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Inhibitory Fcγ Receptor Is Required for the Maintenance of Tolerance through Distinct Mechanisms

Fubin Li,*† Patrick Smith, † and Jeffrey V. Ravetch †

The inhibitory FcγR FcγRIIB is widely expressed on B cells, dendritic cells (DCs), and myeloid effector cells and modulates a variety of Ab-driven in vivo functions. Although it has been established that FcγRIIB plays an important role in the maintenance of peripheral tolerance, the responsible cell-specific FcγRIIB expression remains to be determined. In this study, we generated mice with selective deletion of FcγRIIB in B cells, DCs, and myeloid effector cells and evaluated these novel strains in models of tolerance and autoimmune diseases. Our results demonstrate that mice with selective deletion of FcγRIIB expression in B cells and DCs have increased Ab and T cell responses, respectively, and display enhanced susceptibility to disease in distinct models, suggesting that FcγRIIB expression in distinct cellular populations contributes to the maintenance of peripheral tolerance through different mechanisms. The Journal of Immunology, 2014; 192: 3021–3028.

The loss of tolerance to self-Ags with the resultant accumulation of autoantibodies and pathogenic immune complexes is a hallmark of autoimmune diseases and contributes to their pathological sequelae with resulting morbidity and mortality. Genetic and biochemical studies have identified multiple tolerance checkpoints that appear to be dysregulated in autoimmune diseases such as lupus and are thus considered to be targets for the development of therapeutic approaches to either prevent or cure this disease. One such candidate, the inhibitory FcγRIIB, was initially identified as a member of the wider FcγR family, functioning to balance the ability of IgG immune complexes to activate myeloid effector cells through engagement of the activation members of the FcγR family (1, 2). This inhibitory receptor is widely expressed on cells of the immune system, including myeloid populations, dendritic cells (DCs), and B cells. It functions to gate signaling from ITAM-containing activation receptors by recruiting phosphatases, such as SHIP, and thus set thresholds for immune complex stimulation of inflammatory responses. On B cells, however, in addition to its ability to gate activation responses triggered by the BCR, cross linking of FcγRIIB in the absence of an activation partner resulted in apoptosis and accounted for the role of FcγRIIB in limiting the accumulation of plasma cells (3, 4). Genetic disruption of the gene for FcγRIIB on the mixed C57BL/6 and 129/Sv background resulted in the spontaneous accumulation of autoantibodies, followed by progression to glomerular disease and premature mortality, identifying the gene as an epistatic modifier of lupus susceptibility (5). In a similar manner, immunization of a nonpermissive, H-2b strain of mice, such as B6 with bovine collagen type I or IV, resulted in loss of tolerance with the development of anti-mouse collagen Abs and the subsequent development of arthritis and a Goodpasture disease-like phenotype, respectively (6, 7). More recently, studies with FcγRIIB-deficient mice derived from B6 embryonic stem (ES) cells confirmed that FcγRIIB deficiency sensitizes C57BL/6 mice to collagen-induced arthritis (CIA) (8). Previous studies also demonstrated that mouse strains susceptible to the development of autoimmunity, such as NZW, BXB6, and NOD, displayed defective regulation of FcγRIIB expression, the result of polymorphisms in the promoter region of the gene, leading to a failure of the normal pattern of upregulation upon B cell activation (9). The autoimmunity-associated polymorphic allele encoding FcγRIIB was recently knocked into C57BL/6 mice and resulted in a reduction of autoimmune phenotypes, including the development of more severe CIA (10). Tolerance could be restored in these lupus-susceptible strains by restoring wild-type (WT) levels of FcγRIIB on B cells by gene transfer (11). Combining FcγRIIB dysregulation with other genetic modifiers of autoimmunity, such as yaa, MD45, or MRL/lpr, resulted in exacerbation of autoimmune disease (12–14).

Similar defects in FcγRIIB expression or function were described in human systemic lupus erythematosus populations in which it had been observed that >50% of lupus patients fail to upregulate FcγRIIB upon B cell activation (15). A promoter polymorphism affecting the regulation of FcγRIIB has been identified in some systemic lupus erythematosus populations in which the common haplotype −386G→120T is replaced by −386C/−120A (16). In addition to defects in the appropriate regulation of the FcγRIIB gene, a polymorphism has been identified in the transmembrane region of the gene I232T (17), which results in a hypomorphic mutation that fails to mediate inhibitory signaling and thus compromises this function of FcγRIIB (18–20). Confirming the importance of this hypomorphic allele in maintaining tolerance
was the observation that hematopoietic stem cells derived from patients homozygous for the I232T polymorphism, when transplanted into immunodeficient recipient mice, resulted in reconstituted immune systems that failed to maintain tolerance and developed anti-DNA Abs (21).

Therefore, defects in FcγRIIB function and regulation have emerged as a common feature of lupus and other autoimmune diseases, contributing both to disease susceptibility and progression. However, the relative contributions of FcγRIIB expression in different cellular compartments, such as B cells, DCs, and myeloid effector cells, to these phenotypes have not been firmly established. In the current study, we have investigated the contributions of FcγRIIB expression in B cells, DCs, and myeloid effector cells to the maintenance of peripheral tolerance through the analysis of mice conditionally deleted for this receptor in these immune cells.

