Severe Immunodeficiency Associated with a Human Immunodeficiency Virus 1 NEF/3′-Long Terminal Repeat Transgene

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Summary

We have generated several transgenic mouse strains carrying a human immunodeficiency virus 1 (HIV-1) NEF/3′ long terminal repeat (LTR) transgene under control of a T cell–specific promoter–enhancer element, showing a depletion of $CD4^+$ T cells in the thymus and periphery. The immunological functions of the line with the most dramatic changes in lymphocyte populations, B6/338L, were analyzed in greater detail. The presence of the transgene in the heterozygous animal is associated with a dominant severe immunodeficiency. Older animals develop lymphadenopathy and splenomegaly. $CD4^+CD8^+$ and $CD4^+CD8^-$ single positive thymocytes already are depleted in these mice at the earliest stages in ontogeny, and peripheral T cells are reduced in frequency and present cell surface marker expression, which is characteristic for memory and activated T cells. The immunological response of B6/338L mice to several viral infections is also greatly impaired. Thus, the HIV-1 NEF/3′ LTR as transgene in T cells can cause immunodeficiency and disease with striking similarities to a known retrovirus-induced immunodeficiency called murine AIDS (H. C. Morse III, S. K. Chattopadhyay, M. Makino, T. N. Frederickson, A. W. Hügin, and J. W. Hartley. 1992. *AIDS.* 6:607).

Infection of C57BL/6 mice with a mixture of viruses recovered from the Duplan Laterjet strain of MuLVs induces in certain strains of mice a severe immunodeficiency disease called murine AIDS (MAIDS)[1] which has similarities to AIDS in humans (1). The virus responsible for the profound immunodeficiency is a defective MuLV termed BM5d (2) or Du5H (3). The disease is characterized by pronounced splenomegaly and lymphadenopathy, by polyclonal activation and proliferation of T and B cells (1, 4) and markedly impaired responses to mitogenic and specific antigenic stimuli (5), as well as enhanced susceptibility to infection (6) and late stage lymphomas. The pathogenesis is still unclear as is that caused by HIV-1.

The *nef* gene is found in all primate lentiviruses, HIV-1, HIV-2, and simian immunodeficiency viruses (SIV), but is highly polymorphic. However, in contrast to other viral regulatory genes, *tat* and *rev*, *nef* is dispensable for virus growth in vitro (7) and remains without a precisely defined function. The Nef protein is encoded by a single open reading frame (ORF) located at the 3′ end of the HIV-1 genome, partially overlapping the 3′ LTR. It is found as a 27-kD protein NH_2-terminally myristoylated and associated with cytoplasmic membrane structures (8). Nevertheless, free nonmyristoylated, 25-kD forms initiated at an internal AUG codon (9) also have been detected in the cytoplasm of infected cells. Early mutational experiments demonstrated a participation of Nef in downregulation of HIV-1 expression (10), hence its name "negative factor". However, other reports using identical *nef* genes or gene products were unable to detect any negative effect on HIV-1 replication (11). Moreover, recent experiments show that natural *nef* alleles accelerate HIV-1 replication in primary T-lymphocytes (12), and that the natural HIV-1, ELI *nef* allele promotes efficient HIV-1 infection in primary mononuclear cells (13).

It has been demonstrated that Nef can influence the expression of two cellular proteins, CD4 and IL-2 (14–16). Two different mechanisms of downregulation by Nef seem to be involved. IL-2 production in response to antigen receptor–mediated stimulation is inhibited at the transcriptional level (16), whereas cell surface expression of CD4 in Nef-expressing cells is downregulated by a posttranscriptional mechanism (15). The relevance of these effects in the pathogenesis of AIDS is not yet clear.

[1] *Abbreviations used in this paper:* LCMV, lymphocyte choriomeningitis virus; MAIDS, murine AIDS; ntg, nontransgenic; ORF, open reading frame; SIV, simian immunodeficiency virus; tg, transgenic; VSV, vasicular stomatitis virus.

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As a first step to better understanding the biological activity of Nef in vivo, Kestler et al. (17) have studied SIV_{MAC} infection of rhesus monkeys with infectious molecular clones bearing different *nef* genes. Their results suggest that the SIV *nef* gene is necessary for efficient replication and full pathogenesis in infected rhesus monkeys, which implies for Nef a function as a positive factor in vivo.

In this paper, we describe transgenic (tg) animals as a model system to examine Nef function in vivo. To study the influence of Nef expression on T cell maturation and function, tg mice were generated carrying the HIV-1 *nef* gene under control of a T cell–specific regulatory element. In different independent tg lines, phenotypic changes in lymphocyte populations of thymus and peripheral lymphoid organs were observed at various degrees, mainly affecting the $CD4^+$ T cell lineage.

Materials and Methods

Mice. C57BL/6, (C57BL/6 × DBA/2)F_1, and pseudopregnant Moro mice were purchased from Biological Research Laboratories, Ltd. (Fuellinsdorf, Switzerland). C57BL/6-PL-*Thy1*^{a}/Cy were obtained from The Jackson Laboratory (Bar Harbor, ME).

Generation of TCR-NEF Tg Mice. The mouse TCR β chain enhancer–promotor element (18) was constructed by fusing a 5.5-kb BamHI/KpnI fragment containing the mTCR β chain core enhancer elements (19) to a 1.8-kb BamHI/KpnI fragment containing the mVβ8.3 TCR β chain promotor. This regulatory control element was fused to a PCR fragment comprising nucleotides (nt) 7956–9484 of the HIV-1, Bru genome (20) containing the complete *nef* ORF. The construct was injected as a 9-kb SmaI fragment into (C57BL/6 × DBA/2) × (C57BL/6 × DBA/2) zygotes according to protocols described by Bluethmann and Steinmetz (21). Tg founder mice were mated to C57BL/6J mice to establish permanent inbred Tg lines. The transgene transmission was followed by Southern blot analysis and PCR analysis.

PCR Analysis. Amplifications of mouse tail DNA (22) were performed using the GeneAmp kit (Perkin-Elmer AG, Kuesnacht, Switzerland) according to the manufacturer's instructions. Transgene-specific primers used were N29 (5′-ATGGGTGGCAAGTGGTCAAAAAGT-3′) 5′ primer and N30 (5′-TCAGCAGTTCTTGAAGTACTCCGG-3′) 3′ primer. Amplifications were done in 35 cycles of 30 s at 95°C; 45 s at 69°C, and 1 min at 72°C. Tg offspring showed a 621-bp transgene-specific band upon agarose gel electrophoresis.

RNA PCR. For RNA isolation, tissues were removed and quickly frozen in liquid nitrogen and stored at −80°C until analyzed. RNA was extracted by a modified single-step method (23). 1–5 μg total RNA was used as a template with oligo p(dT)12-18 as primer and Moloney-murine leukemia virus (M-MLV) H^- RT (GIBCO BRL, Gaithersburg, MD) in a cDNA synthesis reaction at 42°C according to the manufacturer's instructions. After additional RNase H (Pharmacia, Dübendorf, Switzerland) treatment at 37°C, cDNA was used in a PCR amplification. Transgene-specific primer sets used were oligonucleotides N205 (5′-CCTGCAGACCCACCTCCCAACC-3′) 5′ primer/N507 (5′-GCGCGCCGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGAAGCAC-3′) 3′ primer, and N201 (5′-CGAGGGGACCCGACAGGCCCG-3′) 5′ primer/N507 3′ primer, yielding products of ~1.3 and ~1.2 kb in length for amplifications 1 and 2, respectively. Amplifications were done for 30–40 cycles using cycles of 30 s at 95°C; 1 min at 66°C; and 1.5 min at 72°C. For control amplifications, TCR $C\beta_2$ chain mRNA (24)–specific primers oligonucleotide sets N210 (5′-ACCCTCAGGCCTACAAGGAGAGC-3′)/N207 (5′-GCAGGAAAATCTATGGCCAGGGTG-3′) and N211 (5′-CTACTGCCTGAGCAGCCGCCTG-3′)/N209 (5′-GGTGAAGAACGGCTCAGGATGCATA-3′) were used, yielding products of 410 and 360 bp in length for amplifications 1 and 2, respectively.

