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FCγRIIB ON MYELOID CELLS RATHER THAN B CELLS PROTECTS FROM COLLAGEN INDUCED ARTHRITIS

ABSTRACT

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 not known.

In this study, we analysed the specific role of the inhibiting FcγRIIb on B lymphocytes and in the myeloid cell compartment in the development of arthritis induced by immunization with either bovine or chicken collagen type II. Although their anti-mouse collagen autoantibody titers were similar, full FcγRIIb KO but not B cell specific FcγRIIb KO mice showed a significantly increased incidence and severity of disease compared to Wt 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 to the corresponding disease parameters in WT control mice.

We concluded that, although anti-mouse collagen autoantibodies are a prerequisite for the development of collagen induced arthritis, their presence is not sufficient for disease development. FcγRIIb on myeloid effector cells, as a modulator of the threshold for the initiation of downstream antibody effector pathways, plays a dominant role in the susceptibility to collagen induced arthritis, whereas FcgRIIb on B cells, as a regulator of antibody production, has only a minor effect on disease susceptibility..

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INTRODUCTION

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

We and others have shown that FcγRs are crucial players in the pathogenesis of RA [3, 4] as well as arthritis in mice [5-8]. crosslinking of FcγRs, leukocyte receptors for IgG, with IgG immune complexes ((IC) results in the initiation of cellular activation pathways [9]. Mice have four different FcγR classes. FcγRI, FcγRIII and FcγRIV are multi subunit receptors that mediate activation signals via their common γ-chain [10]. FcγRIIb is a single chain receptor, which inhibits cell activation upon co-engagement with activating FcγR by ICs [11-15]. The balance of activatory and inhibitory signals determines the outcome of FcγR signaling in myeloid effector cells, such as: macrophages, neutrophils, dendritic cells and mast cells. On B cells, co-engagement of FcγRIIb and the B-cell receptor (BCR) down-regulates the production of antibodies.

FcR γ-chain KO mice, which are deficient in 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 KRN serum induced arthritis, Proteoglycan-Induced Arthritis (PGIA) and with AIA [16-18] FcγRIII KO 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 moAb-induced arthritis models on mixed 129/C57Bl6 background [16, 20]. On the contrary, the role of FcγRI and FcγRIII in AIA and immune complex-mediated arthritis were found to be redundant [6,21].

FcγRIIb-KO mice possess an impaired control over Ab responses and exhibit a hyper-responsive phenotype in several *in vivo* models of inflammation [22, 23]. FcγRIIb KO DBA/1 mice develop more severe CIA than wild type littermates [7] . Moreover, FcγRII KO mice have displayed enhanced-disease activity compared to wild type controls in the K/BxN serum-induced arthritis model [23]. In contrast to wild type C57Bl6 mice, FcγRIIb-KO mice on C57Bl6 background are susceptible to arthritis induced by immunization with bCII, suggesting a role for FcγRIIb in controlling immunological tolerance [24]. The function of FcγRIIb as a immunological checkpoint was further supported by the observation that FcγRIIb KO mice generated in 129 derived ES cells and backcrossed for more than 6 generations into C57BL/6, spontaneously developed lethal lupus-like disease [25]. However, we have recently shown that FcγRIIb KO mice generated by gene targeting in C57BL/6 ES cells are not autoimmune [23]. In contrast, in the presence of the *yaa* autoimmune-susceptibility locus, the C57BL/6 FcgRIIb KO mice do developed lupus-like disease indicating that FcγRIIb is simply a modulator of autoimmunity determined by other genetic loci [23].

Importantly, the C57BL/6 FcγRIIb KO mice are still susceptible to CIA [23] just as the previously generated FcγRIIb KO on mixed 129/C57BL/6 background [24].

For the understanding of the molecular and cellular basis of the susceptibility to CIA of FcγRIIb-deficient mice on C57BL/6 background, an analysis of the cell-type specific role of FcγRIIb in the disease is required. Therefore, we generated C57Bl6 strains that in addition to the presence of two floxed alleles of the FcγRIIb gene, express the recombinase Cre either in B cells (CD19Cre), or in all myeloid cells (CEBPalphaCre). These cell-type specific FcγRIIb KO mouse models enabled us to discriminate between the immune regulatory role of FcγRIIb on B cells, controlling antibody production, and its regulatory role in the downstream-antibody effector pathways of myeloid-effector cells. Our analysis of CIA development in these mice revealed that the immune regulatory function of FcγRIIb on myeloid effector cells exerts a dominant function in the protection against CIA, whereas the role of FcγRIIb on B cells barely affects the predisposition for CIA.

