T–B Cell Interaction Inhibits Spontaneous Apoptosis of Mature Lymphocytes in Bcl-2–deficient Mice

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Summary

Bcl-2 expression is tightly regulated during lymphocyte development. Mature lymphocytes in Bcl-2–deficient mice show accelerated spontaneous apoptosis in vivo and in vitro. Stimulation of Bcl-2–deficient lymphocytes by anti-CD3 antibody inhibited the spontaneous apoptosis not only in T cells but also in B cells. The rescue of B cells was dependent on the presence of T cells, mainly through CD40L and interleukin (IL)-4. Furthermore, we generated Bcl-2–deficient mice transgenic for a T cell receptor or an immunoglobulin, both specific for chicken ovalbumin, to test for antigen-specific T–B cell interaction in the inhibition of the spontaneous apoptosis. The initial T cell activation by antigenic peptides presented by B cells suppressed apoptosis in T cells. Subsequently, T cells expressed CD40L and released ILs, leading to the protection of B cells from spontaneous apoptosis. These results suggest that the antiapoptotic signaling via CD40 or IL-4 may be largely independent of Bcl-2. Engagement of the Ig alone was not sufficient for the inhibition of B cell apoptosis. Thus, the physiological role of Bcl-2 in mature lymphocytes may be to protect cells from spontaneous apoptosis and to extend their lifespans to increase the opportunity for T cells and B cells to interact with each other and specific antigens in secondary lymphoid tissues. Bcl-2, however, appears to be dispensable for survival once mature lymphocytes are activated by antigen-specific T–B cell collaboration.

Apoptosis, a type of physiological cell death, is observed throughout phylogeny (1). In mammals, apoptosis has been extensively documented, particularly during lymphoid and neuronal development. However, little is known about the molecular mechanisms that control apoptosis (2). The product of the *bcl-2* protooncogene, a mammalian homologue of *ced-9* in *Caenorhabditis elegans*, has been considered a key negative regulator of apoptosis (3). Deregulated expression of Bcl-2 promotes cell survival by preventing apoptosis in many but not all types of cells. For example, forced expression of Bcl-2 can inhibit apoptosis in cytokine-dependent cell lines upon withdrawal of the cytokines (4, 5). However, it has been unclear that Bcl-2 is essential for the signaling of the cytokines to inhibit cell death.

Endogenous Bcl-2 is normally expressed in long-lived cells such as mature lymphocytes and neurons (6, 7). During lymphocyte development, Bcl-2 expression is tightly regulated. In T cells, $CD4^-8^-$ (double-negative) thymocytes express Bcl-2, while Bcl-2 is downregulated at the $CD4^+8^+$ (double-positive) stage when thymic positive and negative selections occur. Bcl-2 is subsequently reexpressed in $CD4^+8^-$ or $CD4^-8^+$ (single-positive) thymocytes and can be further induced upon activation by anti-CD3 mAb (8, 9). In B cells, pro-B cells express Bcl-2, whereas Bcl-2 is downregulated among pre-B cells and IgM^+D^- immature B cells. Thereafter, IgM^+D^+ mature B cells highly express Bcl-2 (10). In the peripheral lymphoid system, immunization leads to the formation of germinal centers in which Bcl-2 expression ceases in dividing B cells that undergo somatic hypermutation (6, 11). After selection for high affinity Ig, Bcl-2 is reexpressed in plasma cells (12) and memory B cells (13). Of note, germinal center B cells ($Bcl\text{-}2^-$) show more spontaneous apoptosis than circulating B cells ($Bcl\text{-}2^+$) (14). The spontaneous apoptosis of germinal center B cells can be suppressed by CD40 stimulation. Since reexpression of Bcl-2 follows the CD40 stimulation, it has been suggested that the upregulation of Bcl-2 may be essential for the prevention of apoptosis caused by CD40 stimulation. Kinetics studies, however, have suggested that the induction of Bcl-2 by CD40 stimulation might be secondary to the survival signal through CD40 (15). Thus, requirement for Bcl-2 in the survival signal of B cells mediated by CD40 has been controversial.

Abnormalities occurring in *bcl-2* gene–disrupted mice are consistent with the notion that Bcl-2 is a major regulator of the protection from apoptosis in mature lymphocytes. $Bcl\text{-}2^-$

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mature lymphocytes show significantly increased apoptosis in vivo and in vitro, while lymphocyte development in Bcl-2–deficient mice appears intact (16–18). In a previous report, we could not detect any obvious defect in Bcl-2$^-$ B cell survival in vitro, although the number of B cells clearly decreased in vivo (16, 17). By means of more sensitive assays, we could find a significantly higher degree of apoptosis in Bcl-2$^-$ mature B cells in vitro (this report). Preliminary experiments suggested that stimulation by the anti-CD3 antibody could block T cell apoptosis in Bcl-2$^-$ mice (16). Here, we show that anti-CD3 stimulation inhibited apoptosis, not only in T cells, but also in B cells. This effect was confirmed by a specific antigen instead of anti-CD3 by using Bcl-2$^-$ mice with transgenic TCR or Ig. This inhibition of apoptosis appears to be dependent on cognate T–B cell interaction and T cell activation. In the absence of Bcl-2, B cells present antigenic peptide with class II MHC to T cells, resulting in inhibition of T cell apoptosis and in activation, which protects B cells from apoptosis mainly through CD40 and lymphokines. Our data suggest that the physiological role of Bcl-2 in mature lymphocytes appears to be the protection of cells from spontaneous apoptosis and the extension of their lifespans until they are activated by specific antigens. This may be important for increasing the opportunity for T cells and B cells to interact with each other and specific antigens in secondary lymphoid tissues. Once activated, however, T and B cells become more resistant to spontaneous apoptosis in a Bcl-2–independent fashion.

