HIV-1 Nef Disrupts Maturation of CD4+ T Cells through CD4/Lck Modulation

Pavel Chrobak, Marie-Chantal Simard, Nathalie Bouchard, Thomas Mutushi Ndolo, Joël Guertin, Zaher Hanna, Vibhuti Pavel Chrobak, Marie-Chantal Simard, Nathalie Bouchard, Thomas Mutushi Ndolo, Joël Guertin, Zaher Hanna, Vibhuti Dave and Paul Jolicoeur

*J Immunol* 2010; 185:3948-3959; Prepublished online 8 September 2010;
doi: 10.4049/jimmunol.1001064
http://www.jimmunol.org/content/185/7/3948

Supplementary Material

http://www.jimmunol.org/content/suppl/2010/09/07/jimmunol.1001064.DC1

References

This article cites 63 articles, 22 of which you can access for free at:
http://www.jimmunol.org/content/185/7/3948.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
HIV-1 Nef Disrupts Maturation of CD4+ T Cells through CD4/Lck Modulation

Pavel Chrobak,* Marie-Chantal Simard,† Nathalie Bouchard,* Thomas Mutushi Ndolo,* Joël Guertin,* Zaher Hanna,*†,‡ Vibhuti Dave,†,‡ and Paul Jolicoeur*,*†,‡

The HIV-1 Nef protein is a major determinant of HIV-1 pathogenicity. It has been found to induce thymocyte depletion, but the mechanisms involved are not completely understood. Also, nothing is known about its effects on thymocyte selection. We used the CD4C/HIVNef transgenic (Tg) mice, which develop a profound CD4+ T cell lymphopenia, to study their thymic development. We report that HIV-1 Nef causes depletion of double-positive thymocytes and impairs selection and lineage commitment of CD4+ single-positive thymocytes. This latter defect could be relieved by increasing the affinity of the TCR–MHC interaction or by allowing CD4+ T cell maturation to proceed in absence of the CD4 tail, in double-Tg (Nef × CD4aileless) mice or in the presence of constitutively active Tg LckY505F. These rescue strategies also resulted in reversal of peripheral CD4+ T cell lymphopenia. Our data indicate that impairment of Lck-mediated CD4 coreceptor signaling by Nef is an important in vivo mechanism of HIV-1 pathogenesis. *The Journal of Immunology, 2010, 185: 3948–3959.

During thymopoesis, immature T cell precursors undergo sequential, tightly regulated maturation steps (1). Briefly, double-negative (DN) CD4+CD8– thymic precursors (∼2%) give rise to double-positive (DP) CD4+CD8+ thymocytes (∼80%), which subsequently undergo selection, lineage commitment, and differentiation into CD4+ and CD8+ single-positive (SP) T cells. These latter steps are primarily determined by TCR signaling, which, in the thymus, is greatly dependent on the interaction of CD4 and CD8 with MHC class II and class I, respectively, expressed on stromal cells and on the activity of Lck bound to CD4 and CD8 cytoplasmic tails (2). According to the kinetic signaling model, TCR signaling strength determines the number of thymocytes that undergo selection, whereas the duration of TCR signaling determines lineage choice (3). Lineage commitment into the CD4 lineage requires proper upregulation of CD4 cell surface expression during maturation (4).

HIV-1 infection has a major impact on thymic structure and function (5). HIV-1 (6, 7) and SIV (8–10) can infect thymocytes in vivo and in vitro (11, 12), leading to various thymic abnormalities observed both in humans and in various model systems. In humans, thymic atrophy is frequent (13, 7). Depletion of DP and CD4+ SP thymocytes and an increase of DN (CD4–CD8–) thymocytes have been reported (7). In a small proportion of HIV-1-infected children, the loss of both peripheral CD4+ and CD8+ T cells appears to result from severe loss of DP cells (14). Similar data have been obtained from SIV-infected macaques (9, 15), HIV-1-infected SCID-hu mice (16–18), and HIV-1- or Nef-expressing fetal thymic organ cultures (19, 20). Finally, in transgenic (Tg) mice expressing Nef under the regulation of various T cell-specific regulatory sequences, depletion of DP, CD4+ SP, and CD8+ SP thymocytes could be documented (21–23).

Although HIV-1 is thought to deplete thymocytes by cell killing (17), in some models thymic hypocellularity was not accompanied by increased apoptosis (19, 24) and appeared to have arisen from abnormal thymic development (21, 19, 18). However, data on the effect of HIV-1 on thymic T cell selection are missing from these models. We therefore relied on the CD4C/HIVNef Tg mouse model generated by our group (25) to investigate the nature of Nef-induced thymic defects. These Tg mice express HIV Nef under the control of human CD4 regulatory elements in cells that are normally targeted by HIV-1 in humans (immature and mature CD4+ T cells, macrophages, and dendritic cells). They exhibit a complex phenotype that mimics human pediatric AIDS very closely. A cardinal feature in these mice is a profound CD4+ T cell lymphopenia (25). They also develop thymic atrophy, loss of DP and CD4+ SP thymocytes, and enhanced responsiveness of their thymocytes to anti-CD3 stimulation in vitro (25).

