Bcl-xL Displays Restricted Distribution during T Cell Development and Inhibits Multiple Forms of Apoptosis but Not Clonal Deletion in Transgenic Mice
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Summary
The survival of T lymphocytes is tightly controlled during development. Here, we show that Bcl-xL, 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-xL in thymocyte apoptosis, we generated mice overexpressing an Eμ-bcl-xL transgene within the T cell compartment. Constitutive expression of Bcl-xL resulted in accumulation of thymocytes and mature T cells in lymphoid organs. Thymocytes overexpressing Bcl-xL 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-xL was unable to block clonal deletion of thymocytes reactive with self-superantigens or H-Y antigen. These studies demonstrate that Bcl-2 and Bcl-xL, 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-xL 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 suggests 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.
(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-xL, a product of bcl-x, another gene of the bcl-2 family (26, 27). Like Bcl-2, Bcl-xL 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-xL 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-xL-deficient chimeric mice remains undetermined.

In these studies, we determined the distribution of the endogenous Bcl-xL in thymocytes and T cells and examined the effect of constitutive expression of bcl-xL in the T cell lineage. We showed that thymocytes and peripheral T cells from transgenic Eμ-bcl-xL mice accumulated in the animal and exhibited prolonged survival in vitro. Furthermore, thymocytes overexpressing bcl-xL exhibited increased survival after exposure to a diverse array of death-promoting stimuli that include glucocorticoid hormones, ionizing radiation, calcium ionophore, and activation through CD3 molecules. In addition, we demonstrated that tolerance was maintained despite constitutive bcl-xL expression since deletion of self-reactive lymphocytes was unaffected in the thymus and peripheral lymphoid organs. Finally, we showed that Bcl-2 and Bcl-xL 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-Eh-bcl-xL Transgene and Generation of Transgenic Mice.** To target bcl-xL to the lymphoid compartment, the human bcl-xL 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-xL 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-xL 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 ionomycin (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, Cockeyesville, MD). After 3 d of culture, 1 μCi of [3H]thymidine (Amersham Corp., Arlington Heights, IL) was added, cells were harvested, and [3H]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-4-4), 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-xL expression by flow cytometry was performed as previously described for Bcl-2 (32), using a mouse IgG2b anti-Bcl-xL 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) staining as previously described (33). Briefly, a pellet of 5 × 10^6 cells was resuspended in 600 μl of H,O 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-xL 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-
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, Inc.) diluted 1:250,000 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-xL is the only Bcl-x protein detected by Western blot analysis using polyclonal antisera (34). To examine the expression of the endogenous Bcl-xL protein in subsets of thymocytes, cells were labeled with anti-Bcl-x mAb, anti-CD4, and anti-CD8 antibodies. The expression of Bcl-xL 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-xL protein was not detected in unstimulated peripheral CD4+ or CD8+ T cells (Fig. 1). This difference in Bcl-xL expression was quantified by calculating the ratio of mean channel fluorescence of Bcl-xL 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-xL 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-xL in Thymocytes and Peripheral T Cells. We developed a transgenic mouse model overexpressing Bcl-xL to assess its effects on T cell development. The bcl-xL 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-xL in lymphoid tissues were identified and used to establish transgenic lines. Each line was assessed for expression of Bcl-xL 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-xL 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-xL protein was overexpressed in the thymus of bcl-x-169 (fivefold) and bcl-x-86 mice (two fold) when compared with the endogenous levels of Bcl-xL in normal thymocytes. Quantification was obtained by densitometry scanning and normalized to the reference gene β-tubulin. Moreover, Bcl-xL was not detected in the spleen and lymph nodes of normal mice but was expressed in bcl-xL-transgenic animals (Fig. 2 B). Lastly, the bcl-xL 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-xL observed in control mice (data not shown).

Constitutive Expression of Bcl-xL Leads to Accumulation of Thymocytes and Peripheral T Cells. The expression of the bcl-xL 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 × 106 vs. 60 × 106 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-xL 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 × 106 in control to 68 × 106 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-xL 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-xL expression, respectively. For splenic CD4+ and CD8+ T cells, histograms representing background and Bcl-xL staining overlap. Results are representative of three different experiments.
Figure 2. Ep-Bcl-xL transgene construct and Western blot analysis of Bcl-xL expression. (A) A 0.75-kb cDNA fragment containing the coding region of human bcl-xL was inserted into the EcoRI site of the ER-SV40 cassette (14). Epi is the immunoglobulin heavy enhancer and SV40 the early SV40 promoter. (B) Expression of Bcl-xL 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-xL cDNA (26) is shown as a positive control indicated as Bcl-xL. 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-xL expression during T cell development resulted in uniform accumulation of thymocytes, and its effect on thymocytes correlated with the amount of Bcl-xL expressed in the thymus.

