Immune regulation by receptors for IgG

Boross, P.

Chapter 3

Destructive arthritis in the absence of both FcγRI and FcγRIII

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Destructive Arthritis in the Absence of Both FcγRI and FcγRIII[1]

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Fc receptors for IgG (FcγR) have been implicated in the development of arthritis. However, the precise contribution of the individual FcγR to joint pathology is unclear. In this study, the role of the different FcγR was assessed both in an active and in a passive mouse model of arthritis by analyzing disease development in double and triple knockout (KO) offspring from crosses of FcγRI KO, FcγRIII KO, FcγRI/III double KO, or FcR γ-chain KO with the FcγRII KO on C57BL6 background, which is susceptible for collagen-induced arthritis (CIA). In the active CIA model, onset was significantly delayed in the absence of FcγRIII, whereas incidence and maximum severity were significantly decreased in FcγRI/II/III triple KO but not in FcγRII/III double KO and FcγRI/II double KO mice as compared with FcγRII KO animals. Remarkably, fully destructive CIA developed in FcγRI/II/III triple KO mice. In contrast, FcR γ/FcγRII double KO mice were resistant to CIA. These findings were confirmed with the passive KRN serum-induced arthritis model. These results indicate that all activating FcγR play a role in the development of arthritis, mainly in the downstream effector phase. FcγRIII is critically required for early arthritis onset, and FcγRI can substantially contribute to arthritis pathology. Importantly, FcγRI and FcγRIII were together dispensable for the development of destructive arthritis but the FcR γ-chain was not, suggesting a role for another FcR γ-chain associated receptor, most likely FcγRIV. In addition, FcγRII plays a negative regulatory role in both the central and effector phase of arthritis. ***The Journal of Immunology,*** **2008, 180: 5083–5091.**

It has been well established that Abs are associated with rheumatoid arthritis (RA)[3] (1). However, their direct involvement in disease initiation and progression remains a matter of debate. The promising results with anti-CD20 therapy, targeting Ab-producing B cells in RA, suggest a direct role for this cell type in arthritis (2). The important role of B cells and pathogenic Abs has been confirmed in a large variety of arthritis models in mice including collagen-induced arthritis (CIA) and the K/BxN serum-induced arthritis model (3–5).

CIA is the most widely used animal model of arthritis because it resembles the key features of human RA. Disease is induced by immunization with bovine type II collagen (bCII) in susceptible mouse strains (6). This results in the emergence of bCII-specific T cells and high titers of specific autoantibodies. K/BxN mice, a TCR transgenic mouse crossed onto the non-obese diabetic (NOD) background, develop spontaneous arthritis due to the presence of high titers of autoantibodies directed against glucose-6-phosphate isomerase (GPI) (7, 8). In both models, arthritis can be transferred by sera from sick animals or by purified anti-bCII Abs (anti-bCII moAb-induced arthritis) or anti-GPI Abs, indicating a crucial role for pathogenic immune complexes (IC) in arthritis (4, 9).

IgG-IC crosslinks FcγR, leukocyte receptors for IgG, resulting in the initiation of cellular activation pathways (10). Studies of RA and arthritis in animal models demonstrate that FcγR are crucial players in the pathogenesis of arthritis (11–16). It has been shown that activation of infiltrating or resident cells in the joint leads to severe cartilage destruction, such as matrix metalloproteinase-induced damage or chondrocyte death (17). Activating FcγR have been implicated in this process in the Ag-induced arthritis (AIA) model (13, 18).

Four types of FcγR have been identified in the mouse. FcγRI, FcγRIII, and FcγRIV are multi-subunit receptors that mediate activation signals via the common γ-chain when crosslinked by ICs (19, 20). FcγRII is a single-chain receptor, which inhibits cell activation upon co-engagement with activating FcγR by ICs (21). The balance of activating and inhibiting signals determine the outcome of FcγR signaling in multiple cell types. Dysregulation of this complex system may lead to the emergence of autoimmunity (22).

FcR γ-chain KO mice that lack the expression of all activating FcγR, and C5 KO mice, are substantially protected from CIA and K/BxN serum-induced arthritis (11, 12), indicating that complement and the activating FcγR are indispensable in both models. The crucial role of the activating FcγR has been confirmed with proteoglycan (PG)-induced arthritis and with AIA (23, 24). FcγRIII KO mice show greatly diminished disease activity in CIA on DBA/1 background and in the passive K/BxN serum- and anti-bCII moAb-induced arthritis on mixed 129/C57BL6 background

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[3] Abbreviations used in this paper: RA, rheumatoid arthritis; CIA, collagen-induced arthritis; bCII, bovine type II collagen; KO, knockout; GPI, glucose-6-phosphate isomerase; IC, immune complex; AIA, Ag-induced arthritis; PG, proteoglycan; K/BxN, KRN.

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(11, 25, 26), whereas in AIA, the role of FcγRI and FcγRIII were found to be redundant (27).

FcγRII KO mice have an impaired control over Ab responses and exhibit a hyper responsive phenotype in several in vivo models of inflammation (21). FcγRII KO DBA/1 mice develop more severe CIA than wild-type littermates (12). Moreover, in contrast to wild-type C57BL6 mice, FcγRII KO mice on C57BL6 background are susceptible for the induction of CIA, indicating a role for FcγRII in controlling tolerance (28). Furthermore, FcγRII KO mice show similar (11) or enhanced disease activity compared with wild-type controls (29) in the K/BxN serum-induced arthritis model.

