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G12/G13 Family G Proteins Regulate Marginal Zone B Cell Maturation, Migration, and Polarization

Stefan Ricken,* Antonia Sassmann,* Susanne Herroeder,*† Barbara Wallenwein,* Alexandra Moers,* Stefan Offermanns,* and Nina Wettschureck2*

G protein-coupled receptors play an important role in the regulation of lymphocyte functions such as migration, adhesion, proliferation, and differentiation. Although the role of G12 family G proteins has been intensively studied, no in vivo data exist with respect to G12/G13 family G proteins. We show in this study that mice that lack the G protein α-subunits Gna12 and Gna13 selectively in B cells show significantly reduced numbers of splenic marginal zone B (MZB) cells, resulting in a delay of Ab production in response to thymus-independent Ags. Basal and chemokine-induced adhesion to ICAM-1 and VCAM-1, two adhesion molecules critically involved in MZB localization, is normal in mutant B cells, and the same is true for chemokine-induced migration. However, migration in response to serum and sphingosine 1-phosphate is strongly increased in mutant MZB cells, but not in mutant follicular B cells. Live-cell imaging studies revealed that Gna12/Gna13-deficient MZB cells assumed more frequently an ameboid form than wild-type cells, and pseudopod formation was enhanced. In addition to their regulatory role in serum- and sphingosine 1-phosphate-induced migration, G12/G13 family G proteins seem to be involved in peripheral MZB cell maturation, because also splenic MZB cell precursors are reduced in mutant mice, although less prominently than mature MZB cells. These data suggest that G12/G13 family G proteins contribute to the formation of the mature MZB cell compartment both by controlling MZB cell migration and by regulating MZB cell precursor maturation. The Journal of Immunology, 2006, 177: 2985–2993.

Immune cells express a variety of G protein-coupled receptors that can signal through G proteins of the G12/G13 family, such as lysophospholipid receptors, prostanoid receptors, or nucleotide receptors (1). The activated α-subunits of G12 and G13, Gna12 and Gna13, interact with specific Rhos guanine nucleotide exchange factors (RhoGEFs)3 (2) to mediate activation of the small GTPase RhoA (3, 4), thereby controlling cellular processes such as shape change, contraction, or motility (5, 6). However, because no pharmacological inhibitors of G12/G13 are available, and because constitutively G12/G13-deficient mice die before birth (7), the in vivo function of this G protein family in the immune system is unclear.

Indirect evidence for a role of G12/G13 in immune cells stems from studies in mice lacking Lsc, a RhoGEF activated by G12/G13 family G proteins (8). These mice show abnormalities in B cell functions (9–11), especially in marginal zone B (MZB) cell homeostasis. MZB cells are specialized B lymphocytes that are located between splenic white and red pulp, the so-called marginal zone. Due to their localization at this site of early Ag contact and due to their high proliferative capacity, MZB cells play an important role in the rapid defense against bloodborne bacterial Ags (12, 13). The molecular mechanisms involved in MZB cell entry into and retention within the marginal zone are only partly understood. Adhesion of MZB cells to marginal sinus stromal cells critically depends on interaction between lymphocyte integrins, especially LFA-1 (αLβ2) or αβ7, and their respective ligands on stromal cells, ICAM-1 and VCAM-1 (14), respectively.

To study the role of G12/G13 in B cell functions, we used the Cre/loxP system to generate B cell-specific Gna12/Gna13-deficient mice. We show here that migration and polarization in response to serum or sphingosine 1-phosphate (SIP) is enhanced in Gna12/Gna13-deficient MZB cells, but not in deficient follicular B cells. Together with an impaired maturation of splenic precursor MZB cells, this disinhibition of migration might underlie the loss of mature MZB cells observed in B cell-specific Gna12/Gna13-deficient mice in vivo.

Materials and Methods

Animals

B cell-specific Gna12/Gna13 double-deficient (CD19-Cre+/−; Gna13fl/fl; Gna12−/−) mice, Gna12−/− mice, Gna13−/− mice (CD19-Cre−/−; Gna13fl/fl; Gna12−/−); B cell-specific Gna12-deficient mice (CD19-Cre−/−; Gna13fl/fl; Gna12−/−), and littermate controls (CD19-Cre−/−; Gna13fl/fl; Gna12+/−) were generated by intercrosses of CD19-Cre+/−; Gna13fl/fl; Gna12−/− mice. Animals were kept on a mixed C57 × 129Sv background with a predominant contribution of the C57BL6/N strain and were housed under specific pathogen-free conditions. Animal experiments were in accordance with Institutional Animal Care and Use Committee Regulations. Genotyping for Gna13fl/fl and Gna12−/− alleles as well as for Gna12+/− and Gna12−/− alleles was performed as described previously (15). Primers for the detection of the CD19-Cre transgene were 5′-CCC AGA AAT GCC AGA TTA CGG C-3′, 5′-AAC CAG TCA ACA CCC TCC C-3′, and 5′-CCA GAC TAG ATA CAG ACC AG-3′.

