Immunity, Vol. 3, 273–282, September, 1995, Copyright © 1995 by Cell Press

A Role for the Orphan Steroid Receptor Nur77 in Apoptosis Accompanying Antigen-Induced Negative Selection

Barbara J. Calnan, Shannan Szychowski, Francis Ka-Ming Chan, Dragana Cado, and Astar Winoto
Department of Molecular and Cell Biology
Division of Immunology and Cancer Research Laboratory
469 Life Science Addition
University of California, Berkeley
Berkeley, California 94720–3200

Summary

The transcription factor Nur77, an orphan member of the nuclear hormone receptor superfamily, is highly expressed during T cell receptor–signaled apoptosis, suggesting a possible role for Nur77 in negative selection. We examined this by generating two sets of transgenic mice. In one set of mice, a dominant-negative Nur77 mutant is constitutively expressed and the other in which wild-type Nur77 protein is constitutively expressed in developing thymocytes. We report that inhibition of endogenous Nur77 by the dominant-negative mutant perturbed T cell development and inhibited antigen-induced negative selection in F5 T cell receptor transgenic mice. Constitutive expression of wild-type Nur77 protein induced apoptosis in developing thymocytes, resulting in a decreased number of thymocytes and mature T cells. Together, these data support a role for Nur77 in the downstream signaling events in antigen-induced negative selection.

Introduction

Helper and cytotoxic T cells express T cell antigen receptor (TCR) composed of an α and β chain and recognize peptides of foreign antigen bound to molecules encoded in the major histocompatibility complex (MHC). The ability of these cells to discriminate between self-antigens and foreign antigens in the context of self-MHC results from two selection processes that occur during T cell development. The process of positive selection promotes maturation of thymocytes expressing TCR recognizing self-MHC, whereas negative selection results in apoptosis of self-reactive cells (for review, see Nossal, 1994; Robey and Fowlkes, 1994; von Boehmer, 1994, and references therein).

Positive and negative selection of thymocytes occurs at an intermediate stage in T cell development, after rearrangement of TCR α and β chain genes and transition of thymocytes from an early $TCR^-CD4^-CD8^-$ double-negative stage to a stage in development in which thymocytes express low levels of TCR and both CD4 and CD8 coreceptor molecules. $TCR^{lo}CD4^+CD8^+$ double-positive thymocytes expressing TCR that recognizes self-MHC molecules undergo positive selection mediated by TCR and coreceptor molecules. Positively selected thymocytes down-regulate expression of CD4 or CD8, up-regulate TCR expression, and mature to either $TCR^{hi}CD4^+CD8^-$ class II MHC-restricted helper T cells or $TCR^{hi}CD4^-CD8^+$ class I MHC-restricted cytotoxic T cells. Double-positive thymocytes expressing TCR with too high of an affinity/avidity for self-peptide/MHC undergo negative selection, resulting in death by apoptosis (for review, see Nossal, 1994; Robey and Fowlkes, 1994; von Boehmer, 1994).

Immature thymocytes at the double-positive stage in development have a lifespan of several days. If they fail to express TCR or express TCR that does not recognize self-MHC molecules, they fail to receive maturation and survival signals associated with positive selection, and die by apoptosis. Neglected double-positive thymocytes represent the vast majority of thymocytes. These cells and negatively selected thymocytes are cleared from the thymus by thymic macrophages when they become apoptotic (Savill et al., 1993; Surh and Sprent, 1994; Veis et al., 1993).

The *nur77* gene was identified in a differential screen of T cell hybridomas for genes expressed during TCR-signaled apoptosis, suggesting that it may play a role in negative selection (Liu et al., 1994; Woronicz et al., 1994). The *nur77* (*NGFI-B*) gene was originally characterized as a gene expressed in response to mitogenic signals in NIH 3T3 cells and in response to both mitogenic and differentiative signals in the PC12 neuronal cells line (Hazel et al., 1988; Milbrandt, 1988; Ryseck et al., 1989). In T cell hybridomas, expression of the *nur77* gene is rapidly induced in response to TCR signaling. A high level of Nur77 protein expression is maintained throughout the apoptotic process in these cells (Woronicz et al., 1994). Injection of mice with anti-CD3 antibodies induces apoptosis in thymocytes and a high level of Nur77 expression, demonstrating a correlation between *nur77* expression and apoptosis of thymocytes (Woronicz et al., 1994). Nur77 has been shown to be required for TCR-signaled apoptosis in T cell hybridomas. Stable expression of a dominant-negative Nur77 protein or transient expression of antisense Nur77 RNA has been shown to inhibit TCR-signaled apoptosis of these cells (Liu et al., 1994; Woronicz et al., 1994).

The Nur77 protein belongs to the steroid receptor superfamily of ligand-dependent transcription factors (Hazel et al., 1988; Milbrandt, 1988; Ryseck et al., 1989). The domain organization of proteins in this family includes an N-terminal transactivation domain, a Zn finger DNA-binding domain, and a C-terminal ligand-binding domain. The DNA-binding domain of Nur77 contains an additional element, termed an A box, that recognizes 5′ A residues in the Nur77 DNA binding site (5′-AAAAGGTCA-3′) (Wilson et al., 1991, 1992, 1993b). Among members of this family, the Nur77 protein is unusual in that it appears to bind its target element as a monomer (Wilson et al., 1993a). A ligand for Nur77 has yet to be identified, which has led to its classification as an orphan steroid receptor (Davis et al., 1993; Fahrner et al., 1990; Paulsen et al., 1992; Yoon and Lau, 1993).

Although Nur77 has been shown to be required for TCR-signaled apoptosis in T cell hybridomas, it is not clear whether the molecular mechanisms leading to the death of T cell hybridomas are similar to the mechanisms initiated during the process of negative selection. In addition, the function of the Nur77 protein in TCR-signaled apoptosis is not understood. Here, we examined a role for the Nur77 protein in the process of antigen-induced negative selection. We report that a dominant-negative Nur77 protein inhibits antigen-induced negative selection of thymocytes in vivo. The dominant-negative protein does not block positive selection of thymocytes but perturbs T cell development, causing an increase in the number of mature $CD4^+CD8^-$ thymocytes. We also report that constitutive Nur77 expression induces apoptosis in developing thymocytes. Together, these results support a role for Nur77 in the downstream events in antigen-induced negative selection.

Results

Generation of Nur77 Transgenic Mice

We examined a role for the orphan steroid receptor Nur77 in the process of negative selection from two perspectives. We reasoned if Nur77 regulated genes involved in the downstream events in negative selection, that a dominant-negative mutant of the Nur77 protein would inhibit negative selection; whereas, constitutive Nur77 expression might induce apoptosis in thymocytes. We generated two sets of transgenic mice based on this rationale. In one set of mice, a dominant-negative Nur77 mutant, containing the Zn finger A box DNA-binding domain through the C-terminal ligand-binding domain of Nur77, is expressed in developing thymocytes. This dominant-negative Nur77 protein is the same one that has been shown to block TCR-signaled apoptosis of T cell hybridomas (Woronicz et al., 1994). In a second set of mice, wild-type Nur77 protein is constitutively expressed in thymocytes. We refer to the mice as Nur77–ZnCT and Nur77–FL mice and to the protein products of the transgenes in the same manner. The T cell–specific lck proximal promoter regulates transgene expression in both sets of mice (Figure 1A). Expression from this promoter has been shown to be very strong in immature thymocytes, down-regulated as thymocytes mature, and relatively inactive in mature T cells (Allen et al., 1992).

