

Research article

Open Access

Treatment with recombinant interferon- β reduces inflammation and slows cartilage destruction in the collagen-induced arthritis model of rheumatoid arthritis

Judith van Holten¹, Kris Reedquist¹, Pascale Sattonet-Roche², Tom JM Smeets¹, Christine Plater-Zyberk², Margriet J Vervoordeldonk¹ and Paul P Tak¹

¹Division of Clinical Immunology and Rheumatology, Academic Medical Center/University of Amsterdam, The Netherlands

²Serono Pharmaceutical Research Institute, Geneva, Switzerland

Corresponding author: Paul P Tak (e-mail: P.P.Tak@amc.uva.nl)

Received: 6 Nov 2003 Revisions requested: 12 Dec 2003 Revisions received: 18 Feb 2004 Accepted: 19 Feb 2004 Published: 23 Mar 2004

Arthritis Res Ther 2004, **6**:R239-R249 (DOI 10.1186/ar1165)

© 2004 van Holten *et al.*, licensee BioMed Central Ltd. This is an Open Access article: verbatim copying and redistribution of this article are permitted in all media for any purpose, provided this notice is preserved along with the article's original URL.

Abstract

We investigated the therapeutic potential and mechanism of action of IFN- β protein for the treatment of rheumatoid arthritis (RA). Collagen-induced arthritis was induced in DBA/1 mice. At the first clinical sign of disease, mice were given daily injections of recombinant mouse IFN- β or saline for 7 days. Disease progression was monitored by visual clinical scoring and measurement of paw swelling. Inflammation and joint destruction were assessed histologically 8 days after the onset of arthritis. Proteoglycan depletion was determined by safranin O staining. Expression of cytokines, receptor activator of NF- κ B ligand, and c-Fos was evaluated immunohistochemically. The IL-1-induced expression of IL-6, IL-8, and granulocyte/macrophage-colony-stimulating factor (GM-CSF) was studied by ELISA in supernatant of RA and osteoarthritis fibroblast-like synoviocytes incubated with IFN- β . We also

examined the effect of IFN- β on NF- κ B activity. IFN- β , at 0.25 μ g/injection and higher, significantly reduced disease severity in two experiments, each using 8–10 mice per treatment group. IFN- β -treated animals displayed significantly less cartilage and bone destruction than controls, paralleled by a decreased number of positive cells of two gene products required for osteoclastogenesis, receptor activator of NF- κ B ligand and c-Fos. Tumor necrosis factor α and IL-6 expression were significantly reduced, while IL-10 production was increased after IFN- β treatment. IFN- β reduced expression of IL-6, IL-8, and GM-CSF in RA and osteoarthritis fibroblast-like synoviocytes, correlating with reduced NF- κ B activity. The data support the view that IFN- β is a potential therapy for RA that might help to diminish both joint inflammation and destruction by cytokine modulation.

Keywords: antibodies, cytokines, inflammation, rheumatoid arthritis

Introduction

Interferons are a family of naturally secreted proteins with potent immunomodulatory functions [1]. They are divided into two groups, type I IFNs (IFN- α and - β) and type II IFN (IFN- γ) [2,3]. Generally, IFN- β and IFN- γ are thought to play opposing roles in the regulation of inflammatory responses: IFN- γ promotes inflammatory responses, whereas IFN- β has mainly anti-inflammatory properties.

IFN- β downregulates the proinflammatory cytokines IL-1 β and tumor necrosis factor α (TNF- α) and enhances IL-10 and IL-1 receptor antagonist production by lymphocytes *in vitro* [4–6], increases IL-1 receptor antagonist production by fibroblast-like synoviocytes (FLS) [7], inhibits T-cell proliferation and migration, and prevents contact-dependent T-cell activation of monocytes [8]. IFN- β also suppresses IFN- γ production and class II major

BSA = bovine serum albumin; CIA = collagen-induced arthritis; DMEM = Dulbecco's modified Eagle's medium; ELISA = enzyme-linked immunosorbent assay; FCS = fetal calf serum; FLS = fibroblast-like synoviocytes; GM-CSF = granulocyte/macrophage-colony-stimulating factor; HRP = horseradish peroxidase; IFN = interferon; IL = interleukin; MHC = major histocompatibility complex; NF = nuclear factor; OA = osteoarthritis; PBS = phosphate-buffered saline; RA = rheumatoid arthritis; RANKL = receptor activator of NF- κ B ligand; TNF = tumor necrosis factor.

histocompatibility complex expression by activated peripheral blood mononuclear cells [9]. Recent studies have also found that IFN- β enhances expression of transforming growth factor β 1 and transforming growth factor β 1 receptor type II by peripheral blood mononuclear cells [10].

In a study in the murine collagen-induced arthritis (CIA) model, utilized extensively to evaluate novel forms of therapy for rheumatoid arthritis (RA), DBA/1 mice were injected intraperitoneally with fibroblasts expressing IFN- β , resulting in continuous IFN- β delivery *in vivo*, before or after the onset of CIA [11]. A single injection of IFN- β -secreting fibroblasts was sufficient to prevent arthritis or ameliorate existing disease. A study of four rhesus monkeys with CIA suggested a marked beneficial effect of daily injections with IFN- β [4]. So far, little detailed cellular or molecular analysis has been performed to determine the mechanism of IFN- β action in the CIA models.

In a pilot study, six children with juvenile rheumatoid arthritis were treated with IFN- β for 16 weeks. All tolerated the treatment well and met the criteria for a 30% response to treatment; three of the six met the 50% response criteria [12]. Additionally, evaluation of 11 patients participating in a pilot study in RA showed that IFN- β treatment significantly reduced synovial cell infiltration, as well as IL-1 β and IL-6 expression in the synovial tissue [13].

In addition to the anti-inflammatory effects of IFN- β , a novel role in the maintenance of bone homeostasis has recently been described. RANKL (receptor activator of NF- κ B ligand) stimulation of osteoclast precursor cells results in their differentiation into mature bone-resorbing osteoclasts [14]. RANKL stimulation simultaneously induces c-Fos-dependent IFN- β expression by osteoclasts. Subsequent IFN- β signaling inhibits osteoclastogenesis, in part through negative feedback signalling to c-Fos [14,15]. Mice deficient in IFN- β and IFN receptor display pronounced osteopenia, and exogenous IFN- β treatment can prevent lipopolysaccharide-induced osteopenia in mice. This novel protective role of IFN- β might be important in the prevention of bone erosions, a major problem in the treatment of RA.

We have studied whether daily systemic administration of exogenous IFN- β in CIA in mice could have a beneficial effect on disease activity, despite the short half-life of the compound. Specifically, we examined the effect of IFN- β on osteoclastogenesis in the arthritis model. *In vitro* experiments were conducted to determine the effect on the IL-1-induced production of IL-6, IL-8, and granulocyte/macrophage-colony-stimulating factor (GM-CSF) by FLS in RA and osteoarthritis (OA). Since gene expression of proinflammatory cytokines is known to be under control of the common transcription factor NF- κ B [16], we studied the effect of IFN- β on NF- κ B activity.

