Human Immunodeficiency Virus nef Gene Expression Affects Generation and Function of Human T Cells, But Not Dendritic Cells

By Bruno Verhasselt, Evelien Naessens, Chris Verhofstede, Magda De Smedt, Sigrid Schollen, Tessa Kerre, Dominique Vanhecke, and Jean Plum

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Human immunodeficiency virus (HIV)-infected individuals develop an acquired immune deficiency syndrome (AIDS) due to loss in their lymphocyte numbers and cellular defects in T cells and antigen-presenting cells (APC). HIV infection of the thymus results in deficient replenishment of the peripheral naive T-cell pool. The HIV nef gene was shown to be important for progression towards AIDS and cellular depletion of the infected thymus. Here, we demonstrate by retroviral gene transfer that nef expression, in the absence of other HIV genes, impaired human thymic T-cell development. Thymocytes were generated in reduced numbers and downmodulated CD4 and CD8β cell surface expression. T cells grown from nef-expressing thymocytes were hyperproliferative in vitro upon T-cell receptor triggering. Mature dendritic cells (DC) were functional and had normal surface CD4 levels despite nef expression. Thus, nef expression alone may contribute to AIDS development by reduced T-cell generation and T-cell hyperresponsiveness.

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MATERIALS AND METHODS

Monoclonal Antibodies, Flow Cytometry, and Cell Sorting

Mouse antihuman monoclonal antibodies used were: CD8α (Becton Dickinson Immunocytometry Systems, Mountain View, CA), CD8β (Immunotech, Marseille, France), CD14 (Becton Dickinson), CD25 (Becton Dickinson), CD27 (PharMingen, San Diego, CA), CD40 (PharMingen), CD80 (Becton Dickinson), anti-αβ (Immunotech), anti-MHC-I (antihuman histocompatibility leukocyte antigens [HLA]-A, -B, -C; PharMingen) and as described previously.23,24 For Annexin-V staining, cells were stained with Annexin-V-vitro (Boehringer Mannheim, Mannheim, Germany) plus streptavidin-phycocerythrin (Becton Dickinson). The cells were analyzed on a FACSscan or FACSCalibur flow cytometer (Becton Dickinson). Flow cytometry and cell sorting was performed as described previously.25 Forward light scattering, orthogonal scattering, and 3 or 4 fluorescence signals were stored and analyzed using the CellQuest software (Becton Dickinson).

Cell Culture and Purification of Progenitor Cells

Jurkat and MOLT-4 (American Type Culture Collection, Rockville, MD), Sup-T1 (AIDS Research and Reference Reagent Program, NIH, Bethesda, MD), and other cells were cultured in complete Iscove's modified Dulbecco's medium (IMDM) as described previously.24 T-cell culture, DC functional assay, and human-mouse fetal thymus organ culture (FTOC)25 were performed in complete IMDM containing 10% heat-inactivated human AB serum (BioWhittaker, Walkersville, MD). CD34+ CD4- CD8- CD3- (ISP4+) and CD34+ CD4- CD8- CD3- (CD34+) thymocytes were isolated by sorting freshly isolated thymocytes stained for CD34-allophycocyanin (APC), CD4-phycoerythrin (PE), CD8α-fluorescein isothiocyanate (FITC), and CD3-FITC. CD34+ cord blood (CB) cells were purified by cell sorting as described previously.24 Purity of sorted cells was always at least 99%. Child thymi, removed during cardiac surgery, and CB samples were obtained and used following the guidelines of the Medical Ethical Commission of the University Hospital of Ghent.

