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CD4 cell surface downregulation in HIV-1 Nef transgenic mice is a consequence of intracellular sequestration

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The Nef gene product is a regulatory protein of HIV whose biological function is poorly understood. Nef has been thought to have a negative effect on viral replication *in vitro* but has been shown in studies with SIV to be necessary in the establishment of viraemia *in vivo*. *In vitro* studies in various human cell lines have shown that Nef downregulates the expression of cell surface CD4 and thus could have effects on the immune response. We have generated four transgenic mouse lines, with constructs containing two different Nef alleles under the control of CD2 regulatory elements to examine the interaction of Nef with the host immune system *in vivo*. In adult transgenic mice we have found marked downregulation in the level of CD4 on the surface of double positive thymocytes and a decrease in the number of CD4$^+$ T cells in the thymus. Functional analyses have revealed a decrease in the total activation of transgenic thymocytes by anti-CD3ϵ antibody. By specific intracellular staining of T cells in such mice we have found CD4 colocalizing with a Golgi-specific marker. These results strongly suggest a Nef mediated effect on developing CD4 thymocytes resulting from interference of Nef in the intracellular trafficking or post-translational modification of CD4.

Key words: AIDS/CD4/HIV/Nef/transgenic mice

Introduction

HIV infection causes progressive deterioration of the cell mediated immune system characterized by loss of CD4$^+$ T cells leading eventually to AIDS. The mechanism by which CD4$^+$ cells are depleted is not clear. Virus-induced cytolysis (Gallo *et al.*, 1984) or HIV-specific cytotoxic T cell responses (Siciliano *et al.*, 1988) could cause direct killing of these cells. HIV infection of T cell precursors in the thymus or peripheral pools may halt their maturation and/or proliferation (Schnittman *et al.*, 1990; Weiss, 1993). Alternatively, viral gene expression could deplete CD4$^+$ cells, not by direct killing, but by affecting host cell gene products and perturbing host cell functions. To examine whether viral gene products can interfere with host cell functions *in vivo* we have studied the effect of HIV-1 Nef expression on CD4$^+$ cells.

Nef is encoded by an open reading frame overlapping the 3′ HIV LTR (Guy *et al.*, 1987) and displays a high degree of polymorphism between HIV isolates (Ratner *et al.*, 1985). Up to 80% of the early, multiply spliced class of viral transcripts encode Nef (Robert-Guroff *et al.*, 1990), yet its function is unclear. The 27 kDa myristylated protein is expressed at a very high level early in the HIV life cycle (Haseltine, 1991) and is found in the cytoplasm (Franchini *et al.*, 1986). While Nef shows some sequence homology to G proteins (Guy *et al.*, 1987), it seems unlikely that it has either GTP-binding or GTP-hydrolysing properties (Backer *et al.*, 1991). Evidence for (Ahmad and Venkatesan, 1988) and against (Hammes *et al.*, 1989) the Nef protein having a negative regulatory effect on HIV LTR transcription has been reported. It has been shown that Nef is non-essential for viral replication in cultured T cell lines (Hammes *et al.*, 1989; Kim *et al.*, 1989) although it has been observed to accelerate viral replication in primary lymphocytes (de Ronde *et al.*, 1992). Extensive evidence to support the essential role of Nef in immunodeficiency virus replication has been obtained in SIV infected rhesus monkeys. A full length SIV Nef product was shown to be necessary for viral replication and subsequent SIV pathogenesis (Kestler *et al.*, 1991). These data directly demonstrate that there is a strong selective pressure for a functional Nef protein *in vivo*.

While Nef may provide an important function in the life cycle of the virus, it may have an adverse effect on host cell function. *In vitro*, the Nef protein, in the absence of other HIV sequences, has been shown to downregulate the levels of cell surface CD4 on human T cell lines (Guy *et al.*, 1987, 1990; Garcia and Miller, 1991; Garcia *et al.*, 1993). A post-translational mechanism has been postulated since downregulation has been observed, not at the level of mRNA but in surface CD4 protein. CD4 was found to be localized in the cytoplasm (Garcia and Miller, 1991). Nef has also been found to downregulate mouse and simian cell surface CD4 suggesting a common mechanism of action (Garcia *et al.*, 1993). Since Nef has such effects on established mature human CD4$^+$ T cell lines, the relevance of such downregulation should be established *in vivo*. It is of considerable interest to determine the effect of Nef on developing T cells as they acquire the ability to be infected by HIV through surface expression of CD4. If the Nef protein downregulates CD4 *in vivo*, it could have a marked effect on the immune system of an infected individual. Since Nef has been demonstrated to downregulate mouse CD4 *in vitro*, we report here the generation of a Nef transgenic mouse model to examine the consequences of CD4 downregulation in primary cells on the developing immune system. We have made transgenic mice containing two different alleles of the Nef gene under the control of the regulatory elements of the human CD2 gene and its locus control region (LCR) (Greaves *et al.*, 1989). The results from these mice demonstrate statistically significant cell surface CD4 downregulation on double positive (DP) thymocytes and decreases in the number of CD4 single positive (SP) thymocytes. These changes in CD4$^+$ popula-

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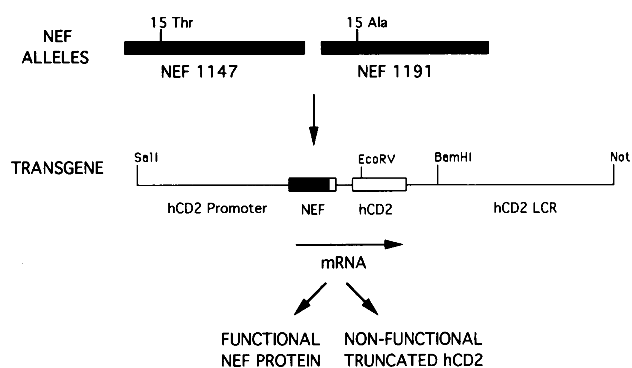

Fig. 1. Nef transgene constructions. The HIV-1 Nef alleles 1147 and 1191 were cloned into a blunted *Eco*RI site upstream of the ATG codon in exon 1 of the human CD2 expression cassette containing the CD2 promoter, shortened coding region and the 3′ LCR. The Nef alleles differ at amino acid 15, threonine in 1147 and alanine in 1191. The unique *Eco*RV site in the coding region of the hCD2 gene was previously removed so as to yield only a truncated non-functional CD2 protein.

tions correlate with a decrease in T cell activation as demonstrated by proliferation assays with anti-CD3ϵ antibody. Such effects appear to be the consequence of intracellular retention of CD4 during thymocyte development as demonstrated by colocalization of CD4 with a Golgi marker but not with a marker for endoplasmic reticulum (ER). Overall, we examine and discuss the effects of the HIV Nef protein on T cell development in transgenic mice and the intracellular fate of CD4.

