bcl-x Exhibits Regulated Expression During B Cell Development and Activation and Modulates Lymphocyte Survival in Transgenic Mice

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Summary

We have assessed during B cell development, the regulation and function of *bcl-x*, a member of the *bcl-2* family of apoptosis regulatory genes. Here we show that Bcl-x_L, a product of *bcl-x*, is expressed in pre-B cells but downregulated at the immature and mature stages of B cell development. Bcl-x_L but not Bcl-2 is rapidly induced in peripheral B cells upon surface immunoglobulin M (IgM) cross-linking, CD40 signaling, or LPS stimulation. Transgenic mice that overexpressed Bcl-x_L within the B cell lineage exhibited marked accumulation of peripheral B cells in lymphoid organs and enhanced survival of developing and mature B cells. B cell survival was further increased by simultaneous expression of *bcl-x_L* and *bcl-2* transgenes. These studies demonstrate that Bcl-2 and Bcl-x_L are regulated differentially during B cell development and activation of mature B cells. Induction of Bcl-x_L after signaling through surface IgM and CD40 appears to provide mature B cells with an additional protective mechanism against apoptotic signals associated with antigen-induced activation and proliferation.

Naturally occurring cell death is common during B cell maturation and is accomplished by apoptosis, a morphologically defined process that is widespread during embryogenesis and postnatal development (1). During B lymphocyte differentiation, it is estimated that as few as one tenth of the daily 3–5 × 10^7 newly formed B cells is incorporated into the peripheral mature pool (2, 3). Death appears to be the fate of most B cell precursors with the majority of the cell loss occurring during the transition from large, cytoplasmic μ chain–producing B cell precursors to small pre-B cells (4, 5). Because rearrangement of Ig genes is a stochastic process, many B cell precursors fail to complete successful assembly of H and L Ig chains and appear to undergo cell death in the bone marrow (BM)[1] (4, 5). Later in development, IgM^+IgD^- immature B cells undergo further selection based on the specificity of their IgM surface receptors. Newly formed B cells that recognize self-antigens are eliminated by apoptosis in the BM or are functionally inactivated (6). In the periphery, IgM^+IgD^+ mature B cells can undergo T cell–dependent affinity maturation of their surface antigen receptors after encounter with antigen. This process involves a high rate of somatic mutations in the rearranged Ig genes during antigen-induced proliferation in germinal centers (7). Mature B cells exhibiting high affinity for antigen are preferentially selected whereas those displaying low affinity for antigen die by apoptosis (8, 9).

The intracellular mechanisms that implement and regulate apoptosis are still poorly understood, but it is thought that cell death is controlled by a genetic program induced within the dying cell (1, 10–12). Little is known about the signals that regulate developmental cell death in the B cell lineage. Because the selection of B cell precursors is largely based on the production of functional H and L chain Ig receptors, it has been hypothesized that failure to express a pre-B cell receptor complex leads to cell death (13–15). Later in development, signaling through surface IgM is critical for the elimination of B cells activated by self-antigens in the BM, whereas the Fas receptor appears to play a major role in the deletion of activated B cells in the periphery (16). Beyond those signals that regulate B cell death during development, apoptosis can be induced in B lym-

[1] *Abbreviations used in this paper:* BM, bone marrow; CD40L, CD40 ligand; HRP, horseradish peroxidase; MCF, mean channel fluorescence.

The first two authors contributed equally to this work.

phocytes by a wide variety of stimuli including exposure to cytotoxic drugs, γ-irradiation, glucocorticoids, or withdrawal of growth factors (17–19).

The *bcl-2* protooncogene was the first member of an expanding family of genes that suppresses the apoptotic mechanism (20). Enforced expression of *bcl-2* in lymphoid cells prevents or delays apoptosis induced by multiple stimuli (17, 18, 21). A role for Bcl-2 in the selection of B lymphocytes was suggested by its pattern of expression during B cell development (22, 23). Analysis of Bcl-2–deficient mice has demonstrated that Bcl-2 is essential for the maintenance of mature B and T lymphocytes (24, 25). In addition, Bcl-2 appears to function as a survival signal for positive selection of B cells in germinal centers (8) and for recruitment or maintenance of high affinity memory B cells (26, 27).

Because mice deficient in Bcl-2 exhibited normal maturation of the B cell lineage (24, 25), we hypothesized that genes other than *bcl-2* could function as survival signals during the selection and maturation of B cell precursors. A candidate is *bcl-x*, a member of the *bcl-2* family of apoptosis-regulatory genes. In the human, two distinct *bcl-x* mRNAs (*bcl-x*$_L$ and *bcl-xs*) that encode proteins with different biological function were identified (28). In the mouse, *bcl-x*$_L$ is the dominant *bcl-x* mRNA expressed in embryonic and postnatal tissues including primary lymphoid organs (29, 30). Like Bcl-2, the product of *bcl-x*$_L$ localized to mitochondria and perinuclear envelope (29) and can inhibit the apoptotic death of hematopoietic cell lines after growth factor withdrawal (28). Analyses of *Bcl-x*–deficient chimeric mice demonstrated significant alteration in the maturation of B and T cell precursors (31). However, the biological basis for the altered phenotype observed during B cell maturation in *bcl-x* mutant mice was unclear. In the present studies, we sought to determine the regulation and function of *bcl-x* during B cell development.

Our results indicate that the expression of Bcl-x$_L$ is essentially limited to pre-B cells, a narrow stage of development characterized by extensive clonal selection. In contrast, its functional homologue, Bcl-2, was downregulated in pre-B cells but expressed in pro-B and mature B cells (23). Constitutive expression of Bcl-x$_L$ promoted accumulation of B cell precursors and mature B cells in the animal and enhanced B cell survival in vitro. Our results demonstrate that Bcl-x$_L$, but not Bcl-2, was upregulated after surface IgM cross-linking, CD40 signaling, or LPS stimulation. Given that Bcl-2 and Bcl-x$_L$ were coexpressed in mature B cells after activation, we examined the effect of simultaneous expression of *bcl-2* and *bcl-x*$_L$ transgenes. Expression of both Bcl-2 and Bcl-x$_L$ led to the enhanced accumulation of B cells in the animal and increased the protection against anti-IgD–induced cell death in vivo. The implications of these findings for physiological pathways of B cell development and survival are discussed.

