Fc γ RI Up-Regulation Induced by Local Adenoviral-Mediated Interferon- γ Production Aggravates Chondrocyte Death during Immune Complex-Mediated Arthritis

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Using various Fc γ R-deficient mice, we have obtained suggestive evidence that Fc γ RI on macrophages is responsible for severe cartilage destruction during arthritis mediated by immune complexes (ICs). This role of Fc γ RI is pronounced in the presence of activated Th1 cells and a likely Th1 cell-derived cytokine mediating up-regulation of Fc γ RI expression is interferon (IFN)- γ . We now investigated whether local overexpression of IFN- γ using an adenoviral vector is able to elevate cartilage destruction during experimental immune complex-mediated arthritis (ICA) and to what extent this process is Fc γ RI-mediated. IFN- γ overexpression during ICA had no significant effect on the total cell mass infiltrating the knee joint. However, a higher percentage of macrophages expressing markers for a proinflammatory phenotype was found and these macrophages were situated in close proximity of the cartilage surface. Interestingly, cartilage destruction as studied by matrix metalloproteinase (MMP)-mediated proteoglycan damage (VDIPEN expression), chondrocyte death, and erosion was significantly increased. This effect of IFN- γ was only found in the presence of ICs, as IFN- γ overexpression during zymosan-induced arthritis, which is not IC-dependent, did not lead to severe cartilage destruction. These results imply a crucial role for ICs and the IgG-binding receptors in the aggravation of cartilage damage by IFN- γ . Local overexpression of IFN- γ induced increased Fc γ RI mRNA levels in synovium. To study whether this up-regulation of Fc γ RI mediates aggravation of cartilage destruction, ICA was raised in Fc γ RI^{-/-} and their wild-type controls. IFN- γ resulted in elevated VDIPEN expression, which was still present in Fc γ RI^{-/-}. Of great interest, chondrocyte

death remained low in Fc γ RI^{-/-}. These results indicate that IFN- γ overexpression deteriorates cartilage destruction in the presence of ICs and that Fc γ RI is crucial in the development of chondrocyte death. (*Am J Pathol* 2003, 163:743–752)

Rheumatoid arthritis is characterized by chronic inflammation and cartilage destruction. Macrophages play a key role in the onset and progression of rheumatoid arthritis. Elegant studies performed by Breshnihan and colleagues^{1,2} have shown that the abundance and activation of macrophages in the inflamed synovial membrane and pannus correlates closely with the severity of cartilage destruction in rheumatoid arthritis.

Macrophages are present in the synovial intimal layer, which covers the inside of diarthrodial joints. Experimental studies in our laboratory have shown that synovial-lining macrophages are involved in onset, propagation, and exacerbation of experimental arthritis mediated by immune complexes (ICs).^{3–5}

IgG-containing ICs are abundantly found in rheumatoid arthritis synovium⁶ and are thought to be involved in activation of infiltrated and resident hematopoietic cells. ICs can activate macrophages by binding to Fc receptors for IgG (Fc γ Rs).^{7,8} Three classes have been described in the mouse: the high-affinity receptor Fc γ RI, and the two low-affinity receptors Fc γ RII and Fc γ RIII.⁹ Fc γ RI and Fc γ RIII trigger cell activation through a common γ -chain that contains an immunoreceptor tyrosine-based activation motif.^{10–12} In contrast, Fc γ RII contains an immunoreceptor tyrosine-based inhibitory motif that inhibits via co-crosslinking activation signals through immunoreceptor tyrosine-based activation motif-containing receptors.^{13,14} Murine macrophages express all three classes of Fc γ Rs.

Recently, we have found that Fc γ RI is involved in cartilage destruction during experimental arthritis mediated by ICs¹⁵ and this role seemed to be even more pro-

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nounced when T cells are also involved, as in the chronic antigen-induced arthritis.¹⁶ The T cell subsets mediating antigen-induced arthritis are not exactly defined yet. However, this model shows similarities with the collagen type II-induced arthritis,^{17–19} in which Th1 cells are of importance. One of the most characteristic mediators primarily released by Th1 cells is interferon (IFN)- γ . IFN- γ has a wide variety of proinflammatory actions such as activation of macrophages to produce inflammatory mediators and promoting the killing of intracellular organisms.^{20–22} IFN- γ is also known to induce a marked up-regulation of Fc γ RI expression.^{23–25}

