

Enforced Bcl-2 Expression Inhibits Antigen-mediated Clonal Elimination of Peripheral B Cells in an Antigen Dose-dependent Manner and Promotes Receptor Editing in Autoreactive, Immature B Cells

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Summary

The mechanisms that establish immune tolerance in immature and mature B cells appear to be distinct. Membrane-bound autoantigen is thought to induce developmental arrest and receptor editing in immature B cells, whereas mature B cells have shortened lifespans when exposed to the same stimulus. In this study, we used E μ -*bcl-2*-22 transgenic (Tg) mice to test the prediction that enforced expression of the Bcl-2 apoptotic inhibitor in B cells would rescue mature, but not immature, B cells from tolerance induction. To monitor tolerance to the natural membrane autoantigen H-2K^b, we bred 3–83 μ δ (anti-K^{k,b}) Ig Tg mice to H-2^b mice or to mice expressing transgene-driven K^b in the periphery. In 3–83 μ δ /*bcl-2* Tg mice, deletion of autoreactive B cells induced by peripheral K^b antigen expression in the liver (MT-K^b Tg) or epithelia (KerIV-K^b Tg), was partly or completely inhibited, respectively. Furthermore, Bcl-2 protected peritoneal B-2 B cells from deletion mediated by acute antigen exposure, but this protection could be overcome by higher antigen dose. In contrast to its ability to block peripheral self-tolerance, Bcl-2 overexpression failed to inhibit central tolerance induced by bone marrow antigen expression, but instead, enhanced the receptor editing process. These studies indicate that apoptosis plays distinct roles in central and peripheral B cell tolerance.

Apoptosis is an essential element in the development and homeostasis of many tissues. In the immune system, a number of important processes are regulated through the control of cell death and survival (1–4). Among these processes is immunological tolerance in which encounter with ligands that signal through antigen receptors affect the cell's subsequent survival (5, 6). The lifespans of lymphocytes in vivo vary widely, from 1–2 d to many weeks (7). Self-antigen can shorten the lifespan of reactive B cells in a number of ways: by promoting cell death through developmental arrest (8, 9), by increasing cell turnover (10, 11), by putting cells at a competitive disadvantage with nonautoreactive cells for unknown resources (12), by making the cells sensitive to Fas

ligand-mediated killing by T cells (13), or apparently through direct induction of apoptosis (11, 14–18).

One regulator of lymphocyte survival is the Bcl-2 protein (1), which is highly expressed in long-lived lymphocytes and is poorly expressed in cells destined to turn over rapidly (19–26). Bcl-2, and the closely related Bcl-x_L (27), are key protein regulators of apoptosis. Overexpression or inappropriate expression of these proteins can play a role in lymphoma development and can allow the continued survival of cells that would otherwise be lost through apoptosis (28–36). However, some forms of apoptosis, including Fas-mediated killing of certain lymphoid cells (37), are not inhibitable by Bcl-2. An important physiological

role for Bcl-2 is evident from the phenotype of *bcl-2*-deficient mice, which manifest a catastrophic apoptotic loss of mature lymphocytes (38, 39).

The ability of Bcl-2 to block B cell tolerance has been studied in several systems in which ligands that bind to the B cell receptor (BCR)¹ can stimulate cell death *in vivo* or *in vitro* (9, 15, 33, 40–48). In some cases, these data have been contradictory, suggesting that the ability of Bcl-2 overexpression to block tolerance-mediated apoptosis may be contingent upon the precise quality of the BCR-mediated signal and perhaps other signals that may differ in certain experimental systems. In the well-characterized surface Ig⁺ B lymphoma WEHI-231, cross-linking of the BCR with anti-immunoglobulin antibody leads to cell death (for review see reference 18). In some studies (43, 44), but not others (40), transfection of *bcl-2* expression constructs protected the cells from BCR-mediated cell death. When transgenic (Tg) mice with enforced B cell overexpression of Bcl-2 were analyzed for B cell tolerance, bone marrow tolerance was perturbed in one study (9), but not in another (46), whereas peripheral tolerance was inhibited (15, 46). Similarly, germinal center B cells have been shown to be sensitive to killing induced by acute ligation of sIg, and this death could be inhibited by enforced Bcl-2 expression in one study (48), but not in another (47). These studies and others focusing on T cell tolerance (3, 4) have suggested that the ability of Bcl-2 to protect autoreactive lymphocytes from cell death may be contingent upon many factors, including the developmental stage of the lymphocyte as well as the nature of the antigen stimulus.

Resting B cells encountering tissue-specific membrane-bound antigen in the periphery are eliminated, suggesting that in this case, peripheral tolerance is mediated by an apoptotic process (14). In contrast, it has been shown in several systems that immature B cells encountering membrane-bound or nuclear self-antigens in the bone marrow are blocked in their development (8, 9) and either undergo receptor editing (49, 50) or are eliminated (50–52). Our recent finding that *in vitro* tolerance induction of immature B cells stimulates intense receptor editing with little immediate cell death (53, 54) suggests that antigen-induced programmed cell death makes only a minor contribution to central tolerance. Assuming that Bcl-2 is a key cell death regulator in B cells, this model predicts that enforced Bcl-2 expression can block clonal elimination of mature, but not immature, self-reactive B cells. To test this, peripheral and central tolerance in 3–83 $\mu\delta$ (anti-H-2^{k,b}) Ig Tg mice were compared to that occurring in 3–83 $\mu\delta$ /E μ -*bcl-2-22* double-Tg mice, in which Bcl-2 is constitutively expressed in B-lineage cells.

Materials and Methods

Mice. MT-K^b mice, in which K^b antigen expression is directed to hepatocytes by the sheep metallothionein promoter

¹Abbreviations used in this paper: BCR, B cell receptor; BrdU, 5-bromo-2'-deoxyuridine; CD, central deleting; ND, nondeleting; Tg, transgenic.

