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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

Masahiro Kitabatake,* Teppei Toda,* Kazuhiko Kuwahara,* Hideya Igarashi,*1 Mareki Ohtsuji,† 2 Hiromichi Tsurui, † Sachiko Hirose, † and Nobuo Sakaguchi*

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 Kit67-negative centrocytes at germinal centers (GCs), and in purified B220+Foxp3+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.

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tigen stimulation activates naive 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 processes.

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 transcription 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) (g5prTg) 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 fused with P1U1 myeloma cells to generate hybridoma cell lines. A clonal cell line secreting mAb against G5PR clone (IgG1) recognized the GST-G5PR 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 G5PRTg 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 icck 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'-GGTGAATTTGTCGAACCTTG-3'; human growth hormone-Rv, 5'-TCTATTCGACACCCTTCCA-3'. G5PR Tg mice and littermates (8-12 wk) were immunized with the TD-Ag SRBC (Nippon Biotest Lab., Tokyo, Japan) or nitrophenyl chicken y-globulin (NP-CGG; Biosearch Technologies, Novato, CA). All mice were maintained under specific pathogen-free conditions in the Center for Animal Resources and Biosearch Technologies, Novato, CA). The experiments were approved by the Laboratory Animal Ethics Committee of the University of Tokyo and performed in accordance with the guidelines of the Japanese Ministry of Education, Culture, Sports, Science and Technology.

Immunohistochemical analysis

Spleens were surgically excised from the mice, embedded in O.C.T. Compound (Sakura Finetech, Tokyo, Japan), and frozen immediately in liquid nitrogen. The frozen block was sliced into 6-m thick sections, using a Cryotome (Thermo Shandon, Cheshire, UK). Sections were fixed in 4% paraformaldehyde in PBS for 10 min, blocked with 3% FBS in PBS, and stained with the following reagents: anti-G5PR mAb, bio-PNA, FITC-anti-Ki67, bio-anti-CD35 (BD Biosciences, Franklin Lakes, NJ), and incorporated biotinylated anti-mouse IgG1 Ab and anti-C3 mAb. The sections were then amplified by PCR with Z-taq (Takara Bio, Ohtsu, Japan), using the primers 5'-GTGAATTTGTCGAACCTTG-3' and 5'-TCTATTCGACACCCTTCCA-3'. The amplified product was subjected to Southern blot analysis to confirm the presence of the transgene. The number of g5pr-expressing cells was determined by counting the number of brown spots in the sections. The sections were analyzed with a fluorescence microscope (Olympus, Tokyo, Japan). For the Z-stacks, the digital images were serially captured every 1-μm slice. For the detection of immune complexes in the kidney, sections were fixed in 4% paraformaldehyde for 10 min, blocked with Fc block (anti-CD16/32 mAb; BD Biosciences), and stained with Alexa 488-conjugated anti-mouse IgG Ab and anti-C3 mAb.

Flow cytometric analysis and cell sorting

Cell surface staining was performed with various combinations of mAbs after blocking with anti-CD16/32 mAb (eBioscience). The reagents used were as follows: FITC-anti-CD11b (BioLegend), FITC-anti-CD3, FITC-anti-CD4, FITC-anti-B220, PE-anti-CD5, PE-anti-CD25, PE-anti-CD95, PE-anti-CD20, allophycocyanin-conjugated anti-B220, allophycocyanin-conjugated anti-IgM (eBioscience), FITC-anti-GL7, PE-anti-Fas, PE-anti-CD1-PE, PECy7-anti-Fas, bio-anti-CD43, bio-anti-CXCR5 (BD Biosciences), PE-4-hydroxy-3-iodo-5-nitrophenylacetyl (NIP; kindly provided by Dr. Toshihiko Takemori, Riken Research Center for Allergy and Immunology, Yokohama City, Kanagawa, Japan), and streptavidin-PerCP-Cy5.5 (BD Biosciences). After washing, flow cytometric analysis was performed on a FACS Calibur, using CellQuest software (BD Biosciences), and data were analyzed with FlowJo software (Tree Star, Ashland, OR). Spleen 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 B-1a (CD5+ B220+), GC B cells (non-GC (B220′ Fas GL7) B cells), and Btg3 (CD4+PD-1+CXCR5) B 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 U/ml penicillin G, 100 μg/ml streptomycin (Meiji Seika, Tokyo, Japan), and 10 mM 2-ME (Invitrogen). For retroviral infection, g5pr cDNA with a 3xFLAG-tag sequence at the 5'-end was inserted into the Sall site of a pB-FIRE-GFP retroviral vector (Agilent Technologies, Santa Clara, CA) and transfected into PLATE-E ecotropic retrovirus packaging cells (kindly provided by Dr. Toshio Kitamura, University of Tokyo, Tokyo, Japan). After 2 d, retrovirus was recovered from culture supernatant, combined with 8 μg/ml polybrene (Sigma-Aldrich, St. Louis, MO), and used to infect WEHI-231 cells. GFP+ cells were sorted using a JSAN Cell Sorter System. For in vitro stimulation, cells were cultured at a density of 2 × 10^6 cells/ml with 10 μg/ml of Ab (ab)2 fragment of anti-IgM Ab (αIgM; ICN Biomedicals, Costa Mesa, CA) and/or 1 μg/ml anti-CD40 mAb (αCD40; purified from LB429 culture supernatant) at several time points. After 24 h stimulation, cultures were pulsed with [3H]-thymidine deoxyribose (Meiji Seika, Tokyo, Japan) or [3H]-thymidine (specific activity 1 Ci/mmol, [3H]-TdR; Nacalai Tesque, Kyoto, Japan). The lysate was subjected to SDS-PAGE 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, Shiga, Japan), using the primers 5′-GGTTAGCGTGGCACACAGC-3′ and 5′-GATTCTCTCGTAATTCCTG-3′ for g5pr, and 5′-CCAAGGCAACGGTGAAGGA-3′ and 5′-TCTTCATGTGTCCTAGGACCA-3′ for β-actin. Quantitative RT-PCR (qRT-PCR) was carried out using TaqMan gene expression assays (biotin, Mm00437796, g5pr, Mm01257828; β-actin, Mm00607939; Invitrogen) using the ABI7500 and Sequence Detection System software (Life Technologies, Grand Island, NY). The fold change in the copy number of the transcripts was calculated using the 2^(-ΔΔCt) method, with β-actin as the internal control.

