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Downregulation of the T-Cell Receptor by Human Immunodeficiency Virus Type 2 Nef Does Not Protect against Disease Progression▽

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Chronic immune activation is thought to play a major role in human immunodeficiency virus (HIV) pathogenesis, but the relative contributions of multiple factors to immune activation are not known. One proposed mechanism to protect against immune activation is the ability of Nef proteins from some HIV and simian immunodeficiency virus strains to downregulate the T-cell receptor (TCR)-CD3 complex of the infected cell, thereby reducing the potential for deleterious activation. HIV type 1 (HIV-1) Nef has lost this property. In contrast to HIV-1, HIV-2 infection is characterized by a marked disparity in the disease course, with most individuals maintaining a normal life span. In this study, we examined the relationship between the ability of HIV-2 Nef proteins to downregulate the TCR and immune activation, comparing progressors and nonprogressors. Representative Nef variants were isolated from 28 HIV-2-infected individuals. We assessed their abilities to downregulate the TCR from the surfaces of CD4 T cells. In the same individuals, the activation of peripheral lymphocytes was evaluated by measurement of the expression levels of HLA-DR and CD38. We observed a striking correlation of the TCR downregulation efficiency of HIV-2 Nef variants with immune activation in individuals with a low viral load. This strongly suggests that Nef expression can influence the activation state of the immune systems of infected individuals. However, the efficiency of TCR downregulation by Nef was not reduced in progressing individuals, showing that TCR downregulation does not protect against progression in HIV-2 infection.

The majority of humans infected with human immunodeficiency virus type 1 (HIV-1) progress relentlessly toward immunodeficiency, whereas simian immunodeficiency virus (SIV) infection in the natural hosts, Old World monkeys, rarely causes disease (9). It was recently shown that HIV-1 and its simian ancestor, SIVcpz, have one distinctive characteristic that may contribute to pathogenesis. In contrast to the Nef proteins of other immunodeficiency viruses, HIV-1 and SIVcpz Nef proteins are unable to downregulate the T-cell receptor (TCR) from the surfaces of infected cells (1, 22). Schindler and colleagues proposed that TCR downregulation protects the host from the impact of chronic immune activation (22), which is increasingly thought to play a major role in HIV-1 disease progression (7). In most cases, SIVsmm infection of sooty mangabeys leads to high viral loads without evidence of immunodeficiency or CD4 depletion, and this is associated with very low levels of immune activation (25). CD4 depletion without immunodeficiency has been reported in a minority of SIVsmm-infected sooty mangabeys. However, this CD4 depletion is not associated with major immune activation or viral-load increase (26). Immunodeficiency associated with CD4 depletion was reported in only one case (18). Schindler et al. discovered that in sooty mangabeys showing a loss of $CD4^+$ T cells, the Nef protein of the infecting SIVsmm was less efficient at TCR downregulation (22), suggesting that the CD4 depletion in sooty mangabeys is linked to the loss of this function, together with a loss of major histocompatibility complex class I downregulation (23). Following transmission to humans in West Africa, SIVsmm zoonosis gave rise to HIV-2 infection, identified in patients with AIDS in 1986 (10). HIV-2 infection can lead to a clinical picture indistinguishable from AIDS caused by HIV-1, but in general, the progress to clinical immunodeficiency is slower than in HIV-1 infection: this appears to be due to an unusually high proportion of HIV-2-infected long-term nonprogressors (8, 21). Although the few HIV-2 *nef* alleles that have been studied so far are capable of TCR downregulation, this has not been systematically evaluated in relation to disease progression. Here, we present data from a well-characterized community cohort followed in Caio in Guinea-Bissau since 1989 (27), in which the abilities of *nef* alleles from the infecting HIV-2 strains to downregulate the TCR could be studied in relation to immune activation and disease status.

MATERIALS AND METHODS

Ethics statement. Study participants provided informed consent. Ethical approval was obtained from the Gambian Government/MRC Ethics Committee, from the Republic of Guinea Bissau Ministry of Health, and from the Oxford Tropical Research Ethics Committee, Oxford, United Kingdom.

