Bim regulates B-cell receptor-mediated apoptosis in the presence of CD40 signaling in CD40-pre-activated splenic B cells differentiating into plasma cells

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Abstract

B-cell receptor (BCR)-mediated apoptosis is critical for B-cell development and homeostasis. CD40 signaling has been shown to protect immature or mature B cells from BCR-mediated apoptosis. In this study, to understand the fate of CD40-pre-activated splenic B cells stimulated by BCR engagement in the presence of CD40 signaling, murine splenic B cells were cultured with anti-Igκ and anti-CD40 antibodies after pre-activation with anti-CD40 antibody. We found that apoptosis was induced in the cultured B cells even in the presence of CD40 signaling during the 3–4 days cultivation. We detected up-regulation of Bim expression followed by Bax activation in this apoptotic process and cessation of the apoptosis in Bim-deficient B cells, indicating that Bim is a key regulator of the BCR-mediated apoptosis in the presence of CD40 signaling in CD40-pre-activated B cells. Importantly, this BCR-mediated apoptosis in CD40-pre-activated B cells was shown to be induced at the initiation of plasma cell differentiation at around the preplasmablast stage, and Bim-deficient B cells cultured under these conditions differentiated into plasma cells. Additionally, transforming growth factor-β was found to protect CD40-pre-activated B cells from BCR-mediated apoptosis in the presence of CD40 signaling. Our identified BCR-mediated apoptosis, which is unpreventable by CD40 signaling, suggests a potential mechanism that regulates the elimination of peripheral B cells, which should be derived from nonspecific T-dependent activation of bystander B cells and continuous stimulation with antigens including self-antigens in the presence of T cell help through CD40.

Keywords: anti-Igκ, bax, blimp-1, TGF-β1

Introduction

In the immune system, B cells play an essential role in adaptive immunity by producing antibodies, secreting cytokines and presenting antigens to T cells. B-cell receptor (BCR) signaling is essential to the development, survival, differentiation and immune responsiveness of B cells. Apoptosis mediated by BCR engagement is one of the most important physiological mechanisms controlling selection and regulation of the B-cell pool in B-cell development and maintenance of B-cell homeostasis (1). During the development of immature B cells or the activation of mature B cells, BCR engagement promotes apoptosis-inducing signals in the absence of survival signals such as CD40-mediated signals (2). BCR-mediated apoptosis has been also thought to delete anti-self specificity from the immune repertoire that leads to unresponsiveness against self (3).

CD40, a member of the tumor necrosis factor receptor superfamily, is constitutively expressed on mature B cells. By being ligated with CD40 ligand (CD40L) which is expressed mainly on activated T cells and acts as a co-stimulatory signal, CD40 transmits a co-stimulatory signal to initiate and control signaling events essential for T-dependent plasma cell differentiation (PCD) (4). CD40 rescues immature and mature B cells from BCR-induced apoptosis (2, 5–8). Combined signals delivered from surface Ig and CD40 have been shown to override the BCR-mediated apoptotic signaling and maintain germinal center B-cell survival (6, 9, 10), which are critical for the development of high-affinity antibody-producing memory B cells and long-lived plasma cells.

Bim, one of the pro-apoptotic BH3-only proteins, is expressed in many hematopoietic cells and is vital for the maintenance of homeostasis in the hematopoietic system as a barrier against autoimmune disease (11). Alternative splicing produces at least three isoforms of Bim in lymphocytes: BimEL (extra long), BimL (long) and BimS (short), all of which can induce apoptosis with different potency (12). Following an apoptotic stimulus, BimEL and BimL interact with anti-apoptotic
BCR-mediated apoptosis in CD40-activated B cells

Bcl-2 family members, including Bcl-2, Bcl-xL and Mcl-1 on mitochondria, resulting in the activation of Bax and/or Bak, pro-apoptotic Bcl-2 family members, which is needed for caspase activation and cell death. Analyses of Bim-deficient mice have shown that Bim is required for controlling the homeostasis and activation of murine B cells (13). Moreover, BCR-induced apoptosis is reduced in immature and mature B cells from Bim-deficient mice, and the deletion of autoreactive B cells is also inhibited (14). In an anti-HEL Ig/HEL double transgenic mouse model of B cell tolerance, loss of Bim inhibits the deletion of autoreactive B cells in vivo (15). Thus, Bim is a major initiator of apoptosis in autoreactive B cells induced by BCR cross-linking.

