gp49B-Mediated Negative Regulation of Antibody Production by Memory and Marginal Zone B Cells

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gp49B-Mediated Negative Regulation of Antibody Production by Memory and Marginal Zone B Cells

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The rapid Ab responses observed after primary and secondary immunizations are mainly derived from marginal zone (MZ) and memory B cells, respectively, but it is largely unknown how these responses are negatively regulated. Several inhibitory receptors have been identified and their roles have been studied, but mainly on follicular B cells and much less so on MZ B, and never on memory B cells. gp49B is an Ig superfamily member that contains two ITIMs in its cytoplasmic tail, and it has been shown to negatively regulate mast cell, macrophage, and NK cell responses. In this study, we demonstrate that gp49B is preferentially expressed on memory and MZ B cells. We show that gp49B−/− mice produce more IgM after a primary immunization and more IgM and IgG1 after a secondary immunization than gp49B+/+ mice in T cell–dependent immune responses. Memory and MZ B cells from gp49B−/− mice also produce more Abs upon in vitro stimulation with CD40 than those from gp49B+/+ mice. The in vitro IgM production by MZ B cells from gp49B−/+, but not gp49B−/−, mice is suppressed by interaction with a putative gp49B ligand, the integrin αβ, heterodimer. In addition, gp49B−/− mice exhibited exaggerated IgE production in the memory recall response. These results suggest that plasma cell development from memory and MZ B cells, as well as subsequent Ab production, are suppressed via gp49B. In memory B cells, this suppression also prevents excessive IgE production, thus curtailing allergic diseases.

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The online version of this article contains supplemental material.

Abbreviations used in this article: AFC, Ab-forming cell; alum, aluminum hydroxide; B1-8 8 k, B1-8 IgH knock-in; CGG, chicken γ-globulin; Fo, follicular; GC, germinal center; IGB, induced GC B; MB, induced memory-like B; MZ, marginal zone; NP, (4-hydroxy-3-nitrophenyl) acetyl; TD, T cell dependent; TI, T cell independent; TI-II, T cell-independent type II.

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BCR-mediated calcium signaling and physiologically regulates the expansion of B1 cells, which express higher levels of Siglec-G than other B cell subsets (22). Thus, the roles played by inhibitory receptors on B cells have become evident, but less is known about their involvement in MZ B cells (23), and no inhibitory receptors on memory B cells have so far been studied in rodent models. Because MZ B and memory B cell responses are characterized by a rapid burst of Ab production, strict regulation would seem to be required.

In this study, we demonstrate that memory and MZ B cells express gp49, a 49-kDa membrane protein belonging to the Ig superfamily (24). There are two subtypes of gp49, gp49A and gp49B, which have significant homology in their extracellular domains (25). We demonstrate in this article that gp49B (also termed Lilrb4) is dominantly expressed on memory and MZ B cells. Although gp49A has a short cytoplasmic tail that lacks any specific signaling motifs, gp49B contains two ITIMs in its cytoplasmic tail that mediate inhibition of cellular activation by recruiting SHP-1 and SHP-2 (26–29). gp49B has been shown to bind to the integrin αi and βi (αβiβi), which is expressed on a variety of cells including activated T cells, and this interaction inhibits IgE-dependent degranulation of mast cells (30–32). Other studies showed that gp49B is also expressed on activated T cells and NK cells, and regulates IFN-γ production (32–34). However, the function of gp49B on any B cell subset has not been determined.

In this article, we show that gp49B−/− mice produce higher amounts of IgM after a primary immunization and of IgM, IgG1, and IgE after a secondary immunization than gp49B+/+ mice. In vitro analyses suggest that gp49B suppresses CD40-mediated Ab production through interaction with the αiβi integrin. Thus, we have found a novel regulatory mechanism that attenuates Ab production by memory and MZ B cells, and this mechanism may be required to control the rapid and intensive Ab responses common to these cells.

Materials and Methods

Mice and immunizations

C57BL/6 mice were purchased from Sankyo Labo Service. B1-8 IgH knockouts (B1-8 iko) mice (35) backcrossed to congenic C57BL/6-Cd45.1 strains were used where indicated. We used heterozygous B1-8 knock-in mice, in which the endogenous IgH allele is effectively excluded (36). gp49B−/− mice (37) and µMT mice (38) were also used. All mice were maintained in our mouse facility under specific pathogen-free conditions, and treated under the protocols approved by the Animal Care and Use Committee of the Tokyo University of Science. Mice were immunized i.p. with 100 μg (4-hydroxy-3-nitropheryl) acetyl (NP)3-ε-chicken γ-globulin (CGG) precipitated in aluminum hydroxide (alum) or 50 μg NP3-Ficoll (Biosearch Technologies) and boosted i.v. with 25 μg soluble NP3-CGG unless otherwise noted. To induce IgE responses, mice were primed s.c. with 150 μg NP3-CGG precipitated in alum, 25 μg per site into bilateral lower flanks and the base of tail, and 50 μg s.c. into the neck. In the secondary immunization, 25 μg each of soluble NP3-CGG was injected i.p. and into the scruff of the neck s.c., and 12.5 μg per site into bilateral lower flanks s.c. (75 μg in total per immunization). Mice were i.v. administered 25 μg each of soluble NP3-CGG as the tertiary immunization, to induce active anaphylaxis, and rectal temperature of the mice was measured thereafter.