(55), and KerIV-K^b mice, in which K^b is expressed in epithelial cells under the control of the keratin IV promoter (56), were bred several generations onto the B10.D2 background (H-2^d) before breeding with other Tg or H-2 congenic mice. 3–83 $\mu\delta$ Tg mice, which express IgM and IgD forms of the 3–83 antibody, have been described (14). 3–83 $\mu\delta$ mice were backcrossed a minimum of 10 times onto the B10.D2 background. C57BL/6(H-2^b), C3H-H-2^{o2}/SfSn (C3H.OH: K^dD^k), and B10.D2/nSnJ (H-2^d) mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice overexpressing the human *bcl-2* gene in B-lineage cells were Tg lines E μ -*bcl-2-22* (34) and *bcl-2*-Ig NL (36). These mice were backcrossed a minimum of three generations to B10.D2 and subsequently bred with 3–83 $\mu\delta$ Tg mice. The phenotypes of mice bearing either of the *bcl-2* transgenes were indistinguishable in our study. Unless otherwise stated, the experiments described made use of the E μ -*bcl-2-22* line. The 3–83 $\mu\delta$ /RAG-1 knockout mice (57) were bred with E μ -*bcl-2-22* Tg mice. F₂ crosses were set up to generate *bcl-2/3-83 $\mu\delta$ /RAG-1^{-/-}* mice with all transgenes hemizygous except where noted. All mice were bred and maintained under specific pathogen-free conditions in the animal care facility at the National Jewish Medical and Research Center (NJMRC, Denver, CO).

Mouse Typing. PCR typing for 3–83 $\mu\delta$, K^b, and *bcl-2* transgenes was as described previously (14). RAG-1-deficient mice were identified by their lack of T cells in flow cytometric analysis of peripheral blood.

Antibodies, Immunofluorescence, Cell Lines, and Intraperitoneal Injection Assays. Preparation of lymphoid cells, antibody reagents, fluorochrome conjugation, flow cytometry analysis, cell lines, and intraperitoneal injection assay methods were as previously described (58). In brief, the A/J splenocyte \times Sp2/0 hybridoma 4549 (which expresses K^b) and Sp2/0 (H-2^d) parent hybridoma were washed and resuspended at either 5×10^7 or 5×10^8 cells/ml in PBS and injected intraperitoneally into 3–83 $\mu\delta$ /H-2^d or 3–83 $\mu\delta$ /H-2^d/*bcl-2* Tg mice. Approximately 16 h later, the peritoneal cells were harvested with 10 ml HBSS, washed twice, and stained for the presence of Id-reactive antibodies.

Cell Culture. Spleen or lymph node cells were cultured in RPMI-1640 medium supplemented with 10% FCS and 50 μ M 2-mercaptoethanol \pm 50 μ g/ml LPS (Sigma Chemical Co., St. Louis, MO). The cell concentration was adjusted depending on the number of 3–83 B cells in the organ according to the following guidelines: 10^6 cells/ml for H-2^d 3–83 Tg, H-2^d *bcl-2/3-83* double-Tg, and *bcl-2/3-83 $\mu\delta$ /KerIV-K^b* triple-Tg mice; and $3-5 \times 10^6$ cells/ml for H-2^b 3–83 Tg, H-2^b *bcl-2/3-83 $\mu\delta$* , 3–83/MT-K^b, 3–83/KerIV-K^b double-Tg, and *bcl-2/3-83 $\mu\delta$ /MT-K^b* triple-Tg mice. In some experiments, lymph node and spleen cells were T cell depleted with anti-Thy1 antibodies and rabbit complement and cultured at 5×10^5 cells/ml. Culture supernatants were harvested on days 3 and 7 after initiation of culture and Ig concentrations determined by ELISA, as described (14).

5-bromo-2'-deoxyuridine Incorporation Assay. 5-bromo-2'-deoxyuridine (BrdU; Sigma Chemical Co.) was provided in filtered, deionized drinking water at a concentration of 1 mg/ml for 7 d. Staining for the incorporation of BrdU was described previously (59).

Results

Enforced Bcl-2 Expression Blocks B Cell Deletion Induced by Acute Antigen Administration at Low Dose, but Not at High Dose. The 3–83 $\mu\delta$ Ig transgene encodes a BCR that is reactive to a number of MHC class I alloforms including

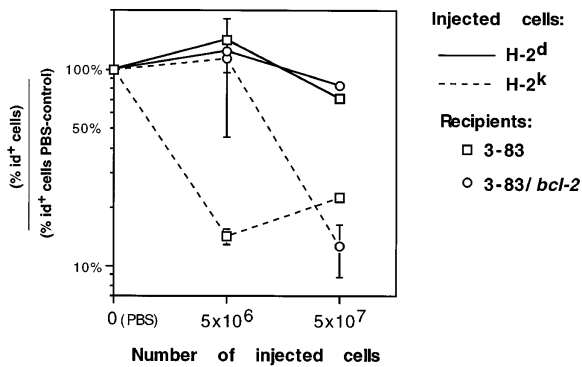


Figure 1. Enforced Bcl-2 expression inhibits B cell deletion induced by acute challenge with membrane antigen in an antigen dose-dependent manner. NDTg and NDTg/*bcl-2* mice were injected intraperitoneally with either PBS control, K^k-expressing tumor cells, or K^d tumor cells, and the peritoneal cells were analyzed 16 h later for the loss of 3-83 B cells using 54.1 anticonotype and anti-IgM antibodies. The data from three experiments are presented as mean ± SEM. In some data points, error bars are not apparent because of their small range.

