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**FcγRIIB regulates autoantibody responses by limiting marginal zone B cell activation**

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**Introduction**

FcγRIIB is an inhibitory receptor expressed throughout B cell development. Diminished expression or function is associated with lupus in mice and humans, in particular through an effect on autoantibody production and plasma cell (PC) differentiation. Here, we analyzed the effect of B cell– intrinsic FcγRIIB expression on B cell activation and PC differentiation. Loss of FcγRIIB on B cells in Fcgr2b–conditional KO (Fcgr2b-cKO) mice led to a spontaneous increase in autoantibody titers. This increase was most striking for IgG3, suggestive of increased extrafollicular responses. Marginal zone (MZ) B cells had the highest expression of FcγRIIB in both mice and humans. This high expression of FcγRIIB was linked to increased MZ B cell activation, ERK phosphorylation, and calcium flux in the absence of FcγRIIB triggering. We observed a marked increase in IgG3+ PCs and B cells during extrafollicular PC responses in Fcgr2b-cKO mice. The increased IgG3 response following immunization of Fcgr2b-cKO mice was lost in MZ-deficient Notch2 Fcgr2b–double KO mice. Importantly, patients with systemic lupus erythematosus (SLE) had a decrease in FcγRIIB expression that was strongest in MZ B cells. Thus, we present a model in which high FcγRIIB expression in MZ B cells prevented their hyperactivation and ensuing autoimmunity.

**Results**

Increased spontaneous autoantibody IgG3 responses in Fcgr2b-cKO mice. We first characterized the spontaneous autoantibody production in mice with a B cell–specific FcγRIIB conditional KO (Fcgr2b-cKO). As reported previously, we observed increased anti-dsDNA IgG, which was present in mice at around 4–5 months of age (Figure 1A and Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI157250DS1). Interestingly, when analyzing the IgG subclasses, we observed a significant increase only in IgG3 anti-dsDNA (Figure 1B). Using a flow cytometric assay we developed to assess anti-nuclear antibody–positive (ANA+) PCs (31), we observed an increased frequen-
cy of ANA‘IgG’ PCs in the spleens of Fcgr2b-cKO mice (Figure 1, C and D). In line with the serum data, the increase in ANA‘IgG’ PCs was only significant in the IgG3 subclass (Figure 1E). The frequency of ANA‘PCs within other isotypes or in the BM was not increased (Supplemental Figure 1, A–F). The increase in ANA‘IgG3’ PCs in the spleen suggested an extrafollicular B cell response.

Since we previously showed that increases in ANA‘IgG’ PCs in patients with SLE and lupus-prone mice occur through aberrant IgG PC differentiation rather than as a result of an antigen-specific tolerance defect (31), we also analyzed tolerance checkpoints for ANA‘B cells and PCs in Fcgr2b-cKO mice. B cell–intrinsic FcγRIIB deficiency did not affect the percentage of ANA‘mature naive B cells or more immature B cell subsets in the BM or spleen (Supplemental Figure 2, A–C and Supplemental Figure 3). In contrast, we detected a specific increase in the frequency of splenic IgG PCs and serum levels of total IgG (Supplemental Figure 1, G–I, M, and N). Again, the most prominent increase was observed in the IgG3 subclass, both in PCs and in serum titers (Figure 1, F and G, and Supplemental Figure 1, J–L and O–R).

Together, these results indicate that spontaneous (auto)antibody production occurred through enhanced differentiation or survival of IgG3 PCs.

Fcgr2b-cKO mice spontaneously displayed an increased frequency of resting IgG3 B cells (Figure 1H and Supplemental Figure 4, A–E). IgG3 is usually derived from B-1 cells or MZ B cells, and increased numbers of peritoneal B-1 cells have been reported in complete Fcgr2b−/− mice (24). Most IgG3 B cells in Fcgr2b-cKO mice had a B-2 (CD19+ B220+) phenotype and were CD5− (Figure 1, H and I). In addition, the frequencies of B-1 or B-1b cells in the spleen and peritoneum were unaffected in Fcgr2b-cKO mice (Supplemental Figure 4, F–M), whereas MZ B cell frequencies were increased in Fcgr2b−/− mice (Supplemental Figure 4, N and O).

