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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 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, BXSB, 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 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 exacerbated 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 Fcgr2b+ and Fcgr2bΔ alleles

To generate Fcgr2b germline and conditional knockout mice from B6 ES cells, two homologous arms cloned from the Fcgr2b 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′-AGGATCCAGAATCTCAGTGAGCCTTG-3′ and 5′-ATATGTCGACTGGCGGCTTTTCG-3′ and cloned into the SphI/XhoI sites of pEasyFlox. The third loxP site was inserted to create the Fc gr2bΔ allele, 134 bp upstream to the EC1 domain. Transfection of B6 ES cells with the targeting vector and the sub-selected 5′-homologous arm, 134 bp upstream to the EC1 exon. Transfection of B6 ES cells with the targeting vector and the sub-selected sequence and screening were performed in The Rockefeller University Gene Targeting Facility. Clones containing the targeted Fcgr2b allele (Fcgr2bΔneo) 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 Fcgr2b allele, and a band of 10.5 kb would identify the targeted Fcgr2bΔ allele (Supplemental Figs. 1A, 1B). Positive clones that contained the locus inserted into the HindIII site in the 5′ homologous arm (confirmed by PCR and sequencing) were selected for microinjection into C57BL/6 embryos, and chimeric male offspring were bred to C57BL/6 females for germline transmissions, which were confirmed by Southern blots and PCR.

Mice

WT C57BL/6 mice were purchased from Taconic Farms. Mice carrying Fcgr2bΔ or Fcgr2bΔ alleles were generated from B6 ES cells (Supplemental Fig. 1). Mb1Cre/Fcgr2bΔneo, Cg1Cre/Fcgr2bΔneo, CD11cCre/Fcgr2bΔneo, and LysMCre/Fcgr2bΔneo mice were generated by crossing mice carrying Fcgr2bΔ alleles to Mb1Cre mice (23), Cg1Cre 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 Fcgr2bΔ allele and gave rise to Mb1Cre/Fcgr2bΔ/Δ and CD11cCre/Fcgr2bΔ/Δ mice. B6.Fcgr2bΔ/Δ (N12) were obtained from Taconic Farms. Fcer1g−/− FcγRIIB−/− mice had been described previously (27). All mice were maintained in The Rockefeller University Comparative Biology Center. All experiments were performed in compliance with federal laws and institutional guidelines and were 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 Fcgr2b, blood cells and splenic single-cell suspensions were prepared and depleted for erythrocytes and stained with fluorescent-conjugated anti-CD19 (1D3), anti-ALK1 (PK136), anti-CD1b (M1/70), anti-CD1c (B1L3), 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 IgG1+ and IgG2a+ cells in peripheral lymphoid tissues, mice were treated with 100 μg NP-chicken γ globulin (CGG) in aluminum hydroxide (alum) and analyzed 12 d later. Splenic single-cell suspensions depleted for erythrocytes were stained with fluorescent-conjugated anti-I-B220 (RA3-6B2), anti-γ6 (Jo2), anti-IgG1 (AS-85-1), and anti-FcγRIIB/III (2.4G2). To analyze FcγRIIB levels in thiolglycolate-elicited macrophages, mice were i.p. injected with 2 ml thiglycollate. Seven days later, peritoneal cavity cells were harvested by gentle flushing with cold PBS and stained with fluorescent-conjugated F4/80 (BM8), CD11b (M1/70), and anti-FcγRIIB (Ly17.2). Abs. Macrophages were defined as CD11b+/F4+80+ cells.

Ab response

Mice were immunized i.p. with 100 μg NP65-CGG emulsified in CFA on day 0 and boosted with 100 μg NP65-CGG emulsified in IFA on day 28. Levels of NP-specific IgG on days 0, 14, and 28 were determined by ELISA using a BSA-plated wells (BD Biosciences) using serum samples from mice treated with 2 ml thiglycollate. Seven days later, peritoneal cavity cells were harvested by gentle flushing with cold PBS and stained with fluorescent-conjugated F4/80 (BM8), CD11b (M1/70), and anti-FcγRIIB (Ly17.2). Abs. Macrophages were defined as CD11b+/F4+80+ cells.

