Bcl-x_L Displays Restricted Distribution during T Cell Development and Inhibits Multiple Forms of Apoptosis but Not Clonal Deletion in Transgenic Mice

By Didier A. M. Grillot, Ramón Merino, and Gabriel Núñez

From the Department of Pathology, University of Michigan, Ann Arbor, Michigan 48109-0608

Summary

The survival of T lymphocytes is tightly controlled during development. Here, we show that Bcl-x_L, a protein homologue of Bcl-2, is highly regulated in the thymus in a pattern different than that of Bcl-2. The maximum expression was in $CD4^+CD8^+$ thymocytes, a developmental stage where Bcl-2 is downregulated. To assess the role of Bcl-x_L in thymocyte apoptosis, we generated mice overexpressing an Eμ-*bcl-x* transgene within the T cell compartment. Constitutive expression of Bcl-x_L resulted in accumulation of thymocytes and mature T cells in lymphoid organs. Thymocytes overexpressing Bcl-x_L exhibited increased viability in vitro and were resistant to apoptosis induced by different signals, including glucocorticoid, γ irradiation, calcium ionophore, and CD3 cross-linking. However, Bcl-x_L was unable to block clonal deletion of thymocytes reactive with self-superantigens or H-Y antigen. These studies demonstrate that Bcl-2 and Bcl-x_L, two functionally related proteins, are regulated independently during T cell development. In contrast to Bcl-2, which has been implicated in the maintenance of mature T cells, Bcl-x_L appears to provide a survival signal for the maintenance of more immature $CD4^+CD8^+$ thymocytes before positive selection.

Lymphoid development is regulated by an array of cellular processes that include proliferation, differentiation, and cell death. Cell death is accomplished by apoptosis, a morphologically defined process that is widespread during embryogenesis and postnatal development (1). Apoptosis serves to ensure the selection of appropriate lymphoid populations during thymic development and to dampen the immune response in peripheral tissues (2, 3). It is estimated that as many as 97% of the T cell precursors die during thymic development through apoptosis (4). The assembly of TCR genes is largely a random process resulting in a large fraction of developing T cells that fail to assemble functional TCRs and are thought to be eliminated by apoptosis (4). Developing $CD4^+CD8^+$ thymocytes that express functional TCRs are further selected by positive and negative events involving the interaction of TCRs with peptides complexed with MHC molecules (5, 6). Negative selection is a major mechanism to establish self-tolerance. Specifically, $CD4^+CD8^+$ thymocytes that bear TCRs with high affinity for self-peptide–MHC complexes are eliminated by apoptosis (2, 3, 6). Developing T cells that successfully complete intrathymic selection processes are exported to the periphery as mature $CD4^+$ or $CD8^+$ T lymphocytes.

The recognition of antigenic peptides by TCRs in the context of MHC molecules is the fundamental event shaping the fate of developing T cells. A variety of stimuli and cellular interactions can trigger the death of T cells by activating the apoptotic process. These include deprivation of essential growth factors, signaling through cell surface receptors other than TCRs, and exposure to agents such as glucocorticoids, calcium ionophore, or genotoxic drugs (1). The mechanisms that mediate and regulate apoptosis are still poorly understood, but it is thought that cell death is controlled by a genetic program that is induced within the dying cell (1, 7). There is increasing evidence that a major function of the death program is to activate proteins that act as effectors of the apoptotic mechanism (8, 9). However, apoptosis is further regulated by a set of genes that function as repressors of cell death (8).

The *bcl-2* protooncogene was the first member of an expanding family of genes that suppress the apoptotic mechanism (10). Constitutive expression of *bcl-2* in lymphoid cells prevents or delays apoptosis induced by multiple stimuli (11–14). A role for Bcl-2 in the selection of T lymphocytes was suggested by its highly restricted cellular distribution within the thymus (15–18). Recent evidence suggest that Bcl-2 plays a role in positive selection in the thymus (19–22). The ability of Bcl-2 to influence negative selection in the thymus is controversial. Some investigators have found that enforced expression of *bcl-2* was inefficient in protecting self-reactive thymocytes from clonal deletion

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/95/12/1973/11 $2.00
Volume 182 December 1995 1973–1983

(13), but others have reported partial abrogation of clonal deletion but not self-tolerance by *bcl-2* overexpression (14, 19, 23).

Mice deficient in Bcl-2 exhibit normal maturation of both B and T cell lineages, indicating that Bcl-2 is not essential for lymphoid development (24, 25). A plausible explanation is that proteins other than Bcl-2 can function as survival signals during the selection and maintenance of T lymphocytes. A candidate is Bcl-x_L, a product of *bcl-x*, another gene of the *bcl-2* family (26, 27). Like Bcl-2, Bcl-x_L localizes to mitochondrial membranes and perinuclear envelope (26) and inhibits the apoptotic death of hematopoietic cell lines after growth factor withdrawal (27). In mice, *bcl-*x_L is the dominant *bcl-x* mRNA expressed in embryonic and postnatal tissues, including the thymus (26, 28). Recent analyses of chimeric mice with a disrupted mutation of *bcl-x* demonstrate a major role for *bcl-x* during lymphoid development (29). However, the biological basis for the altered phenotype observed during T cell maturation in Bcl-x–deficient chimeric mice remains undetermined.

In these studies, we determined the distribution of the endogenous Bcl-x_L in thymocytes and T cells and examined the effect of constitutive expression of *bcl-*x_L in the T cell lineage. We showed that thymocytes and peripheral T cells from transgenic Eμ-*bcl-*x_L mice accumulated in the animal and exhibited prolonged survival in vitro. Furthermore, thymocytes overexpressing *bcl-*x_L exhibited increased survival after exposure to a diverse array of death-promoting stimuli that include glucorticoid hormones, ionizing radiation, calcium ionophore, and activation through CD3 molecules. In addition, we demonstrated that tolerance was maintained despite constitutive *bcl-*x_L expression since deletion of self-reactive lymphocytes was unaffected in the thymus and peripheral lymphoid organs. Finally, we showed that Bcl-2 and Bcl-x_L proteins exhibited discordant cellular distribution in the thymus and peripheral T cells. The implications of these findings for physiological pathways of T cell selection and survival are discussed.

Materials and Methods

Mice and Injections. C57BL/6, BALB/c, CBA/Ca and CBA/J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Animals used for these studies were between 8 and 12 wk of age (unless otherwise indicated). C57BL/6 mice expressing the α and β transgenes for the H-Y TCR were a gift of Dr. Harald von Boehmer (Basel Institute for Immunology, Basel, Switzerland) (30). They were bred and maintained in a pathogen-free environment at the University of Michigan. Glucocorticoids were administered by injection of 2 mg i.p. of dexamethasone (American Regent Laboratories, Inc., Shirley, NY). Hamster anti–mouse CD3 (clone 145-2C11; 100 μg) or anti-DNP (clone UC8-1B9; 100 μg), provided by Dr. R. Miller (University of Michigan), were injected intraperitoneally.

Construction of the SV40-Eμ-bcl-x_L *Transgene and Generation of Transgenic Mice.* To target *bcl-*x_L to the lymphoid compartment, the human *bcl-*x_L cDNA was cloned under the regulatory control of the SV40 promoter and immunoglobulin heavy chain enhancer using a SV40-EH cassette (31). The 2.4-kb fragment containing the SV40-EH-*bcl-*x_L insert was microinjected into F2 hybrid zygotes from (C57BL/6 × SJL/J)F1 parents at a concentration of 2–3 ng/μl. After overnight incubation, the eggs that survived to a two-cell stage were transferred to day 0.5 postcoitum pseudopregnant CS-1 females. 3 wk after birth, genomic DNA was prepared from tail tissue, and the incorporation of the human *bcl-*x_L transgene was assessed by dot blot analysis or PCR as previously described (27).

Cell Preparations. Murine lymphoid organs were placed in sterile PBS, and the tissue was disrupted with the tip of the plunger of a sterile 1-ml syringe. Cells were washed in cold PBS and centrifuged three times at 400 *g* for 5 min to remove cellular aggregates. Single-cell preparations were counted and assessed for viability by trypan blue exclusion. The cells were cultured at 37°C in RPMI supplemented with 2 mM L-glutamine, 10^{-5} M 2-ME, and 10% heat-inactivated FCS (Hyclone Laboratories Inc., Logan, UT). In some experiments, cells were incubated with dexamethasone (American Regent Laboratories, Inc.) or ionomycine (Sigma Chemical Co., St. Louis, MO) or irradiated before culture by exposure to a Co source at 250 rad. Cell proliferation in triplicate cultures was determined by adding Con A (10 μg/ml) to 4 × 10^5 lymphocytes in 96-well microplates (Falcon Plastics, Cockeysville, MD). After 3 d of culture, 1 μCi of [^{3}H]thymidine (Amersham Corp., Arlington Heights, IL) was added, cells were harvested, and [^{3}H]thymidine incorporation was measured in a scintillation counter (Beckman Instruments, Inc., Fullerton, CA).

