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FcγRIIb on Myeloid Cells Rather than on B Cells Protects from Collagen-Induced Arthritis

A. Seda Yilmaz-Elis,*†‡ Javier Martin Ramirez,*† Patrick Asmawidjaja,†‡ Jos van der Kaa,* Anne-Marie Mus,†‡ Maarten D. Brem,†‡ Jill W. C. Claassens,* Cor Breukel,* Conny Brouwers,* Sara M. Mangsbo,§‡ Peter Boross,*∥‡ Erik Lubberts,†‡ and J. Sjef Verbeek*‡

Extensive analysis of a variety of arthritis models in germline KO mice has revealed that all four receptors for the Fc part of IgG (FcγR) play a role in the disease process. However, their precise cell type–specific contribution is still unclear. In this study, we analyzed the specific role of the inhibiting FcγRIIb on B lymphocytes (using CD19Cre mice) and in the myeloid cell compartment (using C/EBPαCre mice) in the development of arthritis induced by immunization with either bovine or chicken collagen type II. Despite their comparable anti-mouse collagen autoantibody titers, full FcγRIIb knockout (KO), but not B cell–specific FcγRIIb KO, mice showed a significantly increased incidence and severity of disease compared with wild-type control mice when immunized with bovine collagen. When immunized with chicken collagen, disease incidence was significantly increased in pan-myeloid and full FcγRIIb KO mice, but not in B cell–specific KO mice, whereas disease severity was only significantly increased in full FcγRIIb KO mice compared with incidence and severity in wild-type control mice. We conclude that, although anti-mouse collagen autoantibodies are a prerequisite for the development of collagen-induced arthritis, their presence is insufficient for disease development. FcγRIIb on myeloid effector cells, as a modulator of the threshold for downstream Ab effector pathways, plays a dominant role in the susceptibility to collagen-induced arthritis, whereas FcγRIIb on B cells, as a regulator of Ab production, has a minor effect on disease susceptibility. *The Journal of Immunology, 2014, 192: 5540–5547.

Collagen-induced arthritis (CIA) is the most widely used animal model of arthritis and resembles human rheumatoid arthritis (RA) in its key features. Disease is induced by immunization with either bovine type II collagen (bCII) or chicken CII (cCII) in CIA-prone strains (1, 2). This results in the emergence of CIA-specific autoreactive T cells and high titers of specific autoantibodies against murine CII (mCII).

We and others have shown that the leukocyte receptors for IgG, FcγRs, are important players in the pathogenesis of RA (3, 4) and of arthritis in mice (5–8). Crosslinking of FcγRs with IgG immune complexes (IC) results in the initiation of cellular activation pathways (9). Mice have four different FcγR classes. FcγRI, FcγRII, and FcγRIII are multisubunit receptors that mediate activation signals via their common γ-chain (10). FcγRIb is a single-chain receptor that inhibits cell activation upon coengagement with activating FcγR by ICs (11–15). The balance of activating and inhibitory signals determines the outcome of FcγR signaling in myeloid effector cells, such as macrophages, neutrophils, dendritic cells (DCs), and mast cells. On B cells, coengagement of FcγRIb and the BCR downregulates the production of Abs.

FcγR γ-chain knockout (KO) mice, which are deficient for the expression of all activating FcγRs, are almost completely protected from CIA (7), indicating that activating FcγR are indispensable in this arthritis model. The crucial role of the activating FcγR has been confirmed with K/BxN serum-induced arthritis, proteoglycan-induced arthritis, and Ag-induced arthritis (16–18). FcγRIII K0 mice have shown greatly diminished disease activity in CIA on DBA/1 background (19) as well as in the passive K/BxN serum– and anti-bCII mAb–induced arthritis models on mixed 129/C57BL/6 background (16, 20). However, our previous results show that in an FcγRIIb KO background, FcγRIIb deficiency results only in a delay in CIA development, whereas deficiency of both FcγRI and FcγRIII in FcγRIIb KO background results in a strong decrease in incidence but a less well-consistent decrease in severity of the disease (5). Some FcγRI/II/III KO mice, expressing only FcγRIV, develop full-blown CIA with strong bone destruction, suggesting that reduction of the number of activating FcγR mainly increases the threshold for the induction of the disease. The role of FcγRIIb and FcγRI was found to be redundant in Ag-induced arthritis and immune complex (IC)–mediated arthritis (6, 21). These combined results illustrate the complexity of the role of the different FcγR in arthritis.