K^k, D^k, and K^b, but fails to bind to H-2^d; thus, 3-83μδ/H-2^d mice contain a virtually monoclonal B cell population bearing the 3-83 BCR and are called nondeleting (ND)Tg mice (14). To probe the ability of enforced Bcl-2 expression to block tolerance to acute intraperitoneal antigen challenge, we injected NDTg and NDTg/*bcl-2* mice with antigen-bearing cells, a protocol that stimulates rapid apoptosis of fully mature antigen-specific peritoneal B cells (15, 16).

Injection of hybridoma cells bearing the high-affinity K^k antigen consistently led to massive loss (~80%) of the 3-83μδ peritoneal B cells over a 16-h period, whereas injection of control H-2^d cells did not (Fig. 1). At low antigen dose (5 × 10⁶ cells), NDTg/*bcl-2* B cells resisted deletion induced by the K^k antigen (Fig. 1); however, this protection from death afforded by Bcl-2 overexpression was overcome with a 10-fold increased antigen dose (Fig. 1).

Enforced Bcl-2 Expression Partially Blocks B Cell Deletion Induced by Hepatocyte-targeted K^b and Completely Blocks B Cell Deletion Induced by Epithelial Cell-targeted K^b. To study peripheral B cell tolerance to natural chronic autoantigen exposure, we generated 3-83μδ mice bearing MT-K^b or KerIV-K^b transgenes, which target cell surface expression of the K^b protein to hepatocytes or epithelia, respectively (55, 56). Relative to antigen-free mice, antigen-bearing 3-83μδ mice had profoundly reduced B cell numbers in the lymph nodes (Fig. 2 A, top; Fig. 2 B, compare H-2^d to MT-K^b and KerIV-K^b), and substantial, but on average, incomplete deletion of the B cells in the spleen (Fig. 2 C, top; Fig. 2 D and reference 14). Control mice bearing antigen on all tissues (Fig. 2, H-2^b) exhibited the phenotype of central B cell tolerance in which antigen-reactive cells were absent from the spleen. In the mice that demonstrated peripheral B cell deletion (3-83μδ/MT-K^b or 3-83μδ/KerIV-K^b), the remaining splenic cells manifested rapid turnover as assessed by their BrdU uptake over a 1-wk labeling period (Fig. 3). It is important to note that the MT-K^b/3-83μδ mice showed a more profound tolerance than KerIV-K^b/3-

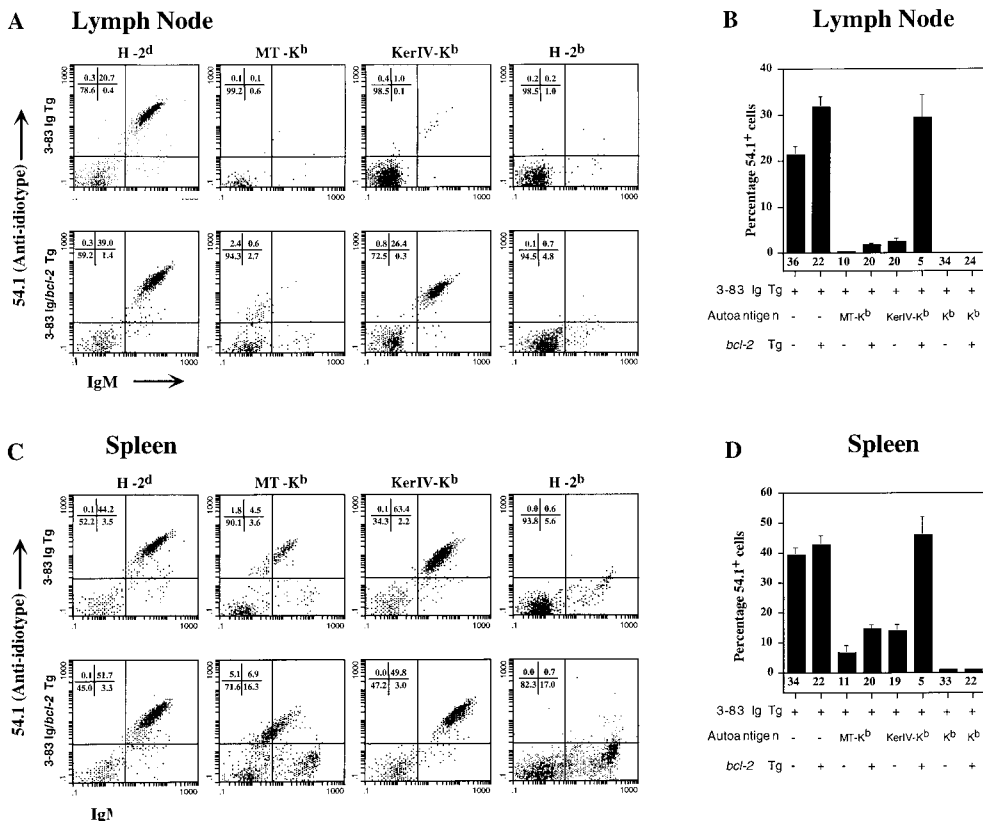


Figure 2. Enforced Bcl-2 expression blocks peripheral clonal deletion in 3-83μδ mice. (A and C) 3-83μδ Tg mice (top) or 3-83μδ/*bcl-2* Tg mice (bottom) bearing the indicated self-antigens were analyzed for Id⁺ B cells in lymph node (A) and spleen (C). (B and D) The compiled FACS[®] data as percentage of IgM⁺/Id⁺ cells in the lymphoid analysis gate is shown. Data are presented as mean ± SEM. The numbers below each bar represent the number of mice analyzed per group.

