Role of Activatory Fc γ RI and Fc γ RIII and Inhibitory Fc γ RII in Inflammation and Cartilage Destruction during Experimental Antigen-Induced Arthritis

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IgG-containing immune complexes, which are found in most RA joints, communicate with hematopoietic cells using three classes of Fc receptors (Fc γ RI, -II, -III). In a previous study we found that if a chronic T-cell-mediated antigen-induced arthritis (AIA) was elicited in knee joints of FcR γ -chain-deficient mice that lack functional Fc γ RI and Fc γ RIII, joint inflammation was comparable but severe cartilage destruction was absent. We now examined the individual role of the stimulatory Fc γ RI and Fc γ RIII and inhibitory Fc γ RII in inflammation and functional cartilage damage in knee joints with AIA using Fc γ RI-, Fc γ RII-, and Fc γ RIII-deficient mice. Three weeks after immunization with the antigen-methylated bovine serum albumin (BSA), cellular (T-cell responses as measured by lymphocyte proliferation) immunity raised against mBSA was comparable in all groups examined. Humoral (total IgG, IgG1, IgG2a, and IgG2b levels) immunity against mBSA was comparable in Fc γ RI-/- and Fc γ RIII-/- but higher in Fc γ RII-/- if compared to controls. Joint swelling as measured by ^{99m}Tc uptake at days 1, 3, and 7 was similar in Fc γ RI-/- and Fc γ RIII-/- mice and significantly higher in Fc γ RII-/. Chronic inflammation and cartilage damage (depletion of proteoglycans, metalloproteinase (MMP)-induced neoepitopes, and matrix erosion) was studied histologically in total knee joint sections stained with hematoxylin or safranin-O. Histologically, at day 7 after AIA induction, exudate and infiltrate in the knee joint was similar in Fc γ RI-/- and Fc γ RIII-/- and significantly higher (230% and 340%) in Fc γ RII-/- mice if compared to controls. Aggrecan breakdown in cartilage caused by MMPs and, which is related to severe irreversible cartilage erosion, was further studied by immunolocalization of MMP-mediated neoepitopes (VDIPEN) and image analysis. MMP-induced neoepitopes determined in various cartilage layers (tibia and femur) were pri-

marily inhibited in Fc γ RI-/- (79 to 87% and 87 to 88%, respectively) and comparable in Fc γ RIII-/. VDIPEN neoepitopes were much higher (82 to 122% and 200 to 250%, respectively) in Fc γ RII-/- mice. Initial depletion of proteoglycans was similar (60 to 100%) in all groups. In the chronic phase, cartilage matrix erosion in the lateral and medial tibia was significantly elevated in Fc γ RII-/- (222% and 186%, respectively) but not in Fc γ RI-/- or Fc γ RIII-/- mice. These results suggest that during T-cell-mediated AIA, Fc γ RI and Fc γ RIII act in concert in acute and chronic inflammation whereas Fc γ RI is the dominant FcR involved in severe cartilage destruction. Fc γ RII is a crucial inhibiting factor in acute and chronic inflammation and cartilage erosion. (*Am J Pathol* 2001, 159:2309–2320)

Chronic inflammation and destruction of cartilage and bone are main characteristics of rheumatoid arthritis (RA).¹ IgG-containing immune complexes (ICs), present in large amounts in joints of most RA patients have been suggested to be major pathogenic factors in RA, responsible for initiation and persistence of the inflammatory cascade and its resulting destruction of the cartilage.² Apart from ICs T cells have shown to also be important in amplification of arthritis^{3,4} and may enhance inflammatory reactions merely induced by ICs.

Immune complexes containing IgG, the dominant immunoglobulin in the circulation, communicate with synovial cells via cellular receptors for IgG that belong to the IgG superfamily.^{5–7} Murine phagocytic effector cells express three different classes of IgG receptors (Fc γ RI, -II, -III).^{8,9} Fc γ RI and Fc γ RIII are hetero-oligomeric complexes in which ligand-binding α chains are associated with the signal-transducing γ -chain. This γ -chain is required for their assembly and triggering of various effector functions including phagocytosis,¹⁰ antigen-presenting function,¹¹ antibody-dependent cytotoxicity,¹² and the release of inflammatory mediators.¹³ These effector

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functions are regulated by an immunoreceptor tyrosine-based activation motif within the γ -chain.¹⁴

The third receptor class for IgG, Fc γ RII is a single α -chain receptor and contains an immunoreceptor tyrosine-based inhibitory motif-containing cytoplasmic domain that by co-ligation of the immunoreceptor tyrosine-based activation motif receptor, inhibits cellular activation signals through the recruitment of the inositol phosphatase SHIP.¹⁵ Fc γ RII has been shown to be a negative regulator of Fc γ RIII in IgG IC-triggered inflammation.¹⁶