In the present study we investigated whether local overexpression of IFN- γ using an adenoviral vector aggravates cartilage destruction in a Fc γ RI-dependent manner. Local overexpression of IFN- γ induced only deterioration of cartilage destruction during immune complex-mediated arthritis (ICA), whereas no effects were found when IFN- γ was overexpressed during zymosan-induced arthritis (ZIA), which is an IC-independent model. As IFN- γ is able to up-regulate Fc γ RI, Fc γ RI mRNA levels were detected in synovium. An increase of Fc γ RI mRNA levels was found and to define the role of Fc γ RI in the deterioration of cartilage destruction when IFN- γ was overexpressed, we used selective Fc γ RI-deficient mice. Our findings indicate that local overexpression of IFN- γ aggravates cartilage destruction only in presence of ICs, and that chondrocyte death is mediated by Fc γ RI-dependent processes.

Materials and Methods

Animals

C57BL/6 mice were purchased from Charles River Lab (Sulzfeld, Germany). Fc γ RI^{-/-} mice (Dr. Verbeek) were backcrossed to the BALB/c background for four generations.²⁶ Homozygous mutants and their wild-type controls (10 to 12 weeks old) were used in the experiments. Mice were fed a standard diet and tap water *ad libitum*.

Overexpression of IFN- γ in Vivo Using an Adenovirus

The recombinant adenovirus-encoding murine IFN- γ (AdIFN- γ) was generated as described before.²⁷ As control adenovirus AdeGFP, encoding green fluorescent protein, was used. Knee joints of naive mice were intra-articularly injected with 6 μ l of phosphate-buffered saline (PBS) or with 6 μ l of either AdIFN- γ or AdeGFP ($1 \cdot 10^7$ pfu). At different time points, patellae with adjacent synovium were dissected in a standardized manner²⁸ and synovium biopsies were taken using a biopsy punch with a diameter of 3 mm. Total RNA was extracted in 1 ml of TRIzol reagent²⁹ (Invitrogen, Carlsbad, CA) and used for quantitative polymerase chain reaction (PCR) as described below. PBS, AdIFN- γ , or AdeGFP were intra-articularly injected 1 day before arthritis induction.

Induction of Immune Complex-Mediated and Zymosan-Mediated Arthritis

Polyclonal antibodies directed against lysozyme were intravenously injected into mice. These antibodies were raised in rabbits. ICA was then induced by injecting 3 μ g of PLL-Lysozym in 6 μ l of pyrogen-free saline into the right knee joint. Zymosan arthritis was induced by injecting 180 μ g of sterilized zymosan into the right knee joint.

Joint Swelling

Joint swelling was determined by ^{99m}Tc uptake measurements of the knee joint at days 1 and 3 after arthritis induction.³⁰ Briefly, mice were injected with 12 μ Ci of ^{99m}Tc and sedated with 4.5% chloral hydrate. After 30 minutes, the amount of radioactivity was determined by external gamma counting. Arthritis was scored as the ratio of ^{99m}Tc uptake in the right (R) and the left (L) knee joint. R:L ratios >1.1 were taken to indicate significant swelling of the right knee.

Histology of Arthritic Knee Joints

Total knee joints of mice were isolated at day 3 after induction of arthritis. For standard histology, joints were decalcified, dehydrated, and embedded in paraffin. Sections of 7- μ m thick were made and stained with hematoxylin and eosin. Serial sections were scored by two observers on decoded slides. Inflammation was graded on a scale from 0 (no inflammation) to 3 (severe inflamed joint) as influx of inflammatory cells in synovium and joint cavity. Chondrocyte death was scored as the amount of empty lacunae expressed as percentage of total amount of cells within the cartilage layers. Cartilage erosion was scored by expressing the amount of eroded cartilage as percentage of the total cartilage surface. Chondrocyte death and erosion were determined using cartilage surfaces of the lateral femur-tibia, and medial femur-tibia and data shown are the mean chondrocyte death and erosion expression present in these cartilage layers.

