EXTENDED REPORT

GM-CSF neutralisation suppresses inflammation and protects cartilage in acute streptococcal cell wall arthritis of mice

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Objective: The pathogenic involvement of granulocyte-macrophage colony-stimulating factor (GM-CSF) in arthritis has been put forward. We have investigated the therapeutic effect of GM-CSF neutralisation in the streptococcal cell wall (SCW) arthritis model in mice. In this model, the pathogenic contribution of tumour necrosis factor (TNF) α is minor and is expressed only on joint swelling, whereas cartilage proteoglycan depletion is independent of this cytokine.

Methods: Acute monoarthritis was induced by injection of SCW bacterial extracts to mouse knees. Treatments (mAb 22E9 at 300, 100, 30 μ g; or Enbrel 300 μ g) were given twice intraperitoneally 2 h before and 3 days after disease induction. Swelling was assessed by 99m Tc uptake into knees on days 1 and 2. Local cytokine levels were determined in patellae washouts on day one. Proteoglycan loss from cartilage was scored on histological sections at termination on day four.

Results: Treatment with anti-GM-CSF mAb 22E9 showed a dose-related efficacy by decreasing swelling that was significant at the 300 and 100 μ g doses in comparison to isotype control, and comparable to dexamethasone (5 mg/ml). Proteoglycan loss from cartilage was also significantly reduced by mAb 22E9 300 μ g ($p=0.001$). This reduced proteoglycan loss observed after GM-CSF neutralisation was not seen after TNF α -blockade with Enbrel. Similarly, levels of interleukin 1 β in joints were reduced after treatment with 22E9 mAb ($p=0.003$) but not in mice receiving Enbrel.

Conclusions: Our findings show a pathogenic role for GM-CSF in this arthritis model, support the therapeutic potential of neutralising this cytokine, and may indicate therapeutic activity of an anti-GM-CSF mAb in TNF α -independent disease situations.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a 23 kDa glycoprotein with a four alpha helical bundle structure that binds to a heterodimeric receptor composed of subunits belonging to the type 1 cytokine receptor family.¹ GM-CSF was originally described as a potent stimulus of the growth and differentiation of granulocyte and macrophage precursors *in vitro*.^{2,3} Subsequent studies showed that GM-CSF also stimulates proliferation and activation of mature immune cells as well as of antigen-presenting dendritic cells.^{4–7} Genetic ablation experiments in mice showed that, despite a previously ascribed role as colony-stimulating factor for blood-borne cells, GM-CSF is not required for steady-state haematopoiesis.⁸ It is however essential for functional activity of macrophage subpopulations such as those involved in clearing surfactant in the lung and responding to certain kinds of infection or immune responses. GM-CSF is now recognised as a key activator of the innate arm of the immune system and as such involved in chronic stages of inflammatory and autoimmune diseases where macrophages, neutrophils, granulocytes, eosinophils and dendritic cells contribute to tissue damage and disease progression.⁹

Rheumatoid arthritis is a chronic destructive disease characterised by joint inflammation leading to erosions of articular cartilage and subchondral bone. Numerous inflammatory cells, including macrophages and neutrophils, when activated, release an array of inflammatory cytokines and destructive enzymes that infiltrate the synovial membrane and joint space in patients with rheumatoid arthritis.^{10,11} Published work has established that GM-CSF is produced in rheumatoid arthritis synovium^{12,13} and that elevated levels of this cytokine can be measured in rheumatoid arthritis synovial fluid,¹⁴ suggesting that this cytokine may play a role in the pathogenesis of the disease. In support of this hypothesis are the findings in mouse

collagen-induced arthritis (CIA), showing that treatment with a neutralising anti-GM-CSF mAb decreases disease severity,¹⁵ and that GM-CSF deficient mice have a reduced susceptibility to disease induction.¹⁶ Further support is provided by studies reporting that GM-CSF injection into mice exacerbates CIA¹⁷ and that GM-CSF treatment corrects neutropenia in patients with Felty's syndrome or patients with rheumatoid arthritis after chemotherapy induced flares of disease severity.^{18,19}