Results

CD2 Nef transgenic mice express Nef in thymocytes and peripheral T cells

Four transgenic lines of mice were produced with a construct containing the human CD2 promoter and LCR element and a 621 bp Nef fragment (Figure 1). Two different alleles of the HIV-1 Nef gene were used to examine the effects of Nef *in vivo*, 1147 with threonine at amino acid position 15 and 1191 with an alanine at position 15. *In vitro* studies show CD4 downregulation with both alleles (Guy *et al.*, 1990) but only the 1147 allele is phosphorylated at position 15. DNA from the four lines; A (1147), F (1147), B (1191) and D (1191) was analysed by Southern blot analysis and copy numbers were determined to be 6, 25, 48 and 26 respectively (Figure 2A). Slot blot RNA analysis demonstrated expression of the Nef transgene in all four lines (Figure 2B). Expression was tissue specific and observed only in the thymus and spleen (Figure 2B). Quantitation of RNA by probing for glucose-6-phosphate dehydrogenase transcripts and phosphorimaging demonstrated that expression levels were consistent with copy number dependent expression in the four different lines. For A:F:B:D having transgene copy numbers 6:25:48:26 respectively, the ratio of RNA expression levels in thymus was 6:22:30:22. Western blot analysis confirmed expression of Nef protein in the spleen (Figure 2C) and the thymus (not shown) of all four transgenic mouse lines.

Thymocytes and peripheral T cell populations are altered in Nef transgenic mice

To examine whether Nef had any effects on the T cells of the transgenic mice, FACS analysis was performed on cells from the thymus and the peripheral lymphoid organs; spleen

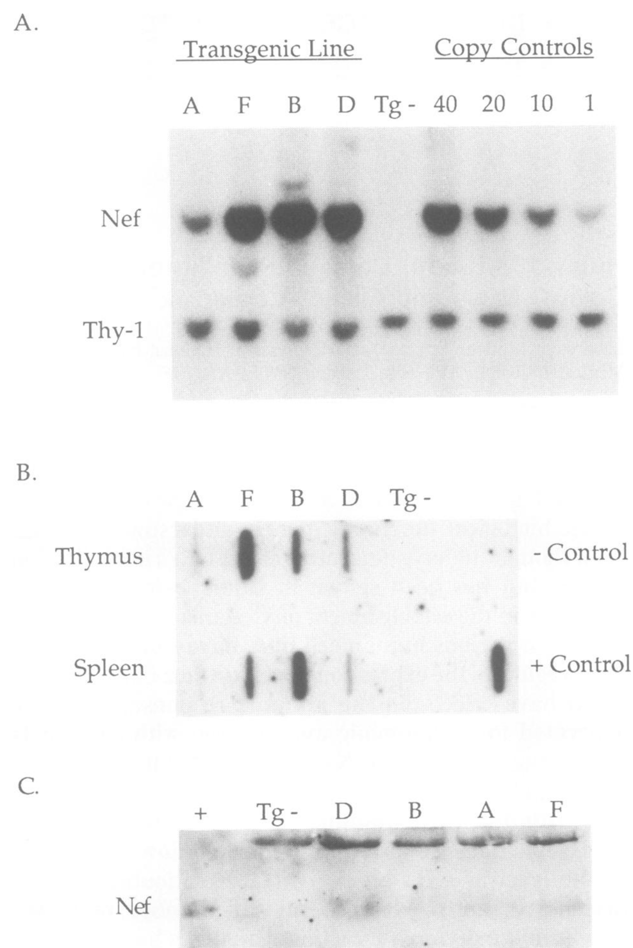

Fig. 2. Molecular characterization of Nef transgenic lines. **(A)** Transgene copy number determined by Southern blot analysis. Genomic DNA was cut with *Hin*dIII, probed with Nef and Thy-1 specific probes and signal compared with plasmid copy controls. Tg− is a non-transgenic littermate control. **(B)** RNA slot blot analysis of spleen and thymus RNA probed with a Nef specific fragment. Tg− refers to RNA from the thymus and spleen of a non-transgenic littermate. The positive control is RNA from the Nef expressing cell line Nef CRIP L (Schwartz *et al.*, 1992). **(C)** Nef specific Western blot of spleen lysates from transgenic lines and non-transgenic littermate control immunoprecipitated with two Nef-specific antisera. The positive control is lysate from the Nef CRIP L cell line. After immunoprecipitation and SDS−PAGE the samples were transferred to nitrocellulose, probed with a monoclonal antibody (mAb) to Nef (AE6) and visualized by ECL and autoradiography.

and lymph nodes. Antibodies specific for CD4 and CD8 were used for analysis of distinct T cell subpopulations. Figure 3 shows representative FACS analysis for transgenic and non-transgenic littermates. In all lines we observed a decrease in the percentage of CD4 single positive (SP) thymocytes and a concomitant increase in the percentage of CD4/CD8 double positive (DP) cells. While the percentages of double negative (DN) and SP CD8 cells remained similar in lines A and D and line B (Figure 3B), the percentages of CD8 SP cells slightly decreased and the DN cells increased in line F (Figure 3A). Similar statistically significant changes were found in all lines (Table I) when comparisons were made of data from 12 litters of mice (three transgenic and three non-transgenic littermates per experiment). Cell counts of the total number of thymocytes in lines A, B and D showed a decrease of 10% on average, but a large decrease (~70%) in thymocyte cell number was

