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

Stefan Rieken,* 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 G 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 Gα12 and Gα13 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 Gα12/Gα13-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, Gα12/Gα13 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 Gα12/Gα13 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, Gα12 and Gα13, interact with specific Rho guanine nucleotide exchange factors (RhOGEFs) (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 Gα12/G13 are available, and because constitutively Gα13-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 homoeostasis. 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 or 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 Gα12/Gα13-deficient mice. We show here that migration and polarization in response to serum or sphingosine 1-phosphate (SIP) is enhanced in Gα12/Gα13-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 Gα12/Gα13-deficient mice in vivo.

Materials and Methods

Animals

B cell-specific Gα12/Gα13 double-deficient (CD19-Cre/+; Gna13fl/fl, Gna12−/−) mice, Gα12−/−mice, Gα12−/−; Gna13−/−). B cell-specific Gα12-deficient mice (CD19-Cre/+; Gna13fl/fl, Gna12−/−), Gα12−/−; Gna13−/−), and littermate controls (CD19-Cre/+; Gna13fl/fl, Gna12−/−) were generated by intercrosses of CD19-Cre/+; Gna13fl/fl, Gna12−/−). Animals were kept on a mixed C57 × 129Sv background with a predominant contribution of the C57BL/6N strain and were housed under specific pathogen-free conditions. Animal experiments were performed in accordance with Institutional Animal Care and Use Committee Regulations. Genotyping for Gna13fl/− and Gna12−/− alleles as well as for Gα12−/− and Gα12−/− alleles was performed as described previously (15). Primers for the detection of the CD19-Cre transgene were 5′-CCC AGA AAT GCC AGA TTA CG-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 femora 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 cultured 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 Biotech), and membrane bound proteins were extracted as described earlier (17). Extracts from 10⁶ 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-Gaα₃/δ (Ab 1/1000; Santa Cruz Biotechnology), followed by incubation with HRP-conjugated secondary Ab (1/1000; Sigma-Aldrich). Chemiluminescence was developed using the ECLplus kit (Amersham Biosciences). After stripping, blots were reprobed with Ab directed against α-tubulin (clone DM1α; 1/1000; Sigma-Aldrich) for loading control.

**Flow cytometry**

B and T lymphocyte subsets were analyzed using a three-color FACSCalibur flow cytometer and CellQuestPro software (BD Biosciences) using FITC-labeled Abs directed against B220/CD45R, Ly-5.1, CD21, and IgD; PE-labeled Abs against IgM, CD3ε, CD23, CD24, CD43; 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**

Giems 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 the analysis of B cells directed against mucosal adhesion molecule-1 (MAdCAM-1) (MECA-367; BD Biosciences; 1/30), mouse CD106 (VCAM-1) (Chemicon; 1:30), mouse macrophage receptor 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. For the isolation of MZB cells, a positive selection procedure was used. B cells 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 kept in RPMI 1640/10% FCS/1% L-glutamine (Invitrogen Life Technologies) overnight at 37°C and 5% CO₂, and then allowed to migrate through 5-μm pore Transwell inserts (Corning) at a density of 2 × 10⁶ spleen cells/μl for 20–40 min. Cell numbers were expressed as proportion of input. All experiments were done in triplicate.

