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*Chapter 4*

Joint inflammation and chondrocyte death become  
independent of Fcγ receptor type III by  
local overexpression of interferon-γ during  
immune complex-mediated arthritis

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## Joint Inflammation and Chondrocyte Death Become Independent of Fc $\gamma$ Receptor Type III by Local Overexpression of Interferon- $\gamma$ During Immune Complex-Mediated Arthritis

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**Objective.** It has previously been shown that the onset and the degree of joint inflammation during immune complex (IC)-mediated arthritis depend on Fc $\gamma$  receptor type III (Fc $\gamma$ RIII). Local adenoviral overexpression of interferon- $\gamma$  (IFN $\gamma$ ) in the knee joint prior to onset of IC-mediated arthritis aggravated severe cartilage destruction. In Fc $\gamma$ RI<sup>-/-</sup> mice, however, chondrocyte death was not enhanced by IFN $\gamma$ , whereas matrix metalloproteinase (MMP)-mediated aggrecan breakdown was markedly elevated, suggesting a role for the activating Fc $\gamma$ RIII in the latter process. We undertook this study to determine the role of Fc $\gamma$ RIII in joint inflammation and severe cartilage destruction in IFN $\gamma$ -stimulated IC-mediated arthritis, using Fc $\gamma$ RIII<sup>-/-</sup> mice.

**Methods.** Fc $\gamma$ RIII<sup>-/-</sup> and wild-type (WT) mice were injected in the knee joint with recombinant adenovirus encoding murine IFN $\gamma$  (AdIFN $\gamma$ ) or with adenovirus encoding enhanced green fluorescent protein 1 day prior to induction of IC-mediated arthritis. Histologic sections were obtained 3 days after arthritis onset to study inflammation and cartilage damage. MMP-mediated expression of the VDIPEN neopeptide was detected by immunolocalization. Chemokine and Fc $\gamma$ R

expression levels were determined in synovial washouts and synovium, respectively.

**Results.** Injection of AdIFN $\gamma$  in naive knee joints markedly increased levels of messenger RNA for Fc $\gamma$ RI, Fc $\gamma$ RII, and Fc $\gamma$ RIII. Upon IFN $\gamma$  overexpression prior to induction of IC-mediated arthritis, joint inflammation was similar in Fc $\gamma$ RIII<sup>-/-</sup> and WT mice. The percentage of macrophages in the knee joint was increased, which correlated with high concentrations of the macrophage attractant macrophage inflammatory protein 1 $\alpha$ . Furthermore, IFN $\gamma$  induced 2-fold and 3-fold increases in chondrocyte death in WT controls and Fc $\gamma$ RIII<sup>-/-</sup> mice, respectively. Notably, VDIPEN expression also remained high in Fc $\gamma$ RIII<sup>-/-</sup> mice.

**Conclusion.** IFN $\gamma$  bypasses the dependence on Fc $\gamma$ RIII in the development of IC-mediated arthritis. Furthermore, both Fc $\gamma$ RI and Fc $\gamma$ RIII can mediate MMP-dependent cartilage matrix destruction.

Rheumatoid arthritis (RA) is a chronic inflammatory synovitis characterized by synovial hypertrophy and synovial pannus formation with accompanying destruction of juxtaarticular cartilage and bone (1). Macrophages play a major role in the arthritis process by releasing multiple factors such as proinflammatory cytokines and tissue-degrading enzymes, and several studies have shown that the number of macrophages in the joints of RA patients correlates well with joint inflammation (2) and cartilage damage (3,4).

IgG-containing immune complexes (ICs) are abundantly present in the synovium of most RA patients and play a dominant role in the activation of macrophages (5,6). Fc $\gamma$  receptors (Fc $\gamma$ R) on macrophages interact with IgG-containing ICs (7,8). These receptors for the Fc portion of the IgG molecule play a central role in immune-mediated tissue injury due to their ability to

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recruit effector immune cells (9). Three classes of Fc $\gamma$ R are distinguished on hematopoietic cells: the high-affinity receptor Fc $\gamma$ R type I (Fc $\gamma$ RI [CD64]) and the low-affinity receptors Fc $\gamma$ RII (CD32) and Fc $\gamma$ RIII (CD16). Fc $\gamma$ RI and Fc $\gamma$ RIII are activating receptors associated with a dimer of a signal transduction subunit, the FcR  $\gamma$ -chain, which contains an immunoreceptor tyrosine-based activation motif. The single-chain Fc $\gamma$ RII is an inhibitory receptor containing an immunoreceptor tyrosine-based inhibitory motif in its cytoplasmic domain (10). In a recent study using Fc $\gamma$ RI<sup>-/-</sup> and Fc $\gamma$ RIII<sup>-/-</sup> mice, we found that during IC-mediated arthritis, Fc $\gamma$ RIII mainly regulates the inflammatory response, whereas Fc $\gamma$ RI is more prominently involved in chondrocyte death and cartilage matrix erosion via activation of matrix metalloproteinases (MMPs) (11,12).