Flow cytometry

Single-cell suspensions from spleens were depleted of RBCs by ammonium chloride lysis, incubated with anti-FcγRII/III Ab (2.4G2) to block FcγRs, and stained for 30 min on ice in PBS containing 0.5% BSA, 2 mM EDTA, 0.05% sodium azide with different combinations of the following Abs and reagents conjugated with FITC, PE, biotin, allophycocyanin, PE-Cy7, allophycocyanin-Cy7, or Pacific Blue: anti-IgG1 (Southern Biotech), anti-IgD and CD45.2 (Beckman Coulter or Biologend), anti-CD45R/B220 (Biologend or eBioscience), anti-pp49 (eBiosciences), anti-IgM (Biologend, eBiosciences, or Southern Biotech), streptavidin and anti-CD138 (Biolegend or BD Biosciences), anti-CD43 (57) and CD21 (BD Biosciences), anti-CD23, CD19, CD5, CD38, FcεRia, CD45.1, integrin αi and integrin βi (Biologend), and NP3-BSA labeled with biotin or Alexa 647. All samples were analyzed using a FACS Calibur flow cytometer or FACS-CantoII (BD Biosciences). The data were analyzed using FlowJo (Tree Star).

Cell purification and culture

For B cell isolation, non-B cells were removed from spleen cells by incubation with the mixture of biotinylated Abs against CD4, CD8, DX5, Ter-119 (Biologend), and CD43 (7; BD Biosciences), followed by sequential negative sorting using the MACS system (Miltenyi Biotec) and iMag system (BD Biosciences). Fo and MZ B cells were sorted from pooled spleen cells of mice (at least two) using FACS Aria II (BD Biosciences). For memory B cell purification, splenic B cells sorted from gp49B+/+ or gp49B−/− B1-8 ki CD45.1 mice were transferred into C57BL/6 mice and the recipients were sacrificed on the next day. For adoptive transfer, gp49B−/− mice from 15 recipient mice were stained with biotin anti-CD45.2, and CD45.2+ cells were depleted using the MACS system. From the remaining cells, memory B cells (CD45.1+ B220+ NP3 CD38IgG1+) were sorted using FACS Aria II as shown in Supplemental Fig. 3C. These procedure yielded >95% purity of corresponding B cell subsets. Feeder cells were removed from cells of the induced GC B (iGB) cell culture by staining with anti-TCRδ (Biologend) Ab, followed by depletion using the iMag system (BD Biosciences).

Purified B cells were cultured with 10 μg/ml (or otherwise indicated) anti-CD40 (Southern Biotech), 10 μg/ml LPS (Sigma), or 10 μg/ml anti-IgM (Jackson ImmunoResearch) in RPMI 1640 medium (Sigma) supplemented with 10% FBS, 5.5 × 10−5 M 2-ME, 10 mM HEPES, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies) in a humified atmosphere at 37°C with 5% CO2.

Generation of BM chimeric mice

BM cells from mice (at least three) of each indicated genotype, mixed at the indicated ratios, were injected i.v. (5 × 106 cells/mouse) into lethally irradiated mice (5.5 Gy, twice at 3-h intervals) of the indicated genotype. At least 8 wk after the transfer, the chimeric mice were used for immunization.

Adoptive transfer

The indicated number of B cells purified from the indicated strains of mice was transferred i.v. into C57BL/6 or µMT mice, which were then immunized with NP-CCG/alum or soluble NP-CCG on the next day, as indicated.

ELISA and ELISPOT assays

NP-specific Ab-forming cells (AFCs) were detected by ELISPOT assay using the MultiScreen 96-well filtration plate (Millipore) coated with 10 μg/ml NP3-BSA in triplicate, as described previously (39). Anti-NP IgM spots were revealed by HRP-conjugated goat anti-mouse IgM Ab (Southern Biotech) in conjunction with 3-aminio-9-ethylcarbazole substrate (Dako). NP-specific IgM and IgG1 were detected by ELISA as described previously (39). Total IgM was detected using 96-well plates coated with goat anti-mouse IgM Ab and with BSA as a blocking reagent. To measure NP-specific IgE titters, we coated plates with purified rat anti-mouse IgE (BD Biosciences), and bound Abs were revealed with biotinylated NP3-BSA and HRP-conjugated streptavidin (Southern Biotech).

Western blotting

Cell samples were lysed in 1 × SDS-PAGE sample buffer, sonicated, boiled, and used for SDS-PAGE, followed by Western blotting. The following primary polyclonal Abs were used: rabbit anti-phospho-p44/p42 MAPK (ERK1/2; Cell Signaling), rabbit anti-p44/p42 MAPK (Cell Signaling), rabbit anti–phospho-Akt (Ser473; Cell Signaling), and rabbit anti-Akt (Cell Signaling). Goat anti-rabbit IgG-HRP (Zymed) was used as a secondary Ab. ECL Western Blotting Detection Reagents (Amersham) were used for detection.

