Protein phosphatase subunit G5PR is needed for inhibition of B cell receptor–induced apoptosis

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B cell receptor (BCR) cross-linking induces B cell proliferation and sustains survival through the phosphorylation–dependent signals. We report that a loss of the protein phosphatase component G5PR increased the activation-induced cell death (AICD) and thus impaired B cell survival. G5PR associates with GANP, whose expression is up-regulated in mature B cells of the peripheral lymphoid organs. To study G5PR function, the \( G5pr \) gene was conditionally targeted with the CD19–Cre combination (\( G5pr^{+/−} \) mice). The \( G5pr^{+/−} \) mice had a decreased number of splenic B cells (60% of the controls). \( G5pr^{+/−} \) B cells showed a normal proliferative response to lipopolysaccharide or anti-CD40 antibody stimulation but not to BCR cross-linking with or without IL-4 in vitro. \( G5pr^{+/−} \) B cells did not show abnormalities in the BCR-mediated activation of Erks and NF-\( \kappa \)B, cyclin D2 induction, or Akt activation. However, \( G5pr^{+/−} \) B cells were sensitive to AICD caused by BCR cross-linking. This was associated with an increased depolarization of the mitochondrial membrane and the enhanced activation of c-Jun NH\(^2\)-terminal protein kinase and Bim. These results suggest that G5PR is required for the BCR-mediated proliferation associated with the prevention of AICD in mature B cells.

Antigen (Ag) binding to the specific B cell receptor (BCR) triggers a series of multiple signal transduction pathways by the initial activation of tyrosine kinase Lyn, which thus causes the phosphorylation of the BCR complex comprised of the membrane form IgM and its accessory molecules Ig\( \alpha \) and Ig\( \beta \) (1–3). The tyrosine phosphorylation of molecules with SH2 domains, including Lyn and Syk tyrosine kinases, leads to the activation of the adaptors and the enzymes involved in the cascade reactions of various signal transduction pathways (4–6). Recent experiments have suggested that the BCR signals for cell proliferation are organized as major signal transduction pathways via Ras/MEK-dependent Erk activation and Carma-1–dependent NF-\( \kappa \)B up-regulation leading to c-myc/Stat5 activation, both of which ultimately induce cyclin D2 activation (7).

BCR cross-linking also activates signal transduction pathways involved in both the inhibition of apoptosis and the activation-induced cell death (AICD) (8–10), which are potentially associated with the selection and maintenance of Ag-reacted B cells during the maturation process in the germinal center (GC). The BCR-mediated signals induce the survival of B cells, which is a process associated with the activation of Akt (11–13). Conversely, BCR-mediated signals induce B cell apoptosis through the alterations of Bcl-2, Bcl-X\(_L\), and mitogen-activated protein kinases (MAPKs), thus resulting in the activation of caspase-3, caspase-7, and poly(ADP-ribose) polymerase (14–16). Recent studies suggest that the depolarization of the mitochondrial membrane potential (\( \Delta \Psi m \)) is an essential event in the BCR-mediated apoptosis of immature (bone marrow) and mature GC–B cells (14, 17–19). The alteration of \( \Delta \Psi m \) depends on the nascent synthesis of the proteins involved in the permeability change of the mitochondrial membrane (13, 17, 20). Mitochondrial dysfunction initiates the release of cytochrome c and Apaf-1, thus leading to the activation of caspases, which are cysteine proteases associated with apoptosis (21).

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Abbreviations used: Ab, antibody; Ag, antigen; AICD, activation-induced cell death; BCR, B cell receptor; \( \Delta \Psi m \), mitochondrial membrane potential; GC, germinal center; JNK, c-Jun NH\(^2\)-terminal protein kinase; MAPK, mitogen-activated protein kinase; PP, protein phosphatase; SRBC, sheep RBC; TUNEL, Tdt-mediated dUTP-biotin nick-end labeling.
To investigate the BCR-mediated signals of mature B cells, we studied the function of the protein phosphatase (PP) component G5PR. The G5pr gene was identified by the yeast two-hybrid screening method (22) as an associated component of GANP that is up-regulated in GC–B cells (23–25). G5PR binds not only to GANP but also to the catalytic subunit of PP2Ac and PP5. G5PR is homologous to the regulatory subunit of PP2A, carrying the region of the EF hand that serves as a Ca\(^{2+}\)-binding motif. To study the function of G5PR in the regulation of B cell proliferation and differentiation, we prepared gene knockout (G5pr\(^{f/f}\)) mice by conditional targeting in B cells with a combination of the floxed-G5pr gene and CD19-Cre—knock-in mice. G5pr\(^{f/f}\) mice showed a deficit in the BCR-mediated signal transduction associated with B cell survival, which may imply a unique regulatory mechanism of BCR-mediated signal transduction in mature B cells of the peripheral lymphoid organs.

**RESULTS**

**CD19-Cre-mediated B cell specific inactivation of G5pr**

The loxP sequences were introduced upstream of exon I and downstream of exon II by a homologous DNA recombination (Fig. 1 A). Southern blot analysis showed the loxP sites to be targeted at the G5pr locus of the floxed (8.9 kb) band in comparison with that of wild type (1.8 kb; Fig. 1 B). The mice carrying a floxed G5pr allele (G5pr\(^{f/f}\)) were crossed with CD19-Cre mice to obtain CD19-Cre/G5pr\(^{f/f}\) (G5pr\(^{-/-}\)) and littermate CD19-Cre/G5pr\(^{f/wt}\) (G5pr\(^{+/-}\)) mice in order to compare B cell activation. The gross appearance and growth of G5pr\(^{-/-}\) mice were apparently normal. The genomic DNAs, obtained from splenic B cells by MACS sorting, showed the alleles of wild-type (11.8 kb), targeted (F; 8.9 kb), and deleted (Δ; 4.5 kb) bands with probe A in a Southern blot analysis with HindIII digestion (Fig. 1, A and C). G5pr\(^{-/-}\) B cells did not show any G5pr transcripts (Fig. 1 D), thus indicating that the mutant mice lacked the G5pr gene.