Materials and methods

Animals

Male DBA/1 mice 10–12 weeks of age were purchased from Bomholdgård (Ry, Denmark). They were maintained in a pathology-free animal facility at the Serono Pharmaceutical Research Institute (Geneva, Switzerland). Water and food were provided *ad libitum* and all experiments were approved by the Institutional Animal Care and Use Committee of Switzerland.

Induction and treatment of arthritis in mice

A solution of bovine collagen type II (2 mg/ml in 0.05 M acetic acid [Chondrex, Redmond, WA, USA]) emulsified in an equal volume of complete Freund's adjuvant (2 mg/ml of *Mycobacterium tuberculosis*; strain H37Ra [Difco Laboratories, Detroit, MI, USA]) was used to induce arthritis [17]. The mice were immunized intradermally at the base of the tail with 100 μ l of emulsion (100 μ g collagen). Arthritis usually developed between days 28 and 40 after immunization.

Mice were scored visually for the appearance of arthritis. They were considered to have arthritis when significant changes in redness and/or swelling were noted in the digits or other parts of the paw. For each mouse, day 1 of arthritis represents the first day that clinical arthritis was detected in that mouse. The animals were randomly assigned to one of four groups, in which they were treated intraperitoneally with 0.25, 1.25, or 2.5 μ g recombinant IFN- β per injection or with saline as a control. Treatment was started at the first clinical sign of disease. All groups were treated daily for 7 days. Thereafter, the mice were killed by cervical dislocation and the hind paws were collected and used for further analysis. The *in vivo* experiments were performed with 8–10 mice per group and were repeated twice to ensure reproducibility.

Evaluation of arthritis activity

Mice were inspected daily for signs of arthritis by an independent observer who was not aware of the treatment. Swelling was quantified by measuring the thickness of the hind footpad of the first arthritic paw with a caliper. Clinical scores were assessed using an established macroscopic system ranging from 0 to 3.5: 0 = normal, 1 = slight swelling and/or erythema, 2 = pronounced edematous swelling, 3.5 = maximal swelling and joint deformities with ankylosis [18]. The cumulative score for all four paws of each mouse was used as arthritis score (maximum of 14 per mouse) to represent overall disease severity and progression in an animal. After 7 days of treatment, mice were killed and their paws were processed for histopathological evaluation.

Histology

Arthritic paws were fixed in 10% buffered formalin and decalcified in 15% EDTA in buffered formalin (5.5%). The

paws were then embedded in paraffin and 5- μ m sagittal serial sections of whole hind paws were cut and stained with hematoxylin and eosin and examined for the degree of synovitis and bone erosions by microscopic evaluation in a blinded manner as described earlier [19]. Bone erosions were scored using a semiquantitative scoring system from 0 to 4 (0 = no erosions, 4 = extended erosions and destruction of bone). Sections were also stained with safranin O-fast green to determine the loss of proteoglycans. Safranin O staining was scored with a semiquantitative scoring system (0–3), where 0 represents no loss of proteoglycans and 3 indicates complete loss of staining for proteoglycans [20].

Immunohistochemistry for cell markers and cytokine detection in synovial tissue

Immunohistochemical staining on serial sections was performed to detect CD3-positive T cells (Novocastra Lab Ltd, Newcastle, UK), and CD22-positive B cells (Southern Biotech, Birmingham, AL, USA). Cytokine staining was performed with the following goat polyclonal antibodies: anti-TNF- α (SC-1351), anti-IL-1 β (SC-1251), anti-IL-6 (SC-1265), anti-IL-10 (SC-1783), and anti-IL-18 (SC-6179) (all purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA). In addition, sections were stained for RANKL and c-Fos (R&D Systems Europe Ltd, Abingdon, UK). For control sections, the primary antibody was omitted or irrelevant immunoglobulins were applied. The sections were washed between all steps with phosphate-buffered saline (PBS).

Paraffin-embedded sections (5 μ m) were dewaxed and dehydrated in a gradient of alcohols. Endogenous peroxidase activity was quenched with 0.3% H₂O₂ and 0.1% sodium azide in PBS. Antigen retrieval was performed by heating the sections for 5 minutes at 95°C in 10 mM citric acid, pH 6.0, or 1 mM EDTA buffer, pH 8.0. The primary antibodies were diluted in PBS containing 1% BSA and 10% normal mouse serum at the following dilutions: anti-CD3 (1:20), anti-CD22 (1:20), anti-TNF- α (1:10), anti-IL-1 β (1:60), anti-IL-6 (1:60), anti-IL-10 (1:80), anti-IL-18 (1:60), anti-RANKL (1:20), and anti-c-Fos (1:800) and incubated overnight at 4°C. Thereafter, the sections were incubated with horseradish peroxidase (HRP)-conjugated swine antigoat antibody (1:320, Tago, Burlingame, CA, USA) or goat antirat HRP (1:100, Southern Biotech, Alabama, USA) in PBS/1% BSA for 30 min at room temperature. Subsequently, the slides were incubated for 15 min with biotinylated tyramine and for 30 min with HRP-conjugated streptavidin. HRP activity was detected using hydrogen peroxide as substrate and aminoethylcarbazole as dye. Sections were briefly counterstained with Mayer's hemalum solution [13].

All sections were analyzed in a blinded manner by two independent observers. After immunohistochemical

staining, expression of the different markers in the synovial tissue of all ankle and knee joints present was scored semiquantitatively on a 5-point scale [21]. A score of 0 represented minimal expression, while a score of 4 represented abundant expression of a marker. Minor differences between the observers were resolved by mutual agreement [22].

***In vitro* studies of the effects of IFN- β on cytokine production by FLS**

FLS were isolated from RA and OA synovial tissue obtained by arthroscopy. Small-bore arthroscopy (2.7-mm arthroscope; Storz, Tuttlingen, Germany) was performed under local anesthesia on the inflamed joint. FLS from three RA and three OA patients were prepared as described previously [23]. Cells used at passages 3 through 6 were seeded at 400,000 cells/well in a 6-well plate and incubated for 24 hours in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) at 37°C. The next day, DMEM/10% FCS was replaced by DMEM/0.5% FCS for 24 hours, followed by another 48 hours in the presence or absence of 125 pg/ml of IL-1 β together with various concentrations of IFN- β in DMEM/1% FCS. Experiments were performed three times in duplicate. Thereafter, cells were stained with trypan blue and cell viability was assessed for potential toxic effects of IFN- β on FLS. Supernatant was removed and stored at -20°C until use. Supernatant, obtained as described above, was analyzed for secreted IL-6, TNF- α , IL-12p40, GM-CSF (all from R&D Systems Europe Ltd), IL-18 (MBL Ltd, Nagoya, Japan), and IL-8 (CLB, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) by sandwich ELISA.