Immunohistochemical Staining of Polymorphonuclear Cells (PMNs)

Sections were stained as described earlier using NIMP-R14, a specific rat anti-mouse PMN monoclonal (diluted 1:50).³ Primary antibodies were detected using rabbit anti-rat peroxidase. Finally, sections were counterstained with hematoxylin. The percentage of PMNs was determined in two representative locations of the synovial lining and joint cavity. A total of 100 cells was counted and the amount of brown-stained cells was expressed as percentage PMNs of the total cell population.

Immunohistochemical Staining of Myeloid-Related Proteins (MRPs)-8 and -14

Sections were stained as described earlier using a final antibody concentration of 1 μ g/ml.^{31–33} Primary antibody

ies were detected using peroxidase-conjugated second-stage antibodies against rabbit IgG (Dianova, Hamburg, Germany). Finally, sections were counterstained with Mayer's hematoxylin (Merck, Germany). The percentage of MRP-8- and MRP-14-positive cells was determined in two representative locations of the synovial lining and joint cavity. A total of 100 cells was counted and the amount of red-stained cells was expressed as percentage of activated macrophages of the total cell population.

Immunohistochemical VDIPEN Staining

Sections were digested with proteinase-free chondroitinase ABC (0.25 U/ml in 0.1 mol/L Tris-HCl, pH 8.0; Sigma, Zwijndrecht, The Netherlands) to remove the side chains of PGs. Subsequently, sections were treated with 1% H₂O₂, 1.5% normal goat serum, and affinity-purified rabbit anti-VDIPEN IgG.^{34–36} Thereafter, sections were incubated with biotinylated goat anti-rabbit IgG, and avidin-streptavidin-peroxidase (Elite kit; Vector, Burlingame, CA). Development of the peroxidase staining was performed. Counterstaining was done with orange G (2%). Areas of immunostaining were expressed as percentage of the total cartilage surface. The cartilage layers of the lateral femur-tibia and medial femur-tibia were used to determine the percentage of VDIPEN expression and data shown are the mean of these cartilage surfaces.

Measurement of IFN- γ by Enzyme-Linked Immunosorbent Assay

To determine the levels of IFN- γ in washouts, patellae with adjacent synovium were isolated in a standard manner and incubated in RPMI 1640 medium (Gibco BRL, Breda, The Netherlands) for 1 hour at room temperature. IFN- γ levels in the supernatants were measured using a specific sandwich enzyme-linked immunosorbent assay. The capture antibody, monoclonal rat-anti-mouse IFN- γ (Pharmingen, San Diego, CA), was coated overnight in a 96-well plate. After incubating with the supernatants, wells were washed three times and incubated using a biotinylated antibody, rat-anti-mouse IFN- γ (Pharmingen, San Diego, CA). The second antibody was detected using poly-horseradish peroxidase and subsequently developed using 3,3',5,5'-tetramethyl benzidine/ureum peroxidase solution. Absorbance was measured at 492 nm. The cytokine concentration in the samples was calculated as pg/ml using recombinant murine IFN- γ as standard in the calibration curve.

Determination of MIP-1 α and KC Levels

To determine levels of KC and MIP-1 α in patellae washouts, patellae were isolated in a standard manner and incubated in RPMI 1640 medium (Gibco BRL, Breda, The Netherlands) for 1 hour at room temperature. Chemokine levels were determined using the BioPlex system from BioRad (Hercules, CA) in combination with multiplex cy-

Table 1. Primers for Detection of Murine Fc γ RI mRNA

Primer sequence	
GAPDH	
Up	5'-GGC-AAA-TTC-AAC-GGC-ACA-3'
Low	5'-GTT-AGT-GGG-GTC-TCG-CTC-CTG-3'
Fc γ RI	
Up	5'-ACA-CAA-TGG-TTT-ATC-AAC-GGA-ACA-3'
Low	5'-TGG-CCT-CTG-GGA-TGC-TAT-AAC-T-3'

Primer sequences for quantitative PCR on synovium samples.

tokine and chemokine kits (catalog no. 10-plex is 171-F11100) for the Luminex multianalyte system.

Quantitative Detection of Fc γ RI mRNA Using Reverse Transcriptase-PCR

Specific mRNA-level for Fc γ RI was quantified using the ABI/PRISM 7000 Sequence Detection System (ABI/PE, Foster City, CA). Briefly, 1 μ g of synovial RNA was used for reverse transcriptase-PCR. mRNA was reverse-transcribed to cDNA using oligodT primers and 1/20 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 GAPDH and Fc γ RI was amplified using specific primers (Biolegio, Malden, The Netherlands) for GAPDH and Fc γ RI (Table 1) at a final concentration of 300 nmol/L. Relative quantification of the PCR signals was performed by comparing the cycle threshold value (Ct) of the Fc γ RI gene in the different samples after correction of the GAPDH content for each individual sample to rule out confounding by variation of the RNA purification and reverse transcriptase step.