Cartilage damage starts with the reversible process of proteoglycan depletion mediated by aggrecanases. If cartilage destruction continues, irreversible collagen fiber degradation occurs. Stromelysin and collagenase are the main MMPs involved in this process (13–15). MMPs are secreted in an inactive form by chondrocytes, stored in the cartilage matrix, and activated after further cleavage (16). MMP activation is primarily found when experimental arthritis is elicited by ICs, which suggests an important role for the IC-binding Fc $\gamma$ R in this process.

Cartilage destruction is more pronounced in T cell-dependent arthritis models, indicating that Th1 cytokines might be of importance. One of the typical Th1 cytokines secreted by T cells is interferon- $\gamma$  (IFN $\gamma$ ). Local overexpression of IFN $\gamma$  during IC-mediated arthritis resulted in more severe cartilage destruction as found in enhanced MMP-mediated proteoglycan degradation, chondrocyte death, and erosion (17). In Fc $\gamma$ RI-deficient mice, chondrocyte death remained low even when IFN $\gamma$  was overexpressed, suggesting a crucial role for Fc $\gamma$ RI (17). However, MMP-mediated cartilage destruction was enhanced by IFN $\gamma$  in arthritic knee joints of Fc $\gamma$ RI<sup>-/-</sup> mice, indicating that Fc $\gamma$ RIII compensates for the absence of Fc $\gamma$ RI.

In the present study, we investigated the particular role of Fc $\gamma$ RIII in joint inflammation and cartilage destruction during IFN $\gamma$ -enhanced IC-mediated arthritis. We found that IFN $\gamma$  aggravates MMP-mediated cartilage damage mediated by activating Fc $\gamma$ RI and Fc $\gamma$ RIII. Furthermore, we showed that both activating Fc $\gamma$ R are redundant in initiating MMP-mediated cartilage destruction, but we confirmed a specific role for Fc $\gamma$ RI in mediating chondrocyte death.

## MATERIALS AND METHODS

**Animals.** Fc $\gamma$ RIII<sup>-/-</sup> mice, deficient for the  $\alpha$ -chain of Fc $\gamma$ RIII, were backcrossed to the C57BL/6 background for 12 generations (18). Homozygous mutants and their wild-type (WT) controls (10–12 weeks old) were used in the experiments. Mice were fed a standard diet and tap water ad libitum. Ethical approval was obtained from the local research ethics committee.

**In vivo overexpression of IFN $\gamma$  using an adenovirus.** The recombinant adenovirus encoding murine IFN $\gamma$  (AdIFN $\gamma$ ) was generated as described previously (19). Adenovirus encoding enhanced green fluorescent protein (AdeGFP) was used as a control. Knees of naive mice were injected intraarticularly with 6  $\mu$ l phosphate buffered saline (PBS) or with 6  $\mu$ l of either AdIFN $\gamma$  or AdeGFP ( $1 \times 10^7$  plaque-forming units). At different time points, patellae with adjacent synovium were dissected in a standardized manner (20), and biopsy samples of synovium were obtained using a biopsy punch with a diameter of 3 mm. Total RNA was extracted in 1 ml TRIzol reagent and used for quantitative polymerase chain reaction (PCR) as described below. PBS, AdIFN $\gamma$ , or AdeGFP was injected intraarticularly 1 day prior to arthritis induction.

**Induction of IC-mediated arthritis.** IC-mediated arthritis was passively induced by injecting 3  $\mu$ g poly-L-lysine-lysozyme into the knee joints of mice that had previously (16 hours earlier) received intravenous injections of polyclonal antibodies directed against lysozyme. These antibodies were raised in rabbits.

**Histology of arthritic knee joints.** Total knee joints of mice were isolated 3 days after arthritis onset. Joints were decalcified, dehydrated, and embedded in paraffin. Tissue sections (7  $\mu$ m) were stained with hematoxylin and eosin. Histopathologic changes were scored using the following parameters. Inflammation was graded on a scale of 0 (no inflammation)–3 (severely inflamed joint) as influx of inflammatory cells (inflammatory cell mass) in the synovium and joint cavity. Chondrocyte death was scored as the amount of empty lacunae, expressed as a percentage of the total amount of cells within the cartilage layers.

**Immunohistochemical detection of macrophage marker F4/80.** F4/80, a murine macrophage membrane antigen, was detected using a specific rat anti-mouse F4/80 IgG (21). Primary antibodies were detected using rabbit anti-rat IgG and avidin-horseradish peroxidase conjugate. Finally, sections were counterstained with hematoxylin. The percentage of macrophages was determined at 2 representative locations of both the synovial lining and joint cavity in 3 different sections of each knee joint. Using a magnification of 200 $\times$ , the percentage of F4/80-positive cells of the inflammatory cell mass present in the visual field was determined using an arbitrary scale of 0–4 (0 = 0%; 1 = 1–25%; 2 = 26–50%; 3 = 51–75%; 4 = 76–100%).

