Stromal cells and osteoclasts are responsible for an exacerbated Collagen-Induced Arthritis in IFN-β deficient mice

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Abbreviations

CII, collagen type II; CIA, collagen-induced arthritis; IFN-β, interferon beta; IFN-β-/-, IFN-β deficient; IFN-β-/+, heterozygous for IFN-β gene; IFN-β+/+, homozygous for IFN-β gene; RA, rheumatoid arthritis and ab, antibody.

Abstract

Objective: Clinical trials using Interferon-β (IFN-β) in the treatment of rheumatoid arthritis have shown conflicting results. The objective of this study was to understand the mechanisms of IFN-β in arthritis at a physiological level.

Methods: Collagen-induced arthritis (CIA) was induced in IFN-β deficient and control mice. The role of IFN-β was investigated in both the priming and effector phase of the disease. The effect of IFN-β deficiency on synovial cells, macrophages and fibroblasts from pre-immunized mice was analyzed by flow cytometry, immunohistochemistry and ELISA. Differences in osteoclast maturation was determined in situ by histology of arthritic paws and by *in vitro* maturation studies of naïve bone marrow cells. The importance of IFN-β producing fibroblasts was determined by transferring fibroblasts into mice at the time of CIA immunization.

Results: Mice lacking IFN-β had a prolonged disease with a higher incidence compared to control mice. IFN-β deficiency was found to influence the effector-, but not the priming phase of arthritis. IFN-β deficient mice had a greater infiltration of CD11b cells and greater TNF-α production *in vivo*, and both macrophages and fibroblasts were more activated *in vitro*. Moreover, IFN-β deficient mice generated a greater number of osteoclasts *in vitro*, and mice immunized for arthritis, but not naïve mice, had a greater number of osteoclasts *in vivo* compared to control mice. Importantly, IFN-β competent fibroblasts were able to ameliorate arthritis in IFN-β deficient recipients.

 Conclusion: Our data indicates that IFN-β is involved in regulating the activation state of osteoclasts and stromal cells including macrophages and fibroblasts but that it has little effect on T cells.

Introduction

Interferons (IFN) are potent cytokines that are classified into either type I or type II interferons. IFN-α and IFN-β belong to type I and are considered to be anti-inflammatory. Type I interferons bind to the same receptor complex, which consists of two transmembrane proteins (1, 2). The binding of IFN-α and IFN-β to the receptor has been shown to have distinct biological functions (3-6).

Apart from the anti-viral properties, IFN-β has been shown to have wide variety of developmental and imunomodulating effects. IFN-β has been shown to be involved in the development of B cells, neutrophils and osteoclasts as well as in inhibition of apoptosis of leucocytes (7-10). However, it is the immunomodulating effects of IFN-β that have been of greatest interest in terms of its therapeutic use. IFN-β has been shown to down-regulate pro-inflammatory cytokines such as TNF-α and IL-1β, as well as increase the secretion of anti-inflammatory mediators such as IL-10 and IL-1 receptor antagonist (11). IFN-β has also been implicated in reducing T cell proliferation as well as in down regulation of MHCII on APC (12, 13). Due to these anti-inflammatory properties of IFN-β, this cytokine has been studied and used in various human immune disorders such as multiple sclerosis (MS) and cancer (14, 15). There has been a positive response with IFN-β treatment of MS patients and it is presently the most effective treatment used for this autoimmune disease.

In recent years there has been an interest in determining the possible beneficial effects of IFN-β in rheumatoid arthritis (RA). IFN-β has been investigated in mice and monkeys with promising results (15, 16), and IFN-β has been shown to regulate osteoclastogenesis in mice (9, 13, 17). Moreover, in a small clinical trial, administration of IFN-β lead to a significant reduction in the expression of IL1-β, MMP-1 and TIMP-1 in the synovial lining and also a reduction in CD3+ T cell infiltration (18). This study also showed that *in vitro* RA-fibroblast-like synoviocytes also had a decreased MMP-1 expression when treated with IFN-β. Together these findings indicate that IFN-β may have a protective effect on joint destruction. In line with this, a recent report showed that IFN-β is highly expressed in the synovium of RA patients, compared to patients with osteoarthritis and reactive arthritis (19).

Despite the early success of IFN-β treatment in animal models as well as in small clinical trials, recent clinical studies have shown limited effect (13, 18, 20, 21). These inconsistent results may, however, have several explanations. Firstly, animal models

differ from human RA in several aspects, such as in the level of T cell infiltration into arthritic joints. Secondly, treatment protocols used in animal model of RA, including administration of very high amounts of IFN-β and administration of retrovirus or transformed fibroblasts that produce IFN-β, are difficult to extrapolate to humans. Consequently, there is a need to gain an enhanced understanding of the mechanism of action of IFN-β at a physiological level. The present study utilized IFN-β deficient mice (IFN-β-/-) in comparison to control heterozygous mice (IFN-β-/+) in order to determine the effect of IFN-β in the murine model for RA, collagen induced arthritis (CIA).

CIA is one of the most commonly used animal model for RA, and is induced in mice by injecting heterologous collagen type II (CII) in an adjuvant, leading to a disease resembling RA (22). T cells have been shown to play an important role in the pathology of CIA (23), possibly by the production of pro-inflammatory cytokines and by providing help to B cell. Although CIA is T cell dependent, T cells are primarily involved in the priming phase of the disease whereas the effector phase is driven by B cells producing anti-CII antibodies that cross-react to mouse CII (24). Histological changes associated with CIA involve the infiltration of neutrophils, macrophages and T cells into the synovia. There is also pannus formation and activation of stromal cells, such as fibroblasts and macrophages. These histological changes are all believed to contribute to the pathogenesis of CIA.