Increased extrafollicular PC responses. IgG3 has been associated primarily with extrafollicular PC responses derived from MZ

Figure 1. Increased spontaneous autoantibody IgG3 responses in Fcgr2b-cKO mice. Female control (Ctr) and Fcgr2b-cKO mice were bred and maintained until experiments at 10–12 months of age, at which point (auto)antibodies in serum and PCs in spleen were characterized. ANA reactivity of PCs was established using flow cytometry. (A and B) dsDNA ELISA for total IgG and IgG subclasses in serum from Fcgr2b-cKO mice. (C) Representative example of ANA staining in IgG and IgM PCs in spleen. (D and E) Frequency of ANA‘IgG’ PCs in spleen, total IgG (D), and by IgG subclass (E). (F) Representative example of IgM and IgG3 staining in total PCs. IgG3+ cells are indicated in green. (G) Frequency of IgG3 PCs in spleen separated by subclass. (H) Frequency of IgG3+ B cells in control and Fcgr2b-cKO mice. (I) Representative example of staining strategy for B-1 and B-2 cells in spleen. (J) Percentage of splenic IgG3+ B cells with a B-1 or B-2 phenotype, respectively, gated as in I. (K) Representative example of staining for B220 and CDS in total IgG3+ B cells compared with B-1, FO, and MZ B cells in spleen. Data are shown as the median, with each symbol representing an individual mouse (A, B, D, E, G, H, and J) (n = 12–17 per group pooled from 2–3 independent experiments). *P < 0.05 and ***P < 0.001, by Mann-Whitney U test.
or B-1 cells (32–34). B-1 cells produce natural antibodies and can even produce these in the absence of antigen stimulation; and MZ B cells can be directly activated by antigens with repeated epitopes and TLRs and act as a first line of defense against blood-borne pathogens (24, 35, 36). Both of these cell types have been associated with extrafollicular humoral responses that are independent of cognate T cell help. Therefore, we analyzed the extrafollicular response to immunization with prototypical T-independent and B-1 cells (32–34). B-1 cells produce natural antibodies and can even produce these in the absence of antigen stimulation; and MZ B cells can be directly activated by antigens with repeated epitopes and TLRs and act as a first line of defense against blood-borne pathogens (24, 35, 36). Both of these cell types have been associated with extrafollicular humoral responses that are independent of cognate T cell help. Therefore, we analyzed the extrafollicular response to immunization with prototypical T-independent and
Figure 3. Phenotype and activation of MZ B cells in Fcgr2b-cKO mice and humans. (A) Representative example of staining for FcγRIIB in different B cell subsets and IgG3+ B cells in the spleen. Panel on the right shows staining in Fcgr2b-cKO cells. (B) FcγRIIB staining intensity in different B cell subsets from control mice. (C) Schematic of the experimental approach used to investigate the effect of FcγRIIB on B cell activation. Using intact anti-IgM, both the BCR and FcγRIIB are engaged (top); using Fab2 anti-IgM (middle) or Fcgr2b-cKO cells (bottom), only the BCR is engaged. (D–G) Sorted FO and MZ B cells were stimulated with 3 μg/mL anti-IgM for 20 hours. Activation was measured by flow cytometry. Representative examples (D) and summary (E–G) of the expression of CD80, CD86, and IAd. (H and I) Representative examples and summary of expression of CD32 analyzed by flow cytometry using different human B cell subsets. (J) qPCR for Fcgr2z2 in sorted human B cell subsets. Relative expression was normalized to polr2a, after which ∆∆Ct was calculated compared with naive B cells. (K–M) Sorted human naive and MZ-like B cells were stimulated with 3 μg/mL intact anti-IgM or equimolar concentrations (2 μg/mL) of Fab2 anti-IgM for 20 hours or were left untreated as controls. Upregulation of HLA-DR, CD80, and CD86 was measured by flow cytometry. The percentage of inhibition was calculated for each donor (the median percentage of inhibition is indicated). Data are shown as the median, with each symbol representing an individual mouse or human (n = 6 per group for B, n = 5–7 per group for D–F, n = 6–10 per group for I–M; data were pooled from 2–5 independent experiments; except for the data in B, which were from 1 experiment). **P < 0.01, and ***P < 0.001, by 1-way ANOVA with Bonferroni’s post hoc test (B, I, and J) or 2-way ANOVA with Bonferroni’s post hoc test (E–G and K–M). mem, memory B cells.

To address these findings in humans, we analyzed the expression and inhibitory function of FcγRIIB in various B cell subsets from healthy donors. IgM+CD27+ B cells have been proposed as the human equivalent of MZ B cells in mice (MZ-like B cells) (37). We isolated these cells by FACS and confirmed their MZ-like phenotype by high SOX7 and low HOPX expression, relative to conventional naive and IgG+ memory B cells (37) (Supplemental Figure 7, A and B). Next, we analyzed the expression of FcγRIIB in MZ-like B cells, naive B cells, and IgG and IgA memory B cells. As in mice, the human MZ-like compartment had the highest expression of FcγRIIB on both the protein and RNA level (Figure 3, H–J). MZ-like B cells were also more strongly activated than were naive B cells by BCR crosslinking using Fab2 anti-IgM across a range of concentrations (Supplemental Figure 7, C–E). We next analyzed the effect of FcγRIIB-BCR crosslinking in MZ-like B cells compared with naive B cells and observed a stronger inhibitory effect of FcγRIIB engagement on MZ-like B cells than on naive B cells (Figure 3, K–M, and Supplemental Figure 7F). For example, although CD86 upregulation by BCR engagement was similar between naive B cells and MZ B cells (Supplemental Figure 7H), the inhibitory effect of FcγRIIB on MZ-like B cells returned CD86 expression close to baseline levels (Figure 3M). Upregulation of HLA-DR and CD80 upon BCR crosslinking was also stronger in MZ-like B cells (Supplemental Figure 7, F and G), and FcγRIIB-mediated inhibition was most pronounced in this subset (Figure 3, K and L).