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

|                | Transgenic mice | Control littermates |
|----------------|----------------|---------------------|
|                | n (X 10^6)     | %                   | n (X 10^6)     | %                   |
| bcl-x-169      |                |                     |                |                     |
| Total cells    | 60.0 ± 2.0*†   | 2.9 ± 0.3           | 41.2 ± 2.1†    | 2.8 ± 0.4           |
| CD4^-CD8^-     | 1.7 ± 0.2      | 47.9 ± 2.4†         | 36.0 ± 1.9†    | 87.5 ± 0.7†         |
| CD4^-CD8^+     | 3.9 ± 0.8†     | 4.6 ± 0.4†          | 2.1 ± 0.1†     | 0.6 ± 0.1†          |
| CD4^+CD8^-     | 0.7 ± 0.1†     | 1.4 ± 0.4           | 1.2 ± 0.1†     | 1.7 ± 0.2           |
| CD4^+CD8^+     | 43.0 ± 2.2†    | 2.0 ± 0.5           | 1.2 ± 0.5      | 2.7 ± 0.9           |
| bcl-x-86       |                |                     |                |                     |
| Total cells    | 68.0 ± 3.1†    | 56.6 ± 2.3†         | 36.1 ± 1.4†    | 84.0 ± 0.6          |
| CD4^-CD8^-     | 1.4 ± 0.4      | 3.9 ± 0.8†          | 2.0 ± 0.9†     | 4.6 ± 0.9           |
| CD4^-CD8^+     | 56.6 ± 2.3†    | 5.7 ± 1.0           | 1.7 ± 0.2      | 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.

The size of the peripheral T cell pool was increased by deregulated bcl-xL. The total number of lymphocytes in bcl-x-169 mice increased in lymph nodes (from 10 x 10^6 in control littermates to 29 x 10^6) and spleen (from 61 x 10^6 to 85 x 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-xL expression in the T cell lineage increases the number of thymocytes and mature CD4 and CD8 T cells in peripheral organs.
Table 2. Lymphoid Populations in Lymph Nodes and Spleen of bcl-x-169- and bcl-86-transgenic Mice

| Lymphoid Populations in Lymph Nodes and Spleen of bcl-x-169-and bcl-86-transgenic Mice |
|-----------------------------------------------|
| Transgenic mice | Control littermates |
|-----------------|---------------------|
|                  | n (X 10^6) | % | n (X 10^6) | % |
| **bcl-x-169**   |             |   |             |   |
| Lymph node      |             |   |             |   |
| CD4*            | 29.0 ± 4.0^* | 10.0 ± 1.5^† | 48.8 ± 3.7^† | 22.6 ± 0.3^† |
| CD8*            | 14.2 ± 3.0^† | 2.3 ± 0.3^†  | 24.5 ± 0.3^† | 1.6 ± 0.3^†  |
| B220*           | 5.8 ± 1.6   | 3.8 ± 0.6   | 38.1 ± 0.2^† | 38.1 ± 0.2^† |
| Spleen          |             |   |             |   |
| CD4*            | 85.0 ± 9.9^† | 61.0 ± 8.5^† | 25.4 ± 0.7^† | 16.9 ± 1.0^† |
| CD8*            | 21.0 ± 3.0^† | 10.3 ± 2.0^† | 12.0 ± 0.1^† | 5.8 ± 1.1^†  |
| B220*           | 37.5 ± 9.9  | 32.9 ± 5.5  | 44.1 ± 7.4   | 54.0 ± 3.5   |
| **bcl-x-86**    |             |   |             |   |
| Lymph node      |             |   |             |   |
| CD4*            | 20.0 ± 2.0^† | 11.0 ± 2.0^† | 34.7 ± 0.7^† | 18.9 ± 0.2^† |
| CD8*            | 6.9 ± 1.0^†  | 2.1 ± 0.3^†  | 28.2 ± 2.2^† | 1.8 ± 0.5^†  |
| B220*           | 5.6 ± 0.4^†  | 18.3 ± 4.0^† | 4.7 ± 0.6    | 43.0 ± 3.0^† |
| Spleen          |             |   |             |   |
| CD4*            | 80.0 ± 3.0^† | 61.0 ± 1.0^† | 20.1 ± 0.9^† | 14.7 ± 1.8^† |
| CD8*            | 16.1 ± 1.5^† | 9.0 ± 2.0^†  | 12.3 ± 1.4^† | 4.4 ± 1.1^†  |
| B220*           | 9.8 ± 1.4^†  | 4.4 ± 1.1^†  | 43.9 ± 6.1^† | 7.2 ± 1.6^†  |

*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-CD8 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-xL 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-xL 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-G0 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 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 B).

