Bcl-x_L and Bcl-2 Repress a Common Pathway of Cell Death

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Summary

The effect of Bcl-x_L upon the developmental death of T cells was assessed by generating transgenic mice that expressed Bcl-x_L within all thymocyte subsets. Bcl-x_L protected thymocytes from a variety of apoptotic stimuli, including γ irradiation, glucocorticoids, and anti-CD3 treatment. Bcl-x_L altered thymocyte maturation, resulting in increased numbers of $CD3^{int/hi}$ and $CD4^-8^+$ thymocytes. Overall, the phenotype of Bcl-x_L transgenics was essentially indistinguishable from a Bcl-2 transgenic model. Overexpression of Bcl-x_L or Bcl-2 resulted in the down-regulation of the other molecule, providing further evidence of their reciprocal regulation. In a genetic test of redundancy, the Bcl-x_L transgene rescued mature T cells in Bcl-2 null mice. Immunoprecipitation indicated that Bcl-x_L, like Bcl-2, heterodimerized with the death-promoting molecule Bax in thymocytes. This in vivo model argues that Bcl-x_L, like Bcl-2, functions in a common pathway to repress cell death.

Regulated cell death is indispensable for the proper differentiation and maintenance of homeostasis in multiple lineages. The creation of a functional immune system is particularly dependent upon the selective survival and apoptotic demise of specific cells during lymphocyte maturation (1, 2). The susceptibility to death for a developing lymphocyte after the same stimulus varies markedly at different stages of development. Bcl-2 is one molecule that helps to determine the cell-autonomous susceptibility to apoptosis. For example, mature CD4 or CD8 single-positive (SP)[1] medullary thymocytes are resistant to apoptotic signals and possess high levels of the death repressor molecule, Bcl-2. In contrast, the $CD4^+8^+$ double-positive (DP) cortical thymocytes lack Bcl-2 and are highly vulnerable to a wide variety of apoptotic stimuli (3, 4).

Recently, an expanding family of Bcl-2 homologues has been identified. They share homology that is principally, but not exclusively, clustered within two conserved domains, entitled Bcl-2 homology 1 and 2 (BH1 and BH2) (5, 6). This includes Bax, which heterodimerizes with Bcl-2 and counters its activity. The ratio of Bcl-2/Bax can determine whether a given cell will execute or ignore an apoptotic stimulus (7). Single–amino acid substitutions within the BH1 or BH2 domain of Bcl-2 disrupted Bcl-2/Bax heterodimers, but not Bcl-2/Bcl-2 homodimers. Bcl-2 mutants that were unable to heterodimerize with Bax no longer inhibited apoptosis (6). This indicates that this family possesses both positive and negative regulators of death and that these proteins function at least in part through protein–protein interactions.

Given the existence of one regulatory pair, Bcl-2 and Bax, that can promote or repress apoptosis, an important question arises as to the rationale for further family members. An additional member, Bcl-x, has also been shown to regulate apoptosis in cell lines (8). A long form, Bcl-x_L, which possesses BH1 and BH2, will repress cell death. A short RNA species noted in humans, Bcl-x_S, lacks the BH1 and BH2 domains and has been noted to counter the protective effect of Bcl-2. It is of interest that species specificity in the alternatively spliced product, Bcl-x_S, has been noted. Bcl-x_S is readily detected in human but not in murine thymocytes, while Bcl-x_L is present in the mouse thymus (9, 10).

Prior studies of gain-of-function Bcl-2 transgenic mice noted effects of this death repressor upon both thymocyte survival and maturation. When Bcl-2 was uniformly expressed within all thymocytes including DP cells, it conferred resistance to glucocorticoids, γ irradiation, and T cell–specific stimuli such as anti-CD3 treatment. However, the levels of Bcl-2 were unable to block the negative selection deletion of thymocytes (11, 12). Subsequently, a Bcl-2 transgene was introduced into genetic models in which thymocyte maturation was blocked.

[1] *Abbreviations used in this paper:* BH1, Bcl-2 homology 1; BH2, Bcl-2 homology 2; DN, double negative; DP, double positive; SP, single positive.

Bcl-2 rescued $CD8^+$ thymocytes in MHC class I −/− and α/β TCR mice but had no effect on CD4 lineage maturation. Moreover, α/β TCR transgenic mice revealed that Bcl-2 was up-regulated at the $CD4^+8^+$ stage during positive selection (13). Studies with Bcl-2–deficient mice have indicated that Bcl-2 is not solely required as a single member for the completion of T cell development. Instead, Bcl-2 −/− mice are unable to maintain homeostasis demonstrating apoptotic loss of both lymphocyte lineages postnatally (14).

To test whether Bcl-x_L would repress cell death in normal development and affect maturation, we generated a Bcl-x_L transgenic model. We wished to explore whether Bcl-x_L functions through a separate pathway or impacts a common pathway shared with Bcl-2. To compare the effects of Bcl-x_L with those of Bcl-2, we generated mice using the same lck proximal promoter to express transgenic Bcl-x_L. Despite the alternating normal pattern of expression of Bcl-2 and Bcl-x_L in the thymus (3, 10, 15), the phenotype of gain-of-function Bcl-x_L transgenics was remarkably similar to Bcl-2 transgenics. Moreover, both repressor molecules heterodimerized with Bax in vivo. These in vivo data favor a model in which Bcl-x_L and Bcl-2 function in a common pathway to regulate cell death.