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The online version of this article contains supplemental material.

Abbreviations used in this article: bCII, bovine type II collagen; cCII, chicken CII; CIA, collagen-induced arthritis; CII, collagen type II; DC, dendritic cell; IC, immune complex; KO, knockout; mCII, autoantibodies against murine CII; qPCR, quantitative PCR; RA, rheumatoid arthritis; wt, wild-type.
FcyRIIb KO mice have increased Ab responses and exhibit a hyperresponsive phenotype in several in vivo models of inflammation (22, 23). FcyRIIb KO DBA/1 mice develop more severe CIA than wild-type (wt) littermates (7). Moreover, FcyRIIb KO mice display increased disease activity compared with wt controls in the K/BxN serum-induced arthritis model (23). In contrast to wt C57BL/6 mice, FcyRIIb KO mice on C57BL/6 background are highly susceptible to arthritis induced by immunization with bCII, suggesting a role for FcyRIIb in controlling immunological tolerance (24). The function of FcyRIIb as a immunological checkpoint was further supported by the observation that FcyRIIb KO mice generated in 129-derived embryonic stem cells and backcrossed for more than six generations into C57BL/6 background spontaneously developed lethal lupus-like disease (25). However, we have recently shown that FcyRIIb KO mice generated by gene targeting in C57BL/6 embryonic stem cells are not autoimmune (23). In contrast, in the presence of the yaa autoimmune-susceptibility locus, pure C57BL/6 FcyRIIb KO mice do develop lupus-like disease, indicating that FcyRIIb is only a modulator of autoimmunity determined by other genetic loci (23).

Nevertheless, the pure C57BL/6 FcyRIIb KO mice are still susceptible to CIA (23), just as the previously generated FcyRIIb KO mice on mixed 129/C57BL/6 background (24).

We have two explanations for the increased susceptibility to CIA of FcyRIIb-deficient C57BL/6 mice. One explanation is that the absence of FcyRIIb from B cells results in increased omCII autoantibody titers compared with the omCII titers in wt C57BL/6 mice when immunized with CII in CFA. These higher omCII autoantibody titers are sufficient for the initiation and progression of CIA. The alternative explanation is that the absence of FcyRIIb on myeloid effector cells lowers the threshold for downstream Ab effector pathways. Effector cells expressing activating FcyR require far less autoantibodies to become highly activated. This lower threshold is sufficient for the initiation and progression of the disease after immunization with CII in CFA, an immunization protocol known to break tolerance in C57BL/6 mice (26). To test these two hypotheses, we generated C57BL/6 strains that express the recombinase Cre, either exclusively in B cells (CD19Cre) (27) or in almost all myeloid cells (C/EBPαCre) (28), in addition to the presence of two floxed alleles of the FcyRIIb gene (23). These cell-type–specific FcyRIIb KO mouse models enable us to discriminate between the immune regulatory role of FcyRIIb on B cells, controlling Ab production, and its negative regulatory role in the downstream Ab effector pathways of myeloid effector cells. Our analysis of CIA development in these mice revealed that the immune regulatory function of FcyRIIb on myeloid effector cells exerts a dominant function in the protection against CIA, whereas the role of FcyRIIb on B cells, notwithstanding its established regulatory function in Ab production, barely affects the development and progression of the disease.

Materials and Methods

Mice

The generation of mice with floxed alleles of the fcyr2b gene, further referred to as FcyRIIbKO mice, and with FcyRIIb KO alleles, further referred to as FcyRIIbKO−/− mice, both in the pure C57BL/6 background, has been described previously (23). By crossing the FcyRIIbKO−/− mouse with transgenic mice expressing Cre under control of either the CD19 promoter (27) (kindly provided by Ari Waisman, Cologne, Germany) or the C/EBPα promoter (28) (kindly provided by Ivo Touw, Rotterdam), mouse strains have been generated that lack the inhibiting FcyRIIb exclusively on B cells or the myeloid cell compartment, respectively. In CD19Cre × FcyRIIbKO mice, further referred to as CD19Cre mice, the deletion of FcyRIIb from B cells is close to 100% without off-target recombination (29). In C/EBPαCre × floxed FcyRIIb mice, further referred to as C/EBPαCre mice, there is excellent deletion of FcyRIIb from circulating monocytes and granulocytes and a majority of the splenic macrophages and DCs, with no effect on B cell expression of FcyRIIb (Supplemental Fig. 1).

