Primary HIV-1 Nef Alleles Show Major Differences in Pathogenicity in Transgenic Mice

Elena Priceputu1#, Zaher Hanna*,1,2,4#, Chunyan Hu1, Marie-Chantal Simard1, Patrick Vincent1, Steffen Wildum5, Michael Schindler5, Frank Kirchhoff5 and Paul Jolicoeur*1,3,4.

Laboratory of Molecular Biology, Clinical Research Institute of Montreal, 110 Pine Avenue West, Montreal, Quebec, H2W 1R7, Canada 1; Departments of Medicine 2 and Microbiology and Immunology, Université de Montréal, Montreal, Quebec, H3C 3J7, Canada; Division of Experimental Medicine, McGill University, Montreal, Quebec, Canada; Institute of Virology, University of Ulm, Germany 5.

# These two authors contributed equally to this work.

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* Correspondence to:

Dr Paul JOLICOEUR, MD, Ph.D.
Clinical Research Institute of Montreal
110 Pine Avenue West
Montreal, Quebec H2W 1R7
CANADA
Tel.: (514) 987-5569
Fax: (514) 987-5794
E-mail: paul.jolicoeur@ircm.qc.ca

Dr Zaher HANNA, Ph.D.
Clinical Research Institute of Montreal
110 Pine Avenue West
Montreal, Quebec H2W 1R7
CANADA
Tel.: (514) 987-5571
Fax: (514) 987-5604
E-mail: Zaher.Hanna@ircm.qc.ca
ABSTRACT

We previously reported that the HIV-1 NL4-3 Nef is necessary and sufficient to induce a severe AIDS-like disease in transgenic (Tg) mice when expressed under the regulatory sequences of the human CD4 gene. We have now assayed additional Nef alleles (SF2, JR-CSF, YU10x and NL4-3T71R), including some from long-term non-progressors (AD-93, 032an, 039nm) in the same Tg system and compared their pathogenicity. All these Nef alleles downregulated cell surface CD4 in human cells in vitro and, except NefYU10x, in Tg CD4+ T cells. Depletion of double-positive (DP) and single-positive (SP) thymocytes occurred with all alleles, but was less pronounced in NefYU10x Tg mice. Loss of peripheral CD4+ T cells was observed with all alleles, but was minimal in NefYU10x Tg mice. In Nef032an and NefSF2 Tg mice, T cell loss was severe despite lower levels of Tg expression, suggesting a higher virulence of these alleles. All Nef alleles, except NefYU10x and NefNL4-3(T71R), induced an enhanced activated/memory and apoptotic phenotype. Also, all could interact with and/or activate PAK2, except NefJR-CSF. Organ (lung and kidney) diseases were present in NefNL4-3(T71R), Nef032an, Nef039nm and NefSF2 Tg mice, despite very low levels of Tg expression for this latter strain. However, no or minimal organ disease developed in NefYU10x, NefAD-93 and NefJR-CSF Tg mice respectively, despite high levels of Tg expression. Our data show that important differences in the pathogenicity of various Nef alleles can be scored in Tg mice. Interestingly, our results also revealed that some phenotypes can segregate independently, such as CD4+ T cell depletion vs activation as well as severe depletion of thymic vs peripheral CD4+ T cells. Therefore, expression of Nef alleles in Tg mice under the CD4C regulatory elements represents a novel assay for measuring their pathogenicity. Because of the very high similarity of this murine AIDS-like disease with human AIDS, this assay may have a predictive value regarding the behavior of Nef in infected human individuals.
INTRODUCTION

The development of AIDS following infection with HIV-1 varies widely, but occurs after a relative long latency in most adults (median 8-10 years) (8, 27, 63, 77). In children infected at birth, AIDS develops with a bimodal distribution, after either a very short (median 4.1 months) or longer (median 6.1 years) incubation period (5, 90, 93). However, in some individuals, the progression of the disease course is very slow and AIDS develops after a much longer period (>12 years after infection for adults and >8 years after neonatal infection for children) (10, 78). These HIV-1 infected individuals are designated slow progressors or long-term non-progressors (LTNP) (24). On the contrary, in other HIV-infected individuals AIDS develops very quickly. These fast progressors are found in both pediatric (5, 80, 87, 93) and adult (43, 63, 72, 76, 77, 80) cohorts. As with many other viral diseases, it is likely that the severity and time course of AIDS development reflect either individual host genetic susceptibility/resistance or virus pathogenicity or both (26). The host genes could control levels of virus replication, immune response to HIV-1 as well as the signaling pathways with which the viral proteins may interfere. The best example of such genetic susceptibility is that of the CCR-5 gene, coding for one of the major co-receptors for HIV-1 infection (73). A homologous deletion of CCR-5 (Δ32-CCR-5), found in ~1% of the Caucasian population, confers nearly complete protection against HIV-1 infection in vivo (18, 42, 65, 89). The most evident case of low viral pathogenicity of HIV-1 in humans was documented in the Sydney blood bank cohort in which eight HIV-1 infected individuals, all LTNP, were infected with a Nef-defective HIV-1 strain by a single blood donor (17, 59). Studies of this cohort and of a few other individuals infected with HIV-1 strains containing deletions or functional defects in Nef (12, 29, 53, 85, 88) strongly suggested that Nef is a major determinant of pathogenicity in humans.
Work with experimental animal models also confirmed the different pathogenicity of various SIV nef alleles (23), as well as the low pathogenicity of nef deleted SIV strains in macaques (51, 67) and of Nef-mutated HIV-1 strains in SCID-hu mice (2, 48). Finally, expression of HIV-1 or SIV Nef in transgenic (Tg) mice was shown to have very important pathological consequences (7, 20, 21, 35, 47, 57, 64, 94, 96).

In vitro comparison of various nef alleles and their effect on viral replication and downregulation of CD4 or MHC class I (MHC-I) molecules has revealed a wide range of potency (4, 13, 14, 16, 28, 30, 69, 100, 104). Correlation could be established, although not by all groups (74), between the extent of Nef-induced CD4 downregulation and viral replication and disease progression (11, 13, 30), suggesting that differences in Nef function may have a major impact on viral pathogenicity. However, similar head-to-head in vivo comparison has been reported for only two nef alleles in Tg mice, using the same promoter (7). Moreover, the few available Tg mouse models of HIV-1 expression exhibiting immune defects following expression of the same Nef\textsuperscript{NL4-3} allele (35, 96) were constructed with different regulatory elements and expressed Nef in different cell subpopulations, making direct comparison difficult.

The CD4C/HIV Tg mice expressing only the Nef protein of the HIV-1 strain NL4-3 develop a severe AIDS-like disease in the absence of virus replication (34, 35, 45). These mice express Nef in immature CD4\textsuperscript{+}CD8\textsuperscript{+} and CD4\textsuperscript{+}CD8\textsuperscript{-} thymocytes, in peripheral CD4\textsuperscript{+} T cells and in cells of the macrophage/dendritic cell (DC) lineage [peritoneal, alveolar and tissue macrophages, Kupffer cells, DC] (34, 35, 82). The disease involves pathological changes of immune structures: thymic and lymphoid organ atrophy, expansion of marginal zone, decreased formation of germinal centers and of follicular dendritic cell network (35, 82, 83). Also, immune functions are significantly impaired: downregulation of CD4 cell surface, preferential loss of
CD4+ T cells, increase of B cell numbers, activation of both CD4+ T cells and B cells, hypergammaglobulinemia, loss of LN DC, accumulation of CD11c+ CD11bHI DC population, impaired DC function, lower helper CD4+ T cell function have been documented (35, 82, 83, 84, 103). In addition, these Tg mice show an increased susceptibility to C. Albicans (15). Finally, kidney (cystic dilatation, focal segmental glomerulosclerosis, interstitial nephritis), lung (lymphocytic interstitial pneumonitis) and heart (cardiomyocytis, cardiomyolysis) diseases develop (35, 46, 49). These pathological and functional changes are very similar to those found in human AIDS.

The large number of Nef-induced phenotypes which can be observed in these CD4C/HIV Tg mice represents a novel in vivo assay for Nef functions. However, it remained to be established whether Nef from other HIV strains would be equally pathogenic when expressed in Tg mice with the same CD4C regulatory elements. Here, we used this Tg mouse model to compare the pathogenicity of six additional nef alleles derived from different HIV-1 infected individuals with distinct clinical courses.
MATERIALS AND METHODS

Proviral and Nef expression constructs for in vitro assays. For functional analysis, HIV-1 nef alleles were cloned into the bicistronic pCG expression vector (31) or a proviral HIV-1 NL4-3 construct coexpressing Nef and the enhanced green fluorescent protein (eGFP) via an internal ribosome entry site (IRES). Generation of the pCG vectors and the NL4-3 IRES-eGFP constructs carrying a defective (nef-) nef reading frame or the NL4-3 nef-allele has been previously described (91, 92). PCR amplification and sequencing of nefs from three individuals with long-term nonprogressive (LTNP) infection (032an-93, 039nm-94, AD-93) was carried out as described (11, 52). The JR-CSF (56) and YU10x (61, 62) HIV-1 DNA clones were obtained respectively from Drs. Irvin S.Y. Chen and Yoshio Koyanagi or from Dr. Beatrice Hahn and George Shaw, through the NIH AIDS Research and Reference Reagent Program. The SF2 (60) DNA clone was obtained from Drs. Louise Poulin and Dr. Jay Levy. All nef alleles were cloned into the bicistronic pCG vector and the proviral HIV-1 NL4-3 IRES-eGFP construct using unique XbaI (pCG vector) or HpaI (proviral construct) and MluI (both) restriction sites flanking the nef ORF essentially as described previously (75, 91, 92). Sequence analysis confirmed that the recombinant proviral constructs contained the correct nef genes and verified the absence of undesired changes.