Murine streptococcal cell wall (SCW) arthritis is an acute animal model of arthritis that can be induced by a single intra-articular injection of bacterial cell wall fragments into a knee joint of a naive mouse.²⁰ It has been shown that tumour necrosis factor (TNF) α and interleukin (IL)1 β play a different role in SCW arthritis. Although TNF α mediates joint swelling, its role in cartilage destruction is nil or minor, whereas IL1 β is critically involved.²¹ The aim of the present study was to extend previously published reports and to further validate GM-CSF as a therapeutic target for inflammatory diseases particularly for patients with rheumatoid arthritis whose disease is TNF α -independent. To this end, we have neutralised endogenous GM-CSF with the rat anti-mouse GM-CSF mAb 22E9 and investigated the effect of the treatment on inflammation and on articular cartilage. Levels of selected cytokines and chemokines in joints were also measured.

MATERIALS AND METHODS

Animals

Male C57/BL6 mice were obtained from Charles River (Sulzfeld, Germany). The mice were housed in filter top cages, and water

Abbreviations: CIA, collagen-induced arthritis; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; PBS, phosphate-buffered-saline; SCW, streptococcal cell wall; TNF, tumour necrosis factor

Figure 1 Effect of treatment with different doses of mAb 22E9 and dexamethasone on knee joint swelling. Streptococcal cell wall (SCW) arthritis was induced by injection of 25 µg SCW fragments into the right knee, whereas the left knee received the vehicle phosphate-buffered-saline. Treatments were given intraperitoneally 2 h before SCW injection into knees. Joint swelling was measured on days 1 and 2 after SCW injection by ^{99m}Tc uptake into knees and is shown expressed as right/left (R/L) knee ratio. A ratio of >1.1 is considered as joint swelling. Statistical analysis was performed according to the Mann Whitney U test comparing treatment groups to the rat IgG2a isotype antibody control groups, respectively. Symbols are: * $0.05 > p > 0.01$, ** $0.01 > p > 0.001$, *** $p \leq 0.001$. Two independent experiments were performed with five animals per group in (A), and 7 animals per group in (B). ip, intraperitoneally; NS, not significant.

and food were provided ad libitum. The mice were used at the age of 10–12 weeks. All animal procedures were approved by the institutional ethics committee.

SCW preparation and induction of SCW arthritis

Streptococcus pyogenes T12 organisms were cultured overnight in Todd-Hewitt broth. Cell walls were prepared as described previously.²⁰ The resulting $10\,000\times g$ supernatant was used throughout the experiments. The preparation contained 11% muramic acid. Unilateral arthritis was induced by intra-articular injection of 25 µg SCW (rhamnose content) in 6 µl phosphate-buffered-saline (PBS) into the right knee joint of naive mice. As a control, PBS was injected into the left knee joint as described previously.²²

Reagents and treatment protocol

GM-CSF was neutralised using mAb 22E9 (MM500CS, Perbio Science, Bonn, Germany). Etanercept (Enbrel; Wyeth Pharma, Münster, Germany) was used for TNF α blockade. Several studies have reported the therapeutic activity of this human soluble TNF α receptor Fc fusion protein in various mouse models, including CIA.^{23–28} Rat immunoglobulin (Ig G2a) isotype control (BLD-400 516-bulk, Biozol Diagnostica, Eching, Germany) was used as a control for mAb 22E9. Humira (Abbott, Wiesbaden-Delkenheim, Germany) the human IgG1 that does not cross-react with mouse TNF α has been used as a control for etanercept (Enbrel, a human IgG1 construct). For the treatments with proteins (mAbs and recombinant protein) mice used for assessment of joint swelling and cytokine levels were treated once intraperitoneally 2 h before the intra-articular administration of SCW fragments. Mice used on day four for analysis of joint histopathology were re-treated intraperitoneally on day 3, after induction of disease with SCW fragments. Dexamethasone (Sigma-Aldrich, Taufkirchen, Germany) at 2 mg/kg was given daily starting 2 h before the induction of disease.