Materials and Methods

Mice and Injections. C57BL/6 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. The Ig-*bcl-2* transgenic mice (32) were a gift from Dr. S. Korsmeyer (Washington University, St. Louis, MO). Mice were bred and maintained in a pathogen-free environment at the University of Michigan Animal Facilities. To study the effects of dexamethasone on BM B cell populations, animals received a single i.p. injection of 2 mg of dexamethasone (American Regent Laboratories, Shirley, NY) or PBS as control. BM cells from two femurs were harvested 48 h later and stained as described below. To analyze the effect of anti-IgD treatment on B cell populations, mice were treated according to the following schedule (33): 3 mg of anti-IL-7 mAb (M25 clone; 34) injected intraperitoneally three times per week starting 2 wk before injection of anti-IgD antibody and continuing for 1 wk; 1 mg of anti-CD4 mAb (GK1.5 clone; 35) once a week injected intravenously starting 2 wk before injection of anti-IgD antibody and continuing for 1 wk; 1 mg of anti-FcγRII mAb (24G2 clone; 36) injected intravenously along with the injection of anti-IgD antibody; 1 mg of anti-IgD mAb (HBδ6 clone; 37) injected 1 wk before killing. The number of BM and peripheral B cell populations was determined by flow cytometry as described below. To assess the efficiency of anti-IgD treatment, the expression of IgD on B cells was analyzed by flow cytometric analysis 2 d after injection of anti-IgD mAb. After this treatment, IgD molecules were saturated to the same extent in all groups of mice (data not shown).

Construction of 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 IgH enhancer using a SV40-EH cassette (21). The 2.4-kb fragment containing the SV40-EH-*bcl-x*$_L$ insert was microinjected into F_2 hybrid zygotes from (C57BL/6 × SJL/J)F_1 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 by PCR as previously described (28).

Cell Preparations. B cell–enriched populations were purified from spleens of normal and transgenic mice after in vitro treatment with anti-Thy-1.2 mAb (HO-13.4 clone, a rat IgM anti-mouse Thy-1.2 mAb) and complement (Cedarlane Laboratories, Ltd., Hornby, ON, Canada). The resulting cell populations were >90% sIgM$^+$ and <1% CD4$^+$ or CD8$^+$. To analyze the expression of Bcl-x or Bcl-2 after B cell activation in normal mice, B cell–enriched spleen cells were cultured in RPMI supplemented with 2 mM L-glutamine, 10^{-5} M 2-MER, and 10% heat-inactivated FCS (Hyclone Laboratories, Logan, UT) at a concentration of 5 × 10^6 cells/ml in the presence of different concentrations of affinity-purified F(ab′)$_2$ goat anti-mouse IgM polyclonal antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), 1 μg/ml recombinant CD40L (38), or 10 μg/ml LPS (Sigma Chemical Co., St. Louis, MO). At several time points, cells were harvested, washed twice in PBS, and cell lysates prepared as described below. Protein concentration in lysates was determined using a protein detection kit (Pierce Chemical Co., Rockford, IL). For cell viability assays, B cell–enriched spleen cells were cultured in triplicate at a concentration of 10^6 cells/ml in 96-well microtiter plates. Viable cells were determined at different days by trypan blue exclusion.

Antibodies. FITC-labeled anti-CD4 (clone H129.19) and PE-conjugated anti-CD8 (clone 53-6.7) mAbs were purchased from GIBCO BRL Research Laboratories (Gaithersburg, MD). The following antibodies were obtained from PharMingen (San Di-

ego, CA): biotinylated anti-CD4 (clone RM-4-5), biotin- and PE-conjugated anti-CD45R (B220) (clone RA3-6B2), PE-conjugated anti-CD24 (HSA) (clone M1/69), PE- and FITC-labeled anti-IgM (clone R6-60.2), FITC-labeled anti-IgD^b (clone 217-170), and FITC-labeled anti-CD43 (leukosialin) (clone S7). The streptavidin-RED670™ reagent was bought from GIBCO-BRL Research Laboratories.

Flow Cytometric Analysis. Single cell suspensions from spleen, LN, and BM were prepared as previously described (39). Cells (10^6) were incubated with different combinations of antibodies specific for cell surface markers in 100 μl of PBS-1% BSA for 30 min on ice and washed twice with PBS-1% BSA. When indicated, 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 was counted using Lysys II software (Becton Dickinson & Co., Mountain View, CA). Analysis of Bcl-x expression in B cell progenitors was performed by flow cytometry as previously described for Bcl-2 (23), using a mouse IgG3 anti-Bcl-x mAb (40) followed by a biotin-conjugated goat anti–mouse IgG3 (Caltag Laboratories, South San Francisco, CA) and streptavidin-RED670™.

Western Blot Analysis. Bcl-x_L or Bcl-2 expression was determined by Western blot analysis as previously described (23). Briefly, proteins (25–100 μg/sample) were transferred to nitrocellulose membranes by electrophoresis and then incubated at 4°C overnight with a rabbit anti-Bcl-x polyclonal serum (41) diluted 1/500, 4C11, a rat anti–mouse Bcl-2 (42), or with a mouse anti-β-Tubulin antibody (Sigma Chemical Co.) in 1.5% milk-TBS (Tris-buffered saline) with 30 μl of normal goat serum (Sigma Chemical Co.) After five washes in TBS with 0.05% of Tween 20, the membrane was incubated for 1 h at room temperature with horseradish peroxidase (HRP)–conjugated goat anti–rabbit, goat anti–rat, or goat anti–mouse antibodies (Jackson ImmunoResearch Laboratories, Inc.) diluted 1/50,000 in 1.5% milk-TBS. The reaction was developed by enhanced chemiluminescence using the ECL kit (Amersham Corp., Arlington Heights, IL).

Results

Bcl-x_L Expression Is Highly Regulated during B Cell Development in Normal Mice. The expression of Bcl-x proteins was assessed in developing and mature B cells by three-color flow cytometric analysis using different combinations of antibodies directed to specific surface markers and a mouse anti-Bcl-x mAb (40). The Bcl-x protein was detected at very low levels in B220^{dull}CD43$^+$ pro-B cells, upregulated in IgM$^-$B220^{dull} pre-B cells, and downregulated again in IgM$^+$HSA^{high} immature B cells (Fig. 1). Bcl-x was not detected in mature B cells from spleen (Fig. 1). This difference in Bcl-x expression was quantified by calculating the ratio of mean channel fluorescence (MCF) of Bcl-x to MCF of control Ig in each subpopulation. The MCF ratio for pro-B cells was 1.6 ± 0.3, increased to 3.0 ± 0.2 for pre-B cells and decreased again for immature B cells to 1.7 ± 0.2. For mature B cells from the BM and spleen, the ratio was 1.1 ± 0.2. To confirm these observations and to determine which form of Bcl-x was expressed, populations of BM B cells enriched in B220$^+$IgM$^-$ (pro- and pre-B cells) and B220$^+$IgM$^+$ (immature and mature B cells) were sorted at high purity by flow cytometry and cell lysates analyzed by Western blot analysis. Bcl-x_L was the only *bcl-x*