Retroviral Gene Transfer

The marker gene Enhanced Green Fluorescent Protein (EGFP) was placed 3' of an internal ribosome entry site (ires) sequence in the L2RS retroviral vector.25 In this Nef+ control vector (Fig 1A), an EcoRI-BamHI fragment containing the complete HIV-1 NLA-3 nef gene26 amplified by polymerase chain reaction (PCR) (Platinum Taq, Life Technologies, Paisley, Scotland) from the pNL4-3 plasmid (AIDS Research and Reference Reagent Program) was inserted 5' of the IRES sequence (Fig 1B) to generate the Nef+ vector. Direct sequencing (ABI, Perkin Elmer, Foster City, CA) confirmed that the clone used contained the published NL-4 nef gene sequence. Retroviral supernatants were prepared as described previously.23 The Nef- and Nef+ batches used in this report contained approximately 2.5 × 10⁵ and 6 × 10⁵ transducing units/mL, respectively, titrated on Jurkat cells. For generation of T-cell progenitor cells, transduced once, 1 day (CD34+ CB cells, cultured in medium supplemented with stem cell factor [SCF, 100 ng/mL], flt3/ilk-2 ligand [FL, 100 ng/mL], thrombopoietin (20 ng/mL) or 2 days (ISP4+ or CD34+ thymocytes, cultured in medium supplemented with SCF [10 ng/mL] and IL-7 [10 ng/mL]) after initiation of the culture (all cytokines from R&D Systems Europe, Abingdon, UK). For generation of DCs, sorted CD34+ CB cells were transduced once 2 days after initiation of the culture as described below. For transduction, cells were seeded on RetroNectin (Takara Biomedicals, Otsu Shiga, Japan) coated culture plates with half of the medium volume replaced by retroviral supernatants, supplemented with cytokines to keep final cytokine concentrations constant. After 3 days of culture, cells were harvested to determine transduction efficiency and used in subsequent assays.

Immunoblotting

Cell lysates were run on 4% to 12% Bis-Tris polyacrylamide gels (Nupage, Novex, San Diego, CA) in 2-(N-morpholino) ethanesulfonic acid buffer in reducing conditions and proteins were blotted on polyvinylidene fluoride membranes (Novex). Blots were stained with the HIV-1 Nef monoclonal antibody EH-1 (AIDS Research and Reference Reagent Program) and antiserum IgG alkaline phosphatase conjugate (Sigma, Steinheim, Germany).

Generation of Cell Lineages and Functional Assays

Thymocytes. About 30,000 ISP4+ or 10,000 CD34+ thymus or CB cells were used in 7 to 30 day FTOC as described previously24 to study in vitro thymic repopulation. To study in vivo thymic repopulation, about 50,000 ISP4+ or 20,000 CD34+ CB cells were injected in the human thymus of SCID-hu mice that were irradiated (0.2 Gy, 0.04 Gy/minute) the day before. SCID-hu mice were obtained by transplantation of human fetal liver and thymus about 5 months before as described previously.21 Inherent to this model, the magnitude of engraftment of the thymus, as measured by the number of donor-derived thymocytes recovered several weeks after injection of progenitor cells, is highly variable27 (and our own unpublished observations). Therefore, calculation of precursor expansion is not possible. Human fetal tissue was obtained following the guidelines of the Medical Ethical Commission of the University Hospital of Ghent. Animals were treated according to the guidelines of the Laboratory Animal Ethical Commission of the University Hospital of Ghent. After 3 weeks (thymi injected with ISP4+ cells) or after 4 to 7 weeks (thymi injected with CD34+ CB cells), mice were killed and thymocytes were phenotyped and in some experiments sorted for EGFP, CD3, and CD27 expression. Sorted thymocytes from thymi injected with CD34+ CB cells were stimulated with phorbol myristate acetate (PMA; 2 ng/mL, Sigma) + phytomengglutinin (PHA; 2 µg/mL, Murex, Dartford, United Kingdom) as described previously23 to induce cytokine expression. To induce proliferation, thymocytes were stimulated with PHA + IL-2 (Boehringer Mannheim) as described previously22 or with anti-CD3 antibody (OKT3, American Type Culture Collection) + PMA as described below.

T cells. SCID-hu thymocytes from thymi injected with transduced ISP4+ or CD34+ CB cells were sorted as described above and seeded on freshly isolated and irradiated (2.5 Gy) allogeneic peripheral blood mononuclear cells (PBMCs) in medium supplemented with PHA + IL-2 as described previously.23 After 14 days of culture, cells were harvested, phenotyped, and used to induce cytokine expression (7 hours of culture, assay by reverse transcribed [RT]-PCR) or proliferation (36 hours of culture, assay by β-counting of DNA incorporated [3H-thymidine after overnight pulsing) in plain flat bottom wells or wells coated with OKT3, in medium with or without PMA.

