Growth-factor receptor-bound protein-2 (Grb2) signaling in B cells controls lymphoid follicle organization and germinal center reaction

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Grb2 (growth-factor receptor-bound protein-2) is a signaling adaptor that interacts with numerous receptors and intracellular signaling molecules. However, its role in B-cell development and function remains unknown. Here we show that ablation of Grb2 in B cells results in enhanced B-cell receptor signaling; however, mutant B cells do not form germinal centers in the spleen after antigen stimulation. Furthermore, mutant mice exhibit defects in splenic architecture resembling that observed in B-cell-specific lymphotoxin−β-deficient mice, including disruption of marginal zone and follicular dendritic cell networks. We find that grb2−/− B cells are defective in lymphotoxin−β expression but not to chemotaxis. Grb2 executes this function by connecting the CXCR5 signaling axis in B cells as a nonredundant pathway that controls lymphoid follicle organization and germinal center reaction. Loss of Grb2 has no effect on B-cell chemotaxis to CXCL13, indicating that Grb2 executes this function by connecting the CXCR5 signaling pathway to lymphotoxin expression but not to chemotaxis.

Results

Grb2−/− Mutation Impairs B-Cell Maturation and Enhances B-Cell Responses to Activating Stimuli. To determine the function of Grb2 in B cells, we generated B-cell-specific grb2 knockout mice.
(Grb2BKO) mice by crossing grb2 floxed mice to CD19-Cre transgenic mice (Fig. S1) (21). Grb2 deficiency did not perturb B-cell development at an early stage (Table S1 and Fig. S2), but impaired further maturation of B cells after immature B cells egressed from the bone marrow (BM) into the spleen. The peripheral transitional T1 and T2 B cells in mutant mice were significantly reduced compared with that of WT mice (Fig. L4 and Table S1). As a result, follicular B cells declined to ~70% of the number in WT mice (Fig. L4 and Table 1), and the subset of mature recirculating B cells (B220+IgM+/-) in the mutant BM was only 40% of the WT counterpart (Table S1 and Fig. S2). We noted that the populations of marginal-zone B cells and B1 B cells were only mildly altered in the spleen of mutant mice. These results indicate that Grb2 exerts a differential regulatory role in B-cell lineage development, with follicular B cells being most profoundly affected by the Grb2BKO mutation.

To evaluate the role of Grb2 in B-cell activation, we compared the proliferative responses of grb2+/- and WT B cells to various activating stimuli. We found that mature B cells exhibited enhanced proliferation in response to anti-IgM or anti-IgM plus anti-CD40 (twofold), or IL-4 plus anti-CD40 (threelfold) (Fig. 1B). When B cells were stimulated with LPS, proliferation responses of both mutant and WT B cells were drastically increased, and even then, mutant B cells showed stronger proliferation (Fig. 1B). These same stimuli also induced increased proliferation in grb2+/- immature B cells, even though responses of both WT and mutant immature B cells to these stimuli were overall much lower than that of mature B cells (Fig. S3).

Next, we sought to determine which BCR signaling event was interfered with by Grb2 deficiency. BCR stimulation activates the tyrosine kinase Lyn, which then phosphorylates immunoreceptor tyrosine-based activation motifs (ITAM) in Igα and Igγ. The phosphorylated ITAMs recruit and activate Src homology 2 (SH2) domains of downstream molecules, including BLNK/p65 and PLCγ2 (25). We first examined whether Grb2 deficiency affected proximal BCR signaling. We found that while the kinetics and the level of tyrosine phosphorylation of total proteins in grb2+/- and WT B cells were comparable, the active form of Lyn phosphorylation in mutant B cells was slightly reduced than that of WT B cells after anti-IgM stimulation (Fig. S4). When distal signaling transduction molecules were examined, we found that phosphorylation of PLCγ2, activation of MAP kinases Erk1/2, p38, and JNK, and mobilization of Ca2+ were all significantly elevated in grb2+/- B cells (Fig. 1C and D). These data indicate that Grb2 is a negative regulator and regulates BCR signaling at the top of the BCR signaling cascade.

