# 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-κB, 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 Igα and Igβ (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-κB 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 (ΔΨm) is an essential event in the BCR-mediated apoptosis of immature (bone marrow) and mature GC–B cells (14, 17–19). The alteration of ΔΨm 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).

The online version of this article contains supplemental material.

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 ($G5pr^{-/-}$) mice by conditional targeting in B cells with a combination of the floxed-*G5pr* gene and CD19-*Cre*– knock-in mice. $G5pr^{-/-}$ 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 ($G5pr^{F/wt}$) 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 *Hin*dIII 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*
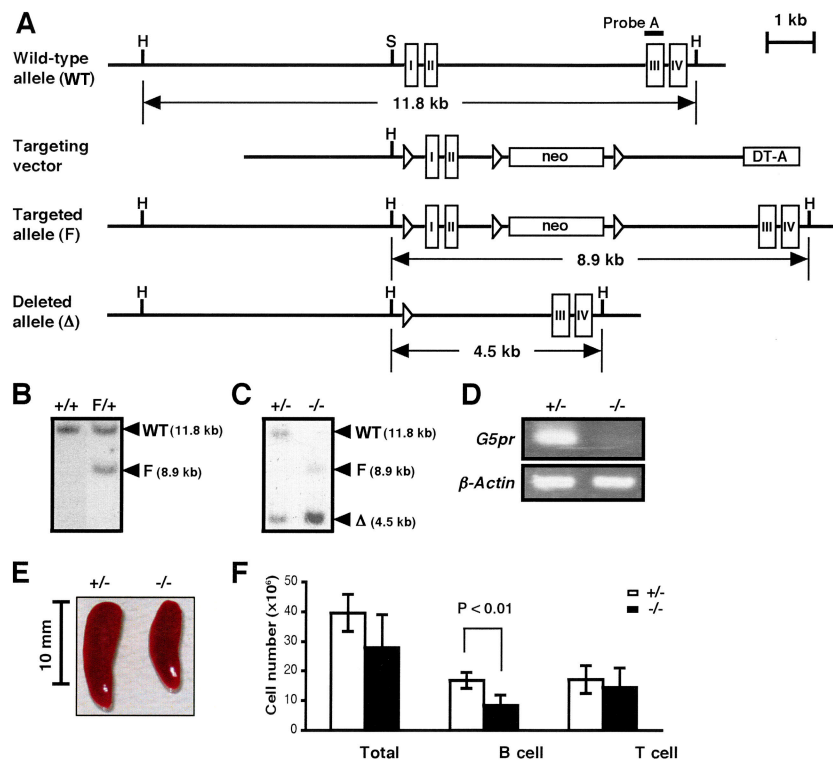