Assessment of equilibrium dissociation constant of mAb 22E9

The kinetic association and dissociation rate constants (k_a , k_d) of mAb 22E9 to recombinant murine GM-CSF were investigated using surface plasmon resonance spectroscopy (Biacore, Uppsala, Sweden). Binding curves were determined with up to 8 concentrations of mAb 22E9 ranging from 10 µg/ml to 1 pg/ml purified protein. The independent fitting of the raw data resulted in dissociation and association rate constants that were used to calculate the equilibrium dissociation constant (K_d).

Assessment of the biological activity of mAb 22E9 (IC₅₀)

The biological activity of mAb 22E9 was determined in a cell-based proliferation inhibition assay using the FDCP1 cell line. FDCP1 cells (DSMZ ACC 368) were propagated in Basal Iscove Medium (Biochrom, with L-glutamine) with 10% heat-inactivated fetal calf serum in the presence of 0.2 ng/ml murine IL3 (Strathmann AG, Hamburg, Germany). For assessment of the biological activity of mAb 22E9, FDCP1 cells were seeded at a density of 0.9×10^4 cells/well in RPMI 1640 substituted with 10% fetal calf serum in a 96-well flat bottom microtest plate after washing twice with $1\times$ PBS. Proliferation of the FDCP1 cells was stimulated by mGM-CSF (Strathmann) at a final concentration of 1 ng/ml. For neutralisation of the mGM-CSF dependent proliferation, mAb 22E9 was added in a dilution series with final concentrations ranging from 10 µg/ml to 0.05 ng/ml. After incubation for 48 h at 37°C in 5% CO₂, the proliferative status of the FDCP-1 cells was determined by adding WST-1 reagent (Roche, Penzberg, Germany). Viable cells were quantitated by measuring the absorbance at 450 nm. Data were analysed and fitted for half-maximal inhibition of proliferation (IC₅₀) using the software package GraphPadPrism V.4.

Measurement of joint swelling

Swelling was quantified by the ^{99m}Tc -uptake method.²⁹ This method measures by external gamma counting the accumulation of a small radioisotope at the site of inflammation owing to local increased blood flow and tissue swelling. The severity of swelling is expressed as the ratio of the ^{99m}Tc -uptake in the right (inflamed) over the left (control) knee joint. All values exceeding 1.10 were assigned as joint swelling.

Cytokine and chemokine measurements

Levels of several cytokines and chemokines, including IL1 β , IL6, TNF α , RANTES, keratinocyte-derived chemokine (KC) and macrophage-inflammatory protein-1 α (MIP-1 α) were determined in patellae washouts, as described previously.³⁰ Patellae with surrounding synovial tissue, isolated from inflamed knee joints 2 h post-SCW administration, were cultured in RPMI 1640 medium containing 0.1% bovine serum albumin (200 μl /patella) for 1 h at room temperature. For adequate comparison, sizes of specimen were standardised to 6 mm using a biopsy puncher (Miltex, York, Pennsylvania, USA). At the end of the incubation, supernatants were harvested and centrifuged for 5 min at 1000 g . Cytokine and chemokine levels were determined using the Luminex multi-analyte technology. We used the BioPlex system from BioRad (Munich, Germany) in combination with multiplex cytokine and chemokine kits.

Histological analysis

Mice were sacrificed by cervical dislocation on day four. Whole knee joints were removed and fixed in 4% formaldehyde for 7 days before decalcification in 5% formic acid and processing for paraffin embedding. Tissue sections (7 μm) were stained with H&E or safranin O/fast green. Histopathological changes in the knee joints were scored in the patella/femur region on five semi-serial sections spaced 140 μm apart. Scoring was performed on coded slides by two separate observers, using the following parameters. In the H&E stained slides, the amount of cells infiltrating the synovial lining was scored from 0 to 3. Proteoglycan depletion was scored in the safranin O/fast green-stained slides on a scale from 0 to 3 (ranging from stained cartilage to fully destained cartilage).