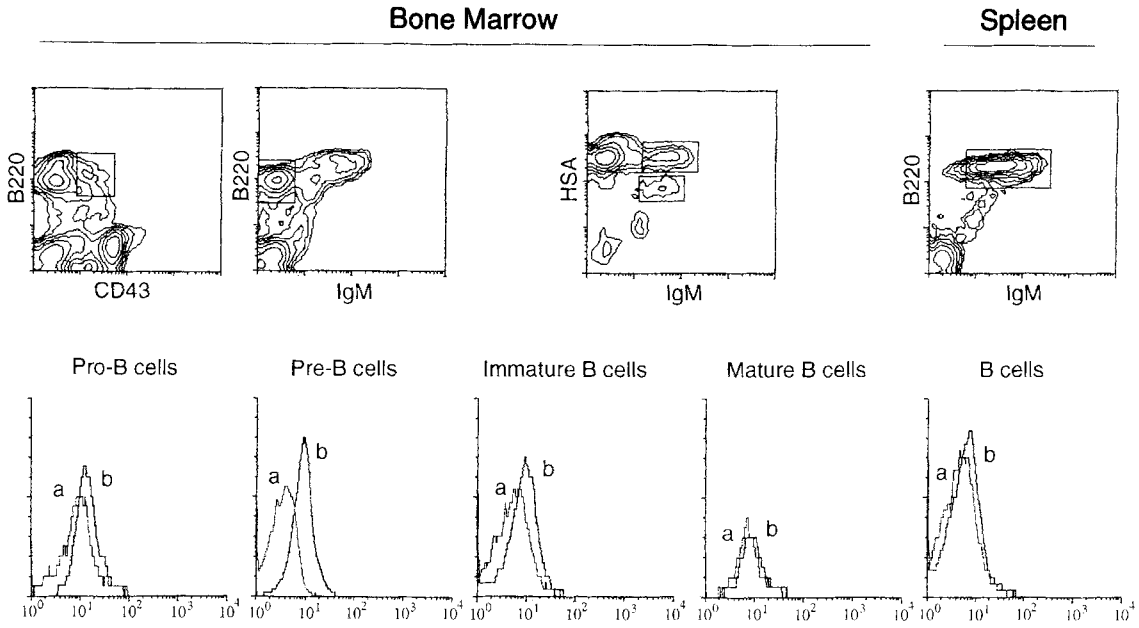

Figure 1. Expression of endogenous Bcl-x during B cell development. BM and spleen cells from 8-wk-old C57BL/6 mice were labeled with FITC-conjugated anti-IgM or anti-CD43, PE-conjugated anti-B220 or anti-HSA, or biotinylated anti-IgD antibodies. Cells were fixed with PBS containing 2% paraformaldehyde, permeabilized with saponin, labeled with anti-Bcl-x mAb or isotype-matched control antibody, and analyzed by flow cytometry. (*Top*) Contour plots of BM cells stained with different combinations of mAbs. BM cell populations were determined as follows: pro-B cells (IgM$^-$CD43^{low}B220$^+$), pre-B and pro-B cells (IgM$^+$B220^{low}), immature B cells (IgM$^+$HSA^{high}), and mature cells (IgM$^+$HSA^{low}). (*Bottom*) Intensity of Bcl-x staining (histogram *b*) in the B cell populations gated in the upper panels. Background staining with control mouse IgG3 is shown for comparison (histogram *a*). Results are representative of three different experiments.

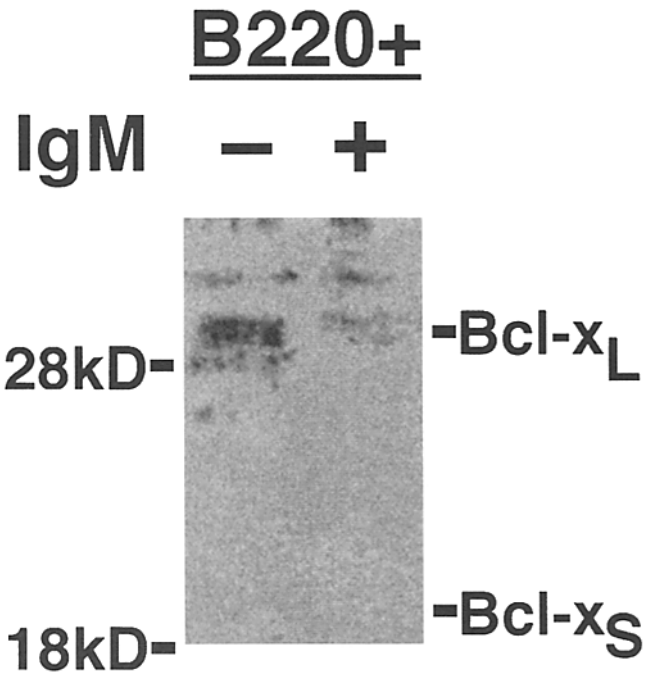

Figure 2. Bcl-x_L is the *bcl-x* product expressed during B cell development. $B220^+$-IgM^- and $B220^+$-IgM^+ B cell populations were sorted from BM by flow cytometry. The expression of Bcl-x in cell lysates from these fractions (25 μg of protein each lane) was assessed by Western blot analysis using a polyclonal anti-Bcl-x antibody followed by HRP goat anti–rabbit serum.

