EXTENDED REPORT

Skewed balance in basal expression and regulation of activating v inhibitory Fcγ receptors in macrophages of collagen induced arthritis sensitive mice

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Background: Recently, it has been found that collagen type II arthritis susceptible mouse strains are hyperreactive to immune complexes (ICs), locally deposited into their knee joints.

Objective: To investigate whether this strain specific knee joint hyperreactivity is related to a disturbed regulation of activatory and inhibitory FcγR on their macrophages before and after stimulation with ICs.

Methods: Macrophages from collagen induced arthritis susceptible strains (DBA/1 and B10.RIII) and non-susceptible strains (C57BL/6 and BALB/c) were compared. FcγR levels on macrophages were detected at protein level by flow cytometric analysis and at mRNA level by reverse transcriptase-polymerase chain reaction. Macrophages were stimulated with ICs, and production of cytokines and enzymes was measured at different times.

Results: On synovial and peritoneal macrophages of DBA/1 mice a higher basal FcγRII and III expression was found, which was skewed towards the activating FcγRIII. In B10.RIII macrophages, however, FcγRIII levels were much lower. Regulation of FcγR mRNA levels in macrophages was tested after stimulation with ICs for one and three days. DBA/1 and B10.RIII macrophages showed a prolonged up regulation of activating FcγRI and III, whereas the inhibiting FcγRII was significantly down regulated compared with non-susceptible strains. In line with this, DBA/1 and B10.RIII macrophages showed a higher interleukin 1 (IL1) and matrix metalloproteinase (MMP) production after IC exposure, whereas IL6 production was significantly reduced.

Conclusions: This study indicates that macrophages derived from collagen type II arthritis susceptible mice show a dysregulated FcγR expression before, and even more clearly, after activation by ICs involved in inflammation and cartilage degradation, resulting in prolonged expression of activatory FcγRI and III, down regulation of inhibitory FcγRII and increased release of IL1 and MMP.

Rheumatoid arthritis (RA) is characterised by chronic inflammation in many joints, resulting in severe cartilage destruction. Immune complexes (ICs) are found in large amounts in RA synovium and are thought to be involved in activation of infiltrated and resident haematopoietic cells.^{1,2} The macrophage is a dominant cell type in synovial tissue, and a strong correlation was found between the number of these cells and cartilage destruction.^{3–5} Experimental studies have shown that synovial lining macrophages participate in the onset, propagation, and exacerbation of IC mediated experimental arthritis.^{6–10} Macrophages communicate with ICs using Fc receptors which recognise the constant part of IgG. In the mouse three classes of FcγR have been described: FcγRI, FcγRII, and FcγRIII.^{11–13} In mice, FcγRI and III have been described as activating receptors, whereas FcγRII is an inhibiting receptor which can down regulate activation by co-ligation with either FcγRI or III.^{14–17} The balance between activating and inhibiting receptors regulates intracellular signalling and has been shown to determine susceptibility to immune complex driven diseases^{18–22} elicited in the skin, lung, and kidney.

Previously we have found that mouse strains that are susceptible for collagen type II arthritis (DBA/1, B10.RIII) are hypersensitive to ICs.²³ Arthritis induction by local deposition of ICs in the knee joint resulted in a severe and chronic inflammation and massive cartilage damage. Control strains which are not sensitive for collagen induced arthritis (CIA) induction (C57BL/6 and BALB/c) developed only a moderate and transient arthritis and no irreversible cartilage damage. As, in this arthritis model, ICs first meet the lining layer which covers the inside of the knee joint and which predominantly

consists of macrophages,^{9,24} these findings suggest that local handling of ICs by synovial macrophages may be different in the CIA sensitive and non-sensitive mouse strains. Hyper-responsiveness of macrophages in response to ICs might be due to an intrinsic defect, resulting in differences in basal expression of FcγR, or there might be a different regulation in activatory and inhibitory FcγR. This may have consequences for the response of macrophages after stimulation by ICs. Upon stimulation these cells release inflammatory mediators like interleukin 1 (IL1) and collagenase,^{25,26} which have also been shown to be very important in promotion of joint inflammation and cartilage destruction within this model, and IL6 which is described as a mediator that inhibits cartilage degradation.²⁷

Recently, we found that macrophages of DBA/1 mice show a higher expression of FcγRII/III on the membrane than C57BL/6 mice.²⁸ However, no distinction was made between FcγRII and III. Based on these findings, the hypothesis was formed that susceptibility to CIA may be determined by the balance of FcγRs on macrophages.