Materials and Methods

Mice. A 0.8-kb cDNA of human Bcl-x_L (8) was inserted into the lck-hGH vector (16) to generate the transgenic animals as previously described (11). *lck^{pr}-bcl-x_L* transgenic mice bred on the C57BL6/J background were maintained in a pathogen-free animal facility at Washington University (St. Louis, MO). Bcl-2–deficient animals (14) were bred to *lck^{pr}-bcl-x_L* transgenic mice in the same facility.

Cell Preparations. Single-cell suspensions were made from lymphoid organs after lysing red blood cells with Tris-NH_4Cl buffer and counted on a hemocytometer by trypan blue exclusion. Splenic T cells were purified by immunomagnetic negative selection with anti-HSA, anti-B220, and anti-Mac-1 as previously described (13). The purity of splenic T cells was usually 85–90%, determined by anti-CD3 staining.

Western Blot Analysis. Single-cell suspensions were lysed in 150 mM NaCl, 10 mM Tris, pH 7.4, and 1% Triton X-100 buffer. After quantitation of the amount of protein using the Bradford method (17), 35 μg of protein was run in each lane of a 12.5% SDS–polyacrylamide gel. Blots were developed with enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL) or diaminobenzidine.

Antibodies. The mAbs 3F11 (hamster IgG anti–murine Bcl-2) and 4D2 (hamster IgG anti–murine Bax) were used as purified antibody prepared by protein A chromatography. 13.6 is a polyclonal antiserum (rabbit IgG) raised against human Bcl-x that cross-reacts with murine Bcl-x. 7B2 is an mAb recognizing human and murine Bcl-x (murine IgG). The details of antibody production are described elsewhere (18). All other mAbs used in this study were purchased from Pharmingen (San Diego, CA). Secondary antibodies were biotinylated goat anti–hamster or anti–rabbit antibodies obtained from Caltag Laboratories (San Francisco, CA).

Viability Assay. Cells were plated at 10^6 cells/ml in DME supplemented with 5% FCS (GIBCO BRL, Gaithersburg, MD). At each time point, the cells were collected and viability was determined by propidium iodide exclusion.

In Vivo Anti-CD3 Injection. 50 μg of affinity-purified anti-CD3 (clone 2C11; Pharmingen Inc.) or vehicle control (PBS) was injected intraperitoneally into 8–12-wk-old transgenic and control littermates. Thymi were removed 48 h after treatment. Single-cell suspensions were made in cold medium and stained for surface CD4 and CD8 within 2 h.

Immunoprecipitation. Fresh thymocytes were washed twice in serum-free, methionine-free DME (GIBCO BRL). Metabolic labeling was then performed with 40 μCi/ml of Trans^{35}S-label (ICN Biomedicals, Inc., Costa Mesa, CA) for 3–4 h. Cells were harvested and lysed in an NP-40 isotonic lysis buffer with protease inhibitors (142.5 mM KCl, 5 mM $MgCl_2$, 10 mM Hepes, pH 7.2, 0.25% NP-40) by nutation for 30 min in the cold room. Immunoprecipitation was performed as previously described (7). ^{35}S-labeled bands were quantitated by PhosphorImager scanning (Molecular Dynamics, Inc., Sunnyvale, CA).

Results

Generation of Transgenic Mice. A transgenic construct was created by inserting the human Bcl-x_L cDNA downstream of the lck proximal promoter (16). The 3′ untranslated portion of the construct possessed hGH exons, introns, and a poly(A) addition site to ensure proper processing of the transcript (Fig. 1 *A*). *lck^{pr}-Bcl-x_L* lines 12 and 24, which expressed high levels of Bcl-x_L, were propagated. The majority of experiments were done with the highest expressing line 12 and confirmed with line 24.

Transgene expression was analyzed by Western blot and flow cytometry. Bcl-x_L was highly expressed in both thymus and spleen. The purified splenic T cell subpopulation showed a marked increase in Bcl-x_L (Fig. 1 *B*). Flow cytometry using permeabilized thymocytes indicated that Bcl-x_L levels were uniformly greater in transgenic than in normal thymocytes (Fig. 1 *C*). Three-color flow cytometric analysis revealed comparable levels of Bcl-x_L in double-negative (DN) ($CD4^-8^-$), DP ($CD4^+8^+$), and CD4 SP or CD8 SP thymocytes (data not shown).

Bcl-x_L Alters Thymocyte Maturation. All transgenic animals had an increase in $CD3^{int/hi}$ and a reciprocal decrease in $CD3^{lo}$ thymocytes compared with control littermates (Fig. 2 *A*). Parallel changes were seen in the α/β T cell subset of transgenic animals, but no alteration was found in γ/δ T cells (data not shown). An increase in intermediate $CD4^+8^{lo}$ and $CD4^{lo}8^+$ thymocytes, which represent a transitional stage in thymic maturation, was also consistently noted in transgenic animals (Fig. 2 *B*). Moreover, all *lck^{pr}-bcl-x_L* mice had an increase in CD8 SP thymocytes, while some also displayed an increase in the CD4 cells (Fig. 2 *B*, Table 1). Although overexpression of Bcl-x_L did not substantially increase the total number of thymocytes, it altered the distribution of T cell subsets (Table 1). The ratio of CD4 to CD8 SP thymocytes was skewed to 1.9 in transgenic versus 3.5 in control mice. In addition, the total number of splenic lymphocytes was uniformly higher in Bcl-x_L transgenic mice. The absolute number of B cells was unchanged. Flow cytometric analysis demonstrated an increase in T cells as well as an increased percentage of CD8 SP cells in the spleen of the transgenics (Fig. 2 *C*). Thus, constitutive overexpression