Statistical Analysis

Differences between experimental groups were tested for significance using the Mann-Whitney *U*-test with the statistic program GraphPad Prism 3.0. *P* values <0.05 were considered significant.

Results

Kinetics of IFN- γ Overexpression in Naive Knee Joints

AdIFN- γ was injected into the right knee joint of naive mice and subsequently IFN- γ levels were measured in washouts from synovium specimen taken at 6, 24, 48, and 72 hours after injection. IFN- γ was already detected at 6 hours (1230 pg/ml), reached its maximum at 24 hours (2870 pg/ml), and waned thereafter. After 48 hours, IFN- γ was below detection level. Control eGFP adenoviral vector did not induce IFN- γ production.

Because IFN- γ is a proinflammatory cytokine, this short-lasting IFN- γ production within the joint may on

Figure 1. Histology of naive knee joints 24 hours after injection of AdeGFP (A) or AdIFN- γ (B) ($10 \cdot 10^7$ pfu). Injection of both AdeGFP and AdIFN- γ resulted in a mild thickening (arrows) of the synovial lining (S), whereas no inflammatory cell mass was found in the joint cavity (J). Original magnifications, $\times 100$ (A, B), $\times 200$ (insert).

itself lead to inflammation. Using ^{99m}Tc uptake, no swelling was measured in knee joints that received AdIFN- γ or AdeGFP (data not shown). Histology of total knee joint sections showed no inflammatory cell mass in the joint cavity and only a mild activation of the synovial lining was induced by both adenoviruses (Figure 1).

IFN- γ Overexpression Has No Effect on the Amount of Infiltrating Cells, but Increases the Population of Activated Macrophages in the Joint Cavity

To investigate whether local IFN- γ production, in the presence of ICs, leads to enhanced joint inflammation, we injected PBS, AdeGFP, and AdIFN- γ , 1 day before induction of ICA. Injection of either AdIFN- γ or AdeGFP resulted in a 30% decrease in joint swelling, compared to mice that had received PBS (Table 2). Three days after ICA induction, swelling in the AdIFN- γ group and PBS group was comparable (Table 2). The inflammatory cell mass in joint cavity (exudate) and synovium (infiltrate) at day 3, was similar in the PBS, AdeGFP, and AdIFN- γ groups (Table 2).

To study whether IFN- γ overexpression altered the composition of the cell mass, PMNs were stained (Figure 2A). The percentage of PMNs infiltrated in the synovium was similar in the PBS, AdeGFP, and AdIFN- γ group (± 45 to 50%). Interestingly, in the joint cavity the percentage of infiltrated PMNs was significantly lower in the AdIFN- γ group (50%) compared to the PBS and AdeGFP groups (70%), indicating that macrophages are more abundantly present in the AdIFN- γ group.

As stimulation of macrophages with IFN- γ modulates chemokine production,^{37,38} we measured macrophage inflammatory protein (MIP)-1 α , which attracts monocytes³⁹ and KC, a neutrophil attractant.³⁹ IFN- γ overexpression in naive joints, resulted in 220-pg/ml MIP-1 α levels after 3 days, injection of AdeGFP induced only 25 pg/ml. KC levels were not up-regulated by IFN- γ (30 pg/ml), and were similar as found after AdeGFP injection (20 pg/ml). These enhanced MIP-1 α levels might explain the increase of infiltrating macrophages found.