In this study, we further investigated the biologic basis of Nef-mediated perturbations of thymic cell subsets in CD4C/HIVNef Tg mice. We report that Nef depletes DP thymocytes by a CD4-independent pathway and impairs the generation of CD4+ SP thymocytes through defective Lck-mediated CD4 coreceptor function.

Materials and Methods

**Mice**

The CD4C/HIVMucG (designated in this study as CD4C/HIVNef) (25), AND TCR (26), CD4 tailless (27), and distal Lck promoter driving constitutively active LckF505 (DLGF) (28) Tg mice have been described. The CD4C/HIVNef Tg mice were bred on C3H (H-2k) background (Harlan Laboratories, Indianapolis, IN). The CD45.1 congenic C3H (H-2k) mice were generated in our laboratory. The AND TCR Tg mice (C57BL/6, H-2b), expressing MHC class II-restricted Vα11.1Vβ3+ TCR specific for pigeon and moth cytochrome c were purchased from The Jackson Laboratory (Bar Harbor, ME). The CD4 tailless Tg mice were obtained from Dr. Kim Bottomly (Yale University, New Haven, CT) and were maintained on a CD4–gene–deficient background.

**Abbreviations used in this paper:** B6, C57BL/6; DLGF, distal Lck promoter driving constitutively active LckF505; DN, double-negative; DP, double-positive; FL, fetal liver; HSA, heat stable Ag; IVKA, in vitro kinase assay; KO, knockout; Lin , lineage-negative; MFI, mean fluorescence intensity; p-Lck, phosphorylated Lck; pLN, peripheral lymph node; SP, single-positive; Tg, transgenic.

The online version of this article contains supplemental material.

Received for publication April 1, 2010. Accepted for publication July 31, 2010.

Address correspondence and reprint requests to Dr. Paul Jolicoeur and Dr. Pavel Chrobak, Clinical Research Institute of Montreal; Department of Medicine and Division of Experimental Medicine, McGill University, Montreal, Quebec, Canada.
background. The DLGF Tg mice (line A16924) were obtained from Dr. P. José Alberola-Ila (Oklahoma Medical Research Foundation, Oklahoma City, OK). Animals were kept in a specific pathogen-free animal facility. Animal studies followed guidelines set by the Canadian Council on Animal Care and were approved by the Clinical Research Institute of Montreal Animal Care Committee.

**Abs and reagents**

For flow cytometry, PE-, CyCh-, biotin-, allophycocyanin-, or FITC-conjugated Abs against mouse CD4, CD8a, CD8b, TCRβ, CD24, CD25, CD26, CD69, Vc11, Vβ3, Thy 1.2, Gr1, CD11c, CD11b, Dx5, anti-human CD4, as well as isotypic control Abs rat IgG2a, rat IgG2b, Armenian hamster IgG1, streptavidin PE-, PE-Cy7- or allophycocyanin-conjugated were purchased from Cedarlane Laboratories (Burlington, Ontario, Canada) or BD Biosciences (San Jose, CA). For FcR detection, anti-CD3 Abs and reagents (4G10) were purchased from BD Biosciences (San Jose, CA). For CD28, anti-CD28 Abs (7C10) and reagents (BD Biosciences) were used. Purified serum produced in our laboratory or rabbit preimmune serum followed by goat anti-rabbit IgG PE (Cedarlane Laboratories) were used (29). Purified (hybridoma) or biotinylated (BD Biosciences) anti-CD3 (145-2C11), anti-CD4 mAb (GK1.5, BD Biosciences), anti-TCRβ mAb (H57-597, BD Biosciences), and streptavidin (Zymed, San Francisco, CA) were used for thymocyte stimulation. Abs used for immunoblotting were: anti-p-Y (4G10), anti-Lck (3A5) mAb (Santa Cruz Biotechnology, Santa Cruz, CA), anti-p-Src PY394 (Cell Signaling Technology, Beverly, MA), anti-GAPDH (6C5) mAb (Abcam, Cambridge, MA), anti-actin (Sigma-Aldrich, St. Louis, MO), goat anti-mouse IRDye 800 (LI-COR, Lincoln, NE), and anti-mouse IgG (H+L) Alexa Fluor 680 (Cedarlane Laboratories). For Lck in vitro kinase assay (IVKA), anti-Lck (3A5) mAb and polyclonal anti-Lck serum were used for immunoprecipitation and Western blotting, respectively.

**FACS analysis**

Flow cytometry was performed as previously published (30). DN thymocytes were phenotyped as CD4+ CD8+ lineage-negative (Lin-) (B220-, Gr1-, Cd5-, TCRβ-, TCRγδ, CD11b-, CD11c-). For intracellular Ag detection, a protocol from BD Biosciences was followed, using Cytofix/Cytoperm and Perm/Wash solutions (BD Biosciences). Acquisition was performed on a FACSscan or FACS calibur (BD Biosciences). Data were analyzed using the CellQuest Pro (BD Biosciences) software. Cell sorting was performed on a MoFlo cell sorter (DakoCytomation, Carpinteria, CA).

**IVKA**

For the IVKA, cell lysates were immunoprecipitated with anti-Lck Abs, and an in vitro kinase reaction was run for 1.5 h with the resuspended immunoprecipitates, essentially as previously described (29), with minor modifications (1% Triton X-100 for cell lysis, protein A-coupled agarose beads).