**Figure 1. Generation of a CD19–Cre–mediated G5PR–deficient mouse.** (A) Targeting of the *G5pr* gene. (top model) Genomic organization of the *G5pr* gene locus (WT). H, *Hin*dIII; S, *Sal*I. The bar indicates the probe used for Southern blots. The *loxP* sites are indicated by triangles. (bottom models) 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 *Hin*dIII-digested DNAs with the indicated probe. Lengths of the respective *Hin*dIII fragments are shown. DT-A represents a selection cassette for diptheria toxin-A. (B) Germ line transmission of a *G5pr*-targeted allele. Tail DNAs from wild-type and $G5pr^{F/wt}$ littermates were digested with *Hin*dIII, followed by Southern blot analysis. (C) CD19-Cre–mediated deletion of exons I and II flanked with *loxP* sites. DNAs of splenic B cells purified from $G5pr^{+/-}$ and $G5pr^{-/-}$ littermates were digested with *Hin*dIII, 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 (B220[Low] CD43^{+}) was relatively high but that of pre–B cells and immature B cells (B220[Low] CD43^{-}) decreased from 31 to 22.7% of the control B cells. The mature B cell (B220[High] CD43^{-}) population was as low as 2.72% in $G5pr^{-/-}$ mice ($\sim$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 (IgM[Low] IgD[High]) population decreased more remarkably (from 31.3 to 20.8%) than the transitional-2 (IgM[High] IgD[High]) B cell population (from 9.88 to 7.08%). Transitional-1 (IgM[High] IgD[Low]) 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^{+} CD21[Int] CD23[High]). 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
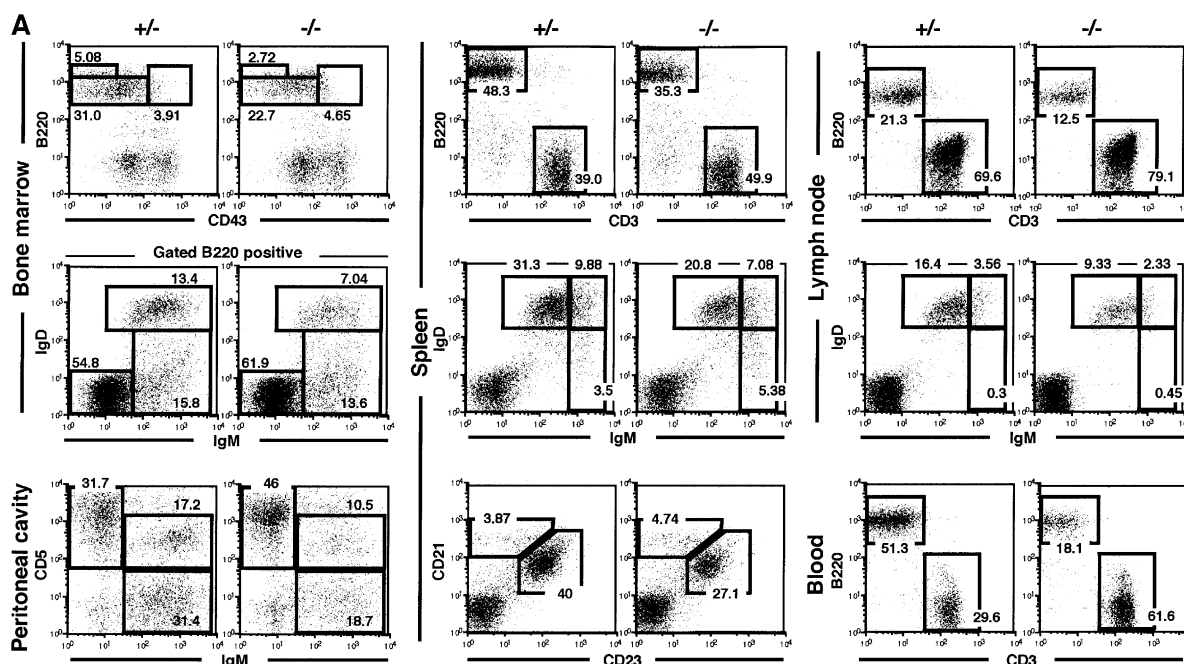


**Figure 2 (continues on next page)**

**Figure 2.   B cell development in $G5pr^{-/-}$.** (A) Flow cytometric analysis of cells from the bone marrow, spleen, axillary lymph node, peritoneal cavity, and peripheral blood. The numbers indicate the relative percentages of cells within the indicated gates. Bone marrow contains (top) pro–B cells (B220^{+} CD43^{+}), pre–B cells and immature B cells (B220[Low] CD43^{-}), and mature recirculating B cells (B220[High] CD43^{-}) and (middle) pro–B cells and pre–B cells (B220^{+} IgM^{-} IgD^{-}), immature B cells (B220^{+} IgM^{+} IgD[Low/-]), and mature recirculating B cells (B220^{+} IgM^{+} IgD[High]). The

spleen contains (top) B cells (B220^{+}) and T cells (CD3^{+}); (middle) transitional type-1 B cells (IgM[High] IgD[Low/-]), transitional type-2 B cells (IgM[High] IgD[High]), and mature B cells (IgM[Low] IgD[High]); and (bottom) marginal zone B cells (CD21[High] CD23[Low/-]) 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 (IgM[High] IgD[High]) and mature B cells (IgM[Low] IgD[High]). The peritoneal cavity contains B-1a cells (CD5^{+} IgM^{+}) and the peripheral blood contains B cells (B220^{+}) and T cells (CD3^{+}).