Materials and Methods

Generation of mice carrying FcγR2b−/− and FcγR2bΔ alleles

To generate FcγR2b germ line and conditional knockout mice from B6 ES cells, two homologous arms cloned from the FcγR2b locus of C57BL/6 genomic DNA were inserted into an ES cell–targeting vector (Supplemental Fig. 1). The 5′ homologous arm, a 8.5-kb DNA fragment containing the exons coding for the S2, extracellular (EC1), EC2, and transmembrane domains of FcγRIIB, was generated by PCR (Expand Long Template PCR, Roche) using primers 5′-CCCATCAGATGAAACAGTTAAGGTGTCGCAGAACCTATGGGGCCCACCTTACAGGAAATA-3′ and 5′-ATATTCTTGCGGCCGCCATTTTC-GAGTCGACAACACTATGGGGCCCACCTTACAGGAAATA-3′ and cloned into the ClaI/NotI sites of the pEasyFlox vector. A loxP-neo-loxP cassette encoding neomycin-resistant gene (neo) was inserted after this 8.5-kb fragment in the NotI/SalI sites of pEasyFlox, and its location in respect to the gene would place it 1300 bp downstream of the transmembrane exon (exon 5) in intron 5. The 3′ homologous arm of the targeting vector, a 4.3-kb DNA fragment containing the exons coding for the three intracellular domains IC1, IC2, and IC3, was generated by PCR using Long Template PCR primers (Roche) using primers 5′-GCCGTCGACAAACAATGTTGCCCCACCTTACAGGAAATA-3′ and 5′-ATAGTCTCTGAGTGTCTCTTACTCTACATCTGTAACAGGAG-3′ and cloned into the ClaI/Sall sites of pEasyFlox. The third loxP site was inserted into the HindIII site in the 5′ homologous arm, 134 bp upstream to the EC1 exon. Transfection of B6 ES cells with the targeting vector and the subsequent selection and screening were performed in The Rockefeller University Gene Targeting Facility. Clones containing the targeted FcγR2b allele (FcγR2bΔneo) were identified by Southern blot analysis of EcoRV-digested genomic DNA with a probe that hybridizes outside of the targeting vector. Based on the design of the targeting vector, a hybridized band of 13.6 kb would identify the WT FcγR2b allele, and a band of 10.5 kb would identify the targeted FcγR2bΔneo allele (Supplemental Figs. 1A, 1B). Positive clones that also contain the loxP site into the HindIII site in the 5′ homologous arm, confirmed by PCR and sequencing, were selected for microinjection into C57BL/6 embryonic, and chimeric male embryos were bred to C57BL/6 females for germline transmissions. The offspring carrying the FcγR2bΔneo allele identified by Southern blot were crossed to B6 mice expressing Cre under the control of the CMV immediate early enhancer chicken β-actin hybrid promoter (22) for the deletion of the FcγR2b sequences between the two distal loxP sites to create the FcγR2b−/− allele. To create the FcγR2bΔNeo allele, ES cells carrying the FcγR2bΔNeo allele were transiently transfected with a Cre-expressing plasmid and screened for deletion of the loxP-flanked neo cassette by PCR specific for the resulting FcγR2bΔ allele using primers pR2loxA, 5′-AATTGCCGCGCGCGGATCCGATATACTCTG-3′ and pR2deleta4.2, 5′-TGCGTCTCAATCCGAGGACCCGATTTTCTACACCCACTTCT-3′ (Supplemental Figs. 1A, 1C). The deletion of the neo cassette was confirmed by two PCR reactions using primers pNeo-F1, 5′-GGCTTCCGCGGCGCACTTTCCGCTTGAGATG-3′ and pR2deleta4.2 or primers pR2loxA and pNeo-R1, 5′-GCCGATGTCGTGTGTTGGTTGCAGCATAG-3′. ES clone 39 carrying the FcγR2bΔ allele was selected for microinjection into C57BL/6 embryos, and chimeric male offspring bred to C57BL/6 females gave successful germline transmissions, which were confirmed by Southern blots and PCR. Mice

WT C57BL/6 mice were purchased from Taconic Farms. Mice carrying FcγR2b−/− or FcγR2bΔ alleles were generated from B6 ES cells (Supplemental Fig. 1). MbiCre/FcγR2bΔNeo, CgCre/FcγR2bΔNeo, CD11cCre/FcγR2bΔNeo, and LysMCre/FcγR2bΔNeo mice were generated by crossing mice carrying FcγR2bΔ alleles to Mb1Cre mice (23), CgCre mice (24), CD11cCre mice (25), and LysMCre mice (26), respectively, which have been backcrossed to the B6 background at least 10 times. In some mice, Mb1Cre and CD11cCre also mediated germline deletion of one FcγR2bΔ allele and gave rise to MbiCre/FcγR2bΔΔ and CD11cCre/FcγR2bΔΔ mice. B6.FcγR2bΔΔ/− (N12) were obtained from Taconic Farms. FcεR1γ−/− FcγR2b−/− mice had been described previously (27). All mice were maintained in The Rockefeller University Comparative Bioscience Center. All experiments were performed in compliance with federal laws and institutional guidelines and had been approved by The Rockefeller University Institutional Animal Care and Use Committee.