Patients. Twenty-eight antiretroviral-naïve subjects, described in Table 1, were recruited from a community cohort in Caio, Guinea Bissau, established in 1989 (27). Plasma samples were screened for HIV antibodies and virus loads, and stabilized whole-blood samples were used for CD4 count analysis as described elsewhere (17). Subjects with HIV-1/HIV-2 dual status were excluded from the study. The plasma virus load was determined using reverse transcription-PCR with long terminal repeat-specific primers, with a lower limit of detection of 100 copies/ml. HIV-2 progressing subjects (Ps) were defined as having a plasma viral

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TABLE 1. Patient descriptions

Patient	Age (yr)	Sex[a]	First seropositivity (yr)	2003		2006	
				PVL[b]	CD4[c]	PVL	CD4
N2A4	83	Fem	1989	<100	717	NA[e]	NA
N2A7	72	Fem	1996	<100	638	NA	NA
N2A8	67	Fem	1989	<100	821	NA	NA
N2A9	71	Fem	1989	<100	667	<100	685
N2B7	38	Fem	1996[d]	<100	629	NA	NA
N2B9	33	Fem	1996	<100	526	116	500
N2C6	41	Fem	1996[d]	<100	826	<100	715
N2D7	60	Male	1996	<100	1,212	393	905
N2D8	49	Fem	1996	<100	747	<100	1,240
N2E10	60	Fem	1989	<100	1,086	<100	1,705
N2F8	79	Fem	1989	<100	935	1,343	615
P2A12	83	Fem	1989	24,002	703	523	460
P2B5	59	Fem	1989	2,247	770	2,653	160
P2C11	63	Fem	1996	7,456	501	<100	170
P2C12	74	Male	1996	1,350	631	1,999	580
P2C5	59	Fem	1996[d]	2,259	597	684	875
P2C7	46	Fem	1996[d]	3,247	737	NA	NA
P2C9	75	Fem	1996[d]	2,298	664	14,104	800
P2E5	70	Fem	1989	1,781	1,193	387	450
P2E6	57	Male	1996	7,260	1,355	23,889	1,075
P2E9	36	Male	1996[d]	4,111	1,151	1,014	560
P2F10	33	Fem	1989	19,576	859	1,608	430
P2F12	38	Fem	1996[d]	6,792	533	NA	NA
P2F4	43	Male	1989	1,655	1,131	22,446	350
P2F5	52	Male	1996	4,366	508	1,587	755
P2F7	74	Fem	1989	10,129	551	NA	NA
P2G10	54	Male	1989	90,184	677	NA	NA
P2G8	59	Fem	2003	3,212	756	NA	NA
NPs[f]	59.4			<100	800	322	909
Ps[f]	57.4			11,290	783	5,916	555

[a] Fem, female.
[b] PVL, plasma viral load in copies per milliliter.
[c] CD4, Peripheral blood absolute CD4 count in cells per μl.
[d] Previously tested negative.
[e] NA, not applicable.
[f] Means.

load above 1,000 copies per ml, and nonprogressing subjects (NPs) were defined as having an undetectable viral load for more than 9 years; all of the patients had CD4 counts above 500 per μl. These criteria are based on the reliability of the HIV-2 RNA plasma viral load as a predictor of disease progression (2). CD4 counts in the two groups were compared in 2003 and 2006. While the CD4 counts of NPs did not change significantly, those of Ps were significantly decreased in 2006 (Wilcoxon signed rank test; NPs, $P = 0.9375$; Ps, $P = 0.0269$).