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
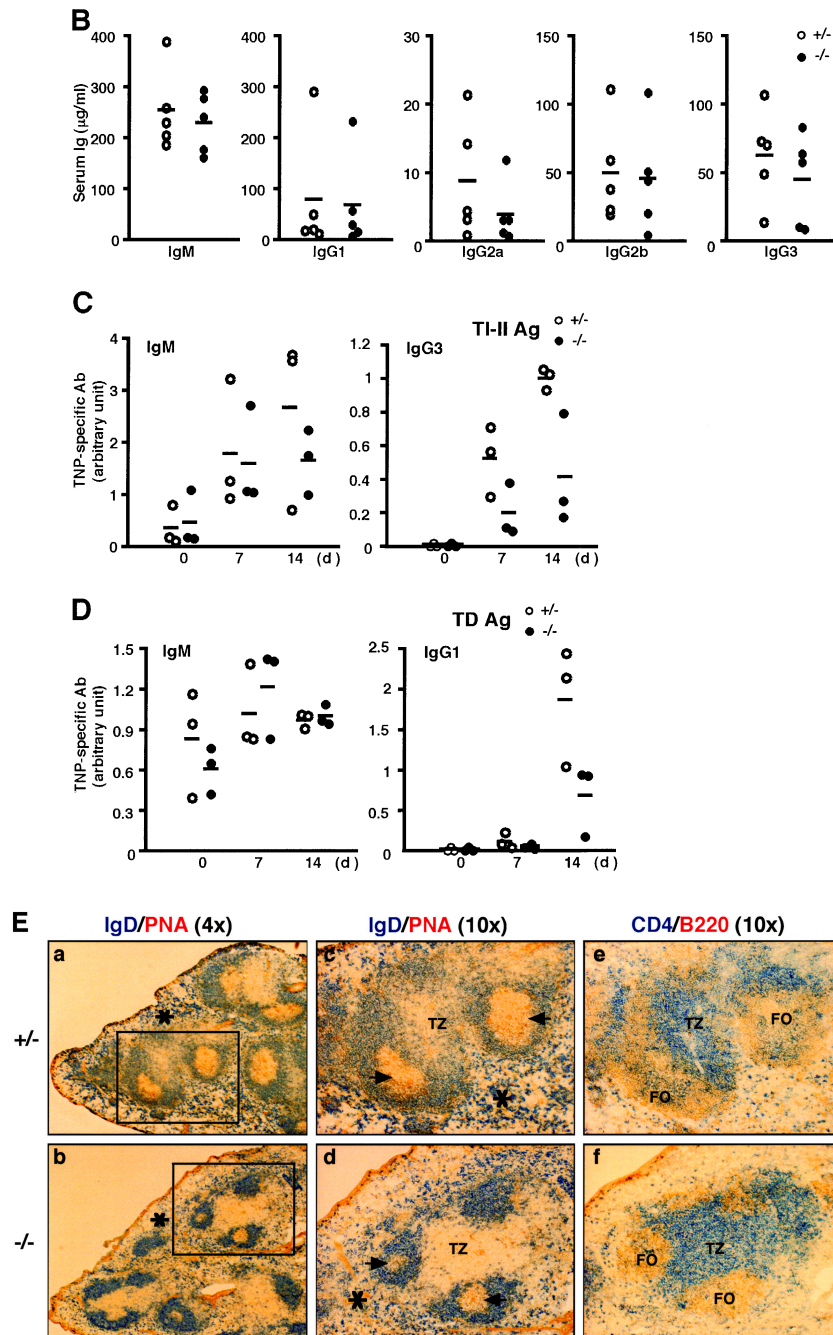


Figure 2 (continued from previous page)