Many in vitro studies have shown that CD40 signaling blocks the apoptosis of immature and mature B cells induced by BCR engagement, and the protective role of CD40 signaling in this process was evaluated by culturing B cells upon simultaneous stimulation with anti-IgM antibody and CD40L/anti-CD40 antibody (2, 5, 16, 17). In some cases, however, naive B cells are stimulated by CD40 first in vivo (18–21). Such stimulation poses a risk of antigen-nonspecific B-cell responses, including production of autoreactive antibody (22), and so must be tightly controlled. In this study, to understand the fate of CD40-pre-activated B cells after simultaneous stimulation via BCR and CD40, we examined the viability of murine splenic B cells cultured with anti-Igκ and anti-CD40 antibodies after pre-activation with anti-CD40 antibody and found that apoptosis was induced by BCR engagement even in the presence of CD40 signaling. It may be significant to clarify whether this BCR-mediated apoptosis, which is unpreventable by CD40 signaling, in CD40-pre-activated B cells is involved in the maintenance of peripheral tolerance.

Methods

Mice and cell lines

C57BL/6 mice were obtained from CLEA Japan Inc. (Tokyo, Japan). Bim−/− mice, on the genetic background of C57BL/6, were kindly provided by Dr Andreas Strasser and Philippe Bouillet (the Walter and Eliza Hall Institute of Medical Research). All mice were maintained under specific pathogen-free conditions at the Experimental Research Center for Infectious Diseases at the Institute for Virus Research, Kyoto University and used at 8–12 weeks of age. Procedures involving animals and their care were conducted according to the guidelines for animal treatment at the Institute of Laboratory Animals (Kyoto University).

Murine mature B-cell lymphoma-derived A20 cells were obtained from the American Tissue Culture Collection. Murine immature B-cell lymphoma-derived WEHI-231–5 cells, a clone from WEHI-231, were a gift from Dr Takashi Tsubata (Tokyo Medical and Dental University). All cells were cultured in RPMI1640 medium containing 10% FCS and 50 μM β-mercaptoethanol.

Reagents and antibodies

A mAb to mouse CD40 (clone HM40-3, kindly provided by Dr Hideo Yagita, Juntendo University) was purified from the serum-free culture medium by ammonium sulfate precipitation and gel filtration.

Splenic B-cells preparation and cell culture

Splenic resting B cells were purified to 96% purity by depletion of CD43+ cells using MACS magnetic beads specific for CD43 (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer’s instructions. Purified B cells were cultured at 1 × 10^6 cells ml^-1 in RPMI1640 medium supplemented with 10% FCS, 20 mM Hepes, 50 μM β-mercaptoethanol, 50 units ml^-1 penicillin and 50 μg ml^-1 streptomycin. Anti-CD40 antibody (HM40-3) was added to the cultures at a concentration of 10 μg ml^-1. Anti-Igκ antibody (Cell Lab goat anti-mouse kappa, Beckman Coulter, Fullerton, CA, USA) was added at a concentration of 5 μg ml^-1. Although F(ab')2 fragment of anti-IgM antibody has often been used previously, we used anti-Igκ antibody, which shows much stronger apoptosis-inducing activity in splenic B cells and WEHI-231 cells.

Quantification of apoptotic cells

Cultured cells were collected by centrifugation and fixed in 70% EtOH for 1 h. After being washed with PBS, cells were treated with 100 μg ml^-1 RNaseA (Nacalai tesque, Tokyo, Japan) for 15 min at 37°C and subsequently stained with 25 μg ml^-1 propidium iodide (PI) (Nacalai tesque) in PBS. DNA content was analyzed using a FACS Canto II flow cytometer (BD Biosciences, San Jose, CA, USA) and FlowJo software (Tree Star, Ashland, OR, USA). The data from 30 000 cells were collected and analyzed. Subdiploid (sub-G1) populations were calculated as the population of apoptotic cells.

Flow cytometry and cell sorting analysis

For cell surface staining, cell suspensions were incubated with anti-mouse CD16/CD32 (2.4G2, Mouse BD Fc Block, BD Pharmingen, San Diego, CA, USA) or 10% normal rabbit serum (Sigma-Aldrich, Saint Louis, MO, USA) to prevent nonspecific binding of antibodies. Cells re-suspended in PBS were incubated with optimal amounts of labeled antibodies as described. Antibodies used for the experiments included Allophycocyanin (APC)-conjugated anti-CD138 (251–2), FITC-conjugated anti-mouse CD45R/B220 (ra3-6B2), biotin-anti-mouse IgE (BD Pharmingen) and biotin-anti-mouse IgG1, IgG2b and IgA (Southern Biotechnology, Birmingham, AL, USA). APC-streptavidin (Molecular Probes, Eugene, OR, USA) was used to detect biotin-conjugated antibodies. Labeled cells were analyzed on a FACS Canto II flow cytometer (BD Biosciences) and cell sorting was carried out on a FACS Aria cell sorter (BD Biosciences).

