Interleukin 17, a Nitric Oxide-Producing Cytokine with a Peroxynitrite-Independent Inhibitory Effect on Proteoglycan Synthesis

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ABSTRACT. ***Objective.*** To compare the potency of 2 cytokines, interleukin 17 (IL-17) and IL-1ß, on rat cartilage proteoglycan synthesis with special attention to nitric oxide (NO) and peroxynitrite formation.

Methods. Chondrocytes in alginate beads were stimulated with human recombinant (rh) IL-17 (0.03 to 300.0 ng/ml) and/or rhIL-1ß (0.25 to 25.0 ng/ml) in the presence or not of L-NMMA or CuDips. Alternatively, rats were injected with either IL-17 (10.0 µg) or IL-1ß (1.0 µg) into each knee joint. NO concentrations were determined by a spectrofluorimetric assay, proteoglycan synthesis by $^{35}SO_4^{2-}$ incorporation, peroxynitrite generation by immunostaining for 3-nitrotyrosine, and IL-1ß mRNA expression by reverse transcription-polymerase chain reaction.

Results. IL-17 inhibited proteoglycan synthesis and increased NO production, both *in vitro* and *in vivo*, without inducing expression of IL-1ß mRNA in cartilage. Additive effects were observed when IL-17 was combined with low concentrations of IL-1. Surprisingly, a similar NO synthesis between IL-1 and IL-17 led to a less suppressive effect of IL-17 on cartilage anabolism than with IL-1. Both *in vitro* and *in vivo*, peroxynitrite formation was extensive with IL-1ß, but negligible or nonexistent with IL-17. L-NMMA and CuDips completely corrected the suppressive effect of IL-1ß on proteoglycan synthesis, unlike with IL-17.

Conclusion. These data showed that NO is weakly involved in the IL-17 mediated inhibition of proteoglycan synthesis in rat. NO overload may not be predictive of any inhibitory effect on cartilage anabolism, but instead superoxide is a key regulator of NO contribution to chondrocyte dysfunction. Since IL-17 is a NO-producing cytokine with additive effects when combined with IL-1, it may play a pivotal role in cartilage destruction during rheumatoid arthritis, for which infiltrating cells produce high levels of superoxide and proinflammatory cytokines. (J Rheumatol 2002; 29:2602–10)

Key Indexing Terms:
CYTOKINES CARTILAGE NITRIC OXIDE
SUPEROXIDE ANION PEROXYNITRITE

Rheumatoid arthritis (RA) is an inflammatory joint disease characterized by progressive destruction of articular cartilage and resorption of subchondral bone. Pathologic hallmarks of affected joints are synovial cell proliferation and infiltration of synovium with macrophages, neutrophils, and lymphocytes[1], which release large amounts of proinflammatory cytokines[2] such as interleukin 1ß (IL-1ß). Indeed, high levels of IL-1ß were detected in synovial fluid (SF) of patients with RA, and increasing evidence reveals its key role in bone damage[3] and cartilage erosion[2].

Several other proinflammatory cytokines have been detected in joints from patients with RA[4]. IL-17[5,6], which is secreted only by CD4+, CD45RO+ memory T cells[7], was shown to share many properties in common with IL-1ß. Thus, by inducing the production of proinflammatory cytokines in articular cells[8] and macrophages[9], this T cell derived cytokine is believed to contribute to the active proinflammatory pattern of RA[5]. In addition, IL-17 was shown to stimulate expression of matrix metalloproteinases (MMP) *in vitro*[10-12], although the enzymes were present predominantly in their latent form[12]. IL-17 was also able to promote osteoclastic bone resorption[13,14]. Therefore, IL-17 may be involved in joint destruction associated with RA. Its contribution is further supported by the presence of its receptor in synovium, especially vascular endothelial cells, and in chondrocytes of patients with RA[15], and by the ability

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of the soluble form of this receptor to reduce joint damage in the collagen induced arthritis model[16]. Although evidence suggests a pathophysiological role of IL-17 in RA, it remains unclear if this cytokine acts as an effector rather than a promoter of the arthritic process.