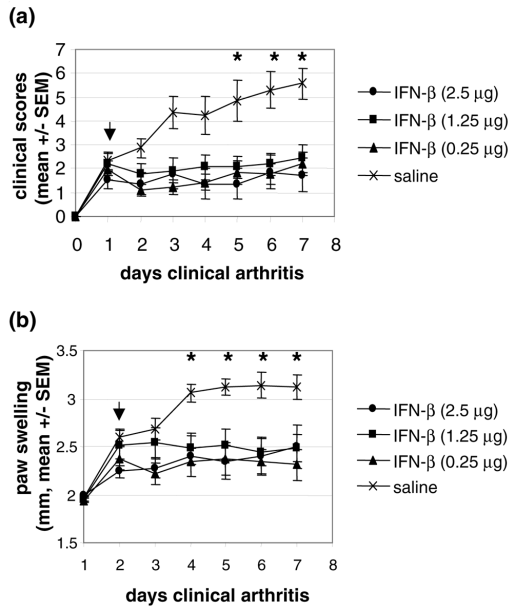
Evaluation of apoptotic induction in FLS by IFN- β

FLS were seeded at 100,000 cells/well and incubated for 18 hours in the presence or absence of 125 pg/ml of IL-1 β together with various concentrations of IFN- β or 150 μ M H₂O₂. Cells were trypsinized and counted, and cytopins were performed. After the slides were dried, Diff-Quik staining (Dade Behring, Düringen, Switzerland) was performed and the percentage of pyknotic nuclei was calculated by microscopic analysis to represent the amount of apoptosis. This technique is comparable to the Pappenheim technique by Giemsa–May–Grünwald staining.

FLS transfection and NF- κ B activation analysis

FLS from RA patients were transfected with 1 μ g each of NF- κ B/luciferase reporter construct and a renilla luciferase construct under the control of the thymidine kinase promoter. FLS cultured at 50,000 cells/well were transfected with 2 μ g of DNA mix and 6 μ l of Fugene 6 transfection reagent (Roche, Indianapolis, IN, USA) in DMEM overnight. Thereafter, transfected FLS were incubated for 18 hours in the presence or absence of

Figure 1



Effect of systemically delivered IFN-β at the onset of arthritis in mice with collagen-induced arthritis. **(a)** Clinical score (mean ± SEM) was assessed on a scale of 0 to 3.5 (as described in Materials and methods), and **(b)** hind paw swelling (mean ± SEM) of the first arthritic paw was monitored during the course of disease using calipers. On day 7 after the start of the treatment, differences between all treatment groups and the control group were statistically significant ($P = 0.005$) (Kruskal–Wallis test). Arrows mark the first day of treatment. *Statistically significant difference. SEM, standard error of the mean.

125 pg/ml of IL-1β together with various concentrations of IFN-β before measurement of luciferase activity (Dual Luciferase Assay System; Promega, Leiden, The Netherlands). NF-κB-dependent luciferase activity was normalized to renilla luciferase activity to account for potential variations in transfection efficiency and/or nonspecific transcriptional effects. Experiments were performed three times.

Statistical analysis

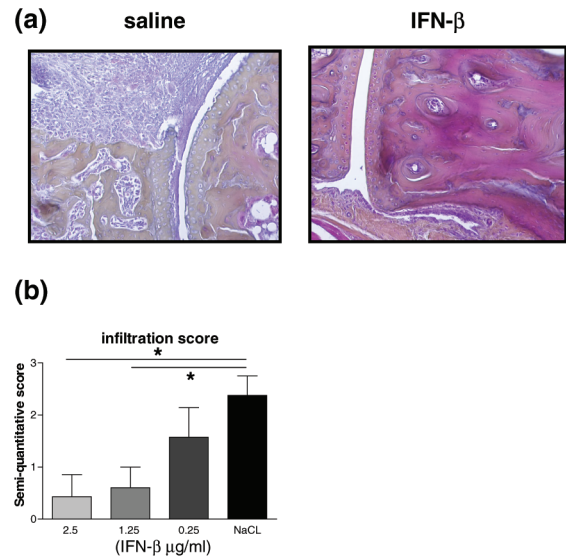
The following nonparametric tests were used: the Kruskal–Wallis test for several group means (comparing histologic scores and the scores for expression of different markers in more than two therapy groups), followed by the Mann–Whitney *U* test for comparison of two groups [22].

Results

IFN-β therapy inhibits arthritic activity and synovial inflammation

To examine whether IFN-β therapy could be effective in the treatment of CIA, mice were given a daily intra-peritoneal injection with various dosages of IFN-β or saline for 7 days. All animals were treated at the first clinical sign

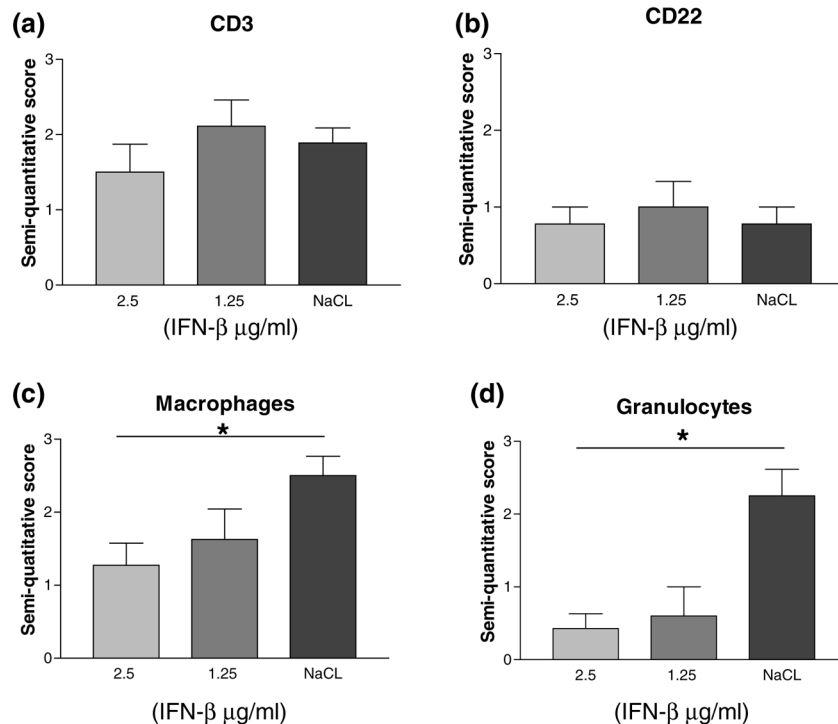
Figure 2



Representative histologic staining (hematoxylin and eosin; ×100) of the ankle joints in mice with collagen-induced arthritis (CIA). At day 7 after the start of treatment with IFN-β, mice were sacrificed and subjected to histopathological examination. **(a)** In the control CIA mice treated with saline, massive cellular infiltration and erosion of bone were observed in the ankle joint. In CIA mice treated with the highest dose of IFN-β (2.5 μg per injection per mouse), limited hyperplasia of the intimal lining layer and cell infiltration of the synovial sublining were detected and a decrease in bone erosions was observed. **(b)** Infiltration of inflammatory cells of the joints was scored from 0 to 4 in a blinded manner as described in Materials and methods. A significant reduction in inflammatory cells was observed for the treatment groups treated with 2.5 μg and 1.25 μg IFN-β in comparison with controls ($P = 0.04$). *Statistically significant difference.

of disease. Although disease started on different days after immunization, we did not observe a relation between clinical response and time of onset of disease. All groups treated with IFN-β showed a 50% decrease in arthritis score and an approximately 70% decrease in paw swelling compared to controls ($P = 0.005$) (Fig. 1a,b). There was no clear dose dependency, suggesting that all dosages were in the therapeutic range with regard to effects on clinical signs of arthritis. An additional experiment in which animals were treated at the first clinical sign of disease for 14 days showed the same beneficial effect of treatment (data not shown).