Antibodies. FITC-labeled anti-CD4 (clone H129.19) and PE-conjugated anti-CD8 (clone 53-6.7) mAbs were purchased from GIBCO BRL (Gaithersburg, MD). The following antibodies were obtained from PharMingen (San Diego, CA): biotinylated anti-CD4 (clone RM-4-5), biotinylated anti-CD3-ϵ (clone 145-2C11), biotinylated and PE-conjugated anti-CD45R (B220) (clone RA3-6B2), PE-conjugated CD24 (heat stable antigen) (clone M1/69), FITC-labeled anti-IgM (clone R6-60.2), FITC-labeled anti-CD43 (leukosialin) (clone S7), FITC-labeled anti-Vβ2 TCR (clone B20.6), FITC-labeled anti-Vβ6 TCR (clone RR4-7), FITC-labeled anti-Vβ8.1, 8.2 TCR (clone MR5-2), FITC-labeled anti-Vβ11 TCR (clone RR3-15). The streptavidin RED670 reagent was purchased from GIBCO BRL.

Flow Cytometric Analysis. 10^6 cells were incubated with the indicated antibodies in 100 μl of PBS with 1% BSA for 30 min on ice and washed twice with PBS/1% BSA. When indicated, 25 μl of streptavidin RED670 was used for three-color analysis. Cells were analyzed with a FACScan® flow cytometer and a minimum of 3 × 10^4 events per sample were counted using Lysys II software (Becton Dickinson & Co., Mountain View, CA). Analysis of Bcl-x expression by flow cytometry was performed as previously described for Bcl-2 (32), using a mouse IgG2b anti–Bcl-x mAb (Transduction Lab., Lexington, KY), followed by a biotinylated goat anti–mouse IgG2b (CALTAG Laboratories, South San Francisco, CA). Apoptosis levels in thymocytes were assessed by propidium iodide (PI)[1] staining as previously described (33). Briefly, a pellet of 5 × 10^5 cells was resuspended in 600 μl of H_2O containing 50 μg/ml of PI, 0.1% Triton X-100 (Sigma Chemical Co.), and 0.1% sodium citrate and then incubated at 4°C overnight in the dark. Cells were analyzed by flow cytometry.

Western Blotting Analysis. Bcl-x_L protein expression was determined by Western blotting as previously described (32). Briefly, proteins were transferred to nitrocellulose membranes by electrophoresis and then incubated at 4°C overnight with a rabbit anti-

[1] *Abbreviations used in this paper:* HRP, horseradish peroxidase; PI, propidium iodide; TBS, Tris-buffered saline.

Bcl-x polyclonal serum (provided by Dr. Craig Thompson, University of Chicago, Chicago, IL) diluted 1:500 in 1.5% milk Tris-buffered saline (TBS) with 30 μl of normal goat serum (Sigma Chemical Co.) or with a mouse anti-β-tubulin antibody (Sigma Chemical Co.) at 1 μg/ml diluted in 1.5% milk-TBS. After five washes in TBS with 0.05% Tween 20, the membrane was incubated for 1 h at room temperature with goat anti-rabbit antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted 1:50,000 in 1.5% milk-TBS or with goat anti-mouse antibody conjugated to HRP (Jackson ImmunoResearch Laboratories) diluted 1:25,000 in 1.5% milk-TBS. Bound antibody was detected by chemiluminescence using the ECL Western Blot kit (Amersham Corp.).

Results

Discordant Regulation of Bcl-x and Bcl-2 during T Cell Development. In thymic tissue of normal mice, Bcl-x_L is the only Bcl-x protein detected by Western blot analysis using polyclonal antisera (34). To examine the expression of the endogenous Bcl-x_L protein in subsets of thymocytes, cells were labeled with anti–Bcl-x mAb, anti-CD4, and anti-CD8 antibodies. The expression of Bcl-x_L was low in $CD4^-CD8^-$ cells, increased in $CD4^+CD8^+$ immature thymocytes, and downregulated in more mature $CD4^+CD8^-$ or $CD8^+CD4^-$ thymocytes (Fig. 1). Furthermore, the Bcl-x_L protein was not detected in unstimulated peripheral $CD4^+$ or $CD8^+$ T cells (Fig. 1). This difference in Bcl-x_L expression was quantified by calculating the ratio of mean channel fluorescence of Bcl-x_L and that of control Ig in each subpopulation of thymocytes and peripheral T cells. The mean channel fluorescence ratio for $CD4^-CD8^-$, $CD4^+CD8^-$, and $CD4^-CD8^+$ thymocytes was similar (1.9 ± 0.2, 2.0 ± 0.4 and 2.0 ± 0.3, respectively), increased for $CD4^+CD8^+$ (4.4 ± 0.5), and decreased for peripheral $CD4^+$ and $CD8^+$ T cells (1.0 ± 0.1). Thus, the expression of Bcl-x_L is tightly controlled during T cell development and differs significantly from that previously observed for Bcl-2 (15–18).

Generation of Transgenic Mice Expressing Deregulated bcl-x_L in Thymocytes and Peripheral T Cells. We developed a transgenic mouse model overexpressing Bcl-x_L to assess its effects on T cell development. The *bcl-x_L* transgene was under the control of the IgH chain enhancer, which is known to target genes to lymphoid cells (31, 35, and Fig. 2 *A*). Seven founders expressing Bcl-x_L in lymphoid tissues were identified and used to establish transgenic lines. Each line was assessed for expression of Bcl-x_L protein by Western blot analysis using a polyclonal antibody reactive with the murine and human Bcl-x proteins. Two lines (*bcl-x*-169 and *bcl-x*-86) that exhibited restricted expression of the *bcl-x_L* transgene to the T cell compartment were further characterized. Targeted expression of transgenes driven by the SV40 promoter and IgH enhancer to the T lineage has been reported in all major thymocyte subsets and peripheral T cells (14, 36). As shown in Fig. 2 *B*, the Bcl-x_L protein was overexpressed in the thymus of *bcl-x*-169 (five-fold) and *bcl-x*-86 mice (two fold) when compared with the endogenous levels of Bcl-x_L in normal thymocytes. Quantification was obtained by densitometry scanning and normalized to the reference gene β-tubulin. Moreover, Bcl-x_L was not detected in the spleen and lymph nodes of normal mice but was expressed in *bcl-x*–transgenic animals (Fig. 2 *B*). Lastly, the *bcl-x_L* transgene was not found in purified populations of splenic B cells and in several nonlymphoid organs, including brain, liver, kidney, and lung, as determined by comparison to endogenous levels of Bcl-x_L observed in control mice (data not shown).

Constitutive Expression of Bcl-x_L Leads to Accumulation of Thymocytes and Peripheral T Cells. The expression of the *bcl-x_L* transgene in the thymus increased the number of total thymocytes. Table 1 shows the results obtained for transgenic mice compared with control littermates. In *bcl-x*-169, the total number of thymocytes increased by 46% (41.2×10^6 vs. 60×10^6 in transgenic mice), and the three major subsets of thymocytes ($CD4^+CD8^+$, $CD4^+CD8^-$, and $CD4^-CD8^+$) were augmented over control littermates. In *bcl-x*-86 mice whose thymocytes expressed less Bcl-x_L protein than *bcl-x*-169 (Fig. 2 *B*), the total number of thymocytes at 6 wk was similar to nontransgenic littermates (data not shown). However, by 10 wk of age, the total number of thymocytes increased by 58% (from 43×10^6 in control to 68×10^6 in transgenic mice) and displayed a slight increase in all thymocyte subsets when compared with the effects observed in *bcl-x*-169. Thus, constitutive