MATERIALS AND METHODS

Mice

The generation of mice with a floxed allele of the *fcgr2b* gene in C57BL/6 background has been described previously [23]. By crossing this mouse strain with transgenic strains expressing either the CD19Cre [26], (kindly provided by Ari Waisman, Cologne) or the CEBPalphaCre, [27] kindly provided by Ivo Touw, Rotterdam), mouse strains have been generated which lack the inhibiting FcgRIIb exclusively on B cells or the myeloid cell compartment respectively. The deletion of FcγRIIb from B cells is close to 100% without of target recombination in CD19Cre X floxed FcγRIIb mice [28]. In CEBPalphaCre X floxed FcγRIIb mice there is excellent deletion of FcγRIIb from circulating monocytes and granulocytes and a majority of the splenic macrophages and dendritic cells, with no effect on B cell expression of FcγRIIb. [29]

All mice were backcrossed, bred and maintained in the SPF 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 FELASA rules and the animals were found to be pathogen free according to FELASA criteria. All experimental protocols were approved by the local ethical committee.

Induction and clinical evaluation of CIA

Bovine or chicken collagen type II (bCII and cCII) (MD Biosciences or Sigma) was dissolved in 0.1 M acetic acid overnight at 4°C at a concentration of 2 mg/ml. Male mice were immunized by subcutaneous injection (s.c.) at the tail base with 100 µg bCII or cCII emulsified in Complete Freund's Adjuvant (CFA, Difco) and boosted on day 28 with 100 µg bCII or cCII in Complete Freund's Adjuvant (CFA) by s.c. injection at the neck. By starting from day 14 onwards, mice were inspected and scored in a

blind manner, three times a week. Disease progress 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.

Anti-mCII antibody titers

Blood was collected from mice by retro-orbital bleeding on the indicated days (day 0, 7, 28) after immunization with either bCII or cCII and Ab titers were determined by ELISA. Immuno-Maxisorp plates were coated with 5 µg/ml mCII (Chondrex, WA, USA) overnight at 4 °C. After washing with PBS-0.05% Tween20, the plates were blocked with PBS/10% FBS (fetal bovine serum) for 1 hour at room temperature. The plates were then washed and incubated with serially diluted mouse serum for 3 hours at room temperature. After washing, the plates were subsequently treated with 0.5 mg/ml of one of the following detection Abs: biotinylated goat anti-mouse total IgG and IgG2a (SouthernBiotech,AL, USA) for 1 hour at room temperature. After washing, the plates were incubated with streptavidin-peroxidase (Sanquin, the Netherlands) for 30 min at room temperature. Following washing step, TMB substrate solution (Therma Scientific, MA, USA) and H_2O_2 was added to the wells and the coloring reaction was stop by adding 0.5M H_2SO_4. The reaction was detected at 405 nm. Mouse collagen type II-specific Ab titers were compared to a reference of pooled sera of arthritic mice and assigned an arbitrary value.

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 of Trizol reagent (Invitrogen, USA) using blender Ika Ultra-Turrax T8 (IKA-Werke GmbH, Germany) and RNA was isolated according to the manufacturer's protocol. RNA concentration and quality was assessed on Nanodrop Spectrophotometer (Therma Scientific, MA, USA). RNA integrity was further checked in 1.5 % agarose gel prepared in Rnase-free conditions.

Quantitative PCR and cytokine measurements

About 1 µg of total RNA was used for first strand cDNA synthesis with random hexamer primers (Roche Applied Science, Netherlands). qPCR reactions containing FastStart Universal SYBR Green Master mix (Roche Applied Science, Netherlands) and 10 pmole of each qPCR primer and 5 µL of 10times diluted cDNA in a 20 µL total volume for each sample were performed on the Roche LightCycler 48. Data were normalized to values of beta-actin gene. The primer sets used in qPCR reactions were: mouse beta-actin forward primer TGCGTGACATCAAAGAGAAG and reverse primer

GATGCCACAGGATTCCATA , IL-6 forwar primer GAGGATACCACTCCCAACAGACC and reverse primer, AAGTGCATCATCGTTGTTCATACA, MIP1α forward primer ATG AAGGTCTCCACCACTGC and reverse primer GATGAATTGGCGTGGAATCT, IL1-β forward primer CAACCAACAAGTGATATTCTCCATG and reverse primer GATCCA CACTCTCCAGCTGCA, IL-10 forward primer GGTTGCCAAGCCTTATCGGA and reverse primer ACCTGCTCCACTGCCTTGCT, MCP-1 forward primer CCCAATG AGTAGGCTGGAGA and reverse primer TCTGGACCCATTCCTTCTTG, MCP-2 forward primer TAAGGCTCCAGTCACCTGCT and reverse primer TCTGGAAAAC CACAGCTTCC, IFN-γ forward primer ATGAACGCTACACACTGCATC and reverse primer CCATCCTTTTGCCAGTTCCTC

Statistics

Incidence, median clinical score and severity score among three or more groups along time were compared by two-way ANOVA. The mean humoral responses displayed by three or more unmatched groups were compared by the nonparametric test one-way ANOVA.. Results were considered significant if $p < 0.05$ and represented by *. If, $0.005 > p < 0.01$, this value was represented by **. If $p < 0.005$, this value was represented by ***. The terms high, little or mild represented values higher than μ + 2SD, lower than μ – 2SD or values comprised in the middle range of these previous criteria, respectively. Wt strain was considered the reference control.

