

The effects of interleukin-18 on rat articular chondrocytes: a study of mRNA expression and protein synthesis of proinflammatory substances

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Introduction

The destruction of articular cartilage matrix by inflamed synovial tissue is a characteristic finding of many types of arthritis. The biomechanical integrity of the cartilage matrix is dependent upon a balance between the anabolic and catabolic activities of chondrocytes. Externally and internally produced proinflammatory mediators activate chondrocytes to degrade the matrix, making cartilage more susceptible to injury. Unfortunately, the potential for cartilage matrix repair is limited. Certain cytokines, namely interleukin (IL)-1 β and tumour necrosis factor (TNF)- α , play major roles in cartilage degradation [1,2]; however, others might similarly be involved [3]. One cytokine of interest is IL-18, a member of the IL-1 cytokine family. IL-18 is released as a propeptide, cleaved by IL-1 β -converting enzyme to a biologically active 18 kDa product. IL-18 is expressed by a

Summary

Interleukin (IL)-18 is a potent stimulator of immunity and augments the severity of type II collagen-induced arthritis (CIA) in rats and mice by enhancing T helper 1 (Th1) cell activation, which increases the production of proinflammatory cytokines and arthritogenic antibodies. In this study, we show that recombinant IL-18 (rIL-18) also has a direct effect on normal rat chondrocytes maintained *in vitro* inducing them to produce proinflammatory factors including IL-6, regulated upon activation normal T cell expressed and secreted (RANTES), prostaglandin E₂ (PGE₂) and prostaglandin F_{2 α} (PGF_{2 α}) in a dose- and time-dependent manner. The production of matrix metalloproteinase (MMP)-13, nitric oxide (NO), tumour necrosis factor (TNF)- α and IL-1 β were also enhanced, although less intensely. Neutralizing polyclonal anti-rIL-18 antibodies effectively blocked the production of IL-6, PGE₂ and RANTES, as well as mRNA expression for the same products in addition to IL-18 and TNF- α . In contrast, neutralizing antibodies to IL-1 β , TNF- α and IL-6 were ineffective in suppressing any of these products. Together, these findings suggest that IL-18 may play an important, possibly direct, role in mediating cartilage injury, which might not be amenable to treatment with currently utilized anti-cytokine agents. These findings suggest further that IL-18 antagonists might prove beneficial as anti-inflammatory and chondroprotective agents in the treatment of arthritis, and that the development of such agents for human use is worth consideration.

Keywords: anti-IL-18 antibody, cartilage, chondrocyte, IL-18, proinflammatory cytokine

variety of cells, including monocytes/macrophages, dendrocytes, synovial fibroblasts, osteoblasts and chondrocytes [4–7]. IL-18 possesses immunoregulatory activities, including the stimulation of interferon (IFN)- γ production by T helper 1 (Th1) and natural killer cells [8,9], as well as other activities that promote inflammation, e.g. the enhancement of TNF- α , IL-1 α , IL-1 β and IL-6 production [10], the up-regulation of intercellular adhesion molecule (ICAM)-1 [11], the enhancement of NO and cyclooxygenase 2 (COX-2) production [6], and the recruitment and activation of neutrophils [12,13]. Thus, not unexpectedly, IL-18 is linked firmly to the destruction of cartilage and bone [3].

With regard to joint injury, Gracie *et al.* [14] have reported higher levels of IL-18 mRNA and protein in the joints of patients with rheumatoid arthritis (RA) than patients with osteoarthritis (OA). In using the collagen-induced arthritis model, it has been shown that IL-18 enhances the incidence

and severity of joint injury in rats and mice [12,15,16]. In contrast, IL-18 knock-out mice develop clinically and histologically milder arthritis at a reduced incidence [15]. *In vitro* studies using normal human cartilage show that IL-18 enhances NO, IL-6 and glycosaminoglycan production [6], suggesting that similar events might occur *in vivo*. Recently, IL-18-induced joint inflammation has been shown to occur independently of IL-1 although it may remain, in part, dependent on TNF- α [17].

