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Lentiviral Nef Proteins Manipulate T Cells in a Subset-Specific Manner

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ABSTRACT

The role of the accessory viral Nef protein as a multifunctional manipulator of the host cell that is required for effective replication of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) *in vivo* is well established. It is unknown, however, whether Nef manipulates all or just specific subsets of CD4$^+$ T cells, which are the main targets of virus infection and differ substantially in their state of activation and importance for a functional immune system. Here, we analyzed the effect of Nef proteins differing in their T cell receptor (TCR)-CD3 downmodulation function in HIV-infected human lymphoid aggregate cultures and peripheral blood mononuclear cells. We found that Nef efficiently downmodulates TCR-CD3 in naive and memory CD4$^+$ T cells and protects the latter against apoptosis. In contrast, highly proliferative CD45RA$^+$ CD45RO$^+$ CD4$^+$ T cells were main producers of infectious virus but largely refractory to TCR-CD3 downmodulation. Such T cell subset-specific differences were also observed for Nef-mediated modulation of CD4 but not for enhancement of virion infectivity. Our results indicate that Nef predominantly modulates surface receptors on CD4$^+$ T cell subsets that are not already fully permissive for viral replication. As a consequence, Nef-mediated downmodulation of TCR-CD3, which distinguishes most primate lentiviruses from HIV type 1 (HIV-1) and its *vpu*-containing simian precursors, may promote a selective preservation of central memory CD4$^+$ T cells, which are critical for the maintenance of a functional immune system.

IMPORTANCE

The Nef proteins of human and simian immunodeficiency viruses manipulate infected CD4$^+$ T cells in multiple ways to promote viral replication and immune evasion *in vivo*. Here, we show that some effects of Nef are subset specific. Downmodulation of CD4 and TCR-CD3 is highly effective in central memory CD4$^+$ T cells, and the latter Nef function protects this T cell subset against apoptosis. In contrast, highly activated/proliferating CD4$^+$ T cells are largely refractory to receptor downmodulation but are main producers of infectious HIV-1. Nef-mediated enhancement of virion infectivity, however, was observed in all T cell subsets examined. Our results provide new insights into how primate lentiviruses manipulate their target cells and suggest that the TCR-CD3 downmodulation function of Nef may promote a selective preservation of memory CD4$^+$ T cells, which are critical for immune function, but has little effect on activated/proliferating CD4$^+$ T cells, which are the main targets for viral replication.

Primate lentiviruses manipulate infected CD4$^+$ T cells in sophisticated ways in order to facilitate viral replication, and the accessory viral Nef protein is perhaps the most versatile modulator of the host cell (1, 2). The importance of Nef is evident from the finding that defective *nef* genes are associated with greatly attenuated viral replication in simian immunodeficiency virus (SIV)-infected macaques (3) and exceedingly low viral loads and long-term nonprogressive infection in human immunodeficiency virus type 1 (HIV-1)-infected humans (4, 5). The HIV-1 Nef protein performs a striking variety of activities, including downmodulation of CD4, CD28, and major histocompatibility complex class I (MHC-I), as well as enhancement of viral infectivity and replication (1, 2). HIV-1 Nef proteins also manipulate cell signaling pathways and modulate the interaction between T cells and antigen-presenting cells (6, 7). Finally, HIV-1 Nefs enhance the responsiveness of T cells to stimulation, and this effect may contribute to the high levels of immune activation and apoptosis that drive progression to AIDS (8–11).

Since *nef*-defective SIVmac and HIV-1 infection is associated with lack of or strongly delayed disease progression, Nef has long been considered a viral "pathogenicity" factor (12, 13). However, all primate lentiviruses encode Nef, and at least some of them replicate to high levels in their natural simian hosts without causing disease (14, 15). Thus, the association of efficient viral replication with the development of immunodeficiency seems to be a consequence of poor virus-host adaptation in recent or experimental hosts of primate lentiviruses. Indeed, some Nef functions may help these viruses to replicate efficiently in their natural simian hosts without causing disease (14, 16). Specifically, most Nef proteins block the responsiveness of virally infected T cells to

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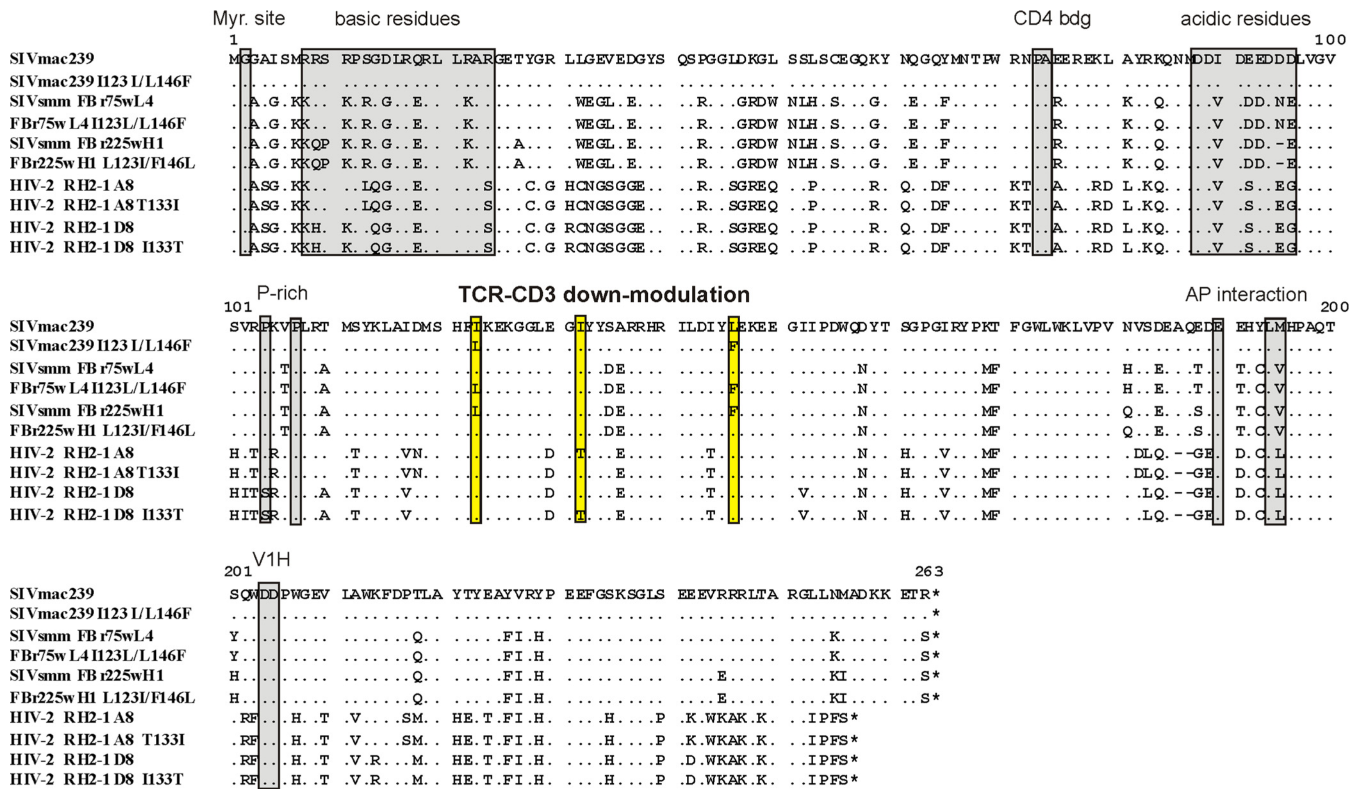

FIG 1 Alignment of Nef amino acid sequences differing specifically in the TCR-CD3 downmodulation function. The SIVmac239 Nef is shown on top for comparison. The myristoylated N terminus, a basic N-proximal region, the CD4 interaction site, the acidic region, and the PxxP motif, as well as the ExxxL/V/M adaptor protein interaction site in the flexible C-loop region, are indicated. Amino acid positions in the core region specifically involved in downmodulation of TCR-CD3 are highlighted by yellow bars. Dashes indicate gaps introduced to optimize the alignment.

stimulation by downmodulation of T cell receptor (TCR)-CD3 from the cell surface (11, 17–19). Exceptions are Nefs of HIV-1 and its *vpu*-containing SIV counterparts that lack this function entirely (11, 19). The efficiency of Nef-mediated downmodulation of TCR-CD3 correlates with high, stable CD4$^+$ T cell counts in sooty mangabeys that are naturally infected with SIV and do not develop disease (20) and in viremic HIV-2-infected individuals (21). Thus, most lentiviral Nef proteins may help the virally infected hosts to maintain normal CD4$^+$ T cell homeostasis by suppressing the activation and apoptosis of infected CD4$^+$ T cells.

The effect of Nef on cell surface expression of TCR-CD3 (11, 17–19) and the costimulatory CD28 receptor (22, 23) together with the manipulation of intracellular signaling pathways may help the virus to establish levels of CD4$^+$ T cell activation high enough to allow effective virus production but also low enough to avoid cell death prior to the completion of the viral replication cycle. It is well established that different CD4$^+$ T cell subsets that are targets of HIV and SIV infection differ fundamentally in their state of activation, their susceptibility to virus infection, and their importance for maintaining critical immune functions (24–26). It is currently unknown, however, whether differences in the ability of primate lentiviral Nefs to modulate CD4$^+$ T cell activation may affect the viral cell tropism and whether Nef manipulates all or just specific subsets of infected CD4$^+$ T cells. In the present study, we show that Nef-mediated downmodulation of TCR-CD3 is particularly effective in memory T cells and protects them against apoptosis. In contrast, modulation of TCR-CD3 as well as CD4 was ineffective in activated/proliferating CD45RA$^+$ CD45RO$^+$ T cells. Thus, the TCR-CD3 downregulation function of Nef, which is a hallmark of SIVs that do not cause disease in their natural hosts, efficiently protects memory CD45RA$^-$ CD45RO$^+$ CD4$^+$ T cells, which have the capacity of self-renewal and are thus important for the maintenance of a functional immune system (24–26), but hardly affects highly proliferating T cells, which are main producers of infectious virus.

MATERIALS AND METHODS

Proviral constructs. The generation of HIV-1 (NL4-3 based) proviral constructs carrying functional or disrupted *nef* genes followed by an internal ribosome entry site (IRES) and the enhanced green fluorescent protein (eGFP) gene has been described previously (11, 27). Splice-overlap extension PCR was used to replace the HIV-1 NL4-3 *nef* allele with the *nef* genes shown in Fig. 1. The integrity of all PCR-derived inserts was confirmed by sequence analysis. The *nef*-defective control constructs contained a mutation of the initiation codon and premature stop codons at positions 3 and 40 of the HIV-1 NL4-3 *nef*.

Cell culture. 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum. Peripheral blood mononuclear cells (PBMCs) from healthy human donors were isolated using lymphocyte separation medium (Biocoll separating solution; Biochrom), stimulated for 3 days with phytohemagglutinin (PHA) (1 μg/ml), and cultured in RPMI 1640 medium with 10% fetal calf serum (FCS) and 10 ng/ml interleukin-2 (IL-2) prior to infection.