RESULTS

B cell specific FcγRIIb KO mice did not show an increase in incidence and severity of CIA compared to wild type control mice

C57BL/6 mice deficient for FcγRIIb on B cells (CD19Cre), full FcγRIIb KO mice and floxed FcγRIIb Wt control mice immunized with bCII were monitored for the development of arthritis in two independent experiments (Table I) for 72 days. Figure 1

Table I. Mice were immunized by either bCII or cCII collagen in four collagen-induced arthritis experiments.

	n	immunization
Experiment 1 & 2		bCII in CFA
FcgRIIb fl/fl	28	
FcgRIIb KO	19	
CD19CrexFcgRIIb fl/fl	24	
Experiment 3 & 4		cCII in CFA
FcgRIIb fl/fl	22	
FcgRIIb KO	21	
CD19CrexFcgRIIb fl/fl	12	
cEBPαCrexFcgRIIb	14	

shows the disease incidence (panel A) and the median severity (panel B) of the different genotypes. As expected disease incidence and severity were significantly higher in full FcγRIIb KO mice compared to floxed FcγRIIb wildtype (Wt) controls. However, the B-cell specific FcγRIIb KOs did not show a significantly increased incidence and severity compared to floxed Wt controls.

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.

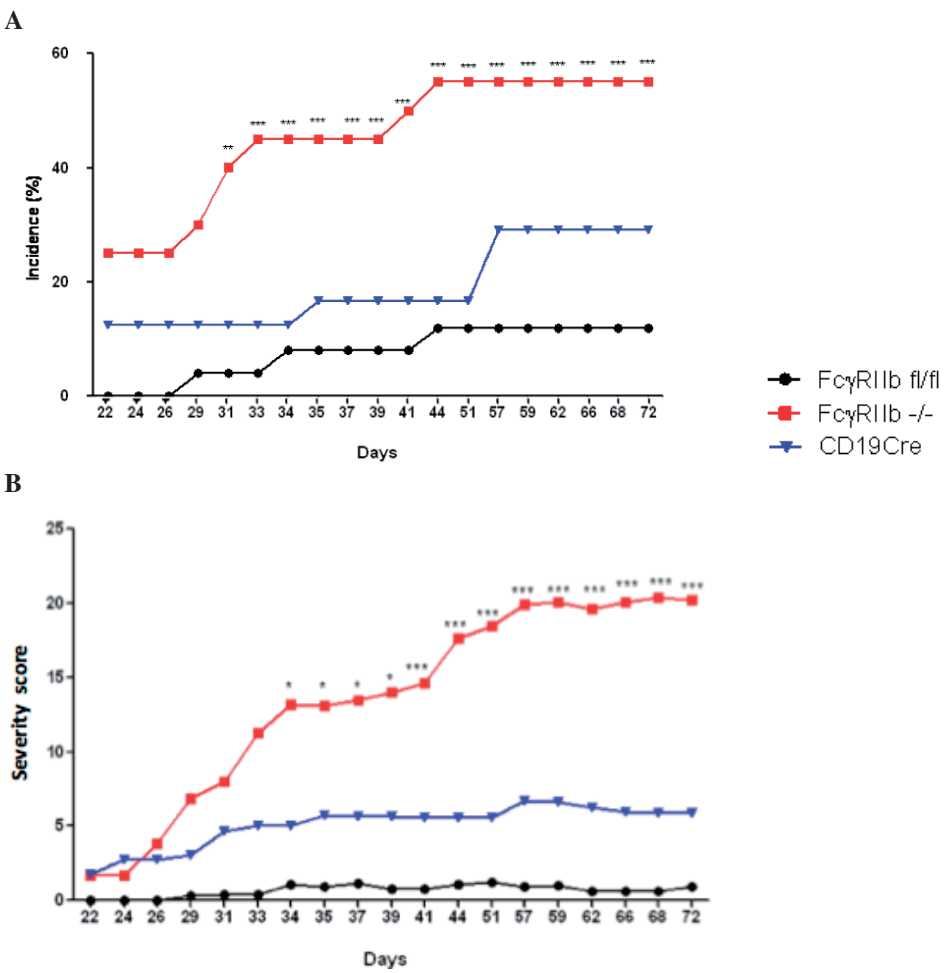

FIGURE 1. Development of bovine CII induced arthritis in B cell specific FcγRIIb KO mice. A. Incidence of arthritis is the percentage of all mice of the same genotype showing arthritic symptoms at a given time point after immunization with bCII in CFA. B. Arthritis index indicates the median severity score from all sick mice with the same genotype on a given time point after immunization with bCII in CFA. Merged data from two separate experiments are shown; group sizes are given in Table 1, asterisks indicate significant difference (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) as compared to floxed FcγRIIb control mice

Anti-mCII autoantibody titers were similar in B-cell specific FcγRIIb KO and wild type control mice

Anti-murine CII (mCII) autoantibody titers in serum of full and B-cell specific FcγRIIb KO mice and Wt FcγRIIb$^{fl/fl}$ control mice, immunized with bovine CII, were determined by ELISA one week after the boost. Full FcγRIIb KO mice and B-cell specific mice showed a similar increase in anti-mCII IgG antibody titers compared to Wt control mice. However the increase was not statistically significant (fig.2A). These results suggest that FcγRIIb deficiency does not lower the threshold for the loss of immunological tolerance in C57BL/6 mice. There was also no significant difference in anti-mCII IgG2a titers between the different phenotypes (Fig 2B).