Our previous report showed that IL-18 plays a strong proinflammatory role in rat collagen-induced arthritis (CIA) [16]. This effect was associated with enhanced Th1 responses, as demonstrated by increased cytokine and anti-CII antibody production. Neutralizing anti-IL-18 antibodies attenuated these changes in addition to arthritis severity and incidence. To ascertain whether IL-18 also has a direct effect on articular cartilage, we stimulated normal rat chondrocytes *in vitro* with IL-18 and measured the expression of mRNA and production of key proinflammatory mediators that cause tissue injury. Our studies described here show that chondrocytes release substantial amounts of proinflammatory substances. Moreover, antibodies specific for IL-18, but not other cytokines, suppressed the production of these products. These data indicate that IL-18 may have a direct effect on chondrocytes resulting in cartilage matrix injury.

Materials and methods

Preparation of rat recombinant IL-18

Total RNA was extracted from rat lymphoid node cells with TRIzol Reagent (Life Technologies, Grand Island, NY, USA). First-strand cDNA synthesis was performed with a cDNA cycle kit (Invitrogen, San Diego, CA, USA). Primer set pairs designed from a rat IL-18 sequence data were used to clone IL-18 (sense, ATGGCTGCAATACCAGAAGAAG; anti-sense, ACTTTGATGTAAGTTAGTAAG). The polymerase chain reaction (PCR) product was cloned into the pET-19b expression vector, expressed in *Escherichia coli*, and purified as a histidine-tagged fusion protein. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), Pierce gelcode blue staining and limulus assays were used to confirm rIL-18 purity, as reported earlier [16].

Chondrocyte isolation and culture

Articular cartilage was pooled from the shoulders, knees and hips of 4–5-week-old BB rats. Chondrocytes were isolated by enzymatic digestion using 0.25% trypsin/ethylenediamine tetraacetic acid (EDTA) solution for 30 min followed by 4 h digestion with 0.2% collagenase in Dulbecco's modified Eagle medium (DMEM)/F-12 medium containing 10% fetal calf serum (FCS) and penicillin/streptomycin [18]. The freshly isolated chondrocytes were washed, suspended in complete medium, cultured at 1×10^5 cells/ml in six-well

plates for 7–10 days, and the medium changed every 3 days. On reaching confluence, chondrocytes were treated with trypsin/EDTA and reseeded into 24-well plates for cytokine measurement or 10-cm culture plates for mRNA studies. Complete medium was replaced with medium containing 0.2% FCS for 12–24 h before rIL-18 stimulation. To ensure the chondrocyte phenotype, only first-passage cells were studied. Fibroblast and dendritic cells were not observed in the cultures.

Measurement of cytokines

Chondrocyte monolayers were incubated in 24-well plates for 6, 24 and 48 h with rat rIL-18 or medium alone. The supernatants were divided into aliquots and cytokines measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA).

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). Chondrocytes grown in 10 ml culture dishes were stimulated *in vitro* with rIL-18 (100 ng/ml of medium) for 0–48 h. After cell lysis with TRIzol reagent (Invitrogen), total RNA was isolated following the manufacturer's instructions. First-strand cDNA synthesis was performed with a cloned avian myeloblastosis virus (AMV) first-strand cDNA synthesis kit (Invitrogen). Conventional PCR was performed using primer pairs purchased from BioSource (Camarillo, CA, USA) in a 50- μ l reaction mixture containing 5 μ l of 10 \times PCR buffer, 4 μ l of 2.5 mM deoxyribonucleoside (dNTP), 2 μ l of primer pairs, 4 μ l of 25 mM MgCl₂, 10 μ l of Q-Solution (Qiagen, Chatsworth, CA, USA), 0.3 μ l of HotStar *Taq* DNA polymerase (Qiagen) and 1 μ l of cDNA product. The reaction utilized a DNA thermal cycler (Perkin-Elmer, Irvine, CA, USA) for 35 cycles at 94°C for 45 s, 60°C for 45 s and 72°C for 2 min, with an initial activation step at 95°C for 15 min. PCR products were resolved by electrophoresis using a 1.5% agarose gel.

Measurement of mRNA expression by quantitative RT-PCR (qPCR)

For transcript quantification, 1 μ g of total RNA was used for cDNA synthesis and diluted at a 1- μ g RNA to 50 μ l RT-mix ratio. qPCR reactions were performed in an ABI Prism 7900 HT Sequence Detection System (ABI Applied BioSystems, Foster City, CA, USA). Primers, probes and reagents for the reverse transcription and PCR were purchased from ABI Applied BioSystems. Results were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and expressed as fold increase *versus* control using the 2- $\Delta\Delta$ Ct method.