Flow cytometry

To analyze the levels of FcγRIIB in WT and mutant mice with germine or conditional knockout of FcγR2b, blood samples and splenic single-cell suspensions were prepared and depleted for erythrocytes and stained with fluorescent-conjugated anti-CD19 (1D3), anti-NK-1.1 (PK136), anti-CD11b (M1/70), anti-CD11c (BHL), anti-Gr1 (RB6-8C5), and biotin-conjugated anti-FcγRIIB (Ly17.2) or mouse IgG2a isotype control (M2A15; Invivogen) Abs in the first step and streptavidin-allophycocyanin in the second step. DAPI was added to exclude dead cells before samples were analyzed using a BD LSRII (BD Biosciences). Acquired data were analyzed using FlowJo (Version 7.05 for Windows; Tree Star). To analyze FcγRIIB expression in the splenic marginal zone and T cells, total splenocytes were stained with fluorescein isothiocyanate-conjugated anti-CD19 (1D3) or IgG-Fc Abs (Jackson ImmunoResearch Laboratories) and tetramethylbenzidine (TMB) substrates.

Analysis of autoimmune phenotypes

Mice were monitored for survival for 10 mo. Proteinuria levels were monitored using Chemstrip 2 GP strips (Roche) monthly, and mice with proteinuria levels ≥100 mg/dl were considered sick. Mice were monitored weekly for 11 wk. To analyze mouse collagen-specific IgG levels, spleen and kidney single-cell suspensions were prepared and stained for fluorescence-activated cell sorting analysis with anti-mouse IgG (H+L) Abs (Bethyl Laboratories) were used as capture Abs, and IgG were detected by HRP-conjugated goat anti-mouse IgG-Fc (1:5000; Jackson ImmunoResearch Laboratories) and TMB substrate. To analyze the levels of total IgM and IgG in sera by ELISA, 100 μl of serum samples were analyzed, and IgG were analyzed by ELISA and IgG were detected with HRP-conjugated goat anti-mouse IgG-Fc (1:5000; Jackson ImmunoResearch Laboratories) and TMB substrate. To analyze the level of IgG antibodies in sera for peptide-specific and anti-mouse Ig H4L Abs (Bethyl Laboratories) were used as capture Abs. Since the majority of patients had anti-nuclear Abs (ANAs) of IgG class, 1:200 diluted serum samples were applied to REAADS ANA Test plates (Corgenix), incubated at room temperature for 1 h, and washed five times with PBS with 0.05% Tween-20. IgG Abs were detected with HRP-conjugated goat anti-mouse IgG-Fc (1:5000; Jackson ImmunoResearch Laboratories) and TMB substrate. To analyze the level of IgG antibodies in sera for peptide-specific and non-peptide-specific Abs, sera were analyzed by ELISA and IgG were detected with HRP-conjugated goat anti-mouse Ig Fc (1:5000; Jackson ImmunoResearch Laboratories) and TMB substrate. C57BL/6 mice were immunized with 100 μg NP26-BSA, 1:106 diluted serum samples were analyzed, and IgG were analyzed by ELISA and IgG were detected with HRP-conjugated goat anti-mouse IgG-Fc (1:5000; Jackson ImmunoResearch Laboratories) and TMB substrate. To analyze the level of IgG antibodies in sera for peptide-specific and non-peptide-specific Abs, sera were analyzed by ELISA and IgG were detected with HRP-conjugated goat anti-mouse Ig Fc (1:5000; Jackson ImmunoResearch Laboratories) and TMB substrate. C57BL/6 mice were immunized with 100 μg NP26-BSA, 1:106 diluted serum samples were analyzed, and IgG were analyzed by ELISA and IgG were detected with HRP-conjugated goat anti-mouse IgG-Fc (1:5000; Jackson ImmunoResearch Laboratories) and TMB substrate. C57BL/6 mice were immunized with 100 μg NP26-BSA, 1:106 diluted serum samples were analyzed, and IgG were analyzed by ELISA and IgG were detected with HRP-conjugated goat anti-mouse IgG-Fc (1:5000; Jackson ImmunoResearch Laboratories) and TMB substrate.

K/BxN arthritis model

Experiments using the K/BxN arthritis model were performed as described previously (29). Mice were injected with 200 μl K/BxN sera i.p. and monitored for the development of arthritis for 4 weeks. The majority of mice (0–12) were recorded as their arthritis clinical scores.
OT-I T cell expansion experiments were performed using a modified protocol (30). Briefly, on day 21, 2 million CFSE-labeled CD45.1+ OT-I T cells enriched by MACS-negative selection were adoptively transferred through i.v. injection into WT, Fcgr2b<sup>−/−</sup>, or Fcgr2b conditional knockout mice with CD11cCre. On day 0, each mouse received 150 µg rabbit anti-OVA IgG through i.v. injection, followed by 2.5 µg OVA 4 h later. The concentration of CD45.1<sup>+</sup>CD8<sup>+</sup>Va<sup>+</sup> cells in blood was analyzed 3 d later by FACS.