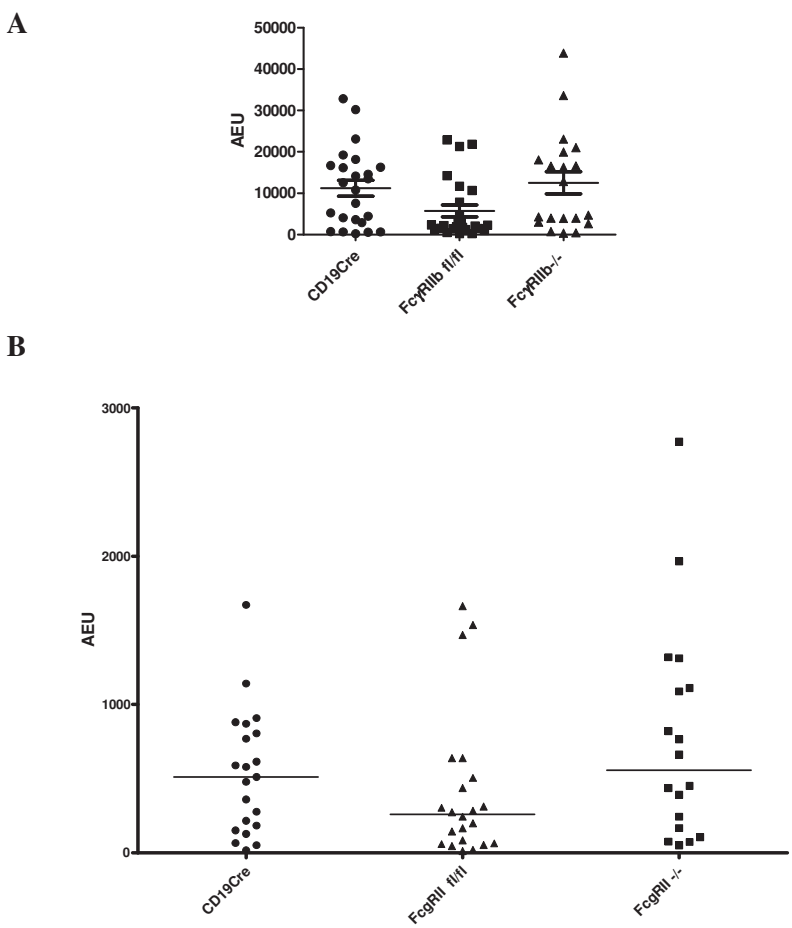

FIGURE 2. Humoral response against mCII in B cell specific FcγRIIb KO mice, full FcγRIIb KO mice and floxed FcγRIIb Wt control mice after immunization with bCII. Blood was collected from mice at day 28 after immunization (one week after the boost) and the concentration of total IgG (Panel A) and IgG2a (Panel B) anti-mouse CII was determined by sandwich ELISA.

In full but not B cell specific FcγRIIb KO mice high anti-mCII autoantibody titers were associated with high incidence of severe CIA

In Table II, an overview of the anti-mCII autoantibody titers, the incidence and severity of arthritis per genotype is presented. The genotypes are divided into three sub-groups: mice with high, intermediate and low (or not detectable) anti-mCII titers. Within these sub-groups the disease incidence and severity was determined (Table II). 50% of the FcγRIIb KO and 58 % of the B-cell specific FcγRIIb KO mice developed high anti-mCII titers. From the mice with high titers only 14% of the B-cell specific FcγRIIb KO mice showed a severe CIA phenotype whereas 60% of the full FcγRIIb KO had severe disease. Moreover, 57% of the B-cell specific FcγRIIb KO but only 10% of the full FcγRIIb KO with high anti-mCII titers did not develop disease. From the mice with intermediate anti-mCII titers only some full FcγRIIb KO mice developed severe (29%) or moderate (29%) disease. In B cell specific FcγRIIb KO mice and FcγRIIb$^{fl/fl}$ Wt control mice intermediate anti-mCII titers were not sufficient to induce arthritis. Taken together we concluded that mainly deficiency of FcγRIIb on other cell types than B cells is responsible for the high susceptibility of full FcγRIIb mice to CIA induced by immunization with bCII.

Significantly increased incidence and severity 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 (cEBPαCre). Because the induction of CIA by

Table II. Anti-mouse Collagen auto-antibody titers (aCII) and incidence and severity of CIA in cell type specific FcγRIIb KO mice after immunization with bovine CII.