Recently we found that activating Fc γ R (Fc γ RI and Fc γ RIII) were crucial in severe cartilage destruction during antigen-induced arthritis (AIA).¹⁷ Irreversible cartilage destruction within this model occurs through enzymatic cleavage by metalloproteinases (MMPs) of cartilage constituents. These Zn-dependent endopeptidases are capable of cleaving aggrecan and collagen type II, the main components of cartilage, which leads to severe cartilage erosion.^{18–20} Various MMPs (MMP-1, -2, -3, -7, -8, -9, -13) have been found to cleave aggrecan between amino residues Asn341-Phe342 resulting in the neopeptide FVDIPEN that remains in the cartilage.²¹

AIA elicited in knee joints of FcR γ -chain $-/-$ lacking functional Fc γ RI and Fc γ RIII showed similar synovial inflammation if compared to controls at day 7 after arthritis induction. Nevertheless, severe cartilage destruction as MMP-mediated matrix destruction and erosion was fully absent in arthritic FcR γ -chain $-/-$ knee joints. These results suggest that Fc γ RI and/or Fc γ RIII are of crucial importance in severe cartilage destruction within this model. Fc γ RIII has been suggested as the most likely candidate in IC-mediated joint inflammation.²² We now investigated the involvement of activating Fc γ RI and Fc γ RIII and the inhibitory Fc γ R in severe cartilage destruction seen during AIA. Expression of MMP-induced aggrecan neopeptides and erosion of the cartilage matrix was investigated in the knee joints of arthritic mice by histology and immunolocalization. Our findings indicate that Fc γ RI and not Fc γ RIII is the dominant activatory Fc receptor involved in severe cartilage destruction in a model in which T cells play a dominant role. In contrast, Fc γ R is involved in inhibition of severe cartilage destruction within this model and may be a new therapeutic target to combat severe cartilage destruction.

Materials and Methods

Animals

Fc γ RIII $-/-$ mice were made deficient for the ligand-binding α -chain of Fc γ RIII (Dr. Verbeek) and were backcrossed to the C57BL/6 background for 12 generations.²³ Fc γ RI $-/-$ were made deficient for the ligand-binding α -chain of Fc γ RI (Dr. Verbeek) and were backcrossed to BALB/c for six generations.²⁴ Fc γ RII $-/-$ were developed by Dr. Takai (Sendai, Japan)²⁵ in the 129 SV (H-2b) and C57BL6 (H-2b) background. Control C57BL/6 and 129SV/C57BL/6 hybrids were derived from Jackson Laboratories (Bar Harbor, ME) and bred in our own facilities. Homozygous mu-

tants and their wild-type controls, 10 to 12 weeks old, were used in the experiments.

Antibody Determination in Serum

Methylated BSA-specific antibodies of various isotypes (total IgG, IgG1, IgG2a, IgG2b, IgG3) were measured in sera of individual mice with an enzyme-linked immunosorbent assay (ELISA). Antigen was coated on microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) at a concentration of 100 μ g/ml. Antibody titers were assessed by twofold serial dilution of the sera followed by detection of bound mouse Ig with 1:500 diluted peroxidase-conjugated rabbit anti-mouse Ig (Miles Laboratories Inc., Elkhart, IN, USA). O-Phenylenediamine (1 mg/ml; Sigma, Zwijndrecht, The Netherlands) was used as substrate for peroxidase, and the antibody titer was determined by using 50% of the maximal extinction as an end point. Sera of Fc γ RI $-/-$, Fc γ RII $-/-$, and Fc γ RIII $-/-$ mice were compared to sera of their wild-type controls. In each group at least 10 mice were tested.

T-Cell Proliferation

Mouse spleen cells were isolated and washed in RPMI supplemented with 10% fetal calf serum, glutamine (2 mmol/L), and pyruvate (1 mmol/L). Erythrocytes were lysed by treatment of the cells with a 0.16 mol/L NH_4Cl solution in 0.17 mol/L Tris, pH 7.2, for 5 minutes. After two washes in RPMI, the cells were plated on plastic T flasks (75 mm^2) from Falcon Plastics, Oxnard, CA. After 60 minutes of incubation at 37°C, the nonadherent cells were harvested by aspiration and two 4- to 5-ml RPMI washes of the adherent cells. One hundred μ l of RPMI containing 1×10^5 T-cell-enriched spleen cells were placed in each well of a sterile, U-bottomed polystyrene microculture plate (Costar, Cambridge, MA). Antigens or mitogens were added in another 100 μ l to give a total volume of 200 μ l, and final concentrations of antigen of 25, 12.5, and 6.25 μ g/ml. Cultures were maintained at 37°C in a humidified atmosphere of 2% CO_2 and 98% air for 4 days. Sixteen hours before harvesting, 1 μ Ci of [^3H]-thymidine (6.7 Ci/mmol; New England Nuclear, Boston, MA) was added in 25 μ l of RPMI. Cultures were harvested with a cell harvester (Tomtec) and [^3H]-thymidine incorporation was determined.