How does Grb2 negatively regulate BCR signaling? It has been previously shown that Grb2 is associated with CD22, a membrane plasmic tail by Lyn, which then recruits tyrosine phosphatase SHP-1 to the membrane to suppress BCR downstream signaling (26). How does Grb2 negatively regulate BCR signaling? It has been previously shown that Grb2 is associated with CD22, a membrane receptor that negatively regulates BCR signaling (26). Upon BCR stimulation, CD22 becomes tyrosine phosphorylated at its cytoplasmic tail by Lyn, which then recruits tyrosine phosphatase SHP-1 to the membrane to suppress BCR downstream signaling molecules, including BLNK and Igα/Igδ (27). Therefore, we examined this negative regulatory circuit of BCR signaling. We found that tyrosine phosphorylation of CD22 in the mutant B cells was reduced compared with that occurring in WT cells (Fig. 1E). These results thus indicate that Grb2 is involved in the regulation of CD22 tyrosine phosphorylation, and suggest that Grb2 may control BCR signaling by integrating the CD22-SHP-1 negative-feedback loop in B cells.

Fig. 1. Altered development and antigen-receptor signaling of grb2+/- B cells. (A) Dot plots show B220+ gated splenic cells of WT and Grb2BKO mice. (Top) Mature (AA4.1+CD24+) and immature (AA4.1+CD24+) B cells. (Middle) transitional T1 (CD21+CD23-) and T2 (CD21+CD23+) B cells within the AA4.1+CD24+ immature B-cell population. (Bottom) marginal-zone B (CD21+CD23+), follicular B (CD21+CD23+), and B1 B (CD21+CD23+) cells within the AA4.1+CD24+ mature B cells. Percentages of each subset are indicated in the plots. (B) Purified B cells (B220+AA4.1+CD24+CD21+) were stimulated with anti-IgM F(ab′)2, anti-CD40, IL-4, and LPS, and either alone or in combination as indicated. Cell proliferation was measured by [3H]-thymidine incorporation; error bars with SD (n = 3). (C) Intracellular Ca2+ in B cells was measured by fluorescence intensity of Fluo-4 vs. Fura-red (n = 8). (D and E) Purified B cells from WT or Grb2BKO mice were stimulated with anti-IgM for various periods. Tyrosine phosphorylation of PLCγ2 and CD22 was determined by immunoprecipitation followed by Western blotting against phosphotyrosine (4G10). Active forms of Erk1/2, JNK, and p38 were directly determined using specific antibodies against individual phosphorylated kinases. The results represent more than three independent experiments.
of high-affinity anti-NP antibody occurred normally in mutant mice (Fig. S5B), despite the apparent lack of splenic GCs. The mutant mice also elicited normal recall responses (Fig. S5C), suggesting that the development of memory B cells and long-lived plasma cells is intact in Grb2−/− mice.

Our serological findings that Ig class switch and antibody-affinity maturation occurred normally in Grb2−/− mice in the absence of splenic GCs suggested that these processes might take place in other tissues than in the spleen, or in grb2−/− GC B cells could not express the characteristic cell-surface markers. We, therefore, examined the GC structure in both spleen and lymph nodes by immunohistology with anti-CD35 antibodies. The GCs in WT mice (Fig. 3A and B) were characterized by the expression of the characteristic cell-surface markers CD35 and CD38. In contrast, neither PNA+ B cells nor CD35+ FDC clusters. In contrast, neither PNA+ B cells nor CD35+ FDC clusters were detected in the spleen of mutant mice (Fig. 3C). These data demonstrate that B-cell–specific depletion of grb2 only affects GC formation locally in the spleen. The observed Ig switch recombination and antibody-affinity maturation in mutant mice can be attributed to normal GC reaction in lymph nodes. In addition to the absence of FDC networks were detected in the spleen of mutant mice (Fig. 3D and E) and Grb2−/− mice (gray bars) were shown as either CD95+PNA+ population in contour plots or CD95+GL7+ cells in dot plots. (C) Splenocytes were stained with lineage markers (CD4, CD8, Gr1, F4/80), anti-B220, NP, and anti-CD38. (Upper) B220 vs. NP staining of gated-lineage marker negative (Lin−) splenocytes. The percentages of Lin−NP-binding B220− cells are indicated. The frequencies of IgG1+CD38− memory (Mem) and IgG1+CD38+ GC subsets within the antigen specific (B220+NP−) B-cell compartment are shown in the dot plots (n = 7). (D) Numbers of NP-specific IgM and IgG secreting plasma cells in the spleen of WT (white bars) and Grb2−/− (gray bars) mice. Each symbol represents a mean value of triplicate samples of an individual mouse.