Antibodies Used in Flow Cytometry Analysis. The following antibodies against different antigens were used. CD4 (GK1.5, Becton Dickinson & Co., Mountain View, CA; and RM-4-5, Pharmingen, San Diego, CA), CD8 (53-6.7, Becton Dickinson & Co.), Thy1.2 (53-2.1, Pharmingen), Thy1.1 (MRC OX-7; Serotec Ltd., Kidlington, Oxford, UK), Ly-1 (CD5) (53-7.3, Pharmingen), CD3ϵ (145-2C11, Pharmingen), Ly5 (B220) (CD45RA) (RA3-6B2, Pharmingen), Ly6C (AL-21, Pharmingen), Pgp-1 (CD44) (IM 7, Pharmingen), IgE-R (CD23) (B3B4, Pharmingen), heat stable antigen (HSA) (J11d, Pharmingen), leukocyte cell adhesion molecule 1 (LECAM-1) (Mel-14, Pharmingen), α/β TCR (H57-597, Pharmingen), γ/δ TCR (GL3, Pharmingen), Vβ5.1 5.2 (MR 9-4, Pharmingen), Vβ3 (KJ-25, Pharmingen), Vβ17a (KJ-23, Pharmingen), Vβ11 (RR3-15, Pharmingen), H-$2K^b$ (AF6-885, Pharmingen), H-$2K^d$ (SF1-1.1, Pharmingen), 3G11, and 6C10 (25).

Mitogenic Stimulation of Lymphocytes In Vitro. Lymphocytes isolated from spleen and LNs were depleted of erythrocytes by hypotonic lysis. T cells were purified by treatment with anti-J11D and anti-class II antibody plus complement as described by Muralidhar et al. (4) or enriched by nylon wool columns. Cells were cultivated in IMDM medium supplemented with 10% FCS (Hyclone Laboratories, Logan, UT), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin, 5×10^{-5} M 2-ME, and 10 mM Hepes in 96-well microtiter plates at 4×10^5 cells per well. Mitogen concentrations used were 5 μg/ml Con A, 50 μg/ml LPS, and 10 μg/ml SEB (all from Sigma Chemical Co., St. Louis, MO). After 48 h, the cultures were pulsed for at least 8 h with [^{3}H]thymidine (Amersham, Zurich, Switzerland) and harvested for counting. Mean and SEs of triplicate cultures were determined.

Neutralizing Anti-Vasicular Stomatitis Virus Antibody Responses. Mice were infected intravenously with 2×10^6 PFU vasicular stomatitis virus (VSV) in a volume of 0.2 ml. Blood samples were collected on days 4, 9, and 14 after infection. Anti-VSV neutralizing titers of the sera were assayed in 96-well plates as described by Roost et al. (26). IgG antibody titers were determined after reduction with 0.1 M 2-ME.

Immune Response to Lymphocytic Choriomeningitis-WE Infection. Mice were infected by subcutaneous injection of 30 PFU lymphocytic choriomeningitis virus (LCMV, WE strain) into footpads. Footpad swelling was monitored by measurement with spring-loaded calliper as described by Zinkernagal et al. (27). Increase in footpad thickness is expressed as a percentage of control value. Footpads of noninjected mice served as control. Animals were killed after 23 d and LCMV-WE titers in spleen, liver, and blood were determined by plaque assay (27).

Bone Marrow Reconstitution. 24 h before the bone marrow transfer, B6-PL-Thyla mice (6–8-weeks-old), were irradiated with 850 rad at 103 rad/min from a ^{127}Cs animal irradiator. Animals were maintained under specific pathogen-free conditions. The drinking water was supplemented with 100 mg/liter neomycin and 10 mg/liter polymixin (Sigma Chemical Co.). As radiation controls, 6–8-wk-old C57BL/6 mice were treated in parallel but not reconstituted with bone marrow. 3-wk-old B6/338L tg or B6 nontransgenic (ntg) mice of the same litter were killed. Bone marrow was isolated by flushing the tibias and femurs with sterile medium (RPMI 1640, 25 mM Hepes, 0.3% BSA). Mature T and B cells were depleted by treatment with polyclonal rabbit anti–mouse Ig (Pharmingen), monoclonal rat anti-Thy1.2 (Pharmingen) antibodies,

and complement-mediated lysis. 0.2 ml cell suspension (5×10^7 cells/ml) was injected intravenously into lethally irradiated mice. Donor and recipients were sex matched.

Results

Generation of Tg Lines and Analysis of the B6/338L Transgene Locus. As illustrated in Fig. 1 *A*, the transgene construct contained a 1.5-kb PCR fragment of the HIV-1, Bru genome (20), which was inserted downstream of the mouse TCR β chain enhancer (19) and Vβ8.3 promoter to target expression of the transgene to lymphoid cells (18). After zygote microinjection, several independent tg lines were generated, three of which showed phenotypic changes, such as loss of CD4 expression in lymphocyte populations, thymic atrophy, and reduced life span, but to different degrees. An example of a typical CD4/CD8 staining pattern of thymocytes from two different tg lines is given in Fig. 2. The most dramatic effects were observed in line B6/338L. Already in the heterozygous state, offspring of line B6/338L were characterized by a profound immunodeficiency and a high mortality rate when kept under nonspecial pathogen free (SPF) conditions. Most animals died at 4–6-mo-old, whereas ntg littermates had normal life expectancies. Similarly, homozygous B6/305 offspring showed a high mortality rate when kept under non SPF conditions, indicating impaired immune responses in these animals.

Tg line B6/338L was the only one with a single copy of the transgene integrated. All other lines carried multiple copies of the transgene. However, the B6/338L integrated transgene was characterized by a smaller 4.5-kb band as compared with a 8.5-kb band in the other tg lines, that hybridized in Southern blots to a Nef-specific probe (Fig. 1 *B*). More detailed analysis by Southern blotting and PCR revealed a rearrangement of the original injected construct as depicted in Fig. 1 *C*. The three components of the transgene construct were present but in a different organization. This organization, however, resembles the natural configuration of the mouse TCR β chain locus where the enhancer is located downstream of the TCR β chain gene. A likely explanation for the rearrangement is a partial deletion of the 5′ and 3′ ends of two tandem copies during the integration process.

To determine whether the *nef* ORF and the Vβ8.3 promoter of the B6/338L transgene were still intact, fragments were cloned by PCR amplification from either genomic DNA or from thymic cDNA templates and sequenced. No mutations to the original *nef* gene and Vβ8.3 promoter sequence used for zygote injections or to transgenes cloned from other tg lines were found (data not shown).