Abbreviations: APMA, p-aminophenylmercuric acetate; CIA, collagen induced arthritis; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme linked immunosorbent assay; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HAGG, heat aggregated gammaglobulins; IC, immune complex; IL, interleukin; MHC, major histocompatibility complex; MMP, matrix metalloproteinase; RA, rheumatoid arthritis; RT-PCR, reverse transcriptase-polymerase chain reaction

The objective of this study was, firstly, to investigate whether mice that are susceptible to collagen type II arthritis, express higher basal levels of FcγRs in macrophages present in the synovial lining and peritoneal cavity. Secondly, whether there is a difference in regulation of stimulatory and inhibitory FcγR on macrophages of these strains after stimulation with ICs. Thirdly, we investigated whether the variation of FcγR expression on macrophages resulted in differences in release of proinflammatory mediators, like cytokines and enzymes upon stimulation with ICs.

MATERIAL AND METHODS

Animals

Male C57Bl/6, C57Bl/10 and DBA/2 mice were obtained from Jackson, Bar Harbor, USA. Male DBA/1 mice were purchased from Bomholdgård, Ry, Denmark. Male B10.RIII and BALB/c mice were obtained from Charles River Institute, Sulzfeld, Germany. Mice were used between the age of 8 and 12 weeks and were fed a standard diet and tap water freely.

Antibodies

Rat anti-FcγRII/III antibodies were obtained from BD PharMingen, San Diego, CA, USA. Mouse anti-FcγRII antibodies²² were obtained by spinnerflask culture of K9-361 hybridoma which was kindly given by Professor Dr RE Schmidt, Hannover, Germany. Rabbit IgG (Sigma-Aldrich Chemie, Steinheim, Germany) was used for the production of heat aggregated gammaglobulins (HAGG).

Immunohistochemistry

Total knee joints were dissected from naïve mice and embedded in OCT compound (Sakura Finetek Europe BV, Zoeterwoude, The Netherlands). Cryostat sections were cut using a tungsten D-type knife and collected on tape. Sections were dried and fixed using acetone vapour. 2.4G2 antibody was used in a final concentration of 2 µg/ml to detect FcγRs. Biotinylated rabbit-antirrat (Vector Laboratories Inc, Burlingame CA, USA) and avidin-biotin complex-horseradish peroxidase complexes were used to detect the primary antibody. Staining was completed using 3, 3'-diaminobenzidine as substrate.

Macrophage isolation

Mice were killed using ether vapour to prevent intraperitoneal bleeding. Ten millilitres of ice cold Dulbecco's modified Eagle's medium (DMEM) + 10% fetal calf serum (FCS) + 1% pyruvate was injected intraperitoneally. Using a thick needle (1.1 mm) the medium containing peritoneal cells was re-collected and kept on ice. In addition, the peritoneal cells were allowed to adhere to plastic flasks for 1.5 hours and, subsequently, the attached macrophages were harvested by scraping the plastic surface with a rubber policeman. The cells were put into 24 well plates (Costar, Acton MA, USA), 1 ml per well at a concentration of 2×10^6 cells/ml. After a four day adjustment period HAGG were added to the culture medium (100 µg/ml). Culture medium was isolated after 24 and 48 hours. HAGG were made by heating 10 mg/ml rabbit IgG (Sigma-Aldrich Chemie, Steinheim, Germany) to 63°C for 30 minutes. After heating, the solution was centrifuged at 12 000 rpm for 10 minutes. The concentration of HAGG in the supernatant was determined by reading the absorbance at 280 nm.