Virus stocks and in vitro infectivity. For virus production, 293T cells were transfected by the calcium phosphate method with 10 µg of the proviral constructs and the p24 antigen concentrations were quantified as described previously (11). Virus infectivity was determined on P4-CCR5 (81) and TZM-bl (19) cells using the β-galactosidase screen from TROPIX as described previously (75).
**Receptor modulation in vitro.** Jurkat T cells or HeLa CIITA cells were transfected with the pCG vectors or transduced with HIV-1 proviral constructs both coexpressing Nef and eGFP as described previously (54, 75, 92). Nef-mediated down- or up-regulation of cellular receptors was quantified by flow cytometry, along with the expression of the eGFP reporter molecules as described previously (11, 54, 75, 92). These include CD4, MHC-I and CD28 measured in Jurkat T cells and class II MHC (MHC-II) and the invariant chain (Ii) associated with immature MHC-II molecules on HeLa CIITA cells (98). Briefly, four ranges of green fluorescence representing no, low, medium, and high levels of GFP and, indirectly, Nef expression were defined. For quantitative fluorescence-activated cell sorting analysis, the mean channel numbers of red fluorescence obtained for cells transfected with a control construct expressing GFP only were divided by the corresponding numbers obtained for cells coexpressing Nef and GFP to calculate the values for down- or up-modulation, respectively. The same ranges of green fluorescence were used in all calculations.

**Generation of Tg mice.** The nef fragments were amplified from the corresponding proviral DNA by PCR using with primers harboring a 5′ Mlu I and 3′ NotI site respectively, to generate unique sites for convenient cloning: #585 (5′-ACGCGTATGGGTGGCAAGTGGTCAAAA-3′) and #584 (5′-GCGGCCGCTTAGTTCTTGTAGTACTCCGG-3′) for AD-93; #585 and #582 (5′-GCGGCCGCTTCAGCAGTTCTTGTAGTACTC-3′) for both 032an and for 039nm; #585 and #725 (5′-GCGGCCGCTCAGCAGTCCTTGTAGTACTC-3′) for JR-CSF; #1009 (5′-ACGCGTATGGCTGGATGGCCTACTGTA-3′) and #1010 (5′-GCGGCCGCTCGAGGTCATCAGTTCTTGTAGTACTC-3′) for YU10x; and #585 and #583 (5′-GCGGCCGCTTCAGCAGTCTTTGTAGTACTC-3′) for SF2. The threonine residue at position 71 of the HIV-1^NL4-3^ Nef protein was mutated to arginine (T71R). This mutation was
produced using PCR site-directed mutagenesis on a SacI-BamHI HIV-1 fragment subcloned in the pBS KS vector as described previously (37, 40) using mutational primer #857 (GTACCTGAGGTCTGACTGGAA) containing C, replacing G, at nt 8998 to produce the Nef\textsuperscript{NL4-3(T71R)} mutation. The identity of each allele and of the T71R mutation was confirmed by sequencing. Each of the \textit{MluI-NotI} fragment was then incorporated into the CD4C/HIV\textsuperscript{MutG} DNA backbone, thus replacing Nef\textsuperscript{NL4-3(WT)}. The CD4C/HIV\textsuperscript{MutG} (designated CD4C/HIV-Nef\textsuperscript{NL4-3}) Tg DNA construct has been described previously (35). This Tg DNA harbors a complete HIV-1\textsubscript{NL4-3} genome in which all the known open reading frames (ORF), except that of Nef, have been interrupted by mutations. These Tg sequences have the capacity to express only the Nef gene. Each linearized transgene was purified and inoculated into 1-day old (C57BL/6 X C3H) F\textsubscript{2} embryos to generate Tg mice, as described (34, 35). A minimum of two independent founder (F) Tg mice faithfully expressing each transgene were generated. Tg lines were established by breeding as heterozygote for the transgene on the C3H/HeN background.

\textbf{Transgene expression.} Northern blot analysis, using the whole HIV-1 genome as a probe, was used to assess HIV-1 RNA transgene expression, as previously described (34, 35, 37). The \textsuperscript{32}P-labeled bands were detected with the PhosphoImager screen and scanned with the StormImaging unit (Amersham Biosciences). Semiquantitation of the RNA bands was estimated with the ImageQuant software (Amersham Bioscience). The ratio of HIV-specific 8.8 kb signal relative to the actin-specific signal was obtained. In addition, transgene RNA expression was estimated by real-time quantitative RT-PCR, as described previously (103). For the reverse transcription (RT) reaction, total RNA (1 to 2 µg) isolated from thymuses was used as a template in a 20-µl reaction mixture containing 1x FIRST-strand buffer, as described by the manufacturer (Invitrogen), with Moloney murine leukemia virus reverse transcriptase and 1 mmol of pd(N)6 random
primers/liter. After incubation at 42°C for 1.5 h, the enzyme was inactivated at 99°C for 5 min and the product was diluted to 40 µl for amplification. Four µl were used for quantitative PCR amplification with a Quantitect probe PCR kit (QIAGEN) with a reaction mixture containing 10µl SYBERGREEN PCR Master mix, 1.6µl of each primer, at 10 pMol/µl, 4µl template DNA (cDNA) in 2.8 µl RNAse-free H2O. Quantitative PCR was performed with an MX4000 multiplex quantitative PCR instrument (Stratagene). Amplification was performed with a reaction volume of 20 µl for 40 cycles (45 s at 95°C, 45 s at 60°C). The primers used were specific for a 150-bp amplicon of fully spliced HIV-1 transcripts and a 100-bp amplicon of the S16 as internal standard. Primers: forward primer (oligo 77) located in Ex1 of human CD4 gene: CCCCCACTGGGCTCCTGGTTGCAGC; reverse primer for HIV (strain NL4-3), CAGTCGCCGCCCCTCGCCTCTT at nt 743 upstream of the major 5’-splicing junction; for mouse S16, forward primer (oligo 2432) AGGAGCGATTTGCTGGTGTGG and reverse primer (oligo 2433) GCTACCAGGGCCTTTGAGATG. The specificity, sensitivity, and reproducibility of the quantitative PCR assays were verified by using cDNA prepared from the thymuses of CD4C/NefNL4-3(WT)Tg mice used as positive control.

Detection of Nef protein was done by Western blot analysis, as described previously (34, 35), using various Nef antibodies. The rabbit anti-Nef antibody used (1:2000) was prepared by injection of GST-Nef fused protein as described previously (40). The monoclonal anti-Nef AE6 (32), EH1 and 6.2 antibodies were obtained from Drs James Hoxie, Kai Krohn and Vladimir Ovod, through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID.

**Histological analysis.** Histological assessment on lymphoid and nonlymphoid organs was carried out essentially as previously described (34, 35). Semiquantitative assessment of the
histological phenotypes was done as previously described (36). The score 1 was given to diseased kidneys with 1-5 glomeruli affected with their tubules; 2, for 10-50% surface area affected; 3, for >50% surface affected and 4, for almost all nephrons affected. The individual scores were added up and divided by total number of mice analyzed to obtain average scores.

**Flow Cytometry of primary mouse cells.** Flow cytometry was performed on a FACSscan and FACS Calibur (Becton BD Dickinson, San Jose, CA) using antibodies specific for various cell surface markers: CD4, CD8, TcRαβ and Thy1.2 for T cells and B220, Mac-1 for B cells and macrophages, respectively, and CD25, CD44, CD45RB, CD69, CD62L for T cell activation, as described previously (35, 103). Cell Quest software (Becton BD Dickinson) was used for analysis.

**Apoptosis Analysis.** This procedure was performed as described previously (84). Apoptosis/death of CD4⁺ T cells was evaluated by Cell Quest software using two different techniques. First, cells were stained with annexinV and PI to detect early apoptotic cells (single-positive, annexinV⁺ PI⁻) and dead cells (double-positive, annexinV⁺ PI⁺). Alternatively, 7AAD staining (membrane impermeable dye) was used to detect apoptotic and dead cells gating on high and intermediate 7AAD⁺ cells. In both techniques, fragments and necrotic cells were excluded by gating on high and intermediate FSC excluding low FSC, low and high SSC. Both techniques provided comparable data.

**Purification of CD4⁺ T cells.** CD4⁺ T cells were purified by cell sorting, by positive selection, as previously described (103). Lymph node (LN) cells were resuspended for 20 min in blocking buffer (PBS, 20% FBSI (fetal bovine serum, Gibco/BRL, Life Technologies). Staining was performed with PE-coupled anti-CD4 on ice for 40 min. CD4⁺ T cells were sorted by gating on the PE-positive population using the MoFlo cell sorter (MoFlo, Colorado, USA).
**In Vitro Kinase Assay (IVKA).** Immunoprecipitation was first performed with rabbit anti-Nef polyclonal antibodies followed with IVKA on the immunoprecipitated pellet. A detailed protocol for this technique has previously been published (102). $^{35}$S-methionine labeling of proteins from thymocytes was described previously (102).

**Statistics.** Statistical analyses (ANOVA, Sigmastat and Student's t-test) were performed as previously described (84, 103).
RESULTS

Functional activity of primary HIV-1 nef alleles in vitro

Eight nef alleles derived from HIV-1-infected individuals were assayed for their ability to modulate CD4, CD28, CXCR4, MHC-I, MHC-II and Ii surface expression. The primary AD-93, 032an-93 and 039nm-94 nef alleles were derived from individuals with long-term non-progressive HIV-1 infection (11, 52). Four nef genes were obtained from well characterized molecular clones, NL4-3 (1), SF2 (3, 14, 60), JR-CSF (48, 56) and YU10x (61, 62, 104). SF2, JR-CSF and YU10x HIV-1 were derived from AIDS patients. A variant of the NL4-3 Nef containing a substitution (T71R) at amino acid residue 71 in the conserved proline-rich region was also included in the analysis. This was done because this amino acid residue is frequently found in primary HIV-1 isolates (29), and because it has been proposed that this variation in Nef might affect T cell activation and viral replication (25), although this could not be confirmed in a subsequent study (86). Alignment of Nef amino acid sequences showed a high conservation of known functional domains and no obvious inactivating point mutations (Fig. 1).