In the present study, we have used IFN-β-/- mice in order to determine the role of IFN-β in the CIA model. We found that while the T cell compartment appeared unaffected by IFN-β deficiency, fibroblasts, macrophages and osteoclasts located in the joints were more activated, compared to IFN-β-/+ mice. Therefore, we postulate that IFN-β deficiency leads to severe arthritis where stromal cells and osteoclasts perpetuate the disease. Pinpointing the mechanism of IFN-β is of importance in determining if IFN-β can be used as a treatment for arthritis and which patient group would benefit most from this treatment.

Materials and Methods

Mice

The generation of IFN-β deficient mice has been described previously (6). The mice were screened for the deletion of IFN-β by PCR from tissue (tail or toe of mice) as previously described (25). Mice were backcrossed to the B10.RIII strain for seven and twelve generations by crossing IFN-β-/+ with B10.RIII mice. Mice were bred and kept at the conventional animal facility at the Section for Medical Inflammation Research; Lund University and all experiments had animal ethics committee approval. Unless stated otherwise male mice aged between 8 and 16 weeks were used.

Antigens

CII was prepared from calf cartilage by pepsin digestion as described earlier (26). Peptides were synthesized as described previously (27). CII was denatured by heating at 65°C for 20 minutes before usage in *in vitro* proliferation assays.

Immunization, scoring and anti-CII antibody ELISA

For arthritis experiments, mice (8-14 per group) were immunized intradermally in the tail base with 100 μg CII, emulsified 1:1 in incomplete Freud's adjuvant (IFA, Difco, Detroit). For *in vitro* lymphocyte assays, mice were immunized in the tail base and each hind footpad with 60 μg of CII in IFA at each location. Arthritis was evaluated by visual scoring using an extended scoring protocol (28), ranging from 1 to 15 for each paw with a maximum score of 60 per mouse. Each arthritic toe and knuckle was scored as 1, with a maxium of 10 per paw. An arthritic ankle or midpaw were given a score of 5.The anti-CII antibody response was determined by measuring the level of CII-specific antibodies in serum collected 121 days post immunization. The amounts of total anti-CII IgG as well as the IgG1 and IgG2a isotypes were determined through quantitative ELISA as previously described (29).

Collagen Antibody-Induced Arthritis (CAIA)

Arthritis in 4-month-old mice (9-12 per group) was induced injecting (intravenously) an anti-CII monoclonal antibody cocktail of CIIC1 and M2139 (9 mg/mouse) as described previously (30) without LPS booster. After 48 hours, clinical signs of arthritis were observed and the arthritis was monitored for 72 days using the scoring protocol described as above.

Proliferation and Cytokine Production Assays

Ten days after immunization, cells from the draining inguinal and popliteal lymph nodes were prepared and re-stimulated *in vitro* in order to determine antigen-specific cell proliferation and IFN-γ response as described previously (31, 32). Six to ten mice per group were used. For determination of anti-CD3 T cell responses, spleen cells from naïve or immunized (10 days prior) mice were seeded at a concentration of 1 x10^6 cells per well in plates pre-coated with anti-CD3 (clone 145-2C11, from our hybridoma collection) and incubated for 48 hours, before pulsing with 1uCi ^{3}H-thymidine (Amersham Int.,) for 15-18 hours. Five mice per group were used.

Macrophage preparation and culture

Spleens were removed 10 days post immunization, and macrophages were enriched and stimulated as preciously described (25), 8-10 mice per group were used. Supernatant were collected after 36 hr incubation and assayed for cytokine content using ELISA. The production of TNF-α was determined using the recommended paired antibodies and protocol of BD PharMingen. The plates were read using a fluorometer (Wallac, Boston MA).

Synovia preparation and culture

Mice (24-25 per group) were immunized for arthritis as described in the immunization section above. Thirty days post immunization mice, hind legs were removed and the synovia of the knees were dissected out pooled and placed in 1.6 mg/mL collagenase type 4 (Worthington Biochemical corporation, Lakewood, NJ) and 0.1% DNaseI (Sigma-Aldrich) in Dulbeccos MEM Medium, and incubated for 1 hour at 37°C. The cells were left untreated or first primed with 10 U/ml of IFN-γ for 60 min, then incubated for 36 hours with 50 ng/ml of LPS.

The expression of surface markers on the synovial cells was determined by Flow cytometry using the following conjugated antibodies; anti-ICAM-FITS (clone 3E2, BD PharMingen), anti-VCAM-biotinylated (Clone 429, BD PharMingen), anti-CD11b-PE (clone M1/70, BD PharMingen), anti-Ly6-G-APC (clone Rb6-8C5, BD PharMingen), anti-MHC II-FITS (clone Y3P, our hybridoma collection) and anti-macrophage antigen F4/80-biotinylated (clone F4/80, our hybridoma collection). In order to determine TNF-α production, monosine 3uM/ml, (Sigma-Aldrich, St Louis, MO, USA) was added 6 hours prior to staining. Intra-cellular staining was then performed using BD Cytofix/Cytoperm solution and protocol (Beckton Dickinson)

using an un-conjugated anti-TNF-α antibody (clone XT22, BD PharMingen) followed by a biotinylated secondary goat anti-rat antibody (Jackson Immuno Research).

Immunohistochemistry

IFN-β-/- and IFN-β-/+ mice (5 per group) were immunized for arthritis and on day 40; mice were killed and paws were dissected and decalcified with EDTA (for 2-3 weeks). The paws were then embedded in OTC Compound (Sakura Finetek Europe B.V) and snap-frozen in isopentane on dry ice. Staining of sides was performed as previously described (25) Diaminobenzidine 50 mg/ml (Saveen biotech AB, Ideon, Sweden) was used for detection and slides were counter stained with hematoxylin. In all studies, the numbers of positive cells were determined blindly by calculating the mean count of 5 distinct areas per section.