Virus stocks and infections. To generate viral stocks, 293T cells were transfected with the X4 and R5 HIV-1 Nef/eGFP constructs as described previously (11) All HIV-1 constructs contained intact *env* genes and were thus replication competent following the first round of infection. The medium was changed after overnight incubation, and virus was harvested 24 h later. Residual cells in the supernatants were pelleted, and the supernatants were stored at −80°C. Virus stocks were quantified using a p24 antigen capture assay provided by the NIH AIDS Research and Reference Reagent Program. For infection experiments, 1×10^6 PBMCs were incubated with 50 ng p24 of virus stocks at 37°C for 4 to 6 h. Infected PBMCs were further incubated in RPMI 1640 medium with 10% FCS and 10 ng/ml IL-2. All results were derived from at least three different PBMC donors.

Human lymphocyte aggregate cultures (HLACs). Human tonsil tissues from routine tonsillectomies were obtained within 5 h of excision and processed as previously described (10, 28). In brief, tonsils were minced and cultured in 96-well U-bottom polystyrene plates (2×10^6 cells/well) in medium (200 μl/well) consisting of RPMI 1640 supplemented with 10% FCS and antibiotics. All HIV-1 infections were performed using virus stocks containing 0.5 ng p24 antigen. Cells were incubated with the virus for 12 to 16 h, washed extensively, and supplemented with fresh medium.

Flow cytometric analysis. CD4, TCR-CD3, MHC-I, CD28, and eGFP reporter expression in human PBMCs transduced with HIV-1 (NL4-3) constructs coexpressing Nef and eGFP was measured as described previously (11), and T cell activation markers were measured by standard fluorescence-activated cell sorter (FACS) staining using CD69 (BD Pharmingen, clone FN50) and CD25 (BD Pharmingen, clone M-A251) monoclonal antibodies (MAbs). For quantification of Nef-mediated modulation, the levels of receptor expression (red fluorescence) were determined for cells expressing a specific range of eGFP. The extent of downmodulation or induction (*n*-fold) was calculated by dividing the mean fluorescence intensity (MFI) obtained for cells infected with the *nef*-negative NL4-3 control viruses by the corresponding values obtained for cells infected with viruses coexpressing *nef* and the eGFP gene. For T cell subset analysis, stimulated human PBMCs or unstimulated HLACs were stained with the following combinations of antibodies: CD3-BD Horizon V450, CD4-peridinin chlorophyll protein (PerCP), CD45RA-phycoerythrin (PE)-Cy7, CD45RO-allophycocyanin (APC), CCR5-APC-Cy7, and CD62L/CCR7-PE. All antibodies were from BD Company. Cells were analyzed using the BD FACSCanto II with FACSDiva software.

Apoptosis in PBMCs and HLACs. PBMCs were first stimulated with PHA (1 μg/ml) for 3 days. Subsequently, the cells were cultured in RPMI 1640 (with 10% FCS and 10 ng/ml IL-2), infected with various HIV-1 eGFP/Nef constructs, and cultured for another 2 days. Thereafter, the PBMCs were treated a second time with PHA for another 3 days. Infected HLACs were cultured without stimulation as described previously (10). The frequency of virally infected apoptotic cells was determined using the annexin V (AnV) apoptosis detection kit (BD Bioscience) as recommended by the manufacturer.

Cell sorting. Sorting of naive, double-positive, and memory $CD4^+$ T cells from stimulated PBMCs, both infected and uninfected, was performed via a FACSaria flow cytometer (BD). Cells were initially gated on the basis of light scatter, followed by positive staining of CD3 and CD4. $CD3^+$ $CD4^+$ T cells with a naive (Tn), activated/proliferating double-positive (Tdp), or memory (Tm) phenotype were further gated on the basis of CD45RA and CD45RO expression. Phenotypic characterization of each T cell subset was performed immediately after sorting and at daily intervals. All cells were cultured in RPMI 1640 (with 10% FCS and 10 ng/ml IL-2) for further experiments.

Cell differentiation and proliferation assay. To examine their differentiation, sorted $CD4^+$ Tn, Tdp, and Tm cells were cultured for 3 days and stained with the following combination of antibodies: CD3-BD Horizon V450, CD4-PerCP, CD45RA-PE-Cy7, and CD45RO-APC. For the analysis of proliferation rates, sorted cells were labeled with CellTrace carboxyfluorescein succinimidyl ester (CFSE) immediately after sorting according to the manufacturer's protocol and were cultured for 3 days. Differentiation and proliferation were measured using the BD FACSCanto II with FACSDiva software.

Virus production and infectivity. Sorted $CD4^+$ Tn, Tdp, and Tm cells were infected with HIV-1 NL4-3 IRES-eGFP constructs, and the culture supernatants were collected at 3 days postinfection. Viral p24 capsid antigen was measured by an enzyme-linked immunosorbent assay (ELISA) provided by the NIH AIDS Research and Reference Reagent Program. Virus infectivity was determined using TZM-bl cells as described previously (29).

Microscopy. Sorted $CD4^+$ Tn, Tdp, and Tm cells were infected with *env*-defective HIV-1 NL4-3 IRES-eGFP virus pseudotyped with the vesicular stomatitis virus G glycoprotein (VSV-G) or mock treated as described previously (11). After overnight incubation, residual virus was removed by extensive washing with phosphate-buffered saline (PBS). Cells were cultivated on microscopy slides, and images were acquired on an LSM 710 confocal microscope (Carl Zeiss, Germany).

Statistical analysis. Groups were compared using a two-tailed Student *t* test. The PRISM package version 4.0 (Abacus Concepts, Berkeley, CA, USA) was used for all calculations.

RESULTS

Generation of HIV-1 constructs differing in the TCR-CD3 downmodulation function of Nef. To examine whether Nef-mediated downmodulation of TCR-CD3 may affect viral tropism for different T cell subsets, we cloned 10 *nef* alleles differing in this activity (Fig. 1) into a replication-competent HIV-1 (NL4-3-based) proviral vector designed to coexpress Nef and eGFP from bicistronic RNAs (Fig. 2A). In these constructs, expression of all viral genes is mediated by the wild-type (wt) HIV-1 long terminal repeat (LTR) promoter, and infected cells coexpress Nef and eGFP at correlating levels (11). Our collection encompassed five *nef* alleles from HIV-2, SIVmac, and SIVsmm. SIVsmm is the direct precursor of HIV-2 and replicates to high levels in its natural sooty mangabey (*Cercocebus atys*) host without causing disease (14). Recently, we identified amino acid substitutions in the core region of HIV-2 (I133T) and SIVsmm (I123L/L146F) Nefs that specifically disrupt their CD3 downmodulation function (21, 30). An analogous mutant SIVmac239 Nef containing alterations of I123L, L146F, and D158N was generated by site-directed mutagenesis (Fig. 1). Analysis of these *nef* alleles in the context of HIV-1 may not fully recapitulate the effects in SIV-infected monkeys. However, utilization of HIV-1 constructs that differ by only a few mutations in Nef that specifically affect the TCR-CD3 downmodulation activity ensured that the observed phenotypes were indeed due to differences in Nef function and not due to variations in other viral properties. Flow cytometric analyses confirmed that the amino acid changes in the core region of Nef affected modulation of TCR-CD3 but not CD4, CD28, and class I MHC surface expression (Fig. 2B). Altogether, five pairs of *nef* alleles that differed specifically in the CD3 downmodulation function were used in the present study (Fig. 1). For simplification, we designated the five SIV and HIV-2 Nefs that downmodulate TCR-CD3 (i.e., wt SIVmac239, SIVsmm FBr75wL4, FBr225wH1 L123I/F146L, HIV-2 RH2-1A8 T133I, and HIV-2 RH2-1D8) "CD3d" Nefs and the five *nef* alleles lacking this function (i.e., SIVmac239 I123L/L146F, FBr75wL4 I123L/L146F, SIVsmm FBr225wH1, HIV-2 RH2-1A8, and HIV-2 RH2-1D8 I133T) "CD3h" Nefs. As controls, we used the parental NL4-3 Nef, which is inactive against TCR-CD3 and less potent than SIV or HIV-2 Nefs in modulating the CD28 receptor (Fig. 2B), and an otherwise isogenic *nef*-defective HIV-1 NL4-3 IRES-eGFP construct.

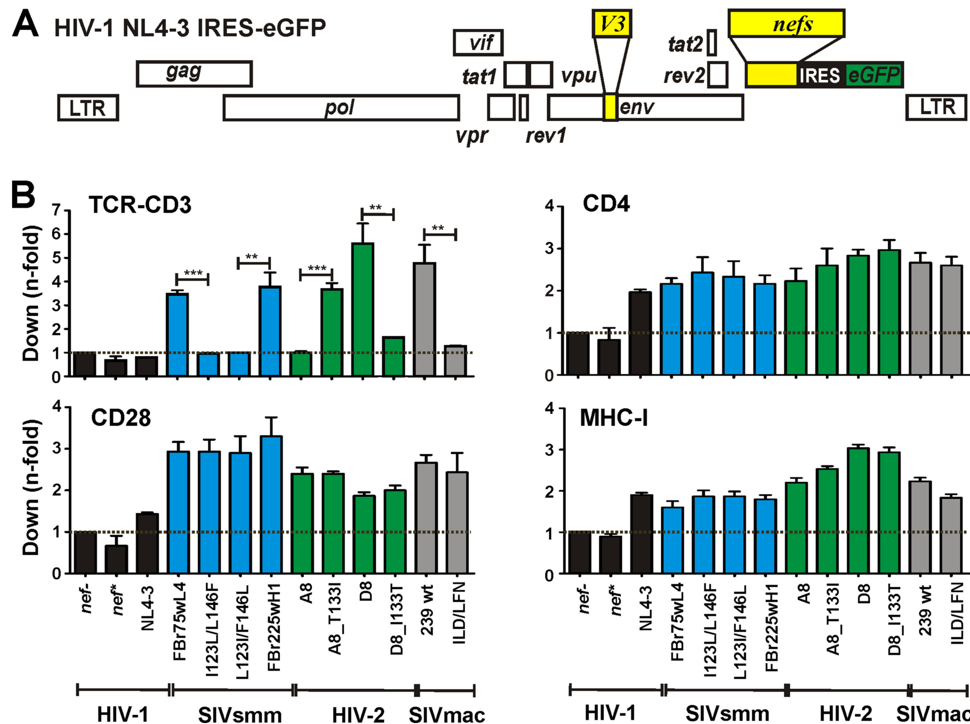

FIG 2 HIV-1 constructs expressing *nef* alleles differing in TCR-CD3 downmodulation. (A) Schematic presentation of proviral HIV-1 NL4-3 IRES-eGFP constructs carrying various *nef* alleles and containing the V3 loop regions of the wt CXCR4-tropic NL4-3 virus or the CCR5 tropic 92th014.12 HIV-1 strain (29). (B) Quantification of Nef-mediated downmodulation of TCR-CD3, CD4, CD28, and MHC-I on PBMCs infected with HIV-1 Nef/eGFP constructs. Data show mean values ± SEM derived from three independent experiments.