We examined the ability of the eight nef alleles to modulate CD4, MHC-I and Ii surface expression. The first two Nef activities are well established (58, 79, 95), whereas the latter has been described more recently and might enable HIV-1 to inhibit functional MHC-II antigen presentation (98). Cells transduced with the proviral NL4-3 construct containing a defective nef gene did not show significant alterations in the surface levels of MHC-I or Ii (Fig. 2A, panels 2b, c). In contrast, CD4 expression was diminished in nef-defective HIV-1-infection because Env and Vpu also down-modulate this receptor (Fig. 2A, panel 2a). At low and medium levels of eGFP and hence viral gene expression, however, CD4 downmodulation was more efficient in the presence of Nef (Fig. 2B, left panel). All HIV-1 nef alleles analyzed modulated the surface
expression of cellular receptors albeit with differential efficiency. For example, the AD-93 Nef was only marginally active in modulating CD4, MHC-I and Ii (Fig. 2A, panel 6). The YU10x Nef also showed little activity in modulating CD4 and Ii but efficiently down-modulated MHC-I (Fig. 2A, panel 8). The eight HIV-1 Nef alleles analyzed also showed differential activity in upregulating Ii. The NL4-3, 039nm, AD-93 and YU10x Nefs were poorly effective at enhancing Ii surface expression, whereas the SF2, 032an and JR-CSF Nef strongly upregulated its expression (Fig. 2B, right panel). Interestingly, replacing arginine by threonine at position 71 (T71R) of Nef$^{NL4-3}$ enhanced its activity in upregulating Ii (Fig. 2B, right panel). Vpu- and Env-mediated CD4 downmodulation to some extent complicated the quantitative analysis of this Nef function in the proviral context, particularly at high levels of viral gene expression (Fig. 2B, left panel). Therefore, we also investigated CD4 downmodulation in Jurkat cells transiently transfected with bicistronic vectors coexpressing the eight HIV-1 nef alleles and GFP. As expected from the results obtained using the proviral constructs, all nef alleles modulated CD4 surface expression, albeit with differential efficiency (Fig. 2C). In both cases, the JR-CSF Nef showed the highest and the YU10x and AD-93 Nefs the weakest activity (compare Fig. 2B, left panel and Fig. 2C). At medium levels of GFP and (corresponding indirectly to Nef expression), the efficiencies of CD4 downmodulation in Jurkat cells expressing Nef alone or in the context of the HIV-1 provirus correlated very well ($R^2=0.73$; $P=0.007$). Finally, we also examined the effects of Nef on CXCR4 and MHC-II surface expression. Both receptors were, however, only marginally downmodulated (up to 3-fold) by the eight nef alleles investigated (data not shown).

To analyze the ability of these HIV-1 Nef alleles to enhance virion infectivity, we generated viral particles by transient transfection of 293T cells and infected two indicator cell lines with virus stocks containing normalized quantities of the p24 antigen. All nef alleles
enhanced HIV-1 infectivity for TZM-bl cells (Fig. 2D, left panel). This cell line is susceptible for both wild-type and nef-defective HIV-1 infection although Nef enhances viral infectivity about 3-fold. In contrast, the effects of Nef were much stronger in P4-CCR5 cells. Viral particles produced in the presence of Nef were about 40-fold more infectious for P4-CCR5 cells (Fig. 2D, right panel). All HIV-1 Nef alleles enhanced infection of P4-CCR5 cells but the JR-CSF Nef was much less effective than the remaining nef genes analyzed (Fig. 2D, right panel).

**Generation of CD4C/HIV-Nef Tg mice expressing different Nef alleles**

The pathogenicity of the Nef alleles studied in vitro was then assayed in vivo in Tg mice. Each of these nef alleles replaced Nef from the NL4-3 strain (Nef$^{NL4-3}$) in the HIV$^{MutG}$ cassette in which all the other known open reading frames (ORF), except that of Nef, have been interrupted by mutations (35). The nef alleles were expressed under the control of the chimeric human CD4 promoter and mouse CD4 enhancer regulatory elements (CD4C) to generate CD4C/HIV-Nef$^{AD-93}$, CD4C/HIV-Nef$^{032an}$, CD4C/HIV-Nef$^{039nm}$, CD4C/HIV-Nef$^{JR-CSF}$, CD4C/HIV-Nef$^{YU10x}$, CD4C/HIV-Nef$^{SF2}$ and CD4C/HIV-Nef$^{NL4-3(T71R)}$ Tg mice (Fig. 3). For all transgenes, at least two independent founder (F) lines faithfully expressing the transgene were established and studied.

Expression of the transgene was first assayed by Northern blot analysis on RNA of different organs from mice of each founder line. This analysis revealed the presence of the three major HIV-1 RNA transcripts, mainly in lymphoid tissues [thymus (Fig. 4A), spleen and lymph nodes (LN) (data not shown)]. Expression was not detected in other organs [heart, brain, liver, testis, muscle] by this technique (data not shown), although previous work with more sensitive techniques [(15, 34, 38, 49, 82) and unpublished data] has shown that these CD4C regulatory elements allow expression in tissue macrophages and T cells of almost every organ. This expression faithfully reflects the specificity of the CD4C promoter documented previously (34,
Semi-quantitative analysis showed that the levels of RNA expression varied among founder lines, as expected, most likely reflecting distinct integration sites of the transgene. This was confirmed by quantitative real-time RT-PCR on RNA extracted from the thymus of mice from each nef allele (Fig. 4A). Detection of Nef protein was done by Western blotting with various anti-Nef antibodies, using the one recognizing best each individual Nef allele. This analysis showed expression of Nef protein in thymus of mice of at least one high expressor Tg line of each allele (Fig. 4B). However, the use of different anti-Nef antibodies and the different sequences of each Nef allele precluded a direct comparison of the levels of Nef protein expression between these alleles. Therefore, we relied on Tg RNA expression in the thymus, as detected by Northern blot analysis, for comparison.

**Tg mice expressing different Nef alleles, including those from LTNP, develop CD4+ T cell depletion, associated with T cell activation and apoptosis**

We previously reported that Tg mice expressing the NefNL4-3 allele showed loss of thymocytes, depletion of peripheral CD4+ T cells and downregulation of CD4 cell surface molecules (35). Similarly, all Tg mice expressing various Nef alleles at sufficient levels, showed loss of thymocytes (Table 1). This was most apparent in Tg lines expressing higher levels of the transgene, as expected. However, loss of thymocytes was less severe in CD4C/HIVYU10x, CD4C/HIVJR-CSF and CD4C/HIV039nm Tg mice, despite relatively high levels of Nef expression in these lines.

Further analysis was performed by FACS on thymocytes from Tg mice expressing each Nef allele. Consistent with previous data on NefNL4-3 -expressing Tg mice (34, 35, 103), depletion of double-positive (DP) CD4+CD8+ and single-positive (SP) CD4+CD8- thymocytes was observed in all Tg lines expressing higher levels of the transgene, although such decrease
was not as pronounced in Tg mice expressing Nef\textsuperscript{YU10x}, Nef\textsuperscript{JR-CSF} or Nef\textsuperscript{039nm} (Table 1). Interestingly, large loss of DP and SP CD4\textsuperscript{+} thymocytes was even documented in Tg lines expressing lower levels of Nef\textsuperscript{SF2}, Nef\textsuperscript{032an} and Nef\textsuperscript{NL4-3(T71R)} RNA (Table 1), suggesting a high pathogenicity of these alleles. Intriguingly, in Tg mice expressing Nef\textsuperscript{032an} or Nef\textsuperscript{NL4-3(T71R)}, the loss of total and DP thymocytes was delayed, being not as marked in younger (1.5-2 month-old) mice (relative to other Nef alleles), but becoming severe as the animals get older (~4-6 month-old). Loss of thymocytes was accompanied by a downregulation of CD4 cell surface molecule in all Tg mice expressing higher levels of Nef alleles, except those expressing Nef\textsuperscript{YU10x} (Table 1). The lack of CD4 downregulation in CD4\textsuperscript{+} T cell thymocytes expressing Nef\textsuperscript{YU10x} and the very low depletion of these cells is unlikely to reflect lack of protein expression, since mice of the higher expressor founder were found to express reasonable protein levels relative to those detected in CD4C/HIV-Nef\textsuperscript{NL4-3} Tg mice (Fig. 4B). Rather, this allele appears to be defective in the CD4 downregulation function both in human Jurkat cells (Fig. 2B) and in primary mouse cells (Table 1 and Fig. 5A).

A similar FACS analysis on cells of peripheral lymphoid organs (mesenteric and peripheral LN) showed depletion of peripheral CD4\textsuperscript{+} T cells in Tg mice expressing higher levels of each of the Nef alleles tested, although it was more modest in those expressing Nef\textsuperscript{YU10x} (Table 2). The downregulation of cell surface CD4 was also apparent in peripheral CD4\textsuperscript{+} T cells from all Tg mice expressing the transgene at higher levels, except those expressing Nef\textsuperscript{YU10x} and Nef\textsuperscript{032an} (Table 2, and see also the FACS profile for a representative mouse in Fig. 5B). The lack of downregulation of CD4 in peripheral cells from Nef\textsuperscript{032an} Tg mice was intriguing. It was unlikely to represent an intrinsic lack of CD4 downregulation potential, since Tg thymocytes expressing this allele showed CD4 downregulation and this nef allele was capable to
downmodulate CD4 in vitro (Fig. 2). It may arise from the lack of sufficient Nef expression, since expression was low in these founders. To test this latter possibility directly, we measured the levels of HIV-1 Tg RNA expression using Q-RT-PCR, on sorted peripheral CD4+ T cells from CD4C/HIV-Nef^032an (F95581, F95582) Tg mice relative to those from CD4C/HIV-Nef^NL4-3 Tg mice. This analysis showed indeed very low levels of Tg RNA in these mice (50 to 100–fold lower in both founders relative to Nef^NL4-3-expressing cells) (data not shown). Loss of CD8+ T cells both in the thymus and peripheral organs could also be documented at a later stage of the disease in most animals, as previously reported for Nef^NL4-3-expressing Tg mice (34, 35, 103). The number of B220+ (B cells) and Mac1+ (macrophages) cells were sometimes increased in the spleen of these Tg mice (data not shown).

