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

Jérôme Feldmann,1,2* Aleksandra Leligdowicz,1 Assan Jaye,1 Tao Dong,2 Hilton Whittle,1 and Sarah L. Rowland-Jones1,2

MRC Laboratories, Fajara, P.O. Box 273, The Gambia,1 and MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 0DW, United Kingdom2

Received 16 June 2009/Accepted 24 September 2009

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-naive 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 those with plasma viral loads above 2,000 copies/ml.
evolutionary distances were calculated in Tree-puzzle (24) software using the intrapatient consensus. Sequence alignments were made in Clustal X (16). The limiting dilution and sequenced from peripheral blood proviral DNA obtained in Biosciences). The data were analyzed using FlowJo (Tree Star). Gating on buffered saline solution before analysis using a FACSCalibur flow cytometer (BD Biosciences). The cells were washed twice and fixed with a 2% paraformaldehyde–phosphate-fluorescence-activated cell sorter (FACS) lysing solution (BD Biosciences). The T-cell surface activation marker expression using anti-HLA-DR–fluorescein isothiocyanate-, CD38-phycoerythrin (PE)-, CD4-peridinin chlorophyll protein-, and CD8 lymphocytes were stimulated 4 h after Amaca 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.

**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...
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 ($4.7 \pm 1.4$ for Ps versus $3.9 \pm 2.0$ 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.
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 gradual 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 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.

ACKNOWLEDGMENTS

J.F. was supported by a long-term fellowship from HFSPO and A.L. by the Rhodes Trust, and the study was funded by the Medical Research Council United Kingdom.

We thank all the donors for their samples and the field workers in Caio for their help in collecting them. We are particularly grateful to Tim Vincent for his long service as head of the Caio field station. We also thank Xiaoning Xu for the HIV-1 Nef controls.

REFERENCES

7. Rouleau, J.-F. was supported by a long-term fellowship from HFSPO and A.L. by the Rhodes Trust, and the study was funded by the Medical Research Council United Kingdom.

We thank all the donors for their samples and the field workers in Caio for their help in collecting them. We are particularly grateful to Tim Vincent for his long service as head of the Caio field station. We also thank Xiaoning Xu for the HIV-1 Nef controls.

ACKNOWLEDGMENTS

J.F. was supported by a long-term fellowship from HFSPO and A.L. by the Rhodes Trust, and the study was funded by the Medical Research Council United Kingdom.

We thank all the donors for their samples and the field workers in Caio for their help in collecting them. We are particularly grateful to Tim Vincent for his long service as head of the Caio field station. We also thank Xiaoning Xu for the HIV-1 Nef controls.