Detection of FcγR mRNA levels using reverse transcriptase-polymerase chain reaction (RT-PCR)

mRNA levels for three different FcγRs (FcγRI, II, and III) were detected by a semiquantitative method. In brief, peritoneal macrophages were isolated and treated as described above and at days 1 and 3 the culture medium was removed and total RNA was extracted from the adherent cells using 1 ml TRIzol reagent.

RT-PCR was performed using 1 µg of RNA. Oligo-DT primers were used for reverse transcription of mRNA and 1/20 of the cDNA was used in one PCR amplification. PCR was done at a final concentration of 200 µmol/l dNTPs, 0.1 µmol/l of each primer, and 1 unit Taq polymerase (Life Technologies, Breda, The Netherlands) in standard PCR buffer. The message for murine FcγRI, II, and III was amplified using the following primers (Eurogentec, Seraing, Belgium): upper 5'-TGT-GCC-CCA-GTA-CTG-TCA-CT-3' and lower 5'-AAG-TGG-TTA-ATG-CCA-CCA-AGG-3' for FcγRI, upper 5'-AAT-CCT-GCC-GTT-CCT-ACT-G-3' and lower 5'-GCC-AAG-GGA-GAC-TAA-ATG-TG-3' for FcγRII, upper 5'-TTC-CAA-AGG-TGT-GGT-CA-3' and lower 5'-CAG-AAA-TCA-CTC-TCA-GAT-C-3' for FcγRIII. There was a pause in the PCR reaction after 20, 23, 26, 29, 32, 35, 38, 41, and 44 cycles at the very end of the extension phase (72°C) and 5 µl samples were taken. PCR products were separated on 1.6% agarose and stained with ethidium bromide. Each PCR was performed at least twice. The results are presented as the cycle number in which the first detectable amount of DNA appears on the agarose gel. The number of cycles in which this occurs in the unstimulated cells is subtracted from the number of cycles in the stimulated group, resulting in the net effect of stimulation by HAGG. Samples were compared after correction for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) content for each individual sample to rule out confounding by variation of the RNA purification and the RT step.

Flow cytometric analysis

Peritoneal macrophages of different strains were isolated from the peritoneal cavity by a lavage with ice cold DMEM/10% FCS/1% pyruvate. 5×10^6 cells/100 µl were incubated with 2.4G2 (5 µg/ml). Subsequently cells were washed and incubated with mouse adsorbed mouse-antirrat F(ab)₂ fragments labelled with FITC (Jackson Immunoresearch Laboratories Inc, West Grove, PA). Flow cytometric analysis was performed using a Coulter Epics XL/XL-MCL (Coulter electronics Ltd, Mijdrecht, The Netherlands). A negative control was obtained by omitting the first antibody and substituting 2.4G2 for the isotype matched irrelevant antibodies (DAKO, Glostrup, Denmark). FcγRII was detected using anti-Ly17.1 from clone K9-361 which was biotinylated using a biotin labelling kit (Roche Diagnostics, Mannheim, Germany) and subsequently detected using streptavidin-FITC (DAKO, Denmark). To detect FcγRIII the binding of 2.4G2 to FcγRII was blocked using unlabelled anti-Ly17.1, according to a method described by Schiller *et al.*²² As a control, all measurements were performed with both isotype controls (IgG2b), and omission of the first antibody. Incubation of cells with normal mouse serum had no effect on binding of the FITC labelled secondary antirrat-IgG antibody, excluding cross reactivity of this antibody with murine IgG. The window was set in such a way that >95% of cells were F4/80 positive, indicating that >95% of cells were macrophages.

Measurement of IL1α and IL6

IL1α and IL6 activity was measured using specific sandwich enzyme linked immunosorbent assays (ELISAs). The capture antibody, monoclonal rat-antimouse IL1α or IL6 (R&D Systems Europe, Oxon, UK), was coated overnight in a 96 well plate. After incubation with the (diluted) culture supernatants or standard concentrations of IL1α or IL6, wells were washed three times and incubated using a biotinylated detection antibody, polyclonal goat antimouse IL1α or IL6 (R&D Systems). The second antibody was detected using poly-horseradish peroxidase and subsequently developed using o-phenylenediamine.

