed. However, although SIV Nef mutants that are apparently exclusively impaired in class I MHC downregulation have been described and analyzed in the SIV/macaque model (47, 60, 61), it is a difficult task to selectively disrupt other Nef functions. In each case, the analysis of Nef mutants that are impaired in multiple related functions also provides related information on viral pathogenicity. Since these functions are mediated by similar Nef surfaces, they will usually be selected for in a linked manner in the infected host. Furthermore, therapeutic agents that disrupt one of these activities will most likely also affect the others. However, it seems that only agents that disrupt molecular interactions of Nef that are critical for all of these in vitro functions will effectively attenuate viral replication.

ACKNOWLEDGMENTS

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