Unimpaired Thymic and Peripheral T Cell Death in Mice Lacking the Nuclear Receptor NGFI-B (Nur77)

Stephen L. Lee, Robin L. Wesselschmidt, Gerald P. Linette, Osami Kanagawa, John H. Russell, Jeffrey Milbrandt*

T cell hybridomas require the immediate-early gene NGFI-B (*nur77*) for T cell receptor (TCR)–mediated apoptosis, a model for negative selection of self-reactive T cells. TCR-mediated death was examined in mice bearing an NGFI-B loss-of-function mutation, either by administration of antibodies to CD3 (anti-CD3) or in two well-characterized transgenic models expressing self-reactive TCRs. Both the extent and the rate of thymocyte death were unimpaired. Anti-CD3–induced death was normal in $CD4^+$ peripheral T cells, in which death is mediated predominantly by the Fas signaling pathway. Thus, no unique requirement for NGFI-B is observed for thymic or peripheral T cell death.

Programmed cell death is vital for eliminating unwanted cells in multicellular organisms, and its dysregulation is now widely recognized in autoimmune and neoplastic disease pathogenesis (*1*). In self-reactive T cells, the molecular events that occur during programmed death are largely unknown, but may be related to the apoptotic death observed upon activation through the TCR-CD3 complex in immature thymic T cells (*2*, *3*), antigen-stimulated mature peripheral T cells (*4*), and T cell hybridomas (*5*). Recently, the immediate-early gene NGFI-B (*nur77*) was identified by differential hybridization as an mRNA induced in T cell hybridomas or in thymocytes undergoing apoptosis (*6*, *7*). Originally identified in nerve growth factor (NGF)–treated PC12 pheochromocytoma cells (*8*) and in serum-stimulated fibroblasts (*9*), NGFI-B encodes an orphan member of the nuclear receptor superfamily. Several lines of evidence implicate NGFI-B in the programmed death of T cells. First, NGFI-B expression is correlated with TCR-mediated apoptosis (*6*, *7*). Second, NGFI-B mRNA is expressed in thymic medulla where negative selection occurs (*10*, *11*). Third, and most important, blocking NGFI-B with a dominant-negative (*6*) or antisense construct (*7*) prevents TCR-mediated apoptosis in T cell hybridomas.

To investigate the processes that require NGFI-B in vivo, we used homologous recombination to mutate NGFI-B in D3 embryonic stem (ES) cells (Fig. 1A) (*12*). Gene-targeted ES cells were injected into blastocysts to produce chimeric mice, and intercross matings of resultant heterozygotes produced offspring homozygous for the mutation (Fig. 1B) (*13*). RNA blots hybridized with NGFI-B probe demonstrated no detectable transcript in homozygous mutant animals (Fig. 1C). We thus refer to the homozygous mutants as NGFI-$B^{-/-}$ mice. Expression of the closely related gene *nurr-1* (RNR-1) (*14*) was faint and was not increased in the NGFI-$B^{-/-}$ background.

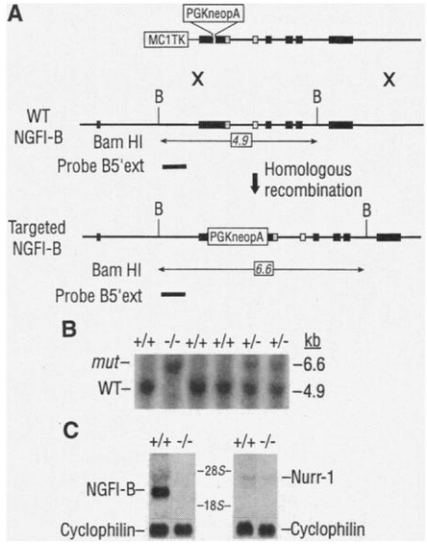

Fig. 1. Targeted inactivation of the NGFI-B gene. (**A**) Schematic representation of the targeting vector pB(PGKneopA)TK, wild-type, and mutated NGFI-B gene, indicating predicted restriction fragment lengths when probed with the external probe B5′ext. (**B**) Southern (DNA) blot of Bam HI–digested tail DNA from a litter derived by heterozygous intercross mating, probed with an NGFI-B genomic fragment external to the homologous arms of the targeting vector. Mutants (−/−) are homozygous for the 6.6-kb mutated allele (*mut*) and have lost the 4.9-kb wild-type allele (WT). (**C**) RNA blot of thymus total RNA (15 μg) from wild-type (+/+) or homozygous mutant (−/−) NGFI-B mice probed with a 400–base pair (bp) NGFI-B 3′ untranslated region probe, a 900-bp Nurr-1 probe, or a full-length cyclophilin probe to control for loading.