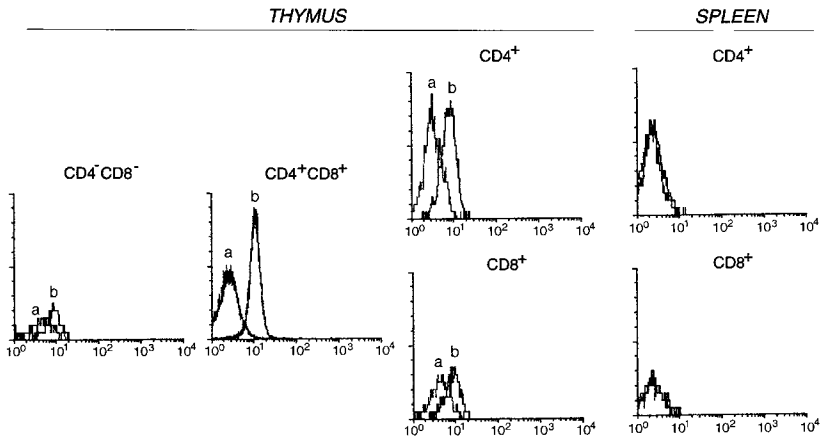

Figure 1. Flow cytometric analysis of murine endogenous Bcl-x expression. Thymocytes and spleen cells from 8-wk-old C57BL/6 mice were labeled with FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 antibodies. Cells were fixed with PBS containing 2% paraformaldehyde, labeled with anti-Bcl-x mAb or isotype-matched control antibody, and analyzed by flow cytometry. Bcl-x_L expression was assessed in different T cell populations. Data are presented as a frequency histogram with fluorescence intensity on the horizontal axis and relative cell number on the vertical axis. *a* and *b* represent background level and Bcl-x_L expression, respectively. For splenic $CD4^+$ and $CD8^+$ T cells, histograms representing background and Bcl-x_L staining overlap. Results are representative of three different experiments.

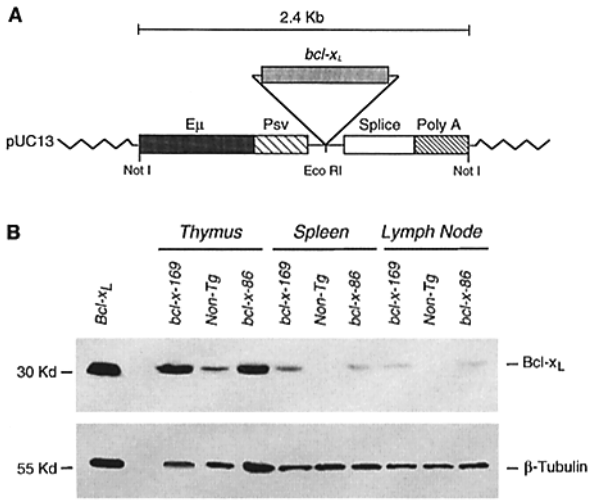

Figure 2. Eμ-*bcl-x$_L$* transgene construct and Westen blot analysis of Bcl-x$_L$ expression. (*A*) A 0.75-kb cDNA fragment containing the coding region of human *bcl-x$_L$* was inserted into the EcoRI site of the Eμ-SV40 cassette (14). Eμ is the immunoglobulin heavy enhancer and SV40 the early SV40 promoter. (*B*) Expression of Bcl-x$_L$ was assessed by Western blot analysis with a polyclonal anti-Bcl-x antibody followed by goat anti–rabbit serum conjugated to HRP. Lysates from 10^6 cells were loaded in each lane. In the left lane, lysate from FL-5.12 cells transfected with the murine *bcl-x$_L$* cDNA (26) is shown as a positive control indicated as Bcl-x$_L$. As a control, expression of β-tubulin was also assessed with a mouse anti-β-tubulin antibody followed by goat anti–mouse serum conjugated to HRP.

Bcl-x$_L$ expression during T cell development resulted in uniform accumulation of thymocytes, and its effect on thymocytes correlated with the amount of Bcl-x$_L$ expressed in the thymus.

The size of the peripheral T cell pool was increased by deregulated *bcl-x$_L$*. The total number of lymphocytes in *bcl-x*-169 mice increased in lymph nodes (from 10×10^6 in control littermates to 29×10^6) and spleen (from 61×10^6 to 85×10^6) (Table 2). The increase in lymphoid cells reflected an accumulation of both CD4 and CD8 mature T cells. The increase in T cells was more pronounced in lymph nodes (5.5-fold) than in spleen (2-fold). Similar effects on peripheral T cells were observed in *bcl-x*-86 mice. However, the accumulation of mature T cell subpopulations was less pronounced in *bcl-x*-86 than in *bcl-x*-169 mice. Whereas the percentage of B cells in peripheral lymphoid organs fell drastically, the absolute number of B cells did not differ significantly between transgenic and control mice. Furthermore, different subsets of developing and mature B cells were unaltered in transgenic mice, indicating that the phenotypic changes mirrored the pattern of transgene expression (data not shown). Thus, in contrast to Bcl-2 (13, 14), constitutive Bcl-x$_L$ expression in the T cell lineage increases the number of thymocytes and mature CD4 and CD8 T cells in peripheral organs.

Bcl-x$_L$ Increases the Survival of Thymocytes and Peripheral T Cells In Vitro. To assess the effect of *bcl-x$_L$* on their survival, thymocytes from *bcl-x$_L$*–transgenic and control littermates were cultured in RPMI medium supplemented with 10% FCS, and their viability was assessed by trypan blue exclusion. There was an improved survival of transgenic thymocytes compared with control thymocytes. By day 6 of culture, 50% of thymocytes from *bcl-x*-169 and 36% from *bcl-x*-86 mice were still viable, whereas <5% of the control thymocytes survived (Fig. 3). Similarly, splenic T cells from *bcl-x*–transgenic mice exhibited increased survival com-

Table 1. *Thymocyte Populations in bcl-x-169–and bcl-x-86–transgenic Mice*

	Transgenic mice		Control littermates	
	n ($\times 10^6$)	%	*n* ($\times 10^6$)	%
bcl-x-169				
Total cells	60.0 ± 2.0*‡		41.2 ± 2.1‡	
$CD4^-CD8^-$	1.7 ± 0.2	2.9 ± 0.3	1.1 ± 0.5	2.8 ± 0.4
$CD4^+CD8^+$	47.9 ± 2.4‡	79.8 ± 1.3‡	36.0 ± 1.9‡	87.5 ± 0.7‡
$CD4^+CD8^-$	4.6 ± 0.4‡	7.6 ± 0.3‡	1.8 ± 0.3‡	4.3 ± 0.5‡
$CD4^-CD8^+$	2.1 ± 0.1‡	3.5 ± 0.1‡	0.6 ± 0.1‡	1.4 ± 0.1‡
bcl-x-86				
Total cells	68.0 ± 3.1‡		43.0 ± 2.2‡	
$CD4^-CD8^-$	1.4 ± 0.4	2.0 ± 0.5	1.2 ± 0.5	2.7 ± 0.9
$CD4^+CD8^+$	56.6 ± 2.3‡	83.3 ± 0.7	36.1 ± 1.4‡	84.0 ± 0.6
$CD4^+CD8^-$	3.9 ± 0.8‡	5.7 ± 1.0	2.0 ± 0.9‡	4.6 ± 0.9
$CD4^-CD8^+$	1.2 ± 0.1‡	1.7 ± 0.2	0.7 ± 0.1‡	1.6 ± 0.1

*Results were obtained by two-color flow cytometric analysis of thymocytes simultaneously labeled with FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 antibodies. Values shown represent the mean ± SD for 10 animals.
‡Differences between transgenic and control littermates were statistically significant ($p < 0.01$) as assessed by Student's *t* test. *bcl-x*-169–transgenic and control littermates were 6 wk old. *bcl-x*-86–transgenic and control littermates were 10 wk old.