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. Kidney sections from mice were stained with daily 5% chicken or rabbit type II collagen emulsified in adjuvant (IFA with 4 mg/ml NP26-BSA) on day 0. IgG1 and IgG2a Abs were detected with HRP-conjugated goat anti-mouse IgG-Fc (1:100) and TMB substrate. To analyze mouse collagen-specific IgG levels, 1:100,000 diluted serum samples were used for the analysis of IgG levels, and 1:100,000 diluted serum samples were used for the analysis of IgM levels, and 1:1,000,000 diluted serum samples were used for the analysis of IgD levels, and 1:5000 diluted serum samples were used for the analysis of IgE levels. All antibodies were purchased from Jackson ImmunoResearch Laboratories.

CIA experiments were performed as described (28). Briefly, mice were immunized intradermally with 100 μg bovine or chicken type II collagen emulsified in adjuvant (IFA with 4 mg/ml Mycobacterium tuberculosis H37 Ra) to induce arthritis. Arthritis incidences were monitored weekly for 11 wk. To analyze mouse collagen-specific IgG response, serum samples were diluted 5000-fold and analyzed with a mouse IgG anti-mouse collagen type II ELISA kit (MD Bioproducts).

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.e. and monitored for the development of arthritis for 15 days. Animals with arthritis were scored (0–3) were assigned to each paw of individual mice depending on the severity of arthritis, and the summed scores of four paws of individual mice (0–12) were recorded as their arthritis clinical scores.

FcγRIIB contributes to tolerance through distinct mechanisms

To characterize the contribution of FcγRIIB to tolerance in vivo, we monitored using Chemstrip 2 GP strips the arthritis clinical scores in FcγRIIB−/− and WT C57BL/6 mice. WT mice were monitored for the development of arthritis for 6 d. Arthritis clinical scores were monitored using Chemstrip 2 GP strips (Roche) monthly, and mice with arthritis levels >3 were considered sick. Kidney sections from mice were stained with daily 5% chicken or rabbit type II collagen emulsified in adjuvant (IFA with 4 mg/ml NP26-BSA) on day 0. IgG1 and IgG2a Abs were detected with HRP-conjugated goat anti-mouse IgG-Fc (1:100) and TMB substrate. To analyze mouse collagen-specific IgG levels, 1:100,000 diluted serum samples were used for the analysis of IgG levels, and 1:5000 diluted serum samples were used for the analysis of IgM levels, and 1:100,000 diluted serum samples were used for the analysis of IgD levels, and 1:5000 diluted serum samples were used for the analysis of IgE levels, and 1:100,000 diluted serum samples were used for the analysis of IgF levels, and 1:5000 diluted serum samples were used for the analysis of IgM levels.

CIA experiments were performed as described (28). Briefly, mice were immunized intradermally with 100 μg bovine or chicken type II collagen emulsified in adjuvant (IFA with 4 mg/ml Mycobacterium tuberculosis H37 Ra) to induce arthritis. Arthritis incidences were monitored weekly for 11 wk. To analyze mouse collagen-specific IgG response, serum samples were diluted 5000-fold and analyzed with a mouse IgG anti-mouse collagen type II ELISA kit (MD Bioproducts).
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+CD8+Va2+ 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 Fcgr2bfl/fl 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 Fcgr2bfl/fl 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+ and IgG1<sup>−</sup> GC B cells, as defined by B220<sup>+</sup>Fas<sup>−</sup>IgG1+ and B220<sup>+</sup>Fas<sup>−</sup>IgG1<sup>−</sup> cells, respectively. As shown in Fig. 1B, FcyRIIB expression was reduced in the majority of both IgG1+ 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 (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>+/+</sup>) and Fcgr2b heterozygous (Fcgr2b<sup>+/−</sup>) mice had comparable primary and secondary IgG responses, whereas these responses in Fcgr2b<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>−/−</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-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>−/−</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 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; 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>−/−</sup> mice with Mb1Cre-mediated selective deletion of Fcgr2b in B cells developed arthritis (p < 0.001), as did 3 of 10 Fcgr2b<sup>−/−</sup> mice with CD11cCre (p < 0.01). As heterozygous Fcgr2b mice (Fcgr2b<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>−/−</sup> mice
with LysMCre did not develop statistically significant disease, excluding the contribution of CD11cCre activity in monocytes to the phenotype observed in Fcgr2b<sup>−/−</sup> mice with CD11cCre (Figs. 1A, 1C). 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<sup>−/−</sup> 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<sup>−/−</sup> mice (Fig. 6A, 6B), which is in sharp contrast to the resistance of Fcgr2b<sup>−/−</sup> 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 arthritogenic 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 (FcεR1<sup>−/−</sup> Fcgr2b<sup>−/−</sup>) mice, whereas in Fcgr2b<sup>−/−</sup> 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 FcγRIIB levels in this passive autoantibody transfer model of inflammation.