Statistics

All statistical analyses were performed in Prism 5 for Windows (version 5.04; GraphPad), one-way ANOVA with Dunnett post hoc test was used in Figs. 2, 4A, and 7 to compare all groups to the WT or Fcgr2b<sup>fl/fl</sup> control group, one-way ANOVA with Tukey post hoc test was used in Fig. 3 and Supplemental Fig. 3, and χ<sup>2</sup> test was used in Figs. 4B, 5, and 6B and Supplemental Fig. 2 to compare every group to the Fcgr2b<sup>fl/fl</sup> control group.

Results

Generation of Fcgr2b germline and conditional knockout mice from B6 ES cells

Fcgr2b germline and conditional knockout mice were generated by crossing a mouse strain with loxP-flanked Fcgr2b alleles (Fcgr2b<sup>fl</sup>) derived from B6 ES cells (Supplemental Fig. 1) to CagCre (22), Mb1Cre (23), Cg1Cre (24), CD11cCre (25), and LysMCre (26) B6 mice. Mb1Cre is expressed in all B cells, whereas Cg1Cre expression is restricted to GC and post-GC B cells. CD11cCre is primarily expressed in DCs, and LysMCre is expressed in most myeloid effector cells. As shown in Fig. 1A, homozygous Fcgr2b<sup>fl/fl</sup> mice have equivalent FcγRIIB expression as WT C57BL/6 mice, confirming that the inserted loxP sites in the Fcgr2b<sup>fl/fl</sup> allele have no effect on the expression of FcγRIIB. Mb1Cre/Fcgr2b<sup>fl/fl</sup> mediated specific and efficient deletion of Fcgr2b in all B cells examined; in contrast, Cg1Cre/Fcgr2b<sup>fl/fl</sup> did not delete Fcgr2b in resting B cells. To determine which B cell populations delete Fcgr2b in Cg1Cre/Fcgr2b<sup>fl/fl</sup> mice, Fcgr2b<sup>fl/fl</sup> mice with Cg1Cre were immunized with NP-CGG in alum and examined for FcγRIIB expression in IgG1<sup>+</sup> and IgG1<sup>+</sup>GC B cells, as defined by B220<sup>+</sup>Fas<sup>+</sup>IgG1<sup>+</sup> and B220<sup>+</sup>Fas<sup>+</sup>IgG1<sup>+</sup>GC B cells, respectively. As shown in Fig. 1B, FcγRIIB expression was reduced in the majority of both IgG1<sup>+</sup> and IgG1<sup>+</sup>GC B cell subsets, consistent with previous studies showing that Cg1Cre expression is restricted to GC and post-GC B cells (24). CD11cCre/Fcgr2b<sup>fl/fl</sup> mediated efficient deletion of Fcgr2b in DCs (CD11c<sup>hi</sup>), as well as in some monocytes (CD11c<sup>int</sup>). LysMCre/Fcgr2b<sup>fl/fl</sup>-mediated
deletion of Fcgr2b was detectable in monocyte and thioglycollate-elicited macrophages (Fig. 1A, 1C), but not in B cells or DCs. However, the deletion efficiency of LysMCre was estimated to be only 20–60%, as has been reported previously for some other genetic systems (31, 32). These data indicate that we have generated B6 mice with a conditional knockout of Fcgr2b in B cells (MbiCre), GC, and post-GC B cells (Cg1Cre), DCs and some monocytes (CD11cCre), and monocytes and macrophages (LysMCre).

FcγRIIB expression in B lineage cells regulates Ab responses

To determine the effect of germline and conditional knockouts of Fcgr2b in different immune cells on the primary and secondary thymic-dependent Ab response, levels of NP-specific IgG were analyzed in mice immunized and boosted with the model Ag NP-CGG. As shown in Fig. 2, WT (Fcgr2b<sup>fl/fl</sup>) and Fcgr2b heterozygous (Fcgr2b<sup>β/−</sup>) mice had comparable primary and secondary IgG responses, whereas these responses in Fcgr2b<sup>−/−</sup> mice were significantly enhanced (<i>p</i> < 0.0001), consistent with previous studies (8, 33). Analyses of Fcgr2b conditional knockout lines showed that only mice with MbiCre have an increase of primary IgG Ab response equivalent to that of Fcgr2b<sup>−/−</sup> mice. Significant increase of secondary IgG responses were observed in both the MbiCre and Cg1Cre lines, whereas CD11cCre- and LysMCre-mediated deletion of Fcgr2b had no significant effect on either primary or secondary IgG responses. These results suggested that the effect of FcγRIIB deficiency on IgG Ab responses is B cell intrinsic, and FcγRIIB functions as a negative regulator in both primary and secondary IgG responses. Although FcγRIIB expressed in resting B cells is important to limit the primary Ab response, FcγRIIB expressed in B lineage cells at the GC or post-GC stages is important to inhibit the secondary response.

FcγRIIB expression in DCs regulates T cell responses

FcγRIIB expression in DCs has been reported to regulate T cell responses, and we confirmed these reports (30). As shown in Fig. 3, in response to OVA immune complexes, significantly enhanced CD8 T cell response was observed in mice with either germline knockout or DC-specific knockout of Fcgr2b, suggesting that FcγRIIB expression in DCs could contribute to the maintenance of tolerance through regulating T cell responses.