**Fig. 2.** Impairment of GC reaction in Grb2−/− spleen. Data were obtained from WT and Grb2−/− mice (8- to 10-wk-old) at day 10 after NP-KLH immunization. (A) (Left) Contour plots show BrdU+IgG− and BrdU+IgG+ populations of the B220−-gated cells. (Right) Frequencies and absolute numbers of BrdU− B cells in WT (white bars) and Grb2−/− (gray bars) mice are shown as mean with SD (n = 7). (B) PNA− IgG+ plasma cells is intact in Grb2BKO mice. A mean value of triplicate samples of an individual mouse. (C) PNA− IgG+ plasma cells is intact in Grb2BKO mice. A mean value of triplicate samples of an individual mouse.
A

B

C

Fig. 4. Regulation of CXCR5 signaling and LT expression by Grb2. (A) IgM^+IgD^+ naive splenic B cells were purified from WT and Grb2^βK0^ mice by FACS. Transcripts of LTA, LTB, and TNFα were quantified by qRT-PCR and are shown as relative units after being normalized to the expression of β-actin in the corresponding samples; P < 0.005, n = 5. (B) Splenocytes were stained with anti-B220 and LTB-R-Ig (n = 7). The percentages of cells within each quadrant are indicated in the plots. (C) Splenocytes were rested on ice for 8 h to disengage the chemokine signaling. Cells were then stimulated with either 3 μg/mL of CXCL13 or 10 μg/mL of anti-CD40 at 37 °C for 16 h and then subjected to LTB-R-Ig and anti-B220 staining. Shown are percentages of LT-positive (B220^+LTB-R-Ig^+) B cells in a B220^+^ gated population. n = 4.

Enforced LTβ expression restores splenic defects in Grb2^βK0^ mice. To further test whether enforced expression of LTβ in Grb2^βK0^ mice is sufficient to restore the FDC networks and GC reaction, we reconstituted Grb2^βK0^ BM cells with either murine stem cell virus (MSCV) or LTβ-expressing MSCV retroviral vector and generated BM chimeras. At day 11 after immunization, we examined GC B cells by flow cytometry and spleen architecture by immunofluorescent staining. As shown in Fig. 6, both GCs and FDC networks were fully restored in the BM chimeras transduced with LTB-MSCV but not with empty MSCV vector. This result clearly shows that impaired LTβ expression in Grb2^βK0^ mice is responsible for the defects of splenic architecture and GC reaction caused by Grb2 deficiency.

Discussion
Grb2 has been asserted to be a key adaptor for multiple cellular functions by virtue of its physical association with a variety of signaling proteins. Our study indicates that Grb2 controls a branch of CXCR5 signaling in B cells by transcriptionally controlling β2-transducin-like expression in Grb2^βK0^ B cells. This finding suggests that the GC defect caused by the Grb2^βK0^ mutation can be rectified by the presence of WT B cells that presumably provide the necessary LT signals for the genesis of FDC networks and pattern formation of lymphoid follicles.

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Discussion
Grb2 has been asserted to be a key adaptor for multiple cellular functions by virtue of its physical association with a variety of
receptors and their downstream signaling molecules. However, its function in the immune system has not been fully characterized. While Grb2 positively regulates TCR signaling, it acts as a negative regulator in B-cell activation. Although the detailed mechanism by which Grb2 negatively regulates BCR signaling is still unclear, our observation of a reduction of CD22 phosphorylation in grb2−/− B cells after BCR stimulation may provide an explanation. CD22 is an inhibitory receptor for BCR signaling. It has been shown that Lyn phosphorylates CD22 after BCR stimulation. Phosphorylated CD22 then recruits the tyrosine phosphatase SHP-1 to the CD22/BCR complex to exert suppressive effects on Ca2+ flux and MAPK activation. Thus, it is likely that Grb2 is required for strengthening the Lyn-CD22-SHP-1-negative-feedback loop. Our data showing that the active form of Lyn was slightly reduced in the mutant B cells is consistent with this notion.