**Immunohistochemical staining of myeloid-related proteins (MRPs) 8 and 14.** Sections were stained as described earlier using a final antibody concentration of 1  $\mu$ g/ml (22). Primary antibodies were detected using peroxidase-conjugated second-stage antibodies against rabbit IgG (Dianova, Hamburg, Germany). Finally, sections were counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany).

**Immunohistochemical VDIPEN staining.** Active MMPs can cleave proteoglycans, resulting in the neoepitope VDIPEN, which can be detected by specific monoclonal antibodies. VDIPEN expression indicates the presence of active MMPs, which also degrade collagen fibers, resulting in severe cartilage damage. To detect VDIPEN, sections were digested with proteinase-free chondroitinase ABC (0.25 units/ml in 0.1M Tris HCl, pH 8.0; Sigma, Zwijndrecht, The Netherlands) to remove the side chains of proteoglycans, followed by incubation with affinity-purified rabbit anti-VDIPEN IgG (23). The primary antibody was detected using biotinylated goat anti-rabbit IgG and avidin–streptavidin–peroxidase (Elite kit; Vector, Burlingame, CA). Counterstaining was done with orange G (2%). Areas of immunostaining were expressed as a percentage of the total cartilage surface.

**Quantitative detection of Fc $\gamma$ R messenger RNA (mRNA) using reverse transcriptase–PCR (RT–PCR).** Levels of specific mRNA for Fc $\gamma$ RI, Fc $\gamma$ RII, and Fc $\gamma$ RIII were detected using the ABI/PRISM 7000 Sequence Detection System (Applied Biosystems/Perkin Elmer, Foster City, CA). Briefly, 1  $\mu$ g of synovial RNA was used for RT–PCR. Messenger RNA was reverse transcribed to complementary DNA (cDNA) using oligo(dT) primers; 1/100 of the cDNA was used in one PCR amplification. PCR was performed in SYBR Green Master Mix using the following amplification protocol: 2 minutes at 50°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C, with data collection in the last 30 seconds. Message for murine Fc $\gamma$ RI, Fc $\gamma$ RII, and Fc $\gamma$ RIII was amplified using the following primers (Biologio, Malden, The Netherlands) at a final concentration of 300 nmoles/liter: for Fc $\gamma$ RI, forward 5'-ACA-CAA-TGG-TTT-ATC-AAC-GGA-ACA-3' and reverse 5'-TGG-CCT-CTG-GGA-TGC-TAT-AAC-T-3'; for Fc $\gamma$ RII, forward 5'-GAC-AGC-CGT-GCT-AAA-TCT-TGC-T-3' and reverse 5'-GTG-TCA-CCG-TGT-CTT-CCT-TGA-G-3'; for Fc $\gamma$ RIII, forward 5'-GAC-AGG-CAG-AGT-GCA-GCT-CTT-3' and reverse 5'-TGT-CTT-CCT-TGA-GCA-CCT-GGA-T-3'. Relative quantification of the PCR signals was performed by comparing the cycle threshold ( $C_t$ ) value of the Fc $\gamma$ R genes in the different samples after correction of the GAPDH content for each individual sample.

**Determination of macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ ) and keratinocyte-derived chemokine (KC) levels.** To determine levels of KC (which is chemotactic for polymorphonuclear neutrophils [PMNs]) and MIP-1 $\alpha$  (which is chemotactic for macrophages) in patella washouts, synovial specimens were isolated in a standard manner, incubated in 200  $\mu$ l RPMI 1640 medium (Gibco BRL, Breda, The Netherlands) for 1 hour at room temperature, and then weighed. Chemokine levels were determined using the BioPlex system from Bio-Rad (Hercules, CA) for the Luminex multianalyte system (Bio-Rad). Chemokine levels were expressed as pg/mg synovium.

**Statistical analysis.** Differences between experimental groups were tested for significance using the Mann-Whitney U test. *P* values less than 0.05 were considered significant.

## RESULTS

**IFN $\gamma$ -induced up-regulation of all 3 Fc $\gamma$ R in the synovial lining.** Since activating Fc $\gamma$ R expressed on synovial macrophages are important in the onset of

**Table 1.** Fc $\gamma$ R mRNA levels in naive knee joints injected with AdeGFP or AdIFN $\gamma$  at different time points\*