(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 Ca$^{2+}$ flux. *G5pr*$^{-/-}$ B cells showed a normal level of Ca$^{2+}$ 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)
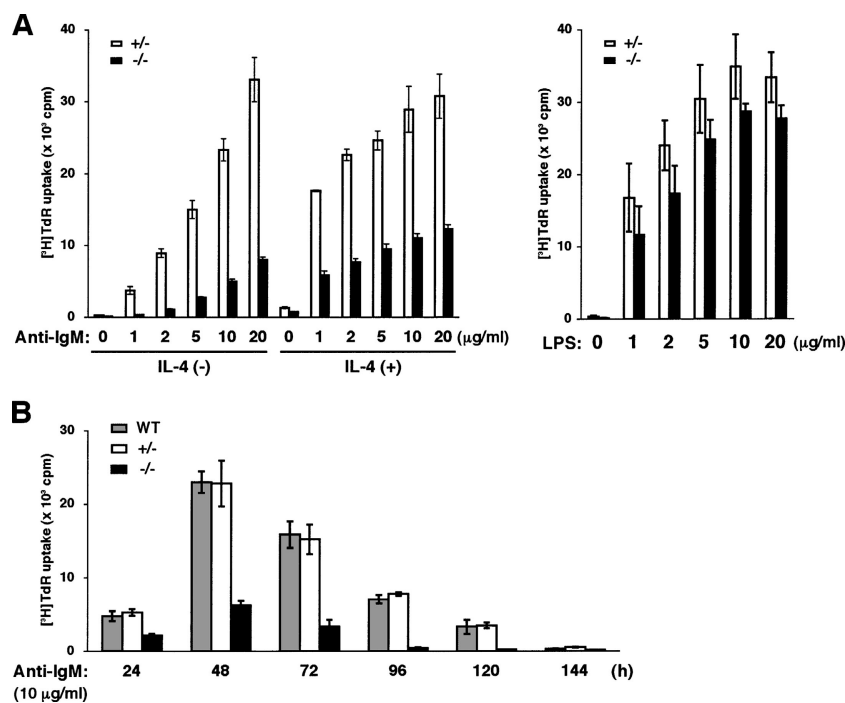


**Figure 3. B cell proliferation in response to the mitogenic stimuli in vitro.** (A) Splenic B cells were cultured for 48 h in the presence of medium alone, anti-IgM F(ab')$_2$ Ab, or 100 U/ml IL-4 (left) or LPS (right). B) Kinetics of the responses of WT, *G5pr*$^{+/-}$, and *G5pr*$^{-/-}$ B cells to anti-IgM Ab indicated a similar response curve with the peak at 48 h after stimulation. [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.
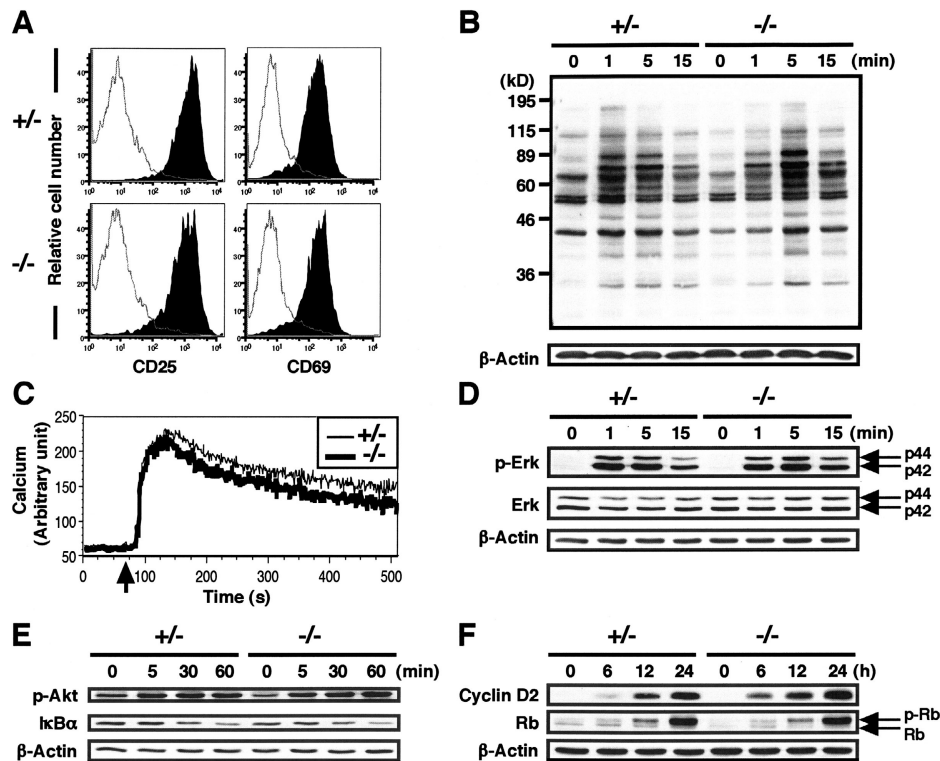
**Figure 4. BCR-mediated signaling in G5PR-deficient B cells.** (A) The activation of $G5pr^{+/-}$ and $G5pr^{-/-}$ B cells in response to 10 μg/ml anti-IgM F(ab')$_2$ Ab as assessed by the up-regulation of CD25 and CD69. Histograms represent CD25 or CD69 expression with (black plot) and without (white 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 phosphorylated 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.