In the light of observations in other IC-mediated disease models, such as hemolytic anemia (30, 31), anaphylaxis, bacterial infections, AIA (31), and in anti-tumor immunotherapy (32, 33), it is surprising that so far no role for FcγRI could be defined in most arthritis models e.g., CIA and the K/BxN serum-induced arthritis. In addition, a prominent role has been described for the recently identified receptor for IgG, FcγRIV in a variety of Ab-mediated immune responses, such as experimental immune thrombocytopenia (20), anti-tumor responses (33), and nephrotoxic nephritis (34) but so far not in arthritis. Experiments performed with single FcγR KO mice may not be suitable to define the highly redundant role of the individual FcγR in arthritis. By establishing C57BL6 strains that in addition to FcγRII lack either FcγRI or FcγRIII or both, the role of individual FcγR in CIA could be assessed. Our results show for the first time that in addition to FcγRIII, FcγRI can contribute to the pathology of CIA. Interestingly, destructive arthritis developed even in the absence of both FcγRI and FcγRIII but not in the absence of the FcRγ-chain, suggesting a role for another γ-chain associated receptor, most likely FcγRIV, in the downstream effector phase of CIA. This could be confirmed in the passive K/BxN serum-induced arthritis model with the same set of KO mice. Furthermore, the results obtained from two different models of arthritis show that FcγRII plays a regulatory role in both the central and the downstream effector phase of arthritis.

Materials and Methods

Mice

The generation of mice deficient for FcγRI (31) and FcγRIII (35) has been described previously. FcγRII KO (21) and FcR γ-chain KO (19) mice were kindly provided by Drs. T. Takai (Tohoku University, Sendai, Japan) and T. Saito (RIKEN Research Center for Allergy and Immunology, Yokohama, Japan), respectively. By intercrossing the three parental single KO strains, a FcγRI/II/III triple KO strain was generated, which was subsequently backcrossed six generations onto C57BL6 background. From the offspring of the intercrosses of the $n = 6$ heterozygous triple KO mice, the different homozygous single, double, and triple KO mice were selected. Their genotypes were routinely checked by PCR and/or FACS analysis. The KRN TCR transgenic strain was a generous gift of Drs. D. Mathis and C. Benoist (Harvard Medical School, Boston, MA) and was maintained on C57BL6 background. B10g7 mice were purchased from Taconic, NY. All mice were backcrossed, bred, and maintained in the SPF unit, and experiments were conducted 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 Federation of European Laboratory Animal Science Associations rules, 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 (MD Biosciences) was dissolved in 0.1 M acetic acid overnight at 4°C at a concentration of 2 mg/ml. Age-matched male mice were immunized at the tail base with 100 μg bCII emulsified in CFA (Difco) and boosted on day 28 with 100 μg bCII in IFA at the same location. Starting from day 14, mice were inspected and scored in a blinded manner three times a week. Disease progress was evaluated visually using an extended scoring protocol (36). In brief, each limb was assigned a score of 0–15 on the basis of the number of joints affected, so that a mouse could reach a total score of 60. An arthritic toe and knuckle was 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.

Passive arthritis induction by K/BxN-serum transfer

K/BxN-serum pools were prepared from arthritic mice generated from the cross between the KRN and B110g7 mice (carrying the NOD-derived g^7 MHC allele) at the age of 6 wk. Arthritis was induced in the recipient strain by i.p. injection (10 μl serum/g body weight) at days 0 and 2. A clinical score was assigned to each mouse as described above. Ankle thickness was measured by a caliper and compared with the baseline value (5).

Anti-bCII Ab titers

Blood was collected from mice by retro-orbital bleeding on the indicated days (day 42 or 60) after immunization, and Ab titers were determined by ELISA. Immuno-Maxisorp plates were coated with 2 μg/ml bCII (Chondrex; MD Biosciences) overnight at 4°C. After washing with PBS-0.05% Tween20, the plates were blocked with PBS/10% milk for 2 h at 4°C. The plates were incubated overnight at 4°C with serially diluted mouse serum. After washing, the plates were subsequently treated with one of the following detection Abs: biotin-conjugated anti-mouse IgG2b (developed with SA-HRP conjugate), HRP-conjugated anti-mouse IgG1, HRP-conjugated anti-mouse IgG2a (BD Biosciences), or HRP-conjugated anti-mouse IgG (Southern Biotechnology Associates). Then, 3,3′,5,5′-tetramethilbenzidine (Sigma-Aldrich) was used as substrate and reaction was detected at 405 nm. bCII-specific Ab titers were compared with a reference of pooled sera of arthritic mice and assigned an arbitrary value.

Anti-GPI ELISA

Recombinant mouse GPI-GST and KRNxNOD serum were a kind gift of Christophe Benoist (Harvard Medical School, Boston, MA). Immunomaxisorp plates were coated with GPI-GST (5 μg/ml) in PBS at 4°C overnight. Plates were blocked with PBS-1% BSA and washed with PBS before incubation with serially diluted serum samples in PBS at room temperature for 1 h. After washing with PBS-1% BSA, titers of isotype-specific anti-GPI were revealed using the detection Abs and substrate as for the anti-bCII ELISA.

Histology and x-ray analysis of arthritic knee joint

Total knee joints were dissected and fixed in 5% phosphate buffered formalin, decalcified in 5% buffered formic acid, dehydrated, and embedded in paraffin. Tissue sections spacing 140 μm representing the whole knee joints were mounted on gelatin coated slides. H&E staining was performed to study the inflammatory cells. To study PG depletion from the cartilage matrix, sections were stained with safranin-*O* and subsequently counterstained with Fast Green. Three adjacent sections showing all cartilage layers (patella/femur and tibia) were used for scoring. Severity of joint inflammation was determined using an arbitrary score (0–3) based on the influx of inflammatory cells (inflammatory cell mass) in the synovium and joint cavity.