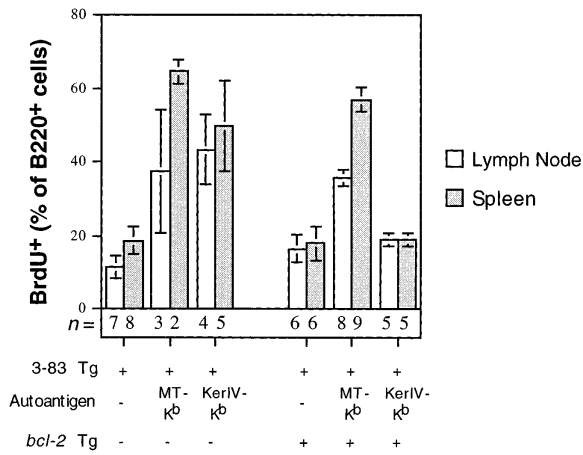


Figure 3. Effect of *bcl-2* transgene on B cell turnover in peripheral antigen-expressing 3–83 $\mu\delta$ Tg mice. Mice were fed BrdU-containing water for 7 d followed by staining of isolated lymph node and spleen cells with anti-BrdU and anti-B220 antibodies. Data are presented as percentage (mean \pm SEM) of B220 $^{+}$ cells that were BrdU positive. Numbers of mice analyzed are indicated below bars. Mice lacking antigen were H-2 d ND controls.

83 $\mu\delta$ mice, and had more complete elimination of Id $^{+}$ B cells in the lymph nodes (Fig. 2) and of Id $^{+}$ antibodies in the serum (Fig. 4 C).

In *bcl-2/3-83 $\mu\delta$ /MT-K b* and *bcl-2/3-83 $\mu\delta$ /KerIV-K b* triple-Tg mice, enforced Bcl-2 expression partially or completely blocked peripheral B cell deletion to liver and skin antigens, respectively, and, on average, increased numbers of Id $^{+}$ cells in both the lymph nodes and spleen (Fig. 2, A and C, *bottom*; Fig. 2, B and D). Interestingly, in the *bcl-2/3-83 $\mu\delta$ /MT-K b* mice in which Bcl-2 protection was partial, the high rate of turnover of the autoreactive B cells was unchanged, whereas in the *bcl-2/3-83 $\mu\delta$ /KerIV-K b* triple-Tg mice in which B cell deletion was effectively blocked, B cell turnover was also normalized, and only \sim 20% of the B cells had incorporated BrdU over a 1-wk period (Fig. 3). Furthermore, lymph node cells from *bcl-2/3-83 $\mu\delta$ /KerIV-K b* triple-Tg, but not those from *bcl-2/3-83 $\mu\delta$ /MT-K b* mice, acquired the ability to respond to LPS stimulation (Fig. 4 A). The ability of lymph node and spleen cells to secrete 3–83 antibodies in vitro was consistent with the levels of 3–83 antibodies measured in the sera of these mice, as Id $^{+}$ antibody levels were negligible in the *bcl-2/3-83 $\mu\delta$ /MT-K b* mice, but were significant in the *bcl-2/3-83 $\mu\delta$ /KerIV-K b* triple-Tg mice (Fig. 4 C). The only differences noted between *bcl-2/3-83 $\mu\delta$ /KerIV-K b* triple-Tg mice and *bcl-2/3-83 $\mu\delta$* ND controls were a consistent twofold reduction in surface IgM expression in the *bcl-2/3-83 $\mu\delta$ /KerIV-K b* mice, suggesting antigen encounter that did not result in deletion (Fig. 2, A and C), and a lower percentage of cells expressing CD21 (data not shown). In both *bcl-2/3-83 $\mu\delta$ /KerIV-K b* and *bcl-2/3-83 $\mu\delta$ /MT-K b* mice, the B220 $^{+}$ B cells coexpressed the maturation marker CD23 (data not shown). Collectively, these data demonstrated that Bcl-2 overexpression could inhibit peripheral B cell tolerance to both acute and chronic antigen exposure, but the degree of pro-

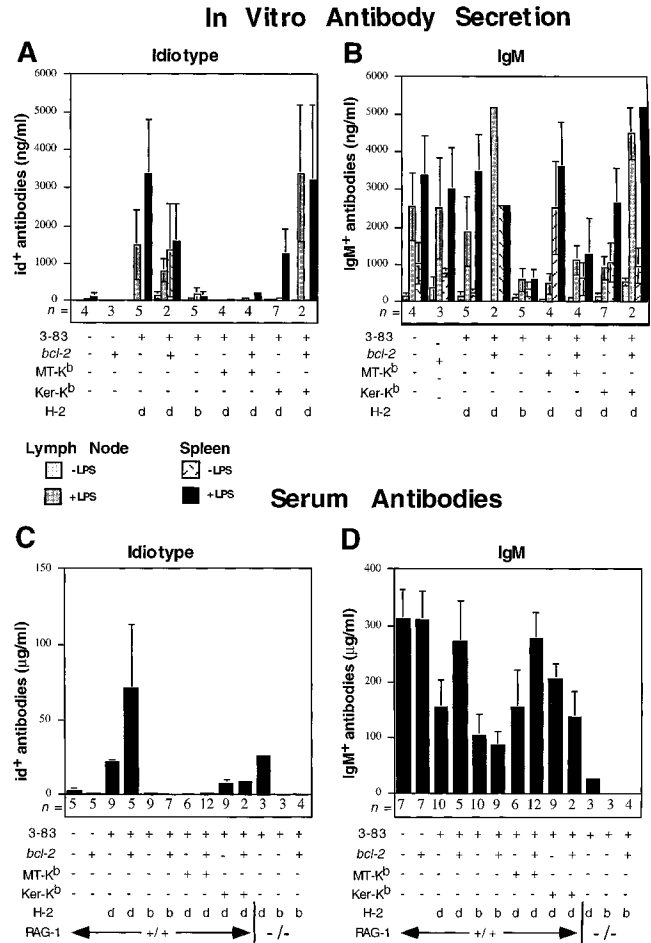


Figure 4. In vitro and in vivo analysis of antibody secretion. Lymph node and spleen cells from mice of the indicated genotypes were cultured in the presence or absence of 50 $\mu\text{g/ml}$ LPS. After 3 d of culture, supernatants were analyzed by ELISA for Id $^{+}$ (A) and IgM (B) antibodies. Antibody amounts were quantitated using 3–83 IgM transfectoma supernatant control. Similarly, Id $^{+}$ (C) and IgM (D) serum antibodies were quantitated by ELISA. Data are presented as mean \pm SEM with number of mice (*n*) analyzed shown. The table below the graph shows Tg genotypes (3–83, *bcl-2*, MT-K b , and/or KerIV-K b) and H-2 haplotypes (*d*, no antigen; *b*, antigen). In C and D the RAG-1 genotype of analyzed mice (+/+ or -/-) is indicated.

tection varied depending upon the dose or anatomical location of the antigenic stimulus.