Induction of Experimental Arthritis

Mice were immunized with 100 μ g of methylated BSA (mBSA, Sigma) emulsified in 100 μ l of Freund's complete adjuvant. Injections were divided over both flanks and footpath of the forelegs. Heat-killed *Bordetella pertussis* (RIVM, Bilthoven, The Netherlands) was administered intraperitoneally as an additional adjuvant. Two subcutaneous booster injections with 50 μ g of mBSA/CFA were given in the neck region 1 week after the initial immunization.²⁶ Two weeks after these injections, arthritis was induced by intra-articular injection of either 15 μ g (Fc γ RII $-/-$) or 60 μ g (Fc γ RIII $-/-$, Fc γ RI $-/-$, and

Fc γ RIII $-/-$) of mBSA in 6 μ l of saline into the right knee joint, resulting in chronic arthritis. The approval to induce arthritis in mice was given by the local ethical committee.

^{99m}Tc Uptake Measurements

Joint inflammation was measured by ^{99m}Tc pertechnetate uptake in the knee joint. This method has earlier been shown to correlate well with histological findings.²⁷ Briefly, mice were injected intraperitoneally with 12 μ Ci of ^{99m}Tc and subsequently sedated with chloralhydrate. Thirty minutes thereafter, γ -radiation was assessed by use of a collimated Na-I-scintillation crystal and the knee in a fixed position. Arthritis was scored as the ratio of the ^{99m}Tc uptake in the right (R) and the left (L) knee joint. R:L ratios >1.1 were taken to indicate inflammation of the right knee joint.

Histology

Total knee joints were dissected, fixed in phosphate-buffered formalin (pH 7.4), decalcified in 5% buffered formic acid, and subsequently embedded in paraffin wax. Semiserial frontal whole knee joint sections (7 μ m) were stained with hematoxylin and eosin (H&E) or Safranin-O and Fastgreen. Histological parameters (joint inflammation, proteoglycan depletion, and erosion) were scored by two independent observers in a blinded manner.

Determination of Proteoglycan Depletion

Total knee joint sections were stained with Safranin-O and Fastgreen. Loss of red staining from various cartilage layers (femur and tibia), which is related to loss of proteoglycans, was determined using an arbitrary scale from 0 to 3. Normal cartilage and cartilage fully depleted of proteoglycans was taken as a 0 and 3 value, respectively.

Immunolocalization of MMP-Induced Neoepitope

For immunohistochemical analysis, sections were deparaffinized, rehydrated, and digested with chondroitinase ABC (0.25 U/ml, 0.1 mol/L Tris-HCL, pH 8.0; Sigma) for 1 hour at 37°C, to remove chondroitin sulfate from the proteoglycans. Sections were then treated with 1% H₂O₂ in methanol for 20 minutes and subsequently 5 minutes with 0.1% (v/v) Triton X-100 in phosphate-buffered saline (PBS). After incubation with 1.5% (v/v) normal goat serum for 20 minutes, sections were incubated with affinity-purified anti-VDIPEN IgG overnight at 4°C. These antibodies were kindly given by Irwin Singer and Ellen Bayne (Merck Research Laboratories, Rahway, NJ) and have been extensively characterized before.^{28,29} In addition, sections were incubated with biotinylated goat anti-rabbit IgG and binding detected using avidin-peroxidase staining (Elite kit; Vector Laboratories, Inc., Burlingame, CA). Development of the peroxidase product was done using

nickel enhancement and counterstaining was done with orange G (2%) for 5 minutes.

Immunolocalization of Aggrecanase-Induced Epitopes

Undecalcified cryosections were digested with proteinase-free chondroitinase ABC (0.25 U/ml Tris-HCl, pH 8.0) for 1 hour at 37°C to remove chondroitin sulfate from the PG. Subsequently, sections were fixed with periodate-lysine-paraformaldehyde fixative for 20 minutes. Sections were then treated with 1% H₂O₂ for 20 minutes followed by 5 minutes with 0.1% Triton X-100 in PBS. After incubation with 1.5% normal goat serum for 20 minutes, sections were incubated for 18 hours with the primary antibody recognizing the sequence NITEGE.³⁰ Then sections were incubated with biotinylated goat anti-rabbit IgG and were detected using avidin-peroxidase staining. Development of the peroxidase product was done using nickel enhancement. Counterstaining was done with orange G.

Immunolocalization of IgG

Paraffin-embedded total knee joint sections were pre-treated with chondroitinase ABC and additionally stained with goat anti-mouse IgG peroxidase overnight. Development of the peroxidase product was done using diaminobenzidine (0.5 mg/ml). Sections were counterstained with H&E.