To exclude the presence of a MAIDS-inducing defective retrovirus in the tg lines, Southern blots were screened with a BM5-defective, retrovirus-specific probe (100-bp SalI-XbaI fragment of plasmid pGEN-3 BM5d/Dp12a; 28). No BM5d-specific hybridization pattern as described by Chattopadhyay

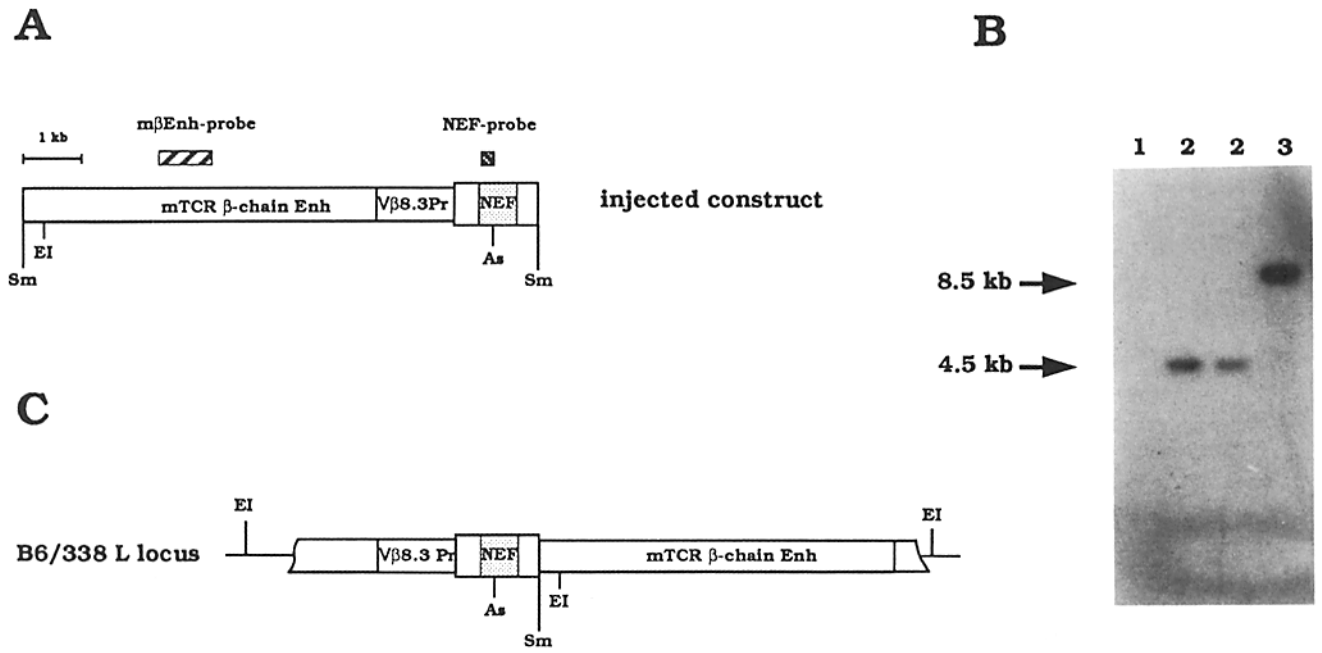

Figure 1. The TCR-NEF transgene construct and the B6/388L integration locus. (*A*) The mouse TCR β chain enhancer-promoter element was constructed by fusion of a 5.5-kb BamHI/KpnI fragment containing the mTCR β chain core enhancer elements (19) to a 1.8-kb BamHI/KpnI fragment containing the mVβ8.3 TCR β chain promoter. This control element was hooked up to a PCR fragment comprising nt 7956–9484 of the HIV-1 Bru genome (20) containing the complete *nef* ORF. (*B*) Southern blot analysis of genomic DNA digested with EcoRI/Asp718 from: (lane *1*) ntg; (lane *2*) B6/338L; and (lane *3*) B6/305 littermates with a *nef*-specific probe. No crosshybridization of the *nef*-specific probe with endogenous retroviral sequences in tg or ntg littermates was observed. (*C*) The B6/338L locus. Schematic illustration of the B6/338L transgene locus as deduced from Southern blot analysis with different restriction enzymes hybridized to *nef* or TCR β chain enhancer–specific probes (19), and PCR analysis with different transgene-specific oligonucleotides.

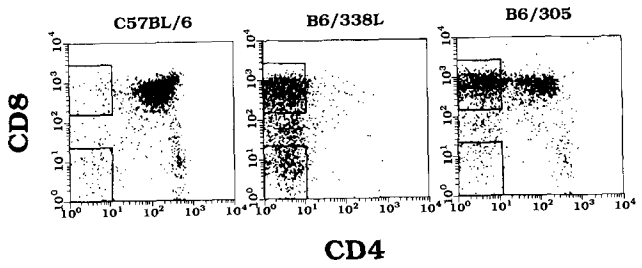

Figure 2. CD4/CD8 staining pattern of tg and ntg thymocytes. Cells were labeled with PE-conjugated anti-mouse CD4 and biotinylated anti-mouse CD8, and subsequently with Streptavidin-Tandem by standard procedures and analyzed on a FACScan® instrument (Becton Dickinson & Co.).

et al. (28) was detected (data not shown). The *nef*-specific hybridizing probe used for identification of tg offspring did not recognize any endogenous MuLV sequences (see Fig. 1 *B*).

Transgene Expression. Transgene expression was monitored by a RNA-PCR method using primer pairs specific for transgene-derived messages (see Materials and Methods). An example of an ethidium bromide–stained agarose gel electrophoresis of *nef*-specific amplification products derived from total thymic RNA templates of B6/338L mice is given in Fig. 3 *A*. PCR products were cloned and sequenced to confirm the specificity of the amplification. A *nef*-specific 1.2-kb fragment was visible in tg samples only. TCR β chain messages could be detected in most lymphoid samples, using mTCR $C\beta_2$ gene–specific primers (Fig. 3 *B*).

RNA samples of different organs from B6/338L mice of various stages were analyzed. An overview of transgene expression in lymphoid organs is given in Table 1. *nef* mRNA could be detected in the thymuses of B6/338L mice at all time points analyzed, even at the earliest embryonic stages, indicating that the first intrathymic precursors already seemed to express the transgene. At day 91, no analysis could be made because in these mice a thymus was not present anymore. In LN samples only, a weak signal was obtained at day 29, whereas all other samples were negative. However, in spleen, strong positive signals were obtained at days 29 and 45. Spleen samples of older and younger animal were negative. In bone marrow and liver samples, no positive signal was ever obtained. All RNA samples of lymphoid organs from day 15 in ontogeny onwards were positive for TCR β chain message (data not shown). Other organs tested, such as lung, heart, brain, kidney, and skin, as well as all samples from ntg littermates, were negative for *nef*-specific transcripts (data not shown). Preliminary experiments showed that similar distribution of Nef expression—strong expression in the thymus, lower expression in the peripheral lymphoid organs—was detected in B6/305 mice.

Thymocyte Populations. Macroscopic analysis of B6/338L offspring revealed signs of thymic atrophy in animals younger than 8 wk old. In animals older than 8 wk old, the thymus often completely disappeared with time. In addition, older mice developed lymphadenopathy and splenomegaly.