Materials and Methods

Animals. All mice used in this study were maintained in the specific pathogen-free animal facility at Washington University. Generation of *bcl-2* gene–disrupted mice was described elsewhere (16, 17). Heterozygous mutant mice (*bcl-2*$^{+/-}$) were crossed with chicken OVA (cOVA)[1]-specific TCR transgenic mice (19) or Ig transgenic mice (20). By intercrossing F1 generation mice, we obtained *bcl-2*$^{-/-}$ TCR or Ig transgenic mice. Similarly, we made *bcl-2*$^{-/-}$ *lpr* (*fas*$^{-/-}$) double-mutant mice. The mice that were killed were 2–3-wk old. All experiments were performed with littermate control animals.

Reagents. All purified and fluorescent dye–conjugated antibodies in this study were purchased from PharMingen (San Diego, CA), except MR-1 (anti–mouse CD40L) (21), a gift of Dr. R. J. Noelle (Dartmouth Medical School, Hanover, NH). RU-486 was a gift of Dr. J. D. Ashwell (National Institutes of Health, Bethesda, MD). Recombinant mouse IL-1α, IL-2, IL-4, IL-7, and IFN-γ were purchased from Genzyme Corp. (Cambridge, MA). cOVA protein was purchased from Sigma Immunochemicals (St. Louis, MO). cOVA protein was multimerized by glutaraldehyde cross-linking (20). cOVA peptide (323-339) was synthesized as described (19, 22).

Cell Culture. Medium was RPMI 1640 (GIBCO BRL, Gaithersburg, MD) with 10% FCS, 14 mM Hepes (pH 7.3), 2 mM L-glutamine, 0.1 mM MEM nonessential amino acid solution, 0.1 mM sodium pyruvate, 20 U/ml penicillin G, 20 μg/ml streptomycin sulfate, and 25 μM 2-ME (GIBCO BRL). For most cultures, 3×10^5 cells were placed in the well of a 96-well v-bottom plate (Costar Corp., Cambridge, MA) at 37°C/6% CO_2 for 20 h. For the 72-h cultures, 100 U/ml rIL-4 was added in culture for the first 48 h and was removed 24 h before the end of culture. In some experiments, subpopulations of lymphocytes were purified. For the purification of B cells, adherent cells were removed by a brief spin at 1,000 rpm for 2 min and after incubation for 1 h at 37°C/6% CO_2. Nonadherent spleen cells were treated with anti–Thy-1 antibody and complement lysis. Viable cells were harvested by Lympholyte M gradient separation according to the manufacturer's protocol (Cedarlane Laboratories Ltd., Ontario, Canada). T cell lines established from the cOVA-specific TCR transgenic mice were used in some cases (20).

Flow Cytometry Analysis. Staining with propidium iodide or ethidium bromide (EtBr; Sigma) to detect apoptotic cells was performed as described (23, 24). To discriminate T and B cell death, staining by anti–Thy-1.2–FITC or anti–B220–FITC were combined with EtBr staining. All analyses were carried out using FACScan® (Becton Dickinson & Co., Mountain View, CA). Percentage of EtBr-positive cells in each population were defined as percentage of EtBr. For comparative purposes, data were normalized in some experiments as follows:

$$\%\ \textit{specific death} = \frac{(\%\ \text{EtBr in treated Bcl-2}^- \text{ cells} - \%\ \text{EtBr in treated Bcl-2}^+ \text{ cells})}{(\%\ \text{EtBr in untreated Bcl-2}^- \text{ cells} - \%\ \text{EtBr in untreated Bcl-2}^+ \text{ cells}).}$$

With any treatment used in this study, the percentage of EtBr in treated and untreated Bcl-2$^+$ cells was comparable (difference was usually within 15%).

Results

Inhibition of Apoptosis in Bcl-2$^-$ Mature Lymphocytes by Anti-CD3 Stimulation. Thymocytes (80–90% immature T cells, 10–20% mature T cells), spleen cells (~10% mature T

[1] *Abbreviations used in this paper:* cOVA, chicken OVA; CsA, cyclosporin A; EtBr, ethidium bromide.

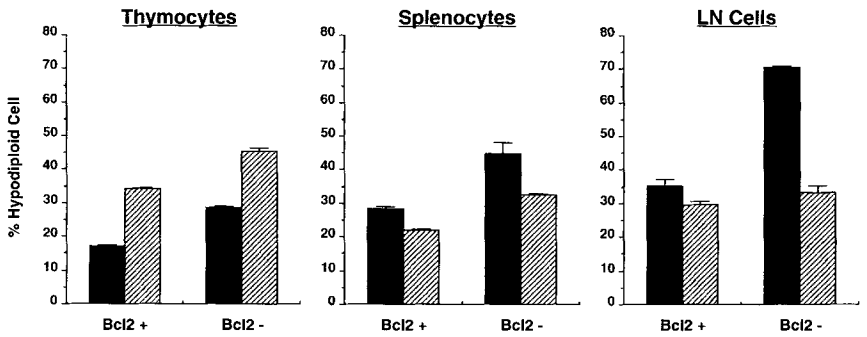

Figure 1. Spontaneous apoptosis in in vitro culture and its inhibition by anti-CD3 mAb. Cells isolated from thymus (*left*), spleen (*middle*), and lymph nodes (*right*) were cultured for 20 h in the absence (*black*) or presence (*hatched*) of anti-CD3 mAb (145-2C11; 1 μg/ml), followed by staining with hypotonic fluorescent solution (23), and the percentage of hypodiploid cells was determined. The means of triplicated culture with SDs are shown.