Table 2. *Lymphoid Populations in Lymph Nodes and Spleen of bcl-x-169–and bcl-86–transgenic Mice*

	Transgenic mice		Control littermates	
	n (× 10^6)	%	*n* (× 10^6)	%
bcl-x-169				
Lymph node	29.0 ± 4.0*‡		10.0 ± 1.5‡	
$CD4^+$	14.2 ± 3.0‡	48.8 ± 3.7‡	2.3 ± 0.3‡	22.6 ± 0.3‡
$CD8^+$	7.1 ± 0.5‡	24.5 ± 0.3‡	1.6 ± 0.3‡	15.5 ± 0.5‡
$B220^+$	5.8 ± 1.6		3.8 ± 0.6	38.1 ± 0.2‡
Spleen	85.0 ± 9.9‡		61.0 ± 8.5‡	
$CD4^+$	21.0 ± 3.0‡	25.4 ± 0.7‡	10.3 ± 2.0‡	16.9 ± 1.0‡
$CD8^+$	10.2 ± 1.0‡	12.0 ± 0.1‡	5.8 ± 1.1‡	9.5 ± 0.6‡
$B220^+$	37.5 ± 9.9	44.1 ± 7.4	32.9 ± 5.5	54.0 ± 3.5
bcl-x-86				
Lymph node	20.0 ± 2.0‡		11.0 ± 2.0‡	
$CD4^+$	6.9 ± 1.0‡	34.7 ± 0.7‡	2.1 ± 0.3‡	18.9 ± 0.2‡
$CD8^+$	5.6 ± 0.4‡	28.2 ± 2.2‡	1.8 ± 0.5‡	16.4 ± 1.8‡
$B220^+$	3.7 ± 0.4	18.3 ± 4.0‡	4.7 ± 0.6	43.0 ± 3.0‡
Spleen	80.0 ± 3.0‡		61.0 ± 1.0‡	
$CD4^+$	16.1 ± 1.5‡	20.1 ± 0.9‡	9.0 ± 2.0‡	14.7 ± 1.8‡
$CD8^+$	9.8 ± 1.4‡	12.3 ± 1.4‡	4.4 ± 1.1‡	7.2 ± 1.6‡
$B220^+$	35.1 ± 4.0	43.9 ± 6.1‡	39.2 ± 1.3	64.3 ± 10‡

*Results were obtained by three-color flow cytometric analysis. Cells from spleen and inguinal and axillary lymph nodes were simultaneously labeled with FITC-conjugated anti-CD4, PE-conjugated anti-CD8, and biotinylated anti-B220 antibodies. Values shown represent the mean ± SD for 10 animals.

‡Differences between transgenic and control littermates were statistically different ($p < 0.01$) as assessed by the Student's *t* test.

pared with T cells from control littermates (data not shown). However, the capacity of both transgenic and control splenic T cells to proliferate in response to Con A in culture was virtually identical (data not shown).

Bcl-x_L Protects Thymocytes against Dexamethasone, γ Irradiation, and Calcium Ionophore–induced Cell Death In Vitro. Glucocorticoid hormones are known to cause a rapid depletion of thymocytes by apoptosis (37). To evaluate whether *bcl-x_L* inhibits DNA fragmentation associated with glucocorticoid-induced apoptosis, thymocytes were treated with 0.1 μM of dexamethasone in culture for up to 18 h. Flow cytometric analysis revealed that dexamethasone-treated thymocytes displayed a sub-Go DNA peak, which is known to correspond to fragmented DNA in thymocytes (38). At 6 h, only 10% of thymocytes from *bcl-x*-169 mice were apoptotic in the presence of dexamethasone compared with 75% of control thymocytes. At 18 h, 40% of the dexamethasone-treated thymocytes from *bcl-x*-169 mice contained fragmented DNA as assessed by staining of nuclei with PI versus 95% of dexamethasone-nontransgenic thymocytes (Fig. 4 *A*). A lesser level of protection against dexamethasone-induced DNA fragmentation was observed for thymocytes from *bcl-x*-86 at 6 and 18 h of culture (Fig. 4 *A*).

Because glucocorticoids and irradiation appear to induce thymocyte apoptosis in part by different pathways (9), the ability of *bcl-x_L* to protect against irradiation-induced cell death was evaluated in thymocyte cultures. At a dose of 250 rad, >35% and 80% of control thymocytes displayed fragmented DNA at 6 and 18 h, respectively, after irradiation (Fig. 4 *B*). However, thymocytes from *bcl-x*-169 mice were remarkably resistant to irradiation (Fig. 4 *B*). Thymocytes from *bcl-x*-86 mice were also more resistant to irradiation than cells from control littermates (Fig. 4 *B*).

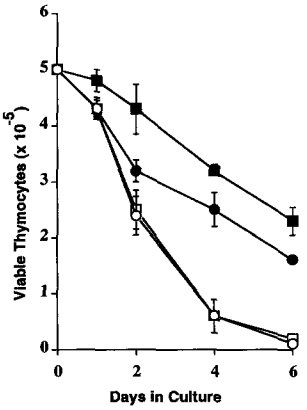

Figure 3. Expression of the transgene *bcl-x_L* increases thymocyte viability in vitro. Thymocytes from transgenic *bcl-x*-169 (*solid squares*) and *bcl-x*-86 (*solid circles*) and their respective control littermates (*open squares* and *open circles*) were cultured in 96-well flat-bottomed plates at 10^6 cells/ml in RPMI with 10% FCS. From day 1 to day 6, viability of thymocytes was assessed by trypan blue exclusion. All data points represent the mean of triplicate cultures ± SD for three independent experiments.

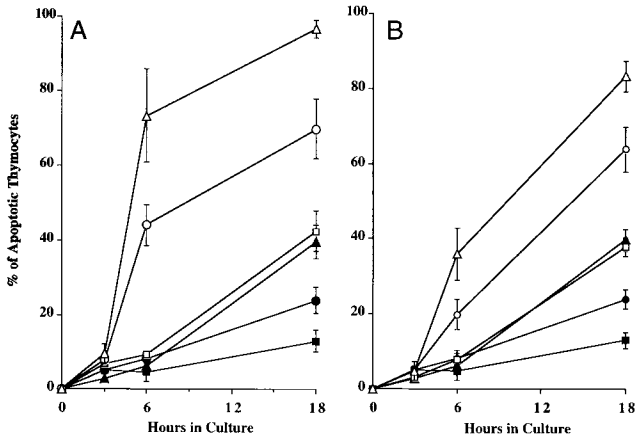

Figure 4. Bcl-x_L protects thymocytes against dexamethasone- and γ irradiation–induced apoptosis in vitro. (*A*) Thymocytes from transgenic *bcl-x*-169 (*squares*), *bcl-x*-86 (*circles*), and control mice (*triangles*) were cultured at 10^6 cells/ml (24-well plates) in the presence (*open symbols*) or absence (*solid symbols*) of 1 μM of dexamethasone. At 0, 3, 6, and 18 h, cells were fixed with paraformaldehyde, permeabilized with Triton X-100, and assessed for nuclear DNA content by PI staining. Cell were analyzed by flow cytometry. Data represent mean ± SEM of apoptotic cells in triplicate cultures for three independent experiments. (*B*) Thymocytes from mice transgenic for *bcl-x*-169 (*squares*), *bcl-x*-86 (*circles*), and control mice (*triangles*) were γ irradiated (*open symbols*) or not (*solid symbols*) at 250 rad and then cultured in vitro for 18 h at 10^6 cells/ml in 24-well plates. The percentage of apoptosis at 0, 3, 6, and 18 h was determined by analysis of the nuclear DNA content by PI staining. Data represent mean ± SD of apoptotic cells in triplicate cultures for three independent experiments.

In addition, thymocytes from *bcl-x*–transgenic mice were resistant to death induced by calcium ionophore. After 10 h of exposure to 2 μg/ml of ionomycin, 88 ± 5% of the control thymocytes were apoptotic versus 9 ± 2% of the thymocytes from *bcl-x*-169 mice.

Bcl-x_L Protects Thymocytes against Dexamethasone-induced Cell Death In Vivo. To assess the effects of *bcl-x_L* on thymocyte survival in vivo, *bcl-x*-169–transgenic and control littermates received 2 mg i.p. of dexamethasone by injection, and thymocyte populations were examined 48 h later by flow cytometric analysis. Dexamethasone treatment eliminated 70 ± 10% of total thymocytes from control mice compared with PBS-treated animals (from 45 × 10^6 to 14 × 10^6). In contrast, transgenic thymocytes were markedly protected from dexamethasone, with a decrease of only 15 ± 3% (from 61 × 10^6 to 51 × 10^6). Further analysis revealed that $CD4^+CD8^+$ thymocytes from control mice were almost entirely eliminated by dexamethasone, whereas 72% of this population remained viable in *bcl-x*–transgenic animals (Fig. 5). In paralleled experiments, treatment with dexamethasone deleted equally $B220^{low}IgM^-$ B cells from transgenic and control animals, demonstrating that the protection conferred by *bcl-x_L* was lineage specific and mirrored transgene expression (Fig. 5).

Bcl-x_L Protects against Anti-CD3–induced Apoptosis In Vivo. Triggering of $CD4^+CD8^+$ thymocytes with anti-CD3 induces apoptosis in vitro and in vivo (2, 39). To assess Bcl-x_L ability to block anti-CD3–induced apoptosis, transgenic mice and control littermates were injected with anti-CD3, and their thymuses were examined 48 h later. As shown in Table 3, 73% of $CD4^+CD8^+$ thymocytes from *bcl-x*-169 mice were still present 48 h after anti-CD3 treatment, whereas only 2% of this population remained in nontransgenic animals. As has been shown by others (2, 39), double-negative and single-positive thymocyte populations were largely unaffected by anti-CD3 treatment (Table 3). Hence, overexpression of Bcl-x_L can block anti-CD3–induced apoptosis.