Comparison of the PBMC and HLAC systems. To explore the ability of Nef to modulate the expression of surface receptors on different $CD4^+$ T cell subsets, we utilized both peripheral blood mononuclear cells (PBMCs) and human lymphocyte aggregate cultures (HLACs). PHA-activated PBMCs contain the relevant $CD4^+$ target T cell populations of HIV-1 and are commonly used to examine virus infection and Nef function. However, these PBMC cultures do not fully recapitulate the conditions encountered by HIV-1 *in vivo*, where many cells are resting or minimally activated. Thus, we also utilized unstimulated HLACs prepared from freshly dissected human tonsils. HLACs closely recapitulate the conditions in lymphoid organs (10, 28), which are the primary sites of HIV replication and contain the vast majority of the body's $CD4^+$ T cells. Importantly, HLACs can be productively infected with HIV-1 in the absence of artificial mitogens and thus allow analysis of Nef function under conditions closely mimicking the *in vivo* situation (10). To characterize both experimental systems, we first determined the fractions of $CD3^+$ $CD4^+$ T cells with a naive (Tn, $CD45RA^+$ $CD45RO^-$), activated/proliferating double-positive (Tdp, $CD45RA^+$ $CD45RO^+$), or memory (Tm, $CD45RA^-$ $CD45RO^+$) phenotype in uninfected PBMC cultures and HLACs (Fig. 3A). We then further distinguished between central (Tcm, $CD62L^+$) and effector (Tem, $CD62L^-$) memory T cells. Our results showed that the fraction of Tn cells was significantly lower (20.5% ± 2.6% versus 41.0% ± 2.6%; $P < 0.0001$) (mean ± standard error of the mean [SEM]; $n = 6$ to 8) and the fraction of Tm cells significantly higher (58.9% ± 3.9% versus 40.7 ± 6.9%; $P = 0.0308$) in PBMCs than in HLACs (Fig. 3A, right panels). In both culture systems, Tcm cells were substantially more frequent (>85%) than Tem cells (<15%). A total of 16.6% ± 2.0% and 8.6% ± 1.0% of $CD4^+$ T cells expressed both CD45RA and CD45RO in PBMCs and HLACs, respectively. Notably, these $CD45RA^+$ $CD45RO^+$ Tdp cells expressed substantially higher levels of CCR5, the main coreceptor of HIV-1 and SIV entry, than Tn and Tm cells (Fig. 3A and B).

Activated/proliferating Tdp cells are main targets of HIV-1 infection. To examine the susceptibility of different T cell subsets to HIV-1 infection, PBMCs and HLACs were exposed to R5- and X4-tropic HIV-1 IRES-eGFP constructs containing various *nef* genes. It has been previously shown that these HIV-1 reporter constructs replicate efficiently in infected PBMC cultures (19, 21). We found that HIV-1 infected (eGFP-positive [$eGFP^+$]) cells were readily detectable at 3 days postinfection (Fig. 3C). Exposure of PBMCs and HLACs to X4 virus resulted in slightly higher infection rates (about 5% and 0.4%, respectively) than exposure to R5 virus (about 3% and 0.2%, respectively) (Fig. 3C and D) because a larger fraction of $CD4^+$ T cells expresses CXCR4 than CCR5 (31). Intact *nef* genes increased viral infection about 3- to 8-fold, irrespective of the TCR-CD3 downmodulation function and the viral coreceptor tropism (Fig. 3D).

Most HIV-1-infected $eGFP^+$ cells (60% to 80%) in PBMC cultures and HLACs were Tm cells (Fig. 4A). In HLACs, about half of these were $CD62L^-$ Tem cells, although this subset represented only ~14% of total RA^- RO^+ Tm cells (Fig. 4B). RA^+ RO^- Tn cells were largely refractory and activated/proliferating Tdp cells were highly susceptible to HIV-1 infection (Fig. 4A). In HLACs, Tdp represented 22.9% ± 4.3% (X4) and 31.8% ± 5.2% (R5) of HIV-1-infected cells but just 8.6% ± 2.5% of uninfected $CD4^+$ T

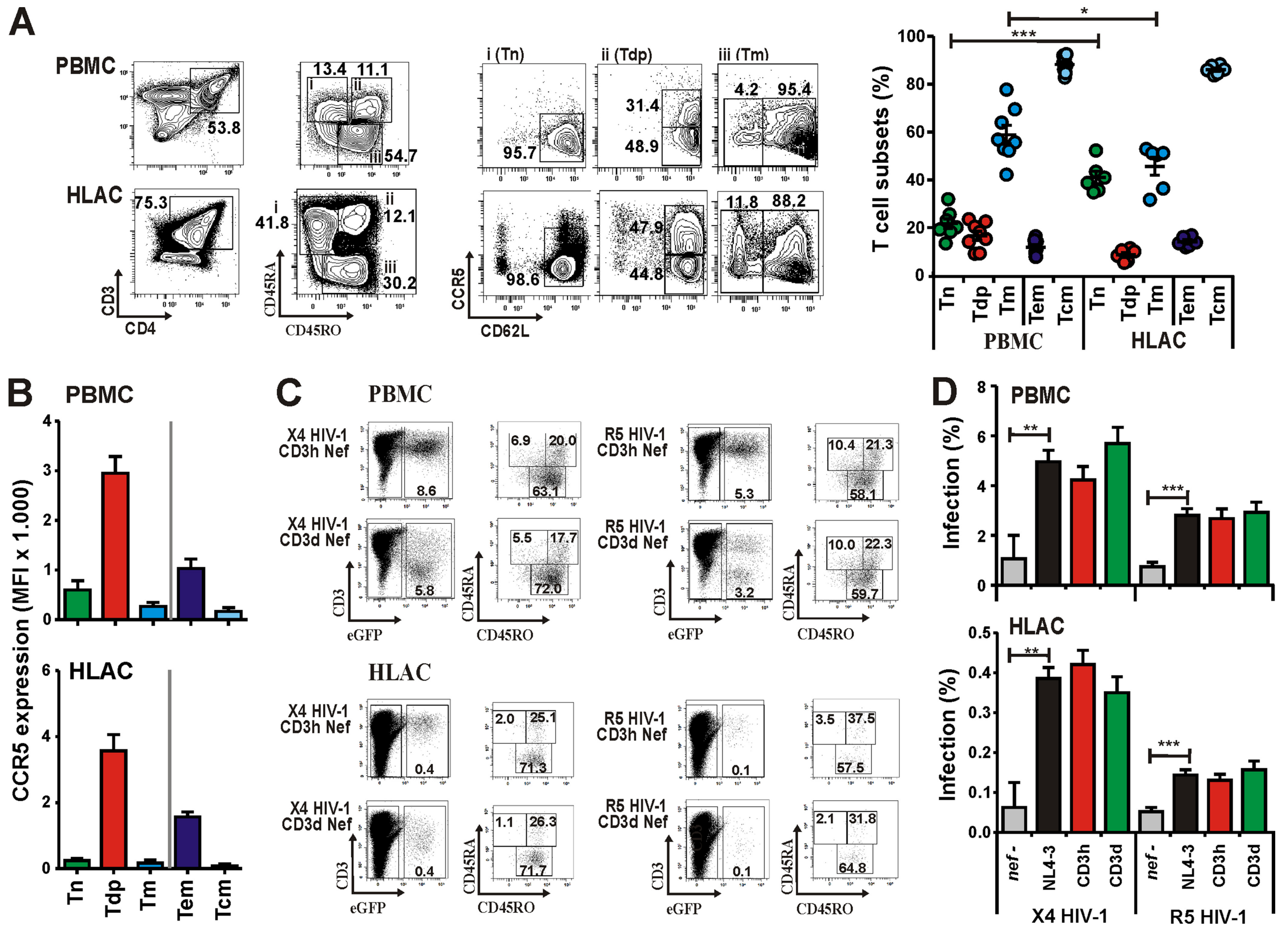

FIG 3 Characterization of uninfected and HIV-infected CD4$^+$ T cell subsets in HLACs and PBMC cultures. (A) Expression of the surface markers CD45RA, CD45RO, CCR5, and CD62L on CD4$^+$ T cells from representative PBMC and HLACs. Numbers give percentages of cells in the respective gates. The right panel gives the mean percentages (±SEM; $n = 8$) of the different T cell subsets in the cultures. (B) Mean fluorescence intensities (MFIs) of CCR5 expression in different T cell subsets. (C) PBMCs or HLACs were infected with X4- and R5-tropic HIV-1 IRES-eGFP constructs and analyzed by flow cytometric analysis. Shown are examples of primary data obtained for viral constructs expressing *nef* alleles that are inactive (CD3h) or active (CD3d) in downmodulation of TCR-CD3. Numbers give the percentage of cells in the respective gate. (D) Frequencies of virally infected (eGFP$^+$) cells after exposure to X4 and R5 HIV-1 constructs differing in their *nef* genes. Virus stocks containing normalized quantities of p24 antigen were used for infection. Values in all panels are averages (±SEM) from at least three independent experiments and 6 to 14 infections. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

cells (Fig. 4A). As mentioned above, HLACs were infected in the absence of artificial mitogens and allow the analysis of HIV-1 infection in a lymphoid microenvironment that closely mimics the *in vivo* situation. Overall, the rates of HIV-1 infection of different T cell subsets correlated well between R5 and X4 HIV-1 constructs as well as between PBMC cultures and HLACs (Fig. 4C). However, the R5 HIV-1 constructs were more effective than X4 viruses in infecting RA$^+$ RO$^+$ Tdp cells in HLACs (26.5% ± 1.6% versus 16.3 ± 1.3% [$n = 13$]; $P < 0.0001$), whereas the opposite was observed in PBMCs (22.9% ± 1.2% versus 31.8% ± 1.4% [$n = 13$]; $P < 0.0001$) (Fig. 4D). This difference may be due to particularly high levels of CCR5 expression by Tdp in HLACs (Fig. 3A and B). Taken together, these results showed that CD4$^+$ Tdp and Tem cells are preferential targets of R5 and X4 HIV-1 infection in both HLACs and PBMC cultures.