Analysis of NGFI-$B^{-/-}$ animals showed that they are born at expected Mendelian frequencies and develop without overt differences in size, growth rate, or behavior. The gross appearance of lymphoid organs was normal. Histological analysis of the thymus showed a normal architecture containing a distinct cortex and medulla, and the spleen showed normal distribution of red and white pulp (*15*). Analysis of $CD4^+$ and $CD8^+$ T cell subsets in thymus and spleen cells showed no difference between wild-type and NGFI-$B^{-/-}$ animals, suggesting that T cell development was normal (*15*). Mutant animals have been maintained in both specific pathogen-free and nonsterile facilities for 1 year without developing clinical symptoms of infection, autoimmunity, or neoplasia.

To examine the requirement for NGFI-B in TCR-mediated apoptosis, we assessed anti-CD3–induced death of immature $CD4^+CD8^+$ thymocytes (*3*). In vivo administration of anti-CD3 (145-2C11) resulted in a significant reduction of the total number of thymocytes and predominantly

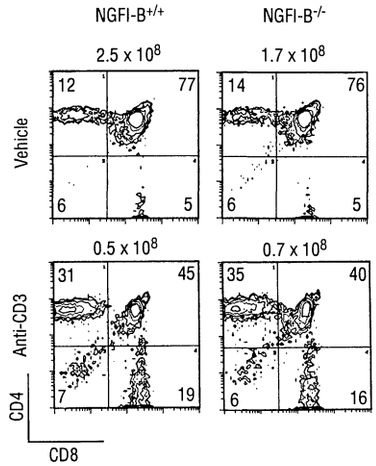

Fig. 2. Selective loss of $CD4^+CD8^+$ thymocytes in both NGFI-$B^{+/+}$ and NGFI-$B^{-/-}$ mice after in vivo administration of anti-CD3. NGFI-$B^{-/-}$ or NGFI-$B^{+/+}$ mice between 6 to 10 weeks of age were injected intraperitoneally with 250 μg of anti-CD3 (145-2C11) or vehicle alone (phosphate-buffered saline) as described (*3*). Forty hours later, thymi were removed, single-cell suspensions prepared, and cell counts determined from ammonium chloride–treated cells. Mean total cellularity is displayed above representative two-dimensional plots derived from flow cytometric analysis of CD4 and CD8 cell surface markers (*23*). Numbers within each quadrant indicate mean subpopulation percentages.

S. L. Lee, O. Kanagawa, J. Milbrandt, Departments of Pathology and Internal Medicine, Washington University School of Medicine, St. Louis, MO 63110, USA.
R. L. Wesselschmidt, Division of Bone Marrow Transplantation and Stem Cell Biology, Departments of Medicine and Genetics at Jewish Hospital, Washington University School of Medicine, St. Louis, MO 63110, USA.
G. P. Linette, Howard Hughes Medical Institute, Departments of Pathology and Medicine, Washington University School of Medicine, St. Louis, MO 63110, USA.
J. H. Russell, Department of Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, MO 63110, USA.
*To whom correspondence should be addressed.

affected the $CD4^+CD8^+$ population (Fig. 2). This reduction in $CD4^+CD8^+$ cell numbers was similar in both NGFI-$B^{-/-}$ and NGFI-$B^{+/+}$ animals (Table 1).