To verify expression of the dominant-negative protein in Nur77–ZnCT transgenic mice and to compare the levels of dominant-negative protein and endogenous Nur77 protein, an electrophoretic mobility shift assay was performed with a probe containing the Nur77 DNA binding site. Nur77–ZnCT protein has previously been shown to bind to the Nur77 site with high specificity (Wilson et al., 1991, 1992; Woronicz et al., 1994). Line 18 and 15 Nur77–ZnCT transgenic mice were found to express relatively high levels of Nur77–ZnCT protein in thymocytes compared with the level of endogenous Nur77 protein that is induced by stimulating thymocytes with ionomycin and phorbol myristate acetate (Figure 1B). A mobility shift binding assay was also used to verify Nur77–ZnCT protein expression

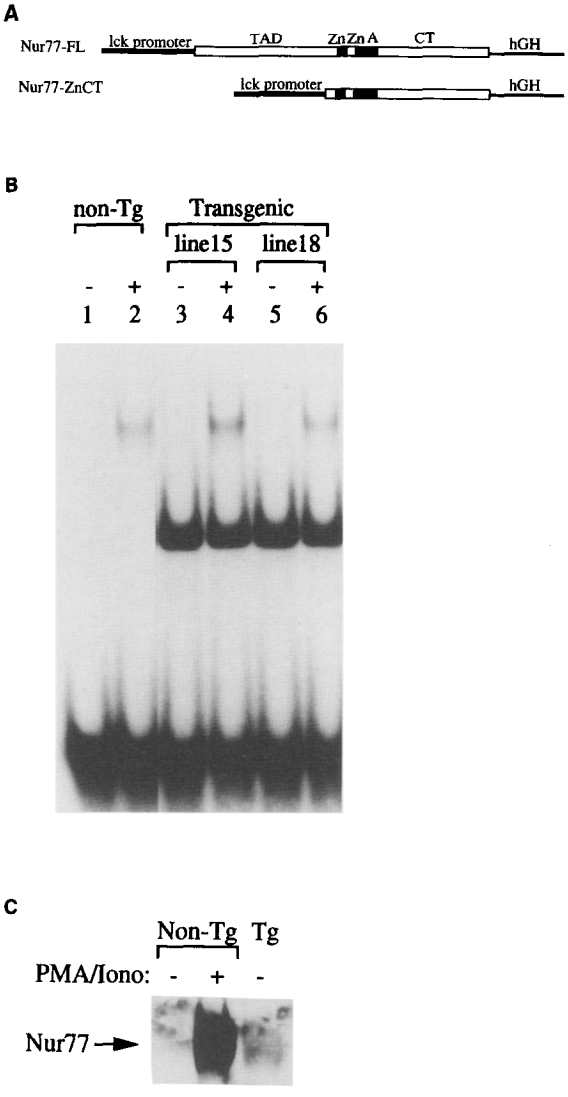


Figure 1. Diagrams of the Nur77 Transgenes and Analyses to Detect Protein Products of the Transgenes in Thymocytes from Transgenic Mice

(A) Nur77–FL (upper) and Nur77–ZnCT (lower) transgenes. The lck-proximal promoter regulates transgene expression and sequences from the human growth hormone gene (hGH) facilitate mRNA processing. Functional domains of the wild-type Nur77 protein include an N-terminal transactivation domain (TAD), a Zn finger A box DNA-binding domain (Zn Zn A), and a C-terminal ligand-binding domain (CT).

(B) An electrophoretic mobility shift assay using the Nur77 DNA binding site detected Nur77–ZnCT protein binding activity in thymocytes from Nur77–ZnCT transgenic mice and endogenous Nur77 protein binding activity in thymocytes stimulated for 2 hr with ionomycin and PMA, indicated by a plus. Data shown is from line 18 and line 15 mice. Complexes containing the dominant-negative protein are distinguished from those containing endogenous Nur77 based on a difference in mobility.

(C) Western blot analysis using 2C1 anti-mouse Nur77 monoclonal antibody to detect Nur77 expression in thymocytes from Nur77–FL transgenic mice. Nur77 was detected in unstimulated thymocytes from transgenic mice but not in unstimulated thymocytes from non-transgenic mice, confirming that Nur77 protein in transgenic animals is expressed from the transgene. Thymocytes in which expression of endogenous Nur77 was induced by stimulation with .5 μM ionomycin and 10 ng/ml PMA served as a positive control, and is indicated by a plus.

in peripheral T cells. Lower levels of the protein were observed in peripheral T cells, consistent with the known decrease in activity of lck proximal promoter in mature T cells (data not shown).

Constitutive expression of full-length Nur77 protein in thymocytes from Nur77–FL transgenic mice was confirmed by Western blot analysis of whole cell extract using 2E1 anti-mouse Nur77 monoclonal antibody (Fahrner et al., 1990). Full-length Nur77 protein was detected in whole cell extract from unstimulated transgenic thymocytes but not in extract from unstimulated nontransgenic thymocytes (Figure 1C). Since *nur77* is an activation-induced gene in T cells, stimulation of thymocytes with ionomycin and PMA or with anti-CD3 antibodies is usually required to detect Nur77 protein in thymocytes, indicating that Nur77 protein detected in unstimulated transgenic thymocytes is protein expressed from the transgene. The DNA binding activity of Nur77 protein in transgenic thymocytes was verified by an electrophoretic mobility shift assay (data not shown). We concentrated our analysis on line 5 of these mice. A second line shows similar results.

Expression of a Dominant-Negative Nur77 Protein Perturbs T Cell Development

We first examined Nur77–ZnCT transgenic mice for any effect of the dominant-negative protein on T cell development. Lymphocyte counts were determined in these mice and T cell development analyzed by flow cytometry based on expression of the coreceptor molecules CD4 and CD8 and on CD3 expression for TCR levels. Nur77–ZnCT transgenic mice were found to have a moderate reduction in thymocyte number, which correlated with a reduction in the absolute number of $CD4^+CD8^+$ thymocytes in these mice (Figures 2A and 2B). These mice were also found to have an increase in the absolute number of mature $CD4^+CD8^-$ thymocytes, reflected as an increase in the number of cells expressing high levels of CD3. An apparent increase in the percentage of mature $CD4^-CD8^+$ thymocytes was observed but is not real in terms of a change in the absolute number of these cells (Figures 2A and 2B). The phenotypic changes observed in heterozygous Nur77–ZnCT transgenic mice were found to be more pronounced in homozygous transgenic mice, indicating a dosage effect of the dominant-negative protein on T cell development (Figure 2A). The same phenotypic changes were observed in both line 18 and line 15 Nur77–ZnCT transgenic mice (Figure 2B). Although the dominant-negative Nur77 protein perturbed thymocyte development, the number of T cells in the lymph nodes and spleen and the ratio of CD4 to CD8 cells in Nur77–ZnCT mice were found to be approximately the same as in nontransgenic littermate controls (data not shown).