CD3 modulation is associated with slightly increased frequencies of HIV-infected Tcm cells. While most primate lentiviral Nefs suppress the activation of infected CD4$^+$ T cells by downmodulating the TCR-CD3 complex (11, 19), HIV-1 Nef is unable to perform this function and may even render infected cells hyperresponsive to stimulation (8, 9). We thus hypothesized that lack of TCR-CD3 downmodulation may facilitate productive virus infection in CD4$^+$ T cells whose preexistent level of activation is near the threshold of activation required for effective virus replication. To test this hypothesis, we compared the rates of infection for the HIV-1 constructs expressing Nef proteins that are active (CD3d) or inactive (CD3h) in this function. As shown in Fig. 5A, the inability of Nef to downmodulate TCR-CD3 was associated with slightly increased frequencies of virally infected (eGFP$^+$) Tdp cells and decreased numbers of eGFP$^+$ Tm cells. This difference was observed in both PBMCs and HLACs and was independent of the viral coreceptor tropism. Further examination revealed that lack of Nef-mediated downmodulation of TCR-CD3 (CD3h Nefs) specifically reduced the percentage of virally infected

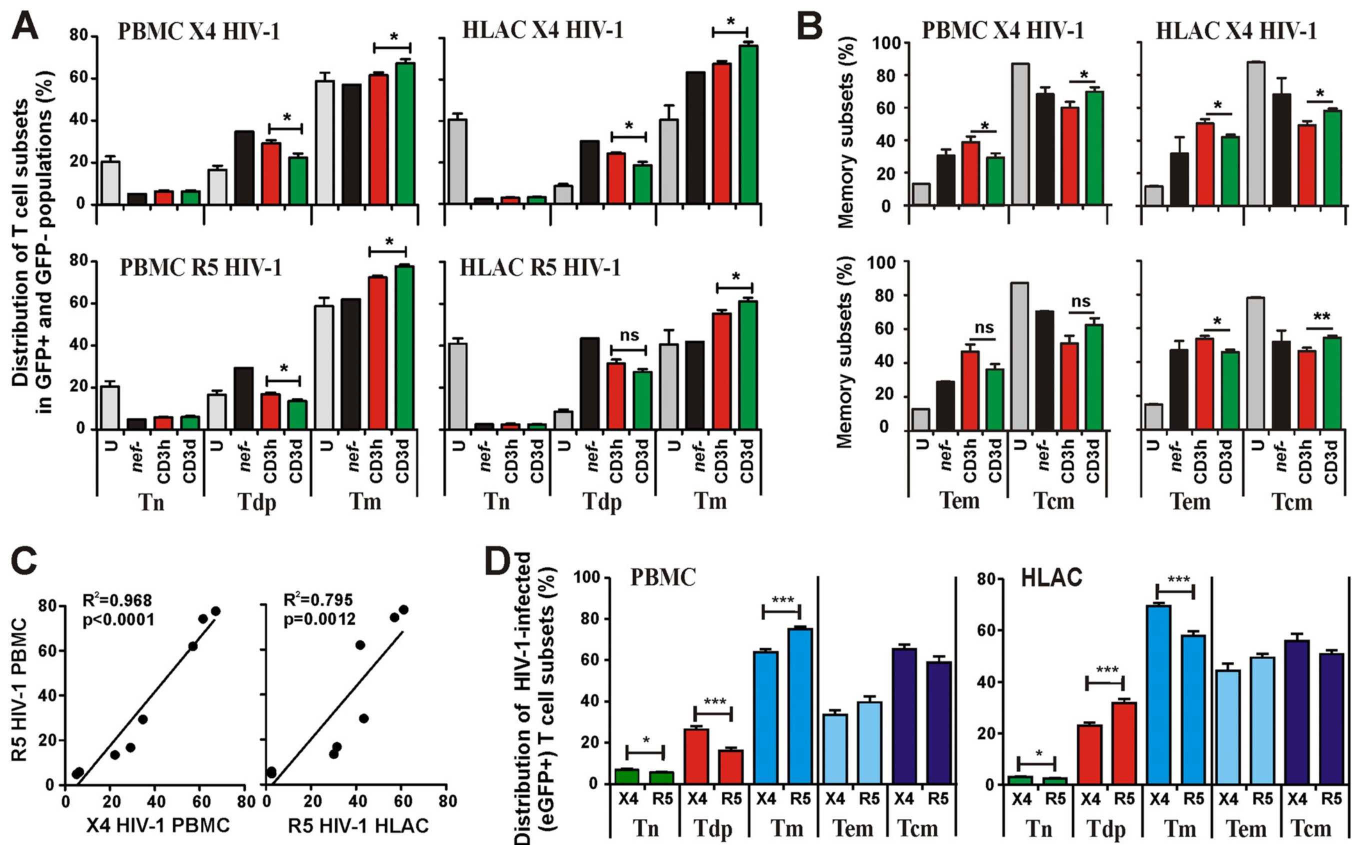

FIG 4 Effect of TCR-CD3 downmodulation on the viral tropism for different T cell subsets. (A and B) Virus constructs were grouped based on the expression of *nef* alleles that do or do not downmodulate TCR-CD3. A construct containing a defective *nef* gene (*nef*−) was used as control. The distributions of Tn, Tdp, and Tm cells (A) and of effector (Tem) and central (Tcm) memory T cells (B) in the uninfected (U) (GFP^-) and virally infected (GFP^+) populations was determined. The gates used to calculate these percentages are shown in Fig. 2C. Shown are mean values (±SEM; $n = 6$). *, $P < 0.05$. The same virus stocks were used in the PBMC and HLAC experiments. Similar results were obtained in three independent experiments using cells or tissues from different donors. (C) Correlation between the infection of different T cell subsets in PBMCs infected with R5 HIV-1 with the percentages in X4 HIV-1 (left)- or R5 HIV-1 (right)-infected HLACs. Percentages were derived from the results shown in panels A and B. (D) Fractions of the indicated subsets of T cells infected with X4 or R5 HIV-1 constructs.

$CD62L^+$ Tcm cells but enhanced the proportion of $eGFP^+$ $CD62L^-$ Tem cells (Fig. 5B). Altogether, the effects of CD3 downmodulation on viral infection of different T cells subsets were modest but were observed for both X4 and R5 HIV-1 constructs and in HLACs as well as PBMC cultures.

Ineffective Nef-mediated downmodulation of TCR-CD3 in $CD4^+$ Tdp cells. It has been previously shown that Nef-mediated downmodulation of TCR-CD3 is highly effective in X4 HIV-1-infected PBMC cultures (11). In agreement with these results, about 80% of PBMCs infected with X4 or R5 HIV-1 constructs expressing CD3d SIVsmm, SIVmac, or HIV-2 Nefs expressed low levels of CD3 (Fig. 5A). Some HIV-1 infected ($eGFP^+$) T cells, however, maintained high levels of TCR-CD3 cell surface expression that were similar to those observed in cells infected with wt HIV-1 strains, with the highest proportion (∼50%) observed in HLACs infected with R5 viruses (Fig. 5A, right panels). To determine possible subset-specific differences in the susceptibility of $CD4^+$ T cells to Nef-mediated downmodulation of TCR-CD3, we next analyzed the composition of T cell subsets in the CD3high and CD3low populations of virally infected $eGFP^+$ cells. We found that the CD3high (CD3-H) population contained up to 60% of activated/proliferating Tdp cells, whereas the CD3low (CD3-L) population generally contained ∼80 to 90% of RA^- RO^+ Tm cells (Fig. 5B). Furthermore, the CD3low population contained a higher proportion of $CD62L^-$ Tem cells (30 to 60%) than the CD3high population (14 to 25%) (Fig. 5B). These striking differences in the composition of infected T cells in the CD3high and CD3low populations were highly reproducible and were observed irrespective of the viral coreceptor tropism and the culture system. Thus, Nef-mediated downmodulation of TCR-CD3 is effective in naive and memory T cells (60% to 90%) but not in activated/proliferating Tdp cells (<30%) that are highly permissive for HIV-1 infection (Fig. 5C).

The effect of Nef on CD4 cell surface expression is also T cell subset specific. We next examined whether primate lentiviral Nef proteins also modulate surface expression of the CD4 molecule in a T cell subset-specific manner. Predictably, most T cells (>90%) infected with *nef*-deleted HIV-1 expressed CD3, and about 60% of these were positive for CD4 (Fig. 6A and B). In contrast, most T cells (>80%) infected with an HIV-1 construct expressing the parental NL4-3 Nef were positive for CD3 but CD4 negative, whereas T cells infected with a virus expressing the SIVmac239 Nef were usually negative for both CD3 and CD4 (79.5% ± 3.9%) (Fig. 6A and B). Similar results were obtained using a *vpu*-defec-