**ELISA and immunization**

Mice were immunized with 20 μg of trinitrophenyl (TNP)-Filcoll (BiosearchTech) as described (18). Basal and TNP-specific IgG isoforms were determined using ELISA mouse Ig quantification kits (Bethyl) and (BiosearchTech) as described (18). Basal and TNP-specific Ig isotypes were determined using ELISA mouse Ig quantification kits (Bethyl) and (BiosearchTech) as described (18). Basal and TNP-specific Ig isotypes were determined using ELISA mouse Ig quantification kits (Bethyl) and (BiosearchTech) as described (18).
Sequence detection system (Applied Biosystems). The resulting bands were normalized against β-actin (5’-TGACGGTGACATCCGTAAGAC-3’/5’-TGCTAGGAGCCAGACGCTAA-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 (Gna13fl/fl) (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-Crefl/+;Gna13fl/fl 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-Crefl/+;Gna13fl/fl 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-Crefl/+;Gna13fl/fl mice to constitutively Gα12-deficient mice, which are themselves without phenotype (21). B cell-specific Gα12/Gα13 double knockout mice (B-Gα12/Gα13-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 Gα12/Gα13-deficient (B-Gα12/Gα13-DKO) mice. a, Western blot with a Gα13-specific Ab performed on extracts of B and T cells from control mice (CD19-CreCrefl/+;Gna13fl/fl) and B cell-specific Gα13-deficient mice (CD19-Crefl/+;Gna13fl/fl). Reblot with Ab against α-tubulin as loading control. b, Representative examples for the flow cytometric analysis of splenic MZB cells (MZB; CD45R/B220high, CD21int, CD23high) and follicular B cells (FoB; CD45R/B220high, CD21high, CD23low) and follicular B cells (FoB; CD45R/B220high, CD21high, CD23low) in control mice (left) and B-Gα12/Gα13-DKOs (right) after staining with Abs directed against CD45R/B220 and CD9. MZB cells are CD45R/B220high, CD9high, and CD23low; IgMhigh, IgDlow cells encircling IgMlow, IgDhigh follicles (22), depending on the adult tissue of origin. c, Representative examples for the flow cytometric analysis of MZB cells (MZB; CD45R/B220high, CD21int, CD23high) and follicular B cells (FoB; CD45R/B220high, CD21high, CD23low) in control mice (left) and B-Gα12/Gα13-DKOs (right) after staining with Abs directed against CD45R/B220 and CD9. MZB cells are CD45R/B220high, CD9high, and CD23low; IgMhigh, IgDlow cells encircling IgMlow, IgDhigh follicles (22), depending on the adult tissue of origin. d, Absolute numbers of follicular (left) and MZB cells (right) in control mice (black), constitutively Gα12deficient (Gα12KO; dark gray), B cell-specific Gα12-deficient (B-Gα12-KO; light gray), and B-Gα12/Gα13-DKO (white) mice (n = 6 per group). e, Representative 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.
restricted to B cells, it is possible that stromal defects, either due to abnormal organization of MAdCAM-1-positive endothelial cells or reduced MZB cell numbers, we also determined the absolute numbers of follicular and MZB cells in constitutively Gα12/Gα13-deficient mice as compared to normal (Fig. 1c). Histological analysis of splenic sections confirmed the strong reduction of MZB cells and found that MZB cells were not only relatively, but also absolutely reduced in B-Gα12/Gα13-DKO mice (WT, 1.7 ± 0.1 × 10⁶ per spleen; DKO, 0.54 ± 0.1 × 10⁶ per spleen), whereas total numbers of follicular B cells were increased (WT, 12.5 ± 0.2 × 10⁶ per spleen; DKO, 18.1 ± 0.7 × 10⁶ per spleen) (Fig. 1d).

To investigate the relative contributions of Gα12 and Gα13 to the reduction in MZB cell numbers, we also determined the absolute numbers of follicular and MZB cells in constitutively Gα12-deficient (CD19-Cre⁺/−; Gna13−/−; Gna12−/−) and B cell-specific Gα12-deficient (CD19-Cre⁺/−; Gna13−/−; Gna12tcr) mice. Although Gα12-deficient mice did not differ from control animals, B cell-specific Gα12-deficient mice showed a significant reduction of MZB cell numbers, although less prominent than in B-Gα12/Gα13-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 Gα12/Gα13 double deficiency is in this model restricted to B cells, it is possible that stromal defects, either due to constitutive Gα12 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).

To test the proliferative capacity of Gα12/Gα13-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 basal 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-Gα12/Gα13-DKOs.

Loss of MZB cells in B-Gα12/Gα13-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

![Figure 2](https://example.com/figure2.png)

**FIGURE 2.** B cell proliferation and Ab production is impaired in B cell-specific Gα12/Gα13-deficient (B-Gα12/Gα13-DKO) mice. a, [³H]Thymidine incorporation in B lymphocytes from control mice (■) and B-Gα12/Gα13-DKOs (□) was determined after stimulation with 0, 10, 30, or 100 ng/ml LPS (n = 3 experiments). b, TNP-specific Ig levels in control mice (■) and B-Gα12/Gα13-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 B220pos; CD21neg; HSAhigh T1 B cells, but a significant reduction of B220pos; CD21high; HSAhigh 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 (CD9high; IgMhigh; IgDlow) and T2 MZB precursors (CD9high; IgMhigh; IgDhigh) (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

**FIGURE 3.** Bone marrow B cell maturation in B cell-specific Gα12/Gα13-deficient (B-Gα12/Gα13-DKO) mice. a–c, Representative examples (left) and statistical evaluation (right) of bone marrow B cell populations as determined by flow cytometry after staining with Abs directed against CD43 and CD45R/B220 (a), CD43, Ly-51, and HSA (b), or CD43, IgD, and IgM (c). Cells in b and c were selected for CD45R/B220 expression by MACS before flow cytometry. Cells were gated as indicated and PreB cell subsets A–F were defined as described by others (44) (n = 3 experiments). ■ Control mice; □ B-Gα12/Gα13-DKOs.