Since CD4+ T cells expressing some of these nef alleles are as depleted as those expressing Nef^NL4-3 and since these latter cells were previously reported to exhibit an activated/memory-like phenotype (103), we investigated whether CD4+ T cells expressing some Nef alleles were also activated. Triple staining of peripheral LN cells from Tg mice expressing various nef alleles was performed for CD4, CD8 and either CD25, CD44, CD69, CD45RB or CD62L cell surface markers. This FACS analysis revealed that in Tg lines expressing each nef allele tested, except Nef^YU10x and Nef^NL4-3(T71R), the percentage of CD4+ T cells expressing CD44^high, CD45RB^low, CD62L^low was higher than in non-Tg mice and that a larger proportion of them expressed CD69 and CD25 (Fig. 5D and data shown for one representative mouse in Fig. 5C). This activated/memory-like phenotype is similar to that previously reported for CD4+ T cells expressing Nef^NL4-3 (103). It is worth noticing that the activated phenotype of Nef^032an-expressing CD4+ T cells developed despite the absence of downregulation of CD4 and the very low levels of Nef expression in these cells.
As shown before, Tg CD4\(^+\) T cells expressing Nef\(^{NL4-3}\) exhibit an enhanced apoptotic phenotype (84). A FACS analysis performed with annexinV or 7AAD labeling revealed that Tg mice expressing all these Nef alleles, except those expressing Nef\(^{YU10x}\) or Nef\(^{NL4-3(T71R)}\) showed a significantly higher proportion of apoptotic/dead CD4\(^+\) T cells (Fig. 5E).

We have recently reported that Nef\(^{NL4-3}\) binds to and activates PAK2 when expressed in various immune cell populations of Tg mice (102). A similar experiment was performed on Tg thymocytes and macrophages of Tg mice expressing each Nef allele. Anti-Nef immunoprecipitation was followed by IVKA and the \(^{32}\text{P}\)-labeled substrates, separated on SDS-PAGE, were visualized by autoradiography. All the tested Nef alleles, except Nef\(^{JR-CSF}\), were found capable of binding to and activating a Nef-associated kinase (NAK) (Fig. 6). Moreover, the \(^{32}\text{P}\)-labeled substrates had very similar migration patterns to those obtained with Nef\(^{NL4-3}\)-expressing cells, strongly suggesting that this NAK is PAK2, as previously documented (102). The lack of mouse PAK2 activation by Nef\(^{JR-CSF}\) confirms previous results in human cells in vitro (28). Interestingly, we found that PAK2 was activated reproducibly more strongly in Nef\(^{SF2}\)-expressing macrophages than in thymocytes, while the converse was true for Nef\(^{YU10x}\). Low expression levels may not totally explain this differential activation. Indeed, another Nef allele (032an) expressed at levels as low as Nef\(^{SF2}\) in thymocytes induced a very strong PAK2 activation. Moreover, Nef\(^{YU10x}\) induced very weak PAK2 activation in macrophages, despite being expressed at equivalent levels as wild-type Nef\(^{NL4-3}\) in these cells (see below). Together, these results suggest the presence of cell-specific factor(s) able to module the PAK2-Nef interaction in an allele-specific manner. To investigate whether the lack of IVKA observed in Nef\(^{JR-CSF}\)-expressing extracts was related to the inability of the anti-Nef Ab to immunoprecipitate this protein, we performed anti-Nef immunoprecipitation and SDS-PAGE migration on Nef\(^{JR-CSF}\)
and control Nef\textsuperscript{NL4-3(WT)}-expressing thymocytes labeled with $^{35}$S-methionine. This experiment revealed substantial levels of the $^{35}$S-labeled Nef\textsuperscript{JR-CSF} proteins, although not as high as those of Nef\textsuperscript{NL4-3(WT)} proteins (Fig. 6B). Thus, the inability to bind to and/or to activate PAK2 appears to be intrinsic to the JR-CSF Nef allele, and may be caused by the presence of leucine at position 201 (equivalent to 191 in Nef\textsuperscript{NL4-3}), as shown (28). Therefore, this Nef-dependent PAK2 activation appears to be a conserved function among most different Nef alleles.

These results indicate that most of the Nef alleles tested in Tg mice affect CD4$^+$ T cells in a similar way as Nef\textsuperscript{NL4-3}, previously reported (34, 35, 84, 102, 103). Only the Nef\textsuperscript{YU10x} allele appears to be significantly less pathogenic for Tg CD4$^+$ T cells than all other alleles tested, despite being apparently expressed at levels higher than other Nef alleles.

**Nef alleles differ in their ability to induce AIDS-like lung and kidney organ diseases in Tg mice.**

Tg mice and their non-Tg littermates from each line of Nef allele were monitored for clinical signs of disease (hypoactivity, ruffled hair, weakness, loss of body weight and early death). Tg mice from different lines expressing Nef\textsuperscript{NL4-3 (T71R)}, Nef\textsuperscript{032an} or Nef\textsuperscript{039nm} all exhibited a mortality rate close to or higher than that observed in Tg mice expressing Nef\textsuperscript{NL4-3(WT)}, while Tg mice expressing another Nef allele (Nef\textsuperscript{SF2}) showed minimal mortality (Fig. 7A). For these latter Nef\textsuperscript{SF2} Tg mice, this may be related to the relatively low levels of Nef RNA expression. It is worth noticing that the high mortality rate of CD4C/HIV-Nef\textsuperscript{NL4-3(T71R)} Tg mice occurs despite lower HIV RNA expression than in CD4C/HIV-Nef\textsuperscript{NL4-3(WT)} Tg mice, suggesting a higher pathogenicity for this variant. For all Tg lines of each Nef allele that exhibited high mortality (032an, 039nm, SF2, T71R), a good correlation could be observed, for founders expressing the same allele, between shorter survival and higher level of transgene HIV RNA expression in thymocytes. One notable exception was the Tg line F101376 of the Nef\textsuperscript{T71R} allele: mice from this
line had a shorter life span than those from the F101374 or F101371 lines, despite showing equivalent or lower transgene expression in thymocytes. Since organ, in particular kidney, disease largely responsible for poor survival, segregates independently of T cell loss [(36, 37, 102) and Table 4] and can develop in absence of CD4+ T cells (103), this suggests that other subset(s) of cells critical for organ disease induction express(es) the transgene. In line F101376, the site of transgene insertion may favor enhanced transcription through the CD4C regulatory elements in this particular cell subset yet to be identified.

Histological evaluation of tissues from Nef032an, Nef039nm, NefNL4-3(T71R) or NefSF2-expressing Tg mice revealed pathological changes indistinguishable from those previously observed in CD4C/HIV-NefNL4-3 Tg mice (34, 35) (Fig. 7A, Table 3). Phenotypes include loss of architecture and atrophy of lymphoid organs, as well as kidney (interstitial nephritis, focal segmental glomerulosclerosis, microcystic dilatation) and lung (lymphocytic interstitial pneumonitis) diseases (Fig. 7A; Table 3). These pathological lesions affect various percentages of Tg mice, and were of different severity, depending on the individual allele and its level of expression (Fig. 7C). This was especially evident in NefSF2 Tg mice which showed typical lesions despite its low levels of expression. Importantly, novel phenotypes not previously documented in Tg mice expressing the NefNL4-3 allele, were not observed in these Tg mice.

Therefore, the pathological changes previously observed in Tg mice expressing the NefNL4-3 allele were not restricted to this single allele, but could also be elicited with other primary Nef alleles. In fact, Tg mice expressing some nef alleles from LTNP (032an, 039nm) or other nef alleles (SF2 and NL4-3(T71R)) appear to be phenocopies of CD4C/HIV-NefNL4-3 Tg mice.

Intriguingly, Tg mice expressing NefJR-CSF, NefAD-93 or NefYU10x RNA at comparable or even higher levels than NefNL4-3 or Nef032an behave differently. Mice from both founder lines of
each of the CD4C/HIV-Nef$^{YU10x}$, CD4C/HIV-Nef$^{AD-93}$ or CD4C/HIV-Nef$^{JR-CSF}$ Tg strain had a life-span comparable to that of their non-Tg littermates, free of apparent clinical signs of disease, for the whole period of observation (up to 12 months) (Fig. 7B). Histological examination of their organs showed pathological lesions in their lymphoid organs (LN and spleen) (loss of architecture and atrophy) as severe as those observed in Tg mice expressing the Nef$^{NL4-3}$ allele, but in not in all mice (Table 3 and data not shown). However, these Tg mice were free of (Nef$^{YU10x}$, Nef$^{AD-93}$) or had only minimal (Nef$^{JR-CSF}$) lung and kidney diseases (Fig. 7B, C), despite that transgene RNA expression in the thymus was comparable or slightly higher than that of CD4C/HIV-Nef$^{NL4-3}$ or CD4C/HIV-Nef$^{032an}$ Tg mice (Fig. 4A).

The CD4C regulatory elements used to express the different alleles have been previously shown to be specific for immature and mature CD4$^+$ T cells and cells of the macrophage/dendritic lineage. To ascertain that the lack of development of the kidney and lung diseases in the CD4C/HIV-Nef$^{YU10x}$ and CD4C/HIV-Nef$^{JR-CSF}$ Tg mice was not the consequence of a lack of expression of the transgene in myeloid cells, we measured the levels of transgene RNA in macrophages of these Tg mice. Transgene RNA expression in Nef$^{YU10x}$ (F92751) or Nef$^{JR-CSF}$ (F104995) Tg macrophages was found to be comparable to that in Nef$^{NL4-3}$-expressing macrophages (data not shown), suggesting that the poor capacity of these alleles to elicit organ diseases in Tg mice is unlikely to be related to low transgene expression in myeloid cells, but rather to their low intrinsic virulence.