Measurement of collagenase/gelatinase

Enzyme activity in the culture supernatants of the peritoneal macrophages was measured using an assay specific for

Table 1 Arbitrary score of immunohistochemical staining of naive knee joint of C57BL/6, BALB/c, B10.RIII, and DBA/1 mice

C57BL/6	BALB/c	B10.RIII	DBA/1
++	++	++	++++

Intensity and extent of immunohistochemical staining of the synovial lining was scored arbitrarily. Note that only DBA/1 mice show a higher level of FcγRII/III staining.

gelatinase/collagenase (EnzChek gelatinase/collagenase assay kit, molecular probes, Leiden, The Netherlands). The assay is based on the cleaving of a fluorescent substrate which can be digested by all collagenases and gelatinases. Briefly, culture supernatants or known concentrations of collagenase were incubated with the fluorescent gelatine substrate. To assess whether enzymes were present in the supernatants in an inactive form, supernatants were previously incubated with 1 mM APMA (*p*-aminophenylmercuric acetate, Sigma Chemical Co, St Louis, MO, USA) for one hour at room temperature. Cleaving of the substrate was monitored by a fluorometer (Fluostar Galaxy, BMG Labtechnologies GmbH, Offenburg, Germany) at 37°C. Enzyme activity was calculated by comparing the rate of substrate digestion of the specimen with that of the collagenase standard. To assess linearity of the assay serial dilutions of some specimens were made. By adding a specific matrix metalloproteinase (MMP) inhibitor, the metal chelating 1,10-phenanthroline (Molecular Probes), the specificity of the assay was determined.

RESULTS

Disturbance of the balance of basal FcγR expression on resident macrophages derived from CIA sensitive mice

In a previous study we showed that synovial lining cells of CIA sensitive DBA/1 mice express higher levels of FcγRII/III than C57BL/6 mice.²⁸ In the present study we additionally detected FcγRII/III on lining cells of knee joints of another CIA sensitive strain, B10.RIII. Unexpectedly, FcγRII/III was low and did not differ from that seen in C57BL/6 or BALB/c, which are both CIA non-sensitive (table 1). As immunolocalisation is at best a semiquantitative method for detecting protein, we next investigated macrophages from the peritoneal cavity to quantify the differences we found in basal FcγR expression. FcγRs were detected and quantified using 2.4G2 and FACS analysis on resident peritoneal macrophages. As previously described,²⁸ macrophages of DBA/1 mice showed a higher expression of FcγRII/III than macrophages from non-sensitive C57BL/6. Macrophages derived from B10.RIII mice now also showed raised FcγR levels, although much lower than found in DBA/1 (50% in B10.RIII v 83% in DBA/1). As expected, FcγRII/III expression by BALB/c macrophages was similar to that of C57BL/6 mice. As an extra control, FcγR expression on genetically more comparable, but CIA insensitive, mouse strains (DBA/2 for DBA/1 mice, and C57BL/10 for B10.RIII mice) was detected. The levels of FcγR in both strains were comparable with those in C57BL/6 and BALB/c mice. Mean (SD) fluorescence for FcγRII/III was 260 (26) for DBA/2 and 230 (50) for C57BL/10 compared with 240 (90) and 250 (110) for C57BL/6 and BALB/c (not shown).

Interestingly, on peripheral blood monocytes of the various strains no differences were found in basal levels of FcγR expressed (fig 1A), indicating that differences in FcγR expression develop during the maturation of monocytes into macrophages.