All mice were backcrossed, bred, and maintained in the specific pathogen-free unit, and experiments were carried out at the experimental unit of the laboratory animal facility of the Leiden University Medical Center. The health status of the animals in both units was monitored over time according to the rules of the Federation of European Laboratory Animal Science Associations, and the animals were found to be pathogen-free according to Federation of European Laboratory Animal Science Associations criteria. All experimental protocols were approved by the local ethical committee.

Induction and clinical evaluation of CIA

bCII and cCII (MD Biosciences or Sigma-Aldrich) was dissolved in 0.1 M acetic acid overnight at 4°C at a concentration of 2 mg/ml. Male mice were immunized by s.c. injection at the tail base with 100 µg bCII or cCII emulsified in CFA (Difco) and boosted on day 28 with 100 µg bCII or cCII in CFA by s.c. injection at the neck. By starting from day 14 onward, mice were inspected and scored in a blind manner, three times a week. Disease progression was evaluated visually using an extended scoring protocol (30). In brief, each limb was assigned a score of 0–15 on the basis of the number of the affected joints, so that a mouse could reach a total score of 60. An arthritic toe and knuckle were scored as 1, with a maximum of 10 per paw. An arthritic ankle or mid paw was given a score of 5. Mice with two legs reaching the maximal score were euthanized, and their end score was carried forward in the analysis. A total score >30 was considered as severe and in the range of 10–30 as moderate.

amCII Ab titers

Blood was collected from mice by retro-orbital bleeding on the indicated days (days 0, 7, and 28) after immunization with either bCII or cCII and mouse-specific anti-CII Ab titers were determined by ELISA. Immuno-Maxisorp plates were coated with 5 µg/ml mCII (Chondrex, Redmond, WA) overnight at 4°C. After washing with PBS-0.05% Tween 20, the plates were blocked with PBS/10% FBS for 1 h at room temperature. The plates were then washed and incubated with serially diluted mouse serum for 3 h at room temperature. After washing, the plates were subsequently incubated with 0.5 mg/ml of one of the following detection Abs: biotinylated goat anti-mouse total IgG and IgG2a (Southern Biotechnology Associates, Birmingham, AL) for 1 h at room temperature. After washing, the plates were incubated with streptavidin-peroxidase (Sanquin, Amsterdam, The Netherlands) for 30 min at room temperature. Following a washing step, TMB substrate solution (Thermo Scientific, Waltham, MA) and H2O2 were added to the wells, and the coloring reaction was stopped by adding 0.5 M H2SO4. The reaction was detected at 405 nm. mCII-specific Ab titers were compared with a reference of pooled sera of arthritic mice and assigned an arbitrary value.

Histological assessment

For standard histology, isolated joints were fixed for 4 d in 10% formalin, decalcified in 5% formic acid, and subsequently dehydrated and embedded in paraffin. Standard frontal sections, 7 mm in size, were mounted on SuperFrost slides (Menzel-Gläser). H&E and Safranin O staining were performed to study the joint pathologic features. The severity of arthritis in the joints was scored as joint inflammation based on the amount of inflammatory cells present in the joint and scored on a scale of 0–3 (in which 0 is none, 1 is mild, 2 is moderate, and 3 is maximal pathology) on five serial sections spaced 140 mm apart. Scoring was performed in a blinded manner by two independent observers (n = 3).

Total RNA isolation from mouse joints

The whole arthritic joints, which were harvested when the mice developed maximum score, including synovium, adjacent tissues, and bones, were pulverized in liquid nitrogen using mortar and pestle. The tissues were further homogenized in 1 ml TRIzol reagent (Invitrogen) using blender Ika Ultra-Turrax T8 (IKA-Werke, Staufen, Germany), and RNA was isolated according to the manufacturer’s protocol. RNA concentration and quality was assessed by a Nanodrop Spectrophotometer (Thermo Scientific). RNA integrity was checked in 1.5% agarose gel prepared in RNase-free conditions.