The effect of IFN-β treatment on synovial inflammation was also assessed by histology. A marked reduction in the number of inflammatory cells was found for all IFN-β-treated groups (Fig. 2a): a statistically significant ($P = 0.04$) reduction of inflammatory cells was observed in mice treated with the 1.25- or 2.5-μg doses; there was also a tendency towards decreased cell infiltration in the mice treated with the 0.25-μg dose, but this did not reach

Figure 3

T-cell, B-cell, macrophage, and granulocyte expression in the synovium of mice with collagen-induced arthritis, after 7 days of IFN- β therapy, detected by immunohistochemistry. Sections were scored for CD3 and CD22 as described in Materials and methods. Macrophages and granulocytes were evaluated by morphology. **(a)** CD3 expression. **(b)** CD22 expression. **(c)** Macrophage expression. **(d)** Granulocyte expression. No significant differences between treated animals and controls were observed for CD3 and CD22 expression ($P = 0.4$ for both). Statistically significant differences were observed for macrophage and granulocyte expression between IFN- β -treated mice and saline-treated mice ($P = 0.04$ and 0.009 , respectively). *Statistically significant difference.

statistical significance (Fig. 2b). While the total numbers of infiltrating T and B lymphocytes were similar between control and IFN- β -treated mice ($P = 0.4$) (Fig. 3a,b), we observed an approximately 50% reduction in the number of macrophages ($P = 0.04$) (Fig. 3c) and a 70% decrease in the number of granulocytes ($P = 0.009$) (Fig. 3d) in the mice treated with the highest dose (2.5 μg) of IFN- β .

IFN- β treatment inhibits cartilage and bone degradation

Next, cartilage destruction was assessed by safranin O staining on paraffin sections of joints from CIA mice treated with the two highest doses of IFN- β , or with saline as control. Scores for proteoglycan staining were similar in the group given 1.25 μg IFN- β /day and the control group (Fig. 4b). However, in CIA mice treated with the highest dose of IFN- β (2.5 μg IFN- β /day) we observed a 70% decrease in scores for proteoglycan depletion in cartilage ($P = 0.03$).

Bone destruction was assessed by staining of paw sections with hematoxylin and eosin to assess erosion scores. Analysis of the ankle and knee joints revealed a 77% reduction in the mean scores for bone erosions in

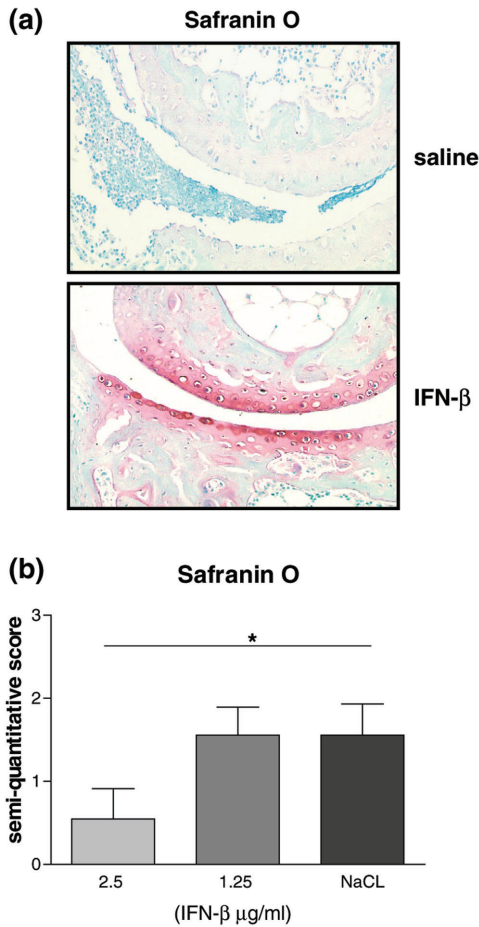
mice treated with the two highest doses of IFN- β in comparison with controls ($P = 0.02$), whereas the lowest treatment dose did not result in a significant decrease in bone erosions (Fig. 5a).

Having shown the protective effect of IFN- β treatment on bone degradation, we analyzed the number of cells positive for RANKL and c-Fos, molecules that are intimately involved in osteoclast function. We found a 50% decrease in the number of RANKL-positive cells ($P = 0.07$) and a 50% reduction in the number of c-Fos-positive cells ($P = 0.04$) in the animals treated with the highest dose of IFN- β (Fig. 5b,c, respectively). In addition, we found a positive correlation between osteoclast-like cells defined by morphology and the number of cells expressing c-Fos (data not shown).

IFN- β modulates the cytokine profile in inflamed synovial tissue.

To provide more insight into the mechanism by which IFN- β therapy exerts its beneficial effects, we examined cytokine expression at the site of inflammation. Several proinflammatory cytokines play a crucial role in the

