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Transgenic Overexpression of G5PR That Is Normally Augmented in Centrocytes Impairs the Enrichment of High-Affinity Antigen-Specific B Cells, Increases Peritoneal B-1a Cells, and Induces Autoimmunity in Aged Female Mice

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To investigate signals that control B cell selection, we examined expression of G5PR, a regulatory subunit of the serine/threonine protein phosphatase 2A, which suppresses JNK phosphorylation. G5PR is upregulated in activated B cells, in Ki67-negative centrocytes at germinal centers (GCs), and in purified B220+CD11c+GL7+ mature GC B cells following Ag immunization. G5PR rescues transformed B cells from BCR-mediated activation-induced cell death by suppression of late-phase JNK activation. In G5PR-transgenic (G5PRTg) mice, G5PR overexpression leads to an augmented generation of GC B cells via an increase in non-Ag–specific B cells and a consequent reduction in the proportion of Ag-specific B cells and high-affinity Ab production after immunization with nitrophenyl-conjugated chicken γ-globulin. G5PR overexpression impaired the affinity–maturation of Ag-specific B cells, presumably by diluting the numbers of high-affinity B cells. However, aged nonimmunized female G5PRTg mice showed an increase in the numbers of peritoneal B-1a cells and the generation of autoantibodies. G5PR overexpression did not affect the proliferation of B-1a and B-2 cells but rescued B-1a cells from activation-induced cell death in vitro. G5PR might play a pivotal role in B cell selection not only for B-2 cells but also for B-1 cells in peripheral lymphoid organs. The Journal of Immunology, 2012, 189: 1193–1201.

A

rtigen stimulation activates naïve B cells expressing Ag-specific BCRs in peripheral lymphoid organs. Activated B cells proliferate rapidly and undergo secondary Ig V region diversification with somatic hypermutation and class-switch recombination in the germinal center (GC) (1–3). B cells differentiate as proliferating centroblasts in the GC dark zone and are found as cell cycle-arrested centrocytes in the GC light zone (4–6). GC B cells expressing high-affinity and isotype-switched BCRs are thought to be selected at the centrocyte stage by follicular dendritic cells (FDCs) presenting opsonized Ag complexes on complement receptors, thus enriching these B cells and allowing for production of high-affinity Abs against the immunizing Ag (7, 8).

During the maturation of Ag-specific B cells, centrocytes that properly react with opsonized Ag complex presumably receive survival signals from follicular Th (Tfh) cells in GCs (8). BCR stimulation induces either B cell proliferation or activation-induced cell death (AICD) through various BCR-mediated signaling pathways, including levels of intracellular Ca2+ mobilization and activation of MAPKs and NF-κB, which are regulated by the phosphorylation status of various signaling molecules (9–12). In BCR-mediated MAPK signaling, ERK is involved in cell proliferation (13, 14), and JNK and p38 are involved in stress response and cell death (15–17). Many studies have suggested that B cell selection is attributable to a balanced regulation of activation strengths between cell survival and apoptotic signals (18–21).

We sought to identify molecules that are expressed or upregulated selectively at the centrocyte stage and regulate the phosphorylation status of BCR-mediated signal transduction molecules. G5PR, a B′′ regulatory subunit of the serine/threonine protein phosphatase 2A, is upregulated in mature B cells after BCR crosslinking via Btk signaling (22, 23). B cell-specific gspr knockout mice show impaired B cell maturation, but no apparent abnormalities in BCR-mediated proliferation and related downstream signals, including tyrosine phosphorylation, Ca2+ influx, and MAPK or cyclin D2 activation (22). B cells in these knockout mice displayed prolonged activation of JNK and an increase in Bim phosphorylation after BCR crosslinking, resulting in depolarization of the mitochondrial membrane and an increased sen-
sitivity to BCR-mediated apoptosis. These results suggest that G5PR is necessary to maintain B cells by suppressing BCR-mediated AICD through regulation of the phosphorylation status of JNK and the proapoptotic molecule Bim.

In this study, we observed that G5PR expression is markedly augmented in the centrocytes of GCs after immunization with T cell-dependent Ag (TD-Ag) in mice. Mature GC B cells showed a marked increase in g5pr transcript in vivo, suggesting a positive role for G5PR in the selection of B cells in the peripheral lymphoid organs. To determine the role of G5PR upregulation in B cell survival, we generated mice expressing a g5pr transgene (Tg) (G5PR Tg) selectively in B cells and examined the effect of G5PR overexpression on the generation of high-affinity Ag-specific B cells.