Western blot analysis

Total cell extract was obtained after lysis in lysis buffer [0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 150 mM NaCl and 50 mM Tris–HCl (pH8.0) containing a protease inhibitor cocktail (Nacalai Tesque)]. Cell lysate was resolved by SDS–PAGE followed by western blotting. Antibodies for caspase-3 (Cell Signaling, Danvers, MA, USA), Bim (14A8; Chemicon, Temecula, CA, USA), Bcl-2 (BD Pharmingen), Bcl-xL (Cell Signaling), Mcl-1 (sc-819; Santa Cruz Biotechnology, Santa
Detection of conformational change in Bax

Cells were suspended in 10 mM HEPES-KOH (pH 7.4) containing 150 mM NaCl, 1% 3-[(3-cholamidopropyl)-dimethylammonio] propanesulfonic acid and the protease inhibitor cocktail. Activated Bax was immunoprecipitated from cell lysate with the anti-Bax mAb (6A7; BD PharMingen), and immunoprecipitates were then subjected to western blotting with an anti-Bax polyclonal antibody (Santa Cruz).

Real-time PCR and semi-quantitative RT-PCR

For real-time PCR analysis, total RNA was prepared using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions and converted to cDNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster, CA, USA). cDNA from each sample (20–100 ng) was subjected to real-time PCR using the ABI StepOne TM Real-Time PCR system with a SuperScript TM III platinum SYBR Green One-Step quantitative reverse transcription (qRT)-PCR Kit (Invitrogen, Carlsbad, CA, USA) and primers (5’-CGACAGTCTCAGGAGGAACC-3’ as the forward and 5’-CCTCTCCATACCAACGGA-3’ as the reverse) specific for mouse bim described previously (23). Each value was normalized to the level of the GAPDH transcript and the expression level of mRNA relative to that in CD40-pre-activated cells was calculated.

For semi-qRT-PCR, cultured B cells were sorted and subjected to RT-PCR according to standard procedures. Briefly, total RNA was isolated with Sepasol RNAi Super G (Nacalai tesque) according to the manufacturer’s instructions. RT-PCR was carried out using a ReverTra Ace qPCR-RT Kit according to the manufacturer’s instructions. Semi-quantitative PCR was carried out with 2 ng of cDNA and primers described previously (24). The relative mRNA levels were measured with Hprt (Hypoxanthine guanine phosphoribosyl transferase).

Analysis of cellular morphology

Splenic B cells of Bim-/- mice, cultured with anti-Igκ and anti-CD40 antibodies for 4 days after CD40 pre-activation, were collected by cell sorting with a FACS Aria cell sorter (BD Biosciences). The sorted cells were spun onto glass slides by cytopsin (900 r.p.m., 3 min), air-dried and stained with Wright-Giemsa (Sigma-Aldrich).

RNA interference

To establish Bim knockdown (KD) A20 cells, we used a lentivirus-based vector CSII-U6-CMV-EGFP that can express short hairpin RNA (shRNA) under the control of the U6 promoter and also expresses egfp gene under the control of the CMV promoter. The mouse bim shRNA expression vector was constructed by inserting the mouse bim cDNA (NM_207680) sequence from nucleotide (nt) 247 to 265 (the sequence was included in three major isoforms of bim mRNA) (25) into CSII-U6-CMV-EGFP. As a negative control, an shRNA expression vector against lacZ was generated. Lentiviruses-containing shRNA expression vectors were prepared as described previously (26). Culture supernatants-containing viral particles were collected, concentrated 20-fold by centrifuging at 6000 r.p.m. for 12–16 h at 4°C and added to the medium for infection of cells for 24 h. Infected cells were selected as GFP-positive cells through sorting on a FACS Aria cell sorter (BD Biosciences).