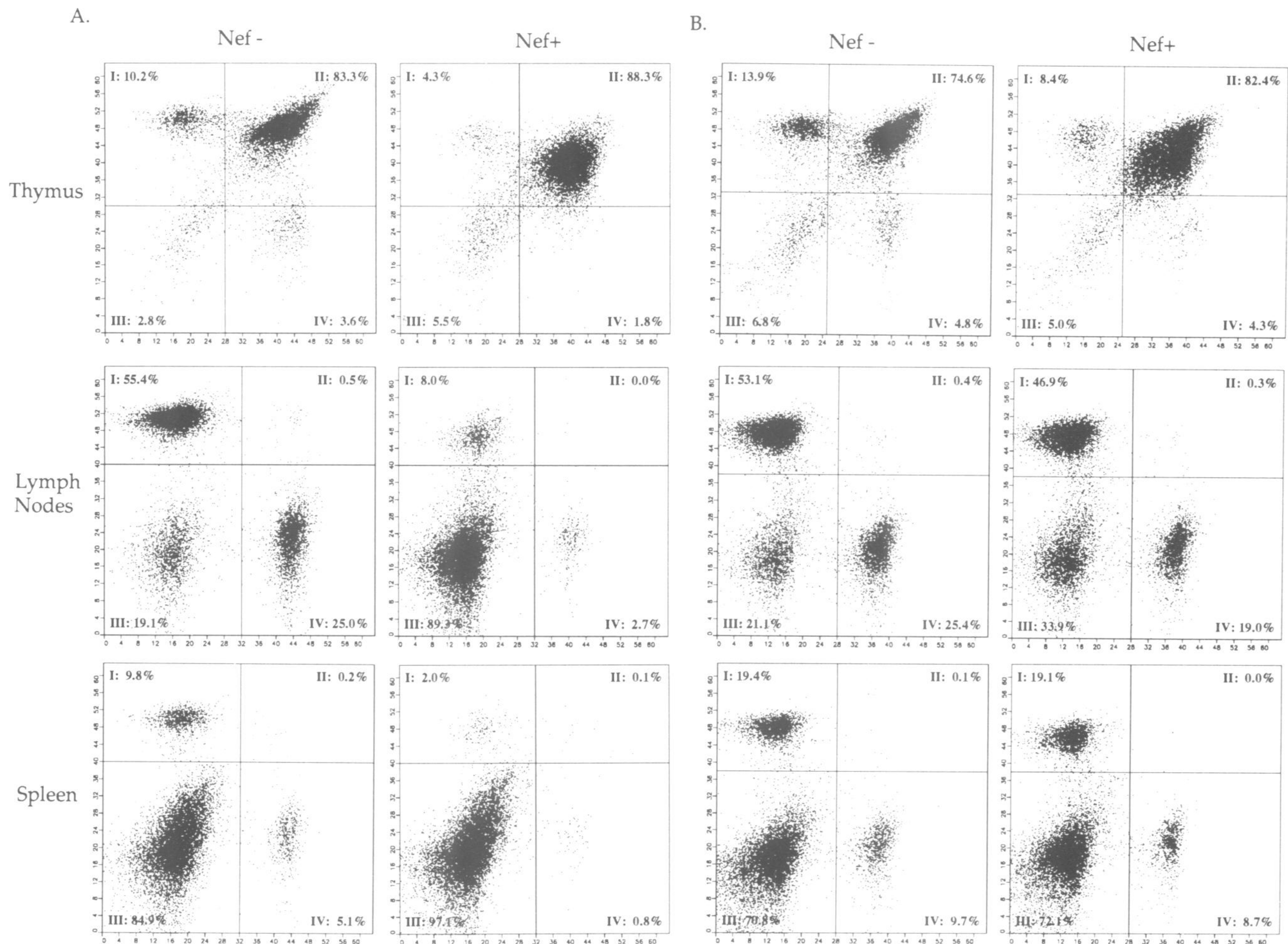

Fig. 3. Distribution of CD4 and CD8 cell subsets in lymphoid tissues of CD2 Nef transgenic mice. Thymocytes, lymph node cells and spleen cells from transgenic, (**A**) line F, 1147 allele and (**B**) line B, 1191 allele, and non-transgenic littermates were stained for CD4 (PE ordinate) and CD8 (FITC abscissa). 10^4 cells were analysed in each sample using the Becton Dickinson FACScan. Relative fluorescence intensities are shown on a logarithmic scale, with percentages of double negative, double positive and CD4/CD8 single positive cells indicated.

Table I. CD4/CD8 subset changes in CD2-Nef transgenic thymocytes

	Age (weeks)	%CD4 SP		%CD4/CD8		%CD8 SP	
		Nef−	Nef+	Nef−	Nef+	Nef−	Nef+
1147-A	2.0	9.1 ± 1.6	7.3 ± 0.2	86.9 ± 1.6	88.0 ± 1.0	1.4 ± 0.2	1.1 ± 0.3
	8.0	14.0 ± 3.1	11.1 ± 1.6	77.9 ± 3.7	81.1 ± 2.4	2.7 ± 0.9	2.6 ± 1.2
	13.5	16.0 ± 3.2	10.2 ± 1.0	76.8 ± 3.7	82.3 ± 1.7	2.8 ± 0.7	2.1 ± 0.4
	14.5	13.4 ± 3.5	8.4 ± 1.1	78.1 ± 7.0	85.3 ± 1.9	1.4 ± 0.4	1.2 ± 0.1
1147-F	5.0	9.2 ± 0.6	2.3 ± 0.6	83.1 ± 1.4	91.7 ± 1.6	2.9 ± 1.0	0.6 ± 0.4
	7.0	12.1 ± 1.4	2.5 ± 0.7	81.3 ± 1.9	90.0 ± 2.1	3.1 ± 0.1	0.9 ± 0.3
	9.0	12.0 ± 1.9	3.3 ± 1.0	81.2 ± 2.1	90.2 ± 1.7	2.6 ± 0.8	0.6 ± 0.3
1191-B	3.0	10.2 ± 1.1	6.7 ± 1.1	83.3 ± 0.7	88.2 ± 1.2	2.6 ± 0.1	2.1 ± 0.6
	11.0	12.3 ± 2.1	9.3 ± 1.4	77.8 ± 3.1	82.2 ± 3.5	2.5 ± 1.0	2.0 ± 0.4
	12.0	13.0 ± 3.4	8.5 ± 2.0	77.3 ± 6.5	83.4 ± 3.3	2.7 ± 1.2	2.3 ± 0.7
	17.5	10.1 ± 1.3	8.0 ± 3.1	80.7 ± 3.5	81.9 ± 6.2	2.3 ± 0.3	3.0 ± 3.2
1191-D	5.0	11.2 ± 2.0	8.3 ± 0.8	79.3 ± 3.1	83.4 ± 0.5	3.0 ± 0.5	2.3 ± 0.4
	6.0	11.8 ± 2.0	6.6 ± 1.1	80.1 ± 4.7	85.8 ± 1.7	2.9 ± 0.6	2.2 ± 0.9
	7.0	15.1 ± 3.6	8.0 ± 1.3	75.1 ± 3.9	84.1 ± 2.0	4.0 ± 0.5	2.5 ± 0.5

Thymocytes from three transgenic and three non-transgenic mice from various aged litters were stained with anti-CD4 and anti-CD8 antibodies. 10^4 stained cells per thymus were analysed on a Becton Dickinson FACScan. Double negative, double positive, CD4 single positive and CD8 single positive populations were gated and displayed as a percentage of total thymocytes. The mean value and standard deviation for non-transgenic and transgenic mice are shown.

observed in line F (Table II). The greatest reduction in absolute numbers for line F was observed in the CD4 SP subset and resulted in 15-fold fewer cells. The total number of CD8 SP cells was decreased 12-fold while the numbers of DN cells were not significantly changed. In the D transgenic line although the reduction in total number of

Table II. Absolute number of thymocytes $\times 10^7$/subset in Nef transgenic mice

Line	Total	DN	DP	CD4	CD8
F Tg−	22.0 ± 2.8	0.74 ± 0.11	18.1 ± 2.5	2.54 ± 0.17	0.67 ± 0.07
F Tg+	6.2 ± 2.9	0.37 ± 0.23	5.6 ± 2.7	0.17 ± 0.11	0.05 ± 0.02
D Tg−	21.8 ± 2.3	1.42 ± 0.18	17.3 ± 2.1	2.44 ± 0.34	0.65 ± 0.13
D Tg+	18.4 ± 3.4	1.10 ± 0.29	15.4 ± 2.8	1.51 ± 0.24	0.42 ± 0.12

Representative litters from line F (1147) and line D (1191) are shown in Table II. Thymocytes from three transgenic and three non-transgenic littermates were accurately counted. The thymocytes were then stained with anti-CD4 and anti-CD8, analysed by FACS, populations gated and the percentage of each cell type displayed. Absolute numbers of each of the four thymic subsets (DN, DP, CD4 SP and CD8 SP) were calculated for each individual mouse and averaged per litter. The standard deviation of each mean is shown. Statistical analysis by two-paired *t*-test reveal significant differences between numbers of non-transgenic and transgenic CD4 SP cells for line F ($P < 0.001$) and line D ($P < 0.02$). The differences between the means from the other populations are not significant at the 5% level except for the DP and CD8 SP cells of line F.