	Total α bovine CII IgG titers (%)			
		CIA severity (%)		
	high	severe	mild	low/no
FcgRIIb -/-	50	60	30	10
CD19Cre x FcgRIIb fl/fl	58.3	14.3	28.6	57.14
FcgRIIb fl/fl	24	0	50	50
	Titer level middle (%)	CIA severity (%)		
		severe	mild	low/no
FcgRIIb -/-	35	28.6	28.6	42.9
CD19Cre x FcgRIIb fl/fl	20.8	0	0	100
FcgRIIb fl/fl	32	0	0	100
	Titer level low/no (%)	CIA severity		
		severe	mild	low/no
FcgRIIb -/-	15	0	0	100
CD19Cre x FcgRIIb fl/fl	20.8	0	0	100
FcgRIIb fl/fl	44	0	0	100

immunization with bCII was clearly ineffective in C57BL/6 background we used cCII and performed two independent experiments (Table I). Both incidence and severity were increased in full- and B cell specific FcγRIIb KO mice and Wt FcγRIIb$^{fl/fl}$ controls after immunization with cCII compared to the same disease parameters in these genotypes after immunization with bCII. Figure 3 shows the incidence (panel A) and median severity (panel B) of CIA in pan-myeloid, B cell specific and full FcγRIIb KO mice and floxed FcγRIIb Wt control mice immunized with cCII. As expected, full FcγRIIb KO mice showed a significantly increased incidence and severity compared to floxed FcγRIIb Wt control mice. In the pan-myeloid specific FcγRIIb KO mice, CIA incidence was similar to disease incidence in full FcγRIIb KO mice whereas the disease severity was not significantly increased. The results from the immunization of B cell specific FcγRIIb KO mice with cCII confirmed the previous results with bCII. Neither incidence nor severity was significantly increased in these mice compared to the same disease parameters in Wt control mice. (Fig.3A and 3B).

Anti-mCII IgG2a autoantibody titers were significantly increased in full FcγRIIb KO mice immunized with cCII

Anti-murine CII (mCII) autoantibody titers in serum of full-, pan-myeloid- and B-cell specific FcγRIIb KO mice and Wt FcγRIIb$^{fl/fl}$ control mice, immunized with chicken CII were determined by ELISA one week after the boost. Full FcγRIIb-, pan-myeloid and B-cell specific FcγRIIb KO mice showed a similar increase in total anti-mCII IgG antibody titers compared to Wt control mice. However the increase was not statistically significant. (Fig.5A). However, full FcγRIIb KO mice showed significantly increased anti-mCII IgG2a titers compared to FcγRIIbfl/fl Wt control mice (Fig 5B). This might explain the higher severity of disease in full FcγRIIb KO mice compared to pan-myeloid FcγRIIb KO mice.

In pan-myeloid FcγRIIb KO mice high and intermediate anti-mCII autoantibody titers were associated with high incidence of severe CIA

In full and pan-myeloid FcγRIIb KO mice but not B cell specific FcγRIIb KO mice high anti-mCII autoantibody titers were associated with high incidence and strong severity of CIA after immunization with cCII, 52% of the FcγRIIb KO, 43% of the pan-myeloid and 36 % of the B-cell specific FcγRIIb KO mice developed high anti-mCII titers. From the mice with high titers 50% of the B-cell specific FcγRIIb KO mice showed a severe CIA phenotype whereas 91% of the full FcγRIIb KO and 83% of the pan-myeloid FcγRIIb KO mice had severe disease. 50% of the B-cell specific FcγRIIb KO but only 9% of the full FcγRIIb KO and 17% of the pan-myeloid FcγRIIb KO mice with high titers developed intermediate disease. From the mice with intermediate anti-mCII titers 40% of the full and pan-myeloid FcγRIIb KO mice developed severe disease but none of the B cell specific FcγRIIb KO mice (Table III). These results indicate that FcγRIIB on myeloid effector cells but not B cells plays a dominant role in the protection against CIA.