Increased spontaneous ANAs in mice with GC/post-GC B cell–specific deletion of FcγRIIB

Because different autoimmune phenotypes have been reported in Fcgr2b knockout mice of different genetic backgrounds in previous studies (5, 8), we analyzed the Fcgr2b<sup>−/−</sup>mice generated from B6 ES cells in this study. FcγRIIB deficiency resulted in modest but significant increases in IgG ANA titers in 10-mo-old B6 mice (<i>p</i> < 0.01), with ~25% displaying relatively high titers of ANAs (Fig. 4A). IgG ANAs were detectable in some Fcgr2b<sup>−/−</sup> mice by immunofluorescence staining of Hep-2 human ECs (Fig. 4B). When Fcgr2b conditional-knockout mice were analyzed for ANA IgG Abs, we found that conditional knockout of Fcgr2b by Cg1Cre in GC/post-GC B cells recapitulated the Fcgr2b germine-knockout phenotype, in contrast to CD11cCre- or LysMCre-mediated conditional knockouts (Fig. 4A), suggesting the FcγRIIB expression in GC or post-GC B cells is responsible for inhibiting the development of spontaneous autoantibodies.

Increased arthritis incidence in mice with B cell– or DC-specific deletion of Fcgr2b on a nonpermissive background

Previous studies have shown that Fcgr2b knockout mice are more susceptible to induced autoimmune diseases (6–8). To study the impact of germline or conditional knockout of Fcgr2b on the maintenance of tolerance, mice were analyzed in a bovine type II CIA (bCIA) model, which is normally nonpermissive in B6 mice because their H-2<sup>b</sup> background does not support sustained T cell and subsequent Ab responses against bovine type II collagen required to initiate and perpetuate bCIA (34–36). We found that FcγRIIB deficiency can sensitize B6 mice in this otherwise resistant model. As shown in Fig. 5, Fcgr2b<sup>−/−</sup> B6 mice are highly susceptible to bCIA (13 of 19; <i>p</i> < 0.0001), in contrast to the resistant WT B6 mice (0 of 23). The contribution of cell-specific FcγRIIB expression to the maintenance of tolerance was evaluated in this model using Fcgr2b conditional-knockout mice. As shown in Fig. 5, 9 of 20 Fcgr2b<sup>β/−</sup> mice with MbiCre-mediated selective deletion of Fcgr2b in B cells developed arthritis (<i>p</i> < 0.001), as did 3 of 10 Fcgr2b<sup>β/−</sup> mice with CD11cCre (<i>p</i> < 0.01). As heterozygous Fcgr2b mice (Fcgr2b<sup>β/−</sup>) generally did not develop arthritis, although a trend toward disease is suggested (2 of 17 mice, not statistically significant different from WT mice; Supplemental Fig. 2), heterozygous conditional-knockout mice with MbiCre or CD11cCre were also analyzed, and similar results were obtained (Supplemental Fig. 2). In contrast, Fcgr2b<sup>β/−</sup> mice were immunized with NP-CGG in CFA on day 0 and boosted with NP-CGG in incomplete adjuvant on day 28 (indicated by arrows). NP-specific IgG levels were analyzed on days 0, 14, and 42 by ELISA and presented as O.D. values (mean ± SD). Representative of two independent experiments (five mice per group). ***<i>p</i> < 0.001, ANOVA with Dunnett post hoc.

**FIGURE 2.** T-dependent IgG responses in WT and mutant mice with germine, partial, or conditional knockout of Fcgr2b. Mice were immunized with NP-CGG in CFA on day 0 and boosted with NP-CGG in incomplete adjuvant on day 28 (indicated by arrows). NP-specific IgG levels were analyzed on days 0, 14, and 42 by ELISA and presented as O.D. values (mean ± SD). Representative of two independent experiments (five mice per group). ***<i>p</i> < 0.001, ANOVA with Dunnett post hoc.

**FIGURE 3.** Immune complex–induced CD8 T cell responses in WT and mutant mice with germine or conditional knockout of Fcgr2b. The expansion of OT-I T cells in mice of the indicated genotypes in response to OVA immune complex was analyzed in blood 3 d after immunization and presented as OT-I T cell concentrations. Symbols represent values from individual mice, and horizontal lines represent the means. Representative of two independent experiments (five to six mice per group). *<i>p</i> < 0.05, **<i>p</i> < 0.01, ***<i>p</i> < 0.001, ANOVA with Tukey post hoc.
FIGURE 4. Spontaneous ANAs in WT and mutant mice with germline or conditional knockout of Fcgr2b. (A) Levels of ANAs of IgG classes in 10-mo-old WT and mutant mice with germline or conditional knockout of Fcgr2b (16–27 mice/group) were analyzed by ELISA and presented as O.D. values (symbols represent O.D. values of individual mice, and thick horizontal lines represent the means). (B) Increased ANA levels in some Fcgr2b−/− mice. Hep-2 human ECs were stained with 1:100 diluted sera from 8- to 9-mo-old WT and Fcgr2b−/− mice, followed by FITC-conjugated goat anti-mouse IgG. IgG ANAs were detected in about 30% of WT mice were positive in this analysis (p < 0.05, χ² test). Original magnification ×100. **p < 0.01, ***p < 0.001, ****p < 0.0001, χ² test (versus the Fcgr2b+/+ mice).

