Inhibition of Nur77/Nurr1 Leads to Inefficient Clonal Deletion of Self-Reactive T Cells

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Summary

The Nur77/Nurr1 family of DNA binding proteins has been reported to be required for the signal transduction of CD3/T cell receptor (TCR)–mediated apoptosis in T cell hybridomas. To determine the role of this family of DNA-binding proteins in thymic clonal deletion, transgenic (Tg) mice bearing a dominant negative mutation were produced. The transgene consisted of a truncated Nur77 (ΔNur77) gene encoding the DNA-binding domain of Nur77 ligated to a TCR-β enhancer resulting in early expression in thymocytes. Apoptosis of $CD4^+CD8^+$ thymocytes mediated by CD3/TCR signaling was greatly inhibited in the ΔNur77 Tg mice, compared with non-Tg littermates, after treatment with anti-CD3 or anti-TCR antibody in vivo and in vitro. Clonal deletion of self-reactive T cells was investigated in ΔNur77-D^b/HY TCR-α/β double Tg mice. There was a five-fold increase in the total number of thymocytes expressing self-reactive D^b/HY TCR-α/β in the ΔNur77–TCR-α/β double Tg male mice. Deficient clonal deletion of self-reactive thymocytes was demonstrated by a 10-fold increase in the $CD4^+CD8^+$ thymocytes that expressed Tg TCR-α/β. There was an eight-fold increase in $CD8^+$, D^b/HY TCR-α/β T cells in the lymph nodes (LN) of ΔNur77-D^b/HY TCR-α/β double Tg compared with D^b/HY TCR-α/β Tg male mice. In spite of defective clonal deletion, the T cells expressing the Tg TCR were functionally anergic. In vivo analysis revealed increased activation and apoptosis of T cells associated with increased expression of Fas and Fas ligand in LN of ΔNur77-D^b/HY TCR-α/β double Tg male mice. These results indicate that inhibition of Nur77/Nurr1 DNA binding in T cells leads to inefficient thymic clonal deletion, but T cell tolerance is maintained by Fas-dependent clonal deletion in LN and spleen.

Clonal deletion and clonal anergy are the primary mechanisms for induction of self-tolerance in T cells (1). During thymic maturation, thymocytes bearing self-reactive TCR undergo clonal deletion and are eliminated by programmed cell death or apoptosis (2, 3). Thymocytes with intermediate to high density levels of expression of the TCR undergo negative selection at the $CD4^+CD8^+$ (double positive [DP][1]) stage of thymocyte development (4–8). Downmodulation of the TCR, CD4, and/or CD8 molecules on the surface of T cells has been proposed as a mechanism for escape from negative selection (8–12). Thus, the TCR generates signals that are capable of mediating negative selection of thymocytes by clonal deletion. However, the signaling mechanisms required for negative selection of thymocytes remain unknown.

Several molecules and pathways known to be of importance in apoptosis have been described in the thymus; however, their contribution to clonal deletion and tolerance induction remains controversial (1). Although knockout of p53 leads to decreased sensitivity of murine thymocytes to radiation-induced apoptosis, negative selection remains intact (13–15). Fas is a cell surface receptor that mediates apoptosis by interaction with a specific ligand and is expressed on most murine thymocytes (16–19). Al-

[1]*Abbreviations used in this paper:* AICD, activation induced cell death; BrdU, bromodeoxyuridine; ΔNur77, dominant negative mutation of Nur77; DP, double positive; FasFP, Fc-Fas fusion protein; M33, D^b/HY TCR anticlonotypic mAb; NBRE, NGFI-B response element; NGF, nerve growth factor; Tg, transgenic; Tg71, TCR transgenic mice expressing the D^b/HY TCR; TINUR, transcriptionally inducible nuclear receptor; TUNEL, terminal digoxigenin nucleotide endlabeling.

though mutant Fas antigen and Fas ligand cause autoimmune disease in *lpr/lpr* and *gld/gld* mice, respectively (18–20), no major negative selection defects have been found in *lpr/lpr* mice (21–25). Therefore, it is unlikely that Fas antigen is directly involved in negative selection in the thymus.

Bcl-2 prevents thymocyte apoptosis that is induced by radiation, steroids, and other chemicals (26–28). Expression of *bcl-2* has been reported to be decreased in $CD4^+8^+$ thymocytes, but not in mature thymocytes, and has been proposed to play a role in inhibition of negative selection in the thymus (29). However, *bcl-2* knockout mice do not exhibit excessive clonal deletion in the thymus (30) and, conversely, *bcl-2* transgenic (Tg) mice do not exhibit a major defect in negative selection or T cell tolerance (28, 31–33). Double Tg *bcl-2*+D^b/HY TCR mice show that constitutive expression of *bcl-2* increases the survival of thymocytes in the absence of positive selection (34–36). The *bcl-2* transgene also reduces the efficiency of negative selection, but the mature peripheral T cells that appear in increased numbers are not autoreactive. Thus, although *bcl-2* can play a role in both positive and negative selection, tolerance is maintained by a mechanism that can bypass *bcl-2*.

Nerve growth factor (NGF)I-B/Nur77 is a growth factor–inducible member of the steroid/thyroid hormone receptor superfamily originally identified in NGF-treated P12 pheochromocytoma cells (37) and in serum-stimulated fibroblasts (38). NGFI-B/Nur77 is transcriptionally regulated as an immediate-early gene and is rapidly activated by phosphorylation after stimulation with serum or NGF (39, 40). NGFI-B/Nur77 has a centrally located, highly conserved DNA-binding domain containing two zinc fingers and a transcriptional *trans*-activating domain (41–47). NGFI-B/Nur77 gene is expressed in thymic medulla and is rapidly upregulated in T cell hybridomas and thymocytes after treatment with anti-CD3 or anti-TCR, and this expression has been correlated with induction of apoptosis (48, 49). Blocking of NGFI-B with either a dominant negative truncated (48) or antisense (49) NGFI-B/Nur77 gene prevented TCR/CD3 signaling-mediated apoptosis in T cell hybridomas. There are at least two gene families with an identical NGFI-B response element (NBRE) AAAGGTCA (50). The first member of this family, referred to as Nur77 (mouse), NGFI-B (rat), and NAK-1 (human) peaks 1 h after stimulation of the PEER T cell line. The second member, referred to as Nurr1 (mouse), regenerating liver nuclear receptor-1 (rat), and transcriptionally inducible nuclear receptor (TINUR [human]) peaks 24 h after stimulation and correlates with apoptosis. Although Nur77 has been shown to be important in T cell signaling and apoptosis (48, 49), other signaling proteins can also contribute to T cell maturation and apoptosis. This was recently demonstrated in a Nur77 mutant mouse, in which T cells did not exhibit defective apoptosis after anti-CD3 cross-linking and exhibited normal T cell development and apoptosis in D^b/HY TCR Tg mice (51). The present experiments utilize a truncated Nur77 transgene capable of expressing high levels of a nonfunctional DNA-binding protein to block the NBRE in T cells. The results indicate that the truncated form of the dominant negative mutation of Nur77 (ΔNur77) gene binds to the NBRE, inhibits TCR/CD3 signaling-mediated apoptosis, and also interferes with selection and clonal deletion in the thymus of ΔNur77-D^b/HY TCR double Tg mice.

Materials and Methods

Production of Tg Mice Expressing the Truncated Nur77 Gene. The mouse Nur77 1,200-bp cDNA fragment corresponding to nucleotides (nt) 794-1993 and encoding the truncated mouse Nur77 at residues from 229 for Met to 601 for Phe was amplified by reverse transcription, using normal mouse thymus RNA as a template. The primers used for PCR were 5′-*CCACC*ATGCCAGCAGCTTTC-3′ and 5′-*GGATCC*GTGGGCTATAGGCT-3′ (complementary to nt 1977-1993). The italicized nucleotides are not derived from the mouse Nur77 cDNA sequence but are added to a eukaryotic translation consensus sequence (underlined) in front of a AUG for Met or constitute the sequence (underlined) for the BamHI restriction site. The amplified Nur77 cDNA fragment was directly subcloned into PCR TA vector (Invitrogen, San Diego, CA). The insert Nur77 cDNA was confirmed by a standard DNA sequencing technique. The truncated Nur77 capable of binding to the homologous Nur77 DNA response element was excised with BamHI and cloned into p913CβE1E vector (52, 53). The TCR β chain enhancer has been demonstrated to result in T cell–specific expression. The expression constructed was excised and used to produce Tg mice as previously described (53). TCR-α/β TCR Tg mice reactive with D^b/HY antigen (Tg71) were obtained as previously described (54) and backcrossed with the ΔNur77 Tg mice.