T-cell activation marker expression. Fresh whole blood was stabilized in a 5:1 ratio with TransFix (Cytomark) for 2 to 14 days and used for determination of T-cell surface activation marker expression using anti-HLA-DR–fluorescein isothiocyanate-, CD38-phycoerythrin (PE)-, CD4-peridinin chlorophyll protein-, and CD8-allophycocyanin-titrated monoclonal antibodies (BD Pharmingen). One hundred microliters of TransFix-preserved blood was incubated with the antibody cocktail for 30 min in the dark. The red blood cells were lysed using 1:10 fluorescence-activated cell sorter (FACS) lysing solution (BD Biosciences). The cells were washed twice and fixed with a 2% paraformaldehyde–phosphate-buffered saline solution before analysis using a FACSCalibur flow cytometer (BD Biosciences). The data were analyzed using FlowJo (Tree Star). Gating on lymphocytes and $CD4^+$ and $CD8^+$ T-cell populations was performed individually for each patient. Gating for $HLA\text{-}DR^+$ and $CD38^+$ populations was done using collective quadrant gates based on $HLA\text{-}DR^+$ and $CD38^+$ expression on $CD4^+$ or $CD8^+$ T cells of HIV-negative subjects, as described by Hanson et al. (8).

TCR downregulation by Nef. 129 unique HIV-2 *nef* alleles were amplified by limiting dilution and sequenced from peripheral blood proviral DNA obtained in 2003 from 28 individuals. For each patient, one unique allele was chosen (among 3 to 18 sequences) as the complete coding sequence that was closest to the intrapatient consensus. Sequence alignments were made in Clustal X (16). The consensus sequences were obtained in Jalview (3), and maximum-likelihood evolutionary distances were calculated in Tree-puzzle (24) software using the Tamura-Nei 1993 substitution model. These sequences were inserted in a pIRES2-GFP vector (Clontech, California). In addition, the five alleles most distant from the consensus were cloned from five patients for comparison. These constructs were then transfected by Amaxa technology in isolated $CD4^+$ T cells from healthy donor buffy coats by negative selection ($CD4^+$ T-cell isolation kit; Miltenyi Biotec, Germany). After 24 h, the cells were analyzed by flow cytometry for green fluorescent protein (GFP) and TCR expression (anti-TCR pan-αβ–PE; Beckman-Coulter Immunotech, France). The TCR downregulation efficiency was calculated as 1 minus the ratio of the geographic mean of TCR-associated fluorescence (PE mean fluorescence intensity [MFI]) in GFP-expressing cells to the PE MFI in GFP-negative fractions.

To evaluate the impact of Nef expression on T-cell activation, the lymphocytes were stimulated 4 h after Amaxa electroporation by coated anti-CD3 (R&D; Clone UCHT1; 5 μg/ml) and anti-CD28 (R&D; clone 37407; 2 μg/ml) for 24 h and then fixed and stained by anti-CD69.

Statistics. The statistical analyses were performed using GraphPad Prism version 4.0b for Mac (GraphPad Software, San Diego, CA).

RESULTS AND DISCUSSION

Twenty-eight HIV-2-infected subjects were selected from the Caio cohort. These individuals were designated NPs (all of whom had been seropositive for at least 10 years) and Ps on the basis of 2003 viral-load measurements, either below the level of detection (<100 copies/ml) ($n = 11$) or above 1,000 copies/ml ($n = 17$). In HIV-2 infection, the viral load remains undetectable in the latent phase, and a consistently detectable viral load has been shown to predict disease progression (2). Accordingly, the CD4 counts of Ps were significantly lower in 2006 than in 2003, while they remained stable in NPs (Wilcoxon signed rank test; NPs, $P = 0.9375$; Ps, $P = 0.0269$). All the patients were seropositive for HIV-2 and seronegative for HIV-1 on several occasions during follow-up. Because CD4 depletion can induce homeostatic proliferation and activation, we selected individuals with CD4 counts above 500 per mm^3 of blood in 2003. The activation levels of peripheral CD4 T lymphocytes in HIV-2 patients were evaluated as the percentage of $CD4^+$ cells expressing both the activation markers HLA-DR and CD38 (Fig. 1). In accordance with other studies of individuals living in rural Africa (4, 8, 19), basal immune activation levels were high and were significantly increased by HIV-2 infection. In the selected patients, as well as in the rest of the HIV-2 cohort, immune activation levels for both CD4 and CD8 lymphocytes were correlated with the viral load in Ps and showed a significant difference between NPs and Ps (Fig. 1 and data not shown). *nef* variants representative of the pool of sequences found in each patient were introduced into primary CD4 T cells from healthy donors, and the efficiency of downregulation of the TCR was measured by flow cytometry (Fig. 2). The representative allele was chosen as the complete coding sequence that was evolutionarily closest to the consensus of available sequences for each patient (between 3 and 18 sequences). Most (31/34) of the studied HIV-2 *nef* variants were able to down-modulate the TCRs from the surfaces of transfected CD4 T cells to some extent, showing that this function of HIV-2 *nef* is highly conserved in vivo. However, the efficiency of the TCR downregulation by functional alleles varied greatly, ranging from 38 to 76% decrease of TCR surface expression (mean, 61.9% ± 27%). We found only one allele that was deficient for both CD4 and TCR downregulation, strongly suggesting that the great majority of the studied HIV-2 *nef* variants are physiologically functional.