Cell preparation

Single-cell suspensions from spleen, lymph node, Peyer’s patch, or thymus were prepared as described (16). Bone marrow cells were obtained by flushing femurs with PBS. Blood was obtained by retro-orbital bleeding into heparinized tubes. For white blood cell counts, red cells were lysed in 388 mM NH4Cl, 29.7 mM NaHCO3, and 25 μM Na2EDTA for 2 min, and...
intact cells were counted in a Neubauer chamber. To enrich lymphocytes from splenocyte suspension or whole blood, we used Lympholyte M or Lympholyte Mammal, respectively (Cedarlane Laboratories).

**Western blotting**

Splenic B and T cells from control and mutant mice were isolated by magnetic cell sorting (Miltenyi Biotec), and membrane bound proteins were extracted as described earlier (17). Extracts from 10^7 cells per lane were electrophoresed on 10% SDS-PAGE gels and blotted onto nitrocellulose membranes according to standard protocols. Membranes were incubated overnight at 4°C with polyclonal anti-Gaα1 Abs (1/1000; Santa Cruz Biotechnology), followed by incubation with HRP-conjugated secondary Abs (1/2000; Sigma-Aldrich). Chemiluminescence was developed using the ECLplus kit (Amersham Biosciences). After stripping, blots were reprobed with Ab directed against α-tubulin (clone DM1a; 1/1000; Sigma-Aldrich) for loading control.

**Flow cytometry**

B and T lymphocyte subsets were analyzed with a three-color FACSCalibur flow cytometer and CellQuestPro software (BD Biosciences) using FITC-labeled Abs directed against B220/CD45R, Ly-51, CD21, and IgG; PE-labeled Abs against IgM, CD3ε, CD23, CD24; biotinylated Abs against B220/CD45R, CD1d, CD9, CD11a, CD18 (all BD Biosciences). Rat IgG2a isotype control and PerCP-labeled streptavidin were also from BD Biosciences. In some cases, B cells were preselected with CD45R/ B220 beads (Miltenyi Biotec) before flow cytometry. The absolute numbers of T and B cell subsets in different immune organs were calculated based on the total number of cells as determined by manual counting in a modified Neubauer chamber.

**Histological, immunohistochemical, and immunocytochemical staining**

Giemsa staining was performed on 4-μm paraffin sections according to standard protocols. For immunohistochemical staining of spleens, 16-μm cryosections were fixed in acetone for 2 min, washed twice in PBS, blocked with 5% goat serum (Vector Laboratories) in PBS for 30 min and incubated for 1 h at room temperature or overnight at 4°C with FITC-anti-IgD and TRITC-anti-IgM Abs (BD Biosciences). For double fluorescence staining for MOMA-1 and CD1d, sections were first stained with rat anti-MOMA-1 and TRITC-anti-rat IgG (DakoCytomation), followed by staining with anti-CD1d-Biotin and FITC-conjugated streptavidin (BD Biosciences). After washing twice in PBS, slides were mounted in Mowiol (Calbiochem/EMD Biosciences) and analyzed on a Leica DC3000 microscope. For triple color staining, Abs directed against one adhesion molecule (1/AdCAM-1) (MCA367A; BD Biosciences; 1/30), mouse CD106 (VCAM-1) (Chemicon; 1:30), mouse macrophage receptor with collagenous structure (MARCO) (Sero tec; 1/1000), MOMA-1 (Sero tec; 1/100, mouse ER-TR9 (BMA Biomedicals; 1/30) was visualized with biotinylated anti-rat IgG Abs (DakoCytomation; 1/1000) and the Peroxidase Substrate DAB kit (Vector Laboratories). Staining with goat anti-mouse ICAM-1 Ab (R&D Systems; 1/10) was detected with the goat IgG Vectastain Elite ABC kit (Vector Laboratories).

**Proliferation assays**

Untouched B cells were isolated by negative selection with a B cell isolation kit (Miltenyi Biotec) from Lympholyte M-purified splenocytes. A total of 1 × 10^7 B cells was stimulated in 96-well U-bottom culture plates with LPS (Sigma-Aldrich) at various concentrations between 1 and 100 ng/ml (6). Cells were cultured for 32 h at 37°C and 5% CO₂ before pulsing the cultures with 1 μCi/well [3H]thymidine (MP Biomedical) for 16 h. [3H]Thymidine incorporation was measured by scintillation counting. All experiments were done in triplicate.