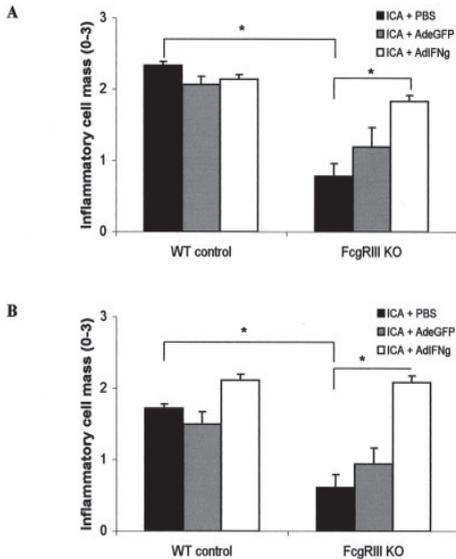
Receptor, treatment	Six hours	Day 1	Day 3	Day 7
Fc $\gamma$ RI				
AdeGFP	1.6	0.5	0	0
AdIFN $\gamma$	4.0	6.2	6.5	3.7
Fc $\gamma$ RII				
AdeGFP	0.3	0.5	0	0
AdIFN $\gamma$	0.1	1.3	1.6	0.5
Fc $\gamma$ RIII				
AdeGFP	0.6	0.5	0	0
AdIFN $\gamma$	0.1	2.6	2.8	1.9

\* Values are changes in the cycle threshold value ( $\Delta C_t$ ). Shown are expression profiles of Fc $\gamma$  receptor type 1 (Fc $\gamma$ RI), Fc $\gamma$ RII, and Fc $\gamma$ RIII mRNA levels after injection of adenovirus encoding enhanced green fluorescent protein (AdeGFP) or recombinant adenovirus encoding murine interferon- $\gamma$  (AdIFN $\gamma$ ) in synovium samples isolated at different time points. Synovium samples from 4 knee joints were pooled in each experiment, and mRNA was isolated. The  $C_t$  values for Fc $\gamma$ RI, Fc $\gamma$ RII, and Fc $\gamma$ RIII in naive knee joints were subtracted from the  $C_t$  values for these Fc $\gamma$ R at different time points after injection.  $C_t$  values were corrected for GAPDH content for each individual sample. Data are the mean of 2 experiments.

IC-mediated arthritis, we first investigated the ability of IFN $\gamma$  to regulate Fc $\gamma$ R expression in the synovium. AdIFN $\gamma$  or the control AdeGFP virus was injected into naive knee joints of C57BL/6 mice, and levels of mRNA for the activating Fc $\gamma$ RI and Fc $\gamma$ RIII and the inhibiting Fc $\gamma$ RII were detected in synovial specimens. Injection of the control virus resulted in a slight increase in the mRNA level for Fc $\gamma$ RI, but not in that for Fc $\gamma$ RIII, and the mRNA level for Fc $\gamma$ RI returned to baseline 3 days after injection (Table 1).

When AdIFN $\gamma$  was injected, IFN $\gamma$  was found in synovial washouts at a high level (2,870 pg/ml) on day 1, but was already undetectable on day 2. This high peak of IFN $\gamma$  resulted in a significant increase in Fc $\gamma$ RI mRNA as soon as 6 hours after injection ( $\Delta C_t = 4$ ), and this remained high until day 7 ( $\Delta C_t = 3.7$ ) (Table 1). In contrast, Fc $\gamma$ RIII mRNA levels were not yet elevated at 6 hours, but increased significantly thereafter. Moderate levels of Fc $\gamma$ RIII were found both at 24 hours and at 7 days after injection ( $\Delta C_t = 2.6$  and 1.9, respectively), but these were clearly lower than levels of Fc $\gamma$ RI. IFN $\gamma$  also induced up-regulation of inhibitory Fc $\gamma$ RII mRNA on days 1 and 3 ( $\Delta C_t = 1.3$  and 1.6, respectively).

**IFN $\gamma$  bypasses IC-mediated joint inflammation in Fc $\gamma$ RIII-deficient mice, resulting in inflammatory cell mass similar to that found in WT controls.** In a previous study, we found that Fc $\gamma$ RIII was the dominant activating receptor involved in the onset of IC-mediated arthritis, since cell influx was largely blocked in

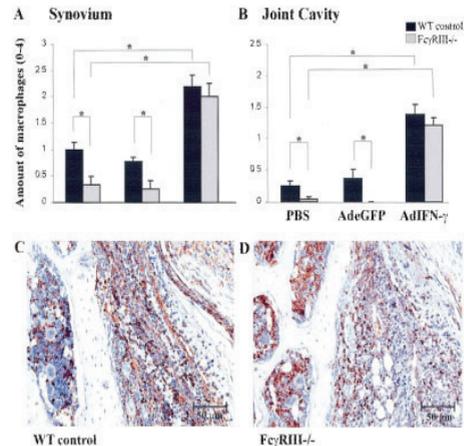


**Figure 1.** Inflammation in arthritic knee joints of wild-type (WT) control and Fc $\gamma$  receptor type III-deficient (Fc $\gamma$ RIII<sup>-/-</sup>) mice determined as the amount of inflammatory cells in the synovium (A) and in the joint cavity (B) using an arbitrary scale of 0–3 (0 = none; 1 = minor; 2 = moderate; 3 = maximal). The inflammatory cell mass was significantly increased in Fc $\gamma$ RIII<sup>-/-</sup> mice after injection with recombinant adenovirus encoding murine interferon- $\gamma$  (AdIFN $\gamma$ ). Values are the mean and SEM (n = 6 mice). \* =  $P < 0.05$  by Mann-Whitney U test. ICA = immune complex-mediated arthritis; PBS = phosphate buffered saline; AdeGFP = adenovirus encoding enhanced green fluorescent protein; KO = knockout.