Southern Blot Analysis of the ΔNur77 Transgene. Tail DNA was prepared and digested with the indicated restriction enzymes. Approximately 10 μg of the digested DNA was separated on a 0.7% agarose gel, blotted to a nylon membrane, and hybridized with a ^{32}P-labeled full-length Nur77 cDNA probe.

Nuclear Extract Preparation. Nuclear extract was prepared from single cell suspensions of thymocytes of ΔNur77 Tg mice and of mice having no transgene, respectively, as described (55). Thymocytes were cultured in media or stimulated with 100 ng/ml PMA and 500 μg/ml ionomycin (Calbiochem-Novabiochem Corp., San Diego, CA) for 6 h. The protein concentration was determined by bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL), and aliquots were frozen at −80°C until an electrophoretic mobility shift assay was performed.

Electrophoretic Mobility Shift Assay. Oligonucleotides containing the NBRE were synthesized at the University of Alabama at Birmingham (Oligonucleotide Core Facility). They are sense strand 5′-GGAGTTTTAAAAGGTCATGCTCAATTT-3′ and antisense strand 5′-GGAAATTGAGCATGACCTTTTAAAACT-3′ (44). The sense and antisense strands were mixed in equal molar amounts, annealed by heating to 100°C for 2 min and slowly cooled at 37°C for 4 h; 20 ng of the double stranded oligonucleotides were endlabeled with α-[^{32}P]dCTP by the Klenow fragment reaction. The unincorporated α-[^{32}P]dCTP was removed by passing the reaction through a sodium-tris-EDTA select-D G25 column (5 prime →3 prime, Inc., Boulder, CO). A 0.1-ng (~20,000 cpm) radioactive probe was added to 20 μg of extract protein solution, which had been mixed with 2 μg of poly(dI-dC) and reaction buffer (10 mM Tris, pH 7.5, 1 mM dithiothreitol, 100 mM KCl, 1 mM EDTA, 0.2 mM PMSF, 1 mg/ml BSA, and 5% glycerol) at 25°C for 10 min (56). In the competition as-

say, a 100-fold excess of cold (unlabeled) probe was added to the radiolabeled probe before they were placed into the reaction mixture. After incubation at 25°C for 20 min, the samples were fractionated on a nondenaturing polyacrylamide gel in 0.25 tris borate EDTA, 5% glycerol. After electrophoresis, the gel was dried by vacuum and autoradiography was carried out at −70°C overnight.

Northern Blot Analysis. Total RNA was isolated from the thymus, transferred onto nylon nitrocellulose membranes, and probed with a 1.2-kb Nur77 cDNA fragment or β-actin as control.

Antibodies. Anti-CD3 (clone 145.2C11), anti-CD4 (clone GK 1.5), anti-CD8 (clone 53-47), anti-TCR (clone H57), and anti-Fas (clone Jo2) were purchased from PharMingen (San Diego, CA). The D^b/HY TCR anticlonotypic mAb M33 was produced as previously described (24).

Induction of Apoptosis In Vivo and In Vitro. Thymocyte apoptosis was induced in vivo by daily injection of 50 μg i.p. anti-CD3 or anti-TCR antibody for 3 d and was analyzed 12 h after the last dose, or it was induced by a single injection of 10 μg i.p. dexamethasone and analyzed 12 h later. For in vitro induction of apoptosis, the thymocytes were incubated for different time periods on 6-well flat bottom plates (Corning, Corning, NY) that had been coated with either 10 μg/ml anti-CD3 or cultured in the presence of 10 μM dexamethasone (Sigma Chemical Co., St. Louis, MO).

Expression of CD3ζ. Single cell suspensions of thymocytes were cultured in 6-well flat bottom plates with either anti-CD3 or control antibody for 5 min followed by protein extract preparation. Cells (10^6) were lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 0.5% Triton X–100, 1 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) and equivalent amounts of total cellular protein lystates (20 μg) were separated on 10% SDS-polyacrylamide gels, blotted to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA), and incubated with antiphosphotyrosine antibody. The blots were counterstained with goat anti–mouse IgG conjugated with alkaline phosphatase, and incubated with nitro blue tetrazolium (17 mg/ml) and 5-bromo-4-chloro-3-indolylphosphate (33 mg/ml) substrate in 0.1 M Tris-HCl (pH 9.5) (Sigma Chem. Co.).

T Cell–mediated Cytotoxicity. Peripheral $CD8^+$ T cells were purified from LN cells using a murine CD8 T cell enrichment column (R&D Systems, Inc., Minneapolis, MN). The purified $CD8^+$ T cells were stimulated with irradiated spleen cells obtained from C57BL/6 male mice in the presence of 50 U/ml of IL-2 for 3 d. Viable cells were collected by centrifugation over Ficoll. Con A–stimulated spleen cells from C57BL/6 male or female mice were labeled with ^{51}Cr and mixed with stimulated $CD8^+$ T cells at the indicated ratio. After incubation for 8 h, the ^{51}Cr released in the supernatants was measured using a gamma counter (Packard Industries, Meriden, CT) and the specific release calculated using the standard method.

Assay for Fas Ligand Activity. Unstimulated LN T cells were incubated with ^{51}Cr-labeled Fas^+ C57BL/6-+/+ A20 target cells at different effector/target ratios in the presence and absence of 10 μg/ml of a Fc-Fas fusion protein (FasFP, 57). Release of ^{51}Cr into the supernatant was assessed 8 h later using a gamma counter. The specific release was calculated using standard methods.

Proliferative Response of D^b/HY-reactive T Cells. Peripheral $CD8^+$ T cells, purified as described above, were cultured in the presence of 50 U/ml of murine IL-2 (Genzyme Corp., Cambridge, MA) for the indicated time with irradiated (3,300 rad) syngeneic spleen cells obtained from C57BL/6 male or female mice. For measurement of proliferation, 1 μCi of [^{3}H]thymidine was added at 16 h before harvest.

Flow Cytometry Analysis. Single cell suspensions of thymocytes or LN cells were labeled with optimal concentrations of FITC-conjugated anti-CD8, PE-conjugated anti-CD4, or PE-conjugated anti-Fas (PharMingen) and biotin-conjugated M33 (24) followed by Tandem-Strepavidin. Viable cells (10,000/sample) were analyzed by flow cytometry on a FACScan® (Becton Dickinson & Co., Mountain View, CA) equipped with logarithmic scales and the data processed in a Hewlett-Packard (Palo Alto, CA) computer. The number of cells in each population was

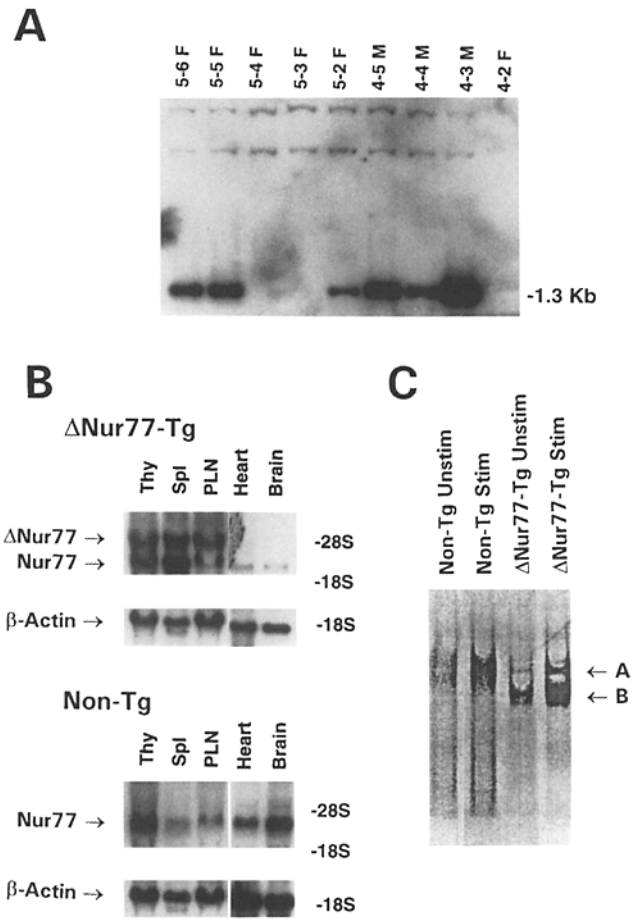


Figure 1. Characterization of ΔNur77 Tg mice. (*A*) Detection of the transgene by Southern blot analysis. Southern blot analysis of tail DNA carried out after digestion with BamHI revealed six lines of ΔNur77 Tg mice. BamHI digestion liberated a 1.3-kb cDNA corresponding to the Nur77 DNA-binding domain and also higher molecular weight endogenous genomic Nur77 DNA fragments at ~8 and 6 kb. Different lines of Tg founder mice are designated according to sex (*M*, male; *F*, female) as 4-3M, 4-4M, 4-5M, 5-2F, 5-5F, and 5-6F. Experiments were carried with the line drive from 4-4M. (*B*) Expression of the truncated ΔNur77 RNA transcript in ΔNur77 Tg mice. RNA was prepared from thymus (*Thy*), spleen (*Spl*), peripheral LN (*PLN*), heart, and brain of ΔNur77 Tg mouse (line 4-4M) or non-Tg control mice. Non-Tg control mice expressed a full-length Nur77 transcript with a mol wt of ~3.7 kb in the lymphoid organs as well as nonlymphoid organs. ΔNur77 Tg mice expressed the 3.7-kb endogenous Nur77 transcript as well as an ~5.5-kb ΔNur77 transcript in tissue containing T cells. (*C*) Gel shift assay of Nur77 protein. The NBRE was endlabeled and incubated with the nuclear extract derived from unstimulated and stimulated thymocytes of non-Tg or ΔNur77 Tg mice. Non-Tg and ΔNur77 Tg mice exhibited an identical size higher gel shift band (A). ΔNur77 Tg mice also exhibited a unique lower gel shift band (B).