**ELISA and immunization**

Mice were immunized with 20 μg of trinitrophenyl (TNP)-Ficoll (BiosearchTech) as described (18). For ICAM-1, cells were coated with 50 μg/ml goat-anti-human-IgG-Fc Ab (Jackson ImmunoResearch) at 4°C overnight, washed twice with PBS, incubated with 2.5 μg/ml mouse ICAM-1-F2 (R&D Systems) in PBS for 2–3 h, and finally blocked with 0.5% BSA/PBS (100 μl per well for each step). Uncoated wells were treated with 0.5% BSA/PBS only. For VCAM-1, wells were incubated for 1 h at 37°C with 100 μl/well 3 μg/ml human VCAM-1 (R&D Systems) in carbonate buffer and blocked with 0.5% BSA/PBS for 30 min before use. CXCL12 (Chemicon) or CXCL13 (R&D Systems) were coinjected together with ICAM-1 or VCAM-1 at a concentration of 0.5 μg/ml. Freshly isolated splenic lymphocytes were incubated for 30 min in RPMI 1640 medium (Invitrogen Life Technologies) with 10 mM HEPS and 10% heat-inactivated (Invitrogen Life Technologies) at 37°C and 5% CO₂ to remove contaminating macrophages. Nonadherent cells were washed twice, counted, and resuspended at a density of 1 × 10^6 cells/ml in RPMI 1640/10 mM HEPS/0.5% FCS, and 100 μl of cell suspension was applied per well. After 30 min of adhesion at 37°C/5% CO₂, nonadherent cells were discarded and adherent cells were detached in RPMI 1640/5 mM EDTA for 2–3 min at 37°C. Cells were washed twice with RPMI 1640/5% FCS and resuspended in 200 μl of PBS, and data were acquired for 1 min. The absolute number of follicular (CD45R/B220−, CD21lin, CD23inh) and marginal zone (CD45R/B220−, CD21inh, CD23inh) B cells was calculated and expressed as proportion of input into adhesion assay.

**Migration assays**

For chemotaxis assays, Lympholyte M-purified splenocytes were preincubated in RPMI 1640/10 mM HEPS/0.1% BSA for 30 min at 37°C and 5% CO₂, and then allowed to migrate through 5-μm pore size Transwell inserts (Corning) at a density of 2 × 10^5 splenocytes/100 μl per well. The lower wells contained 600 μl of RPMI 1640/10 mM HEPS/0.1% BSA or with or without 100 mM CXCL12 (Chemicon), CXCL13 (R&D Systems), S1P (Biodrug lysophosphatidic acid, Biomedical), and 10% FCS (Invitrogen Life Technologies) or mouse serum. Mouse serum was prepared from wild-type (WT) mouse blood. After 3 h of incubation at 37°C and 5% CO₂, Transwell plates were kept on ice for 20 min and centrifuged at 180 × g for 3 min, inserts were discarded, and cells were stained for B cell subpopulations as described above. Cell numbers were expressed as proportion of input. All experiments were done in triplicate.

**Live-cell imaging**

For the isolation of follicular B cells, untouched B cells were isolated by negative selection with a B cell isolation kit (Miltenyi Biotec) from Lympholyte M-purified splenocytes. In a second step, CD23-positive follicular B cell were isolated by positive selection using biotinylated mouse monoclonal anti-CD23 Abs (BD Biosciences) and anti-Biotin-Microbeads (Miltenyi Biotec). For the isolation of MZB cells, biotinylated anti-CD23 Abs were added to the Ab mixture used during negative selection to remove CD23-positive B cells in addition to non-B cells. In a second step, MZB cells were positively selected with PE-labeled anti-CD21/CD35 Abs (BD Biosciences) and anti-PE-Microbeads (Miltenyi Biotec). The purity of cell preparations was usually between 93 and 98% as determined by flow cytometry. Cells were suspended in RPMI 1640/10 mM HEPS/0.1% BSA at a concentration of 1 × 10^6/ml in the absence or presence of 10% FCS or 5% Sip. Cells were allowed to adhere to Transwell inserts coated with 4% FCS (Greiner Bio-One) at 37°C and 5% CO₂ for 20–40 min, and cell motility was recorded for 8 min with a Leica DM IRE2 inverted fluorescence microscope and Leica FW4000 software (1 frame every 10 s). Cells were then fixed for 10 min in 4% parafomaldehyde, washed twice in PBS, permeabilized with 0.1% Triton X-100 in PBS for 15 min, washed twice in PBS, and incubated for 1 h in AlexaFluor BODIPY FL Phallacidin (Invitrogen Life Technologies). After washing three times in PBS, cells were mounted in Mowiol 4–88 (Calbiochem) and analyzed on a Leica DM LB microscope with DC 300F camera. Cell motility in movies was classified by an investigator blinded to treatment and genotype as follows: 1) non-adherent (round, floating); 2) adherent immobile; 3) adherent with filopodia formation; 4) adherent with ameboid shape and motility, stationary; 5) adherent with ameboid shape and motility, mobile. Around 100 randomly chosen cells were evaluated per movie.

**Quantitative RT-PCR**

MZB cells were isolated by magnetic cell sorting as described for live-cell imaging and mRNA was prepared with a RNeasy Mini kit (Qiagen). Reverse transcription was performed according to standard protocols. LPA receptor subtypes were amplified with the following primers: S1P₁: 5’-CCCACTACCCCCAGATTCC-3’/5’-CTCTCCCTCCCTCCTCCT-3’; S1P₂: 5’-AAGGCTTAGAAAAGATAAGGAGAGT-3’/5’-ACTGCCCCAGAAGATGTGA-3’; S1P₅: 5’-AGTCCCGAGGACTCTACTAA-3’/5’-CCACAGAGTTAGAACCAGATGGTGA-3’ with the Platinum SYBR Green qPCR SuperMix UDG (Invitrogen Life Technologies) and an ABI PRISM 7700
Sequence detection system (Applied Biosystems). The resulting bands were normalized against β-actin (5′-TGACGGTGACATCCGTAAA GA-C-3′/5′-TGCTAGGAGCCAGACGATGA-3′).