**In vitro thymocyte stimulation**

Thymocytes from Tg and non-Tg mice were plated at a concentration of 2.5 × 10^6 cells per milliliter in Iscove 10% FBS complete medium on 96-well flat-bottom plates precoated (1 h at 37°C) with different concentrations of anti-CD3e (145-2C11) or anti-TCRβ (H57-597) mAbs and were incubated at 37°C for 17 h. After this incubation period, cells were cell-surface detergent (with detergent) or mild lysis buffer, containing protease inhibitors. Subsequently, extracts were loaded on 8–12% SDS gels and then transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Blocking was performed in 5% BSA at 4°C. Incubation with primary Abs (anti-p-Y 4G10, anti-Lck 3H5, anti-p-Src PY394), anti-actin, or anti-GAPDH was done for 2 h at room temperature. Secondary Abs were coupled to Alexa Fluor 680 or 800. Signals were revealed using the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE).

**RT-PCR**

The RT-PCR assays for quantification of transgene-specific HIV RNA were performed as described previously (30).

**Statistical analysis**

Statistical analysis was performed in most cases using the paired two-tailed Student t test. In case of comparison of ratios, a one-sample t test was used.

**Results**

**Early depletion of CD4+ SP but not DP thymocytes in young CD4C/HIV^Nef^ Tg mice**

Nef expression in C3H CD4C/HIV^Nef^ Tg mice is strong in the thymus and in peripheral CD4+ T cells (25). Thymocytes from adult (6- to 8-wk-old) CD4C/HIV^Nef^ Tg mice showed CD4+ and CD8α cell surface downregulation and a depletion of total, DP, and mature TCRβ cells with a preferential profound loss of the TCRβCD4+ SP subset (25, 30) (Fig. 1A–D). The degree of the CD4 downregulation increased as the Tg thymocyte population matured (mature CD4+CD8α SP > TCRβ DP > total DP) (Fig. 1A, 1B). Although CD4- and CD8α cell surface downregulation and TCRβ CD4+ SP depletion were already apparent in young (1.5- and 3-wk-old) Tg mice (Fig. 1D and data not shown), the number of total, DP, and TCRβCD8α SP Tg thymocytes was normal in the first 3 wk of life but only significantly decreased by 6–8 wk (Fig. 1C, 1D and data not shown). Peripheral CD4+ T cell lymphopenia, present in adult Tg mice (30), was also already apparent in 1.5-wk-old Tg mice (Fig. 1E, 1F). These data suggest that distinct mechanisms are involved in Nef-mediated CD4+ SP and DP thymocyte depletion.

**The depletion of CD4+ SP but not DP thymocytes of CD4C/HIV^Nef^ Tg mice can be transferred by FL cell transplantation**

To determine whether Tg thymic phenotypes are cell-autonomous or are due to an abnormal thymic stroma, FL cells from Tg and non-Tg embryos were transplanted into lethally irradiated C3H non-Tg or Tg hosts (Fig. 2). FACS profiles showed that most (99%) thymocytes were donor-derived (expressing cell surface CD45.1) (Fig. 2A) and that Tg thymocytes expressed Nef (Fig. 2B). In addition, this analysis revealed a downregulation of CD4 and a decrease of CD4+ SP thymocytes (Fig. 2C, 2D). TCRβCD4+ thymocytes (Supplemental Fig. 1C), as well as peripheral lymph node (pLN) CD4+ T cells (data not shown), suggested a T cell-autonomous impairment of generation of Tg CD4+ SP T cells. The numbers of DN and DP thymocytes upon transfer of Tg FL into non-Tg hosts were normal (Fig. 2D), suggesting that early Tg progenitors (prior to the DP stage) were competent in the context of FL cell transplantation. However, the numbers of total and DP thymocytes were surprisingly only decreased in Tg hosts transplanted with Tg FL cells (Fig. 2D), consistent with a role for stromal cells in DP thymocyte depletion.

**The different kinetics of CD4+ SP and DP thymocyte depletion together with the different requirements for Tg stroma further strongly suggest distinct mechanisms for the loss of these two populations.**

CD4C/HIV^Nef^ Tg mice show a normal rate of DP production

The DP thymocyte depletion in adult Tg mice led us to investigate the DN to DP transition. We documented Tg RNA expression in Lin+ DN thymocytes by RT-PCR (see below) as well as the expression of Tg human CD4 and GFP in this population in CD4C/HIV^Nef^ Tg mice (data not shown).
shown). Analysis of the Lin- DN compartment of CD4/HIVNef Tg mice revealed that Tg mice had similar numbers of DN1 (CD44+CD25-), DN2 (CD44+CD25+), DN3 (CD25+CD44-), and DN4 (CD25-CD44-) thymocyte subsets as non-Tg mice (Fig. 3A,3B). Furthermore, assessment of proliferation of DP and Lin- DN thymocytes by BrdU incorporation revealed no difference between Tg and non-Tg mice (Fig. 3C). These data suggest that early thymocyte maturation, including the DN3 to DN4 transition, may not be impaired and that b selection is likely to proceed well in these Tg mice.