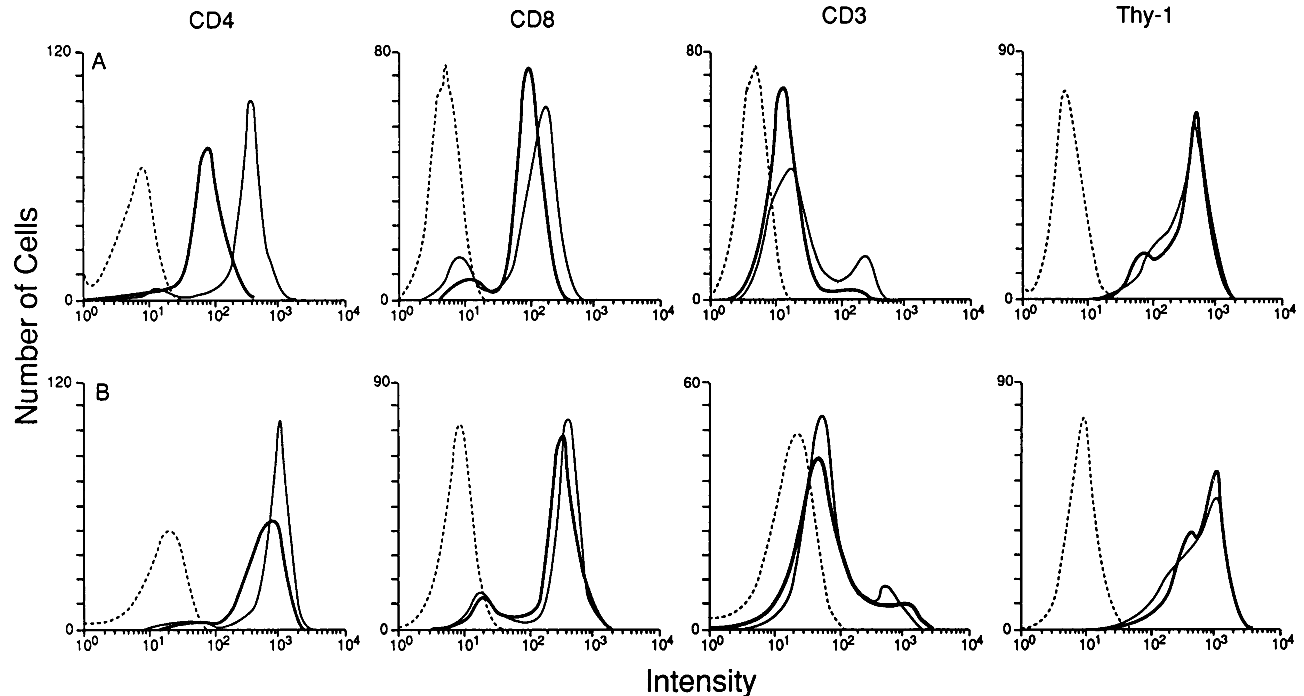

Fig. 4. Levels of expression of CD4 and other T cell surface markers on Nef transgenic thymocytes. Thymus cells from non-transgenic and transgenic littermates from **(A)** line F, 1147 allele and **(B)** line B, 1191 allele were stained with anti-CD4, CD8, CD3 and Thy-1 antibodies. Histogram plots show number of cells (ordinate) and intensity of fluorescence (logarithmic scale, abscissa) for non-transgenic cells (solid line), transgenic cells (thick line) and no stain controls (dotted line).

thymocytes was less than that in line F, again a significant decrease was observed in the CD4 SP subset.

When peripheral T cell populations were examined, variable decreases in CD4 SP cells in the lymph nodes and spleen were seen for lines A, B and D. However, line F exhibited large decreases in the percentage of CD4 SP cells in the lymph nodes. Normally non-transgenic lymph nodes contained 51.1% (± 3.4) CD4 SP while only 14.7% (± 6.7) were found in Nef transgenic lymph nodes, representing a 3.5-fold decrease. A decrease from 23.5% (± 1.9) to 8.5% (± 3.9) was observed in the CD8 SP subset. In addition, the total number of cells in the spleen was unchanged in the A, B and D lines but decreased in line F by a factor of 1.5 in the CD4 SP compartment. Thus, Nef gene expression consistently results in a decrease of the absolute number of CD4 SP cells in the thymus, and in one line this effect extends to the peripheral lymphoid organs.

Nef expression results in a downregulation of CD4 on the surface of developing T cells

Since others have found downregulation of CD4 on T cell lines transfected with the 1147 and 1191 alleles of the Nef gene, we investigated whether T cells in Nef transgenic mice also downregulate CD4 *in vivo*. FACS histogram analysis revealed that surface levels of CD4 were decreased in thymocytes from Nef transgenic mice (Figure 4). The downregulation was observed in all four lines (Table III), for both Nef alleles. As controls, antibodies specific for CD8, CD3 and Thy-1 were used to examine the specificity of the downregulation effects. With both Nef transgenic alleles, CD8 levels were found to be slightly decreased and the normally high CD3 expressing population of thymocytes was found to be greatly reduced in line F and less so in the other three lines. This loss of CD3 high cells correlates well with the loss of CD4 SP cells in the thymus. Thy-1 levels did not change, thus demonstrating the specific effects of Nef.

To determine which subset of cells was downregulated for CD4 levels, we performed histogram analysis of gated CD4 SP and DP thymocyte populations. In 12 litters of mice, consistent downregulation ranging from 17 to 72% of normal levels of surface CD4 was observed on all DP populations. Thymocytes from the F line showed the greatest levels of downregulation. Some downregulation (but not to the extent in the DP population) was also seen on SP cells ranging up

Table III. Cell surface marker downregulation in CD2-Nef transgenic thymocytes

	T cell subset Age (weeks)	% Change in CD4 levels		% Change in CD8 levels	
		SP	DP	SP	DP
1147-A	2.0	−25.4	−41.6	−0.6	−21.7
	8.0	+2.7	−16.9	+4.4	−7.9
	13.5	−21.8	−39.7	−11.7	−16.2
	14.5	−21.2	−44.2	−8.4	−20.9
1147-F	5.0	−46.0	−72.1	−22.0	−28.3
	7.0	−38.2	−73.4	−16.8	−31.5
	9.0	−30.9	−68.9	−28.7	−24.6
1191-B	3.0	N/C	−33.8	−8.0	+11.3
	11.0	−1.4	−29.4	−10.0	−27.6
	12.0	−24.8	−42.8	+5.7	−20.8
	17.5	−27.6	−47.2	−17.0	−25.6
1191-D	5.0	N/C	−36.0	+9.0	−16.0
	6.0	+5.0	−36.0	+9.4	−7.0
	7.0	−16.9	−40.6	+3.4	−18.6

Thymocytes from three transgenic and three non-transgenic mice from various aged litters were stained with anti-CD4 and anti-CD8 antibodies. 10^4 stained cells per thymus were analysed on a Becton Dickinson FACScan. DN, DP, CD4 SP and CD8 SP populations were gated and then displayed as a single parameter (CD4 or CD8) histogram with cell number (ordinate) against log of fluorescence intensity (abscissa). The mean fluorescence value for each population was obtained. These values were averaged for transgenic and non-transgenic littermates and the difference between the two is shown as a percentage of the average fluorescence for the non-transgenic mice. N/C, no change.

to a 28% decrease from normal levels. When lymph node T cells were examined, some but not consistent downregulation of CD4 was observed only in line F (data not shown), suggesting that CD4 low expressing cells rarely leave the thymus for the periphery.