**Figure 1.** Generation of a CD19-Cre-mediated G5PR-deficient mouse. (A) Targeting of the G5pr gene. (top model) Genomic organization of the G5pr gene locus (WT). H, HindIII; S, SalI. The bar indicates the probe used for Southern blots. The loxP sites are indicated by triangles. (bottom models) The targeting vector, the targeted allele (F), and the deleted allele (Δ). The G5pr-deleted allele was generated by the Cre-mediated deletion of loxP-flanked exons I and II. Correct targeting was verified by a Southern blot analysis of HindIII-digested DNAs with the indicated probe. Lengths of the respective HindIII fragments are shown. DT-A represents a selection cassette for dipheria toxin-A. (B) Germ line transmission of a G5pr-targeted allele. Tail DNAs from wild-type and G5pr\(^{f/wt}\) littermates were digested with HindIII, followed by a Southern blot analysis. (D) CD19-Cre-mediated disruption of the G5pr gene. RNA was extracted from splenic B cells isolated from G5pr\(^{+/-}\) and G5pr\(^{-/-}\) littermates and subjected to RT-PCR using exons spanning specific primers designed from exons II and IV of the G5pr gene. (E) Spleens from G5pr\(^{+/-}\) and G5pr\(^{-/-}\) mice. (F) Columns show mean cell numbers of total nucleated cells, B cells, and T cells in the spleens. The number of cells was calculated based on the proportion of B cells and T cells determined as B220\(^+\) and CD3\(^+\), respectively. Error bars represent SEM. A significant difference in cell numbers between G5pr\(^{+/-}\) and G5pr\(^{-/-}\) mice was indicated (P < 0.01).
transcription in the splenic B cells. The spleen was small in G5pr−/− mice and the B220+ B cell population decreased to approximately half that of the littermate G5pr+/− mice (P < 0.01; Fig. 1, E and F).

Reduction in the number of mature B cells in G5pr−/− mice CD19 is expressed on late pro–B cells and BCR+ B cells. We evaluated the B cell development in lymphoid organs of G5pr−/− mice. In the bone marrow, the proportion of pro–B cells (B220low CD43+) was relatively high but that of pre–B cells and immature B cells (B220low CD43−) decreased from 31 to 22.7% of the control B cells. The mature B cell (B220high CD43−) population was as low as 2.72% in G5pr−/− mice (~50% of the control littermate G5pr+/− mice). The recirculating B cells with the IgM+ IgD+ phenotype also decreased (from 13.4 to 7.04%; Fig. 2 A). In the spleen, the proportion of B cells dropped considerably (from 48.3 to 35.3%), and the T cell population increased (from 39 to 49.9%; Fig. 2 A). The maturation of B cells was affected in G5pr−/− mice. The mature B cell (IgMlow IgDhigh) population decreased more remarkably (from 31.3 to 20.8%) than the transitional-2 (IgMhigh IgDhigh) B cell population (from 9.88 to 7.08%). Transitional-1 (IgMhigh IgDlow) B cells, as a newly migrated population from the bone marrow, were less affected or even tended to increase (from 3.5 to 5.38%) in G5pr−/− mice, thus suggesting different sensitivities to the defect of G5PR in these B cell populations. In addition, G5pr−/− mice showed a decrease (from 40 to 27.1%) in the number of follicular mature B cells (B220+ CD21int CD23high). The B cells decreased in the mature B cell population of the axillary lymph node (from 16.4 to 9.33%) and the peripheral blood (from 51.3 to 18.1%) of G5pr−/− mice (Fig. 2 A). These results suggested that the G5PR deficiency affected the maturation or life span of B cells in the periphery. Many gene knockout mice lacking B cell signal–related molecules displayed an abnormal CD5+ B-1a cell development in the peritoneal cavity (26–29). Likewise, G5pr−/− mice showed a decrease of B-1a (IgM+ CD5+) cells (from 17.2 to 10.5%), thus suggesting that G5PR is also necessary for the differentiation of B-1a cells or their maintenance.

Antibody (Ab) responses of G5pr−/− mice G5pr−/− mice did not show any abnormalities in serum Igs of various classes in comparison with the control littermates under nonimmunized conditions (Fig. 2 B), nor did they display an impairment in Ag-specific IgM responses against spleen contains (top) B cells (B220+), and T cells (CD3+); (middle) transitional type-1 B cells (IgMhigh IgDlow), transitional type-2 B cells (IgMhigh IgDhigh), and mature B cells (IgMlow IgDhigh); and (bottom) marginal zone B cells (CD21high CD23int) and follicular B cells (CD21+ CD23+). The lymph node contains (top) B cells (B220+), and T cells (CD3+) and (bottom) transitional type-2 B cells (IgMhigh IgDlow) and mature B cells (IgMlow IgDhigh). The peritoneal cavity contains B-1a cells (CD5+ IgM−) and the peripheral blood contains B cells (B220+).
both TNP-Ficoll and TNP-keyhole limpet hemocyanin (Fig. 2, C and D). However, the IgG responses against both Ags were slightly decreased in the $G5pr^{-/-}$ mice at 14 d after immunization. The GC formation examined after immunization with sheep RBCs (SRBCs) showed that $G5pr^{-/-}$ mice displayed similar numbers of lymphoid follicles with

(B) Ig levels of various classes were measured by ELISA with the serum of nonimmunized mice. Specific Ab responses were measured after immunization with either TI-II Ag (C) or TD Ag (D). TNP-specific Abs were measured, and the results for TI-II Ag (IgM and IgG3) and TD Ag (IgM and IgG1) are shown. Horizontal lines represent mean Ab titers. (E) Immunization with TD Ag and SRBCs created GCs in the spleen comparable with those in control mice. The spleen sections were studied with G5PR2/2 mice (b, d, and f) in comparison with those of control mice (a, c, and e). Higher magnification images, outlined by boxes in panels a and b, show control mice (c and e) and mutant mice (d and f). PNA$^+$ GCs (brown), IgD$^+$ B cells (blue), B220$^+$ B cells (brown), and CD4$^+$ T cells (blue) are shown. Arrows indicate typical GCs, and asterisks indicate extrafollicular regions. TZ, T cell zone; FO, follicular region.
IgD⁺ B cells at 10 d, whereas the size of each mature PNA⁺ GC area was small (Fig. 2 E, b and d). The number of IgD⁺ B cells decreased in the extrafollicular regions of the spleens in these mice (Fig. 2 E, as indicated by asterisks in b and d in comparison with the control mice in a and c). In contrast, the T cell zones appeared to be normal (Fig. 2 E, e and f).

**Impaired cell proliferation of G5pr⁻/⁻ B cells induced by BCR cross-linking**

The B cell proliferation activity was measured in vitro after stimulation with anti-IgM Ab or LPS. BCR cross-linking could not induce the proliferation of G5pr⁻/⁻ B cells over a wide range of Ab concentrations in comparison with the control G5pr⁺/⁺ B cells, and the addition of 100 U/ml IL-4 did not cause the proliferation of G5pr⁻/⁻ B cells to recover at 48 h after stimulation (Fig. 3 A, left); however, LPS stimulation induced the proliferation of G5pr⁻/⁻ B cells at a comparable level to that of control B cells (Fig. 3 A, right). The lower response of the G5pr⁻/⁻ B cells was not caused by the different kinetics of cell proliferation (Fig. 3 B). The peak thymidine incorporation was observed at 48 h after BCR cross-linking in both G5pr⁻/⁻ and control B cells. No marked difference was found between the control B cells of G5pr⁺/⁺ and wild-type mice. These results suggested that a loss of G5PR impaired BCR-induced cell proliferation or survival, which was not completely compensated by IL-4 costimulation.

