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 lympho- toxin-β-deficient mice, including disruption of marginal zone and follicular dendritic cell networks. We find that grb2−/− B cells are defective in lympho- toxin-β expression. Although lympho- toxin can be up-regulated by chemokine CXCL13 and CD40 ligand stimulation in wild-type B cells, elevation of lympho toxin expression in grb2−/− B cells is only induced by anti-CR40 but not by CXCL13. Our results thus define Grb2 as a nonredundant regulator 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 lympho toxin expression but not to chemotaxis.

B-cell signaling and activation | follicular dendritic cell development | germinal center development | regulation of lympho toxin expression | chemokine signaling

Germinak center (GC) reaction is critical for effective humoral immune responses and for the generation of memory B lymphocytes. This process requires close interactions between antigen-specific B and T cells, and follicular dendritic cells (FDCs) (1–3). Multiple B-cell intrinsic signals are involved in orchestrating this process. The signal that initiates GC formation is delivered through the B-cell receptor (BCR), with a group of signaling proteins modulating the strength of BCR signaling and immunological synapse formation (4–6). These proteins include Lyn, SHP1, and DOCK8, as well as the coreceptor CD19, which collectively drive B-cell activation, proliferation, and differentiation to either memory B cells or antibody-producing plasma cells in GCs (7–9). In addition to BCR signaling, signals derived from the surrounding niche induce activated B cells to undertake the GC differentiation pathway and support them to complete the GC maturation program. B-cell–expressing molecules that are involved in this process include ICOSL, CD40, PD-1 ligands, and cytokine receptors (10–13). However, how these signaling pathways converge in B cells to elicit an optimal GC response is not known.

Only a small fraction of antigen-activated B cells will differentiate into GC cells. It is not clear what selects a B-cell to commit to the GC fate, although it is suggested that B cells compete for antigens, cognate T-cell help, and for other growth and survival signals from the specialized follicular microenvironment to establish GCs. Thus, the precise localization of B cells during immune responses is decisive for GC maturation. Chemokine receptors CXCR5 and CXCR4, lympho toxin (LT), and TNF play a crucial role in organizing B-cell follicles and GCs (14). Of a particular interest, it has been shown that activation of CXCR5 induces naive B cells to produce LT that is required for establishing the FDC network and GC formation (15). Currently, how the CXCR5 signal is connected to LT expression still remains elusive.

The mammalian growth-factor receptor-bound protein-2 (Grb2) is a simple adaptor that consists of one central SH2 domain flanked by two SH3 domains (16). It is broadly expressed in many tissues and is essential for embryo development and multiple cellular functions (17–19). Grb2 has been identified as a major mediator in Ras-MAPK activation induced by numerous receptors because of its association with son of sevenless, a GDP-GTP exchange factor for Ras (18–20). Studies using T-cell–specific Grb2-deficient mice show that Grb2 plays a broad role in early T-cell development as both thymic-positive and -negative selection are impaired in the absence of Grb2. Grb2 is a positive regulator for TCR signaling, and it does not directly affect the activities of Ras and Erk1/2, but rather amplifies the activity of Lck, a member of the Src family of tyrosine kinases that functions at the top of the TCR signaling cascade (21).

In chicken B cells, it has been reported that Grb2 regulates Ca2+ influx upon BCR activation (22). Mice deficient in the hematopoietic-adapter protein downstream of kinase-3 (Dok-3), an adapter that recruits Grb2 to the membrane, exhibit enhanced BCR-induced Ca2+ mobilization (23, 24), suggesting that Grb2 may participate in BCR signaling also in mammalian B cells (for review, see refs. 18 and 19). However, the physiological role of Grb2 in B-cell development and function remains unclear. Here we show that Grb2 negatively regulates BCR signaling, grb2−/− B cells are hyperreactive; however, mutant splenic B cells did not form GCs. We find that Grb2 is necessary for CXCL13-induced LT expression in B cells. Our findings establish the CXCL13/CXCR5-Grb2-LT signaling axis in B cells as a nonredundant pathway that controls lymphoid follicle organization and GC reaction.

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.


The authors declare no conflict of interest.

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(Grb2−/−) 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 impaired further maturation of B cells after immature 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 Grb2−/− 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 (threefold) (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 Igβ. The phosphorylated ITAMs recruit and activate Lyn, leading to subsequent tyrosine phosphorylation of signaling effector molecules, including BLNK/Igα 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 was 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 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. 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.