Enforced Bcl-2 Expression Does Not Block Central B Cell Tolerance. The E μ -*bcl-2-22* transgene drives expression of functionally active Bcl-2 in immature bone marrow B cells that normally lack Bcl-2 expression (46). To test the effect of this transgene on central B cell tolerance, we bred H-2 b mice to 3–83 $\mu\delta$ and *bcl-2/3-83 $\mu\delta$* mice, generating central deleting (CD)Tg and CDTg/*bcl-2* mice, respectively. Like CDTg mice, CDTg/*bcl-2* mice lacked Id $^{+}$ B cells in the peripheral lymphoid organs (Fig. 2, H-2 b ; Table 1) and Id $^{+}$ antibodies in the sera (Fig. 4 C). In addition, no Id $^{+}$ antibodies were found in the supernatants of LPS-stimulated spleen cells from CDTg or CDTg/*bcl-2* mice, whereas NDTg and NDTg/*bcl-2* controls had significant levels of Id $^{+}$ antibodies in both the sera and LPS culture supernatants (Fig. 4 A;

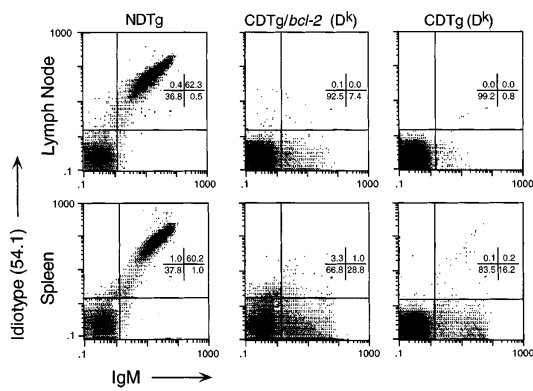


Figure 5. Enforced Bcl-2 expression fails to block central deletion induced by the ultralow affinity 3–83 ligand D^k. Lymph node and spleen cells from mice of the indicated genotypes were double stained for the presence of B cells bearing the 3–83 Tg BCR with 54.1 anti-Id and anti-IgM antibodies. The substantial population of IgM⁺ B cells present in the CDTg mice were clonotype⁻ and are presumably the result of receptor editing. The large percentage of B cells in the ND control in this experiment reflects the RAG deficiency of this particular mouse.

data not shown). Since previous central tolerance studies investigating Bcl-2 overexpression used high-affinity antigens ($K_A \sim 10^9 M^{-1}$), we tested whether or not Bcl-2 overexpression had perhaps a subtle, antigen affinity-dependent effect on tolerance induction by generating CDTg/*bcl-2* mice expressing the D^k class I molecule to which 3–83 has very low affinity ($K_A \sim 10^4 M^{-1}$; reference 58). Again the CDTg/*bcl-2* mice had no detectable increase of Id⁺ B cells in the peripheral lymphoid organs (Fig. 5) nor IgM idiotype in the serum (data not shown).

Bcl-2 Overexpression Allows Survival of a Population of Tolerant, Immature IgM⁻, B220⁺ B Cells in the Peripheral Lymphoid Organs. In some CDTg/*bcl-2* mice (13/21), a population of IgM⁻, B220⁺ B cells was detected in the spleen and, to a

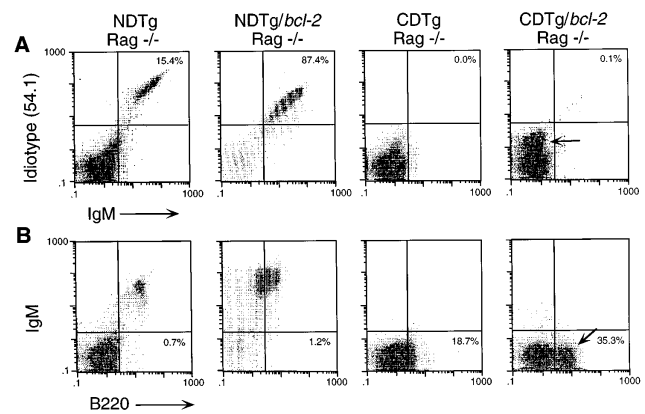


Figure 6. Appearance of immature B cells in spleen of CD (H-2^b) *bcl-2*/RAG-1-deficient mice. Spleen cells from mice of the indicated genotypes were stained for presence of 3–83 B cells with anticonotype (54.1) and anti-IgM antibodies (A), and anti-IgM and anti-B220 antibodies (B). The immature B cells have low levels of Id and lack detectable sIgM (*arrows*). The NDTg control used in this particular experiment was 3–83 μ d homozygous, which consistently show reduced B cell populations. Data shown represent one of five similar experiments.