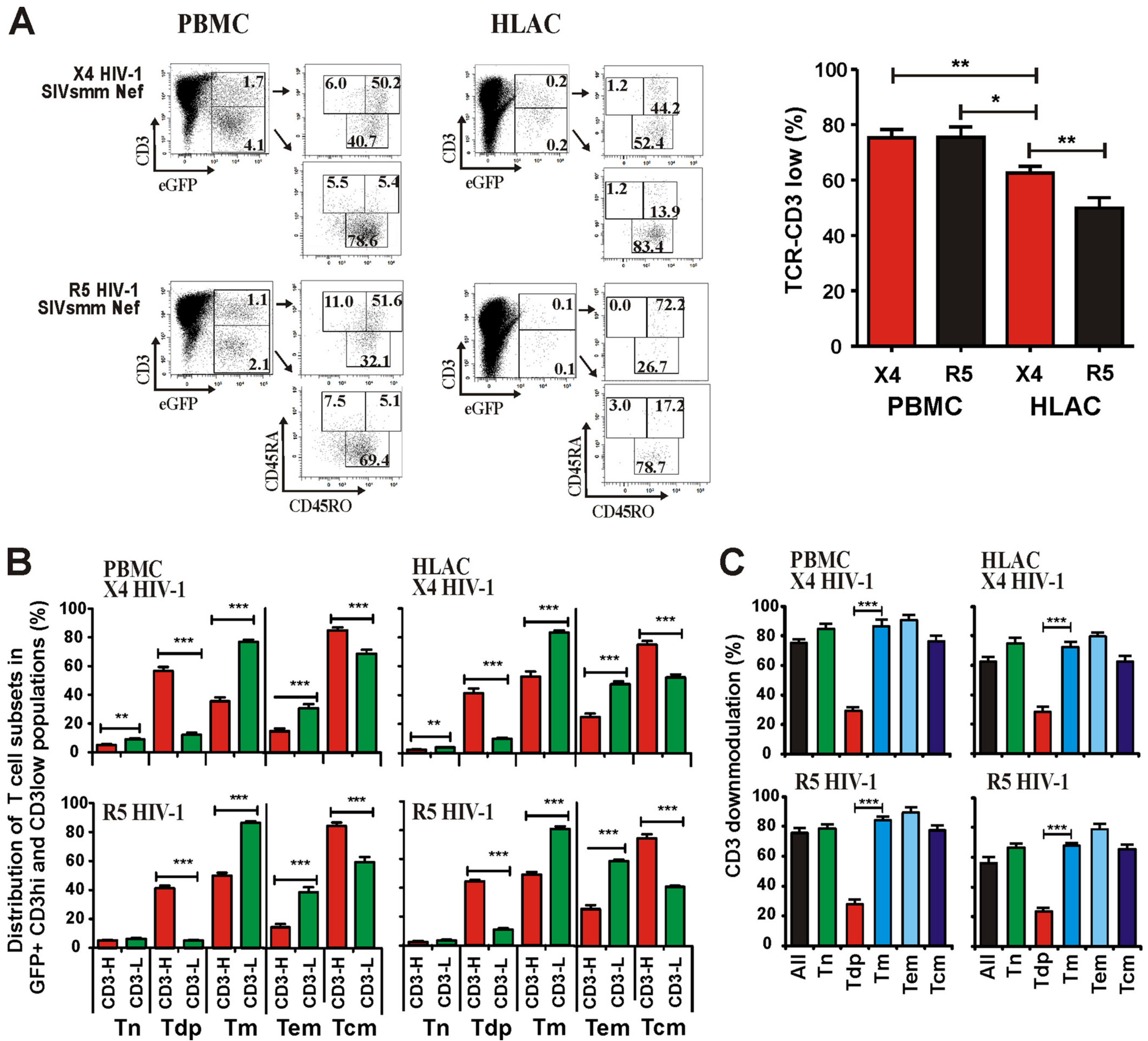

FIG 5 Nef-mediated modulation of TCR-CD3 is inefficient in Tdp cells. (A) PBMC cultures and HLACs were infected with X4 or R5 HIV-1 IRES-eGFP constructs expressing *nef* alleles capable of downmodulating TCR-CD3 and analyzed by flow cytometric analysis. HIV-1-infected $CD4^+$ T cells were grouped based on their CD3 expression levels and further examined for expression of CD45RA, CD45RO, and CD62L. Numbers give percentages in the respective gates. The right panel indicates the percentage of virally infected T cells in the CD3low gate. All bar diagrams represent mean percentages (±SEM; $n = 6$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (B) Frequencies of the indicated T cell subsets in the virally infected ($eGFP^+$) cell populations expressing high (red) or low (green) levels of TCR-CD3. (C) Efficiency of Nef-mediated downmodulation of TCR-CD3 in different T cell subsets.

tive viral backbone except that the frequency of $CD4^+$ cells increased since Vpu is capable of degrading the CD4 receptor (Fig. 6B, right panel). Thus, in agreement with our previous data on TCR-CD3 (Fig. 5A), CD4 downmodulation was observed in most (~80%) but not all HIV-1-infected $CD4^+$ T cells. Next, we determined the composition of virally infected T cell subsets that differed in their TCR-CD3 and CD4 expression levels. The results showed that 48.9% ± 8.1% of virally infected cells in the $CD4^+$ $CD3^+$ gate but only 3.8% ± 2.0% of those in the $CD4^-$ $CD3^-$ gate were comprised of RA^+ RO^+ Tdp cells (Fig. 6C). In contrast, only 48.7% ± 8.5% of HIV-1 infected ($eGFP^+$) cells in the $CD4^+$ $CD3^+$ gate but 91.0% ±3.6% of T cells in the $CD4^-$ $CD3^-$ double-negative/low population represented RA^+ RO^- Tm cells (Fig. 6C). These results were confirmed in a *vpu*-defective viral backbone (Fig. 6C) and showed that the effect of Nef on CD4 surface expression is also T cell subset specific.

To exclude the possibility that the activity of the IRES element may depend on the subset of virally infected T cells, we also examined proviral constructs expressing Nef-eGFP fusions. The results showed that Nef-eGFP fusions downmodulate CD4 and (in the

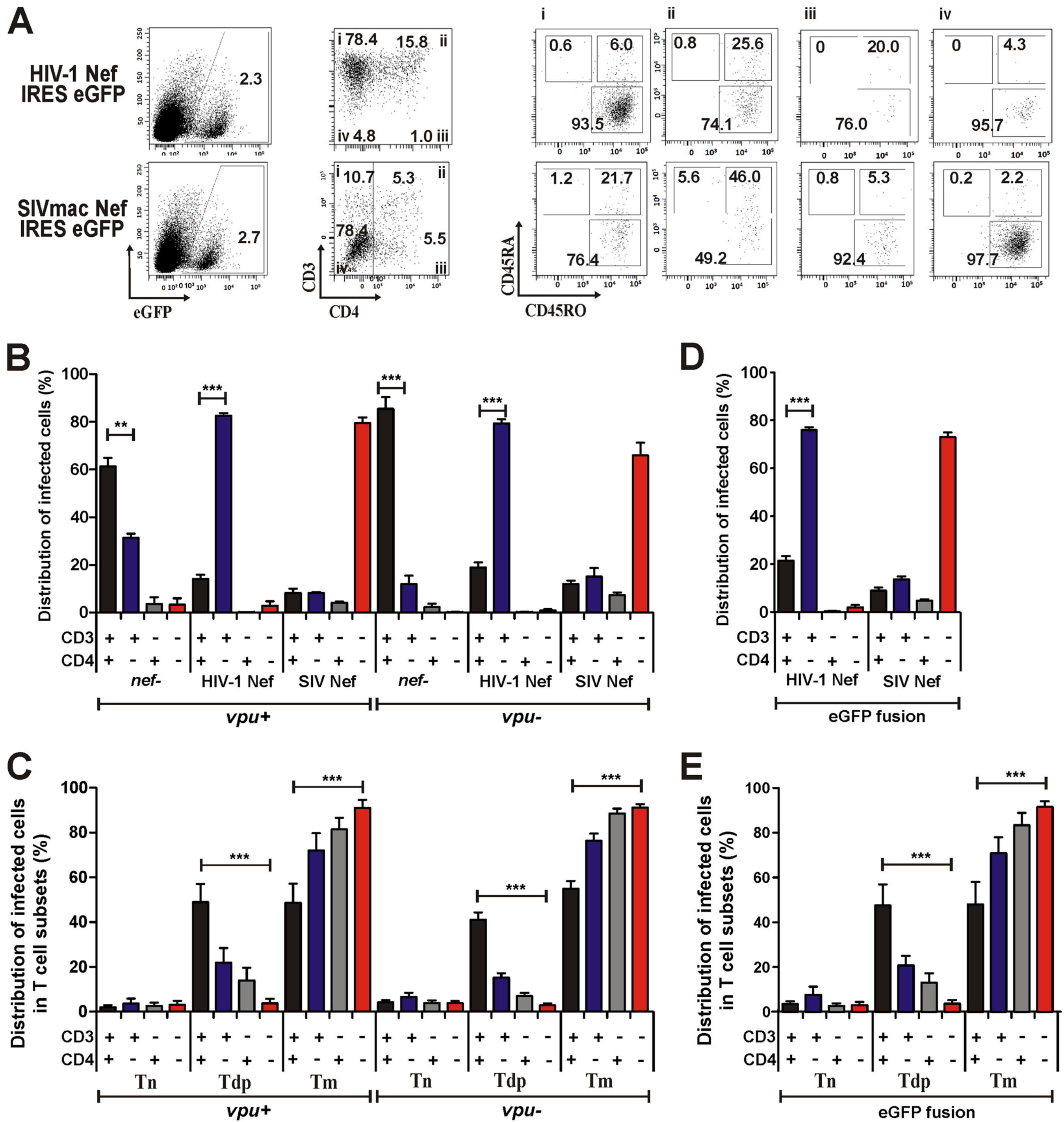

FIG 6 Nef modulates both TCR-CD3 and CD4 in a T cell subset-specific manner. (A) PBMCs were infected with X4 HIV-1 IRES-eGFP constructs expressing the parental NL4-3 or SIVmac239 Nef proteins. Virally infected (eGFP$^+$) cells were first examined for CD3 and CD4 expression, and subsequently cells in the four indicated gates were examined for the levels of CD45RA and CD45RO expression to distinguish Tn, Tdp, and Tm subsets. (B) Frequency of CD3- and/or CD4-expressing PBMCs infected with wild-type and *vpu*-defective X4 HIV-1 IRES-eGFP constructs differing in their *nef* genes. All panels show mean percentages (±SEM) of infected T cells in the respective gate measured in at least three experiments. (C) Expression of CD3 and/or CD4 by the indicated subsets of T cells infected with *vpu*-positive (left) or *vpu*-negative (right) HIV-1 NL4-3 constructs expressing eGFP and the SIVmac239 Nef protein via an IRES element. (D) Frequency of CD3- and/or CD4-expressing PBMCs infected with X4 HIV-1 constructs expressing fusions between the HIV-1 NL4-3 or SIVmac239 Nefs and eGFP. (E) Expression of CD3 and/or CD4 by the indicated subsets of T cells infected with an HIV-1 NL4-3 construct expressing a mac239 Nef-eGFP fusion.

case of SIVmac239 Nef) TCR-CD3 in ~80% of infected T cells (Fig. 6D) and that this effect is highly effective in Tm cells but not in Tdp cells (Fig. 6E). Thus, the different CD4 and CD3 downmodulation efficacies in Tm and Tdp cells were confirmed in the absence of the IRES element and are most likely relevant in HIV-1 infection *in vivo*.

TCR-CD3 downmodulation is associated with low levels of T cell apoptosis. To examine the effect of Nef on the activation of virally infected $CD4^+$ T cells, we measured the induction of CD69 as an early marker, and the IL-2 receptor (CD25) as a late marker, for cellular activation in HIV-1-infected PBMC cultures. In agreement with previous studies using X4 HIV-1 NL4-3 *nef*-IRES-eGFP constructs (11, 21), Nef-mediated downmodulation of TCR-CD3 was associated with suppression of T cell activation, although the differences in the expression of CD69 and CD25 were relatively modest for the overall population of virally infected cells (Fig. 7A and B). To examine the effect of TCR-CD3 surface expression in more detail, we next quantified the expression levels of T cell activation markers in the CD3high and CD3low populations of the same PBMC cultures infected with HIV-1 constructs capable of CD3 downmodulation. These analyses showed that the levels of CD69 and CD25 expression in the CD3low T cell population were similar to those found in uninfected T cells (Fig. 7A and B). In contrast, expression of T cell activation markers in the CD3high population was exceedingly high. The CD3low population contains mainly (~90%) Tm cells (Fig. 5B). Thus, Nef-mediated downmodulation of TCR-CD3 efficiently prevents the activation of memory T cells.

Increased programmed death of T cells is a hallmark of pathogenic lentiviral infections and may represent a key contributor to immune exhaustion and disease progression. We found that PBMCs infected with *nef*-defective X4 or R5 HIV-1 constructs showed about 3- to 4-fold-higher levels of apoptosis (measured as the percentage of annexin V-positive cells) than uninfected cells (Fig. 7C). These levels were similar in PBMCs infected with HIV-1 constructs expressing CD3h Nefs (X4, 30.0% ± 2.3%; R5, 29.0% ± 2.1% [$n = 5$]) but significantly reduced in cells expressing CD3d Nefs (X4, 24.2% ± 1.6%; R5, 24.0% ± 1.5% [$n = 5$]) (Fig. 7C). Since Nef may trigger apoptosis of uninfected bystander $CD4^+$ T cells (32), we always determined the levels of apoptotic T cells in both the uninfected ($eGFP^-$) and HIV-1-infected ($eGFP^+$) T cells. However, apoptosis increased only in virally infected cells, with substantial differences between the CD3high and CD3low T cell populations (Fig. 7C). For example, the average frequency of apoptotic cells in the CD3high population of X4 HIV-1-infected constructs was 45.8% ± 4.1%, while only 15.3% ± 0.9% of cells in the CD3low population were apoptotic. Similar results were obtained with R5 HIV-1 (Fig. 7C).