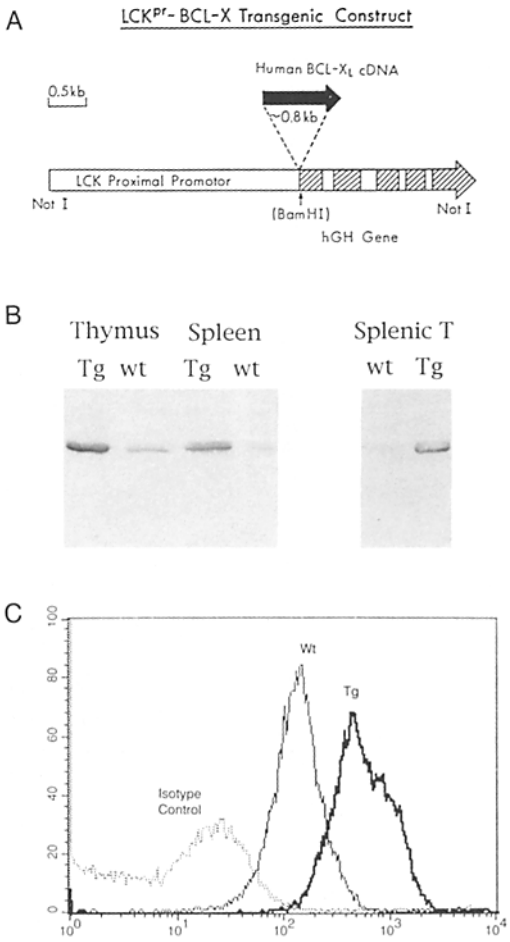

Figure 1. Bcl-x_L construct and expression analysis. (*A*) The human Bcl-x_L cDNA was inserted at the BamHI site under the control of lck proximal promoter (17). Hatched boxes in the 3′ untranslated region represents the exons of the human growth hormone (*hGH*) gene. (*B*) Western blot analysis of transgene expression. Cell lysates were prepared from single-cell suspensions of thymus or spleen. Splenic T cells were purified using negative selection with immunomagnetic beads. 35 μg of protein lysate from transgenic line 12 or wild-type (*wt*) cells was loaded in each lane. The anti–Bcl-x_L polyclonal antibody 13.6 was used to detect the presence of both the endogenous murine and the human transgenic Bcl-x_L proteins. The immunoblot was developed with diaminobenzidine. (*C*) Flow cytometry histogram quantifying the Bcl-x_L protein in permeabilized thymocytes from wild-type and *lck^pr-bcl-x_L* transgenic (*Tg*) mice stained with the 7B2 anti–Bcl-x mAb.

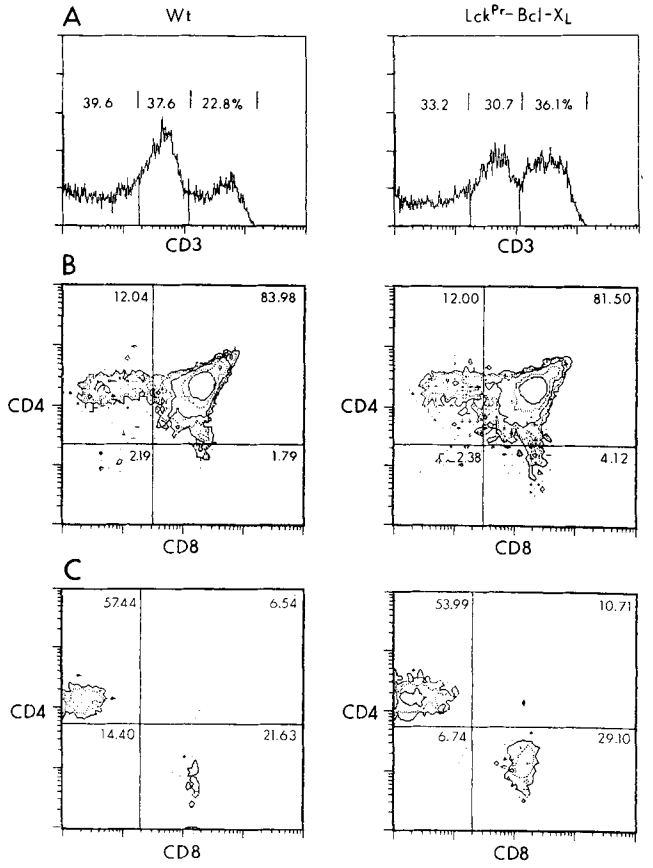

Figure 2. Bcl-x_L alters thymocyte maturation. (*A*) Single-cell suspensions of thymocytes from 5-wk-old wild-type (*Wt*) and *lck^pr-bcl-x_L* transgenic mice were stained with FITC-conjugated anti-CD3 mAb. The percentages of $CD3^{neg}$, $CD3^{lo}$, $CD3^{int/hi}$ cells are indicated at the top of the histograms. (*B*) Flow cytometric analysis of thymocytes, and (*C*) CD3-gated splenic T cells from wild-type and transgenic animals for CD4 and CD8 expression. The percentages of each subset are shown at the right upper corner of each quadrant.

of Bcl-x_L altered thymocyte maturation in a pattern similar to that of Bcl-2 transgenic mice (11), resulting in a marked excess of CD8 SP cells.