**Statistics**

Data are displayed as mean ± SEM. Comparisons between two groups were performed with Student’s t test. Multiple comparisons were performed with ANOVA and Bonferroni’s post hoc test.

**Results**

To generate B cell-specific Gα13-deficient mice, we mated animals in which the gene coding for Gα13, Gna13, is flanked with loxP sites (Gna13ΔΔ) (19) to the B cell-specific CD19-Cre line provided by K. Rajewsky (Harvard Medical School, Boston, MA) and R. C. Rickert (Burnham Institute, La Jolla, CA) (20). Degree and specificity of Cre-mediated recombination in CD19-Cre<sup>-/-</sup>:Gna13<sup>fl/fl</sup> mice were determined by Western blot with an Ab directed against Gα13 (Fig. 1a). Although Gα13 protein was almost absent in B cells of CD19-Cre<sup>-/-</sup>:Gna13<sup>fl/fl</sup> mice, protein levels in T cells were unchanged. Because the closely related Gα12 has been shown to partly compensate for defects in Gα13-deficient mice (21), we crossed CD19-Cre<sup>-/-</sup>:Gna13<sup>fl/fl</sup> mice to constitutively Gα12-deficient mice, which are themselves without phenotype (21). B cell-specific Gα12/Gα13 double knockout mice (B-Gα<sub>12</sub>/Gα<sub>13</sub>-DKO) were viable and fertile, and showed no gross morphological abnormalities of immune organs. Numbers of total B cells were normal in lymph nodes, bone marrow, and blood, but increased in the spleen (Table I).

**FIGURE 1.** MZB cells are reduced in B cell-specific Gα<sub>12</sub>/Gα<sub>13</sub>-deficient (B-Gα<sub>12</sub>/Gα<sub>13</sub>-DKO) mice. a, Western blot with a Gα13-specific Ab performed on extracts of B and T cells from control mice (CD19-Cre<sup>-/-</sup>:Gna13<sup>fl/fl</sup>) and B cell-specific Gα<sub>13</sub>-deficient mice (CD19-Cre<sup>-/-</sup>:Gna13<sup>ΔΔ</sup>). Reblot with Ab against α-tubulin as loading control. b, Representative examples for the flow cytometric analysis of splenic MZB cells (MZB; CD45R/B220<sup>pos</sup>, CD21<sup>high</sup>, CD23<sup>low</sup>) and follicular B cells (FoB; CD45R/B220<sup>pos</sup>, CD21<sup>int</sup>, CD23<sup>high</sup>) in control mice (left) and B-Gα<sub>12</sub>/Gα<sub>13</sub>-DKOs (right) after staining with Abs directed against CD45R/B220 and CD9. MZB cells are CD45R/B220<sup>pos</sup>, CD21<sup>high</sup>, CD9<sup>high</sup>, IgM<sup>high</sup>, IgD<sup>low</sup> cells encircling IgM<sup>low</sup>, IgD<sup>high</sup> follicles. c, Representative examples for the flow cytometric analysis of splenic MZB cells (MZB; CD45R/B220<sup>pos</sup>, CD21<sup>high</sup>, CD23<sup>low</sup>) and follicular B cells (FoB; CD45R/B220<sup>pos</sup>, CD21<sup>int</sup>, CD23<sup>high</sup>) in control mice (left) and B-Gα<sub>12</sub>/Gα<sub>13</sub>-DKOs (right) after staining with Abs directed against CD45R/B220 and CD21. MZB cells are CD45R/B220<sup>pos</sup>, CD21<sup>high</sup>, CD23<sup>low</sup> CD9<sup>high</sup>. d, Absolute numbers of follicular (left) and MZB cells (right) in control mice (black), constitutively Gα<sub>13</sub>-deficient (Gα13KO; dark gray), B cell-specific Gα<sub>12</sub>-deficient (B-Gα<sub>12</sub>-KO; light gray), and B-Gα<sub>12</sub>/Gα<sub>13</sub>-DKO (white) mice (n = 6 per group). e, Representative photomicrographs of splenic sections from control mice (left) and B-Gα<sub>12</sub>/Gα<sub>13</sub>-DKO (right) after Giemsa staining (top; light blue ring encircling the dark blue follicle are MZB cells), after immunofluorescence staining with TRITC-anti-IgM- and FITC-anti-IgD Abs (middle; IgM<sup>low</sup>, IgD<sup>high</sup> cells encircling the dark blue follicle are MZB cells), after immunofluorescence staining with TRITC-anti-IgM- and FITC-anti-IgD Abs (middle; IgM<sup>low</sup>, IgD<sup>high</sup> cells encircling the dark blue follicle are MZB cells), and after immunofluorescence staining for MOMA-1 (red, stains marginal sinus metallophilic macrophages) and CD1d (green, stains MZB cells) (bottom) (magnification, ×100). f, Representative photomicrographs of splenic sections from control mice (left) and B-Gα<sub>12</sub>/Gα<sub>13</sub>-DKO (right) after immunohistochemical staining with Abs directed against MadCAM (stains endothelial cells lining the marginal sinus) (top), or MARCO (stains marginal zone macrophage subpopulations) (bottom). Magnification, ×100. *, p < 0.05; ***, p < 0.0001.
The splenic follicular structure was roughly normal, but detailed analysis revealed a strong reduction of MZB cell numbers in B-Gna12/Gna13-DKOs (Fig. 1, b–e). Flow cytometric analysis after staining with Abs directed against CD45R/B220, CD21, and CD23 revealed a reduction of MZB cells to 22.5 ± 3% of normal (Fig. 1b), and after staining for CD45R/B220 and CD9 to 25.2 ± 6.2% of normal (Fig. 1c). We calculated total numbers of follicular and MZB cells and found that MZB cells were not only relatively, but also absolutely reduced in B-Gna12/Gna13-DKO (WT, 17.1 ± 0.7 × 10^6 per spleen; DKO, 0.54 ± 0.1 × 10^6 per spleen), whereas total numbers of follicular B cells were increased (WT, 12.5 ± 0.2 × 10^6 per spleen; DKO, 18.1 ± 0.7 × 10^6 per spleen) (Fig. 1d).