Depletion of PG as detected by the loss of red safranin-*O* staining from various cartilage layers (patella and femur) was determined using an arbitrary scale of 0–3. Normal cartilage was scored 0, whereas a cartilage fully depleted of PGs was scored as 3. Erosion was defined as ruffling of the cartilage surface expressed as percentage of impaired cartilage surface of the total cartilage surface. All variables were scored by an experienced investigator.

At the end of the experiments mice were anesthetized and x-ray images were taken.

Statistics

Statistical differences between the KO strains for onset of arthritis and maximum severity were assessed by Student's *t* test. Differences in histological parameters and in serum levels of Abs were assessed with Mann-Whitney *U* test. Arthritis incidence was compared using the Fisher's exact test. A value of $p < 0.05$ was considered significant.

Results

Clinical course of CIA in combined FcγR KO mice

Mice deficient for different combinations of FcγR, established on C57BL6 background, and immunized against bCII were observed

Table I. *Collagen-induced arthritis course in four separate experiments*[a]

	n	Day of Onset Mean ± SEM	Incidence	Max Severity Score Mean ± SD
Experiment 1				
DBA/1	8	34.4 ± 2.7	7/8 (87.5%)	27.3 ± 9.9
FcγRII KO	6	39.4 ± 5.1	5/6 (83.3%)	31.4 ± 20.8
Experiment 2				
FcγRII KO	12	40.4 ± 9.7	7/12 (58.33%)	42.2 ± 19.7
FcγRI/II KO	20	35.6 ± 6.7	13/20 (65%)	41.7 ± 19.1
FcγRII/III KO	23	75.4 ± 7.3*	13/23 (56.52%)	28.9 ± 20.6
FcγRI/II/III KO	17	68.9 ± 12.6*	6/17 (35.29%)	27.5 ± 25.4
Experiment 3				
FcγRI/II KO	12	29.6 ± 6.9	11/12 (91.6%)	35.9 ± 19.9
FcγRII/III KO	15	53.6 ± 8.9*	15/15 (100%)	40.5 ± 17.5
FcγRI/II/III KO	20	60.3 ± 13.2*	8/20 (40%)	15.6 ± 11.5
C57BL6	10	72.0 ± 1*	3/10 (30%)	7.6 ± 6.3
Experiment 4				
C57BL6	9	40	1/9 (11.1%)	3
FcγRII KO	23	28.3 ± 2.7	20/23 (86.9%)	42.2 ± 5.1
FcRγ/FcγRII KO	41	N.A.	0/41 (0%)	N.A.

[a] Mice were immunized with bCII in CFA as described in *Materials and Methods* and monitored for the development of arthritis. Four independent experiments were performed, and mice were inspected blindly three times a week. Incidence stands for the number of mice affected per total number of mice in a group. Arthritic indices only represent sick mice. Asterisk indicates significant differences (*, $p < 0.05$) as compared with FcγRI/II double KO groups (Student's *t* test) N.A.: Not applicable.

for the development of arthritis for at least 125 days. An overview of the four separate experiments (experiments 1–4) using FcγR KO mice is presented in Table I. In Experiment 1, FcγRII KO mice were susceptible to CIA and exhibited a disease course similar to DBA/1 mice confirming published results (Table I, data not shown) (28). Fig. 1 shows that the relative disease incidence and severity between KO strains were similar in experiments 2 and 3. In experiment 3, FcγRII/III double KO mice developed arthritis more rapidly compared with experiment 2, which probably reflects variation between the two immunizations.

FcγRII KO mice developed severe arthritis with an early onset and with a cumulative incidence (experiments 1 and 2) of 66.6% (12/18), similar to previous studies (28). Incidence and severity in FcγRI/II double KO mice were similar to that in FcγRII KO mice (Table I; Fig. 1, *A–D*). We observed attenuated disease progression in FcγRII/III double KO mice. However, at the end point, incidence in both experiments 2 and 3 and severity in experiment 3 were comparable to the incidence and severity of FcγRII KO and FcγRI/II double KO mice (Table I, Fig. 1, *A–D*). FcγRI/II/III triple KO mice developed arthritis with a significantly lower cumulative