Quantitative PCR and cytokine measurements

About 1 µg total RNA was used for first-strand cDNA synthesis with random hexamer primers (Roche Applied Science, The Netherlands).

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Quantitative PCR (qPCR) reactions containing FastStart Universal SYBR Green Master mix (Roche Applied Science, Almere, The Netherlands) and 10 pM each qPCR primer and 5 μl 10 times diluted cDNA in a 20 μl total volume for each sample were performed on the Roche LightCycler 48 (Roche). Data were normalized to values of the β-actin gene. The forward and reverse primer sets used for the different transcripts are: mouse β-actin, 5'-TGGCGTAGACATCAAAGAGA-3' and 5'-AGGCTCCAGTCACCTGCT-3'; IL-6, 5'-GAAGTATCCCTCGAACAGACC-3' and 5'-AACTGCCATCATGTTGCTACA-3'; MIP-1α, 5'-ATGAGGTTCTCCACCCACTGC-3' and 5'-GATGAAATTGGCTGGAATCT-3'; IL-β, 5'-CAACCAAAAGTATCCCTGCATG-3' and 5'-GATCCACACTTCCTCAGCTGCA-3'; IL-10, 5'-GGTTGCAAGCCTTGAGA-3' and 5'-ACCTGCTCAGTGGCTTGTCTG-3'; MCP-1, 5'-CCCAATGAGTAGGCTGGAATCT-3' and 5'-ATGAAGGTCTCATTGCTTCA-3'; IFN-γ, 5'-ATGAAAGCTACACTGCATC-3' and 5'-CCATCCAACTGCATC-3'.

Results

B cell–specific FcγRIIb KO mice did not show an increase in incidence and severity of CIA compared with wt control mice after immunization with bCII

C57BL/6 mice deficient for FcγRIIb on B cells (CD19Cre), full FcγRIIb KO mice (FcγRIIb<sup>-/-</sup>), and floxed FcγRIIb wt (FcγRIIb<sup>fl/fl</sup>) control mice immunized with bCII were monitored for the development of arthritis for 72 d. Fig. 1 shows the disease incidence (Fig. 1A) and mean severity (Fig. 1B) of the different genotypes. As expected, disease incidence and severity were significantly higher in FcγRIIb<sup>fl/fl</sup> mice compared with FcγRIIb<sup>fl/fl</sup> control mice. However, the CD19Cre mice did not show a significantly increased incidence and severity compared with FcγRIIb<sup>fl/fl</sup> control mice.

These results indicate that deficiency exclusively on B cells is not sufficient to increase the susceptibility of C57BL/6 mice to arthritis induced by immunization with bCII.

Anti-mCII autoantibody titers were significantly increased in CD19Cre and FcγRIIb<sup>-/-</sup> mice compared with FcγRIIb<sup>fl/fl</sup> control mice after immunization with bCII

amlII autoantibody titers in serum of FcγRIIb<sup>-/-</sup>, CD19Cre, and FcγRIIb<sup>fl/fl</sup> control mice, immunized with bCII, were determined by ELISA 1 wk after the boost. CD19Cre and FcγRIIb<sup>-/-</sup> mice showed a similar small but significant increase in amlII IgG Ab titers compared with FcγRIIb<sup>fl/fl</sup> control mice (Fig. 2A). There was no significant difference in amlII IgG2a titers among the different genotypes (Fig. 2B).

In FcγRIIb<sup>-/-</sup> but not CD19Cre mice, intermediate amlII autoantibody titers are sufficient to induce severe CIA

In Table I, the association between amlII autoantibody titers and incidence and severity of autoimmune arthritis in CD19Cre and FcγRIIb<sup>-/-</sup> mice is shown. The genotypes are divided into three subgroups: mice with high, intermediate, and low (or not detectable) amlII titers. Within these subgroups, the percentage of mice with and without disease was determined. From the mice with intermediate amlII titers, only FcγRIIb<sup>-/-</sup> mice developed disease. In CD19Cre mice and FcγRIIb<sup>fl/fl</sup> control mice, intermediate anti-mCII titers were not sufficient to induce arthritis. Besides this