Interestingly, IL-1ß and IL-17 were shown to induce the production of nitric oxide (NO) in cultured chondrocytes[10,17-20]. High concentrations of nitrate and nitrite, the stable endproducts of NO, were detected in SF of patients with RA[21]. This overproduction was ascribed to the expression of the inducible form of NO synthase (iNOS) in synoviocytes and chondrocytes[22]. *In vitro* studies with NO donors or NOS inhibitors suggested further that NO disrupted the balance between the biosynthetic and the degrading activities of chondrocytes through induction of chondrocyte apoptosis[23], inhibition of type II collagen[24] or proteoglycan synthesis[25], and activation of MMP[26]. Animal models confirmed its pathophysiological relevance as an early mediator of the inhibitory effect of IL-1ß on proteoglycan synthesis[27,28]. Although IL-17 is a NO-producing cytokine, its effect on cartilage metabolism remains unclear: if the catabolic effects of IL-17 (MMP induction, glycosaminoglycans release[12,29]) are well documented, contradictory data were reported on cartilage anabolism (proteoglycan synthesis) due to differences in the experimental conditions used. Indeed, proteoglycan synthesis was shown to be unchanged in patellar cartilage after intraarticular injection of IL-17 in mice[29], whereas it was inhibited in murine intact cartilage incubated *in vitro* with this cytokine[30].

We investigated the effect of IL-17 on proteoglycan synthesis through a dual *in vitro* and *in vivo* approach in the rat, using IL-1ß as a reference. Special attention was given to the pathophysiological relevance of NO and superoxide anion, due to their ability to combine into peroxynitrite. We observed that IL-17 inhibited proteoglycan synthesis whatever the experimental conditions, independently from IL-1ß. Surprisingly, despite its high potency to stimulate NO production, IL-17 suppressed cartilage anabolism with a much lower potency than IL-1ß, and this inhibitory effect occurred mainly through NO-independent pathways in rat. Indeed, superoxide may be a key regulator of NO contribution to chondrocyte dysfunction: peroxynitrite generation, which was detected extensively in rat cartilage after exposure to IL-1ß, but to a much lesser degree after exposure to IL-17, was shown to participate in the IL-1 mediated inhibition of proteoglycan synthesis. The use of distinct pathways to reduce cartilage anabolism may explain the additive effect observed in this study with low concentrations of IL-1 and IL-17, and may have pathophysiological relevance in the context of cartilage damage.

MATERIALS AND METHODS

Reagents. Human recombinant (rh) IL-1ß (10^7 U/mg) (lipopolysaccharide < 1 EU/µg) and rhIL-17 (LPS < 1 EU/µg) were provided by Peprotech (Tebu, Le Perray-en-Yvelines, France). Products for molecular and cell biology were obtained from Gibco BRL (Cergy Pontoise, France). Copper (II) 3,5-diisopropylsalicylate hydrate (CuDips), N^w-monomethyl-L-arginine (L-NMMA), 2,3-diaminonaphthalene (DAN), Trizol, and pronase were purchased from Sigma (St. Quentin Fallavier, France). Collagenase, NADPH, and nitrate reductase (*Aspergillus niger*) were from Boehringer Mannheim (Gagny, France). Soluene 350 and scintillation liquid (hionic fluor) were obtained from Packard (Rungis, France) and radiolabeled sodium sulfate was from Amersham (Les Ulis, France). Rabbit polyclonal antibody for iNOS was provided by Santa Cruz Biotechnology (Tebu) and antibody for nitrotyrosine by Upstate Biotechnology (Euromedex, Souffelweyersheim, France). RDO rapid decalcifier was obtained from Apex Engineering (Plainfield, IL, USA). Immunostaining reagents (Novostain super ABC kit) were purchased from Novocastra (Kington, UK).