Materials and Methods

mAb against G5PR

Recombinant G5PR protein with GST (GST-G5PR) was expressed using an *Escherichia coli* expression system and purified with Glutathione Sepharose 4B (GE Healthcare, Buckinghamshire, U.K.) (24). Lewis rats were immunized with the protein together with Freund’s complete or incomplete (for boosting) adjuvant. Splenocytes from immunized animals were cultured in the presence of 3 mM 2-mercaptoethanol and screened by PCR according to standard procedures (26) and recognized by ELISA. The specificity of the mAb was confirmed by its recognition of the G5PR protein (53 kDa) introduced in g5pr-cDNA-transfected WEHI-231 cells.

Mice and generation of G5PR Tg mice

C57BL/6 and New Zealand Black (NZB) mice were purchased from Kyudo (Fukuoka, Japan). For the Tg vector, g5pr cDNA was inserted into the BamHI site of p1026x vector (kindly provided by Dr. Satoshi Takaki, National Center for Global Health and Medicine, Shinjuku-ku, Tokyo), which contains the murine *lck* proximal promoter, *immunoglobulin H*-chain gene intronic enhancer, and mutated nontranslational form of human growth hormone gene (25). G5PR Tg mice were established on a C57BL/6 background according to standard procedures (26) and screened by PCR using primers for the human growth hormone gene: human growth hormone-Fw, 5'-GTGAAATTGTTCAGCAATCT-3' and human growth hormone-Rv, 5'-TCTATTCCGACACCCTCACA-3'. G5PR Tg mice and litters (8–12 wk) were immunized with the TD-Ag SRBC (Nippon National Center for Animal Resources and Development regulations (Center for Animal Resources and Development, Biosearch Technologies, Novato, CA). All mice were maintained under the following conditions in the Center for Animal Resources and Development, University of Tokyo. All procedures were carried out according to the manufacturer's protocol. Cell sorting of peritoneal B1a (CD45R+B220+), peritoneal non-GC (B220+; Fas GL7+) B cells, and Tfr (CD4+PD-1+CXCR5+) cells was performed using the JSAN Cell Sorter System (Bay Bioscience, Kobe, Japan).

Cell culture, retroviral infection, and in vitro stimulation

WEHI-231 B cells and primary B-1 and B-2 cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated FCS (Thermo Trace, Waltham, MA), 2 mM L-glutamine, 50 μM 2-ME (Wako, Osaka, Japan), 200 μM penicillin G potassium, 100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO), and 10 mM HEPES (Invitrogen). Retroviral infection, g5pr cDNA with a 3XFLAG-tag sequence at the 5'-end was inserted into the Sall site of a pFB-1RES-GFP retroviral vector (Agilent Technologies, Santa Clara, CA) and transfected into PLAT-E (Adgentic Technologies, Santa Clara, CA) and/or 1 μg/ml anti-Cd40 mAb (sCd40; purified from LB429 culture supernatant) at several time points. After 24 h stimulation, cultures were washed, and cultures were fixed and stained with propidium iodide (PI), and flow cytometric analysis was performed on a FACScalibur, using CellQuest software (BD Biosciences), and data were analyzed with FlowJo software (Tree Star, Ashland, OR). Splenocyte B-2 cells were isolated using a mouse B cell isolation kit and an autoMACS separator (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s protocol. Cell sorting of peritoneal B1a (CD45R+B220+), GC (B220+Fas GL7+) B cells, and non-GC B cells were assessed for apoptotic cells by staining with Annexin V-FITC, 7-AAD, and HRP-conjugated streptavidin (eBioscience, San Diego, CA), and flow cytometric analysis was performed on a FACScalibur, using CellQuest software (BD Biosciences), and data were analyzed with FlowJo software (Tree Star, Ashland, OR). Splenocyte B-2 cells were isolated using a mouse B cell isolation kit and an autoMACS separator (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s protocol. Cell sorting of peritoneal B1a (CD45R+B220+) and/or 1 μg/ml anti-IgM or 5 μg/ml LPS in 96-well plates for 48 h and pulsed with [3H]-thymidine deoxyribose (TdR) during the last 16 h of the culture. After stimulation, cells were harvested onto glass fiber filters (Applied Biosystems), and incorporation of [3H]-TdR was measured using a scintillation counter (MicroBeta 1450; Wallac, Turku, Finland).