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**FIGURE 1.** Development of bCII-induced arthritis in B cell–specific FcγRIIb KO mice. (A) Incidence of arthritis is the percentage of all mice of the same genotype showing arthritic symptoms at a given time point after immunization with bCII in CFA. Incidence among the different groups was compared by performing the χ² contingency test. (B) Arthritis index indicates the mean severity score from all mice that developed disease with the same genotype at a given time point after immunization with bCII in CFA. Group sizes are as follows: FcγRIIb<sup>fl/fl</sup>, n = 28; FcγRIIb<sup>-/-</sup>, n = 19; and CD19Cre, n = 24. Asterisks indicate significant difference (*p < 0.05, **p < 0.01, ***p < 0.001) as compared with FcγRIIb<sup>fl/fl</sup> control mice.

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**FIGURE 2.** Humoral response against mCII in CD19Cre, FcγRIIb<sup>-/-</sup>, and FcγRIIb<sup>fl/fl</sup> control mice after immunization with bCII. Blood was collected from mice at day 28 after immunization (1 wk after the boost), and the concentration of total IgG (A) and IgG2a (B) amlII was determined by sandwich ELISA. Distributions regarding titer levels were normalized by log transformation. Geometrical means were compared by using Kruskal-Wallis test. Asterisks indicate significant difference (*p < 0.05) as compared with FcγRIIb<sup>fl/fl</sup> control mice. AEU, arbitrary ELISA units.
Table I. Association between total IgG αmCII autoantibody titers and incidence of CIA in CD19Cre and FcγRIIb<sup>−/−</sup> mice after immunization with bCII

<table>
<thead>
<tr>
<th>Phenotype/Genotype</th>
<th>High Titers</th>
<th>CIA</th>
<th>Intermediate Titers</th>
<th>CIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>FcγRIIb&lt;sup&gt;−/−&lt;/sup&gt; (n = 19)</td>
<td>53% (10/19)</td>
<td>90% (9/10)</td>
<td>35% (7/19)</td>
<td>57% (4/7)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD19Cre (n = 24)</td>
<td>58% (14/24)</td>
<td>43% (6/14)</td>
<td>21% (5/24)</td>
<td>0% (0/5)</td>
</tr>
<tr>
<td>FcγRIIb&lt;sup&gt;fl/fl&lt;/sup&gt; (n = 28)</td>
<td>21% (6/28)</td>
<td>50% (3/6)</td>
<td>29% (8/28)</td>
<td>0% (0/8)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Significant difference (p < 0.05) as compared with FcγRIIb<sup>fl/fl</sup> control mice (χ² test).

statistically significant difference between FcγRIIb<sup>−/−</sup> and CD19Cre mice with intermediate anti-mCII titers, the mice with high anti-mCII titers showed clearly the same trend. A total of 53% of the FcγRIIb<sup>−/−</sup> and 58% of the CD19Cre mice, but only 21% of the FcγRIIb<sup>fl/fl</sup> control mice, developed high anti-mCII titers. From these mice with high titers, only 43% of the CD19Cre mice and 50% of the FcγRIIb<sup>fl/fl</sup> control mice developed CIA, whereas 90% of the FcγRIIb<sup>−/−</sup> mice had disease (Table I). Taken together, we concluded that, although deficiency of FcγRIIb on B cells resulted in significantly increased anti-mCII autoantibody titers, mainly deficiency of FcγRIIb on other cell types than B cells is responsible for the high susceptibility of the FcγRIIb<sup>−/−</sup> mice to CIA induced by immunization with bCII.

Significantly increased incidence of CIA in pan-myeloid– but not B cell–specific FcγRIIb KO mice immunized with cCII

To explore the role of FcγRIIb on myeloid cells in the susceptibility to CIA, we induced CIA in pan-myeloid FcγRIIb KO mice (C/EBPaCre). Because the induction of CIA by immunization with bCII was clearly ineffective in the C57BL/6 background, we used cCII for this experiment. Both incidence and severity were increased in FcγRIIb<sup>−/−</sup>, CD19Cre, and FcγRIIb<sup>fl/fl</sup> control mice after immunization with cCII compared with the same disease parameters in these genotypes after immunization with bCII (Figs. 1, 3).