Measurement and Characterization of Cartilage Erosion

Erosion and chondrocyte death was determined in total knee joint sections stained with H&E. Erosion was detected as ruffling of the cartilage surface and was only mild at day 7 after AIA induction. Ruffling of the cartilage surface was determined using an arbitrary scale of 0 to 3. Normal cartilage surface and maximal ruffling within this experiment was taken as a 0 and 3 value, respectively.

Results

Role of the Activatory Fc γ RIII and Inhibitory Fc γ RII in Acute and Chronic Joint Inflammation during AIA

As the absence of a particular Fc γ R may alter the immunological response against methylated BSA during immunization, thereby impairing the onset and course of arthritis, we first tested cellular and humoral immunity to mBSA, 3 weeks after immunization. Cellular immunity as measured by spleen lymphocyte proliferation against mBSA showed no significant differences between knock-outs and their controls (Figure 1; B, D, and F). In addition,

Figure 1. Humoral and cellular immunity in FcγRI^{-/-}, FcγRIII^{-/-}, and FcγRII^{-/-} mice, and their wild-type controls was determined 7 days after induction of AIA. Humoral immunity was determined by measuring levels of various isotypes of antibodies (total IgG, IgG1, IgG2a, IgG2b) raised against mBSA using ELISA. Data of ELISA are the mean of the determination in sera of 10 mice. Mean is expressed as two-log values using 50% of the maximal extinction as an endpoint (**A**, **C**, and **E**). Cellular immunity was determined by measuring T-cell proliferation in the presence of mBSA. ³H-thymidine incorporation was measured in cpm of spleen T lymphocytes derived from arthritic mice that were stimulated with various concentrations of mBSA (25, 12.5, 6.25, 3.1, and 0 μg/ml) (**B**, **D**, and **F**). The results were expressed as stimulation indexes (ratio stimulation with and without antigen) and are the mean of eight animals. Significance was tested using the Wilcoxon rank test (*, *P* < 0.05).

humoral immunity was measured by ELISA. Total IgG, IgG1, IgG2a, IgG2b, and IgG3 anti-mBSA levels were high but not significantly different in immunized FcγRI^{-/-} and FcγRIII^{-/-} if compared to their controls (Figure 1, A and C). In sera of FcγRII^{-/-} immunized mice however, total IgG, IgG2a, and IgG3 anti-mBSA were fourfold higher and IgG1 was even eightfold higher. IgG2b anti-mBSA was not significantly different (Figure 1E).

To investigate the role of a particular FcγR in joint inflammation, AIA was elicited by injection of 60 μg of mBSA directly into the knee joints of immunized FcγRI^{-/-}, FcγRIII^{-/-}, and FcγRII^{-/-} mice and their wild-type controls. Because FcγRIII^{-/-} has been described as a main regulator of IC diseases, we expected a down-regulation of swelling in FcγRIII^{-/-} mice but knee swelling was not significantly different from controls at all time points measured (Figure 2B).

The course of knee joint swelling in FcγRI^{-/-} was also comparable (Figure 2A) suggesting that FcγRI and FcγRII are redundant with respect to joint inflam-

mation. Subsequently we investigated the role of FcγRII in joint inflammation. Because FcγRII^{-/-} mice have been shown to be highly vulnerable to ICs, injection of 60 μg of mBSA into the knee joint may be too high and for that reason we also injected a lower (15 μg) mBSA dose. Injection of 15 μg of mBSA into knee joints of FcγRII^{-/-} mice resulted in a significantly higher knee joint swelling if compared to controls (2.4 versus 1.7) at day 1 but no difference was found anymore at day 4 or day 7 after AIA induction (Figure 2C). Injection of 60 μg of mBSA led to a much higher swelling at day 1 (3.0 versus 2.0) and compared to controls was still significantly higher at day 7 after AIA induction (2.0 versus 1.1) (Figure 2D).

In addition, cellular infiltration and exudate in the knee joint was studied by histology. At day 7 after induction of AIA, total knee joint sections were made and stained with H&E. In FcγRI^{-/-} and FcγRIII^{-/-} arthritic knees, similar exudate and infiltrate was measured in all animals studied (Figure 3, A and B). In arthritic knees of FcγRII^{-/-}

Figure 2. R:L ratios of ^{99m}Tc uptake at various days (1, 4, and 7) after intra-articular injection of mBSA in knee joints of mBSA-immunized FcγRI-/- (A), FcγRIII-/- (B), and FcγRII-/- (C and D) mice, and their wild-type controls. In knees of FcγRI-/- and FcγRIII-/- mice, 60 µg of mBSA was injected (A and B) and in knees of FcγRII-/- mice, 15 or 60 µg of mBSA (C and D) was injected. Values represent the mean $\pm$ SD of 10 mice. Data were evaluated using the Wilcoxon rank test (*, $P \leq 0.05$). Note the significantly higher joint swelling in FcγRII-/- mice, after injection of 15 µg of mBSA at day 1 (C) and after injection of 60 µg of mBSA at days 1, 4, and 7 (D).

deficient mice, both exudate and infiltrate were found to be significantly elevated (180% and 242% in the 60-µg group, respectively) (Figure 3D). This reached significance only in mice injected with the high dose of mBSA (Figure 3; D, E, and F).