Fibroblast preparation and culture

Fibroblasts were prepared from IFN-β-/- and IFN-β-/+ mice that had shown clinical signs of arthritis for at least one week. The fibroblasts were prepared by removing the skin and muscle from the hind legs (8-10 mice per group) and grinding them in a mortar in a solution of 0.25% Trypsin in PBS. Cells were then incubated for 30 min at 37°C, washed with DMEM containing 10% FCS, incubated for a further 90 minutes in 0.1% collagenase. The fibroblasts were subjected to a minimum of 6 passages (detachment by 0.5% trypsin in 5mM EDTA) to obtain a pure culture. To analyze the phenotype of the fibroblasts, 1×10^4 cells per well were seeded into 48 well plates in DMEM containing 10% FCS and cultured for 4 days before detaching cells with cell dissociation media (Sigma-Aldrich). The IL-6 content in the supernatant of the cultured fibroblasts was determined using the recommended paired antibodies and protocol of BD PharMingen. The expression of cell-surface markers on fibroblasts was determined by Flow cytometry, using the following antibodies; anti-ICAM-FITC, anti-VCAM-biotinylated, anti-MHC II-FITC, anti-CD40-PE (clone 3/23, BD PharMingen), anti-CD44-biotinylated (clone IM7.8.1, BD PharMingen), anti-CD71-PE (clone C2, BD PharMingen, and biotinylated anti-IFN-gRα chain (clone GR20, BD PharMingen).

Transfer of Fibroblasts

Fibroblasts were detached from culture bottles using EDTA/trypsin and washed with PBS and a single cell suspension was obtained by passing the fibroblasts through

a 23G needle. The fibroblasts were then injected periarticularly into the joints of mice (total 2 x 10^6 fibroblasts/mouse) at 6 injection sites, metacarpal, metatarsal and ankle joints. At the same time the mice (8-10 mice per group) were immunized for CIA as described earlier.

In vitro generation of osteoclasts

Bone marrow cells were obtained from the tibia of four IFN-β-/- and four IFN-β-/+ mice, by removing the bone ends and flushing with αMEM (GIBCO BRL, Life Technologies). Non-adherent cells were washed and 2.5x10^5 cells per well were seeded into a 48 well plate and M-CSF (10ng/mL, R&D) was added. Three days later media was removed and fresh media containing M-CSF (10ng/ml) plus rmRankL (100ng/mL, PeproTech, UK) was added and cells were cultured for a further 3-4 days. Bone marrow cells incubated with M-CSF alone were used as negative control. The osteoclasts were visualized using TRAP staining according to Beckton Dickinson Technical Bulletin #445: "Tartrate Resistant Acid Phosphatase (TRAP) staining of osteoclasts". Cells were counter stained with hematoxylin. Osteoclasts were classified as multi-nucleated and TRAP positive. In all studies, the numbers of positive cells were determined by calculating the mean count from 5 fields of views per well.

In situ determination of osteoclasts using TRAP staining

Naïve IFN-β-/- and IFN-β-/+ mice, aged between 8-16 weeks or older than 1.5 years, as well as pre-immunized IFN-β-/- and IFN-β-/+ mice (40 days prior, 4-6 mice per group) were sacrificed and paws were dissected. The paws were fixed in 4% phosphate buffered formaldehyde for 24 hours at 4°C, decalcified with EDTA (for 2-3 weeks), embedded in paraffin and sectioned at a thickness of 5μm. The sections were re-hydrated and stained for TRAP as described above. All joints in the section were counted and joints which contained one or more osteoclast was counted as affected. Thereafter, the number of affected joints per total number of counted joints was determined individually in order to compare the two groups of mice.

Statistics

Frequency of arthritis was analyzed by the X^2 test and the Mann-Whitney U test was used in all other statistical analyzes.

Results

Mice deficient in IFN-β have an exacerbated CIA in the chronic phase of the disease

There has recently been an interest in addressing whether IFN-β has an ameliorating effect on arthritis but the results from these investigations have been conflicting. We therefore decided to investigate the effect of IFN-β deficiency on arthritis in the CIA mouse model. IFN-β-/- mice were backcrossed to the B10.RIII background for 7 generations and then investigated for arthritis susceptibility. There was no difference in the incidence, day of onset or severity of arthritis between mice heterozygote for IFN-β deficiency (IFN-β-/+) and IFN-β wild type littermates (IFN-β+/+; data not shown) and hence both groups were pooled for subsequent comparison with the group of IFN-β-/- mice. Although there was no difference in day of onset, IFN-β-/- mice were more susceptible to CIA and developed an exacerbated disease compared to control mice. Relapses of arthritis were also observed in IFN-β-/- mice and they had a tendency for a higher anti-CII antibody response (Figure 1A, Table 1). A similar disease profile was also observed when mice that had been backcrossed for 12 generations were used in CIA (Table 1).

T cell response to CII is not affected by a lack of endogenous IFN-β

As the CIA model is T cell dependent, it was feasible that the IFN-β-/- mice had an exacerbated arthritis due to an increased T cell response to CII. Furthermore, it has been previously reported that IFN-β affects the proliferative response of T cells (8, 33). In agreement with a recent report (8) naïve IFN-β-/- spleen cells (and lymph node cells, data not shown) were found to have a significantly greater proliferative response when stimulated with anti-CD3, compared to control mice (Figure 1B). To evaluate whether IFN-β-/- mice also had an increased antigen-specific proliferative response, mice were immunized with CII and cells were subsequently re-stimulated *in vitro* with bovine CII and the immunodominant peptides 607-621 and 442-456. However, there was no significant difference between the IFN-β-/- and control mice in their proliferative response (Figure 1C and D) or in production of IFN-γ (data not shown). Similar results were also observed in mice immunized 3 weeks prior (data not

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shown). Furthermore, the detected difference in anti-CD3 stimulation of naïve mice (Figure 1B) was not observed in immunized mice (Figure 1C).