Furthermore, we studied the activation state of macrophages using MRP-8 and MRP-14 as markers. These S100 proteins are associated with an activated phenotype of macrophages present in inflammatory sites. In the synovial layer, the percentage of macrophages express-

Table 2. Inflammatory Response 1 and 3 Days after ICA Induction

	Joint swelling		Inflammatory cell mass	
	Day 1	Day 3	Joint cavity	Synovium
ICA + PBS	1.3 $\pm$ 0.1	1.3 $\pm$ 0.1	1.4 $\pm$ 0.1	1.6 $\pm$ 0.1
ICA + AdeGFP	1.2 $\pm$ 0.1	1.2 $\pm$ 0.1	1.4 $\pm$ 0.1	1.5 $\pm$ 0.1
ICA + AdIFN- γ	1.2 $\pm$ 0.1	1.3 $\pm$ 0.1	1.6 $\pm$ 0.2	1.6 $\pm$ 0.2

Inflammation was determined using joint swelling and inflammatory cell mass in the joint cavity and synovium. Joint swelling of the right inflamed knee joint was quantified using the right:left ratio of ^{99m}Tc uptake in both knee joints. The amount of inflammatory cells in the joint cavity (exudate) and in the synovial layer (infiltrate) were scored in a blinded fashion by two independent observers using an arbitrary scale from 0 to 3 (0, no; 1, minor; 2, moderate; 3, maximal). Note that both joint swelling and inflammatory cell mass were not significantly different between the PBS, AdeGFP, and AdIFN- γ group. Data are the mean $\pm$ SEM of 12 mice and significance was evaluated using the Mann-Whitney *U*-test (*, $P < 0.05$).

Figure 2. The percentage of PMNs (A) and MRP-8-expressing macrophages (B) in the joint cavity (exudate) and synovial lining (infiltrate) 3 days after ICA induction in the PBS, AdeGFP, and AdIFN- γ groups. Note the significantly lower percentage of PMNs and the significant increase of MRP-8-expressing macrophages in the exudate of the AdIFN- γ group. Values represent the mean $\pm$ SEM of 12 mice. Data were evaluated using the Mann-Whitney *U*-test (*, $P < 0.05$).

ing MRP-8 was similar in all groups (40 to 45%) (Figure 2B). However, in the joint cavity the percentage MRP-8-positive macrophages was somewhat, although significantly, increased in the AdIFN- γ group (50% versus 35 to 40% in controls) (Figure 2B). Intriguingly, these MRP-8-expressing macrophages were clustered in the proximity of the cartilage surface. MRP-14 expression on cells in the synovial lining and joint tissue was identical with MRP-8 expression and followed the same pattern as described above in all groups (data not shown).

Local Overexpression of IFN- γ during ICA Results in Aggravation of Severe Cartilage Destruction

In addition, we studied the impact of IFN- γ on cartilage destruction in ICA. MMP-mediated cartilage damage (VDIPEN immunostaining), chondrocyte death, and surface erosion were used as histological parameters. Strikingly, injection of AdIFN- γ and subsequent induction of ICA resulted in a twofold to threefold increase in VDIPEN expression in the cartilage matrix, when compared to both control groups [Figure 3, A and D (AdIFN- γ) versus B and C (controls)]. Furthermore, chondrocyte death as measured by the percentage of empty lacunae within the cartilage layers was two times higher in presence of IFN- γ , compared to the PBS group [Figure 3, A and G (AdIFN- γ) versus E (PBS)] and even four times elevated when compared to the AdeGFP-injected group [Figure 3, A, G (AdIFN- γ), and F (AdeGFP)].

Matrix erosion was determined using the cartilage surfaces of the lateral and medial femur-tibia plateau. Erosion was almost absent in the PBS and AdeGFP group, in line with the early time point chosen (Figure 3; A, E, and F). In contrast, local overexpression of IFN- γ induced erosion, which was found on all cartilage surfaces (Figure 3, A and G). Our results indicate that during an arthritis mediated by ICs, IFN- γ overexpression significantly aggravates irreversible cartilage destruction.

Aggravation of Cartilage Destruction by IFN- γ Is IC-Dependent

To further investigate whether the aggravating effect of IFN- γ on cartilage destruction is specific for ICs, we also induced zymosan arthritis (ZIA). Twenty-four hours before ZIA induction, mice were injected with either PBS, AdeGFP, or AdIFN- γ . IFN- γ resulted in increased number of inflammatory cells in the joint cavity (exudate) at day 3, whereas the infiltrate in the synovium was comparable with both control groups (Table 3). VDIPEN expression, chondrocyte death, and erosion were completely absent in all groups (Figure 4; A to F). These results show that local overexpression of IFN- γ in the knee joint during a non-IC-dependent model, does not elicit irreversible cartilage destruction.