Prolonged Survival of Transgenic Thymocytes. We next asked whether Bcl-x_L altered the survival and susceptibility of thymocytes to apoptosis. Since we had previously generated Bcl-2 transgenic mice using the same promoter, we were able to compare the effects of Bcl-x_L and Bcl-2 on thymocyte cell death. Thymocyte suspensions from *lck^pr-bcl-2*, *lck^pr-bcl-x_L*, and wild-type littermates were plated in 5% FCS (Fig. 3 *A*). After 100 h, 80% of both Bcl-x_L and Bcl-2 transgenic thymocytes were viable, whereas <10% of the normal thymocytes survived. Similarly, splenic T cells from both transgenics also revealed improved survival in vitro (data not shown). The survival between Bcl-x_L– and Bcl-2–expressing thymocytes or splenic T cells was similar.

Bcl-x_L Protects Thymocytes from Glucocorticoid and Radiation-induced Apoptosis. The protective ability of overexpression of Bcl-x_L to several different apoptotic stimuli in thymocytes was tested. Both glucocorticoid treatment and low-dose irradiation are known to cause a rapid depletion of immature thymocytes by apoptosis. Thus, thymocytes were treated either with 100 nM dexamethasone (Fig. 3 *B*) or 225 rad of γ irradiation (Fig. 3 *C*). By 48 h, most of the normal thymocytes were dead, whereas 50–60% of either *lck^pr-bcl-x_L* or *lck^pr-bcl-2* thymocytes were still viable (Fig. 3 *C*). The transgenic thymocytes were resistant to anti-Fas Ab treatment in a similar matter (data not shown).

lck^pr-bcl-x_L Thymocytes Are Resistant to Anti-CD3–induced Apoptosis In Vivo. To assess whether Bcl-x_L was able to

Table 1. *Bcl-x_L Alters T Cell Maturation*

	Transgenic			Wild type		
Total thymocytes ($\times 10^6$)	93.5 ± 35.4			77.4 ± 35.1		
Splenic T cells ($\times 10^6$)	27.1 ± 8.8			9.8 ± 2.5		
	CD4	CD8	CD4/CD8 ratio	CD4	CD8	CD4/CD8 ratio
Percentage of total thymocytes	16.1 ± 5.7	9.8 ± 4.6	1.9 ± 0.7	10.4 ± 3.3	3.4 ± 1.2	3.5 ± 1.8
Percentage of total splenic cells	26.3 ± 7.4	15.4 ± 4.3	1.7 ± 0.5	21.7 ± 6.4	8.9 ± 2.8	2.5 ± 0.3

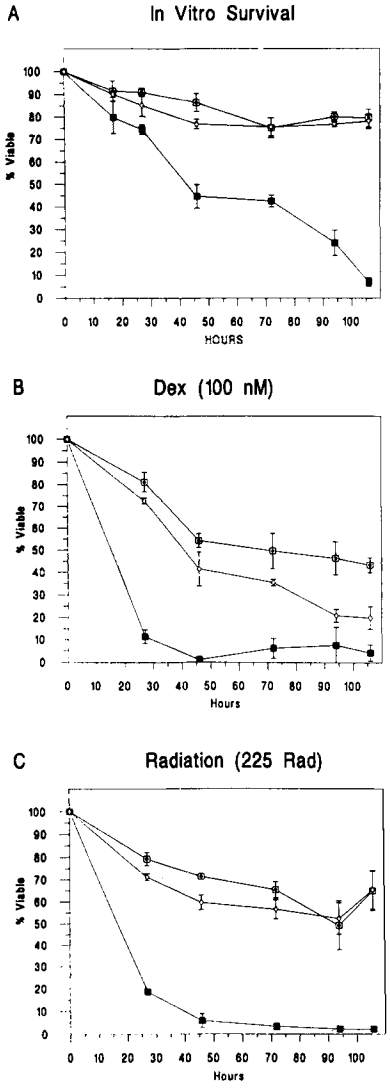

Figure 3. Bcl-x_L prolongs thymocyte survival in vitro and protects from glucocorticoid and γ irradiation–induced apoptosis. Thymocytes from wild-type (■), *lck^{pr}-bcl-x$_L$* (◙), and *lck^{pr}-bcl-2* (◇) mice were plated at 106 cells/ml in DMEM with 5% FCS (*A*), with 100 nM dexamethasone (*B*), or with 225 rad γ irradiation (*C*). Cell viability was determined by propidium iodide exclusion.

