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BCR-crosslinking induces a transcription of protein phosphatase component G5PR that is required for mature B-cell survival ☆

Faisal Mahmudul Huq Ronny [a,1], Hideya Igarashi [a,b,1], Nobuo Sakaguchi [a,b,*]

[a] Department of Immunology, Graduate School of Medical Sciences, Kumamoto University, 1-1-1, Honjo, Kumamoto 860-8556, Japan
[b] Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency, Japan

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Abstract

BCR-crosslinking triggers activation-induced cell death (AICD) selectively in the restricted stage of B-cell differentiation. We examined the transcription of a protein phosphatase subunit G5PR in immature and mature B-cells, because absence of this factor augmented cell sensitivity to AICD, associated with increased activation of JNK and Bim. BCR-crosslinking-induced *G5pr* transcription in AICD-resistant mature splenic $IgM^{lo}IgD^{hi}$ B-cells but not in AICD susceptible immature $IgM^{hi}IgD^{lo}$ B-cells. Thus, *G5pr* induction correlated with the prevention of AICD; High in mature splenic $CD23^{hi}$ B-cells but low in immature B-cells of neonatal mice, sub-lethally irradiated mice, or *xid* mice. Lack of *G5pr* upregulation was associated with the prolonged activation of JNK. The *G5pr* cDNA transfection protected an immature B-cell line WEHI-231 from BCR-mediated AICD. The differential expression of G5PR might be responsible for the antigen-dependent selection of B-cells.

Keywords: BCR signal; Apoptosis; Mature B-cell; Immature B-cell

B-cells generated in the primary lymphoid organs express BCRs that are created randomly by the rearrangement of *immunoglobulin* genes during early B-cell differentiation in the fetal liver and adult bone marrow [1]. The newly generated immature B-cell pool contains self-reactive B-cell clones which have to be eliminated before exporting to the peripheral lymphoid organs by mechanisms such as clonal deletion, anergy [2–4], and receptor editing [5,6]. Immature B-cells are defined by the expression of a surface phenotype such as $B220^{lo}IgM^{hi}IgD^{lo}$ in the bone marrow and spleen, especially in neonatal mice up to day 4 after birth [7,8]. These immature B-cells are more sensitive than mature B-cells to activation-induced cell death (AICD) following BCR-crosslinking in vitro [9–12]. The immature B-cells differentiate into mature B-cells expressing a $B220^{hi}IgM^{lo}IgD^{hi}CD23^{hi}$ phenotype in peripheral lymphoid organs and undergo maturation of BCR-affinity and class switching [13,14]. During these maturation processes, the antigen (Ag)-driven B-cells are selected for specificity and high affinity to specific Ags in peripheral lymphoid organs.

BCR-crosslinking induces AICD selectively in immature B-cells, suggesting the existence of a unique regulatory mechanism of BCR-induced AICD in B-cells. Recently, we identified a protein phosphatase component designated G5PR that associates with the catalytic subunits of protein phosphatases such as PP2A and PP5 [15]. These protein

☆ *Abbreviations*: AICD, activation-induced cell death; Ab, antibody; Ag, antigen; APC, Allophycocyanin; BCR, B-cell receptor; BTK, Bruton's tyrosine kinase; JNK, C-Jun N-terminal kinase; FITC, fluorescein isothiocynate; GFP, green fluorescent protein; PE, phycoerythrin; RT-PCR, reverse transcription-PCR; TUNEL, terminal deoxynucleotidyltransferase (TDT) dUTP nick end labeling assay; *xid*, X-linked immunodeficiency.

* Corresponding author. Fax: +81 96 373 5138.
E-mail address: nobusaka@kaiju.medic.kumamoto-u.ac.jp (N. Sakaguchi).

[1] These two authors contributed equally to this work.

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phosphatases are present in cells as complexes associated with various subcomponents [16,17]. Loss of G5PR resulted in increased B-cell sensitivity to BCR-induced AICD. As a consequence, the number of mature B-cells in peripheral lymphoid organs of *G5pr*-gene knockout mice (B-*G5pr*$^{-/-}$ established by the conditional targeting strategy in B-cells using *flox* sequences in combination with *CD19-Cre* knockin mice [18]) decreased to 60% of that of the control mice. However, G5PR$^{-/-}$ B-cells had no abnormalities in BCR-mediated proliferation signaling, with normal activation of tyrosine phosphorylation, Ca^{2+} influx, activation of the MAPK pathway, and NF-κB activation, leading to Cyclin D2 activation. Nevertheless, there was an increase of BCR-induced apoptosis detected by the TUNEL assay, increased mitochondrial membrane depolarization, and prolonged activation of JNK with increased phosphorylation of Bim. These results suggested that G5PR controls the BCR-induced AICD presumably by regulating the phosphorylation state(s) of various signal transduction, pro-apoptotic, and anti-apoptotic molecules in B-cells.

Here, we studied the level of *G5pr* expression in B-cells at various differentiation stages following BCR-crosslinking and correlated this with proliferation and AICD responses. The results showed that G5PR is an activation-induced phosphatase component preferentially expressed in the IgM^{lo}IgD^{hi}CD23^{hi} mature B-cell subset, which would be positively selected following Ag-stimulation.

Materials and methods

Mice. C57BL/6J and CBA/CaHN-*Btk*^{xid}/J mice were purchased from The Jackson Laboratory (Bar Harbor, MA). RAG1/*Green Fluorescent Protein* (*GFP*) gene knockin mice were generated as described previously [19]. All mice were maintained in the Center for Animal Resources and Development (CARD), Kumamoto University. Newborns were examined within 4 days after birth.