DC. Sorted CD34+ CB cells were cultured26 for 5 days (transduction 2 days after initiation of the culture) in complete IMDM supplemented with SCF (100 ng/mL), FL (100 ng/mL), granulocyte macrophage colony-stimulating factor (GM-CSF, 5 ng/mL), and tumor necrosis factor α (TNFα, 10 ng/mL) to generate DC precursors, and for a further 9 days in complete IMDM supplemented with GM-CSF, TNFα, and IL-4 (20 ng/mL) to generate mature DCs. Sorted transduced mature DCs (EGFP+ HLA-DR+ “CD14”) were seeded in various numbers in 96-well round bottom plates and gamma irradiated (2 Gy). On irradiated DCs, 20,000 freshly isolated allogeneic PBMCs (ratio DCs/PBMCs numbers: 1/1 to 1/729) were cocultured for 6 days and then pulsed overnight to assay proliferation by β-counting of DNA incorporated [3H-thymidine.

RESULTS

Retroviral-Mediated Transfer of nef

To investigate the effect of nef gene expression on the development and function of human T cells and DCs, we
constructed retroviral vectors containing the marker gene EGFP, without (Nef<sup>-</sup>, Fig 1A) or with (Nef<sup>+</sup>, Fig 1B) the HIV NL4-3 nef gene. Due to the IRES, Nef<sup>+</sup> transduced cells expressed both Nef and EGFP proteins from 1 bicistronic messenger RNA (mRNA). As expected, semiquantitative RT-PCR on sorted Nef<sup>+</sup> transduced cells showed a linear correlation between the amount of mRNA for Nef and EGFP fluorescence intensity (data not shown). Nef<sup>+</sup> transduced cells expressed a +/- 27 kD Nef protein as shown by immunoblotting (Fig 1C). We transduced established T-cell lines to demonstrate the biological activity of the Nef protein. Sup-T1 cells still expressed CD4 and MHC class I after transduction with Nef<sup>+</sup> retroviral supernatant at the same level as nontransduced cells (Fig 2). In contrast, in Nef<sup>-</sup> transduced cells, expression levels of both of these surface molecules were up to 10 times lower in function of increasing EGFP expression (Fig 2). Similar results were obtained with MOLT-4 and Jurkat T cell lines (data not shown).