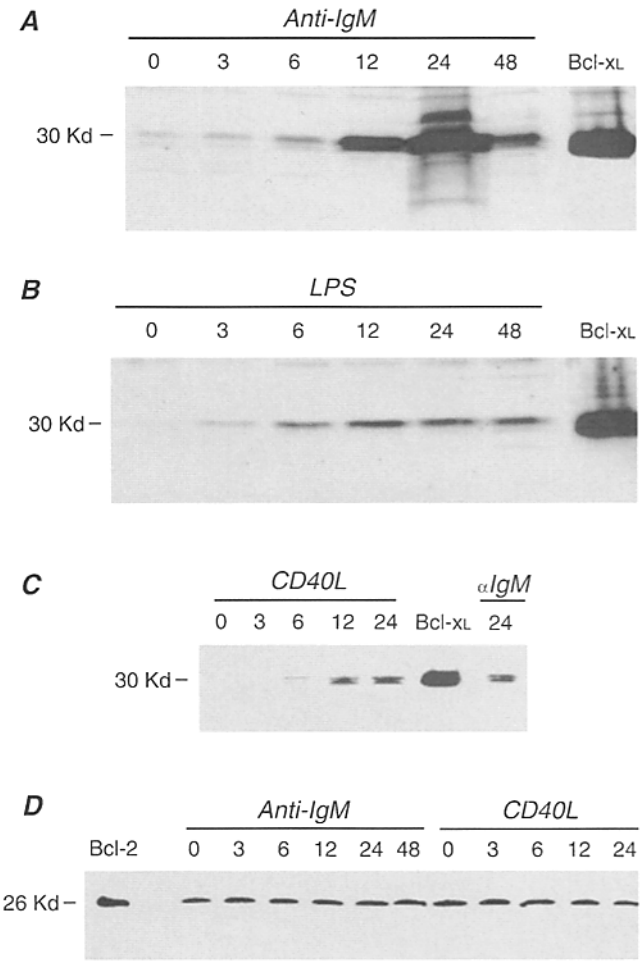

Figure 3. Activation of mature B cells with anti-IgM antibody, LPS, or CD40L induces Bcl-x_L but not Bcl-2 expression. B cell enriched populations were obtained from spleens of normal mice and stimulated in vitro with 10 μg/ml of $F(ab')_2$ goat anti–mouse IgM polyclonal antibody (*A* and *D*), 10 μg/ml of LPS (*B*), or 1 μg/ml recombinant CD40L (*C* and *D*). After 3, 6, 12, 24, or 48 h of incubation, cells were harvested and the expression of Bcl-x in cell lysates (50 μg of protein in each lane) was assessed by Western blot analysis using a polyclonal anti-Bcl-x antibody (*A–C*) or 4C11, a rat anti-Bcl-2 mAb (*D*) followed by HRP goat anti–rabbit or anti–rat serum. In *C*, the last lane represented a lysate from cells stimulated with anti-IgM antibody for 24 h to allow comparison between Bcl-x and Bcl-2 expression. In the experiment shown in *C*, activation of B cells with anti-IgM and CD40L was performed in parallel. Activation of B cells with LPS (*B*) was performed in a separate experiment.

product detected in preparations of developing and mature B cells (Fig. 2). The 30-kD Bcl-x_L protein was expressed in lysates from $B220^+IgM^-$ B cell precursors but downregulated in more mature $B220^+IgM^+$ B cells (Fig. 2). These results demonstrate that Bcl-x_L is the *bcl-x* product expressed during B cell development. Furthermore, the expression pattern of Bcl-x_L is predominantly restricted to pre-B and differs considerably from that previously reported for Bcl-2 (23, 43).

Bcl-x_L but Not Bcl-2 Is Induced in Mature B Cells after Activation. Resting B cells from peripheral lymphoid tissues express Bcl-2 but are devoid of Bcl-x protein (23 and Fig. 1). To assess whether Bcl-x is regulated during cellular activation, purified populations of splenic B cells were stimulated with purified $F(ab)'_2$ anti-IgM antibody or LPS, two signals that induce activation and proliferation of B cells (44). Bcl-x_L was clearly upregulated by 6 h and reached maximum expression at 24 h after surface IgM cross-linking (Fig. 3 *A*). The Bcl-x product was detected as a doublet of 29–31 kD in agreement with previous reports (41, 45). Induction of Bcl-x_L with anti-IgM was dose dependent and reached a plateau with 10 μg/ml of anti-IgM antibody (data not shown). Similarly, stimulation of B cells with the mitogen LPS upregulated Bcl-x_L with kinetics comparable to that observed with anti-IgM (Fig. 3 *B*). Similarly, signaling through CD40 using recombinant CD40 ligand (CD40L) induced Bcl-x_L in splenic B cells (Fig. 3 *C*). The level of Bcl-x_L induced by CD40 signaling was very similar to that observed after anti-IgM cross-linking (Fig. 3 *C*). Induction of Bcl-xL with anti-IgM or CD40L was specific in that both B cell activators failed to modulate Bcl-2 expression (Fig. 3 *D*). Thus, peripheral B cells that constitutively produce Bcl-2 can be induced to express Bcl-x_L after signaling through surface IgM receptors, stimulation by LPS, or CD40.

Generation of Transgenic Mice Expressing Constitutively Bcl-x_L in the B Cell Lineage. We developed a transgenic mouse model to assess the effects of Bcl-x_L overexpression in the animal. The *bcl-x_L* human cDNA was placed under the control of the IgH enhancer (Fig. 4 *A*), which is known to target genes to lymphoid cells (21, 46). Three founder mice