**Grb2−/− Mice Have Disrupted Lymphoid Follicles and Cannot Form GCs in the Spleen.** To directly assess the impact of Grb2 deficiency on T-cell–dependent (TD) antibody responses, we immunized mice with a TD antigen NP-KLH (4-hydroxy-3-nitrophenoxyacetyl–keyhole limpet hemocyanin). At day 10 after immunization, WT B cells had undergone extensive clonal expansion and some of the antigen-activated B cells differentiated into GC cells (28). Grb2−/− mice generated an equivalent number of BrdU+ B cells in the spleen during 4-h BrdU pulse, even though they have ~40% fewer mature B cells compared with controls. Thus, proliferating B cells were proportionally increased ~twofold in Grb2−/− mice (Fig. 2A). Although mutant B cells responded strongly to antigen stimulation, these cells did not give rise to GC cells (Fig. 2B). We further analyzed the antigen-specific B-cell compartment for the generation of IgG1−/−IgG3−/− GC and the IgG1−/−IgG3−/− memory B cells (29, 30) and found that both subsets were reduced in the spleen of the mutant mice (Fig. 2C). As a consequence of the enhanced antigen-activated B-cell proliferation but an impaired GC reaction, Grb2−/− mice generated more NP-specific IgM-producing and fewer IgG-producing plasma cells in the spleen (Fig. 2D).

To determine whether the lack of GC responses impaired long-term humoral immunity, we tested primary and recall responses to TD antigens in Grb2−/− and WT mice. When the serum level of NP-specific antibodies was measured at different time points during the primary immune response, we found that the NP-specific IgM titer was slightly higher in the mutant mice compared with that of controls (Fig. S5A). To our surprise, anti-NP responses of other Ig isotypes including IgG1, IgG2b, IgG2c, and IgG3 were unaffected by the loss of Grb2 (Fig. S5A). Generation
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 Grb2BKO mice.

Our serological findings that Ig class switch and antibody-affinity maturation occurred normally in Grb2−/−B cells in the absence of splenic GCs suggested that these processes might take place in other tissues than in the spleen, or grb2−/−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 that highlights FDC clusters in the light zone of the GC. At day 10 after immunization with sheep red blood cells (SRBC), WT mice formed large GCs in both the spleen and lymph nodes (Fig. 3 A–D), visualized as PNA+ B-cells and CD35+ FDC clusters. In contrast, neither PNA+ B-cells nor FDC networks were detected in the spleen of mutant mice (Fig. 3 A and B). Interestingly, the GC structure was intact in lymph nodes of Grb2BKO mice (Fig. 3 C and D). These data demonstrate that B-cell-specific deletion 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 in the spleen, further inspection of the spleen follicles revealed that the marginal zone was also severely disrupted in Grb2BKO mice (Fig. 3E). This result thus indicates that Grb2 is responsible for general organization of the spleen follicles.

**Naive grb2−/− B Cells Are Deficient in LTβ Expression.** The phenotypes of Grb2BKO mice, such as defective marginal zone, diminished FDC networks, and failure of GC formation in the spleen but unaffected lymph-node architecture, bear a striking resemblance to that of B-cell–specific LTβ-deficient mice (31–33). We hence decided to examine whether Grb2 was required for the expression of LTβ and other immediate members of the TNF family. Quantitative RT-PCR analysis shows that although grb2−/− and WT naive B cells (IgM+ IgD+) had comparable levels of LTα and TNF-α transcripts, LTβ expression was clearly defective in the mutant B cells compared with the WT counterparts (Fig. 4A). When cell-surface LT was examined by a soluble form of the LT receptor, LTβR-Ig, which binds to the membrane-anchored LTα1β2, we could not detect LT on the cell surface of grb2−/− B cells (Fig. 4B). These results demonstrate that Grb2-mediated signaling is critical for LT expression in naive B cells.

**Grb2 Connects CXCR5 Signaling to LT Expression.** Previous studies have shown that CXCR5 signaling is essential for the expression of LT on naive B cells to control the formation of lymphoid follicles (15), whereas CD40L and other stimulatory molecules further up-regulate LT expression on activated lymphocytes during immune responses (34, 35). To determine whether Grb2 connected signaling from CXCR5 or CD40 to LT up-regulation in B cells, we examined LT expression after stimulating splenic B cells with the CXCR5 ligand CXCL13 or antibody to CD40. We found that anti-CD40 treatment drastically up-regulated LT expression on both WT and grb2−/− B cells (Fig. 4C). In contrast, CXCL13 induced LT expression only in WT but not in grb2−/− B cells (Fig. 4C and Fig. S6), demonstrating that Grb2 is an essential adaptor downstream of the CXCR5 signal-transduction pathway leading to LT expression. It is important to note that chemotaxis of grb2−/− B cells to either CXCL13 or CXCL12 works in the spleen, further inspection of the spleen follicles revealed that the marginal zone was also severely disrupted in Grb2BKO mice (Fig. 3E). This result thus indicates that Grb2 is responsible for general organization of the spleen follicles.
stimulation was normal (Fig. S7). These results indicate that Grb2 specifically controls a branch of CXCR5 signaling in B cells that induces LTβ transcription but not chemotaxis.