Up-Regulation of Fc γ RI in Synovium by IFN- γ

Because we recently found an important role for Fc γ RI in mediating cartilage destruction, we focused on the expression of this receptor. After injection of AdIFN- γ in knee joints, synovial specimens were isolated at different time points (6 hours, 1, 3, and 7 days) and mRNA levels of Fc γ RI were detected. In naive knee joints Fc γ RI mRNA could not be detected, whereas 6 hours after AdIFN- γ injection, Fc γ RI mRNA level was markedly increased (Δ Ct = 3.97). At day 1, maximal values were found, which remained high until day 7 after injection (Figure 5). Injection of PBS had no effect on Fc γ RI mRNA levels, whereas AdeGFP levels showed an increase at 6 hours (Δ Ct = 1.59), which was decreased at day 1 and completely absent after 3 days (Figure 5).

IFN- γ -Induced Aggravation of Chondrocyte Death is Fc γ RI-Dependent

Because the presence of IFN- γ induced an increase of Fc γ RI mRNA, we further investigated whether this receptor contributed to the aggravation of cartilage destruction using Fc γ RI-deficient mice and their respective wild-type controls. The IFN- γ effect in these wild-type controls was primarily as expected. Fc γ RI^{-/-} and wild-type controls received PBS, AdeGFP, or AdIFN- γ , 24 hours before ICA induction. Joint inflammation was similar in both wild-type controls and Fc γ RI^{-/-} (Table 4). Unexpectedly, IFN- γ enhanced VDIPEN expression was still present in Fc γ RI^{-/-} (Figure 6A). In the Fc γ RI^{-/-} related wild-type controls, IFN- γ greatly increased chondrocyte death, re-

A) Cartilage destruction 3 days after ICA induction

Figure 3. Cartilage destruction, measured as VDIPEN expression (**A–D**), chondrocyte death, and erosion of the cartilage matrix (**A, E–G**), in the PBS, AdeGFP, and AdIFN- γ group 3 days after ICA induction. IFN- γ overexpression resulted in a significant increase in VDIPEN expression (**A** and **D** versus control **B** and **C**), chondrocyte death (**A** and **B** versus control **D** and **E**), and erosion [**A** and **G** (arrows) versus control **D** and **E**]. Values represent the mean $\pm$ SEM of 12 mice. Data were evaluated using the Mann-Whitney *U*-test (**, $P < 0.0001$). Original magnifications, $\times 200$.

sulting in 35% empty lacunae. Of great interest, chondrocyte death remained low in Fc γ RI $^{-/-}$ (3%) (Figure 6B). Unfortunately, erosion of the cartilage surface could not be detected in these wild-type controls after IFN- γ overexpression, hampering analysis of Fc γ RI involvement.

Table 3. Inflammatory Response 3 Days after Zymosan Arthritis Induction

	Inflammatory cell mass	
	Joint cavity	Synovium
ZIA + PBS	0.9 $\pm$ 0.1	1 $\pm$ 0.1
ZIA + AdeGFP	1.3 $\pm$ 0.2	1.2 $\pm$ 0.2
ZIA + AdIFN- γ	1.7 $\pm$ 0.1*	0.8 $\pm$ 0.2

The amount of inflammatory cells in the joint cavity (exudate) and in the synovium (infiltrate) were scored in a blinded fashion by two independent observers using an arbitrary scale from 0 to 3 (0, no; 1, minor; 2, moderate; 3, maximal). Note that the inflammatory cell mass is significantly increased in the AdIFN- γ group, when compared to the PBS and AdeGFP group. Data are the mean $\pm$ SEM of eight mice and significance was evaluated using the Mann-Whitney *U*-test (*, $P < 0.05$).

Discussion

In the present study we demonstrate that overexpression of IFN- γ results in increased chondrocyte death by up-regulation of Fc γ RI. We locally overexpressed IFN- γ in two different arthritis models using an adenoviral vector and found that only in the presence of ICs cartilage damage was aggravated. Using Fc γ RI-deficient mice, it was confirmed that chondrocyte death is mediated by Fc γ RI and that up-regulation of this receptor by IFN- γ directs deterioration of chondrocyte death.