lesser extent, in the lymph nodes. To further characterize this population in a context in which receptor editing could not occur, we generated CDTg/*bcl-2*/RAG-1-deficient mice (57). Again, no strongly Id⁺ cells were detected in the peripheral lymphoid organs (Fig. 6 A; Table 1), but a significant population of IgM⁻, B220⁺, Id^{lo} cells was detected in the spleen of the CDTg/*bcl-2*/RAG-1^{-/-} mice (Fig. 6, A and B, note *arrows*), and a similar population was detected to a lesser extent in CDTg/RAG-1^{-/-} mice without Bcl-2 overexpression. These IgM⁻, B220⁺ cells were CD23⁻, κ^{lo} , IgD^{lo}, and CR1/2⁻ (data not shown) which is indicative of immature B cells as has been previously reported in Bcl-2 overexpressing Ig Tg mice (9). Also

Table 1. Effect of Bcl-2 Overexpression on Tolerance Induction and Receptor Editing in 3-83 Immunoglobulin Transgenic Mice

Genotype	Lymph Node			Spleen		
	54.1 ⁺	IgD ^a , λ^+	B220 ⁺ , IgM ⁻	54.1 ⁺	IgD ^a , λ^+	B220 ⁺ , IgM ⁻
	<i>Cell content ($\times 10^5$)</i>					
NDTg +/+	82 ± 16 (12)	0.6 ± 0.2 (11)	1.6 ± 0.4 (10)	280 ± 38 (11)	1.8 ± 0.7 (11)	9.9 ± 2.5 (11)
NDTg/ <i>bcl-2</i> +/+	160 ± 40 (9)	1.6 ± 0.4 (9)	6.3 ± 1.2 (9)	350 ± 70 (9)	4.4 ± 1.3 (9)	17 ± 7 (9)
NDTg -/-	30 ± 2 (2)	0 (1)	0.7 ± 0.7 (2)	230 ± 40 (3)	0.7 ± 0.3 (2)	15 ± 7 (3)
NDTg/ <i>bcl-2</i> -/-	580 (1)	0 (1)	2.1 (1)	870 (1)	0 (1)	5.3 (1)
CDTg +/+	0.3 ± 0.1 (19)	3.3 ± 0.6 (19)	2.3 ± 0.4 (17)	5.8 ± 1.2 (19)	36 ± 5 (20)	31 ± 8 (19)
CDTg/ <i>bcl-2</i> +/+	0.6 ± 0.2 (19)	14 ± 3 (18)	9.9 ± 2.5 (16)	6.1 ± 1.7 (17)	66 ± 19 (19)	57 ± 17 (17)
CDTg -/-	0.1 ± 0.1 (5)	0.1 ± 0.1 (3)	3.8 ± 1.4 (5)	1.9 ± 1.2 (5)	0 ± 0 (4)	45 ± 15 (5)
CDTg/ <i>bcl-2</i> -/-	0.8 ± 0.4 (6)	0.1 ± 0.1 (4)	11 ± 6 (6)	5.3 ± 3.3 (6)	0.2 ± 0.2 (5)	160 ± 28 (6)

Absolute number of idiotype-positive (54.1⁺), “edited” (IgD^a, λ^+), and immature (B220⁺, IgM⁻) B cells in 3-83 immunoglobulin transgenic mice in the presence (CDTg) or absence (NDTg) of antigen in the bone marrow. In the genotype column +/+ refers to normal levels of RAG-1 whereas -/- refers to RAG-1 homozygous knock-out mice. All numbers shown are averages ± SEM divided by 10^5 . The numbers in parenthesis refer to sample size.

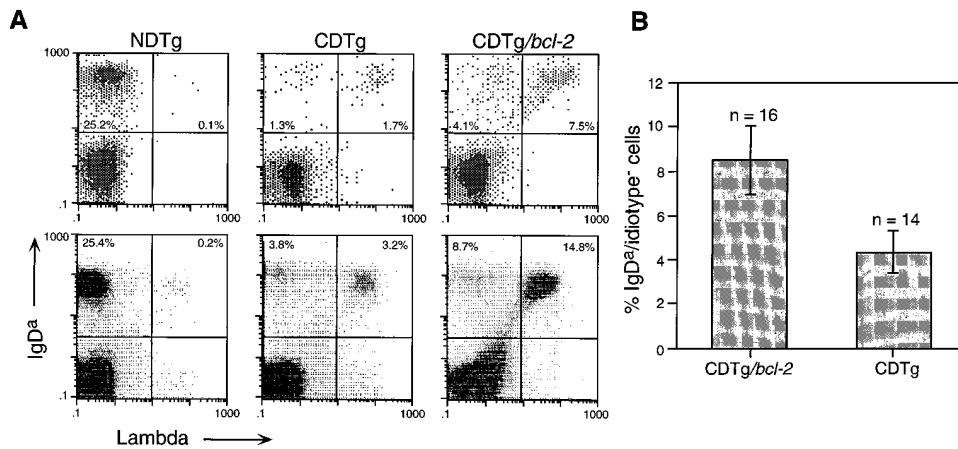


Figure 7. Increased percentage of IgD^a, λ⁺ cells in CDTg mice with enforced Bcl-2 expression. 3–83μδ Tg mice were bred with *bcl-2* Tg mice in the presence (CDTg) or absence (NDTg) of bone marrow antigen expression. To determine the extent of receptor editing, lymph node cells were double stained for Tg heavy chain (anti-IgD^a) and endogenous light chain (anti-λ). (A) Each row illustrates FACS analysis from independent experiments. (B) Summary data showing percentage of IgD^a-positive, Id⁻ cells in both CDTg and CDTg mice with Bcl-2 overexpression. Data are presented as mean ± SEM.

indicative of immature B cells, these cells expressed RAG-2 messenger RNA transcripts (data not shown). These cells failed to secrete antibodies in LPS cultures and in vivo, as no antibodies were detected in the serum (Fig. 4 C). In CDTg/RAG-1^{-/-} mice, no serum IgM was detected because RAG deficiency prevented development of Id⁻ B cells (Fig. 4 D). These splenic immature B cells were short lived, as ~50% of the cells had incorporated BrdU⁺ after 1 wk compared to only ~20% of B220⁺ cells from non-Tg or NDTg mice (data not shown). Thus, in contrast to its effect on peripheral tolerance, the *bcl-2* transgene appeared to have only a subtle effect on central B cell tolerance, allowing a subset of autoreactive nonfunctional, immature B cells to survive in the spleen for a short time.