Cell line and cell culture. IgM^{+}IgD$^{lo/-}$ immature B-cell line WEHI-231 was maintained as described previously [20] in RPMI-1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (M.A. Bioproduct, Walkersville, MD), 2-mercaptoethanol (5×10^{-7} M), streptomycin (100 μg/ml), penicillin (100 U/ml), and 2 mM L-glutamine (Flow Lab, Northern Island) in the 5% CO_2-humidified incubator at 37 °C.

Cell preparation. Spleen B-cells were purified by magnetic beads based negative selection method (MACS) using B-cell isolation kit from Miltenyi (Cologne GmbH). For autoreconstitution assays, mice were pre-treated with sub-lethal γ-irradiation (500 rad) as described [21,22].

Flow cytometry and cell sorting. Purified B-cells were stained with FITC- or APC-anti-mouse IgM Ab, PE-anti-mouse IgD Ab, APC-anti-mouse CD45R Ab, and in some cases with biotin-anti-mouse CD23 Ab (e-Biosciences, San Diego, CA) combined with streptavidin–PerCP–Cy5.5 (BD Biosciences, San Jose, CA). After washing, flow cytometric analysis was performed using the software of FACSCalibur, CellQuest (Becton Dickinson, Franklin Lakes, NJ), and Flowjo (Tree Star, Ashland, OR). Cell sorting was performed on a JSAN cell sorter system (BayBio Science, Kobe).

Proliferation and cell death assay. Cell proliferation was estimated with the CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI). Briefly, 1×10^5 cells were cultured for 48 h in a 96-well plate in triplicate with or without 10 μg/ml of rabbit anti-IgM F(ab′)$_2$ Ab (ICN, Irvine, CA) followed by the addition of the reagent containing tetrazolium compound [3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; MTS] for 4 h. The intensity of colored formazan was measured by the absorbance at 495 nm. Apoptosis associated change of DNA contents was determined by staining with propidium iodide (PI). Increase of subdiploid DNA was measured by flow cytometry.

RT-PCR. Total RNAs from the spleen B-cells and cell lines were prepared with TRIZOL LS reagent (Life Technologies, Gaithersburg, MD). For RT-PCR, each 3 μg of the RNA was reverse transcribed for 1 h at 42 °C using Geneamp RNA PCR Kit (Perkin-Elmer Cetus, Foster City, CA) [23]. PCR primer sequences were as follows: *mouse G5pr* specific forward (5′-ATGGACTGGAAAGACGTGCTTCGCC-3′) and the reverse (5′-TCATGTGTCATCAAGATCTGCAGAGTTCTC-3′) primers [15], *mouse* β-*actin* forward (5′-CCTAAGGCCAACCGTGAAAAG-3′) and the reverse (5′-TCTTCATGGTGCTAGGAGCCA-3′) primers [23], and *mouse Pax5* specific forward (5′-CTACAGGCTCCGTGACGCAG-3′) and the reverse (5′-GTCTCGGCCTGTGACAATAGG-3′) primers [24].

Western blot analysis. Spleen B-cells (1×10^6/ml) were stimulated with anti-IgM F(ab′)$_2$ Ab and were 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, and 1 μg/ml aprotinin) [20]. Western blot analysis was carried out with Abs as follows: anti-phospho JNK1/2 and anti-Bim Abs (Cell Signaling Technology, Beverly, MA, and Stressgen Bioreagents, Victoria BC, Canada). Each membrane was re-probed with anti-β-actin Ab (Sigma–Aldrich, St. Louis, MD).

Caspase-3 activity. An active form of caspase-3, consisting of 17- and 12-kDa subunits derived from a 32-kDa proenzyme, was detected with FITC-conjugated monoclonal Ab specific to active caspase-3 (BD Biosciences) by flow cytometry according to the manufacturer's protocol.

Introduction of exogenous G5pr expression in AICD-sensitive B-cell line. The pCXN2-*G5pr* expression vector constructed with the cDNA encoding full-length *G5pr* protein was transfected into WEHI-231 cell line (2×10^6 cells) with Nucleofector (Amaxa, Gaithersburg, MD) according to the optimized condition determined by the company (program; C-05). To enrich the transfectants with transient *G5pr* expression, the pCXN2-*EGFP* was co-transfected with a fivefold less amount (pCXN2-*G5pr:* pCXN2-*EGFP* = *5:1*) and incubated for 24 h in RPMI1640 complete medium. After sorting of transected cells into GFP^{+} and GFP^{-} fractions, their sensitivities to AICD were examined by stimulation with anti-IgM Ab (10 μg/ml) for 20 h. The cell death assay was performed by staining with PI. The transfectant with pCXN2 vector only was used as a control.

Results

BCR-crosslinking induces the transcription of G5pr in mature spleen B-cells

First, we investigated the expression of *G5pr* in B-cells after BCR-crosslinking in vitro. Splenic B-cells were stimulated with anti-IgM Ab in vitro and their expression of *G5pr* transcripts was examined by RT-PCR. *G5pr* transcription gradually increased at 3 h and peaked at 24 h following BCR-crosslinking. This is shown in comparison with the control transcripts of *Pax5* and β-actin in Fig. 1A.