Bcl-x_L Fails to Protect Thymocytes from Clonal Deletion Induced by Endogenous Superantigens. An important form of thymocyte apoptosis is the deletion of developing T cells that express high-affinity TCRs for self-antigens (2, 3). Signaling through CD3 in $CD4^+CD8^+$ is thought to transduce signals that mimic those of negative selection, and Bcl-x_L is able to block anti-CD3–induced apoptosis. Therefore we determined the ability of the *bcl-x_L* transgene to overcome the deletion of self-reactive T cells. We evaluated the deletion of T cells reactive with superantigen Mls-1

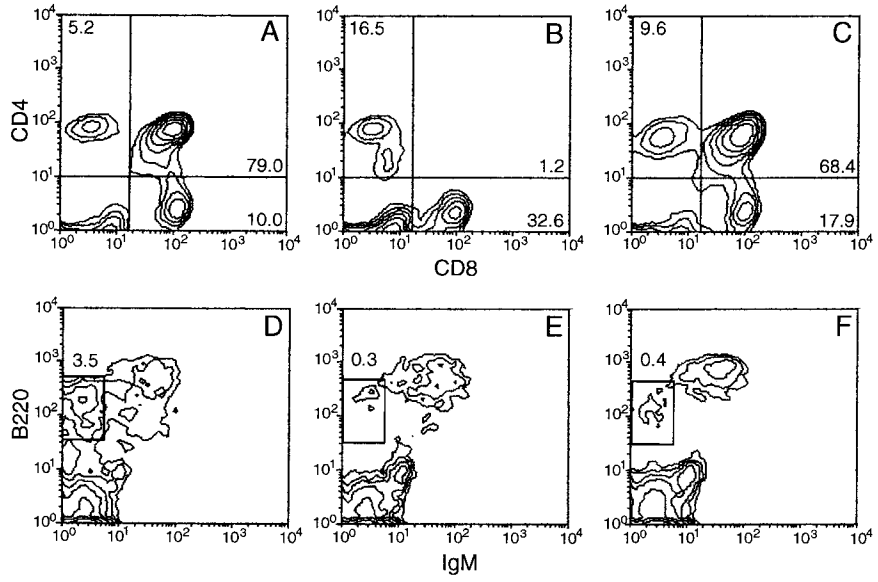

Figure 5. Overexpression of Bcl-x_L blocks thymocyte dexamethasone-induced cell death in vivo. Transgenic *bcl-x*-169 mice (*C* and *F*) and control littermates (*B* and *E*) were injected i.p. with 2 mg of dexamethasone and compared with control mice injected with PBS (*A* and *D*). 48 h later, thymocytes (*A–C*) were recovered and stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 antibodies and analyzed by flow cytometry. The mean percentage of cells is given for each subpopulation. Bone marrow cells (*D–F*) were collected at the same time and stained with FITC-conjugated anti-IgM and biotinylated anti-B220 antibodies. Pro- and pre-B cells (IgM^-B220^{low}) were gated in region R1 (defined by the small rectangle in *D–F*). The percentage of this population is indicated. The same experiment was repeated twice with three mice in each experiment with similar results. Identical results were obtained with mice from *bcl-x*-86 (data not shown).

Table 3. *Bcl-x_L Protects against Anti-CD3–induced Apoptosis In Vivo*

Cell populations	*bcl-x*-169 Transgenic mice		Control littermates	
	Control*	Anti-CD3*	Control	Anti-CD3
		× 10^6		
Total cells	61.0 ± 8.0‡	48.0 ± 6.0‡	40.0 ± 5.0‡	9.0 ± 2.0‡
$CD4^-CD8^-$	2.8 ± 0.8	3.0 ± 0.2	1.6 ± 0.7	2.0 ± 0.5
$CD4^+CD8^+$	48.8 ± 4.3‡	35.6 ± 5.4‡	34.5 ± 2.0‡	0.7 ± 0.1‡
$CD4^+CD8^-$	4.9 ± 0.5	4.5 ± 0.3	2.6 ± 0.8	3.8 ± 0.5
$CD4^-CD8^+$	3.2 ± 0.8	3.4 ± 0.3	1.1 ± 0.2	0.8 ± 0.3

*Transgenic and control littermates were injected with 100 μg of anti-CD3 (clone 145-2C11) or 100 μg of anti-DNP (clone UC8-1B9) i.p. as control and killed 48 h later. Results were obtained by three-color flow cytometric analysis of thymocytes simultaneously labeled with FITC-conjugated anti-CD4, PE-conjugated anti-CD8, and biotinylated anti-CD3 antibodies. Values shown represent the mean ± SD for six animals.
‡Differences between control- and anti-CD3–treated cells were statistically significant ($p < 0.01$) as assessed by Student's *t* test.

and an endogenous superantigen presented in the context of the MHC class II molecule I-E. When Mls-1 is expressed in the thymus, T cells bearing TCRs containing $V\beta6^+$, $V\beta7^+$, $V\beta8.1^+$, or $V\beta9^+$ regions are deleted (40–43). Similarly, expression of a specific superantigen in association with I-E induces the deletion of T cells bearing $V\beta11^+$, $V\beta5^+$, or $V\beta17a^+$ regions (44, 45). Transgenic mice in the C57BL/6 × SJL ($H\text{-}2^b$) background that do not express Mls-1 or I-E were crossed to CBA/J mice that are homozygous for Mls-1 and I-E. Flow cytometric analysis revealed that mature thymocytes expressing Vβ6 or Vβ11 in their TCRs were completely deleted in [CBA/J × (C57BL/6 × SJL)-*bcl-x*-169]F1 mice (Table 4). There was a compensatory increase in thymocytes bearing $V\beta2^+$ in [CBA/J × (B6 × SJL)] F1 compared with B6 × SJL mice, which has been reported previously by others (46). Consis-

Table 4. *Overexpression of bcl-x_L Does Not Block Clonal Deletion Induced by Mls-1^a or I-E*

Mice	*bcl-x* tg	Mls-1^a	Percentage of $V\beta6^+$ in thymocyte populations		
			$CD4^+$	$CD8^+$	$CD4^+CD8^+$
[CBA/J × (B6 × SJL)]	+	+	0.2 ± 0.0*	0.2 ± 0.0	5.1 ± 0.1
[CBA/J × (B6 × SJL)]	−	+	0.3 ± 0.0	0.0 ± 0.0	4.6 ± 0.2
B6 × SJL	−	−	7.6 ± 0.2	9.4 ± 1.0	4.3 ± 0.3
Mice	*bcl-x* tg	I-E	Percentage of $V\beta11^+$ in thymocyte populations		
			$CD4^+$	$CD8^+$	$CD4^+CD8^+$
[CBA/J × (B6 × SJL)]	+	+	0.0 ± 0.0	0.3 ± 0.3	5.1 ± 0.3
[CBA/J × (B6 × SJL)]	−	+	0.1 ± 0.0	0.3 ± 0.0	5.5 ± 0.1
B6 × SJL	−	−	5.1 ± 0.6	6.6 ± 0.5	4.7 ± 0.3
Mice	*bcl-x* tg		Percentage of $V\beta2^+$ in thymocyte populations		
			$CD4^+$	$CD8^+$	$CD4^+CD8^+$
[CBA/J × (B6 × SJL)]	+		11.4 ± 0.5	15.5 ± 1.2	3.3 ± 0.4
[CBA/J × (B6 × SJL)]	−		11.9 ± 0.8	20.3 ± 1.1	3.1 ± 0.5
B6 × SJL	−		3.8 ± 0.2	3.5 ± 0.1	2.5 ± 0.2

*Results were obtained by three-color flow cytometric analysis of thymocytes simultaneously labeled with biotinylated anti-CD4, PE-conjugated anti-CD8, and different FITC-conjugated anti-Vβ antibodies. Values shown represent the mean ± SD for five animals. Percentage differences between *bcl-x*-169–transgenic and control littermates were not statistically different by Student's *t* test.