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 IκB 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 obvi-

ous 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 the $G5pr^{-/-}$ 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
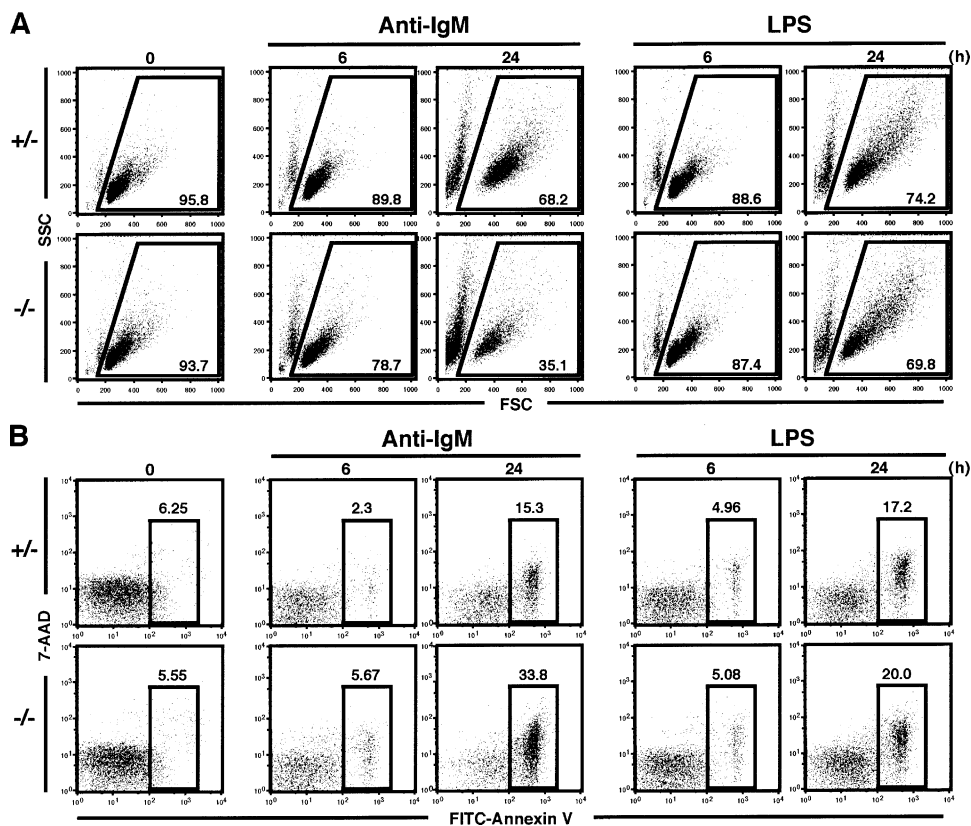
**Figure 5.   Cell death of G5PR–deficient B cells.** Splenic B cells from either *G5pr$^{+/-}$* or *G5pr$^{-/-}$* mice were stimulated with 10 μg/ml anti-IgM F(ab')$_2$ Ab. (A) Living cells were identified with forward scatter (FSC) and side scatter (SSC) on the FACS profile. (B) Apoptotic cells were monitored as annexin V–FITC positive cells. Lymphoid gating was set by the parameters of SSC and FSC. The number in each box indicates the percentage of cells in that box based on the gated sample.