Nef expression in thymocytes leads to decreased selection and altered lineage commitment of MHC class II-restricted CD4+ T cells

Thymocytes undergoing selection at the DP to SP transition show upregulation of TCR and CD69 and are characterized phenotypically as TCRhiCD69hi (32). In Tg animals and in Tg → non-Tg transplanted mice (Supplemental Fig. 1), we found a decreased percentage of TCRhiCD69hi cells, indicating that fewer Tg thymocytes were undergoing selection. Moreover, downregulation of CD69 and CD5 was found on TCRhiCD4+ SP but not TCRhiCD8+ SP cells (Supplemental Fig. 1), revealing a defect specific to CD4+ SP cells. Because the levels of cell surface CD5 on thymic T cells have been found to correlate with the strength of TCR signaling (33), these results suggest that Tg CD4+ T cells receive weaker TCR signals during maturation.

To better characterize the impact of Nef on CD4+ T cell generation and to determine the role of antigenic stimulation in CD4+ T cell lymphopenia, we bred CD4C/HIVNef Tg mice with MHC class II-restricted AND TCR Tg mice. On a C57BL/6 (B6) background, AND+CD4+ T cells undergo lower-affinity selection following interaction with I-A\(^b\). Adult thymuses from double-Tg (AND 3 CD4/HIVNef) mice showed downregulation of CD4, more prominent on V\(\alpha_{11}\)hiCD4+ SP than on V\(\alpha_{11}\)hi DP thymocytes (Fig. 4A,4B). The double-Tg thymuses had equal numbers of V\(\alpha_{11}\)hi DN thymocytes (Fig. 4C) but were hypocellular (Fig. 4C) and had a decrease of V\(\alpha_{11}\)hi DP cells and an almost complete absence of V\(\alpha_{11}\)hiCD4+ SP cells (Fig. 4A,4C). In addition, the frequency and absolute number of mature V\(\alpha_{11}\)hi heat stable Ag (HSA)low T cells (Fig. 4A) and the frequency of V\(\alpha_{11}\)hiCD69hi thymocytes (Fig. 4A) were lower. Also, the CD5 levels on V\(\alpha_{11}\)hi thymocytes were decreased (Fig. 4E). These results indicate weaker TCR signaling and a decreased selection of CD4+ SP cells in double-Tg (AND 3 CD4/HIVNef) mice.

Interestingly, mature T cells (V\(\alpha_{11}\)hiHSA\(^{low}\)) generated in the thymuses of adult double-Tg (AND × CD4/HIVNef) mice consisted mainly of CD8+ SP cells, which were increased in their absolute number despite the decrease of the DP thymocyte cellularity (Fig. 4A,4C). This observation indicates an altered lineage commitment of MHC class II-restricted T cells in these mice. According to the kinetic signaling model, TCRhiCD4+CD8- intermediate cells...
undergo lineage commitment after positive selection and become CD4+ SP T cells if TCR signaling continues (3); otherwise, they become CD8+ T cells. The upregulation of cell surface CD4 is critical for this event (4). In young (1.5-wk-old) mice, thymic cellularity and the number of Vα11hi DP thymocytes were comparable between single- and double-Tg mice, whereas Vα11hiCD4+ SP thymocytes were absent and Vα11hiCD8+ SP thymocytes were increased (Fig. 4D). An identical pattern of CD69 and CD5 marker analysis was also observed at this young age, as in the adult (5- to 8-wk-old) group (data not shown). Interestingly, in thymuses of young double-Tg mice, we also found an increase of mature (Vα11hiHaβ4hi) CD4-CD8- T cells (data not shown). These cells were similar to those in AND single-Tg mice: they were CD5low (unlike CD4+ or CD8+ T cells), were more prominent in very young mice, and likely belong to the γδ T cell lineage (34).

Analysis of pLN and spleen also revealed a dramatic decrease of Vα11hiCD4+ T cells and an increase of the Vα11hiCD8+ and Vα11hiCD4-CD8- T cells in both young and older double-Tg (AND × CD4/HiNef) mice (Fig. 4F, 4G).

These results confirm the presence of a DP to CD4+ SP maturation block and the maintenance of the DP population at an early age in Nef Tg mice. They also reveal a lineage commitment defect and show that Nef-mediated depletion of CD4+ T cells occurs independently of antigenic stimulation.

**Higher-affinity selection partially rescues the depletion of CD4+ T cells**

The severe CD4 downregulation on CD4+ Tg thymocytes and especially the lower expression of CD5 on Vα11+ Tg thymocytes (Fig. 4E) suggested that they may be selected under low-avidity interactions. Therefore, we next studied single- (AND Nef-) and double-Tg (AND Nef) mice bred on the (B6 × B10BR) F1 background, where the presence of I-Ek in the thymus leads to the preferential deletion of Vα11hiCD4+ SP T cells (35). Selection on this background is thought to be less dependent on CD4 coreceptor signaling (35). A higher percentage and absolute cell number of AND Tg Vα11hiCD4+ SP thymocytes were observed in (B6 × B10BR) F1 than in B6 double-Tg mice (Fig. 5A–C). The average percentage and number of Vα11hi DP thymocytes were lower (Fig. 5A–C), most likely reflecting more efficient CD4+ SP generation from DP thymocytes on the (B6 × B10BR) F1 background. Furthermore, the percentage and absolute cell number of Vα11hiCD8+ SP thymocytes were decreased (Fig. 5A–C). In the periphery, a partial reversal of CD4+ T cell depletion was also observed in the spleen and, to a smaller extent, in the pLN (Fig. 5D). An increase in Vα11hiCD8+ T cells (similar to that observed in the B6 background) and a decrease of Vα11hiCD4+ CD8- T cells were also present (Fig. 5D).