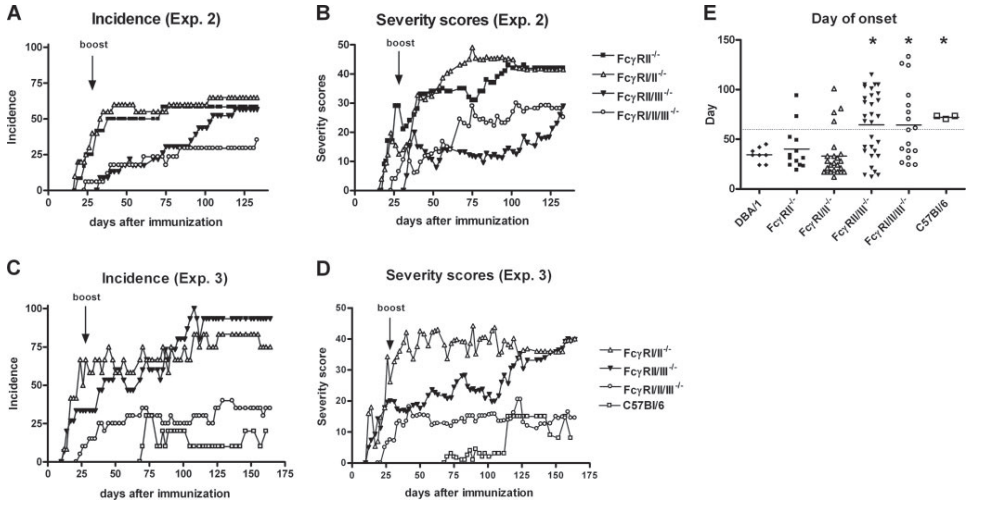

FIGURE 1. Development of CIA in FcγR KO mice. The incidence of arthritis (*A* and *C*) is the percentage of all mice showing arthritic symptoms at a given time point after immunization with bCII in CFA. Arthritis index (*B* and *D*) indicates the mean severity score from all the sick mice in a group on a given day. Results from two separate experiments (2 and 3) are shown; group sizes are given in Table I. *E*, Disease onset of affected mice from CIA experiments 1–3 is plotted and their mean is given. Asterisks indicate significant difference (*, $p < 0.05$) as compared with FcγRII KO and FcγRI/II double KO groups (Student's *t* test).

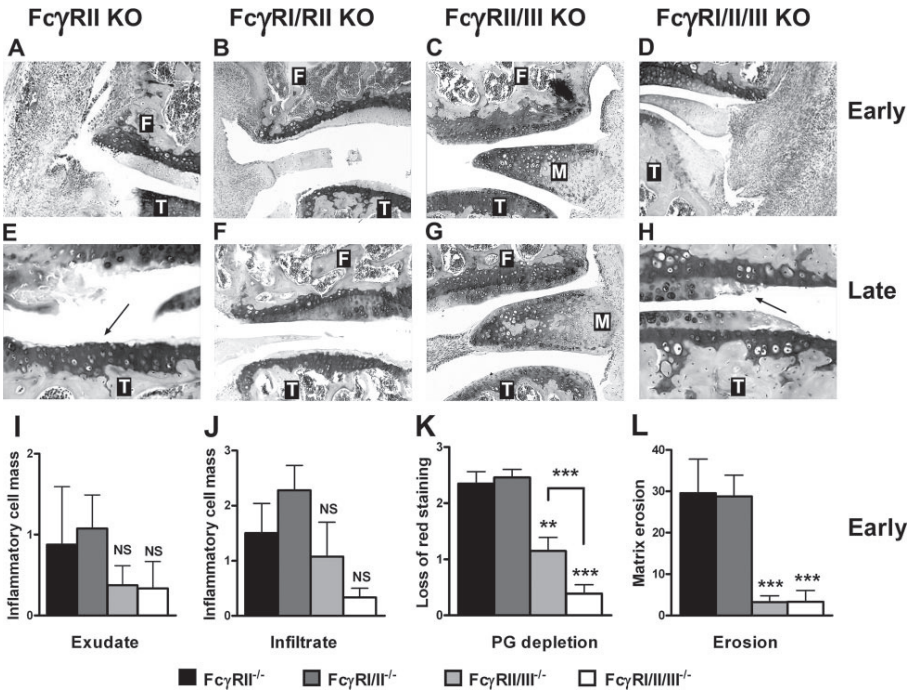

FIGURE 2. Histology of CIA in FcγR KO mice. Representative Safranin *O*-stained knee sections from sick FcγR KO mice are shown (*A–H*). Mice suffering from arthritis for <2 wk (*A–D*; early) or from 60 to 100 days (*E–H*; late). Quantification of histological parameters (*I–L*) in arthritic knee joints in mice with <2 wk of arthritis is shown. Sections were scored for features of inflammation and cartilage destruction using an arbitrary scale (see *Materials and Methods*). Cellular mass in the joint cavity (exudate (*I*)), and in the synovial layer (infiltrate (*J*)) was graded using an arbitrary scale of 0–3 (0 = no cells, 1 = few cells, 2 = moderate, and 3 = maximal within the experiment). Cartilage destruction was measured as PG depletion (*K*) and cartilage erosion (*L*). Joint structures are labeled as follows: femur (F), tibia (T), and the meniscus (M). (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, NS: non significant by Mann-Whitney *U* test) Magnification of original photographs: ×100 (*A–D*, *F*, and *G*) or ×400 (*E* and *H*). The arrows indicate damage of cartilage surface.

incidence (37.8%, 14/37), compared with FcγRI/II double KO (24/32, 75%) and FcγRII/III double KO mice (28/38, 73.7%) ($p < 0.05$, Fisher's exact test). In experiment 2 and 3, maximum severity was also significantly lower in FcγRI/II/III triple KO mice compared with the other three FcγR KO strains (Table I; $p < 0.05$, Student's *t* test). Arthritis in FcγRI/II/III triple KO mice typically affected fewer joints but was clinically similar to the arthritis in the other strains.

The majority of the arthritic FcγRII KO and FcγRI/II double KO mice (83.7% and 84%, respectively) developed arthritis with an early onset (defined as < day 60), similar to DBA/1 mice (Fig. 1*E*). Combined data from experiments 1–3 showed significantly delayed onset of arthritis in FcγRII/III double KO and FcγRI/II/III triple KO mice compared with FcγRII KO or FcγRI/II double KO mice ($p < 0.05$, Student's *t* test). Approximately half of the

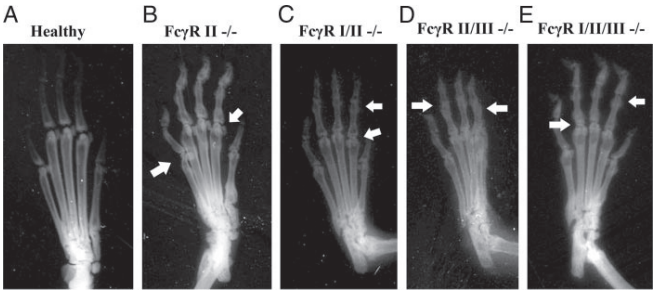

FIGURE 3. Bone destruction in FcγR KO mice in CIA. Representative x-ray images from FcγR KO mice with CIA between 11 and 13 wk (*B–E*). Clear signs of bone destruction can be observed in all genotypes, including the FcγR I/II/III triple KO (E), compared with intact bone surface in healthy animals (*A*).