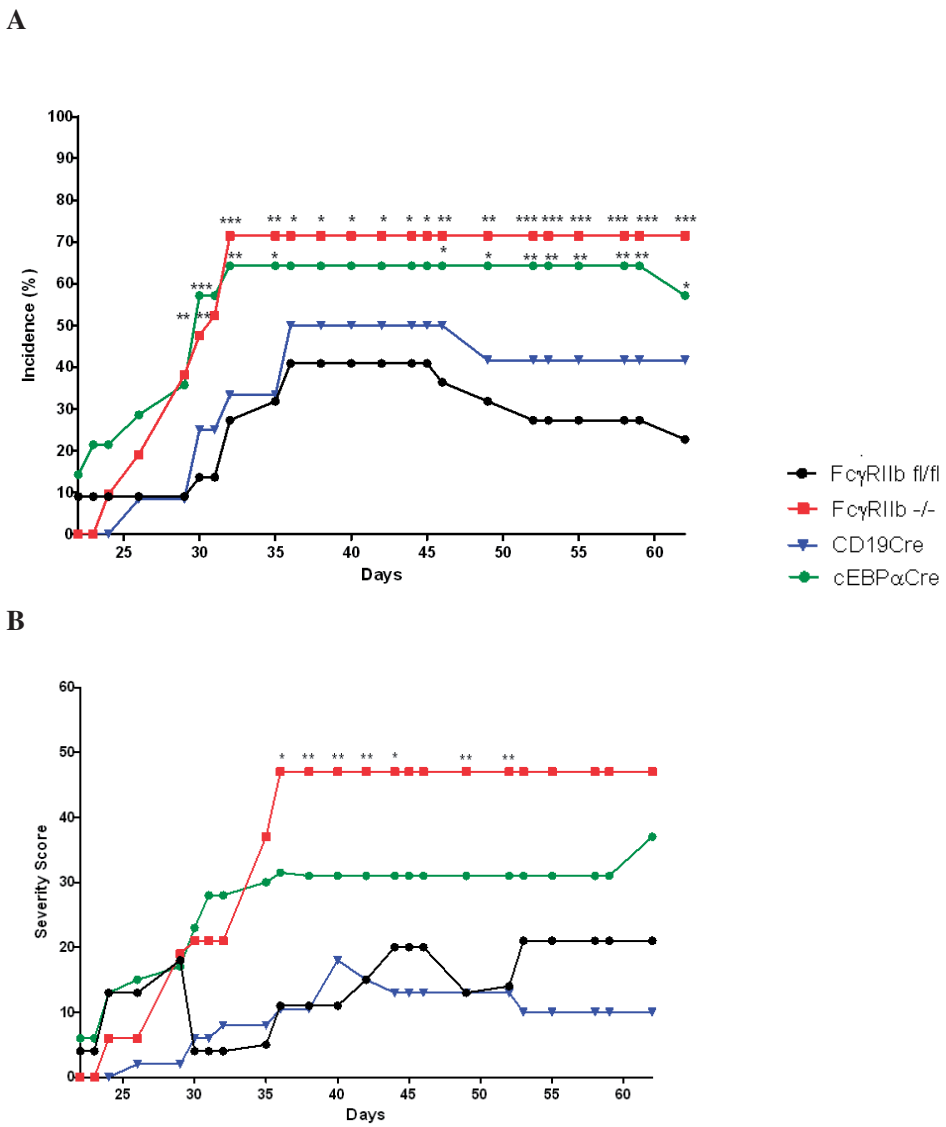

FIGURE 3. Development of CIA in pan-myeloid and B cell specific FcγRIIb KO mice. A. Incidence of arthritis is the percentage of all mice of the same genotype showing arthritic symptoms at a given time point after immunization with cCII in CFA. B. Arthritis index indicates the median severity score from all sick mice with the same genotype on a given time point after immunization with cCII in CFA. Merged data from two separate experiments are shown; group sizes are given in Table 1, asterisks indicate significant difference (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) as compared to floxed FcγRIIb control mice.

Cytokine and chemokine expression profile of inflamed joints

Joints from all mice of each genotype that developed highly severe arthritis were isolated and processed for mRNA quantification by qPCR. A non-immunized group of C57BL/6