Results

BCR engagement induces apoptotic cell death in CD40-pre-activated splenic B cells even in the presence of CD40 signaling

It is well known that CD40 signaling rescues immature and mature B cells from apoptosis induced by BCR engagement in vitro. In previous analyses of CD40-mediated protection against BCR engagement-induced apoptosis, cell viability was examined within 2 days in vitro culture with simultaneous stimulation of BCR and CD40 (2, 5, 16, 17). However, it has not been identified yet whether CD40 signaling can also effectively protect CD40-pre-activated B cells, which were shown to be induced by the T cell-dependent activation of bystander B cells (21, 22), from BCR-mediated apoptosis. Here, to evaluate the viability of CD40-pre-activated B cells under the stimulation of BCR and CD40, splenic resting B cells from wild-type (WT) mice were cultured with anti-Igκ and anti-CD40 antibodies for 4 days after pre-activation with anti-CD40 antibody for 24 h, and their viability was determined everyday by analysis using PI. We found that the number of subdiploid cells, a hallmark of apoptosis, was significantly increased after 2 days cultivation, and more than 80% of cells died after 4 days cultivation (Supplementary Figure 1 is available at International Immunology Online and Fig. 1A). BCR and CD40 dual signals were reported to synergistically induce marked proliferation of B cells (27–29). To exclude the possibility that the cell death is induced by excessive growth of B cells co-stimulated by BCR and CD40 signals, we cultured CD40-pre-activated B cells under conditions where the cell number was adjusted to 5 × 10⁶ cells ml⁻¹ every 24 h to keep the cell density constant. Cell death was shown to be induced regardless of the cell number adjustment (Fig. 1B). In relation to this cell death phenomenon, the activation of caspase-3 was coordinately observed after activation of caspase-3 was coordinately observed after activation of caspase-3. In contrast to CD40-pre-activated splenic B cells without pre-activation of CD40 (Supplementary Figure 2 is available at International Immunology Online), indicating that pre-activation with anti-CD40 is required for effective induction of apoptosis. All the data indicate that BCR-mediated apoptotic cell death is specifically induced in CD40-pre-activated bystander B cells even in the presence of CD40 signaling.
Data from one of three representative experiments is shown. (casp-3) by western blotting. Actin was analyzed as a loading control.

Figure 1, available at International Immunology Online. (B) To avoid overgrowth-induced cell death, primary splenic B cells were cultured by adjusting the cell number every 24 h to $5 \times 10^5$ cells mL$^{-1}$ under the same condition as in (A). The apoptotic cells were assessed and represented as in (A). The graphs in (A and B) represent the mean of the same condition as in (A). The apoptotic cells were assessed and represented as in (A). The graphs in (A and B) represent the mean of three independent samples and the error bars indicate the SD. The apoptotic cells were assessed and represented as in (A). The graphs in (A and B) represent the mean of three independent samples and the error bars indicate the SD.

To determine the mechanism underlying the induction of apoptosis in CD40-pre-activated B cells after BCR engagement in the presence of CD40 signaling, we first examined the expression level of Bim, which was reported to play a critical role in controlling homeostasis and the activation of murine B lymphocytes (13). Quantitative PCR analysis revealed the expression of bim mRNA to be increased in CD40-pre-activated B cells co-stimulated with anti-Ig$\alpha$ and anti-CD40 antibodies (Fig. 2A). Western blot analysis of Bim protein revealed that the expression of BimEL and BimL was induced within half a day and continued to increase for 3–4 days (Fig. 2B). The smallest form of Bim, BimS, was hardly observed. These results indicate that Bim expression is up-regulated in CD40-pre-activated B cells after BCR engagement in the presence of CD40 signaling and correlated well with the onset of apoptosis (Fig. 1A). In splenic B cells activated with anti-CD40 alone, however, expression of BimEL and BimL was also induced after 2–4 days cultivation, while apoptosis was not substantially induced. These results suggest that Bim regulates the induction of apoptosis while other factors regulating apoptosis is also involved in the induction of apoptosis.

Bim is reported to induce apoptosis by activating pro-apoptotic molecules of the Bcl-2 family, Bax and/or Bak, which are generally inhibited by anti-apoptotic Bcl-2 family members (e.g., Bcl-2, Bcl-xL, and Mcl-1) (33–35). We next investigated the expression of these anti- and pro-apoptotic Bcl-2 family members by western blotting. The expression level of Bcl-2 was not increased and remained low in the cells treated with anti-Ig$\alpha$ and anti-CD40 antibodies compared with those treated with anti-CD40 antibody alone. The expression level of Bcl-2 was inversely correlated with the induction level of apoptosis. In contrast, expression levels of Bcl-xL and Mcl-1 increased for 1–2 days in the culture with anti-Ig$\alpha$ and anti-CD40 antibodies and then decreased rapidly after 3 days (Fig. 2C). As the expression of Bax did not change in the presence or absence of anti-Ig$\alpha$ antibody (Fig. 2D), we speculated that Bax must be activated during the cultivation. Activation of Bax was examined by detecting a conformational change to the active form of Bax, which is provoked by apoptosis-inducing stimuli (36). An immunoprecipitation experiment using an antibody specific for active Bax revealed that Bax is activated in CD40-pre-activated B cells after 3 days cultivation with anti-Ig$\alpha$ and anti-CD40 antibodies but not in B cells after cultivation with anti-CD40 antibody alone (Fig. 2E). All the results suggest that both up-regulation of Bim expression and inhibition of Bcl-2 expression are involved in induction of the apoptosis in CD40-pre-activated splenic B cells after co-stimulation with anti-Ig$\alpha$ and anti-CD40 antibodies.

