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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 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 II 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, BXS, and NOD, displayed protective 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 number 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, MDA5, 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 Fcγr2bfl/fl alleles

To generate Fcγr2b germline 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′-CTCTCTGCAAGGTCACT-3′

and

5′-ATATTCTTCTGGCACCCTTGTTCAGACTGTTGAAACTG-3′

and cloned into the SalI/XhoI sites of pEasyFlox. The third loxP site was inserted into the homologous arm, 134 bp upstream to the EC1 exon. Transfection of B6 ES cells with the targeting vector and the sub-sequences between the two distal loxP sites to create the Fcγr2bfl allele (Supplemental Figs. 1A, 1B) was 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, hybridized bands of 13.6 kb and 9 kb were identified by PCR and sequencing) were selected for microinjection into C57BL/6 embryos, and chimeric male offspring were bred to C57BL/6 females (Supplemental Figs. 1A, 1C). The deletion of the Fcγr2bfl 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 blot analysis of genomic DNA with a probe that hybridizes outside of the targeting vector.

Mice

WT C57BL/6 mice were purchased from Taconic Farms. Mice carrying Fcγr2bfl/fl or Fcγr2bfl/fl alleles were generated from B6 ES cells (Supplemental Fig. 1). Mb1Cre/Fcγr2bfl/fl, CgCre/Fcγr2bfl/fl, CD11cCre/Fcγr2bfl/fl, and LysMCre/Fcγr2bfl/fl mice were generated by crossing mice carrying Fcγr2bfl/fl 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 Fcγr2bfl allele and gave rise to Mb1Cre/Fcγr2bfl/fl and CD11cCre/Fcγr2bfl/fl mice. B6.Fcγr2b fl/fl (N12) were obtained from Taconic Farms. Fc γr2b fl/fl 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 germline or conditional knockout of Fcγr2b, blood cells and splenic single-cell suspensions were prepared and depleted for erythrocytes and stained with fluorescent-conjugated anti-CD19 (1D3), anti-CD11b (M1/70), anti-CD11c (BL3), anti-Gr1 (RB6-8C5), and biotin-conjugated anti-FcγRIIB (Ly17.2) or mouse IgG2a isotype control (MG2a15; Invitrogen) 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 lymphoid and myeloid lineages, spleens were prepared by digestion with cold PBS and stained with fluorescein-conjugated anti-FcγRIIB mAb (B8/8a8) and biotinylated anti-mouse IgG1 mAb (A1G11). CD11b+ myeloid populations were analyzed using a BD FACSCanto II (BD Biosciences).

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. To analyze levels of anti-nuclear Abs (ANAs) of IgG class, 1:200 diluted serum samples were applied to READAS 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 (1:1,000; Jackson ImmunoResearch Laboratories) or tetramethylbenzidine (TMB) substrates.

Analysis of immune systems that failed to maintain tolerance and developed anti-DNA Abs (21).

RIIB CONTRIBUTES TO TOLERANCE THROUGH DISTINCT MECHANISMS

Based on the design of the targeting vector, hybridized bands of 13.6 kb and 9 kb were identified by PCR and sequencing) were 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 blot analysis of genomic DNA with a probe that hybridizes outside of the targeting vector. Based on the design of the targeting vector, hybridized bands of 13.6 kb and 9 kb were identified by PCR and sequencing) were 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 blot analysis of genomic DNA with a probe that hybridizes outside of the targeting vector. Based on the design of the targeting vector, hybridized bands of 13.6 kb and 9 kb were identified by PCR and sequencing) were 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 blot analysis of genomic DNA with a probe that hybridizes outside of the targeting vector. Based on the design of the targeting vector, hybridized bands of 13.6 kb and 9 kb were identified by PCR and sequencing) were 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 blot analysis of genomic DNA with a probe that hybridizes outside of the targeting vector. Based on the design of the targeting vector, hybridized bands of 13.6 kb and 9 kb were identified by PCR and sequencing) were 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 blot analysis of genomic DNA with a probe that hybridizes outside of the targeting vector. Based on the design of the targeting vector, hybridized bands of 13.6 kb and 9 kb were identified by PCR and sequencing) were 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 blot analysis of genomic DNA with a probe that hybridizes outside of the targeting vector. Based on the design of the targeting vector, hybridized bands of 13.6 kb and 9 kb were identified by PCR and sequencing) were 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 blot analysis of genomic DNA with a probe that hybridizes outside of the targeting vector. Based on the design of the targeting vector, hybridized bands of 13.6 kb and 9 kb were identified by PCR and sequencing) were 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 blot analysis of genomic DNA with a probe that hybridizes outside of the targeting vector. Based on the design of the targeting vector, hybridized bands of 13.6 kb and 9 kb were identified by PCR and sequencing) were 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 blot analysis of genomic DNA with a probe that hybridizes outside of the targeting vector. Based on the design of the targeting vector, hybridized bands of 13.6 kb and 9 kb were identified by PCR and sequencing) were 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 blot analysis of genomic DNA with a probe that hybridizes outside of the targeting vector. Based on the design of the targeting vector, hybridized bands of 13.6 kb and 9 kb were identified by PCR and sequencing) were 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 blot analysis of genomic DNA with a probe that hybridizes outside of the targeting vector.
OT-I T cell expansion