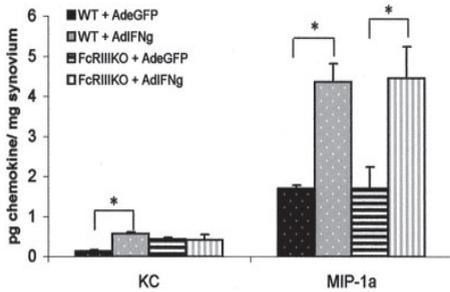
Fc $\gamma$ RIII<sup>-/-</sup> mouse arthritic knee joints (11). Injection of AdIFN $\gamma$  into Fc $\gamma$ RIII<sup>-/-</sup> mouse knee joints followed by induction of IC-mediated arthritis led to a 100% increase of the inflammatory cell mass on day 3, to a level comparable with that found in WT control mouse arthritic knee joints. In contrast, inflammation in PBS- or control AdeGFP virus-injected Fc $\gamma$ RIII<sup>-/-</sup> mouse arthritic knee joints remained inhibited compared with that in WT control mouse arthritic knee joints (Figure 1). These results indicate that IFN $\gamma$  bypasses inhibition of joint inflammation.

In addition, we investigated whether the composition of the inflammatory cell mass was similar in Fc $\gamma$ RIII<sup>-/-</sup> and WT control mouse arthritic knee joints. Macrophages, the dominant cell type involved in cartilage destruction within this arthritis model, were detected using an antibody directed against F4/80. The

activation state of the infiltrating inflammatory cells was determined using the markers MRP-8 and MRP-14, which are associated with an activated phenotype of cells present at sites of inflammation. Using an arbitrary scale of 0–4, we found that the amount of macrophages in Fc $\gamma$ RIII<sup>-/-</sup> mouse arthritic knee joints injected with PBS or AdeGFP was low, both in the joint cavity and in the synovium, compared with the amount of macrophages found in arthritic knee joints of WT mice. However, in IFN $\gamma$ -accelerated arthritis in Fc $\gamma$ RIII<sup>-/-</sup> and WT control mouse arthritic knee joints, the percentage of macrophages was similar (Figures 2A and B). Furthermore, it was found that the amount of MRP-8-positive cells, both in the joint cavity and in the synovium, was comparable in Fc $\gamma$ RIII<sup>-/-</sup> mice and their WT controls after injection of AdIFN $\gamma$  (Figures 2C and D). MRP-14 expression on cells in the synovial lining and in the joint cavity was identical to MRP-8 expression (data not shown).

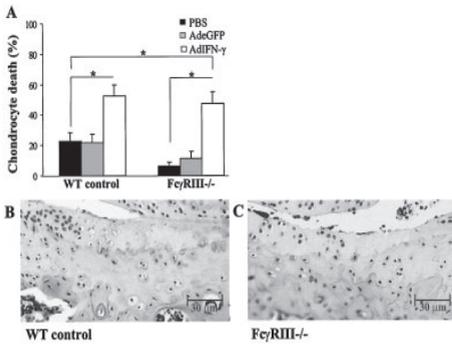


**Figure 2.** Macrophages in the synovial lining (A, infiltrate) and in the joint cavity (B, exudate) in WT control and Fc $\gamma$ RIII<sup>-/-</sup> mice 3 days after induction of immune complex-mediated arthritis. Macrophages were detected using an antibody against F4/80, and the percentage of F4/80-positive cells was quantified using an arbitrary scale of 0–4 (0 = 0%; 1 = 1–25%; 2 = 26–50%; 3 = 51–75%; 4 = 76–100%). Note that after injection of AdIFN $\gamma$ , the percentages of macrophages were comparable in WT control and Fc $\gamma$ RIII<sup>-/-</sup> mice, whereas injection of PBS or AdeGFP resulted in significantly fewer macrophages in Fc $\gamma$ RIII<sup>-/-</sup> mice. Values are the mean and SEM (n = 6 mice). \* =  $P < 0.05$  by Mann-Whitney U test. Representative sections (C and D) show localization of myeloid-related protein 8, which was comparable in arthritic knee joints of WT control mice (C) and Fc $\gamma$ RIII<sup>-/-</sup> mice (D) 3 days after induction of immune complex-mediated arthritis (original magnification  $\times 200$ ). See Figure 1 for definitions.