We checked that TCR downregulation by HIV-2 *nef* variants

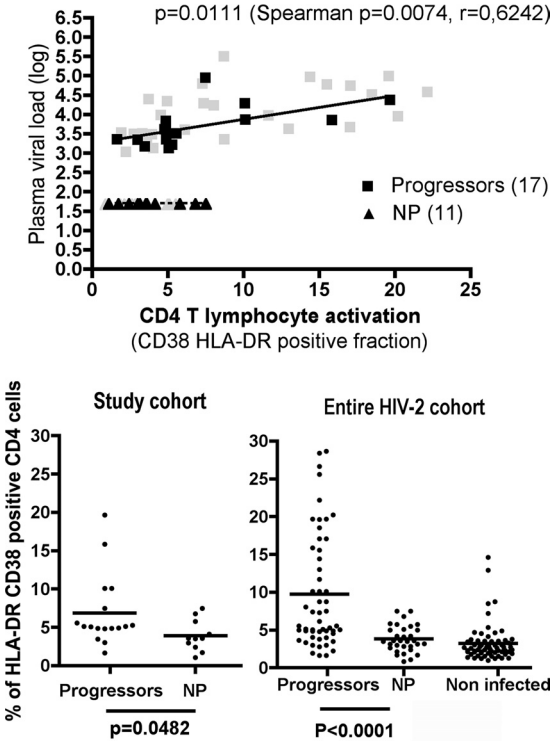

FIG. 1. Immune activation in HIV-2 patients. CD4 T-lymphocyte activation levels in patients were measured as the percentage of CD38/HLA DR double-positive $CD4^+$ T lymphocytes in peripheral blood of HIV-2-infected individuals. The upper panel shows the correlation of the percentage of CD38/HLA-DR double-positive $CD4^+$ cells with plasma viral loads in copies per milliliter (both measures were done in the same samples). The entire cohort of HIV-2-infected individuals is indicated in gray, whereas individuals selected for the study are shown in black. Correlation was evaluated using a two-tailed Spearman rank correlation test and linear regression. The lower panel shows CD4 activation levels in Ps (mean, 6.87%), NPs (mean, 3.83%), and non-infected individuals (mean, 3.23%) using the Prism Mann-Whitney test (two-tailed) for the studied individuals. The entire HIV-2 cohort (three groups) was analyzed by one-way analysis of variance.