Dramatic changes in thymocyte populations of B6/338L mice were demonstrated by subjecting thymocytes to FACS® analysis (Becton Dickinson & Co.). The total number of thymocytes isolated from 6–8-wk-old mice was reduced 5–10-fold compared with ntg littermates. Furthermore, the $CD4^+CD8^+$ cells, which normally constitute the majority of the thymocytes, were almost completely lacking in B6/338L mice (Fig. 2). In contrast, the largest population were $CD8^+CD4^-$ thymocytes, which represented only a minor fraction in ntg littermates. However, only a minority of these $CD8^+CD4^-$ cells expressed high levels of CD3 and low levels of HSA, two characteristic markers of mature $CD8^+$ cells (data not shown). Most of these cells showed cell surface marker expression found on $CD4^+CD8^+$ cells in normal mice except for their lack of CD4 expression. The $CD4^-CD8^-$ population and cells expressing intermediate levels of CD8 were found to be increased as well. No $CD4^+CD8^-$ thymocytes were detected (Fig. 2). In other TCR-NEF tg lines, B6/305 (Fig. 2) and B6/553 (data not shown), a similar depletion of $CD4^+$ cells in the thymus was observed, but to a lesser degree. Compared with ntg littermates, total $CD4^+$ thymocyte numbers were decreased 200–300-, 15–20-, and 8–10-fold in heterozygous B6/338L, B6/305, and B6/553 mice, respectively.

Thymocyte Populations during Ontogeny. Single cell suspensions of thymus from prenatal stages of B6/338L mice were analyzed by FACS® to determine at what time point of development the depletion of the $CD4^+CD8^+$ and $CD4^+CD8^-$ thymocyte population occurred. As shown in Fig. 4, at day 15 of gestation, first $CD4^-CD8^{low}$, $CD4^{low}CD8^-$, and $CD4^{low}CD8^{low}$ thymocytes were detected in ntg thymus, whereas the overall majority of tg thymocytes was

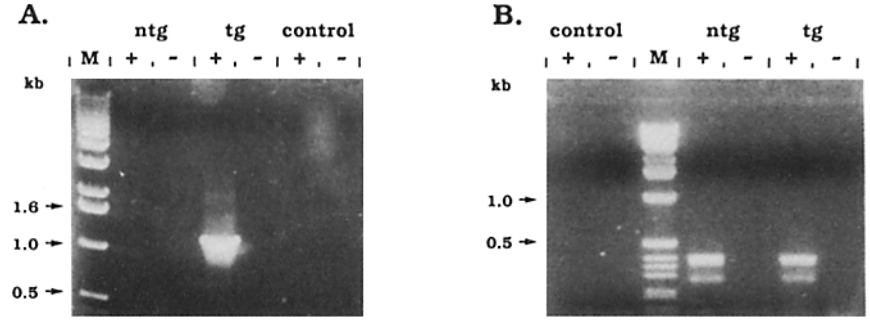

Figure 3. PCR amplification of transgene and mTCR β chain messages. Agarose gel electrophoresis of (*A*) 2nd PCR products with transgene-specific primers and (*B*) 2nd PCR products with mTCR β chain C_2-specific primers. Sizes of control DNA fragments are shown in kilobases. Lanes (*M*) DNA size marker (1-kb ladder, Gibco BRL); (*ntg*) total thymus RNA from ntg day 16 embryos; (*tg*) total thymus RNA from tg day 16 embryos; (control) no RNA; (+) addition of reverse transcriptase (RT) to cDNA mix; (−) no addition of RT to cDNA mix. For details of the RNA-PCR procedure, refer to Materials and Methods.

Table 1. *Overview of Transgene Expression in B6/338L Mice*

	Prenatal						Postnatal								
Tissue	E13	E14	E15	E16	E17	E18	d1	d3	d6	d10	d14	d21	d29	d45	d91
Thymus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	ND
Liver	–	–	–	–	–	–	–	ND	ND	ND	ND	ND	–	–	–
Spleen	ND	ND	–	–	–	–	–	–	–	–	–	–	+	+	–
LN	ND	ND	ND	ND	ND	ND	ND	–	–	–	–	–	±	–	–
Bone marrow	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	–	–	–	–

1–5 μg of total RNA from different tissues was used as template for RNA-PCR amplification with transgene-specific primers. (+) Expression; (–) no expression; (±) very weak expression.

$CD4^-CD8^-$. A $CD4^+CD8^+$ thymocyte population was present in ntg thymus on day 16 of gestation, when the depletion of this population was already prominent in tg thymus. Only very low CD4 expression was observed on tg thymocytes. 1 or 2 d before birth, at day 18 of gestation, the overall majority of ntg thymocytes had the $CD4^+CD8^+$ phenotype, whereas in tg thymus, larger populations of $CD4^-CD8^+$ and $CD4^-CD8^-$ cells were observed.

These results suggest that transgene expression leads to a depletion of CD4-positive thymocytes already at the earliest stages in embryogenesis. The mechanism that leads to this effect, however, is still unclear.

Thymic Populations in C57BL/6 Mice Reconstituted with tg Bone Marrow. Reconstitution experiments were performed to investigate the role of B6/388L bone marrow–derived hematopoietic cells versus those of thymus and lymphoid organs in the generation of the severe immunodeficiency of the B6/388L tg mouse line. B6-PL-Thy1[a] mice expressing the Thy1.1 allele on T cells were chosen as recipients to distinguish T cells derived from the donor bone marrow of B6/338L or B6/ntg mice expressing the Thy1.2 allele from those derived from the endogenous bone marrow. 6–8-wk-old B6-PL-Thy1[a] mice were lethally irradiated and reconstituted with bone marrow of 3-wk-old heterozygous B6/338L tg mice or B6/ntg control mice of the same litter, as described in Materials and Methods.

Reconstituted mice survived irradiation and some of them were killed 8 and 12 wk after transfer for thymocyte analysis. Mice reconstituted with B6/ntg bone marrow showed normal ratios of $CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^-CD8^+$, and $CD4^+CD8^-$ thymocytes (Fig. 5 *A*). In contrast, almost complete depletion of $CD4^+CD8^+$ and $CD4^+CD8^-$ cells was seen in mice reconstituted with B6/338L bone marrow. The increase of the $CD4^-CD8^-$ and $CD4^-CD8^+$ popula-

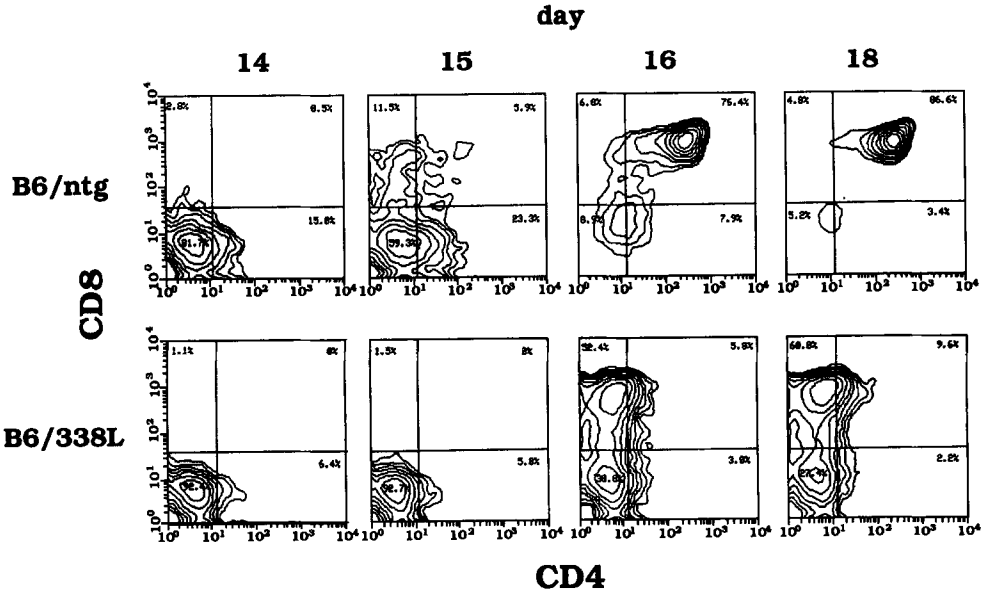

Figure 4. CD4/CD8 staining pattern of ntg and tg thymocytes from fetal thymus at day 14–16 and day 18 of gestation. Timed matings with B6/338L males and C57BL/6 females were set up. The day at which a vaginal plug was observed was taken as day 0. Pregnant females were killed at day 13–18 of gestation. The fetal thymuses of 6–10 embryos per time point were removed. Genomic DNA samples were prepared from tail biopsies, and by PCR analysis tg and ntg embryos were identified. Single cell suspensions of thymocytes were stained with PE-conjugated anti-mouse CD4 and biotinylated anti-mouse CD8, subsequently with Streptavidin-Tandem, and analyzed by flow cytometry. (*Top*) The time point of gestation.