To examine further T cell development in Nur77–ZnCT transgenic mice, expression patterns of the developmental markers CD69, HSA, and CD5 were analyzed by flow cytometry. In normal mice, expression of CD5 increases and expression of HSA decreases after positive selection of thymocytes (Bendelac and Schwartz, 1991; Crispe and Bevan, 1987; Godfrey and Zlotnik, 1993; Ramsdell et al., 1991; Wilson et al., 1988). CD69 is an activation marker transiently up-regulated as the result of positive selection (Bendelac et al., 1992; Swat et al., 1993). In thymocytes from Nur77–ZnCT transgenic mice, CD69, HSA, and CD5 were found to be expressed in a normal pattern and at the same levels as in nontransgenic littermate controls. Thus, the dominant-negative Nur77 pro-

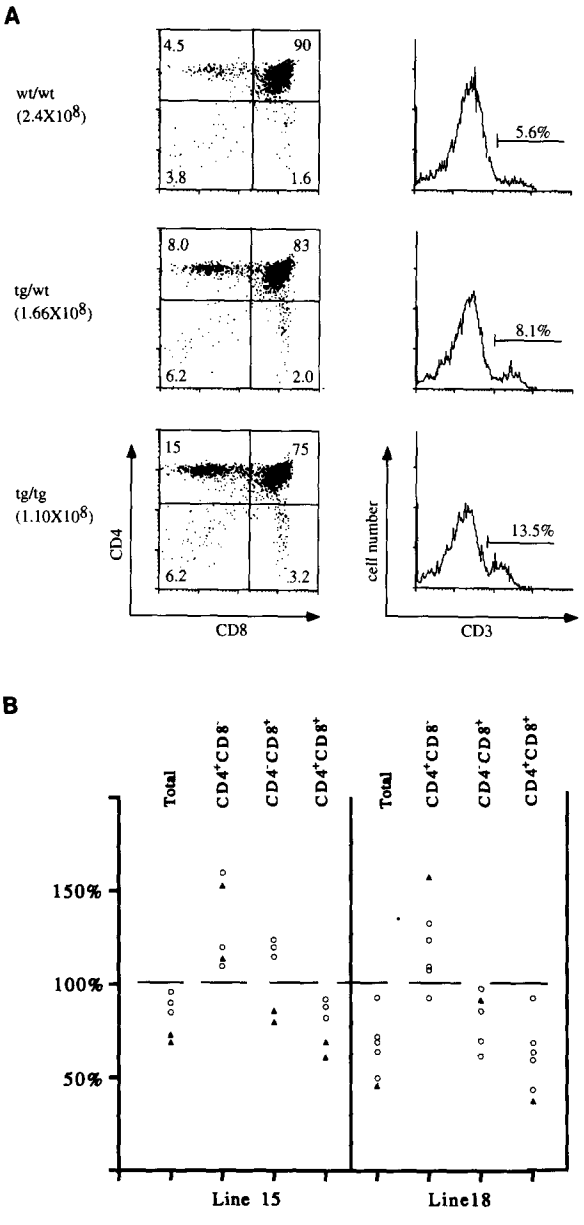


Figure 2. Analyses of Nur77–ZnCT Transgenic Mice for Changes in T Cell Development

(A) Flow cytometric analysis of thymocytes from line 18 Nur77–ZnCT transgenic mice. CD4, CD8, and CD3 expression on thymocytes from a nontransgenic (wt/wt), heterozygous (tg/wt), and homozygous (tg/tg) mouse. Data shown is from 5-week-old male littermates.

(B) Absolute number of thymocytes in the various thymocyte subsets in Nur77–ZnCT transgenic mice expressed as a percentage of the number in a nontransgenic same-sex littermate control. Data is from line 18 and line 15 mice 3–6 weeks old. Each point represents the difference between a transgenic and nontransgenic control. Open circles are data from Nur77–ZnCT heterozygous mice and triangles are data from homozygous mice.

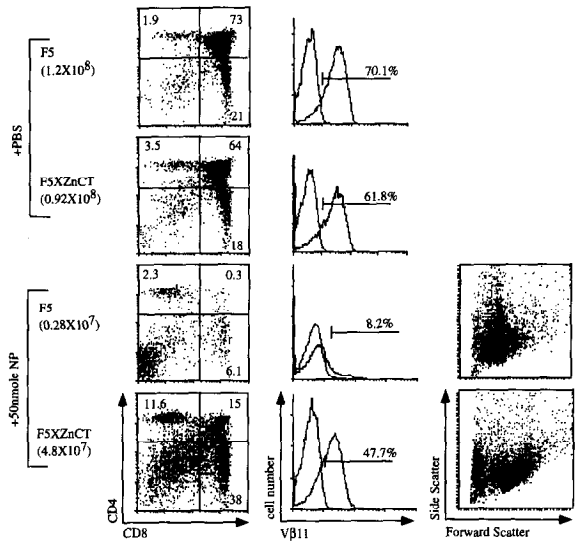


Figure 3. Antigen-Induced Deletion Is Inhibited in F5/Nur77–ZnCT Mice

Flow cytometric analysis of CD4, CD8, and Vβ11 staining of thymocytes from F5 TCR single-transgenic and F5 × ZnCT double-transgenic mice after injection of PBS or 50 nmol of nucleocapsid peptide daily for 4 days. Forward versus side light scatter of ungated thymocytes is shown. The total number of thymocytes after injection of PBS or peptide is indicated. In the experiment shown, mice were 6-week-old male littermates. The experiment was repeated three times with similar results. In each experiment, same-sex littermates were used.

tein does not block positive selection of thymocytes but does cause a change in the steady-state distribution of thymocytes among the various subsets of thymocytes.

Antigen-Induced Negative Selection Is Inhibited by a Dominant-Negative Nur77 Protein

After characterizing T cell maturation in Nur77–ZnCT transgenic mice, we crossed these mice with F5 TCR transgenic mice to examine an effect of the dominant-negative Nur77 protein on antigen-induced negative selection in vivo. F5 TCR transgenic mice provided conditions where negatively selected cells could be identified and, in the event of a partial inhibition of negative selection, where the timing of deletion could be controlled. In F5 mice, the majority of thymocytes express a transgenic F5 TCR (Mamalaki et al., 1992, 1993). This receptor contains Vα4 and Vβ11 TCR segments and recognizes an influenza virus nucleocapsid peptide bound to D^b class I molecules. In the absence of nucleocapsid peptide, F5 thymocytes undergo positive selection on D^b, skewing development towards production of $CD4^-CD8^+$ cells. For the purposes of our analysis, negative selection of immature F5 thymocytes could be induced by injection of nucleocapsid peptide (Mamalaki et al., 1992, 1993). Thus, in the event of a partial inhibition of negative selection, the difference in the extent of deletion in F5 single-transgenic and F5/Nur77–ZnCT double-transgenic mice could be compared.

F5 single-transgenic and F5/Nur77–ZnCT double-transgenic mice were injected intraperitoneally with 50 nmol of NP peptide in phosphate-buffered saline (PBS) once a day for 4 days. Littermate controls were injected with PBS for the same period. On the fifth day, thymocytes from experimental and control mice were analyzed by flow cytometry for Vβ11, CD4, and CD8 expression. In PBS-injected F5 single-transgenic and F5/Nur77–ZnCT double-transgenic control mice, most thymocytes expressed Vβ11 and the majority of mature thymocytes were $CD4^-CD8^+$ cells (Figure 3). In experimental F5 single-transgenic mice, injection of nucleocapsid peptide resulted in a severe reduction in total number of thymocytes. In addition, most thymocytes remaining in these mice were apoptotic, as measured by flow cytometry as a decrease in forward light scatter and an increase in side light scatter of the cells (Figure 3). There were very few $CD4^+CD8^+$ cells remaining after peptide injection, and of the total cells remaining only a small percentage expressed Vβ11 (Figure 3). In contrast, the population of thymocytes remaining in F5/Nur77–ZnCT double-transgenic mice after injection of nucleocapsid peptide consisted primarily of viable cells, of which approximately 15% were $CD4^+CD8^+$ cells and 47.7% expressed Vβ11 in the experiment shown (Figure 3). A difference in the extent of deletion clearly demonstrates an inhibition of antigen-induced negative selection by the dominant-negative Nur77 protein. A summary of experiments is shown in Table 1.