Immunoblotting

Matrix metalloproteinase (MMP)-13 production was detected by Western blotting. Aliquots of concentrated

culture media from 10-ml dishes were resolved on 7.5% SDS-PAGE gel, then electrotransferred to nitrocellulose membrane at 250 mA for 1 h. Non-specific antibody binding was blocked with 5% non-fat dry milk in Tris-buffered saline (TBS)/0.1% Tween 20 at 25°C for 2 h. After washing, membrane was incubated overnight at 4°C with 1 : 300 diluted anti-MMP-13 monoclonal antibody (mAb) (Calbiochem, La Jolla, CA, USA). The membrane was washed and reacted with peroxidase-conjugated goat anti-mouse immunoglobulin (IgG) (Sigma, St. Louis, MO, USA) at 25°C for 2 h. Immunoreactive bands were visualized using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA), and exposure to CL-Xposure film (Pierce).

Inhibition of rIL-18-induced cytokine production with anti-cytokine antibodies

Antibodies to rIL-18 were produced in rabbits and IgG isolated from a protein-A column [16]. Chondrocytes were cultured for 24 h in 10-cm dishes with 100 ng/ml of rIL-18 and increasing amounts of anti-IL-18 or normal rabbit IgG. Cytokines in the supernatant were measured by ELISA. For inhibition of mRNA expression, cells were cultured with 100 ng/ml of rIL-18 and 5 µg/ml of anti-rIL-18 IgG or normal IgG. Efforts were made to inhibit cytokine mRNA expression with antibodies to IL-6 (2 µg/ml), IL-1β (10 µg/ml) and TNF-α (10 µg/ml) antibodies (R&D Systems). These products and concentrations are non-toxic and are twofold (or greater) than those reported by others to be effective in comparable systems [19,20], or described in information provided by the supplier. Total RNA was isolated from the aforementioned cultures after 6 h exposure to rIL-18 and antibody and subjected to qPCR study and the data were evaluated by 2-delta delta Ct analysis.

Results

Rat rIL-18 stimulates chondrocytes to express mRNA for of proinflammatory mediators

Conventional RT-PCR was used to ascertain the expression of mRNA for β-actin, IL-1β, TNF-α, IL-6, regulated upon activation normal T cell expressed and secreted (RANTES), inducible macrophage type nitric oxide synthase (iNOS), IL-10, IFN and rIL-4 by chondrocytes cultured 2–48 h with rIL-18. Data presented in Fig. 1 show that IL-18 stimulated mRNA expression of all these substances except IFN-γ and IL-4. Expression levels were highest at 4–6 h and decreased after 24–48 h culture. IL-10 mRNA was weakly expressed at 4–6 h. Only small amounts of IL-1β, IL-6, RANTES and iNOS mRNA were detected in unstimulated cultures.

These results were verified by qPCR, as shown in Fig. 2. Message for TNF-α and IL-1β was 844 and 499-fold greater in cultures stimulated with rIL-18 for 2 h than control. Cultures maintained for 2, 4, 6, 24 and 48 h demonstrated that