In vitro experiments. Chondrocyte isolation and culture conditions. Normal articular cartilage was obtained from Wistar male rats (130–150 g) (Charles River, Saint-Aubin-les-Elbeuf, France) killed under dissociative anesthesia [ketamine (Rhône-Mérieux, France) and acepromazine (Sanofi Santé Animale, France)]. Articular cartilage pieces were dissected aseptically from femoral head caps and chondrocytes were obtained by sequential digestion with pronase and collagenase according to the method of Kuettner, *et al*[31]. Chondrocytes (6×10^6/ml) were encapsulated into alginate beads as described[32]. Chondrocyte beads were maintained in Dulbecco's modified Eagle medium/Nut Mix F12 (DMEM/Ham's F12) supplemented with L-glutamine (2 mM), gentamycin (50 µg/ml), amphotericin B (0.25 µg/ml), and heat inactivated fetal calf serum (FCS) (10%) in a humidified atmosphere of 5% CO_2 at 37°C for one week before experiment.

Study design. A dose ranging study with IL-17 (0–300 ng/ml) and a costimulation with IL-17 (0.3–3 ng/ml) and IL-1ß (0.25–0.5 ng/ml) versus cytokine alone were performed on chondrocytes beads. The contribution of IL-1ß in IL-17 effects was evaluated by reverse transcription-polymerase chain reaction (RT-PCR) using cartilage explants (femoral head caps) stimulated for 10 h with 30 ng/ml IL-17 into DMEM/Ham's F12 containing FCS (2.5%). Since IL-1ß is known to induce its own expression in chondrocytes, this cytokine (2.5 ng/ml) was used as a positive control for the *in vitro* RT-PCR analysis. To evaluate the contribution of NO and reactive oxygen species (ROS) to the inhibitory effect of cytokines on proteoglycan synthesis, L-NMMA (10^{-3} M), a NOS inhibitor known to be active on the constitutive and the inducible isoforms[33], and CuDips (10^{-5} M), a lipid-soluble SOD mimic, were used in the presence of either IL-17 (30 ng/ml) or IL-1ß (2.5 ng/ml). In all experiments, chondrocytes were placed into DMEM/Ham's F12 containing FCS (2.5%) that was changed daily. Supernatants collected after 24 and 48 h incubation were stored at –20°C until nitrite determination. Chondrocyte beads, stimulated for 48 h, were used further for proteoglycan synthesis assay. The formation of nitrotyrosine, used as an index of peroxynitrite generation, was determined in chondrocytes exposed to cytokines for 12 h in phenol-red-free DMEM/Ham-s F12.

In vivo experiments. Animals. Male Wistar rats (150–175 g) were housed under controlled temperature and lighting conditions, with food and water *ad libitum* according to the guidelines for confinement of laboratory animals. Animals were acclimatized to the laboratory environment for one week before experiment. All surgical procedures were performed under anesthesia.

Experiment design. Rats were injected in both knee joints with IL-17 (0.1–1.0 or 10.0 µg/knee) or IL-1ß (1 µg/knee) in a volume of 50 µl sterile saline. Rats injected with saline alone served as controls. In one experiment, body temperature was measured by biotelemetry[34] for a 48 h period postinjection. In a second experiment, rats were killed 10 or 48 h after cytokine administration. At 10 h, SF, synovium, femoral condyles, and tibial plateaus were collected. SF was absorbed by means of small pieces of filter paper (Schleicher & Schuell, Prolabo, France) as described[28]. The

paper pieces were left for 24 h at room temperature in ultrapure water and stored at –20˚C until nitrite/nitrate determination. Synovium were immediately frozen at –80˚C for RNA extraction and RT-PCR analysis, and femoral condyles and tibial plateaus were processed for immunohistochemical analysis. Forty-eight hours after IL-17 or IL-1ß injection, proteoglycan synthesis was measured by *ex vivo* incorporation of $^{35}SO_4^{2-}$ into cartilage of patellae.