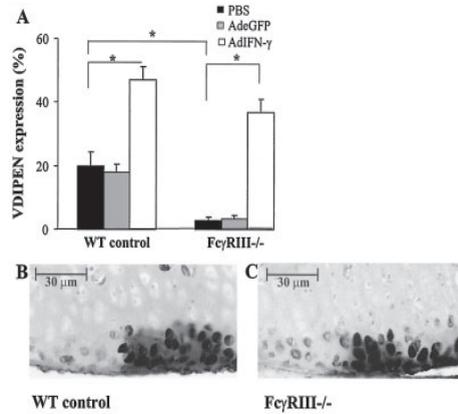


**Figure 3.** Levels of keratinocyte-derived chemokine (KC) and macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ ) measured in patella washouts from arthritic knee joints of WT control and Fc $\gamma$ RIII $^{-/-}$  mice injected with AdeGFP or AdIFN $\gamma$ . Note that IFN $\gamma$  induced a significant up-regulation of MIP-1 $\alpha$  both in WT control mice and in Fc $\gamma$ RIII $^{-/-}$  mice. Values are the mean and SEM ( $n = 5$  mice). \* =  $P < 0.05$  by Mann-Whitney U test. See Figure 1 for other definitions.

**Complete restoration of chemokine production in Fc $\gamma$ RIII $^{-/-}$  mice during IFN $\gamma$ -driven IC-mediated arthritis.** In the presence of IFN $\gamma$ , the amount of inflammatory cells found in arthritic knee joints of WT control and Fc $\gamma$ RIII $^{-/-}$  mice was comparable. IFN $\gamma$  overexpression increased the influx of macrophages. We also investigated macrophage and neutrophil chemokine



**Figure 4.** Lack of involvement of Fc $\gamma$ RIII in regulating chondrocyte death in IFN $\gamma$ -stimulated immune complex-mediated arthritis. Chondrocyte death after injection of PBS, AdeGFP, or AdIFN $\gamma$  in WT control and Fc $\gamma$ RIII $^{-/-}$  mice was determined 3 days after arthritis onset (A). Note that chondrocyte death was significantly enhanced by IFN $\gamma$  both in Fc $\gamma$ RIII $^{-/-}$  mice and in WT control mice. Values are the mean and SEM ( $n = 6$  mice). \* =  $P < 0.05$  by Mann-Whitney U test. Representative sections show chondrocyte death in the cartilage layer of AdIFN $\gamma$ -injected arthritic knee joints of WT control mice (B) and Fc $\gamma$ RIII $^{-/-}$  mice (C) (original magnification  $\times 400$ ). See Figure 1 for definitions.



**Figure 5.** Enhancement of matrix metalloproteinase (MMP)-mediated cartilage destruction by IFN $\gamma$  in arthritic knee joints of Fc $\gamma$ RIII $^{-/-}$  mice. MMP-mediated proteoglycan damage (measured as VDIPEN expression) after injection of PBS, AdeGFP, or AdIFN $\gamma$  in WT control and Fc $\gamma$ RIII $^{-/-}$  mice was determined 3 days after arthritis onset (A). Note that IFN $\gamma$  significantly increased VDIPEN expression both in Fc $\gamma$ RIII $^{-/-}$  mice and in WT control mice. Values are the mean and SEM ( $n = 6$  mice). \* =  $P < 0.05$  by Mann-Whitney U test. Representative sections show VDIPEN expression in AdIFN $\gamma$ -injected arthritic knee joints of WT control mice (B) and Fc $\gamma$ RIII $^{-/-}$  mice (C) (original magnification  $\times 400$ ). See Figure 1 for other definitions.

production in the arthritic knee joints. MIP-1 $\alpha$  (which is chemotactic for macrophages) and KC (which is chemotactic for PMNs) protein levels were determined in synovial washouts using the BioPlex method. Knee joints injected with AdIFN $\gamma$  showed a significant up-regulation of MIP-1 $\alpha$  (from 1.5 pg/mg synovium to 4.5 pg/mg synovium), whereas levels of KC remained low ( $< 1$  pg/mg synovial tissue) (Figure 3), which may explain the elevated macrophage influx. No significant differences were found between Fc $\gamma$ RIII $^{-/-}$  and WT control mouse synovial washouts (4.3 pg/mg synovial tissue and 4.5 pg/mg synovial tissue, respectively).

**Lack of involvement of Fc $\gamma$ RIII in regulating chondrocyte death in IFN $\gamma$ -stimulated IC-mediated arthritis.** Since the amount and activation state of macrophages in the early phase of IC-mediated arthritis are similar in Fc $\gamma$ RIII $^{-/-}$  and WT control mice in the presence of IFN $\gamma$ , we further investigated whether IFN $\gamma$  also bypasses Fc $\gamma$ RIII in late-phase cartilage destruction. Chondrocyte death is a characteristic feature in late-phase IC-mediated arthritis and is one of the causes of irreversible cartilage destruction. Chondrocyte death

was determined in knee joints by measuring empty lacunae as a percentage of the total amount of chondrocytes in various cartilage layers. Injection of AdIFN $\gamma$  significantly increased chondrocyte death (by up to 50%) in cartilage layers of WT control and Fc $\gamma$ RIII $^{-/-}$  mice (Figure 4).