Next, we examined the induction of *G5pr* after purification of immature and mature B-cells from the spleen by sorting cells with IgM^{hi}IgD^{lo} and IgM^{lo}IgD^{hi} phenotypes (Fig. 1B). *G5pr* expression was not induced by BCR-crosslinking in IgM^{hi}IgD^{lo} B-cells, which underwent AICD without proliferating. In contrast, IgM^{lo}IgD^{hi} B-cells, that showed marked proliferation and less AICD, also had strong *G5pr* expression 48 h after BCR-crosslinking (Figs. 1C–E).

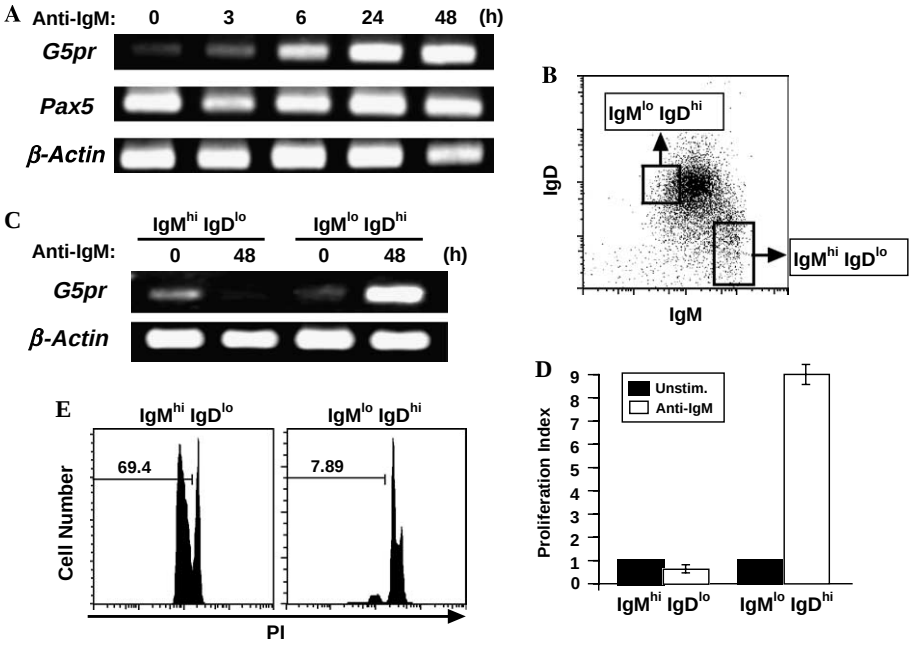


Fig. 1. Induction of *G5pr* in $IgM^{lo}IgD^{hi}$ B-cells. (A) Spleen B-cells (C57BL/6 mice) purified by negative selection with MACS magnetic beads were stimulated with 10 μg/ml of goat-anti mouse IgM $F(ab')_2$ Ab for the indicated period and the transcription of *G5pr* was evaluated by RT-PCR. The total RNA was isolated from the stimulated B-cells and RT-PCR was performed with the sets of gene-specific primers as described in Materials and methods. *Pax5* was used as the control. (B) Purified spleen B-cells were stained with FITC-anti-mouse IgM and PE-anti-mouse IgD Abs, and analyzed by flow cytometry with lymphoid gating using forward and side scatters. (C) Cells were further sorted into either $IgM^{lo}IgD^{hi}$ mature or $IgM^{hi}IgD^{lo}$ immature B-cell populations and the sorted B-cells were cultured at 1×10^5 cells/well for 48 h with or without 10 μg/ml of goat-anti-mouse IgM $F(ab')_2$ Ab. The induction of *G5pr* expression was determined by RT-PCR. (D) The cell proliferation was measured with Cell Proliferation Assay Kit. Proliferation index was calculated in comparison with unstimulated cells. (E) After 48 h culture, apoptotic cells of two B-cell populations were analyzed by flow cytometry with propidium iodide staining to measure DNA contents. The cells of subdiploid fractions were calculated as apoptotic cells.

BCR-induced G5pr upregulation is selective for mature spleen B-cells that are resistant to AICD in peripheral lymphoid organs

To investigate whether the upregulation of *G5pr* is seen only in B-cells that are resistant to BCR-induced AICD, we examined neonatal spleen B-cells with a virgin/transitional phenotype, which are extremely sensitive to BCR-induced AICD. The neonatal spleen B-cell population, mostly consisting of $IgM^{hi}IgD^{lo}$ cells (Fig. 2A), failed to proliferate on BCR-crosslinking (Fig. 2B) and was highly sensitive to AICD (56.7% versus 8.8% in adult spleen B-cells; Fig. 2C) [25]. The loss or absence of *G5pr* upregulation was correlated with the hyper-phosphorylation of JNKs and Bim. This further supports the notion that G5PR regulates the BCR-mediated AICD pathway in B-cells [18]. We examined whether neonatal spleen B-cells and immature $IgM^{hi}IgD^{lo}$ B-cells of adult mice had similar *G5pr* upregulation and activation states of JNKs and Bim. It was found that BCR-crosslinking did not induce *G5pr* upregulation in neonatal spleen B-cells (Fig. 2D). Interestingly, JNKs (JNK1 and JNK2) were constitutively strongly activated in neonatal spleen B-cells. BCR-crosslinking slightly induced further activation of JNKs in neonatal B-cells (Fig. 2F) but did not cause prolonged activation of Bim (Fig. 2E). The activation status of JNKs and Bim has not been previously established in neonatal B-cells. Koncz et al. [26] reported that BCR-mediated signals induced JNK phosphorylation in autoreconstituted spleen B-cells but this phosphorylation was not long lasting, ceasing after 2 h as in mature B-cells. Here, neonatal spleen B-cells manifested continued JNK phosphorylation upon BCR-crosslinking, suggesting that the survival pathways are not identical in neonatal and adult immature B-cells.