Superantigen-Induced Negative Selection Is Not Inhibited by a Dominant-Negative Nur77 Protein

To examine the role of Nur77 in negative selection by superantigens, we crossed Nur77–ZnCT transgenic mice to CBA/J mice. The presence of the retroviral superantigens $Mls1^a$ and $Mls2^a$ in CBA/J mice as well as expression of MHC class II I-E molecules normally leads to deletion of T cells carrying Vβ3, Vβ6, and Vβ11, but not of T cells carrying Vβ8.2/Vβ8.3 (Herman et al., 1991). In addition,

Table 1. Inhibition of Negative Selection by a Dominant-Negative Nur77 Protein

Experiments	Transgenic Mice	Number of viable cells after injection with 50 nmol of NP peptide	Percent of $CD4^+CD8^+V\beta11^+$ cells
1	F5	2.8×10^6	<0.10%
	F5 × Nur77–ZnCT	4.8×10^7	15.8%
2	F5	6.0×10^6	1.7%
	F5 × Nur77–ZnCT	1.7×10^7	38.6%
3	F5	1.3×10^7	<0.3%
	F5 × Nur77–ZnCT	4.8×10^7	5.4%

Table 2. Percentage of Vβ3, Vβ5, Vβ6, Vβ11, and Vβ8 Thymocytes in I-E$^-$ and I-E$^+$ Nontransgenic (Tg$^-$) and Dominant-Negative Nur77 Transgenic (Tg$^+$) Mice

Mice		$CD4^+CD8^+$					$CD4^+CD8^-$				
Tg	I-E	Percent of Vβ3$^+$	Percent Vβ5$^+$	Percent of Vβ6$^+$	Percent Vβ8$^+$	Percent of Vβ11$^+$	Percent of Vβ3$^+$	Percent of Vβ5$^+$	Percent of Vβ6$^+$	Percent of Vβ8$^+$	Percent of Vβ11$^+$
−	−	1.7	0.7	1.7	2.3	1.7	2.8	4.6	6.8	15.8	4.6
+	−	1.3	0.9	1.6	2.0	1.5	3.3	5.3	6.7	14.8	4.8
−	+	2.50 (0.36)	1.47 (0.40)	2.80 (0.26)	3.50 (0.61)	1.37 (0.38)	0.1 (0.0)	0.1 (0.0)	0.1 (0.0)	15.7 (0.20)	0.1 (0.0)
+	+	2.50 (0.26)	1.17 (0.15)	2.90 (0.40)	3.73 (0.57)	1.30 (0.61)	0.1 (0.0)	0.1 (0.0)	0.1 (0.0)	15.6 (1.20)	0.1 (0.0)

Numbers represent the mean percentage of $CD4^+CD8^+$ and $CD4^+CD8^-$ thymocytes bearing each Vβ. For superantigen-positive I-E$^+$ mice, data represent three independent animals. Standard deviations are shown in parentheses.

the presence of Etc-1 retroviral antigens in C57BL/6 mice leads to deletion of Vβ5$^+$ T cells in I-E$^+$ mice (Woodland et al., 1990). We examined the percentages of Vβ3$^+$, Vβ5$^+$, Vβ6$^+$, and Vβ11$^+$ $CD4^+CD8^+$ double-positive and $CD4^+CD8^-$ single-positive thymocytes (Table 2) and Vβ3$^+$, Vβ5$^+$, Vβ6$^+$, and Vβ11$^+$ $CD4^+$ lymph node cells (Table 3) in wild-type and transgenic offspring from this cross. We did not detect any increase in the percentage of these cells in transgenic offspring, indicating that deletion by superantigens is not blocked by the dominant-negative Nur77 mutant. These results, together with the results of the F5 deletion experiment (above), suggest that either the molecular mechanisms of deletion by superantigens and antigens are different, or that the dominant-negative Nur77 mutant is only able to delay apoptosis in negatively selected thymocytes, as suggested by the F5 experiment. Thus, we may not be able to detect a partial inhibition of negative selection under steady-state conditions.

Constitutive Nur77 Expression Results in a Reduction in Thymocyte Number

We next examined Nur77–FL transgenic mice to determine whether constitutive Nur77 expression induces apoptosis in developing thymocytes. Nur77–FL mice were found to have a 5- to 20-fold reduction in number of thymocytes, a reduction in number of lymph node cells, and normal numbers of splenocytes (Figure 4). Population of thymocytes from these mice were found to contain a large number of granular cells, as measured by flow cytometry as an increase in side light scatter, suggesting an apoptotic morphology (Figure 5A). All 5 males examined between 3–6 weeks old and 1 of the 5 females examined in this age group contained a population of granular thymocytes, although all Nur77–FL transgenic mice examined had a reduction in thymocyte number. A population of granular thymocytes has not been observed in older mice of either sex, suggesting that accumulation of these cells is age and sex dependent (see Discussion).

To verify that granular thymocytes in Nur77–FL transgenic mice were apoptotic, TUNEL analysis (Gavrieli et al., 1992) was performed to identify cells with fragmented DNA. This analysis confirmed that granular thymocytes were in fact apoptotic (Figure 5B). Thus, constitutive Nur77 expression induces apoptosis in developing thymocytes, leading to a reduction in thymocyte number.

To examine T cell development in Nur77–FL transgenic mice, thymocytes from these mice were examined by flow cytometry for expression of the coreceptor molecules CD4 and CD8 and for CD3. In thymi containing a population of granular cells, there were very few immature $CD4^+CD8^+$ and mature $CD4^+CD8^-$ and $CD4^-CD8^+$ thymocytes (Figure 5A). In thymi that did not contain the granular population, although there were fewer thymocytes, the relative percentages of $CD4^+CD8^+$ and mature $CD4^+CD8^-$ and $CD4^-CD8^+$ thymocytes were higher (Figure 5A). CD3 profiles reflected the decrease in percentages of immature $CD4^+CD8^+$ and mature $CD4^+CD8^-$ and $CD4^-CD8^+$ thymocytes (Figure 5A). These data demonstrate that the effect of constitutive Nur77 protein expression is more severe

Table 3. Percentage of Vβ3, Vβ5, Vβ6, Vβ11, and Vβ8 CD4+ Lymph Node Cells in I-E- and I-E+ Non-Transgenic (Tg-) and Dominant-Negative Nur77 Transgenic (Tg+) Mice

CD4+CD8-						
Tg	I-E	Percent Vβ3+	Percent Vβ5+	Percent Vβ6+	Percent Vβ8+	Percent Vβ11+
-	-	4.2	3.1	9.3	16.4	5.6
+	-	3.7	3.7	9.0	15.7	4.7
-	+	0.27 (0.12)	0.53 (0.12)	1.10 (0.30)	16.70 (0.78)	0.37 (0.12)
+	+	0.23 (0.06)	0.43 (0.23)	0.93 (0.35)	16.23 (0.15)	0.50 (0.17)

Numbers represent the mean percentage of CD4+ lymph node T cells bearing each Vβ. For superantigen-positive I-E+ mice, data represent three independent animals. Standard deviations are shown in parentheses.

in some mice than others. The severity of the phenotype correlates with an accumulation of granular apoptotic thymocytes in the thymus.

To examine further T cell development in these mice, thymocytes were analyzed for the developmental marker CD69, CD5, and CD25. Expression of CD69 and CD5 was normal on thymocytes from Nur77–FL mice. However, low levels of CD25, the α chain of the interleukin-2 (IL-2) receptor, were detected on CD4+CD8+ thymocytes (data not shown). Expression of CD25 is normally down-regulated as thymocytes undergo expansion during the transition from the CD4-CD8- stage to the CD4+CD8+ stage in development (Godfrey and Zlotnik, 1993). This observation raises the possibility of a problem during the CD4-CD8- to CD4+CD8+ transition (see Discussion).

Lymph node cells and splenocytes from Nur77–FL mice were examined by flow cytometry for expression of CD4 and CD8. Although transgenic mice 3–6 weeks old had populations of both CD4 and CD8 T cells (lymph node data is shown in Figure 6), the percentages of these cells were low compared with the percentages in nontransgenic littermate controls. Consistent with the reduction in the number of CD4 and CD8 T cells, the percentage of CD3+ cells was found to be low (Figure 6). Interestingly the number of CD4 and CD8 T cells declined with age (Figure 6, lymph node data). The reduction in T cell number was observed in both the lymph nodes and spleen of Nur77–FL transgenic mice. These data demonstrate that Nur77–FL mice have fewer peripheral T cells and that the number of these cells declines over time.