OT-I T cell expansion experiments were performed using a modified protocol (30). Briefly, on day −1, 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>Vα2<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 FcyRIIB 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 FcyRIIB. 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 FcyRIIB 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> cells, respectively. As shown in Fig. 1B, FcyRIIB expression was restored 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>high</sup>), as well as in some monocytes (CD11c<sup>int</sup>), LysMCre/Fcgr2b<sup>fl/fl</sup>-mediated...

FIGURE 1. Expression profiles of FcyRIIB in WT and mutant mice with germline or conditional knockout of Fcgr2b. (A) Histogram profiles showing the expression of FcyRIIB in the indicated cell types of the indicated mice. FcyRIIB levels were analyzed in B cells (CD19<sup>+</sup>), monocytes (CD11b<sup>+</sup>NK1.1<sup>−</sup>Gr1<sup>−</sup>/Gr2<sup>−</sup>SSC<sup>−</sup>), and neutrophils (CD11b<sup>+</sup>NK1.1<sup>−</sup>Gr1<sup>−</sup>/Gr2<sup>−</sup>SSC<sup>−</sup>) in the peripheral blood and DCs (CD11c<sup>high</sup>) in the spleen in WT C57BL/6 mice and mice with germline or conditional knockout of Fcgr2b. (B) The gating strategy for non-GC B cells and IgG1<sup>+</sup> and IgG1<sup>−</sup> GC B cells and histogram profiles showing Cg1Cre-mediated deletion of Fcgr2b in these cells. Mice with the indicated genotypes were treated with NP-CGG in alum and analyzed 12 d later for the expression of FcyRIIB in splenic non-GC B cells (B220<sup>+</sup>Fas<sup>−</sup>) and IgG1<sup>+</sup> and IgG1<sup>+</sup> GC B cells, and histogram profiles showing Cg1Cre-mediated deletion of Fcgr2b in these cells. Mice with the indicated genotypes were treated with NP-CGG in alum and analyzed 12 d later for the expression of FcyRIIB in splenic non-GC B cells (B220<sup>+</sup>Fas<sup>−</sup>) and IgG1<sup>+</sup> and IgG1<sup>+</sup> GC B cells (B220<sup>+</sup>Fas<sup>−</sup>IgG1<sup>+</sup> and B220<sup>+</sup>Fas<sup>−</sup>IgG1<sup>+</sup>, respectively). (C) Histogram profiles showing FcyRIIB levels in thioglycollate-elicited macrophages (CD11b<sup>+</sup>F4/80<sup>+</sup>) isolated from mice of the indicated genotypes. Representative of two independent experiments with three mice per group.
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 (Mb1Cre), 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>b<sup>+/−</sup></sup>) mice had comparable primary and secondary IgG responses, whereas these responses in Fcgr2b<sup>b<sup>−/−</sup></sup> mice were significantly enhanced (p < 0.001), consistent with previous studies (8, 33). Analyses of Fcgr2b conditional knockout lines showed that only mice with Mb1Cre have an increase of primary IgG Ab response equivalent to that of Fcgr2b<sup>b<sup>−/−</sup></sup> mice. Significant increase of secondary IgG responses were observed in both the Mb1Cre 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 response, 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>b<sup>−/−</sup></sup>-knockout 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 (p < 0.01), with ~25% displaying relatively high titers of ANAs (Fig. 4A). IgG ANAs were detectable in some Fcgr2b<sup>b<sup>−/−</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 germline-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 FcγRIIB 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>b<sup>−/−</sup></sup> B6 mice are highly susceptible to bCIA (13 of 19; p < 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>b<sup>−/−</sup></sup> mice with Mb1Cre-mediated selective deletion of Fcgr2b in B cells developed arthritis (p < 0.001), as did 3 of 10 Fcgr2b<sup>b<sup>−/−</sup></sup> mice with CD11cCre (p < 0.01). As heterozygous Fcgr2b mice (Fcgr2b<sup>b<sup>+/−</sup></sup>) mice 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 Mb1Cre or CD11cCre were also analyzed, and similar results were obtained (Supplemental Fig. 2). In contrast, Fcgr2b<sup>b<sup>−/−</sup></sup> mice

**FIGURE 2.** T-dependent IgG responses in WT and mutant mice with germline, partial, or conditional knockout of FcγRIIB. 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). ***p < 0.001, ANOVA with Dunnett post hoc.