Enforced Bcl-2 Expression Increases the Number of Id⁻ B Cells. Bcl-2 overexpression increased the numbers of nonautoreactive, Id⁻ B cells that developed in CD H-2^b/3–83μδ Tg mice by two- to fivefold (Fig. 7 B; Table 1). In contrast to the results with the mice bearing antigen targeted to peripheral tissues in which autoreactive B cells with enforced Bcl-2 expression were spared, the B cells appearing in the spleen and lymph nodes of CDTg/*bcl-2* mice were nonautoreactive and had undergone receptor editing (Figs. 2 and 5). One clear indication of receptor editing was the appearance of B cells bearing both the Tg heavy chain, as detected with anti-IgD^a, and endogenously encoded light chains, detected with λ chain-specific antibody (Fig. 7 A, and reference 49). The increase in the percentages of “edited” cells was the result of an increase in the total number of these cells in the lymphoid organs (Table 1). Thus, Bcl-2 overexpression apparently enhanced receptor editing or allowed survival of cells that had undergone receptor editing, or both.

Altered Ig-λ/κ Ratio Suggests that Bcl-2 Overexpression Promotes Receptor Editing. The elevated frequency of nonautoreactive B cells in the CDTg/*bcl-2* mice could theoretically have been the result of expansion or prolonged survival of B cells in the peripheral lymphoid organs. This simple explanation would predict that the relative frequencies of B cells bearing endogenous Ig-λ and Ig-κ light chains would be the same in both CDTg and CDTg/*bcl-2* mice.

To test this notion, we measured the frequencies of Id⁻ B cells that expressed κ or λ light chains. Fig. 8 A shows that most of the increase in peripheral B cells of the CDTg/*bcl-2* mice could be accounted for by an increase in λ⁺ B cells, suggesting that the *bcl-2* transgene influences B cells undergoing light chain gene rearrangement. Interestingly, this elevated λ expression was also observed in *bcl-2* Tg mice lacking Ig transgenes (Fig. 8 B), which consistently had a significantly higher percentage (~17%) of λ⁺ B cells compared to non-Tg mice (~6% λ⁺).

Discussion

This study provides insight into the mechanisms by which Bcl-2 can perturb B cell tolerance. First, we have found that natural self-proteins expressed on epithelial cells can mediate B cell tolerance by accelerating cell death through a Bcl-2-inhibitable pathway. We have also found that foreign antigens, administered acutely into the perito-

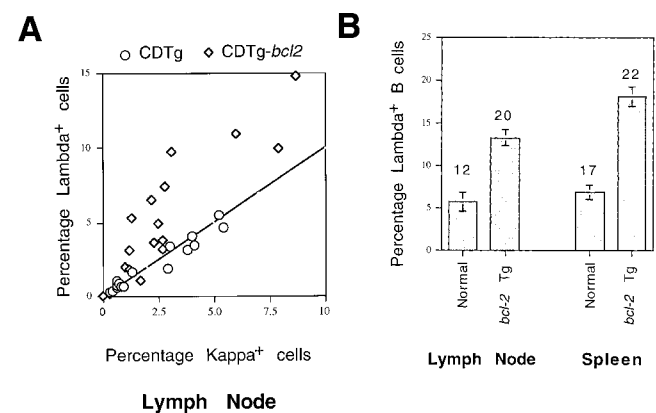


Figure 8. Bcl-2 overexpression increases λ/κ ratio of B cells in both 3–83 CDTg mice and non-Ig Tg mice. (A) Lymph node cells from CDTg and CDTg/*bcl-2* mice were double stained with anti-IgD^a and anti-λ antibodies. Percentage of lymph node cells staining positive for IgD^a and negative for λ were scored as κ positive. (B) Increased percentage of λ-expressing B cells in (non-Ig Tg) *bcl-2* Tg mice. The percentage of IgM⁺ cells that were also λ positive was determined. Data presented as mean ± SEM. Numbers above bars reflect number of mice examined.

neal cavity, can cause rapid B cell deletion that is rescued in an antigen dose-dependent manner by enforced Bcl-2 expression. Finally, we have made the novel observation that enforced Bcl-2 expression in immature B cells promotes receptor editing.

Consistent with the notion that tolerance mechanisms differ in immature and mature B cells, Bcl-2 overexpression was able to abrogate peripheral B cell tolerance, while only subtly altering central B cell tolerance. The ability of Bcl-2 to confer protection from deletion induced by peripherally expressed membrane self-antigen suggests that mature, autoreactive B cells are tolerized by apoptosis induction. These results are partly in agreement with the studies of Honjo's group (15, 46), who studied the effects of intraperitoneal injections of antiimmunoglobulins and self-erythrocytes, rather than foreign antigens, on peritoneal B cells that were largely of the B-1 lineage. The signals regulating apoptosis and immune tolerance in B-1 and B-2 subsets are likely to differ (5, 6, 18, 60). In our 3–83 Tg mice, the Id⁺ B cells, including those in the peritoneal cavity, are almost entirely B-2 B cells. Thus, our data are clear evidence for deletion of peritoneal B-2 B cells upon antigen encounter *in vivo*, which is inhibited by Bcl-2 overexpression in a dose-dependent manner. No such dose dependence was found in the study by Nisitani et al. (46), but analogous results have been obtained in *bd-2* transfected WEHI-231 cells in which anti-IgM-mediated death could be blocked at low but not high antibody dose (43). These differences may reflect a difference in the density of tolerogenic antigen expression, in BCR affinity for antigen, or in the relative tolerance susceptibility of B-1 and B-2 B cells.