It has been shown that a strong BCR signal in immature B cells may lead to apoptosis, a mechanism being used to eliminate autoreactive B cells. Notably, Grb2BKO mice show about 60% loss of immature B cells at T1 and T2 stages, despite normal B-cell genesis. Because Grb2 deficiency enhances BCR signaling without affecting BAFF dependent survival of B cells, it is possible that a strong BCR signal in grb2−/− immature B cells assumes negative selection and purge ~60% of immature B cells in the periphery of Grb2BKO mice. Alternatively, it is also likely that the splenic microenvironment of Grb2−/− B cells is less supportive for grb2−/− B cells. Consistent with this idea, we observed that grb2−/− B cells were specifically increased compared with the WT counterpart in Grb2BKO and WT irradiated BM chimeras (Fig. S8), suggesting that the presence of WT B cells may correct the defective spleen environment (such as the lack of FDCs and perhaps also other accessory cells). More experiments are needed to clarify this issue.

While progressing through the T2 stage, maturing B cells migrate to and reach the B-cell follicles. After encountering antigens and with T-cell help, mature follicular B cells initiate GC reaction and develop into either memory B cells or long-lived plasma cells. While much progress has been made in the past decades, the mechanism that governs B cells into these two developmental pathways has not been fully understood. It is surprising that Grb2 deficiency in B cells abrogates GC formation in the spleen, even though mutant B cells respond to antigens vigorously. Our further studies demonstrate that Grb2-mediated signaling is not involved in dictating the GC-cell fate of antigen-activated B cells; rather, it delivers signals to induce chemokine CXCR5 to induce expression of proper lymphokine that is essential for the development of proper lymphoid follicle structures, including the marginal zone, FDC networks, and GCs. Indeed, grb2−/− B cells are able to form GCs when coexisting with WT B cells, suggesting that the CXCR5-LTβR-LT signaling axis in WT naive B cells is required for laying out functional spleen architecture that accommodates mutant B cells to undergo the GC maturation program. Because grb2−/− B cells can form GCs, our results thus indicate that reverse signaling from the membrane-anchored LT, if any, is not required for the GC reaction.

Why is the defective development of FDC and GC confined to the spleen but not to lymph nodes of Grb2BKO mice? One explanation for such a phenotype is that deletion of Grb2 in B cells impairs LT expression only in B cells but not in other lineages of cells. It is known that different secondary lymphoid organs require different LT- and LTβR-expressing hematopoietic and stromal cell lineages for proper organogenesis. For example, the architecture of the spleen but not of lymph nodes relies on B-cell-derived LT (31–33). On the other hand, LT produced by lymphoid tissue-inductor cells during days 11 to 16 gestation is pivotal for lymph node development (36, 37); however, lack of lymphoid tissue-inductor cells did not affect the formation of splenic B-cell follicles (38). We will be interested to determine whether Grb2 also functions in other cell lineages to control LT expression for the formation of lymphoid follicles and GCs in lymph nodes. It should also be noted that although our results establish Grb2 as an essential integrator of CXCR5 signaling pathways, there are phenotypic differences between Grb2BKO mice and CXCL13−/− or CXCR5-mutant mice, as the latter show more distorted follicular organization and a diminishment—but not absence—of GC reaction in the spleen (15, 39, 40). In vitro data have shown that chemokines other than CXCL13 can also up-regulate LT expression in naive B cells (15). It is therefore likely that ablation of CXCL13−/− B cells or IL-4/IL-15 signaling from all chemokine receptors to LT expression (41), hence completely eliminating splenic FDC networks and GCs.