**FIGURE 3.** Immune complex–induced CD8 T cell responses in WT and mutant mice with germline or conditional knockout of FcγRIIB. 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). *p < 0.05, **p < 0.01, ***p < 0.001, ANOVA with Tukey post hoc.
with LysMCre did not develop statistically significant disease, excluding the contribution of CD11cCre activity in monocytes to the phenotype observed in Fcgr2bfl/fl 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. 


to the phenotype observed 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 Fcgr2bfl/fl 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 (FcγR1g−/− 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 germline 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 autoimmune diseases and the role of the FcγRIIB have been extensively studied in mouse models. The FcγRIIB is a key regulator in the maintenance of tolerance, particularly in B cells and dendritic cells.

**Discussion**

The development of autoimmune diseases has been studied in several FcγRIIB-deficient mouse models. Initially, in Fcγ2b−/− mice derived from 129/Sv ES cells and backcrossed to either the B6 (B6.Fcγ2b129−/−) or BALB/c background (5), and more recently in Fcγ2b−/− mice derived from B6 ES cells (8). The Fcγ2b−/− mice independently generated from B6 ES cells (Fcγ2bB6+129−/−) showed significantly attenuated lupus-like phenotypes (proteinuria) as compared with the backcrossed B6. Fcγ2b129−/− (N12) mice (Supplemental Table I), consistent with the report of Bolland and Revetch et al. (5). 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.Fcγ2b129−/− mice based on the analysis of B6. Fcγ2b129−/− mice with different lengths of 129/Sv DNA segments around the targeted Fcγ2b gene in a spontaneous arthritis model and an induced tolerance model (37, 38). Although these studies supported the conclusion from the early studies that Fcγ2b 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.Fcγ2b129−/− mice at the same time, we also observed several autoimmune phenotypes in Fcγ2bB6+−/− 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 diseases in Fcγ2b−/− 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 Fcγ2b-deficient mice to defined cellular populations difficult. The collection of Fcγ2b 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 Fcγ2b 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 Fcγ2b in myeloid effector cells by LysMCre or CEBPαCre. However, our analysis of Fcγ2bB6+−/− 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 Fcγ2b by B cell–specific Mb1Cre leads to significantly enhanced primary and secondary Ab responses, and the deletion of Fcγ2b by GC and post-GC B cell–specific Cg1Cre specifically enhanced the secondary Ab response, consistent with the timing when these Cres 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 Fcγ2bB6+−/− 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 Fcγ2b 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

**FIGURE 6.** Susceptibilities of mice with germline or conditional knockout of Fcγ2b to the cCIA. (A) The development of cCIA in WT (Fcγ2bB6+−/−) and the indicated mutant mice with germline or conditional knockout of Fcγ2b (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 Fcγ2bB6+−/− mice with LysMCre. WT, Fcγ2bB6+−/−, and Fcγ2bB6+−/− mice with LysMCre and FcγR-deficient (Fcε1g−/−, Fcγ2b−/−) mice were treated with K/BxN sera and monitored for the development of arthritis. Arthritis clinical scores (mean ± 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 Fcgr2b by Mb1Cre and Cg1Cre, respectively, resulted in different autoimmune phenotypes. Although hypersensitive to CIA, Fcgr2b<sup>fl/fl</sup> mice with Cg1Cre are not susceptible to bCIA, in contrast to Fcgr2b<sup>fl/fl</sup> mice with Mb1Cre. Although this could be due to the different impact of Mb1Cre- and Cg1Cre-mediated Fcgr2b deletion on immune responses, it might be also related to the difference in these two different arthritis models. Although both CIA models require robust T cell and Ab responses to initiate and perpetuate arthritis, the H<sup>2</sup>B<sup>6</sup> background of B6 mice only support such responses against chicken, not bovine type II collagen (34–36). The fact that conditional knockout of Fcgr2b in APCs (DCs and B cells) resulted in the break of tolerance in the bCIA model suggests that these conditional knockouts of Fcgr2b 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 Fcgr2b after B cell activation only results in increased secondary Ab response that is sufficient to enhance autoimmune response in the permissive CIA model in which T cell tolerance is already broken. This notion is supported by increased anti-mouse type II collagen IgG levels in Fcgr2b<sup>fl/fl</sup> mice with Cg1Cre (Supplemental Fig. 5).

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 FcγR can promote DC maturation and T cell responses (43–45). Previous studies with B<sub>6</sub> Fcgr2b<sup>b29</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 contributes 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 FcγRIIB can result in significant difference 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 FcγR2b 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 DR5 (51, 52), and these conditional FcγR2b knockout mice might also be 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.