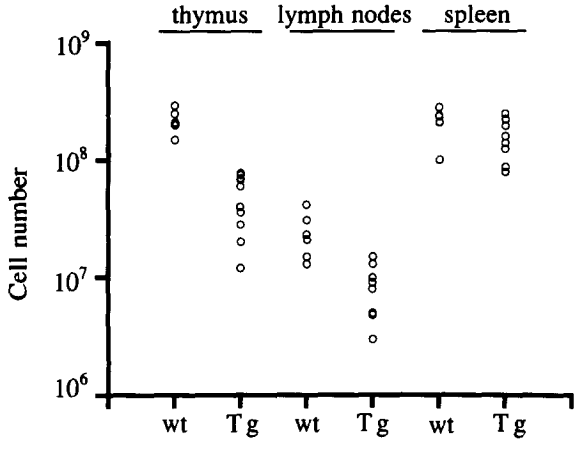


Figure 4. Total Number of Thymocytes, Lymph Node Cells, and Splenocytes in Nur77 FL Transgenic Mice 3–6 weeks old

The numbers in transgenic (Tg) and nontransgenic littermate controls (wt) are compared.

Discussion

We have investigated a role for the orphan steroid receptor Nur77 in the process of negative selection using a dominant-negative mutant of the Nur77 protein to inhibit endogenous Nur77 function. Transgenic mice were generated in which the dominant-negative Nur77 protein (Nur77–ZnCT) was expressed in developing thymocytes. These mice were then crossed with F5 TCR transgenic mice and negative selection induced by injection of antigenic peptide. The extent of deletion of F5 thymocytes in F5 single-transgenic and F5/Nur77–ZnCT double-transgenic mice was compared. The dominant-negative protein was found to inhibit apoptosis significantly, providing in vivo evidence supporting a role for the Nur77 protein in antigen-induced negative selection.

The role of Nur77 in the molecular mechanism of negative selection by superantigens was examined by analyzing deletion in MMTV+ I-E+ dominant-negative Nur77 transgenic mice. Expression of the dominant-negative Nur77 protein did not rescue Vβ3+, Vβ5+, Vβ6+, or Vβ11+ thymocytes from deletion by superantigens. Thus, the mechanisms of apoptosis in superantigen- and antigen-induced negative selection may be different. However, whereas the timing of deletion by peptide antigen was controlled, deletion by superantigens was examined under steady-state conditions. Thus, the possibility of a role for Nur77 in the process of superantigen deletion cannot be entirely ruled out for the following reasons. First, if the level of dominant-negative Nur77 protein is not sufficient to block endogenous Nur77 function entirely, apoptosis of negatively selected cells may only be delayed. Second, signals delivered during negative selection may result in commitment of a cell to the death pathway. Therefore, inhibiting the downstream events in negative selection may not necessarily result in maturation of negatively selected thymocytes but may instead only delay the death of these cells.

To determine whether Nur77 expression alone could activate mechanisms leading to apoptosis of thymocytes, Nur77 protein was constitutively expressed in developing

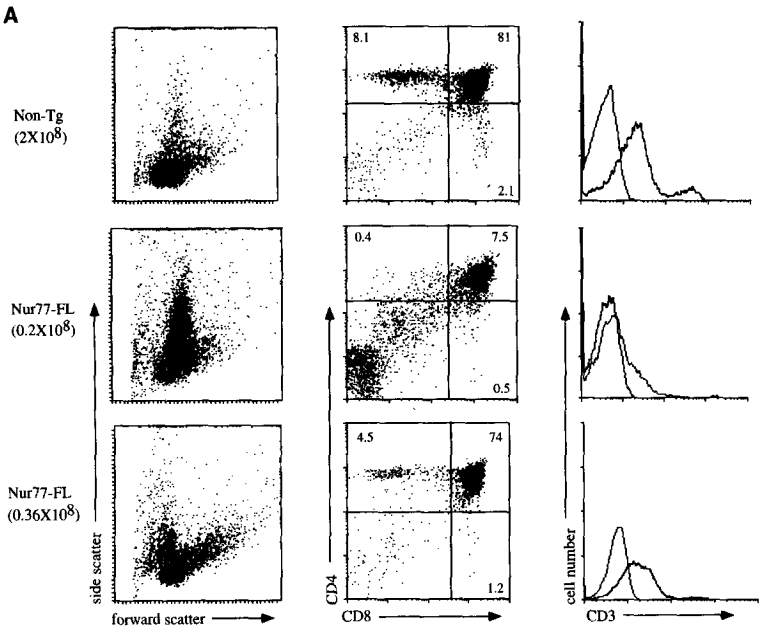
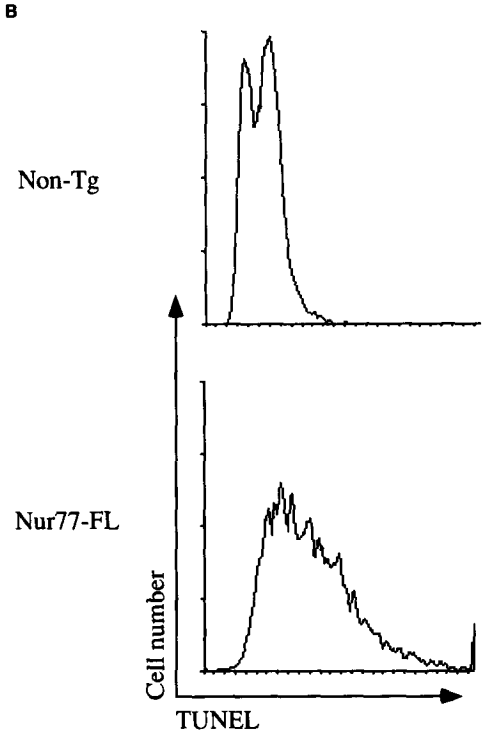


Figure 5. Severity of the Phenotype in the Nur77–FL Mice Correlates with the Presence of a Population of Granular Thymocytes

(A) Representative FACS plots of CD4, CD8, and CD3 expression on thymocytes from Nur77–FL transgenic mice comparing the severity of phenotypes. The thymus of some transgenic mice contain a large number of granular cells, as measured by an increase in forward and side light scatter. The presence of this population correlates with severity of the phenotype in the thymus.

(B) TUNEL staining of thymocytes from a Nur77–FL transgenic mice confirms that granular thymocytes are apoptotic



thymocytes. Expression of Nur77 resulted in a significant reduction in number of thymocytes and lymph node T cells. A population of thymocytes with a granular apoptotic morphology was observed by flow cytometry and confirmed as apoptotic using TUNEL. An accumulation of apoptotic thymocytes is somewhat surprising, since apoptotic cells are normally cleared very rapidly from the thymus by resident macrophages (Savill et al., 1993; Surh and Sprent, 1994; Veis et al., 1993). The presence of apoptotic cells in the thymus of some Nur77–FL mice suggests that either the rate of apoptosis has exceeded the capacity of macrophages to clear dead cells or that these cells have not yet been identified as apoptotic by macrophages.

The effects of Nur77 expression appeared to be more pronounced in some mice than others. Our analysis to date indicates that the phenotype is more severe in young males. An increase in severity of the phenotype may result from additional effects of hormones during sexual maturation.