Injection of AdIFN- γ in naive knee joints induced a short-lasting although high peak of IFN- γ . This might be explained by the ability of IFN- γ to shut off the CMV promotor of the adenovirus.⁴⁰ We found that 1 day after injection of AdIFN- γ only a very mild synovial inflammation was induced, indicating that this concentration of IFN- γ was not able to attract large amounts of inflammatory cells. This is in line with a previous study in which recombinant mouse IFN- γ was injected in the peritoneal cavity, and no chemotactic activity for mouse macro-

Figure 4. Cartilage destruction after zymosan arthritis. Cartilage destruction, measured as VDIPEN expression (**A–C**), chondrocyte death, and erosion of the cartilage matrix (**D–F**), in the PBS, AdeGFP, and AdIFN- γ group, respectively, 3 days after ZIA induction. No VDIPEN expression (**C** versus control **A** and **B**), chondrocyte death, and erosion (**F** versus control **D** and **E**) were found. Original magnifications, $\times 200$.

phages or neutrophils was found.⁴¹ When triggers such as IC or zymosan were additionally injected into the joint, a large amount of inflammatory cells infiltrated probably regulated by IL-1⁴² and chemotactic factors.⁴³ Surprisingly, IFN- γ did not alter the amount of infiltrated cells in ICA, whereas in ZIA the inflammatory cell mass in the joint cavity was increased. A possible explanation for this discrepancy might be that IFN- γ elevates joint inflammation dependent on the trigger and receptors involved to induce arthritis. Although AdIFN- γ injection before ICA induction did not contribute to the amount of infiltrated cells, yet the type of infiltrated cells was markedly altered.

The percentage of macrophages was significantly higher in the IFN- γ stimulated joint. This is in line with our finding that IFN- γ elevated levels of MIP-1 α , a potent chemokine of macrophages, but not of KC, which is dominant for attracting PMNs.³⁹ Macrophages in IC-mediated arthritis expressed MRP-8 and -14, which are markers for a proinflammatory phenotype. MRP-8- and MRP-14-expressing macrophages have shown to be the major source of IL-1 and respiratory burst under inflammatory conditions *in vivo*.^{44,45} Because the abundance and activation of macrophages is closely correlated to the severity of cartilage destruction,^{1,2} we further studied this parameter.

mRNA levels of Fc γ RI in synovium

Figure 5. Expression profile of Fc γ RI mRNA levels after injection of PBS, AdeGFP, and AdIFN- γ in synovium specimens at different time points (6 hours, day 1, day 3, and day 7). The Ct value of Fc γ RI in synovium at 0 hours was subtracted from the Ct values for Fc γ RI at different time points after injection. Ct values were corrected for GAPDH content for each individual sample.

Table 4. Inflammatory Response in Fc γ RI^{-/-} Compared to WT Controls after Injection of PBS, AdeGFP, or AdIFN- γ 3 Days after ICA Induction

	Inflammatory cell mass	
	Joint cavity	Synovium
PBS		
WT control	0.7 $\pm$ 0.3	1.7 $\pm$ 0.3
Fc γ RI ^{-/-}	1.1 $\pm$ 0.1	1.9 $\pm$ 0.3
AdeGFP		
WT control	0.9 $\pm$ 0.2	1.6 $\pm$ 0.3
Fc γ RI ^{-/-}	0.7 $\pm$ 0.2	1.6 $\pm$ 0.2
AdIFN γ		
WT control	1.1 $\pm$ 0.2	1.7 $\pm$ 0.3
Fc γ RI ^{-/-}	1.1 $\pm$ 0.1	1.3 $\pm$ 0.2

The amount of inflammatory cells in the joint cavity (exudate) and in the synovium (infiltrate) were scored in a blinded fashion by two independent observers using an arbitrary scale from 0 to 3 (0, no; 1, minor; 2, moderate; 3, maximal). Note that the inflammatory cell mass is not significantly different between the PBS, AdeGFP, and AdIFN- γ group. Data are the mean $\pm$ SEM of six mice and significance was evaluated using the Mann-Whitney *U*-test (*, *P* < 0.05).

Figure 6. Cartilage destruction, measured as VDIPEN expression (A) and chondrocyte death (B) after injection of PBS, AdeGFP, and AdIFN- γ in Fc γ RI $^{-/-}$ and their wild-type controls 3 days after ICA induction. Note that IFN- γ resulted in increased VDIPEN expression in both wild-type controls and Fc γ RI $^{-/-}$. IFN- γ -enhanced chondrocyte death was not found in Fc γ RI $^{-/-}$. Values represent the mean $\pm$ SEM of six mice. Data were evaluated using the Mann-Whitney *U*-test (*, $P < 0.05$; **, $P < 0.0001$).