Assay for nitrite and nitrite/nitrate. Nitrite and nitrate levels were determined in synovial fluid after nitrate conversion into nitrite with nitrate reductase. Briefly, each sample was incubated at room temperature for 10 min in a final volume of 500 µl with 40 µM NADPH and 0.28 IU/ml nitrate reductase, both prepared in 20 mM Tris-HCl (pH 7.6). Nitrite obtained after enzymatic reduction or nitrite accumulated in culture supernatants was assessed by adding 100 µl DAN reagent (0.05 mg/ml in 0.62 N HCl). The reaction was stopped by 50 µl NaOH (2.8 N), and the formation of 2,3-diaminonaphthotriazole was measured by spectrofluorimetry (excitation 363 nm, emission 426 nm). Standard curves were produced under the same experimental conditions using nitrate solutions (0.5–50 µM) for nitrite and nitrate determination, or nitrite solutions (0.5–50 µM) for nitrite assay only.

Proteoglycan synthesis. 1. In chondrocytes beads. Chondrocytes were pulsed in complete culture medium (2.5% FCS) with 10 µCi/ml $Na_2{}^{35}SO_4$ for 4 h at 37˚C in a 5% CO_2 atmosphere. After 5 washing steps with 0.15 M NaCl, alginate beads were digested overnight at 40˚C in soluene 350. The ^{35}S-proteoglycan was measured by liquid scintillation counting after addition of hionic fluor.

2. In patellar cartilage. Patellae were pulsed for 3 h at 37˚C in a humified 5% CO_2 atmosphere with 0.6 µCi/ml $Na_2{}^{35}SO_4$ in RPMI Hepes 1640 medium supplemented with 2 mM L-glutamine, 100 µg/ml streptomycin, and 100 IU/ml penicillin. After 5 washing steps in saline, patellae were fixed overnight at room temperature in 0.5% cetylpyridinium chloride dissolved in 10% formalin buffer. Patellae were then decalcified for 6 h in 5% formic acid, and the central parts were punched out from the surrounding tissues. The central and peripheral specimens of patellae were processed further as described above for chondrocyte beads.

Immunohistochemical analysis. 1. In chondrocytes. At the end of incubation (12 h), cells were fixed in methanol/acetone (1/1) for 10 min at –20˚C, permeabilized with Triton X-100 (0.5%) for 45 min at room temperature, then immersed for 1 h in immune goat serum. Chondrocytes were incubated subsequently with a rabbit anti-nitrotyrosine polyclonal antibody (1.5 µg/ml) for 2 h at 37˚C, then incubated with biotinylated goat anti-rabbit IgG for 45 min at room temperature. Signal was then amplified with preformed avidin-biotinylated horseradish peroxidase complexes for 45 min at room temperature and staining was developed with 3,3'-diaminobenzidine (0.05% in hydrogen peroxide). Chondrocytes incubated without anti-nitrotyrosine antibody or with immune rabbit serum (1.5 µg/ml) served as controls. All slides were counterstained with eosin.

2. In cartilage. Femoral condyles and tibial plateaus were fixed in 4% paraformaldehyde for at least 24 h, decalcified for 45 min in RDO, post-fixed for 24 h in paraformaldehyde, then embedded in paraffin. Paraffin sections, 5 µm thick, were deparaffinized in toluene, and hydrated in a graded series of ethanol. Cartilage tissues were digested with chondroitinase ABC (0.25 U/ml in PBS at pH 6) for 90 min at 37˚C. Cells were further processed as described above for chondrocytes, except that antibodies against nitrotyrosine and iNOS were used at 10 and 1.3 µg/ml, respectively.