Proliferation assay

MZ B cells (1 × 105/well) were cultured in 96-well flat plates (BD Falcon) as described earlier for 48 h, with the last 8 h in the presence of [3H]thymidine (1 μCi/well; PerkinElmer). Incorporated [3H]thymidine was counted in a BetaPlate scintillation counter (Wallac, Gaithersburg, MD). These experiments were performed in triplicate.
Preparation of 3T3 cells expressing integrin $\alpha_B\beta_2$

The integrin $\beta_2$ gene was retrovirally transduced into BALB/c 3T3 cells, which express only $\alpha$ chain intracellularly (data not shown). Mouse integrin $\beta_2$ cDNA was cloned into pMX-ires-rafC2 vector (40). The vector was transfected into PLAT-E packaging cell line (41) by using Lipofectamine 2000 (Invitrogen), and the supernatant was added to BALB/c 3T3 fibroblasts (clone A31, provided by RIKEN BRC) in the presence of DOTAP Liposomal Transfection Reagent (Roche).

iGB cell culture and iMB cell generation

iGB cell culture and iMB cell generation were performed as previously described (42). In brief, purified B cells from CD45.1+ mice were cultured for 4 d on irradiated 40%L cell feeder layer with rIL-4 (1 ng/ml; Peprotech). Then the expanded B (6GB) cells were injected i.v. into iγ-irradiated (6.5 Gy) C57BL/6 mice. The iGB cell–derived (CD45.1+) B cells (iMB cells) were sorted from pooled spleens of 10 recipient mice by FACS Aria II.

Quantitative real-time PCR

RNA preparation, cDNA synthesis, and quantitative real-time PCR were performed, and data were presented as described previously (42). Gene expression was normalized to that of gapdh. The primer sets were used: gpdh sense: 5′-GGAGAAACCTGCCAAGATGATGG-3′; gpdh antisense: 5′-CCCTGTCGTCGTAAGCACTTT-3′; gp49A sense: 5′-CAAGTTTCTACATCCAAG-3′; gp49A antisense: 5′-CTATTGAGTGCCTGAACGAC-3′; gp49B sense: 5′-ACCAAGTTCAATTGCATTGG-3′; gp49B antisense: 5′-GTGGTAGGATGCCTGACTCT-3′; Blimp1 sense: 5′-GAACACCTGCTTTACGTAGCTG-3′; Blimp1 antisense: 5′-AGTGTAGACCTTACCGATGAG-3′.

Statistical analysis

Statistical analysis was performed using the two-tailed unpaired Student $t$ test. When the normality test failed, a Wilcoxon–Mann–Whitney $U$ test was performed by using R software.

Results

gp49B is selectively expressed on memory B and MZ B cells

To identify molecules selectively expressed on memory B cells, we performed microarray analysis using in vivo–induced memory-like B cells, termed iMB cells (42), and naive Fo B cells for comparison. We have shown previously that the iMB cells are functionally equivalent to, but far more abundant than, bona-fide memory B cells (42). Among genes encoding cell-surface receptors that are highly preferentially expressed on iMB cells, we focused on gp49B, because we have also found it to be one of the most differentially expressed genes in memory B cells as compared with naive B, GC B, and plasma cells in a published data set (43). We confirmed by quantitative RT-PCR that the expression of gp49B transcripts was markedly higher in iMB cells than in Fo B cells, whereas the expression of gp49A did not significantly differ (Fig. 1A). Using an available gp49 mAb (H1.1), which recognizes a common epitope on gp49A and gp49B (33), we found that gp49 protein was expressed on iMB cells at a higher level than naive B cells (Fig. 1B).

We next examined the expression of gp49 on bona-fide memory B cells, as well as other B cell subsets taken from mice immunized with a typical T-dependent Ag, NP-CGG in alum. Expression of gp49 was higher on NP-binding IgG1+ memory B cells than on GC or naive B cells (Fig. 1C), whereas on plasma cells was as low as on naive B cells (Fig. 1D). We also found that, among spleen B cells from unimmunized mice, MZ B cells expressed more abundant gp49 than Fo B cells (Fig. 1E). Finally, we demonstrated that gp49B is the major subtype expressed on MZ B cells and memory B cells, because the gp49 staining was mostly lost on these cells from gp49B−/− mice (Fig. 1F).

B cell development and memory B cell formation are normal in gp49B−/− mice

Although three strains of gp49B-null knockout mice have been published, so far profiles of B cell subsets have not been reported in any of these mice (37, 44, 45). Therefore, we analyzed B cell subsets in lymphoid organs by flow cytometry. The numbers of Fo B and MZ B cells in the spleen (Fig. 2A) were equivalent between gp49B+/+ and gp49B−/− mice. The numbers of pro-B, pre-B, immature B, and mature B cells in the bone marrow were also normal in gp49B−/− mice (Supplemental Fig. 1A). These data indicate that early B cell development is largely normal in the absence of gp49B.