cross-linking was caused by the alteration of B cell survival. *G5pr$^{-/-}$* B cells showed a reduction in the number of live cells at 6 h after BCR cross-linking in comparison with the control *G5pr$^{+/-}$* 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$^{-/-}$* B cells for 24 h (Fig. 5 B). Notably, *G5pr$^{-/-}$* 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$^{-/-}$* B cells in a manner similar to that of control *G5pr$^{+/-}$* B cells (Fig. 5, A and B). We thus conclude that the hypoproliferation of *G5pr$^{-/-}$* B cells resulted from an increased susceptibility to BCR–mediated AICD.

### Increased mitochondrial membrane depolarization induced by BCR cross-linking in *G5pr$^{-/-}$* B cells

We examined the DNA fragmentation of *G5pr$^{-/-}$* B cells by propidium iodide staining. The number of dead cells increased ∼6 times in *G5pr$^{-/-}$* 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$^{+/-}$* B cells. In comparison with the controls, *G5pr$^{-/-}$* 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 $\Delta\Psi$m (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$^{-/-}$* 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).
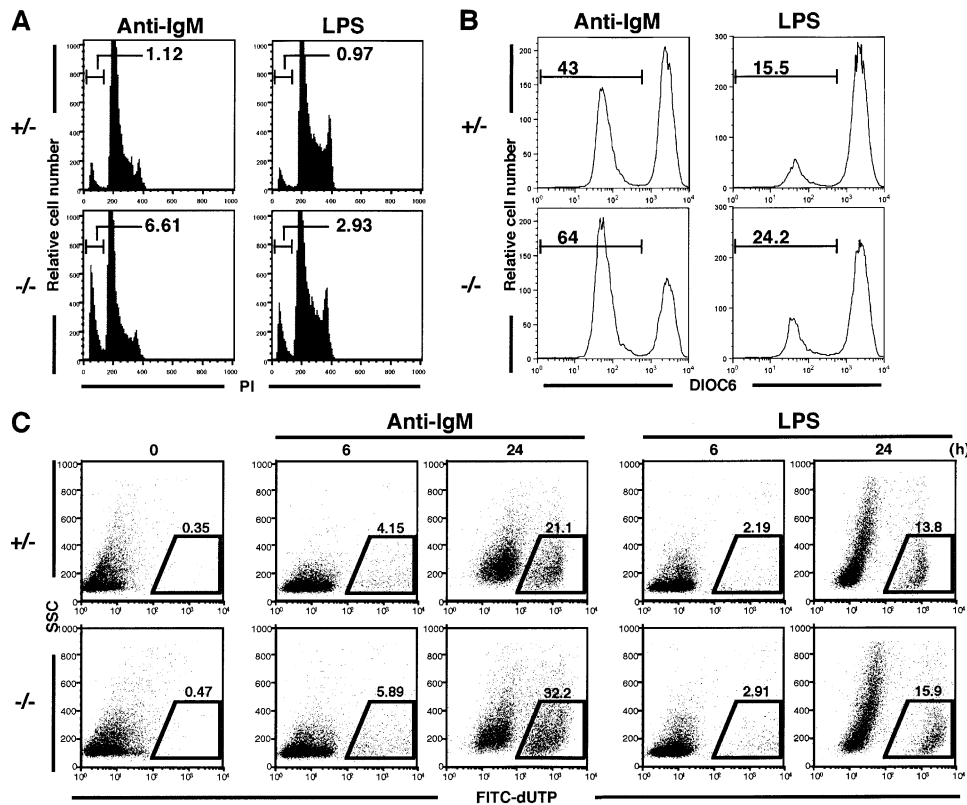
**Figure 6.** **Mitochondrial apoptotic machinery in G5PR–deficient B cells.** (A) 10⁶ B cells stimulated either with 10 μg/ml anti-IgM F(ab')₂ Ab or 10 μg/ml LPS for 24 h were lysed in hypotonic buffer containing propidium iodide, and nuclear DNA contents were analyzed. The number in each box indicates the percentage of cells in that box based on the gated sample. (B) For the quantitation of ΔΨm, 10⁶ splenic B cells were stimulated either with 10 μg/ml anti-IgM F(ab')₂ Ab or 10 μg/ml LPS for 24 h and subjected to staining with the ΔΨm-sensitive dye DiOC₆. The proportion of depolarized cells is shown by the percentage in the histogram. (C) 10⁶ splenic B cells were stimulated with anti-IgM F(ab')₂ Ab or LPS and were harvested and characterized by TUNEL assay. FITC-dUTP–incorporated cells were measured by flow cytometry. The number in each box indicates the percentage of cells in that box based on the gated sample.