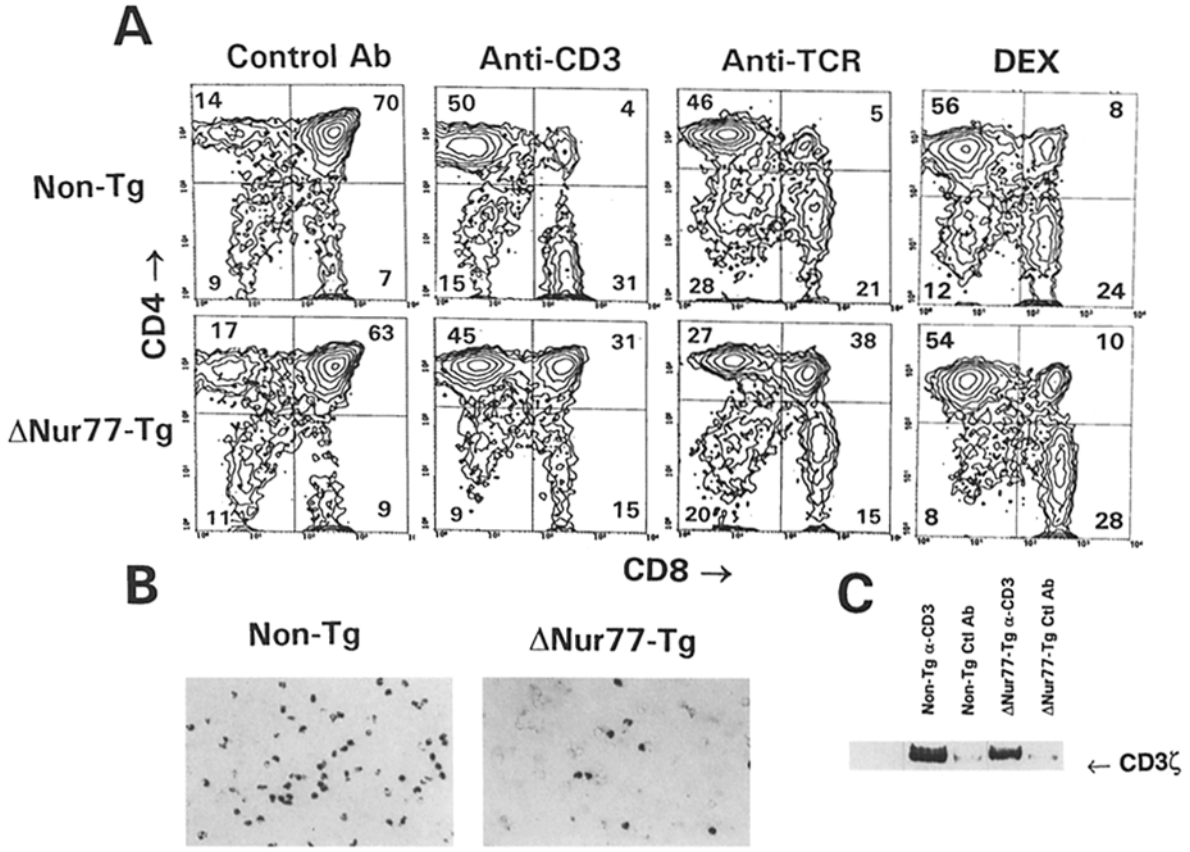


Figure 2. Decreased deletion of $CD4^+8^+$ thymocytes after anti-CD3 or anti-TCR treatment in ΔNur77 Tg mice. Non-Tg or ΔNur77 Tg mice were injected with 50 mg i.p. of either control or anti-CD3 or anti-TCR antibody daily for 3 d and analyzed on day 4. Non-Tg or ΔNur77 Tg mice were injected with dexamethasone (10 μM i.p.) and analyzed 12 h later. (*A*) Flow cytometric analysis of thymocyte subpopulation. Thymocyte suspensions were labeled with anti-CD4 or anti-CD8 and analyzed by flow cytometry analysis. Percent thymocytes in each of the CD4 and CD8 subpopulations as defined by the cursors are shown on the graph. These data represent a typical flow cytometry analysis that was carried out using at least five different mice. (*B*) TUNEL staining of the thymocytes from anti-CD3–treated non-Tg and ΔNur77 Tg mouse. The thymocytes were prepared as above. 10^5 thymocytes were cytospun onto the slide and stained for apoptosis by the TUNEL technique. (*C*) Tyrosine phosphorylation of the CD3ζ chain after anti-CD3 cross-linking. Single cell suspensions of thymocytes were incubated with 10 ug/ml of either control antibody or anti-CD3 antibody for 5 min, followed by Western blot analysis of CD3ζ using an antiphosphotyrosine antibody.

determined by quadrant analysis of contour graphs. 10,000 viable cells were analyzed by FACScan®.

Apoptosis Analysis by Terminal Digoxigenin Nucleotide Endlabeling staining. The in situ nick translation method of DNA staining was used for in situ determination of apoptotic cells according to the published method, with slight modifications (58). Briefly, 10^5 cells were cytospun onto poly-L-lysine pretreated slides, fixed in 10% formalin for over 30 min, and the cells subjected to proteinase K digestion (10 μg/ml at room temperature for 20 min). After extensive washing with ddH_2O, a reaction mix containing 0.5 μm/ml TdT, 10 μM digitonigen modified-dUTP, and TdT buffer was applied to the slide. The slides were incubated at 37°C for 1 h. The poly-dUTP tail, which was synthesized at the broken ends of DNA, was detected by alkaline phosphatase–conjugated antidigitonigen antibody and NBT/BCIP substrate. At least 200 cells were counted using light microscopy.

Bromodeoxyuridine Incorporation In Vivo and In Situ Staining. Bromodeoxyuridine (BrdU) was purchased from Sigma Chemical Co. and was diluted to 5 mg/ml in PBS (59). Mice received i.p. injections with 1 mg BrdU/mouse at 6-h intervals for four doses and were killed 1 h after the last injection when LN were removed and frozen in OCT. After frozen section, the slides were fixed in ice-cold ethanol (70%) for 20 min, and the DNA denatured by incubation at room temperature for 20 min in 3 N HCl with 0.5% Tween 20 (Sigma Chemical Co.). The slides were then incubated 3–5 min with 0.5 ml 0.1 M sodium borate buffer, pH 8.5, followed by two further washes in PBS. The slides were then incubated at room temperature for 30 min with FITC-conjugated anti-BrdU mAb (Boehringer Mannheim, Indianapolis, IN), washed in PBS, and mounted. Slides were examined and photographed using an Argon ion laser scanning confocal microscope (model 1000; Molecular Dynamics, Sunnyvale, CA).