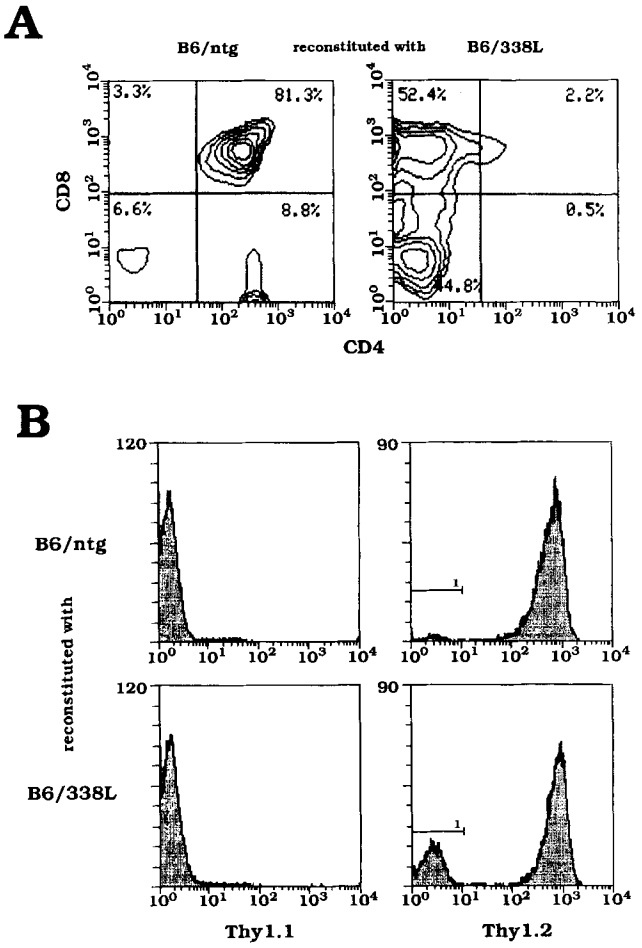

Figure 5. Thymocyte populations of reconstituted B6/Thy1[a] mice. Cells from B6/Thy1a mice were labeled (*A*) with PE-conjugated anti-mouse CD4 and biotinylated anti-mouse CD8, and subsequently with Streptavidin-Tandem; or (*B*) with FITC-conjugated anti-mouse Thy1.1 and PE-conjugated anti-mouse Thy1.2 antibodies 8 wk after reconstitution with B6/338L tg or ntg bone marrow.

tion, as well as their CD3ε and HSA expression pattern is analogous to that found in thymuses of B6/338L mice (data not shown). Total thymocyte numbers of these mice were reduced three to sixfold when compared with the control mice. In the thymuses of both types of reconstituted mice, only very few cells could be detected as expressing Thy1.1, and most of them were Thy1.2 positive (Fig. 5 *B*). Thy1.2 staining also revealed the presence of a proportion of Thy1.2-negative/Thy1.1-negative cells in mice reconstituted with B6/338L bone marrow, which was not found in mice reconstituted with B6/ntg bone marrow.

Taken together, these experiments demonstrate that the phenotypic changes in the thymocyte population of B6/338L tg mice are inherent to bone marrow–derived cells and are not due to a defect of thymic epithelium as found, for example, in nude mice.

Peripheral Lymphocyte Populations. To address the question of whether these changes in thymus (Fig. 6, *A* and *B*) would influence the distribution of the peripheral lymphocyte population, lymphocytes were isolated from LNs and spleens and analyzed by FACS®. As mentioned earlier, B6/338L mice developed lymphadenopathy and splenomegaly starting at about 6–8 wk after birth. Expansion of lymphocytes continued throughout their life span, as the total number of cells in LNs and spleen increased up to 10-fold over controls. The percentage of T cells in the periphery was reduced 5–10-fold, as demonstrated by Thy1 or CD4/CD8 staining (Fig. 6, *C* and *D*), mainly because of a large increase of the B cell population. The actual number of T cells in older mice was reduced only two- to three-fold because of the enlargement of lymphocyte populations. The ratio of $CD4^+$ to $CD8^+$ cells, about 2:1 in normal mice, was as low as 0.5:1. B6/338L peripheral T cells also showed major phenotypic alterations compared with ntg controls. Their expression of CD8 and CD3 was reduced. A substantial fraction of the $CD8^+$ cells is most probably NK cells, as they were $CD3^-$ (Fig. 6 *G*) and expressed the murine NK cell marker NK1.1 (data not shown).

In the mouse system, naive T cells can be distinguished from memory or antigen-triggered T cells by differential expression of the cell surface markers Pgp-1 (CD44) and Mel-14 (LECAM-1) (29). Naive T cells express low levels of Pgp-1 and high levels of Mel-14; memory or activated T cells express high levels of Pgp-1 and low levels of Mel-14. In control mice, only a minor population expressed high levels of Pgp-1 and low levels of Mel-14 (Fig. 6, *H* and *I*), whereas in B6/338L mice, the majority of peripheral T cells had this phenotype (Fig. 6, *H* and *I*). Recently Hayakawa and Hardy (25) described four subsets of peripheral $CD4^+$ T cells according to their differential expression of the 6C10 and 3G11 antigens, which show differential growth potential and discriminate virgin and memory or activated T cells. The $3G11^+6C10^-$ subset was the predominant population in ntg mice. In B6/338L mice, a clear shift towards the double negative subsets was observed (Fig. 6, *E* and *F*). Taken together, these data show that the changes in tg thymocyte populations lead to a drastic depletion of peripheral T cells. In addition, they indicate that as in the MAIDS mice (1, 4) the peripheral T cells in B6/338L mice show cell surface marker expression characteristic of memory or activated T cells, whereas virgin T cells seem to be absent.

In Vitro Stimulation of Peripheral Lymphocytes. To study the functional capacity of B6/338L lymphocytes in vitro, lymphocytes were prepared from LNs and spleen and stimulated with different mitogens. For cell proliferation assays, either total cells (Fig. 7, *A* and *B*) were used or T cells were enriched (Fig. 7 *C*) to compensate differential proliferation due to the different frequencies of T cells in B6/338L mice. Lymphocytes isolated from spleen or LN showed only weak proliferation upon stimulation with Con A or SEB (Fig. 7, *A* and *B*) even when IL-2 was included in the culture medium. The LPS response was drastically reduced when using spleen cells (Fig. 7 *A*), but, only a two to three-fold reduction was observed with LN cells (Fig. 7 *B*). Cultures with adjusted numbers of T cells showed a six to eight-fold reduction in response to Con A stimulation (Fig. 7 *C*).