These results are in agreement with published data that C57BL/6 mice are more susceptible to CIA induced by immunization with cCII than to CIA induced by immunization with bCII (26). Fig. 3 shows the incidence (Fig. 3A), mean severity (Fig. 3B), and histology (Fig. 3C) of CIA in C/EBPaCre, CD19Cre, FcγRIIb<sup>−/−</sup>, and FcγRIIb<sup>fl/fl</sup> control mice immunized with cCII. As expected, FcγRIIb<sup>−/−</sup> mice showed a significantly increased incidence and severity compared with FcγRIIb<sup>fl/fl</sup> control mice. In C/EBPaCre, mice, CIA incidence was similar to disease incidence in FcγRIIb<sup>−/−</sup> mice, whereas the disease severity was not significantly increased compared with disease severity in FcγRIIb<sup>fl/fl</sup> control mice. The results from the immunization of CD19Cre mice with cCII confirmed the previous results with bCII. Neither incidence nor severity was significantly increased in these mice compared with the same disease parameters in FcγRIIb<sup>fl/fl</sup> control mice (Fig. 3A, 3B). Fig. 3C shows representative histological pictures of affected knee joints. Histological score of joint inflammation, based on the number of inflammatory cells present in the joint, was in agreement with the outer severity score based on the number of affected joints and ankle thickening (Supplemental Table I).

Severity of CIA correlates well with the cell type–specific deficiency of FcγRIIb in mice immunized with cCII

In Table II, the association between genotype and severity of CIA is shown. FcγRIIb<sup>fl/fl</sup> control mice (normal FcγRIIb expression), CD19Cre mice (FcγRIIb exclusively absent on B cells), C/EBPaCre (FcγRIIb absent on almost all myeloid cells), and FcγRIIb<sup>−/−</sup> mice (FcγRIIb absent on all cells) were immunized with cCII, and the severity of CIA was determined in mice that developed a detectable total IgG αmCII titer. A χ² test for trend, taking into account the three categories (severe, moderate, and no CIA was performed for the high susceptibility of the FcγRIIb<sup>−/−</sup> mice to CIA induced by immunization with bCII.
Titers, 67% of the Fcγ showed significantly increased 23% of the Fcγ significantly higher expressed in C/EBP C57BL/6 control mice (Fig. 5A, 5C, 5E). IL-6 and MCP-2 were 83% of the CD19Cre, and 59% of the Fcγ protection against CIA. Effector cells but not B cells plays a dominant role in the (Table II). These results strongly suggest that Fcγ on myeloid effector cells but not on B cells plays a dominant role in the protection against CIA.

Cytokine and chemokine expression profile of inflamed joints
From each genotype, joints of four or more mice with the highest disease severity were isolated and processed for mRNA quantification by qPCR. A nonimmunized group of C57BL/6 mice (n = 3) was used as control. IL-1β, IL-10, and MIP-1α were significantly higher expressed in FcγRllb−/− mice compared with untreated wt C57BL/6 control mice (Fig. 5A, 5C, 5E). IL-6 and MCP-2 were significantly higher expressed in C/EBPαCre mice and FcγRllb−/− mice (Fig. 5B, 5D). No difference was seen between the different genotypes in MCP-1 expression (Fig. 5F). These results reflect the overall higher inflammation in FcγRllb−/− mice and to a lower extent the C/EBPαCre mice compared with CD19Cre and FcγRllb0/0 control mice. In all genotypes, there was a large variation in the expression of IFN-γ (Fig. 5G).

Discussion
FcγRllb−/− mice backcrossed into the C57BL/6 background are highly susceptible to CIA. omCII autoantibodies are a prerequisite for the development of CIA. Our results show that for all genotypes (CD19Cre, C/EBPαCre, FcγRllb−/−, and FcγRllb0/0), there is a direct correlation between omCII autoantibody titers and disease incidence. None of the mice with low or undetectable omCII autoantibody titers developed disease, whereas the highest incidence of disease was found in mice with high autoantibody titers.