To discriminate between the activating FcγRIII and the inhibiting FcγRII we additionally used another antibody (K9-361) which is directed only against FcγRII. By using this antibody to block the binding of 2.4G2 to FcγRII, FcγRIII can also

Figure 1 Expression of FcγRs on macrophages of C57BL/6, BALB/c, DBA/1, and B10.RIII mice. The expression of FcγRII/III was significantly higher in DBA/1 and B10.RIII mice than in the other strains. However when FcγR expression was tested on peripheral blood mononuclear cells (PBMC) of these different strains, no difference in expression was found (A). When K9-361 was used to detect FcγRII, we found higher expression of this receptor in DBA/1 and B10.RIII mice (B). To detect FcγRIII, the binding of anti-FcγRII/III (2.4G2) to FcγRII was blocked using K9-361. DBA/1 mice express high levels of FcγRIII than the other strains (C).

be detected. Interestingly, macrophages from DBA/1 mice showed both a higher FcγRIII (200%) and FcγRII (50%) expression if compared with macrophages from non-sensitive mice, so the balance between FcγRII and III was skewed towards FcγRIII. On B10.RIII macrophages, FcγRII was raised by 50% but unexpectedly FcγRIII expression did not differ significantly if compared with BALB/c and C57BL/6 (figs 1B and C). Also DBA/2 mice and C57BL/10 mice expressed comparable levels of FcγRII and III with those of C57BL/6 and BALB/c (not shown). The negative control for the flow cytometric analysis, substitution of the first antibody with an isotype control (IgG2b), was negative in all experiments.

Regulation of FcγRI, II, and III at mRNA level by macrophages after stimulation with HAGG

As basal levels of the activating FcγRIII on CIA sensitive B10.RIII macrophages were comparable with those of non-sensitive mice, differences in FcγR expression may evolve after stimulation with ICs. As ICs bind firmly to FcγRs, it is not possible to measure FcγR expression using FACS and for that reason semiquantitative RT-PCR was used. Peritoneal macrophages of the different strains were stimulated with HAGG for one and three days and we determined to what extent mRNA levels of the subclasses of FcγR (FcγRI, II, and III) were regulated.

At day 1 after stimulation, activating FcγRs were strongly up regulated. FcγRI (fig 2A) and FcγRIII (fig 2C) were both clearly up regulated in macrophages of all strains. In contrast with the activatory receptors, the inhibitory FcγRII was down regulated in all strains. Interestingly, macrophages from the

Figure 2 Regulation of expression of Fc γ RI (A), II (B), and III (C) by macrophages of C57BL/6, BALB/c, DBA/1, and B10.RIII after stimulation with HAGG. The number of PCR cycles needed to detect these receptors in unstimulated cells was subtracted from the number needed in stimulated cells, after correction for GAPDH content. Note that CIA sensitive mice show a prolonged up regulation of Fc γ RI and III and a prolonged down regulation of Fc γ RII, whereas BALB/c and C57BL/6 mice show an up regulation of Fc γ RII at a later time.

hyperresponsive B10.RIII strain showed a much lower expression of the inhibitory Fc γ RII (fig 2B).

Moreover, at day 3 after stimulation, Fc γ RI and III expression normalised to basal levels in C57BL/6 and BALB/c mice, but strikingly in DBA/1 and B10.RIII, macrophages still showed a high expression of these receptors (between eight and 64 times). In contrast, Fc γ RII was still up regulated at this time in C57BL/6 and BALB/c (respectively eight and two times), whereas this receptor was still down regulated in DBA/1 macrophages (eight times). This indicates that macrophages of CIA sensitive mice after stimulation with ICs show a prolonged up regulation of activating Fc γ Rs and a down regulation of inhibiting Fc γ Rs.

IL1 and IL6 production by C57BL/6, BALB/c, DBA/1, and B10.RIII after stimulation with HAGG

To investigate whether the higher basal Fc γ R expression and skewing of Fc γ R regulation towards activatory receptors by macrophages from CIA sensitive mice is a functional difference, we determined cytokine production by macrophages after stimulation with HAGG. We chose IL1 and IL6, two cytokines that are involved in inflammation and respectively promote and inhibit cartilage destruction. At day 1 after stimulation the production of IL1 α was significantly enhanced in DBA/1 mice in comparison with controls (fig 3A).