We next analyzed late B cell development during an immune response. Two weeks after immunization with NP-CGG in alum, the number of NP-specific GC B cells determined by flow cytometry, and the size and frequency of GCs determined by histological analysis were equivalent between the spleens of gp49B+/+ and gp49B−/− mice (data not shown). Ninety days after the primary immunization, we did a secondary immunization. The numbers of NP-specific IgG1+ memory B cells just before the secondary immunization, or after 5 d, did not differ significantly between gp49B+/+ and gp49B−/− mice (Fig. 2B). To ask whether the lack of gp49B intrinsically affects memory B cell formation, we generated mixed bone marrow chimeric mice by transferring equal numbers of bone marrow cells of CD45.2+ gp49B+/− and congenic CD45.1+ gp49B+/+ mice into lethally irradiated μMT mice (Supplemental Fig. 1B). These mice were immunized with NP-CGG, and their spleen cells were analyzed 4 and 14 wk later. At both time points, the proportion of gp49B+/− (CD45.2+) cells in the NP-specific IgG1+ memory B cell fraction was comparable with that in the naive B cell fraction (Supplemental Fig. 1C). These data indicate that memory B cells that lack gp49B develop and are maintained normally.

Enhanced Ab production in gp49B−/− mice during immune responses

Memory and MZ B cells share the common feature of responding to TD Ags more quickly than Fo B cells to produce Abs. Given that the development of memory and MZ B cells in gp49B−/− mice is normal, we next examined Ab production in these mice during a TD immune response. After primary immunization with NP-CGG/alum, gp49B−/− mice produced NP-specific IgM and IgG1 at similar levels as gp49B+/+ mice on day 7, but had significantly increased levels of IgM on day 14 (Fig. 3A). Upon secondary challenge with NP-CGG on day 50, gp49B−/− mice produced significantly higher levels of NP-specific IgM and IgG1, with the latter extending over 2 wk after the challenge (Fig. 3A). Because MZ B cells are the major source of plasma cells in response to T cell–dependent type II (TI-II) Ag (8), we also tested TI-II immune response of gp49B−/− mice. After primary immunization with model TI-II Ag, NP-Ficoll, NP-specific IgM was significantly higher in gp49B−/− mice than in wild-type mice (Supplemental Fig. 2A).

Because gp49B is also expressed on dendritic cells and activated T cells, it was unclear at first whether the enhanced Ab production by gp49B−/− mice is due to a B cell defect. Therefore, we generated mixed bone marrow chimeric mice in which B cells were derived from gp49B+/+ or gp49B−/− mice, and the vast majority of other leukocytes were from gp49B+/+ mice. The mouse group in which B cells lacked gp49B produced a significantly larger number of NP-specific IgM-secreting AFCs than the control mouse group in response to NP-CGG and NP-Ficoll (Fig. 3B, Supplemental Fig. 2B). This result indicates that B cell defect is responsible for the augmented primary IgM responses to TD and TI-II Ags in gp49B−/− mice.

To determine whether the enhanced memory-recall response in gp49B−/− mice is also due to a B cell–intrinsic defect, we transferred splenic B cells of NP-primed gp49B+/+ or gp49B−/− mice to lethally irradiated congenic CD45.2+ mice. As shown in Supplemental Fig. 2C, the memory responses were similar between the two groups of mice, which indicates that the enhanced memory response in gp49B−/− mice is due to a B cell–intrinsic defect.

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gp49B REGULATES MEMORY/MZ B CELL Ab PRODUCTION

FIGURE 1. gp49B is expressed on memory and MZ B cells. (A and B) iMB cells (CD19+ CD21high CD23low) B cells (gating shown in the right panel) were purified from spleens of recipient mice (C57BL/6, CD45.2+) transferred with iGB cells (42) 2 wk previously. (A) Real-time PCR analysis of gp49A and gp49B mRNA in Fo B cells sorted from C57BL/6 mice [as shown in (E)] and iMB cells sorted from recipient mice. Gene amplification was normalized to that of the control gapdh gene, and the gene expression in iMB cells is presented as that of the control gp49A gene. (B) The expression of gp49 protein on the iMB and naive B cells from the recipient mice (gating shown in left panel) was analyzed by flow cytometry. (C) The expression of gp49 on naive, GC, and memory B cells from spleens of mice immunized with NP-CGG 4 wk earlier. The following populations were gated as shown in the left three panels: naive B cells, B220+ CD19+ IgG1+ NP+; GC B cells, B220+ CD19+ IgG1+ NP+ CD38low; and memory B cells, B220+ CD19+ IgG1+ NP+ CD38high. (D) The expression of gp49 on naive B and plasma cells from spleens of C57BL/6 mice immunized with NP-CGG/alum 5 d before the analysis (right panel). Gating strategy for the naive B cells (B220+ CD138-) and plasma cells (B220high CD138+) is shown in the left two panels. (E) The expression of gp49 on mature Fo and MZ B cells from spleens of naive C57BL/6 mice (right panel). Gating strategy for the Fo (B220+ CD21+ CD23high) and MZ B cells (B220+ CD21high CD23low) is shown in the left two panels. (F) The expression of gp49 on Fo, MZ, and memory B cells from gp49B+/+ (black line) and gp49B−/− (shaded histogram) mice. The memory B cells were purified from mice immunized with NP-CGG/alum 4 wk before the analysis. All data are representative of two to five independent experiments.