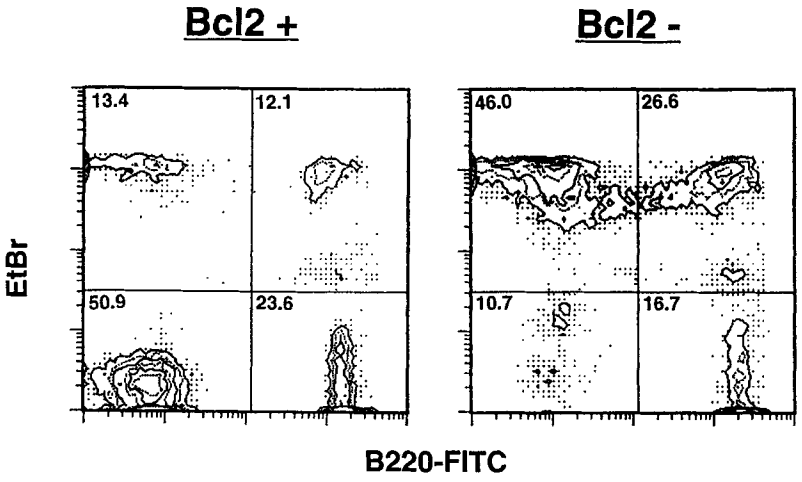

Figure 2. Both T and B lymphocytes undergo apoptosis in vitro. Lymph node cells from Bcl-2$^+$ (*left*) and Bcl-2$^-$ (*right*) mice were placed in culture for 20 h, then stained with B220-FITC and EtBr. Note the significant increase in EtBr$^+$ cells, representing apoptotic cells, in not only the B220$^-$ fraction, but also the B220$^+$ fraction of the Bcl-2$^-$ lymph node cells.

cells, 50% mature B cells, 40% non–T, non–B cells), and lymph node cells (~60% mature T cells, 35% mature B cells) were prepared from Bcl-2$^+$ and Bcl-2$^-$ animals in the same litter. Cells were placed in culture for 20 h with or without anti-CD3 mAb (1 μg/ml). After culture, cells were stained by a hypotonic fluorescent solution containing 50 μg/ml propidium iodide (23), and the percentage of hypodiploid cells, representing apoptotic cells, was assayed (Fig. 1). In thymocytes, apoptosis in Bcl-2$^-$ cells was moderately higher than in Bcl-2$^+$ cells. Since >80% of thymocytes do not (or barely) express Bcl-2 normally, the difference between Bcl-2$^+$ and Bcl-2$^-$ probably result from apoptosis of mature thymocytes, which normally express Bcl-2 (8, 9). In contrast, immature thymocytes express Bcl-x, which is a member of the *bcl-2* gene family and may prevent apoptosis of immature thymocytes (25, 26). Bcl-2$^-$ peripheral lymphocytes showed a significant increase in apoptosis (Fig. 1). Of note, the increase in apoptosis was more evident in lymph node cells than in spleen cells, suggesting that differences in cell populations may affect apoptosis (as discussed below).

The observed dramatic apoptosis does not appear to require any specific induction or stimuli. First, we failed to block this apoptosis by addition of RU-486, a potent inhibitor of glucocorticoid receptors (data not shown). Second, we generated *bcl-2*$^{-/-}$ *lpr/lpr* mice to test for the requirement of signaling via Fas for the induction of apoptosis. Peripheral lymphocytes lacking both Bcl-2 and Fas showed the same high degree of apoptosis as did those lacking Bcl-2 only, while less apoptosis was observed in Bcl-2$^+$/Fas$^+$ and Bcl-2$^+$/Fas$^-$ control cells (data not shown). For simplicity, here we designate this accelerated apoptosis observed in Bcl-2$^-$ cells as "spontaneous apoptosis." The spontaneous apoptosis in Bcl-2$^-$ mature lymphocytes could be inhibited by anti-CD3 treatment in the periphery, while anti-CD3 treatment induced increased apoptosis among thymocytes (Fig. 1).

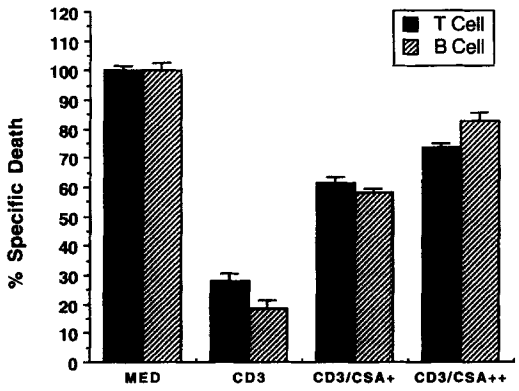

Figure 3. Anti-CD3 stimulation inhibits the spontaneous apoptosis of mature T and B cells. Lymph node cells were placed in culture and stained in the combination with B220-FITC and EtBr as shown in Fig. 2. Percentage of specific death in T cells (*black bars*) and B cells (*hatched bars*) was calculated as described in Materials and Methods. Cells were cultured with (*CD3*) or without (*MED*) 1 μg/ml anti-CD3 mAb. Cyclosporin A was added at the concentration of 0.1 μM (*CSA+*) or 1 μM (*CSA++*) with anti-CD3 mAb. CsA alone did not affect the viability of lymphocytes in the concentration used (data not shown). The means of triplicated culture with SDs are shown.