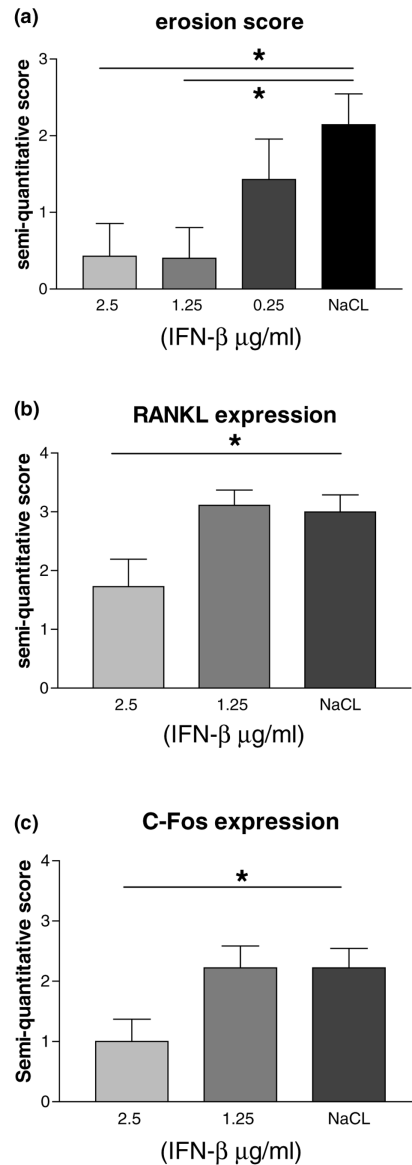
Figure 4



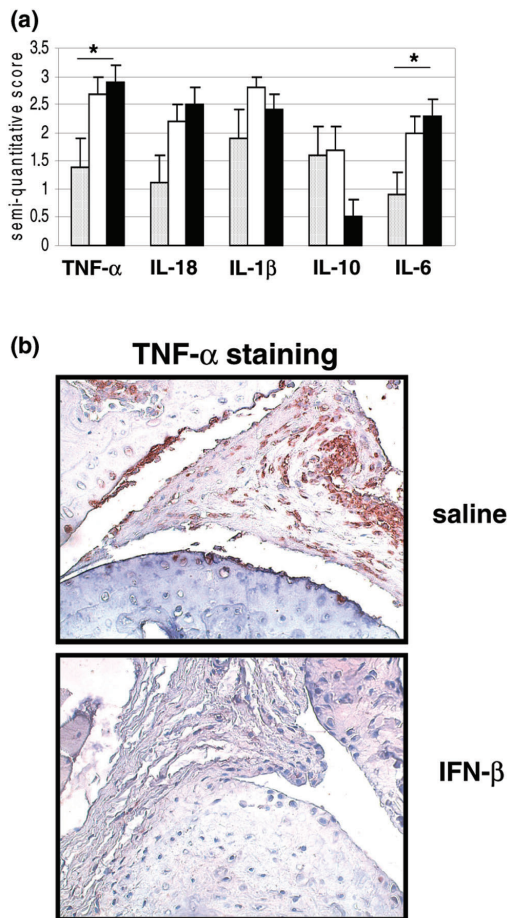
Representative histologic staining with safranin O-fast green ($\times 100$) of the ankle joint of mice with collagen-induced arthritis (CIA) mice after daily IFN- β therapy for 7 days. **(a)** (Upper panel) In CIA mice treated with saline as controls, hardly any safranin O staining was observed in the ankle joints. (Lower panel) In CIA mice treated daily with 2.5 μg IFN- β , significantly less loss of safranin O staining was observed, indicating inhibition of cartilage breakdown. **(b)** Histologic analysis of cartilage in CIA in mice after 7 days of IFN- β therapy. Hind paw sections were stained with safranin O-fast green, which stains the cartilage proteoglycans. Sections were scored in a blinded manner on a 4-point scale as described in Materials and methods. Significantly less loss of safranin O staining was observed in the animals treated with IFN 2.5 μg than in controls ($P = 0.03$), indicating inhibition of cartilage destruction. *Statistically significant difference.

pathogenesis of RA, including IL-1, TNF α , and IL-6 [24], while the anti-inflammatory cytokine IL-10 has been found to be protective [25]. Recently, the presence of IL-18 in RA synovium and its role in the development and maintenance of inflammatory arthritis have been shown [26]. To determine whether alterations in the expression of these mediators might contribute to the protective effect of IFN- β treatment on the development of CIA in mice, we performed an immunohistochemical analysis of cytokine expression on paraffin sections of mouse paws. The

Figure 5



Histologic analysis of bone erosions in mice with collagen-induced arthritis (CIA) after treatment with IFN- β . Mice were treated with the indicated concentrations of IFN- β for 7 days starting when the first clinical signs of arthritis were observed. Hind paws were taken for histology. **(a)** Bone erosions were scored in a blinded manner on a scale of 0 to 4 as described in Materials and methods. A significant reduction in bone erosions was observed in the animals treated with 2.5 μg and 1.25 μg IFN- β per injection/mouse in comparison with controls ($P = 0.02$); results are means \pm SEM. Immunohistochemical analysis of the number of cells positive for RANKL (receptor activator of NF- κB ligand) and c-Fos after 7 days of IFN- β treatment in CIA mice. Sections were scored on a 4-point scale. **(b)** RANKL staining; a decrease of RANKL-positive cells was observed in the group of animals treated with 2.5 μg IFN- β , although not statistically significant ($P = 0.07$). **(c)** c-Fos staining; the number of c-Fos-positive cells was significantly reduced in the mice treated with daily 2.5 μg per injection IFN- β ($P = 0.04$). *Statistically significant difference. SEM, standard error of the mean.

Figure 6

(a) Cytokine expression measured by immunohistochemistry after 7 days of systemic IFN- β treatment in mice with collagen-induced arthritis (CIA). Tumor necrosis factor (TNF)- α , IL-18, IL-1 β , IL-10, and IL-6 were scored (mean \pm SEM) in a blinded manner on a scale of 0 to 4 as described in Materials and methods. A statistically significant reduction of the proinflammatory cytokines TNF- α and IL-6 ($P = 0.03$ and 0.02 , respectively) was observed in the mice treated with 2.5 μ g IFN- β in comparison with controls. The expression of IL-18 and IL-1 β was reduced in the animals treated with the highest dose of IFN- β , and the expression of IL-10 was higher than in controls. These differences did not reach statistical significance. No clear-cut differences were observed in the animals treated with 1.25 μ g IFN- β in comparison with controls. Bars represent mice treated with 2.5 μ g IFN- β (grey), 1.25 μ g IFN- β (white) or saline (black). **(b)** Representative immunohistologic staining showing TNF- α expression in the ankle joints in CIA mice after 7 days of IFN- β treatment, assessed by immunohistochemistry. (Upper panel) TNF- α expression in CIA mice treated with saline; abundant expression of TNF- α was observed in the ankle joint. (Lower panel) TNF- α expression in CIA mice treated with 2.5 μ g IFN- β ; in the group treated with the highest dose of IFN- β , only a few positive cells for TNF- α were observed. *Statistically significant difference. SEM, standard error of the mean.

expression of IL-6 and of TNF- α was decreased (50% and 55% respectively) in mice treated with 2.5 μ g IFN- β for 7 days in comparison with saline-treated mice ($P = 0.02$ and $P = 0.03$, respectively). IL-18 and IL-1 β expression

also tended to be lower in IFN- β -treated animals, but the differences did not reach statistical significance. IL-10 expression was increased by approximately 70% in the synovium of IFN- β -treated mice (Fig. 6). No significant effects were observed for the lower dosage of IFN- β .

IFN- β reduces cytokine expression in FLS

In vitro experiments revealed that IL-1 β -induced production of IL-6, IL-8, and GM-CSF in RA as well as OA FLS was inhibited by IFN- β (Fig. 7). No clear differences between RA and OA FLS were found. The highest reduction (40%, 65%, and 65%, respectively) ($P = 0.0005$, 0.0007 , and 0.003 , respectively) was found for the dosage of 1.22 μ g/ml IFN- β , corresponding to the injection dosage required for optimal serum concentration of IFN- β in healthy human volunteers [27].

To exclude the possibility that the observed differences in cytokine secretion were the result of increased cell death, apoptotic cells were counted at the end of each experiment using Diff-Quik staining of cytopspins. Although treatment of FLS with hydrogen peroxide readily induced apoptosis, no significant induction of apoptosis was observed after IFN- β treatment (data not shown).