**Enhancement of MMP-mediated cartilage destruction by IFN $\gamma$  in arthritic knee joints of Fc $\gamma$ RIII $^{-/-}$  mice.** We also determined the extent of cartilage breakdown mediated by MMPs, which have previously been shown to be responsible for induction of severe irreversible breakdown of the cartilage matrix. MMP-mediated cartilage damage in arthritic knee joints was determined using immunolocalization of neopeptides in proteoglycans (VDIPEN expression) and was scored in various cartilage layers within the knee joint. Local overexpression of IFN $\gamma$  resulted in marked VDIPEN expression both in WT control mouse knee joints and in Fc $\gamma$ RIII $^{-/-}$  mouse knee joints (45% and 35%, respectively, in the total cartilage surface) (Figure 5) compared with knee joints that had received PBS or AdeGFP before onset of IC-mediated arthritis.

## DISCUSSION

In the present study, we demonstrated that the Fc $\gamma$ RIII dependency of joint inflammation during IC-mediated arthritis can be bypassed by local overexpression of IFN $\gamma$ . Furthermore, we showed that both activating Fc $\gamma$ RI and Fc $\gamma$ RIII are able to initiate MMP-mediated cartilage damage, and we thereby confirmed the specific linkage between activation of Fc $\gamma$ RI and chondrocyte death.

In a previous study using Fc $\gamma$ RIII $^{-/-}$  mice, we found that the onset of IC-mediated arthritis is highly Fc $\gamma$ RIII dependent, whereas IC-mediated arthritis was not inhibited in Fc $\gamma$ RI $^{-/-}$  mice (11). Here we show that local IFN $\gamma$  expression in the knee joint can bypass this Fc $\gamma$ RIII dependency. Synovial lining macrophages, which determine the onset of IC-mediated arthritis (24–26), express low levels of Fc $\gamma$ RIII, whereas Fc $\gamma$ RI is not expressed. IFN $\gamma$  induces strong up-regulation of activating Fc $\gamma$ RI and, to a lesser extent, Fc $\gamma$ RIII, on macrophages (27). In accordance with this is our finding that local overexpression of IFN $\gamma$  in the knee joint significantly enhanced Fc $\gamma$ RI expression in the synovium. These results led us to speculate that when Fc $\gamma$ RI is highly expressed, as in IFN $\gamma$ -stimulated IC-mediated arthritis, joint inflammation could be induced by this receptor.

In the present study, the control virus also in-

duced a slight increase in Fc $\gamma$ RI expression. This was probably due to production of low amounts of IFN $\gamma$  by macrophages, as a response to the adenovirus (28). This enhanced Fc $\gamma$ RI expression can also account for the somewhat higher cell influx found in arthritic knee joints injected with AdeGFP compared with PBS-injected arthritic knee joints.

T cells or T cell-derived cytokines are also able to regulate Fc $\gamma$ R expression on macrophages either directly (29) or indirectly by producing cytokines like IFN $\gamma$  (30). This can explain why during a T cell-mediated arthritis such as antigen-induced arthritis (AIA), joint inflammation has been shown to follow an Fc $\gamma$ RI-dependent pathway (12), whereas Fc $\gamma$ RIII dependency is completely lost. In contrast, in non-T cell IC-mediated arthritis models, such as the K/BxN model (31) or our passive IC model (11), joint inflammation was highly Fc $\gamma$ RIII dependent. The increase in joint inflammation in Fc $\gamma$ RIII $^{-/-}$  mice after onset of IFN $\gamma$ -stimulated IC-mediated arthritis was not due to a direct effect of IFN $\gamma$ , since overexpression of IFN $\gamma$  in naive knee joints induced a negligible amount of joint inflammation (17).

Since the percentage of macrophages is related to the severity of cartilage destruction (3,4), and no difference in inflammatory mass was present between WT control and Fc $\gamma$ RIII $^{-/-}$  mice when IFN $\gamma$  was overexpressed, comparison of cartilage damage between these groups was simplified. MMPs mediate severe cartilage destruction found in IC-mediated arthritis. Interleukin-1 induces chondrocytes to release latent MMPs that are stored in the cartilage matrix (32,33). Moreover, synovial macrophages and fibroblasts are also involved in the production of latent MMPs (34). Activation of proMMPs leads to destruction of proteoglycans and type II collagen fibers that form the cartilage matrix (13–15). The factors involved in activation of proMMPs are still not identified. However, recent studies using Fc $\gamma$ R-deficient mice have shown that Fc $\gamma$ R are crucial in activation of latent MMPs (11,12).