Table 5. *Overexpression of bcl-x_L Does Not Block H-Y–induced Clonal Deletion*

Mice*		Thymocyte populations				
H-Y	Bcl-x_L	Total	$CD4^-CD8^-$	$CD4^+CD8^+$	$CD4^-CD8^+$	$CD4^+CD8^-$
		$\times 10^6$		%		
+	+	9.0 ± 1.0‡	73.0 ± 9.0	1 ± 0.9	0.8 ± 0.3	15.9 ± 2.3
+	−	10.0 ± 2.0	70.0 ± 9.7	5 ± 2.4	0.6 ± 0.3	16.6 ± 3.0
−	−	40.0 ± 8.0	8.0 ± 0.5	85 ± 8.0	3.1 ± 0.2	4.0 ± 0.2

*bcl-x-169–transgenic mice and control littermates were [C57BL/6 H-Y tg × (C57BL/6 × SJL)]F1 males.
‡Results were obtained by two-color flow cytometric analysis of thymocytes simultaneously labeled with FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 antibodies. Values shown represent the mean ± SD for five animals. Percentage differences between transgenic and control littermates were not statistically significant as determined by Student's *t* test.

tent with the previous results, peripheral T cells bearing Vβ6 or Vβ11 were similarly deleted in transgenic and control littermates (data not shown). Thus, constitutive expression of *bcl-x_L* during T cell development fails to abrogate clonal deletion induced by Mls-1 or endogenous superantigen in the context of I-E MHC.

Bcl-x_L Does Not Block Thymocyte Clonal Deletion Induced by H-Y Antigen. Because presentation of superantigens to T cells differs from that of classical peptides (47), the ability of *Bcl-x_L* to inhibit clonal deletion of reactive T cells specific to the male H-Y antigen presented in the context of H-2 D^b MHC class I molecules (30, 48) was assessed by mating C57BL/6 mice homozygous for a H-Y α/β-TCR transgene (30) to heterozygous *bcl-x*-169–transgenic mice. In the H-Y male offspring, $CD4^+CD8^+$ and $CD4^-CD8^+$ thymocytes from both *bcl-x_L*− and non-*bcl-x_L*–transgenic mice were markedly deleted, compared with those from non-H-Y C57BL/6 male mice (Table 5). The number of $CD4^+$-$CD8^-$ thymocytes from both types of H-Y TCR–transgenic mice was essentially identical to that of non-transgenic controls, since clonal deletion induced by H-Y antigen is restricted to MHC class I molecules (30, 48). Thus, Bcl-x_L fails to abrogate clonal deletion of T cells reactive to self- H-Y antigen.

Discussion

These studies demonstrated that *bcl-x_L* can inhibit several forms of T cell death in thymocytes and/or peripheral T cells. These include the spontaneous apoptosis in the absence of growth factors in vitro and the apoptosis induced by glucocorticoids, γ irradiation, calcium ionophore, and anti-CD3 antibody. All of these stimuli have been shown to induce apoptotic cell death in thymocytes (13, 14). We also showed that endogenous Bcl-x_L expression is regulated during T cell development in a pattern different from that of Bcl-2. A major role for *bcl-x* in thymocyte development has been demonstrated in chimeric mice that are deficient in Bcl-x (29). In these studies, the absence of Bcl-x_L preferentially affected immature populations of thymocytes and pre-B cells (29). Our results provide an explanation for the findings in the T cell lineage in that Bcl-x_L was expressed predominately in $CD4^+CD8^+$ thymocytes, the developmental stage particularly affected by the absence of Bcl-x (29). Deregulated Bcl-x_L expression led to a perturbation of T cell homeostasis characterized by accumulation of thymocytes and mature $CD4^+$ and $CD8^+$ T cells in all lymphoid organs. Remarkably, despite this alteration, Bcl-x_L deregulation failed to inhibit clonal deletion of self-reactive T cells against endogenous superantigens and H-Y antigen.

The basis for the failure or poor ability of Bcl-x_L and Bcl-2 to block clonal deletion compared with T cell death induced by other stimuli, including anti-CD3, is unclear. During negative selection, thymocytes bearing TCRs with high affinity for a self-antigen are deleted (6). Signaling through CD3 in $CD4^+CD8^+$ cells is thought to transduce signals that mimic those of negative selection. However, clonal deletion of thymocytes appears to involve CD4–CD8, CD28, and other cellular interactions in addition to the CD3–TCR complex (49, 50). Thus, it is possible that self-peptide–MHC/TCR signaling during negative selection results in a powerful death signal that cannot be abrogated by the levels of Bcl-2 or Bcl-x_L produced by the transgenes. It is also possible that certain signaling pathways such as those responsible for clonal deletion involve distinct death effector molecules that may not be inhibited by Bcl-2 or Bcl-x_L. Experimental evidence for different intracellular mediators of T cell death has been recently provided for glucocorticoids, radiation, and TCR–CD3 engagement (9). For example, thymocyte apoptosis triggered by exposure to γ irradiation or certain DNA-damaging agents but not glucocorticoids requires the tumor suppressor protein p53 (51, 52). Another example is the requirement of nur77 for TCR-mediated apoptosis, whereas it does not play a role in either glucocorticoid- or radiation-induced death (53, 54).

Constitutive expression of *bcl-2* and *bcl-x_L* protected thymocytes from a number of apoptotic stimuli. However, in contrast to our results with *bcl-x_L* mice, a Eμ-*bcl-2* transgene targeted to the thymus did not result in increased numbers of thymocytes or peripheral T cells despite ade-

quate expression of Bcl-2 in the thymus and peripheral lymphoid organs (14). The reason for the altered T cell homeostasis in Eμ-*bcl-x*$_L$–transgenic mice as opposed to the lack of effect observed in Eμ-*bcl-2* mice is unclear. However, mice that are transgenic for a construct containing *bcl-2* downstream of the proximal promoter of the lck tyrosine kinase gene (lck^{pr}) showed an accumulation of T cells (mainly CD8$^+$) (13), suggesting that both *bcl-x* and *bcl-2* can affect T cell homeostasis. Nevertheless, given the different phenotype observed between Eμ-*bcl-x* and Eμ-*bcl-2*–transgenic mice, it can not be excluded that Bcl-x$_L$ is more effective than Bcl-2 in countering some forms of T cell death operating in vivo. For example, Bcl-x$_L$ has been shown to be more efficient than Bcl-2 in protecting the immature B cell line WEHI 231 from apoptosis induced by certain chemotherapeutic agents (55).

Bcl-2 and Bcl-x$_L$ display a discordant expression pattern during T cell development. Bcl-x$_L$ is primarily expressed in CD4$^+$CD8$^+$ thymocytes, downregulated in CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ thymocytes, and undetectable in mature T cells. In contrast, Bcl-2 is expressed at high levels in early CD4$^-$CD8$^-$ T cell precursors, medullary CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ thymocytes and peripheral T cells, but downregulated in cortical CD4$^+$CD8$^+$ thymocytes (15–18). After the submission of this manuscript, another study was published (56) that showed that Bcl-x$_L$ is primarily expressed by CD4$^+$CD8$^+$ thymocytes and confirmed the essential role of *bcl-x* during thymic development (29). Bcl-x$_L$ can protect thymocytes from various apoptotic stimuli. Indeed, our results have shown that constitutive expression of Bcl-x$_L$ resulted in an accumulation of thymocytes belonging to all major subsets and mature T cells in the periphery. Thus, it is likely that its expression serves to maintain CD4$^+$CD8$^+$ thymocytes before positive selection. Because Bcl-x$_L$ is downregulated in more mature single-positive thymocytes and mature T cells, we suggest that CD4$^+$CD8$^+$ thymocytes that are not positively selected downregulate Bcl-x$_L$ later in development and fail to upregulate Bcl-2. In contrast, the subpopulation of CD4$^+$CD8$^+$ thymocytes that undergo positive selection is known to upregulate Bcl-2, a survival signal associated with positive selection (19–22). Thus, these results suggest that Bcl-2 acts later in development than Bcl-x$_L$ and functions as a survival switch for CD4$^+$-CD8$^+$ thymocytes during positive selection.