Effector phase of the disease affected Lack of IFN-β

Data so far indicated that deficiency of IFN-β did not have an effect in the priming phase of CIA. Instead the exacerbated disease of IFN-β-/- mice may be explained by events occurring in the effector phase. The effector phase of CIA is mediated via arthritogenic anti-CII antibodies, which can be mimicked by using the acute and T cell independent Collagen Antibody Induced Arthritis (CAIA) model (34). IFN-β-/- and control mice were subjected to passive transfer of the disease using two collagen-specific monoclonal antibodies. As shown in Table 1, IFN-β-/- were indeed found to developed a more severe and prolonged disease, compared to control mice, indicating that IFN-β operates in the inflammatory phase in the joints.

Augmented activation *in vitro* of peripheral and synovial APCs in IFN-β-deficient mice

Since the enhanced arthritis in the IFN-β-/- mice was not due to an increase in T cell proliferation and could not be explained by an increase in anti-CII antibody production, the exacerbation had to be due to other cells. We therefore investigated cells that could be activated by MHC class II restricted T cells.

Initial investigation of spleen macrophage population showed a significant increase in TNF-α production (Figure 2A), following 48 hrs of culture *in vitro* in the presence of both IFN-γ and LPS, but no alteration in the level of IL-1β or IL-10 production (data not shown). However, as splenic macrophages are distant from the site of joint inflammation, we therefore aimed to investigate whether the macrophages as well as other cells in the synovia were more activated in the IFN-β-/- mice. Mice were immunized and 30 days later the synovia was extracted and stimulated *in vitro* with IFN-γ and LPS.

Following activation, flow cytometry analyzes of synoviocytes from IFN-β-/- and IFN-β-/+ mice revealed that the IFN-β-/- synoviocytes included a greater number of macrophages; these increased slightly after stimulation (Table 2). To our surprise, we did not see an increase in TNF-α intracellular staining when the synovial cells were stimulated with IFN-γ and LPS. This result was in contrast to the macrophages

derived from the spleen (Figure 2A). This could be due to a kinetic problem. However, in the synovial population there was a greater intra-cellular expression of TNF-α in IFN-β-/- synoviocytes compared to control cells, both before and after stimulation. There was also an increase in ICAM-1 positive and ICAM-1 VCAM-1 double positive cells after stimulation, compared to stimulated control cells (Table 2).

Interestingly, after 36 hours cultivation there was a greater number of neutrophils (Ly6-G and CD11b positive cells) in IFN-β-/- synovial population, which increased after stimulation (Table 2). However, fresh synoviocytes originating from IFN-β-/+ mice had a greater number of neutrophils compared with synoviocytes from IFN-β-/- (34.7% and 25.8% respectively). This was an expected result as IFN-β-/- mice have previously been shown to have a reduced number of neutrophils (8).

Increased infiltration and fibroblast activation in synovia of IFN-β-/- mice

To investigate the phenotype of joint inflammation during the effector phase of CIA, hind and fore paws were harvested 40 days post immunization and were evaluated by immunohistochemistry. In agreement with the above data (Table 2 and Figure 2A), infiltrated areas of IFN-β-/- mice contained more CD11b+ cells and greater amount of TNF-α, compared to control mice (Figure 2B-D).

To determine whether fibroblast were also affected by IFN-β deficiency, fibroblasts were prepared from mice with clinical signs of arthritis lasting for at least one week, and then subjected to 6 passages of trypsination *in vitro* before analyzes. Fibroblasts from the IFN-β-/- mice (IFN-β-/-FB) produced 10-fold higher amount of IL-6 compared to fibroblasts from control mice (control-FB) when both cultured for 4 additional days (Figure 3A, YY-axis). Flow cytometry analyzes of the fibroblasts also showed that the IFN-β-/-FB were more activated than control-FB in terms of expression of CD44 and ICAM and had a slightly increased in expression of CD40 (Figure 3A, Y-axis).

IFN-β competent fibroblasts are able to protect IFN-β deficient mice from CIA

To investigate if the increased activation status *in vitro* of IFN-β-/-FB would also have an impact *in vivo* during an inflammatory attack directed to the joints, we next conducted fibroblast transfer experiments. Neither IFN-β-/-FB, nor control-FB could induce arthritis when injected into the knee (1 x 10^5 cells per knee) of irradiated or

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non-irradiated B10.RIII mice (data not shown). However, transfer of control-FB (6 joint injection sites with a total of 2 x 10^6 per mouse) into IFN-β-/- mice resulted in a significant protection from subsequent induction of CIA, compared to IFN-β-/- mice injected with IFN-β-/-FB (Figure 3 B-C). The IFN-β-/- mice that had received control-FB had an arthritis profile similar to control IFN-β-/+ mice that had received control-FB, demonstrating the importance of FB in this model possibly via production of IFN-β.

There is a difference in osteoclast generation *in vitro* and *in vivo* in mice deficient for IFN-β

Another important synovial cell that contributes to the arthritic process is osteoclasts, which degrade cartilage and bone. It has previously been shown that IFN-β deficient and IFN-βR knockout mice have a greater capacity to generate osteoclasts *in vitro* and that these mice have an intrinsic bone erosion disorder *in vivo* (9). We therefore analyzed osteoclastogenesis in IFN-β-/- mice both *in vitro* and *in vivo*. Bone marrow from IFN-β-/- mice generated more osteoclasts than bone marrow from control mice *in vitro* (Figure 4A-C). However, TRAP staining analyzes did not suggest an increase in osteoclastogenisis *in vivo* in either 4 or 16 months old naïve IFN-β-/- mice compared to age matched naïve control mice (data not shown). Therefore, in contrast to the previous report (9), the IFN-β-/- mice on the B10.RIII background did not show signs of an intrinsic bone erosion disorder. Analyzes of arthritic joints showed a clear increase in the number of osteoclast in IFN-β-/- mice, compared to control mice (Figure 4D), suggesting that IFN-β plays an important role in down-regulating inflammation-mediated osteoclastogenis.