To investigate the relative contributions of Gna12 and Gna13 to the reduction MZB cell numbers, we also determined the absolute numbers of follicular and MZB cells in constitutively Gna13-deficient (CD19-Cre^{-/-}; Gna13^{−/−}; Gna12^{−/−}) and B cell-specific Gna13-deficient (CD19-Cre^{+/−}; Gna13^{−/−}; Gna12^{+/−}) mice. Although Gna13-deficient mice did not differ from control animals, B cell-specific Gna13-deficient mice showed a significant reduction of MZB cell numbers, although less prominent than in B-Gna12/Gna13-DKOs (Fig. 1d).

Histological analysis of splenic sections confirmed the strong reduction of MZB cells both in Giemsa staining and after immunohistochemical staining with IgM and IgD Abs (Fig. 1e). To investigate whether MZB cell translocation into follicles contributes to the phenotype, we performed double stainings for the MZB cell marker CD1d and MOMA-1, which labels marginal sinus macrophages at the inner side of the marginal zone (22). These stainings confirmed the reduction of MZB cells, but we did not detect significant translocation into CD1d-positive cells into follicles (Fig. 1e).

Although Gna12/Gna13 double deficiency is in this model restricted to B cells, it is possible that stromal defects, either due to constitutive Gna12 deficiency or due to changes secondary to a primary B cell defect, contribute to loss of MZB cells. For example, abnormal organization of MadCAM-1-positive endothelial cells (10) or of MARCO-positive marginal zone macrophages (23) has been shown to contribute to altered MZB localization. However, we found no differences in the organization of MadCAM-1- or MARCO-positive cells between control and mutant mice (Fig. 1f), suggesting that the observed phenotype is B cell autonomous. In addition, we performed immunohistochemical stainings for ICAM-1 and VCAM-1, the two relevant adhesion molecules for MZB cells (14), as well as additional markers for marginal zone macrophage subpopulations such as MOMA-1 (22) and ER-TR9 (24), but also here failed to detect significant differences between the genotypes (data not shown).

The proliferative capacity of Gna12/Gna13-deficient B cells, we performed proliferation assays with LPS, which stimulates at low concentrations preferentially MZB cells. Proliferation in response to 10 and 30 ng/ml was significantly reduced in mutant B cells, whereas at 100 ng/ml LPS the difference was not significant (Fig. 2a). This dose-dependent defect most likely reflects the loss of the highly proliferative MZB cell population. Because MZB cells are known to critically contribute to rapid Ig production in response to thymus-independent Ags, we determined the levels of TNP-specific Ig isotypes after immunization with the thymus-independent Ag TNP-Ficoll. We found that, in mutant mice, production of TNP-specific IgM and IgG3, but not of IgG2a, was significantly reduced 7 days after immunization (Fig. 2b), whereas total Ig levels were normal (data not shown). The differences between the genotypes were less prominent 14 days after immunization (data not shown), suggesting that thymus-independent responses are delayed in B-Gna12/Gna13-DKOs.

Loss of MZB cells in B-Gna12/Gna13-DKOs might be due to abnormal B cell maturation, reduced adhesion, or altered migration. Bone marrow cell numbers were normal in mutant mice (Table I), and flow cytometric analysis of bone marrow B cell precursors showed no major abnormalities (Fig. 3, a–c). We next investigated transitional B cells in the spleen, of which two types exist: type I