MZ-like B cells have been reported to be more prone to activation; we now show that they also exerted stronger inhibition of activation through FcγRIIB, an effect that was observed in both mice and humans.

FcγRIIB inhibits MZ B cell activation through inhibition of Erk phosphorylation and calcium flux. We next analyzed the phosphorylation of signaling molecules downstream of the BCR and FcγRIIB (Figure 4A). We chose to study Syk, SHIP1, and Erk1/2 signaling. Syk is an early B cell signaling molecule that is not...
inhibited by FcγRIIB or SHIP1, the phosphatase activated by FcγRIIB engagement. Erk1/2 is downstream of FcγRIIB/SHIP1 signaling and is important for B cell activation and PC differentiation (38). Consistent with the increased upregulation of activation markers, we found increased phosphorylation of Syk and Erk1/2 in murine MZ B cells compared with FO B cells stimulated with Fab′2 anti-IgM (Figure 4, C and D). There was strong inhibition of Erk1/2 phosphorylation in MZ B cells in the presence of FcγRIIB engagement with intact anti-IgM antibodies, but no inhibition of Syk phosphorylation (Figure 4, B–D). A significant decrease in Erk1/2 phosphorylation was only observed in MZ B cells and not in FO B cells (Figure 4D). Furthermore, MZ B cells...
exhibited increased SHP1 phosphorylation following combined BCR-FcγRIIB crosslinking compared with FO B cells (Figure 4E). Similar results for Erk1/2 phosphorylation were obtained when we studied the absence of FcγRIIB engagement in Fcγ2b-cKO mice compared with control mice (Figure 4, F and G). Again, this difference was only observed in MZ B cells.

In human MZ-like B cells, we likewise observed increased phosphorylation of Erk1/2 following BCR triggering in MZ-like cells compared with naive B cells (Supplemental Figure 8, A and B). FcγRIIB engagement strongly inhibited p-Erk in MZ-like B cells. Decreased Erk phosphorylation was also observed in naive B cells (Figure 4, H–J). MZ-like B cells compared with naive B cells had increased calcium flux upon BCR triggering (Supplemental Figure 9), and the calcium flux, particularly the peak flux, was more strongly inhibited by FcγRIIB in MZ-like B cells than in naive B cells (Figure 4, K–M).

These results point to a strong inhibitory effect of FcγRIIB on the activation of MZ B cells through decreased phosphorylation of Erk1/2 and decreased calcium flux.

IgG3 responses in Fcγ2b-cKO mice are derived from MZ B cells. Because our results suggest that MZ B cells are a likely source for the increased IgG3 response in Fcγ2b-cKO mice, we generated double-KO (dKO) mice that lacked Notch2 and FcγRIIB in B cells, resulting in large reductions in MZ B cell numbers (39) and B cell-specific FcγRIIB deficiency. These mice exhibited deficiency of MZ B cells (~80% reduction), whereas FO B cells and B-1 cell numbers were maintained (Figure 5, A and B, and Supplemental Figure 9, A–C). Similar to what has been previously described (32), Notch2-cKO mice had decreased NP-specific IgM and IgG serum levels and PC numbers, but there was no effect of FcγRIIB on the IgM response in either the presence or absence of Notch2 (Supplemental Figure 9, D and F). More important, the increased IgG response to NP-Ficoll that we observed in Fcγ2b-cKO was completely reversed in the dKO mice lacking both Notch2 and FcγRIIB in B cells (Supplemental Figure 9, E and G). Furthermore, the increase in NP-IgG3 serum titers and NP-IgG3+ B cells and PCs generated by FcγRIIB deficiency was absent in dKO mice (Figure 5, C–F). IgG3+ B-2 B cells, but not IgG3+ B-1 B cells, were significantly reduced in MZ-deficient dKO mice (Supplemental Figure 9, H and I). Finally, we analyzed spontaneous autoantibody production triggered by deficiency of FcγRIIB in MZ-deficient mice. The increased IgG and IgG3 anti-dsDNA observed in FcγRIIB-cKO mice was abolished in dKO mice lacking both FcγRIIB and MZ B cells (Figure 5, G–I). Together, these results show that MZ B cells were responsible for enhanced extrafollicular (auto)antibody responses in Fcγ2b-cKO mice.