**No apparent alteration of BCR-mediated signal transduction pathways of B cell proliferation**

We examined the alteration of the BCR-mediated signal transduction for B cell proliferation in vitro. BCR cross-linking induced an up-regulation of the activation markers CD25 and CD69 on G5pr⁻/⁻ B cells quite similarly to the levels of the control B cells (Fig. 4 A). The BCR signaling cascade leading to cell activation is initiated by the activation of tyrosine kinase and the tyrosine phosphorylation of various secondary signal transduction molecules (1, 4, 5, 30–32). G5pr⁻/⁻ B cells caused a normal tyrosine phosphorylation reaction after BCR cross-linking in vitro and there was no apparent difference in the entire tyrosine phosphorylation profile between G5pr⁺/⁺ and G5pr⁻/⁻ B cells that would account for the impairment in BCR-induced cell proliferation (Fig. 4 B). The initial tyrosine phosphorylation induces PLC-γ2 activation, which thus induces the downstream signal transduction pathways involved in protein kinase C activation, subsequently leading to generation of Ca²⁺ flux. G5pr⁻/⁻ B cells showed a normal level of Ca²⁺ flux both in amplitude and duration (Fig. 4 C). These results demonstrated that the proximal events of BCR-mediated signaling appeared normal in G5pr⁻/⁻ mice.

The activation of the MAPK pathway (Erk1 and Erk2) is essential for the BCR-mediated signal transduction leading to B cell proliferation (33). Both Erk1 (p44) and Erk2 (p42) stimulation. [³H]thymidine was added for the final 6 h. The results are representatives of three experiments performed in triplicate. The incorporation of [³H]thymidine is shown as counts per min (cpm) with the SD of the triplicate culture samples per each.

**Figure 3.** B cell proliferation in response to the mitogenic stimuli in vitro. (A) Splenic B cells were cultured for 48 h in the presence of medium alone, anti-IgM F(ab')₂, Ab, or 100 U/ml IL-4 (left) or LPS (right). B) Kinetics of the responses of WT, G5pr⁺/⁺, and G5pr⁻/⁻ B cells to anti-IgM Ab indicated a similar response curve with the peak at 48 h after stimulation.
were promptly phosphorylated at 1 min after the BCR cross-linking of G5pr+/− B cells (Fig. 4 D). There was also no difference in the Erk protein expression. BCR cross-linking induces the activation of PI3K, which regulates downstream signal transductions through the interaction with Gab-1 and/or BCAP (34, 35). PI3K is upstream of Akt and NF-κB, and these pathways are implicated in the inhibition of BCR-mediated apoptotic signals (36–38). We investigated whether the impairment of cell proliferation by BCR cross-linking was attributed to the disruption of these two pathways in G5pr−/− B cells; however, the B cells showed normal responses of Akt phosphorylation to BCR cross-linking (Fig. 4 E). Similarly, the NF-κB pathway assessed by IκBα degradation showed no change in comparison with G5pr+/− B cells. The major downstream target of these signals is cyclin D2, which promotes the cell cycle progression of B cells (7). BCR cross-linking induced cyclin D2 and phosphorylation of Rb in G5pr−/− B cells at levels comparable with control B cells (Fig. 4 F), thus indicating that G5pr−/− B cells did not show obvious defects in the above-mentioned BCR-mediated signal transduction pathways involved in B cell proliferation.

G5PR is associated with PP2Ac as one of the possible components for phosphatase regulation or for target specificities (22). The phosphatase activity was measured with the synthetic substrate using the PP2Ac immunoprecipitate from B cells after BCR cross-linking for 1 h. The PP2A activities were slightly lower in G5pr−/− B cells than in the control B cells (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20050637/DC1). Usually, it is rare to detect a clear difference in the PP2A activity in lymphocytes because of a low expression of phosphatases (39) and the presence of multiple regulatory subunits, which often disturb the identification of selective regulatory activity for various target molecules.

Enhanced BCR-mediated apoptosis in G5pr−/− B cells
We evaluated the mortality of B cells in vitro to examine whether the poor proliferation of G5pr−/− B cells after BCR

Figure 4. BCR-mediated signaling in G5PR-deficient B cells. (A) The activation of G5pr+/− and G5pr−/− B cells in response to 10 μg/ml anti-IgM F(ab′)2 Ab as assessed by the up-regulation of CD25 and CD69. Histograms represent CD25 or CD69 expression with (black plot) and without (white plot) stimulation. (B) A Western blot analysis for tyrosine-phosphorylated proteins. The isolated splenic B cells from G5pr+/− and G5pr−/− littermates were stimulated with anti-IgM F(ab′)2 Ab. Cell lysates were subjected to Western blotting with antiphosphotyrosine Ab (4G10) and reprobed with anti–β-actin Ab for controls. (C) A calcium Flux analysis in response to BCR cross-linking. A flow cytometry analysis depicting calcium flux (y axis) as a function of time (x axis) in the cells stimulated with 10 μg/ml anti-IgM F(ab′)2 Ab. The arrow indicates the time point of stimulation. (D) BCR-induced Erk phosphorylation was monitored by a Western blot analysis using phosphorlated Erk-specific Ab. The entire Erk expression was also monitored as an internal control. Erk1 (p44) and Erk2 (p42) were indicated. (E) Time course of Akt phosphorylation and the degradation of IκBα. (F) BCR cross-linking–dependent expression of cyclin D2 and phosphorylation of Rb. The phosphorylated Rb was detected as shifted bands because of a slower mobility than nonphosphorylated Rb. 10^6 B cells were stimulated with 10 μg/ml anti-IgM F(ab′)2 Ab. Each membrane was reprobed with anti–β-actin Ab as control.
cross-linking was caused by the alteration of B cell survival. G5pr<sup>-/-</sup> B cells showed a reduction in the number of live cells at 6 h after BCR cross-linking in comparison with the control G5pr<sup>+/+</sup> B cells. The reduction became more obvious at 24 h after stimulation (from 68.2 to 35.1%; Fig. 5 A). The fraction containing both annexin V single-positive cells (early phase) and annexin V/7-amino-actinomycin D double-positive cells (late phase) increased from 15.3 to 33.8% during the stimulation of G5pr<sup>-/-</sup> B cells for 24 h (Fig. 5 B). Notably, G5pr<sup>-/-</sup> B cells did not show any difference in the spontaneous cell death (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20050637/DC1). On the other hand, LPS prevented the cell death of G5pr<sup>-/-</sup> B cells in a manner similar to that of control G5pr<sup>+/+</sup> B cells (Fig. 5, A and B). We thus conclude that the hypoproliferation of G5pr<sup>-/-</sup> B cells resulted from an increased susceptibility to BCR-mediated AICD.