To clarify whether the deletion of Bim prevents this BCR-mediated apoptosis, we cultured splenic B cells from Bim$^{−/−}$ mice with the same stimuli for 4 days and assessed apoptosis. As expected, Bim-deficient B cells were sufficiently rescued from apoptosis induced by BCR engagement in the presence of CD40 signaling (Fig. 2F). Consequently, all the results provide the evidence that BCR-mediated apoptosis in the presence of CD40 signaling in CD40-pre-activated B cells is induced through up-regulation of Bim expression followed by Bax activation.

CD40-pre-activated Bim-deficient B cells differentiate into plasma cells after the cultivation with anti-Ig$\alpha$ and anti-CD40 antibodies

Once activated, naive B cells show potency to differentiate into plasma cells and secrete antibodies in vitro in response
to a variety of factors. While the stimulation of B cells through BCR and CD40 synergistically initiates the program of B-cell activation, it has not been clear whether this activation is sufficient to lead to PCD. In order to understand at which phase in PCD apoptosis is initiated in CD40-pre-activated B cells, we examined PCD in WT- and Bim-deficient B cells after 3 or 4 days cultivation with anti-Ig and anti-CD40 antibodies by staining for CD138 (syndecan-1), a marker expressed on mature plasma cells at high levels. While few of the cultured WT B cells showed CD138⁺ (Fig. 3, upper panel), a larger population of CD138⁺ cells was observed among Bim-deficient B cells after 3 days cultivation, and the number of CD138⁺ cells was increased on day 4 (Fig. 3, lower panel). These observations suggested that BCR-mediated apoptosis in the presence of CD40 signaling was initiated at the primary stage of PCD in CD40-pre-activated B cells cultured with anti-Ig and anti-CD40 antibodies.

Bim-dependent apoptosis was shown to be induced by BCR engagement in the presence of CD40 signaling in CD40-pre-activated WT B cells before their differentiation into mature plasma cells (Fig. 3). By contrast, Bim-deficient B cells exhibited some distinguishing phenotypes of plasma cells; however, they were not clarified to differentiate properly into functional mature plasma cells. To investigate whether Bim-deficient B cells cultured with anti-Ig and anti-CD40 antibodies express several genes associated with PCD, we sorted CD138⁺ B cells from Bim⁻/⁻ or WT mice were pre-activated with anti-CD40 antibody for 24 h, and subsequently cultured with anti-Ig antibody in the presence or absence of anti-Ig antibody for the periods indicated. The percentage of apoptotic cells was assessed as shown in Supplementary Figure 1, available at International Immunology Online. The graph represents the mean of three independent samples where the error bars indicate SD. The data are representative of three independent experiments.
BCR-mediated apoptosis in CD40-activated B cells

Fig. 3. CD40-pre-activated Bim-deficient B cells cultured with anti-Igκ and anti-CD40 antibodies differentiate into plasma cells. CD40-pre-activated Bim-deficient and WT splenic B cells were cultured with anti-CD40 antibody in the presence or absence of anti-Igκ antibody for 3 or 4 days and assessed for the expression of CD138 in a B220+ population. The numbers in the upper right quadrants indicate the percentage of B220+ CD138+ cells. Data are representative of three independent experiments.

essential regulators for PCD (37–39), were observed (Fig. 4B). In addition, initiation of Xbp-1 and Igκ expression, which was reported to be associated with progression of PCD and high immunoglobulin secretion (40), were also observed (Fig. 4B). However, these cells failed to up-regulate Irf-4 expression, which is also essential for PCD and Ig secretion (41). We also examined the morphology of the CD138+ and CD138− Bim-deficient cells by staining with Wright-Giemsa, compared with small resting B cells (Fig. 4C). CD138+ cells showed a typical morphology of mature plasma cells with a considerable nucleus-to-cytoplasm ratio, basophiliccytoplasm and eccentric nucleus, whereas CD138− cells exhibited the morphology of activated B cells with an increased cell volume, big nucleus and less abundant cytoplasm (Fig. 4C). These results support our notion that Bim-deficient B cells have the ability to differentiate into plasma cells in response to co-stimulation of BCR and CD40 but exhibit incomplete PCD.