Diminished expression of FcγRIIB in MZ-like B cells from patients with SLE. Previous studies have shown diminished expression of FcγRIIB on CD27+ B cells from patients with SLE (9, 10). However, these cell populations were not studied in further detail, and CD27+ cells comprise both switched memory B cells as well as MZ-like B cells. We therefore wanted to assess the expression of FcγRIIB in MZ-like B cells from patients with SLE. We performed high-dimensional spectral flow cytometry, which allowed a detailed identification of more than 10 B cell subsets, in combination with staining for CD32B/C, on PBMCs from patients with SLE (n = 15) and healthy donors (n = 10). The patients’ characteristics are shown in Supplemental Table 2.

In line with previous studies, patients with SLE showed decreased expression of CD32B/C on CD27+ B cells, but not on CD27– B cells (Figure 6A). Next, B cell subsets were clustered using FlowSOM and visualized on a uniform manifold approximation and projection (UMAP) projection (Figure 6B). MZ B cells were identified in cluster 08, characterized as CD27+, IgM+, IgD–, CD21hi, CD24lo, and CD38lo B cells (Supplemental Figure 10). Likewise, other B cell subsets were identified according to published characteristics (40). Interestingly, we observed a significant decrease in CD32B/C expression in MZ-like B cells (Figure 6, C–E). Although some other cell populations (activated naive B cells, DN1 B cells, resting switched memory B cells, and plasmablasts) also exhibited some decrease in expression of CD32B/C, this difference was no longer significant after correction for multiple testing (Figure 6F). Similar results were obtained using conventional gating (data not shown).

Interestingly, decreased expression of CD32B/C was already observed in the MZ precursor (MZP) population, gated as CD27 CD32bhiCD24loCD45RBhi, further characterized in a previous report as IgM+CD21+CD1cint (37) (Figure 6G and Supplemental Figure 11).

MZ-like B cells also exhibited significantly increased expression of the activation marker CD80 in patients with SLE compared with healthy donors (Figure 6, H and I). The increased expression of CD80 was not seen in other B cell populations (Supplemental Figure 12). No difference in other activation markers (CD40, CD69, CD86, HLA-DR) was observed (data not shown). CD80 expression in MZ-like B cells was inversely correlated to levels of CD32B/C (Figure 6J), suggesting that the lower expression of Fcγ-RIIB was associated with higher activation of MZ B cells in SLE, as we demonstrated in the murine studies.

In summary, our results show increased extrafollicular responses in B cell-intrinsic Fcγ-RIIB-deficient mice characterized by a major increase in MZ-derived IgG3 responses. MZ B cells are highly sensitive to inhibition through FcγRIIB, which can be explained by the high expression of FcγRIIB in MZ B cells compared with expression levels in other B cell subsets. High FcγRIIB expression resulted in strong inhibitory signaling through FcγRIIB in MZ B cells, an effect that we observed in both mice and humans. Finally, patients with SLE exhibited a marked decrease in FcγRIIB expression in B cells, most strongly in MZ-like B cells.

Discussion

In this study, we analyzed B cell tolerance and extrafollicular PC differentiation in B cell-intrinsic FcγRIIB-deficient mice (16). We have identified spontaneous PC differentiation in Fcγ2b-cKO mice, leading to increased serum autoantibody levels, in particular of the IgG3 isotype. In addition, Fcγ2b-cKO mice showed increased IgG3 responses following immunization, which was MZ B cell dependent. In both mice and humans, MZ B cells had the highest expression of FcγRIIB; this high expression was associated with increased FcγRIIB-mediated inhibitory effects on Erk phosphorylation and calcium signaling in vitro. Importantly, we showed that MZ B cells from patients with SLE had reduced expression of FcγRIIB that was associated with increased expression of the activation marker CD80. Thus, B cell-intrinsic FcγRIIB deficiency was linked to increased extrafollicular responses through an effect specifi-
ANA+ IgG PCs, suggestive of increased B cell activation and PC differentiation rather than an antigen-specific tolerance defect. This is also in line with the increased responses to immunization we observed with NP-Ficoll and NP-CGG in Fcgr2b-cKO mice. Of note, these mice did not develop a fulminant lupus phenotype, despite the presence of autoreactive PCs in the spleen and auto-

cally on MZ B cells. In patients with SLE, reduced expression of FcγRIIB may contribute to increased activation of MZ B cells and subsequent autoantibody production.