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**Table 1. Lymphocyte numbers in different organs from control mice and B cell-specific Gna12/Gna13-deficient mice (B-Gna12/Gna13-DKO)**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>B-Gna12/Gna13-DKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen (n = 12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total lymphocytes</td>
<td>38.2 ± 4.7</td>
<td>45.5 ± 4.7*</td>
</tr>
<tr>
<td>CD45R/B220^+</td>
<td>16.8 ± 1.8</td>
<td>23.2 ± 2.5*</td>
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<tr>
<td>CD3e^+</td>
<td>9.3 ± 1.4</td>
<td>11.8 ± 1.2</td>
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<tr>
<td>BloodP (n = 5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total leukocytes</td>
<td>7.5 ± 0.1</td>
<td>7.7 ± 0.1</td>
</tr>
<tr>
<td>CD45R/B220^+</td>
<td>2.4 ± 0.3</td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td>CD3e^-</td>
<td>2.2 ± 0.9</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>Lymph node (n = 6)</td>
<td></td>
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<tr>
<td>Total cells</td>
<td>3.1 ± 0.5</td>
<td>3.1 ± 0.6</td>
</tr>
<tr>
<td>CD45R/B220^+</td>
<td>0.9 ± 0.1</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>CD3e^-</td>
<td>2.1 ± 0.1</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>Thymus (n = 5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cells</td>
<td>157 ± 34</td>
<td>129 ± 31</td>
</tr>
<tr>
<td>CD4^-/CD8^-</td>
<td>2.8 ± 0.8</td>
<td>3.3 ± 1.5</td>
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<tr>
<td>CD4^-/CD8^+</td>
<td>127 ± 7</td>
<td>113 ± 2.8</td>
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<tr>
<td>CD8^-</td>
<td>16.1 ± 5.7</td>
<td>12.0 ± 4.4</td>
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<tr>
<td>Bone marrow (n = 8)</td>
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<tr>
<td>Total cells</td>
<td>13.0 ± 1.9</td>
<td>16.4 ± 3</td>
</tr>
<tr>
<td>CD45R/B220^+</td>
<td>2.5 ± 0.2</td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td>CD3e^-</td>
<td>0.9 ± 0.5</td>
<td>1.1 ± 0.6</td>
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* Data represent multiples of 10^6 cells.

^+ Cell numbers per milliliter of blood.

^+ Cell numbers per femur.

^+ p < 0.05 vs control.

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**FIGURE 2. B cell proliferation and Ab production is impaired in B cell-specific Gna12/Gna13-deficient (B-Gna12/Gna13-DKO) mice.** a, [3H]Thymidine incorporation in B lymphocytes from control mice (■) and B-Gna12/Gna13-DKOs (□) was determined after stimulation with 0, 10, 30, or 100 ng/ml LPS (n = 3 experiments). b, TNP-specific IgG in control mice (■) and B-Gna12/Gna13-DKOs (□) before (p0) and 7 days after (d7) immunization with the thymus-independent Ag TNP-Ficoll (n = 8–14). Horizontal lines indicate mean values. *, p < 0.05; **, p < 0.005 vs control.
transitional B cells (T1) are recent immigrants from the bone marrow and develop into type 2 transitional B cells (T2), which in turn differentiate into recirculating mature B cells (25, 26). In addition, the T2 pool was shown to contain MZB cell precursors (27). Flow cytometric analysis with Abs directed against CD21, heat-stable Ag (HSA), and CD45R/B220 did not show major abnormalities with respect to B220<sup>pos</sup>; CD21<sup>neg</sup>; HSA<sup>high</sup> T1 B cells, but a significant reduction of B220<sup>pos</sup>; CD21<sup>high</sup>; HSA<sup>high</sup> cells (Fig. 4a), which have been suggested to comprise both T2 cells and MZB cells (25, 28). To test whether this is solely due to a reduction of mature MZB cells or also to a reduction of T2 MZB precursors, we performed additional stainings to differentiate between mature MZB cells (CD9<sup>high</sup>, IgM<sup>high</sup>; IgD<sup>low</sup>) and T2 MZB precursors (CD9<sup>high</sup>, IgM<sup>high</sup>; IgD<sup>high</sup>) (25, 29). We found that not only absolute numbers of mature MZB cells were reduced in mutant mice (34 ± 2% of normal; p = 0.002), but also T2 MZB precursor cells.
VCAM-1 was altered in G12/G13-DKO mice, and we tested whether chemokine-mediated adhesion to ICAM-1 or VCAM-1 between the two genotypes (Fig. 5a). Chemokines and their G protein-coupled receptors are important regulators of integrin-mediated adhesion in lymphocytes (30, 31), and the chemokines CXCL12 and CXCL13 have been shown to regulate integrin-dependent entry of B cells into peripheral lymph nodes or Peyer’s patches (32). Because an involvement of G12/G13 in chemokine receptors signaling has recently been suggested (33), we tested whether chemokine-mediated adhesion to ICAM-1 or VCAM-1 was altered in Gα12/Gα13-deficient B cells. We did not find significant differences with respect to basal (Fig. 5a) or chemokine-induced adhesion to ICAM-1 or VCAM-1 between the two genotypes (Fig. 5b and data not shown), suggesting that the loss of MZB cells in B-Gα12/Gα13-DKOs is not to be attributed to differences in basal or chemokine-mediated adhesion.