**Nef Expression Hampers Human T-Cell Development**

The most immature thymocytes susceptible for HIV infection in vivo are immature CD4 single positive cells (CD4<sup>+</sup>CD3<sup>-</sup>CD<sup>8</sup><sup>-</sup>; ISP4<sup>+</sup>).<sup>13,14</sup> the precursors of DP immature thymocytes and single positive (SP) mature thymocytes and T cells. To study the effect of nef expression on human T-cell development, ISP4<sup>+</sup> cells were isolated by cell sorting and transduced with Nef<sup>-</sup> or Nef<sup>+</sup> retroviral supernatant (Fig 3A). T-cell development from transduced progenitor cells was assayed in vitro in FTOC and in vivo after injection in the human thymus of SCID-hu mice. After 1 week of FTOC, Nef<sup>-</sup> transduced ISP4<sup>+</sup> cells developed into DP cells with comparable cell membrane CD4, CD8β, and the TCR-associated CD3 expression levels as nontransduced cells (Fig 3B), as EGFP expression does not hamper T-cell development.<sup>24,25</sup> In contrast, most Nef<sup>+</sup> transduced thymocytes expressed reduced CD4, CD8β (Fig 3B), and MHC class I levels (data not shown) and were skewed towards higher CD3 levels, all correlating with EGFP and thus with Nef levels. Gating on EGFP<sup>+</sup> cells (Fig 3C) showed that the Nef<sup>-</sup> transduced cells were mainly CD4<sup>dim</sup>-CD8β<sup>dim</sup>-CD8<sup>a</sup>, so that the DP fraction of Nef<sup>-</sup> transduced thymocytes was significantly reduced (Table 1). Mean fluorescence intensities of Nef<sup>-</sup> versus Nef<sup>+</sup> transduced thymocytes (Fig 3C) were for CD4, 125 versus 26; for CD8β, 299 versus 165; for CD3, 95 versus 124. Precursor expansion, ie, the ratio of output transduced thymocyte number to input transduced progenitor number (about 1,500 cells) was on the average 10.0 (range, 6.2 to 17) for Nef<sup>-</sup> transduced cells compared with 2.5 (range, 1.3 to 3.4), with Nef<sup>+</sup> transduced cells (Table 1). In addition, unlike the transduced ISP4<sup>+</sup> start population, relatively more Nef<sup>+</sup> transduced thymocytes expressed intermediate EGFP levels compared with Nef<sup>-</sup> transduced cells, possibly indicating a selective disadvantage for cells expressing high levels of nef (Fig 3). The fraction of CD3<sup>+</sup> cells expressing CD4 was diminished (Fig 3C and Table 1). On Nef<sup>-</sup> transduced thymocytes, CD8<sup>α</sup> expression was less reduced than CD8β expression, and similar to Nef<sup>-</sup>, most Nef<sup>+</sup> transduced CD3<sup>+</sup> cells expressed an αβ TCR (data not shown). On Nef<sup>+</sup> thymocytes, αβ TCR expression increased with EGFP fluorescence intensity, similar to CD3. A minor subset of Nef<sup>+</sup> transduced thymocytes expressing normal CD4 and CD8β levels was present (Fig 3C). After additional weeks of culture, the differences in thymocyte generation and phenotype in FTOC started with Nef<sup>-</sup> compared with Nef<sup>+</sup> transduced ISP4<sup>+</sup> precursors were even more pronounced (Table 1). After 3 weeks of FTOC, almost no Nef<sup>-</sup> DP cells were observed (Fig 3D and Table 1). The few remaining Nef<sup>+</sup> cells were mostly CD3<sup>+</sup>CD4<sup>dim</sup>-CD8<sup>α+</sup> and either CD8β<sup>+</sup> or CD8β<sup>-</sup>. Thus, generation of DP thymocytes from ISP4<sup>+</sup> precursors decreases with increasing nef expression.
We injected transduced ISP4⁺ precursor cells in human thymi of SCID-hu mice to study in vivo thymopoiesis. Three weeks after injection of transduced ISP4⁺ thymocytes, few EGFP⁺ thymocytes could be recovered from the thymic graft. However, phenotypical alterations of thymocyte development were similar to those observed in FTOC (Fig 4). To generate sufficient mature transduced thymocytes, we chose to repopulate the human thymus with transduced CB CD34⁺ precursors. These cells, which are more readily transducible and have a higher proliferative potential than ISP4⁺ cells, can generate transduced thymocytes in this model⁶ (and our own unpublished observations). One month after injection of CD34⁺ CB precursors, Nef⁺ transduced thymocytes expressed decreased CD4 levels in function of increasing EGFP expression (Fig 5A). Similar to the experiments with ISP4⁺ cells, most Nef⁺ transduced thymocytes were CD4dim⁻CD8β⁺dim and upregulated CD3 (Fig 5B). Mean fluorescence intensities of Nef⁻ versus Nef⁺ transduced thymocytes (Fig 5B) were for CD4, 109 versus 21; for CD8β, 307 versus 139; for CD3, 50 versus 113. Moreover, a part of the Nef⁺ transduced CD3⁺ thymocytes were CD27⁺, a marker previously shown to correlate with acquisition of functionality by human thymocytes. Apoptosis was absent in bright EGFP⁺ thymocytes in SCID-hu thymi injected with either Nef⁻ transduced (data not shown) or Nef⁺ transduced (Fig 6A) CB precursors. EGFP expression was only detected in viable cells, as propidium iodide staining cells were

### Table 1. Precursor Expansion and Phenotype of Thymocytes Generated in FTOC From Nef⁻ and Nef⁺ Transduced ISP4⁺ Precursors

<table>
<thead>
<tr>
<th>Precursor expansion</th>
<th>Weeks of Culture</th>
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<tr>
<td></td>
<td>1</td>
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<tr>
<td>Nef⁻</td>
<td>Nef⁺</td>
</tr>
<tr>
<td>Precursor expansion</td>
<td>10.0 ± 4.0</td>
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<tr>
<td>Phenotype†</td>
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<tr>
<td>CD4⁻CD8β⁺</td>
<td>93.2 ± 1.2</td>
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<td>CD4⁺CD8βdim⁻</td>
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</tr>
<tr>
<td>CD4dim⁻CD8β⁺</td>
<td>1.1 ± 0.4</td>
</tr>
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<td>CD4dim⁻CD8βdim⁻</td>
<td>1.7 ± 0.6</td>
</tr>
<tr>
<td>CD3⁻CD4⁺</td>
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</tr>
<tr>
<td>CD3⁺CD8β⁺dim⁻</td>
<td>61.9 ± 1.1</td>
</tr>
<tr>
<td>CD3⁺CD8β⁺</td>
<td>1.3 ± 0.6</td>
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</table>

†Phenotype, percentage of cells of corresponding phenotype on total thymocyte population. For CD4 and CD8 (β, +, expression above level delimited by quadstat as in Fig 3D; -, expression below level delimited by quadstat as in Fig 3B; dim, expression level between – and +. For CD3, + and –, expression above or below level delimited by quadstat as in Fig 3B.