Thymocytes in Nur77–FL mice appear to follow a normal sequence of developmental events with one exception. Low levels of CD25 are detected on $CD4^+CD8^+$ thymocytes. In normal mice, expression of CD25 is downregulated before proliferation of thymocytes during a transition from the $CD4^-CD8^-$ stage to the $CD4^+CD8^+$ stage in development. Low levels of CD25 have been observed on $CD4^+CD8^+$ thymocytes in ζ chain–deficient mice (Crompton et al., 1994). The possibility was raised that thymocytes in ζ chain–deficient mice had undergone fewer rounds of proliferation during the $CD4^-CD8^-$ to $CD4^+CD8^+$ transition. CD25 expression was therefore not "diluted" from the surface of thymocytes through cell division during this transition. The same possibility is raised here but probably results from different reasons. Preliminary data suggest that thymocytes in Nur77–FL transgenic mice are undergoing apoptosis during the $CD4^-CD8^-$ to $CD4^+CD8^+$ proliferative transition. If Nur77 expression has an inhibitory effect on proliferation of thymocytes during this transition, it could explain both the expression of low levels of CD25 on double-positive thymocytes and apoptosis of some cells during this transition. Thus, apoptosis of thymocytes during the double-negative to double-positive transition is one possible explanation for the reduction in thymocyte numbers in Nur77–FL transgenic mice.

In addition, Nur77–FL transgenic mice have a decline in number of peripheral T cells. By 6 weeks of age, the

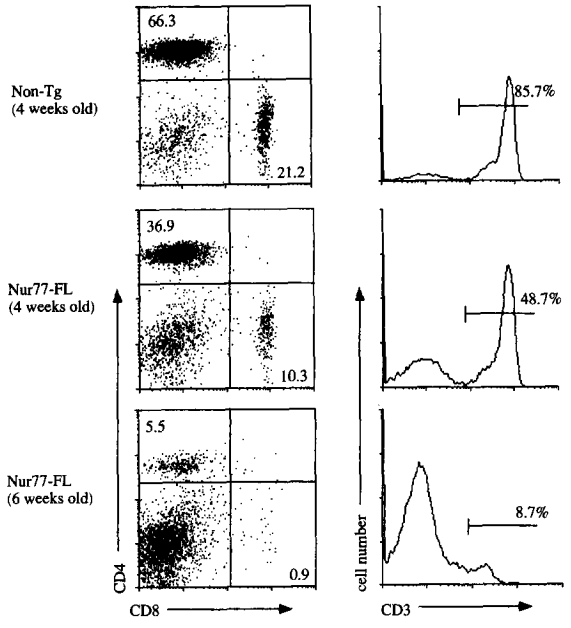


Figure 6. Representative FACS Plots of CD4, CD8, and CD3 Expression on Lymph Node Cells from Nur77 Transgenic Mice

Numbers of T cells decline with age.

number of mature T cells remaining in these mice is very low. There are several possible explanations for this decline in T cell number. One is that T cells developing in these mice are less viable, and as the mice age the rate of T cell turnover in the periphery exceeds the rate of thymocyte maturation, resulting in a steady decline in T cell numbers. A second possibility is that, because of a problem in thymocyte development, fewer T cells are maturing in Nur77–FL transgenic mice. These cells in the periphery are then attempting to increase in number and die because of an inhibitory effect of Nur77 on proliferation.

We have examined our data in light of recent suggestions that Nur77 may regulate expression of Fas or the Fas ligand (Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995). Fas is a member of a family of receptors that includes the tumor necrosis factor (TNF) receptor (for review, see Crispe, 1994; Nagata and Golstein, 1995). Signals delivered through Fas induce apoptosis in cells and an interaction between Fas and the Fas ligand has been shown to mediate TCR-signaled apoptosis of T cell hybridomas, leukemic T cells, and primary T cells growing in culture. Fas is constitutively expressed on immature $CD4^+CD8^+$ thymocytes and on mature $CD4^+CD8^-$ and $CD4^-CD8^+$ thymocytes (for review, see Crispe, 1994; Nagata and Golstein, 1995), whereas Fas ligand expression is inducible in thymocytes. One possible model for negative selection involving Fas, the Fas ligand, and Nur77 would argue that induction of Nur77 expression during negative selection leads to up-regulation of the Fas ligand and cell autonomous suicide in negatively selected cells. A variation on this model is that negatively selected cells become susceptible to Fas-mediated apoptosis and are killed by cells expressing the Fas ligand. However, experiments with *lpr/lpr* mutant mice, which do not express Fas, suggests that Fas is not required for negative selection (Kotzin et al., 1988; Singer and Abbas, 1994; Singer et al., 1989; Zhou et al., 1991) and argue against a model for negative selection involving Fas. We have examined Fas levels on Nur77–FL transgenic thymocytes and Nur77–ZnCT transgenic thymocytes and have found no difference between the levels of Fas on these cells and on nontransgenic thymocytes. Experiments are in progress to examine Fas ligand expression on negatively selected F5/Nur77–ZnCT double-transgenic thymocytes and on Nur77–FL transgenic thymocytes.

We conclude by proposing a hypothesis for Nur77 function in antigen-induced negative selection. Although downstream targets of Nur77 have yet to be identified in T cells, we would like to propose that Nur77 may negatively regulate a process X required for proliferation and differentiation. During antigen-induced negative selection, a high affinity/avidity interaction between TCR and self-peptide/MHC may result in an induction of a high level of Nur77 protein. A strong inhibition by Nur77 of this process X in negatively selected thymocytes may result in apoptosis. Expression of the dominant-negative Nur77 protein in developing thymocytes may have partially relieved an inhibitory effect of Nur77 on proliferation and differentiation. This effect was seen as a change in the steady-state distribution of thymocytes among the various subsets and an inhibition of negative selection. Constitutive expression of wild-type Nur77 may have increased the level of inhibition, causing apoptosis in thymocytes.

Nur77 may have a similar function in neuronal cells in their response to proliferative and differentiative signals, as Nur77 expression has been correlated with both proliferation and differentiation in the neuronal cell line PC12. In addition, a role for Nur77 as a negative regulator has implications for the response of peripheral T cells to TCR signaling. Nur77 may be part of the downstream mechanisms in mature T cells that determine whether T cells respond to TCR signaling by proliferating, becoming anergic, or undergoing apoptosis. In HIV-infected individuals, Nur77 may be part of the mechanisms that lead to a decline in $CD4^+$ T cells over time. Identifying downstream targets of Nur77 will further our understanding of the molecular mechanisms in positive and negative selection of thymocytes and the connections between proliferation, differentiation, and apoptosis.

Experimental Procedures

Construction of Transgenes

An HgAI–EcoRV cDNA fragment encoding the Zn fingers through the C terminus of murine Nur77 was isolated from the plasmid N10 (Ryseck et al., 1989), the ends of the fragment filled in with Klenow, and the fragment cloned into SmaI in the vector pEVRF (Matthias, et al., 1989) by ligation of blunt ends. The cDNA was cloned in frame with a thymidine kinase (TK) ATG codon in pEVRF. TK–Nur77–ZnCT sequence was isolated from pEVRF by digestion with SstI, the ends were filled in with Klenow, and the fragment cloned into the BamHI site in the plasmid p1017 by ligation of blunt ends after filling in plasmid ends. TK–Nur77–ZnCT sequence was cloned upstream from the lck proximal promoter and regulatory sequences from the human growth hor-

mone gene contained in p1017, generating the Nur77–ZnCT transgene. To construct the Nur77–FL transgene, the complete Nur77 cDNA coding sequence was isolated from the plasmid N10 by digestion with EcoRI and cloned into the BamHI site in p1017 by blunt-end ligation after filling in insert and plasmid ends. Nur77–ZnCT and Nur77–FL transgene sequences were isolated from p1017 by digestion with Not1 and purified from plasmid sequence.