In addition to their role in integrin-mediated adhesion, chemokines are important regulators of lymphocyte migration (34, 35). To study the role of G12/G13 in B cell migration, we let splenocytes migrate toward CXCL12 and CXCL13 in Transwell assays, but did not find significant differences with respect to chemokine-induced migration between the genotypes (Fig. 6). Interestingly, Gα12/Gα13-deficient MZB cells showed strongly increased migration toward fetal calf serum or adult mouse serum (Fig. 6a), whereas serum-induced migration was not altered in follicular B cells (Fig. 6b). A potent promigratory stimulus for MZB cells is the lysophospholipid S1P (36), which is present at high concentrations in blood and serum. Because S1P receptors can couple to G12/G13 (37), we investigated migration toward S1P in G12/G13-deficient B cells and found that, also in response to S1P, migration was disinhibited in MZB cells (Fig. 6a), but not in follicular B cells (Fig. 6b).

In contrast, the lysophospholipid LPA, which is also present in high concentrations in the blood, did not induce significant migration in either genotype and cell type (Fig. 6c). We next investigated whether the dose-response curve for S1P-induced migration was shifted to the left in deficient MZB cells, but found that in both genotypes the maximal response was at 100 nM (Fig. 6c), and that the migratory response of mutant cells was enhanced at almost all concentrations investigated. MZB cells have been shown to express the S1P receptor subtypes S1P1, S1P3, and S1P4 (36), and we tested whether expression of S1P receptor subtypes on deficient MZB cells was affected. Quantitative RT-PCR did not reveal differences in the relative expression of the subtypes S1P1, S1P3, and S1P4 (Fig. 6d).

![FIGURE 5](image-url) 

**FIGURE 5.** Normal basal and agonist-induced adhesion in MZB cells and follicular B (FoB) cells from B cell-specific Gα12/Gα13-deficient (B-Gα12/Gα13-DKO) mice. a, Basal adhesion to uncoated, ICAM-1- and VCAM-1-coated wells (n = 4 experiments). b, Effect of coimmobilized chemokines CXCL12 and CXCL13 (0.5 μM each) on adhesion to ICAM-1 (n = 4 experiments). Data are displayed as percent of input. ■, Control mice; □, B-Gα12/Gα13-DKO; ρ, untreated sample. *, p < 0.05 vs untreated.

![FIGURE 6](image-url) 

**FIGURE 6.** Abnormal agonist-induced migration in MZB cells from B cell-specific Gα12/Gα13-deficient (B-Gα12/Gα13-DKO) mice. a and b, Migration of MZB cells (a) and follicular B (FoB) cells (b) from control mice (■) and B-Gα12/Gα13-DKOs (□) toward medium alone (basal), or medium containing CXCL12, CXCL13, S1P, LPA (100 nM each), calf serum (FCS), or mouse serum (MS) (10% each) in Transwell migration assays. Data are shown as percentage of cell input (n = 3 experiments). c, Migration of control and mutant MZB cells in response to different concentrations of S1P (n = 3 experiments). d, Relative expression of S1P receptor subtypes in control and deficient MZB cells as determined by quantitative RT-PCR. Data were normalized to β-actin expression. ■, Control mice; □, B-Gα12/Gα13-DKOs. *, p < 0.05; **, p < 0.005 vs control.
To allow a more detailed analysis of the migratory behavior of mutant cells, we studied follicular and MZB cells from control and mutant mice by live-cell imaging (Fig. 7 and data not shown). In the absence of serum or agonist, no significant differences between control and G_{12/13}-deficient MZB cells or follicular B cells were observed (data not shown). However, in the presence of 10% FCS, the proportion of cells showing ameboid motility was significantly increased in mutant MZB cells as compared with control cells, and the proportion of nonadherent cells was significantly reduced (Fig. 7, a and c). In contrast, no differences were found between control and G_{12/13}-deficient follicular B cells (Fig. 7e). Also, after treatment with 100 nM S1P motility of mutant MZB cells was significantly higher than that of control cells (Fig. 7, b and d), whereas follicular B motility in response to S1P did not differ (Fig. 7f). We finally investigated polarization of control and mutant MZB cells by phalloidin staining for F-actin. Three experiments revealed that mutant MZB cells formed significantly more and bigger F-actin positive protrusions (“pseudopods”) in response to serum and S1P (Fig. 8), suggesting that regulation of polarization is abnormal in mutant MZB cells.

**Discussion**

We show in this paper that B cell-specific inactivation of G_{12} in constitutively G_{12/13}-deficient mice results in a strong reduction of splenic MZB cells in vivo. This phenotype might be due to G_{12/13} double deficiency in B cells or to G_{12} deficiency in splenic stromal or endothelial cells, but the fact that G_{12/13}-deficient mice themselves are without phenotype, together with the finding that organization of MadCAM-positive endothelial sinus or marginal zone macrophage populations is undisturbed, indicates that the loss of MZB cells is a B cell-autonomous defect. Such a B cell-autonomous defect might involve abnormal maturation, altered adhesive functions, or abnormal migration. We did not detect abnormalities in bone marrow B cell progenitor populations, but flow cytometric analysis of splenic transitional B cells revealed that not only mature MZB cells are reduced in B-G_{12/13}-DKOs, but also T2 precursor MZB cells. This finding suggests that G_{12/13} family G proteins play a role in the maturation of MZB cell precursors, but it is not clear which G_{12/13}-coupled receptors mediate this process. However, the reduction of precursor cells is less significant than that of mature MZB cells, suggesting that also other defects, e.g., in adhesion or migration, contribute to the in vivo phenotype.