with LysMCre did not develop statistically significant disease, excluding the contribution of CD11cCre activity in monotypes to the phenotype observed in Fcgr2b+/− mice with CD11cCre (Figs. 1A, 5). The significantly increased bCIA incidence in Fcgr2b conditional-knockout mice with either Mb1Cre or CD11cCre demonstrated that multiple cell compartments, including B cells and DCs, are involved in the development of CIA, and the regulation of these cells by FcγRIIB is critical to the maintenance of tolerance.

Increased arthritis severity in mice with GC/post-GC B cell–specific deletion of Fcgr2b on a permissive background

We also studied the impact of germline and conditional knockout of Fcgr2b on the maintenance of tolerance in the chicken type II CIA (cCIA) model. In contrast to the bCIA model, cCIA is permissive in B6 mice because a robust and sustained T cell response can be mounted against chicken type II collagen (34–36). As shown in Fig. 6A and 6B, whereas WT mice developed only mild arthritis, Fcgr2b−/− mice developed significantly more severe arthritis, consistent with previous reports (10). Analysis of conditional-knockout lines in this model showed that selective deletion of Fcgr2b in GC/post-GC B cells is sufficient to recapitulate the exacerbated arthritis phenotype in Fcgr2b−/− mice (Fig. 6A, 6B), which is in sharp contrast to the resistance of Fcgr2b+/− mice with Cg1Cre to bCIA (Fig. 5). Therefore, FcγRIIB expression in GC and/or post-GC B cells plays an important role in inhibiting autoimmunity in permissive models, but not in nonpermissive models.

Exacerbated arthritis in mice with selective deletion of Fcgr2b in myeloid effector cells in response to adoptively transferred arthritic sera

In previous studies, FcγRIIB has been shown to play an important role in modulating Ab-mediated effector functions by setting thresholds for immune complex activation of myeloid effector cells, which has been hypothesized to contribute to the maintenance of tolerance (8). We tested this hypothesis in a passive autoimmune model in which K/BxN autoreactive sera are adoptively transferred into mice with germline deletion of Fcgr2b or conditional deletion of Fcgr2b in myeloid effector cells by LysMCre. As shown in Fig. 7, administration of K/BxN sera leads to the development of arthritis in WT, but not FcγR-deficient (FcεRIg−/− Fcgr2b−/−) mice, whereas in Fcgr2b−/− mice, the development of arthritis was accelerated and exacerbated, consistent with the notion that although activating FcγRs is required for the development of K/BxN serum-induced arthritis, FcγRIIB negatively regulates Ab-triggered inflammation. LysMCre-mediated deletion of Fcgr2b, although not complete (Fig. 1A, 1C), resulted in near-total recapitulation of the effect of Fcgr2b germine deletion (Fig. 7), suggesting that the myeloid effector cells responsible for joint inflammation are very sensitive to Fcgr2b levels in this passive autoantibody transfer model of inflammation.

FIGURE 5. Susceptibilities of mice with germline or conditional knock-out of Fcgr2b to bCIA. Accumulative bCIA incidences in WT (Fcgr2b+/+) and the indicated mutant male mice with germline or conditional knockout of Fcgr2b are presented. n values are the numbers of mice in each group. Data are combined from three independent experiments with similar results. **p < 0.01, ****p < 0.0001, χ² test (versus the Fcgr2b+/+ mice).

FIGURE 6. Spontaneous ANAs in WT and mutant mice with germline or conditional knockout of Fcgr2b. (A) Levels of ANAs of IgG classes in 10-mo-old WT and mutant mice with germline or conditional knockout of Fcgr2b (16–27 mice/group) were analyzed by ELISA and presented as O.D. values (symbols represent O.D. values of individual mice, and thick horizontal lines represent the means). (B) Increased ANA levels in some Fcgr2b−/− mice. Hep-2 human ECs were stained with 1:100 diluted sera from 8- to 9-mo-old WT and Fcgr2b−/− mice, followed by FITC-conjugated goat anti-mouse IgG. IgG ANAs were detected in about 30% of WT mice were positive in this analysis (p < 0.05, χ² test). Original magnification ×100. **p < 0.01, ***p < 0.001, ****p < 0.0001, χ² test (versus the Fcgr2b+/+ mice).