Statistical analysis

Differences between experimental groups were tested using the Mann Whitney U test and GraphPad Prism 4 software. The following convention was used: *0.05 > p > 0.01; **0.01 > p > 0.001; and *** p $\leq$ 0.001.

RESULTS

Characteristics of anti-murine GM-CSF mAb 22E9

Quantitative binding data for mAb 22E9 to recombinant murine GM-CSF (rmGM-CSF) produced in *Escherichia coli* were obtained by plasmon resonance spectroscopy. mAb 22E9 had an association rate constant of $k_a = 4.6 \times 10^5 \text{ M/s}$, a dissociation rate constant of $k_d = 2.5 \times 10^{-5} \text{ s}^{-1}$, and an equilibrium binding constant of $K_D = 5.4 \times 10^{-11} \text{ M}$.

A cell-based proliferation inhibition assay using the FDCP1 cell line was used to determine the neutralising biological activity of mAb 22E9. Half-maximal inhibition of FDCP1 cell proliferation (IC₅₀) by mAb 22E9 was seen at $2.0 \times 10^{-11} \text{ M}$.

GM-CSF neutralisation is anti-inflammatory in the SCW model

The effect of neutralising endogenous GM-CSF was investigated by intraperitoneal injection of mAb 22E9 2 h before intra-articular injection of SCW fragments into knee joints. Joint swelling was measured by the ratio of ^{99m}Tc uptake between the SCW-injected knee and the PBS-injected knee on days 1 and 2 post-SCW injection. In a first experiment ($n = 5$), an antibody dose-related significant inhibition of joint swelling was observed on both day 1 and day 2 after treatment with 100 μg ($p = 0.01$ and $p = 0.008$ on days 1 and 2 respectively) and 300 μg mAb 22E9 ($p = 0.008$ on days 1 and 2; fig 1A). This was confirmed in a second independent experiment ($n = 7$) in which lower doses of 30 μg mAb 22E9 as well as dexamethasone at 5 mg/kg were added as positive controls. The 30- μg dose of mAb 22E9 was not sufficient to considerably decrease joint swelling on either day, whereas dexamethasone was as potent as the 300- μg dose of the anti-GM-CSF antibody (fig 1B). The anti-inflammatory activity of GM-CSF neutralisation was compared with TNF α neutralisation using Enbrel at the same protein concentration as mAb 22E9 (300 μg). Enbrel showed efficacy at decreasing joint swelling in the acute model of SCW arthritis only at day 2 (fig 2). Treatment with mAb 22E9 was significantly more effective than Enbrel at decreasing joint swelling on days 1 and 2 ($p = 0.008$ and $p = 0.05$ for days 1 and days 2, respectively). In these and all subsequent experiments, respective isotype control antibodies for 22E9 and Enbrel were not found to be effective.

GM-CSF neutralisation reduces inflammatory cell influx to synovium and proteoglycan loss from cartilage

Histopathological sections from the different groups of mice were prepared after termination of the experiment on day 4. The influx of inflammatory cells into synovium was scored visually on H&E-stained sections. Proteoglycan content in

Figure 2 Effect of granulocyte-macrophage colony-stimulating factor neutralisation by mAb 22E9 (300 μg) and tumour necrosis factor α -blockade by Enbrel (300 μg) on joint swelling as measured by ^{99m}Tc uptake into knees. Disease induction, treatments, measurements and symbols are as described in the legend to fig 1. Statistical analysis was performed according to the Mann Whitney U test comparing treatment groups to the rat IgG2A or human IgG1 isotype antibody control groups, respectively. The experiment was performed twice with five mice tested per group.