**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 means). (B) Increased ANA levels in some Fcgr2b<sup>−/−</sup> mice. Hep-2 human ECs were stained with 1:100 diluted sera from 8- to 9-mo-old WT and Fcgr2b<sup>−/−</sup> mice, followed by FITC-conjugated goat anti-mouse IgG. IgG ANAs were detected in about half (9 of 18) of Fcgr2b<sup>−/−</sup> mice, whereas none of 5 WT mice were positive in this analysis (p < 0.05, χ² test). Original magnification ×100. **p < 0.01, ANOVA with Dunnett post hoc comparing each group to the WT group.

**FIGURE 5.** Susceptibilities of mice with germline or conditional knockout of Fcgr2b to bCIA. Accumulative bCIA incidences in WT (Fcgr2b<sup>+/+</sup>) 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<sup>+/+</sup> mice).
FIGURE 6. Susceptibilities of mice with germline or conditional knockout of FcγRIIb to the cCIA. (A) The development of cCIA in WT (FcγRIIbfl/fl) 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.

Discussion

The development of autoimmunity has been studied in several FcγRIIB-deficient mouse models, initially in FcγRIIb−/− 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 (FcγRIIbfl/fl) showed significantly attenuated lupus-like phenotypes (proteinuria and premature mortality) as compared with the backcrossed B6. FcγRIIb−/− (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.FcγRIIb−/− mice based on the analysis of B6. FcγRIIb−/− mice with different lengths of 129/Sv DNA segments around the targeted FcγRIIb 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. 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).

FIGURE 7. Accelerated development of K/BxN serum-induced arthritis in FcγRIIb−/− mice with LysMCre. WT, FcγRIIb−/−, and FcγRIIbfl/fl mice with LysMCre and FcyR-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.

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 FcγRIIb 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).

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 FcγRIIb by B cell–specific Mb1Cre leads to significantly enhanced primary and secondary Ab responses, and the deletion of FcγRIIb 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 FcγRIIb−/− 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 FcγRIIb 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...
a number of autoimmune phenotypes, including the development of more severe chicken CIA (10).

Interestingly, selective deletion of FcγR2b by Mb1Cre and Cg1Cre, respectively, resulted in different autoimmune phenotypes. Although hypersensitive to cCIA, FcγR2bδ/δ mice with Cg1Cre are not susceptible to bCIA, in contrast to FcγR2bδ/δ mice with Mb1Cre. Although this could be due to the different impact of Mb1Cre- and Cg1Cre-mediated FcγR2b 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 H2 backgrounds of B6 mice only support such responses against chicken, not bovine type II collagen (34–36). The fact that conditional knockout of FcγR2b in APCs (DCs and B cells) resulted in the break of tolerance in the bCIA model suggests that these conditional knockouts of FcγR2b 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 FcγR2b 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 FcγR2bδ/δ 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 FcγRIIB can promote DC maturation and T cell responses (43–45). Previous expression in DCs can inhibit T cell response (42), presumably by inhibiting myeloid effector cells are very sensitive to the FcγRIIB expression in multiple cellular compartments, we demonstrated that FcγR2bδ/δ mice with Mb1Cre in defined cellular compartments, we demonstrated that FcγR2b 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 be also useful to dissect the contribution of cell-specific FcγRIIB to the activities of these Abs.

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Disclosures

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References