Diminished Bcl-2 expression in cortical CD4$^+$CD8$^+$ thymocytes has been proposed to play a facilitating role in the death of thymocytes during clonal selection (16–18, 57). Indeed, CD4$^+$CD8$^+$ thymocytes are more sensitive to multiple forms of apoptosis, including those induced by glucocorticoids, γ irradiation, and anti-CD3 signaling, than other thymocyte populations that express Bcl-2 (18, 39, 58). Given that overexpression of Bcl-x$_L$ can confer protection against multiple forms of apoptosis, it is intriguing that CD4$^+$CD8$^+$ thymocytes are sensitive to apoptosis, despite expression of endogenous Bcl-x$_L$ protein. A possible explanation is that simultaneous expression of Bcl-2 and Bcl-x$_L$ is more effective than Bcl-x$_L$ or Bcl-2 alone in protecting thymocytes from apoptosis. An alternative nonexclusive interpretation is that the ability of endogenous Bcl-x$_L$ to protect CD4$^+$CD8$^+$ thymocytes from death signals is countered by intracellular inhibitory proteins. There is accumulating evidence that the function of Bcl-2 and Bcl-x$_L$ is modulated by several interacting proteins that include Bax (59), Bad (60), and Bag-1 (61). Interestingly, two proteins of the Bcl-2 family, Bad and Bax, are produced by thymocytes and can form heterodimers with Bcl-2 and Bcl-x$_L$ (60, 62). In tissue culture experiments, susceptibility of cells to death signals appears to be determined by competing dimerizations among the different proteins of the Bcl-2 family expressed by the cell (60). Furthermore, Bad binds more strongly to Bcl-x$_L$ than to Bcl-2, and its interaction with Bcl-x$_L$ promotes cell death (60). Although the precise regulation of Bad or Bax during thymocyte development is unknown, it is tempting to speculate that endogenous Bcl-x$_L$ in CD4$^+$CD8$^+$ is countered by interacting proteins, resulting in an increased susceptibility to cell death. Based on the present studies and those by others, we would propose a hypothetical model of T cell survival in which the threshold to apoptosis is controlled by a balance among survival proteins such as Bcl-2 or Bcl-x$_L$, their inhibitory partners, and the "strength" of the death signal. In this model, endogenous levels of Bcl-x$_L$ in CD4$^+$CD8$^+$ thymocytes maintain T cell survival but are not sufficient to protect cells from death signals. Our results suggest that overexpressed Bcl-x$_L$ can effectively compete for inhibitory proteins or compensate for Bcl-2 downregulation and promotes thymocyte survival.

We thank Dr. Craig Thompson for providing the rabbit polyclonal anti–Bcl-x antibody, Dr. Richard Miller for the anti-CD3 and anti-DNP mAbs, Dr. Harald von Boehmer for the H-Y–transgenic mice, Dr. Suzanne Cory for the Eμ-SV40 cassette, and Dr. Michael Clarke, Dr. Phil Simonian, Mary Benedict, and Dr. Maribel Gonzalez-Garcia for critical review of the manuscript. We thank the University of Michigan Transgenic Core for their excellent support.

This work was supported by National Institutes of Health (NIH) grants CA64556 and P60-AR20557 to G. Nuñez. D. A. M. Grillot was supported by a fellowship from the Swiss National Science Foundation and R. Merino by a fellowship from the Leukemia Research Foundation. G. Nuñez is the recipient of Reseach Career Development Award (K04 CA64421) from the NIH.

Address correspondence to Dr. Gabriel Nuñez, Department of Pathology, MSRBII C558, 1500 W. Medical Center Drive, University of Michigan, Ann Arbor, MI 48109-0608.

Received for publication 3 May 1995 and in revised form 5 July 1995.

References

1. Ellis, R.E., J.Y. Yuang, and H.R. Horvitz. 1991. Mechanisms and functions of cell death. *Annu. Rev. Cell Biol.* 7:633–698.
2. Smith, C.A., J.T. Williams, R. Kingston, E.J. Jenkinson, and J.J.T. Owen. 1989. Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic culture. *Nature (Lond.).* 337:181–184.
3. Surh, C.D., and J. Sprent. 1994. T-cell apoptosis detected in situ during positive and negative selection in the thymus. *Nature (Lond.).* 372:100–103.
4. Rothenberg, E.V. 1992. The development of functionally responsive T cells. *Adv. Immunol.* 51:85–214.
5. Von Boehmer, H. 1994. Positive selection of lymphocytes. *Cell.* 76:219–228.
6. Nossal, G.J.V. 1994. Negative selection of lymphocytes. *Cell.* 76:229–239.
7. Wyllie, A.H., R.G. Morris, A.L. Smith, and D. Dunlop. 1984. Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. *J. Pathol.* 142:67–77.
8. Williams, G.T., and C.A. Smith. 1993. Molecular regulation of apoptosis: genetic controls on cell death. *Cell.* 74:777–779.
9. Osborne, B.A., and L.M. Schwartz. 1994. Essential genes that regulate apoptosis. *Trends Cell Biol.* 4:394–399.
10. Nuñez, G., and M.F. Clarke. 1994. The Bcl-2 family of proteins: regulators of cell death and survival. *Trends Cell Biol.* 4:399–403.
11. Vaux, D.L., S. Cory, and J.M. Adams. 1988. Bcl-2 gene promotes haematopoietic cell survival and cooperates with c-myc to immortalize pre-B-cells. *Nature (Lond.).* 335:440–442.
12. Nuñez, G., L. London, D. Hockenbery, M. Alexander, J.P. McKearn, and S.J. Korsmeyer. 1990. Deregulated Bcl-2 gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell lines. *J. Immunol.* 144:3602–3610.
13. Sentman, C.L., J.R. Shutter, D. Hockenbery, O. Kanagawa, and S.J. Korsmeyer. 1991. bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. *Cell.* 67: 879–888.
14. Strasser, A., A.W. Harris, and S. Cory. 1991. bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship. *Cell.* 67:889–899.
15. Pezzella, F., A.G. Tse, J.L. Cordell, K.A. Pulford, K.C. Gatter, and D.Y. Mason. 1990. Expression of the bcl-2 oncogene protein is not specific for the 14;18 chromosomal translocation. *Am. J. Pathol.* 137:225–232.
16. Gratiot-Deans J., L. Ding, L.A. Turka, and G. Nuñez. 1993. bcl-2 proto-oncogene expression during human T cell development. Evidence for biphasic regulation. *J. Immunol.* 151: 83–91.
17. Veis, D.J., C.L. Sentman, E.A. Bach, and S.J. Korsmeyer. 1993. Expression of the Bcl-2 protein in murine and human thymocytes and in peripheral T lymphocytes. *J. Immunol.* 151:2546–2554.
18. Andjelic, S., N. Jain, and J. Nikolic-Zugic. 1993. Immature thymocytes become sensitive to calcium-mediated apoptosis with the onset of CD8, CD4, and the T cell receptor expression: a role for bcl-2? *J. Exp. Med.* 178:1745–1751.
19. Strasser, A., A.W. Harris, H. von Boehmer, and S. Cory. 1994. Positive and negative selection of T cells in T-cell receptor transgenic mice expressing a bcl-2 transgene. *Proc. Natl. Acad. Sci. USA.* 91:1376–1380.
20. Gratiot-Deans, J., R. Merino, G. Nuñez, and L.A. Turka. 1994. Bcl-2 expression during T cell development: early loss and late return occurs at specific stages of commitment to differentiation and survival. *Proc. Natl. Acad. Sci. USA.* 91: 10685–10689.
21. Hogquist, K.A., S.C. Jameson, W.R. Heath, J.L. Howard, M.J. Bevan, and F.R. Carbone. 1994. T cell receptor antagonist peptides induce positive selection. *Cell.* 76:17–27.
22. Linette, G.P., M.J. Grusby, S.H. Hedrick, T.H. Hansen, L.H. Glimcher, and S.H. Korsmeyer. 1994. Bcl-2 is upregulated at the $CD4^+CD8^+$ stage during positive selection and promotes thymocyte differentiation at several control points. *Immunity.* 1:197–205.
23. Siegel, R.M., M. Katsumata, T. Miyashita, D.C. Louie, M.I. Greene, and J.C. Reed. 1992. Inhibition of thymocyte apoptosis and negative selection in transgenic mice. *Proc. Natl. Acad. Sci. USA.* 89:7003–7007.
24. Veis, D.J., C.M. Sorenson, J.R. Shutter, and S.J. Korsmeyer. 1993. Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys and hypopigmented hair. *Cell.* 75:229–240.
25. Nakayama, K., K. Nakayama, I. Negishi, K. Kuida, Y. Shinkai, M.C. Louie, L.E. Fields, P.J. Lucas, V. Stewart, F.W. Alt, and D.Y. Loh. 1993. Disappearance of the lymphoid system in Bcl-2 homozygous mutant chimeric mice. *Science (Wash. DC).* 261:1584–1588.
26. Gonzalez-Garcia, M., R. Perez-Ballestero, L. Ding, L. Duan, L.H. Boise, C.B. Thompson, and G. Nuñez. 1994. Bcl-x_L is the major Bcl-x mRNA form expressed during murine development and its product localizes to the outer mitochondria. *Development.* 120:3033–3042.
27. Boise, L.H., M. Gonzalez-Garcia, C.E. Postema, L. Ding, T. Linsten, L.A. Turka, X. Mao, G. Nuñez, and C.B. Thompson. 1993. bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell.* 74:597–608.
28. Fang, W., J.J. Rivard, D.L. Mueller, and T.W. Behrens. 1994. Cloning and molecular characterization of mouse bcl-x in B and T lymphocytes. *J. Immunol.* 153:4388–4398.
29. Motoyama, N., F. Wang, K.A. Roth, H. Sawa, K.-I. Nakayama, K. Nakayama, I. Negishi, S. Senju, Q. Zhang, S. Fujii, and D.Y. Loh. 1995. Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. *Science (Wash. DC).* 267:1506–1510.
30. Kisielow, P., H. Bluthmann, U.D. Staerz, M. Steinmetz, and H. von Boehmer. 1988. Tolerance in T-cell receptor transgenic mice involves deletion of non mature $CD4^+CD8^+$ thymocytes. *Nature (Lond.).* 333:742–746.
31. Strasser, A., S. Whittingham, D.L. Vaux, M.L. Bath, J.M. Adams, S. Cory, and A.W. Harris. 1991. Enforced BCL-2

expression in B-lymphoid cells prolongs antibody responses and elicits autoimmune disease. *Proc. Natl. Acad. Sci. USA.* 88:8661–8665.