Western blot analysis

Cells were lysed with TNE buffer (1% Nonidet P-40, 150 mM NaCl, 10 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, p-IkBa, p-JNK, p-Jun (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 10 μg/ml NP2-BSA or NP25-BSA (Bioresearch). After washing, the captured Ab was incubated 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 Ab**

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. Relative anti-NP Ab titer were determined 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 in 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+ T cells in the follicular region (Fig. 1Be and Fig. 1Bf). Some G5PR+ cells seem to attach to the CD4+ FDCs (Fig. 1Bc, Fig. 1Bd). G5PR+ 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+ FDCs (Fig. 1Bc, Fig. 1Bd). G5PR+ cells coexpressed IgG1 in GCs (Fig. 1Bg and Fig. 1Bh) and IgM and/or CD35 in centrocytes. G5PR+ cells seem to attach to the CD4+ FDCs (shown by a merged yellow signal) and interacted with FDCs, as shown in the tricolor image (white). To confirm 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).

**FIGURE 1.** G5PR upregulation in centrocytes. (A and B) C57BL/6 mice were immunized with SRBC for 10 d, and the follicular region of their spleens was analyzed by immunohistochemistry. (A) Immunohistochemistry for G5PR/IgD (a) and immunofluorescent staining for G5PR/IgD/PNA (b), G5PR/IgD/CD35 (c), G5PR/IgD/CD11b (d), and G5PR/IgD/CD11c (e). (B) The sections were stained for visualization of G5PR/Ki67/IgD (a, b), G5PR/Ki67/CD35 (c, d), G5PR/IgD/CD4 (e, f), and G5PR/CD35/IgG1 (g, h). Scale bars, 100 μm. The results are representative of three independent experiments. (C) The GC sections were stained with G5PR/CD35 and are shown as Z-stack images. (D and E) Measurement of g5pr transcripts by qRT-PCR. (D) Spleen B cells were stimulated with αIgM and/or αCD40 for 48 h in vitro. The amount of g5pr transcript was compared 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 (Supplemental 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 IκBα 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-
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 G5PR<sup>Tg</sup> mice that overexpress g5pr transcripts in lymphoid cells under the lck promoter, IgH-
chain gene promoter, and intronic enhancer elements (Fig. 3A). Increased G5PR expression was confirmed in G5PR<sup>Tg</sup> 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<sup>Tg</sup> mice. (A) Peritoneal cavity cells were isolated from young (8–12 wk) and aged (>40 wk) G5PR<sup>Tg</sup> mice or littermates and stained with anti–CD5-PE, anti–CD11b-FITC, and anti-B220 allophycocyanin-conjugated Abs. The proportion of B-1a (CD<sup>5</sup>CD<sup>11b</sup>+/B220+), B-1b (CD<sup>5</sup>CD<sup>11b</sup>−B220+), and B-2 (CD<sup>5</sup>CD<sup>11b</sup>−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 (CD<sup>5</sup>B220+) cells of G5PR<sup>Tg</sup> 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 (CD<sup>5</sup>B220+) cells of G5PR<sup>Tg</sup> 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<sup>Tg</sup> 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<sup>Tg</sup> 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<sup>Tg</sup> mice and littermates were stained with periodic acid-Schiff (PAS), anti-IgG Ab or anti-C3 Ab, respectively. Scale bars, 100 μm.
splenic B cells from G5PRTg 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 G5PRTg 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 G5PRTg mice with TD-Ag SRBC, we observed an altered formation of PNA+ splenic GCs surrounded by IgD+ 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+Fas+GL7+ 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 G5PRTg 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 G5PRTg mice compared with WT mice, but the low-affinity Ab tiers measured by NP25-BSA were significantly higher in G5PRTg mice than in WT mice (Fig. 4D). The overall affinity of the sera was calculated by the ratio of each measurement, NP2/NP25. These results suggest that increased G5PR expression in G5PRTg 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.