Grb2BKO mutation selectively affects humoral immunity in the spleen but not lymph nodes. It remains unclear whether such a differential regulation has any biological impact on immunity against pathogens or autoantigens. It is generally believed that lymph nodes normally mediate immunity against pathogens from their draining peripheral tissues. In contrast, the spleen encounters mostly blood-borne antigens. Interestingly, B-cell–specific LTβ−/− deficient mice, which have normal lymph nodes but a similar defect in the splenic architecture as Grb2BKO mice, fail to develop effective humoral responses against a low dose of vesicular stomatitis virus (33). In this regard, splenic GC reaction may play a more profound role in immune responses against those blood-borne pathogens. In addition to immunity against pathogens, we have noticed that Grb2BKO mice produce high titers of anti-dsDNA IgM and have a high incidence of antibody deposits in kidneys, but do not develop lupus-like nor other autoimmune diseases. It is therefore worthwhile to examine whether lack of splenic GC formation in these mice may contribute to autoimmune responses.

In summary, our data demonstrate that Grb2 plays an important role in B cells via integrating various signaling transduction pathways of the BCR and CXCR5. Our Grb2BKO mice thus provide a useful model to further dissect the signalosomes downstream of these receptors. Importantly, we additionally reveal a Grb2-containing CXCR5 signalosome, distinct from those required for chemotaxis, which regulates LT expression. In this regard, targeting Grb2 may differentially modify chemokine receptor signaling and immune responses to achieve more selective therapeutic goals.

Materials and Methods

Mice, Adoptive Transfer, and Immunization. grb2−/− mice (generated in our laboratory, have been back crossed to C57BL/6 for more than 12 generations, and further crossed to CD19−Cre transgenic mice to generate B-cell–specific grb2 knockout (grb2−/−CD19−Cre heterozygous) mice, termed here as Grb2BKO mice. Grb2BKO mice were healthy with no obvious abnormality. To generate BM chimeras, 105 BM cells from WT B6.SJL and Grb2−/− mice were injected intravenously, either alone or at 1:1 ratio, into 750 Rad- irradiated B6.SJL recipients (The Jackson Laboratory). To immunize mice with TD antigens, 50 μg of NP-βKLL (Biosearch Technologies) precipitated in Inject Alum (Pierce) were injected into mice intraperitoneally. To immunize mice with SRBC, 200 μL of PBS-washed 10% SRBC were injected into each mouse intraperitoneally. All animals were housed in the specific pathogen-free barrier facility at Columbia University in accordance with institution approved protocols.

Biochemistry and Antibodies. For Western blot analysis, 50 to 100 μg of total proteins were loaded and size-fractionated on a 12% SDS-PAGE gel by electrophoresis and transferred to a PVDF membrane. For immunoprecipitation, 1 to 2 μg of antibody and 50 μL of protein G agarose beads was added to total cell lysate from 2 to 5 × 106 cells and rotated at 4 °C overnight. After washing, proteins were denatured by boiling and fractionized on 12% SDS-PAGE gel. Immunoblotting was performed according to our previous protocol (42). The following antibodies were used for biochemical study: anti-IgM F(ab)2, (Biosource); anti-phosphotyrosine (4G10) (Upstate Bio-tech); anti-p-Erk, anti-ERK1/2, anti-p-INA, anti-INA, anti-p-38, anti-IgG2, and anti-PLCγ2 (Santa Cruz); and anti-p-38 (Biosource).

In Vitro 3H-Thymidine Incorporation Assay. Mature (B220AA4.1CD24−) splenic B cells were purified by FACS sorting, and seeded in triplicates to a 96-well plate at 5 × 104 cells per well, in the presence of goat anti-IgM F(ab), (Organon Teknica Corp.; 10 μg/mL), anti-IgM F(ab), plus anti-CD40 (BD Pharmingen; 10 μg/mL), or IL-4 (Biosource; 20 U/mL), anti-CD40 plus IL-4, or LPS (Sigma-Aldrich; 30 μg/mL). Forty-eight hours later, 3H-thymidine (1 μCi) was added to each well and incubated for an additional 6 h. Cells were harvested on a cell harvester and 3H-thymidine incorporation was measured on a β-counter.
Flow Cytometry and BrdU Labeling. Single-cell suspensions were prepared from total BM, spleen, or mesenteric lymph nodes. After staining, cells were analyzed using a FACSCalibur or LSR II. The following antibodies were used for the staining: anti-B220, anti-CD21, anti-CD23, anti-CD24, anti-CD38, anti-GL7, anti-AA4.1, anti-igG1 (BD Pharmingen); anti-igM, anti-igG (Southern Biotech); PNA (Vector); NP-PE (gift from M. Shlomchik, Yale University, New Haven, CT). For cell-surface LT staining, cells were incubated for 1 h on ice with staining solution containing 10% goat and rat serum to block Fc receptors. After washing, cells were incubated with 50 µL of LT-ig (10 µg/mL) on ice for 45 min. Stained cells were further revealed by anti-human IgG (e Bioscience). For BrdU-labeling, mice were injected with BrdU (1 mg per mouse) intraperitoneally. Four hours later, mice were killed for analysis. BrdU staining was performed using a kit (BD Pharmingen) according to the manufacturer’s instructions.