Exacerbated arthritis in mice with selective deletion of Fcgr2b in myeloid effector cells in response to adoptively transferred arthritic sera

In previous studies, FcγRIIB has been shown to play an important role in modulating Ab-mediated effector functions by setting thresholds for immune complex activation of myeloid effector cells, which has been hypothesized to contribute to the maintenance of tolerance (8). We tested this hypothesis in a passive autoimmune model in which K/BxN autoreactive sera are adoptively transferred into mice with germline deletion of Fcgr2b or conditional deletion of Fcgr2b in myeloid effector cells by LysMCre. As shown in Fig. 7, administration of K/BxN sera leads to the development of arthritis in WT, but not FcγR-deficient (FcεRIg−/− Fcgr2b−/−) mice, whereas in Fcgr2b−/− mice, the development of arthritis was accelerated and exacerbated, consistent with the notion that although activating FcγRs is required for the development of K/BxN serum-induced arthritis, FcγRIIB negatively regulates Ab-triggered inflammation. LysMCre-mediated deletion of Fcgr2b, although not complete (Fig. 1A, 1C), resulted in near-total recapitulation of the effect of Fcgr2b germine deletion (Fig. 7), suggesting that the myeloid effector cells responsible for joint inflammation are very sensitive to Fcgr2b levels in this passive autoantibody transfer model of inflammation.
The development of autoimmunity has been studied in several FcγRIIB-deficient mouse models, initially in *FcgRIIB*−/− mice derived from 129/Sv ES cells and backcrossed to either the B6 (B6.FcγRIIB−/−) or BALB/c background (5), and more recently in FcγRIIB−/− mice derived from B6 ES cells (8). The FcγRIIB−/− mice we independently generated from B6 ES cells (FcgRIIB−/−) showed significantly attenuated lupus-like phenotypes (proteinuria and premature mortality) as compared with the backcrossed mice derived from B6 ES cells (FcgRIIB−/−). This is also consistent with the finding that in addition to FcγRIIB deficiency, the 129/Sv-derived Sle16 locus may be involved in the autoimmune phenotype in B6.FcγRIIB−/− mice based on the analysis of B6.FcγRIIB−/− mice with different lengths of 129/Sv DNA segments around the targeted FcgRIIB gene in a spontaneous arthritis model and an induced tolerance model (37, 38). Although these studies supported the conclusion from the early studies that FcγRIIB is an epistatic modifier of autoimmunity, it also demonstrated additional susceptibility factors contributed by 129/Sv genes may have contributed to the severe proteinuria and premature mortality phenotypes observed in B6.FcγRIIB−/− mice (5). At the same time, we also observed several autoimmune phenotypes in FcgRIIB−/− mice, including the increased spontaneous ANA levels and susceptibility to bCIA or cCIA. These results, together with the previously reported moderate glomerulonephritis phenotype and increased incidence of anti–glomerular basement membrane disease in FcγRIIB−/− mice derived from B6 ES cells (8, 39), confirmed that in B6 mice, FcγRIIB plays an important role in the maintenance of tolerance.

FcγRIIB is the most widely expressed of all FcγRs and found on essentially all lymphoid and myeloid subsets, with the exception of T and NK cells. This wide expression pattern has made the assignment of specific phenotypes of FcγRIIB-deficient mice to defined cellular populations difficult. The collection of FcγRIIB conditional-knockout strains generated in this study has provided us an opportunity to dissect the contribution of cell-specific FcγRIIB expression to a long list of FcγRIIB functions proposed based on the studies using FcgRIIB germline-knockout mice. In this study, we focused on the function of cell-specific FcγRIIB and its impact on the maintenance of tolerance. It has been hypothesized in a recent study that the contribution of FcγRIIB deficiency to autoimmunity is mainly through the regulation of Ab effector pathways, such as immune complex–mediated inflammation (5).

This seems to be true in models that involve adoptive transfer of autoimmune Abs, such as the serum transfer K/BxN arthritis model in this study or the NTN model used in a previous study (40), as immune complex–mediated inflammation was enhanced in mice with selective deletion of FcgRIIB in myeloid effector cells by LysMCre or CEBPαCre. However, our analysis of FcgRIIB−/− mice with LysMCre in CIA models, in which autoreactive Abs are actively induced, does not support this hypothesis, as these mice are not more susceptible than WT mice to either bCIA or cCIA (data not shown). In contrast, we demonstrated that FcγRIIB expression in both B cells and DCs is important for the maintenance of tolerance in the bCIA model and that the contribution of B cell and dendritic FcγRIIB expression to the maintenance of tolerance might be based on different mechanisms.

We found that the effect of FcγRIIB deficiency on IgG Ab responses is B cell intrinsic, and FcγRIIB functions as a negative regulator in both primary and secondary IgG responses, suggesting that the activation of both resting B cells and memory B cells, in the primary and secondary responses, respectively, are both regulated by FcγRIIB. The deletion of FcgRIIB by B cell–specific Mb1Cre leads to significantly enhanced primary and secondary Ab responses, and the deletion of FcgRIIB by GC and post-GC B cell–specific Cg1Cre specifically enhanced the secondary Ab response, consistent with the timing when these Cre become active. FcγRIIB deficiency may contribute to increased Ab response by promoting B cell activation during the early stage and plasma cell survival during the late stage of B cell differentiation (3, 4, 33). The increased Ab responses in FcgRIIB−/− mice with Mb1Cre or Cg1Cre could contribute to the increased autoimmune phenotypes. These studies are consistent with the previous study showing that overexpression of a B cell–specific FcgRIIB transgene suppressed T-dependent IgG responses, spontaneous lupus, and chicken CIA phenotypes (41) and a more recent study showing that FcγRIIB expression from the autoimmunity-associated polymorphic allele was specifically reduced in GC B cells and resulted in

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**FIGURE 6.** Susceptibilities of mice with germline or conditional knockout of FcγRIIB to the cCIA. (A) The development of cCIA in WT (FcγRIIB−/−) and the indicated mutant mice with germline or conditional knockout of FcγRIIB (11 to 12 mice/group), expressed as average arthritis clinical scores (mean ± SEM), is presented. (B) The distribution of the maximum arthritis clinical scores observed in the mice in (A) is presented. The p values were calculated by χ² test.