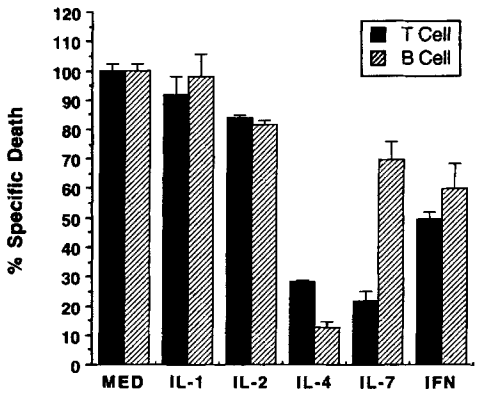

Figure 4. Effect of cytokines on the spontaneous apoptosis in Bcl-2$^-$ lymphocytes. Lymph node cells were cultured for 20 h in medium alone (*MED*) or with IL-1α, IL-2, IL-4, IL-7, and IFN-γ. All cytokines were used at 100 U/ml. Staining was carried out as in Fig. 2, and the percentage of specific death in T cells (*black bars*) and B cells (*hatched bars*) is shown. The means of triplicated culture with SDs are shown.

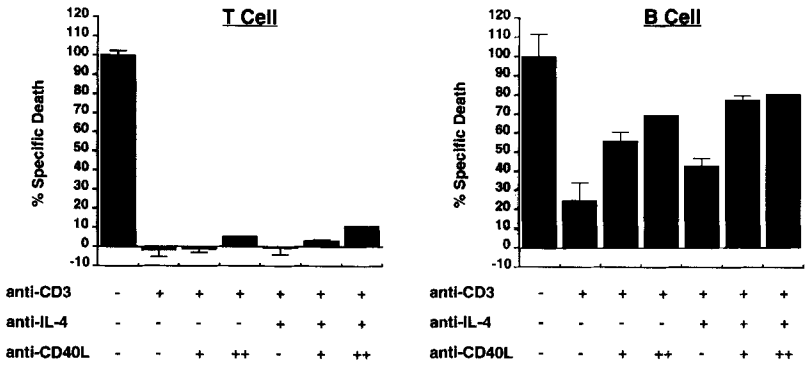

Figure 5. Synergistic inhibition of anti-CD3–induced protection of Bcl-2$^-$ lymphocytes by anti–IL-4 and anti-CD40L antibodies. Cultures were done as in Fig. 3, except for the presence of anti–IL-4 (1 μg/ml), anti-CD40L (+; 10 μg/ml, ++; 50 μg/ml), or both in some cases. Percentage of specific death in T cells (*left*) and B cells (*right*) was depicted. The means of triplicated culture with SDs are shown, except for 50 μg/ml anti-CD40L treatment (in duplicate).

Anti-CD3 Stimulation Inhibits both T and B Cell Apoptosis. To determine which lineage of cells underwent apoptosis, dual-color FACS® analyses with FITC-labeled antibodies and EtBr were performed (24). As shown in Fig. 2, both B220$^+$ and B220$^-$ lymph node cells showed significantly greater apoptosis in Bcl-2$^-$ cells than in Bcl-2$^+$ cells. Since most lymph node cells consist of T and B cells, B220$^-$ cells primarily represent T cells. Consistent with this, the result of Thy-1–FITC/EtBr staining of lymph node cells was reciprocal to that of B220-FITC/EtBr (data not shown). The observed T cell death was more pronounced than B cell death among Bcl-2$^-$ lymph node cells (Fig. 2). Since T cell content in the lymph node is much higher than that in the spleen, this may explain why spontaneous apoptosis was more evident previously in Bcl-2$^-$ lymph node cells than in spleen cells.

Unexpectedly, anti-CD3 treatment inhibited the spontaneous apoptosis, not only in Bcl-2$^-$ T cells, but also in Bcl-2$^-$ B cells (Fig. 3). This rescue effect of anti-CD3 stimulation was dependent on the coexistence of T and B cells (as shown in Fig. 7), and was reversed by the addition of cyclosporin A (CsA) in a dose-dependent manner (Fig. 3). Antibody-induced inhibition of the spontaneous apoptosis appeared to be specific to anti-CD3: anti-CD2, anti-CD4, anti-CD8, or anti–Thy-1 mAb did not affect the spontaneous apoptosis (data not shown). These results eliminate the possibilities that nonspecific cross-linking of T and B cells inhibited the spontaneous apoptosis, or that signaling via the Fc receptor is solely responsible for the inhibition of B cell death. Thus, we hypothesized that certain molecule(s) on the T cells, either membrane-bound or secreted, might be induced by anti-CD3, and mediate the inhibition of B cell death.

IL-4 Blocks Spontaneous Apoptosis in Bcl-2$^-$ T and B Cells. Some ILs or cytokines can inhibit lymphocyte death under certain conditions. These include IL-1 (27, 28), IL-2 (28–31), IL-4 (32–34), and IFN-γ (35, 36). To test whether these soluble factors may inhibit the spontaneous apoptosis due to the lack of Bcl-2, recombinant mouse ILs were added into cultures (Fig. 4). We failed to find any significant inhibitory effect on the spontaneous apoptosis with IL-1 or IL-2. In contrast, IL-4 clearly inhibited both T and B cell apoptosis. IL-7, which induces growth of mature T cells and early differentiation of B cell precursors, remarkably inhibited T cell apoptosis. Although IL-7 also affected B cell apoptosis, the degree of inhibition was less prominent. This suggests that the survival of T cells may not be sufficient for the avoidance of B cell apoptosis. In addition, IFN-γ showed moderate inhibition of T and B cell apoptosis (Fig. 4), although increased concentrations of IFN-γ could not further block the spontaneous apoptosis (data not shown). It is possible that a subpopulation of cells was rescued by IFN-γ.