Enhanced Ab production by gp49B−/− MZ and memory B cells

We then asked whether the augmented primary IgM response in gp49B−/− mice is attributable to the defect in MZ B cells. To address this, we transferred MZ or Fo B cells from gp49B−/− or gp49B+/+ mice into naive recipients together with CGG-primed wild-type spleen cells, as a source of memory T cells. The recipient mice were challenged with soluble NP-CGG on the next day. As shown in Fig. 3C, the recipients of gp49B−/− B cells produced larger amounts of Ag-specific IgG1 (anti-NP 13.6 IgG1), the majority of which was high affinity (anti-NP 1.1 IgG1), than the recipients of gp49B+/+ B cells. As a negative control, recipients of naive B cells did not respond to the same soluble Ag. These data indicate that gp49B expressed on memory B cells directly suppresses Ab production by these cells upon Ag restimulation.

FIGURE 2. Normal B cell development and memory B cell formation in gp49B−/− mice. (A) The frequency (representative data, center panel) and the number (right panel) of Fo (B220+ CD21+ CD23high) and MZ (B220+ CD21high CD23low) B cells (gating shown in the left and center panels) in the spleens of unimmunized gp49B+/+ and gp49B−/− mice. n = 4. (B) gp49B+/+ and gp49B−/− mice were immunized with NP-CGG/alum and boosted with soluble NP-CGG 3 mo later. Gating strategy for memory B cells (B220+ Dump IgG1+ NP+ CD38low; Dump: CD138, FcεRI, Ter119) is shown by the left three panels. (Right panel) Numbers of memory B cells in the spleen of each mouse 3 mo after the primary immunization (preboost) and 5 d after the booster immunization. n = 3.
transferred (Supplemental Fig. 3A). These data suggest that MZ B cells, but not Fo B cells, are responsible for the augmented IgM production upon primary immunization in gp49B^{2/2} mice.

Next, we assessed whether the lack of gp49B directly affected MZ B cells in vitro. Sorted MZ and Fo B cells were stimulated 4 d with anti-CD40 Ab or LPS, and IgM in the culture supernatants was estimated by ELISA. gp49B^{2/2} MZ B cells produced nearly 3-fold more IgM than gp49B^{+/+} cells after stimulation with anti-CD40 Ab, but not significantly more upon LPS stimulation (Fig. 4B). After 3 d of culture with anti-CD40 Ab, gp49B^{2/2} MZ B cells generated more plasma cells than gp49B^{+/+} cells, as assessed by flow cytometry and ELISPOT assays (Fig. 4C, 4D).

FIGURE 3. Augmented Ab responses to TD Ag in gp49B^{2/2} mice. (A) gp49B^{+/+} and gp49B^{2/2} mice received a primary immunization with NP-CGG/alum and 50 d later, they were boosted with soluble NP-CGG (n = 6). Anti-NP IgM (left panels) and IgG1 (right panels) concentrations in the sera 7 or 14 d after the primary (top panels) and the secondary (bottom panels) immunizations were determined by ELISA. (B) Pooled BM cells from gp49B^{+/+} or gp49B^{2/2} mice and μMT mice, mixed at a ratio of 1:4, or BM cells of μMT mice alone, were transferred (5 x 10^6 cells/mouse) i.v. into lethally irradiated C57BL/6 mice. Ten weeks later, chimeric mice were immunized with NP-CGG/alum (left panel). Seven days after the immunization, the numbers of anti-NP IgM AFCs in the spleens of the mice were determined by ELISPOT (right panel). n = 4 (μMT alone: n = 3). (C) Purified B cells (1 x 10^6) from pooled spleens of NP-CGG–primed or unimmunized gp49B^{+/+} or gp49B^{2/2} mice (five for each group), and pooled spleen cells (1 x 10^7) from three C57BL/6 mice primed with 100 μg CGG/alum 30 d earlier were cotransferred i.v. into C57BL/6 mice (primed B cells: n = 5; naive B cells: n = 3). One day after the transfer, the recipient mice were immunized with soluble NP-CGG (left panel). Concentrations of anti-NP,13,6 IgG1 (center panel) and anti-NP,1, IgG1 (right panel) Abs in the sera 7 d after immunization were determined by ELISA. *p < 0.05, **p < 0.01. Data are representative of two to three independent experiments.

FIGURE 4. gp49B suppresses Ab production by MZ and memory B cells. (A) Fo or MZ B cells sorted from pooled spleens of gp49B^{+/+} or gp49B^{2/2} mice were transferred i.v. into μMT mice (2 x 10^6 cells/mouse, left panel), and the recipients (n = 6) were immunized with NP-CGG/alum on the next day. IgM titers in the sera 7 or 14 d after immunizations were determined by ELISA (center and right panels). (B–E) MZ B cells sorted from pooled spleens of gp49B^{+/+} or gp49B^{2/2} mice were unstimulated (−) or stimulated with anti-CD40, anti-IgM Abs, or LPS, as indicated. IgM in culture supernatants was detected by ELISA on day 4 (B). The frequency of plasma cells (CD138+) in the cultures was analyzed by flow cytometry on day 2 and 3 (C). The numbers of IgM-secreting AFCs in the cultures were estimated by ELISPOT on day 3 (D). [3H]thymidine incorporation was determined on day 2 (E). (F) gp49B^{+/+} or gp49B^{2/2} NP-binding cells (IgG1 memory, Fo, and MZ B cells) were purified as shown in Supplemental Fig. 3C, 3D. Sorted cells (10,000 cells/well) were stimulated with anti-CD40 Ab for 7 d in vitro, and the concentration of NP-specific IgG1 in the culture supernatant was determined by ELISA. *p < 0.05, **p < 0.01. All cultures were performed in triplicate. Data are representative of two to three independent experiments. n.s., not significant.
However, proliferation upon stimulation with anti-CD40 Ab, anti-IgM, or LPS did not significantly differ between gp49B+/+ and gp49B−/− MZ B cells (Fig. 4E). In contrast, gp49B+/+ and gp49B−/− Fo B cells produced equivalent levels of IgM after stimulation with anti-CD40 Ab or LPS (Supplemental Fig. 3B).