**FIGURE 4.** Peripheral B cell maturation in B cell-specific Gα12/Gα13-deficient (B-Gα12/Gα13-DKO) mice. a, Representative examples (left) and statistical evaluation (right) of transitional B cells of the spleen as determined by flow cytometry after staining with Abs directed against CD45R/B220, CD21, and HSA. CD45R/B220-positive splenocytes were isolated by magnetic cell sorting and stained for IgD, IgM, and CD9. Per sample, 10^5 CD9-positive cells were analyzed. The absolute numbers of mature MZB cells (IgM^high; CD9^low) and T2 MZB precursor cells (prec MZB) in the spleen. CD45R/B220-positive splenocytes were isolated by magnetic cell sorting and stained for IgD, IgM, and CD9. Per sample, 10^5 CD9-positive cells were analyzed. The absolute numbers of mature MZB cells (IgM^high; CD9^low) and T2 MZB precursor cells (prec MZB) were calculated by multiplying the fraction of each B cell subset by the total number of CD9^pos^ cells (n = 4 experiments). ■ Control mice; □ B-Gα12/Gα13-DKOs. *, p < 0.05; **, p < 0.005.
VCAM-1 was altered in G12/G13-deficient B cells, and it is interesting that G12/G13-dependent entry of B cells into peripheral lymph nodes or Peyer’s patches has recently been suggested (33). We tested whether chemokine-mediated adhesion to ICAM-1 or VCAM-1 or chemokine receptors signaling has recently been suggested (33), we tested whether chemokine-mediated adhesion to ICAM-1 or VCAM-1 between the 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/α13-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/α13-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. 6a, a and b). Interestingly, Gα12/α13-deficient MZB cells showed strongly increased migration toward fetal calf 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 Gα12/α13 (37), we investigated migration toward S1P in Gα12/α13-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).

We retained mature MZB cells in the marginal zone critically depends on integrin-mediated adhesion to ICAM-1 and VCAM-1 (14). We therefore tested basal adhesion of control and Gα12/α13-deficient follicular and MZB cells to uncoated, ICAM-1-coated, or VCAM-1-coated dishes, but did not find significant differences 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/α13-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/α13-DKOs is not to be attributed to differences in basal or chemokine-mediated adhesion.

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**FIGURE 5.** Normal basal and agonist-induced adhesion in MZB cells and follicular B (FoB) cells from B cell-specific Gα12/α13-deficient (B-Gα12/α13-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/α13-DKO s, p, untreated sample; *, p < 0.05 vs untreated.

**FIGURE 6.** Abnormal agonist-induced migration in MZB cells from B cell-specific Gα12/α13-deficient (B-Gα12/α13-DKO) mice. a and b, Migration of MZB cells (a) and follicular B (FoB) cells (b) from control mice (■) and B-Gα12/α13-DKO s (□) 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/α13-DKO s, 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 \( \text{G}_{\alpha_{12}}/\text{G}_{\alpha_{13}} \)-deficient MZB cells or follicular B cells were observed (data not shown). However, in the presence of 10% FCS, the proportion of cells showing amoeboid 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 \( \text{G}_{\alpha_{12}}/\text{G}_{\alpha_{13}} \)-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 B-cell 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 \( \text{G}_{\alpha_{13}} \) in constitutively \( \text{G}_{\alpha_{12}}/\text{G}_{\alpha_{13}} \)-deficient mice results in a strong reduction of splenic MZB cells in vivo. This phenotype might be due to \( \text{G}_{\alpha_{12}}/\text{G}_{\alpha_{13}} \) double deficiency in B cells or to \( \text{G}_{\alpha_{12}} \) deficiency in splenic stromal or endothelial cells, but the fact that \( \text{G}_{\alpha_{12}}/\text{G}_{\alpha_{13}} \)-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-\( \text{G}_{\alpha_{12}}/\text{G}_{\alpha_{13}} \)-DKOs, but also T2 precursor MZB cells. This finding suggests that \( \text{G}_{\alpha_{12}}/\text{G}_{\alpha_{13}} \) family G proteins play a role in the maturation of MZB cell precursors, but it is not clear which \( \text{G}_{\alpha_{12}}/\text{G}_{\alpha_{13}} \)-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 chemokinemediated 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 \( \text{G}_{i} \) family G proteins (38), and not through \( \text{G}_{\alpha_{12}}/\text{G}_{\alpha_{13}} \).

However, we found that \( \text{G}_{\alpha_{12}}/\text{G}_{\alpha_{13}} \)-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 chemotransactant 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...
matured of precursor cells, contribute to reduced numbers of MZB cells observed in vivo.

Disclosures
The authors have no financial conflict of interest.

References