FcγRII/III double KO and FcγRI/II/III triple KO mice (56.6% and 47%, respectively) developed arthritis with late onset (>day 60) (Fig. 1*E*). In addition, FcRγ/FcγRII double KO mice, lacking functional expression of all known FcγR, were fully resistant to CIA (Table I, data not shown).

A background of spontaneously developing cartilage destruction was observed in some aged (>8 mo) nonimmunized FcγRII KO mice but not in aged C57BL6 mice (data not shown). Moreover, some C57BL6 mice immunized with bCII were scored positive for the development of arthritis at later stage. However, in all cases the effects were very mild. Therefore, we concluded that in the chosen experimental conditions also at late time points (>60 days) development of severe arthritis was dependent on the absence of FcγRII and immunization with bCII.

Taken together, the incidence and maximum severity in CIA were significantly decreased in FcγRI/II/III triple KO mice as compared with FcγRII KO, FcγRI/II, and FcγRII/III double KO animals. In addition, the onset of arthritis was significantly delayed in the absence of FcγRIII. Moreover, we have found that FcR γ-chain is indispensable for the development of CIA in agreement with previous results (12).

Histological analysis of CIA in FcγR KO mice

The characteristic histological features of arthritis, representing different stages of disease progression, such as cell infiltrate (neutrophils, macrophages, and lymphocytes), exudate, proliferation of the synovium, PG depletion, erosion of cartilage, and bone destruction were observed in all arthritic mice analyzed independent from their genotype. Arthritis developed in mice deficient for multiple FcγR (FcγRI/II, FcγRII/III, and FcγRI/II/III), and carried all the histological hallmarks of CIA as observed in FcγRII KO mice. Representative images of knee sections from diseased animals are shown in Fig. 2, *A–H*.

Knee joints that were affected for the same period of time (less than 2 wk) were selected for in depth analysis from three to four arthritic mice of each FcγR KO genotype. Sections from each knee were scored for the degree of PG depletion and cartilage erosion, reflecting early and advanced cartilage damage, respectively.

Exudate and infiltrate were both decreased in the absence of FcγRIII (Fig. 2, *I* and *J*), but the differences were not statistically significant. Both PG depletion and cartilage erosion were significantly decreased in FcγRII/III double and FcγRI/II/III triple KO mice, as compared with FcγRII and FcγRI/II double KO mice ($p < 0.01$, Mann-Whitney *U* test) (Fig. 2, *K* and *L*).

It is noteworthy to point out that we observed mild erosion of the cartilage in knee joints of aged nonimmunized FcγRII KO mice, which suggests that spontaneous autoimmune arthritis can develop in these mice, in addition to the reported spontaneous systemic lupus erythematosus-like phenotype (37).

It has been suggested that destruction of cartilage and bone are independent processes (38), therefore, x-ray analysis was performed to assess bone destruction. After prolonged disease (12–13 wk), severe bone damage and malformation in the joints was observed in the arthritic animals of all FcγR KO genotypes (Fig. 3).

Taken together, these results indicate that although FcγRI and FcγRIII are both involved in the early arthritis pathology; together, they are not absolutely required for the development of fully destructive arthritis in FcγRII KO mice on C57BL6 background.

Anti-collagen Ab titers

It has been shown that FcγRII KO mice exhibit higher specific Ab titers after immunization compared with wild-type mice (21), and elevated anti-collagen titers have been observed during CIA, especially in sick FcγRII KO mice on C57BL6 background (28).

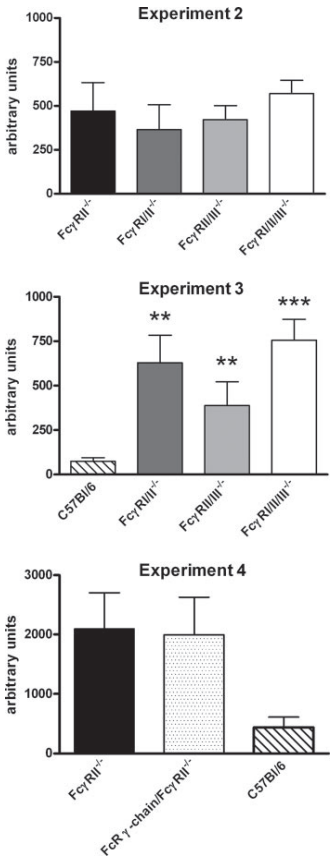

FIGURE 4. Humoral response against collagen type II in FcγR KO mice during CIA. Blood was collected from mice at day 42 (experiments 2 and 3) or 60 (experiment 4) after immunization and the concentration of total IgG anti-bCII was measured. The mean Ab concentration of IgG (± SD) is shown. Bars are labeled as follows: black–FcγRII KO, dark gray – FcγRI/II double KO, light gray – FcγRII/III double KO, white – FcγRI/II/III triple KO, dotted – FcR γ/FcγRII double KO, and striped – C57BL6. Ab levels in mice lacking FcγRII were significantly increased compared with WT controls (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, Mann-Whitney *U* test).