Because glucocorticoids and irradiation appear to induce thymocyte apoptosis in part by different pathways (9), the ability of bcl-xL 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-xL 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.

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In addition, thymocytes from bcl-x-transgenic mice were resistant to death induced by calcium ionophore. After 10 h of exposure to 2 \( \mu \)g/ml of ionomycin, 88 \( \pm \) 5% of the control thymocytes were apoptotic versus 9 \( \pm \) 2% of the thymocytes from bcl-x-169 mice.

**Bcl-xL Protects Thymocytes against Dexamethasone-induced Cell Death In Vivo.** To assess the effects of bcl-xL 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 \( \pm \) 10% of total thymocytes from control mice compared with PBS-treated animals (from 45 \( \times \) 10^6 to 14 \( \times \) 10^6). In contrast, transgenic thymocytes were markedly protected from dexamethasone, with a decrease of only 15 \( \pm \) 3% (from 61 \( \times \) 10^6 to 51 \( \times \) 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-xL-transgenic animals (Fig. 5). In parallel experiments, treatment with dexamethasone deleted equally B220^+IgM^− B cells from transgenic and control animals, demonstrating that the protection conferred by bcl-xL was lineage specific and mirrored transgene expression (Fig. 5).

**Bcl-xL Protects against Anti-CD3-initiated Apoptosis In Vivo.** Triggering of CD4^+CD8^+ thymocytes with anti-CD3 induces apoptosis in vitro and in vivo (2, 39). To assess Bcl-xL 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-xL can block anti-CD3-induced apoptosis.

**Bcl-xL 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-xL is able to block anti-CD3-induced apoptosis. Therefore we determined the ability of the bcl-xL transgene to overcome the deletion of self-reactive T cells. We evaluated the deletion of T cells reactive with superantigen Ms-1.

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**Figure 4.** Bcl-xL 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. Cells 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.

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**Figure 5.** Overexpression of Bcl-xL 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^+^) 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).
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 Vβ6+, Vβ7+, Vβ8.1+, or Vβ9+ regions are deleted (40–43). Similarly, expression of a specific superantigen in association with I-E induces the deletion of T cells bearing Vβ11+, Vβ5+, or Vβ17a+ regions (44, 45). Transgenic mice in the C57BL/6 X SJL (H-2b) 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 X (C57BL/6 X SJL)-bcl-x-169) F1 mice (Table 4). There was a compensatory increase in thymocytes bearing Vβ2+ in (CBA/J X (B6 X SJL)] F1 compared with B6 X SJL mice, which has been reported previously by others (46). Consis-

### Table 3. *Bcl-x, Protects against Anti-CD3–induced Apoptosis In Vivo*

| Cell populations | *bcl-x*-169 Transgenic mice | Control littermates |
|------------------|-----------------------------|---------------------|
|                  | Control*                    | Anti-CD3*           | Control | Anti-CD3 |
| 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 ± 4.3β                 | 35.6 ± 5.4β        | 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 UC8-1139) 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.

### Table 4. Overexpression of *bcl-x, Does Not Block Clonal Deletion Induced by Mls-1* or I-E

| Mice                      | bcl-x tg | Mls-1 | Percentage of Vβ6+ in thymocyte populations |
|----------------------------|----------|-------|--------------------------------------------|
| [CBA/J X (B6 X SJL)]       | +        | +     | 0.2 ± 0.0*                                  |
| [CBA/J X (B6 X SJL)]       | −        | +     | 0.3 ± 0.0                                  |
| B6 X SJL                   | −        | −     | 7.6 ± 0.2                                  |

| Mice                      | bcl-x tg | I-E   | Percentage of Vβ11+ in thymocyte populations |
|----------------------------|----------|-------|--------------------------------------------|
| [CBA/J X (B6 X SJL)]       | +        | +     | 0.0 ± 0.0                                  |
| [CBA/J X (B6 X SJL)]       | −        | +     | 0.1 ± 0.0                                  |
| B6 X SJL                   | −        | −     | 5.1 ± 0.6                                  |