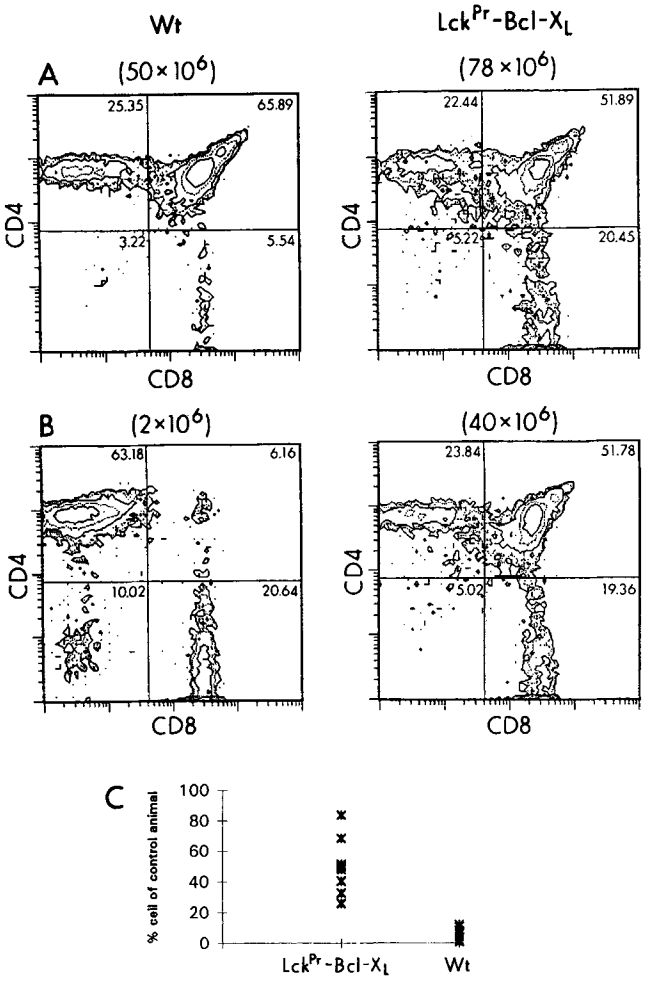

Figure 4. Anti-CD3 induced apoptosis. Total thymocytes from 12-wk-old mice were analyzed 48 h after in vivo injection of (*A*) PBS or (*B*) anti-CD3 mAb for cell number (*in parenthesis*) and thymocyte subsets by surface CD4 and CD8. (*C*) Combined data from a total of six independent experiments and >10 mice in each category (ages 8–12 wk old). Each experiment compares transgenics with littermate controls as wild type (*Wt*). Plotted values represent the percentages of thymocytes remaining after in vivo treatment with anti-CD3 Ab, in which 100% represents the average thymocyte number after treatment with PBS.

block a T cell–specific apoptotic signal, an anti-CD3 mAb was injected intraperitoneally into transgenic mice and control littermates. 48 h after treatment, thymi were harvested and compared with mice treated with PBS alone (Fig. 4 *A*). After anti-CD3 administration, the number of DP thymocytes was decreased substantially (90%) in wild-type animals. However, *lck*^{pr}*-bcl-x*$_L$ mice were resistant to in vivo anti-CD3 treatment in the DP population (Fig. 4 *B*). While the extent of protection varied, all transgenic mice had substantially more thymocytes after in vivo anti-CD3 treatment compared with their wild-type littermates in six independent experiments (Fig. 4 *C*).

Reciprocal Down-regulation of Endogenous Bcl-x$_L$ *and Bcl-2.* A previous report had shown that endogenous Bcl-2 was down-regulated in the B cells of animals bearing the Bcl-2-Ig transgene (19). A similar down-regulation of endogenous Bcl-2 was also observed in *lck*^{pr}*-bcl-2* transgenic mice using 3F11, an mAb specific for mouse Bcl-2 (Fig. 5). It is noteworthy that, in both the thymocytes and splenic T cells of *lck*^{pr}*-bcl-x*$_L$ mice, the endogenous Bcl-2 (3F11 mAb) protein expression was down-regulated. In parallel, endogenous Bcl-x$_L$ (13.6 Ab) expression was also decreased in the thymocytes of *lck*^{pr}*-bcl-2* animals (Fig. 5). The minimal amount of Bcl-x$_L$ in splenic T cells was also suppressed in the *lck*^{pr}*-bcl-2* animals. We were unable to determine if endogenous Bcl-x$_L$ was down-regulated in Bcl-x$_L$ transgenic mice since the polyclonal Ab 13.6 recognizes both human and murine Bcl-x$_L$. The reciprocal relationship of Bcl-2 and Bcl-x$_L$ protein levels suggests that their regulation may be coordinated in an inverse fashion.

Bcl-x$_L$ *Rescues Bcl-2–deficient Cells.* The phenotype of the Bcl-x$_L$ gain-of-function transgenics was similar to that of comparable Bcl-2 transgenics. Consequently, we tested whether Bcl-x$_L$ would substitute for Bcl-2 in vivo. *lck*^{pr}*-bcl-x*$_L$ transgenics were mated to Bcl-2–deficient mice to determine whether selective expression of Bcl-x$_L$ would reverse the apoptotic loss of T cells in Bcl-2 null mice. Analysis of peripheral lymphocyte populations in healthy-appearing Bcl-2−/− mice revealed loss of both B and T cell populations (Fig. 6). In contrast, *lck*^{pr}*-bcl-x*$_L$/Bcl-2−/− mice displayed a comparable loss of B cells but retained normal to increased numbers of splenic T cells (Fig. 6). In a total of six independent experiments (mice aged 4–7 wk), all *lck*^{pr}*-bcl-x*$_L$/Bcl-2−/− mice had normalized peripheral T cell populations when compared with their Bcl-2−/− littermates. Thus, Bcl-x$_L$ was able to rescue total splenic T cell numbers in Bcl-2–deficient mice.