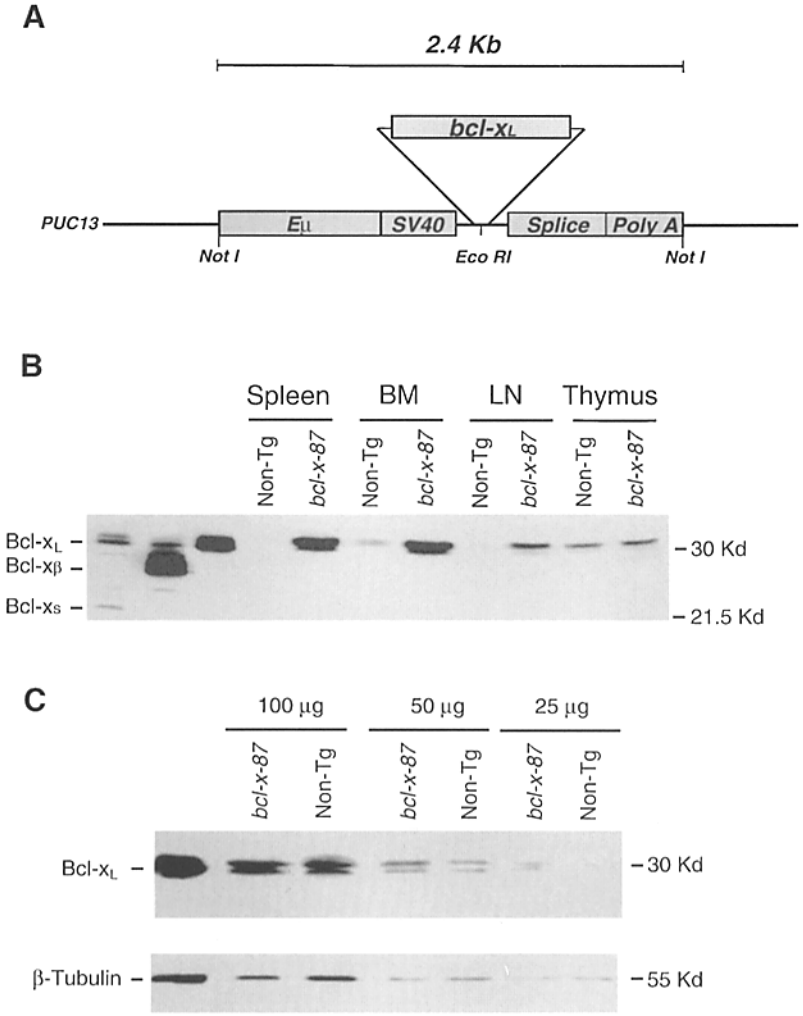

Figure 4. Eμ-*bcl-x*$_L$ transgene construct and Western blot analysis of Bcl-x$_L$ expression. (*A*) A 0.75-kb cDNA fragment containing the coding region of human *bcl-x*$_L$ (*closed box*) was inserted into the EcoRI site of the EμSV40 cassette (21). Eμ is the IgH enhancer and SV40 the early SV40 promoter. (*B*) Expression of Bcl-x$_L$ was assessed by Western blot analysis with a polyclonal anti-Bcl-x antibody followed by HRP goat anti–rabbit serum. Lysates from these cells (50 μg of protein) were loaded in each lane. Lysates from FL5.12 cells stably transfected with the human *bcl-x*$_s$, murine *bcl-xβ*, or murine *bcl-x*$_L$ cDNA (28, 29) are shown in the first three lanes, respectively, as positive controls and indicated as Bcl-x$_L$, Bcl-x$_β$ and Bcl-x$_s$. Notice that FL5.12 cells transfected with *bcl-xs* and *bcl-xβ* also expressed endogenous Bcl-x$_L$. (*C*) Comparison of Bcl-x$_L$ levels between resting B splenic cells from transgenic and activated B cells from control mice. B cells from normal mouse were activated for 24 h with 10 μg/ml of F(ab')$_2$ goat anti–mouse IgM polyclonal antibody. Western blot was performed using lysates containing 100, 50, or 25 μg of total proteins. 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. Densitometry scanning showed that resting splenic B cells from transgenic animals expressed 30% more Bcl-x$_L$ than activated B cells from control mice.

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 (41). One line (*bcl-x*-87) that exhibited restricted expression of the *bcl-x*$_L$ transgene to the B cell compartment was further characterized. As shown in Fig. 4 *B*, Bcl-x$_L$ was the only *bcl-x* product detected by Western blot analysis in the BM and thymus of normal mice. Bcl-x$_L$ protein was overexpressed in the BM of *bcl-x*-87 as compared to the levels of endogenous Bcl-x$_L$ in nontransgenic littermates (Fig. 4 *B*). Moreover, Bcl-x$_L$ was undetectable in the spleen and lymph nodes of normal mice but it was expressed in *bcl-x*-87 animals (Fig. 4 *B*). The *bcl-x*$_L$ transgene was not detected in purified populations of T 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). The relative levels of Bcl-x$_L$ in activated splenic B cells from normal mice and those in resting B cells from transgenic mice were similar when compared by Western blot analysis (Fig. 4 *C*).

Bcl-x$_L$ *Protects Immature and Pre-B Cells against Dexamethasone-induced Cell Death.* Pre-B and immature B cells are highly sensitive to treatment with glucocorticoids (23). To assess the effect of Bcl-x$_L$ overexpression in mice, *bcl-x*-87 transgenic and control littermate animals were treated with 2 mg of dexamethasone or PBS as a vehicle control, and 48 h later the different B cell populations in the BM were quantified by flow cytometry. In accordance with previous results (23), 80% of IgM$^-$B220^{dull} pre-B and 95% IgM$^+$IgD$^-$ immature B cells were preferentially depleted after dexamethasone treatment (Table 1). In contrast, pre-B and immature B cells from *bcl-x*-87 transgenic mice were largely protected (Table 1). For comparison, parallel experiments were performed with *bcl-2* transgenic mice that express Bcl-2 in the B cell lineage (32). As previously reported, overexpression of Bcl-2 protects pre-B and immature B cells against dexamethasone-induced cell death (21, 32, 47). These results indicate that Bcl-x$_L$ can protect developing B cells from glucocorticoid-induced death in a manner indistinguishable from that of Bcl-2 (Table 1).

Bcl-x$_L$ *Increases the Survival of Splenic B Cells In Vitro.* Splenic B cells, when cultured in vitro without growth fac-

Table 1. *Overexpression of Bcl-x_L Protects Pre-B and Immature BM Cells against Dexamethasone (Dex)-induced Cell Death In Vivo*

Mice	Dex	Total	Bone marrow B cell populations ($\times 10^6$)*				
			B220$^+$	Pro-B	Pre-B	Immature	Mature
Control	−	31 ± 3.0	7.8 ± 1.7	1.4 ± 0.2	3.3 ± 0.2‡	1.3 ± 0.2‡	1.8 ± 0.2‡
	+	28 ± 2.0	5.4 ± 1.5	1.0 ± 0.2	0.7 ± 0.2‡	0.1 ± 0.0‡	3.6 ± 0.6‡
bcl-x-87	−	35 ± 5.5	19.0 ± 5.1	2.0 ± 0.1	6.8 ± 2.5‡	3.9 ± 0.5	6.3 ± 0.6‡
	+	36 ± 4.6	19.8 ± 5.2	1.2 ± 0.2	4.0 ± 0.3‡	4.6 ± 0.5	9.9 ± 1.9‡
Ig-*bcl-2*	−	38 ± 5.0	18.0 ± 4.7	1.8 ± 0.2	6.4 ± 1.3‡	3.6 ± 1.0	6.2 ± 1.0‡
	+	40 ± 6.1	19.1 ± 4.0	1.9 ± 0.1	4.0 ± 0.4‡	4.0 ± 0.4	9.2 ± 1.5‡

*Results were obtained by three-color flow cytometric analysis of BM cells from two femurs simultaneously labeled with PE-conjugated anti-B200, biotinylated anti-IGD, and FITC-conjugated anti-IgM or anti-CD43. Cell populations were determined as follows: mature B cells (IgM$^+$IgD$^+$), immature B cells (IgM$^+$IgD$^-$), pre-B cells (IgM$^-$B220^{low}CD43$^-$), pro-B cells (IgM$^-$B220^{low}CD43^{low}). Values represent the mean ± SD for 10 15-wk-old animals.

‡Cell number between control and transgenic animals were significantly different ($p < 0.01$) as determined by the Student's *t* test.

tors, rapidly begin to die by apoptosis (48). To assess the effect of *bcl-x_L* on B cell survival, spleen cells from *bcl-x*-87 transgenic and control littermates were cultured in RPMI medium supplemented with 10% FCS and their viability assessed by trypan blue exclusion from day 1 to 10. There was an improved survival of spleen cells from transgenic mice when compared to spleen cells from control mice (Fig. 5). After 10 d of culture, only 5% of spleen B cells from normal mice were viable whereas 40% of the B cells from *bcl-x*-87 mice survived (Fig. 5). By comparison, we assessed the survival of spleen cells from *bcl-2* transgenic mice (32) (Fig. 5). As previously reported, survival was significantly increased for splenic B cells from *bcl-2* transgenic mice (21, 32) in a manner similar to that observed in *bcl-x*-87 transgenic mice (Fig. 5).