Similar to previous studies of lupus-prone mice performed in our laboratory (31), we found that B cell deficiency of FcγRIIB led to an increase in PCs, without an increase in the fraction of ANA+ IgG PCs, suggestive of increased B cell activation and PC differentiation rather than an antigen-specific tolerance defect. This is also in line with the increased responses to immunization we observed with NP-Ficoll and NP-CGG in Fcgr2b-cKO mice. Of note, these mice did not develop a fulminant lupus phenotype, despite the presence of autoreactive PCs in the spleen and auto-

Figure 5. Combined FcγRIIB and MZ deficiency reverses the enhanced response to antigen challenge and the increase in autoantibody production. (A–F) Female control, Notch2-cKO, Fcgr2b-cKO, and Notch2 Fcgr2b-dKO mice were immunized with NP-Ficoll. Serum and splenocytes were obtained 7 days later. (A and B) Representative examples of MZ B cell frequencies in Fcgr2b-cKO and Notch2 Fcgr2b-dKO mice. (C) Representative examples of intracellular IgG3 and NP staining in splenic PCs. (D) Frequency of NP-specific IgG3+ PCs in spleen, as a percentage of B cells. (E) Levels of NP-specific IgG3 in serum. (F) Frequency of NP-specific IgG3+ B cells in spleen. (G–I) Female control, Notch2-cKO, Fcgr2b-cKO, and Notch2 Fcgr2b-dKO mice were bred and maintained until 6–7 months of age, after which dsDNA antibodies in serum were characterized. dsDNA ELISAs for total IgM, IgG, and IgG subclasses were performed. Data are shown as the median, with each symbol representing an individual mouse (n = 5 mice per group for A–F; n = 13–17 mice per group for G–I). *P < 0.05, **P < 0.01, and ***P < 0.001, by 2-way ANOVA with Bonferroni’s post hoc test.
In contrast to studies with the full FcγRIIB KO (24), we did not observe an effect of B cell–intrinsic FcγRIIB deficiency on B-1 numbers in the spleen or peritoneum, and FcγRIIB expression in B-1 cells was low, suggesting that the effect on the B-1 compart-

Figure 6. Reduced FcγRIIB expression in MZ B cells from patients with SLE. High-dimensional spectral flow cytometry was used to identify multiple B cell subsets within PBMCs from patients with SLE (n = 15) and healthy donors (HD) (n = 10). (A) Expression of CD32B/C in CD27+ and CD27− B cells. (B) Live B cells were clustered using FlowSOM and are shown in a UMAP plot. Live B cells from healthy donors and patients with SLE were concatenated from each donor (n = 250,000 cells per group). (C) Expression of CD32B/C among all B cell clusters in the UMAP plot. (D) Representative example of CD32B/C expression in MZ-like B cells from healthy donors and patients with SLE, including the FMO control. (E) Summary of CD32B/C expression in MZ B cells. (F) Summary of CD32B/C expression in all FlowSOM clusters. (G) Expression of CD32B/C in MZP cells identified by manual gating as live CD19+CD27−IgD−CD38loCD21hiCD24hi. (H) Representative examples of CD80 expression and gating in MZ B cells from healthy donors and patients with SLE. MZ B cells from healthy donors and patients with SLE were concatenated from each donor (n = 25,000 cells per group). (I) Summary of the percentage of CD80+ MZ B cells from healthy donors and patients with SLE. (J) Correlation of the percentage of CD80+ cells and CD32B/C MFI in MZ B cells. Data are shown as the median, with each symbol representing an individual donor (n = 10 for healthy donors; n = 15 for patients with SLE). **P < 0.01 (A) and other P values were determined by Mann-Whitney U test (E–G and I) or Spearman’s rank test (J). Act. Nv, activated naive; Rest. Nv, resting naive; Atyp. UnswM, atypical unswitched memory; Act. SwM, activated switched memory; Rest. SwM, resting switched memory; PB, plasmablast.

antibodies in serum. Complete Fcgr2b-KO mice on the C57BL/6 background spontaneously develop autoantibody titers and develop a fulminant lupus phenotype (11, 16), probably due to combined effects on B cells and myeloid cells (41).
ment was indirect. We now show that the enhancement of NP-Ficoll responses in Fcgr2b-cKO mice was completely reversed in Notch2 Fcgr2b−/−-deficient dKO mice. Since these mice had largely reduced MZ numbers without significant reductions in B1-cells (39), this suggests that the enhanced extrafollicular responses to NP-Ficoll were dependent on MZ B cells.