When cultured in vitro with the proper stimulation, B cells undergo class switch recombination (CSR) to produce antibody with a specific isotype before differentiating into mature plasma cells. In the induction of CSR in vitro, CD40 signaling is essential for B cells to turn on their isotype-switching machinery, and the specificity of CSR to which isotype of Ig is determined by the subsequently provided cytokines. However, it is still unknown whether BCR engagement functions as a CSR factor. We analyzed the specificity of CSR in CD40-pre-activated WT- or Bim-deficient B cells cultured with anti-Igκ and anti-CD40 antibodies after 4 or 4.5 days. We found a distinct population of B cells expressing IgG2b among Bim-deficient B cells, compared with other Ig isoforms (IgM for the control of unswitched Igκ, IgG1 and IgA) (Fig. 4D). Moreover, CSR to IgG2b was also observed in CD40-pre-activated WT B cells cultured with anti-Igκ and anti-CD40 antibodies (data not shown). These results show that the isotype-switching machinery functions normally, and CSR to IgG2b is induced in both WT- and Bim-deficient B cells on co-stimulation with anti-Igκ and anti-CD40 antibodies. BCR-mediated apoptosis of CD40-pre-activated B cells in the presence of CD40 signaling may be initiated at the primary stage of PCD after CSR.

Blimp-1, expressed in plasma cells but absent in the early stages of B-cell ontogeny, is the master regulator of the differentiation of B cells into plasma cells (42, 43). Through western blot analysis of CD40-pre-activated Bim-deficient B cells cultured with anti-CD40 antibody, we confirmed that expression of the Blimp-1 protein increased in Bim-deficient B cells but not in WT B cells (Fig. 4E). All the results indicate

Fig. 4. PCD of CD40-pre-activated Bim-deficient B cells cultured with anti-Igκ and anti-CD40 antibodies. (A) CD40-pre-activated Bim-deficient splenic B cells were cultured with anti-Igκ and anti-CD40 antibodies for 4 days and examined the expression of B220 and CD138. The numbers in the upper right quadrants indicate the percentage of B220+ CD138+ cells. (B) Cells (R1: B220+ and CD138−; R2: B220− and CD138+) were sorted according to the gates depicted in (A) and subjected to a semi-qRT-PCR analysis for the genes indicated. WT- and Bim-deficient resting splenic B cells were used as controls. (C) Sorted populations were stained with Wright-Giemsa after being spun onto slides by cytospin for a morphological analysis, with resting Bim-deficient B cells as a control. The data in (A–C) are representative of three independent experiments. (D) Expression of membrane-bound immunoglobulins with the indicated isotype was examined by FACS analysis in CD40-pre-activated Bim-deficient B cells cultured with anti-Igκ and anti-CD40 antibodies for 4 days (IgM, IgG1 or IgG2b) or for 4.5 days (IgA). Data are representative of 2–3 independent experiments. (E) Western blot analysis. Expression of Blimp-1 protein was analyzed by western blotting for the total protein extracts prepared from CD40-pre-activated Bim-deficient or WT B cells cultured with anti-CD40 antibody in the presence or absence of anti-Igκ antibody for the periods indicated. Data are representative of three independent experiments.
that this BCR-mediated apoptosis in the presence of CD40 signaling may be induced at the initiation of PCD, which is Blimp-1-independent (24).

Murine B-cell lymphoma-derived A20 but not WEHI-231 cells undergo Bim-dependent apoptosis in response to BCR engagement after CD40 treatment