Although this result showed that NGFI-B is not required for anti-CD3–induced death, we further examined αβTCR transgenic mice, in which the majority of thymocytes specifically undergo negative selection. NGFI-$B^{-/-}$ mice were crossed with mice expressing a major histocompatibility complex (MHC) class I–restricted ($H\text{-}2D^b$) αβTCR specific for the male H-Y antigen (*16*). In females, H-Y TCR expression resulted in positive selection of the $CD4^-CD8^+$ lineage. In males, expression of this transgene resulted in the elimination of immature $CD4^+CD8^+$ thymocytes irrespective of NGFI-B genotype (Fig. 3A), thereby reducing the total number of $CD4^+CD8^+$ thymocytes by a factor of 30 (Table 1). Moreover, $CD4^-CD8^{hi}$ lymphocytes indicative of escape from clonal deletion were not detected in peripheral lymph nodes. The previously described $CD4^-CD8^{low}$ lymphocytes were detected, but their relative abundance was comparable between NGFI-$B^{+/+}$ and NGFI-$B^{-/-}$ mice.

We confirmed these observations in a second transgenic model that expresses the class II MHC–restricted TCR specific for pigeon cytochrome c (*17*). These transgenic mice were crossed with NGFI-$B^{-/-}$ and either $H\text{-}2^b$ or $H\text{-}2^s$ MHC haplotypes, and the T cell subsets of their progeny were examined. Positive selection of thymic $CD4^+CD8^-$ cells resulted from interaction of this TCR with $I\text{-}A^b$, as evidenced by a significant increase in $CD4^+CD8^-$ cells. Interaction with $I\text{-}A^s$, however, resulted in negative selection of these cells irrespective of NGFI-B genotype (Fig. 3B and Table 1) (*17*).

To address the possibility that a redundant or parallel mechanism or mechanisms masked the extent but not the rate of apoptosis, as was observed in granzyme B–deficient mice (*18*), we examined the kinetics of thymocyte clonal deletion. In vitro coculture of female H-Y TCR thymocytes with male antigen-presenting cells (APCs) of the $H\text{-}2^b$ haplotype (*19*) resulted in the disappearance of $CD4^+CD8^+$ thymocytes,