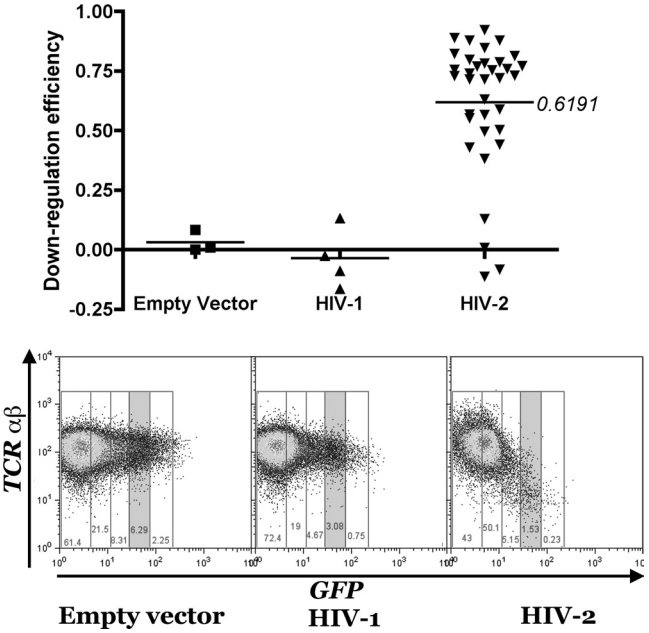

FIG. 2. TCR downregulation by HIV-2 Nef. Primary CD4 T cells expressing various alleles of *nef* were compared for TCR surface expression. The TCR downregulation efficiency was calculated as 1 minus the ratio of the geographic mean of TCR-associated fluorescence (PE MFI) in GFP-expressing cells divided by the PE MFI in GFP-negative fractions (upper panel). In the lower panel, representative FACS plots of TCR and GFP expression are shown. The shaded boxes were used to define the Nef-expressing cells.

was able to inhibit the activation of CD4 T lymphocytes in vitro. CD69 is upregulated for 24 to 48 h by lymphocytes upon stimulation. When *nef*-transfected T cells were stimulated using coated anti-CD3 and anti-CD28, the extent of upregulation of CD69 was inversely correlated with the efficiency of the *nef* variants to downregulate the TCR (Fig. 3, top). Consistent with a direct effect of TCR downregulation on T-cell activation, the CD4 downregulation efficiency in the same cells did not correlate with CD69 upregulation (Spearman; $P = 0.3894$). Remarkably, the in vivo CD4 T-cell activation levels of the NP group were also correlated with the activity of the *nef* variants on TCR expression (Fig. 3, middle). This suggests that for subjects with low viral loads there is a visible impact of TCR downregulation by Nef on T-cell activation in vivo. As in vitro, the CD4 downregulation efficiency of HIV-2 Nef did not correlate with in vivo levels of activated lymphocytes (Spearman; $P = 0.4972$), confirming that the effect of Nef on immune activation is functionally linked to TCR downregulation. The CD8 T-cell activation levels showed no correlation with the TCR or CD4 downregulation efficiency. In the P group, however, no correlation was observed between immune activation in vivo and Nef-induced TCR downregulation. This lack of correlation may be explained by the strong immune activation associated with disease progression in HIV-2-infected individuals, which could mask the effect of TCR downregulation. Indeed, the correlation was significant (Spearman; $P = 0.0022$) in individuals with less than 7.5% CD38/HLA-DR double-positive CD4 cells. This percentage corresponds to the maximum activation level in NPs. Thus, within the NP range of activation levels (4.7 ± 1.4 for Ps versus 3.9 ± 2.0 for NPs), there was a significant correlation between the activation level of peripheral CD4 cells and the efficiency of TCR downregulation by Nef (Fig. 3, bottom). Immune activation in HIV infection is thought to be driven by multiple factors. The robust correlation between the viral load and immune activation in HIV-2-infected individuals strongly suggests that plasma virus is one of them. HIV virions have multiple means to promote immune activation, among which are double-stranded RNA binding to Toll-like receptors (15), binding of gp120 to cell surface receptors (11), Tat internalization by uninfected cells (12), and antigenic stimulation (17). Current studies also show that microbial translocation can be associated with immune activation in HIV-1-infected individuals independently of the viral load (13), which may also be the case in HIV-2-infected individuals. Thus, in HIV-2-infected individuals, the association of immune activation and downregulation of the TCR by Nef is present and visible at low activation levels but is likely masked by the activation induced by viral replication in Ps.