Discussion

IFN-β has been used in the therapy of various diseases such as cancer, viral infections and is one of the few available treatments for MS (14, 35). There has also been a great deal of interest in the possible therapeutic effects of IFN-β in RA but there is a need to gain a better understanding of the mechanisms of IFN-β in arthritis. We have previously shown in EAE, a murine model for MS, that IFN-β has the greatest effects in reducing the activation of macrophages and microglia, with little effect on T cells. Moreover, IFN-β deficiency did not cause a shift in the T helper phenotype (25, 36). In accordance with this, we find in this study that a lack of IFN-β in arthritis lead to a greater activation of stromal cells such as macrophages and fibroblasts, as well as an enhanced generation of osteoclasts in the arthritic joints and that IFN-β had little effect on antigen-specific T cell responses.

IFN-β has not only been shown to have anti-inflammatory effects but it has also been suggested to be involved in development, homeostasis and apoptosis of several cell populations such as osteoclasts, T cells, neutrophils and B cells (8). Osteoclasts are cells that degrade bone and are vital in maintaining bone homeostasis; however excess osteoclastogenesis in the arthritic joints leads to a net loss of cartilage and bone. Takayanagi *et al*. elegantly showed the importance of IFN-β in osteoclastogenesis, with a clear increase in osteoclastogenesis both *in vitro* and *in vivo* in naïve mice lacking the IFN-β receptor and in IFN-β-/- mice (37). In the current study, we also show *in vitro* that IFN-β-/- mice have an enhanced osteoclastogenesis. However, we did not see an increase in osteoclastogenesis *in vivo* in naïve mice, but instead found that immunized IFN-β-/- mice have a significant increase in the number of osteoclasts, indicating that IFN-β in an arthritic joint would be beneficial in reducing the amount of joint destruction.

IFN-β has been shown to be involved in apoptosis of T cells and neutrophils (7, 10). IFN-β together with Stromal Cell-Derived Factor 1 (SDF-1or CXCL12) was shown to inhibit apoptosis of T cells located in the joint synovia of human RA patients and was therefore believed to maintain T cells within the joint (7). However, the role of T cells in RA synovia is not known and it is possible that T cells are involved in the priming phase of arthritis but have little role in the effector phase of the disease since T cells have been shown to proliferate poorly and secrete few

cytokines (38). In addition, anti-T cell agents seem to have little influence on ongoing arthritis (39, 40) whereas anti-B cell and anti-monokine reagents such as anti-TNF-α have had considerable therapeutic effects (41, 42). In the present study, the IFN-β-/- mice showed no difference in T cell numbers compared with control mice, indicating that there was no increase in apoptosis in the T cell compartment. Furthermore, there was no difference in antigen-specific T cell proliferation upon re-stimulation *in vitro*. In addition, there was no difference in the degree of T cells infiltration in the synovia of these mice (data not shown).

Neutrophils have been suggested to be involved in both RA and CIA (10, 43). In line with this, IFN-β has been shown to prevent apoptosis of neutrophils (10) and IFN-β-/- mice have previously been shown to have a reduced number of neutrophils (8). Indeed, in the current study, a reduced number of neutrophils in both spleen and lymph nodes were observed (data not shown). However, a prominent role of neutrophils was not found since IFN-β-/- mice developed a more exacerbated arthritis. Interestingly, the neutrophil populations in the spleen and synovia of IFN-β deficient mice were able to withstand (and even proliferate) *in vitro* culture (36 hours), which was in contrast to IFN-β-/+ neutrophils (data not shown and Table 2). This indicated that the influence of IFN-β on neutrophil apoptosis *in vitro* differs from that *in vivo*.

In the present study, lack of IFN-β expression seemed to have a prominent effect on stromal cells including macrophages and fibroblasts. Flow cytometry analyzes of synoviocytes showed that, upon LPS and IFN-γ stimulus, there was an up-regulation of CD11b+ cells, an increase in macrophages as well as an up-regulation of the adhesion molecule ICAM-1. This indicates that the stromal cells in the synovia are more readily activated in IFN-β deficient mice, potentially leading to a more chronic arthritic profile.

Fibroblasts have been implicated both in the priming and effector phase of RA pathology (7, 44), thus, targeting fibroblasts should be of therapeutic benefit. In the present study, a lack of IFN-β was associated with fibroblasts having a more active phenotype with an increased IL-6 production. There was also an up-regulation in the cell-surface molecules CD44, CD40 and ICAM-1, all of which are believed to be involved in RA pathology (44). However, as fibroblasts require IFN-β to produce IFN-α (6), it is possible that the phenotype of the IFN-β deficient fibroblast is

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partially due to IFN-α deficiency. Nevertheless, the current study determined that local injection of IFN-β-producing fibroblasts into the joints of IFN-β-/- mice completely reverted the augmented arthritic phenotype to that of control mice. This is an important finding as it shows that physiological levels of IFN-β produced by non-transfected fibroblasts can have a beneficial effect *in vivo*.