T cell activation is decreased in Nef transgenic mice

Mitogen induced or anti-CD3ε mediated activation assays were performed to examine whether downregulation of CD4 or loss of $CD4^+$ cells have negative effects on thymocyte or peripheral T cell activation. Proliferation of thymocytes as measured by [^{3}H]thymidine incorporation after activation via calcium ionophore (ionomycin) and phorbol ester (PMA) revealed small differences between Nef transgenic and non-transgenic cells of both alleles (not shown) demonstrating that the total response of transgenic thymocytes to mitogen is not impaired. However, when cells were activated via the T cell receptor−CD3 complex with anti-CD3ε antibodies and PMA, a measurable difference between transgenic and non-transgenic thymocytes was observed (Figure 5). This decrease in activation was seen in transgenic lines carrying both the Nef 1147 allele and the 1191 allele. In these experiments the shift of the titration curves to the right clearly indicates that more transgenic thymocytes are required to achieve the equivalent activation levels observed in non-transgenic thymocytes. These changes correlate well with the quantitative loss of SP CD4 cells in the transgenic thymuses (Table I). In addition, decreases in CD3ε mediated activation were observed in proliferation assays performed on peripheral T cells from line F (data not shown) and correspond to the decrease in the number of CD4 SP cells in the lymph nodes.

Changes in T cell subsets are a consequence of intracellular sequestration of CD4

In vitro, Nef has been shown to have no effect on the steady-state levels of CD4 mRNA or CD4 protein and the surface downregulation was found to be a consequence of intracellular localization of CD4 (Garcia and Miller, 1991). Thus, we examined thymocytes from Nef transgenic mice for the presence of intracellular CD4. Indirect immunofluorescence was performed on permeabilized thymocytes with anti-CD4 antibody. As shown in Figure 6A, non-transgenic permeabilized cells have normal cell surface expression of CD4 whereas transgenic thymocytes (Figure 6B) express only low levels. Instead, CD4 was observed as a singular brightly staining area within the cytoplasm of Nef transgenic thymocytes. To determine whether CD4 was localized to any particular subcellular compartment, we stained thymocytes with antibodies specific for the endoplasmic reticulum (ER) and the α-mannosidase II protein of the Golgi apparatus (Moremen *et al.*, 1991). Double staining with CD4 and compartment specific antibodies revealed that CD4 was sequestered within the specific region stained by the anti-Golgi (Figure 7A and B) but not the anti-ER antibodies (Figure 7C and D). While CD4 colocalizes with the Golgi marker, due to the coalescence of these organelles in the perinuclear region we cannot rule out an endosomal localization for CD4. Nef expression was found throughout the cytoplasm of transgenic thymocytes (data not shown) as previously described for HIV infected cells (Franchini *et al.*, 1986; Ovod *et al.*, 1992). Since the cytoplasmic domain of CD4 is known to interact directly with the tyrosine kinase $p56^{lck}$ (Shaw *et al.*, 1989; Veillette *et al.*, 1988), double staining with CD4 and $p56^{lck}$ specific antibodies was performed to determine whether the Nef mediated downregulation of CD4 also affected the cellular location of $p56^{lck}$. Although the intensity of membrane associated staining for $p56^{lck}$ does not change between transgenic and non-transgenic thymocytes, intracellularly $p56^{lck}$ appears to colocalize with CD4 in CD4 downregulated cells (Figure 7E and F).

Discussion

We have demonstrated that HIV-1 Nef has an effect on the CD4 subsets of thymocytes which extends to the peripheral T cells in transgenic mice. Due to the CD2 gene regulatory

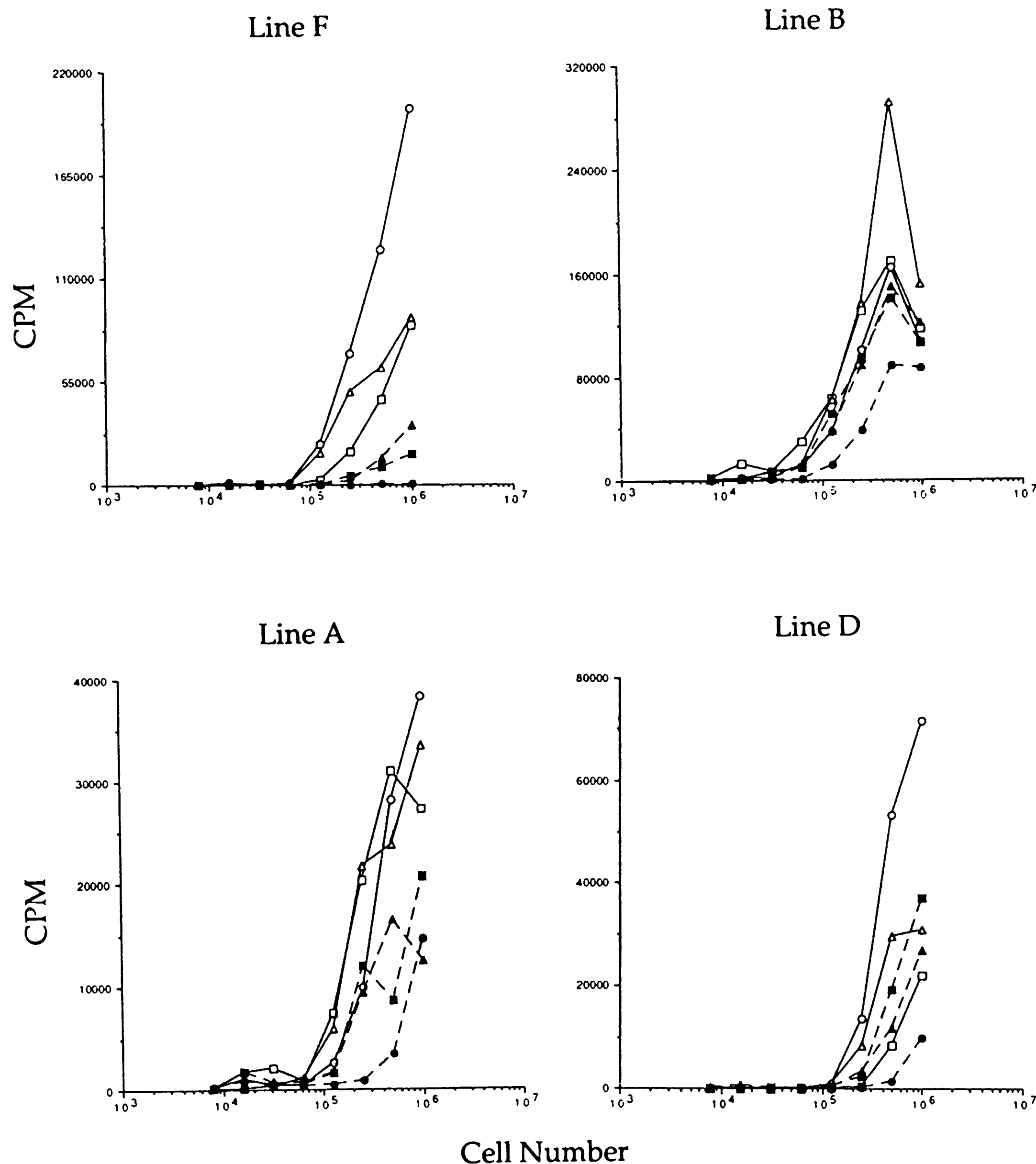

Fig. 5. T cell activation assays. Varying numbers of thymocytes from non-transgenic (open symbols) and transgenic (solid symbols) littermates of Nef lines F, B, A and D were stimulated with anti-CD3ε antibody and PMA and proliferation was measured via [^{3}H]thymidine incorporation. PMA alone produced no activation (not shown). Activation is plotted as [^{3}H] c.p.m. incorporated versus cell number.