Bcl-x$_L$ *Represses Cell Death in Bcl-2–deficient Thymocytes.* Thymocytes from Bcl-2–deficient mice demonstrate accelerated cell death after an apoptotic stimulus (14). We compared the sensitivity to apoptosis of thymocytes from Bcl-2−/− and *lck*^{pr}*-bcl-x*$_L$/Bcl-2−/− mice. Thymocytes plated in media with 5% FCS displayed increased survival for *lck*^{pr}*-bcl-x*$_L$/Bcl-2−/− cells compared with littermate Bcl-2−/− cells (75% vs 45% at 24 h) (Fig. 7 *A*). In addition, thymocytes from *lck*^{pr}*-bcl-x*$_L$/Bcl-2−/− mice were more resistant to 100 nM dexamethasone (Fig. 7 *B*), and 225 rad of γ irradiation

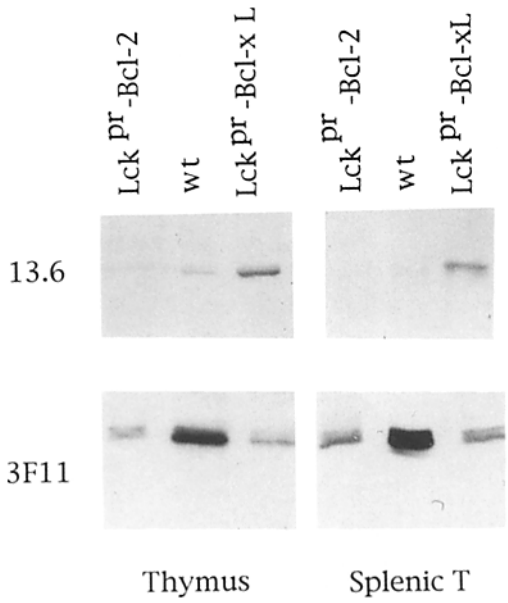

Figure 5. Down-regulation of endogenous Bcl-x$_L$ and Bcl-2. Western blot analysis of Bcl-x$_L$ (13.6 Ab) and murine Bcl-2 (3F11 mAb) in thymocytes and splenic T cells from *lck*^{pr}*-bcl-2*, wild-type (*wt*), and *lck*^{pr}*-bcl-x*$_L$ animals. The 13.6 polyclonal antibody recognizes both human and murine Bcl-x. The 3F11 mAb recognizes the endogenous murine Bcl-2 but not the transgene origin human Bcl-2.

(Fig. 7 *C*) than Bcl-2–deficient cells. Therefore, Bcl-x$_L$ was able to restore viability and repress apoptosis after multiple stimuli in cells lacking Bcl-2.

Bcl-x$_L$ *Also Heterodimerizes with Bax In Vivo.* Since Bcl-x$_L$ would substitute for Bcl-2 in countering cell death, we explored whether they both function in a common pathway. Bcl-2 heterodimerizes with a homologue Bax, which promotes cell death (7). Consequently, we examined thymocytes from normal and *lck*^{pr}*-bcl-x*$_L$ transgenic mice to determine whether Bcl-x$_L$ associates with Bax (Fig. 8). Normal thymocytes, when radiolabeled and immunoprecipitated with an anti–Bcl-x antibody, revealed moderate amounts of Bcl-x$_L$ and some associated Bax (Fig. 8 *A*). It is of potential interest that several other bands were also seen (Fig. 8 *A*). However, only a minority of the total amount of Bax in normal thymocytes was heterodimerized with Bcl-x$_L$. In Bcl-x$_L$ transgenic mice, however, immunoprecipitation of Bcl-x$_L$ coprecipitated substantial portions of endogenous Bax (Fig. 8 *A*). Moreover, immunoprecipitation of Bax demonstrates considerable heterodimerizing Bcl-x$_L$ (Fig. 8 *B*). PhosphorImager analysis of primary and secondary immunoprecipitates indicates that, in thymocytes from normal control mice, only 30% of Bax is heterodimerized with Bcl-x$_L$ (Fig. 8 *A*, *upper panel*), while the supernatant of that immunodepletion reveals that 70% of Bax is unbound (Fig. 8 *A*, *lower panel*). In contrast, in the presence of the Bcl-x$_L$ transgene, a substantial portion of Bax (77%) is heterodimerized with Bcl-x$_L$, while only 23% of Bax is unbound (Fig. 8 *B*). The heterodimerization