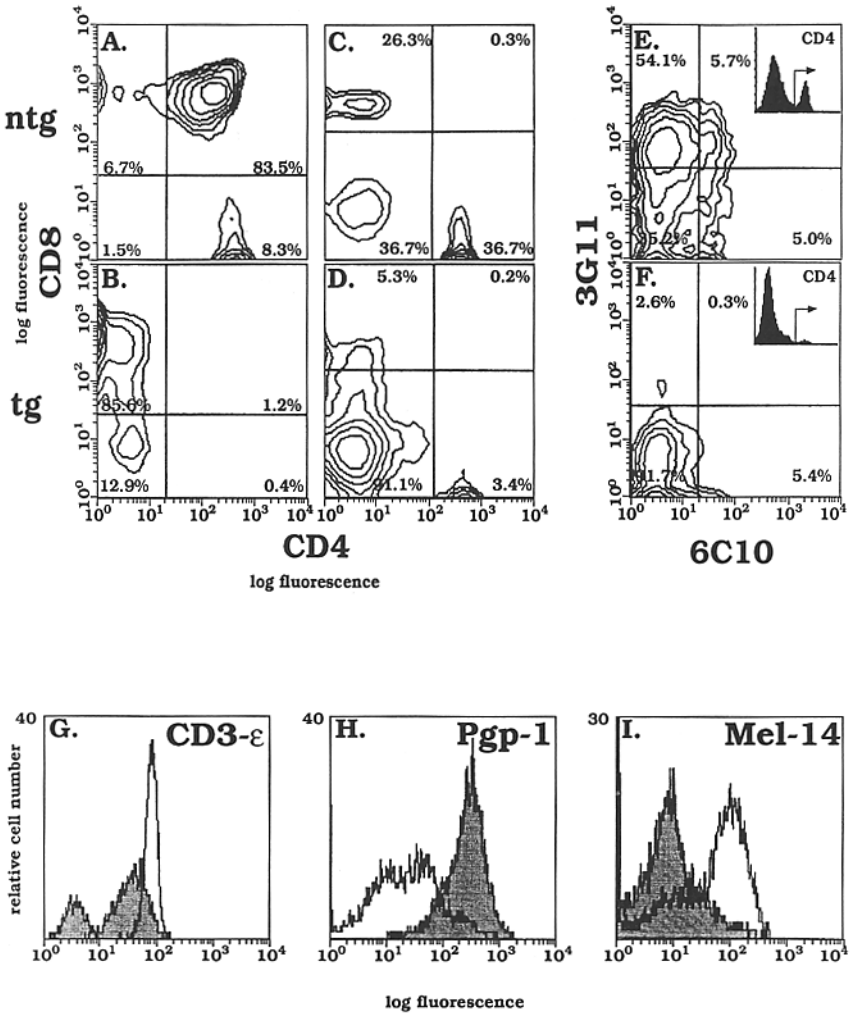

Figure 6. Cell surface marker analysis of cells from different lymphoid tissues of B6/338L mice. Thymocytes of 6-wk-old (*A*) nontransgenic (*ntg*) or (*B*) B6/338L transgenic (*tg*) littermates were stained with PE-conjugated anti-mouse CD4 and biotinylated anti-mouse CD8, and subsequently with Streptavidin-Tandem. CD4/CD8 staining of LN cells of (*C*) ntg or (*D*) tg offspring. Spleen cells were stained with PE-conjugated anti-mouse CD4 and biotinylated mAb 3G11 and FITC-conjugated 6C10. Only $CD4^+$ cells were acquired (*inset*). 3G11/6C10 profile is shown for (*E*) ntg or (*F*) tg mice. LN cells were stained with PE-conjugated anti-mouse CD4, FITC-conjugated anti-mouse CD8, and counterstained with antibodies to (*G*) biotinylated anti-mouse CD3-ε, (*H*) biotinylated anti-mouse Pgp-1 (CD44), or (*I*) biotinylated anti-mouse Mel-14 (LECAM-1). $CD4^-CD8^-$ cells were omitted during acquisition. (*Shaded curves*) The staining of B6/338L cells; (*unfilled curves*) staining of ntg controls.

In Vivo Immune Responses. The in vivo immune responses of B6/338L mice were studied by infection with various viruses. To test whether B6/338L mice were able to generate a normal antiviral humoral response, 8-wk-old mice were infected with VSV. The efficiency of the response was assessed by determination of the serum titer of virus neutralizing antibodies at different time points. In B6/338L mice, anti-VSV IgM responses measured on day 4 after infection were normal, however, the T cell–dependent neutralizing IgG antibodies, determined on days 9 and 14, were reduced when compared with controls (Table 2). These results illustrate the impairment of B6/338L T h cell responses involved in switching anti-VSV antibody responses from IgM to IgG.

$CD8^+$ T cell responsiveness was tested in mice infected with LCMV (27). Subcutaneous inoculation of LCMV (WE strain) into footpads of mice normally induces a virus-specific swelling reaction. The initial major swelling which is maximal around day 8, is mediated by $CD8^+$ T cells, followed by a late minor swelling phase around day 12, which is $CD4^+$ T cell dependent (27). This typical reaction was indeed observed in the control mice. However, B6/338L mice infected with LCMV-WE showed no measurable swelling reaction in the footpads (Table 3). This unresponsiveness again illustrates the functional defect of T cells in B6/338L mice. $CD8^+$ T cells are also crucial for the clearance of LCMV after acute infection (30). As reported previously, in normal mice LCMV virus was detected in the spleen on days 4–6 after infection, but no virus could be demonstrated after day 23 (30). In contrast, B6/338L mice still contained high virus titers on day 23 after subcutaneous infection (Table 3). These results suggest that the $CD8^+$ T cells of B6/338L mice were not able to induce a protective virus-specific CTL response.

Discussion

The data presented here suggest an association of a HIV-1 NEF/3′ LTR transgene with a severe immunodeficiency, as manifested by the lack of responsiveness of tg lymphocytes to mitogenic stimuli, defective immune response to various viral infections and enhanced susceptibility to infections. The similarities to MAIDS and to human AIDS are striking and a further study of this tg mouse line might lead to a better