As the total number of inflammatory cells present in the synovium at day 7 after AIA was not different in FcγRI-/- and FcγRIII-/- and higher in FcγRII-/- mice, we further investigated whether the absence of a particular Fc receptor might influence the type of inflammatory cell present in the joint. The polymorphonuclear leukocyte/macrophage ratio was determined by immunolocalization using NIMP-R14 that stains polymorphonuclear leukocyte specifically. In arthritic knee joints of FcγRI-/-, FcγRIII-/-, and FcγRII-/- mice and their controls, no differences were found in the polymorphonuclear leukocyte/macrophage ratio that was 40:60 in the exudate and 25:75 in the infiltrate (data not shown).

As Fc receptors have been shown to be involved in removal of ICs from various body compartments, the presence of IgG ICs localized in the arthritic joints at day 7 AIA was determined using rabbit anti-murine IgG antibodies. No significant differences in IC deposition was found in the knee joints of FcγRI-/-, FcγRIII-/-, and FcγRII-/- mice and their controls (data not shown).

Activatory FcγRI and FcγRIII and Inhibitory FcγRII Are Not Involved or Redundant in Loss of Proteoglycans from Cartilage Layers in Knee Joints with AIA

Subsequently, we studied the role of FcγRI, FcγRIII, and FcγRII in cartilage damage. The earliest cartilage damage seen during experimental arthritis is loss of proteoglycans from the cartilage matrix that is evident between 24 and 48 hours after AIA induction. Proteoglycan breakdown was measured by determining the loss of red staining in safranin O-stained knee joint sections using an arbitrary scale from 0 to 3. At day 7 after induction of AIA, loss of red staining in the cartilage layers of femur and tibia in control mice injected with 60 µg of mBSA reached almost maximal values (Figure 4; A, B, and D). In arthritic knee joints of FcγRI-/- (Figure 4A), FcγRIII-/- (Figure 4B), and FcγRII-/- (Figure 4D) mice, proteoglycan depletion was not significantly different from their arthritic control groups. Injection of the 15-µg mBSA dose showed lower PG depletion (Figure 4C). Although proteoglycan depletion was slightly higher in the FcγRII-/- mice, this difference did not reach significance.

FcγRI Activates Whereas FcγRII Inhibits the Induction of MMP Cleavage Site Neopeptide VDIPEN during AIA. No Effect of FcγRIII on VDIPEN Expression

Within AIA, MMPs have been shown to be involved in degradation of aggrecan^{31,32} and collagen³³ leading to irreversible cartilage destruction. MMPs degrade aggrecan leaving the C terminal ending with the amino acid sequence VDIPEN that can be detected by specific antibodies around day 5 after induction of AIA.³² For this reason, AIA day 7 was taken to detect VDIPEN expression in the cartilage matrix.

The amount of VDIPEN was measured by determining the area of cartilage expressing VDIPEN using automated image analysis. In all investigated knee joints of wild-type mice injected with 60 µg of mBSA, prominent VDIPEN staining was found in the cartilage layers of tibia and femur (Figure 5; A to D). Strikingly, in arthritic knees of FcγRI-/-, VDIPEN expression was significantly lower in medial and lateral femur (88% and 87%, respectively) and in medial and lateral tibia (79% and 87%, respectively) (Figure 5A and micrographs in Figure 5, E and F). In arthritic knees of FcγRIII-/-, VDIPEN expression was comparable to controls (Figure 5B). In contrast, in FcγRII-/- arthritic joints, VDIPEN was significantly higher in lateral and medial femur and lateral tibia (250%, 200%, and 122% higher, respectively), indicating that FcγRII is involved in MMP-mediated cartilage destruction (Figure 5, C and D and micrographs in Figure 5, G and H). VDIPEN expression was much lower (between 56 to 76%) when 15 µg of mBSA was injected if compared to the 60-µg group but still significantly higher in lateral and medial tibia of FcγRII-/- if compared to their wild-type controls (Figure 5C).