In summary, the present study shows that mice deficient in IFN-β display a chronic arthritic disease with a high incidence. These results may have been expected from previous animal studies using IFN-β treatments. However, we were able to demonstrate that the mechanism of action of IFN-β is not mediated through T cells but rather due to an increased activation of resident cells of the joint; that is fibroblasts, macrophages and osteoclasts. It is likely that IFN-β serves to control the activation state of fibroblasts and in the absence or low levels of IFN-β, fibroblasts become more prone to produce cytokines, chemokines and growth factors that in turn enhance infiltration of inflammatory cells. In fact it has previously been shown that treatment of RA-derived fibroblasts *in vitro* with IFN-β leads to a decreased production of chemokines, such as MMP-1 and MMP-2, as well as of PGE$_2$ (45). The mouse model used in this study thus supported the conclusion that fibroblasts have an important role as producers of IFN-β. We were able to show via transfer of IFN-β competent fibroblasts that increasing the amount of IFN-β indeed has a profound effects on arthritis. This is an important finding as it has recently been proposed that naturally produced IFN-β plays an anti-inflammatory role in RA patients (19).

There is no doubt that IFN-β has potent anti-inflammatory properties. However, the question of which cell type and which phase of arthritis is most receptive to IFN-β treatment remains unclear, possibly explaining the limited success of clinical trials. Therefore, animal models can aid in pinpointing mechanisme(s) of this cytokine in order to determining the most effective treatment protocol and which group of patients would benefit the most.

Acknowledgments

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Tables

Table 1: Arthritis parameters and anti-CII antibody response in IFN-β-/- and control (IFN-β +/+ and -/+) mice[A].

	IFN-β-/-	control	P-value
IgG Total[B] (Units / ml)	56 ± 11	37 ± 8	0.15
IgG1 (Units / ml)	33 ± 5	20 ± 3	0.08
IgG2 (Units / ml)	88 ± 54	26 ± 5	0.08
Incidence[C]	100%	43%	0.02
Duration[D]	63 ± 6	27 ± 6	0.003
Max score[E]	23 ± 3	12 ± 2	0.01
Incidence 12th generation[C]	90 %	40%	0.05
CAIA average score[F]	10.2±2.1	4.2±1.7	0.05
CAIA incidence[F]	90%	40%	0.09
CAIA Duration[E,F]	25.9±1	19.8±2.1	0.04

[A] Except where indicated otherwise, values are the mean ± SD. CII = type II collagen; IFN-β = interferon-β deficient; CAIA= collagen antibody-induced arthritis

[B] Mean total CII-IgG as well as IgG subclass levels in sera collected on day 121 was calculated as arbitrary units/ml using a polyclonal serum.

[C] Incidence was calculated from day 65 after immunization.

[D] The average duration of disease was calculated from the first day the mice showed clinical signs until the end of the experiment (un-affected mice were given 0).

[E] Maximum score of affected mice was calculated as the average of the highest obtained score of mice showing clinical signs of arthritis.

[F] Calculated on 21 days after transfer of CII-specific antibodies

Table 2: Expression levels of cell-surface markers on synovia cells taken from IFN-β-/- and control (control IFN-β-/+) mice[A].

|	IFN-β-/-			Control IFN-β-/+		
	Media	LPS+IFN-γ	Delta[B]	Media	LPS+IFN-γ	Delta[B]
F4/80	20.3	22.1	1.8	17.6	16.6	-1.0
TNF-α	32.8	29.5	-3.3	23.9	18.3	-5.6
CD11b	24.5	27.7	3.2	16.1	19.0	2.9
Ly6-G + CD11b	8.4	11.9	3.5	2.1	2.4	0.3
ICAM-1	17.8	27.9	10.1	31.6	35.0	3.4
VCAM-1	43.5	42.0	-1.5	36.1	28.5	-7.6
ICAM-1+VCAM-1	11.4	19.6	8.2	22.8	23.4	0.6

[A] 24-25 mice per group were pooled and stimulated. The experiment was performed twice with similar results and one representative experiment is shown

[B] Delta expression was calculated by subtracting the expression level of cells cultured in media from cells cultured with LPS+IFN-γ.
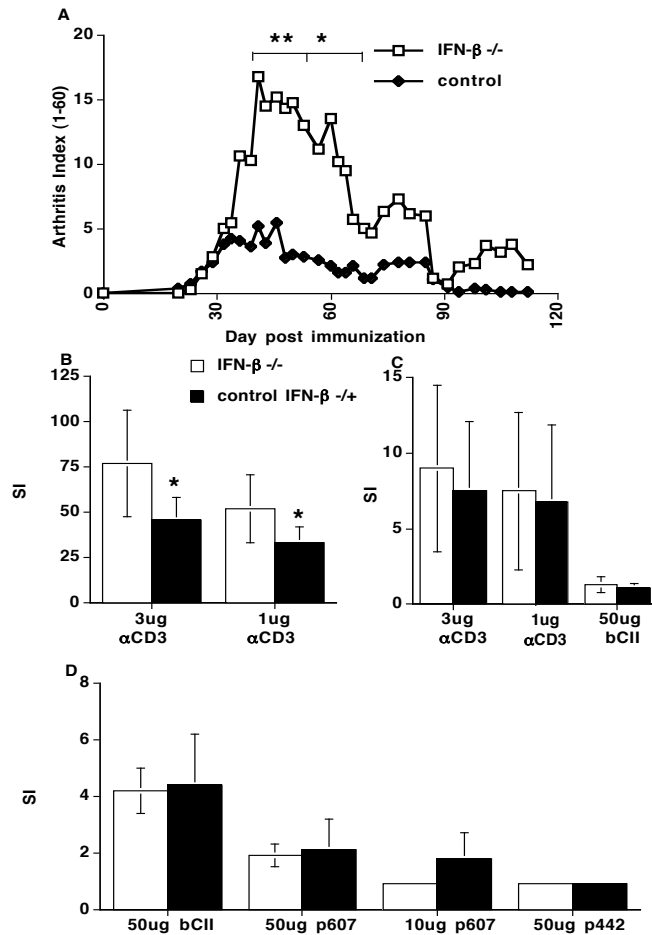
Figure 1: IFN-b deficiency leads to an augmented CIA without altering the antigen specific T cell response