We next examined the effects of Nef on HIV-1-dependent programmed cell death in unstimulated HLACs. Unexpectedly, even *nef* alleles that do not modulate TCR-CD3 reduced the levels of apoptotic cells in HIV-1-infected HLACs, from 60.3% ± 9.1% to 44.1% ± 3.9% (X4) and from 51.7% ± 10.6% to 32.7% ± 6.5% (R5) (Fig. 7D). Indeed, on average, apoptosis was reduced to similar levels in HLACs infected with HIV-1 constructs that were active or inactive in TCR-CD3 downmodulation. Further examination revealed, however, that the CD3high population of virally infected T cells showed very high levels (X4, 69.9% ± 4.6%; R5, 52.3% ± 6.4%) of programmed death, whereas the CD3low population was protected against apoptosis (X4, 20.3% ± 2.0%; R5, 11.9% ± 1.8%) (Fig. 7D). Finally, we analyzed the protective effects of Nef-mediated downmodulation of TCR-CD3 in $CD4^+$ T cells purified from 14 different PBMC donors. CD3 downmodulation reduced the overall levels of apoptosis from 35.4% ± 4.3% to 15.2% ± 2.5% (Fig. 7E). Unexpectedly, this protective effect was more pronounced for the RO^+ $CCR7^+$ Tcm subset (Fig. 7E). Thus, Nef proteins that downmodulate TCR-CD3 efficiently prevent apoptosis in virally infected central memory T cells.

Highly proliferative Tdp cells are main producers of infectious HIV-1. To determine whether different T cell subsets may differ not only in their susceptibility to HIV-1 infection (Fig. 4A and B) but also in the level of virus production on a per-cell basis, we isolated the Tn, Tdp, and Tm cell subsets by flow cytometry sorting from PBMC cultures. Postsorting FACS analyses confirmed that the great majority of Tn and Tm cells (>95%) showed the expected phenotype, whereas the purity of Tdp cells was only about 60% (Fig. 8A), possibly due to the relatively low percentage and rapidly changing phenotypic properties of these cells. Specifically, Tn and Tdp cells rapidly differentiated to Tm cells, whereas the phenotype of Tm cells remained stable (Fig. 8A and B). In agreement with their high state of activation, Tdp cells showed much higher rates of proliferation (64.8% ± 9.4%) than either Tn (26.5% ± 3.1%) or Tm (4.9% ± 1.5%) cells (Fig. 8C).

To examine the efficiency of virus production, T cells were transduced with VSV-G-pseudotyped HIV-1-IRES-eGFP constructs immediately after sorting. VSV-G-pseudotyped virus was used to achieve effective infection of all T cell subsets irrespective of coreceptor expression and Nef function. Flow cytometric analysis showed that the number of wt HIV-1-infected ($eGFP^+$) Tdp cells at 3 days postinfection was only about 2-fold higher (9.3% ± 1.8%; $n = 3$) than those of the Tn and Tm cell subsets (3.5% ± 0.5% and 4.4% ± 1.0%, respectively) (Fig. 9A). In comparison, the levels of p24 antigen detected in the culture supernatants of Tdp cells were about 4- to 6-fold higher than those in Tn and Tm cells, respectively (Fig. 9B). Most strikingly, however, Tdp cells produced about 20-fold more infectious HIV-1 than Tm cells (Fig. 9C). Tn cells showed an intermediate phenotype, most likely because they rapidly differentiated to Tdp and Tm cells (Fig. 8A and B) and may produce HIV-1 during the transition period at the Tdp stage. Of note, Nef enhanced infectious HIV-1 production more efficiently (~10- to 20-fold) than p24 antigen production (~2- to 4-fold), suggesting that Nef increased the infectiousness of progeny HIV-1 virions produced by all three T cell subsets. Most importantly, these results strongly suggest that highly proliferative Tdp cells are main producers of infectious HIV-1.

To investigate the mechanism(s) underlying the different efficiencies of infectious virus production, we first determined the levels of eGFP expression in HIV-1-infected T cells. Since proviral HIV-1 constructs express eGFP together with Nef via the regular LTR promoter and splice sites, they serve as an indicator of the proviral transcriptional activity. We found that intact *nef* genes increased LTR-dependent eGFP expression by ~1.5- to 2-fold irrespective of their TCR-CD3 modulation activity (Fig. 9D). This was unexpected because CD3 downmodulation is associated with reduced T cell activation (11, 21). Nonetheless, the average levels of eGFP expression in the CD3low population of virally infected T cells in PBMC cultures

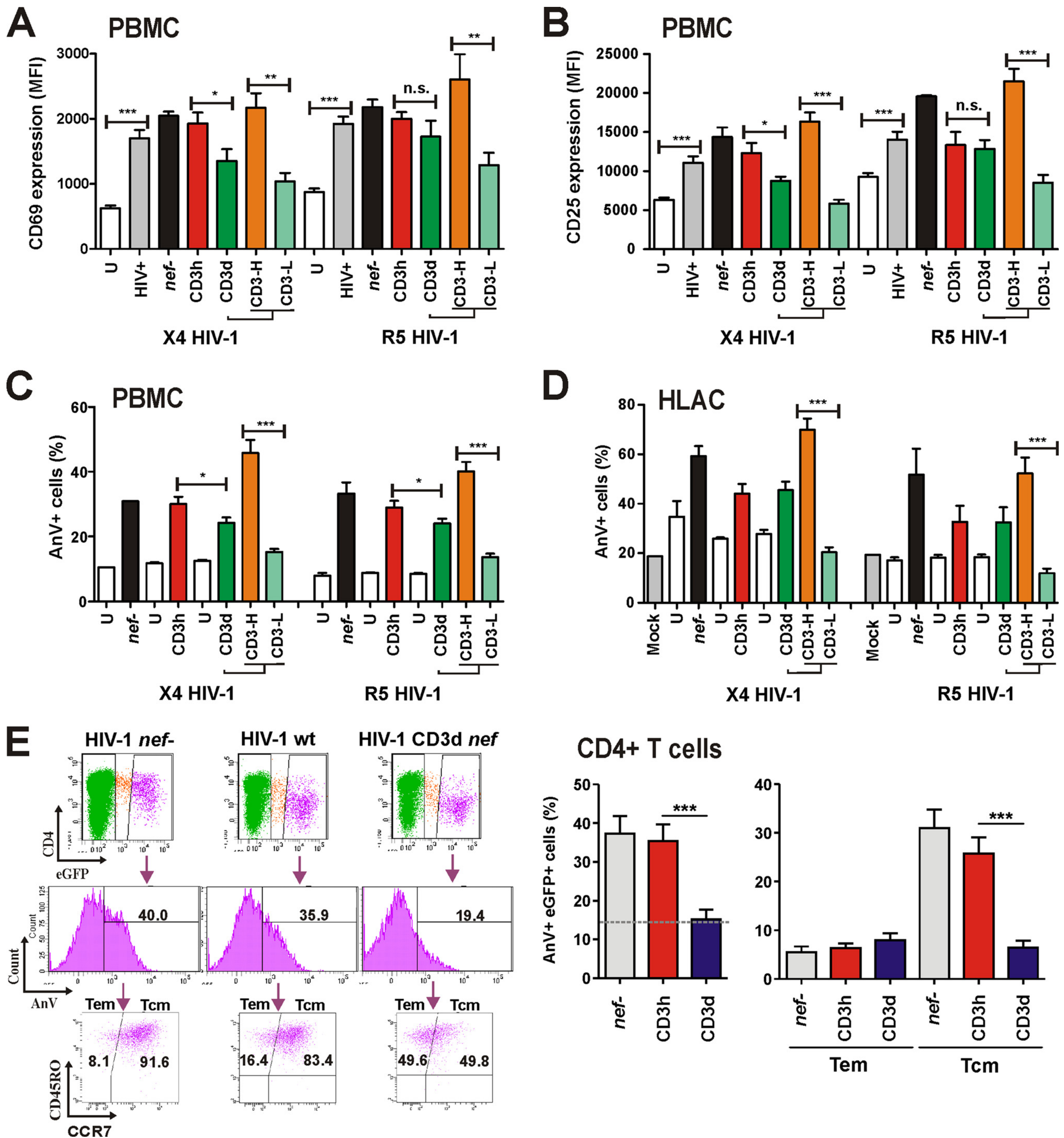

FIG 7 TCR-CD3 downmodulation protects memory T cells against activation and apoptosis. (A and B) Flow cytometric analysis of CD69 (A) and CD25 (B) expression by PBMCs infected with X4 or R5 HIV-1 constructs expressing G1 or G2 *nef* alleles. U, uninfected control cells. (C and D) Frequency of apoptotic cells in PBMCs (C) and HLACs (D). Shown are mean values (±SEM; $n = 6$) in cultures infected with X4 or R5 HIV-1 constructs expressing *nef* alleles that are inactive (CD3h) or active (CD3d) in CD3 downmodulation. For the latter, we also determined the levels of apoptotic (annexin V-positive) cells in the CD3high (CD3-H) and CD3low (CD3-L) populations of HIV-1-infected ($eGFP^+$) T cells. (E) Frequency of apoptotic cells in purified $CD4^+$ T cells infected with HIV-1 IRES-eGFP constructs expressing the NL4-3 (CD3h) or HIV-2 Ben (CD3d) Nef proteins. The left panels shows representative FACS data and the right panels the percentages of virally infected apoptotic (annexin V-positive) cells in the total population and in the Tem and Tcm subsets. Shown are mean values (±SEM) obtained from 14 blood donors.

and HLACs were usually as high as those measured in the CD3high population (Fig. 9E). These analyses also revealed significantly higher levels of eGFP expression in Tdp cells than in Tn and Tm cells (Fig. 9D). Thus, increased proviral gene expression is one reason for effective virus production by Tdp cells. We also examined why Tdp cells produced much higher levels of infectious HIV-1 than Tm cells. Analyses of viral RNA sequences revealed comparably low levels of G-to-A hypermu-