These results indicate that the Nef$^{YU10x}$, Nef$^{AD-93}$ and Nef$^{JR-CSF}$ alleles are respectively non or poorly pathogenic for kidney and lungs of Tg mice when expressed in CD4$^+$ T cells and in cells of the macrophage/dendritic lineage.

A summary of the phenotypes observed in these Tg mice expressing different nef alleles is shown in Table 4.
DISCUSSION

The HIV-1 Nef\textsuperscript{NL4-3} allele induces a severe AIDS-like disease in Tg mice when expressed under the control of the CD4C regulatory elements, in immature and mature CD4\textsuperscript{+} T cells and in cells of the macrophage/dendritic lineage (34, 35). We show here that this property is not unique and restricted to this specific allele, but is shared by other Nef alleles tested in the same \textit{in vivo} assay. It is worth noticing that novel phenotypes, absent in Tg mice expressing the Nef\textsuperscript{NL4-3} alleles, were not observed in Tg mice expressing any of the new nef alleles tested. This strongly suggests that all the phenotypes to be elicited by various nef alleles, when expressed in their natural target cells in Tg mice, have indeed developed in Nef\textsuperscript{NL4-3}-expressing Tg mice. These pathological changes are quite numerous and in fact cover most of the phenotypes associated with this syndrome (AIDS) in humans or in SIV-infected primates, except for pathologies which are known to be caused by pathogens other than HIV or SIV itself.

However, although the seven new nef alleles tested induced CD4\textsuperscript{+} T cell depletion to various degrees, they did not induce identical phenotypes and important differences could be noted. In particular, our data clearly show that the pathogenic potential varies between alleles. For example, despite its robust expression, the Nef\textsuperscript{YU10x} allele induced only a modest loss of thymic and peripheral CD4\textsuperscript{+} T cells, by comparison with other alleles (NL4-3, 039an, AD-93). In contrast, the Nef\textsuperscript{SF2} and Nef\textsuperscript{032an} alleles were able to elicit significant loss of thymic and peripheral CD4\textsuperscript{+} T cells, despite their low expression. The Nef\textsuperscript{SF2} allele harbours an arginine at position 71 which has been shown to significantly enhance viral replication in immature DC/T cells cocultures and to enhance association with T cell signaling molecules (Ick, vav and TcR\textsubscript{ζ}) (25). Our data also suggest that, when levels of Nef expression were taken into account, the mutated Nef\textsuperscript{T71R} NL4-3 allele was more pathogenic in Tg mice than the parental Nef\textsuperscript{NL4-3} wild-type
allele, especially regarding survival and kidney lesions. However, an arginine at this position is not required for the development of an AIDS-like disease in Tg mice, since the Nef\textsuperscript{032an} allele is highly pathogenic in Tg mice although it contains a threonine at the position corresponding to amino acid residue 71 in the NL4-3 Nef (Fig. 1). Thus, the influence of the R to T variation in the proline-rich region of Nef on its pathogenicity appears to be context dependent.

Further comparison between the immune phenotypes studied here also provided interesting hints on Nef pathogenicity. We indeed found an absence of CD4 downregulation on peripheral CD4\textsuperscript{+} T cells expressing Nef\textsuperscript{032an}, most likely reflecting its low expression in these cells. Yet, these cells were activated and depleted and showed enhanced apoptosis. The genetic segregation of these major phenotypes (CD4 downregulation vs activation/apoptosis/depletion) strongly suggests that CD4 downregulation itself on peripheral CD4\textsuperscript{+} T cells is not the cause of their depletion. These results are consistent with our previous observations on some mutants of Nef\textsuperscript{NL4-3} (D174K, RD35/36AA) impaired in their capacity to downregulate CD4, but still competent at inducing an activated/memory-like phenotype and a modest depletion of CD4\textsuperscript{+} T cells (36).

Previous studies have shown that some LTNP are infected with HIV-1 variants expressing nef alleles showing a diminished activity in CD4 downregulation (4, 68, 101). One of the three nef alleles from LTNP studied here (AD-93) also showed a diminished capacity to downregulated human CD4 relative to the NL4-3 allele. We found that in CD4C/HIV-Nef\textsuperscript{AD-93} Tg mice, CD4\textsuperscript{+} T cell depletion was severe despite showing only modest CD4 downregulation. Also, the CD4C/HIV-Nef\textsuperscript{032an} Tg mice did not exhibit much of a downregulation of murine CD4 on peripheral T cells despite showing loss of this T cell population and severe lung and kidney diseases. Thus, effective CD4 downmodulation does not seem sufficient for Nef to cause CD4\textsuperscript{+} T
cell depletion and severe disease in Tg mice.

A second apparently discordant finding observed with two nef alleles and not previously documented with the Nef\textsuperscript{NL4-3} allele is the lack of an activated/memory-like phenotype and of detectable enhanced apoptosis of CD4\(^+\) T cells expressing Nef\textsuperscript{NL4-3(T71R)} (F101374, F101376) and Nef\textsuperscript{YU10x} (F92751). Yet, the Nef\textsuperscript{NL4-3(T71R)}-expressing CD4\(^+\) T cells were modestly depleted to comparable levels as CD4\(^+\) T cells expressing other virulent alleles at comparable low levels (Nef\textsuperscript{032mm}). The genetic segregation of these two phenotypes suggests that the activated state of CD4\(^+\) T cells may not be the single major factor responsible for their Nef-induced depletion. The state of activation of the CD4\(^+\) T cells is now largely interpreted as a plausible mechanism for the CD4\(^+\) T cell loss observed in HIV-1 infected individuals (22, 33, 41, 71, 97). Our results on this Nef\textsuperscript{NL4-3(T71R)} allele suggest alternative, yet unknown, mechanism(s) of cell death induced by HIV-1 Nef in these Tg mice. We have already excluded Fas, FasL, ICE and TNF-RI pathways by showing that depletion of Nef\textsuperscript{NL4-3}-expressing CD4\(^+\) T cells is not abrogated in Tg mice deficient for each of these four genes (84).

The third interesting discordant finding revealed by our study is that thymic and peripheral CD4\(^+\) T cells can be depleted independently following Nef expression. We indeed found that SP CD4\(^+\) thymocytes were depleted only mildly in most CD4C/HIV-Nef\textsuperscript{039nm} Tg mice, while their peripheral CD4\(^+\) T cells were severely depleted. Similarly, in CD4C/HIV-Nef\textsuperscript{AD-93} Tg mice, peripheral CD4\(^+\) T cells appeared more severely depleted than the thymic SP CD4\(^+\) T cells. Assuming that Nef expression levels are not downregulated specifically in SP CD4\(^+\) thymocytes, these results strongly suggest that Nef-mediated depletion of thymic and peripheral CD4\(^+\) T cells can occur independently and possibly through distinct pathways and that some Nef alleles (such as Nef\textsuperscript{039nm} or Nef\textsuperscript{AD-93}) may interact more efficiently than others with
effectors of peripheral CD4$^+$ T cells than of thymic CD4$^+$ T cells. In independent studies of Tg mice expressing some mutants of Nef$^{NL4-3}$ (D174K, RD35/36AA), we previously reached similar conclusions (37). This may partly explain why the thymic function and regenerative capacity may be better conserved and for a longer time, in some HIV-1 infected individuals (55, 70, 71).

The capacity to elicit another organ phenotype, kidney disease (focal segmental glomerulosclerosis, microcystic dilatation, tubulointerstitial nephritis), also distinguishes the seven new alleles tested. Three (YU10x, JR-CSF, AD-93) of the seven nef alleles were totally or almost completely defective at inducing kidney disease, even though their levels of RNA expression were comparable or higher than other Nef alleles causing this disease, including the wild-type Nef$^{NL4-3}$ previously reported (35). This suggests that the molecular or cellular requirements for inducing kidney disease are more stringent than for the loss of CD4$^+$ T cells, since two of these alleles (JR-CSF, AD-93) were able to induce significant CD4$^+$ T cell loss. A similar phenomenon was observed in studies of some mutants of Nef$^{NL4-3}$ in Tg mice, where it was found that mutants had lost more easily their capacity to elicit kidney disease than to induce T cell changes (37). More recently, it was reported that Tg mice expressing the whole genome of HIV-1$^{JR-CSF}$ under the regulation of its own LTR promoter showed only a modest depletion of peripheral CD4$^+$ T cells, but no changes in the thymocyte populations nor any organ disease (99). These results are consistent with the phenotypes observed in our CD4C/HIV-Nef$^{JR-CSF}$ Tg mice i.e. a more severe depletion of peripheral than thymic CD4$^+$ T cells and presence of only minimal organ diseases. The inability of Nef$^{JR-CSF}$ to activate PAK2 may be responsible for some of its milder phenotypes. However, it would have to be hypothesized that such activation is not sufficient or context-dependent, since another Nef allele (YU10x) with a similar phenotype is able to activate PAK2.
Interestingly, the three *nef* alleles from LTNP which were studied in Tg mice were quite pathogenic in this *in vivo* biological assay, while one allele from an AIDS patient (YU10x) had very low virulence. If this Tg assay is of any relevance for predicting pathogenicity in humans, this would suggest that the slow tempo of AIDS development in the LTNP patients from which these *nef* genes were obtained reflects mutations in other viral genes than *nef*, or is caused by a host genetic resistance of these individuals. It may also suggest that some of the HIV-1 variants molecularly cloned from AIDS patients may not fully represent the virulent HIV-1 population causing the disease. This is known to occur and has been well documented in the SIV infected macaque model where reinoculation of viruses is possible (6, 9, 44, 50, 66). The availability of such an *in vivo* Tg assay could then help in classifying LTNP patients in distinct subgroups and to assess the virulence of specific HIV-1 variants obtained from them and from individuals with AIDS. Obviously, such comparative analysis should take into account the specificity of the Tg system: minimal contribution of opportunistic infection, since these mice are kept in specific pathogen-free conditions, presence of a genetic background susceptible for the development of HIVAN (HIV-1 associated nephropathy) and absence of virus replication.