Next, we examined the upregulation of *G5pr* in immature splenic B-cells generated in autoreconstituted RAG1/GFP knockin mice. Fourteen days after sublethal irradiation, these mice had reduced numbers of spleen B-cells (≈1/5 of the untreated littermate) but allowed the generation of newly synthesized immature B-cells in the spleens. Numbers of mature B-cells with a $IgM^{lo}IgD^{hi}$ phenotype decreased to about 1/4 of the control mice (Fig. 3A, C versus A) and, reciprocally, immature B-cells with $IgM^{hi}IgD^{lo}$ phenotype increased about 7 times in comparison to the control (Fig. 3A, D versus B). The GFP marker clearly distinguished the newly synthesized and recruited RAG1/GFP^+ $IgM^{lo}IgD^{hi}$ B-cells from the mature $IgM^{lo}IgD^{hi}$ B-cell subset. Approximately, 90% of $IgM^{lo}IgD^{hi}$ B-cells were newly recruited B-cells in irradiated mice (16.8% in non-irradiated control) irrespective of the surface phenotype of mature B-cells. The spleen B-cells from irradiated mice were functionally immature as evidenced by their low responses. They did not proliferate but underwent cell death upon BCR-crosslinking for 48 h (Figs. 3B and C).

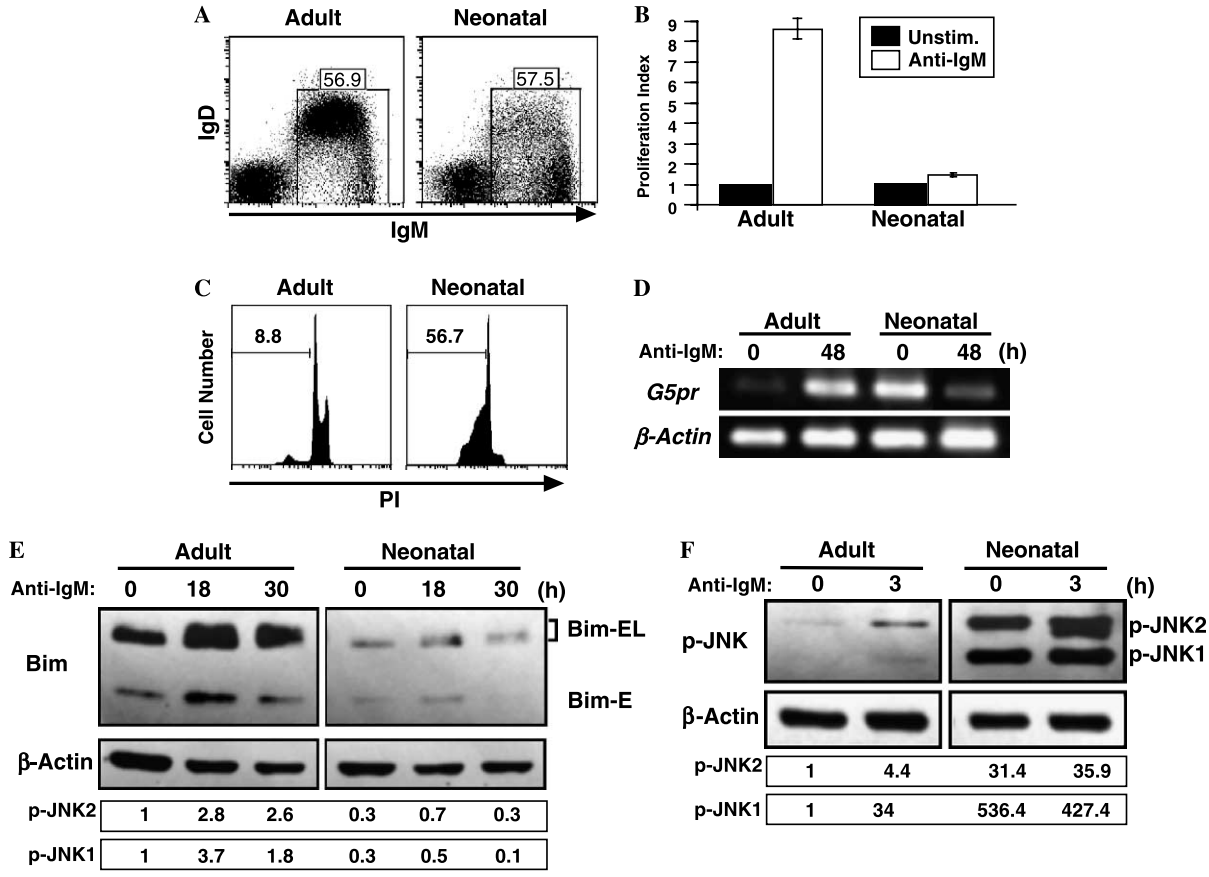


Fig. 2. Induction of *G5pr* upregulation in neonatal B-cells. Spleen B-cells from adult (8–12 weeks old) and newborn (within 4 days old) C57BL/6 mice were used. (A) The purified B-cells as described above were stained with FITC-anti-mouse IgM and PE-anti-mouse IgD Abs. (B) Purified B-cells at 1×10^5 cells/well were stimulated and the cell proliferation was measured as in Fig. 1. Proliferation index was calculated based on the proliferation of unstimulated cells as "1." (C) After 48 h culture, the apoptotic cells were analyzed by flow cytometry. (D) Purified B-cells were stimulated and the RNAs from cells were subjected to RT-PCR for *G5pr* induction. (E) Purified B-cells were stimulated for the time as indicated and the Bim expression was determined by Western blot analysis with anti-Bim Ab. The number in the bottom indicates the relative fold induction of Bim which is corrected with the intensity of β-actin and calculated based on the intensity of unstimulated cells as "1." (F) Purified spleen B-cells were stimulated as described above for 3 h and the expression of phosphorylated JNK was determined by Western blot analysis with anti-phospho JNK-specific Ab. The relative intensity of phospho-JNK was calculated as described above.