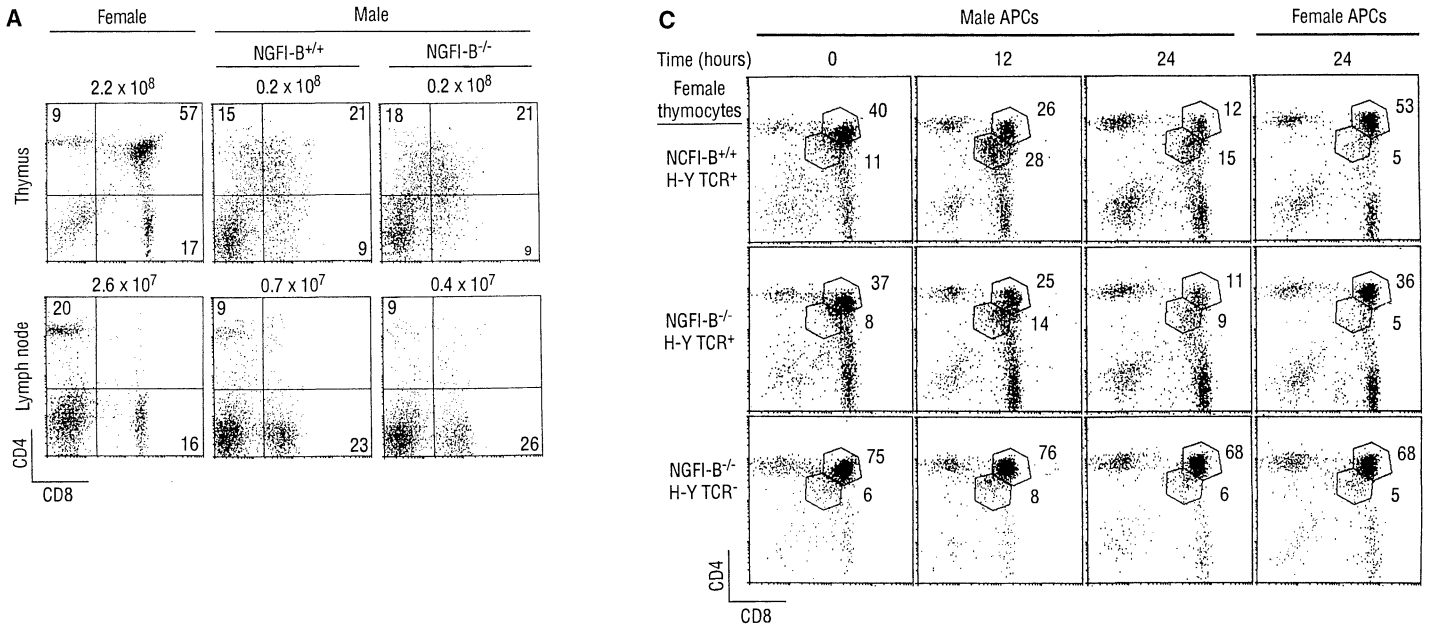

Fig. 3. Negative selection of autoreactive thymocytes in αβTCR transgenic mice. Analysis of thymocyte and lymph node subsets in NGFI-$B^{+/+}$ or NGFI-$B^{-/-}$ mice expressing (**A**) a class I MHC–restricted H-Y TCR transgene or (**B**) a class II MHC–restricted TCR (AND) transgene in either the $I\text{-}A^b$ or $I\text{-}A^s$ genetic background. Mean total cellularity is displayed above representative two-dimensional plots derived from flow cytometric analysis of CD4 and CD8 cell surface markers (*23*). Numbers within each quadrant indicate mean subpopulation percentages. In (B), the proportions of thymocyte subsets in the NGFI-$B^{+/-}$ mice were similar to wild-type proportions reported previously (*17*) and therefore were used as controls. (**C**) Analysis over time of clonal deletion of $CD4^+CD8^+$ H-Y TCR thymocytes by male antigen-presenting cells. In vitro cultures (*19*) of thymocytes (1×10^6) from NGFI-$B^{+/+}$ ($n = 2$) or NGFI-$B^{-/-}$ ($n = 2$) female H-Y TCR transgenic or nontransgenic control mice ($n = 2$) were cocultured in 96-well plates in Iscove's modified Dulbecco's medium containing 10% fetal calf serum, with an equal number of antigen-presenting cells (APCs) that were purified by adherence from cell suspensions of spleens of male or female $H\text{-}2D^b$ mice. At various times, cells were analyzed for CD4 and CD8 cell surface marker expression (*23*). Numbers adjacent to each region indicate the relative percentage of cells in each region.

Table 1. Effect of (I) treatment with anti-CD3, (II) expression of H-Y TCR transgene, or (III) expression of AND TCR transgene on thymus and lymph node T cell subpopulations in NGFI-B wild-type (+/+ or +/−) and knockout (−/−) mice. Absolute numbers are calculated from T cell subpopulation percentages and total thymocyte and lymph node counts for each animal. Values are means ± SD. ND, not determined.

Exp.	NGFI-B genotype	Treatment or transgenic TCR*	*n*	Thymus ($\times 10^7$ cells)				Lymph node ($\times 10^6$ cells)	
				$CD4^+8^+$	$CD4^+8^-$	$CD4^-8^+$	$CD4^-8^-$	$CD4^+8^-$	$CD4^-8^+$
I	+/+	Vehicle	3	18.9 ± 9.1	2.9 ± 1.2	1.1 ± 0.6	1.6 ± 2.4	ND	ND
	−/−	Vehicle	3	12.9 ± 5.2	2.4 ± 0.9	0.8 ± 0.7	1.1 ± 1.5	ND	ND
	+/+	Anti-CD3	3	2.3 ± 0.2	1.7 ± 0.8	1.1 ± 0.7	0.3 ± 0.0	ND	ND
	−/−	Anti-CD3	3	2.7 ± 1.9	2.6 ± 2.0	1.3 ± 1.1	0.5 ± 0.3	ND	ND
II		H-Y Female	1	12.8	2.0	3.8	3.1	5.2	4.2
	+/+	H-Y Male	4	0.4 ± 0.3	0.3 ± 0.2	0.2 ± 0.1	1.2 ± 0.7	0.6 ± 0.6	1.3 ± 1.3
	−/−	H-Y Male	4	0.4 ± 0.3	0.4 ± 0.1	0.2 ± 0.1	1.0 ± 0.2	0.3 ± 0.1	1.0 ± 0.8
III		Nontransgenic	3	15.3 ± 7.0	3.7 ± 2.1	1.6 ± 1.1	5.9 ± 5.0	6.4 ± 0.4	4.1 ± 0.2
	+/−	AND I-A (b/b)	2	10.0 ± 2.2	12.0 ± 1.7	0.9 ± 0.4	3.0 ± 0.6	13.3 ± 3.8	0.4 ± 0.1
	−/−	AND I-A (b/b)	5	10.9 ± 5.6	8.8 ± 3.4	0.8 ± 0.7	1.7 ± 1.0	13.3 ± 6.7	0.5 ± 0.4
	+/−	AND I-A (b/s)	3	3.1 ± 0.5	0.5 ± 0.1	0.4 ± 0.1	2.2 ± 1.1	1.2 ± 0.2	1.7 ± 0.5
	−/−	AND I-A (b/s)	3	3.4 ± 1.0	0.7 ± 0.3	1.3 ± 1.2	3.6 ± 2.4	1.2 ± 0.3	1.9 ± 1.3