Figure 3 Effect of granulocyte-macrophage colony-stimulating factor neutralisation by mAb 22E9 (300 µg) and tumour necrosis factor α -blockade by Enbrel (300 µg) on (A) influx of inflammatory cells into synovium, and (B) proteoglycan loss in articular cartilage. Disease induction was as described in the legend to figure 1. Treatments were given intraperitoneally twice, first 2 h before and then 3 days after intra-articular injection of streptococcal cell wall fragments. Mice were euthanised on day 4 and histological sections were prepared and scored visually. Statistical analysis was performed according to Mann Whitney U test comparing treatment groups to isotype antibody control groups. Results from rat IgG2A and human IgG1 control groups were combined because they did not show a significant difference. Six animals were tested per treatment group in two separate experiments.

cartilage was scored on safranin O/fast green-stained section. Both GM-CSF neutralisation with mAb 22E9 and TNF α neutralisation with Enbrel were significantly efficacious at reducing the influx of inflammatory cells into synovium ($p = 0.008$ and $p = 0.017$, respectively, fig 3A). However, only GM-CSF neutralisation was effective at reducing the loss of proteoglycan from articular cartilage ($p = 0.001$; 22E9 vs isotype control (fig 3B)). Treatment with Enbrel, although efficacious at decreasing the influx of cells into the synovium, did not reduce loss of proteoglycan from cartilage (fig 3B). The effect of the treatments is illustrated on the microphotographs of one representative mouse from each treatment group (fig 4). The intense cartilage stain and good tissue preservation observed in the 22E9-treated mouse (fig 4A) show the protective effect of GM-CSF neutralisation on cartilage preservation. By contrast, figure 4C show the cartilage destruction and reduced staining intensity in mice receiving the IgG2A control mAb. The Enbrel-treated mouse also shows reduction in staining intensity of cartilage (fig 4B). Abundant inflammatory cells infiltrate the synovium and joint space in the isotype control-treated mouse (fig 4C) showing the severe inflammation that is markedly lower in the mice treated with anti-GM-CSF mAb (fig 4A) and Enbrel (fig 4B).

GM-CSF neutralisation reduces production of IL1 β in knee joints

Affected (right) knees were analysed for levels of selected cytokines and chemokines. Only affected knees were analysed

as levels in the non-affected control knees (left) have been found repeatedly to be below the detection limit in several previous experiments.³¹ Analysis showed that GM-CSF neutralisation resulted in a considerable reduction of IL1 β in affected knee joints of mice treated with 22E9 mAb in comparison to the levels detected in joints from mice receiving the isotype control (fig 5; $p = 0.003$, 22E9-treated vs control). By contrast, TNF α blockade with Enbrel had no effect on the levels of IL1 β in joints (fig 5). Levels of IL2, TNF α and GM-CSF were measured but all three were below the sensitivity limits of the assays (ie, 10 pg/ml). Local levels of IL6, KC (a murine IL8 equivalent) and RANTES were measurable but their levels were not influenced by either of the two treatments investigated.

DISCUSSION

Presently, the most effective treatment for rheumatoid arthritis in humans is TNF α neutralisation.^{32–38} However, 30–40% of patients with rheumatoid arthritis are resistant to anti-TNF α therapy, suggesting that in a proportion of patients the disease is TNF α -independent. In SCW arthritis mice, only joint inflammation is TNF α -dependent whereas joint destruction is not blocked by TNF α antagonists.²¹ We have investigated the anti-inflammatory and chondroprotective efficacy of GM-CSF neutralisation in the SCW arthritis model in an attempt to identify a treatment that could potentially be effective in cases of TNF α -independent destructive disease. We have used a rat anti-mouse GM-CSF neutralising mAb and shown that it has a high binding affinity for recombinant mouse GM-CSF and

Figure 4 Photomicrograph of representative knee joints from mice treated with: anti-granulocyte-macrophage colony-stimulating factor mAb 22E9 (A); tumour necrosis factor α antagonist Enbrel (B); and isotype control mAb (C). Whole knees were removed and fixed as described in materials and methods. Frontal sections (7 µm) were stained with safranin O/fast green for visualisation of proteoglycan depletion by diminished red staining of cartilage matrix. Cartilage with well-preserved proteoglycan content is seen in (A), partial proteoglycan depletion (pink layer) is seen in (B) and (C). Note the severe synovial inflammation in (C) and milder inflammation in (A) and (B) (arrowheads). F = femur; P = patella; JS = joint space; C = cartilage, S = synovium (Original magnification 100 $\times$).