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Supplementary Figure 1 Generation of Fcgr2b<sup>fl</sup> and Fcgr2b<sup>b<</sup> mice. (A) Strategy used to generate mice with loxP-flanked Fcgr2b alleles (Fcgr2b<sup>fl</sup>). Shown are schematic maps of the wild-type Fcgr2b allele, targeting vector, targeted alleles before (Fcgr2b<sup>fl/Neo</sup>) and after (Fcgr2b<sup>b</sup>) the loxP-flanked neomycin-resistant gene (neo) cassette is deleted by Cre, as well as the Fcgr2b<sup>b</sup> allele. The exons of the Fcgr2b gene (S1, S2, EC1, EC2, TM, IC1, IC2, IC3) are shown as vertical bars; grey triangles represent loxP sites; wide empty arrow labeled with “NEO” is the neomycin-resistant gene; vertical lines labeled as “RV” represent EcoR V restriction sites and the relevant EcoR V fragments are shown using dashed line labeled with the sizes; spiky circles represent probe for Southern blot; the sites of two forward PCR primers (pR2floxA and pNeo-cF1) and two reverse primers (pNeo-R1 and pR2delta4.2) are shown as bended arrows. The targeting vector is designed to generate a targeted Fcgr2b allele (Fcgr2b<sup>fl/Neo</sup>) with a loxP site inserted between the S1 and EC1 exons, and a loxP flanked neo cassette between the TM and IC1 exons after the desired recombination between the targeting vector and the WT Fcgr2b allele in ES cells. The loxP-flanked neo cassette can be deleted by Cre in ES cells to generate the Fcgr2b<sup>fl</sup> allele that has two loxP sites flanking the region between the S2 and TM exons. The region between the two distal loxP sites can be deleted by Cre to generate the Fcgr2b<sup>b</sup> allele. (B) Identification of ES clones containing the Fcgr2b<sup>fl/Neo</sup> targeted allele (after the endogenous WT Fcgr2b gene recombines with the targeting vector) by Southern blot using EcoR V digested ES cell genomic DNA the probe shown in (A). One of the positive clones, #231 was picked for the downstream experiments. (C) Identification of ES clones with the Fcgr2b<sup>fl</sup> allele (after ES clone #231 was transiently transfected with a Cre-expression construct) by PCR using primers pR2floxA and pRdelta4.2, which generate a 0.5 kb product specific from the Fcgr2b<sup>fl</sup> allele, and a 2.5kb product from the Fcgr2b<sup>fl/Neo</sup> allele, and no product from other alleles. (D) Confirmation of the deletion of the neo cassette by PCR using the indicated primers. Among several clones that are positive for the Fcgr2b<sup>fl</sup> allele and the deletion of neo cassette, #39 was picked for further development of mice carrying the Fcgr2b<sup>fl</sup> allele.
Supplementary Figure 2 Susceptibilities of mice with germline or conditional knockout of Fcgr2b to bovine type II collagen induced arthritis (bCIA). Accumulative bCIA incidences in WT (Fcgr2b<sup>fl/fl</sup>) and the indicated mutant male mice with germline or conditional knockout of Fcgr2b on the heterozygous Fcgr2b<sup>fl</sup> background are presented. “n” values are the numbers of mice in each group. *** p < 0.001, **** p < 0.0001, Chi-square test (vs the “Fcgr2b<sup>fl/fl</sup>” mice).
Supplementary Figure 3. Mouse type II collagen-specific IgG responses in mice with germline or conditional knockout of Fcgr2b in the chicken type II collagen induced arthritis (cCIA) model. Fcgr2b^{fl/fl}, Fcgr2b^{-/-}, and Fcgr2b^{fl/fl}Cg1Cre^{+} mice were treated with chicken type II collagen in adjuvant to induce arthritis. Levels of mouse type II collagen-specific IgG were analyzed two months later by ELISA and presented as O.D. values (mean±s.d. and individual values plotted). *** p < 0.001, ANOVA with Tukey’s post hoc (additional statistical test results: “Fcgr2b^{-/-}” vs “Fcgr2b^{fl/fl}”, p < 0.05 in t-test; “Fcgr2b^{fl/fl}, Cg1Cre^{+}” vs “Fcgr2b^{fl/fl}”, p < 0.01 in F-test).
**Supplementary table**: premature mortality and proteinuria phenotypes in FcγRIIB-deficient mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Age (month)</th>
<th>Total number of mice</th>
<th>Mortality</th>
<th>Sick&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>18</td>
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<sup>a</sup>“sick” is define as proteinuria levels 100 mg/dL or above.