Figures indicate average on experiments with 3 different donors +/- standard deviation. Abbreviation: ND, not determined.

*Precursor expansion: ratio of output thymocyte number to input progenitor number.
never EGFP+ (data not shown). Nef expression is thus not toxic for developing thymocytes. Similar to Nef transduced thymocytes, most Nef transduced thymocytes expressed a TCR-ab, while a small minority expressed a TCR-gd (Fig 6B and C). Expression of other markers as CD1 or CD45RA and development of natural killer cells and DCs in the thymus was not affected by nef expression (data not shown).

**T Cells Expressing nef Are Hyperproliferative Upon CD3 Triggering**

To assess whether nef expressing mature thymocytes were functionally impaired, we purified CD3+CD27+ transduced SCID-hu thymocytes by cell sorting. By semiquantitative RT-PCR, no difference in IL-2 induction could be detected between Nef- and Nef+ transduced thymocytes (data not shown). Both Nef- and Nef+ transduced SCID-hu thymocytes could be expanded on irradiated PHA-stimulated PBMCs. Phenotypically, T cells from these cultures showed CD4 downmodulation (Fig 7A), and neither TCR-αβ (Fig 7A), CD8α, or CD25 expression levels (data not shown) were different from that of Nef- T cells. Few cells were CD8β+ (data not shown). As was the case with freshly sorted CD3+CD27+ transduced thymocytes, IL-2 mRNA could be induced as efficient in Nef+ as in Nef- T cells (data not shown). Proliferation upon TCR triggering was more pronounced in Nef+ transduced cultured T cells (Fig 7B). Unlike Nef-, Nef+ transduced cultured T cells did not require addition of PMA for maximal proliferation in response to immobilized anti-CD3 antibody (Fig 7B). Functional assays were not different after removal of CD8β+dim T cells by cell sorting (data not shown).

**Nef Expression Does Not Affect Generation or Function of DCs**

Besides thymocytes and T cells, also CD4+ APC are targets for HIV virus infection. Activation and aberrant antigen presentation has been reported with HIV-infected APC, such as DCs. To study the role of Nef in this process, we have generated DCs from transduced CD34+ CB cells in suspension culture. After 5 days of culture, Nef transduced cultures contained EGFP+ dendritic cell precursors, expressing CD4. Nef+ transduced DC precursors did not express CD4 (Fig 8A). At day 9, onset of maturation is reflected by CD80 upregulation. On Nef+ transduced DCs, CD4 was concomitantly upregulated (Fig 8B). Most Nef+ transduced mature DCs expressed normal CD4 levels (Fig 8A) in coexpression with CD80 (Fig 8B). In addition, more than 90% of both Nef- and Nef+ transduced mature DCs were CD1a+CD40+CD80+HLA-DR+CD14+ (data not shown). They displayed the same dendritic morphology and were generated in comparable numbers: 20-fold expansion of transduced CD34+ CB precursor cell numbers in 14 days of culture. No defect of Nef+ mature DCs in inducing an alloreponse of PBMCs could be detected (Fig 8C).
By retroviral gene transfer, we expressed the HIV molecular clone NL4-3 nef gene,\(^{26}\) the nef gene used in previous studies on T-cell development in SCID-hu mice\(^{13,15-17}\) and Tg mice,\(^{18,19,21,22}\) in T-cell and DC precursors. We have shown here that expression of the nef gene induced abnormalities of T-cell development. The number of thymocytes generated from ISP4\(^{1}\) cells expressing nef was greatly reduced. Immature thymocytes generated from nef expressing precursor cells were CD4\(^{\text{dim}}\)/CD8\(^{\text{b1/dim}}\), while mature output was mainly CD3\(^{1}\)CD4\(^{2}\)CD8\(^{\alpha1}\)CD8\(^{\beta\text{dim/-}}\). The observed reduced cellularity and phenotypical alterations are similar to those observed in HIV-infected SCID-hu thymus.\(^{13-17}\) The phenotype of Nef\(^{1}\) transduced thymocytes strikingly resembles that of SCID-hu thymocytes productively infected with a pathogenic reporter NL4-3 HIV virus.\(^{13}\) The reporter virus allows detection of HIV gene expression in infected thymocytes in vivo. One month after infection, most HIV-expressing cells are CD4\(^{\text{dim/-}}\)/CD8\(^{\text{b/dim}}\), while a minor subset is DP. As we can reproduce this phenotype by expressing nef as the only HIV gene, phenotypical alterations of infected thymocytes may be primarily due to nef expression. Because nef expression was shown in our experiments to severely diminish T-cell generation from ISP4\(^{1}\) cells, the attenuated pathogenic potential of nef defective HIV in SCID-hu mice\(^{15-17}\) could therefore be primarily due to the lack of direct cellular effects by Nef. Thymocytes expressing nef were not apoptotic or dead. Reduced T-cell generation in our experiments was therefore probably not caused by simple toxicity of Nef, but by a specific function of Nef in thymocytes. In our experiments, the phenotype and cell number of nontransduced thymocytes (EGFP\(^{2}\)) present in the same thymus as Nef\(^{1}\) transduced cells was normal, indicating that nef expression does not affect nontransduced cells. However, in SCID-hu thymus infection, nef...
defective HIV still retains some pathogenicity, indicating that cellular nef expression alone is not responsible for all observed phenomena in HIV infection of the thymus.