In conclusion, our data indicate that the CD4C/HIV Tg mice model system represents a biological *in vivo* assay for *nef* alleles. Although this assay does not score the influence of Nef on viral replication, it scores numerous other phenotypes caused by Nef expression in very distinct T (immature and mature) and myeloid (macrophage/DC) primary cell subpopulations. These phenotypes consist of perturbations of immature and mature T cells as well as severe organ diseases similar to those found in human AIDS. We have shown here that very important different T cell changes may be induced by different Nef alleles. These phenotypes could not have been scored *in vitro*, nor easily in HIV-infected human individuals, highlighting the need to study HIV-1 pathogenesis in primary, unstimulated T cells, and the advantage to use an animal model for such
an investigation. It remains to be determined whether such assay in the mouse has any predictive value regarding the behavior of Nef in infected human individuals.
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LEGENDS TO FIGURES

Figure 1. Alignment of Nef amino acid sequences. The NL4-3 sequence is shown in the upper panel for comparison. Some conserved sequence elements in Nef, the position of some binding (bdg) domains, of the polypurine-tract (PPT) as well as the start of the 3’LTR are indicated schematically. Dots indicate identity with the consensus sequence and dashes indicate gaps introduced to optimize the alignment.

Figure 2. Functional activity of HIV-1 nef alleles in in vitro assays
(A) Jurkat T (lanes a, b) or HeLa CIITA (lane c) cells were transduced with eGFP-expressing HIV-1 NL4-3 particles containing a disrupted nef gene (nef-) or the indicated nef alleles pseudotyped with the VSV-G glycoprotein and analyzed by flow cytometric analysis. The ranges of no, low, medium and high levels of eGFP expression used to calculate receptor modulation are indicated in the upper panel. (B) Quantitative effect of HIV-1 Nefs on CD4, MHC-I and Ii surface expression. Values were determined as described in Materials and Methods. Shown are data derived from the transduced cells presented in panel A. Similar results were obtained in two independent experiments. (C) Quantitative presentation of CD4 downmodulation in Jurkat T cells transiently transfected with bicistronic vectors expressing GFP alone (nef-) or together with Nef. Symbols are shown in panel B. (D) Infectivity of HIV-1 IRESeGFP variants containing the indicated HIV-1 nef alleles. TZM-bl or P4-CCR5 indicator cells were infected in triplicate with 293T cell-derived virus stocks containing 1 ng p24 antigen. Infectivity is shown relative to the recombinant virus containing the NL4-3 nef allele. Similar results were obtained in another independent experiment.

Figure 3. Structure of the CD4C/HIV transgenes expressing various Nef alleles
Different Nef allele fragments Nef\textsuperscript{AD\textasciitilde93}, Nef\textsuperscript{032an}, Nef\textsuperscript{039nm}, Nef\textsuperscript{R-CSF}, Nef\textsuperscript{SF2} and Nef\textsuperscript{YU10x}, as well as the mutant Nef\textsuperscript{NL4\texttextlangle3\textrangle(T71R)} were inserted within the same CD4C/HIV\textsuperscript{MutG} backbone sequences replacing Nef\textsuperscript{NL4\texttextlangle3\textrangle(WT)}. The transgenes were constructed as described in Materials and Methods. Abbreviations: mCD4enh, mouse CD4 enhancer; hCD4 prom, human CD4 promoter; SV40, polyadenylation sequences from SV40; Ex1, CD4 gene exon1; X represents interruption of the ORF of the indicated HIV-1 genes. Restriction sites: A, AatII; Bs, BssHII; S, SstI.

**Figure 4. Expression of HIV-1 Nef in CD4C/HIV-Nef\textsuperscript{allele} Tg mice**

Thymuses from different founder (F) lines of each of the CD4C/HIV-Nef\textsuperscript{allele} transgene were analyzed.

(A) Northern blot analysis of HIV-1 RNA. Total RNAs from thymuses were hybridized with a \textsuperscript{32}P-labeled HIV-1-specific probe. The blots were stripped and rehybridized with \textsuperscript{32}P-labeled actin DNA probe. Also shown below are the semi-quantitative values for the levels of expression of each Nef allele relative to Nef\textsuperscript{NL4\texttextlangle3\textrangle(WT)} (value of 1), measured in Northern blot or in RT-PCR analysis, as described in Materials and Methods. (B) Western blot analysis of protein extracts (100 µg) from thymuses of one month-old Tg and non-Tg littermates, using the indicated anti-Nef antibodies: rabbit polyclonal produced in our laboratory (lab), monoclonal EH1 or 6.2 antibody. Nef\textsuperscript{NL4\texttextlangle3\textrangle(WT)} represents the gene encoded by the CD4C/HIV\textsuperscript{MutG} (F27367) Tg mice previously reported (34) and used as positive controls. The membranes were stripped and reanalyzed with anti-actin antibody.

**Figure 5. Immunophenotypic analysis of thymic and peripheral T lymphocytes from CD4C/HIV-Nef\textsuperscript{allele} Tg Mice**

Thymus (A) and peripheral LN (B) cells from a representative Tg mouse for each allele [CD4C/HIV-Nef\textsuperscript{AD93} (F115820), CD4C/HIV-Nef\textsuperscript{032an} (F95582), CD4C/HIV-Nef\textsuperscript{039nm} (F75581),
CD4C/HIV-Nef^{JR-CSF} (F104995), CD4C/HIV-Nef^{YU10x} (F92751), CD4C/HIV-Nef^{SF2} (F92748), CD4C/HIV-Nef^{NL4-3(T71R)} (F101376) and CD4C/HIV-Nef^{NL4-3(WT)} (F27367) and from a non-Tg littermate were analyzed the same day by flow cytometry for the expression of CD4 and CD8. The percentage of cells found in relevant quadrant is indicated at the left, while the right underlined number refers to the mean fluorescence intensity. Note the presence of two subsets of CD4^{+} T cells (CD4^{high} and CD4^{low}) in pLN cells expressing Nef^{039nm} and to a lesser extent in those expressing Nef^{SF2} and Nef^{JR-CSF}. These correspond respectively to low and high Nef expressing cells, as previously reported in Tg mice expressing Nef^{NL4-3(WT)} (103). These data are representative of at least three independent experiments with 4 to 10 mice. (C) Three-color FACS analysis (CD4-APC, CD8-FITC, CD44-PE) was performed on pLN cells from a representative mouse from CD4C/HIV-Nef^{AD-93} (F115820), CD4C/HIV-Nef^{032an} [F95582 and F95581 (not shown)], CD4C/HIV-Nef^{039nm} [F75581 and F115817 (not shown)], CD4C/HIV-Nef^{YU10x} (F92751), CD4C/HIV-Nef^{JR-CSF} (F104995), CD4C/HIV-Nef^{SF2} (F92748) or CD4C/HIV-Nef^{NL4-3(T71R)} [F101376, F101374 (not shown)] Tg lines and from a non-Tg littermate. Isotype control antibody was used as negative control. Data are shown only for the CD4^{+} T cell population. The results shown are representative of at least 2-3 independent experiments. (D) Table representing ratios (Tg/non-Tg) of the percentage of cells not expressing (negative/low for CD62L and CD45RB) or expressing (high for CD25, CD44, CD69) the indicated cell surface marker, as shown in Panel C. Between 4 and 6 Tg mice of each allelic line along with their respective non-Tg littermates lines were used. (E) Quantitation of apoptotic/dead peripheral CD4^{+} T cells from the same CD4C/HIV-Nef^{allele} Tg mice analyzed in D. LN cells of Tg and non-Tg littermates were analyzed by FACS after staining with anti-CD4 mAb and 7AAD. The data were pooled from Tg and non-Tg mice of each line as in D and
represent ratios (Tg/non-Tg) of the percentage of apoptotic/dead cells among the CD4+ T cells. Statistical analysis was performed with the Student’s t test. *, p<0.05; **, p<0.01; ***, p<0.001.

Figure 6. Interaction of various Nef alleles with NAK in Tg mice

(A) Total protein extracts from thymocytes (250 µg) and macrophages (100 µg) from CD4C/HIV-Nef Tg mice expressing the indicated Nef alleles and from their non-Tg littermates were incubated with rabbit anti-Nef polyclonal antibodies (lab) overnight and subjected to an IVKA using 10 µCi of [γ-32P] ATP for 5 min at room temperature. The phosphorylated proteins were next separated by SDS-PAGE and visualized by autoradiography. (B) Thymocytes from CD4C/HIV-NefJR-CSF, CD3C/HIV-Nef039 and CD4C/HIV-NefNL4-3(WT) Tg and non-Tg mice were metabolically labeled with (35S)-methionine. Lysates were immunoprecipitated with rabbit polyclonal anti-Nef Ab produced in our laboratory. The 35S-labelled proteins were separated by SDS-PAGE and visualized by autoradiography. Note the capacity of the polyclonal anti-Nef Ab used to immunoprecipitate the NefJR-CSF proteins effectively.