As has been reported in the literature, we observed increased BCR-mediated activation of MZ B cells compared with activation of FO or naive B cells, in both humans and mice (42). Importantly, we showed that MZ B cells also had stronger inhibition mediated by FcγRIIB coengagement. In the MZ B cell compartment, FcγRIIB-mediated inhibition was linked to diminished Erk phosphorylation and calcium flux. Previous studies into the effect of FcγRIIB-BCR crosslinking on B cell signaling have revealed quite variable effects, probably depending on the source and B cell subset studied. Most studies have shown no effect on Syk phosphorylation (43, 44). FcγRIIB engagement preferentially recruits SHIP, which acts to hydrolyze phosphatidylinositol(3,4,5)P3 and inositol(1,4,5)P3, downstream of Syk (45). FcγRIIB-BCR crosslinking also leads to earlier closing of calcium channels (44, 46), consistent with our data showing diminished calcium flux. Since we were interested in the difference between MZ B cells and FO B cells, we used primary B cells as opposed to the cell lines that were used in most of the previous studies. Our results show the importance of analyzing signaling in a cell type-specific manner, as different B cell subsets can respond quite differently, and with our studies, we were able to reveal strong inhibitory signaling of FcγRIIB in MZ B cells.

**FCGR2B** risk alleles, which lead to diminished expression and/or inhibitory function of FcγRIIB, predispose individuals to SLE. Furthermore, diminished expression of FcγRIIB on CD27+ B cells and plasmablasts from patients with SLE has been shown irrespective of the presence of risk alleles (9, 10). In these studies, the expression of FcγRIIB was studied only in major B cell populations, and IgM+ and IgG+CD27+ B cells were not distinguished. Here, using high-dimension spectral flow cytometry, we were able to show that this difference was mainly driven by reduced expression in MZ B cells. Furthermore, this difference was already present in circulating MZP B cells (37), suggesting that the dysregulation of CD32B/C expression in MZ B cells may occur during early B cell differentiation in SLE. We and others have recently demonstrated that most patients with SLE exhibit enhanced PC differentiation and that some exhibit increased extrafollicular PC differentiation (28, 30, 47). Our current data show how diminished expression of FcγRIIB can lead to increased extrafollicular B cell activation and autoantibody production. Since MZ B cells are known to have increased autoactivity (31, 48–50), a loss in the regulation of extrafollicular MZ B cell responses through diminished function of FcγRIIB may therefore lead to autoantibody production in SLE.

In summary, we present a model in which high expression of FcγRIIB in MZ B cells was necessary to prevent ongoing activation and thereby functioned as a feedback loop to prevent ensuing autoimmunity, in particular through its effect on extrafollicular MZ responses. As SLE risk alleles for FCGR2B diminish the functionality of FcγRIIB, patients with SLE with a risk allele for FCGR2B may have a greater impairment of MZ B cells and may have the highest levels of MZ-derived autoantibodies and the largest fluctuation in autoantibody titers. We believe these studies move us closer to precision medicine in SLE.

**Methods**

*Mice.* Fcgr2b−/− mice were a gift from Jeffrey Ravetch (The Rockefeller University, New York, New York, USA) (16). CD19cre mice (stock no. 006785), B6.C-H2d (stock no. 000359) were obtained from The Jackson Laboratory and were crossed with Fcgr2b−/− mice to generate CD19cre−/− Fcgr2b+/− H2d+/− (FcγRIIB-cKO) mice. Control mice were either carrying only the Cre allele (CD19cre−/− Fcgr2b+/− H2d+/−) or only the floxed alleles (CD19cre−/− Fcgr2b+/− H2d+/−). To generate MZ-deficient mice, CD19cre−/− control mice and Fcgr2b-cKO mice were crossed with Notch2+/− (The Jackson Laboratory, stock no. 010525) to generate CD19cre−/− Notch2+/− H2d+/− and CD19cre−/− Fcgr2b+/− Notch2+/− H2d+/− mice.

For analysis of spontaneous autoimmunity, mice were kept until 10–12 months of age. For analysis of immunization responses, 8- to 16-week-old female mice were immunized i.p. with 50 μg NP-Ficoll (conjugation ratio, 55:1) in 100 μL saline (Biosearch Technologies) or 100 μg NP-CCG (conjugation ratio, 20–29:1) (Biosearch Technologies) in Imject Alum (Thermo Fisher Scientific) and followed for 7 days (both NP-Ficoll and NP-CCG) and for 42 days (NP-CCG only). For functional in vitro studies of FcγRIIB, spleens of 8- to 16-week-old female mice were used.

Spleens and BM were collected at the indicated time points followed by the formation of a single-cell suspension by mashing over a 70 μm cell strainer. RBC lysis was performed using RBC lysis buffer (BioLegend). Peritoneal cells were obtained by injecting 10 mL HBSS plus 5% FBS, followed by massaging of the peritoneum and withdrawal of the buffer with cells. Serum was obtained through submandibular bleeding followed by centrifugation.