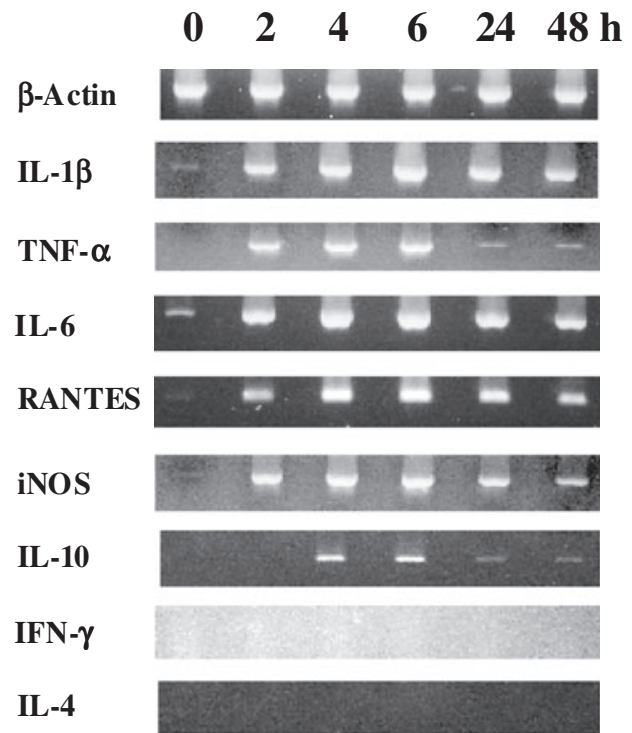


Fig. 1. Conventional reverse transcription-polymerase chain reaction (RT-PCR) analysis of recombinant interleukin (rIL)-18-induced mRNA expression of proinflammatory mediators by rat chondrocytes. Cells were cultured with 100 ng/ml of rat rIL-18 for 2, 4, 6, 24 and 48 h or medium alone (0 h). Total RNA was isolated and reverse transcribed. The PCR products were separated on 1.5% agarose gels and stained with ethidium bromide.

message peaked at 4 or 6 h for all the factors. Message for TNF-α, RANTES, IL-1β, IL-6 and COX-2 peaked at levels 1977, 1553, 711, 290 and 305-fold above control, respectively. Message for MMP-13, IL-12, iNOS, IL-10, IL-18 and MMP-8 were detected at lower peak levels of 125, 35, 13, 10, 6 and 2.1-fold increases, respectively.

Rat rIL-18 stimulates chondrocytes to produce proinflammatory mediators

Exogenous rIL-18 stimulated chondrocytes to secrete proinflammatory mediators and MMP-13 in a time- and dose-dependent manner as measured by ELISA (Fig. 3) or Western blotting (Fig. 4). IL-6, prostaglandin F_{2α} (PGF_{2α}), RANTES, IL-1β, TNF-α and NO appeared in supernatants after 6 h stimulation and reached peak levels at 48 h (245, 20, 120, 2.9, 3.6 and fourfold peak increases, respectively). Prostaglandin E₂ (PGE₂) production was an exception, peaking at 24 h (143-fold increase). IL-6, PGF_{2α}, RANTES, TNF-α and NO production rose with increasing amounts of rIL-18. Although substantial amounts of product were detected with 50 ng/ml of rIL-18, maximal levels were often reached with 500 ng/ml. IL-1β levels peaked with 100 ng/ml of rIL-18, but