Figure 7. Survival and histopathology of CD4/HIV-Nef allele Tg mice

(A) Nef-expressing strains showing shorter survival and/or pathological lesions. Upper panel: Cumulative incidence of mortality. Mice from the indicated Tg lines were observed for a period of up to 12 months. The cumulative incidence of mortality of the different Tg founder lines from each indicated Tg mouse strain, as well as of their non-Tg littermates was plotted as percentage of surviving mice. NefNL4-3(WT) represents the CD4C/HIVMutG [CD4C/HIV-NefNL4-3(WT)] (F27367) Tg mice used as positive controls. The number of animals observed (n) in each indicated Tg line is shown. The number of non-Tg mice represents the sum of control mice for all founders in these lines. Lower panel: Kidney histology of a representative Tg mouse from corresponding Tg lines. All these Tg mice exhibit the typical pathology previously observed.
in CD4C/HIV-Nef$^{NL4-3(WT)}$ Tg mice. Note the tubular dilatation and atrophy, with some cystic changes, tubulointerstitial nephritis as well as focal segmental glomerulosclerosis in all Tg kidneys. (B) No or minimal lung or kidney organ disease in CD4C/HIV-Nef$^{YU10x}$, CD4C/HIV-Nef$^{AD-93}$ and CD4C/HIV-Nef$^{JR-CSF}$ Tg mice. **Upper panel:** Absence or very low mortality. Mice were observed for up to 12 months. The data are presented as in the upper panel A. **Lower panel:** Light microscopic analysis of kidneys from Tg mice. The histological appearance of the kidney from these Tg animals was indistinguishable (Nef$^{YU10x}$, Nef$^{AD-93}$) or showed only minimal lesions (Nef$^{JR-CSF}$) relative to that of non-Tg mice. (C) Semiquantitative assessment of the kidney disease in Tg mice. A score 1 to 4 was assigned to each mouse and the average score for each group is shown.
REFERENCES


cells but are not sufficient to induce an AIDS-like disease in CD4C/HIV transgenic Mice. J. Biol. Chem. **281**:6940-6954.


Table 1
Cell Number in thymocyte subsets from Tg mice expressing different Nef alleles

<table>
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<th>Absolute cell number (X 10^6) b</th>
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<td></td>
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<td>Total</td>
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<tr>
<td>Non-Tg d</td>
<td></td>
<td>94±21</td>
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<td>AD-93</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>F115817</td>
<td>L</td>
<td>70±27</td>
<td>60±25</td>
</tr>
<tr>
<td>F115820</td>
<td>M</td>
<td>16±7</td>
<td>11±6</td>
</tr>
<tr>
<td>032an</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F95581</td>
<td>L</td>
<td>30±17</td>
<td>20±12</td>
</tr>
<tr>
<td>F95582</td>
<td>L-M</td>
<td>53±28</td>
<td>42±21</td>
</tr>
<tr>
<td>039nm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F75581</td>
<td>M-H</td>
<td>70±27</td>
<td>62±21</td>
</tr>
<tr>
<td>F51444</td>
<td>M-H</td>
<td>59±19</td>
<td>44±12</td>
</tr>
<tr>
<td>JR-CSF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F104995</td>
<td>M-H</td>
<td>48±25</td>
<td>37±20</td>
</tr>
<tr>
<td>F104996</td>
<td>L-M</td>
<td>100±21</td>
<td>84±19</td>
</tr>
<tr>
<td>YU10x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F92751</td>
<td>H</td>
<td>88±18</td>
<td>75±17</td>
</tr>
<tr>
<td>F92754</td>
<td>L</td>
<td>101±12</td>
<td>84±11</td>
</tr>
<tr>
<td>SF-2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F92748</td>
<td>L-M</td>
<td>20±10</td>
<td>12±7</td>
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<tr>
<td>F92749</td>
<td>L</td>
<td>58±19</td>
<td>47±18</td>
</tr>
<tr>
<td>NL4-3(T71R)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F101376</td>
<td>L</td>
<td>65±17</td>
<td>49±13</td>
</tr>
<tr>
<td>F101374</td>
<td>L</td>
<td>66±17</td>
<td>57±13</td>
</tr>
<tr>
<td>F101371</td>
<td>L</td>
<td>81±22</td>
<td>71±20</td>
</tr>
<tr>
<td>F86707</td>
<td>L-M</td>
<td>67±17</td>
<td>52±13</td>
</tr>
<tr>
<td>Non-Tg d</td>
<td></td>
<td>104±26</td>
<td>90±23</td>
</tr>
<tr>
<td>NL4-3(WT)</td>
<td>M-H</td>
<td>69±23</td>
<td>58±20</td>
</tr>
</tbody>
</table>
FACS analysis was performed on 4-10 mice (1.5-4 month-old) for each Tg line, including the positive control CD4C/HIV-Net^{NL4-3(WT)} Tg line. Data from Tg mice were always compared to those of their littermates done the same day. Older (4-6 month-old) Tg mice expressing Nef^{032an} (F95582) or Nef^{T71R} (F101376) showed more severe depletion: non-Tg, total thymocytes are 53±7.6 X 10^6, DP are 45±5.9 X 10^6; Nef^{032an} Tg, total thymocytes are 13.8±10 X 10^6, DP are 11±8.9 X 10^6; Nef^{T71R}, total thymocytes are 20.3±9.5 X 10^6, DP are 17.4±8.3 X 10^6, n = 6-8 in each group.

The absolute cell number was calculated by multiplying the total cell number in each organ by the percentage of each cell subset in this organ.

The mean fluorescences for CD4 were obtained by calculating the ratio of CD4 staining in Tg thymuses relative to that of non-Tg thymuses (100%) done in the same experiment, the same day. Mean values were then calculated with the values for each line.

The non-Tg control values were obtained by pooling the results of all non-Tg littermates from different lines.

Significant decrease: p < 0.01 by using Student’s t-test.

Significant decrease: p < 0.05 by using Student’s t-test.

Significant increase: p < 0.05 by using Student’s t-test.
Table 2
Cell Number in T cell subsets of peripheral lymphoid organs from Tg mice expressing different Nef alleles

<table>
<thead>
<tr>
<th>Mouse line</th>
<th>Absolute cell number (X 10⁶)</th>
<th>CD4/CD8 Ratio</th>
<th>Mean Fluorescence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total LN</td>
<td>CD4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Tg</td>
<td>21.5±5.5</td>
<td>10.6±2.5</td>
<td>4.3±0.8</td>
</tr>
<tr>
<td>AD93</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F115817</td>
<td>20.1±18.9</td>
<td>9.8±8.0</td>
<td>3.0±2.5</td>
</tr>
<tr>
<td>F115820</td>
<td>8.4±5.7</td>
<td>1.8±1.2</td>
<td>2.2±1.6</td>
</tr>
<tr>
<td>032an</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F95581</td>
<td>9.7±5.2</td>
<td>2.9±2.0</td>
<td>1.6±0.8</td>
</tr>
<tr>
<td>F95582</td>
<td>7.8±5.4</td>
<td>3.0±2.6</td>
<td>1.6±1.6</td>
</tr>
<tr>
<td>039nm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F75581</td>
<td>5.8±3.0</td>
<td>0.8±0.3</td>
<td>1.1±0.3</td>
</tr>
<tr>
<td>F51444</td>
<td>12.5±7.0</td>
<td>2.4±1.1</td>
<td>2.1±0.6</td>
</tr>
<tr>
<td>JR-CSF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F104995</td>
<td>6.6±4.0</td>
<td>1.4±1.2</td>
<td>1.9±0.8</td>
</tr>
<tr>
<td>F104996</td>
<td>15.3±5.0</td>
<td>3.0±1.4</td>
<td>6.1±2.5</td>
</tr>
<tr>
<td>YU10x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F92751</td>
<td>11.3±2.0</td>
<td>5.3±1.0</td>
<td>3.1±0.6</td>
</tr>
<tr>
<td>F92754</td>
<td>12.9±5.5</td>
<td>6.2±1.8</td>
<td>2.8±0.9</td>
</tr>
<tr>
<td>SF-2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F92748</td>
<td>5.9±4.0</td>
<td>0.3±0.2</td>
<td>0.9±0.1</td>
</tr>
<tr>
<td>F92749</td>
<td>20.3±15.0</td>
<td>9.3±6.0</td>
<td>4.1±3.8</td>
</tr>
<tr>
<td>T71R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F101376</td>
<td>11.5±3.0</td>
<td>5.4±1.0</td>
<td>1.9±0.5</td>
</tr>
<tr>
<td>F101374</td>
<td>9.3±2.0</td>
<td>3.7±1.0</td>
<td>2.4±0.4</td>
</tr>
<tr>
<td>F101371</td>
<td>28.4±5.5</td>
<td>14.1±3.0</td>
<td>5.9±1.0</td>
</tr>
<tr>
<td>F86707</td>
<td>8.8±1.5</td>
<td>1.3±0.3</td>
<td>2.2±0.3</td>
</tr>
<tr>
<td>Non-Tg</td>
<td>19.5±3.5</td>
<td>9.3±2.5</td>
<td>4.3±1.0</td>
</tr>
<tr>
<td>NL4-3(WT)</td>
<td>6.3±3.6</td>
<td>1.2±0.5</td>
<td>1.7±0.9</td>
</tr>
</tbody>
</table>
a FACS analysis was performed on 4-10 mice (1.5-4 month-old) for each Tg line, including the positive control CD4C/HIV-Nef\textsuperscript{NL4-3(WT)} Tg line. Data from Tg mice were always compared to those of their littermates done the same day.

b The absolute cell number was calculated by multiplying the total cell number in each organ by the percentage of each cell subset in this organ.

c The mean fluorescences for CD4 were obtained by calculating the ratio of CD4 staining in Tg thymuses relative to that of non-Tg thymuses (100\%) done in the same experiment, the same day. Mean values were then calculated with the values for each line.

d The non-Tg control values were obtained by pooling the results of all non-Tg littermates from different lines.

e Significant decrease: $p < 0.01$ by using Student’s t-test.

f Significant decrease: $p < 0.05$ by using Student’s t-test.
Table 3
Histopathological assessment of Tg mice expressing different Nef alleles

<table>
<thead>
<tr>
<th>Organ</th>
<th>AD-93</th>
<th>032an</th>
<th>039nm</th>
<th>JR-CSF</th>
<th>YU10x</th>
<th>SF2</th>
<th>NL4-3&lt;sup&gt;T71R&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymus&lt;sup&gt;c&lt;/sup&gt;</td>
<td>F115820 (M)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>F115817 (L)</td>
<td>F95582 (L-M)</td>
<td>F95581 (L-M)</td>
<td>F75581 (M)</td>
<td>F51444 (M)</td>
<td>F104996 (L)</td>
</tr>
<tr>
<td>LN&lt;sup&gt;d&lt;/sup&gt;</td>
<td>9/12</td>
<td>2/7</td>
<td>6/7</td>
<td>1/9</td>
<td>13/13</td>
<td>5/9</td>
<td>2/12</td>
</tr>
<tr>
<td>Lung&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0/14</td>
<td>0/9</td>
<td>11/11</td>
<td>4/11</td>
<td>13/13</td>
<td>9/9</td>
<td>5/12</td>
</tr>
<tr>
<td>Lung&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0/14</td>
<td>0/9</td>
<td>0/11</td>
<td>2/11</td>
<td>3/13</td>
<td>2/9</td>
<td>2/12</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of animals with disease over total number of animals studied for the two founders of each Tg line showing the most severe phenotype. Non-Tg mice (n=86) showed none of these phenotypes.