CD40 and IL-4 Are Mediators of the Inhibition of B Cell Apoptosis by Anti-CD3–activated T Cells. Some reports demonstrated that the stimulation of CD40 on B cells by stimulatory antibody or its natural ligand, CD40L, rescued B cells from apoptosis (14, 15, 37–39). CD40L is specifically expressed on activated T cells, e.g., after stimulation by anti-CD3 mAb (21, 40). To address the possible requirement and role for CD40 and IL-4 in the suppression of spontaneous apoptosis, we performed an inhibition experiment by using blocking antibodies against CD40L and IL-4. Protection of Bcl-2$^-$ T cells from spontaneous apoptosis by anti-CD3 was almost unaffected by the addition of the anti-CD40L and anti–IL-4 mAbs (Fig. 5). In contrast, anti-CD40L

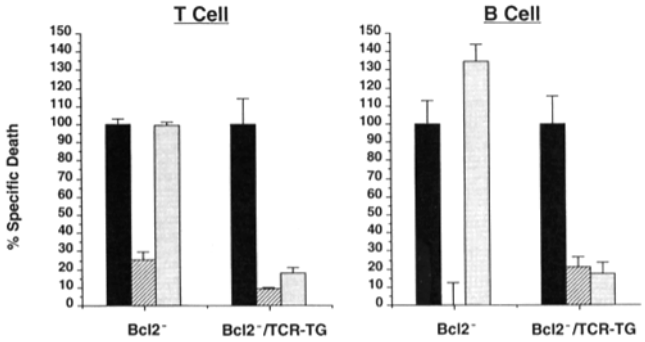

Figure 6. Inhibition of spontaneous apoptosis in Bcl-2$^-$ TCR-transgenic lymphocytes by the specific peptide antigen. Lymph node cells were isolated from Bcl-2$^-$ nontransgenic mice (*Bcl-2$^-$*) or Bcl-2$^-$ TCR transgenic mice (*Bcl-2$^-$/TCR-TG*), which are specific for cOVA (323-339) peptide. Cells were cultured for 20 h in medium alone (*black bars*), with 1 μg/ml anti-CD3 mAb (*hatched bars*), or with 5 μM cOVA (323-339) peptide (*shaded bars*). Percentage of specific death in T cells (*left*) and B cells (*right*) is shown. The means of triplicated culture with SDs are shown. Note that specific inhibition of apoptosis with cOVA peptide was seen only in Bcl-2$^-$ TCR transgenic lymphocytes.