**Increased mitochondrial membrane depolarization induced by BCR cross-linking in G5pr<sup>-/-</sup> B cells**

We examined the DNA fragmentation of G5pr<sup>-/-</sup> B cells by propidium iodide staining. The number of dead cells increased ~6 times in G5pr<sup>-/-</sup> B cells in comparison with the control B cells at 24 h after BCR cross-linking, as measured by the subdiploid fraction (from 1.12 to 6.61%; Fig. 6 A). Next, we investigated the mitochondrial membrane depolarization of B cells after stimulation as an early event of apoptosis, which subsequently increases mitochondrial membrane permeability and facilitates the release of the proapoptotic factor cytochrome c from the mitochondria into the cytosol and is followed by caspase-3 activation (40). A fluorescent dye (DiOC6) that binds to the mitochondrial membrane in living cells was used for the initial change. BCR cross-linking induced membrane depolarization (43%), which was higher than that observed after LPS stimulation (15.5%), in the control G5pr<sup>+/+</sup> B cells. In comparison with the controls, G5pr<sup>-/-</sup> B cells showed much more change (64%) by BCR cross-linking, thus indicating that a lack of G5PR augmented the susceptibility to the BCR-mediated signal transduction, thereby leading to a reduction in ΔΨm (Fig. 6 B).

The single-strand and double-strand DNA breaks were measured using a Tdt-mediated dUTP-biotin nick-end labeling (TUNEL) assay in vitro. TUNEL-positive cells were induced by BCR cross-linking at higher levels in G5pr<sup>-/-</sup> B cells in comparison with control B cells (32.2 vs. 21.1% at 24 h after stimulation), but were not by LPS stimulation (15.9 vs. 13.8%; Fig. 6 C).
Caspase-3 is involved in the BCR-mediated cell death pathways in the immature B cell line WEHI-231 (20, 41–43). We attempted to measure the change in the caspase-3–dependent cell death pathway by detecting the active caspase-3. Anti-Fas Ab induced caspase-3–positive cells (49.1%) as a positive control (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20050637/DC1). However, we did not observe any increase of the active caspase-3 in G5pr/H11002/H11002 B cells by BCR cross-linking for 6 h (3.51%) and 24 h (2.01%; Fig. S3). This was also the case in the control B cells. Only 3.56% of the control B cells showed active caspase-3, which was estimated to be from 5 to 9% of mitochondrial membrane–depolarized cells (43%). These results suggest that BCR-mediated AICD is not dependent on the caspase-3–mediated pathway in mature B cells.

**Prolonged Bim activity and enhanced c-Jun NH₂-terminal protein kinase (JNK) activity in G5pr⁻/⁻ B cells after BCR cross-linking**

Mitochondrial integrity has been proposed to be regulated by the pro- and antiapoptotic members of the Bcl-2 family. The Bcl-2 proteins maintain a balance between the specific homo- and heterodimers for cell survival and the induction of apoptosis on stimulation. BCR cross-linking induces antiapoptotic Bcl-XL and Bcl-2, which play a prominent role in the protection of mature B cells from AICD (44, 45). The transgenic mice of Bcl-XL and Bcl-2 displayed an increased number of B cells in vivo (46–48). We investigated whether the decreased survival of G5pr⁻/⁻ B cells was caused by the down-regulation of Bcl-2 and Bcl-XL. BCR cross-linking induced the up-regulation of Bcl-XL in both the control and G5pr⁻/⁻ B cells, which demonstrated comparable levels with the shift in the size at 24 h after stimulation (Fig. 7 A). A down-regulation of the Bcl-2 expression after BCR cross-linking was nearly equivalent between the control and G5pr⁻/⁻ B cells (Fig. 7 A) (49).

BH3-only proteins have been reported to induce Bax- and Bak-dependent apoptosis under environmental stress (50). Among the BH3-only proteins, Bim has been shown to play a crucial role in the cell death signal induced by BCR cross-linking. Bim⁻/⁻ mice showed a 2–3-fold increase in B cell number (51). The Bim⁻/⁻ B cells were refractory to the
apoptosis induced by BCR cross-linking in vitro (49).

Therefore, we examined whether the expression and/or phosphorylation of Bim on BCR cross-linking was affected in the absence of G5PR by Western blotting (Fig. 7 B). The Bim phosphorylation was detected as p-Bim-EL with nonphosphorylated Bim-EL, Bim-L, and Bim-S subunits. BCR cross-linking induced the expression and phosphorylation of Bim-EL at 6 h, but gradually suppressed their expressions after 12 h in control B cells. G5pr/H11002/H11002 B cells showed a prolonged increase of the expression and phosphorylation of Bim-EL. The increase was marked at 24 h and showed a 5.4-fold increase over the nonstimulated B cells, whereas the control G5pr/H11001/H11002 B cells showed a 2.2-fold increase.

Bim-EL mRNA was induced by BCR cross-linking, suggesting that G5PR regulates the Bim expression also at the transcriptional level (Fig. 7 C). These results indicated that a lack of G5PR caused an abnormal continuation of the expression and phosphorylation of Bim-EL during B cell stimulation by BCR cross-linking, which potentially could lead to a prolonged mitochondrial membrane destabilization.

JNK and p38, which belong to the MAPK family, have been shown to be involved in the BCR-mediated AICD pathway (52). JNK promotes cell death through the phosphorylation and activation of several proapoptotic Bcl-2 family members. In UV-induced apoptosis, JNK phosphorylates the BH-3–only protein Bim, which thus results in the Bax/Bak–dependent activation of the mitochondrial apoptosis pathway (50). We examined the induction of JNK between the control and G5pr−/− B cells. BCR cross-linking induced the expression and phosphorylation of JNK at higher levels in G5pr−/− B cells, especially at 30 min, whereas the induction of phosphorylation of p38 occurred normally (Fig. 7 D). We evaluated the Bim phosphorylation in the presence of the JNK–specific inhibitor SP600125. The JNK inhibitor at 10 μM suppresses the anti-CD40–induced JNK activation, but this treatment caused severe B cell apoptosis after 24 h (unpublished data). We thus chose a dosage of mild effect (2 μM), which did not cause such severe cell death but still suppressed the phosphorylation of JNK at 10 μM after CD40 cross-linking (Fig. 7 D). This treatment did not cause any apparent different in the Bim phosphorylation in G5pr−/− B cells (Fig. 7 E). Whether or not JNK is the upstream regulator of Bim phosphorylation still remains to be elucidated.