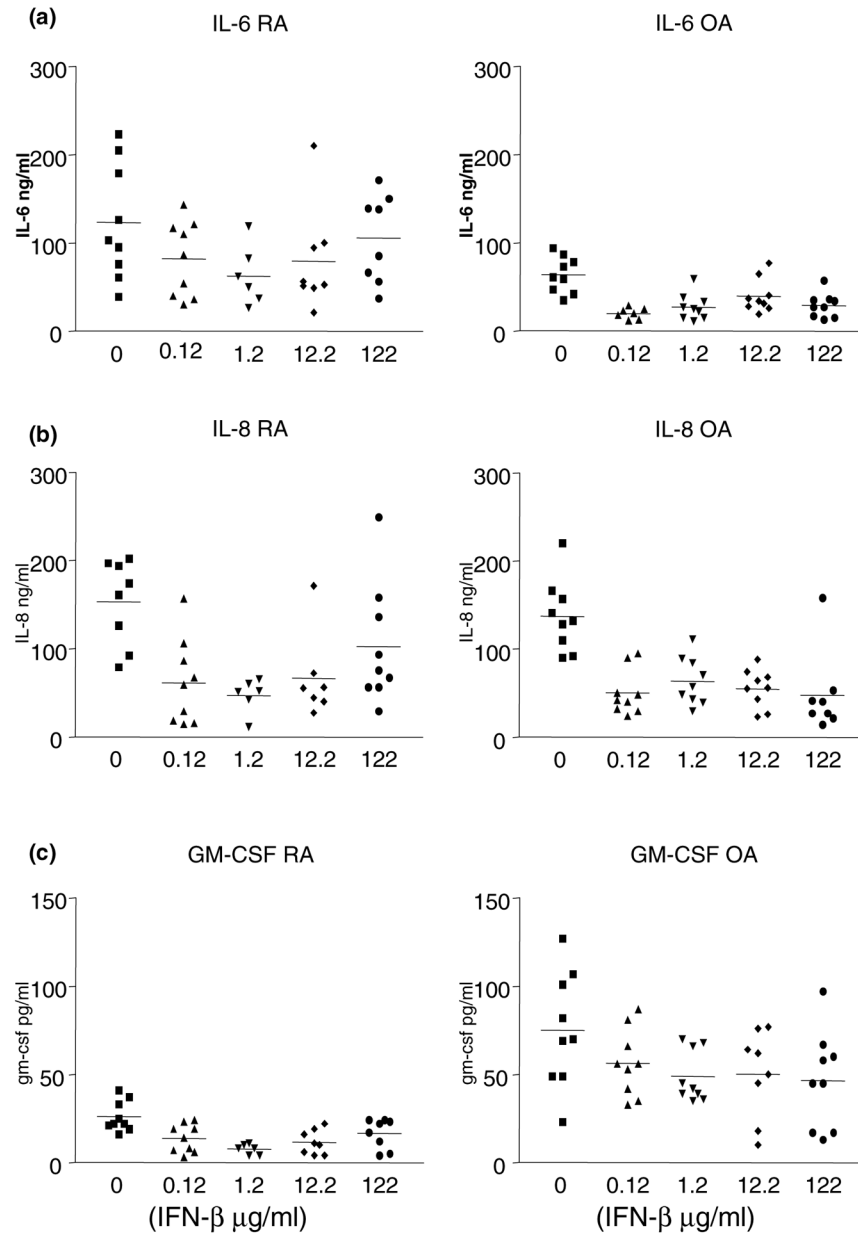
IL-1-dependent production of IL-6, IL-8, and GM-CSF in FLS is critically dependent upon activation and nuclear translocation of NF- κ B. Therefore, we assessed the effects of IFN- β treatment on IL-1-dependent NF- κ B activity after transfection of RA FLS with an NF- κ B-dependent luciferase reporter construct. We observed a tendency towards lower NF- κ B activity with increasing concentrations of IFN- β used (a 45% decrease for 1.22 μ g/ml IFN- β), indicating that the reduced cytokine production we observed may be explained in part by the inhibition of NF- κ B activity by IFN- β in RA FLS (Fig. 8).

Discussion

The results presented in this study demonstrate for the first time that daily administration of exogenous IFN- β starting at the onset of disease in the murine CIA model reduces synovial inflammation and protects against cartilage and bone destruction. The fact that clinical effects, but not histologic changes, were detected at the lowest dosage might be explained by the relative lack of sensitivity to change of semiquantitative histologic analysis. IFN- β treatment also resulted in a reduction in proinflammatory cytokine expression by synovial cells, which could be explained in part by inhibition of NF- κ B activity.

Of importance, histologic examination revealed a reduction in the number of osteoclasts in the animals treated with IFN- β , correlating with a reduction in cartilage and bone destruction, suggesting that osteoclastogenesis is inhibited by the presence of IFN- β . Recent studies by Takayanagi and colleagues have described an essential

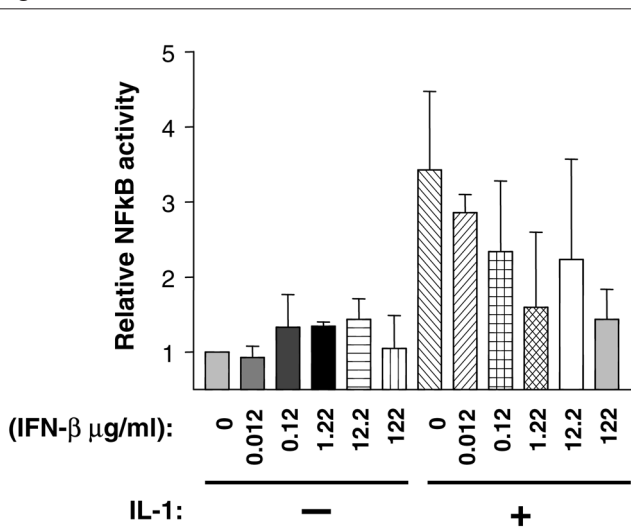
Figure 7



IL-6, IL-8, and GM-CSF (granulocyte/macrophage-colony-stimulating factor) production in synoviocytes from patients with rheumatoid arthritis (RA) and osteoarthritis (OA) after incubation with increasing concentrations of IFN- β , measured in supernatant of fibroblast-like synoviocytes using enzyme-linked immunosorbent assay. Decreased production of (a) IL-6, (b) IL-8, and (b) GM-CSF in RA and OA fibroblast-like synoviocytes after 48 hours' incubation with increasing concentrations of IFN- β .

role for IFN- β in the negative regulation of RANKL- and c-Fos-dependent osteoclast differentiation [14]. In agreement with their predictions, decreases in osteoclastogenesis observed in arthritic mice treated with IFN- β were paralleled by a decrease in the number of RANKL- and c-Fos-positive cells. In addition to effects on osteoclastogenesis, IFN- β might also affect osteoclast activity directly.

Src tyrosine kinase function (and two of its targets, Ras and Cbl) are required for bone resorption by osteoclasts [28–30]. It was previously reported that IFN- β has negative effects on tyrosine kinase signalling pathways in HL-60 cells [31]. Thus, IFN- β treatment may represent a potentially therapeutic strategy in inhibiting bone degradation in arthritis.

Figure 8

Relative NF- κ B activity measured in NF- κ B-transfected rheumatoid arthritis synoviocytes after incubation with increasing concentration of IFN- β . Unactivated fibroblast-like synoviocytes showed low NF- κ B activity. IL-1 β -induced NF- κ B activity revealed a trend towards inhibition after incubation with IFN- β in increasing concentrations.

Further experiments were carried out to determine the effect of IFN- β on the total numbers of T cells, B cells, macrophages, and granulocytes. We found no clear differences in the total numbers of T cells and B cells in the animals treated with IFN- β in comparison with controls as assessed by immunohistochemistry. Recent studies have proposed that endogenous IFN- β production in RA might theoretically promote inflammation, as IFN- β can promote T-cell and neutrophil survival *in vitro* [32]. While our studies did not specifically address the effects of exogenous IFN- β on T-cell survival in the synovial joint, we did not observe an increase in T-cell numbers, and any potential anti-apoptotic effect did not prevent a beneficial therapeutic effect of IFN- β *in vivo*.