*Patients and healthy donors.* Buffy coats from healthy donors were obtained through the Sanquin blood bank (Netherlands). PBMCs were isolated using the standard Ficoll procedure. For signaling experiments, frozen PBMCs obtained from buffy coats were used.

For spectral flow cytometry, heparinized blood was obtained from patients with SLE (n = 15) and healthy donors (n = 10). Patients with SLE were recruited from the Rheumatology outpatient clinic of the Leiden University Medical Center in the Netherlands, and healthy donor samples were obtained through the LUMC Voluntary Donor Service Biobank (Leiden, Netherlands). The age and sex of the healthy donors were selected to reflect the sex and age range of the patients with SLE. PBMCs were isolated using a standard Ficoll procedure and frozen.

**ELISA.** Half-area ELISA Plates (Corning) were coated with 10 μg/mL of NP-2 or NP25-BSA (Biosearch technologies) and anti-mouse IgG, IgM, IgG1, IgG2b, IgG2c, or IgG3 unlabeled antibody (Southern Biotech) overnight at 4°C, or with 100–400 μg/mL sonicated filtered calf thymus DNA (Calbiochem) overnight at 37°C uncovered. Plates were washed with PBS containing 0.05% Tween-20 and blocked with 1% BSA in PBS. Diluted serum samples were incubated for 1.5 hours. Serum samples for anti-DNA IgG and IgG subclasses were diluted 1:100. Serum samples from NP-immunized mice were diluted 1:10,000 (day 7) or 1:50,000 (time course days 0, 14, 28, 42) for NP-specific IgM and IgG, and 1:2500 for NP-specific IgG subclasses. Plates were washed with wash buffer and secondary goat polyclonal alkaline phosphatase-labeled (AP-labeled) anti-mouse IgG, IgM, IgG1, IgG2b,
IgG2c, or IgG3 (Southern Biotech) was added for 1 hour. After washing, plates were developed using phosphatase substrate (MilliporeSigma) dissolved in distilled water with 50 mM NaHCO3 (MilliporeSigma) and 1 mM MgCl2 (MilliporeSigma). Plates were read at a wavelength of 405 nM on a 1430 Multilabel Counter Spectrometer (PerkinElmer).

Flow cytometry. For flow cytometry phenotyping, cells were preincubated on ice for 5 minutes with Fc block (anti-mouse or anti-human) for each staining, except the staining for FcyRIBB. After this, cells were stained for 30 minutes with antibodies against cell membrane proteins followed by washing in 5% FBS in HBSS or 0.5% BSA in PBS. For surface staining, cells were washed and stained in 1% paraformaldehyde (PFA) until acquisition. For intracellular staining, cells were fixed and permeabilized with a Foxp3 transcription factor fixation/permeabilization kit (eBioscience) for 45 minutes on ice. After fixation and permeabilization, the intracellular staining cocktail was incubated for 30 minutes in permeabilization buffer to visualize immunoglobulins and ANA stainings (eBioscience). Cells were then incubated with a mix of antibodies against cell-surface and phospho-specific signaling molecules or isotype controls at room temperature for 60 minutes.

For capillary Western blotting, mouse FO B cells and MZ B cells were sorted and stimulated for 10 minutes with the anti-IgM antibodies (intact 7.5 μg/mL; Fab′2, 2–10 μg/mL). Cells were immediately fixed in 1× Phosphoflow Lyse/Fix buffer (BD). After 10–12 minutes of incubation at 37°C, cells were washed and permeabilized using PhosphoFlow Perm Wash 1 (BD) for 30–60 minutes. Following a 5-minute preincubation with Fc block (BD), cells were then incubated with a mix of antibodies against cell-surface and phospho-specific signaling molecules or isotype controls at room temperature for 60 minutes.
RNA isolation and quantitative PCR. Human naïve, MZ-like, and conventional memory (IgG+ and IgA+) B cells were sorted according to the gating strategy described above. A total of 50,000–200,000 cells per population were lysed in RLT buffer (QIAGEN) with 10 µL/mL 2-ME (Merck), followed by RNA isolation using the RNaseasy Microprep kit (QIAGEN). cDNA was synthesized using iScript (Bio-Rad). Quantitative PCR (qPCR) was performed after preamplification using TaqMan PreAmp Master Mix (Thermo Fisher Scientific) and the TaqMan assays mentioned below. qPCR was performed using TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific) and the following multiplexed VIC and FAM TaqMan assays (all from Applied Biosystems/Thermo): POLR2A VIC-MGB (Hs00172187_ml); ACTB VIC-MGB (Hs01060665_g1); HOPX FAM-MGB (Hs04188695_ml); SOX7 FAM-MGB (Hs00846731_sl); and FCGR2B FAM-MGB (Hs00269610_ml). qPCR was run on a CFX Opus machine (Bio-Rad) using the recommended cycling conditions.