DISCUSSION

Mature B cells in peripheral lymphoid organs are triggered by the signal through BCR, but the complete activation depends on costimulatory signals provided in secondary lymphoid organs. BCR–mediated signal transduction uses non-specific and common pathways that are involved in the proliferation of many kinds of cells. Antigenic stimulation presumably activates B cells through common pathways, but
precisely how the BCR-unique pathway modifies or affects their response remains to be elucidated. Our results suggest that the BCR-mediated signal transduction regulates B cell survival through the phosphatase-binding protein G5PR. We demonstrated that the target of G5PR was the BCR-mediated transduction pathway involved in cell survival at the regulation of JNK and Bim activation.

G5PR regulates the cell survival selectively in the BCR-mediated induction of AICD. The loss of G5PR did not affect the proliferation of B cells stimulated by either LPS or by anti-CD40 stimulation in vitro. We did not find any abnormalities in the major B cell proliferation pathways leading to the activation of cyclin D2, which also supported the hypothesis that G5pr−/− B cells could proliferate normally in response to other stimulations. G5pr−/− mice revealed a similar phenotype to Bam32−/− and cyclin D2−/− mice (27, 28). These mutant mice showed a marked hyporesponsiveness of mature B cells to BCR-mediated proliferation but not to LPS or anti-CD40 stimulation. The number of CD5+ B-1a cells in the peritoneal cavity decreased dramatically in these mice. However, the target of G5PR is obviously different from those described in of previous reports (27, 28). The most sensitive pathway related to the loss of G5PR seemed to be the JNK pathway, whose activity is strictly dependent on the phosphorylation state as the serine/threonine-kinase cascade (53, 54). The selective regulation of BCR-mediated JNK activation might depend on the existence of G5PR in B cells. This might explain the fine tuning of the amplitude of BCR-mediated signal transduction, thus leading to the proliferation and maintenance of B cells in the peripheral lymphoid organs.

Bcl-2 and Bcl-XL, antiapoptotic members of Bcl-2 family proteins, were described to be both phosphorylated and activated by JNKs in vivo (55, 56). The hyperactivation of JNK caused an alteration of Bcl-2 and Bcl-XL, thus resulting in a dysfunction of the mitochondria, including an altered respiration, transmembrane potential, and calcium buffering capacity; however, the expression and phosphorylation of Bcl-2 and Bcl-XL seemed to be largely normal in G5pr−/− B cells.

The BH3-only protein Bim and its relatives appear to be potential targets of JNK in the regulation of mitochondrial apoptosis (57). Bim, which is expressed at high levels in human leukocytes and spleens (58, 59), associates with the dynein light chain of the microtubule-associated dynein motor complex and are released from the complex by specific apoptotic stimuli (60). In our study, JNK inhibitor could not provide the clear evidence that the activation of JNK is the upstream signal for Bim phosphorylation in BCR-mediated AICD.

Bim has been proposed to bind to Bak and Bax, thus causing an allosteric conformational activation to promote mitochondrial membrane depolarization (61). Recently, Chen et al. demonstrated that the BH3 domain derived from Bim physically interacted strongly with Bcl-2, Bcl-XL, Bcl-w, Mcl-1, and A1 in vitro (62). These results suggested that Bim promotes B cell apoptosis by the inactivation of the prosurvival Bcl-2 family rather than the activation of Bak and Bax, as supported by the functional analysis of Bim in TCR signaling. Anti-CD3 cross-linking induces expression of Bim−EL and −L and enhances their binding with Bcl-XL, in thymocytes (63), thus suggesting that Bim association inactivates Bcl-XL in thymocytes, resulting in AICD. This notion was supported by the finding that Bim−/− thymocytes were resistant to TCR-induced apoptosis (63).

Bim−/− B cells were unresponsive to BCR-induced apoptosis (49, 51). On the other hand, Bim overexpression induces apoptosis by mitochondrial dysfunction in Apaf-1−/− mouse embryonic fibroblast cells that lack caspase-9 or caspase-3 activation (64). The Bcl-2−Bim−Bax/Bak pathway, which is involved in the control of lymphocyte apoptosis and survival at the level of the mitochondria, does not necessarily trigger the effector caspase-dependent apoptotic pathway, especially in mature spleen B cells. The caspase-3-dependent pathway, as an effector protease, is presumably inhibited by the normal activation of NF-κB in G5pr−/− B cells, as demonstrated previously (65). Collectively, we hypothesize that the prolonged Bim up-regulation is a major cause of increased sensitivity to AICD in G5pr−/− B cells. G5PR might suppress kinases through the interaction with PPs and/or regulate the expression of Bim. The augmented Bim may induce vigorous disruption of the mitochondrial function, thus resulting in AICD.

The mature B cell numbers markedly decreased in adult G5pr−/− mice, thus suggesting that B cell numbers in peripheral lymphoid organs were maintained by the signals dependent on G5PR and, presumably, mediated through BCR. This may provide information regarding the model proposed by Cancro and Monroe in which the continuous triggering of BCR by self-Ags is essential for the maintenance of the B cell pool in peripheral lymphoid organs (66, 67). Based on their model, B cells need to survive with the subthreshold stimulatory signals that are continuously or intermittently provided to either maintain or expand the B cell number in peripheral lymphoid organs. Almost the entire B cell population was depleted in adult mice at 10–20 d after inducible surface IgM ablation, thus suggesting that the expression of BCR is indispensable for B cell maintenance by transducing the survival signal that promotes cell survival (68).