Typing of Transgenic Mice

Purified transgene DNA was injected into (C57BL6 × CBA)F2 embryos. Transgenic founders were identified by Southern blot analysis of tail DNA and transgenic lines established by outcrossing with C57BL/6J mice. F1 offspring were typed by Southern blot analysis of tail DNA and later generations by polymerase chain reaction using the primers Ceder-2 (5′-GCGGATC CGGGGAGGCATCTGGAGGCTG-3′) and N-ter (5′-GGGGGATCTTGGTGG CGTG-3′) to type Nur77–ZnCT transgenic mice and Ceder-2 and N10/E2 (5′-TTCCATGCCAGCAGCTTTCC-3′) to type Nur77–FL transgenic mice. Offspring of matings between heterozygous Nur77–ZnCT transgenic mice were typed by Southern blot analysis of tail DNA. Transgene copy number was measured by phosphorimaging analysis of endogenous and transgene bands to distinguish heterozygous transgenic mice from homozygous transgenic mice. All mice were maintained in the pathogen-free Berkeley Transgenic Facility.

Negative Selection in Nur77–ZnCT × F5 TCR Transgenic Mice

The MHC haplotype of Nur77–ZnCT transgenic mice was determined by polymerase chain reaction using the following H-2^k and H-2^b primers: EK-5′ CATGGGCATAGAAAGGGCAGTCTTTGA ACT-3′), EA-5′ (5′-AGTCTTCCCAGCCTTCACACTCAGAGGTAC-3′) and EA3′(5′-CATAGCCCCAAATGTCTGACCTCTGGAGAG-3′). F3 heterozygous Nur77–ZnCT mice from line 18 (H-2^b) were crossed with homozygous F5 TCR transgenic mice (H-2^b). An NP peptide (Ala–Ser–Asn–Glu–Asn–Met–Asp–Ala–Met [NP 366–374]) from the nucleocapsid peptide of the influenza virus A/NT/60/68 was used to induce deletion. A control peptide (Ala–Ser–Asn–Glu–Asn–Met–Glu–Thr–Met [NP 366–374]) from the nucleocapsid of the influenza virus A/PR/8/34 did not induce deletion in either F5 single-transgenic or F5/Nur77–ZnCT double-transgenic mice.

Cell Count and Flow Cytometry

Thymus, spleen, and lymph nodes were removed from mice and placed on ice in RPMI medium containing 10% fetal calf serum. Cell suspensions were generated, filtered to remove tissue debris, and cells counted using a hemacytometer. After determining cell count, cells were washed twice with 1 × PBS supplemented with 2% fetal calf serum. Cells/sample (10^6) were stained on ice in 50 μl PBS, 2% fetal calf serum using the following antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-CD8, phycoerythrin (PE)-conjugated anti-CD4 and anti-B220, biotinylated anti-CD8, anti-CD3, anti-Vβ3, Vβ5, Vβ6, and Vβ8.1/8.2 were purchased from Caltag, and FITC-conjugated anti-HSA and biotinylated anti-Vβ11, CD5, and CD25 were purchased from Pharmingen. Streptavidin–Tricolor (Caltag) was used as a second step reagent to detect biotinylated antibodies. Samples were analyzed on a Coulter EPICS XL flow cytometer. FACS plots were generated using WindMIDI developed by J. Trotter at the Salk Institute.

Gel Mobility Shift Assays

Whole cell extract for binding assays was generated as follows: single cell suspensions were made in RPMI medium containing 10% fetal calf serum. Cells were filtered to remove tissue debris and washed once with 1 × PBS. Cells were resuspended in 50–100 μl of lysis buffer (Veis et al., 1993), depending on the number of cells, and incubated on ice for approximately 10 min. Cellular debris was pelleted and extract was removed to a fresh tube and stored at −80°C. To induce expression of endogenous Nur77, between 10^7 and 10^8 thymocytes were plated in 10 ml of RPMI, 10% fetal calf serum and stimulated with 0.5 μm ionomycin and 10 ng/ml PMA for 2 hr at 37°C. Cells were washed once with 1 × PBS following stimulation before resuspension in lysis buffer. Protein concentration was determined by Bradford assay. Electrophoretic mobility shift assays were performed using a ^{32}P-labeled oligonucleotide probe containing a single Nur77 DNA binding site (5′-TCGAGTTTTAAAGGTCATGCTCAA TTTG-3′) (Wilson et al., 1991). Whole cell extract (20 μg) was incubated with labeled probe in 25 μl of binding buffer (Davis et al., 1993) for 25 min at room temperature. Complexes were resolved by polyacrylamide gel electrophoresis on a 4% acrylamide 0.5 × TBE gel.

Western Blot Analysis

Thymocyte whole cell extract was generated by the method of Veiss et. al. (1993) and 50 μg was run in each lane of a 12% polyacrylamide gel for SDS–polyacrylamide gel electrophoresis. Protein was transferred to nitrocellulose and stained with Ponceau to verify that equal amounts of protein had been loaded in each lane. Extract from non-transgenic thymocytes, which had been stimulated with ionomycin and PMA to induce endogenous Nur77 expression, served as a positive control. Nitrocellulose was probed with 2E1 anti-mouse Nur77 monoclonal antibody (a gift of Dr. J. Milbrandt). Blots were developed with enhanced chemiluminescence (DuPont NEN).

TUNEL

TdT labeling of thymocytes was performed according to the instructions of the manufacturers, as follows: Thymocytes were fixed in 1% paraformaldehyde followed by permeabilization with 70% ethanol. Fragmented DNA ends were labeled with digoxigenin-11-dUTP using TdT and detected with a FITC-conjugated anti-digoxigenin antibody. Samples were run on a Coulter EPICS XL flow cytometer.

Acknowledgments

We thank D. Raulet, N. Hong, and A. Lina for critical reading of the manuscript and E. Robey and M. Coles for helpful discussions. We thank D. Kioussis for permission to use F5 TCR transgenic mice and for providing NP peptide, R. Perlmutter for p1017, and P. Schow for technical assistance. This work is supported by National Institutes of Health grant CA66236 (to A. W.), and funds from the Searle Chicago Community Trust and Cancer Research Institute Investigatorship. A. W. is an National Science Foundation Presidential Faculty Fellow.

Received May 31, 1995; revised July 20, 1995.

References

Allen, J. M., Forbush, K. A., and Perlmutter, R. M. (1992). Functional dissection of the lck proximal promoter. Mol. Cell. Biol. *12*, 2758–2768.

Bendelac, A., and Schwartz, R. H. (1991). $CD4^+$ and $CD8^+$ T cells acquire specific lymphokine secretion potentials during thymic maturation. Nature *353*, 68–71.

Bendelac, A., Matzinger, P., Seder, R. A., Paul, W. E., and Schwartz, R. H. (1992). Activation events during thymic selection. J. Exp. Med. *175*, 731–742.

Brunner, T., Mogil, R., LaFace, D., Yoo, N. J., Mahboubi, A., Echeverri, F., Marin, S. J., Force, W. R., Lynch, D. H., Ware, C. F., and Green, D. R. (1995). Cell-autonomous Fas (CD95)/Fas-ligand interaction mediates activation-induced apoptosis in T cell hybridomas. Nature *373*, 441–444.

Crispe, I. N. (1994). Fatal interactions: fas-induced apoptosis of mature T cells. Immunity *1*, 347–349.

Crispe, I. N., and Bevan, M. J. (1987). Expression and functional significance of the J11d marker on mouse thymocytes. J. Immunol. *138*, 2013–2018.

Crompton, T., Moore, M., MacDonald, H. R., and Malissen, B. (1994). Double-negative thymocyte subsets in CD3 epsilon chain-deficient mice: absence of $HSA^+CD44^-CD25^-$ cells. Eur. J. Immunol. *24*, 1903–1907.