A. Arthritic Index, calculated as the mean score of arthritis of IFN-b-/- mice and control mice (IFN-b-/+ and IFN-b+/+ mice). Mice were scored twice weekly, starting from day 14. Data include a total of 8-14 mice per group. * p=0.05, ** p=0.01 versus control mice. B-C. Anti-CD3 response of spleenocytes from either naïve (B) or pre-immunized (C) IFN-b-/- and control IFN-b-/+ mice. Cells were incubated for 48hr before [3H] dThd incorporation. *p=0.05 versus control mice. D. Antigenic specific response of lymphocytes from IFN-b-/- and control IFN-b-/+ mice immunized 10 days prior to in vitro cultivation. Lymphocytes were re-stimulated with 50ug/ml of whole bovine collagen (bCII) or 50 or 10ug/ml of the immunodominant peptide sequence 607-621 of CII (p607) or 50ug of the immunodominant peptide sequence 442-456 of CII (p442). Cells were cultivated for 72hr before [3H] dThd incorporation. Error bars represent standard deviation of the mean. The data shown is one representative experiment out of three.
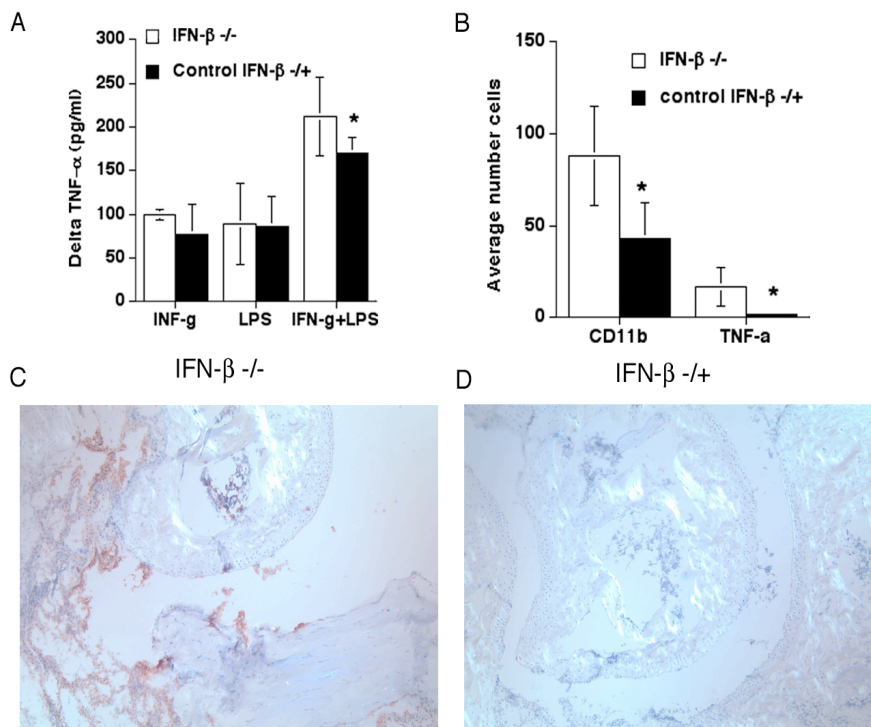
Figure 2: Mice deficient in IFN-β have a greater TNF-α production in spleen-derived macrophages and in joints of pre-immunized mice.

A. Delta production (cytokine production of LPS, IFN-γ, or LPS and IFN-γ minus cytokine production of macrophage cultured in media alone) of TNF-α. The data shown is one representative experiment out of three. B. Number of cells staining positive for CD11b and TNF-α in hind or front paws of IFN-β-/- (n=5) and control (n=5) mice all having similar symptoms of arthritis. For each mouse, five distinct fields on a single sample slide were counted and the average number determined. * p=0.05verus control mice. Error bars represent standard error of the mean C-D. Immunohistochemistry 40 days after immunization of one representative IFN-β-/- (C) and control IFN-β-/+- (D) mouse with comparable clinical symptoms of arthritis at the time of analyzes. Sections were stained for TNF-α with positive cells staining brown (Original magnification x 200).
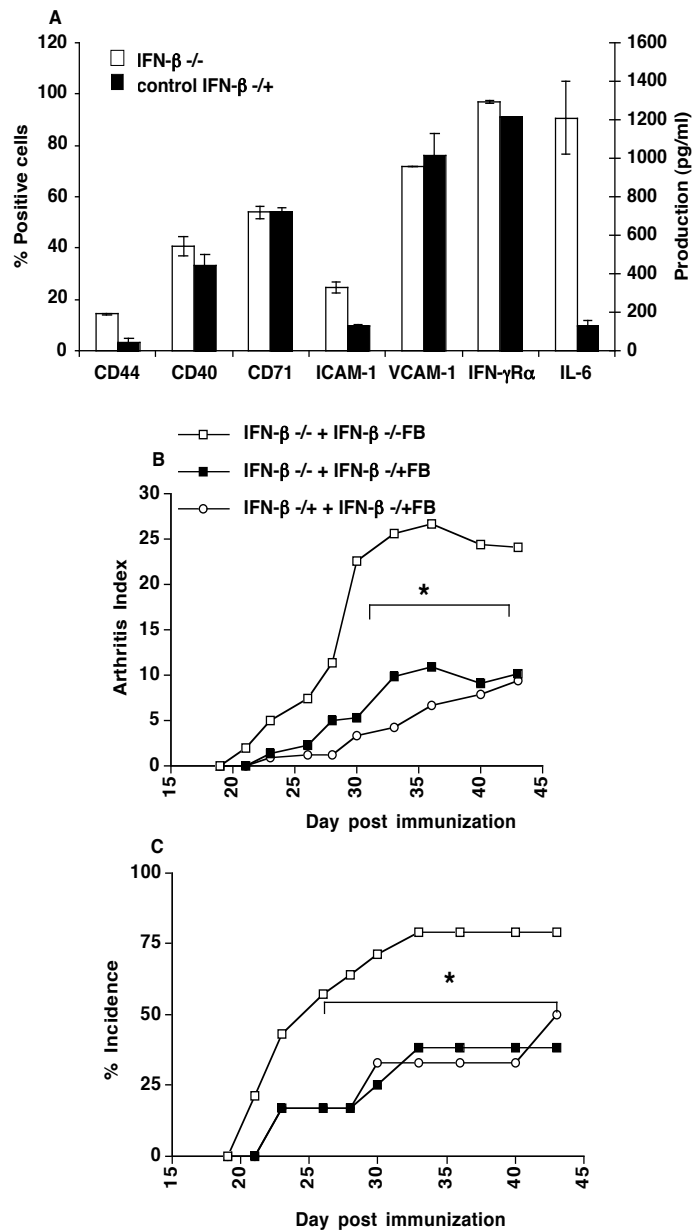
Figure 3: Reconstitution of IFN-β deficient mice with IFN-β competent fibroblasts ameliorates arthritis