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 $G5pr^{-/-}$ 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-X_L and Bcl-2, which play a prominent role in the protection of mature B cells from AICD (44, 45). The transgenic mice of Bcl-X_L 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-X_L. BCR cross-linking induced the up-regulation of Bcl-X_L 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
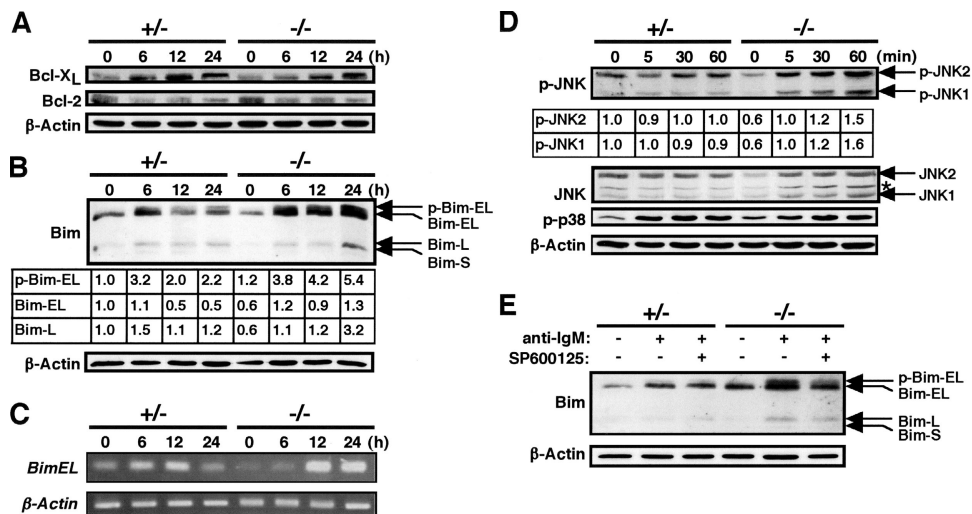
**Figure 7. BCR–mediated regulation of apoptotic and survival signaling molecules in G5PR$^{-/-}$ B cells.** Expression of Bcl-XL or Bcl-2, expression and phosphorylation of Bim, the phosphorylation of JNK or p38, or expression and phosphorylation of Bim in the presence of JNK inhibitor were studied. (A) The membrane was probed with anti–Bcl-XL or anti–Bcl-2 Ab. (B) Three major isoforms of Bim—short type (S), large type (L), and extra-large type (EL)—were detected with anti-Bim Ab. The relative intensities of the bands are shown based on the controls. $10^6$ splenic B cells from both mutant and control mice were stimulated with 10 mg/ml anti-IgM F(ab')2 Ab for the time depicted in the figures. To show the loading amounts, each membrane was reblotted with anti–β-actin Ab. (C) $10^6$ splenic B cells were stimulated with 10 mg/ml anti-IgM F(ab')2 Ab, and total RNAs were isolated, converted to cDNA, and subjected to measure BimEL expression by RT-PCR. (D) BCR-induced JNK and p38 phosphorylation was monitored with phospho-JNK or phospho-p38–specific Ab. The entire JNK expression was monitored as an internal control. JNK1 (p46) and JNK2 (p54) are indicated with arrows. An asterisk indicates a nonspecific band. The relative intensities of bands were shown based on the controls. (E) Splenic B cells were stimulated with 10 mg/ml anti-IgM F(ab')2 Ab for 24 h after incubation with or without 2 mM SP600125 for 30 min. Bim induction and its phosphorylation were detected (B).