Ca2+ Mobilization. Splenic B cells were labeled with Flu-4 and Fura-red (Molecular Probes) according to the manufacturer’s instructions. Cells were then stimulated with anti-igM (Fab) at 37 °C and subjected to FACS analysis. Intracellular Ca2+ concentration was presented as the ratio of fluorescence intensities of Flu-4 vs. Fura-red.

Immunofluorescence Histology. Cryosections of spleen and mesenteric lymph nodes were fixed in 4% paraformaldehyde at room temperature for 10 min, blocked with 10% BSA in PBS containing 0.1% Triton X-100 for 1 h at room temperature, and then stained with corresponding antibodies in PBS containing 0.1% Tween-20 in a humidity chamber at RT for 1 h. Antibodies used are: anti-CD4, anti-CD8, anti-CD35, and B220 (BD Pharmingen); PNA-biotin (Vector); anti-Moma-1 (Serotec Inc.), anti-igM-1 clone (3-1; gift from R. Pelanda, University of Colorado, Denver, CO), streptavidin-Alexa 568 or streptavidin-Cy5 (Molecular Probes). Data were obtained with a Nikon TE-2000E microscope using NIS-Elements 2.3 software.

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Supporting Information
Jang et al. 10.1073/pnas.1016451108

Fig. S1. Specific deletion of grb2 in B cells. (A) Southern blot analysis of grb2 alleles. Bands representing WT, floxed, and deleted grb2 alleles are indicated by arrows. Genotype and cell types of the samples are indicated at the top of the blot. (B) Western blot analysis of grb2 deletion in B cells.

Fig. S2. Normal B-cell generation in the bone marrow (BM) of Grb2^BKO^ mice. Flow cytometry analysis of the BM B-cell subsets in Grb2^BKO^ mice. Shown are dot plots of BM cells stained for B220 and CD43 (Upper), B220 and IgM (Lower). Percentages of each B-cell subset are indicated in the plots.
**Fig. S3.** In vitro proliferation of immature B cells. Immature T1 and T2 (AA4.1^hi^CD24^hi^) B cells were isolated by FACS sorting. Cells were cultured in 96-well plates (5 × 10^5^ per well) in the presence of soluble anti-μ F(ab)_2, IL-4, anti-CD40, and LPS, either alone or in different combinations for 48 h. ^3^H-thymidine (1 μCi per well) was added to the culture for 12 h. ^3^H-thymidine incorporation was determined on a β-counter. Shown are representatives of more than three independent experiments.

**Fig. S4.** B-cell receptor (BCR)-induced tyrosine phosphorylation of total cellular proteins and activation of Lyn kinase. Purified B cells were stimulated with anti-IgM for various periods. Tyrosine phosphorylation of total proteins and activation of Lyn were detected by an anti-phosphotyrosine antibody (4G10) and anti-active Lyn (Lyn-pY508).
In vitro up-regulation of lymphotoxin (LT)-β. Splenic B cells were purified from WT and Grb2BKO mice using the mouse B-cell enrichment kit (StemCell Tech). B cells were rested in RPMI medium with 0.1% FCS at 4 °C overnight, and then stimulated with either 3 μg/mL of CXCL13 or 10 μg/mL of anti-CD40 at 37 °C for 3.5 h. Total RNA was prepared from cell lysates and LTβ transcript was quantified by qRT-PCR and are shown as relative units after normalized to the expression of 18sRNA in the corresponding samples. P < 0.001, n = 3.