32. Merino, R., L. Ding, D.J. Veis, S.J. Korsmeyer, and G. Nuñez. 1994. Developmental regulation of the Bcl-2 protein and susceptibility to cell death in B lymphocytes. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:683–691.
33. Nicoletti, I., G. Migliorati, M.C. Pagliacci, F. Grignani, and C. Ricardi. 1991. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Methods.* 139:271–279.
34. Krajewski, S., M. Krajewska, A. Shabaik, H.-G. Wang, S. Irie, L. Fong, and J.C. Reed. 1994. Immunohistochemical analysis of in vivo patterns of Bcl-x expression. *Cancer Res.* 54:5501–5507.
35. Rosenbaum, H., E. Webb, J.M. Adams, S. Cory, and A.W. Harris. 1989. N-myc transgene promotes B lymphoid proliferation, elicits lymphomas and reveals cross-regulation with c-myc. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:749–755.
36. Brombacher, F., M.C. Lamers, G. Kohler, and H. Eibel. 1989. Elimination of $CD8^+$ thymocytes in transgenic mice expressing an anti-Lyt2.2 immunoglobulin heavy chain gene. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:3719–3726.
37. Cohen, J.J., and R.C. Duke. 1984. Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. *J. Immunol.* 132:38–42.
38. Migliorati, G., I. Nicoletti, M.C. Pagliacci, L. D'Adamio, and C. Riccardi. 1993. Interleukin-4 protects double-negative and CD4 single-positive thymocytes from dexamethasone-induced apoptosis. *Blood.* 81:1352–1358.
39. Shi, Y., R.P. Bissonette, N. Parfrey, M. Szalay, R.T. Kubo, and D.R. Green. 1991. *In vivo* administration of monoclonal antibodies to CD3 T cell receptor complex induces cell death (apoptosis) in immature thymocytes. *J. Immunol.* 146:3340–3346.
40. Kappler, J.W., U. Staerz, J. White, and P. Marrack. 1988. Self-tolerance eliminates T cells specific for Mls-modified products of the major histocompatibility complex. *Nature (Lond.).* 332:35–40.
41. MacDonald, H.R., R. Schneider, R.K. Lees, R.C. Howe, H. Acha-Orbea, H. Festenstein, R.M. Zinkernagel, and H. Hengartner. 1988. T-cell receptor V beta use predicts reactivity and tolerance to Mlsa-encoded antigens. *Nature (Lond.).* 332:40–45.
42. Happ, M.P., D.L. Woodland, and E. Palmer. 1989. A third T-cell receptor β-chain variable region gene encodes reactivity to $Mls\text{-}1^a$ gene products. *Proc. Natl. Acad. Sci. USA.* 86: 6293–6296.
43. Okada, C.Y., B. Holzman, C. Guidos, E. Palmer, and I.L. Weissmann. 1990. Characterization of a rat monoclonal antibody specific for a determinant encoded by the $V_\beta 7$ gene segment. *J. Immunol.* 144:3473–3477.
44. Kappler, J.W., N. Roehm, and P. Marrack. 1987. T cell tolerance by clonal elimination in the thymus. *Cell.* 49:273–280.
45. Bill, J., V.B. Appel, and E. Palmer. 1988. An analysis of T-cell receptor variable region gene expression in major histocompatibility complex disparate mice. *Proc. Natl. Acad. Sci. USA.* 85:9184–9188.
46. White, J., A. Herman, A.M. Pullen, R. Kubo, J.W. Kappler, and P. Marrack. 1989. The V beta-specific superantigen staphylococcal enterotoxin B: stimulation of mature T cells and clonal deletion in neonatal mice. *Cell.* 56:27–35.
47. Dellabona, P., J. Peccoud, J.W. Kappler, P. Marrack, C. Benoist, and D. Mathis. 1990. Superantigens interact with MHC class II molecules outside of the antigen groove. *Cell.* 62:1115–1121.
48. Swat, W., L. Ignatowicz, H. von Boehmer, and P. Kisielow. 1991. Clonal deletion of immature $CD4^+8^+$ thymocytes in suspension culture by extrathymic antigen-presenting cells. *Nature (Lond.).* 335:150–153.
49. Punt, J.A., B.A. Osborne, Y. Takayama, S.O. Sharrow, and A. Singer. 1994. Negative selection of $CD4^+CD8^+$ thymocytes by T cell receptor–induced apoptosis requires a costimulatory signal that can be provided by CD28. *J. Exp. Med.* 179:709–713.
50. Weiss, A., and D.R. Littman. 1994. Signal transduction by lymphocyte antigen receptors. *Cell.* 76:263–274.
51. Lowe, S.W., E.M. Schmitt, S.W. Smith, B.A. Osborne, and T. Jacks. 1993. p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature (Lond.).* 362:847–849.
52. Clarke, A.R., C.A. Purdie, D.J. Harrison, R.G. Morris, C.C. Bird, M.L. Hooper, and A.H. Wyllie. 1993. Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature (Lond.).* 362:849–852.
53. Liu, Z.-G., S.W. Smith, K.A. Mclaughlin, L.M. Schwartz, and B.A. Osborne. 1994. Apoptotic signals delivered through the T-cell receptor of a T-cell hybrid require the immediate-early gene nur77. *Nature (Lond.).* 367:281–284.
54. Woronicz, J.D., B. Calnan, V. Ngo, and A. Winoto. 1994. Requirement for the orphan steroid receptor Nur77 in apoptosis of T-cell hybridomas. *Nature (Lond.).* 367:277–280.
55. Gottschalk, A.R., L.H. Boise, C.B. Thompson, and J. Quintans. 1994. Identification of immunosuppressant-induced apoptosis in a murine B-cell line and its prevention by bcl-x but not bcl-2. *Proc. Natl. Acad. Sci. USA.* 91:7350–7354.
56. Ma, A., J.C. Pena, B. Chang, E. Margosian, L. Davidson, F.W. Alt, and C.B. Thompson. 1995. Bclx regulates the survival of double-positive thymocytes. *Proc. Natl. Acad. Sci. USA.* 92:4763–4767.
57. Nuñez, G., R. Merino, D. Grillot, and M. Gonzalez-Garcia. 1994. Bcl-2 and Bcl-x: regulatory switches for lymphoid death and survival. *Immunol. Today.* 15:582–588.
58. Tadakuma, T., H. Kizaki, C. Odaka, R. Kubota, Y. Ishimura, H. Yagita, and K. Okumura. 1990. $CD4^+CD8^+$ thymocytes are susceptible to DNA fragmentation induced by phorbol ester, calcium ionophore and anti-CD3 antibody. *Eur. J. Immunol.* 20:779–784.
59. Oltvai, Z.N., C.L. Milliman, and S.J. Korsmeyer. 1993. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell.* 74:609–619.
60. Yang, E., J. Zha, J. Jockel, L.H. Boise, C.B. Thompson, and S.J. Korsmeyer. 1995. Bad, a heterodimeric partner for $Bcl\text{-}x_L$ and Bcl-2, displaces Bax and promotes cell death. *Cell.* 80: 285–291.
61. Takayama, S., T. Sato, S. Krajewski, K. Kochel, S. Irie, J.A. Millan, and J.C. Reed. 1995. Cloning and functional analysis of BAG-1: a novel Bcl-2-binding protein with anti-cell death activity. *Cell.* 80:279–284.
62. Yin, X.-M., Z.N. Oltvai, and S.J. Korsmeyer. 1994. BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. *Nature (Lond.).* 369: 321–323.