Preparation of RNA, RT-PCR, and quantitative RT-PCR

Total RNA was purified using an RNeasy Micro Kit (Qiagen, Hilden, Germany). The cDNAs were prepared with SuperScript III (Invitrogen) and then amplified by PCR with Z-taq (Takara Bio, Ohtsu, Japan) and incorporation of [3H]-TdR was measured using a scintillation counter (MicroBeta 1450; Wallac, Turku, Finland).

Western blot analysis

Cells were lysed with TNE buffer (50 mM Tris-HCl, pH 7.5; 1 mM EDTA) containing a protease inhibitor mixture (Nacalai tesque, Kyoto, Japan). The lysate was subjected to SDS-PAGE and then transferred to a nitrocellulose membrane (Protran, GE Healthcare). The membrane was blocked with primary Abs against p-ERK, p-p38, p-Akt, and p-JNK (Cell Signaling Technology, Danvers, MA), Bim

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Anti-dsDNA Abs were detected by ELISA, as described previously (27). Briefly, serially diluted sera were incubated for 1 h at room temperature in 96-well plates coated with 250 ng/dsDNA (Sigma-Aldrich). After washing, the captured Ab was reacted with alkaline phosphatase-anti-mouse-IgG (µ-chain specific; Sigma-Aldrich) in combination with p-nitrophenyl peroxidase substrate (Sigma-Aldrich). The Ag–Ab reaction was measured by the absorbance at 405 nm, using an ImmunoMini NJ-2300 plate reader (System Instruments, Tokyo, Japan). An arbitrary unit for anti-dsDNA titer was defined by the serial dilution of pooled sera from NZB mice (10 mice) >40 wk of age.

Affinity measurement of anti-4-hydroxy-3-nitrophenyl acetyl Abs

To detect 4-hydroxy-3-nitrophenyl acetyl (NP)-specific Abs, serially diluted sera were incubated in 96-well plates coated with 10 µg/ml NP2-BSA or NP25-BSA (Bioresearch). After washing, the captured Ab was incubated with alkaline phosphatase-anti-mouse-IgG (γ-chain specific; Sigma-Aldrich) in combination with p-nitrophenyl peroxidase substrate. The Ag–Ab reaction was calculated by measuring the absorbance at 405 nm, using an ImmunoMini NJ-2300 plate reader. Absolute anti-NP Ab titers were defined by the serial dilution of sera from wild-type (WT) mice. The relative affinity of the anti-NP Ab was estimated by calculating the ratio of the ELISA measurements as anti-NP/anti-NP25.

Immunofluorescence analysis for anti-nuclear Abs

Anti-nuclear Ab was detected using an ANA-HEp-2 Immunofluorescence Kit (ORGENTEC Diagnostika, Mainz, Germany), according to the manufacturer’s protocol. Briefly, 80-fold diluted sera were reacted with HEP2 cells on the slide for 30 min at room temperature. After washing with PBS, Ag–Ab complex was detected with Alexa 488-conjugated anti-mouse IgG Ab. The signal was observed using a fluorescence microscope BZ-8000.

Statistical analysis

Data are presented as the mean ± SD. Statistical analysis was performed by Student t test. A p value < 0.05 was considered statistically significant.

Results

Upregulation of G5PR expression in GC B cells

We prepared a mAb against G5PR for immunohistochemical staining and analyzed the location of G5PR+ cells in the spleen after immunization with SRBC. We observed G5PR+ cells in the follicular region, which contained PNA+ GC B cells surrounded by IgD+ B cells (Fig. 1Aa, Fig. 1Ab), and in the GC region, which contained a CD35+ FDC network (Fig. 1Ac), but not in the splenic white pulp area containing CD11b+ macrophages and CD11c+ dendritic cells (Fig. 1Ad, Fig. 1Ae). G5PR+ B cells could be distinguished from IgD+ B cells and Ki67+ proliferating centroblasts in GCs (Fig. 1Ba, Fig. 1Bb) but could be seen interacting with CD35+ T cells in the follicular region (Fig. 1Be and Fig. 1Bf). Some G5PR+ cells coexpressed IgG1 in GCs (Fig. 1Bg and Fig. 1Bh) and interacted with FDCs, as shown by a merged yellow signal (Fig. 1Bi). We confirmed the interaction further, two-color imaging of the interactions between G5PR+ cells and FDCs (shown by a merged yellow signal) was demonstrated clearly by Z-stack analysis (Fig. 1C).