elements, Nef begins expression in the transgenic mice very early in T cell differentiation while the cells are in the CD4/CD8 DN stage in the thymus and continues in the DP and SP thymocytes as well as in the peripheral T cells (Kamoun *et al.*, 1981; Lang *et al.*, 1988; Owen *et al.*, 1988). We observed a dramatic decrease in the levels of cell surface CD4 on the DP subset of thymocytes and a significant reduction in the number of CD4 SP thymocytes in all four mouse lines. Additionally, we observed a reduction in the peripheral $CD4^+$ T cells of the F transgenic line but no significant loss of $CD4^+$ cells occurred in the periphery of the other three transgenic lines. The variation seen between line F (25 copies of 1147) and line A (six copies of 1147) is most likely due to threshold effects of Nef expression. Others have reported that high levels of Nef expression are required for *in vitro* downregulation of CD4 (Schwartz *et al.*, 1993). Allelic differences are most likely responsible for the variation between line F (25 copies of 1147) and line D (26 copies of 1191) although more transgenic lines are being produced to verify the more potent effects of Nef 1147.

The decrease in CD4 SP cell number could be due to a Nef specific cytopathic effect, as has been indicated previously (Luria *et al.*, 1991; Skowronski *et al.*, 1993). However, we have found Nef RNA and protein in both the thymocytes and splenocytes of the transgenic mice demonstrating that Nef is not directly toxic to T cells (SP or DP). Alternatively, Nef mediated depletion of CD4 SP cells may be a consequence of aberrant positive selection in the thymus. The effect of Nef is most likely to occur early in the T cell differentiation pathway at the DP stage, initiated by the downregulation of CD4. In normal positive selection, DP cells interact with MHC class I and II molecules presented by the cortical thymic epithelium and receive a signal to expand (Berg *et al.*, 1989). Mice deficient in MHC class II (Cosgrove *et al.*, 1991) and mice treated with anti-class

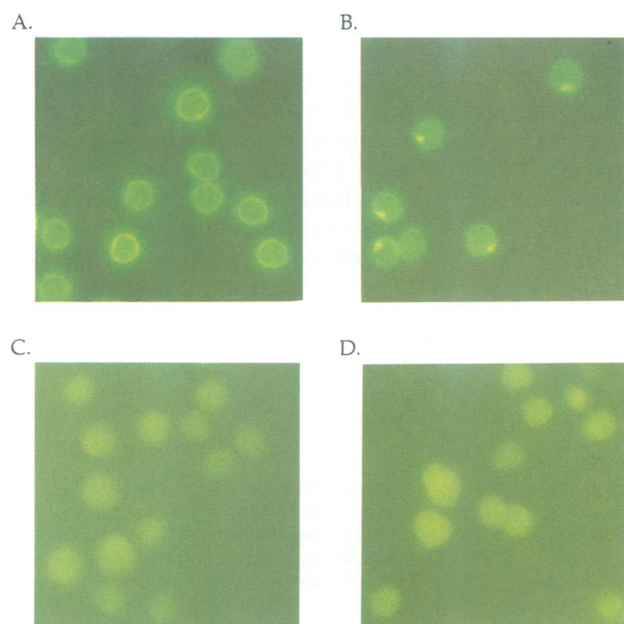

Fig. 6. Indirect immunofluorescent staining of permeabilized thymocytes. (**A**) Non-transgenic and (**B**) transgenic thymocytes from line F littermates were stained with (i) an anti-CD4 rat antibody (45 min room temp.) followed by (ii) an anti-rat FITC-conjugated antibody (45 min room temp.). Cells were visualized using a Zeiss Axiophot fluorescence microscope and 15 s exposures were taken. (**C**) Control thymocytes from a non-transgenic littermate were carried through the staining procedure without addition of either antibody and (**D**) control thymocytes from a transgenic littermate were stained only with the second layer antibody (FITC anti-rat Ig). Exposures were 30 s.

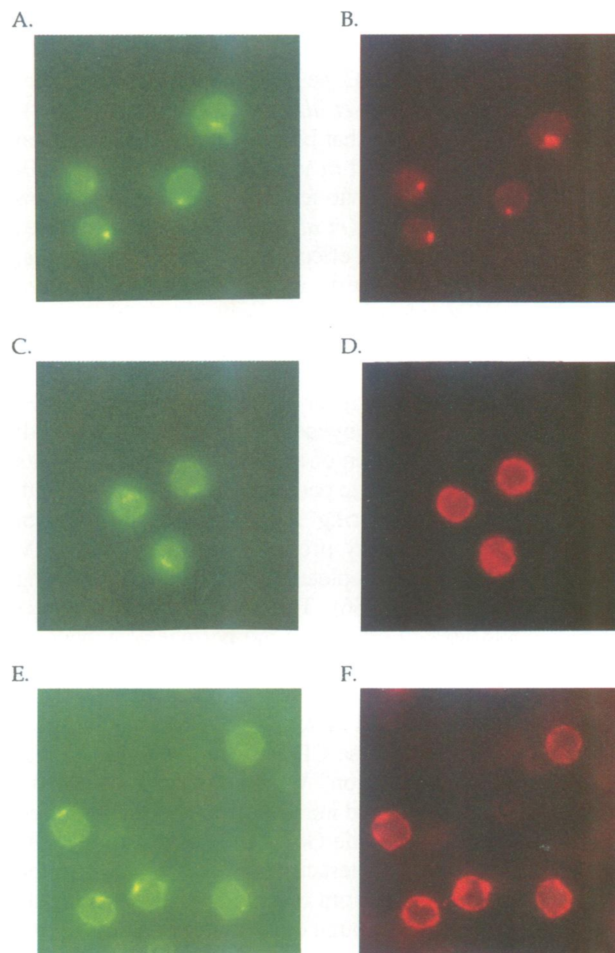

Fig. 7. Subcellular compartmentalization of CD4 and p56^{lck} in Nef transgenic thymocytes. Permeabilized thymocytes from line F Nef transgenic mice were double stained firstly with an anti-CD4 rat mAb detected via a FITC-conjugated anti-rat Ig antibody (**A**), (**C**) and (**E**) followed by either (**B**) rabbit anti-Golgi (anti-α-mannosidase II) antibody or (**D**) an anti-ER rabbit antibody (anti-ERC 55) or (**F**) an anti-p56^{lck} rabbit antibody. The rabbit antibodies were detected via Texas Red-conjugated anti-rabbit Ig antibody. Cells were visualized using a Zeiss Axiophot fluorescence microscope and photographed.

II antibodies (Kruisbeek *et al.*, 1983) show defective development of their CD4$^+$ T cells and clearly demonstrate that CD4 must interact with class II for expansion to occur. Additionally, in mice hemizygous for CD4 which have reduced levels of CD4 on the surface of DP thymocytes (Rahemtulla *et al.*, 1991), decreased numbers of CD4 SP cells have been found in the thymus. These studies along with our results strongly support a Nef mediated effect on positive selection in the thymus through downregulation of cell surface CD4. This could result in an alteration in the interaction of transgenic thymocytes with MHC class II molecules and lead to the maturation of fewer SP CD4 thymocytes.