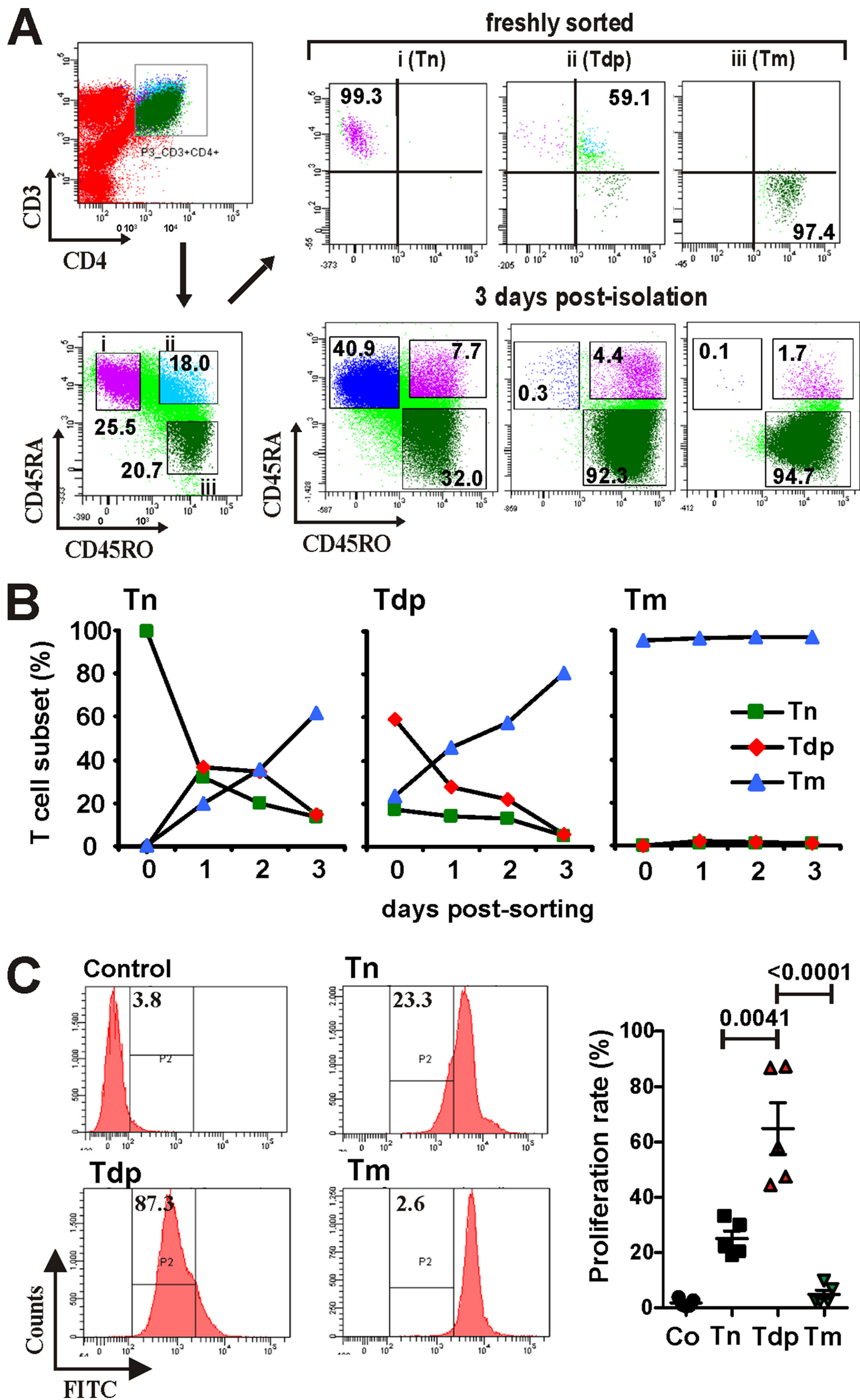

FIG 8 Differentiation and proliferation of different T cell subsets. (A) Gating strategy for the isolation of different T cell subsets and phenotypic characterization immediately after sorting and 3 days later. Note that initially only low numbers of cells were analyzed. Numbers give percentages of the total (100%) analyzed in the respective gates. (B) Composition of T cell subsets immediately after sorting and after up to 3 days of culture. Shown are average values derived from 4 different experiments. (C) Proliferation rates of different T cell subsets. The left panel shows primary FACS data and the right panel the average proliferation rates.

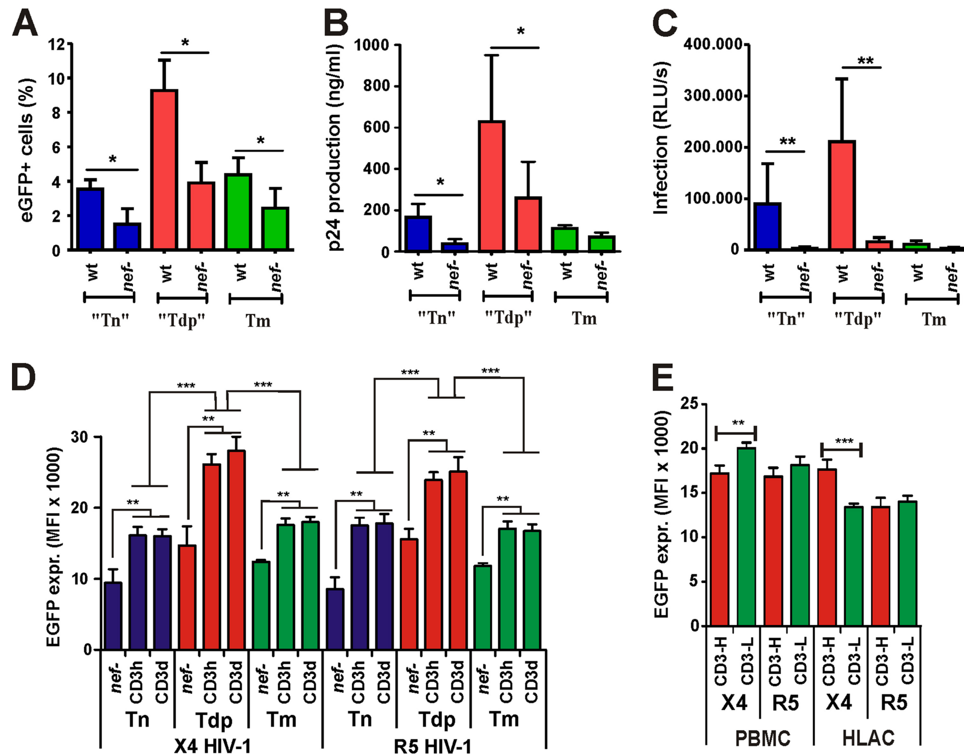

FIG 9 Highly proliferative RA$^+$ RO$^+$ Tdp cells are main producers of infectious HIV-1. (A to C) Percentages of HIV-1 infected (eGFP$^+$) cells in the indicated T cell subsets (A) and p24 core antigen (B) and infectious virus (C) production by the indicated T cell subsets. The indicated T cell subsets were isolated by flow cytometric sorting and infected with VSV-G-pseudotyped HIV-1 NL4-3 constructs containing an intact or disrupted *nef* allele. Tn and Tdp are shown in quotation marks because they rapidly differentiated to Tm cells (Fig. 7A and B). The results represent mean values (±SEM) derived from three independent experiments. (D and E) Levels of eGFP expression on different T cell subsets in PBMC cultures infected with X4 or R5 HIV-1 constructs expressing various *nef* alleles (D) and the CD3high and CD3low populations of virally infected PBMC cultures and HLACs infected with viral constructs capable of CD3 downmodulation. Data represent means (±SEM; $n = 6$ to 12).

tations (data not shown), suggesting that the low infectiousness of HIV virions produced by Tm cells was not due to increased activity of APOBEC3G. We found, however, that wt HIV-1 induced rapid apoptosis in Tm cells, whereas this was not the case in Tn and Tdp cells (Fig. 10A). These results suggested that a significant proportion of HIV-1-infected Tm cells may undergo lysis and release p24 before the completion of the viral replication cycle. Indeed, treatment of HIV-1 infected Tm (but not Tdp) cells with detergents did not further increase the levels of p24 capsid antigen in the culture supernatant (Fig. 10B).

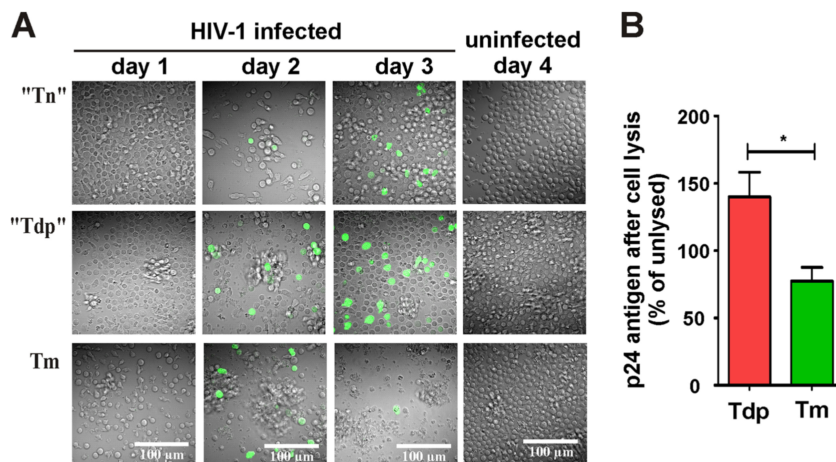

FIG 10 Effect of HIV-1 infection on cell death. (A) Sorted naive, activated, and memory T cells (purified by sorting) were infected with *env*-defective HIV-1 NL4-3 X4-tropic IRES-eGFP virus pseudotyped with VSV-G or left untreated. On the next day, virus was removed by extensive washing with PBS. Cells were cultured on microscopy slides and imaged for eGFP expression for 4 days. Similar results were obtained in two independent experiments. (B) Mean levels of p24 capsid antigen (±SEM) in the supernatants of HIV-1-infected Tdp and Tm cells after cells lysis relative to those measured without lysing the cells (100%) ($n = 3$).