We then examined the effect of gp49B on Ab production by memory B cells in vitro. The mice that were transferred with gp49B+/+ or gp49B−/−, B1-8 ki B cells were immunized with NP-CGG, and donor-derived NP-specific IgG1+ memory B cells were sorted at 4 wk after the immunization, to obtain a sufficient number of memory B cells (Supplemental Fig. 3C). The memory B cells, as well as naive NP-specific Fo and MZ B cells (Supplemental Fig. 3D), were stimulated in vitro with anti-CD40 Ab for 7 d. As shown in Fig. 4F, gp49B+/+ memory B cells produced ∼3-fold more NP-specific IgG1 than gp49B−/− cells, whereas Fo and MZ B cells did not produce detectable amounts of NP-specific IgG1 under the same conditions.

Taken together, these data strongly suggest that gp49B expressed on memory and MZ B cells negatively regulates the differentiation of these cells into plasma cells that produce Abs in the immune response.

**gp49B suppresses MZ B cell Ab production through interaction with integrin αβ3**

It was previously shown that gp49B interacts with the αβ3 integrin heterodimer, and thereby suppresses the degranulation of mast cells (30). We found that the expression of integrin αβ3 was induced on both Fo and MZ B cells after stimulation with anti-CD40 Ab (Fig. 5A), suggesting that the gp49B-mediated suppression of Ab production by MZ B cells in vitro might be caused by the gp49B and integrin αβ3 interaction. Thus, we cultured MZ B cells with anti-CD40 Ab on a feeder layer of BALB/c 3T3 cells expressing integrin αβ3 or not, and measured IgM produced in the culture supernatant (Fig. 5B). As shown in Fig. 5C, Ab production by gp49B+/+ MZ B cells was significantly suppressed on the feeder cells expressing integrin αβ3 as compared with control feeder cells. By contrast, gp49B−/− MZ B cells were not suppressed by the integrin αβ3-expressing feeder cells. This result indicates that gp49B suppresses Ab production by MZ B cells through its interaction with integrin αβ3.

**gp49B attenuates CD40-mediated ERK activation and blimp1 expression**

CD40 signaling is known to promote proliferation, Ab production, and survival of B cells (46–48). Earlier mentioned results suggest that gp49B may specifically suppress a CD40 signaling pathway that promotes B cell differentiation to plasma cells. CD40 signaling induces the activation of ERK and PI3K signaling, which are also important for the plasma cell differentiation (49, 50). Therefore, we compared the phosphorylation of ERK and AKT on gp49B+/+ and gp49B−/− B cells after the stimulation with anti-CD40. To obtain the B cells expressing both gp49B and integrin αβ3, we cultured splenic B cells on a feeder layer of 40LB cells that express exogenous CD40L and BAFF (42) (Fig. 6A). Then these cells were stimulated with anti-CD40 after starvation. Although the phosphorylation of Akt was comparable in gp49B+/+ and gp49B−/− cells, the levels of the induced Erk phosphorylation were higher in gp49B+/+ cells than in gp49B−/− cells (Fig. 6B). In addition, the expression of blimp1, the master regulator of plasma cell development (51) known to be induced by ERK, was more strongly induced by anti-CD40 stimulation in gp49B−/− cells than in gp49B+/+ cells (Fig. 6D). In contrast, the levels of Erk phosphorylation and blimp1 expression were equivalent between gp49B+/+ and gp49B−/− B cells after stimulation with anti-IgM (Fig. 6C, 6D). Collectively, our results suggest that gp49B attenuates a CD40 signaling pathway leading to Erk activation and the following plasma cell differentiation.