Immunological tolerance has to be broken for the development of these autoantibodies. In CIA, this is achieved by immunization with CII in CFA, resulting in detectable omCII autoantibody titers (in this study, in 50 [bCII] to 60% [cCII] of FcγRllb0/0 mice). It has been postulated by Fukuyama et al. (31) that FcγRllb on

Table II. Association between total IgG omCII autoantibody titers and incidence and severity of CIA in CD19Cre, C/EBPαCre, and FcγRllb−/− mice after immunization with cCII

<table>
<thead>
<tr>
<th>Phenytype/Genotype</th>
<th>+ omCII titer</th>
<th>Severe CIA</th>
<th>Moderate CIA</th>
<th>No Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>FcγRllb0/0 (n = 22)</td>
<td>59% (13/22)</td>
<td>23% (3/13)</td>
<td>23% (3/13)</td>
<td>54% (7/13)</td>
</tr>
<tr>
<td>CD19Cre (n = 12)</td>
<td>83% (10/12)</td>
<td>20% (2/10)</td>
<td>40% (4/10)</td>
<td>40% (4/10)</td>
</tr>
<tr>
<td>C/EBPαCre (n = 14)</td>
<td>79% (11/14)</td>
<td>64% (7/11)</td>
<td>18% (2/11)</td>
<td>18% (2/11)</td>
</tr>
<tr>
<td>FcγRllb−/− (n = 21)</td>
<td>100% (21/21)</td>
<td>67% (14/21)</td>
<td>5% (1/21)</td>
<td>29% (6/21)</td>
</tr>
</tbody>
</table>

The percentage of mice with severe, moderate, or no disease was determined in mice of the different genotypes with detectable omCII titers. Severe disease is >30 on a scale of 0–60, whereas moderate disease is in the range of 10–30.
B cells is a late checkpoint in the maintenance of B cell tolerance. Therefore, B cell–specific FcγRIIb deficiency may lower the threshold for breaking immune tolerance, resulting in an increased incidence of high αmCII autoantibody titers after immunization with CII in CFA, which drives the disease. In addition, FcγRIIb on B cells plays a prominent role in a negative-feedback mechanism that regulates Ab production. In the absence of FcγRIIb, Ab titers are increased irrespective of their specificity (22, 23). Indeed, compared with FcγRIIbfl/fl mice, CD19Cre mice showed significantly increased αmCII autoantibody titers when immunized with bCII, and we observed the same trend when these mice were immunized with cCII. Our experiments do not allow us to determine whether the enhanced αmCII autoantibody titers are the consequence of a lower threshold for breaking immune tolerance or the consequence of the absence of the negative-feedback mechanism on B cells that controls Ab production. Whatever the mechanism may be, the impact of B cell–specific FcγRIIb deficiency on disease susceptibility and progression was restricted. Although in CD19Cre mice, both incidence and severity of CIA after immunization with bCII and incidence after immunization with cCII showed the same trend—an increase compared with the same parameters in FcγRIIbfl/fl control mice—this was not significant, indicating that the increase in autoantibody titers in CD19Cre mice was insufficient to increase their susceptibility to CIA.

Although αmCII autoantibodies are a prerequisite for the development of CIA, their presence is not sufficient for disease development, because only ∼50% of wt mice with high αmCII autoantibody titers developed disease. This suggests that, besides the presence of autoantibodies, additional changes are required to trigger the development of full-blown disease. In contrast to the small effect of B cell–specific deficiency of FcγRIIb on disease susceptibility, the absence of FcγRIIb on almost all cells of the myeloid compartment (C/EBPαCre) resulted in a significantly increased incidence of CIA. This result indicates that FcγRIIb is a strong modulator of the threshold for the initiation of downstream Ab effector pathways in myeloid effector cells. We hypothesize that in FcγRIIb−/− mice, FcγRIIb deficiency results in enhanced myeloid effector cell responses and impaired IC clearance, which lowers the threshold for the induction of CIA by αmCII autoantibodies. It is striking that these observations in the autoimmune CIA model are very similar to our previous observations in the non-autoimmune–accelerated nephrotoxic nephritis model. In nephrotoxic nephritis, CD19Cre mice develop higher Ab titers without an increase in disease, whereas C/EBPαCre mice develop exacerbated disease (32).
The results with the CIA model confirm our previous conclusions about the role of FcγRIIB in two models of lupus-like disease (23). In these spontaneous autoimmune disease models in the C57BL/6 background, the presence of either the FcγRIIB-flanking 129-derived Sle16 or the yaa locus, both associated with autoimmunity, cause the development of anti-nuclear Ab autotibodies without pathology. In the autoimmune disease model presented in this study, immunization with CII in CFA initiated the development of cCI autoantibodies in 50 (bCII) to 60% (cCII) of the C57BL/6 FcγRIIBfl/fl control mice, resulting in mainly mild to moderate CIA in only 11 (bCII) to 27% (cCII) of these mice. In both the spontaneous (lupus) and the initiated (CIA) autoimmune disease models, incidence and severity of disease strongly increase in the absence of FcγRIIB, indicating that FcγRIIB acts as a downstream regulator of autoimmune disease. In contrast to FcγRIIB−/− mice on a mixed 129/C57BL/6 background (25), FcγRIIB−/− mice on a pure C57BL/6 background develop spontaneously neither autoantibodies nor autoimmune disease (23).