No inducible expression of *G5pr* was observed in these newly synthesized and reconstituted B-cells (Fig. 3D).

G5pr upregulation is downstream of the BTK pathway and is restricted to the mature IgD^{hi}CD23^{hi} B-cell subset

In peripheral lymphoid organs, immature B-cells further differentiate into a functionally mature B-cell subset. The later stage of B-cell maturation also depends on BCR-crosslinking as demonstrated by the impairment of X-linked immunodeficient (*xid*) B-cells [27,28]. Spontaneous mutation of BTK found in *xid* mice (CBA/N) results in a slight reduction of mature B-cells, which have an impaired response to BCR-crosslinking [29]. We purified splenic B-cells with IgD^{hi}CD23^{hi} and IgD^{hi}CD23^{lo} phenotypes. The IgD^{hi}CD23^{hi} B-cells are more mature because they express low levels of RAG1/GFP in contrast to the high-level expression of GFP in the IgD^{hi}CD23^{lo} B-cells (Figs. 4A and B). The *xid* B-cells with a mature IgD^{hi}CD23^{hi} phenotype did not proliferate (Fig. 4C) but underwent AICD on BCR-crosslinking (Fig. 4D) in contrast to wild-type B-cells. Upregulation of *G5pr* transcripts was not induced in *xid* B-cells despite their mature IgD^{hi}CD23^{hi} B-cell phenotype (Fig. 4E). These results not only confirmed that G5PR is inducible in functionally mature B-cells but also suggested that proximal events of BCR-signal transduction, in particular, downstream of BTK, are needed for induction of *G5pr*.

JNK activation induced by BCR-crosslinking is independent of BTK [30]. However, when we compared the kinetics of JNK phosphorylation in B-cells from C57BL/6 and CBA/N mice, phospho-JNK1 and JNK2 were detectable for a longer period in *xid* B-cells than in C57BL/6 B-cells (Fig. 4F). The BTK-dependent pathway is indispensable for *G5pr* induction in mature B-cells: the expression and activity of G5PR may be involved in a pathway inhibiting signals of JNK activation. In contrast to JNK phosphorylation, Bim expression was hard to