In a previous study we found that production of bioactive IL1 by DBA/1 macrophages was also higher than with C57BL/6. IL1 β production could not be detected (not shown). At day 2 after HAGG stimulation, IL1 α levels were still significantly raised in supernatants of DBA/1 and also B10.RIII macrophages now produced higher levels of IL1 α than did C57BL/6 and BALB/c, indicating that IL1 production is prolonged. As a control, peritoneal macrophages derived from FcR γ -chain^{-/-} knockout mice which fail to express functional activatory Fc γ Rs were used. These macrophages did not produce detectable amounts of IL1 or IL6 after two days of stimulation with HAGG, indicating that production of these cytokines after stimulation with HAGG is completely Fc γ R mediated (data not shown).

In contrast with IL1, IL6 levels were significantly lower in culture supernatants of peritoneal macrophages of CIA sensitive than in non-sensitive strains (fig 3B). Moreover, DBA/1 macrophages produced significantly lower levels of IL6 than

Figure 3 IL1 α (A) and IL6 (B) production by macrophages of C57BL/6, BALB/c, DBA/1, and B10.RIII after stimulation by HAGG. IL1 α production was measured by ELISA and was higher in DBA/1 mice at day 1 and in DBA/1 and B10.RIII at day 2, indicating higher cytokine production after stimulation with HAGG by macrophages of CIA sensitive strains. This is in contrast with IL6 levels produced by peritoneal macrophages after HAGG stimulation. DBA/1 and B10.RIII mice produced significantly less IL6 than the other strains.

Figure 4 Collagenase/gelatinase production by macrophages of C57BL/6, BALB/c, DBA/1, and B10.RIII mice after stimulation with HAGG. Collagenase/gelatinase production was measured at day 2 after stimulation with HAGG, using a specific fluorescent substrate. The activity of the enzyme was measured by following fluorescence at different time points. Basal enzyme activity was subtracted from the levels of enzyme activity after stimulation with HAGG. Interestingly, enzyme activity of DBA/1 mice is largely increased at day 2 after stimulation with HAGG, compared with other strains.

peritoneal macrophages derived from B10.RIII mice. When instead of HAGG, zymosan was used to stimulate macrophages, no differences in cytokine production were found (data not shown).

Production of collagenase by macrophages of C57BL/6, BALB/c, DBA/1, and B10.RIII after HAGG stimulation

To investigate whether stimulation of macrophages with ICs also leads to differences in cartilage degrading enzymes, supernatants were tested for collagenase and gelatinase activity. However, enzyme activity was completely absent in the supernatants of all tested strains. To test whether latent forms of collagenase/gelatinase were present, supernatants were additionally activated by APMA, a chemical which activates pro-MMPs (fig 4). Only DBA/1 macrophages produced high amounts of collagenase, which strongly increased in time after stimulation with HAGGs, resulting in a net activity of 3.0 mU/ml. Enzyme activities released by C57BL/6, BALB/c and B10.RIII macrophages were respectively 0.4, 0.6, and 0.6 mU/ml after correction for the background. When the selective MMP inhibitor *o*-phenanthroline was added to the samples, no enzyme activity was measured. These results indicate that immune complex stimulation strongly activates macrophages of DBA/1 mice to express latent collagenase/gelatinase activity.

DISCUSSION

In previous studies we found that knee joints of mice which are susceptible to collagen type II arthritis are hyperresponsive to induction of experimental IC mediated arthritis,²³ and that macrophages of DBA/1 mice express higher levels of FcγRII/III.²⁸ In the present study we find that macrophages from two CIA sensitive strains express higher levels of activating FcγRs before and/or after stimulation with ICs, whereas the level of the inhibiting FcγRII is lowered after stimulation. As macrophages in the synovium of the joint are crucial factors in the onset and propagation of IC mediated knee joint inflammation, prolonged up regulation of activating FcγRs may well explain the more severe and chronic inflammation found within these joints.