**Enhanced IgE production by gp49B−/− mice upon secondary immunization**

It has been shown that IgG1+ memory B cells are able to undergo secondary class switching to IgE and to generate a high-affinity IgE response upon repeated immunization (52). Alternatively, a recent study suggested that IgE+ memory B cells are generated through a germinal center pathway and produce IgE in the recall response (53). Whatever the case, we considered the possibility that the exaggerated memory B cell response in gp49B−/− mice might also result in a deregulated IgE recall response. To test this possibility, we immunized gp49B+/+ or gp49B−/− mice with NP-CGG s.c. and i.p., routes known to induce an IgE response (54). As compared with gp49B+/+ mice, NP-specific IgE production was slightly elevated in gp49B−/− mice after the primary immunization, but markedly so after the secondary immunization (Fig. 7A). Previously it was reported that the Th2-mediated IgE response to OVA induced by intranasal priming with LPS and OVA is augmented in gp49B−/− mice, and this was suggested to be likely due to a dendritic cell defect (55). To clarify whether the augmented IgE production in gp49B−/− mice in our system was attributable to memory B cells, we generated mixed bone marrow chimeric mice harboring gp49B+/+ or gp49B−/− B cells, with the vast majority of other hematopoietic cells intact. Upon immunization by the same s.c./i.p. protocol, the mice with gp49B+/− B cells produced significantly elevated levels of NP-specific IgE compared with the control mouse group in the recall response upon a challenge 6 wk after the primary immunization (Fig. 7B). Accordingly, when these mice were induced an active anaphylaxis reaction by i.v. injection of NP-CGG as the tertiary immunization, the mice with gp49B−/− B cells showed severer hypothermia than those with gp49B+/+ mice (Fig. 7C). Notably, two of the mice with gp49B−/− B cells died during this response, whereas those with gp49B+/+ B cells were all alive. These results indicate that gp49B suppresses excessive...
IgE production by memory B cells in the recall response, and contribute to prevention of acquired allergic disease.

Discussion
Memory B cells express higher levels of B7-1 and B7-2, and respond to Ags more quickly, than naive B cells (7, 56). In addition, compared with the IgM BCR on Fo B cells, the IgG BCR on IgG+ memory B cells transduces stronger signals and induces robust proliferation, cellular activation, and Ab production (57–59). MZ B cells have increased basal signaling activity, and thus lower threshold for activation, compared with Fo B cells (9, 12, 13). In contrast, GC B cells have inactive BCR signaling caused by in-

FIGURE 6. gp49B suppresses CD40-mediated ERK activation and blimp1 induction. (A) Splenic B cells of gp49B+/+ and gp49B−/− mice were cultured on 40LB cells for 3 d. Then feeder cells were depleted, and the remaining B cells were incubated in medium overnight. The expressions of gp49 and integrin α5β3 after incubation were analyzed by flow cytometry. Control: gp49B−/−-derived B cells stained with isotype-matched control Abs. (B–D) The cells prepared as in (A) were stimulated with anti-CD40 (5 μg/ml) or anti-IgM (5 μg/ml) Abs for the indicated time periods. The cell lysates from the anti-CD40- (B) or anti-IgM-stimulated (C) cells were analyzed by Western blotting to detect phosphorylated and total ERK1/2 and Akt proteins, as indicated. RNA samples from the cells stimulated for 8 h, or unstimulated (−), were subjected to real-time PCR analysis. Blimp1 mRNA amplification was normalized to that of the control gtpdh mRNA, and the normalized values of gp49B−/− cells are presented as relatives to those of gp49B+/+ cells (D). Data are representative of two independent experiments.

FIGURE 7. Exaggerated IgE production in gp49B−/− mice in the memory recall response. (A) gp49B+/+ and gp49B−/− mice received a primary immunization with NP-CGG/alum s.c., and were boosted 3 wk later with soluble NP-CGG s.c. and i.p. as detailed in Materials and Methods. Serum anti-NP IgE concentrations on the indicated days after immunization were determined by ELISA. n = 9. Data are representative of two independent experiments. (B) BM chimeric mice containing gp49B+/+ or gp49B−/− B cells were generated as shown in Fig. 3B. Ten weeks later, these chimeric mice were immunized and then boosted 6 wk after that with NP-CGG, as in (A). Serum anti-NP IgE concentrations were determined as in (A), n = 9. (C) The mice shown in (B) were injected i.v. with soluble NP-CGG 5 mo after the boost immunization, and rectal temperature of these mice was measured at the indicated time after the tertiary immunization. Two gp49B−/− mice died as indicated by dagger (†). * p < 0.05, **p < 0.01.
creased phosphatase activity and are not activated but rather undergo apoptosis upon BCR stimulation in vivo (60–62). Because of their elevated responsiveness with lower activation thresholds, memory and MZ B cells might need a strong regulatory system distinct from that of naive Fo or GC B cells. In this report, we demonstrated that gp49B is expressed at significantly higher levels on memory and MZ B cells compared with Fo or GC B cells, and regulates plasma cell differentiation and Ab production of memory and MZ B cells during TD immune responses. Thus, such “activation-prone” cells among B-lineage cells appear to be specially equipped with the gp49B negative regulator.

The number of MZ B and other B-lineage cells was normal in gp49B+/− mice, as was the number of memory B cells after primary and secondary immunization. In addition, gp49B+/− memory B cells were normally developed in early and late phases of the immune response in mixed BM chimeric mice. Thus, gp49B is not involved in the development or the maintenance of memory and MZ B cells. In contrast, the production of IgM was increased in gp49B−/− mice after primary immunization. It is known that MZ B cells dominantly contribute to primary IgM production during TD immune responses (10), which was confirmed by our B cell transfer experiments (Fig. 4A). We demonstrated that gp49B+/− MZ B cells, but not Fo B cells, produce more IgM Ab than gp49B+/+ cells upon immunization, suggesting that gp49B negatively regulates Ab production by MZ B cells in the primary response. It is of note that the enhanced IgM response was evident on day 7 after primary immunization with either TD or TI-II Ags in the bone marrow chimera models where gp49B was largely intact in non-B cells, whereas it was not on day 7, but only after 2 wk in gp49B−/− mice (Fig. 3A and 3B and Supplemental Fig. 2A, 2B). Further study is necessary to solve this apparent inconsistency, but it would be possible to explain that the early IgM response is negatively regulated by some non-B cells, the function of which is attenuated by gp49B.