These data demonstrate that an increase in selection affinity can partially reverse the Nef-mediated selection and lineage commitment defect of MHC class II-restricted CD4+ SP thymocytes and partially rescue CD4+ T cell lymphopenia in the periphery.

**Partial reversal of CD4+ SP T cell lymphopenia in double-Tg (CD4Tailless × CD4/HiNef) Tg mice**

The partial reversal of SP CD4+ T cell lymphopenia by higher-affinity selection and the well documented importance of CD4 levels in the maturation of CD4+ T cells (36) led us to test the contribution of CD4 to the CD4+ T cell lymphopenia in Nef Tg mice.
Because CD4 downregulation by Nef requires the CD4 cytoplasmic tail (37, 38) and because a CD4tailless transgene could rescue CD4+ T cell development in CD4 knockout (KO) mice (27), we hypothesized that CD4tailless, unable to be downregulated by Nef, may also prevent CD4+ SP T cell lymphopenia. Double-Tg (CD4tailless × Nef+) mice were generated on a CD4 KO background. The presence of the CD4tailless Tg dramatically increased the percentage of and partially rescued the Nef-induced depletion of TCRhiCD4+ SP thymocytes (Fig. 6A, 6B). As expected, these latter cells now showed no downregulation of cell surface CD4tailless (Fig. 6C). Unexpectedly, for a reason not clear at this time, CD4tailless remained slightly downregulated on DP and HSA+TCR CD4+CD8+ SP thymocytes (Fig. 6C and data not shown) and on peripheral TCR CD4+CD8+ cells (Fig. 6A). We also observed a normalization of the percentage of TCR CD469th cells (Fig. 6D) as well as a reversal of CD5 downregulation (Fig. 6D) on TCR CD4+ SP thymocytes. These data suggest normalization of thymic maturation of CD4+ SP T cells. However, the Nef-induced depletion of total thymocytes, even present in CD4 KO Nef+ Tg mice, was not rescued by the CD4tailless transgene (Fig. 6B), indicating no effect on DP depletion. To rule out a decrease of HIV transgene expression as a mechanism of the rescue of TCR CD4+ SP T cell depletion, HIV Tg expression was quantified by RT-PCR on total RNA from Lin− DN and DP thymocytes and by Western blot of proteins from total thymocytes. With both techniques, Tg expression was found to be similar in these cell subsets of double- (CD4tailless × Nef+) and single-Tg (Nef+) mice (Fig. 6E, 6F).

In the periphery (spleen and LN), relative to appropriate Nef− controls, the number of CD4+ T cells (corresponding to TCR CD8+ cells) (Fig. 6A, 6B) and the CD4/CD8 ratio (Fig. 6A) were also higher in Nef−CD4tailless+ than those in Nef−CD4WT Tg mice. This partial rescue was not caused by the absence of transgene

**FIGURE 4.** Compromised thymic maturation in double (AND TCR × CD4/HIVNef+) Tg mice. Single- (AND Nef−) and double-Tg (AND Nef+) mice (B6 background) were analyzed for thymic and peripheral FACS profiles at the age of 1.5 and 5–8 wk after birth. A, CD4 versus CD8 profiles were obtained on total and Vo11thHSA+ thymocytes in 6-wk-old mice. Numbers alone represent percentages, and those in parentheses mean absolute cell numbers ± SD. B, MFI of CD4+ on DP Vo11th DP and Vo11th CD4+ SP thymocytes was compared between 5- to 8-wk-old (AND × Nef+) double- (Nef+) and AND single- (Nef−) Tg mice. C and D, Absolute cell numbers of distinct thymic populations were determined in 5- to 8-wk-old (C) or 1.5-wk-old (D) mice. E, CD5 marker profiles (full lines) and isotype controls (dashed lines) on distinct thymic subpopulations from 1.5- to 8-wk-old single- (AND−Nef− [normal line]) and double-Tg (AND−Nef+ [bold line]) mice were compared based on MFI. Numbers represent Tg/non-Tg ratios (mean ± SD). F and G, Absolute numbers of peripheral T cell populations of 5- to 8-wk-old (F) or 1.5-wk-old (G) mice. Data are presented as mean ± SD. Statistical analysis was performed using the Student t test for absolute cell numbers and using the one-sample t test for evaluation of maturation markers. *p < 0.5; **p < 0.01; ***p < 0.001.
expression, because the levels of HIV RNA in sorted spleen or LN CD4+ T cells were comparable (or even higher) to those from (Nef+ CD4WT) Tg mice, as assessed by RT-PCR (data not shown). These data demonstrate that the downregulation of CD4 cell surface and/or the CD4 tail-associated signaling pathways have an important role in the Nef-mediated CD4+ SP T cell lymphopenia. However, these events do not appear to be significantly involved in the loss of total thymocytes. Together, these results further support the view that CD4+ SP and DP thymocytes are lost by different mechanisms.