*See text for description of transgenes.

which proceeded at the same rate in both NGFI-$B^{+/+}$ and NGFI-$B^{-/-}$ cultures (Fig. 3C). Furthermore, the transient appearance of $CD4^{low}CD8^{low}$ cells that precedes apoptosis (*19*) was also comparable between genotypes. No reduction was observed when either nontransgenic thymocytes or female APCs were used, indicating that the reduction was specific for TCR-antigen interaction.

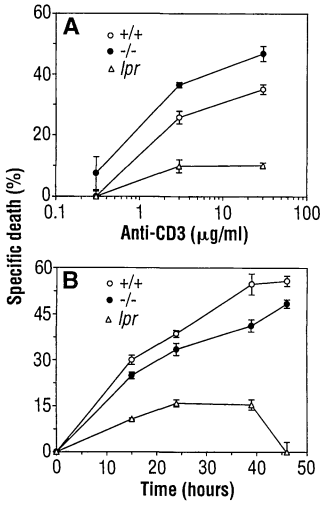

Fig. 4. Cell death of wild-type, NGFI-$B^{-/-}$, and *lpr/lpr* T cells stimulated with immobilized anti-CD3. $CD4^+$ single-positive splenocytes were prepared and activated with staphylococcal enterotoxin A (0.5 μg/ml) as described (*4*). Three to 4 days after a secondary or tertiary weekly stimulation, viable cells were harvested by a density step gradient (Ficoll/Hypaque, 1.077 g/ml) and plated at 3×10^5 per milliliter in the presence of recombinant human interleukin-2 (IL-2, 50 U/ml) (Cetus). After 30 to 48 hours, viable cells were again harvested, and resuspended at 8×10^5 per milliliter in the presence of recombinant human IL-2 (50 U/ml). Cell death was assessed by trypan blue dye exclusion (**A**) 30 hours after plating 0.1 ml in wells (96-well) precoated with control or anti-CD3 at indicated concentrations or (**B**) at various times after plating in wells precoated with control or anti-CD3 (10 μg/ml). Specific death (%) denotes death in anti-CD3 wells normalized to controls as previously described (*4*).

It was recently demonstrated that T cell hybridomas require the Fas pathway for TCR-mediated death, suggesting that hybridomas model peripheral rather than thymic T cell death (*20*). To investigate this possibility, we examined the activation-induced death of $CD4^+$ peripheral T cells, in which death is mediated primarily by the Fas pathway (*21*). No significant difference was observed in sensitivity to or kinetics of anti-CD3–induced cell death in NGFI-$B^{+/+}$ or NGFI-$B^{-/-}$ T cells. In contrast, cells from *lpr/lpr* mice, which are defective in Fas signaling (*4*), were significantly resistant (Fig. 4, A and B). These observations in vitro correlate with disease susceptibility in vivo; whereas *lpr/lpr* mice of similar genetic background typically develop a lymphadenopathy and splenomegaly within 10 to 20 weeks, NGFI-$B^{-/-}$ mice maintained in a nonsterile environment exhibited normal spleen and lymph node cellularity and remained free of autoimmune disease symptoms for over 1 year (*15*).