In the present study we focused on expression of FcγRI, II, and III on macrophages of the different strains, before and after stimulation with ICs. The balance between activating and inhibiting FcγRs has been shown to be of utmost importance in the course of different IC mediated diseases elicited in the

skin, kidney, peritoneum, or the lung.^{20, 21, 29-33} Whether activating FcγRI or FcγRIII predominates appears to be related to the body compartment in which inflammation was raised. FcγRI was dominant in the peritoneum and FcγRIII in the skin, kidney, and lung. Recently, we have found that the activating FcγRIII is the most crucial activating receptor in the joint, because induction of IC arthritis in knee joints of FcγRIII knockout mice was completely blocked (manuscript in preparation).

Interestingly, higher basal expression of activating or inhibiting FcγRs was only found on macrophages derived from DBA/1 and B10.RIII strains and not on their blood monocytes, indicating that the difference occurs during maturation of the monocyte into the macrophage. Classically, collagen type II arthritis was restricted to mice with an H-2q and/or H-2r haplotype background. These haplotypes may be related to mechanisms regulating transcription, translation, or expression of FcγRs on the macrophage membrane. The mechanism for higher susceptibility to CIA in strains possessing H-2q and H-2r haplotypes is thought to involve binding of specific collagen type II immunodominant peptides. In the aforementioned IC arthritis these peptides are, at least in the early phase, not present and therefore this mechanism is unlikely to have a dominant role. Apart from major histocompatibility complex (MHC) control, CIA is also under non-MHC polygenic control.^{34, 35} Recently, the C5 gene and the FcγRIIb encoding gene were found to be associated with inducibility of CIA.³⁶ C5a is a crucial mediator in IC mediated inflammation.^{29, 33} Blockade of C5a by CoVF completely prevented onset of IC mediated arthritis.²⁴ Apart from complement, FcγRIIb also is related to inducibility of CIA. C57BL/6 mice which are non-susceptible to CIA, become susceptible when these mice are made deficient for the inhibiting FcγRII.¹⁷ The latter suggests that dysregulation of FcγRs may have serious consequences for arthritis inducibility.

As macrophages covering the inside of the knee joint determine IC mediated inflammation, we detected FcγRs on these cells by immunohistochemistry. Synovial macrophages of CIA sensitive DBA/1, but not of B10.RIII, showed higher FcγR expression than C57BL/6 and BALB/c mice. The detection of murine FcγRs is hampered by the availability of only two specific anti-FcγR antibodies, anti-FcγRII and anti-FcγRII/III, of which anti-FcγRII is not suitable for immunohistochemistry. As essentially the same pattern was found in synovial and peritoneal macrophages we suggest that differences in FcγR expression are strain specific and found in macrophages present in all compartments. The finding that macrophages of B10.RIII mice only, express significantly higher levels of inhibiting FcγRII and not of activating FcγRIII seems to show that the balance between these mice is skewed towards the inhibiting receptor. Because of the lack of an anti-FcγRI antibody, we could not determine whether differences in expression of activating FcγRI exist, which means that it is still possible that the balance between activating and inhibiting FcγRs is skewed on naïve macrophages. When ratios of the mean expression of activating FcγRIII to inhibitory FcγRII are calculated we found a ratio of 1 for DBA/1 macrophages, and 0.5 for C57BL/6, BALB/c, and B10.RIII, indicating that the balance of FcγRIII/FcγRII in B10.RIII mice is comparable with that in C57BL/6 and BALB/c. Please note that absolute values of mean FcγRII and III cannot be compared as factors such as affinity of the monoclonal antibodies may be different. Only the ratios can be compared. The difference in the ratio of basal activating FcγRIII expression to inhibiting FcγRII between DBA/1 mice and B10.RIII mice indicates that hyperreactivity of their macrophages to ICs cannot be explained by a basal skewed balance of FcγRIII and II towards FcγRIII alone.

Apart from differences in basal expression, differences in regulation of FcγRs also may lead to hypersensitivity to ICs. We could not examine the regulation of expression of FcγRs at a protein level after stimulation with HAGG because the HAGG

attached to the cell membrane interfere with binding of the specific antibodies used for FACS analysis. Therefore, RT-PCR was used to determine the regulation of FcγRs after IC stimulation. At mRNA level we showed that macrophages of CIA sensitive mice express higher levels of FcγRI and III and lower levels of FcγRII at day 3 after stimulation, indicating a prolonged up regulation of the activating FcγRs and down regulation of the inhibitory FcγRs. Whether this leads to differences in expression at a protein level remains to be studied.