In Ps, TCR downregulation was not correlated with the

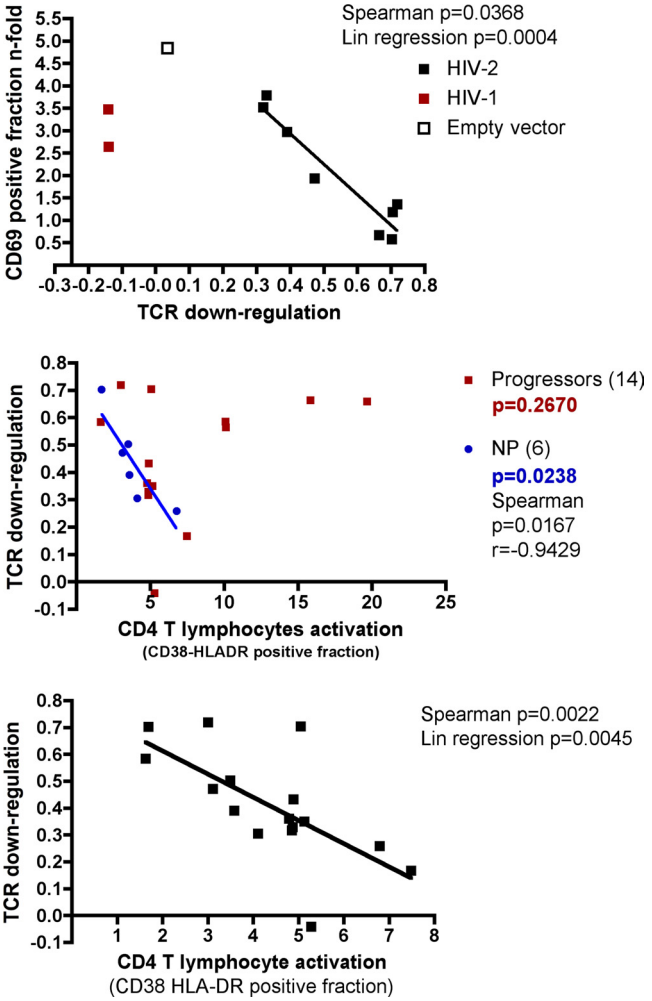

FIG. 3. Correlation of HIV-2 Nef-induced TCR downregulation with in vivo parameters. (Top) In vitro activation. Lymphocyte responses to in vitro activation and TCR downregulation efficiency by HIV-2 Nef were correlated. CD4 T lymphocytes were transfected with Nef iresGFP vectors and then stimulated by anti-CD3/CD28-immobilized antibodies for 24 h. The TCR downregulation efficiency, calculated as before, was correlated with the CD69-positive fraction. The eight *nef* alleles studied (three from NPs and five from Ps) were selected as similarly efficient at downregulating CD4 and as covering the range of TCR downregulation efficiencies. (Middle) In vivo activation. The CD4 T-cell fraction expressing both HLA-DR and CD38 in HIV-2 patients in 2006 was correlated with the efficiency of TCR downregulation by HIV-2 Nef by linear regression (colored values) and a Spearman correlation test. The Ps ($n = 14$) are in red, and the NPs ($n = 6$) are in blue. (Bottom) TCR downregulation and CD4 T-cell activation in the lower activation range. Immune activation was correlated with HIV-2 Nef downregulation efficiency in HIV-2-infected individuals with less than 7.5% CD38/HLA-DR double-positive CD4 cells.