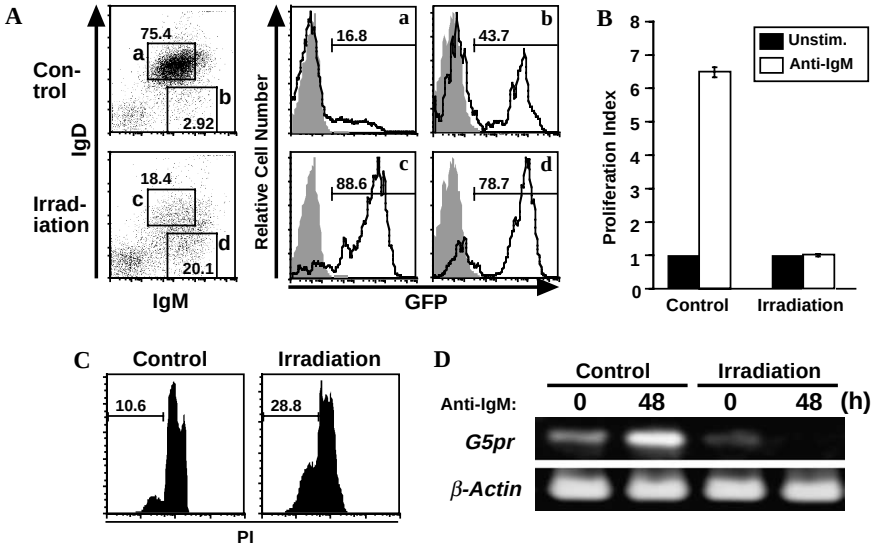


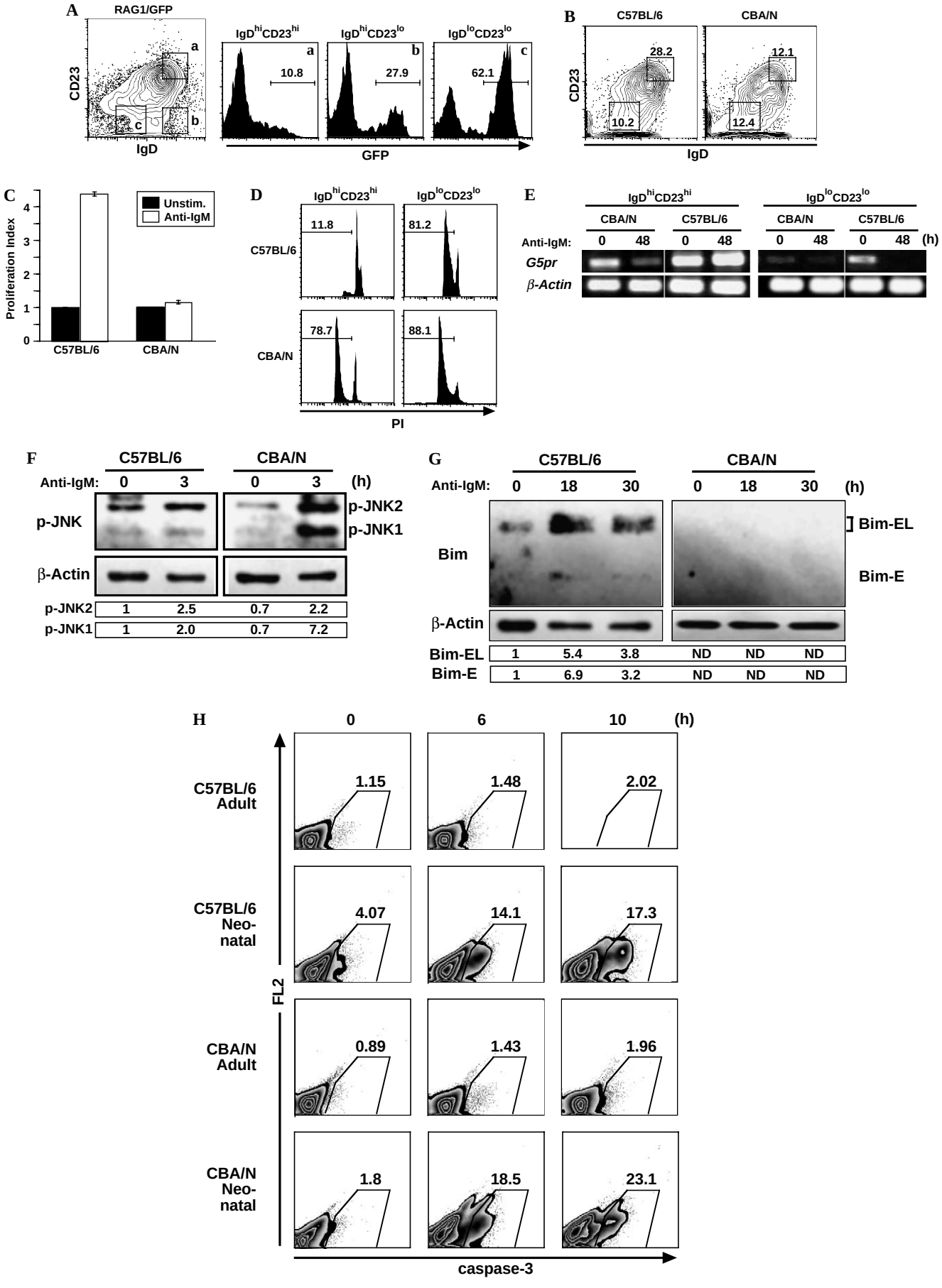
Fig. 3. *G5pr* upregulation in newly synthesized immature B-cells. Adult RAG1/GFP reporter mice were irradiated at 500 rad, and the spleens were removed after 14 days. Non-irradiated reporter mice were used for controls. (A) The purified spleen B-cells were stained with APC-anti-mouse IgM and PE-anti-mouse IgD Abs. The expression of RAG1/GFP signal was analyzed after gating the B-cells as $IgM^{lo}IgD^{hi}$ mature B-cell population (a and c) and $IgM^{hi}IgD^{lo}$ immature B-cell population (b and d). The GFP-negative signal was based on the results of C57BL/6 mice. The number in FACS profile indicates the percentage of cell population. (B) Purified B-cells at 1×10^5 cells/well were stimulated for 48 h and the cell proliferation was measured as described. (C) After 48 h, apoptotic cells were measured by flow cytometry. (D) Purified B-cells stimulated as above were harvested and the RNAs from cells were subjected to RT-PCR.

detect in *xid* B-cells after BCR-crosslinking as similar to the downregulation of Bim observed in neonatal B-cells (Fig. 4G).

The principal pro-apoptotic function of Bim is exerted via its binding to Bcl-2 or other anti-apoptosis family members, leading to mitochondrial depolarization, inducing the release of cytochrome-*C* and activating Apaf-1. Activated Apaf-1 initiates sequential activation of caspase-dependent cell death pathway. $G5PR^{-/-}$ B-cells did not show activation of caspase-3 regardless of facilitated BCR-induced AICD with hyper-activation of Bim, suggesting that G5PR regulates BCR-induced AICD through a mitochondria-dependent but caspase-independent mechanism [18]. We compared caspase-3 activity in the three different B-cell differentiation stages (Fig. 4H). Neonatal B-cells showed a marked increase of active caspase-3 at 6 and 10 h after BCR-crosslinking (14.1% and 17.3%, respectively). However, neither *xid* B-cells (1.43% and 1.96%) nor control wild-type adult B-cells (1.48% and 2.02%) showed such activation of caspase-3. This inability of caspase-3 activation in *xid* B-cells is not an intrinsic defect caused by the lack of Btk-mediated pathway because neonatal B-cells of *xid* mice also showed the similar level of caspase-3 activation (18.5% at 6 h and 23.1% at 10 h after stimulation) compared to that of control wild-type mice. These results clearly demonstrated that BCR-mediated apoptosis pathways involve different sets of cell death signaling pathways in the neonatal B-cells and mature-stage B-cells in the peripheral lymphoid organs.