In this study, we showed that in contrast to FcγRIIB−/− mice, C/EBPαCre mice fail to show a significant increase in mean disease severity compared with FcγRIIBfl/fl control mice. Moreover, in the synovium of C/EBPαCre mice with severe CIA, only two out of seven measured cytokines and chemokines were significantly increased compared with the synovium of untreated control mice, whereas five of these factors were significantly increased in the synovium of FcγRIIB−/− mice. There are several explanations why FcγRIIB−/− mice develop more severe disease compared with C/EBPαCre mice: 1) incomplete deletion of FcγRIIB from myeloid cells in C/EBPαCre mice; 2) involvement of FcγRIIB on another cell type than myeloid cells; and 3) significantly increased cCI IgG2a autoantibody titer compared with FcγRIIBfl/fl control mice in FcγRIIB−/− but not in the C/EBPαCre mice (Fig. 4B). Although the C/EBPαCre mouse is superior in the deletion of the FcγRIIBfl/fl allele from the myeloid cells compared with the widely used LysMCre strain (29), this deletion is still <100% (Supplemental Fig. 1). Because it is unlikely that FcγRIIB on liver sinusoidal endothelial cells or kidney mesangial cells plays a role in susceptibility to CIA, we speculate that deficiency of FcγRIIB on B cells, although on its own not sufficient to increase significantly susceptibility to CIA, synergizes with deficiency on myeloid cells in the development of the stronger CIA phenotype of the FcγRIIB−/− mouse. Among the different IgG subclasses, IgG2a activates downstream effector pathways most effectively because it interacts with the complement system and with all activating Fcγ receptors.

Furthermore, in the absence of FcγRIIB, the absence of FcγRIIα significantly increases susceptibility to CIA, synergizes with deficiency on myeloid cells in the development of the stronger CIA phenotype of the FcγRIIB−/− mouse. Among the different IgG subclasses, IgG2a activates downstream effector pathways most effectively because it interacts with the complement system and with all activating Fcγ receptors. For example, in the absence of FcγRIIB, IgG2a titers are significantly increased in the synovium of CD11cCre mice with severe CIA immunized with cCI but not in the synovium of the FcγRIIB−/− mice with severe disease. However, the cytokines were selectively measured only in mice with severe CIA. Although the exact origin of the cells producing IFN-γ is unknown, and changes in the T cell profile needs further investigation, it means that in these mice, additional events had occurred to lower the threshold for the autotibodies to initiate the development of severe disease. In FcγRIIB−/− mice or C/EBPαCre mice, this threshold has been lowered already by the absence of FcγRIIB on myeloid effector cells. In the presence of FcγRIIB on myeloid effector cells, increasing IFN-γ is another way to downregulate the disease threshold, because IFN-γ upregulates the expression of the activating FcγRI and FcγRIV and concomitantly downregulates the inhibitory FcγRIIB. However, changes in FcγR expression are not the only way to lower the disease threshold, because half of the CD19Cre and FcγRIIBfl/fl control mice with severe disease had little increase of IFN-γ expression.

Also, in humans, besides the development of autoimmune diseases, unknown additional changes are required to trigger the development of full-blown RA. Anti-citrulline autotibodies can be detected in circulation long before the clinical onset of RA (33). Identification of the different pathways that lower the threshold for the induction of the pathology of the disease by autotibodies can be very helpful in the development of new strategies to protect against an autoimmune disease such as RA. The results of our studies suggest that genetically modified mouse models are suitable for the identification of these unknown disease mechanisms.

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Disclosures

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References