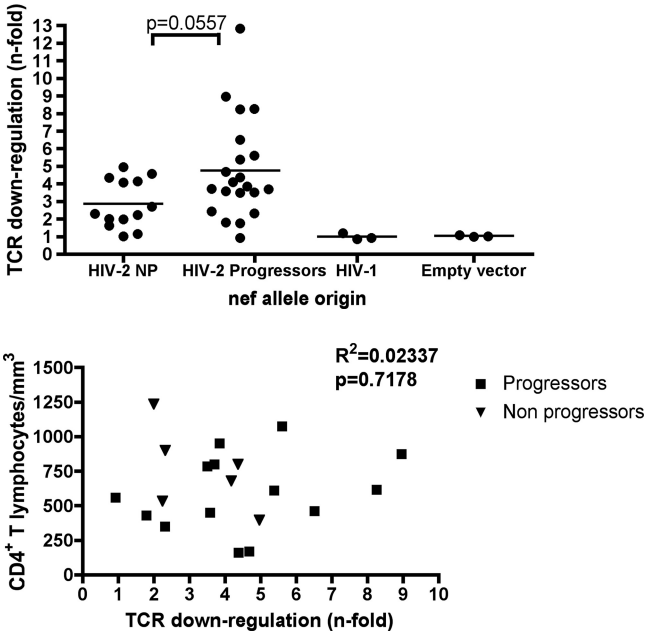

FIG. 4. TCR downregulation does not protect against progression. (Top) TCR downregulation and disease progression. Nef TCR downregulation, expressed as the TCR-associated MFI in Nef-expressing (GFP-positive) cells divided by the TCR-associated MFI in Nef-negative (GFP-negative) cells (*n*-fold TCR downregulation), was compared in P and NP HIV-2-infected individuals by a two-tailed Mann-Whitney test. (Bottom) Absence of correlation between TCR downregulation and CD4 counts. HIV-2 Nef TCR downregulation (*n*-fold) was compared to CD4 counts in 2006. Ps ($n = 14$) and NPs ($n = 6$) are shown. No correlation was found if Ps and NPs were analyzed separately (Spearman; Ps, $P = 0.2464$; NPs, $P = 0.2417$) or with 2003 CD4 counts (Spearman; $P = 0.8156$).

plasma viral load (Spearman; $P = 0.6397$), ruling out a direct role of TCR downregulation in viral replication.

Our results suggest that HIV-2 has the ability to affect immune activation in vivo in NPs, despite undetectable plasma viral loads. If the RNA viral loads in HIV-1 and HIV-2 infections differ significantly, the proviral loads in the peripheral blood are similar (5, 20), and they are approximately five times higher in lymph nodes for both infections (14). Thus, a number of circulating CD4 T cells in infected individuals carry the HIV-2 provirus and may express the *nef* gene independently of the functionality of the other viral genes. Depending on the efficiency of Nef-induced TCR downregulation, these *nef*-expressing CD4 T cells would be more or less susceptible to immune activation. Besides, activation levels were slightly higher in HIV-2 NPs than in uninfected individuals (Fig. 1). This shows that the immune activation is linked to HIV infection and is likely driven by HIV-specific CD4 T cells. As these CD4 T cells are preferentially infected (6), it is very possible that the observed correlation between activation and TCR downregulation is due to the infection and a subsequent graduated inhibition of HIV-2-specific CD4 T cells.

Despite the apparent effect of HIV-2 Nef on immune activation in vivo, the HIV-2 *nef* variants retrieved from Ps did not downregulate the TCR less efficiently than those from NPs. In fact the opposite trend was observed, close to significance (Fig. 4). These results show that, in HIV-2 infection, the loss of control of viral replication seen in Ps is not due to a loss of TCR downregulation efficiency. The correlation of TCR downregulation and immune activation in vivo suggests that this Nef function might help to delay the onset of AIDS in HIV-2 infection. However, the conservation of highly efficient TCR downregulation after progression shows that, in vivo, TCR downregulation does not preclude disease progression in HIV-2 infection. This contrasts with the observation that the

loss of TCR downregulation by Nef seems to be the cause of CD4 depletion in sooty mangabeys (22). CD4 depletion in HIV-2 infection is associated with increased viral replication, with immune activation, and, finally, with immunodeficiency, which is not the case in SIVsmm infection (21, 26). Together with our results, this suggests that in a majority of cases, disease progression in HIV-2 infection is not comparable to CD4 depletion in sooty mangabeys.

Our study also illustrates the complexity of the interaction between immunodeficiency viruses and the immune system. In particular, HIV-2 appears to lead to a complex interplay between the activation state of the immune system and viral replication, with *nef* reducing immune activation while viral replication promotes it.

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