Figure 3. Frontal sections of whole knee joints 7 days after induction of AIA in Fc γ RI $^{-/-}$ (A), Fc γ RIII $^{-/-}$ (B), Fc γ RII $^{-/-}$ 15 μ g (C), and Fc γ RII $^{-/-}$ 60 μ g (D) mice, and their wild-type controls. The amount of cells present in the synovium (infiltrate) and in the joint cavity (exudate) was determined using an arbitrary scale from 0 to 3. 0, No cells; 1, minor; 2, moderate; 3, maximal. The amount of cells was determined by two blind observers. Data are the mean $\pm$ SD of 10 animals. Significance was tested using the Wilcoxon rank test (*, $P < 0.05$). Original magnifications, $\times 100$. F, Femur; T, tibia. Note the significantly higher infiltrate and exudate at day 7 after injection of 60 μ g of mBSA in knee joints of Fc γ RII $^{-/-}$ (D) and photographs F = Fc γ RII $^{-/-}$ versus E = wild-type control) and comparable cell mass in arthritic Fc γ RI $^{-/-}$ (A) and Fc γ RIII $^{-/-}$ (B) mice.

Figure 4. Loss of red staining from cartilage layers of total knee joint sections 7 days after induction of AIA in FcγRI^{-/-} (A), FcγRIII^{-/-} (B), FcγRII^{-/-} 15 μg (C), and FcγRII^{-/-} 60 μg (D) mice. Loss of red staining was scored in tibia and femur using an arbitrary scale from 0 to 3. Data are expressed as mean ± SD of 10 mice and were tested on significance using the Wilcoxon rank test (*, $P < 0.05$). No significant difference in PG loss was found between wild-type controls and FcγRI^{-/-} (A), FcγRIII^{-/-} (B), and FcγRII^{-/-} (C and D) mice. MT, Medial tibia; MF, medial femur; LT, lateral tibia; LF, lateral femur; F, femur; T, tibia. Original magnifications, ×100.

FcγRII Is Involved in Inhibition of Erosion of the Cartilage Matrix

To examine the role of FcγRI, FcγRIII, and FcγRII in severe irreversible cartilage destruction, we also analyzed erosion of the cartilage matrix in paraffin sections. Erosion of the cartilage matrix that is only mild at day 7 after AIA was determined as ruffling of the cartilage surface. No significant differences in matrix erosion were found in knees of FcγRI^{-/-} and FcγRIII^{-/-} mice if compared to their wild-type controls (Figure 6, A and B). In contrast, in knee joints of FcγRII^{-/-} mice, erosion was

enhanced in the 60-μg mBSA group and this difference reached significance in the lateral and medial tibia (222% and 186% higher, respectively) (Figure 6D and micrographs in Figure 6, E and F). In the 15-μg mBSA group, although erosion was somewhat higher, differences did not reach significance (Figure 6C).

Discussion

In a previous study we found that absence of functional FcγRI and FcγRIII is related to acute and sustained inflammation and is also a major determinant of severe destruction of cartilage in AIA. In the present study we show that both FcγRI and FcγRIII are important in synovial inflammation whereas FcγRI is the dominant activatory receptor involved in severe cartilage destruction within this model. In contrast, FcγRII is crucial in negative regulation of both acute and chronic inflammation and late severe cartilage destruction.

Because the absence of a particular FcγR may impair the T- and B-cell-mediated immunological response against the arthritogen mBSA, cellular and humoral immunity against mBSA was determined. T-cell responses against mBSA were however not different in immunized FcγRI^{-/-}, FcγRIII^{-/-} and FcγRII^{-/-} mice and their controls and is in line with earlier studies showing that loss of functional FcγRI and FcγRIII did not perturb T-cell maturation.³⁴ FcγRIII is also expressed on mast cells, and FcγRIII-deficient mice lack IgG-mediated mast cell degranulation.³⁵ However, earlier studies by our laboratory showed that the course of AIA elicited in knee joints of mast cell-deficient (WBB6F1-W/W^v) mice was not significantly different from control littermates, suggesting that mast cells do not play an important role in joint inflammation within this model.³⁶

Measuring humoral immunity, isotype-specific antibody responses against mBSA were not different in FcγRI^{-/-} and FcγRIII^{-/-}, which is in line with earlier studies.³⁷ In immunized FcγRII^{-/-} however, all anti-mBSA isotype antibody titers (with the exception of IgG2b) were fourfold to eightfold elevated if compared to controls. In the present study, we investigated FcγRII^{-/-} on a hybrid SV129/C57BL6 background because this background does not display abnormalities in the immunological status. FcγRII^{-/-} on other backgrounds (eg, C57BL/6) develop autoantibodies of multiple IgG subclasses³⁸ that might enhance the arthritis induced by mBSA immunization. Differences in autoimmune development however suggest that FcγRII in combination with genetic loci may be important in breaking the tolerance not only against an autoantigen such as collagen type IV³⁹ but also against an exogenous antigen such as mBSA. FcγRII may function to maintain tolerance in the periphery and together with genetic loci, gate B-cell activation against various antigens.