As described above, CD40-pre-activated splenic B cells undergo a Bim-regulated apoptosis during the prolonged BCR engagement in the presence of CD40 signaling. We next examined whether the system established here could reproduce apoptosis in murine B-cell lymphoma cell lines, WEHI-231-5 cells, a clone from the immature B-cell lymphoma cell line WHEI-231, which is widely used as a model for the clonal deletion of immature B lymphocytes (2, 44–46), and the mature B-cell lymphoma cell line A20, which shows some germinal center B-cell-like phenotypes, were selected. We found that apoptosis was not significantly induced in the anti-CD40-pre-treated WEHI-231-5 cells cultured with anti-Ig and anti-CD40 antibodies (Fig. 5A) but was significantly induced in the CD40-pre-treated A20 cells just like primary splenic B cells during the cultivation with anti-Ig and anti-CD40 antibodies (Fig. 5B). We therefore speculated that Bim might also regulate apoptosis in A20 cells. To address this issue, we analyzed the expression of anti- and pro-apoptotic Bcl-2 family members including Bim, Bcl-2, Bcl-xL, and Mcl-1 in A20 cells cultured with anti-Ig and anti-CD40 antibodies, anti-CD40 antibody only or anti-Ig antibody only (Fig. 5C) and found the expression levels of BimEL and BimL to be increased in all the cells. The expression level of Mcl-1, which was notably increased after 2 days stimulation with anti-CD40 alone or anti-Ig antibody alone, was not increased by co-stimulation with anti-CD40 and anti-Ig antibodies. The expression levels of Bcl-2 and Bcl-xL in cells co-stimulated with anti-CD40 and anti-Ig antibodies were not significantly lower than those in cells stimulated with anti-CD40 antibody alone or anti-Ig antibody alone. Thus, up-regulation of Bim expression and inhibition of Mcl-1 expression are correlated well with the induction of apoptosis in anti-CD40-pre-treated A20 cells after the co-stimulation with anti-CD40 and anti-Ig antibodies.

To clarify whether Bim is involved in this BCR-mediated apoptosis, KD of Bim expression was performed in A20 cells by using a lentivirus-based RNA interference system to specifically attenuate the expression of Bim (26) (Fig. 5D), and the viability of anti-CD40-pre-treated A20 cells cultured with anti-Ig and anti-CD40 antibodies was examined (Fig. 5E). Down-regulation of Bim expression by RNA interference was observed, and apoptosis, induced by the stimulation with anti-Ig and anti-CD40 antibodies, was dramatically suppressed in shBim (shRNA for Bim)-expressing A20 cells, in contrast to the control shLacZ-expressing cells. These results indicate that BCR-mediated apoptosis in the presence of CD40 signaling is selectively induced in CD40-pre-activated mature B cells in a Bim-dependent manner.

TGF-β1 rescues CD40-pre-activated B cells from BCR-mediated apoptosis in the presence of CD40 signaling

Transforming growth factor (TGF)-β signaling is known to induce IgG2b CSR in murine B cells (47). Moreover, in B cells,
TGF-β also induces the expression of Bim and mediates apoptosis which could be abrogated by CD40 signaling (48). To understand whether TGF-β signaling influences the apoptosis of CD40-pre-activated B cells induced by BCR engagement, we examined the fate of primary splenic B cells cultured with a combination of anti-CD40 antibody, anti-Igκ antibody and TGF-β1 for 4 days after pre-activation with anti-CD40 antibody. Interestingly, TGF-β1 was found to potently rescue B cells from apoptosis induced by the continuous BCR engagement (Fig. 6). Furthermore, CSR to IgG2b was successfully triggered but PCD was poorly induced in CD40-pre-activated B cells cultured with a combination of anti-CD40 antibody, anti-Igκ antibody and TGF-β1 (Supplementary Figure 3 is available at International Immunology Online). These results suggest that TGF-β signaling plays a protective role against BCR-mediated apoptosis in the presence of CD40 signaling in CD40-pre-activated B cells through inhibiting PCD, and this BCR-mediated apoptosis in the presence of CD40 signaling may be induced at the initiation of PCD after CSR.

**Discussion**

Previous studies have shown that CD40 signaling protects immature and mature B cells from BCR-mediated apoptosis in cultured cells simultaneously receiving BCR and CD40 signals (2, 5, 16). In this study, we focused on the protective effect of CD40 signaling on CD40-pre-activated splenic B cells cultured with anti-Igκ and anti-CD40 antibodies. Contrary to an above leading theory, we found that BCR engagement induced apoptosis in CD40-pre-activated splenic B cells (bystander B cells) in the presence of CD40 signaling (Fig. 1). Bim is required for not only BCR-induced apoptosis in immature and mature B cells but also negative selection of autoreactive B cells (13, 14, 49). In this study, by using splenic B cells derived from Bim-deficient mice, we have shown that Bim is involved in the apoptotic process in CD40-pre-activated splenic B cells induced by BCR engagement in the presence of CD40 signaling, and this Bim-induced apoptosis is induced by inhibition of anti-apoptotic Bcl-2 family proteins, such as Bcl-2 and Mcl-1. Furthermore, we demonstrated that this Bim-mediated apoptosis is caused by the activation of Bax (Fig. 2C) and caspase-3 (Fig. 1C). Thus, we provided a new insight that CD40-pre-activated splenic B cells (bystander B cells) undergo apoptosis through activation of Bim, which is caused by BCR engagement even in the presence of CD40 signaling.