Fig. 55. grb2−/− B cells have undergone antibody affinity maturation and produce normal serum levels of antigen-specific IgM and IgG antibodies. (A) Serum levels of NP-specific Ig isotypes of WT (○) and Grb2BKO (●) mice at indicated time points after NP-KLH immunization were measured by ELISA. Each symbol represents the value obtained from one mouse. (B) Relative affinities of IgG1 of WT (white bars) and Grb2BKO (black bars) mice were determined at the indicated time points after antigenic challenge. Bars represent the mean ± SD (n = 5). (C) Serum levels of NP-specific Ig at day 7 after antigenic rechallenge. WT (○) and Grb2BKO (●) mice.

Fig. 56. In vitro up-regulation of lymphotxin (LT)-β. Splenic B cells were purified from WT and Grb2BKO mice using the mouse B-cell enrichment kit (StemCell Tech). B cells were rested in RPMI medium with 0.1% FCS at 4 °C overnight, and then stimulated with either 3 μg/mL of CXCL13 or 10 μg/mL of anti-CD40 at 37 °C for 3.5 h. Total RNA was prepared from cell lysates and LTβ transcript was quantified by qRT-PCR and are shown as relative units after normalized to the expression of 18sRNA in the corresponding samples. P < 0.001, n = 3.
Fig. S7. grb2−/− B cells exhibit normal chemotactic migration to CXCL12 and CXCL13. (A) Dot plots show splenocytes from WT and Grb2BKO mice stained with antibodies against B220 and CXCR5. Histograms show the CXCR5 expression level on WT B cells (shaded) or on grb2−/− B cells (red). (B) Chemotaxis of WT (white bars) or grb2−/− (gray bars) B cells toward CXCL12 and CXCL13 were analyzed by a transwell assay. The input and migrated splenocytes were stained with anti-B220-Alexa 780, and the number of B cells in each population was determined by flow cytometry. The percentages of migration of splenic B cells toward CXCL13 and CXCL12 are shown.

Fig. S8. Preferential expansion of grb2−/− B cells in BM chimeras. BM chimeras were generated by transferring equal numbers (10^6) of BM cells from CD45.1+ WT and CD45.2+ Grb2BKO mice into irradiated CD45.1+ WT recipients (n = 4). Flow cytometry analysis was carried out 2 mo after transplantation. Splenocytes were stained with anti-B220-Alexa 780, anti-CD45.1-Alexa 450, and anti-CD45.2-APC. B220+ and B220− populations are gated, and the cells of Grb2BKO or WT origin were displayed as CD45.2+ and CD45.1+ cells, respectively. The inversed chimerism between B cells and non-B cells indicates that grb2−/− B cells have undergone preferential expansion.

Table S1. Numbers of subset B cells in the bone marrow and spleen

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<tr>
<th></th>
<th>Grb2+/+</th>
<th>Grb2BKO</th>
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<tr>
<td>Bone marrow (N = 6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-B</td>
<td>5.9 ± 2.3</td>
<td>5.8 ± 2.2</td>
</tr>
<tr>
<td>Pro-B</td>
<td>1.6 ± 0.5</td>
<td>1.5 ± 0.2</td>
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<tr>
<td>Immature B</td>
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<td>Recirculating B</td>
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<td>Spleen (N = 8)</td>
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<tr>
<td>Immature B</td>
<td>4.0 ± 1.3</td>
<td>1.8 ± 0.8*</td>
</tr>
<tr>
<td>T1</td>
<td>1.7 ± 0.5</td>
<td>0.7 ± 0.4*</td>
</tr>
<tr>
<td>T2</td>
<td>2.0 ± 0.8</td>
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<tr>
<td>Mature B</td>
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<td>15.7 ± 5.7**</td>
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<tr>
<td>FO</td>
<td>16.4 ± 4.9</td>
<td>9.7 ± 4.2**</td>
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<td>B1</td>
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Numbers shown here represent data from six to eight mice. Bone marrow pro-B, IgM-IgD-B220−CD43−; Pre-B, IgM-IgD-B220−CD43+; immature B, B220−IgM+ IgD−; recirculating B, B220+IgD+. Cell surface markers for splenic B cell subsets are described in the legend to Fig. 1. Shown is mean ± SD. *P < 0.01; **P < 0.05.