In contrast to the lack of effect on T- and B-cell infiltration, morphological analysis revealed a tendency towards a decreased number of macrophages and a significant reduction in the number of granulocytes in animals treated with IFN- β . Although one study has reported that IFN- β induces apoptosis in a monocytic leukaemia cell line [33], on the basis of that study, it is unclear whether IFN- β is influencing monocyte recruitment, survival, and/or retention in the synovial joint.

Alternatively, IFN- β may inhibit inflammatory cell infiltration indirectly, via suppression of FLS and/or monocyte activation. Our data demonstrated statistically significant modulation of proinflammatory and anti-inflammatory cytokine production in animals treated with IFN- β . We found a statistically significant reduction of TNF- α and IL-6

expression and an increase in IL-10 production in the animals treated with the highest dose IFN- β . TNF- α plays an important role in the pathogenesis of CIA as well as RA. Treatment with TNF- α blockade has been shown to be effective in both CIA and RA [34–36]. The reduced expression of IL-6 could also be beneficial, as IL-6 may inhibit bone formation and induce bone resorption through its stimulatory effects in osteoclasts, and it is known that IL-6 knockout mice do not develop bone erosions. Moreover, it has been suggested that treatment with anti-IL-6 receptor antibody may be effective in RA patients [37]. Of interest, IL-10 production was increased in the IFN- β -treated animals. IL-10 may be a potent anti-inflammatory cytokine, achieving the effect through suppression of TNF- α , IL-6, and IL-1 production by activated macrophages [38]. A trend towards clinical improvement has been suggested in RA patients treated with recombinant human IL-10 [39].

It has been shown previously that IFN- β has an inhibitory effect on the production of TNF- α by lipopolysaccharide-stimulated macrophages from mouse bone marrow [11]. In this study, we show for the first time that IFN- β can decrease the production of IL-6, IL-8, and GM-CSF by stimulated FLS from RA and OA patients. Although the molecular signalling mechanism underlying this inhibitory effect requires further elucidation, preliminary evidence presented here suggests that IFN- β acts at least in part via inhibition of NF- κ B activity induced by IL-1.

The potential effects of IFN- β were previously investigated in CIA in mice by IFN- β gene therapy [11]. Fibroblasts from DBA/1 mice were infected with a retrovirus expressing murine IFN- β and were injected intraperitoneally into CIA mice before and after the onset of arthritis, leading to continuous IFN- β delivery. A significant decrease in inflammation was observed after IFN- β gene therapy both before and after the onset of disease. At present, viral and nonviral vectors that are used for gene therapy have limited applications for use in humans. Therefore, our approach using daily injections with murine IFN- β could have the advantage of easily translating results into RA patients. However, it remains to be shown whether the exciting biological effects described in the present study can be achieved in RA patients if IFN- β is administered only three times weekly, in accordance with the regular treatment regimen in patients with multiple sclerosis. A recent pilot study, which was not designed to evaluate clinical efficacy, did not suggest clinical improvement after IFN- β treatment three times weekly [40]. It is conceivable that more frequent injections as in the present study, higher doses, or the use of compounds with a longer half-life is required to induce clinically meaningful effects in RA patients. Obviously, although animal arthritis models are very useful for screening interesting compounds, the results are not necessary identical to those obtained in RA patients.

Conclusion

The marked reduction of CIA in mice, the changes in synovial tissue from mice with CIA after IFN- β therapy, and the effects on synoviocytes from RA patients all support the view that IFN- β treatment has immunomodulating effects and might have a beneficial effect on joint inflammation and, perhaps more importantly, on bone destruction in RA patients.

Competing interests

Dr Tak has received support from Serono for a separate clinical study investigating the use of interferon- β in rheumatoid arthritis patients. Mrs Sattonet-Roche is an employee of Serono. Dr Plater-Zyberk is a former employee of Serono. Dr van Holten has received a research grant from the Serono Pharmaceutical Research Institute.