Using Fc $\gamma$ RIII $^{-/-}$  mice, we demonstrated in the present study that Fc $\gamma$ RI can mediate cartilage destruction by metalloproteinases. Up-regulation of Fc $\gamma$ RI compensated for the absence of Fc $\gamma$ RIII, resulting in comparable amounts of VDIPEN in cartilage layers of Fc $\gamma$ RIII $^{-/-}$  and WT control mice. Earlier, we found that IFN $\gamma$  overexpression in Fc $\gamma$ RI $^{-/-}$  mice during IC-mediated arthritis also enhanced VDIPEN expression (17). Combining these results, it can be concluded that both Fc $\gamma$ RI and Fc $\gamma$ RIII have the potential to mediate MMP-mediated proteoglycan destruction.

Normally, the concentration of IFN $\gamma$ , which pref-

erentially induces Fc $\gamma$ RI expression and, to a lesser extent, Fc $\gamma$ RIII expression, is low during experimental arthritis. However, during T cell-dependent AIA, a shift of Fc $\gamma$ RIII toward Fc $\gamma$ RI was observed. Fc $\gamma$ RI became the dominant receptor involved in MMP-mediated cartilage damage, whereas Fc $\gamma$ RIII dependency was completely lost (12). In the present study, we found that the presence of high amounts of IFN $\gamma$  within the knee joint not only results in a shift in expression levels from Fc $\gamma$ RIII to Fc $\gamma$ RI, but also induces a strong up-regulation of Fc $\gamma$ RIII. This may explain why Fc $\gamma$ RIII still plays a role in MMP-mediated cartilage destruction under these conditions.

Apart from differences in the amount and/or balance of the two activating Fc $\gamma$ R expressed within the synovium, there may also be a difference in the potential of the two receptors to drive severe cartilage destruction. In contrast to Fc $\gamma$ RIII, Fc $\gamma$ RI is a high-affinity receptor for IgG. Binding of IgG-containing ICs results in production of oxygen radicals, which have been shown to be potent regulators of gene activation through redox signaling (35,36). This may be reflected by chondrocyte death, another parameter of severe cartilage damage, which appeared to be significantly aggravated during IFN $\gamma$ -stimulated arthritis. Previously, we found that in Fc $\gamma$ RI<sup>-/-</sup> mice, chondrocyte death remained low even in the presence of IFN $\gamma$  (17), indicating that Fc $\gamma$ RI is the crucial Fc $\gamma$ R mediating this process. The specific role for Fc $\gamma$ RI in chondrocyte death was confirmed in the present study, since IFN $\gamma$  overexpression in Fc $\gamma$ RIII<sup>-/-</sup> mice resulted in high levels of chondrocyte death similar to those in controls. Since Fc $\gamma$ RI is exclusively expressed on macrophages (37), this proves that macrophage activation is crucial in the induction of chondrocyte death.

Binding of IgG to Fc $\gamma$ RI leads to intracellular signaling involving activation of phospholipase D<sub>1</sub>, and eventually leads to activation of NADPH oxidase (38). IFN $\gamma$  itself or products of Fc $\gamma$ RI signaling might further augment NADPH oxidase function. Elevation of the oxidative burst may lead to high concentrations of the relatively long-lived H<sub>2</sub>O<sub>2</sub> (39). H<sub>2</sub>O<sub>2</sub> is able to act on more distant targets; it easily penetrates cell membranes and has been shown to kill cells by apoptosis (40). In accordance with this, overproduction of the glycolytic enzyme glucose oxidase in the knee joint generated high levels of H<sub>2</sub>O<sub>2</sub> and caused severe chondrocyte death (41).

Increased expression of Fc $\gamma$ RI induced by IFN $\gamma$  is also found in RA patients. In a previous study, Quayle et al (42) found that neutrophils isolated from synovial

fluid of RA patients expressed higher levels of Fc $\gamma$ RI, whereas no surface expression of Fc $\gamma$ RI was detected on blood neutrophils either from patients or from healthy controls. This indicates that Fc $\gamma$ RI expression is induced when inflammatory cells enter the diseased joint. Furthermore, it was found that stimulation of neutrophils from healthy controls with RA synovial fluid induced Fc $\gamma$ RI expression, and this stimulating effect could be abrogated by addition of anti-IFN $\gamma$  antibody (42). This increase in Fc $\gamma$ RI expression induced by IFN $\gamma$  may affect the ability to respond to IgG-containing ICs, which are abundantly present in synovial fluid and synovium from RA patients (5,6). The present study highlights the fact that enhanced Fc $\gamma$ RI expression induced by IFN $\gamma$  in arthritic knee joints indeed alters the arthritis response, resulting in increased severity of cartilage destruction in experimental IC-mediated arthritis.

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