**Lower CD4 coreceptor-dependent TCR signaling despite higher Lck activity in CD4C/HIVNef Tg thymocytes**

The CD4 is a coreceptor for TCR and largely transmits its signal by binding to Lck, thus regulating its accumulation in the proximity of the TCR in the immunological synapse (39). Lck is crucial for thymic T cell maturation, especially of the CD4+ T cell lineage (40, 41). Moreover, Nef has been shown to be capable of interacting with Lck, modulating its distribution, trafficking, and function in tumor cells in vitro (42, 43). In Nef+ Tg thymocytes, the levels of total Lck (Fig. 7A), of “active” p-LckY394 (phosphorylated at Y394) (Fig. 7B), as well as of Lck activity (as measured with an IVKA) (Fig. 7C) were constitutively elevated. Consistent with higher Lck activity, a number of tyrosine substrates showed increased phosphorylation (Fig. 7D, E) (25). In addition, relative to non-Tg controls, in vitro TCR stimulation of Tg thymocytes with anti-CD3 mAb alone resulted in enhanced tyrosine phosphorylation (p-Y) of a large number of substrates (Fig. 7F, 7G), as documented previously (25). Unexpectedly, cell surface levels of CD5 and CD69 were comparable in both groups (data not shown). However, CD4 cross-linking resulted in a synergistic increase of p-Y levels in non-Tg but not in Tg thymocytes (Fig. 7F, 7G). Such a decreased

---

**FIGURE 5.** Higher-affinity selection partially rescues the depletion of CD4+ T cells in double-Tg (AND TCR × CD4C/HIVNef) mice bred on the (B6 × B10BR) F1 background. Thymocytes and pLN and spleen cells from 5- to 8-wk-old single- (AND+Nef−) and double-Tg (AND+Nef+) mice bred on the B6 or (B6 × B10BR) F1 background were subjected to FACS analysis. A, A representative CD4 versus CD8 profile on Vo11hi thymocytes is shown. B–D, Percentage (B) or absolute cell numbers (C, D) of thymic (B, C) or pLN and spleen (D) cells relative to Nef− controls (100%). Numbers in FACS plots represent percentages. Statistical analysis was done using the Student t test. *p < 0.05; **p < 0.01; ***p < 0.001.
capacity of CD4 to contribute to TCR signaling in Nef-expressing thymocytes was also observed after stimulation with lower concentrations of anti-CD3 mAb (Supplemental Fig. 2).

Together, these results indicate that Nef activates Lck, possibly by binding to it, but paradoxically prevents CD4 from contributing to TCR signaling, a process thought to be Lck-mediated.

Low levels of constitutively active LckY505F lead to Nef-mediated enhancement of Lck activation and correct thymic CD4+ T cell lymphopenia in Tg mice

The lower response of Nef+ Tg thymocytes to TCR/CD4 engagement suggested a loss of CD4 coreceptor function. Because this function is closely linked with Lck signaling (44), we attempted a rescue experiment by enhancing Lck activity. For this experiment, we used the DLGF Tg mice expressing low levels of LckY505F under the distal Lck promoter (28). These Tg mice exhibit very efficient DP to SP transition and have a high number of CD4+ SP thymocytes (45). The levels of total Lck (Fig. 7A), active p-LckY394 (Fig. 7B), and Lck activity (Fig. 7C) in DLGF Tg thymocytes were only slightly increased relative to those in non-Tg control littermates. However, in double-Tg (DLGF × Nef+) thymocytes, the levels of active p-LckY394 and of Lck activity were further increased relative to those in single-Tg Nef or DLGF thymuses (Fig. 7B, 7C).

In addition, analysis of tyrosine phosphorylated substrates in total thymocyte extracts of double-Tg mice showed the presence of a limited number of highly hyperphosphorylated substrates relative to controls (Fig. 7D, arrows). One of these is likely to be p56 Lck, found to be hyperphosphorylated by other methods (Fig. 7C).

FACS analysis of double-Tg (DLGF × Nef) thymocytes still showed downregulation of cell surface CD4 on DP cells (Fig. 8A, 8B), but this phenotype was partially reversed on CD4+ SP (Fig. 8A, 8B) and peripheral CD4+ (data not shown) T cells. Strikingly, this analysis revealed completely normal percentage and absolute numbers of CD4+ SP cells in double-Tg (DLGF × Nef) Tg mice (Fig. 8A, 8E). The reversal of CD69 downregulation in double-Tg mice (Fig. 8D) suggests a rescue of the thymic selection defect.