Davis, I. J., Hazel, T. G., Chen, R.-H., Blenis, J., and Lau, L. (1993). Functional domains and phosphorylation of the orphan steroid receptor Nur77. Mol. Endocrinol. *7*, 953–964.

Dhein, J., Walczac, H., Baumler, C., Dabatin, K.-M., and Krammer, P. H. (1995). Autocrine T cell suicide mediated by APO-1/(Fas/CD95). Nature *373*, 438–441.

Fahrner, T. J., Carroll, S. L., and Milbrandt, J. (1990). The NGFI-B

protein, an inducible member of the thyroid/steroid receptor family, is rapidly modified posttranslationally. Mol. Cell. Biol. *10*, 6454–6459.

Gavrieli, Y., Sherman, Y., and Ben-Sasson, S. A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J. Cell Biol. *119*, 493–501.

Godfrey, D. I., and Zlotnik, A. (1993). Control points in early T cell development. Immunol. Today *14*, 547–553.

Hazel, T. G., Nathans, D., and Lau, L. F. (1988). A gene inducible by serum growth factors encodes a member of the steroid and thyroid hormone receptor superfamily. Proc. Natl. Acad. Sci. USA *85*, 8444–8448.

Herman, A., Kappler, J. W., Marrack, P., and Pullen, A. M. (1991). Superantigens: mechanism of T cell stimulation and role in immune responses. Annu. Rev. Immunol. *9*, 745–772.

Ju, S.-T., Panka, D. J., Cui, H., Ettinger, R., El-Khatib, M., Sherr, D. H., Stanger, B. Z., and Marshak-Rothstein, A. (1995). Fas(CD95)/FasL interactions required for programmed cell death after T cell activation. Nature *373*, 444–448.

Kotzin, B. L., Babcock, S. K., and Herron, L. R. (1988). Deletion of potentially self-reactive T cell receptor specificities in L3T4-, Lyt2- T cells of lpr mice. J. Exp. Med. *168*, 2221–2229.

Liu, Z.-G., Smith, S. W., McLaughlin, K. A., Schwartz, L. M., and Osborne, B. (1994). Apoptotic signals delivered through the T cell receptor of a T cell hybrid require the immediate-early gene nur77. Nature *367*, 281–284.

Mamalaki, C., Norton, T., Tanaka, Y., Townsend, A. R., Chandler, P., Simpson, E., and Kioussis, D. (1992). Thymic depletion and peripheral activation of class I major histocompatibility complex–restricted T cells by soluble peptide in T cell receptor transgenic mice. Proc. Natl. Acad. Sci. USA *89*, 11342–11346.

Mamalaki, C., Elliott, J., Norton, T., Yannoutsos, N., Townsend, A. R., Chandler, P., Simpson, E., and Kioussis, D. (1993). Positive and negative selection in transgenic mice expressing a T cell receptor specific for influenza nucleoprotein and endogenous superantigen. Dev. Immunol. *3*, 159–174.

Matthias, P., Muller, M. M., Schreiber, E., Rusconi, S., and Schaffner, W. (1989). Eukaryotic expression vectors for the analysis of mutant proteins. Nucl. Acids Res. *17*, 6418.

Milbrandt, J. (1988). Nerve growth factor induces a gene homologous to the glucocorticoid receptor gene. Neuron *1*, 183–188.

Nagata, S., and Golstein, P. (1995). The Fas death factor. Science *267*, 1449–1455.

Nossal, G. J. V. (1994). Negative selection of lymphocytes. Cell *76*, 229–239.

Paulsen, R. E., Weaver, C. A., Fahrner, T. J., and Milbrandt, J. (1992). Domains regulating transcriptional activity of the inducible orphan receptor NGFI-B. J. Biol. Chem. *267*, 16491–16496.

Ramsdell, F., Jenkins, M., Dinh, Q., and Fowlkes, B. J. (1991). The majority of $CD4^+CD8^-$ thymocytes are functionally immature. J. Immunol. *147*, 1779–1785.

Robey, E., and Fowlkes, B. J. (1994). Selective events in T cell development. Annu. Rev. Immunol. *12*, 675–705.

Ryseck, R. P., Macdonald, B. H., Mattei, M. G., Ruppert, S., and Bravo, R. (1989). Structure, mapping and expression of a growth factor inducible gene encoding a putative nuclear hormonal binding receptor. EMBO J. *8*, 3327–3335.

Savill, J., Fadok, V., Henson, P., and Haslett, C. (1993). Phagocyte recognition of cells undergoing apoptosis. Immunol. Today *14*, 131–136.

Singer, G. G., and Abbas, A. K. (1994). The fas antigen is involved in peripheral but not thymic deletion of T lymphocytes in T cell receptor transgenic mice. Immunity *1*, 365–371.

Singer, P. A., Balderas, R. S., McEvilly, R. J., Bobardt, M., and Theofilopoulos, A. N. (1989). Tolerance-related Vbeta clonal deletion in normal $CD4^-CD8^-$, TCR^- alphabeta$^+$ and abnormal lpr and gld cell population. J. Exp. Med. *170*, 1869–1877.

Surh, C. D., and Sprent, J. (1994). T cell apoptosis detected in situ during positive and negative selection in the thymus. Nature *372*, 100–103.

Swat, W., Dessing, M., von Boehmer, H., and Kisielow, P. (1993). CD69 expression during selection and maturation of $CD4^+CD8^+$ thymocytes. Eur. J. Immunol. *23*, 739–746.

Veis, D. J., Sorenson, C. M., Shutter, J. R., and Korsmeyer, S. J. (1993). Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell *75*, 229–240.

von Boehmer, H. (1994). Positive selection of lymphocytes. Cell *76*, 219–228.

Wilson, A., Day, L. M., Scollay, R., and Shortman, K. (1988). Subpopulations of mature murine thymocytes: properties of $CD4^-CD8^+$ and $CD4^+CD8^-$ thymocytes lacking the heat-stable antigen. Cell. Immunol. *117*, 312–326.

Wilson, T. E., Fahrner, T. J., Johnston, M., and Milbrandt, J. (1991). Identification of the DNA binding site for NGFI-B by genetic selection yeast. Science *252*, 1296–1300.

Wilson, T. E., Paulsen, R. E., Padgett, K. A., and Milbrandt, J. (1992). Participation of non-zinc finger residues in DNA binding by two nuclear orphan receptors. Science *256*, 107–110.

Wilson, T. E., Fahrner, T. J., and Milbrandt, J. (1993a). The orphan receptors NGFI-B and steroidogenic factor 1 establish monomer binding as a third paradigm of nuclear receptor–DNA interaction. Mol. Cell. Biol. *13*, 5794–5804.

Wilson, T. E., Padgett, K. A., Johnston, M., and Milbrandt, J. (1993b). A genetic method for defining DNA-binding domains: application to the nuclear receptor NGFI-B. Proc. Natl. Acad. Sci. USA *90*, 9186–9190.

Woodland, D., Happ, M. P., Bill, J., and Palmer, E. (1990). Requirement for cotolerogenic gene products in the clonal deletion of I-E reactive T cells. Science *247*, 964–967.

Woronicz, J. D., Calnan, B., Ngo, V., and Winoto, A. (1994). Requirement for the orphan steroid receptor Nur77 in apoptosis of T cell hybridomas. Nature *367*, 277–281.

Yoon, J. K., and Lau, L. F. (1993). Transcriptional activation of the inducible nuclear receptor gene nur77 by nerve growth factor and membrane depolarization in PC12 cells. J. Biol. Chem. *268*, 9148–9155.

Zhou, T., Bluethmann, H., Eldridge, H., Brockhaus, M., Berry, K., and Mountz, J. D. (1991). Abnormal thymocyte development and production of autoreactive T cells in T cell receptor transgenic autoimmune mice. J. Immunol. *147*, 466–474.