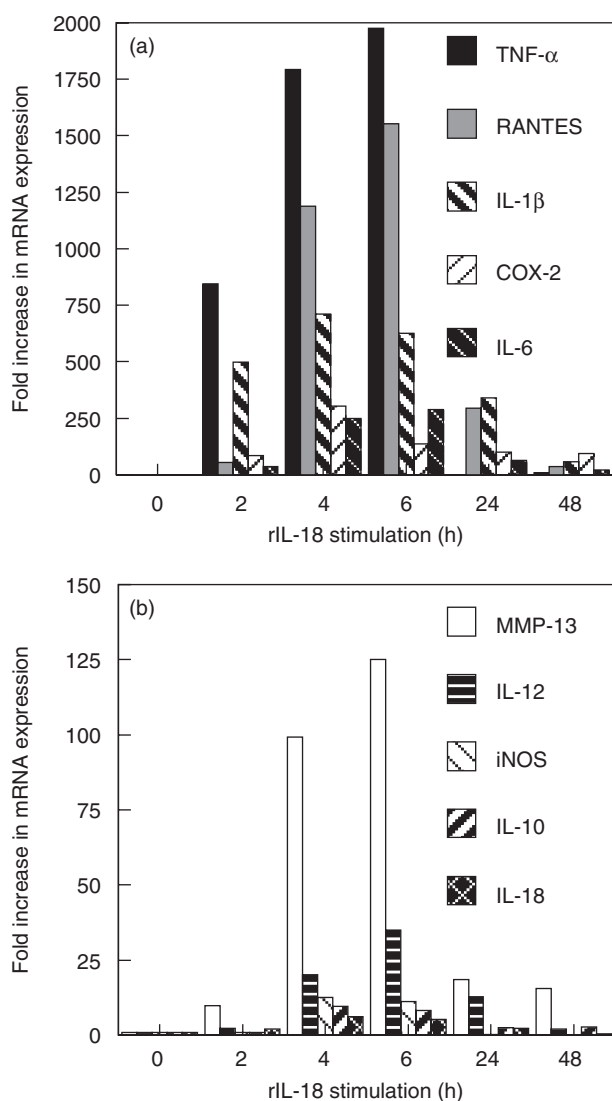


Fig. 2. Real-time polymerase chain reaction (PCR) analysis of recombinant interleukin (rIL)-18-induced mRNA expression of proinflammatory mediators by rat chondrocytes. Cells were cultured with 100 ng/ml of rat rIL-18 for 2, 4, 6, 24 and 48 h or medium alone (0 h). Total RNA was isolated, reverse transcribed, and subjected to quantitative PCR. The results were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase and expressed as x-fold increase *versus* control using the 2 delta-delta Ct method.

fell with 500 ng/ml. MMP-13 was detected as a 60-kDa proenzyme at 24 h and remained elevated at 96 h (Fig. 4). IL-10 and IFN- γ products were undetectable.

Anti-rIL-18 antibody inhibits IL-18 induced chondrocyte mRNA expression and the production of proinflammatory mediators

To determine if the effects of rIL-18 on chondrocytes could be attenuated, anti-rIL-18 IgG was added to cultures stimulated with rIL-18. Message for IL-18, TNF- α , RANTES and

IL-6 fell to levels below 50% of control cultures treated with normal IgG (Fig. 5). Levels of PGE₂, IL-6 and RANTES product also fell in a dose-related manner (Fig. 5); IL-1 β , NO and PGF_{2 α} levels fell, although less notably (data not shown). Efforts to block the actions of rIL-18 on chondrocytes by simultaneously adding anti-IL-6, anti-IL-1 β or anti-TNF- α antibodies were not successful and often produced small increases of the amount message expressed *versus* cultures where normal IgG was added (data not shown). These cultures were maintained for 6 h using the same conditions described in Fig. 6 and in Materials and methods. The cause and biological relevance of the apparent synergistic interaction between rIL-18 and these antibodies are unclear, and might be a topic of future interest.

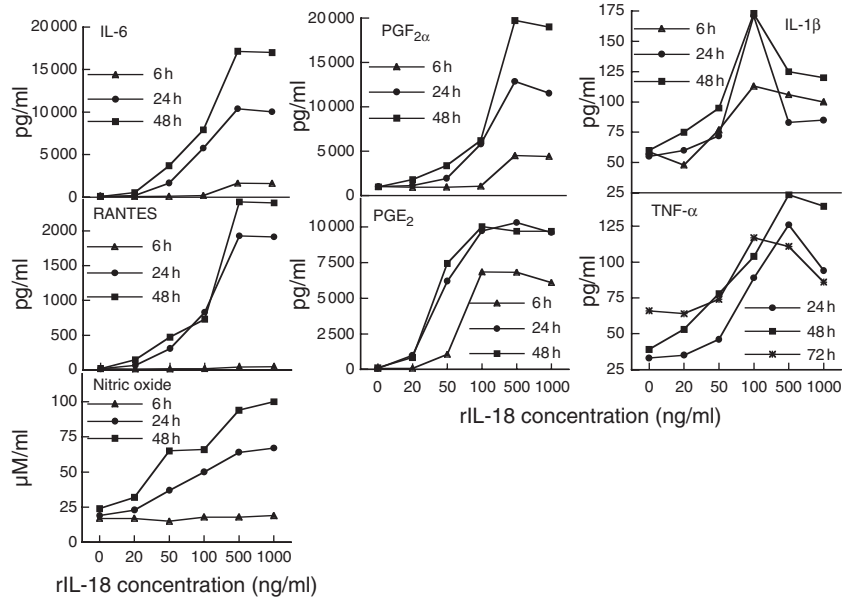
Discussion

Unravelling the complexities of immune-mediated polyarthritides is a challenge, whether investigated in human or in experimental animal systems. Collagen-induced arthritis is initiated by autoreactive anti-CII antibodies, which bind to cartilage, fix complement and trigger synovitis by a variety of pathways. This process occurs whether antibodies are produced actively or administered passively [21,22]. Th1 cells are essential for anti-CII antibody production and infiltrate the synovium to sustain inflammation. All these events lead to the production of proinflammatory cytokines, chemokines and other substances, which promote cartilage injury.