Comparative analysis with that of unstimulated B cells. (E) C57BL/6 mice were immunized with SRBC for 14 d. B220+ FAS+ GL7+ GC B cells were sorted as described in Supplemental Fig. 1. The amount of g5pr transcript was compared with that of B220+ FAS+ GL7+ B cells. Results are shown as the mean ± SD of three independent experiments. *p < 0.05.
We next examined G5PR upregulation in splenic B cells cultured in vitro by qRT-PCR. B cells stimulated with αIgM showed a modest increase in g5pr transcription (Fig. 1D), which was further increased by costimulation with αIgM and anti-CD40 mAb. B220⁺Fas⁺GL7⁺ mature GC B cells purified from SRBC-immunized mice (Supplementary Fig. 1) showed significantly higher g5pr transcription, compared with B220⁺Fas⁻GL7⁻ B cells (Fig. 1E). Thus, G5PR is expressed at a high level in centrocytes that have undergone isotype maturation and are closely interacting with FDCs in GCs.

**Effect of G5PR upregulation upon BCR-mediated AICD in a B cell line**

To assess the effect of G5PR upregulation on B cell survival, we transfected WEHI-231 B cells with a FLAG-tagged G5PR vector coexpressing enhanced GFP using a bicistronic retroviral expression system and compared between cells transduced with this vector and enhanced GFP-only vector (Fig. 2A). The control and G5PR transfectants were examined for sensitivity to BCR-mediated AICD. Increased expression of G5PR significantly enhanced cell survival, as analyzed by PI staining (Fig. 2B).

**Effect of G5PR upregulation on BCR-mediated signal transduction**

The activation of various BCR-mediated signal transduction molecules was examined in G5PR transfectants. Phosphorylation of ERK, p38, Akt, or IkBα was not affected by G5PR expression (Fig. 2C). JNK phosphorylation was similar in control and G5PR transfectants at 5 min after stimulation, but enhanced JNK de-

**FIGURE 2.** Effect of G5PR upregulation on BCR-mediated AICD and signal transduction in WEHI-231 cells. (A) A schematic of the g5pr Tg construct. WEHI-231 cells were transfected with gfp (Ctrl) or flag-g5pr-ires-gfp (G5PR) constructs using a retroviral system, and GFP⁺ cells were sorted (purity > 90%). Expression of the introduced genes was analyzed by Western blotting against FLAG and GFP. β-Actin was used for loading controls. (B) Cell death of G5PR transfectants by BCR crosslinking. Cells were stimulated with αIgM for 24 h, and apoptotic cells in the control or G5PR transfectants were identified by PI staining and flow cytometric analysis. Results are shown as the mean ± SD of five independent experiments. **p < 0.01. (C–F) BCR-mediated activation of signal transduction molecules. Whole-cell lysates from control or G5PR transfectants stimulated with αIgM at the indicated time were blotted using Ab against p-ERK, p-p38, p-Akt, p-IκBα, Bim, and p-c-Jun. β-Actin was used for loading controls. Results are representative of three independent experiments. (G) Expression of bim transcripts. Control or G5PR transfectants were stimulated with αIgM for 12 h, and bim transcripts were measured by qRT-PCR. Results are shown as the mean ± SD of three independent experiments. *p < 0.05.
phosphorylation was seen at 60 min in the G5PR transfectants (Fig. 2D). Among the Bcl-2 family molecules, the expression of antiapoptotic molecules Bcl-2 and Bcl-xL was not altered after stimulation of cells transfected with either vector (data not shown), but G5PR transfectants showed a reduced level of Bim-EL expression at 12 h after αlgM stimulation (Fig. 2E). The phosphorylation of c-Jun, a downstream molecule of JNK, was also lower at 6 h after αlgM stimulation in G5PR transfectants (Fig. 2F). qPCR analysis showed that G5PR overexpression suppressed the augmentation of bim transcription caused by αlgM stimulation (Fig. 2G). Thus, although G5PR upregulation only modestly rescued B cells from rapid BCR-mediated apoptosis, it significantly altered BCR-mediated JNK-c-Jun activation, leading to altered transcription of target bim.