GC−B cells express a high level of Bim and Bax, proapoptosis players, thus indicating that they are at risk to undergo cell death, which might be involved in the selection of Ag-specific B cells. A possible site of B cell selection is the follicular dendritic cell network expressing CR1, which can potentially trap the Ag–Ab complex with complement C3d (69, 70). The high-affinity BCR may well recognize the complex and, thus, would readily receive a signal for the survival and proliferation of B cells with the help of such accessory molecules as CD40 and Blys on the FDC (71). G5PR might regulate another kinase molecule selectively expressed in GC−B cells such as GCK, which is a member of the Ste20 family and selectively activates JNK (72, 73). Studies of such GC−B cell specific molecules in G5pr−/− mice would provide further information regarding the regulation of B cell survival in the secondary lymphoid organs.
MATERIALS AND METHODS

Generation of G5pr<sup>−/−</sup> and CD19<sup>−/−</sup>G5pr<sup>+/−</sup> mice. The CD19<sup>−/−</sup>-Cre-knock-in mouse was provided by R.C. Rickert (The Burnham Institute, La Jolla, CA). The G5pr-foxed mice were generated in collaboration with N. Takeda (Kumamoto University, Kumamoto, Japan). All mice were maintained in the Center for Animal Resources and Development (CARD) at Kumamoto University. A targeting vector was designed to insert the loxP sites and the neomycin-resistant gene Neo<sup>+</sup> into the G5pr genomic locus, the 3′ end of which contains the negative selection marker DT-A (Fig. 1 A). The floxed 0.88-kb Sau3A/BglII fragment contains the ATG start codon. Homologous embryonic stem recombinants screened by PCR were confirmed by Southern blot analysis with a 0.28-kb fragment of the 3′ homologous arm. Five G5pr<sup>−/−</sup> embryonic stem clones were injected into blastocysts (Institute for Cancer Research), and the two independent chimeric males were mated with C57BL/6 females to obtain G5pr<sup>−/−</sup> offspring. The heterozygous mutant mice were maintained for the C57BL/6 background. All mice were tested using the neo2 primer (5′-GCGGCTTGCCTGCCACACCGG-3′ and 5′-GATTCCTCTCGTAATTTCGGTG-3′), respectively. For BimEL, the primers 5′-CTACGAGATCCTCCACATTTC-3′ and 5′-CAGCTCTGTCGCAAATCCGGTC-3′ were used (74).

B cell isolation. B cells were isolated by the depletion of CD43<sup>+</sup> the primers 5′-CTGTGCAATCCGTATC-3′ and 5′-ACCGGATGATGCTTATAGAAGTCGT-3′. B cell-specific, G5PR-deficient (G5pr<sup>−/−</sup>) mice were crossed by G5pr<sup>−/−</sup>/CD19<sup>−/−</sup>-Cre-knock-in mice. Experimentation and animal care was in accordance with the guidelines of the CARD at Kumamoto University.

RT-PCR. cDNAs synthesized using total RNAs were PCR amplified using primers from exons II and IV (5′-GGTTAGGCTCGCCCAACCCGGG-3′ and 5′-GATTCCCTCTCGTAATTTCGGTG-3′), respectively. For BimEL, the primers 5′-CTACGAGATCCTCCACATTTC-3′ and 5′-CAGCTCTGTCGCAAATCCGGTC-3′ were used (74).

B cell isolation. B cells were isolated by the depletion of CD43<sup>+</sup>, CD4<sup>+</sup>, and Tcr-119<sup>+</sup> cells with magnetic beads (MACS; Miltenyi Biotec) and were >90% B220<sup>+</sup> as verified by flow cytometry.

Flow cytometric analysis. Lymphoid cells stained with anti-B220–PE, anti-B220–APC, anti-CD3–biotin, anti-CD5–PE, anti-CD21–FITC, anti-CD19–FITC, anti-IgM–FITC, anti-IgG–PE, and Streptavidin PerCP-Cy5.5 (BD Biosciences) were analyzed by FACS Calibur (Becton Dickinson) and FlowJo (Tree Star, Inc.) software. The apoptosis-associated changes to the plasma membrane were determined with annexin V–FITC and 7-aminoactinomycin D (BD Biosciences).

Immune responses of mice to TI-II Ag and TD Ag. Mice were immunized peritonally with TI-II Ag, TNP conjugated with Ficoll (25 μg/mouse; Southern Biotechnology Associates, Inc.) in PBS or TD Ag, and TNP conjugated with keyhole limpet hemocyanin (20 μg/mouse; Southern Biotechnology Associates, Inc.) in the Freund’s complete adjuvant. Serum IgGs were measured by ELISA (25). GCs and architectures of spleens were analyzed by FACSCalibur using CellQuest (Becton Dickinson) and FlowJo (Tree Star, Inc.) software. The apoptosis-associated changes to the plasma membrane were determined with annexin V–FITC and 7-aminoactinomycin D (BD Biosciences).

In vitro proliferation assay. 10<sup>6</sup> purified splenic B cells/ml in triplicate were cultured for 48 h with anti-IgM F(ab′)2, Ab (anti-IgM; ICN Biomedicals), IL-4 (PeproTech), and the combination of these stimulants or LPS (Sigma-Aldrich). 2 μCi/ml 3<sup>H</sup>thymidine (ICN Biomedicals) was added for the final 6 h.

Ca<sup>2+</sup> mobilization response. Splenic B cells loaded with 4 μM Fluor-3/AM (Dojindo) were stimulated with 10 μg/ml goat anti-IgM Ab (ICN Biomedicals), and the increase of intracellular Ca<sup>2+</sup> mobilization was recorded on live gated cells.

Western blotting. 10<sup>6</sup> splenic B cells/ml were stimulated with 10 μg/ml of anti-IgM Ab (ICN Biomedicals) and lysed in 1% TNE lysis buffer (1% Nonidet P40, 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 μg/ml aprotinin, 1 mM Na<sub>2</sub>VO<sub>3</sub>, and 10 mM NaF) (22). Western blot analysis was performed with anti-phosphotyrosine Ab (4G10; Upstate Biotechnology) and anti-Bcl-2 Ab (StressGen Biotechnology Associates, Inc.). Anti-cyclin D2 and anti-IkBα Abs were obtained from Santa Cruz Biotechnology, Inc. Anti-Rb, anti-Bcl-X<sub>L</sub>, and anti-Bcl-2 Abs were obtained from BD Biosciences. Each membrane was reprobed with anti-β-actin Ab (Sigma-Aldrich). The membranes were developed with Abs specific to phosphorylated proteins of Erk1/2, JNK1/2, p38, and Akt (Cell Signaling Technology). SP600125 (Calbiochem) was used as a JNK inhibitor. The membranes were incubated with the Ab to the nonphosphorylated form of Erk1/2, JNK1/2, or β-actin (Sigma-Aldrich).

Analysis of DNA fragmentation and ΔΨm. 10<sup>6</sup> splenic B cells/ml were stimulated with 10 μg/ml of anti-IgM Ab (ICN Biomedicals) for 24 h, stained with hypotonic DNA staining solution (50 μg/ml propidium iodide, 0.1% sodium citrate, 0.1% Triton X-100), and analyzed. DiOC6 (3,3′-dihexyloxacarbocyanine iodide; Molecular Probes) was added at a concentration of 40 μM, and the cells were incubated at 37°C for 30 min. The DiOC6<sup>+</sup> fluorescence was measured at the FL1 channel.