Because differences in the titers of pathogenic collagen-specific Abs could account for the differences in severity, we analyzed anti-collagen Ab titers in blood samples taken at day 42 (experiments 1 and 2) or 60 (experiment 4) after immunization.

All strains that lack FcγRII exhibited higher total IgG anti-bCII titers, as compared with wild-type mice (Fig. 4). Additional deletion of one or more activating FcγR did not influence total anti-bCII IgG titers, confirming previous studies on CIA in FcR γ-chain KO and FcγRIII KO mice in DBA/1 background (12, 25). ICs which contain Abs of the IgG1 isotype preferentially bind to FcγRIII and FcγRII, whereas IgG2a IC bind with high affinity to

Table II. *K/B×N serum-induced arthritis course in two separate experiments*[a]

Experiment	Strain	Incidence	Day of Onset	Max. AT (mm)	Max. Score
Experiment 1	C57BL6	3/3	1,1,1	1.07 ± 0.5	28.3 ± 11.6
	Fcγ RII KO	3/3	1,1,1	1.52 ± 0.19	51.6 ± 2.9
	Fcγ RI/II KO	3/3	1,1,1	0.64 ± 0.36	33.0 ± 16.8
	Fcγ RII/III KO	5/5	1,2,2,2,4	0.70 ± 0.24	24.6 ± 4.7
	Fcγ RI/II/III KO	5/5	1,2,4,9,11	0.26 ± 0.06	8.0 ± 4.3
Experiment 2	C57BL6	3/3	1,1,1	1.7 ± 0.06	44.0 ± 0.5
	Fcγ RI/III KO	5/5	5,5,5,6,8	0.23 ± 0.06	9.8 ± 4.4
	FcR γ-chain KO	0/5	N.A.	0.05 ± 0.01	N.A.

[a] Average maximum severity scores (Max. score) and average maximum ankle thickening (Max. AT) are given as Mean ± SEM. N.A.: Not applicable.

FcγRI (30, 31, 39). FcγRIV binds IgG2a and IgG2b IC with intermediate affinity (20, 40), therefore, subclass distribution in ICs may affect engagement of the different FcγR. No differences in the isotype distribution of anti-bCII titers in the different FcγR genotypes were found (data not shown). Therefore, it is unlikely that skewed isotype distribution of the anti-bCII titers accounts for differences in arthritis development.

K/BxN serum-induced arthritis

To study the role of FcγR exclusively in the downstream effector phase of arthritis, we turned to the passively induced K/BxN disease model (5, 7). Sera from sick K/BxN mice were transferred into the different FcγR KO mice, and arthritis development was monitored as described in *Materials and Methods*. Results from two separate experiments are summarized in Table II. FcγRII KO mice developed more severe arthritis upon serum injection than wild-type controls (Fig. 5*A*). Disease progression in FcγRI/II double KO mice was similar to that in FcγRII KO mice. FcγRII/III double KO mice developed apparent joint inflammation with a delayed onset and significantly decreased maximum severity compared with FcγRII KO mice ($p < 0.01$, Student's t test). All FcγRI/II/III triple KO mice developed disease, however, with significantly milder maximum severity than FcγRII/III double KO mice ($p < 0.05$, Student's t test) (Fig. 5*A*). In addition, FcγRI/III double KO mice showed mild signs of arthritis with a delayed onset, whereas FcR γ-chain KO mice were completely protected (Fig. 5*B*). These results with the K/BxN serum-induced arthritis model suggest a significant role for another γ-chain dependent receptor, most likely FcγRIV, in the effector phase of arthritis, confirming the specific role of the different activating FcγR in arthritis as defined in the active CIA model.

Discussion

Although extensive investigations with a variety of mouse models of arthritis have shown that FcγR play a prominent role in the chronic inflammation of this autoimmune disease (41), the contribution of the individual activating FcγR, particularly FcγRI and FcγRIV, remains unclear. A detailed analysis of the contribution of the individual activating FcγR in arthritis pathology is hampered by the complexity of the IgG Fc receptor family, which

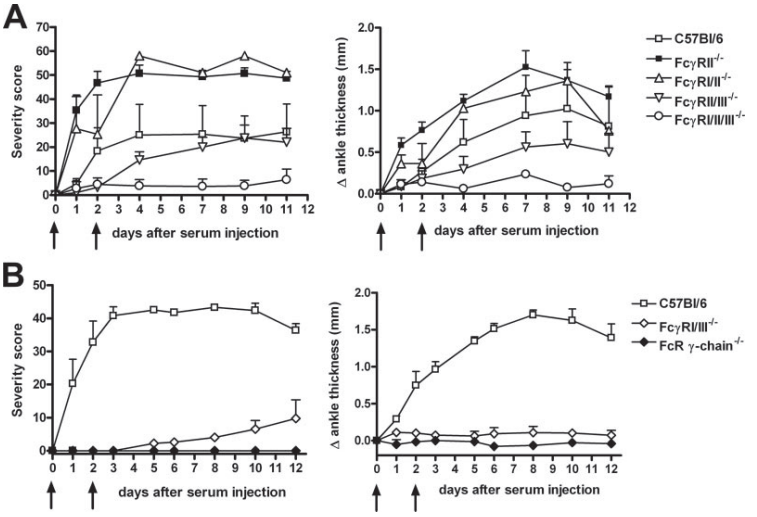

FIGURE 5. Arthritis development in FcγR KO mice after K/BxN serum transfer. Mice were injected with K/BxN sera on days 0 and 2. Arthritis was evaluated by measuring clinical index (score of 0–60) and ankle thickening as described in *Materials and Methods*. Mean ± SEM values are depicted of 3–5 mice/group. Arthritis K/BxN serum was transferred into the following strains: wild type (C57BL6), FcγRII KO, FcγRI/II double KO, FcγRII/III double KO, FcγRI/II/III triple KO (*A*), or wild type, FcγRI/III double KO, FcR γ-chain KO mice (*B*).