IL-18 is produced by activated synovial macrophages and fibroblasts [4] and is believed to be instrumental in joint injury CIA and in RA. We have shown that rIL-18 enhances the incidence and severity of CIA in rats, in addition to the production of IFN- γ , IL-6, TNF- α and IL-2 by splenocytes [16]. Importantly, arthritis severity can be attenuated with neutralizing anti-rIL-18 antibodies or other IL-18 specific antagonists [23]. These findings, plus increased mRNA expression for IL-18 by inflamed rat synovium [16] and the presence of elevated levels of IL-18 in the synovial fluids and tissues of RA patients suggest that IL-18 might participate in cartilage injury [6,14]. This possibility is supported further by the work of Joosten *et al.* [17], who injected wild-type, TNF- α - and IL-1 α,β -deficient C57BL/6 mice intra-articularly with an IL-18-expressing adenovirus vector. Histological examination of the joints of all three strains disclosed pronounced inflammation and cartilage proteoglycan loss; such changes were absent in the joints of control mice injected with non-expressing adenovirus.

The studies reported here show that rat chondrocytes stimulated *in vitro* with rIL-18 produce a variety of proinflammatory substances. IL-6 was the greatest, reaching values 245-fold above control. The importance of IL-6 in the pathogenesis of RA and experimental arthritis is well established. IL-6 is significantly higher in RA synovial fluid than in OA samples [24,25]. In the CIA model, IL-6 knockout mice are protected against arthritis [26,27]. We have also

Fig. 3. Rat recombinant interleukin (rIL)-18 stimulates rat articular chondrocytes to produce a variety of proinflammatory products. The chondrocytes were cultured with increasing amounts of rIL-18 or medium for 6, 24 and 48 h. Levels of IL-6, prostaglandin E₂, prostaglandin F_{2α}, regulated upon activation normal T cell expressed and secreted (RANTES), interleukin (IL)-1β, tumour necrosis factor (TNF)-α and nitric oxide (NO) secreted into the culture supernatant were measured as described in Materials and methods. These products were secreted in a dose- and time-dependent manner.



found that the synovia of rats with CIA express mRNA for IL-6 at levels 37 times greater than messages for IL-1α/β, TNF-α/β, IFN-γ and IL-2; moreover, the synovial production of IL-6 protein was 200 times greater in arthritic rats than non-arthritic rats (our unpublished data). Our work described here, showing that IL-18 stimulates chondrocytes to produce IL-6, IL-1β and TNF-α, complements the work of others who investigated the effects of IL-18 on synovial cells and splenocytes [14,16]. The roles of these mediators in the pathogenesis of RA, and to a lesser degree osteoarthritis, are well established [28,29]. Although IL-1β and TNF-α stimulate IL-6 synthesis [30], we were unable to block IL-6 expression by adding neutralizing antibodies to either cytokine to IL-18-stimulated chondrocyte cultures, suggesting that IL-18 is a potent and possibly an independent activator of IL-6.

Although often thought of as T lymphocyte-related product, synovial fibroblasts and a host of other cells [31] produce RANTES (CCL5). Our studies show that rIL-18-stimulated chondrocytes produce large amounts of RANTES, complementing the work of Alaaeddine *et al.* [32], who found increased RANTES in chondrocytes obtained

from patients with osteoarthritis. RANTES is a strong chemoattractant for eosinophils, monocytes and T lymphocytes, specifically CD4⁺/CD45RO⁺ memory T cells [33,34]. *Ex vivo* studies show that RANTES also stimulates chondrocytes to produce iNOS and IL-6 [32]. RANTES production, like IL-6, is stimulated by IL-1β and TNF-α, which were elevated in our system [32,35]. The importance of RANTES in experimental arthritis has been demonstrated by Plater-Zyberk *et al.* [36], who showed that metRANTES, a chemically modified antagonist of RANTES, attenuates CIA.