**Effect of increased G5PR expression on B cells in vivo**

To explore the effect of G5PR upregulation in B cells of peripheral lymphoid organs, we developed GSPRTg mice that overexpress g5pr transcripts in lymphoid cells under the lck promoter, IgH-

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**FIGURE 3.** BCR-mediated apoptosis of B-2 cells in GSPRTg mice. (A) A schematic diagram of the vector construct of the Tg used to generate GSPRTg mice. Full-length g5pr cDNA was inserted into a p1026x vector containing an Lck proximal promoter and an Eμ enhancer. Spleen B cells were purified from WT and GSPRTg mice. Tg expression was analyzed by RT-PCR for g5pr transcripts and by Western blotting for G5PR protein. Results are representative of two independent experiments. (B) Activation of JNK in splenic B cells. Splenic B cells of WT and GSPRTg mice were stimulated with αlgM for the indicated times. Whole-cell lysates were subjected to SDS-PAGE and analyzed by immunoblotting with anti–p-JNK Ab. Anti–β-actin mAb was used as the internal control. Results are representative of two independent experiments. (C and D) BCR-mediated AICD of splenic B cells. Splenic B cells from female WT or GSPRTg mice (12 wk after birth) were stimulated with αlgM for 24 h. Apoptotic cells were analyzed by induction of active caspase 3 (C) or Annexin VFITC and 7-AAD (D) staining by flow cytometry. (E) Proliferation of splenic B cells. Splenic B cells from female WT or GSPRTg mice (12 wk after birth) were stimulated with αlgM or LPS for 48 h. Incorporation of [3H]-TdR was measured using a scintillation counter. Results are representative of three independent experiments.

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**FIGURE 4.** GC formation and B cell selection in GSPRTg mice. (A) Generation of GC B cells after immunization. The GSPRTg mice and littermates (n = 4) were immunized with SRBC for 10 d, and the spleen sections were analyzed by immunohistochemistry with PNA and IgD staining. Left panel shows the size of each GC by measuring the area of PNA staining. Scale bars, 100 μm. Result is shown as the median ± SD. (B–D) The GSPRTg mice (n = 7) and littermates (n = 6) were immunized with NP-CGG in alum for 14 d, and spleen GC B cells were characterized by flow cytometry. The frequencies of B220+Fas+GL7+ GC B cells (B) and NIP+ GC B cells (C) were compared. NP-specific IgG titers were measured by ELISA plates coated with NP2-BSA (high-affinity) or NP25-BSA (low-affinity), and the relative affinity for NP-specific IgG was calculated by NP2/NP25. Results are shown as the mean ± SD, *p < 0.05, **p < 0.01.
chain gene promoter, and intronic enhancer elements (Fig. 3A). Increased G5PR expression was confirmed in G5PR^g^ murine splenocytes by RT-PCR and Western blot analysis. No obvious difference was observed in the number or proportion of lymphoid cells or in the development of B cells in the bone marrow and the spleen at 8–12 wk after birth, in comparison with that in WT littermates (Supplemental Fig. 2). Although an apparent inhibition of JNK activation was noted after αIgM stimulation (Fig. 3B),