Results

Production of ΔNur77 Tg Mice. Six lines of mice carrying ΔNur77 Tg DNA were produced (Fig. 1 *A*). Digestion of tail DNA with BamHI revealed the expected truncated Nur77 fragment of 1.3 kb, which contained the DNA-binding domain without the transactivation domain, as well as the genomic Nur77 bands of higher molecular weight. The transgene copy number varied from approximately 3 in the line designated 5-2F to 10 in the Tg line designated 4-3M (Fig. 1 *A*). Expression of the truncated ΔNur77 gene in the Tg mice was analyzed by Northern

Table 1. *Comparison of Thymocyte Deletion after Anti-CD3 Treatment in ΔNur77 Tg and non-Tg Control Mice*

	Non-Tg					ΔNurr77 Tg				
Treatment	Total	DN	DP	$CD4^+$	$CD8^+$	Total	DN	DP	$CD4^+$	$CD8^+$
Control Ab	114 ± 10	5.2 ± 2.2	84 ± 12	12 ± 2.3	8.4 ± 1.5	128 ± 12	13 ± 2.4	82 ± 14	21 ± 4	14 ± 3
Anti-CD3	16 ± 21.8	3.5 ± 1.5	0.8 ± 0.3	8.0 ± 1.5	4.8 ± 2.1	68 ± 8	8.1 ± 1.5	30 ± 5	19 ± 6	11 ± 2.5
Percent deletion	86	33	99	33	43	50	38	63	10	24

blot analysis (Fig. 1 *B*). Non-Tg control mice expressed a Nur77 mRNA transcript of ~3.7 kb in both lymphoid and nonlymphoid organs. ΔNur77 Tg mice expressed an additional transcript of 5.5 kb, corresponding to the truncated Nur77 gene, and a minigene including the CTβ enhancer. The transgene transcript was expressed highly in the thymus, spleen, and LN, but was not expressed in the heart or brain. This result indicated that the expression of ΔNur77 controlled by the CTβ enhancer was confined to the lymphoid organs. To determine the ability of ΔNur77 protein to bind the corresponding DNA-binding motif and compete with endogenous Nur77 protein, gel shift assays were performed with a double-strained oligonucleotide containing the Nur77-binding motif, NBRE. Nuclear extracts were prepared from the thymocytes of non-Tg and ΔNur77 Tg mice with and without stimulation with PMA plus ionomycin. In the unstimulated thymocytes from non-Tg mice, there was only minimal expression of the endogenous Nur77 DNA-binding protein indicated by a single high molecular weight gel shift band (Fig. 1 *C*). The expression of endogenous Nur77 protein was greatly increased at 6 h after stimulation. In unstimulated thymocytes of ΔNur77 Tg mice, there was high expression of the ΔNur77 protein, as demonstrated by a lower molecular weight gel shift band. After PMA plus ionomycin stimulation, there was increased expression of both the endogenous Nur77 gel shift band (band A) and the ΔNur77 gel shift band (band B). Both of these gel shift bands were the result of specific interaction with the NBRE because the binding could be competitively inhibited by addition of increasing concentrations of unlabeled DNA containing the NBRE but not by an irrelevant DNA oligonucleotide (data not shown). These results indicate that RNA encoding the truncated ΔNur77-binding protein is specifically expressed in the T cells of the Tg mice, and that the truncated ΔNur77 protein produced in ΔNur77 Tg mice competitively inhibits the binding of endogenous Nur77 to the NBRE.

Inhibition of Anti-TCR/CD3–mediated Thymocyte Apoptosis in ΔNur77 Tg Mice. There was no significant abnormality in the development of thymocytes in ΔNur77 Tg mice (Fig. 2 *A* and Table 1). To characterize the function of the ΔNur77 transgene, we examined whether apoptosis induced by CD3/TCR signaling was impaired in thymocytes of ΔNur77 Tg mice. Non-Tg and ΔNur77 Tg mice were treated in vivo with control, anti-CD3, or anti-TCR antibodies. Anti-CD3 antibody treatment led to depletion of 86% of the total and 99% of the DP thymocytes in non-Tg mice (Table 1) and reduced the percentage of DP thymocytes to 4% (Fig. 2 *A*). In contrast, anti-CD3–induced deletion of thymocytes was less efficient in ΔNur77 Tg mice, with depletion of 50% of the total and 63% of the DP thymocytes (Table 1) and reduced the percentage of DP thymocytes to 31% (Fig. 2 *A*). Depletion of $CD4^+$ and $CD8^+$ thymocytes was also inhibited after CD3 antibody treatment of ΔNur77 Tg mice, whereas deletion of double negative (DN) thymocytes was equivalent in both non-Tg and ΔNur77 Tg mice (Table 1). Although anti-CD3–induced apoptosis was inhibited in ΔNur77 Tg mice, not all pathways of thymocyte apoptosis were inhibited since there was no significant difference in dexamethasone-induced thymocyte deletion comparing non-Tg and ΔNur77

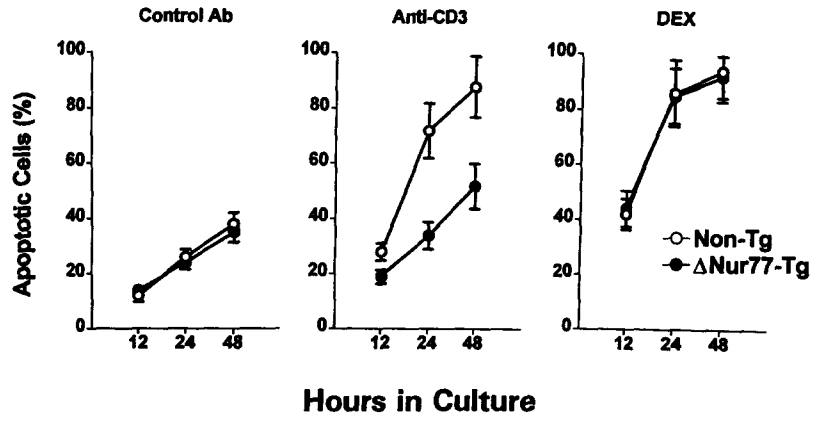
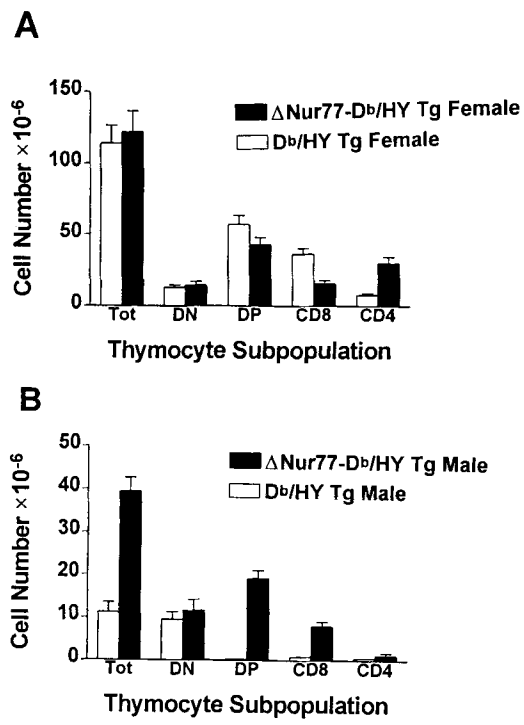


Figure 3. Thymocyte apoptosis induced by anti-CD3 and dexamethasone in vitro. The thymocytes were prepared from non-Tg and ΔNur77 Tg mice. 2×10^6/ml thymocytes were cultured with plate-bound anti-CD3 or control hamster IgG (10 μg/ml) or 10 μM dexamethasone for the indicated time. Apoptosis was determined by TUNEL staining. (○) Non-Tg and (●) ΔNur77 Tg mice.



Tg mice (Fig. 2 *A*). Cell sorting and terminal digoxigenin nucleotide endlabeling (TUNEL) staining verified that anti-CD3 treatment in vivo resulted in extensive apoptosis of thymocytes (Fig. 2 *B*). To determine whether the ΔNur77 transgene interfered with early signaling events after CD3/TCR stimulation, tyrosine phosphorylation of the CD3ζ chain was examined in the peripheral T cells after anti-CD3 cross-linking. There was no significant difference between the extent of tyrosine phosphorylation of the CD3ζ chain in the T cells of both non-Tg and ΔNur77 Tg mice (Fig. 2 *C*).

To determine if the inhibition of anti-CD3–induced depletion of thymocytes by the ΔNur77 transgene was specific for CD3/TCR signaling, or is a nonspecific effect related to an in vivo stress response induced by anti-CD3 treatment, thymocytes obtained from non-Tg and ΔNur77

Figure 4. Effect of ΔNur77 transgene on thymocyte subpopulations. D^b/HY TCR single Tg female and male mice or ΔNur77-D^b/HY double Tg female and male mice were analyzed for expression of CD4 and CD8. The numbers of thymocytes in each subpopulation were derived by multiplying the percent thymocytes in each subpopulation by the total number of thymocytes. These are representative of typical results for at least three mice analyzed individually.