References

- van Holten J, Plater-Zyberk C, Tak PP: **Interferon-beta for treatment of rheumatoid arthritis?** *Arthritis Res* 2002, **4**:346-352.
- Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD: **How cells respond to interferons.** *Annu Rev Biochem* 1998, **67**:227-264.
- De ME, De Maeyer-Guignard J: **Type I interferons.** *Int Rev Immunol* 1998, **17**:53-73.
- Tak PP, Hart BA, Kraan MC, Jonker M, Smeets TJ, Breedveld FC: **The effects of interferon beta treatment on arthritis.** *Rheumatology (Oxford)* 1999, **38**:362-369.
- Rep MH, Hintzen RQ, Polman CH, van Lier RA: **Recombinant interferon-beta blocks proliferation but enhances interleukin-10 secretion by activated human T-cells.** *J Neuroimmunol* 1996, **67**:111-118.
- Rep MH, Schrijver HM, van Lopik T, Hintzen RQ, Roos MT, Ader HJ, Polman CH, van Lier RA: **Interferon (IFN)-beta treatment enhances CD95 and interleukin 10 expression but reduces interferon-gamma producing T cells in MS patients.** *J Neuroimmunol* 1999, **96**:92-100.
- Palmer G, Mezin F, Juge-Aubry CE, Plater-Zyberk C, Gabay C, Guerne PA: **Interferon beta stimulates interleukin 1 receptor antagonist production in human articular chondrocytes and synovial fibroblasts.** *Ann Rheum Dis* 2004, **63**:43-49.
- Jungo F, Dayer JM, Modoux C, Hyka N, Burger D: **IFN-beta inhibits the ability of T lymphocytes to induce TNF-alpha and IL-1beta production in monocytes upon direct cell-cell contact.** *Cytokine* 2001, **14**:272-282.
- Yong VW, Chabot S, Stuve O, Williams G: **Interferon beta in the treatment of multiple sclerosis: mechanisms of action.** *Neurology* 1998, **51**:682-689.
- Ossege LM, Sinden E, Patzold T, Malin JP: **Immunomodulatory effects of interferon-beta-1b in patients with multiple sclerosis.** *Int Immunopharmacol* 2001, **1**:1085-1100.
- Triantaphyllopoulos KA, Williams RO, Taylor H, Chernajovsky Y: **Amelioration of collagen-induced arthritis and suppression of interferon-gamma, interleukin-12, and tumor necrosis factor alpha production by interferon-beta gene therapy.** *Arthritis Rheum* 1999, **42**:90-99.
- Sundel RP, Wallace CA, Zurawski Boston D: **Pilot trial of interferon beta-1a in JRA [abstract].** *Arthritis Rheum* 2001, **44**:s272.
- Smeets TJ, Dayer JM, Kraan MC, Versendaal J, Chicheportiche R, Breedveld FC, Tak PP: **The effects of interferon-beta treatment of synovial inflammation and expression of metalloproteinases in patients with rheumatoid arthritis.** *Arthritis Rheum* 2000, **43**:270-274.
- Takayanagi H, Kim S, Matsuo K, Suzuki H, Suzuki T, Sato K, Yokochi T, Oda H, Nakamura K, Ida N, Wagner EF, Taniguchi T: **RANKL maintains bone homeostasis through c-Fos-dependent induction of interferon-beta.** *Nature* 2002, **416**:744-749.
- Alliston T, Derynck R: **Medicine: interfering with bone remodeling.** *Nature* 2002, **416**:686-687.
- Tak PP, Firestein GS: **NF-kappaB: a key role in inflammatory diseases.** *J Clin Invest* 2001, **107**:7-11.
- Gerlag DM, Ransone L, Tak PP, Han Z, Palanki M, Barbosa MS, Boyle D, Manning AM, Firestein GS: **The effect of a T cell-specific NF-kappa B inhibitor on in vitro cytokine production and collagen-induced arthritis.** *J Immunol* 2000, **165**:1652-1658.
- Plater-Zyberk C, Buckton J, Thompson S, Spaul J, Zanders E, Papworth J, Life PF: **Amelioration of arthritis in two murine models using antibodies to oncostatin M.** *Arthritis Rheum* 2001, **44**:2697-2702.
- Williams RO, Feldmann M, Maini RN: **Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis.** *Proc Natl Acad Sci USA* 1992, **89**:9784-9788.
- Dudler J, Renggli-Zulliger N, Busso N, Lotz M, So A: **Effect of interleukin 17 on proteoglycan degradation in murine knee joints.** *Ann Rheum Dis* 2000, **59**:529-532.
- Tak PP, Smeets TJ, Daha MR, Kluin PM, Meijers KA, Brand R, Meijers AE, Breedveld FC: **Analysis of the synovial cell infiltrate in early rheumatoid synovial tissue in relation to local disease activity.** *Arthritis Rheum* 1997, **40**:217-225.
- Thurkow EW, van der Heijden IM, Breedveld FC, Smeets TJ, Daha MR, Kluin PM, Meijers AE, Tak PP: **Increased expression of IL-15 in the synovium of patients with rheumatoid arthritis compared with patients with Yersinia-induced arthritis and osteoarthritis.** *J Pathol* 1997, **181**:444-450.
- Dayer JM, Beutler B, Cerami A: **Cachectin/tumor necrosis factor stimulates collagenase and prostaglandin E2 production by human synovial cells and dermal fibroblasts.** *J Exp Med* 1985, **162**:2163-2168.
- Firestein GS: **Cytokine networks in rheumatoid arthritis: implications for therapy.** *Agents Actions Suppl* 1995, **47**:37-51.
- Moore KW, O'Garra A, de Waal MR, Vieira P, Mosmann TR: **Interleukin-10.** *Annu Rev Immunol* 1993, **11**:165-190.
- Wei XQ, Leung BP, Arthur HM, McInnes IB, Liew FY: **Reduced incidence and severity of collagen-induced arthritis in mice lacking IL-18.** *J Immunol* 2001, **166**:517-521.
- Buchwalder PA, Buclin T, Trinchard I, Munafò A, Biollaz J: **Pharmacokinetics and pharmacodynamics of IFN-beta 1a in healthy volunteers.** *J Interferon Cytokine Res* 2000, **20**:857-866.
- Miyazaki T, Katagiri H, Kanegae Y, Takayanagi H, Sawada Y, Yamamoto A, Pando MP, Asano T, Verma IM, Oda H, Nakamura K, Tanaka S: **Reciprocal role of ERK and NF-kappaB pathways in survival and activation of osteoclasts.** *J Cell Biol* 2000, **148**:333-342.
- Missbach M, Jeschke M, Feyen J, Muller K, Glatt M, Green J, Susa M: **A novel inhibitor of the tyrosine kinase Src suppresses phosphorylation of its major cellular substrates and reduces bone resorption in vitro and in rodent models in vivo.** *Bone* 1999, **24**:437-449.
- Chiusaroli R, Sanjay A, Hendriksen K, Engsig MT, Horne WC, Gu H, Baron R: **Deletion of the gene encoding c-Cbl alters the ability of osteoclasts to migrate, delaying resorption and ossification of cartilage during the development of long bones.** *Dev Biol* 2003, **261**:537-547.
- Berger LC, Hawley RG: **Interferon-beta interrupts interleukin-6-dependent signaling events in myeloma cells.** *Blood* 1997, **89**:261-271.
- Pilling D, Akbar AN, Girdlestone J, Orteu CH, Borthwick NJ, Amft N, Scheel-Toellner D, Buckley CD, Salmon M: **Interferon-beta mediates stromal cell rescue of T cells from apoptosis.** *Eur J Immunol* 1999, **29**:1041-1050.
- Van Weyenbergh J, Wietzerbin J, Rouillard D, Barral-Netto M, Liblau R: **Treatment of multiple sclerosis patients with interferon-beta primes monocyte-derived macrophages for apoptotic cell death.** *J Leukoc Biol* 2001, **70**:745-748.
- Joosten LA, Helsen MM, van De Loo FA, van Den Berg WB: **Anti-cytokine treatment of established type II collagen-induced arthritis in DBA/1 mice. A comparative study using anti-TNF alpha, anti-IL-1 alpha/beta, and IL-1Ra.** *Arthritis Rheum* 1996, **39**:797-809.
- Moreland LW, Schiff MH, Baumgartner SW, Tindall EA, Fleischmann RM, Bulpitt KJ, Weaver AL, Keystone EC, Furst DE, Meas PJ, Ruderman EM, Horwitz DA, Arkfeld DG, Garrison L, Burge DJ, Blosch CM, Lange ML, MacDonnell ND, Weinblatt ME: **Etanercept therapy in rheumatoid arthritis. A randomized, controlled trial.** *Ann Intern Med* 1999, **130**:478-486.

36. Weinblatt ME, Kremer JM, Bankhurst AD, Bulpitt KJ, Fleischmann RM, Fox RI, Jackson CG, Lange M, Burge DJ: **A trial of etanercept, a recombinant tumor necrosis factor receptor:Fc fusion protein, in patients with rheumatoid arthritis receiving methotrexate.** *N Engl J Med* 1999, **340**:253-259.
37. Nishimoto N, Yoshizaki K, Maeda K, Kuritani T, Deguchi H, Sato B, Imai N, Suemura M, Kakehi T, Takagi N, Kishimoto T: **Toxicity, pharmacokinetics, and dose-finding study of repetitive treatment with the humanized anti-interleukin 6 receptor antibody MRA in rheumatoid arthritis. Phase I/II clinical study.** *J Rheumatol* 2003, **30**:1426-1435.
38. van Roon JA, Lafeber FP, Bijlsma JW: **Synergistic activity of interleukin-4 and interleukin-10 in suppression of inflammation and joint destruction in rheumatoid arthritis.** *Arthritis Rheum* 2001, **44**:3-12.
39. Maini R, Paulus H, Breedveld F, Moreland LW, William St Clair E, Russel A: **rHUIL-10 in subjects with active rheumatoid arthritis (RA): a phase I and cytokine response study [abstract].** *Arthritis Rheum* 1997, **40**:s224.
40. Genovese M, Chakravaty E, Krishnan E, Moreland LW: **A randomized, controlled trial of interferon- β -1a in patients with rheumatoid arthritis: a pilot study.** *Arthritis Res Ther* 2004, **6**:R73-R77.

Correspondence

Paul P Tak, MD, PhD, Division of Clinical Immunology and Rheumatology F4-218, Academic Medical Center, PO Box 22700, 1100 DE Amsterdam, The Netherlands. Tel: +31 20 5662171; fax: +31 20 6919658; e-mail: P.P.Tak@amc.uva.nl