RT-PCR for IL-1ß. Total RNA from synovium (*in vivo*) and cartilage (*in vitro*) was isolated using a commercial phenol-chloroform solution (Trizol). RNA (2 µg) was reverse transcribed for 1.5 h at 37˚C using M-MLV reverse transcriptase (200 U) and hexamer random primers (100 pmol). PCR amplification was then performed with 2 µl of RT products with Taq polymerase (2.5 U) (EurobioTaq). The primers for IL-1ß were selected to amplify a 367 basepair fragment: forward 5'-TGA-AAG-CTC-TCC-ACC-TCA-ATG-G-3'; reverse 5'-TCC-ATG-GTG-AAG-TCA-ACT-ATG-TCC-3' (MWG, Biotech). The conditions for amplification were 45 s at 94˚C, 45 s at 62˚C, and 45 s at 72˚C for 27 cycles. As an internal control, the same RNA preparation was also subjected to RT-PCR analysis for the gene L27, coding for a ribosomal protein, using specific primers that amplified a 225 basepair fragment: forward 5'TCC-TGG-CTG-GAC-GCT-ACT-C-3'; reverse 5'-CCA-CAG-AGT-ACC-TTG-TGG-GC-3'. The PCR products were analyzed by 0.2 µg/ml ethidium bromide staining in 1.5% agarose gel.

Statistics. Data are expressed as mean ± SD, except for *in vivo* experiments (mean ± SEM). Each value is the mean of at least 5 samples analyzed from 3 independent *in vitro* experiments. *In vivo* data were obtained from 6 to 8 samples collected from different animals. The results were analyzed by ANOVA followed by a Fisher test. P values < 0.05 were considered significant.

RESULTS

Dose ranging study with IL-17. In chondrocyte beads, IL-17 decreased proteoglycan synthesis while increasing nitrite level in culture supernatants. Both effects were dose related, but the stimulating effect of IL-17 on NO production plateaued from 3 ng/ml (25 µM), while its inhibitory potency on anabolism increased up to 300 ng/ml (–45%) (Figure 1). As *in vitro*, the intraarticular administration of IL-17 decreased proteoglycan synthesis in a dose dependent manner in patellar cartilage (Figure 2A) while stimulating NO production in SF (Figure 2B).

Comparison of IL-17 and IL-1ß potencies. The intraarticular administration of IL-17 in rats failed to generate any febrile response, even at the highest dose used (10 µg/knee) (Figure 3). In contrast, IL-1ß (1 µg/knee) elicited fever starting at 3 h postinjection, with a maximum increase at 6 h and a return to basal level by 10 h. For proteoglycan synthesis, IL-17

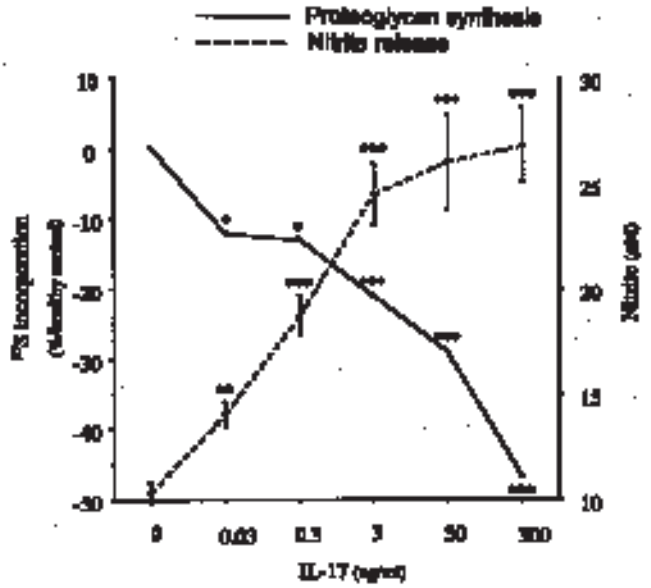

Figure 1. Effect of increasing concentrations of IL-17 on proteoglycan synthesis and nitrite release in chondrocytes encapsulated in alginate beads. Nitrite level as an index of NO production and proteoglycan synthesis determined by ^{35}S incorporation were measured after 48 h incubation with IL-17. Data are mean ± SD or means percentage (n = 5). Comparisons by Fisher's exact test between IL-17 stimulated chondrocytes and controls: *p < 0.05, **p < 0.001, ***p < 0.0001.