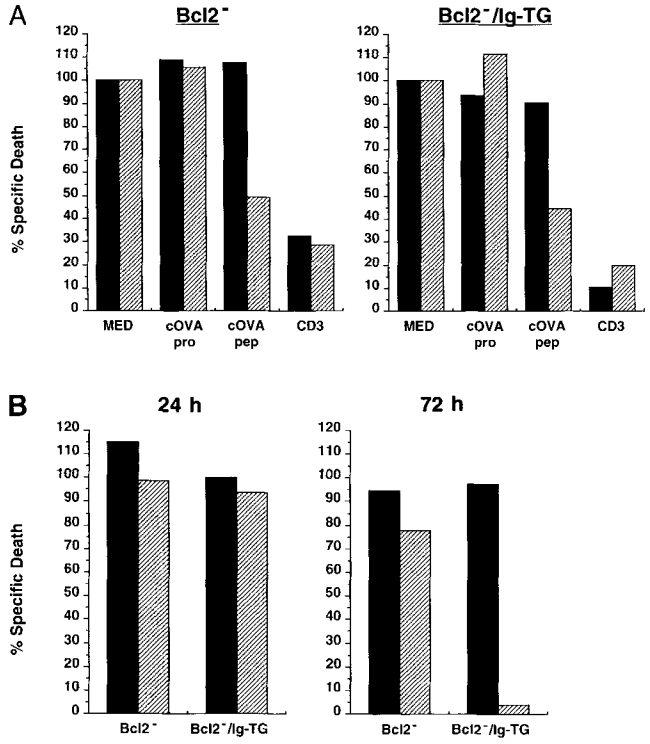

Figure 7. Inhibition of spontaneous apoptosis in Bcl-2$^-$ Ig transgenic B cells by antigen-specific T–B cell collaboration. (*A*) Lymph node cells were cultured in medium alone (*MED*), with 1 μg/ml cOVA protein (*cOVA pro*), 1 μM cOVA (323-339) peptide (*cOVA pep*), or 1 μg/ml anti-CD3 mAb (CD3), in the absence (*black bars*) or presence (*hatched bars*) of TCR transgenic T cells that are specific for cOVA (323-339) peptide. Because of the presence of T cells from Bcl-2$^-$ or Bcl-2$^-$ Ig transgenic mice (*Bcl-2$^-$/Ig-TG*) in the culture, anti-CD3 could suppress the apoptosis of Bcl-2$^-$ B cells regardless of the presence of cOVA peptide-specific transgenic T cells. In contrast, cOVA peptide could inhibit the B cell apoptosis only in the presence of cOVA-specific transgenic T cells. Addition of cOVA protein did not affect B cell apoptosis significantly in short-term (24 h) cultures. (*B*) Inhibition of B cell apoptosis by cOVA protein takes place at a later stage. Purified splenic B cells were prepared as described in Materials and Methods and were cultured for 24 h (*left*) or 72 h (*right*) in the absence (*black bars*) or presence (*hatched bars*) of cOVA-specific TCR transgenic T cells. Cross-linked cOVA protein was added at a concentration of 1 μg/ml. In the 72-h culture, 100 U/ml IL-4 was added for the first 48 h and was then withdrawn by washing. In the 24-h culture, no significant reduction of Bcl-2$^-$ B cell apoptosis was observed in either Bcl-2$^-$ nontransgenic or Ig transgenic B cells, as described above. In contrast, at 72 h, specific inhibition was detected only in the presence of cOVA-specific B cells and cOVA-specific T cells with cOVA protein.

mAb significantly blocked the rescue of B cell apoptosis by anti-CD3 (Fig. 5). Anti–IL-4 synergized with anti-CD40L to inhibit up to 80% of the rescue, but showed only a slight effect by itself. Yet, the inhibition never reached 100%, suggesting that other soluble or membrane-bound molecules may further contribute to the suppression of B cell apoptosis. We conclude that the rescue of Bcl-2$^-$ B cell apoptosis by anti-CD3 stimulation was mediated mainly through CD40 and IL-4. Bcl-2 appears to not be necessary for signaling via CD40 and IL-4 receptor on B cells because the inhibition of apoptosis was observed in Bcl-2$^-$ cells.

Antigen-specific T–B Cell Collaboration Inhibits Spontaneous Apoptosis. To confirm the effect of anti-CD3 stimulation on spontaneous apoptosis under more physiological conditions, we have established a system that recapitulates an antigen-specific collaboration between T cells and B cells. Mice transgenic for TCR and Ig specific for cOVA were bred with *bcl-2$^{+/-}$* mice, and F1 mice carrying the transgenes and mutant alleles of *bcl-2* were intercrossed to generate *bcl-2$^{-/-}$* transgenic mice. The Bcl-2$^-$ transgenic T cells and B cells showed the same dramatic apoptosis as did nontransgenic Bcl-2$^-$ cells (Figs. 6 and 7). Fig. 6 shows apoptosis in lymph node cells from Bcl-2$^-$ TCR transgenic mice. Anti-CD3 treatment suppressed the spontaneous apoptosis of T and B cells from Bcl-2$^-$ nontransgenic mice, as well as those from Bcl-2$^-$ TCR transgenic mice. In contrast, cOVA peptide inhibited the apoptosis only in the TCR transgenic Bcl-2$^-$ T and B cells, but not in nontransgenic Bcl-2$^-$ T and B cells. Since B cells in the culture were not antigen specific, specificity of Ig on the B cells appeared irrelevant to the suppression of apoptosis when cOVA peptide was added. This suggests that the antigen-presenting function of B cells is primarily important in the inhibition of Bcl-2$^-$ T and B cells. Moreover, this also eliminates the possibility that signaling via the Fc receptor on the B cells is necessary for the inhibition of apoptosis in B cell apoptosis.

Using cells from Bcl-2$^-$ Ig transgenic mice, we tested for the inhibition of B cell apoptosis with cOVA protein, cOVA peptide (323-339), or anti-CD3 in the presence and absence of cOVA-specific T cells. The concentration of cOVA protein was carefully determined to maximize Ig-dependent responses and to minimize Ig-independent pinocytosis of cOVA (20). As shown in Fig. 7 *A*, the spontaneous apoptosis of B cells from Bcl-2$^-$ nontransgenic mice and Bcl-2$^-$ Ig transgenic mice was inhibited by anti-CD3

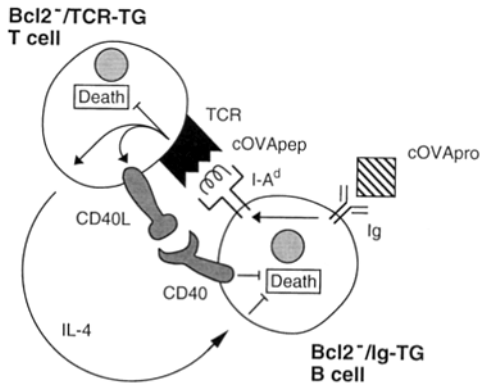

Figure 8. A model for the inhibition of spontaneous apoptosis in Bcl-2–deficient T and B cells by antigen-specific T–B cell collaboration. Bcl-2–deficient Ig transgenic B cells (*Bcl2$^-$/Ig-TG B cell*) capture the cOVA protein (*cOVApro*) through their Igs and process it to present small peptides (*cOVApep*) in the groove of variable domains of class II MHC molecules (*I-A^d*) to Bcl-2–deficient TCR transgenic T cells (*Bcl2$^-$/TCR-TG T cell*). Engagement of the TCR by the peptide–MHC complex confers a signal to suppress T cell apoptosis, induces surface expression of CD40L, and releases ILs, including IL-4. Consequently, signaling via CD40 and the IL-4 receptor blocks the apoptosis in B cells in a Bcl-2–independent manner.

regardless of the presence of cOVA-specific T cells because of the presence of nonspecific T cells in the culture. In contrast, cOVA peptide could inhibit the apoptosis only in the presence of cOVA-specific T cells (Fig. 7 *A*). However, cOVA protein, which engages the transgenic Ig, could not block the spontaneous apoptosis in the transgenic B cells in a 24-h culture (Fig. 7, *A* and *B*). Furthermore, engagement of the Ig by anti-IgM antibody, which is believed to cross-link Igs much more strongly than antigens do, did not rescue the B cells from apoptosis (data not shown). Thus, engagement of the Ig alone is unlikely to be a sufficient signal for the inhibition of B cell apoptosis.