Spectral flow cytometry and clustering analysis. For spectral flow cytometry, cells were stained as described for conventional flow cytometry using with the antibodies listed in Supplemental Table 2. Antibody cocktails were prepared using Brilliant Stain Buffer (BD) Plus (BD). Monocyte blocker (BioLegend) was added to prevent aspecific binding of tandem dyes. To determine the specificity of CD32B/C staining, a fluorescence minus one (FMO) control was taken along for each sample. Cells were washed and fixed with 1% PFA for 20 minutes. Cells were washed again and stored in FACS buffer until acquisition on a 5-laser Aurora (Cytek). Raw spectral data were unmixed using SpectroFlo software (Cytek), after which unmixed fcs files were analyzed in OMIQsoftware. Live B cells were gated, after which B cell populations were gated manually, and expression levels of CD32B/C were obtained. In parallel, live B cells were clustered with FlowSOM (elbow metaclustering) using CD19, CD20, CD21, CD24, CD27, CD38, IgD, and IgM as input parameters. Using the same parameters, cells were projected onto UMAP (neighbors = 15, mindist = 0.2). Expression levels of markers were analyzed in UMAP and heatmaps to identify known B cell subsets (40). Fifteen FlowSOM metaclusters were obtained, among which 3 clusters were very similar in expression patterns and were together designated as resting naïve. One small cluster (<1%) was identified as a non-B cell cluster and excluded from downstream analysis, leaving 12 final B cell clusters.

Data analysis. Analysis of flow cytometric data was performed using the BD FACS DIVA and FlowJo software. Graphing of calcium flux was performed using GraphPad Prism (GraphPad Software) after exporting the binned data for each population to FlowJo. Cells were gated as percentage positive if there were 2 clear populations, whereas median fluorescence intensity (MFI) was used when an entire population showed a shift. All intensities are shown as MFIs. To control for differences in autofluorescent backgrounds or nonspecific binding, isotype controls were used for in vitro activation and PhosphoFlow experiments. No differences in isotype control background were found between the cell populations that were compared. In vitro activation and phosphorylation data are shown for 3 µg/mL intact and 2 µg/mL Fab’2 anti-IgM antibodies. For both mouse and human studies, similar results were obtained with 10 and 15 µg/mL, respectively (data not shown). For in vitro activation and signaling, the percentage of inhibition with intact anti-IgM was calculated per donor as follows: intact anti-IgM/Fab’2 anti-IgM – unstimulated) × 100.

Immunized mice were excluded when they exhibited no serum antibody response compared with baseline (n = 3 for the day-7 NP-CGG response; n = 3 for the day-7 NP-CGG response; n = 1 for the day-7 NP-Ficoll response). One sample for FCγRIIB expression in human B cells was excluded from the qPCR analysis because it was an extreme outlier of protein expression in naïve B cells (Figure S1). Serum samples were excluded because they were extreme outliers (>mean + 4 × SD; n = 3 excluded) in the Notch2 experiments for spontaneous autoantibody production (Figure 5, G–I).

Statistics. Statistical analysis was performed using GraphPad Prism (GraphPad Software). For comparison of ex vivo mouse data with 2 groups, a 2-tailed Mann-Whitney U test was performed. For comparison of multiple groups, 1-way ANOVA with Bonferroni’s post hoc test was used. For 2-group analysis of in vitro activation, a 2-tailed, paired t test was used, and for comparison of 2 categorical variables, 2-way ANOVA with Bonferroni’s post hoc test was used. Comparison of expression levels in healthy donors versus patients with SLE was performed using a 2-tailed Mann-Whitney U test. Correlation was calculated using Spearman’s rank test. P values of less than 0.05 were considered statistically significant.

Study approval. Mice were housed according to Association for Assessment and Accreditation of Laboratory Animal Care (International) (AAALAC) regulations, and all mouse studies were approved the IACUC of The Feinstein Institutes for Medical Research/Northwell health. Studies with buffy coats from healthy donors were performed in accordance with the Declaration of Helsinki, and written informed consent was obtained from all donors. Studies of blood from patients with SLE and healthy donors were approved by the local medical ethics committee (METC-LDD, Leiden, The Hague, Delft, Netherlands), and written informed consent was obtained from all donors.

Author contributions ANB designed and conducted experiments, analyzed data, and wrote the initial manuscript. SM designed and conducted experiments and analyzed data and revised the manuscript. IKS and ALD conducted experiments and analyzed data. JS conceptualized the study, designed and conducted experiments, analyzed data, and wrote the manuscript. The order of the co–first authors was determined by who initiated the study (ANB) and who completed the study (SM).

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