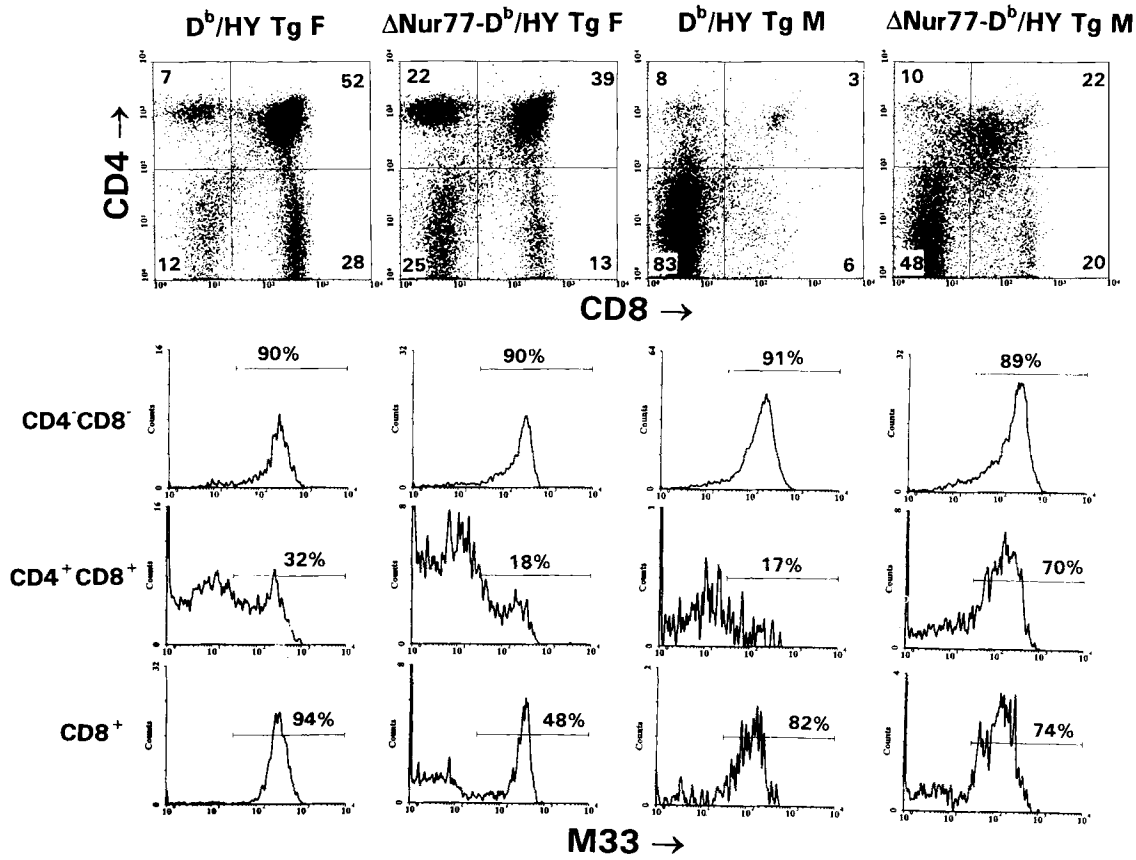


Figure 5. Decreased deletion of $M33^+CD4^+CD8^+$ thymocytes in ΔNur77-D^b/HY TCR double Tg male mice. D^b/HY TCR single Tg female or male mice or ΔNur77-D^b/HY double Tg female or male mice were analyzed for expression of CD4, CD8, and the anti-TCR transgene mAb M33. Percent thymocytes expressing high levels of M33 is shown by the cursor. These are representative of typical results for at least three mice analyzed individually.

Tg mice were cultured in vitro for different lengths of time with either anti-CD3 or dexamethasone (Fig. 3). There was no difference in the numbers of thymocytes undergoing dexamethasone-induced apoptosis, whereas the thymocytes from ΔNur77 Tg mice exhibited decreased apoptosis when cultured with anti-CD3 (Fig. 3). This result strengthens the in vivo data indicating that the inhibition of apoptosis of $CD4^+CD8^+$ thymocytes in ΔNur77 Tg mice was specific for apoptosis induced by CD3/TCR signaling.

Decreased Deletion of $CD4^+8^+$ Thymocytes in ΔNur77-D^b/HY TCR Double Tg Male Mice. The D^b/HY TCR Tg male mouse has been extensively analyzed as a model for analysis of positive and negative selection. The D^b/HY-reactive thymocytes exhibit extensive positive selection in D^b/HY TCR Tg female mice and are extensively deleted at the DP stage of thymocyte development in D^b/HY TCR Tg male mice (60–63). To determine whether interruption of Nur77/Nurr1 function in ΔNur77 Tg mice leads to defective positive and negative selection of thymocytes, H-2^b ΔNur77 Tg mice were backcrossed to D^b/HY TCR Tg C57BL/6 mice. In ΔNur77-D^b/HY TCR double Tg female mice, there were equal numbers of total and DN thymocytes compared with D^b/HY TCR Tg female mice. The ΔNur77 transgene resulted in a significant decrease in the number of DP and $CD8^+$ thymocytes expressing the D^b/HY-reactive Tg TCR (detected by the mAb M33) and a significant increase in the number of CD4 thymocytes expressing an endogenously rearranged TCR (Figs. 4 *A* and 5). Flow cytometric analysis indicated that in ΔNur77-D^b/HY TCR double Tg female mice, only 18% of DP and 48% of CD8 thymocytes expressed the Tg TCR compared with 32 and 94% in D^b/HY TCR Tg female mice in the absence of the ΔNur77 transgene (Fig. 5). This result suggested that the ΔNur77 transgene may have an inhibitory effect on positive selection of thymocytes in female TCR Tg mice.

D^b/HY TCR Tg male mice exhibited a 10-fold decrease in total thymocyte number and nearly complete deletion of DP and $CD8^{bright}$ thymocytes due to negative selection of thymocytes bearing the Tg TCR. Several changes were observed in the thymus of the C57BL/6 ΔNur77-D^b/HY TCR double Tg male mice compared with that of D^b/HY TCR Tg male mice. First, there was a fourfold increase in total thymocytes that comprised a 20-fold increase in $CD4^{low}$ $CD8^{low}$ DP thymocytes and a fivefold increase in CD8 thymocytes (Fig. 4 *B*). Second, increased numbers of DP and CD8 thymocytes in ΔNur77-D^b/HY TCR Tg male mice expressed the Tg TCR. 70% of $CD4^+CD8^+$ thymocytes were also M33 positive in the ΔNur77-Tg71 double Tg male compared with 17% in D^b/HY TCR Tg male mice (Fig. 5). Third, in ΔNur77-D^b/HY TCR Tg male mice, there was the appearance of a substantial number of CD8 thymocytes that exhibited high levels of expression of CD8; 70–80% of these thymocytes expressed the Tg TCR (Fig. 5). This population was absent in the D^b/HY male mice. Taken together, these results indicate that negative

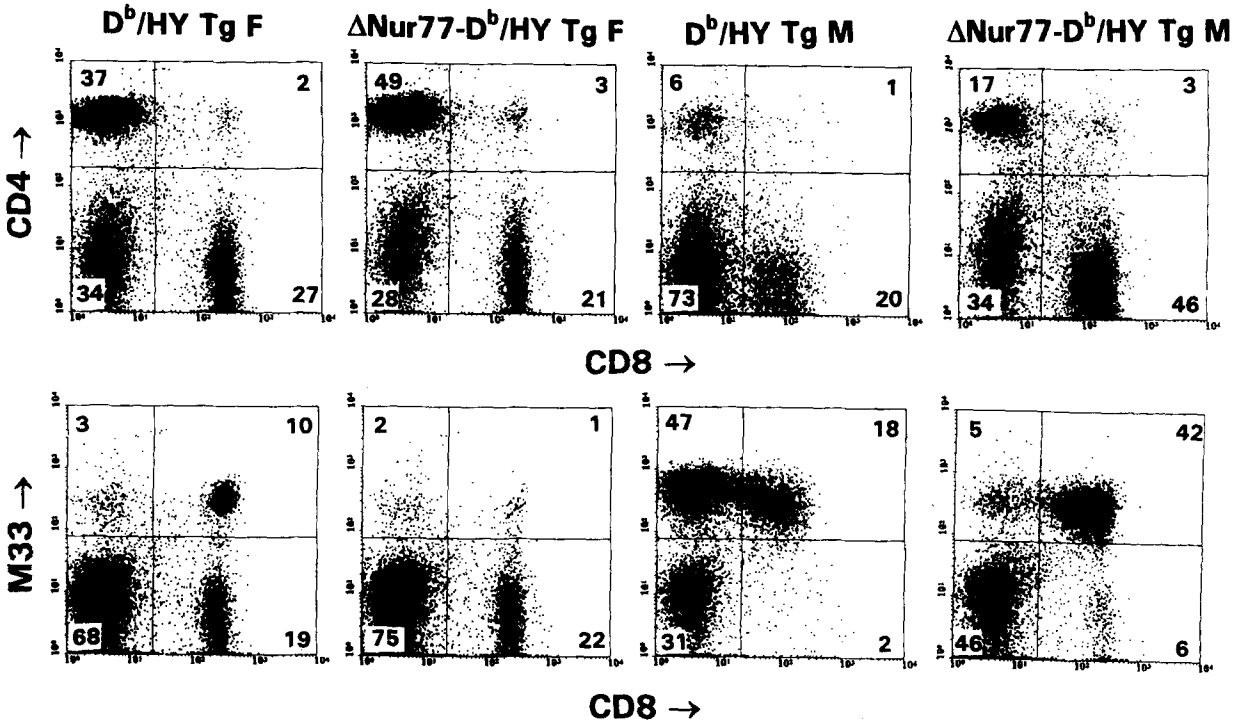


Figure 6. Increased $M33^+CD8^+$ LN T cells in the ΔNur77-D^b/HY TCR double Tg male mice. Single cell suspensions from LN were prepared from D^b/HY TCR single Tg female and male mice or ΔNur77-D^b/HY TCR double Tg male mice. Cells were labeled with anti-CD4, anti-CD8, and M33 recognizing the D^b/HY Tg TCR. Flow cytometry analysis was carried out on 10,000 viable cells. Percentage cells in each subpopulation marked by the gated cursors is indicated. These results are representative of three mice analyzed individually.