Figure 5 Effect of granulocyte-macrophage colony-stimulating factor neutralisation by 22E9 monoclonal antibody (mAb) (300 µg) and of tumour necrosis factor α -blockade by Enbrel (300 µg) on IL1 β the levels in knee joints. Disease induction and treatments were as described in legend to figs 1 and 2. Patellae washouts were obtained 2 h after streptococcal cell wall injections. Groups were statistically compared by the Mann Whitney U test. Five animals were tested per treatment group. Results were confirmed in a second independent experiment.

equally high cytokine-neutralising potential. Our results show a significant dose-related anti-inflammatory activity of GM-CSF neutralisation by mAb 22E9 as measured by the uptake of a radiolabelled molecule into knees, a validated test for quantification of joint swelling.²⁹ The suppression of joint swelling by 22E9 was seen on days 1 and 2 after arthritis induction and was comparable to the anti-inflammatory effect observed with dexamethasone treatment. GM-CSF neutralisation by mAb 22E9 was clearly superior to TNF α neutralisation by Enbrel at the same protein concentration (300 µg) showing that the two anti-inflammatory approaches have different modes of action in the SCW model. Apparently, GM-CSF neutralisation did not act via inhibition of TNF α production but rather via inhibition of other mediators of inflammation.

Many pathogenic features of arthritis rely on the accumulation of inflammatory cells in the joints and the local release of proinflammatory mediators such as cytokines and chemokines, inducing more immune cells to the site of disease. This leads to further increased inflammation, even higher levels of proinflammatory cytokines and destructive enzymes in joints, and ultimately to cartilage and bone destruction.³⁹ One of the major constituents of articular cartilage is aggrecan, a large aggregating chondroitin sulphate proteoglycan. Its loss from cartilage is one of the major causes for cartilage destruction in arthritis.^{40–41} GM-CSF neutralisation significantly reduced the number of inflammatory cells in the synovium of diseased knees, as did neutralisation of TNF α . However, although efficacious at decreasing cell influx, neutralisation of TNF α had no effect on proteoglycan loss, showing that in this model cartilage proteoglycan loss is independent of TNF α ²¹ and probably mediated by factors produced by cells already present in the joint. By contrast, GM-CSF neutralisation was remarkably efficacious at decreasing proteoglycan loss showing a chondroprotective therapeutic activity. Published studies have shown that in several models of arthritis one of the main cytokines controlling cartilage destruction is IL1 β .^{21–42–44} One possible mechanism of the cartilage protective activity of GM-CSF neutralisation could be by reduction of local levels of IL1 β . Indeed, GM-CSF neutralisation by 22E9 decreased levels of IL1 β in joints whereas TNF α neutralisation had no effect on

IL1 β levels, thereby providing a possible explanation for the differential chondroprotective effect of both treatments. GM-CSF treatment had no effect on local levels of other mediators such as the chemokines RANTES and KC or IL6, supporting the predominant role for IL1 β in cartilage destruction.

Increased levels of GM-CSF have been found in rheumatoid arthritis synovium^{12–13} and synovial fluid,¹⁴ suggesting that this cytokine may play a role in the pathogenesis of the disease. Exogenous administration of GM-CSF to patients with rheumatoid arthritis or Felty's patients and CIA mice also increased disease severity.^{17–19} By contrast, in the absence of GM-CSF, such as in GM-CSF knock-out mice, susceptibility to arthritis is reduced¹⁶ and treatment of CIA arthritic mice with anti-GM-CSF mAb decreased disease severity.¹⁵ Our findings in another experimental model of rheumatoid arthritis support the hypothesis that GM-CSF may represent a novel therapeutic target for arthritis. Together with our study in the fully TNF α -independent chronic SCW arthritis model in which treatment was started on week 3 of disease induction (Plater-Zyberk et al, submitted), our data support the fact that neutralisation of GM-CSF is a potential treatment that would not only decrease inflammation but additionally protect cartilage from destruction possibly also in TNF α -independent disease.

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