As the adaptive immunity in FcγRI^{-/-} and FcγRIII^{-/-} is not altered, local expression of both activating FcγR in the joint may drive synovial inflammation and cartilage destruction. Immune complex mediated activation is predominantly regulated by synovial macrophages that have

Figure 5. Expression of VDIPEN staining in knee joints of FcγRI-/- (A), FcγRIII-/- (B), FcγRIIb-/- 15 μg (C), and FcγRII-/- 60 μg (D) mice, and their wild-type controls 7 days after AIA induction. Positive VDIPEN staining was determined in various cartilage layers at an original magnification of ×100 (MT, medial tibia; MF, medial femur; LT, lateral tibia; LF, lateral femur; F, femur; T, tibia) using automated image analysis and expressed in VDIPEN staining per μm² cartilage. VDIPEN expression was significantly lower in cartilage layers of arthritic knee joints of FcγRI-/- (A and micrographs F = FcγRI-/- versus wild-type control = E) but not in FcγRIII-/- mice if compared to wild-type controls (B). VDIPEN expression was elevated in arthritic knee joints of FcγRII-/- (C and D). Difference reached significance in the lateral and medial tibia after injection of 15 μg of mBSA (C) and in the lateral and medial tibia and medial femur after injection of 60 μg of mBSA (D) and photographs H = FcγRII-/- versus wild-type control = G). Data represent the mean ±SD of 10 mice. Original magnifications, ×250.

been shown to drive onset,^{40,41} elongation,⁴² and flare-up⁴³ during AIA. In a previous study we found that mice that express nonfunctional FcγRI and FcγRIII and developing comparable adaptive immunity with controls showed a significantly lower knee joint swelling. In this

study, both FcγRI-/- and FcγRIII-/- mice showed however comparable swelling with controls. These results suggest that FcγRI and FcγRIII present on macrophages act in concert with respect to swelling. In the absence of one receptor its function may be fully com-

Figure 6. Erosion in cartilage layers (LF, lateral femur; LT, lateral tibia; MF, medial femur; MT, medial tibia) of arthritic knee joints at day 7 after AIA induction in Fc γ RI^{-/-}, Fc γ RIII^{-/-}, and Fc γ RII^{-/-} mice, and their wild-type controls. Erosion of the cartilage surface was defined as ruffling of the cartilage surfaces using an arbitrary scale from 0 to 3. In Fc γ RI^{-/-} and Fc γ RIII^{-/-} mice, erosion was mild and comparable to wild-type controls. Erosion was found in 8 of 10 animals in both groups (A and B). Injecting 60 μ g of mBSA in knees of Fc γ RII^{-/-} resulted in elevated erosion in all cartilage layers that reached significance in the lateral and medial tibia (D and F: Fc γ RII^{-/-} and their wild-type control, E). After injection of 15 μ g of mBSA in knee joints of Fc γ RII^{-/-}, erosion was higher but did not reach significance (C). Original magnifications, $\times 100$.

pensated by the other FcR. These receptors that are expressed on mononuclear cells⁴⁴ are involved in the respiratory burst on cross-linking by IgG ICs. As a result these cells release vasoactive products such as oxygen and nitrogen radicals and histamine that are highly involved in vascular leakage leading to enhanced ^{99m}Tc uptake in the joint.

In humans, Fc γ RIIIA has been suggested to play a role in the pathogenesis of RA and a close correlation was found between Fc γ RIIIA expression and the location of both synovitis and extracellular features.⁴⁵ A recent study suggests a dominant role for Fc γ RIIIA in the induction of both tumor necrosis factor- α and interleukin-1 α production by human macrophages in RA after receptor ligation by small ICs.⁴⁶ Furthermore many animal studies have shown that Fc γ RIII is crucial in IC-dependent diseases raised in skin,⁴⁷ kidney,⁴⁸ or lung.⁴⁹ A reverse passive arthus reaction is inhibited within Fc γ RIII^{-/-} mice.²³ Recently, we also found that arthritis elicited by ICs only is also very strongly Fc γ RIII-dependent (Nabbe K, Van Lent

P, Blom AB, Holthuysen A, Verbeek S, Vandenberg W, manuscript in preparation). The reason why Fc γ RIII does not play an important role within AIA may be that apart from ICs T cells also play an important role in chronicity within this model. During AIA, mBSA-specific Th1 cells accumulate in the arthritic joint thereby releasing interferon- γ . Interferon- γ is one of the most potent cytokines involved in up-regulation of Fc γ RI on macrophages.⁵⁰ Local interferon- γ production may cause a shift in Fc γ R expression from Fc γ RIII to Fc γ RI and may explain the redundancy found within this study.

The inhibiting Fc γ RII seems to be of utmost importance in both the acute and chronic phase of synovial inflammation and absence leads to significantly higher inflammation. Fc γ RII is expressed on various hematopoietic cells and is a negative regulator of Fc γ RIII receptor on B cells and macrophages⁵¹ and probably also of Fc γ RI.⁵² Fc γ RII may act as a negative regulator of antibody production and/or local IC triggered activation. As most of

the anti-mBSA isotype titers were elevated, this may be responsible for a higher IC load within the knee joint.