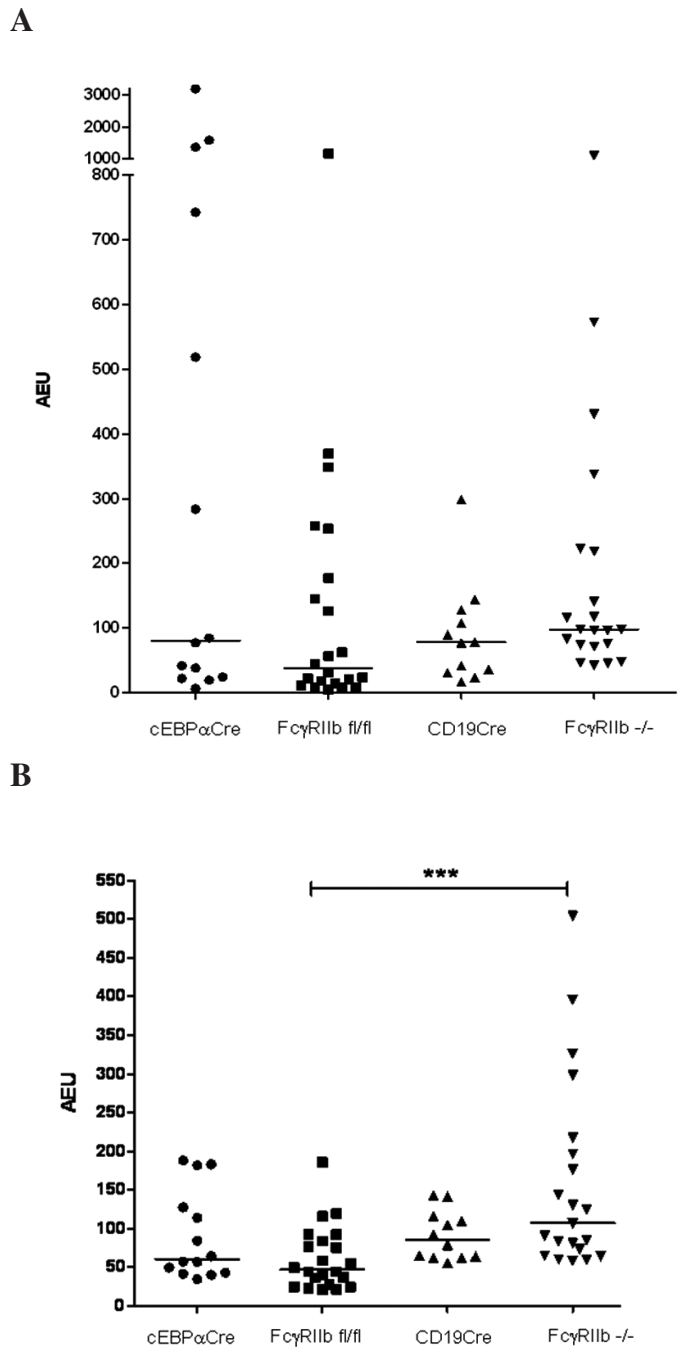

FIGURE 4. Humoral response against mCII in pan-myeloid and B cell specific FcγRIIb KO mice, full FcγRIIb KO mice and floxed FcγRIIb Wt control mice after immunization with cCII. Blood was collected from mice at day 28 after immunization (one week after the boost) and the concentration of total IgG (**Panel A**) and IgG2a (**Panel B**) anti-mouse CII was determined by sandwich ELISA (*** P< 0,05, Kruskal-Wallis test)

mice (n=3) was used as control. IL-1β, IL-6, MCP-2 and MIP-1α were significantly higher expressed in full FcγRIIb KO mice compared to untreated Wt control mice (Figure 7). IL-6 and MCP-2 were significantly higher expressed in the pan-myeloid specific FcγRIIb KO mice. IL-10, was significantly higher expressed in full FcγRIIb KO mice compared to IL-10 expression in untreated Wt control mice. These results reflect the overall higher inflammation in full FcγRIIb KO mice and to a lower extend the pan-myeloid FcγRIIb KO mice compared to B cell specific FcγRIIb KO and FcγRIIb$^{fl/fl}$ Wt control mice.

DISCUSSION

Full FcγRIIb KO mice when backcrossed into C57BL/6 background are highly susceptible to CIA. A prerequisite for disease development is anti-mouse CII antibodies. Immunological tolerance has to be broken for the development of these autoantibodies. It was postulated that FcγRIIB on B cells is a late checkpoint in the maintenance of B cell tolerance. In addition FcγRIIb on B cells plays a role in a negative feedback mechanism that negatively regulates antibody production. Although in B cell specific FcγRIIb KO mice all three measured disease parameters, incidence, severity and autoantibody titers showed the same trend – an increase compared to the corresponding parameters in Wt control mice - this was never significant. We therefore concluded that deficiency

Table III. Anti-mouse Collagen auto-antibody titers (aCII) and incidence and severity of CIA in cell type specific FcγRIIb KO mice after immunization with chicken CII.

	Total αchicken CII IgG titers (%)			
		CIA severity		
	high	severe	mild	low/no
FcgRIIb -/-	52	90.9	9.1	0
CD19Cre x FcgRIIb fl/fl	36	50	50	0
CEBPaCre x FcgRIIb fl/fl	43	83.3	16.7	0
FcgRIIb fl/fl	36	25	25	50
	Titer level middle (%)	CIA severity		
		severe	mild	low/no
FcgRIIb -/-	48	40	0	60
CD19Cre x FcgRIIb fl/fl	23	0	33.3	66.7
CEBPaCre x FcgRIIb fl/fl	36	40	20	40
FcgRIIb fl/fl	23	20	20	60
	Titer level low/no (%)	CIA severity (%)		
		severe	mild	low/no
FcgRIIb -/-	0	0	0	0
CD19Cre x FcgRIIb fl/fl	41	0	0	100
CEBPaCre x FcgRIIb fl/fl	21	0	0	100
FcgRIIb fl/fl	41	0	0	100

A
IL-1β
IL-1β Relative Expression
Ctr
Fcgrllb f/f
CD19Cre
cEBPaCre
Fcgrllb -/-
B
IL-6
IL-6 Relative Expression
Ctr
Fcgrllb f/f
CD19Cre
cEBPaCre
Fcgrllb -/-
C
IL-10
IL-10 Relative Expression
Ctr
Fcgrllb f/f
CD19Cre
cEBPaCre
Fcgrllb -/-
D
MCP-2
MCP-2 Relative Expression
Ctr
Fcgrllb f/f
CD19Cre
cEBPaCre
Fcgrllb -/-