FIGURE 6. Partial reversal of thymic and peripheral CD4+ T cell lymphopenia in double-Tg (CD4Tailless × CD4C/HIV × Nef) mice. (CD4TaillessNef+/CD4+/−), (Nef+/CD4+/−), and (Nef+/CD4+/−) mice together with appropriate Nef- controls were sacrificed at the age of 6–12 wk, and cells from different organs were subjected to FACS analysis. A, CD4 and CD8 profiles of TCRhi thymocytes and splenocytes are presented. Numbers refer to percentages. B, Absolute cell numbers of total thymocytes (total Thy) and of different TCRhiCD4+ T cell subsets were calculated based on cell frequency obtained by FACS and total cell counts. C, MFI of CD4+ on DP and TCRb+CD4+CD8− SP thymocytes was compared. D, Percentage of TCRhiCD69+ cells on total thymocytes and CD5 MFI on TCRb+CD4+CD8− thymocytes. Results were compared with appropriate Nef− Tg negative controls CD4Tailless (Nef−CD4−) or (Nef−CD4+/−) in the way of a Nef− Tg/non-Tg ratio and are expressed as mean ± SD. E, Lin− DN and DP thymocytes were sorted, and Nef was detected by RT-PCR. F, Protein lysates from total thymus were analyzed for Nef and GAPDH expression by Western blotting. Nef expression was normalized to GAPDH and set to a value of 1 in a single-Tg Nef control sample. Data were obtained from 3 to 11 individual mice of each genotype, except for RT-PCR data, which were pooled from three mice of each genotype. Statistical comparison was performed using the Student t test. *p < 0.05; **p < 0.01; ***p < 0.001.
However, some alterations remained in the double-Tg mice. These included an increase of CD8 SP thymocytes (Fig. 8A, 8F), a higher ratio of TCR\textsuperscript{hi}/TCR\textsuperscript{low/neg} CD4 SP T cells (Fig. 8C) (also observed in the periphery [data not shown]) and a decrease, although not statistically significant, of DP thymocytes (Fig. 8E). The significant increase of active Lck signaling in double-Tg (DLGF \times Nef) Tg mice could also explain the remaining alteration of the CD4/CD8 T cell ratio in double-Tg mice, because this ratio has been shown to decrease once certain levels of Lck were exceeded (28). In peripheral lymphoid organs of double-Tg mice, significant reversal of the depletion of total (TCR\textsuperscript{hi} and TCR\textsuperscript{low/neg}) and especially of TCR\textsuperscript{hi} CD4 T cell numbers was also observed (Fig. 8E), whereas...
CD8⁺ T cells were significantly increased (Fig. 8F). This impressive reversal of many phenotypes was not the consequence of decreased Nef expression in double-Tg mice, because strong Nef expression was detected in Lin⁺ DN and DP thymocytes by semiquantitative RT-PCR (Fig. 8G) and by Western blotting (Fig. 8H) in DP thymocytes of double-Tg (DLGF³Nef) mice. These data demonstrate that low levels of LckY505F can overcome the Nef-induced thymic CD4⁺ T cell maturation defect and partially rescue the Nef-induced peripheral CD4⁺ T cell lymphopenia.

Discussion
We report that Nef induces distinct thymic phenotypes in a Tg mouse model of AIDS: a progressive depletion of DP cells and a CD4⁺ SP T cell generation defect. We also provide evidence that the biological basis of these defects is different.

Nef induces DP thymocyte depletion
Although adult CD4C/HIVNef Tg mice show a severe depletion of DP thymocytes (25), we found that the Tg DP cell number was normal early in life (during embryonic development and early adulthood). This suggests that, in young Tg mice, DP cells are generated at a normal rate and that DN to DP transition is not significantly hindered in this context. In addition, we found no evidence of impaired DN to DP transition in adult Tg mice, even at a time when DP depletion does occur. Indeed, Tg mice exhibited normal DN thymocyte distribution and intrathymic proliferation. Moreover, as expected in the presence of an apparently normal DN to DP transition, the depletion of DP thymocytes could not be rescued by the expression of a TCR Tg. These data suggest that Tg DP thymocyte depletion occurs as a consequence of their impaired survival. Our previous report showing that Bcl2 overexpression could partially rescue DP, but not CD4⁺ SP T cell depletion (46), is consistent with this interpretation. Conversely, we have now shown that, with higher-affinity selection, LckY505F and CD4tailless were able to rescue Tg CD4⁺ SP loss but failed to rescue DP T cell depletion. These results suggest that distinct and independent molecular pathways are involved in DP and CD4⁺ SP thymocyte loss. This conclusion is also in agreement with our results from FL cell transplantation. These latter experiments demonstrated that depletion of donor Tg CD4⁺ SP cells is cell-autonomous, while...
suggested a rather complex mechanism for depletion of DP thymocytes (i.e., a mechanism requiring Tg expression in both stromal and DP cells). This Tg-expressing thymic stromal cell population could represent the same nontransplantable cell subset that we recently found to be responsible for organ diseases in CD4C/HIVNef Tg mice (47). Although the identity of the stromal cells in both cases remains unknown, they are most likely of nonhematopoietic origin. Data from the literature suggest that they may represent thymic epithelial cells, which were reported to harbor HIV-1 footprints in vivo (48).

An early onset deletion of total thymocytes was reported in CD2/NeF Tg mice (49). In contrast to our findings in CD4C/HIVNef Tg mice, it was found to result from impaired DN to DP transition and could be abrogated by activated LckY505F. The apparent distinct mechanism of DP cell loss in these two Tg models make a comparison hazardous. These differences are likely to reflect the levels of Nef and/or the promoters used to target its expression.