IgG1 titers were equivalent in gp49B+/− and gp49B−/− mice in the primary response, but higher in gp49B+/− mice in the secondary memory response. In addition, memory B cells derived from gp49B−/− mice produced higher levels of IgG1 Ab after adoptive transfer into recipient mice upon immunization with soluble Ag. Higher production of IgG1 in gp49B−/− mice in the secondary response against soluble Ag is likely due to IgM+ memory B cells. These data indicate that gp49B also regulates the recall response of memory B cells.

We also demonstrated that upon stimulation by CD40 in vitro, gp49B−/− MZ and memory B, but not Fo B cells, generated more Ab-producing plasma cells than gp49B+/+ cells, despite their equivalent proliferation. These results suggest that gp49B regulates differentiation of MZ and memory B cells into plasma cells induced by CD40 stimulation during immune responses. CD40 signaling is known to promote proliferation, Ab production, and survival of B cells (46–48). Thus, gp49B may regulate a specific pathway downstream of CD40, namely, a pathway promoting B cell differentiation to plasma cells. gp49B contains two ITIMs in its cytoplasmic tail and has been shown to inhibit cellular activation by recruiting SHP-1 and SHP-2 in mast cells (26–29). A similar mechanism may regulate the CD40 signaling pathway in MZ and memory B cells.

Although CD40 stimulation is known to activate the NF-κB pathway, the amount of p52/p100 and the degradation of IkBα was equivalent between gp49B+/+ and gp49B−/− B cells after the stimulation with anti-CD40 (data not shown). Instead, the phosphorylation of ERK and the expression of blimp1 were enhanced in gp49B−/− B cells. ERK signaling is essential for the induction of Blimp1 by stimulation with anti-CD40, and ERK signaling plays a critical role for the plasma cell differentiation of memory B cells in the immune response (49). Therefore, gp49B is likely to regulate the plasma cell differentiation by suppressing ERK activation. Because ERK phosphorylation was not affected in gp49B−/− B cells when stimulated with anti-IgM Abs, it is suggested that gp49B regulates ERK activation specifically signaled from CD40.

Although CD40 is generally considered to be involved in TD immune response, our data indicated that gp49B also attenuates IgM production from B cells during TI-II immune responses. Because MZ B cells play a major role in the TI IgM response (8), it is plausible that gp49B negatively regulates Ab production by MZ B cells. Recent study suggests that neutrophils localizing at the peri-MZ induce the Ab production and class switching of MZ B cells by providing BAFF, APRIL, and IL-21 (63). Because these neutrophils also express CD40L, they may activate MZ B cells via CD40L. Thus, gp49B may suppress the signaling from CD40 on MZ B cells in TI immune response, as well as in TD immune response.

We demonstrated that gp49B transduces an inhibitory signal in MZ B cells upon interaction with integrin α5β1, as previously shown for mast-cell degranulation (30). Although gp49B has no RGD sequence, which commonly binds to integrins, it has been suggested to interact with integrin through Leu-Asp-Ser-Gln (30), a sequence that conforms to the integrin-binding consensus motif, alphilastic-Asp/Glu-Ser/Thr-Pro/hydrophilic, found in other Ig superfamily members (64). Integrin α5β1 is expressed on various cell types, and its interaction with ligands is tightly controlled by its adhesion capacity (30), which is typically upregulated after cellular activation (65). Therefore, gp49B-mediated suppression is presumably regulated redundantly by its expression, as well as the activation state of the α5β1 integrin on the cells with which the B cells interact. Considering the additional involvement of various cytokines, among others, the regulation of Ab production by MZ and memory B cells may be more complex in a physiological context than has generally been appreciated.

In this study, we have identified, for the first time as far as we are aware, an inhibitory receptor that is expressed and functions on memory B cells. What would be the physiological significance of gp49B-mediated inhibition of recall Ab response by memory B cells? Peritoneal B1 B cells, which are typically weakly self-reactive, are known to produce autoantibodies in vivo in the absence of inhibitory receptors such as PIR-B or Siglec-G (21, 22). It was reported that, in humans, about one third of circulating IgG+ memory B cells, but much fewer mature naive B cells from healthy donors, express self-reactive BCR encoded by somatically hypermutated V genes (66). As in the case of B1 cells, negative regulation of Ab production by such self-reactive memory B cells would be important to prevent unintentional activation of such cells and the resultant autoimmunity.

Another possibility is that gp49B suppresses IgE production in the recall response, as indicated in this study. The enhanced IgE produced from memory B cells after secondary immunization resulted in exacerbated active anaphylaxis in gp49B−/− mice, perhaps through its augmented deposition on mast cells. Recent reports indicated that both IgE and IgG1 memory B cells might contribute to the production of IgE in the recall response (52, 53, 67). Thus, negative regulation of the production of Ag-specific IgE from the memory B cell pool could prevent immediate hypersensitivity diseases such as asthma and allergy.

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