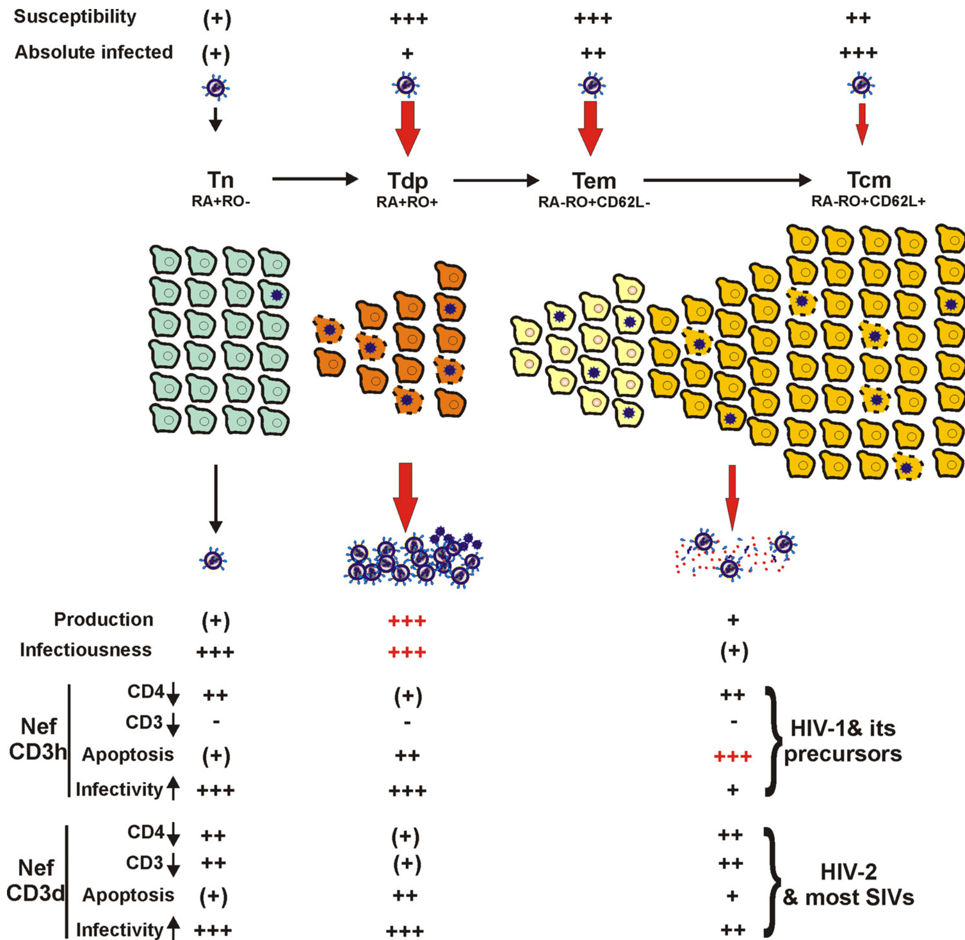

FIG 11 Susceptibility of various T cell subsets to HIV-1 infection and modulation by Nef. Top, highly proliferative Tdp and Tem cells are most permissive for HIV-1 infection, but Tcm cells are more frequent are and the main type of virally infected cells in PBMCs and HLACs. Middle, activated/proliferating RO^+ RA^+ Tdp cells are main producers of infectious HIV-1. Bottom, modulation of different T cell subsets by *nef* alleles that have lost (CD3h) or maintained (CD3d) the CD3 downmodulation function. ↓, downmodulation; ↑, enhancement; −, inactive; (+), poorly active; +, modest activity; ++, high levels of activity; +++, very high levels of activity.

DISCUSSION

The role of the accessory Nef protein as a multifunctional manipulator of the host cell that is required for effective replication of HIV and SIV *in vivo* is well established. However, although various $CD4^+$ T cell subsets differ strongly in their susceptibility to virus infection as well as in their importance for the maintenance of a functional immune system, it is unknown whether Nef manipulates viral host cells in a $CD4^+$ T cell subset-specific manner. Here, we show that most virally infected $CD4^+$ T cells in PBMC cultures and HLACs exhibit an RA^- RO^+ memory phenotype. However, activated/proliferating RA^+ RO^+ Tdp cells produced substantially higher levels of infectious HIV-1 than Tm cells (summarized in Fig. 11). It also came as a surprise that Nef efficiently downmodulates CD4 and TCR-CD3 (in the case of HIV-2 and SIVmac/smm) from the surfaces of Tn, Tem, and Tcm cells but has a much lesser effect on Tdp cells. Thus, while Nef increased virion infectivity in a T cell subset-independent manner, its modulatory effect on cellular receptors preferentially involved the functionality and survival of virally infected $CD4^+$ Tm (and, in particular, Tcm) cells and was much less effective in Tdp cells, which already represent "ideal" targets for effective viral replication.

Initially, we examined a possible role of the CD3 downmodulation function of Nef in the viral replication fitness in different T cell subsets. We hypothesized that lack of Nef-mediated downmodulation of TCR-CD3 may facilitate productive virus infection of T cell populations with a relatively resting phenotype because it allows their activation above the threshold normally required for effective virus replication. In fact, it has recently been shown that a lack of TCR-CD3 downmodulation may facilitate the coreceptor switch from CCR5 to CXCR4 and hence infection of naive T cells (30). We found that lack of the CD3 downmodulation function was associated with slightly increased percentages of HIV-infected $CD62L^-$ Tem and RA^+ RO^+ Tdp cells and reduced frequencies of $CD62L^+$ Tcm cells (Fig. 4A and B). The exact reasons for this phenomenon need further investigation. It is conceivable, however, that efficient differentiation of Tn cells that maintain TCR-CD3 expression to Tdp and Tem cells and (more importantly) high levels of apoptosis in the Tm subset associated with lack of the CD3 downmodulation function may shift the balance of viable virally infected T cells in PBMC cultures or HLACs toward the Tdp and Tem cell subsets.

Previous studies suggested that the *in vivo* life span of the

CD4$^+$ T cell population that accounts for the bulk of viral replication is remarkably similar in HIV-1-infected human individuals and in SIVagm- or SIVsmm-infected monkeys (33–35). This result seemed to contradict the finding that the HIV-1 Nef protein renders virally infected CD4$^+$ T cells hyperresponsive to stimulation and is associated with high levels of apoptosis, whereas SIVagm and SIVsmm Nefs efficiently block the activation, proliferation, and programmed death of virally infected T cells (11, 36). Here, we demonstrate that Nef-mediated downmodulation of TCR-CD3 and suppression of activation and apoptosis are inefficient in highly proliferative RA$^+$ RO$^+$ Tdp cells (Fig. 5C), and we provide evidence that these cells may be main producers of infectious virus *in vivo* (Fig. 9A to C). As a consequence, the already short life span of this T cell subset would hardly be affected by this Nef function but rather would be determined by a combination of the virus cytopathic effect and their high activation status. Our results may thus explain this seeming discrepancy and are consistent with reports suggesting that the bulk of virus replication in both pathogenic and nonpathogenic primate lentiviral infections occurs in highly activated, proliferating CD4$^+$ T cells with a short life span (33–35).

Our analyses of sorted CD4$^+$ T cell subsets that are targets of HIV and SIV infection provide first insights into the rapid dynamics of their differentiation. Further studies on HIV-1 infection and simultaneous monitoring of the phenotypic changes in T cell subsets in unsorted PBMC cultures or HLACs, e.g., by live-cell imaging techniques, are of significant interest but are technically very challenging. Notably, TCR-CD3 and CD28, which are both efficiently downmodulated by HIV-2 and most SIV Nef proteins but not by HIV-1 Nefs (11, 19), play a key role in the activation of T cells by antigen-presenting cells and subsequent differentiation. Thus, it tempting to speculate that these viruses may modulate the differentiation of infected CD4$^+$ T cells and that differences in Nef function may affect these dynamics and possibly the efficacy of the antiviral immune response and the levels of inflammation.

Previous data suggested that low levels of CCR5 expression may protect SIVsmm-infected sooty mangabeys against the loss of Tcm cells (25). The same effect may be achieved by downmodulation of CD4 expression by Tcm cells in SIVagm-infected African green monkeys (37). Our results confirm that Nef-mediated downmodulation of TCR-CD3 protects virally infected T cells against programmed death (11, 21, 36) and further demonstrate that this protective effect is particularly effective in CD4$^+$ Tcm cells. It is conceivable that not only reduced virus infection but also decelerated apoptotic cell death of this specific T cell subset may help the hosts of primate lentiviruses to maintain a functional immune system. Our findings thus add to the growing evidence that selective preservation of CD4$^+$ Tcm cells that have the capacity of self-renewal (26) may be a key mechanism allowing natural hosts of SIV to avoid disease progression despite high levels of viral replication. The preservation of CD4$^+$ T cell homeostasis is beneficial for both the host and the virus, as it protects the host from disease progression while allowing persistent high levels of viral replication in a functional immune system that ensures continuous *de novo* production of viral target cells. Notably, Nef-mediated downmodulation of TCR-CD3 is conserved between most primate lentiviruses (11, 19), and the protective effects were also observed in HLACs, suggesting that they most likely also occur in lymphoid tissues *in vivo*. The clinical outcome of primate lentiviral infections depends on multiple viral and host properties (14–16), and TCR-CD3 downmodulation is insufficient to prevent disease in nonnatural hosts that are highly susceptible to disease, such as SIV-infected macaques. However, the latter does not argue against a role of Nef-mediated downmodulation of TCR-CD3 in reducing damaging high levels of immune activation and decelerating the loss of critical CD4$^+$ Tcm cells. In support of a protective role of this Nef function, it has been shown that the efficiency of CD3 downregulation in HIV-2-infected individuals correlates with high CD4$^+$ T cell counts (21) and reduced levels of immune activation (38). Conversely, lack of this Nef function was associated with loss of CD4$^+$ T cells in natural SIVsmm infection (20).

The CD3high and CD3low populations of virally infected T cells differed strongly in their state of activation (Fig. 7A and B). It is commonly assumed that the transcriptional activity of the HIV-1 LTR promoter correlates with the level of T cell activation. As such, it was unexpected that the CD3high and CD3low populations of virally infected CD4$^+$ T cells showed similar levels of transcriptional proviral activity (as indicated by the mean fluorescence intensities of LTR-driven eGFP expression [Fig. 9E]) while differing substantially with respect to their state of activation. Another unanticipated finding was that intact *nef* genes generally increased the levels of LTR-driven eGFP expression, irrespective of the effect on TCR-CD3 (Fig. 9D). These findings suggest that primate lentiviruses can, at least to some extent, uncouple the transcriptional activity of the viral LTR promoter from the state of cellular activation, possibly because Nef may support NF-κB induction independently of TCR-CD3 signaling (39). However, although similar levels of HIV-1-infected (eGFP$^+$) cells and only modest differences in eGFP expression levels were detected in RA$^+$ RO$^+$ Tdp and RA$^-$ RO$^+$ Tm cells, only the former produced high levels of infectious HIV-1 (Fig. 9C). The underlying mechanisms need further investigation, but our preliminary data suggest that premature cell death prior to completion of the viral replication cycle may contribute significantly to the inefficient production of infectious HIV-1 by Tcm cells.

In summary, our data demonstrate that the Nef proteins of primate lentiviruses manipulate CD4$^+$ T cells in a subset-specific manner (Fig. 11). Downmodulation of CD4 and TCR-CD3 is ineffective in RA$^+$ RO$^+$ Tdp cells, which are already highly permissive for replication, but is very effective in CD4$^+$ Tm and Tcm cells, which are essential for the maintenance of a functional immune system. These results, together with our finding that RO$^+$ RA$^+$ Tdp cells are the main target cells for HIV-1 replication although they comprise less than 20% of total CD4$^+$ T lymphocytes (and although most HIV-1-infected T cells have an RA$^-$ RO$^+$ memory phenotype), provide new insights into how HIV-1 and other primate lentiviruses manipulate the function of their target T cells *in vivo* and how this manipulation may result in CD4$^+$ T cell death, overall immune dysfunction, and progression to AIDS.

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