FIGURE 5. qPCR analysis of a selected panel of cytokines and chemokines expressed in the inflamed joints. Expression levels of mRNAs of the cytokines IL-1β (A), IL-6 (B), IL-10 (C) and IFN-γ (G) and the chemokines MCP-2 (D), MIP-1α (E) and MCP-1 (F) were determined by qPCR analysis (* $p < 0.05$ Kruskal-Wallis test).

of FcγRIIb on only B cells has little impact on the susceptible to CIA of C57BL/6 mice. Our results do not support the hypothesis that FcγRIIB on B cell acts as an important late checkpoint for the maintenance of B cell tolerance confirming our previous data with mouse models of SLE. In contrast to the little effect of B cell specific deficiency of FcγRIIb on disease susceptibility, absence of FcγRIIb on almost all cells of the myeloid compartment strongly increases susceptibility to CIA. This can be explained by an important role of FcγRIIb as a modulator of the threshold for the initiation of downstream antibody effector pathways in myeloid effector cells. In all genotypes there is a direct correlation between anti-mCII autoantibody titers and disease incidence. Irrespective their genotype none of the mice with low or undetectable anti-mCII autoantibody titers developed disease whereas the highest incidence of disease was found in mice with high autoantibody titers. Although that means that anti-mouse collagen autoantibodies are a prerequisite for the development of CIA, their presence is not sufficient for disease development because less than 50% of WT mice with high or intermediate autoantibody titers developed disease. This suggests that besides the autoantibody titers additional changes are required to trigger the development of full blown disease. The incidence of CIA was increased up to 80% in mice deficient for FcγRIIb on almost all myeloid cells. Therefore 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 anti-mCII autoantibodies.

In contrast to full FcγRIIb KO mice, pan-myeloid mice did not show a significant increase in median disease severity compared to Wt controls. Moreover, only two out of seven measured cytokines and chemokines were significantly increased compared to untreated controls in the synovium of pan-myeloid FcγRIIb KO mice with severe CIA whereas five of these factors were significantly increased in full FcγRIIb KO mice. There are several explanations why full FcγRIIb KO mice develop more severe disease compared to pan-myeloid FcγRIIb KO mice: a. incomplete deletion of FcγRIIb from myeloid cells in cEBPαCre X FcγRIIb$^{fl/fl}$ mice; b. involvement of FcγRIIb on another cell type than myeloid cells; c. significantly increased anti-mCII IG2a autoantibody titer compared to Wt control mice in full FcγRIIb KO but not in the pan-myeloid FcγRIIb KO mice. Because it is unlikely that FcγRIIb on LSEC or kidney mesanchial cells plays a role in susceptibility to CIA most likely deficiency of FcγRIIb on B cells, although on its own not sufficient to increase susceptibility to CIA, synergizes with deficiency on myeloid cells in the development of the stronger CIA phenotype of the full FcγRIIb KO mouse. Ig2a is the IgG subclass that most effectively initiates downstream antibody effector pathways because it interacts with all activating FcγR, but not the inhibiting FcγRIIb, and with the complement system. It is unclear why full FcγRIIb KO mice immunized with cCII developed significantly increased IgG2a titers compared to Wt untreated controls and full FcγRIIb KO mice immunized with bCII did not. It is unlikely that FcγRIIb on DC plays an important regulatory role in the immune response against cCII and bCII because we analyzed CIA development in DC specific FcγRIIb KO mice (CD11cCre) and

did not find significant changes in any of the measured disease parameters compared to the corresponding parameters in Wt FcγRIIb$^{fl/fl}$ mice. Moreover, in the pan–myeloid cEBPαCre X FcγRIIb$^{fl/fl}$ KO mice the majority of DCs are FcγRIIb deficient but these mice do not have significantly increased IgG2a anti-cCII autoantibody titers.

Surprisingly the Th1 cytokine IFNγ, associated with increased IgG2a titers, was significantly increased in the synovium of B cell specific FcγRIIb KO mice with severe CIA immunized with cCII but not the full FcγRIIb KO mice. However the cytokines were measured only in the small percentage of mice with severe CIA. That means that in these mice additional events had occurred to lower the threshold for the autoantibodies to initiate the development of full blow disease. In full FcγRIIb KO mice or pan-myeloid FcγRIIb KO 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 IFNγ is another way to down regulate the disease threshold because IFNγ upregulates the expression of the activating FcγRI and FcγRIV and in the mean time down regulates the inhibiting FcγRII. This is not the only way to lower the threshold because half of the B cell specific FcγRIIb KO mice and FcγRIIb$^{fl/fl}$ controls with severe disease had little increase of IFNγ expression.

In humans, anti-citrullin autoantibodies develop long before the clinical onset of RA, suggesting that also in humans unknown additional changes are required to trigger the development of full blown RA [31]. Identification of the different pathways that lower the threshold for the induction of the pathology of RA by auto-antibodies such as anti-citrullin 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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CONFLICT OF INTEREST

The authors have no conflict of interest.

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