<sup>b</sup> The levels of RNA expression in the thymus are provided in parenthesis: L, low; M, medium; H, high. Medium expression is comparable to that of Nef<sub>NL4-3(WT)</sub> (F27367).

<sup>c</sup> Atrophy and disorganization.

<sup>d</sup> Include follicle hyperplasia, fragmentation, involution and relative enlarged paracortex with lymphocytopenia. For the 032an, 039nm, JR-CSF and NL4-3<sup>T71R</sup> Nef alleles, the predominant change is depletion of lymphocytes throughout the LN.

<sup>e</sup> Different degree of glomerulopathy includes FSGS (focal segmental glomerulosclerosis) and tubulo-interstitial nephritis with microcystic dilation.

<sup>f</sup> LIP (lymphocytic interstitial pneumonitis)

<sup>g</sup> Despite high expression in Nef<sub>YU10x</sub> (F92751)-expressing mice, kidney disease was mild.

<sup>h</sup> The low penetrance of disease in Nef<sub>SF2</sub> Tg mice is likely to be related to the low levels of expression in both founder lines relative to those of the Nef<sub>NL4-3(WT)</sub> Tg mice.
Table 4
Summary of phenotypes in CD4C/HIV Tg mice expressing different Nef alleles

<table>
<thead>
<tr>
<th>Nef alleles</th>
<th>In vitro infectivity</th>
<th>Downregulation of CD4</th>
<th>Activated/ Memory</th>
<th>Depletion of T cells</th>
<th>Lung and kidney disease</th>
<th>PAK activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In vitro</td>
<td>In vivo</td>
<td>In vitro</td>
<td>In vivo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thymus</td>
<td>LN</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>DP</td>
<td>CD4⁺</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>NL4-3</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>Yes</td>
<td>+++</td>
</tr>
<tr>
<td>NL4-3(T71R)</td>
<td>++++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>No</td>
<td>+++</td>
</tr>
<tr>
<td>AD-93</td>
<td>++++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>Yes</td>
<td>+++</td>
</tr>
<tr>
<td>032an</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>Yes</td>
<td>++</td>
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<tr>
<td>039nm</td>
<td>+++</td>
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<td>+++</td>
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<td>Yes</td>
<td>+++</td>
</tr>
<tr>
<td>JR-CSF</td>
<td>+/-</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>Yes</td>
<td>+++</td>
</tr>
<tr>
<td>YU10x</td>
<td>++++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>No</td>
<td>+</td>
</tr>
<tr>
<td>SF2</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>Yes</td>
<td>+++</td>
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</table>

a The most severe phenotypes observed in the higher expressor Tg lines are shown for each Nef allele.

b Measured on P4-CCR5 cells (see Fig. 2D).

c The low CD4 downregulation in LN of Nef⁰₃₂an-expressing Tg mice is most likely related to very low levels of expression in these cells.

d Lung and kidney diseases are apparent in Nef⁰₃₂SF²-expressing Tg mice, despite low levels of expression, suggesting high pathogenicity.
**Fig. 1**

<table>
<thead>
<tr>
<th>Myristoylation</th>
<th>length variable region</th>
<th>cleavage</th>
<th>acidic</th>
<th>PxxP Motif</th>
<th>PPT LTR</th>
<th>PAK-bdg</th>
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</thead>
<tbody>
<tr>
<td>NL4-3</td>
<td>NLGKWSSSV IGWPAVRRMK</td>
<td></td>
<td></td>
<td>-AEPAADGV</td>
<td>GAVSRDLEKH</td>
<td>GAITSSNTA</td>
</tr>
<tr>
<td>NL4-3&lt;sup&gt;371&lt;/sup&gt;</td>
<td>I</td>
<td>T</td>
<td>K</td>
<td>V</td>
<td>D</td>
<td>G</td>
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</table>

**Thioesterase-bdg**

<table>
<thead>
<tr>
<th>Thioesterase-bdg</th>
<th>β-COP?</th>
<th>AP-interaction</th>
<th>VIH</th>
</tr>
</thead>
<tbody>
<tr>
<td>NL4-3</td>
<td>LDLWIYHTQV</td>
<td>YFPDWQNYTP</td>
<td>GPGVRYPLTF</td>
</tr>
<tr>
<td>NL4-3&lt;sup&gt;371&lt;/sup&gt;</td>
<td>I</td>
<td>F</td>
<td>E</td>
</tr>
</tbody>
</table>

**Priceputu et al.**
Fig. 4

A

<table>
<thead>
<tr>
<th>Nef</th>
<th>Actin</th>
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<tbody>
<tr>
<td>YU10x</td>
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<tr>
<td>JR-CSF</td>
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</tr>
<tr>
<td>SF2</td>
<td></td>
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<tr>
<td>AD-93</td>
<td></td>
</tr>
<tr>
<td>032an</td>
<td></td>
</tr>
<tr>
<td>039nm</td>
<td></td>
</tr>
<tr>
<td>NL4-3(T71R)</td>
<td></td>
</tr>
<tr>
<td>NL4-3(WT)</td>
<td></td>
</tr>
<tr>
<td>Non-Tg</td>
<td></td>
</tr>
</tbody>
</table>

Northern: 0.1  2.7  1.4  0.5  0.3  0.1  0.7  1.5  1.7  0.6  0.1  0.1  1  1  0.3

Q-RT-PCR: 0.1  4.3  1.3  0.8  0.4  0.1  0.3  0.5  1.7  1.1  0.5  0.2  0.1  1  1  0.1
Fig. 4

Priceputu et al.
Fig. 5

<table>
<thead>
<tr>
<th>Markers</th>
<th>Non-Tg</th>
<th>AD-93</th>
<th>032an</th>
<th>039nm</th>
<th>YU10x</th>
<th>JR-CSF</th>
<th>SF2</th>
<th>T71R</th>
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</thead>
<tbody>
<tr>
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<td>2.7±0.3***</td>
<td>0.8 ±0.1</td>
<td>2.3 ±0.4***</td>
<td>3.3 ±0.3***</td>
<td>1.3 ±0.3</td>
</tr>
<tr>
<td>CD25⁺</td>
<td>1</td>
<td>2.5 ±0.1***</td>
<td>1.9 ±0.3**</td>
<td>2.8±0.3***</td>
<td>0.9 ±0.1</td>
<td>1.7 ±0.1***</td>
<td>2.3 ±0.8*</td>
<td>1.0 ±0.1</td>
</tr>
<tr>
<td>CD44⁺</td>
<td>1</td>
<td>4.9 ±0.4***</td>
<td>2.4 ±0.4***</td>
<td>3.2±0.4***</td>
<td>0.9 ±0.2</td>
<td>3.4 ±0.3***</td>
<td>3.7 ±0.2*</td>
<td>1.2 ±0.4</td>
</tr>
<tr>
<td>CD45RB⁻</td>
<td>1</td>
<td>3.4 ±0.2***</td>
<td>2.4 ±0.5**</td>
<td>3.7±0.7***</td>
<td>1.0 ±0.2</td>
<td>3.4 ±0.2***</td>
<td>5.6 ±1.0***</td>
<td>1.4 ±0.3</td>
</tr>
<tr>
<td>CD62L⁻</td>
<td>1</td>
<td>4.0 ±0.3***</td>
<td>1.7 ±0.3**</td>
<td>2.5±0.3***</td>
<td>1.1 ±0.1</td>
<td>2.4 ±0.2***</td>
<td>2.8 ±0.4***</td>
<td>1.3 ±0.2</td>
</tr>
</tbody>
</table>

**Ratio:** Tg / non-Tg

E

<table>
<thead>
<tr>
<th>Ratio: Tg/non-Tg apoptotic/dead CD4⁺ T cells</th>
<th>Non-Tg</th>
<th>AD-93</th>
<th>032an</th>
<th>039nm</th>
<th>YU10x</th>
<th>JR-CSF</th>
<th>SF2</th>
<th>T71R</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.1 ± 0.3***</td>
<td>2.0 ± 0.2***</td>
<td>2.1 ± 0.3***</td>
<td>1.2 ± 0.3</td>
<td>3.0 ± 0.6***</td>
<td>3.0 ± 0.3***</td>
<td>1.2 ± 0.4</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 6

A

THYMOCYTES

MACROPHAGES

1. IP: Nef
2. IVKA

B

IP:Nef

JR-CSF  Non-Tg  039nm  NL4-3^{WT}

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Fig. 7

A

Nef^{032an}  
Non-Tg (n=45)  
F75581 (n=34)  
F95582 (n=88)  
F95583 (n=7)  
F95585 (n=15)  
Nef^{039nm}  
Non-Tg (n=50)  
F92754 (n=30)  
F92756 (n=30)  
F92758 (n=45)  
Nef^{SF2}  
Non-Tg (n=50)  
F92751 (n=30)  
F92752 (n=30)  
F92753 (n=45)  
Nef^{T71R}  
Non-Tg (n=50)  
F92750 (n=30)  
F92751 (n=30)  
F92752 (n=45)  

B

Non-Tg  
Nef^{YU10x}  
Non-Tg (n=48)  
Nef^{JR-CSF}  
Non-Tg (n=50)  
Nef^{AD}  
Non-Tg (n=48)  

Fig. 7