comprises four different members that show strong functional redundancy. In the present study, the role of the different FcγR in CIA and K/BxN serum-induced arthritis was analyzed in a unique set of C57BL6 KO mice not only deficient for FcγRII but also for either FcγRI, FcγRIII, both, or the FcR γ-chain. The results confirmed a prominent role for FcγRIII, showed for the first time a role for FcγRI, and suggested a substantial contribution of FcγRIV to the downstream effector phase of CIA and in K/BxN serum-induced arthritis.

One of the most striking and consistent features of the FcγRII/III double KO was the delay in onset and attenuation of disease activity in both the CIA and the K/BxN serum-induced arthritis model, whereas at the end point, incidence was not affected (Figs. 1 and 5). In the early phase of CIA, cartilage destruction correlated with the level of inflammation, which was predominantly dependent on FcγRIII (Fig. 2), confirming results from studies in other arthritis models (13, 18). The prominent role of the low affinity FcγRIII can be explained by its 1) broad expression pattern (macrophages, neutrophils, mast cells, NK cells, and NKT cells); 2) relatively high basal expression level; and 3) broad specificity for IgG (IgG1 > IgG2a > IgG2b). In contrast, the expression pattern of FcγRI is restricted to mononuclear cells and that of FcγRIV to mononuclear cells and neutrophils. These cell types also express FcγRIII at high levels. FcγRI binds IgG2a with high affinity (30, 31, 39) and FcγRIV has been shown to bind both IgG2a and IgG2b IC with intermediate affinity in in vitro studies (20). Both receptors show very low affinity to IgG1 IC (20, 31). Moreover, newly formed IgG2a IC have to compete with monomeric IgG2a for binding to the high affinity FcγRI. Therefore, FcγRIII is in general more accessible to IC.

In both CIA and K/BxN serum-induced arthritis, the role of FcγRI became apparent only when FcγRIII was absent, suggesting that FcγRI is involved downstream of FcγRIII in arthritis pathology and that FcγRIII can compensate for the loss of FcγRI but not the other way around. Surprisingly, 35–40% of FcγRI/II/III triple KO mice developed arthritis, characterized by mild cartilage erosion in the early phase and severe cartilage damage in the late phase culminating in destruction of the underlying bone (Figs. 1–3), whereas the FcR γ-chain/FcγRII double KO was fully resistant. Taken together, these results indicate that another FcR γ-chain associated receptor, most likely FcγRIV, is sufficient for the development of CIA and suggest a shift in the requirement for the different activating FcγR in early and late phases of arthritis. Since the FcR γ-chain is also associated with several other receptor molecules, e.g., subset of TCR on NK T cells, subset of γδ TCR on γδ T cells, Pir-A on B cells, macrophages and dendritic cells, and GPIb and GPVI on blood platelets, a role of one of these receptors in CIA cannot be definitively ruled out. However, the observation that in the passive K/BxN model, in which exclusively Ab mediated down stream effector pathways are involved, the FcγRI/III double KO mice showed significant disease, but the FcR γ-chain KO mice did not (Fig. 5) further supports a role for FcγRIV as shown also recently in other IC-mediated diseases (e.g., nephrotoxic nephritis) (34).

The shift in the requirement in activating FcγR (FcγRIII to FcγRI/FcγRIV) in progressive disease possibly reflects a shift in the dominant cell types involved in the different phases of arthritis. FcγRIII is the only activating FcγR expressed on mast cells and NKT cells, both of which are cell types that have been shown to be involved in arthritis (42, 43). The expression of FcγRI and FcγRIV is low on resting macrophages and increases more strongly in response to proinflammatory cytokines than the expression of FcγRIII (20, 44).

On the basis of observations in the passive models of anti-CII Ab- and K/BxN serum-induced arthritis, a four stage model for the development of arthritis has been proposed (14). In stage 1, when autoantibodies have reached a critical titer, circulating IC cause macromolecular vasopermeability by triggering release of inflammatory mediators (e.g., cytokines, chemokines, and vasoactive amines) by cross linking FcγRIII predominantly on mast cells and neutrophils (14). Because the vasculature of the joints appears to be particularly sensitive to vasoactive amines, this allows Ab entry more specifically to the joints (14, 45). This was confirmed with in vivo imaging studies in the K/BxN model (45). In stage 2, Abs that recognize joint-specific structures will be retained in the joints. In stage 3, cartilage-bound IC activate complement resulting in the release of C5a, which activates many different effector cells e.g., macrophages to produce proinflammatory cytokines and chemokines attracting other effector cells. It has been shown that C5a and IFN-γ can strongly up-regulate FcγRI and FcγRIV on the resident and recruited effector cells e.g., macrophages ((18, 20, 46); unpublished results). This may explain why in other models, such as in AIA, where IFN-γ probably plays a more prominent role, a clear shift from FcγRIII toward FcγRI dependency was observed (27). In stage 4, chronic inflammation results in cartilage destruction and ultimately destruction of the underlying bone. The results presented here, demonstrating a prominent role for FcγRIII and a substantial delay in the onset of the disease in the absence of this receptor, together with the published observations that neutrophils (47) and C5 (11) are indispensable in the development of this disease, strongly suggest that in the active model of CIA the same stages can be recognized.