Our results indicate that in macrophages of CIA non-susceptible mice, activation by ICs is normalised after three days despite the presence of ICs. This mechanism probably protects macrophages from hyperactivation. In contrast, in the macrophages of the CIA susceptible strains, FcγRIII remains high and FcγRII low; this does not normalise even after three days of incubation, thus keeping the macrophage in an activated state. This may be regulated by an unidentified locus lying near the region encoding for FcγRIIb on chromosome 1, because this locus is highly associated with the severity of arthritis during CIA.³⁶ Apart from that, activation of macrophages by ICs may lead to production of cytokines like interferon γ,³⁷ transforming growth factor β,³⁸ and IL10,³⁹ which have clear enhancing effects on FcγRI and III expression. A different response on these cytokines may also explain the disturbed FcγR expression and is currently under investigation.

The absence of negative feedback on raised activating FcγRs on the membrane of CIA sensitive macrophages leads to raised and prolonged release of proinflammatory mediators like IL1 and matrix degrading enzymes like gelatinases/collagenases (MMP-1, MMP-8, MMP-13), after stimulation by ICs. The cytokine IL1 has a crucial role in IC mediated arthritis. It plays an important part in the regulation of joint inflammation,²³⁻⁴⁰ inhibits proteoglycan synthesis,²⁵ and regulates MMP mediated cartilage destruction.²⁶ Prolonged higher concentrations of IL1 within the joint during IC mediated arthritis may explain why this arthritis becomes chronic and results in very severe cartilage destruction.²³ The production of IL1 by macrophages of B10.RIII mice after stimulation with ICs is delayed compared with DBA/1. This can be explained by the finding that only DBA/1 mice show a skewed balance on naïve macrophages. In addition, the balance between activating and inhibiting FcγRs is skewed, as a consequence of interaction with ICs, and this change in FcγR expression is not seen until after day 1. When stimuli other than IC based components, streptococcal cell walls, and zymosan, were used no differences in IL1 production could be measured,²³⁻²⁸ strengthening the probability that this phenomenon is FcγR mediated.

Higher MMP expression by macrophages of DBA/1 mice may be the reason why IC arthritis in these mice is much more destructive.²³ Macrophages of B10.RIII mice do not produce detectable levels of pro-MMP within 48 hours after IC stimulation. This might be explained by the delay in IL1 production that was found in this strain. IL1 is an important mediator in the production of pro-MMPs.²⁶⁻⁴¹⁻⁴² The lack of active MMPs in the culture supernatants of all strains suggests that additional factors like oxygen radicals or proteases derived from polymorphonuclear cells, for example, are needed to activate latent MMPs in order to obtain the MMP mediated matrix damage that is found during IC arthritis.

In contrast with IL1α, IL6 production by peritoneal macrophages was significantly lower in the CIA sensitive strains than in C57BL/6 and BALB/c mice. IL6 induces tissue inhibitor of metalloproteinase-1 production and thus blocks collagenase activity.²⁷ Higher IL6 production by B10.RIII macrophages compared with DBA/1 macrophages may also explain why B10.RIII show low gelatinolytic activity.

During RA, a strong correlation has been found between the number of synovial macrophages and severe cartilage destruction.³⁻⁵ Also during RA, a strong up regulation of FcγRs

is found on synovial macrophages within the inflamed joint. Earlier studies by Edwards *et al* reported that FcγRIII is predominantly raised on RA synovial macrophages⁴³ and that activation of this receptor leads to a high release of tumour necrosis factor α.⁴⁴ A genetically determined derailment of activating FcγR regulation on synovial macrophages may, locally in the joints, drive hypersensitivity to ICs, resulting in chronicity of inflammation and severe cartilage destruction. Therapeutic intervention with activating FcγRs may form a new approach to combat this crippling disease.

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