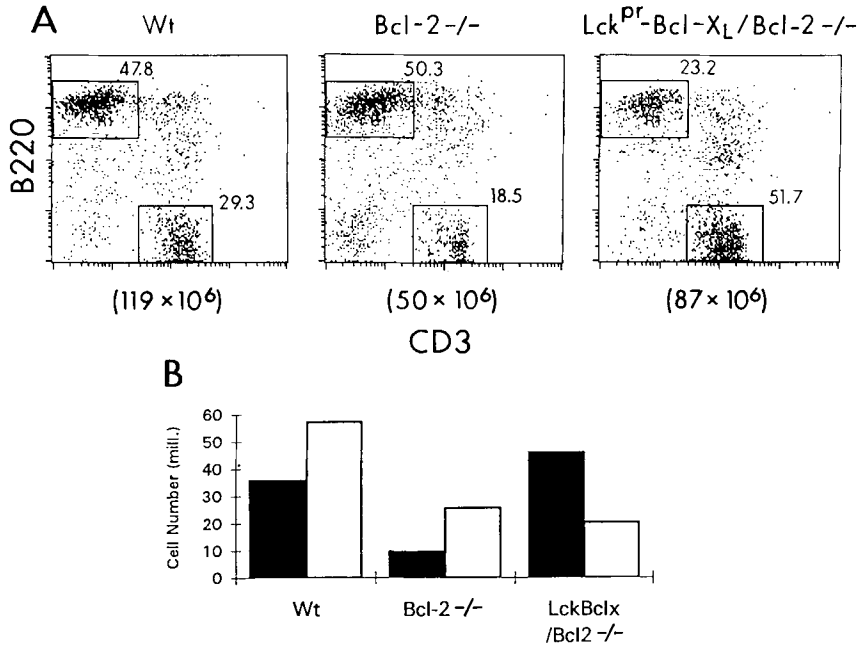

Figure 6. Bcl-x_L substitutes for Bcl-2 function in vivo. (A) Flow cytometric analysis of spleen mononuclear cells from representative, healthy 7-wk-old mice. Total spleen cell numbers of each animal are shown in parenthesis under each panel. Numbers indicating the percentages of total spleen cells that were B ($B220^+$) or T ($CD3^+$) cells are shown next to the boxed populations. (*B*) Graphic presentation of the absolute number (in millions) of splenic T (*closed bar*) and B cells (*open bar*) from representative littermate mice at 7 wk of age.

of >50% of Bax with either Bcl-2 or Bcl-x_L resulted in repression of cell death in a cell line system (20). Thus, transgenic Bcl-x_L as well as Bcl-2 displays substantial heterodimerization in vivo with the death-promoting molecule Bax.

Discussion

We generated a gain-of-function transgenic model to determine whether Bcl-x_L, which has been shown to block apoptosis within certain cell lines (8), would alter normal developmental cell death within an animal model. Overexpressed Bcl-x_L in normal thymocytes inhibited cell death after dexamethasone, γ irradiation, and anti-CD3 treatment. Moreover, lineage-specific expression of Bcl-x_L altered T cell maturation. Increased populations of $CD3^{int/hi}$ thymocytes and intermediate $CD4^+8^{lo}$ and $CD4^{lo}8^+$ cells in transition to becoming SP cells were noted. In addition, a skew to CD8 cells was noted both in the thymus and the periphery. The overall phenotype of the *lck^{pr}-bcl-x$_L$* transgenic mice was nearly indistinguishable from that of *lck^{pr}-bcl-2* transgenics generated with the same promoter (11). Detailed studies of Bcl-2 transgenics indicated that Bcl-2 was able to promote maturation in the absence of critical signals (13). This Bcl-x_L model strengthens the argument that repressing apoptosis may enable differentiation irrespective of the specific molecule that inhibits the death pathway.

Recently, there has been a marked expansion in the number of Bcl-2 family members. This has raised important questions as to whether each molecule has an entirely distinct function or whether they all regulate a common pathway. The similarity of Bcl-2 and Bcl-x_L transgenics suggests that these two molecules, which both repress cell death, might be interchangeable. The selective introduction of Bcl-x_L into the T cells of Bcl-2–deficient mice restored resistance to apoptotic stimuli and rescued mature T cell populations. This rigorously established that Bcl-x_L could substitute for Bcl-2 in maintaining T cell homeostasis. Since the Bcl-x_L transgene was only expressed in T cells, the capacity for Bcl-x_L to replace Bcl-2 in B cells was not assessed. However, the normal physiologic roles of Bcl-2 and Bcl-x_L could prove to be distinct. Bcl-2 displays an on-off-on pattern of expression during T cell development. The most immature DN thymocytes possess Bcl-2, which is down-regulated at the DP stage. Bcl-2 is subsequently reexpressed during positive selection, and the protein remains high in mature CD4 and CD8 thymocytes (3, 13). In contrast, Bcl-x is principally expressed in the cortex and not the medulla (15). Only minimal amounts of Bcl-x_L are found in DN thymocytes, but DP cells have high amounts of Bcl-x_L. Bcl-x_L is no longer expressed in SP thymocytes or peripheral T cells (10). However, it is Bcl-x_L, but not Bcl-2, that responds to activation being reexpressed in mature T cells after TCR engagement (18). It is noteworthy that both Bcl-2 and Bcl-x_L transgenic mice demonstrated a reciprocal down-regulation of the other member. This pattern of regulation is consistent with a sensing mechanism that would help coordinate the alternating patterns of Bcl-2 and Bcl-x_L expression during development. This example emphasizes that homologues that display functional redundancy in genetic complementation tests may normally have slightly different physiologic roles, thereby accounting for their comaintenance during evolution.