**FIGURE 7.** Accelerated development of K/BxN serum-induced arthritis in FcγRIIB−/− mice with LysMCre. WT, FcγRIIB−/−, and FcγRIIB−/− mice with LysMCre and FcγR-deficient (Fcer1g−/− FcγRIIB−/−) mice were treated with K/BxN sera and monitored for the development of arthritis. Arthritis clinical scores (mean ± SD) are presented. Representative of two independent experiments. *p < 0.05, **p < 0.001, ANOVA with Dunnett post hoc comparing each group to the WT group.
a number of autoimmune phenotypes, including the development of more severe chicken CIA (10).

Interestingly, selective deletion of FcγRIIb by Mb1Cre and Cg1Cre, respectively, resulted in different autoimmune phenotypes. Although hypersensitive to cCIA, FcγRIIb<sup>−/−</sup> mice with Cg1Cre are not susceptible to bCIA, in contrast to FcγRIIb<sup>−/−</sup> mice with Mb1Cre. Although this could be due to the different impact of Mb1Cre- and Cg1Cre-mediated FcγR IIb deletion on immune responses, it might be also related to the difference in these two different arthritis models. Although both Cia1 models require robust T cell and Ab responses to initiate and perpetuate arthritis, the H<sup>2</sup>B<sup>−</sup>B<sup>−</sup> background of B6 mice only support such responses against chicken, not bovine type II collagen (34–36). The fact that conditional knockout of FcγR IIb in APCs (DCs and B cells) resulted in the break of tolerance in the bCIA model suggests that these conditional knockouts of FcγR IIb might result in enhanced Ag presentation, which may lead to the observed increase in T cell and primary Ab responses in these mice and autoimmunity. In contrast, Cg1Cre-mediated deletion of FcγR IIb after B cell activation only results in increased secondary Ab response that is sufficient to enhance autoimmune response in the permissive cCIA model in which T cell tolerance is already broken. This notion is supported by increased anti-mouse type II collagen IgG levels in FcγR IIb<sup>−/−</sup> mice with Cg1Cre (Supplemental Fig. 3).

We also confirmed previous studies showing that FcγRIIB expression in DCs can inhibit T cell response (42), presumably by regulating DC maturation and Ag presentation. This is consistent with other studies showing that selective blockade of FcγRIIB can promote DC maturation and T cell responses (43–45). Previous studies using B6.FcγR IIb<sup>−/−</sup> mice in the experimental autoimmune encephalomyelitis model suggested the impact of FcγRIIB expression on T cell response could contribute to the maintenance of tolerance (46). Our study, together with that of van Montfoort et al. (30), established that the increased T cell response in FcγRIIB-deficient B6 mice may contribute to autoimmunity. In addition, FcγRIIB has been previously shown to set thresholds for immune complex–triggered inflammation in a number of animal models presumably by regulating myeloid effector cells. In this study, partial deletion of FcγRIIB on myeloid effector cells leads to significantly exacerbated arthritis triggered by K/BxN sera, suggesting that myeloid effector cells are very sensitive to the regulation by FcγRIIB levels, in agreement with our finding that increased FcγRIIB expression in myeloid effector cells in response to IVIG is responsible for its significant anti-inflammatory effects in vivo (29, 47, 48).

Our data also showed that quantitative changes in immune responses due to selective deletion of FcγR IIb can result in significant differences in autoimmune models. In corroboration with this notion, previous studies have shown a quantitative increase in TLR7 expression due to gene duplication can accelerate the development of autoimmune diseases (49, 50). These findings suggest that the maintenance of tolerance involves many checkpoints that do not qualitatively but quantitatively regulate immune system at various levels, highlighting the importance of the balance in immunoregulatory networks.

Taken together, through the analysis of a collection of novel FcγRIIB conditional-knockout strains with specific deletion of FcγR IIb in defined cellular compartments, we demonstrated that FcγRIIB expression in multiple cellular compartments is required for the maintenance of peripheral tolerance through different mechanisms, and FcγRIIB expression in the same cell lineage (B cells) but at different differentiation stages also has a different impact on the maintenance of tolerance. This collection of FcγRIIB conditional-knockout strains is likely useful to investigate other functions assigned to FcγRIIB. For instance, FcγRIIB coengagement has been recently found to be necessary for the in vivo activities of agonistic Abs to the TNFR family members, such as CD40 and DR3 (51, 52), and these conditional FcγR IIb knockout mice might be also useful to dissect the contribution of cell-specific FcγRIIB to the activities of these Abs.

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Disclosures

The authors have no financial conflicts of interest.

References