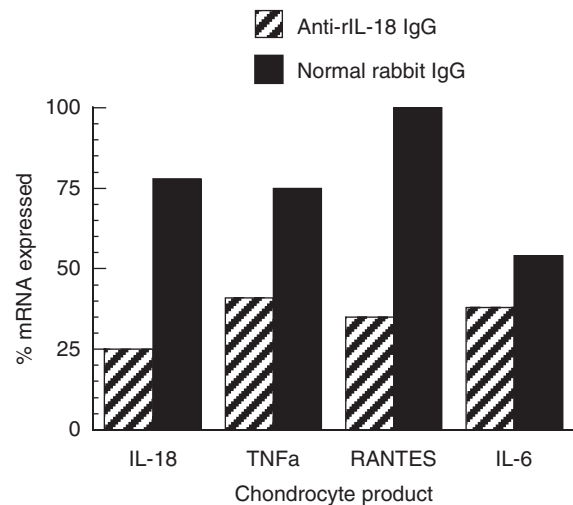


Fig. 5. Anti-recombinant interleukin (rIL)-18 antibodies inhibit the expression of mRNA for proinflammatory mediators by rIL-18-stimulated chondrocytes. Cells were cultured with 5 μg/ml of anti-rIL-18 IgG or normal rabbit IgG and 100 ng/ml of rIL-18 for 6 h. Total RNA was isolated, reverse transcribed and subjected to quantitative polymerase chain reaction analysis. The results are expressed as described in Fig. 3.

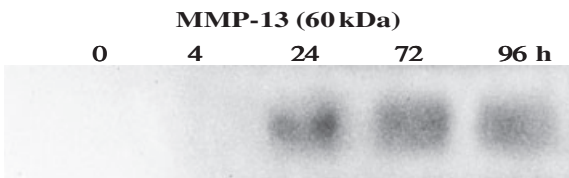


Fig. 4. The production of matrix metalloproteinase-13 by recombinant interleukin (rIL)-18-stimulated chondrocytes demonstrated by Western blotting. Chondrocytes were stimulated with 100 ng/ml of rIL-18 for 4, 24, 72 and 96 h or cultured with medium; cells lysed at 0 h provided a background value.