We have used established genetic and in vitro models to examine the requirement for NGFI-B in TCR-mediated death. In contrast to T cell hybridomas, mice lacking NGFI-B have no measurable defects in TCR-mediated apoptosis in immature thymic or mature peripheral T cells. Other forms of apoptosis such as dexamethasone- and ionizing radiation–induced thymocyte death also proceeded normally without NGFI-B (*15*). These results suggest redundancy by related members of the NGFI-B gene family such as *nurr-1*, NGFI-Bγ (*15*), or by other factors such as c-*myc*, which has also been shown to be required in T cell hybridoma apoptosis (*22*). We conclude that NGFI-B is not a single gene uniquely required for the TCR-mediated death program.

REFERENCES AND NOTES

1. D. L. Vaux, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 786 (1993).
2. C. A. Smith, G. T. Williams, R. Kinston, E. J. Jenkinson, J. J. T. Owen, *Nature* **337**, 181 (1989).
3. Y. F. Shi *et al.*, *J. Immunol.* **146**, 3340 (1991).
4. J. H. Russell, B. Rush, C. Weaver, R. Wang, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 4409 (1993).
5. Y. F. Shi *et al.*, *J. Immunol.* **144**, 3326 (1990).
6. J. D. Woronicz, B. Calnan, V. Ngo, A. Winoto, *Nature* **367**, 277 (1994).
7. Z.-G. Liu, S. W. Smith, K. A. McLaughlin, L. M. Schwartz, B. A. Osborne, *ibid.*, p. 281.
8. J. Milbrandt, *Neuron* **1**, 183 (1988).
9. T. G. Hazel, D. Nathans, L. F. Lau, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8444 (1988).
10. M. A. Watson and J. Milbrandt, unpublished observations.
11. H. Hengartner *et al.*, *Nature* **336**, 388 (1988); C. D. Surh and J. Sprent, *ibid.* **372** 100 (1994).
12. Germline-competent D3 ES cells were passaged, electroporated, and selected for homologous recombination as described (*24*). pB(PGKneopA)TK was constructed from a 7.2-kb Balb/c genomic fragment encompassing exons 2 through 7 of the NGFI-B gene by insertion of a neomycin expression cassette (*25*) into the Sma site in the coding region of the second exon. Homologous recombination of the targeting vector results in the insertion of the neomycin cassette and truncation of the NGFI-B reading frame upstream of the DNA-binding domain.
13. From 360 neomycin-ganciclovir–resistant clones screened, six had incorporated the targeting vector by homologous recombination. Clone B6 yielded chimeras that transmitted the agouti and targeted alleles to their offspring.
14. L. M. Scearce, T. M. Laz, T. G. Hazel, L. F. Lau, R. Taub, *J. Biol. Chem.* **268**, 8855 (1993); S. W. Law, O. M. Conneely, F. J. DeMayo, B. W. O'Malley, *Mol. Endocrinol.* **6**, 2129 (1992).
15. S. L. Lee and J. Milbrandt, data not shown.
16. P. Kisielow, H. Bluthmann, U. D. Staerz, M. Steinmetz, H. von Boehmer, *Nature* **333**, 742 (1988).
17. N. J. Vasquez, J. Kaye, S. M. Hedrick, *J. Exp. Med.* **175**, 1307 (1992).
18. J. W. Heusel, R. L. Wesselschmidt, S. Shresta, J. H. Russell, T. J. Ley, *Cell* **76**, 977 (1994).
19. W. Swat, L. Ignatowicz, H. von Boehmer, P. Kisielow, *Nature* **351**, 150 (1991).
20. J. Dhein, H. Walczak, C. Bäumler, K.-M. Debatin, P. H. Krammer, *ibid.* **373**, 438 (1995); T Brunner *et al.*, *ibid.*, p. 441; S.-T. Ju *et al.*, *ibid.*, p. 444.