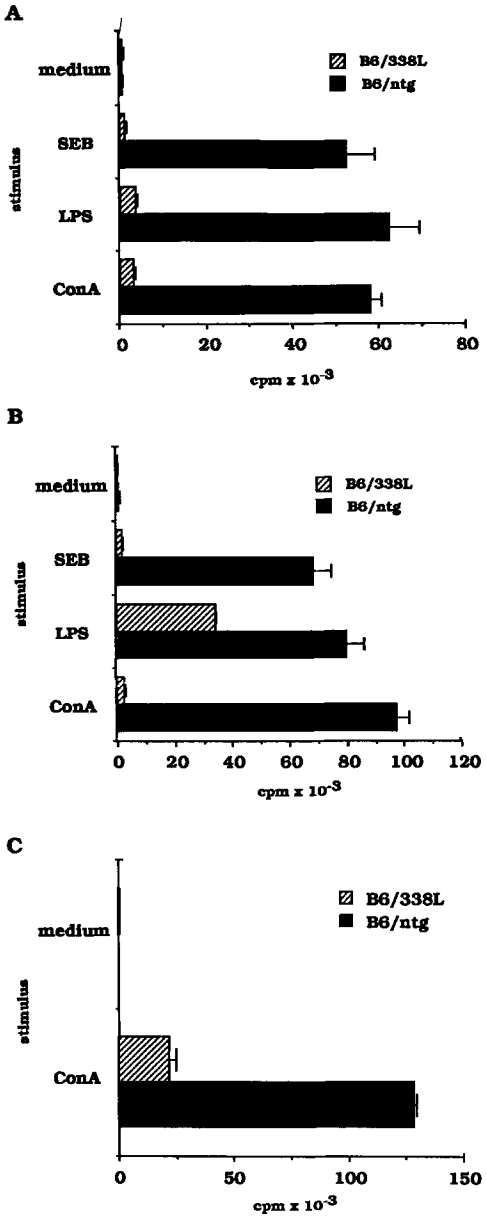

Figure 7. Proliferative responses of B6/338L and ntg lymphocytes to mitogenic stimulation. Lymphocytes isolated from spleen (*A*) and LNs (*B*) were depleted of erythrocytes by hypotonic lysis. (*C*) T cells from LNs were enriched by nylon wool columns. T cell numbers were adjusted according to anti-Thy1.2 FACS® staining. Cells were cultivated in IMDM supplemented with 10% FCS (Hyclone Laboratories), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin, 5×10^{-5} M 2-ME, and 10 mM Hepes in 96-well microtiter plates at 1–4 $\times 10^5$ cells per well. Mitogen concentrations used were 5 μg/ml Con A, 50 μg/ml LPS, and 10 μg/ml SEB. After 48–72 h the cultures were pulsed for at least 8 h with [^{3}H]thymidine and harvested for counting. Mean and SEs of triplicate cultures are shown.

understanding of the pathogenesis of AIDS. Accidental infection of our mice with a MAIDS-inducing retrovirus mixture could be excluded by Southern analysis using a BM5 defective probe as described by Chattopadhyay et al. (28). Whether T cell–specific expression of the HIV-1 *nef* gene product leads directly to the deficiency in thymocyte development as observed in the tg B6/338L mice remains to be proven. Low levels of *nef* transcripts could be detected by RNA-PCR in the thymus of B6/338L mice, as well as in B6/305 mice (data not shown). So far we have not been able to detect the presence of Nef protein in any of the transgenic lines. Work is in progress to improve the sensitivity of the methods used. Nevertheless, the downregulation of CD4 observed in thymocytes of different independent tg lines would fit in with reports demonstrating a downregulation of CD4 from the cell surface in response to Nef expression in transfected human (15) and mouse T cell lines (31).

Differences in the genetic backgrounds of the tg lines due to the (C57BL/6 × DBA/2)F_2 zygotes used for injection could be responsible for the differential effects seen. In the case of MAIDS, for example, it is known that only certain strains of mice develop the immunodeficiency, according to their MHC haplotypes and other genetic determinants (1). Backcrossing of the B6/338L locus into different mouse strains should clarify this point. In addition, the unique organization of the transgene or the integration site can lead to differential expression during embryogenesis and/or differentiation of T cells, and thus may account for the strong effects observed in B6/338L mice versus the less obvious changes in other tg lines. It has been shown for several tg lines that the site of integration can influence the timing and magnitude of transgene expression (32). Another possible explanation for the B6/338L phenotype could be an insertional mutation. Although this cannot be excluded formally, it seems rather unlikely because of the following reasons: first, insertional mutations in tg animals tend to be recessive (32) (the B6/338L phenotype is dominant); and second, two other independent HIV-1 NEF/3′ LTR tg mouse lines show similar effects with respect to CD4 expression, atrophy of the thymus and reduced life spans.

Finally, reconstitution experiments indicate that the B6/338L phenotype can be established in normal C57BL/6 mice by bone marrow transfer. This finding would suggest that the immunodeficiency phenotype in B6/338L mice is caused by a bone marrow–derived defect that leads to an impaired recruitment of precursor T cells in the thymus, a phenomenon that is believed to also occur in AIDS patients.

During the course of the preparation of this manuscript, Skowronski et al. (33) reported the generation of tg mice carrying the *nef* gene under control of the CD3δ promoter–enhancer. Similar to our TCR-NEF mice, these mice show a depletion of CD4-positive thymocytes and peripheral T cells, but to a lesser degree than in our B6/338L mice. In addition, the authors report a hyperreactivity of CD3-NEF1 tg thymocytes to TCR stimulation, however, no data on mitogenic stimulatory capacity of tg peripheral lymphocytes or on the capacity of tg mice to generate immune responses to specific pathogens were presented. The authors suggest that this hyperresponsiveness of the CD3-NEF1 tg thymocytes may lead to the direct elimination rather than the positive selection of these cells, which is manifested in peripheral T cell deple-

Table 2. *In Vivo Analysis of Immune Responses to Infection with Different Viruses I*

	Neutralizing antibody titers ($log_2 \times 40$)					
	Day 4		Day 9		Day 14	
Mouse	IgM/IgG	IgG	IgM/IgG	IgG	IgM/IgG	IgG
B6/338L I	9	<1	7	3	2	<1
B6/338L II	7	<1	*	*	*	*
B6/338L III	8	<1	5	3	2	1
B6/ntg I	8	<1	11	7	11	9
B6/ntg II	7	<1	11	7	9	9
B6/ntg III	8	<1	11	8	9	9

Neutralizing anti-VSV antibody responses of B6/338L or B6/ntg littermates. Mice were infected intravenously with 2×10^6 PFU in 0.2 ml. Blood samples were collected on days 4, 9, and 14 after infection. Anti-VSV neutralizing titers of the sera were assayed in 96-well plates as described elsewhere (26). IgG antibody titers were determined after reduction with 0.1 M 2-ME. One mouse died of VSV (*). This correlates with increased susceptibility to VSV in the absence of an IgG response (34).

Table 3. *In Vivo Analysis of Immune Responses to Infection with Different Viruses II*

	Percent swelling vs. control on day				LCMV-WE clearance by day 23		
Mouse	6	8	13	15	PFU/ spleen	PFU/ liver	PFU/ ml blood
B6/338L I	<5%	<5%	<5%	<5%	1.8×10^5	3.0×10^7	2.5×10^5
B6/338L II	<5%	<5%	<5%	<5%	1.5×10^6	2.4×10^7	3.8×10^4
B6/ntg I	<5%	100%	10%	<10%	$<10^2$	$<10^2$	$<10^2$
B6/ntg II	<5%	90%	15%	<10%	$<10^2$	$<10^2$	$<10^2$

Immune response to LCMV-WE infection. Mice were infected with 30 PFU into footpads. Footpad swelling was monitored by measurement with spring-loaded calliper (27). Increase in footpad thickness is expressed as a percentage of control value. Footpads of noninjected mice served as controls. Animals were killed after 23 d and LCMV-WE titers in spleen, liver, and blood were determined by plaque assay (27).

tion. This is in line with our observations of an unresponsive state of the residual peripheral T cells and impaired immune responses to specific pathogens in B6/338L mice. These unresponsive T cells, whose number is reduced drastically compared with the T cell population of normal mice, may represent a small population of tg thymocyte escaping the deleterious effect of Nef expression and/or CD4 downregulation by a so far undetermined mechanism. Interestingly, in B6/338L as well as CD3-NEF1 tg mice, Nef expression seems to be strongly reduced in peripheral lymphocytes as compared with thymocytes, suggesting the reduction or elimination of transgene expression to be the mechanism to escape elimination in the thymus.