Table 2. *Phenotype of LN T Cell in ΔNur77-D^b/HY Tg Mice*

Mouse	Sex	*n*	Total	M33$^+$ CD8$^+$	M33$^+$ CD8$^-$
Non-Tg	M	5	1.9 ± 0.5*	<0.001	<0.001
ΔNur77 Tg	M	5	2.0 ± 0.4	<0.001	<0.001
D^b/HY	M	5	0.5 ± 0.1	0.09 ± 0.01	0.25 ± 0.03
ΔNur77-D^b/HY	M	5	1.4 ± 0.3	0.68 ± 0.07	0.06 ± 0.01
Non-Tg	F	5	1.8 ± 0.5	<0.001	<0.001
ΔNur77 Tg	F	5	1.9 ± 0.6	<0.001	<0.001
D^b/HY	F	5	1.5 ± 0.3	0.2 ± 0.05	0.08 ± 0.02
ΔNur77-D^b/HY	F	5	1.7 ± 0.4	0.08 ± 0.02	0.06 ± 0.01

* Total of two axillary and two inguinal LN per mouse. All mice were 8–10 wk of age. Total represents the mean ± SEM of five mice per group assayed separately.

‡ Number of LN T cells expressing either the D^b/HY TCR-α/β recognized by the anticlonotypic mAb, M33, or CD8. The number was derived by multiplying the percent gated cells after flow cytometry by the total number of LN cells. The number represents the mean ± SEM of T cells with the indicated surface marker of five mice per group assayed separately.

selection of the autospecific D^b/HY-reactive thymocytes was inhibited in ΔNur77-D^b/HY TCR compared with D^b/HY TCR Tg male mice.

Increased M33$^+$CD8$^+$ LN T Cells in the ΔNur77-D^b/HY TCR Double Tg Male Mice. To determine whether defective thymic clonal deletion leads to the escape of autospecific T cells to the periphery, the phenotype of LN T cells was analyzed. In D^b/HY female mice, most of the CD4$^+$ LN T cells in the D^b/HY TCR Tg female mice were M33$^-$ indicating expression of endogenously rearranged TCR genes (Fig. 6). Approximately 10% of LN cells were M33$^+$ and CD8$^+$. This phenotype was greatly reduced in ΔNur77-D^b/HY TCR Tg female mice in which only 1% of LN cells were CD8$^+$ and M33$^+$, whereas most CD8$^+$ T cells expressed an endogenously rearranged TCR transgene (Fig. 6). In the D^b/HY male mice, 47% of LN cells were CD4$^-$CD8$^-$ but expressed equivalent levels of the M33 TCR transgene as observed in female mice. In these mice, peripheral LN cells expressed downmodulated levels of CD8 and also expressed the D^b/HY TCR transgene (Fig. 6). In the ΔNur77-D^b/HY TCR double Tg male mice, there was a significant increase in the total number of cells, and M33$^+$CD8$^+$ LN T cells, but a decreased number of M33$^+$CD4$^-$CD8$^-$ T cells (Table 2). Compared with D^b/HY Tg male mice, most M33$^+$ T cells expressed intermediate to high levels of CD8 (Fig. 6).

Tolerance of LN T cells in ΔNur77-D^b/HY TCR Tg Male Mice. To determine if the increased numbers of CD8$^+$ M33$^+$ LN T cells in the double Tg male mice exhibited loss of tolerance, proliferation was analyzed using irradiated D^b male stimulator cells (Fig. 7 *A*). The proliferative response was greatly reduced in both D^b/HY and ΔNur77-D^b/HY male compared with female Tg mice (Fig. 7). Specific lysis was assayed using chromium-labeled D^b male target cells (Fig. 7 *B*). There was low specific lysis of target cells by LN T cells from both D^b/HY and ΔNur77-D^b/HY TCR male Tg mice compared with female Tg mice (Fig. 7 *B*). The proliferative and cytotoxic response was specific to D^b/HY antigen as there was no increased specific proliferation or lysis when D^b female cells were used as stimulators or targets (Fig. 7, *C* and *D*).

Increased In Vivo Activation and Apoptosis in the LN of ΔNur77-D^b/HY Mice. In spite of inefficient thymic clonal deletion in ΔNur77-D^b/HY Tg male mice, peripheral T cell tolerance was maintained, suggesting there are alternative mechanisms to maintain T cell tolerance. Fas- and Fas ligand–mediated apoptosis has been shown to play a critical role in activation-induced cell death (AICD) of peripheral T cells (64–67). To determine whether AICD in the periphery compensated for defective thymic clonal deletion, in vivo activation and apoptosis were examined in the LN by BrdU labeling of cycling cells and TUNEL staining, respectively (Fig. 8 *A*). There was increased uptake of BrdU by LN T cells in the ΔNur77-D^b/HY TCR compared with the D^b/HY TCR Tg male mice and D^b/HY TCR Tg female mice with or without the ΔNur77 transgene (Fig. 8 *A*). This increased activation was specific for the D^b/HY antigen as no increased BrdU uptake was observed in ΔNur77-D^b/HY TCR double Tg female mice. In vivo apoptosis was also analyzed by in situ TUNEL staining of LN. There was significantly increased apoptosis in the LN of ΔNur77-D^b/HY TCR male mice compared with D^b/HY TCR single Tg male mice and ΔNur77-D^b/HY TCR double Tg female mice (Fig. 8 *A*). These results indicate that there was increased in vivo activation and apoptosis in the LN of ΔNur77-D^b/HY TCR Tg male mice and suggest that tolerance might be maintained by increased AICD in the periphery of ΔNur77-D^b/HY TCR double Tg male mice.

Fas and Fas ligand expression by LN T cells from D^b/HY TCR single and ΔNur77-D^b/HY TCR double Tg male mice was determined (Fig. 8 *B*). Previous studies have shown that two major T cell populations express the Tg D^b/HY TCR in D^b male mice: one is CD4$^-$CD8$^-$ and the other is CD8^{dull}. Both T cell populations are functionally anergic. Fas was expressed at low level in both T cell populations of D^b/HY male mice. In contrast, Fas expression was increased on the self-reactive M33$^+$ CD8$^+$ T cells but not on the M33$^+$CD8$^-$ T cells of ΔNur77-D^b/HY TCR double Tg male mice. Fas ligand expression was determined by culture of different effector/target ratios of T cells from the LN of different mice with a ^{51}Cr-labeled Fas-sensitive cell line (Fig. 8 *B*). There was increased Fas ligand production by T cells from the ΔNur77-D^b/HY TCR double Tg male mice compared with the D^b/HY TCR Tg male mice that could be inhibited by a soluble FasFP. These results indicate that in ΔNur77-D^b/HY TCR Tg male mice, peripheral tolerance was likely maintained by a Fas-mediated AICD mechanism.