We hypothesized that the processing of the cOVA protein and presentation of the appropriate peptide to T cells in the context of class II MHC may be necessary. To test this hypothesis, we performed long-term (72-h) culture to allow antigen-specific B cells to process the cOVA protein (Fig. 7 *B*). IL-4 was added in culture for the first 48 h to maintain the viability of Bcl-2$^-$ cells, and it was removed 24 h before the end of culture. Without cOVA protein, nontransgenic and Ig-transgenic Bcl-2$^-$ B cells underwent a comparable degree of apoptosis (data not shown). In contrast, the addition of cOVA protein suppressed the B cell apoptosis only in the Ig transgenic Bcl-2$^-$ B cells, but not in the nontransgenic Bcl-2$^-$ B cells (Fig. 7 *B*). This inhibition of apoptosis was dependent on the presence of cOVA-specific helper T cells. This result, specifically observed in Ig-transgenic B cells, minimizes the possibility that cOVA protein was nonspecifically degraded to small peptides by proteases present in the serum. Instead, it is likely that transgenic B cells effectively capture the cOVA protein through the cOVA-specific Ig, and present the cOVA peptide to the cOVA-specific T cells in the context of class II MHC molecules after proper processing of the protein. This is consistent with the kinetics of antigen-dependent B cell activation observed in vitro (20).

Hence, as depicted in Fig. 8, the inhibition of spontaneous apoptosis appears to be dependent on the cognate interaction between T and B cells. B cells capture antigens through their Igs and process them to present small peptides, complexed with class II MHC molecules, to T cells. Activated T cells then express CD40L and release ILs including IL-4. In turn, signaling via CD40 and the IL-4 receptor blocks the apoptosis in B cells.

Discussion

The Bcl-2 oncoprotein functions as an antiapoptotic molecule in many experimental systems (3). The restricted expression of Bcl-2 to long-lived cells also implies the importance of Bcl-2 in the prevention of apoptosis (6). Not only the spatial distribution of Bcl-2 expression, but also the developmental regulation of Bcl-2 expression in certain lineages of cells reinforces the notion that Bcl-2 is an antiapoptosis molecule. Bcl-2 is expressed at early and late stages of T and B lymphocyte development, but it is downregulated in cells undergoing selective processes, including CD4$^+$8$^+$ thymocytes, IgM$^+$D$^-$ immature B cells, and germinal center B cells (8–10, 41). Our data presented here demonstrate that lymphocytes lacking Bcl-2 expression die rapidly. The biphasic expression of Bcl-2 commonly observed in both T and B cell development may render T and B cells susceptible to apoptosis during specific selection stages and protect them from apoptosis at other times. Thus, Bcl-2 appears to be a main regulator of susceptibility to apoptosis in T and B lymphocyte development. There may be a physiological rescue signal from such apoptosis-sensitive Bcl-2$^-$ status. The signaling we described in this paper may be responsible for the rescue.

Recently, we and others have described the requirement for Bcl-2 in the maintenance of the lymphoid system. In Bcl-2$^-$ mice, the initial development of T and B lymphocytes was unexpectedly intact, but mature lymphocytes disappeared (16–18). The Bcl-2$^-$ mature lymphocytes underwent dramatic apoptosis in vivo and in vitro. Since the reduction of the number of lymphocytes becomes apparent with the age of Bcl-2–deficient mice, the changing level of glucocorticoid in the serum may account for the delayed onset of lymphoid abnormalities (42). However, at least in vitro, Bcl-2$^-$ lymphocytes were still susceptible to apoptosis in the presence of 1 μM RU-486, a potent inhibitor of glucocorticoid receptors that clearly inhibited dexamethasone-induced apoptosis in thymocytes in vitro (our unpublished observations). Therefore, the apoptosis observed in vitro could not be explained by the presence of steroids in the FCS. Another possibility is that Bcl-2 might normally counter the signaling via Fas, a mediator of apoptosis. The absence of Bcl-2 may render the Fas-mediated signal constitutive and result in apoptosis. Fas is not expressed on most resting B lymphocytes, however, and Bcl-2$^-$Fas$^-$ B lymphocytes still showed drastic apoptosis. Hence, the apoptosis in Bcl-2$^-$ lymphocytes appears to take place without any inducing stimuli. There is some evidence that Bcl-2 functions in an antioxidant pathway, and the antioxidant ability may be sufficient for the inhibition of apoptosis (43, 44). If this is the case, oxidative substances, which are normally antagonized by Bcl-2, may be produced in the mature lymphocytes. Lack of Bcl-2 might allow such oxidative substances to be accumulated, resulting in cell death. However, we could not observe any significant effect of either *N*-acetyl-L-cysteine or ascorbic acid, which are potent antioxidative agents and effective in inhibition of apoptosis (43, 44), on the spontaneous apoptosis in Bcl-2$^-$ lymphocytes (our unpublished observations).