TUNEL assay. TUNEL analysis for DNA fragmentation was performed using an APODIRECT kit (BD Biosciences). Their double- or single-strand DNA breakages were determined by flow cytometry after incorporation of FITC-dUTP with exogenous DiT.

Online supplemental material. Fig. S1 shows PP activity in G5pr<sup>−/−</sup> B cells. Fig. S2 depicts spontaneous blast formation and apoptosis in vitro. Fig. S3 caspase-3 activation in G5pr<sup>−/−</sup> B cells on BCR cross-linking. Fig. S4 depicts the effect of a JNK-specific chemical inhibitor. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20050637/DC1.

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REFERENCES

1. Cambier, J.C., and J.T. Ransom. 1987. Molecular mechanisms of transmembrane signaling in B lymphocytes. Annu. Rev. Immunol. 5:175–199.

2. Sakaguchi, N., T. Matsuo, J. Nomura, K. Kuwahara, H. Igarashi, and S. Inui. 1993. Immunoglobulin receptor-associated molecules. Adv. Immunol. 54:337–392.

3. Kurosaki, T. 1997. Molecular mechanisms in B cell antigen receptor signaling. Curr. Opin. Immunol. 9:309–318.

4. Cambier, J.C. 1995. Antigen and Fc receptor signaling. The awesome power of the immunoreceptor tyrosine-based activation motif (ITAM). J. Immunol. 155:3281–3285.

5. DeFranco, A.L., J.D. Richards, J.H. Blum, T.L. Stevens, D.A. Law, V.W. Chan, S.K. Datta, S.P. Foy, S.L. Hounihane, M.R. Gold, et al. 1995. Signal transduction by the B-cell antigen receptor. Ann. NY Acad. Sci. 766:195–201.

6. Flav winkel, H., M. Barner, and M. Reth. 1995. The tyrosine activation motif as a target of protein tyrosine kinases and SH2 domains. Semin. Immunol. 7:21–27.

7. Chiles, T.C. 2004. Regulation and function of cyclin D2 in B lymphocyte subsets. J. Immunol. 173:2901–2907.

8. Carey, G.B., D. Donjerkovic, C.M. Mueller, S. Liu, J.A. Hinshaw, L. Tonnetti, W. Davidson, and D.W. Scott. 2000. B-cell receptor and Fas-mediated signals for life and death. Immunol. Rev. 176:105–115.

9. Ranger, A.M., B.A. Malynn, and S.J. Korsmeyer. 2001. Mouse models
of cell death. Nat. Genet. 28:113–118.
10. Ninno, H., and E.A. Clark. 2002. Regulation of B-cell fate by antigen-receptor signals. Nat. Rev. Immunol. 2:945–956.
11. Datta, S.R., A. Brunet, and M.E. Greenberg. 1999. Cellular survival: a play in three Acts. Gene Dev. 13:2905–2927.
12. Lee, H.H., H. Dadgostar, Q. Cheng, J. Shu, and G. Cheng. 1999. NF-kappaB-mediated up-regulation of Bcl-x and Bcl-2/A1 is required for CD40 survival signaling in B lymphocytes. Proc. Natl. Acad. Sci. USA. 96:9136–9141.
13. Eeva, J., V. Postila, M. Mattio, U. Nuutinen, A. Ropponen, M. Eray, and J. Pelkonen. 2003. Kinetics and signaling requirements of CD40-mediated protection from B cell receptor-induced apoptosis. Eur. J. Immunol. 33:2783–2791.
14. Dori, T., N. Motoyama, A. Tokunaga, and T. Watanabe. 1999. Death signals from the B cell antigen receptor target mitochondria, activating necrotic and apoptotic death cascades in a murine B cell line, WEHI-231. Int. Immunol. 11:933–941.
15. Eeva, J., and J. Pelkonen. 2004. Mechanisms of B cell receptor induced apoptosis. Apoptosis. 9:525–531.
16. Eldering, E., and R.A. Vanlier. 2005. B-cell antigen receptor-induced apoptosis: looking for clues. Immunol. Lett. 96:187–194.
17. Berard, M., P. Mondiere, M. Casamayor-Palleja, A. Hennino, C. Bella, and T. Deffrance. 1999. Mitochondria connects the antigen receptor to effector caspases during B cell receptor-induced apoptosis in normal human B cells. J. Immunol. 163:4655–4662.
18. Bouchon, A., P.H. Kramer, and H. Walczak. 2000. Critical role for mitochondria in B cell receptor-mediated apoptosis. Eur. J. Immunol. 30:69–77.
19. Katz, E., M.R. Dechan, S. Seatter, C. Lord, R.D. Sturrock, and M.M. Harriott. 2001. B cell receptor-stimulated mitochondrial phospholipase A2 activation and resultant disruption of mitochondrial membrane potential correlate with the induction of apoptosis in WEHI-231 B cells. J. Immunol. 166:137–147.
20. Graves, J.D., K.E. Draves, A. Craxton, E.G. Krebs, and E.A. Clark. 1998. A comparison of signaling requirements for apoptosis of human B lymphocytes induced by the B cell receptor and CD95/Fas. J. Immunol. 161:168–174.
21. Li, P., D. Nijhawan, I. Budihardjo, S.M. Srinivasula, M. Ahmad, E.S. Alnemri, and X. Wang. 1997. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Genes Dev. 11:479–489.
22. Kono, Y., K. Maeda, K. Kuwahara, H. Yamamoto, E. Miyamoto, K. Yonezawa, K. Takagi, and N. Sakaguchi. 2002. MCM3-binding GANP DNA-primease is associated with a novel phosphatase component Grb2-associated binder 1. J. Biol. Chem. 277:8103–8107.
23. Kuwahara, K., M. Yoshida, E. Kondo, A. Sakata, Y. Watanabe, E. Abe, Y. Kosono, S. Tomiyasu, S. Fujimura, T. Tokuhashi, et al. 2000. A novel nuclear phosphoprotein, GANP, is up-regulated in centrocyes of the germinal center and associated with MCM3, a protein essential for DNA replication. Blood. 95:2321–2328.
24. Kuwahara, K., S. Tomiyasu, S. Fujimura, K. Nomura, Y. Xing, N. Nishiyama, M. Ogawa, S. Imao-Ohmi, S. Izuta, and N. Sakaguchi. 2001. Germinal center-associated nuclear protein (GANP) has a phosphorylation-dependent DNA-primease activity that is up-regulated in germinal center regions. Proc. Natl. Acad. Sci. USA. 98:10279–10283.
25. Kuwahara, K., S. Fujimura, Y. Takahashi, N. Nakagata, T. Takemori, S. Aizawa, and N. Sakaguchi. 2004. Germinal center-associated nuclear protein contributes to affinity maturation of B cell antigen receptor in T cell-dependent responses. Proc. Natl. Acad. Sci. USA. 101:1010–1015.
26. Khan, W.N., F.W. Alt, R.M. Gerstein, B.A. Malynn, I. Larson, G. Rathbun, L. Davidson, S. Muller, A.B. Kantor, L.A. Herzenberg, et al. 1995. Defective cell development and function in Bk-deficient mice. Immunity. 3:283–292.
27. Solosyva, N., W.W. Wu, D. Parry, D. Mahony, E.W. Lam, J. Glassford, G.G. Klaus, P. Sicinska, R. Weinberg, Y.J. Liu, et al. 2000. Cyclin D2 is essential for BCR-mediated proliferation and CD5 B cell development. Int. Immunol. 12:631–638.
sion during B cell development and activation and modulates lymphocyte survival in transgenic mice. J. Exp. Med. 183:381–391.