Discussion

Nur77 mutant mice were recently found to not exhibit defective apoptosis after anti-CD3–induced death and did not exhibit defective thymocyte development or apoptosis after crossing with the D^b/HY TCR Tg mice (51). Results of previous investigators and the present results indicate that inhibition of the DNA binding by overexpression of the NBRE-binding portion of Nur77 results in defective apoptosis (48, 49). Nuclear factors capable of binding to the NBRE consist of several members of a superfamily sharing homology of the DNA-binding domain. The NBRE consensus sequence is AAAGGTCA and is composed of two 5′ adenine nucleotides and the half-site of the estrogen response element (41–47). The second member of this family has been recently isolated from a human lymphoid cell line PEER after induction of apoptosis and is referred to as TINUR in human and Nurr1 in mouse (50). TINUR has a highly homologous DNA-binding domain to Nur77, as do other members of this family described in other species, but there was little homology in the NH_2-terminal effector function region. TCR-mediated signaling results in early induction of Nur77 which peaks after 1 h, and later induction of TINUR which peaks at 24 h in PEER cells (50). Expression of TINUR also correlated with apoptosis which was also maximal 24 h after anti-TCR signaling. The difference in the kinetics of peak expression of Nur77 and TINUR expression leads to the conclusion that different genes may play complementary roles in T cell activation or apoptosis. The preferential inhibition of apoptosis of $CD4^+8^+$ thymocyte by blocking the NBRE, combined with previous results that Nur77 knockout does not inhibit apoptosis of $CD4^+8^+$ thymocytes, support the conclusion that other factors that interact with the NBRE, such as Nurr1/TINUR, play a role in apoptosis of thymocytes.

Although Nurr1 and Nur77 have been shown to be important in TCR-mediated apoptosis in T cell hybridomas in vitro, the importance of Nur77 and Nurr1 in negative selection and clonal deletion in the thymus has not been established. The present results indicate that apoptosis of $CD4^+$ $CD8^+$ (DP) thymocytes after TCR/CD3 signaling is inhibited in the ΔNur77 Tg mice and suggest that this event is dependent on DNA-binding factors, including Nurr1 and Nur77. This is consistent with results of previous investigators who have demonstrated that apoptosis after anti-TCR or anti-CD3 is highly dependent on a functional NGFI-B/Nur77 pathway (48, 49). Negative selection was also analyzed in the D^b/HY TCR Tg male mice. The pres-

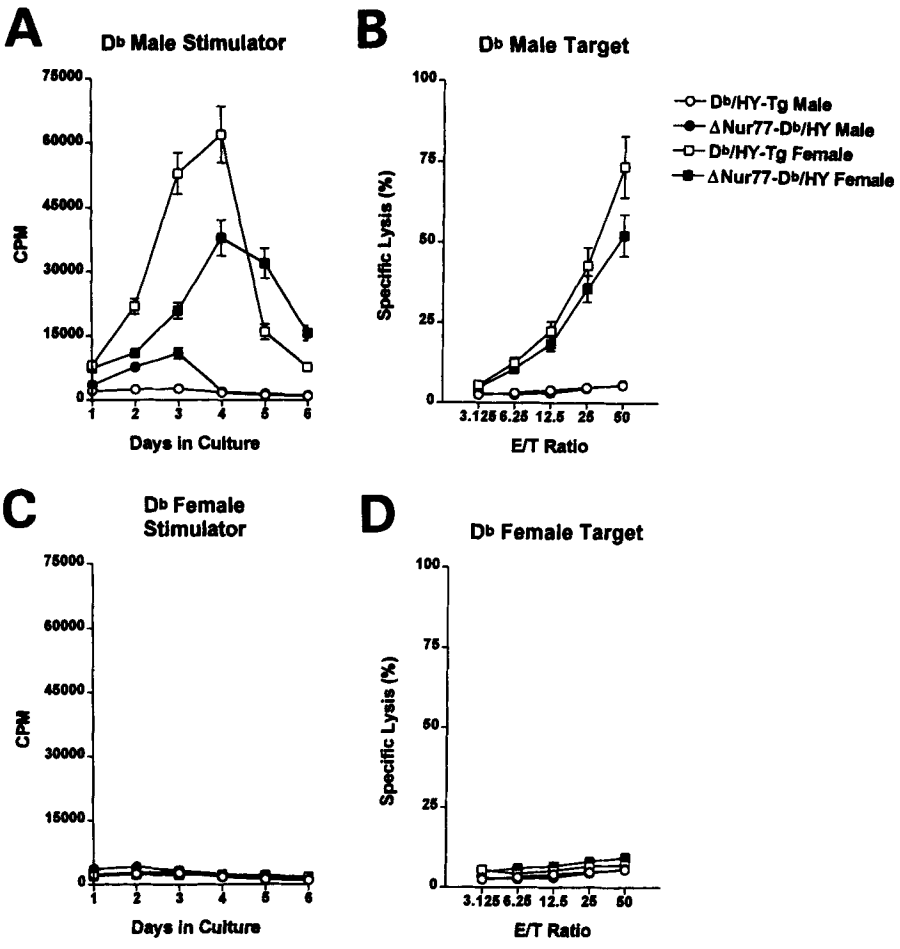
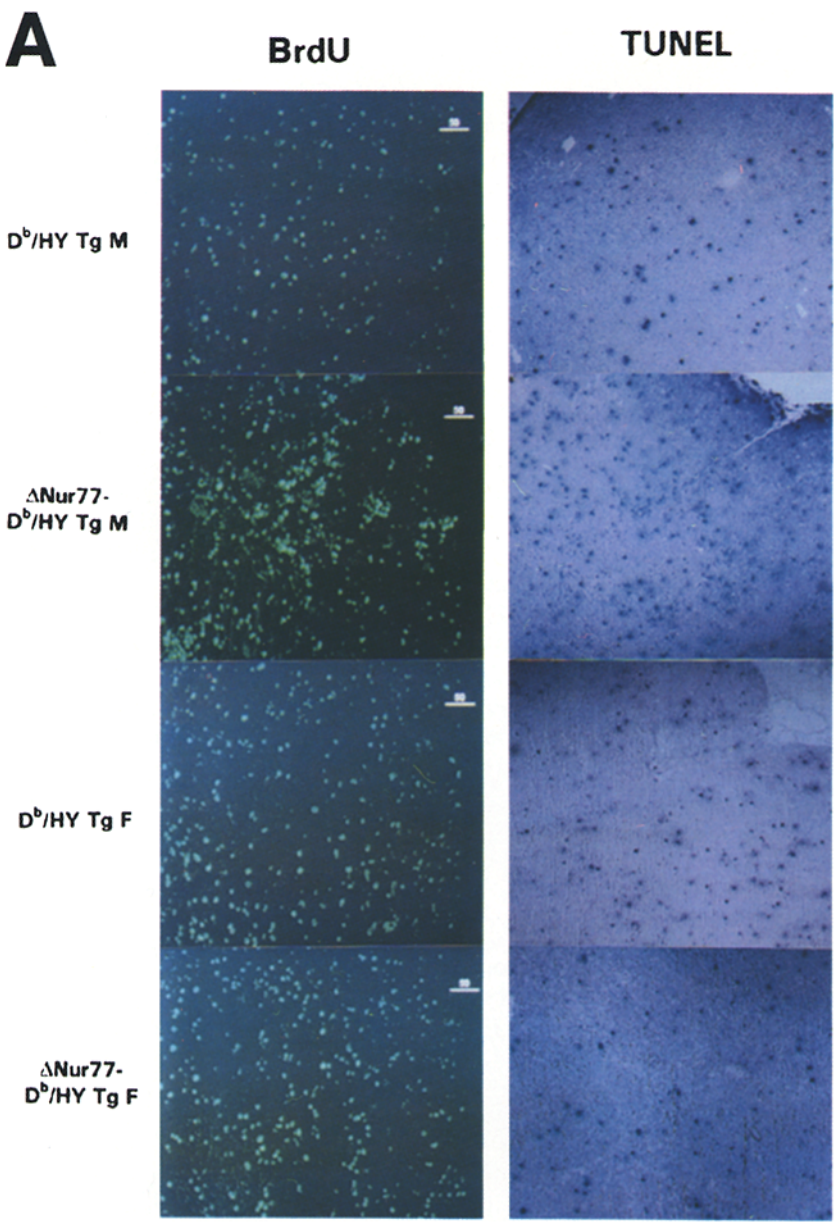


Figure 7. Proliferation and cytotoxicity of LN T cells. LN T cells from the indicated single or double Tg mice were purified by negative selection after passing over a T cell column. Proliferation was assayed after culture of purified LN T cells with irradiated D^b male (*A*) or D^b female (*C*) stimulator cells for the indicated time. Equal numbers of T cells were used at different effector/target ratios to lyse ^{57}Cr-labeled D^b male (*B*) or D^b female (*D*) target cells. Percent specific lysis or proliferation is indicated as the mean ± SEM for at least three individual mice analyzed in triplicate.



ence of the ΔNur77 transgene resulted in increased numbers of $CD4^+CD8^+$ thymocytes and the appearance of more mature $CD8^+$ thymocytes both expressing the autospecific D^b/HY Tg TCR. The increase in the production of $CD4^+8^+$ (DP) thymocytes in D^b/HY male mice was not due to increased positive selection for these cells. This is supported by a decrease in $CD4^+8^+$ thymocytes in the ΔNur77-D^b/HY TCR Tg female mice. The appearance of mature CD8 single positive autospecific thymocytes suggests a deletion defect at a mature stage of thymocyte development. Together, these data strongly suggest that the family of DNA-binding proteins, including Nurr1 and Nur77 is directly involved in signaling of positive and negative selection during T cell development in the thymus.