We have also observed the downregulation of CD8 on thymocytes of all Nef transgenic lines as well as a decrease in CD8 SP thymocyte cell number in line F. The degree of these changes is much less than that found for CD4 and is in agreement with previous findings (Skowronski *et al.*, 1993). The concomitant downregulation of both mouse CD4 and CD8 has been observed previously in Nef transduced cell lines (Garcia *et al.*, 1993). This is in contrast to the human forms, of which only CD4 is downregulated. The human CD4 cytoplasmic domain has been shown to be required for the downregulation (Garcia *et al.*, 1993) and is highly conserved between human and mouse (79%) in amino acid sequence (Littman, 1987). However, the human and mouse CD8 cytoplasmic domains are less conserved (55%) and may be responsible for differences in downregulation between the species.

It has been shown that thymocyte activation assays with anti-CD3ϵ antibody result in proliferation of only SP cells (Havran *et al.*, 1987; Weiss *et al.*, 1987). Differences in thymocyte activation could be due to quantitative changes in the total number of SP cells or the level to which individual SP cells can be activated. Our results concerning the effects of Nef on the *in vivo* immune system indicate that equal numbers of transgenic thymocytes are stimulated to a lesser degree than those from non-transgenic littermates. This is most likely due to the quantitative loss of the CD4 SP subset (which are CD3 high expressing cells). When the levels of activation in the transgenic thymocytes are corrected for the depleted SP cells, they become comparable with those from non-transgenic littermates. Further analysis is required to determine if the activation level of individual thymocytes is also perturbed. These data are in direct contrast to those of others (Skowronski *et al.*, 1993) where, despite large losses in CD4 SP cells, Nef transgenic thymocytes are hyper-activated through anti-CD3 stimulation. The Nef transgene in these mice is controlled via murine CD3 gene regulatory elements which direct expression of Nef much later in T cell ontogeny than CD2 elements (Yagita *et al.*, 1989). Thus developmental expression differences may account for the opposing data. Alternatively, position effects on the transgene

could play a role in variable Nef expression on T cells since, unlike human CD2 (Greaves *et al.*, 1989), no elements have been identified in the CD3 gene to confer position independent expression (Lacy *et al.*, 1983; Lee *et al.*, 1992).

At present, it is known that both DP and SP human fetal thymocytes can be infected *in vitro* (de Rossi *et al.*, 1990; Hays *et al.*, 1992) and that fetal thymus tissue *in utero* harbours HIV (Courgnaud *et al.*, 1991). Since our studies demonstrate that Nef has an effect on DP and SP thymocytes, Nef transgenic mice may serve as useful models of *in utero* or paediatric HIV infection. The thymus probably acts as a site of T cell differentiation and maturation throughout life (Steinmann, 1986) and thus dysfunction of thymopoiesis may be a pathogenic mechanism for HIV as recently suggested in the SCID-hu model (Bonyhadi *et al.*, 1993; Aldrovandi *et al.*, 1993). HIV infection of adult thymus/liver implants affects DP and SP thymocyte percentages and numbers, with the DP population harbouring >90% of the virus. Taken together, these studies may provide clues as to why HIV infected patients become depleted for CD4 cells over a long period of time (Fauci, 1986). The ability of HIV to infect DP cells could lead to effects on the peripheral T cells as we observe in some of the Nef transgenic mice. We are presently examining the response to antigen of CD4 cells in the peripheral lymphoid system of Nef transgenic mice.

Given that Nef does cause CD4 downregulation, what is the mechanism of this action? We have shown that downregulated CD4 is sequestered intracellularly and is colocalized with a marker specific to the Golgi and not one to the ER. Thus, Nef may directly interact with CD4, block it in the cytoplasm and prevent it from reaching the cell membrane or it may act indirectly through other proteins necessary for mediating cell surface appearance of CD4. It is known that HIV gp160 env glycoprotein precursor also causes CD4 downregulation (Stevenson *et al.*, 1988; Crise *et al.*, 1990) and that HIV-1 Vpu induces the rapid degradation of CD4 in the ER of HIV infected cells (Willey *et al.*, 1992). We show here that in the absence of gp160, CD4 is downregulated specifically by Nef and that CD4 colocalizes to the perinuclear region. Further to this, the interaction of CD4 with cytoplasmic tyrosine protein kinase p56^{lck} (Veillette *et al.*, 1988; Shaw *et al.*, 1989) has been examined. It has been shown that p56^{lck} and gp160–CD4 form a ternary complex in the ER (Crise and Rose, 1992). Indirect immunofluorescence of p56^{lck} in the thymocytes from our Nef transgenic mice shows no decrease in p56^{lck} membrane expression but some intracellular colocalization with CD4. It will be necessary to determine whether there is a direct association between Nef, CD4 and p56^{lck}. Considering the differences between Nef and gp160 mediated CD4 downregulation and data showing that Nef is expressed at high levels (Franchini *et al.*, 1986) very early in the HIV life cycle from a Rev independent RNA (Haseltine, 1991), Nef most likely acts to modify CD4 localization before the production of the env precursor gp160. Thus, Nef, gp160 and Vpu could perform different functions at different stages in a viral mechanism to downregulate its CD4 receptor.

Why would the virus need such an elaborate mechanism(s) to downregulate the CD4 receptor? By removing the cell surface receptor, virus producing cells can be protected from superinfection. This phenomenon is known as interference and has been well characterized for retroviruses (Weller *et al.*, 1980; Stevenson *et al.*, 1988; Crise *et al.*, 1990; Heard and Danos, 1991). Other viruses have enzymes to degrade their receptor e.g. influenza virus (Muchmore and Varki, 1987) and coronavirus (Vlasak *et al.*, 1988). Furthermore, it has been demonstrated that high levels of CD4 block HIV virion formation and that downregulation is important for efficient production of infectious virus (Marshall *et al.*, 1992). Thus, our transgenic mice could serve as a useful model to elucidate further the mechanism of Nef mediated CD4 downregulation, to study effects of Nef on the host developing immune system and for the testing of Nef inhibitors that may have a therapeutic effect against HIV replication *in vivo*.

Materials and methods

DNA constructs

The 800 bp *Bam*HI–*Sma*I fragment from either pTG1147 or pTG1191 (gift from Dr B.Guy) was blunted and ligated into a unique blunted *Eco*RI site in the first exon of the p2629 CD2 expression plasmid (gift from D.Kioussis) to give either p2629N47 or p2629N91. A 4.5 kb *Bam*HI–*Not*I fragment containing the 3′ CD2 LCR from p2694 (gift from D.Kioussis) was then ligated into the unique *Bam*HI–*Not*I sites in p2629N47 or p2629N91, resulting in either pCD2Nef1147 or pCD2Nef1191. The 12 kb *Sal*I–*Not*I fragment from these plasmids was prepared for microinjection into (CBA × C57Bl/10) fertilized mouse oocytes as previously described (Grosveld *et al.*, 1987). Positive founder animals were bred with (CBA × C57Bl/10) mice and lines were maintained as heterozygotes.

DNA and expression analysis

Tail DNA (10 μg) from founder animals was analysed by Southern blot analysis after digestion with *Hin*dIII or *Asp*718. DNA was run on a 1% agarose/Tris, acetate, EDTA gel, blotted onto nitrocellulose and probed with a randomly primed 800 bp *Bam*HI–*Sma*I Nef fragment from pTG1147. A 1.2 kb Thy-1.2 fragment was used as a loading control probe. Appropriate amounts of pCD2Nef1147 were added to 10 μg genomic DNA and used as copy number controls. Quantitation was performed on the Molecular Dynamics PhosphorImager.