IFN- γ may directly stimulate chondrocytes resulting in higher expression of latent stromelysin. Apart from that, expression of aggrecan and other core protein genes can decrease.^{46–48} This eventually may lead to enhanced levels of pro-MMPs within the cartilage matrix and decreased proteoglycan synthesis. However, in the present study IFN- γ alone did not induce severe cartilage destruction directly, because injection of AdIFN- γ in naive knee joints failed to induce irreversible cartilage destruction (data not shown) and additional triggers are needed.

When AdIFN- γ was injected before zymosan-induced arthritis, although increased infiltration of inflammatory cells was found, severe cartilage destruction was completely absent. In contrast, when in such an IFN- γ joint, IC-mediated arthritis was induced, a strongly increased MMP-mediated proteoglycan damage, chondrocyte death, and erosion were found. This suggests an important role for IgG-binding Fc γ R. In earlier studies using

Fc γ RI $^{-/-}$, we have found that Fc γ RI is highly involved in mediating severe cartilage destruction during IC-mediated arthritides.^{15,16} In the present study, IFN- γ significantly increased Fc γ RI mRNA levels in the synovium for 7 days which is in line with earlier studies describing up-regulation of Fc γ RI by IFN- γ .^{23–25} This elevated Fc γ RI expression is exclusively present on the macrophage population, because murine PMNs do not express Fc γ RI.⁴⁹ Unfortunately, it was not possible to detect the murine Fc γ RI protein because a specific anti-Fc γ RI antibody is not available. Further proof that elevation of Fc γ RI induced by IFN- γ is responsible for the observed cartilage destruction was obtained by IFN- γ overexpression in mice lacking Fc γ RI.

Chondrocyte death appeared to be specifically mediated by Fc γ RI. Chondrocyte death may be mediated by oxygen radicals. Binding of IgG to Fc γ RI leads to an overkill of the oxidative burst resulting in prominent oxygen radical production.⁵⁰ Apart from that, IFN- γ also regulates the production of nitric oxide by macrophages,⁵¹ which has been shown to induce apoptosis of chondrocytes.⁵² The close interaction between macrophages and the cartilage surface we found makes the above mechanisms highly plausible, because oxygen and nitrogen radicals only produce tissue damage within a short distance. Furthermore, the finding that chondrocyte death is absent in Fc γ RI $^{-/-}$ also indicates that neutrophils are probably not involved, because these cells lack Fc γ RI.⁴⁹

Unexpectedly, we found that IFN- γ still induced cartilage proteoglycan damage mediated by MMPs (VDIPEN epitopes) when Fc γ RI was absent. One explanation may be that in Fc γ RI-deficient mice, the other activating Fc γ RIII is still present and becomes up-regulated after IFN- γ stimulation. As VDIPEN expression was strongly diminished in ICA in both Fc γ RI $^{-/-}$ and Fc γ RIII $^{-/-}$ mice, this indicates that also Fc γ RIII when present in sufficient amounts may significantly contribute to MMP-mediated proteoglycan damage.¹⁵

Eventually activation of MMPs leads to degradation of the collagen type II network and erosion of the cartilage matrix. In contrast to marked erosion after ICA induction in mice with C57BL/6 background, erosion was completely absent in the Fc γ RI $^{-/-}$ but also in their proper controls. As the Fc γ RI $^{-/-}$ are generated in the BALB/c background and the severity of the ICA model is related to the genetic background of the mice,⁵³ this explains the absence of erosion, but hampers evaluation of this aspect at present.

The present study demonstrates that IFN- γ aggravates irreversible cartilage destruction in the presence of ICs implicating an important role for Fc γ RI in mediating chondrocyte death. As ICs and macrophages are abundantly found within the synovia of rheumatoid arthritis patients, local production of IFN- γ within the synovium may induce elevated expression of Fc γ RI on the macrophage, which appears to be a crucial receptor involved in mediating severe cartilage destruction. Fc γ RI may form a new important therapeutic target to combat this crippling disease.

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