After anti-TCR or anti-CD3 cross-linking in vivo, apoptosis of $CD4^+8^+$ thymocytes was inhibited in the ΔNur77 Tg mice. Failure to completely block apoptosis in these mice could be due either to the presence of other apoptosis pathways that do not involve the NBRE or to a "leaky" blockade of the NBRE caused by a lack of complete competition of DNA-binding sites by the truncated, inactive ΔNur77 protein. We favor the first possibility because in vitro, anti-CD3 apoptosis was not significantly greater than control antibody-induced apoptosis (Fig. 3), providing evidence that in ΔNur77 Tg mice, anti-CD3–induced apoptosis via the NBRE is functionally inactivated. Other apoptosis pathways exist in the thymus in vivo and these pathways may operate at different developmental stages of thymocytes or in association with different signaling events in addition to anti-CD3. Increased T cell survival during negative selection has been observed in *bcl-2*/D^b/HY TCR-α/β double Tg mice (34–36). Increased survival of

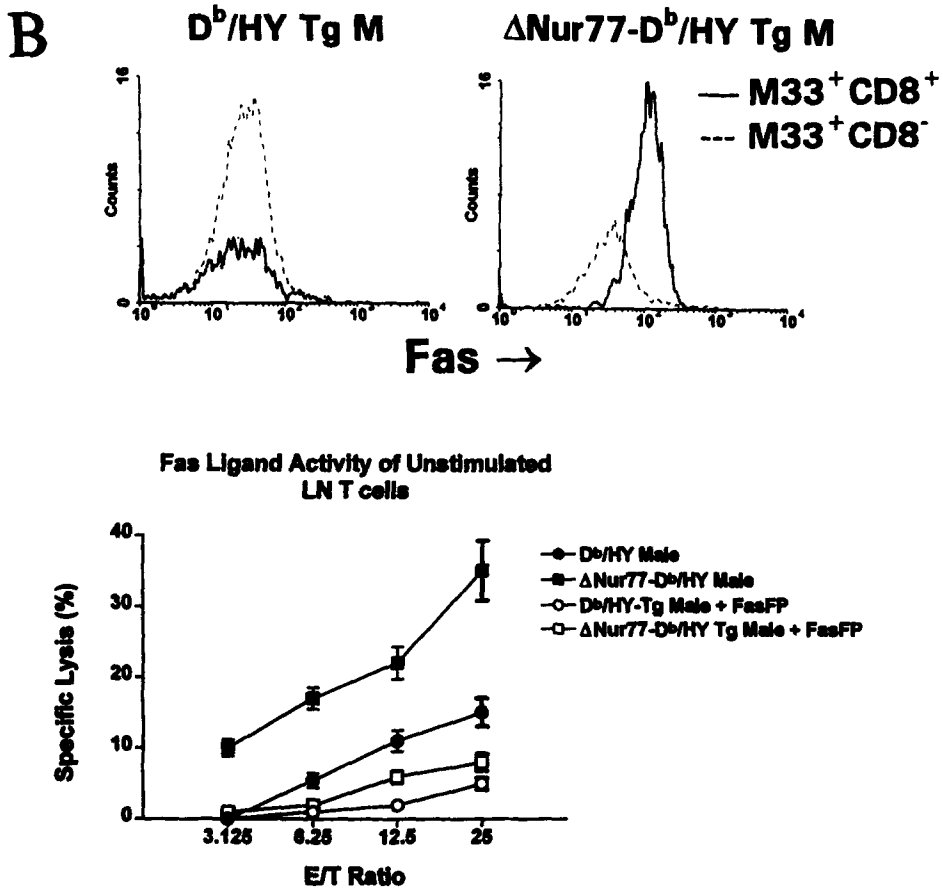


Figure 8. Increased activation and apoptosis of LN cells in D^b/HY TCR and ΔNur77-D^b/HY TCR Tg mice. (*A*) Immunohistochemical analysis for BrdU incorporation and apoptosis. Mice were injected with 1 mg BrdU every 6 h for 24 h. LN sections were analyzed for DNA incorporation of BrdU using the anti-BrdU antibody or for apoptosis using the TUNEL technique. The figure is representative of at least eight LN from three different mice. (*B*) Fas expression and Fas ligand activity. Single cell suspensions were prepared from LN of D^b/HY TCR Tg male and ΔNur77-D^b/HY TCR double Tg male mice. Cells were labeled with antibodies against anti-Fas, anti-CD8, and M33, followed by analysis of 10,000 cells by flow cytometry. Cells were gated into $M33^+CD8^+$ and $M33^+CD8^-$ populations, and Fas expression on each population is shown. These results are representative of three mice analyzed individually. Fas ligand activity by unstimulated LN cells was determined by the extent of lysis of ^{51}Cr-labeled Fas^+ target cells using different effector/target ratios. Specificity for Fas–Fas ligand interaction was determined by the inhibitory effect in the presence of FasFP. Percent specific lysis is indicated as the mean ± SEM for at least three individual mice analyzed in triplicate.

unselected thymocytes and inhibition of negative selection of thymocytes was observed in *bcl-2*/D^b/HY TCR female and male mice (34). Another possibility is that the NBRE might be one factor leading to regulation of apoptosis-related proteins such as *Bcl-Xs* and *Bax* which promote apoptosis; or downregulation of proteins such as Bcl-2 and $Bcl\text{-}X_L$, which inhibit apoptosis (68–71).

Another important pathway of apoptosis for thymocytes is dexamethasone-induced apoptosis (72, 73). The lack of inhibition of dexamethasone-induced apoptosis in the ΔNur77 Tg mice is significant since this indicates that there is no competitive inhibition between the Nur77/Nurr1 orphan steroid receptors either at the level of cytoplasmic steroid binding or at the receptor-DNA binding site with glucocorticoids. This lack of inhibition between dexamethasone apoptosis and Nur77/Nurr1 apoptosis indicates that apoptosis is mediated by separate independent pathways. Taken together, there are multiple independent pathways of thymocyte apoptosis related to TCR/CD3 signaling that may be affected by inhibition of Nurr1/Nur77 interaction with NBRE.

Functional blockade of the NBRE did not greatly inhibit the T cell activation signal after TCR/CD3 cross-linking. There was normal phosphorylation of the CD3ζ chain in ΔNur77 Tg mice. Also, proliferation after TCR/CD3-mediated activation of LN T cells was increased in the ΔNur77-D^b/HY TCR Tg male mice and was only slightly decreased in T cells from ΔNur77 D^b/HY TCR Tg female mice compared with D^b/HY single TCR Tg male and female mice, after culture with D^b male stimulator cells (Fig. 7). Proliferation after stimulation was very low in the D^b/HY male mice, and this was increased approximately 10-fold in the ΔNur77-D^b/HY double Tg male mice. Increased proliferation could not be accounted for by an increase in the number of $M33^+$ $CD8^+$ T cells, which were increased only twofold. These results suggest that alternative pathways to signal proliferation after stimulation through the TCR/CD3 molecules are present in addition to the ΔNur77 pathway, and these result in nearly normal proliferation after TCR/CD8 signaling in the ΔNur77 Tg mice.

In ΔNur77 Tg male mice, there was an increase in $M33^+$ $CD8^+$ T cells in the LN which resulted from inefficient thymic clonal deletion and compensatory increase in LN peripheral expansion followed by clonal deletion. Activation-induced apoptosis of nontolerant T cells is supported by the observation of a specific increase in BrdU incorporation in vivo in Nur77-D^b/HY TCR Tg male mice but not in ΔNur77-D^b/HY TCR Tg female mice lacking the HY antigen. Also, increased T cell activation was associated with increased apoptosis in ΔNur77-D^b/HY TCR Tg male but not ΔNur77-D^b/HY TCR Tg female mice. Fas–Fas ligand interaction has been shown to be an important mechanism for activation-induced apoptosis (64–67,

74, 75). Several lines of evidence indicate that this AICD in ΔNur77-D^b/HY TCR Tg male mice was due to Fas–Fas ligand interaction. First, apoptosis in vitro could be blocked by the FasFP. Second, Fas expression was increased in the M33$^+$, CD8$^+$ T cells, but not in the M33$^+$, CD8$^-$ T cells, of Nur77-D^b/HY TCR Tg male mice, consistent with ongoing AICD using a Fas–Fas ligand pathway. Finally, there was increased Fas ligand production by LN T cells from Nur77-D^b/HY TCR Tg male but not female mice.

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