Tfh cells may play an important role in B cell maturation in GCs, in association with G5PR expression. The g5pr transcripts were upregulated in Tfh cells in G5PRTg mice. However, Ag immunization induced a similar number of Tfh (CD4+PD-1+ CXCR5+) cells in the spleens of G5PRTg mice comparable to that in WT mice (Supplemental Fig. 3). Although we have not neglected the effects of G5PR on Tfh cells in G5PRTg 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 G5PRTg 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+CD11b+B220+) cells was higher than did female WT littermates (Fig. 5F). Female, but not male, G5PRTg mice also produced anti-nuclear Abs (Fig. 5G). Moreover, immunohistochemical staining of the kidneys of aged female G5PRTg mice showed stronger signals for immune complexes with IgG and C3 in glomeruli than were observed in WT mice (Fig. 5H). Male G5PRTg mice did not show such autoantibody production, suggesting that generation of B-1a cells in G5PRTg 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.

FIGURE 6. AICD and JNK phosphorylation in B cells of female NZB mice. (A) Expression of g5pr transcripts in B-1a or B-2 cells of NZB mice. Peritoneal B-1a or splenic B-2 cells were isolated from female NZB mice (n = 3), and g5pr expression was measured by qRT-PCR. Results are shown as the mean ± SD, *p < 0.05. (B) Survival of stimulated B-1a and B-2 cells. B-1a and B-2 cells from NZB mice were stimulated with αlgM for 24 h. Live cells were identified by forward scatter (FS) and side scatter (SS) signals on the flow cytometric profile. Results are representative of three independent experiments. (C) Activation of JNK in B-1a and B-2 cells of NZB mice. B-1a or B-2 cells were stimulated with αlgM for the indicated times. JNK phosphorylation was assessed by Western blotting. Results are representative of two independent experiments.
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 induced by the interaction of B cells with FDCs and Tfh 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 transfected 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 Tfh 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 parietal 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 autoimmunity 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 Src 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^ mouse 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

The authors have no financial conflicts of interest.

References

**Supplementary Figure 1.** Purification of mature GC B cells. C57BL/6 mice were immunized with SRBC for 10 days and then B220<sup>+</sup>Fas<sup>+</sup>GL7<sup>+</sup> mature GC B cells and B220<sup>+</sup>Fas<sup>-</sup>GL7<sup>-</sup> B cells were sorted by JSAN cell sorter. Results are representatives of three independent experiments.
Supplementary Figure 2. Development of B lineage cells in G5PR\textsuperscript{Tg} mice. (A) Total cell numbers of bone marrow, spleen and peritoneal cavity in young and aged G5PR\textsuperscript{Tg} or WT mice (n=5, each group) were counted. Results are shown as the mean ± SD. (B) The proportion of lymphoid lineage cells from bone marrow and spleen were characterized in young and aged groups by flow cytometric analysis; pro-B (B220\textsuperscript{hi}CD43\textsuperscript{lo}), pre-B (B220\textsuperscript{hi}CD43\textsuperscript{lo}), immature/mature bone marrow B (B220\textsuperscript{hi}CD43\textsuperscript{hi}) cells, T (CD3\textsuperscript{+}) cells, whole B (CD19\textsuperscript{+}), follicular B (B220\textsuperscript{+}CD21\textsuperscript{+}CD23\textsuperscript{+}), marginal zone B (B220\textsuperscript{+}CD21\textsuperscript{hi}CD23\textsuperscript{lo}) cells. Results are representatives of three independent experiments.
**Supplementary Figure 3.** Induction of T<sub>FH</sub> cells in the spleen of G5PR<sup>Tg</sup> mice after immunization. (A) The G5PR<sup>Tg</sup> and WT mice (n=4, each group) were immunized with NP-CGG in the alum for 14 days, and whole CD4<sup>+</sup> and T<sub>FH</sub> (CD4<sup>+</sup>PD-1<sup>+</sup>CXCR5<sup>+</sup>) cells in the spleen were characterized by flow cytometric analysis. (B) T<sub>FH</sub> cells were isolated by JSAN cell sorter. The amount of g5pr transcript was compared between G5PR<sup>Tg</sup> and WT mice by qRT-PCR. Results are shown as the mean ± SD.
Supplementary Figure 4. BCR-mediated AICD of splenic B-2 cells from aged group. Splenic B-2 cells were purified from female G5PR$^{Tg}$ or WT mice at aged group (over 40 wk after birth, n=3) were stimulated in vitro with $\alpha$IgM for 24 h. Apoptotic cells with Annexin-V$^+$7-AAD$^+$ were compared. Results are representative of three independent experiments.