Although we did not find differences in the IC load in the knee joints of Fc γ RII $^{-/-}$ and controls at day 7 after AIA using immunolocalization, differences may have been present at earlier time points of this arthritis. Local IC-triggered activation in the joint may further be different. Absence of the Fc γ RII on macrophages has been shown to enhance the release of inflammatory mediators such as interleukin-1 and tumor necrosis factor- α ⁵³ that are highly involved in synovial inflammation. Comparing two doses of mBSA we found that only the high dose (60 μ g) of mBSA was Fc γ RII-dependent during the complete 7-day course of arthritis. Immune complexes drive inflammation by both activating Fc receptors and complement and the two systems seemed to be co-dominant in the early arthus reaction.⁵⁴ Our results suggest that after the arthus, in the developing phase of chronicity of experimental arthritis, higher IC doses may be more FcR-dependent if compared to lower IC doses.

Earlier studies have shown that macrophages are crucial in cartilage destruction seen during AIA.^{38–41} Macrophages may function either indirectly by attracting inflammatory cells or directly as important producers of cytokines⁵⁵ and enzymes⁵⁶ and in RA, activation of macrophages may be T-cell-dependent.^{57,58} During AIA, two phases of cartilage destruction are observed. The first early phase, which is characterized by proteoglycan loss, is mediated by aggrecanase, a member of the ADAMS (a desintegrin and metalloproteinase) family. The second phase that starts at approximately day 5 after AIA induction, is characterized by MMP-mediated matrix destruction resulting in irreversible cartilage erosion.³² Because no difference was found in PG breakdown nor NITEGE staining in cartilage surfaces of arthritic knee joints of Fc γ R $^{-/-}$, Fc γ RIII $^{-/-}$, or Fc γ RII-deficient mice and their wild-type controls (data not shown), this suggests that these receptors are not important or redundant in aggrecanase-induced cartilage damage.

In contrast, MMP-mediated cartilage destruction as measured by VDIPEN staining was significantly lower in Fc γ RI $^{-/-}$ but not in Fc γ RIII $^{-/-}$ arthritic knee joints. The latter indicates that Fc γ RI rather than Fc γ RIII is involved in late severe cartilage destruction. This type of cartilage destruction only occurs in experimental models, containing an IC component.³² Other arthritis models induced by bacterial or yeast cell walls never resulted in severe cartilage destruction and also expression of VDIPEN neopeptides was fully absent.³² As we showed earlier, the activatory FcR are probably involved in activation of latent MMPs present in the cartilage matrix of arthritic knees, binding of FcRI may lead to activation of synovial macrophages leading to selective production of pro-MMP-activating factors. Although erosion was still mild at day 7 after AIA induction, there was a tendency that erosion was lower in cartilage layers of arthritic knees of Fc γ RI $^{-/-}$ mice. Differences may diverge more at later time points when erosion becomes more severe within this model.

In contrast in the absence of Fc γ RII, significantly higher MMP-mediated destruction was found within the cartilage layers of the arthritic joints. First, this may be because of an elevated load of IC within the joint as a result of higher anti-mBSA titers and may explain the higher VDIPEN expression in the 60- μ g mBSA group. Furthermore, macrophages deficient for Fc γ RII produce higher interleukin-1 concentrations on activation with ICs.³⁹ As interleukin-1 is the dominant cytokine involved in VDIPEN expression during AIA⁵⁹ this also may contribute to the higher VDIPEN expression in the cartilage. Moreover the absence of Fc γ RII may elevate mechanisms involved in activation of pro-MMPs into their active form. A higher concentration of enzymes released by macrophages and/or polymorphonuclear leukocytes such as elastase may be responsible for more efficient activation of pro-MMPs.⁶⁰ Whether Fc γ RII contributes to higher joint inflammation and severe cartilage destruction either by elevation of anti-mBSA antibodies and subsequent raise of ICs and/or by elevation of local hypersensitivity of macrophages to ICs is momentarily under investigation by blocking Fc γ RII at the onset of AIA using anti-Fc γ RII antibodies.

The above results suggest that expression of the activatory Fc γ RI on the surface of local cells present in the knee joint during T-cell-dependent AIA is the dominant activatory Fc receptor involved in cartilage destruction whereas the inhibiting Fc γ RII $^{-/-}$ may be an important regulating receptor of severe cartilage destruction. During late phases of human arthritis, monocytes/macrophages are the dominant hematopoietic cells present in the RA joint and a strong correlation between the number of macrophages and erosion of the cartilage matrix was found during RA.⁶¹ Disturbance in the balance between activating Fc γ RI and Fc γ RIII receptors and the inhibiting Fc γ RII receptor on synovial macrophages may have important implications for cartilage destruction during arthritis and the inhibiting Fc γ RII may form a new target for therapeutic intervention of cartilage destruction within this destructive disease.

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