**FIGURE 5.** Increase in peritoneal B-1a cells and an autoimmune phenotype in aged female G5PR^g^ mice. (A) Peritoneal cavity cells were isolated from young (8–12 wk) and aged (>40 wk) G5PR^g^ mice or littermates and stained with anti–CD5-PE, anti–CD11b-FITC, and anti-B220 allophycocyanin-conjugated Abs. The proportion of B-1a (CD5^+^CD11b^+^B220^+^), B-1b (CD5^−^CD11b^+^B220^+^), and B-2 (CD5^−^CD11b^−^B220^+^) cells was analyzed by flow cytometry. Results are representative of three independent experiments (left panel). The proportion of B-1a, B-1b, and B-2 cells is shown as the mean of each group (right panel, n = 5). (B) Proliferation of peritoneal B-1a cells. Peritoneal B-1a (CD5^+^B220^+^) cells of G5PR^g^ mice or littermates (n = 3 per each) were isolated using a JSAN Cell Sorter and stimulated with αIgM and LPS for 48 h. Incorporation of [3H]-TdR was measured as in Fig. 3E. (C) Peritoneal B-1a (CD5^+^B220^+^) cells of G5PR^g^ mice or littermates (n = 6) were isolated using a JSAN Cell Sorter and stimulated with αIgM for 24 h. Apoptotic cells were identified with Annexin V-FITC and 7-AAD staining. Results are representative of four independent experiments. (D) Peritoneal B-1a cells from G5PR^g^ mice or littermates were stimulated with αIgM for the indicated times, and JNK phosphorylation was monitored by immunoblotting for p-JNK. Results are representative of two independent experiments. (E) Peritoneal B-1a cells from G5PR^g^ mice or littermates were stimulated with αIgM for 24 h. Active caspase 3 was stained with mAb and detected by flow cytometry. Results are representative of three independent experiments. Original magnification ×200. (H) Detection of immune complex deposition in the kidney. Kidney sections from female G5PR^g^ mice and littermates were stained with periodic acid-Schiff (PAS), anti-IgG Ab or anti-C3 Ab, respectively. Scale bars, 100 μm.
spleen B cells from G5PR<sup>Tg</sup> mice showed neither an alteration in active caspase 3 (Fig. 3C) nor a difference in BCR-mediated AICD, in comparison with WT B cells (Fig. 3D). The proliferation potential of splenic B cells from G5PR<sup>Tg</sup> mice was similar to that of WT mice following stimulation with αlGm or LPS in vitro (Fig. 3E). These results imply that increased G5PR expression does not cause a marked change in the development of mature peripheral B cells or the survival of B cells under nonimmunized conditions.

However, when we immunized G5PR<sup>Tg</sup> mice with TD-Ag SRBC, we observed an altered formation of PNA<sup>+</sup> splenic GCs surrounded by Ig<sup>D</sup> B cells. The number of GCs was similar but significantly larger in comparison with that in WT mice (Fig. 4A).

Following immunization with NP-CGG in alum, the frequency of mature GC B cells expressing B220<sup>Fas<sup>+</sup>GL7<sup>+</sup></sup> markers was significantly increased, as compared with that in WT mice (Fig. 4B). However, the increase of mature GC B cells did not result in an increase in NIP-specific B cells. Instead, we observed a decrease in strongly Ag-binding B cells in G5PR<sup>Tg</sup> mice (Fig. 4C). The decrease of high-affinity Ag-binding B cells was also confirmed in the sera by a convenient affinity measurement, using the differential ELISA. The levels of high-affinity Abs measured by NP25-BSA were unchanged in G5PR<sup>Tg</sup> mice compared with WT mice, but the low-affinity Ab tiers measured by NP25-BSA were significantly higher in G5PR<sup>Tg</sup> mice than in WT mice (Fig. 4D). The overall affinity of the sera was calculated by the ratio of each measurement, NP<sub>2</sub>/NP<sub>25</sub>. These results suggest that increased G5PR expression in G5PR<sup>Tg</sup> mice affects the selection of Ag-binding B cells in GCs, perhaps by altering the threshold of BCR-mediated signal during the generation of high-affinity Ag-specific B cells.

Th<sub>1</sub> cells may play an important role in B cell maturation in GCs, in association with G5PR expression. The g5pr transcripts were upregulated in T<sub>FH</sub> cells in G5PR<sup>Tg</sup> mice. However, Ag immunization induced a similar number of T<sub>FH</sub> (CD4<sup>+</sup>PD-1<sup>+</sup> CXCR5<sup>+</sup>) cells in the spleens of G5PR<sup>Tg</sup> mice comparable to that in WT mice (Supplemental Fig. 3). Although we have not neglected the effects of G5PR on T<sub>FH</sub> cells in G5PR<sup>Tg</sup> mice, the G5PR overexpression on B cells is most likely the cause of impaired affinity–maturation of Ag-specific GC B cells in vivo.