Nef impairs CD4+ SP thymocyte positive selection and lineage commitment as a consequence of defective CD4/Lck function

The profound depletion of CD4+ SP thymocytes represents an additional thymic defect. This depletion is already present in very young CD4C/HIVNef Tg mice and suggests an impaired maturation/survival of this population. A similar decrease of CD4+ SP thymocytes was observed in other Tg mice expressing HIV-1 Nef under the regulation of CD2 (22) or CD38 (21) regulatory elements but was not further analyzed. Generation of SP thymocytes from DP thymocytes is governed primarily by the character of the TCR signals that the thymocytes received (50, 51). DP thymocytes that receive a TCR signal of appropriate strength first undergo positive selection and become TCRhiCD4+CD8+ intermediately cells. Subsequently, these cells undergo lineage commitment and become CD4+ SP T cells if TCR signaling persists or limit the amount of Lck at the TCR complex. Moreover, Nef-induced CD4 downregulation, which is dependent on direct binding of Nef to the CD4 intracytoplasmic tail (37, 38), is critical for impairment of CD4+ SP maturation. However, depletion of total or DP thymocytes was not reversed by CD4tailless. This result confirms a recent study showing a Nef-mediated increase of Lck activity in 293T and Jurkat cells (58) and extends these observations to in vivo primary thymocytes, a population not easily amenable to experimentation in humans. Moreover, our results are in agreement with the reported activation of another Src-related kinase, Hck, by Nef (60, 61). Secondly, this enhanced signaling possibly reflects the upregulation of TCR/CD3ε observed on DP Nef-expressing thymocytes. Finally, the elevated coreceptor-independent signaling could be related, at least in part, to CD4 downregulation, which most probably increases free Lck in Tg thymocytes, as shown previously in Nef-expressing cultured tumor cells (38) and in CD4/CD8–gene–deficient mice (62). In this latter study, Van Laethem et al. (62) found that coreceptor-independent TCR signaling was enhanced in thymocytes expressing Tg CD4 molecules unable to sequester Lck, as a result of increased levels of CD4-unbound (i.e., “free”) Lck in these cells.

The lower number of CD4+ SP thymocytes coupled with the significantly increased number of CD8+ SP thymocytes generated in young (AND × Nef+) double-Tg mice indicate that Nef also impairs lineage commitment. Interestingly, a recent report demonstrated that failure of MHC class II thymocytes to upregulate CD4 coreceptor during maturation of TCRβ DP thymocytes toward the TCRβCD4+ SP stage resulted in skewed lineage commitment toward CD8+ T cells (4). Consistent with these results, cell surface CD4 levels were found to be progressively downregulated in Nef Tg mice as DP cells matured toward TCRβCD4+ SP thymocytes (Fig. 1B).

Thus, the Nef-mediated CD4 downregulation in thymocytes appears to negatively affect positive selection, most likely by limiting the amount of Lck at the TCR complex. Moreover, Nef impairs lineage commitment of CD4+ T cells by preventing CD4 upregulation during maturation. In contrast, the development of CD8+ T cells in Nef Tg mice is essentially normal (M. Rahim, E. Priceputu, P. Chrobak, and P. Jolicoeur, manuscript in preparation).

CD4+ SP thymocyte depletion is rescurable

We used three complementary approaches to manipulate and enhance the delivery of signals to Tg thymocytes and rescue CD4+ SP T cell deficiency. The first approach involved selection in the presence of LEu in (AND × Nef) double Tg mice bred on a (B6 × B10BR) F1 background. In these conditions of higher-affinity TCR–MHC interaction, the partial rescue of CD4+ SP thymocyte depletion is likely to have occurred, because stronger signals are thought to be less dependent on coreceptor signaling (35).

The second rescue experiment of Nef-induced CD4+ T cell deficiency was achieved with the CD4ailless transgene. This gene has been shown to rescue the loss of CD4+ T cells in CD4–gene–deficient mice in the absence of CD4 tail-associated Lck (27). Interestingly, in this experiment, the normalized CD4/CDS ratio in peripheral lymphoid organs was associated with reversal of the Nef-mediated progressive CD4 downregulation during the DP to TCRβCD4+ SP T cell transition, strongly suggesting a normalized lineage commitment. This result further supports the hypothesis that Nef-induced CD4 downregulation, which is dependent on direct binding of Nef to the CD4 intracytoplasmic tail (37, 38), is critical for impairment of CD4+ SP maturation. However, depletion of total or DP thymocytes was not reversed by CD4ailless. In agreement with this latter result, total thymocytes were depleted in CD4C/HIVNef Tg mice bred on the CD4-gene–deficient background (30). These data are also consistent with other work showing that the level of cell surface CD4 influences T cell maturation (36) and Lck sequestration (62).
Finally, we achieved rescue of Tg CD4+ SP but not DP depletion by expressing low levels of constitutively active LckY505F. This result further strengthened our hypothesis that downregulation of CD4, together with its effects on Lck signaling, is implicated in the presence of Nef significantly enhanced Lck activity, strongly suggesting that Nef binds and/or activates the mutant open form of Lck (LckY505F) much better than wild-type Lck (LckWT). These findings are reminiscent of other in vitro work showing that the C-terminal phosphorylated form of Lck is more activated by Nef than its phosphorylated form (60). However, our results are apparently at odds with data showing that Hck can be activated by its phosphorylated form (61). Nevertheless, our results are ap-