**grb2**−/−**B Cells Are Not Intrinsically Deficient in Differentiation into GC Cells.** The above results let us posit that grb2**−/−** B cells may undergo GC differentiation if the positive-feedback loop of LT and chemokines were restored in the spleen of Grb2**−/−** mice by WT B cells. To test this hypothesis, we established BM chimeras by cotransplanting an equal number of WT (CD45.1+) and WT Grb2BKO (CD45.2+) BM cells into lethally irradiated B6.SJL mice (CD45.1+) and examined GC formation after immunization. In this experimental setting, B cells derived from WT BM stem cells should express LT and, therefore, were expected to restore the splenic FDC networks. We found that indeed, in chimeras that received both WT and Grb2BKO BM cells, splenic GC cells contained not only WT but also Grb2BKO donor cells. (Fig. 5A) BM chimeras with mixed WT and Grb2BKO donor cells. (B) BM chimeras with only Grb2BKO donor cells. n = 4.

**Enforced LTβ Expression Restores Splenic Defects in Grb2BKO Mice.** To further test whether enforced expression of LTβ in Grb2BKO mice is sufficient to restore the FDC networks and GC reaction, we reconstituted Grb2BKO 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 LTβ-MSCV but not with empty MSCV vector. This result clearly shows that impaired LTβ expression in Grb2BKO 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
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−/− mice is less supportive for grb2−/− B cells. Consistent with this idea, we observed that grb2−/− B cells were preferentially 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. Although 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 CXCR5 to induce expression of LT, a well-known chemokine 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-LT-Grb2-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 populations to pinch off lymphoid nodules (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 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 pathogens. 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 higher 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 could elicit more subtle autoimmune reactions.

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-floxed (grb2fl) 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. Grb2fl mice were healthy with no obvious abnormality. To generate BM chimeras, 106 BM cells from WT B6.SJL and Grb2BKO 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 NP30-KLH (Biosearch Technologies) precipitated in Injekt 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-JNK, anti-JNK, anti-p38, anti-Grb2, and anti-PLCγ2 (Santa Cruz); and anti-p-g10 (BioSource).

In Vitro 3H-Thymidine Incorporation Assay. Mice (B220+AA4.1CD42+4+) splenic B cells were purified by FACS sorting, and seeded in triplicates to a 96-well plate at 5 × 105 cells per well, in the presence of goat anti-IgM F(ab), (Organon Teknika 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.
Intracellular Ca\(^{2+}\) concentration was presented as the ratio of Fluo-4 vs. Fura-red.

For BrdU-labeling, mice were injected with BrdU (1 mg per mouse) i.p. 24 h before sacrifice to label newly synthesized DNA. Total splenic cells were isolated by Ficoll density gradient centrifugation. Cells were washed twice and counted. Cells were plated in 96-well plates (50 \(\mu\)L well) and incubated at 37 °C for 1 h. After washing, viable cells were stained with either NP2-BSA or NP30-BSA (Biosearch Technologies; 10 \(\mu\)g/mL) for 1 h. Bound antibody plaques were revealed using a CBR/NIKT kit (Vector) and quantified under a microscope.

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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<sup>BKO</sup> mice. Flow cytometry analysis of the BM B-cell subsets in Grb2<sup>BKO</sup> 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<sup>hi</sup>CD24<sup>hi</sup>) B cells were isolated by FACS sorting. Cells were cultured in 96-well plates (5 × 10<sup>4</sup> 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. <sup>3</sup>H-thymidine (1 μCi per well) was added to the culture for 12 h. <sup>3</sup>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).
Fig. S6. In vitro up-regulation of lymphotoxin (LT)-β. Splenic B cells were purified from WT and Grb2 BKO 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. S5. 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 Grb2 BKO (●) 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 Grb2 BKO (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 Grb2 BKO (●) mice.
**Fig. S7.** *grb2*−/− B cells exhibit normal chemotactant migration to CXCL12 and CXCL13. (A) Dot plots show splenocytes from WT and Grb2KO 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 Grb2KO 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 Grb2KO or WT origin were displayed as CD45.2+ and CD45.1+ cells, respectively. The inverted 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

<table>
<thead>
<tr>
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<th>Grb2+/+</th>
<th>Grb2KO</th>
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<tr>
<td><strong>Bone marrow</strong></td>
<td></td>
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<tr>
<td>Pre-B</td>
<td>5.9 ± 2.3</td>
<td>5.8 ± 2.2</td>
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<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>0.9 ± 0.6</td>
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<tr>
<td>Recirculating B</td>
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<td>0.9 ± 0.6</td>
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<tr>
<td><strong>Spleen</strong></td>
<td></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>
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<tr>
<td>T2</td>
<td>2.0 ± 0.8</td>
<td>0.8 ± 0.3*</td>
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<td>Mature B</td>
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<td>15.7 ± 5.7**</td>
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<td>16.4 ± 4.9</td>
<td>9.7 ± 4.2**</td>
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<td>3.0 ± 1.3</td>
<td>2.4 ± 0.9</td>
</tr>
<tr>
<td>B1</td>
<td>1.9 ± 0.9</td>
<td>2.3 ± 1.1</td>
</tr>
</tbody>
</table>

Numbers shown here represent data from six to eight mice. Bone marrow pro-B, IgM-IgD-B220CD43+; Pre-B, IgM-IgD-B220CD43−; immature B, B220IgM+ IgD−; recirculating B, B220IgD+. 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.