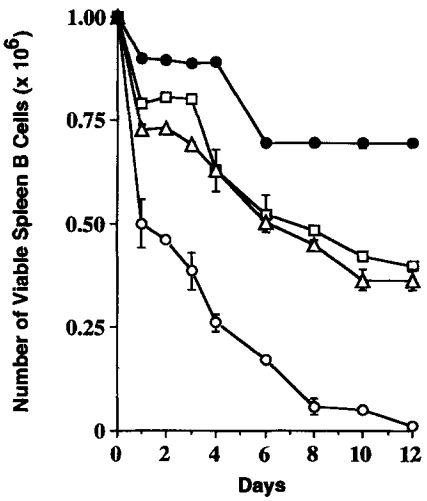

Figure 5. Expression of the *bcl-x_L* transgene increases B cell viability in vitro. Spleen cells were cultured in triplicates in 96-well flat-bottomed plates at a concentration of 10^6 cells/ml in RPMI 10% FCS. From day 1 to 10, viability of spleen B cells was assessed by trypan blue exclusion. Results are representative of four separate experiments. Cells were obtained from *bcl-x*-87 (□), *bcl-2* (△), *bcl-x*-87/*bcl-2* transgenic mice (●), or control littermates (○).

Resting mature B cells constitutively express high levels of Bcl-2 (23) but undetectable levels of Bcl-x_L (Figs. 1, 3, and 4 *B*). However, after activation, Bcl-x_L was induced in splenic B cells whereas Bcl-2 expression remained unchanged (Fig. 3). To determine whether coexpression of Bcl-2 and Bcl-x_L confers an increased resistance to cell death, we compared the capacity of B cells from *bcl-x*-87/*bcl-2* double transgenic mice, *bcl-2* or *bcl-x_L* single transgenic mice, and control littermates to survive in vitro. The viability of splenic B cells coexpressing *bcl-x* and *bcl-2* transgenes was significantly enhanced as compared to that of single *bcl-x* or *bcl-2* transgenic mice (Fig. 5).

Constitutive Expression of Bcl-x_L Leads to Accumulation of Developing and Mature B Cells. The results shown above indicated that the *bcl-x_L* transgene expressed in *bcl-x*-87 mice was functional in pre-B, immature B, and peripheral B cells (Table 1 and Fig. 5). We next assessed whether Bcl-x_L overexpression affected B cell homeostasis. In 5-mo-old *bcl-x*-87 mice, the total number of spleen cells was increased by 108% (283×10^6 vs. 136×10^6 in control mice) and the total number of LN cells was increased by 92% (15.5×10^6 in *bcl-x*-87 mice vs. 7.8×10^6 in control mice; Table 2). This increase was due to an accumulation of B220$^+$ cells in all tissues examined since the number of CD4$^+$ and CD8$^+$ T cells remained practically unchanged (Table 2). In the BM, the total number of B220$^+$ cells was increased by 158% (18.6×10^6 in *bcl-x*-87 mice vs. 7.2×10^6 in control mice). Although pre-B and immature B cells were significantly augmented in *bcl-x*-87 mice, the number of mature IgM$^+$IgD$^+$ B cells was particularly increased when compared to control littermates (Table 2). Given the distinct pattern of expression of Bcl-2 and Bcl-x_L during B cell development, we compared the phenotype of *bcl-x*-87 transgenic animals with that of *bcl-2* transgenic mice (32). It

Table 2. *Accumulation of Mature B Cells in bcl-x-87, bcl-2, and bcl-x-87/bcl-2 Transgenic (tg) Animals*

Cell populations	Number of cells in mice ($\times 10^6$)			
	Control	*bcl-x*-87 tg	*bcl-2* tg	*bcl-x*-87/*bcl-2* tg
BM*	33.0 ± 1.9	34.7 ± 3.5	36.7 ± 2.4	52.0 ± 5.0‖
$B220^+$ cells	7.2 ± 0.5	18.6 ± 2.8§	18.9 ± 1.9§	26.2 ± 2.9‖
Pro-B cells	1.5 ± 0.2	2.0 ± 0.2§	1.9 ± 0.2	2.1 ± 0.2‖
Pre-B cells	2.9 ± 0.3	6.0 ± 1.0§	6.2 ± 0.8§	9.4 ± 1.7‖
Immature B cells	1.2 ± 0.0	4.0 ± 0.2§	4.3 ± 0.4§	4.9 ± 0.5‖
Mature B cells	1.6 ± 0.1	6.6 ± 1.3§	6.5 ± 1.6§	9.8 ± 2.0‖
Spleen‡	156.0 ± 10	263.0 ± 15§	248.0 ± 23§	328.0 ± 42‖
$B220^+$ cells	81 ± 7.1	184.0 ± 12§	171.0 ± 16§	227.0 ± 35‖
$CD4^+$ cells	25.8 ± 2.6	27.4 ± 2.9	28.0 ± 1.9	26.8 ± 3.0
$CD8^+$ cells	20.9 ± 3.1	20.4 ± 1.9	20.6 ± 2.4	22.3 ± 2.5
LN‡	7.8 ± 0.3	15.5 ± 3.3§	17.5 ± 2.0§	20.5 ± 0.5‖
$B220^+$ cells	3.2 ± 0.2	8.3 ± 0.6§	8.8 ± 0.3§	12.0 ± 1.4‖
$CD4^+$ cells	2.5 ± 0.3	3.3 ± 0.7	2.9 ± 0.4	3.5 ± 0.8
$CD8^+$ cells	1.6 ± 0.1	1.8 ± 0.3	1.8 ± 0.1	1.7 ± 0.2

*Results were obtained by three-color flow cytometric analysis of BM cells from two femurs simultaneously labeled with FITC-conjugated anti-IgM or anti-CD43, PE-conjugated anti-HSA, and biotinylated anti-B220 antibodies. Immature B cells were defined as $IgM^+HSA^{high}B220^{low}$, mature B cells as $IgM^+HSA^{low}B220^{high}$, pre-B cells as $IgM^-CD43^-B220^{low}$, and pro-B cells as $IgM^-CD43^{low}B220^{low}$.

‡Analyses of cells from spleen and inguinal and axillary LN were performed by simultaneously labeling with FITC-conjugated anti-CD4, PE-conjugated anti-CD8, and biotinylated anti-CD3 antibodies. Values represent the mean ± SD for 10 15-wk-old animals.