Unexpectedly, the apoptosis observed in Bcl-2$^-$ mature T cells was much more evident than that in Bcl-2$^-$ thymocytes, suggesting the presence of redundant molecule(s) in thymocytes. Bcl-xL, another member of the Bcl-2 family, is expressed in CD4$^+$8$^+$ thymocytes, but not in mature T cells, and protects them from apoptosis (26). Initial reports failed to detect Bcl-xL expression in the human thymus, whereas it was demonstrated that Bcl-xS, which is a dominant negative molecule produced by alternative splic-

ing, is specifically expressed in the thymus (25). However, we detected much more Bcl-xL than Bcl-xS in the mouse thymus, and we have shown that Bcl-xL may be functionally dominant because targeted disruption of *bcl-x* gene resulted in marked increase in apoptosis of $CD4^+8^+$ thymocytes (26). The functional difference between Bcl-2 and Bcl-x has not been clear. Since mature T cells ($Bcl\text{-}2^{high}$, $Bcl\text{-}x^{low}$) have a much longer lifespan than do immature thymocytes ($Bcl\text{-}2^{low}$, $Bcl\text{-}x^{high}$) (45, 46), Bcl-2 may have a stronger effect on the longevity of lymphocytes than Bcl-x. Alternatively, Bcl-xL may have a comparable ability to inhibit apoptosis to Bcl-2, but the presence of Bcl-xS antagonizes this Bcl-xL activity in $CD4^+8^+$ thymocytes. Consequently, in Bcl-2–deficient mice, an opposite phenomenon was observed: mature T cells were more sensitive to apoptosis than to thymocytes. This could be explained by the presence of Bcl-x in thymocytes, but not in mature T cells. Moreover, Bcl-x is reexpressed upon T cell activation (25). Therefore, the inhibition of spontaneous apoptosis in $Bcl\text{-}2^-$ lymphocytes by antigenic stimulation may result from upregulation of Bcl-x. This possibility remains to be tested in Bcl-2/Bcl-x double-mutant mice.

Our data suggest that T–B cell collaboration with specific antigens inhibits the apoptosis in the absence of Bcl-2 (Fig. 8): B cells capture the antigens by their Ig and present the digested peptide in the groove of variable domains of class II MHC molecules. T cells specific for the peptide are activated by the peptide–MHC complex, and express CD40L on their surface as well as releasing ILs including IL-4. In turn, B cells escape from apoptosis with the help of T cells, mainly through CD40 and IL-4, both of which have been shown to inhibit apoptosis in some experimental systems. CsA does not affect signaling via CD40 (47); thus, the inhibition of anti-CD3–induced rescue of $Bcl\text{-}2^-$ B cells by CsA may be the result of disruption of the T cell activation process. CD40 and IL-4 show synergism in many cases, including B cell proliferation (48), secretion of IgE (49–51), and the induction of NF-AT (52). It has been shown that signals via CD40 or IL-4 induce upregulation of Bcl-2 (11, 33). Bcl-2 has therefore been suspected as a key molecule in the pathway. Bcl-2, however, is not necessary for the antiapoptotic activity of CD40 and IL-4 because signaling via CD40 and IL-4 inhibited B cell apoptosis in the absence of Bcl-2. This is consistent with the observation that Bcl-2 induction in germinal center B cells is a relatively late event compared to the suppression of apoptosis (15).

The susceptibility of mature lymphocytes to apoptosis resulting from a lack of Bcl-2 is observed, not only in experimental systems, but also in normal animals. Upon immunization, Bcl-2 is downregulated in germinal center B cells, which become susceptible to apoptosis (41). Interestingly, it has recently been suggested that T cells in the germinal centers are antigen-specific and selectively recruited into the germinal centers (53, 54). Engagement of CD40 on germinal center B cells that lack Bcl-2 expression, by CD40L on activated T cells, suppresses spontaneous apoptosis and upregulates Bcl-2 expression. In Bcl-2–deficient mice, circulating B cells showed high susceptibility to apoptosis, as seen in germinal center B cells of wild-type mice, suggesting that the lack of Bcl-2 is the cause of the accelerated spontaneous apoptosis in germinal center B cells. Moreover, spontaneous apoptosis in both germinal center B cells and circulating B cells in Bcl-2–deficient mice could be suppressed by CD40 stimulation and IL-4. Given that both B cells are similar in characteristics of apoptosis, our data suggest that germinal center B cells with high affinity to antigen after somatic hypermutation can be selectively rescued by activated T cells in an antigen-specific, Bcl-2–independent manner. Bcl-2–independent inhibition of apoptosis during cognate T–B cell collaboration may be critically important in the selection of germinal center B cells.

Deregulated expression of Bcl-2 in bcl-2–IgH fusion minigene transgenic mice resulted in an expanded germinal center cell population, prolonged the lifespan of germinal center cells, and augmented secondary immune responses (55, 56). In contrast, Bcl-2–deficient mice failed to form germinal centers efficiently after immunization, suggesting that the Bcl-2–independent inhibition is not solely sufficient for such secondary responses as germinal center formation (our unpublished observation). Collectively, the physiological function of Bcl-2 in mature lymphocytes may be to protect lymphocytes from spontaneous apoptosis, and to promote their survival until they are activated by specific antigens. Thus, the lack of Bcl-2 may decrease the opportunity for antigen-specific interaction, or it may reduce the ability to sustain the interaction. The assays in vitro using transgenic T and B cells circumvent this problem because of the extremely high efficiency of antigen-specific cognate interaction (20). Taken together, it is likely that both Bcl-2–dependent and –independent inhibition of apoptosis play important roles in lymphocyte development and differentiation.

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