The most obvious candidates with respect to impaired adhesion or migration are chemokine-mediated effects, but we did not find differences in basal or chemokine-induced adhesion or migration. The latter findings are consistent with the notion that chemokine G protein-coupled receptors primarily signal through G_{i} family G proteins (38), and not through G_{12/13}.

However, we found that G_{12/13}-deficient MZB cells show abnormally strong migration toward calf and mouse serum, and also toward S1P. Importantly, this defect was not observed in follicular B cells, and therefore may explain why MZB cell localization and not follicular B cell localization is altered in vivo. S1P, which is present at high concentrations in the blood, is a potent chemotactant for MZB cells, and it seems plausible that any disturbance of the equilibrium between S1P-induced migration toward blood and CXCL13-induced migration toward B cell follicles impairs MZB localization (14, 36). Interestingly, disinhibition of migration toward S1P is less prominent than toward serum, suggesting that also other, yet-undefined serum constituents contribute to abnormal serum-induced migration. However, the actual localization of lost MZB cells is unclear, for the proportion of
We were able to show that increased migration in deficient MZB cells is not associated with abnormal S1P receptor subtype expression. Our dose-response experiments for S1P-induced migration revealed that the maximal response to S1P is not shifted to lower concentrations, but that the migratory response is enhanced at basically all concentrations tested. This indicates that loss of G_{12q}/G_{13} does not affect the potency of S1P, but enhances efficacy of S1P with respect to stimulation of migration. We therefore hypothesize that G_{12q}/G_{13} family G proteins normally exert an inhibitory effect on S1P-induced migration in MZB cells, and that loss of G_{12q}/G_{13} causes disinhibition of S1P-induced promigratory signaling.

The role of the different S1P receptors subtypes in the regulation of MZB cell migration is complex. Compared with follicular B cells, MZB cells show stronger S1P-induced migration, and this coincides with significantly higher levels of S1P1 expression, suggesting that S1P1 is the predominant receptor mediating S1P-induced migration in B cells (36). This notion is in line with the finding that migration toward S1P is abrogated in S1P1-deficient MZB cells, but not in S1P1-deficient MZB cells (36). The S1P3 receptor has been shown to couple both to G_{i} and G_{12q}/G_{13} family G proteins (37). Pharmacological inhibition of G_{i} by pertussis toxin completely abrogates S1P-induced migration in MZB cells (data not shown), whereas genetic inactivation of G_{12q}/G_{13} enhances MZB cell migration toward S1P (Figs. 6 and 7). This suggests that, under normal conditions, S1P3-Gi-mediated stimulation of migration is limited by the concomitant activation of G_{12q}/G_{13}. It cannot be excluded that also the Gi- and G_{12q}/G_{13}-coupled S1P4 receptor (37) is contributing to this process, but the fact that S1P4 expression does not differ between MZB cells and follicular B cells (36) makes a major contribution of S1P4 less likely. The S1P1 receptor has been shown to play an important role in MZB cell homeostasis (36, 39), but because this receptor is predominantly Gi coupled (40, 41), a major contribution to the loss of MZB cells in B-G_{12q}/G_{13}-DKO seems unlikely.

By which mechanism G_{12q}/G_{13} counteracts Gi-induced migration is not completely clear. We show that mutant MZB cells display increased pseudopod formation in response to serum or S1P, and an increased agonist-induced pseudopod formation was also observed in a neutrophil cell line transfected with dominant-negative mutants of G_{12q} and G_{13} (42). The latter study suggested that the agonist, in that case fMLP, activates both Gi and G_{12q}/G_{13} family G proteins, and that the mutual suppression of both pathways allows normal polarization. We therefore hypothesize that S1P and probably other, yet-unknown serum constituents do not only enhance migration through G_{i} family G proteins, but also control pseudopod formation through G_{12q}/G_{13} family G proteins, thereby critically regulating motility in MZB cells. Interestingly, Lsc/p115RhoGEF-deficient mice do not only resemble B-G_{12q}/G_{13}-DKO with respect to reduction of the MZB cell population to approximately one-fourth of normal (9), but also show increased migratory responses of B cells toward serum and S1P (9, 11). Although polarization has not been investigated in B cells from these mice, neutrophil granulocytes lacking Lsc/p115RhoGEF show aberrant pseudopod formation, resulting in increased motility with decreased directionality (43).

Taken together, this study shows that G_{12q}/G_{13} family G proteins are critically involved in the formation and maintenance of the mature MZB cell compartment of the spleen. Our results indicate that G_{12q}/G_{13} negatively regulates polarization and migration toward serum and serum constituents such as S1P specifically in MZB cells, and this defect might, together with the impaired maturation of precursor cells, contribute to reduced numbers of MZB cells observed in vivo.

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