§, ‖Values between single transgenic and control littermates or between double and single transgenic mice, respectively, were statistically different ($p < 0.01$) as assessed by the Student's t test.

is interesting to note that the phenotype and accumulation pattern of B cell populations in the BM, LN, and spleen were similar in *bcl-x_L* and *bcl-2* transgenic animals (Table 2). Because activated B cells can express both Bcl-2 and Bcl-x_L, we tested the possibility of a functional interaction between the two proteins in vivo by mating *bcl-x*-87 and *bcl-2* transgenic mice. Co-expression of both *bcl-x_L* and *bcl-2* transgenes resulted in a significant increase of mature B cells in BM, spleen, and LN as compared to *bcl-x_L* or *bcl-2* transgenic animals (Table 2).

Combined Overexpression of Bcl-x_L and Bcl-2 Can Protect against Anti-IgD–induced Cell Death In Vivo. Cross-linking of membrane IgD on mature B cells, in the absence of T cell help, has been shown to result in B cell death in vivo (33). The anti-IgD model was used to evaluate the ability of Bcl-x_L or Bcl-2 to block Ig receptor-mediated B cell death in the absence of T cell costimulatory function. Transgenic (*bcl-x*-87, *bcl-2*, *bcl-x*-87/*bcl-2*) and nontransgenic mice were treated with anti-IL-7 mAb to block the generation of newly formed B cells (34), with anti-CD4 mAb to block the generation of T cell help (49), and with anti-FcγRII mAb to avoid any potential inhibitory interactions between surface Ig and FcγRII. In both *bcl-x_L* and/or *bcl-2* transgenic mice and control littermates, treatment with anti-IL-7 antibody induced a 90% reduction in the number of pre-B and immature B cells, indicating that overexpression of Bcl-2 and/or Bcl-x_L proteins cannot overcome the inhibitory effect of anti-IL-7 mAb on the generation of newly formed B cells (data not shown). 7 d after anti-IgD treatment, a decrease of 86 ± 3% in the total number of IgM^+ mature B cells was observed in nontransgenic animals (from 81.5×10^6 to 11.2×10^6; Fig. 6), which is in agreement with recently published results (33). It is interesting to note that overexpression of Bcl-x_L or Bcl-2 had little or no effect on anti-IgD–induced B cell death (79 ± 2% reduction for *bcl-2* transgenic mice and 82 ± 5% reduction for *bcl-x*-87 transgenic mice; Fig. 6). In contrast, IgM^+ mature B cells from double *bcl-x*-87/*bcl-2* transgenic animals were partially but significantly protected from anti-IgD–induced cell death when compared to the number of B cells that survived in control, *bcl-x*-87, or *bcl-2* mice (53 ± 6% reduction from 110.2×10^6 to 52.1×10^6). Thus, coexpression of Bcl-2 and Bcl-x_L, but not Bcl-2 or Bcl-x_L alone, partially protected B cells from death induced by surface IgD cross-linking with a high affinity anti-IgD antibody (33).

Discussion

These studies demonstrate that Bcl-x_L is developmentally regulated in the B cell lineage and functions to protect developing and mature B cells from apoptosis. The pattern of Bcl-x_L expression is strikingly different from that of Bcl-2,

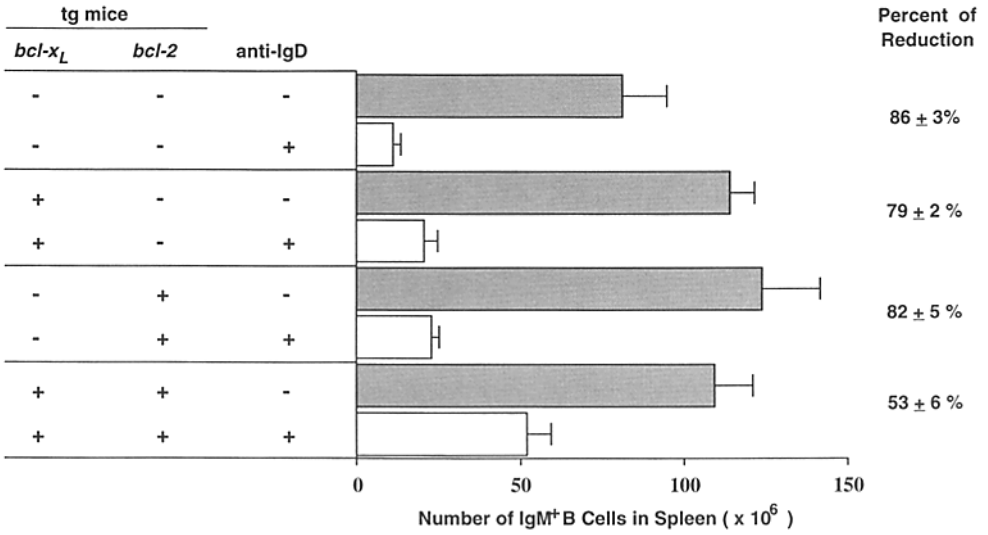

Figure 6. Protection of peripheral B cells from anti-IgD–induced cell death by *bcl-x*-87/*bcl-2* transgenes in vivo. 2-mo-old control and transgenic *bcl-x*-87, *bcl-2*, and *bcl-x-87/bcl-2* mice were treated with anti-IL-7, anti-CD4, and anti-FcγRII mAbs and with anti-IgD mAb (*empty bars*) or PBS (*gray bars*). The number of IgM^+ mature B cells in the spleen was determined by flow cytometric analysis using FITC-conjugated anti-IgM and biotinylated anti-B220 antibodies. Data represent the mean values obtained with four 7 wk-old mice ± SD. The reduction in the number of IgM^+ mature B cells between anti-IgD–treated and PBS-treated animals is indicated for each group as a mean percentage ± SD. The number of mature B cells after anti-IgD treatment in *bcl-x*-87/*bcl-2* mice was statistically different when compared to the number of mature B cells in the other groups of mice ($p < 0.01$ by Student's *t* test). Note that at 7 wk, *bcl-x*-87, *bcl-2*, and *bcl-x*-87/*bcl-2* transgenic mice have not accumulated B cells in the spleen as much as the 15 wk-old mice shown in Table 2.

a functional homologue of Bcl-x_L. Pre-B cells, a developmental stage in which Bcl-2 is downregulated (23), expressed the highest levels of Bcl-x_L. Although the role of Bcl-x_L during B cell development is not fully understood, our studies argue that Bcl-x_L provides a survival signal for the maintenance of pre-B cells and activated mature B cells. Recent studies with chimeric mice have shown a differential defect in the maturation and survival of pre-B cells deficient in Bcl-x without obvious alterations in mature B cells (31). Our studies provide an explanation for the latter findings in that Bcl-x_L is expressed predominantly in pre-B cells, the developmental stage particularly affected by the absence of Bcl-x (31). Furthermore, transgenic mice overexpressing Bcl-x_L exhibited accumulation of immature and mature B cells, implying that Bcl-x_L promotes the maturation of pre-B cells into B cells. Although a specific role for Bcl-x_L on B cell differentiation is possible, the results suggest that Bcl-x_L acts primarily by inhibiting cell death, a function that is essential for the maturation of pre-B cells into mature B cells.