21. S. Nagata and P. Golstein, *Science* **267**, 1449 (1995).
22. Y. Shi *et al, ibid.* **257**, 212 (1992).
23. Thymocytes (1×10^6) were stained with directly conjugated phycoerythrin–anti-CD4 and fluorescein isothiocyanate (FITC)–anti-CD8a (Ly-2) (Pharmingen) in staining buffer [2% fetal calf serum, 0.1% sodium azide in phosphate-buffered saline (PBS)] at 4°C, washed, and live cells (2.5×10^4) as determined by forward- and side-scatter parameters were analyzed by flow cytometric analysis with LYSYS or FACScan Research Software (Becton Dickinson). H-Y transgenic mice were identified by staining of cells with biotinylated antibody to Vβ8.2 (MR14.1) followed by streptavidin-TriColor (CalTag).
24. T. C. Doetschman, H. Eistetter, M. Katz, W. Schmidt, R. Kemler, *J. Embryol. Exp. Morphol.* **87**, 27 (1985); E. Robertson, *Teratocarcinoma and Embryonic Stem Cells: A Practical Approach* (IRL Press Oxford, Washington, DC, 1987); S. L. Lee, L. C. Tourtellotte, R. L. Wesselschmidt, J. Milbrandt, *J. Biol. Chem.* **270**, 9971 (1995).
25. P. Soriano, C. Montgomery, R. Geske, A. Bradley, *Cell* **64**, 693 (1991).
26. We thank L.-A. Tourtellotte, P. Goda, B. Rush, and D. Donermeyer for expert assistance in the generation and analysis of the mice. We also thank A. Bradley for providing the PGKneopA vector, S. Potter for the D3 ES cells, and B. Hayashi for the MR-1.4 antibody. Special thanks to S. M. Hedrick and H. von Boehmer for permission to use their transgenic mice, which were instrumental to much of the analysis, and to P. M. Allen and S. J. Korsmeyer for critical reading and comments. Supported by a grant from Ronald McDonald Children's Charities (J.M.) and from the National Cancer Institute PO1 CA49712 (J.M.). J. M. is an established investigator of the American Heart Association.

7 December 1994; accepted 19 April 1995

A Role for CD5 in TCR-Mediated Signal Transduction and Thymocyte Selection

Alexander Tarakhovsky,* Steven B. Kanner, Joachim Hombach, Jeffrey A. Ledbetter, Werner Müller, Nigel Killeen, Klaus Rajewsky

CD5 is a transmembrane protein that is expressed on the surface of T cells and a subset of B cells. The absence of CD5 rendered thymocytes hyperresponsive to stimulation through the T cell antigen receptor (TCR) in vitro. Selection of T cells expressing three distinct transgenic TCRs was also abnormal in CD5-deficient mice. These observations indicate that CD5 can influence the fate of developing thymocytes by acting as a negative regulator of TCR-mediated signal transduction.

Signal transduction through the TCR determines the outcome of thymic selection (*1*). The T lymphocyte transmembrane protein CD5 is part of a receptor complex that also comprises the TCR-CD3 ζ chain and protein tyrosine kinases (PTKs) (*2*). Activation of T cells results in a rapid phosphorylation of tyrosine residues within the cytoplasmic domain of CD5 (*3*), which is thought to bind Src homology 2 (SH2) domain–containing proteins (*2*).

Previous studies of peripheral T cell activation in vitro revealed a costimulatory effect of antibodies specific for CD5 on TCR-mediated proliferative responses, suggesting that in the absence of CD5, TCR-mediated proliferation of T cells may be reduced (*4*). In contrast, we observed that TCR-mediated proliferative responses of single positive (SP) $CD4^+$ or $CD8^+$ thymocytes, but not peripheral T cells, from CD5-deficient mice (*5*) were greater than those of the corresponding cells from wild-type mice (Fig. 1A) (*6*). However, both control and $CD5^{-/-}$ cells responded equally to stimulation by phorbol 12-myristate 13-acetate (PMA) and ionomycin, which are known to bypass TCR-dependent signal transduction (*7*).