Since GPI is present in the circulation while collagen is not, the GPI-specific serum from K/BxN mice directly forms macromolecular vasopermeability inducing IC in the circulation resulting in a more effective induction of arthritis compared with the induction of arthritis by anti-collagen Abs/anti-serum. The development of anti-CII Ab-induced arthritis is strongly accelerated by i.v. injection of irrelevant IC, which induces the required macromolecular vasopermeability (14). Most likely, in CIA when the boost with collagen is given while anti-collagen Abs are already present in the circulation, IC are formed in the blood, which cause the required macromolecular vasopermeability.

The inhibitory FcγRII regulates the immune response on multiple levels, from Ag presentation through B cell response to activation of effector cells (48, 49). Moreover, we have previously shown that FcγRII contributes substantially to the clearance of IC (50). In CIA, all strains deficient for FcγRII exhibited higher anti-bCII titers compared with wild-type controls, as expected (28). In the K/BxN serum-induced arthritis, FcγRII inhibited the effector phase confirming results of a previous study (29). These data indicate that FcγRII negatively regulates both the central and effector phase of arthritis.

Remarkably, in the substantially different arthritis models of CIA and K/BxN serum-induced arthritis, for each individual FcγR class similar specific roles could be defined.

Furthermore, the absence of activating FcγR did not alter specific Ab levels in CIA, consistent with previous reports (12, 25). Taken together, these observations strengthen the view that activating FcγR are preferentially involved in the end-stage effector phase of arthritis. Since it is reported that IgG1, an isotype that does not interact with FcγRI and FcγRIV (20, 31), is the dominant subclass of anti-GPI IgG in the K/BxN serum (51), a role of FcγRI and FcγRIV in the passive K/BxN serum-induced arthritis model was somewhat unexpected. However, the small but significant response in FcγRIII KO mice, the lack of a response in FcR γ-chain KO mice, and a response in FcγRI KO mice indistinguishable

from the response of wild-type mice upon injection of K/BxN serum we reported in the past (11) suggests that another FcR γ-chain associated receptor is involved (probably FcγRIV), implicating also that another subclass of IgG, most likely IgG2b, may be present in the serum. Moreover, in contrast to the generally used KRN TCR transgenic mouse on NOD background, in the study presented here a KRN on B10 background, congenic for the required MHC g7, was used as a source for arthritic serum. A direct comparison of serum preparations from both mouse strains in an anti-GPI ELISA revealed that the KRNxB10g7 serum contains a substantially increased amount of IgG2a anti-GPI compared with the KRNxNOD serum. In contrast, the anti-GPI IgG1 and the significant IgG2b titers did not differ between the two sera (data not shown).

Up to 40% of the FcγRI/II/III triple KO mice developed CIA with destruction of cartilage and bone in the late phase of the disease indistinguishable from the same type of destruction in FcγRII KO mice. In contrast, K/BxN serum induced a very mild disease, as measured by joint swelling, in FcγRI/II/III triple KO mice compared with the severe disease in FcγRII KO mice. This relative difference between CIA and K/BxN serum-induced arthritis might reflect differences in the contribution of other immune cells like T cells and NK cells in the two arthritis models. In the active chronic CIA model, activated T cells might serve as a source of cytokines, such as IFN-γ, which enhance FcγRI and FcγRIV expression resulting in a stronger contribution of these receptors to the down stream effector pathways.

The results presented here show some discrepancy to the results of previous studies, (12, 25, 52) which have shown a dominant role for FcγRIII and little or no role for the other FcR γ-chain associated activating Fc receptors. This can be explained by the much higher anti-collagen Ab titers of the arthritic mice in the present study due to the absence of FcγRII (Fig. 4). In the model of experimental autoimmune hemolytic anemia, we have shown that at low concentrations of an anti-mouse erythrocyte IgG2a autoantibody the development of the disease fully depends on FcγRIII, whereas at high concentrations of the same Ab disease development depends on FcγRIII and FcγRI (30). Interestingly, the majority of the FcγRII/III double KO mice develop arthritis with a delayed onset, after day 60 (Fig. 1*E*), which indicates that the role of FcγRIII was somewhat overestimated in previous studies in which CIA experiments were terminated on day 60 or 90 (25, 52). Given the chronic nature of RA, human homologues of FcγRI and FcγRIV might therefore play a significant role in the pathology of arthritis.

In conclusion, using two murine arthritis models, the active CIA and the passive K/BxN serum-induced arthritis, in a unique set of FcγR KO strains, we have further elucidated the role of the individual FcγR in arthritis pathology. In addition to a role for FcγRIII, we have identified a significant role for FcγRI, and another FcR γ-chain associated receptor, most likely FcγRIV, in the end-stage effector phase of arthritis.

Human studies did not reveal a strong association between RA and polymorphisms in the human FcγR gene family, except for FcγRIIIA (53). Recently it was shown that copy number polymorphism of the activating FcγRIIIB predisposes to the chronic inflammatory disease of glomerulonephritis in both rats and humans (54). This observation draws attention to copy number polymorphisms as a possible risk factor for the development of arthritis. Our results suggest that it is highly relevant to analyze copy number polymorphisms of all activating FcγR. Gaining a better insight into FcγR function in autoimmune diseases will provide valuable information for the rational design of therapeutics.

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Disclosures

The authors have no financial conflict of interest.

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