| Mice                      | bcl-x tg | CD4+ | CD8+ | CD4+CD8+ |
|----------------------------|----------|------|------|----------|
| [CBA/J X (B6 X SJL)]       | +        | 0.2 ± 0.0 | 0.2 ± 0.0 | 5.1 ± 0.1 |
| [CBA/J X (B6 X SJL)]       | −        | 0.3 ± 0.0 | 0.0 ± 0.0 | 4.6 ± 0.2 |
| B6 X SJL                   | −        | 7.6 ± 0.2 | 9.4 ± 1.0 | 4.3 ± 0.3 |

| Mice                      | bcl-x tg | Percentage of Vβ2+ in thymocyte populations |
|----------------------------|----------|--------------------------------------------|
| [CBA/J X (B6 X SJL)]       | +        | 11.4 ± 0.5                                  |
| [CBA/J X (B6 X SJL)]       | −        | 11.9 ± 0.8                                  |
| B6 X SJL                   | −        | 3.8 ± 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.

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Table 5. **Oveexpression of bcl-xₐ Does Not Block H-Y-induced Clonal Deletion**

| Mice* | Thymocyte populations |
|-------|-----------------------|
|       | Total | CD4⁻CD8⁻ | CD4⁺CD8⁺ | CD4⁻CD8⁺ | CD4⁺CD8⁺ |
| H-Y   | Bcl-xₐ | × 10⁶ | % | % | % |
| +     | +     | 9.0 ± 1.0⁹ | 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-x₁-169-transgenic mice and control littermates were [C57BL/6 × H-Ytg] (C57BL/6 × SJL)₁F₁ 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.

Discussion

These studies demonstrated that bcl-x₁ 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-x₁ 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-x₁ 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-x₁ was expressed predominately in CD4⁺CD8⁺ thymocytes, the developmental stage particularly affected by the absence of Bcl-x (29). Deregulated Bcl-x₁ 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-x₁ 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-x₁ 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⁺ cells with low affinity for a self-antigen (6). In the context of I-EMHC, the expression of Bcl-x₁ fails to abrogate clonal deletion of T cells reactive to self-H-Y antigen.

**Bcl-x₁ 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-x₁ to inhibit clonal deletion of reactive T cells specific to the male H-Y antigen presented in the context of H-2D⁺ MHC class I molecules was assessed by mating C57BL/6 mice homozygous for a H-Y α/β-TCR transgene (30) to heterozygous bcl-x₁-transgenic mice. In the H-Y male offspring, CD4⁺CD8⁺ and CD4⁻CD8⁺ thymocytes from both bcl-x₁⁻ and non-bcl-x₁⁻ 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-x₁ fails to abrogate clonal deletion of T cells reactive to self-H-Y antigen.

**Constitutive expression of bcl-2 and bcl-x₁ protected thymocytes from a number of apoptotic stimuli.** However, in contrast to our results with bcl-x₁ mice, a Epk-bcl-2 transgene targeted to the thymus did not result in increased numbers of thymocytes or peripheral T cells in mice deficient in bcl-x₁ (29). Our results provide an explanation for the findings in the T cell lineage in that Bcl-x₁ was expressed predominately in CD4⁺CD8⁺ thymocytes, the developmental stage particularly affected by the absence of Bcl-x (29). Deregulated Bcl-x₁ 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-x₁ deregulation failed to inhibit clonal deletion of self-reactive T cells against endogenous superantigens and H-Y antigen.

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**Discussion**

These studies demonstrated that bcl-x₁ 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-x₁ 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-x₁ 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-x₁ was expressed predominately in CD4⁺CD8⁺ thymocytes, the developmental stage particularly affected by the absence of Bcl-x (29). Deregulated Bcl-x₁ 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-x₁ deregulation failed to inhibit clonal deletion of self-reactive T cells against endogenous superantigens and H-Y antigen.

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Bcl-x, is downregulated in more mature single-positive thymocytes and 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-xL can confer protection against multiple forms of apoptosis, it is intriguing that CD4^+ CD8^+ thymocytes are sensitive to apoptosis, despite expression of endogenous Bcl-xL protein. A possible explanation is that simultaneous expression of Bcl-2 and Bcl-xL is more effective than Bcl-2 alone in protecting thymocytes from apoptosis. An alternative nonexclusive interpretation is that the ability of endogenous Bcl-xL 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-xL 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-xL (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-xL than to Bcl-2, and its interaction with Bcl-xL 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-xL 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-xL, their inhibitory partners, and the “strength” of the death signal. In this model, endogenous levels of Bcl-xL in CD4^+ CD8^+ thymocytes maintain T cell survival but are not sufficient to protect cells from death signals. Our results suggest that overexpressed Bcl-xL can effectively compete for inhibitory proteins or compensate for Bcl-2 downregulation and promotes thymocyte survival.

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