Tg mice expressing NL4-3 nef driven by transcription control elements of either mouse TCRβ, mouse CD3δ, or human CD4 regulatory sequences have been constructed. The thymic phenotypes of these Tg lines differed considerably: DP thymocytes were either found to be almost absent, present in reduced numbers, or present in normal to even increased numbers. However, all investigators observed a decrease of CD4 expression level and a reduced number of mature CD4+ thymocytes. Upon TCR triggering, proliferation and tyrosine phosphorylation of several substrates involved in TCR signaling was augmented in nef transgenic thymocytes. By contrast, in mice Tg for a different nef allele than that of NL4-3, in which expression is controlled by human CD2 transcription control elements, anti-CD3 + PMA-induced total thymocyte proliferation was reported to be diminished, and DP thymocyte numbers somewhat increased. These conflicting observations could, in part, be due to the difference in transcriptional control or the different nef allele used. Furthermore, observations in murine thymocytes may not mirror HIV Nef function in human thymocytes. Our observation that in vivo generated Nef+ thymocytes contained CD3+CD27+ cells suggested that Nef+ transduced human thymocytes can acquire a mature phenotype, as we have previously shown that CD27 is a marker for functionally maturing human thymocytes. Indeed, with purified transduced CD3+CD27+ thymocytes, we did not observe an effect of nef expression on either proliferation or IL-2 mRNA induction upon in vitro stimulation (data not shown).

In our experiments, thymic output of Nef+ transduced mature CD4+ and CD8+ thymocytes was reduced. HIV infection of ISP4 cells implies that both lineages could suffer from HIV-induced depletion. In accordance with this notion, thymic output of both CD4+ and CD8+ naive T cells is decreased in HIV-infected patients. In HIV-infected individuals, production of both lineages correlates and increases during highly active antiretroviral therapy. The mechanism of reduced T-cell generation from nef-expressing precursor cells is unclear. Theoretically, it could be the result of increased negative selection or of an inefficient positive selection of immature nef expressing thymocytes. We could not show that nef expressing thymocytes are hyperresponsive upon TCR triggering. This argues against Nef-induced negative selection, as negative selection is expected to result from hyperresponsiveness on TCR triggering in thymocytes. Also, nef-expressing thymocytes were not apoptotic (Fig 6A) and did not upregulate Fas ligand levels (data not shown). These results are in line with the observation that HIV NL4-3–infected SCID-hu mice do not show increased thymocyte apoptosis in vivo compared with mock-infected mice.
could be due to Nef-mediated downregulation of CD4 and CD8β. Loss of coreceptors CD4 or CD8β has been shown to hamper positive selection of the respective lineages, at least in mice. Most likely, these receptors are also essential in man, so that inefficient positive selection of human thymocytes expressing nef may contribute to a decreased output of T cells. As some of the domains of NL4-3 nef that are important for pathogenicity in SCID-hu thymus have recently been described, use of relevant nef mutants could provide some insight into the mechanism of reduced thymocyte generation reported here.