These results suggested that B lineage cells undergo BCR-induced AICD through at least two different mitochondria-associated cell death pathways: one in neonatal spleen B-cells with caspase-3 activation and the prolonged hyper-activation of JNKs, and another in mature B-cells of

Fig. 4. Impaired induction of *G5pr* upregulation in *xid* B-cells. (A) Purified spleen B-cells from RAG1/GFP reporter mice were stained with PE-anti-mouse IgD Ab and biotin-conjugated anti-mouse CD23 Ab combined with streptoavidin–PerCP–Cy5.5. The $IgD^{hi}CD23^{hi}$ B-cell population (a), $IgD^{hi}CD23^{lo}$ B-cell population (b), and $IgD^{lo}CD23^{lo}$ B-cell population (c) were gated, and the expression of GFP signal within each gate was compared as histogram. The number in FACS profile indicates the percentage of cells in the population. (B) Purified spleen B-cells from C57BL/6 and CBA/N mice were stained as described. $IgD^{hi}CD23^{hi}$ follicular mature B-cell population and $IgD^{hi}CD23^{lo}$ transitional immature B-cell population were gated and the number in FACS profile indicates the percentage of cell population. (C) B-cells from C57BL/6 and CBA/N mice were stimulated for 48 h and the cell proliferation was measured in comparison with the control cells. (D) B-cell subpopulation gated in (B) was further purified, stimulated for 48 h, and the apoptotic cells were evaluated by flow cytometry. The number in FACS profile indicates the percentage of cells in the cell population. (E) The total RNA was isolated from the sorted B-cells and stimulated as described above. The induction of G5PR upregulation was evaluated by RT-PCR. (F) Purified spleen B-cells from C57BL/6 and CBA/N mice were stimulated similarly. The extent of phosphorylated JNK was determined by Western blot analysis with anti-phospho JNK-specific Ab. The number in the bottom indicates the relative fold induction of phospho JNK which was corrected with the intensity of β-actin and calculated based on the intensity of unstimulated cells as "1." (G) Purified spleen B-cells from C57BL/6 and CBA/N mice were stimulated similarly. The Bim expression was determined by Western blot analysis with anti-Bim Ab and quantity analysis was performed as described in (E). (H) Spleen B-cells from neonatal and adult mice were purified by negative selection with MACS magnetic beads and were stimulated with 10 μg/ml of goat-anti mouse IgM $F(ab')_2$ Ab for the indicated periods and the activated caspase-3 was detected by flow cytometry with FITC-conjugated anti-activated form of caspase-3.


A
RAG1/GFP
CD23
IgD
a
b
c
IgD^hiCD23^hi
IgD^hiCD23^lo
IgD^loCD23^lo
10.8
27.9
62.1
GFP
B
C57BL/6
CBA/N
28.2
12.1
10.2
12.4
CD23
IgD
C
Unstim.
Anti-IgM
Proliferation Index
C57BL/6
CBA/N
D
IgD^hiCD23^hi
IgD^loCD23^lo
C57BL/6
CBA/N
11.8
81.2
78.7
88.1
PI
E
IgD^hiCD23^hi
IgD^loCD23^lo
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β-Actin
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CBA/N
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p-JNK2 1 2.5 0.7 2.2
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C57BL/6 Adult
C57BL/6 Neo-natal
CBA/N Adult
CBA/N Neo-natal
1.15
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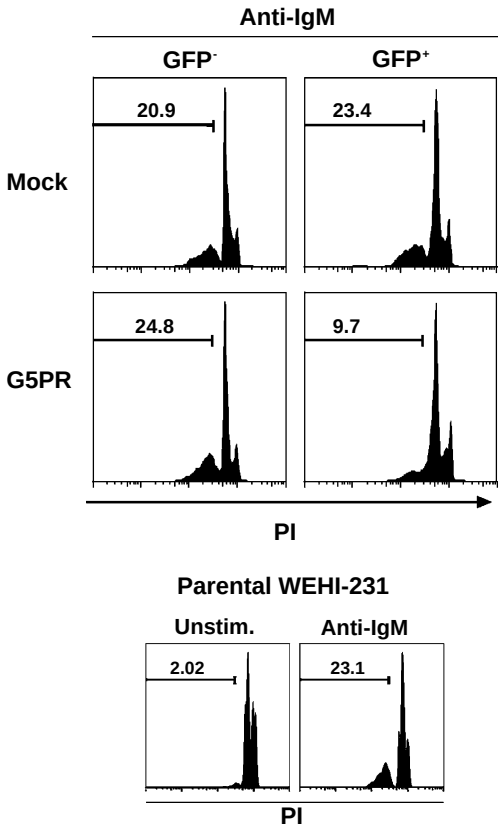

Fig. 5. Effect of G5PR expression on BCR-induced AICD in the B-cell line WEHI-231. Mammalian expression vector encoding either GFP or G5PR protein was co-transfected into WEHI-231 and the GFP^+ cells were purified by cell sorting 24 h later and cultured for another 20 h with 10 μg/ml of goat-anti-mouse IgM $F(ab')_2$ Ab.

adult mice with caspase-3-independent pathway but is associated with activation of Bim and JNKs. The expression of *G5pr* is dependent on the differentiation stage of B-cells and an intact BTK-dependent BCR-signaling pathway in mature B-cells.