**Effect of increased G5PR expression upon the number of peritoneal B-1a cells after aging**

A more dramatic change was observed in the G5PR<sup>Tg</sup> mice after aging. Aged mice (>40 wk), in comparison with WT littermates, showed a marked increase in B-1a cells in the peritoneal cavity (Fig. 5A). The proportion of B-1a (CD5<sup>+</sup>CD11b<sup>+</sup>B220<sup>+</sup>) cells, but not B-1b (CD5<sup>+</sup>CD11b<sup>+</sup>B220<sup>−</sup>) and B-2 (CD5<sup>−</sup>CD11b<sup>−</sup>B220<sup>+</sup>) cells, in the peritoneal cavity increased significantly in aged female G5PR<sup>Tg</sup> mice, although no increase was observed in mice at a young time point. To examine the proliferation capacity of B-1 cells, we isolated sufficient numbers of peritoneal B-1a cells from aged mice only and used them for comparison in the proliferation assay (Fig. 5B). B-1a cells from both G5PR<sup>Tg</sup> and WT mice did not respond to αlGm stimulation but did respond well to LPS stimulation. As LPS is an efficient stimulator of B-1 cells, compared with αlGm Ab in vitro (28), we concluded that the proliferation capacity of B-1a cells is not impaired in G5PR<sup>Tg</sup> mice (Fig. 5B). However, peritoneal B-1a cells, but not splenic B-2 cells, from aged female G5PR<sup>Tg</sup> mice were resistant to BCR-mediated AICD in vitro, in comparison with those of WT mice (Fig. 5C, Supplemental Fig. 4). JNK activation by αlGm stimulation was lower in peritoneal B-1a cells from WT mice, but was further decreased in the G5PR<sup>Tg</sup> mice during the period from 5 to 60 min after stimulation (Fig. 5D). The peritoneal B-1a cells from aged female G5PR<sup>Tg</sup> mice, in comparison with those from WT mice, also showed suppression in activation of caspase 3 after αlGm stimulation in vitro (Fig. 5E), suggesting that the peritoneal B-1a cells from G5PR<sup>Tg</sup> mice are under the continued suppression of BCR-mediated JNK activation, most likely leading to AICD.

As a persistent increase of B-1a cells might be associated with the development of autoimmunity, we examined autoantibody production in the aged G5PR<sup>Tg</sup> mice by ELISA with dsDNA. Female G5PR<sup>Tg</sup> mice showed higher levels of anti-dsDNA Ab than did female WT littermates (Fig. 5F). Female, but not male, G5PR<sup>Tg</sup> mice also produced anti-nuclear Abs (Fig. 5G). Moreover, immunohistochemical staining of the kidneys of aged female G5PR<sup>Tg</sup> mice showed stronger signals for immune complexes with IgG and C3 in glomeruli than were observed in WT mice (Fig. 5H). Male G5PR<sup>Tg</sup> mice did not show such autoantibody production, suggesting that generation of B-1a cells in G5PR<sup>Tg</sup> mice is affected by factors including sex hormone (or hormones) and aging, as has been previously described for B-1 cells from New Zealand Black (NZB) mice (29).

We next measured g5pr transcripts and cell survival potential of B-1a cells from NZB mice by qRT-PCR. B-1a cells from aged female NZB mice showed higher levels of g5pr transcription than did B-2 cells of NZB mice (Fig. 6A). The B-1a cells were more resistant to BCR-mediated AICD in vitro than were the B-2 cells (Fig. 6B), which correlated with lower levels of JNK activation upon BCR crosslinking in the B-1a cells (Fig. 6C).

Taken together, our results show that G5PR is a critical factor for selection of peripheral B cells during the physiological immune response, and abnormal G5PR upregulation is associated with the development of autoimmunity.
Discussion

G5PR is upregulated in mature GC B cells with B220^Fas^GL7^ phenotype during normal immune responses to TD-Ags. These G5PR^{high} B cells are selectively found at the centrocyte area of GCs. Centroblasts expressing Ki67, a marker of high proliferation potential (6), do not show G5PR upregulation. G5PR upregulation occurs in those B cells that have undergone class switching to IgG in the follicular region and appears in the Ki67^{+} region. In addition, G5PR^{high} B cells closely adhere to CD35^{+} FDCs in GCs. These results indicate that G5PR is upregulated markedly in the centrocytes of GCs and can be used as a centrocyte marker in combination with Ki67, the marker for centroblasts. G5PR upregulation was induced by IgM stimulation but was further augmented by αCD40 in vitro, suggesting that G5PR upregulation is reinforced by the interaction of B cells with FDCs and T_{FH} cells in GCs.