Surface expression levels of the TCR-CD3 complex and CD4 or CD8 coreceptors on thymocytes from $CD5^{-/-}$ and control mice are similar (*5*). Hence, CD5 deficiency likely influences signaling downstream of the TCR. The hyperresponsiveness of $CD5^{-/-}$ thymocytes in terms of proliferation was accompanied by moderate, but stably reproducible, increases in Ca^{2+} mobilization associated with thymocyte activation induced by antibodies to CD3, either alone or in combination with antibodies to CD4 (Fig. 1B) (*8*). The increase in Ca^{2+} mobilization in $CD5^{-/-}$ thymocytes was consistent with a three- to fivefold increase in the phosphorylation of phospholipase C–γ1 (PLC-γ1) and the PLC-γ1–associated phosphoprotein pp35/36 (Fig. 1C) (*9*), which control the TCR-mediated activation of protein kinase C and mobilization of Ca^{2+} (*1*, *9*, *10*).

Interaction of the TCR with peptide-loaded major histocompatibility complex (MHC) proteins induces the phosphorylation of TCR subunits and activation of PTKs important in signaling downstream of the TCR (*1*, *11*). The association of the phosphorylated TCR-CD3 ζ chain with the PTK ZAP-70 plays a critical role in T cell activation (*12*). We performed immunoprecipitation from whole-cell lysates of resting and in vitro–stimulated $CD5^{-/-}$ and control thymocytes with antibodies to ZAP-70 and subjected the immunoprecipitates to immunoblot analysis with antibodies to phosphotyrosine (*9*). Unstimulated and activated $CD5^{-/-}$ and control thymocytes contained equal amounts of similarly phosphorylated ZAP-70 protein. One or two phosphoproteins with molecular sizes of 21 kD (pp21) and 23 kD (pp23) coprecipitated with ZAP-70; these proteins are known isoforms of the CD3 ζ chain (*12*) (Fig. 1C). The identity of these phosphoproteins was confirmed with antibodies to the CD3 ζ chain. The extent of phosphorylation of the pp21 ζ isoform was lower in $CD5^{-/-}$ than in control cells. In $CD5^{-/-}$ cells, but not in control cells, cross-linking of CD3 resulted in the induction of the pp23 isoform (Fig. 1C). This isoform of the ζ chain predominated in lysates of $CD5^{-/-}$ thymocytes activated with the combination of antibodies to CD3 and CD4, whereas in control thymocytes the pp23 ζ isoform was only slightly more phosphorylated on tyrosine than the pp21 ζ isoform (Fig. 1C). Both pp21 and pp23 ζ isoforms are induced by TCR ligands that stimulate T cell proliferation and interleukin-2 secretion (that is, agonists), whereas anergy-inducing TCR ligands (that is, antagonists) induce predominantly the pp21 ζ isoform (*12*). It is possible that CD5 regulates the ratio between pp21 and pp23 ζ isoforms by competing with CD3 for common PTKs (*2*), thereby defining the amplitude of TCR-mediated stimulation.

The phosphorylation of the Vav protein, which is critical for TCR-mediated proliferation of T cells (*13*), was markedly increased in both unstimulated and stimulated $CD5^{-/-}$ thymocytes relative to control cells (Fig. 1C). The alterations in phosphorylation were selective: There were no other significant differences between $CD5^{-/-}$ and control thymocytes with respect to the general pattern of TCR-induced phosphorylation of intracellular proteins. Moreover, neither the phosphorylation nor the catalytic activity of the TCR-CD4 associated protein kinase $p56^{lck}$ was altered in nonstimulated or stimulated $CD5^{-/-}$ thymocytes.

The changes in TCR-mediated signal transduction in $CD5^{-/-}$ mice might have been expected to influence thymic selection and affect the fate of developing thymocytes. However, an initial analysis of T cell devel-

A. Tarakhovsky, W. Müller, K. Rajewsky, Institute for Genetics, University of Cologne, 50937 Cologne, Germany.
S. B. Kanner and J. A. Ledbetter, Bristol-Myers Squibb, Pharmaceutical Research Institute, Seattle, WA 98121, USA.
J. Hombach, Institute of Experimental Immunology, Department of Pathology, University of Zurich, CH-8091 Zurich, Switzerland.
N. Killeen, Department of Microbiology and Immunology, University of California, San Francisco, CA 94143, USA.
*To whom correspondence should be addressed.