T cells isolated from adult peripheral blood were found to downregulate CD8β, but not CD8α after transduction with Nef+ supernatant (data not shown). This indicates that decreased expression of CD8β on Nef+ transduced thymocytes is not merely a reflection of the disturbed thymocyte development, but that it is an intrinsic property of Nef. Earlier reports demonstrated that Nef did not downregulate human CD8α in cell lines, but human CD8β was never addressed. A dileucin motif was found to be essential in the CD8 cytoplasmic tail for Nef-mediated CD4 downregulation. Similar to MHC class I, the cytoplasmic tail of CD8β does not contain a dileucin motif. In MHC class I, a tyrosine-based motif present in HLA-A and HLA-B, but not in HLA-C, was shown to be essential for Nef-induced downmodulation. At least 1 tyrosine residue is present in the cytoplasmic tail of all CD8β splice variants, but not in the motif as found in HLA-A and HLA-B. The mechanism of TCR upregulation and coreceptor CD8β downmodulation by Nef+ transduced thymocytes remains elusive.

In contrast to CD3+CD27+ thymocytes, we observed increased proliferation upon TCR triggering in Nef+ transduced cultured T cells compared with Nef+ transduced cells. Nef-induced TCR hyperresponsiveness may promote virus production in quiescent peripheral T cells. Rapid progression towards an AIDS-like syndrome, observed in SIV-infected macaque monkeys, was found to be correlated with a nef allele that allowed SIV infection of resting PBMC cultures and that mediated SIV-induced T-cell activation. Activation of T cells by HIV renders them prone to apoptosis and may contribute to peripheral T-cell loss. It is unclear why cultured T cells, but not mature thymocytes expressing nef, were hyperproliferative upon TCR triggering. Differences in TCR-mediated response in thymocytes, as opposed to T cells, could be at the basis of this observation. Direct activation, including Fas ligand upregulation, of Jurkat T cells by Nef was recently shown to involve association with TCR ζ chain. It remains to be determined whether this holds true for primary T cells.

Hanna et al. constructed mice Tg for the NL4-3 HIV genome under control of human CD4 transcriptional control elements. By use of mutant NL4-3 genomes, they showed that the AIDS-like pathology observed in NL4-3 HIV Tg was entirely due to nef expression. These mice express nef not only in CD4+ thymocytes and T cells, but also in cells of the DC/macrophage lineage. Expression of nef in APC may therefore be significant in disease induction. Deficient or aberrant APC function in HIV infection has been reported and very likely contributes to AIDS. Immature DCs are targets for productive HIV infection, whereas mature DCs are efficient transmitters of HIV to T cells. Here we showed that Nef+ transduced mature DCs were phenotypically similar to Nef+ transduced DCs and were not compromised in inducing an allosresponse in PBMCs. DC generation and maturation in suspension cultures and in the thymus was not affected by nef expression. In contrast to DC precursors, Nef+ transduced mature DCs expressed normal CD4 levels, possibly reflecting reduced endocytotic activity. A minority of the few CD4+ Nef+ transduced thymocytes we observed in our experiments (Figs 3B and 5A) were DCs. By downmodulation of CD4 in immature DCs, nef might prevent or hamper superinfection. Our results suggest that nef does not affect function of mature DCs, which may be relevant for HIV transmission to T cells.

We thus show here a direct effect of nef expression on human T-cell generation and activation in the absence of any other HIV gene. These results may give clues in understanding HIV-induced immunopathology and provide a rationale for therapeutic interventions to block Nef function in vivo.

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REFERENCES

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2817

19. Skowronski J, Parks D, Mariani R: Altered T cell activation and development in transgenic mice expressing the HIV-1 nef gene. EMBO J 12:703, 1993
20. Brady HJ, Pennington DJ, Miles CG, Dzierzak EA: CD4 cell surface downregulation in HIV-1 Nef transgenic mice is a consequence of intracellular sequestration. EMBO J 12:4923, 1993
32. Norment AM, Littman DR: A second subunit of CD8 is expressed in human T cells. EMBO J 7:3433, 1988
38. Du Z, Lang SM, Sasseville VG, Lackner AA, Ilyinskii PO, Daniel MD, Jung JU, Desrosiers RC: Identification of a nef allele that...
causes lymphocyte activation and acute disease in macaque monkeys. Cell 82:665, 1995
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