A. Percent of fibroblasts staining positive for different cell surface markers (Y-axis) and IL-6 production following *in vitro* culture of fibroblasts (YY-axis) from IFN-β-/- and control mice. Error bars represent standard error of the mean. B. And C. Arthritic Index(mean score) and incidence of arthritis in IFN-β-/- mice that had received $2x10^6$ fibroblasts from either IFN-β-/- (IFN-β-/- + IFN-β-/-FB) or from control (IFN-β-/- + IFN-β-/+FB) mice and control IFN-β-/+ mice injected with control IFN-β-/+ fibroblasts (IFN-β-/+ + IFN-β-/+FB). All mice were injected with fibroblasts and immunized with CII on day 0. Mice were scored twice weekly, starting from day 14. Data include a total of 8-10 mice per group pooled from 2 separate experiments, each with balanced groups. * p≤0.05 versus control.
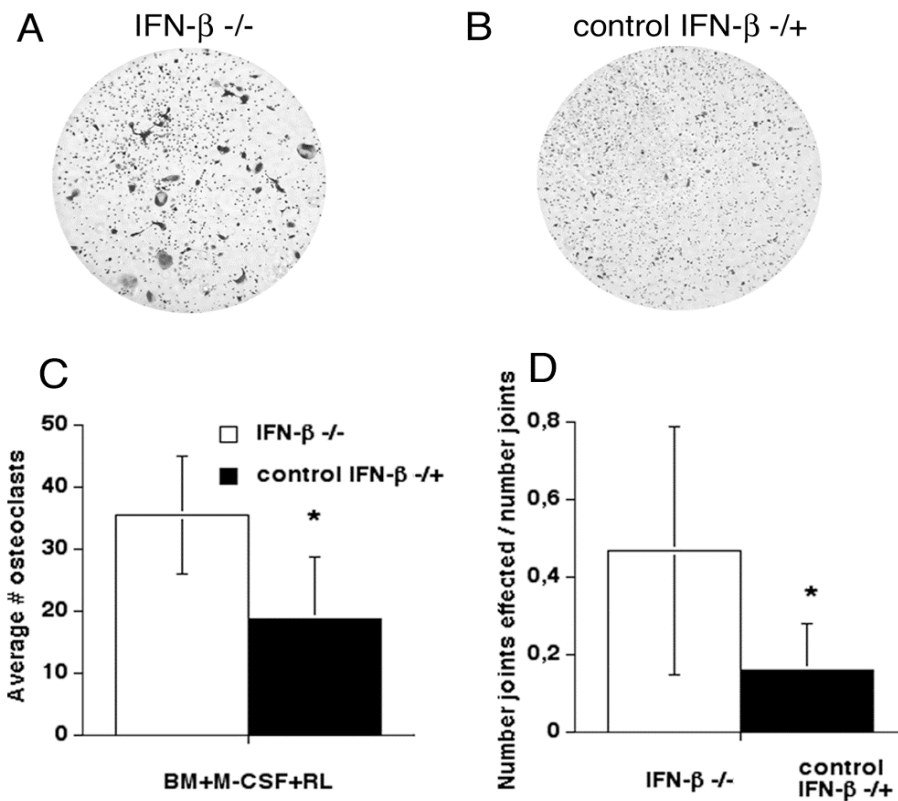
Figure 4: Enhanced generation of osteoclasts in IFN-β deficient mice
A-B *In vitro* generation of osteoclasts from bone marrow cells from either IFN-β-/- (A) or
control IFN-β-/+ (B) mice. Cells were cultured *in vitro* with M-CSF and rmRANKL for 6-7
days in 48 well plates. Original magnification x 100. C. Average number of osteoclasts
generated from bone marrow from 4 IFN-β-/- and 4 control IFN-β-/+ mice. Cells were
seeded in duplicate and 5 fields of view of each well were counted under 200x
magnification. Bone marrow cells from IFN-β-/- or control IFN-β-/+ mice cultured in M-
CSF alone did not support the generation osteoclasts and were used as negative control for
the experiment (data not shown). D. *In vivo* staining of osteoclasts (TRAP positive and
multi-nucleated). Paws from pre-immunized mice (30 days prior), were removed and
prepared for paraffin sectioning and stained for TRAP. All joints in the section were
counted and the joint that contained one or more osteoclast was counted as effected.
Consequently, the graph represents the number of effected joints divided by the total
number of joints counted in both hind and front paws. Graph includes 4-6 mice per group.
Error bars represent standard deviation of the mean. * p≤0.05.