Similar to the results with acute antigen administration, peripheral B cells tolerized by chronic exposure to natural autoantigen were variably rescued by enforced Bcl-2 overexpression. Furthermore, the extent of rescue afforded by the Bcl-2 transgene appeared to be inversely correlated with the degree of deletion in its absence: in MT-K^b mice, which normally show complete deletion of Id⁺ B cells from lymph nodes, enforced Bcl-2 only slightly inhibited deletion, whereas in the KerIV-K^b mice, which may express lower amounts of K^b in a relatively small subset of epithelial cells and in which autoreactive B cells are eliminated at a slower pace than in MT-K^b mice, B cell elimination was fully abrogated by Bcl-2. Because in this study the autoantigen and antibody receptor were kept constant, and only the nature and anatomical location of the antigenic stimulus were altered, the variable outcomes of Bcl-2 overexpression appear to be real effects, dependent on antigen density, concentration, *in vivo* location, or the developmental state of the B cell at time of antigen encounter. These results seem at odds with the paradigm developed from genetic studies in *Caenorhabditis elegans* that places the action of the nematode Bcl-2 homologue *ced-9* at a distal point in the death signaling cascade (61). If this were also true in B cells, one might predict that Bcl-2 should protect from apoptosis over a wide range of antigen doses. It is therefore possible that the function of Bcl-2 is involved with the downstream integration of certain BCR signals. As Bcl-2 can titrate the

death activity of heterodimerization partners, such as Bax (62–64), it is possible that the activity or quantity of these molecules can be affected by antigen signaling. It is interesting in this regard that Bcl-2 is subject to inactivation by inducible serine phosphorylation (65), a process that could conceivably be influenced by BCR signaling. It will be important to determine if higher doses of Bcl-2 can provide protection at higher antigen dose, or if cell death regulators distinct from Bcl-2 become limiting. It is interesting to note in this regard that in one study combined transgene expression of Bcl-2 and Bcl-x_L had an additive, but still incomplete, effect in protection from anti-IgD-mediated B cell elimination (66). An alternative interpretation of our data is that strong tolerogenic signals through the BCR activate both Bcl-2-inhibitable and Bcl-2-resistant death pathways, whereas weaker tolerance signals activate only Bcl-2-inhibitable death pathways.

The observed weak or negligible effect of Bcl-2 overexpression on the rescue from the bone marrow of autoreactive B cells is in agreement with the study of Hartley et al. (9), who showed that Bcl-2 could delay turnover and permit limited peripheralization of immature autoreactive B cells, but could not promote their continued development to maturity. Young et al. have shown that Bcl-2 expression permits ectopic accumulation of pre-B cells in the spleen (67). In RAG-sufficient mice, these peripheral, immature B cells are presumably capable of undergoing receptor editing even in response to antigens expressed exclusively in the periphery. This suggestion is supported by the upregulation of RAG-2 messenger RNA levels in spleens of CDTg/*bd-2*/RAG-1^{-/-} mice, which have a significant population of these immature B220⁺ cells (data not shown), and by the appearance of Id⁻, IgM⁺ B cells in spleens of 3–83/MT-K^b/*bd-2* mice (Fig. 1 C). These Id⁻, IgM⁺ cells express Tg heavy chain (data not shown) and are presumed to be the product of receptor editing. Collectively, these data show that Bcl-2 overexpression can manifest varying tolerance phenotypes depending on the maturational stage at which the B cell encounters autoantigen, promoting receptor editing in immature cells, and rescuing functional, mature cells from apoptosis.

How might Bcl-2 promote receptor editing? The ability of Bcl-2 to permit elevated steady-state numbers of immature and mature cells cannot alone explain the increased proportion of λ⁺ B cells because increased numbers of cells undergoing editing, or surviving after editing, would not be expected to alter the Ig light chain λ/κ ratio. A more likely explanation is that the extended lifespan of autoreactive bone marrow B cells that is conferred by the *bd-2* transgene may provide an extended time window per cell for light chain gene rearrangement. The current models of murine light chain rearrangement suggest that λ rearrangements generally follow κ rearrangement by ~24 h (68), and that most cells that turn over in the bone marrow do so before λ rearrangements are complete (69). This so-called crash-factor (70) suggests that mouse B cells normally have too little time to take full advantage of potential λ rearrangements that could rescue nonfunctional or autoreactive

B cells. We propose that the *bcl-2* transgene expression allows autoreactive B cells more potential rearrangement attempts, promoting the development of more nonautoreactive B cells bearing "edited" BCRs. This is consistent with data concerning transgene-driven Bcl-2 overexpression in thymocytes in which positive selection is enhanced as a result of increased endogenous TCR rearrangements, presumably due to the increased lifespan of the double-positive thymocytes expressing the *bcl-2* transgene (1). The finding that Eu-*bcl-2-22* Tg mice have consistently high percentages of λ^+ B cells suggests that receptor editing is enhanced even in the absence of antibody transgenes. Data from Rolink et al. have indicated that the E μ -*bcl-2-22* transgene prolongs in vitro survival of B cells undergoing light chain

gene rearrangement resulting in high λ/κ ratios in cultured B cells (71).

One implication of our study is that altered or defective apoptosis in B cells encountering self-antigen in the periphery could provide a pool of potentially functional autoreactive B cells. In this study we have observed that the cells rescued from elimination by enforced Bcl-2 expression could sometimes also acquire functional reactivity. Further studies are needed to fully establish their functional capacity, but these results further suggest that although multiple independent levels of regulation are available to limit autoreactivity at each step in differentiation, tolerance to certain autoantigens may be definitively abrogated by a Bcl-2-inhibitable pathway.

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