The present study showed that BCR-mediated apoptosis in the presence of CD40 signaling occurred in CD40-pre-activated splenic B cells after 3–4 days cultivation, a similar time course to the differentiation of B cells into mature plasma cells expressing Ig with a special isotype induced in vitro cultivation with appropriate mitogens or cytokines. We then hypothesized that this apoptotic event reflects in vivo phenomena and prevention of the apoptosis is necessary for appropriate selection to differentiate into plasma cells. To verify our hypothesis, we investigated PCD of CD40-pre-activated splenic B cells derived from WT and Bim−/− mice in our culture system. As expected, most of the WT B cells had a CD138+ phenotype, whereas a large fraction of Bim-deficient B cells were CD138− (Fig. 3), suggesting that BCR-mediated apoptosis in CD40-pre-activated B cells occurs during PCD prior to progression to mature functional plasma cells. We also verified isotype-switching in the cultured B cells from WT and Bim−/− mice, showing that CD40-pre-activated B cells from both mice similarly express IgG2b isotype immunoglobulin after the cultivation with anti-Igκ and anti-CD40 antibodies (Supplementary Figure 4 is available at International Immunology Online). We could reproduce CSR, an early event in PCD, in CD40-pre-activated B cells from both WT and Bim−/− mice, after co-stimulation of BCR and CD40. In addition, TGF-β treatment did not inhibit CSR to IgG2b (Supplementary Figure 3 is available at International Immunology Online) but notably inhibited both PCD and apoptosis. All the results suggest that apoptosis is induced after CSR during PCD. After encountering antigens, B cells undergo multiple rounds of mitotic division and sequentially differentiate into preplasmablasts, then plasmablasts and finally plasma cells. In this differentiation process, the initiation of preplasmablastic differentiation is Bimp-1-independent (24). In this study, we found that Blimp-1 was induced to express in Bim-deficient B cells but not WT B cells cultured with anti-Igκ and anti-CD40 antibodies. This finding helped us to determine the specific stage where the BCR-mediated apoptosis in the presence of CD40 signaling is induced, to be around the differentiation into preplasmablasts. The completion of PCD depends on the coordinated expression of three factors, Blimp-1, IRF-4 and XBP-1 (24). Importantly, we confirmed the up-regulated expression of both Xbp-1 and Blimp-1 but not that of IRF-4. The failure of induction of IRF-4 in CD138+ B cells coincides well with the incomplete PCD in our culture system (Fig. 3 and Fig. 4B). Alternatively, the incomplete PCD, correlated with the failure of up-regulation of IRF-4 expression, may cause apoptosis by reducing anti-apoptotic signals probably retained in normal PCD.

In the development and homeostatic maintenance of B cells, apoptosis is tightly regulated by various cytokines.
Among these cytokines, TGF-β is known to promote apoptosis in resting human B cells, in murine lymphoma-derived WEHI-231 cells and human B-cell lymphoma-derived Ramos cells (50–52). In this study, CD40-pre-activated B cells cultured with anti-IgM and anti-CD40 antibodies were shown to express IgG2b (Fig. 4D). As IgG2b isotype-switching is known to be induced by TGF-β signaling (47), we speculate that TGF-β signal is positively involved in our found apoptotic process. Contrary to our expectation, TGF-β prevented CD40-pre-activated B cells from BCR-mediated apoptosis in the presence of CD40 signaling (Fig. 6). This finding would be relevant to the result of a recent report that TGF-β-producing Foxp3+ follicular regulatory T cells in germinal center contribute to the germinal center reaction and antibody production (53). Additionally, we also examined the effects of other cytokines such as IL-4 and IFN-γ on the BCR-mediated apoptosis in the presence of CD40 signaling. Although reported to act as a potent anti-apoptotic cytokine for B cells and prevent death induced by Ig cross-linking (17), IL-4 did not prevent the BCR-mediated apoptosis in our culture (data not shown). IFN-γ, which is known to promote B-cell death (54), did not significantly affect the BCR-mediated apoptosis in the presence of CD40 signaling (data not shown). Taken together, these cytokines, TGF-β, IL-4 and IFN-γ, may be able to potentially transduce pro-survival or pro-apoptotic signals depending on the status of the cell.

The apoptotic cell death detected in our culture system may provide a clue as to the potential regulatory mechanism to eliminate peripheral B cells, which may be derived by non-specific T-dependent activation of bystander B cells through CD40 during T-dependent normal immune responses (22) and/or by continuous stimulation with antigens including self-antigens in the presence of T-cell help through CD40.

**Supplementary data**

Supplementary data are available at International Immunology Online.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