RNA was prepared using the lithium chloride/urea method (Fraser *et al.*, 1990). For Northern blot analysis (Sambrook *et al.*, 1989) 10 μg of RNA was run on a 1% formaldehyde gel, blotted onto nitrocellulose and probed with a 800 bp *Bam*HI–*Sma*I Nef fragment from pTG1147. For RNA slot blots (Sambrook *et al.*, 1989) 5 μg of RNA was blotted onto nitrocellulose and probed as above. RNA from the Nef producing CRIP L producer cell line (Schwartz *et al.*, 1992) was used as a positive control.

FACS analysis

FACS analysis was used to detect cell surface markers on lymphocytes from transgenic mice. The antibodies used were: a PE-conjugated rat monoclonal antibody (mAb) against murine CD4; a FITC-conjugated rat mAb against murine CD8 [anti-leu-3a (SK) and anti-leu-2a (SK1) from Becton Dickinson, San Jose, CA]; a FITC-conjugated hamster mAb against murine CD3ε (145-2C11, Pharmingen, San Diego, CA) and a FITC-conjugated rat mAb against murine Thy-1.2 (TS from Sigma Chemical Co., St Louis, MO). The thymus, spleen and lymph nodes were removed and homogenized to single cell suspensions in FACS medium (αMEM, 5% FCS, 10 μg/ml Na azide) on ice. Accurate cell counts were obtained and 10^6 cells were washed in 5 ml FACS medium, pelleted and supernatant removed. Antibodies were added at a dilution of 1:200 in FACS medium and incubated for 30 min on ice. Cells were washed once with 5 ml of cold FACS medium, once with 5 ml of cold PBS, fixed in 1% formaldehyde/PBS and filtered through fine gauze. Stained cells were analysed with a Becton Dickinson FACScan cell sorter and the LYSIS II software package.

Immunoprecipitation and Western blot analysis

Single cell suspensions from thymus and spleen of transgenic and non-transgenic mice were prepared. Erythrocytes were removed by lysis in Tris-buffered ammonium chloride. Extracts were prepared by lysing cells in 1 ml of 0.5% Triton X-100, 10 mM Tris pH 7.5, 1 mM EDTA, 0.15 mM NaCl, 10 mg/ml bovine serum albumin, 200 μM PMSF, 5 mM iodoacetamide and 5 μM leupeptin on ice for 15 min. The extracts were cleared by centrifugation (14 000 r.p.m.) for 5 min at 4°C and incubated with 50 μl of normal rabbit serum for 1 h at 4°C and for 30 min with 100 μl of a 10% suspension of protein A–Sepharose in lysis buffer followed by 15 min

centrifugation at 4°C. Anti-Nef antibodies [HIV-1 HXB3 Nef antisera, to the N and C termini (Hammes *et al.*, 1989)] were then added at a 1:250 dilution to each cell lysate and incubated overnight on ice. Following this the extracts were incubated again with 100 μl of 10% suspension of protein A–Sepharose beads for 1 h. The beads were collected by centrifugation for 15 min at 4°C and washed three times in lysis buffer. Pellets were resuspended in reducing sample buffer, heated at 100°C for 5 min and the supernatants recovered. The supernatants were resolved on 15% SDS–polyacrylamide gels and transferred to nitrocellulose filters by electroblotting. Filters were blocked in 5% low fat dried milk dissolved in phosphate buffered saline with 0.1% Tween-20 (PBS-T) at 4°C overnight. After extensive washing in PBS-T the filters were incubated for 1 h at room temperature with a mAb to Nef (AE6) diluted at 1:1000 in PBS. After further washing in PBS-T the filters were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Amersham) at 1:5000 dilution, washed extensively in PBS-T and the immune complexes visualized by the ECL detection system (Amersham) and autoradiography.

T cell proliferation assays

Thymocytes and erythrocyte depleted splenocytes were cultured in 200 μl of αMEM, 10% FCS, 2 mM glutamine, 10 U/ml penicillin, 100 μg/ml streptomycin and 50 μM β-mercaptoethanol. Cells were cultured in microtitre wells from a density of 8×10^3 to 1×10^6 per well for thymocytes and 4×10^3 to 5×10^5 per well for splenocytes. Thymocytes were stimulated with anti-CD3ε (145-2C11) mAb (0.36 μg/well) and 5 ng/ml PMA (Sigma) or with 5 ng/ml PMA and 500 ng/ml ionomycin (Sigma) or 5 ng/ml PMA alone as a control. Splenocytes were stimulated as above except no PMA was added with anti-CD3ε antibodies. Controls contained no PMA. 48 h after stimulation cells were labelled for 16 h with 1 μCi/well of [^{3}H]thymidine (Amersham) before harvest. The incorporated radioactivity was precipitated on glass fibre filter paper and subsequently counted by liquid scintillation.

Immunofluorescence

Indirect immunofluorescence was used to detect the intracellular and surface distribution of CD4 on lymphocytes from transgenic mice. The antibodies used were a rat mAb against murine CD4 (RM-4-5 from Pharmingen, San Diego, CA) detected via a FITC-conjugated goat anti-rat IgG (Calbiochem, La Jolla, CA); a Golgi-specific rabbit anti-α mannosidase II antibody (gift from Dr K.Moreman, Univ. Georgia); an endoplasmic reticulum-specific rabbit antibody, anti-ERC55 (gift from K.Weis and A.Lamond, EMBL, Heidelberg) and an anti-p56^{lck} [RNGS] rabbit antiserum (gift from M.Marsh, University College, London), all three detected via a Texas Red-conjugated goat anti-rabbit IgG (Calbiochem, La Jolla, CA).

Whole thymus and lymph nodes were homogenized to a single cell suspension in PBS and filtered through fine gauze. Approximately 3×10^5 cells were spread on 10 mm poly-lysine coated coverslips and were allowed to attach for 2–3 min. Cells were fixed with 3.7% paraformaldehyde in CSK (100 mM NaCl, 300 mM sucrose, 10 mM PIPES pH 6.8, 3 mM MgCl, 1 mM EGTA pH 6.8) for 10 min with periodic swirling, followed by three 5 min washes with PBS. Cells were permeabilized with 0.5% Triton-X in CSK for 15 min, followed by three 5 min washes with PBS.

To stain the cells the coverslips were first overlaid with 5 μl of block solution (0.8% BSA, 0.1% gelatin in PBS) for 15 min. Diluted antibodies in the same block solution were then applied (10 μl) in the following sequence; (i) anti-CD4 (1:200); (ii) FITC anti-rat IgG (1:100); then for double staining either (iii) anti-Golgi marker (1:1000), or anti-ER marker (1:20), or anti-p56^{lck} (1:50); (iv) Texas Red anti-rabbit IgG (1:100). The incubation period for each antibody was 30 min at room temperature in a humidified chamber with three 5 min washes with 0.05% Tween-20/PBS between each application. Coverslips were mounted with a drop of Univert (BDH, Poole, UK) containing 100 mg/ml of DABCO (Sigma Chemical Co., St Louis, MO) as anti-fading agent, and cells were viewed under a Zeiss Axiophot fluorescence microscope.

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