In the neonatal stage, BCR-crosslinking induces the prolonged activation of JNKs presumably in the absence of *G5pr*. In the mature stage of adult mice, BCR-crosslinking induces a short period activation of JNKs and Bim. The initial induction of Bim might be regulated directly by the BTK-dependent pathway, while the later and prolonged activation of Bim in mature B-cells might be under the regulation of G5PR-dependent phosphatase activity.

G5PR is involved in BCR-mediated AICD in B-cell line

The *G5pr* knockout mice demonstrated that lack of G5PR caused B-cells extremely sensitive to BCR-mediated AICD in vivo and ex vivo [18]. We attempted to examine this effect in a B-cell line WEHI-231 with immature B-cell phenotype that has been characterized as a model of BCR-mediated AICD [31,32]. WEHI-231 cells were co-transfected with pCXN2-*G5pr* and pCXN2-*EGFP*. Then, the GFP^+ cells were enriched by sorting and stimulated with anti-IgM Ab for 20 h. As expected, the GFP^+WEHI-231 cells became resistant to BCR-induced AICD with the decrease of subdiploid apoptotic cells, indicating that exogenous introduction of *G5pr* rescues the cells from AICD (Fig. 5). The GFP only control excluded non-specific effect, and BCR-crosslinking reduced the viability of GFP^- WEHI-231 cells (20.9% of Mock and 24.8% of G5PR transfection) as comparable to the response of parental WEHI-231 cells. Taken, together, it became clear that BCR-induced alteration of G5PR is responsible for sensitivity of B-cells to BCR-mediated AICD.

Discussion

Selection of "useful" B-cells and elimination or silencing of "harmful" B-cells is a main tenet of self-tolerance and this is accomplished at relatively immature stages of B-cell development. The susceptibility of immature B-cells to apoptosis following stimulation via their Ag receptor seems to be essential to exclude auto-reactive B-cells whose receptors recognize "self" Ags. Many intrinsic differences of immature and mature B-cells have been proposed, and BCR-crosslinking of immature B-cells fails to induce various biochemical changes like that of in mature B-cells such as phosphoinositide hydrolysis, upregulation of early immediate gene of *egr-1* and *c-fos* [25], upregulation of MHC-class II molecules [33], and transient phosphorylation of Erks and Akt [21].

Here, we demonstrate that BCR-crosslinking induces the transcription of protein phosphatase component G5PR as an activation-induced molecule in mature B-cells. The upregulation of *G5pr* is correlated with resistance to AICD upon BCR-crosslinking. Immature B-cells from neonatal mice, newly generated transitional B-cells, and follicular B-cells with low CD23 expression do not show such a high level *G5pr* upregulation in vitro. These B-cell subpopulations are all highly sensitive to AICD induced by BCR-crosslinking. The high level upregulation of *G5pr* is selective for the mature B-cell subpopulation with an $IgM^{lo}IgD^{hi}CD23^{hi}$ phenotype, which is resistant to AICD induced by BCR-crosslinking in vitro. These observations are in accordance with the results of studies on $G5pr^{-/-}$ B-cells [18].

G5pr was not upregulated in *xid* B-cells, suggesting that the BCR-induced G5PR upregulation is downstream of BTK signal transduction pathways. However, the expression of Bim was not detected in the *xid* B-cells, although the absence of *G5pr* upregulation mostly correlated with increased and prolonged Bim activation after BCR-crosslinking. Bim activation might be regulated by at least two BCR-mediated pathways involving initial *Bim* transcription and induction of Bim protein in mature B-cells as the earlier event and the modification of Bim by protein phosphorylation or by as yet unknown mechanisms in the later stage of activation following BCR-crosslinking. G5PR might rather be involved in the regulation of the later stage Bim activation or inactivation processes.

The absence of G5PR affected the number of mature B-cells in the peripheral lymphoid organs of B-*G5pr*$^{-/-}$ mice. BTK is located upstream of various pathways involving the Erk-, NF-κB-, and NF-AT pathways, which regulate B-cell development, proliferation, and survival [34,35]. STAT5 has been suggested as a possible target for downstream signaling of BTK [36]. Consistent with this notion, there is a STAT5 binding motif (TTCGGAGAA)(−551 bp) in the 5′-flanking region of the mouse *G5pr* gene (GenBank Accession No. NM021529). It will be important to study the signal transduction pathway in detail that is involved in the upregulation of *G5pr* transcription upon BCR-crosslinking of mature B-cells. The effect of *G5pr* expression was confirmed in a B-cell line WEHI-231. The results suggested that G5PR regulates signal transduction molecules involved in BCR-induced AICD in B lineage cells.

G5pr expression is induced in IgD^{hi} circulating/follicular mature B-cells on BCR-crosslinking but the same stimulus does not induce *G5pr* upregulation in IgD^{lo} transitional immature B-cells. In addition, G5PR deficient B-cells are highly sensitive to BCR-induced cell death [18]. Considering these observations, it is likely that the induced expression of G5PR has a role in protecting Ag-reactive mature B-cells from death during the selection phase in germinal centers, where B-cells with high affinity BCR against specific Ags are enriched. In fact, *G5pr* expression was upregulated in B220^{+}GL7^{+} germinal center B-cells after immunization with T-cell dependent Ag, sheep red blood cells (data not shown). And B-*G5pr*$^{-/-}$ mice showed shrinkage of germinal centers and decreased numbers of extra-follicular B-cells after immunization [18]. These results suggest that B-cell survival is strictly regulated by protein phosphatase activity in mature B-cells during the immune response and is potentially important for the maintenance of the B-cell repertoire in the central and peripheral lymphoid organs.

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