Bcl-x_L and Bcl-2 exhibit a distinct expression pattern implying that these two related proteins play different roles in B cell selection and homeostasis. Bcl-2 but not Bcl-x_L is highly expressed in large $CD43^+$ early B cell precursors that undergo extensive rearrangements of IgH and IgL chain genes (23). Thus, Bcl-2 may play a role in the initial stage of B cell maturation when a diverse pool of pre-B cells is generated through IgH and IgL gene recombination (14, 43). At the end of the pro-B cell stage, Bcl-2 is downmodulated (23). Most of these B cell precursors will die during the transition to pre-B cells (4, 5) with survival of those B cell precursors bearing a functional pre-B cell receptor complex (50, 51). Because Bcl-x_L expression is induced in pre-B cells, it may serve as a survival signal for the small population of pre-B cells that successfully complete a productive rearrangement of their IgG and IgL gene. Later in development, both Bcl-2 and Bcl-x_L are downregulated in immature IgM^+IgD^- cells (23). Deletion of developing B cells bearing self-reactive IgM receptors occurs at the immature B cell stage (52–54). Thus, downregulation of both Bcl-2 and Bcl-x_L may facilitate the elimination of autoreactive B cells by making the cells more vulnerable to death signals associated with negative selection. At the mature stage, B cells express Bcl-2 but not Bcl-x_L (23). Thus, Bcl-2 induction at the mature B cell stage appears to represent a developmental switch involved in the selection and maintenance of peripheral B cells. The continuous expression of Bcl-2 is critical for mature B cells since mice deficient in Bcl-2 are unable to maintain peripheral lymphocytes (24, 25). A very similar pattern of regulated expression for Bcl-x_L has been observed during T cell development (55, 56). The expression of Bcl-x is the highest in $CD4^+CD8^+$ thymocytes and downregulated in mature T cells. Thus, the regulation of *bcl-x* is conserved in both T and B cell lineages.

It is intriguing that Bcl-2 and Bcl-x_L, two structurally related proteins that exhibit similar function, are differently regulated during B cell development. A possible explanation is that Bcl-2 and Bcl-x_L differ in their ability to counter death signals generated at specific stages during development. Bcl-2 and Bcl-x_L share remarkable structural homology and localize to identical intracellular sites, suggesting that they inhibit cell death by a similar biochemical mechanism (29, 57). However, it has been recently shown that Bcl-2 and Bcl-x_L interact differentially with some intracellular targets in vivo (58 and see below). Thus, it is possible that Bcl-2 and Bcl-x_L differ in a subtle manner in their functional ability to prevent cell death. Consistent with this possibility is the observation that Bcl-x_L is more efficient than Bcl-2 in protecting the immature B cell line

WEHI-231 from apoptosis induced by certain chemotherapeutic agents (41). The antiapoptotic functions of Bcl-2 and Bcl-x_L are controlled in part by several interacting proteins (58–60), one of which (Bad) exhibits a differential ability to heterodimerize with Bcl-2 and Bcl-x_L (58). Thus, another possibility is that the expression of the interacting partners of Bcl-2 and Bcl-x_L is differentially regulated during B cell development. In this scenario, developmental regulation of Bcl-2 and Bcl-x_L could reflect differential requirements for complexes between Bcl-2 or Bcl-x_L and their interacting partners to perform specific functions during B cell development.

The signals that govern Bcl-x_L expression during B cell development remain to be established. Because Bcl-x_L is upregulated at the pre-B cell stage, it is possible that signaling through the pre-B cell receptor complex is involved in Bcl-x_L regulation. Mutant mice lacking a functional pre-B cell receptor complex display a block in B cell development that coincides with the massive loss of precursor B cells in normal mice (13). Thus, it has been postulated that the pre-B cell receptor complex transduces signals that result in enhanced survival of pre-B cells (14, 15). A candidate signal is Bcl-x_L since it is upregulated at the pre-B cell stage of development and promotes the survival of developing B cells.

Cellular activation induces intracellular signals such as *c-myc* that are involved in both proliferation and cell death (61, 62). In B lymphocytes, cross-linking with anti-IgM or anti-IgD induces cell proliferation and ultimately cell death (33, 63). In mature B cells, our studies demonstrate that Bcl-x_L is upregulated by cross-linking of IgM receptors and presumably by antigen–IgM interactions. This notion is supported by the recent observation that Bcl-x protein is expressed in proliferating B cells of the germinal center (45), arguing that Bcl-x_L is upregulated during antigen-driven B cell activation in vivo. Similarly, Bcl-x_L was induced after the CD40–CD40L interaction, a signaling pathway known to deliver survival signals to mature B cells (64). As with B cells, Bcl-x_L can be induced after cellular activation in peripheral T cells (65, 66). Thus, under physiological situations, induction of Bcl-x_L in Bcl-2–positive mature B and T cells may serve as a mechanism to counter more effectively the death signals associated with activation and proliferation (61, 62). Consistent with this hypothesis is our observation that accumulation of B cells in the animal and B lymphocyte survival in vitro was further increased by simultaneous expression of *bcl-x_L* and *bcl-2* transgenes. Furthermore, combined overexpression of both Bcl-2 and Bcl-x_L in the absence of T cell costimulatory function protected mature B cells from anti-IgD–induced apoptosis more effectively than either protein alone. This indicates that Bcl-2 and Bcl-x_L can function as autonomous survival proteins in B lymphocytes. Under our experimental conditions, coexpression of Bcl-2 and Bcl-x_L only partially protected peripheral B cells from anti-igD–induced cell death, presumably because of the powerful cell death signal provided by the high affinity anti-IgD antibody (33). The increased survival of B cell expressing both Bcl-2 and Bcl-x_L could be explained by a gene dose effect, implying that resistance to cell death is determined by the relative levels of apoptosis-inhibitory proteins. Preliminary results obtained in our laboratory favor this hypothesis since B cells from double *bcl-2/bcl-2* transgenic mice exhibit in vitro survival greater than heterozygous *bcl-2* transgenic mice and similar to that of *bcl-2/bcl-x_L* transgenic animals (Grillot, D., R. Merino, and G. Nuñez, unpublished observation). Alternatively, the increased survival could be explained by a specific cooperation between Bcl-2 and Bcl-x_L. Regardless of the mechanism, our studies suggest that for certain death stimuli, particularly those associated with cellular activation, coexpression of Bcl-2 and Bcl-x_L may prove critical in determining the fate of certain populations of B cells.

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