G5PR upregulation in centrocytes suggested to us that G5PR may have a cell stage-associated role in the regulation of BCR-mediated AICD for the selection of Ag-reactive GC B cells. Our results from transfecting WEHI-231 cells with a G5PR-overexpressing construct indicate that G5PR upregulation enhances B cell survival by inhibiting JNK phosphorylation. Unlike the case with transformed cells, young G5PR^{Tg} mice did not show marked changes in the number and differentiation of B-lineage cells, and overexpression of G5PR did not cause any significant changes in B cell survival compared with that in WT mice under nonimmunized conditions in vivo or after IgM stimulation of the Tg B cells in vitro.

We investigated whether the effect of G5PR upregulation appears during immune responses or is limited to the later response of BCR-mediated AICD. Upon immunization with TD-Ags, G5PR^{Tg} mice indeed showed an increase in mature GC B cells, but this was characterized by more low-affinity or non-Ag–binding B cells than were seen in WT mice. The induction of T_{FH} cells was not affected in the spleens of Ag-immunized G5PR^{Tg} mice. These findings suggest that the alteration of G5PR expression in GC B cells regulates BCR-mediated signals that might ultimately determine the threshold of high-affinity B cell selection in GCs.

Aged female G5PR^{Tg} mice displayed a marked increase in B-1a cells with resistance to AICD in the peritoneal cavity. It is possible that this resistance is related to altered signal thresholds for cell survival or apoptosis. Many studies have compared BCR-mediated signaling between B-1 and B-2 cells of various mutant mice. CD19-Tg mice display more B-1 cells and autoimmune phenotype (30). B-1 cells show constitutive activation of ERK and NF-AT, and induced the high level of ERK signaling, but not activation of p38 and NF-κB or delayed JNK activation after IgM stimulation (31). Btk-deficient mice exhibit a severe defect in B-1 cells (32). Further, the Scc family tyrosine kinase Lyn is expressed in B-1 cells and is also involved in the hyporesponsive phenotype of B-1 cells (33). These observations suggest that B-1 cells depend more critically on BCR signaling than do B-2 cells in the peripheral lymphoid organs.

In comparison with G5PR-deficient B cells (22), increased G5PR expression reversely affected the JNK pathway leading to BCR-mediated AICD. However, G5PR overexpression resulted in only modest differences in the initial activation of JNK and Bim phosphorylation in B-1a cells. In fact, JNK activation was mildly reduced in peritoneal B-1a cells from aged female G5PR^{Tg} mice at 60 min after IgM stimulation, and this suppression of JNK activation was sustained. These results are in accordance with previous observations that prolonged JNK activation mediates the signal for apoptosis (34). Importantly, G5PR overexpression markedly reduced the level of c-Jun activation induced by IgM stimulation, suggesting that the effect of G5PR overexpression was exerted predominantly via suppression of c-Jun target molecules. This idea may explain why G5PR^{Tg} mice show only a mild phenotype with increased survival of B-1a cells, whereas the survival of B-2 cells of G5PR-deficient mice is severely impaired (22).

B-1 cells of autoimmune-prone mice are resistant to BCR-mediated AICD in vitro (35). A number of studies have attempted to elucidate the molecular mechanism regarding this B-1 cell–specific resistance to apoptosis (31, 36). Our results show that an increased expression of the g5pr gene does not affect the proliferation of B-1a cells in response to LPS stimulation but is associated with increased resistance to BCR-mediated AICD in B-1a cells, which might be associated with the generation of autoantibodies and autoimmunity. Overexpression of the g5pr gene did not cause abnormalities in the cell number and maturation of B cells in young G5PR^{Tg} mice, suggesting that the increase in g5pr transcription might be within a level that can maintain normal proportions of B-1a, B-1b, and B-2 cells in mice. B-1a cells, however, became resistant to BCR-mediated AICD and increased in the peritoneal cavity in aged female G5PR^{Tg} mice, suggesting that the effect of G5PR upon BCR-mediated signaling is regulated by the age and gender. The endogenous G5PR gene is located on chromosome 12 in mice, and the G5PR^Tg is not linked to the X chromosome, as the G5PR^{Tg} mouse colony is maintained by backcrossing male G5PR^{Tg} mice with female WT mice.

Our results provide novel insights into the mechanism of B cell selection in GCs in peripheral lymphoid organs during immune responses to TD-Ags and into the generation of abnormal autoreactive B cell clones during aging. These findings suggest that investigating regulation of g5pr transcription might help further our understanding of the risk factors, onset, and molecular mechanisms involved in development of autoimmunity, potentially also assisting in the design of treatment for autoimmune diseases.

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Disclosures

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References