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References

1. Morse, H.C. III, S.K. Chattopadhyay, M. Makino, T.N. Frederickson, A.W. Hügin, and J.W. Hartley. 1992. Retrovirus-induced immunodeficiency in the mouse: MAIDS as a model for AIDS. *AIDS.* 6:607.
2. Chattopadhyay, S.K., H.C. Morse, III, M. Makino, S.K. Ruscetti, and J.W. Hartley. 1989. Defective virus is associated with induction of murine retrovirus induced immunodeficiency syndrome. *Proc. Natl. Acad. Sci. USA.* 86:3862.
3. Aziz, A., H. Zaher, and P. Jolicoeur. 1989. Severe immunodeficiency induced by a defective murine leukemia virus. *Nature (Lond.).* 338:505.
4. Muralidhar, G., S. Koch, M. Haas, and S.L. Swain. 1992. CD4 T cells in murine acquired immunodeficiency syndrome: polyclonal progression to anergy. *J. Exp. Med.* 175:1589.
5. Mosier, D.E., R.A. Yetter, and H.C. Morse, III. 1985. Retroviral induction of acute lymphoproliferation disease and profound immunosuppression in adult C57BL/6 mice. *J. Exp. Med.* 161:766.
6. Buller, R.M., R.A. Yetter, T.N. Frederickson, and H.C. Morse, III. 1987. Abrogation of resistance to severe mousepox in C57BL/6 mice infected with LP-BM5 murine leukemia viruses. *J. Virol.* 61:383.
7. Terwilliger, E., J.G. Sodroski, C.A. Rosen, and W.A. Haseltine. 1986. Effect of mutations within the 3′ orf open reading frame region of human T cell virus type III on replication and cytopathogenicity. *J. Virol.* 60:754.
8. Franchini, G., M. Robert-Guroff, J. Ghrayeb, N.T. Chang, and F. Wong-Staal. 1986. Cytoplasmic localization of the HTLV III 3′ orf protein in cultured T-cells. *Proc. Natl. Acad. Sci. USA.* 83:5282.
9. Kaminchik, J., B. Bashan, A. Itach, N. Sarver, M. Gorecki, and A. Panelt. 1991. Genetic characterization of human immunodeficiency virus type 1 nef gene product translated in vitro and expressed in mammalian cells. *J. Virol.* 65:583.
10. Luciw, P.A., C. Cheng-Mayer, and J.A. Levy. 1987. Mutational analysis of the human immunodeficiency virus (HIV): the orf-B region downregulates virus replication. *Proc. Natl. Acad. Sci. USA.* 84:1434.
11. Hammes, S.R., E.P. Dixon, M.H. Malim, B.R. Cullen, and W.C. Greene. 1989. Nef protein of human immunodeficiency virus type 1: evidence against its role as transcriptional silencer. *Proc. Natl. Acad. Sci. USA.* 86:9549.
12. de Ronde, A., B. Klarer, W. Keulen, L. Smit, and J. Goudsmit. 1992. Natural HIV-1 nef accelerates virus replication in primary human lymphocytes. *Virology.* 188:391.
13. Terwilliger, E.F., E. Langhoff, D. Gabuzda, E. Zazopoulos, and W.A. Haseltine. 1991. Allelic variation in the effects of the nef gene on replication of human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA.* 88:10971.
14. Guy, B., Y. Riviere, K. Dott, A. Regnault, and M.P. Kieny. 1990. Mutational analysis of the HIV nef protein. *Virology.* 176:413.
15. Garcia, J.V., and A.D. Miller. 1991. Serine phosphorylation–independent downregulation of cell-surface CD4 by nef. *Nature (Lond.).* 350:508.
16. Luria, S., I. Chambers, and P. Berg. 1991. Expression of the type I human immunodeficiency virus Nef protein in T-cells prevents antigen receptor–mediated induction of interleukin-2 mRNA. *Proc. Natl. Acad. Sci. USA.* 88:5326.
17. Kestler, H.W. III, D.J. Ringler, K. Mori, D.L. Panicali, P.K. Sehgal, M.D. Daniel, and R.C. Desrosiers. 1991. Importance of the nef gene for maintenance of high virus loads and for development of AIDS. *Cell.* 65:651.
18. Gray, C. The significance of CD8 in negative selection. 1992. PhD Thesis. University of Basel, Switzerland.
19. Krimpenfort, P., R.D. Jong, Y. Uematsu, Z. Dembic, S. Ryser, H.V. Boehmer, M. Steinmetz, and A. Berns. 1988. Transcription of T cell receptor β-chain genes is controlled by a downstream regulatory element. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:745.
20. Wain-Hobson, S., P. Sonigo, O. Danos, S. Cole, and M. Alizon. 1985. Nucleotide sequence of the AIDS virus, LAV. *Cell.* 40:9.
21. Bluethmann, H., and M. Steinmetz. 1990. Transgenic mice for analysis of T cell development. *In* Immunological Methods. Academic Press, Inc., New York. 311.
22. Laird, P.W., A. Zijderveld, K. Linders, M.A. Rudnicki, R. Jaenisch, and A. Berns. 1991. Simplified mammalian DNA isolation procedure. *Nucleic Acids Res.* 19:4293.
23. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156.
24. Malissen, M., K. Minard, S. Mjolsness, M. Kronenberg, J. Goverman, T. Hunkapiller, M.B. Prystowsky, Y. Yoshikai, F. Fitch, T.W. Mak, and L. Hood. 1984. Mouse T cell antigen receptor: structure and organization of constant and joining gene segments encoding the β polypeptide. *Cell.* 37:1101.
25. Hayakawa, K., and R.R. Hardy. 1991. Murine $CD4^+$ T-cell subsets. *Semin. Immunol.* 123:145.
26. Roost, H.P., S. Claran, R. Gobet, E. Rüedi, H. Hengartner, A. Althage, and R.M. Zinkernagel. 1988. An acquired immune suppression in mice caused by infection with lymphocytic choriomeningitis virus. *Eur. J. Immunol.* 18:511.
27. Zinkernagel, R.M., T. Leist, H. Hengartner, and A. Althage. 1985. Susceptibility to lymphocytic choriomeningitis virus isolates correlates directly with early and high cytotoxic T-cell activity, as well as with footpad swelling reaction, and all three are regulated by H-2D. *J. Exp. Med.* 162:2125.
28. Chattopadhyay, S.K., D.N. Sengupta, T.N. Fredrickson, H.C. Morse III, and J.W. Hartley. 1991. Characteristics and contributions of defective, ecotropic, and mink cell focus–inducing viruses involved in a retrovirus-induced immunodeficiency syndrome of mice. *J. Virol.* 65:4232.
29. Vitetta, E.S., M.T. Berton, C. Burger, M. Kepron, W.T. Lee, and X.-M. Yin. 1991. Memory B and T cells. *Annu. Rev. Immunol.* 9:193.
30. Lehmann-Grube, F., D. Moskophidis, and J. Löhler. 1988.

Recovery from acute virus infection. Role of cytotoxic T lymphocytes in the elimination of lymphocytic choriomeningitis virus from spleens of mice. *Ann. NY Acad. Sci.* 532:238.
31. Garcia, J.V., J. Alfano, and A.D. Miller. 1993. The negative effect of human immunodeficiency virus type I Nef on cell surface CD4 expression is not species specific and requires the cytoplasmic domain of CD4. *J. Virol.* 67:1511.
32. Jaenisch, R. 1988. Transgenic animals. *Science (Wash. DC).* 240:1468.
33. Skowronski, J., D. Parks, and R. Mariani. 1993. Altered T cell activation and development in transgenic mice expressing the HIV-1 nef gene. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:703.
34. Gobet, R., A. Cerny, E. Ruedi, H. Hengartner, and R.M. Zinkernagel. 1988. The role of antibodies in natural and acquired resistance of mice to vesicular stomatitis virus. *Exp. Cell Biol.* 56:175.