49. Enders, A. P. Bouillet, H. Puthalakath, Y. Xu, D.M. Tarlinton, and A. Strasser. 2003. Loss of the proapoptotic BH3-only Bcl-2 family member Bim inhibits BCR-stimulation-induced apoptosis and deletion of autoreactive B cells. J. Exp. Med. 198:1119–1126.

50. Lei, K., and R.J. Davis. 2003. JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis. Proc. Natl. Acad. Sci. U.S.A. 100:2432–2437.

51. Bouillet, P., D. Metcalf, D.C. Huang, D.M. Tarlinton, T.W. Kay, F. Kontgen, J.M. Adams, and A. Strasser. 1999. Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. Science. 286:1735–1738.

52. Graves, J.D., K.E. Draves, A. Craxton, J. Saklatvala, E.G. Krebs, and E.A. Clark. 1996. Involvement of stress-activated protein kinase and p38 mitogen-activated protein kinase in mIgM-induced apoptosis of human B lymphocytes. Proc. Natl. Acad. Sci. U.S.A. 93:13814–13818.

53. Davis, R.J. 2000. Signal transduction by the JNK group of MAP kinases. Cell. 103:239–252.

54. Weston, C.R., and R.J. Davis. 2002. The JNK signal transduction pathway. Curr. Opin. Genet. Dev. 12:14–21.

55. Maundrell, K., B. Antonsson, E. Magnenat, M. Camps, M. Muda, C. Chabert, C. Gillieron, U. Boschert, E. Vial-Knecht, J.C. Martinou, and S. Arkinstall. 1997. Bcl-2 undergoes phosphorylation by c-Jun N-terminal kinase/stress-activated protein kinases in the presence of the constitutively active GTP-binding protein Rac1. J. Biol. Chem. 272:25238–25242.

56. Yamamoto, K., H. Ichijo, and S.J. Korsmeyer. 1999. BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase/stress-activated protein kinase pathway normally activated at G(2)/M. Mol. Cell. Biol. 19:8469–8478.

57. Tournier, C., P. Hess, D.D. Yang, J. Xu, T.K. Turner, A. Nimmul, D. Bar-Sagi, S.N. Jones, R.A. Flavell, and R.J. Davis. 2000. Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. Science. 288:870–874.

58. Hsu, S.Y., P. Lin, and A.J. Hsueh. 1998. BOD (Bcl-2-related ovarian death gene) is an ovarian BH3 domain-containing proapoptotic Bcl-2 protein capable of dimerization with diverse antiapoptotic Bcl-2 members. Mol. Endocrinol. 12:1432–1440.

59. O’Reilly, L.A., L. Cullen, J. Vissvader, G.J. Lindeman, C. Print, M.L. Bath, D.C. Huang, and A. Strasser. 2000. The proapoptotic BH3-only protein bim is expressed in hematopoietic, epithelial, neuronal, and germ cells. Am. J. Pathol. 157:449–461.

60. Puthalakath, H., D.C. Huang, L.A. O’Reilly, S.M. King, and A. Strasser. 1999. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynemor motor complex. Mol. Cell. 3:287–296.

61. Kuwana, T., L. Bouchier-Hayes, J.E. Chipuk, C. Bonzon, B.A. Sullivan, D.R. Green, and D.D. Newmeyer. 2005. BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. Mol. Cell. 17:525–535.

62. Chen, L., S.N. Willis, A. Wei, B.J. Smith, J.J. Fletcher, M.G. Hinds, P.M. Colman, C.L. Day, J.M. Adams, and D.C. Huang. 2005. Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. Mol. Cell. 17:393–403.

63. Bouillet, P., J.F. Purton, D.I. Godfrey, L.C. Zhang, L. Coutsas, H. Puthalakath, M. Pellegrini, S. Cory, J.M. Adams, and A. Strasser. 2002. BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. Nature. 415:922–926.

64. Cheng, E.H., M.C. Wei, S. Weiler, R.A. Flavell, T.W. Mak, T. Lindsten, and S.J. Korsmeyer. 2001. BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. Mol. Cell. 7:675–711.

65. Pham, L.V., A.T. Tamayo, L.C. Yoshimura, P. Lo, and R.J. Ford. 2003. Inhibition of constitutive NF-kappa B activation in mantle cell lymphoma B cells leads to induction of cell cycle arrest and apoptosis. J. Immunol. 171:88–95.

66. Cancro, M.P., and J.F. Kearney. 2004. B cell positive selection: road map to the primary repertoire? J. Immunol. 173:15–19.

67. Monroe, J.G. 2004. Ligand-independent tonic signaling in B-cell receptor function. Curr. Opin. Immunol. 16:288–295.

68. Lam, K.P., R. Kuhn, and K. Rajewsky. 1997. In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. Cell. 90:1073–1083.

69. Caux, C., Y.J. Liu, and J. Banchereau. 1998. Recent advances in the study of dendritic cells and follicular dendritic cells. Immunol. Today. 19:6–8.

70. Tew, J.G., J. Wu, D. Qin, S. Helm, G.F. Burton, and A.K. Szakal. 1997. Follicular dendritic cells and presentation of antigen and costimulatory signals to B cells. Immunol. Rev. 156:39–52.

71. Noelle, R.J., and L.D. Erickson. 2005. Determinations of B cell fate in immunity and autoimmunity. Curr. Dir. Autoimmun. 8:1–24.