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$^{-/-}$* 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$^{+/-}$* 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 apopto-

sis 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 min after CD40 cross-linking (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20050637/DC1). This treatment did not cause any apparent difference 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 nonspecific 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-X$_L$, antiapoptotic members of Bcl-2 family proteins, were described to be both phosphorylated and inactivated by JNKs in vivo (55, 56). The hyperactivation of JNK caused an alteration of Bcl-2 and Bcl-X$_L$, 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-X$_L$ 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-X$_L$, 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-X$_L$ in thymocytes (63), thus suggesting that Bim association inactivates Bcl-X$_L$ 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^{F/wt}* and CD19-*Cre*/*G5pr^{F/F}* mice.** The CD19–Cre–knock-in mouse was provided by R.C. Rickert (The Burnham Institute, La Jolla, CA). The *G5pr*–floxed 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^r* 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 *Sal*I/*Bgl*II 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^{F/wt}* 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^{F/wt}* offspring. The heterozygous mutant mice were maintained for the C57BL/6 background. PCR was performed using the neo2 primer (5′-GCCTGCTTGC-CGAATATCATGGTGGAAAAT-3′) and the 3′ *G5pr* exon3 primer (5′-ACCGGGGTATGGTCTTATAGAACTCGTTTG-3′). B cell–specific, G5PR-deficient (*G5pr^{-/-}*) mice were generated by crossing *G5pr^{F/F}* mice with CD19–*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′-GGTTAGCGTCGCCCAACACG-3′ and 5′-GATTCCTCTCGTAATTTCTG-3′, respectively). For *BimEL,* the primers 5′-CTACCAGATCCCCACTTTTC-3′ and 5′-CAGCTC-CTGTGCAATCCGTATC-3′ were used (74).

**B cell isolation.** B cells were isolated by the depletion of CD43^+, CD4^+, and Ter-119^+ cells with magnetic beads (MACS; Miltenyi Biotec) and were ≥90% B220^+, 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-CD23–biotin, anti-CD25–PE, anti-CD69–FITC, anti-CD43–biotin, anti-IgM–FITC, anti-IgD–PE, and Streptavidin PerCP-Cy5.5 (BD Biosciences) 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-amino-actinomycin D (BD Biosciences).

**Immune responses of mice to TI-II Ag and TD Ag.** Mice were immunized peritoneally 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 Igs were measured by ELISA (25). GCs and architectures of spleens were examined with PNA-biotin (Vector), anti-IgD, anti-CD4, and anti-B220–biotin after immunization with TD Ag and SRBCs for 10 d (23).

**In vitro proliferation assay.** $10^6$ 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 [$^3$H]thymidine (ICN Biomedicals) was added for the final 6 h.

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

**Western blotting.** $10^6$ 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 phenylmethylsulphonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 μg/ml aprotinin, 1 mM Na$_3$VO$_4$, and 10 mM NaF) (22). Western blot analysis was performed with anti-phosphotyrosine Ab (4G10; Upstate Biotechnology) and anti-Bim Ab (StressGen Biotechnology Associates, Inc.). Anti-cyclin D2 and anti-IκBα Abs were obtained from Santa Cruz Biotechnology, Inc. Anti-Rb, anti-Bcl-X$_L$, 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.** $10^6$ 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′-dihexyloxacarbocynine iodide; Molecular Probes) was added at a concentration of 40 nM, and the cells were incubated at 37°C for 30 min. The DiOC6 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 TdT.

**Online supplemental material.** Fig. S1 shows PP activity in *G5pr^{-/-}* B cells. Fig. S2 depicts spontaneous blast formation and apoptosis in vitro. Fig. S3 caspase-3 activation in *G5pr^{-/-}* 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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