The serial, alternating pattern of Bcl-2 and Bcl-x_L expression and the fact that each gain-of-function model appeared essentially identical suggested that both Bcl-2 and Bcl-x_L im-

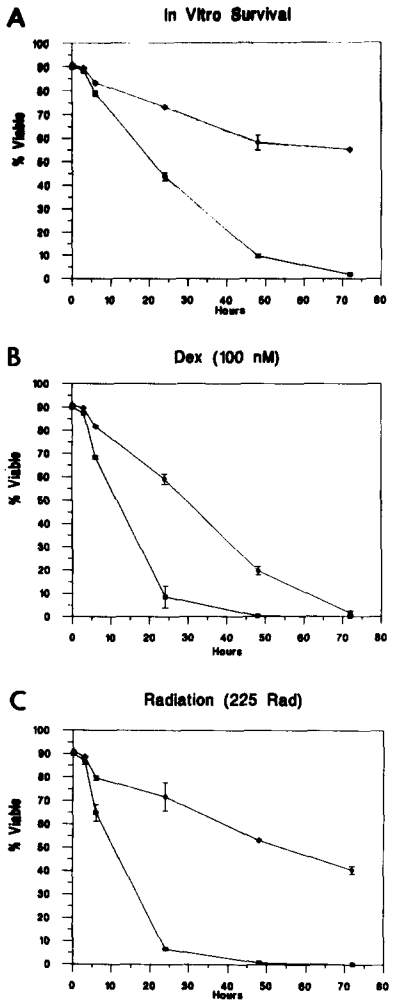

Figure 7. Bcl-x_L restores resistance to apoptotic stimuli in Bcl-2–deficient thymocytes. Fresh thymocytes from Bcl-2 −/− (■) and *lck*^{pr}-*bcl-x*$_L$/Bcl-2−/− mice (◇) were plated at 106 cells/ml in DMEM with 5% FCS (*A*). In *B*, thymocytes were treated with 100 nM dexamethasone, and, in *C*, with 225 rad γ irradiation. A representative of four independent experiments is shown. Animals used in this experiment were 6-wk-old littermates.

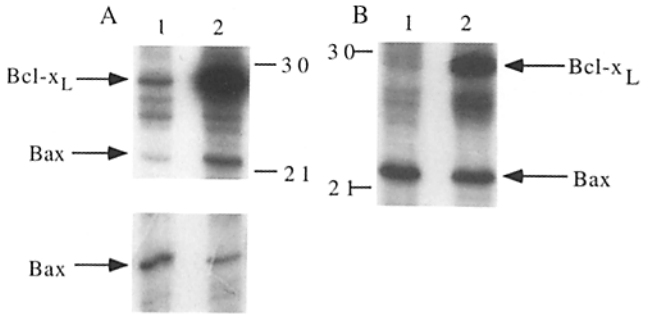

Figure 8. Heterodimerization of Bcl-x_L with Bax in thymocytes. (*A*, *upper panel*) Radiolabeled thymocytes from wild-type (lane *1*) or *lck*^{pr}-*bcl-x*$_L$ mice (lane *2*) were immunoprecipitated with anti–Bcl-x (13.6) polyclonal antibody. A Western blot of this immunoprecipitate confirmed that the 21-kD species was Bax (data not shown). (*A*, *lower panel*) The remaining supernatant from the immunodepletion of Bcl-x_L shown in the upper panel underwent a secondary immunoprecipitation with the 4D2 anti-Bax mAb to assess the residual amount of Bax not heterodimerized with Bcl-x_L. (*B*) A primary immunoprecipitation of the same radiolabeled thymocytes with a polyclonal anti-Bax Ab (10519) that recognizes either Bax homo or heterodimers.

pact a common pathway. The emerging Bcl-2 gene family shares its principal homology within two conserved domains entitled BH1 and BH2 (5). Bcl-2 is known to heterodimerize with a death-promoting family member, Bax (7). Mutagenesis analysis of Bcl-2 indicated that BH1 and BH2 are novel domains that regulate heterodimerization. Point mutations within BH1 and BH2 argue that Bcl-2 must heterodimerize with Bax to repress death (6). Consequently, we assessed whether Bcl-x_L would heterodimerize with Bax in vivo as part of their common effects. In normal thymocytes, only a minority of Bax (30%) was heterodimerized with the modest levels of Bcl-x_L. However, transgenic levels of Bcl-x_L resulted in the majority of Bax (77%) being heterodimerized with Bcl-x_L. In a separate study using a cell line, various levels of Bcl-2, Bcl-x_L, Bad, and Bax were rigorously quantitated and correlated with the susceptibility to apoptosis. When ~50% or more of the Bax in FL5.12 cells was complexed in heterodimers, apoptosis was suppressed after IL-3 deprivation (20). Thus, the difference in the percentage of Bax found in heterodimers with Bcl-x_L within these transgenics is predicted to be functionally significant. The capacity of Bcl-x_L to mimic Bcl-2 and substitute for it in repressing T cell death may relate to its ability to heterodimerize with a substantial portion of Bax. However, only a minority of native Bax (30%) is associated with Bcl-x_L in normal thymocytes. It is possible that other bands noted in the anti–Bcl-x_L immunoprecipitate might represent independent partners for Bcl-x_L that may also prove biologically significant in normal development.

The alternative death repressor molecules of Bcl-2 and Bcl-x_L appear to sequentially regulate cell death at serial stages of T cell development. These studies of transgenic mice indicate that Bcl-x_L and Bcl-2 can repress cell death through a common pathway in which their interaction with Bax is one shared parameter.

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