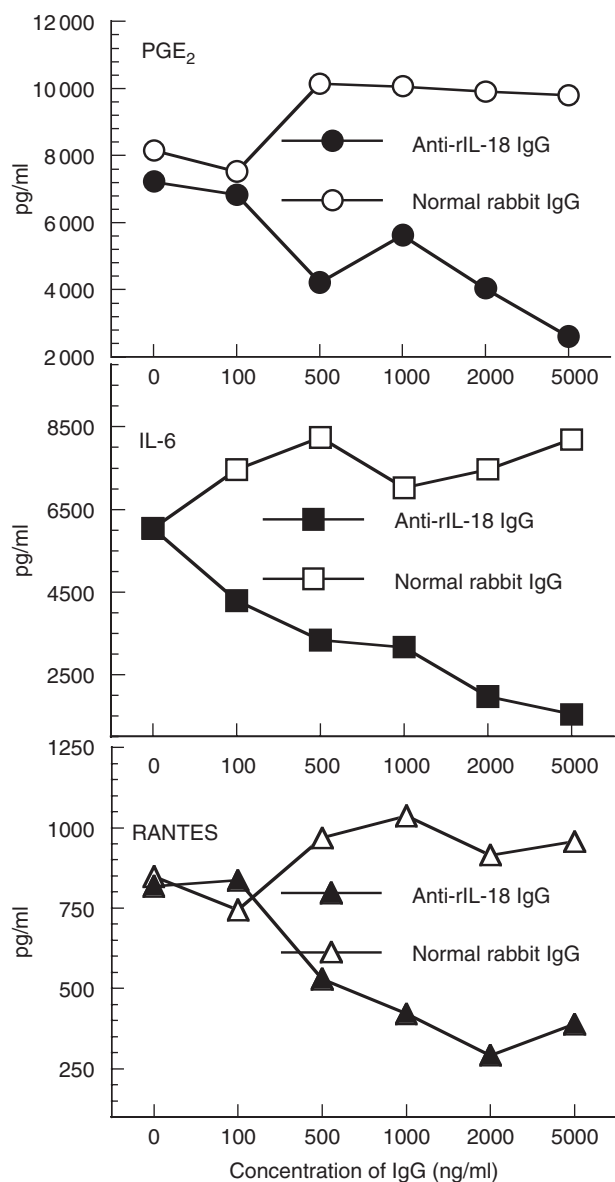


Fig. 6. Anti-recombinant interleukin (rIL)-18 antibodies inhibit the production of proinflammatory mediators stimulated by rIL-18. Chondrocytes cultures were stimulated with 100 ng/ml of rIL-18 and increasing amounts of anti-rIL-18 IgG or normal rabbit IgG added. A commercial enzyme-linked immunosorbent assay system was used to quantify IL-6, prostaglandin E₂ and regulated upon activation normal T cell expressed and secreted (RANTES) released into supernatants collected after 24 h culture. Anti-rIL-18 antibody suppressed the production of proinflammatory mediators in a dose-related manner.

Chondrocytes stimulated with IL-18 also produced large quantities of PGE₂ and PGF_{2α}. PGE₂ is an important mediator of inflammation in RA and OA and triggers osteolysis [37]. PGE₂, which is biologically more potent than most other prostaglandins, rose more rapidly than the other products quantified. Message for COX-2 also rose quickly and remained elevated at 72 h. Levels of PGE₂ exceeded those of non-stimulated controls by 79-fold at 6 h and peaked at

143-fold at 24 h. PGF_{2α} also reached a high level, although more slowly than PGE₂. PGF_{2α} is linked closely to joint injury and is capable of down-regulating TGF-β-induced osteoblast activation [38].

iNOS is active in endothelial cells, synovial cells and chondrocytes, where it generates high levels of NO in response to lipopolysaccharide (LPS), IL-1β, TNF-α and, as shown here, IL-18. The overproduction of iNOS, and consequently NO, occurs in the synovia of rheumatoid patients [39,40] and rats with CIA [41]. Nitric oxide exerts numerous proinflammatory effects, including increased vascular permeability [42], PGE₂ synthesis [43], MMP production, nuclear factor (NF)-κB gene activation and DNA fragmentation. NO also suppresses CII and proteoglycan synthesis and TGF-β-induced chondrocyte proliferation. The potential role of NO in cartilage destruction is thus substantial [44]. Nevertheless, NO possesses some anti-inflammatory properties, including the down-regulation of PGE₂, IL-6 and IL-8 in human chondrocytes [45]. The dual effect of NO might be related to the rate and amount of product generated, in addition to its rate of clearance. Stadler *et al.* [46] have shown that low levels of NO stimulation increased chondrocyte PGE₂ synthesis, whereas a high level was inhibitory. Our studies show that rIL-18 stimulated relatively low levels of iNOS expression, which was accompanied by increased production of PGE₂, suggesting that iNOS was promoting inflammation.

MMPs are zinc-dependent enzymes that degrade extracellular matrix. MMP-13 (collagenase-3) is expressed by synovial cells and chondrocytes and cleaves CII ~10 times faster than MMP-1 [47]. The over-expression of MMP-13 occurs in RA synovium [48] and OA cartilage [49]. Interestingly, mice expressing the MMP-13 transgene develop OA-like changes and transient synovitis, as do mice injected intra-articularly with an MMP-13 expressing adenovirus [50]. In our studies, rIL-18 significantly induced MMP-13 expression by chondrocytes at mRNA and protein levels, indicating that IL-18 mediates matrix degradation.

Lastly, we found that PGE₂, IL-6 and RANTES produced by IL-18-stimulated chondrocytes, as well as message for IL-1β, NO and PGF_{2α}, could be suppressed effectively by neutralizing antibodies to IL-18, but not to IL-1β, TNF-α and IL-6. This observation could have potential clinical relevance. That is, if current and future biological treatments designed to inhibit the actions of IL-1β, TNF-α and IL-6 do not inhibit IL-18 production, it might continue to activate chondrocytes resulting in continued cartilage injury. Together, we believe that the findings reported here support the further investigation of IL-18 as a mediator of cartilage injury and as a target for future therapeutic intervention.

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