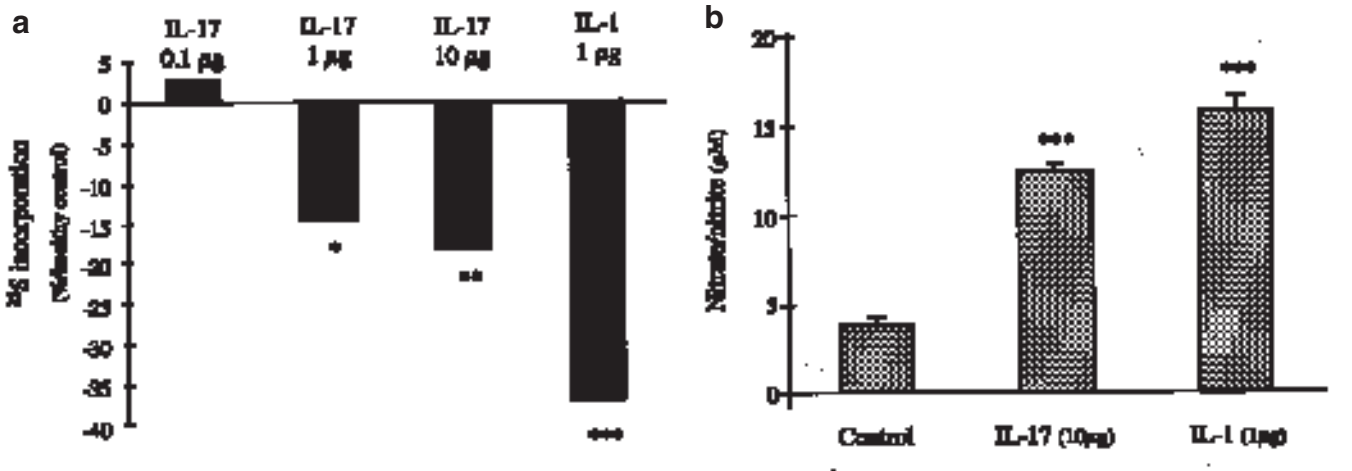

Figure 2. In vivo effect of IL-17 or IL-1ß on proteoglycan synthesis in rat patellar cartilage (a) and on NO production in SF (b); 48 and 10 h, respectively, after a single IA injection of IL-17 (0.1, 1.0, or 10.0 µg/knee) or IL-1ß (1 µg/knee), proteoglycan synthesis was measured by *ex vivo* ^{35}S labeling for 3 h in the central part of patellae, and nitrate and nitrite concentration was determined, as an index of NO production, in SF. Values are expressed as mean percentages (n = 8) (a); or mean ± SEM (n = 8–12) (b). Comparisons by Fisher's exact test between IL-17 or IL-1ß injected rats and healthy controls injected with sterile saline: *p < 0.05, **p < 0.001, ***p < 0.0001.

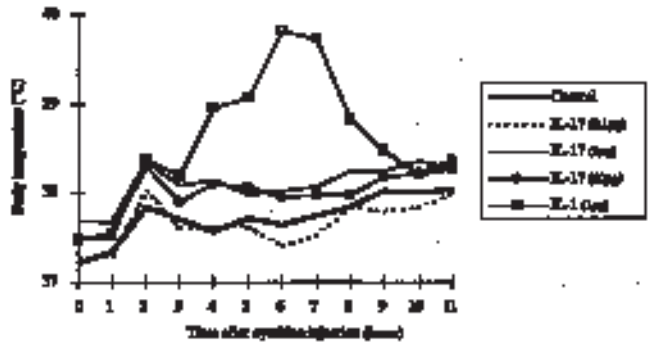

Figure 3. Kinetics of the febrile response (nocturnal temperature) after a single IA injection of IL-17 (0.1, 1.0, 10.0 µg/knee) or IL-1ß (1.0 µg/knee). Values are recorded each hour (n = 4).

was found to be less potent than IL-1ß, despite its similar stimulatory effect on nitrite release. Indeed, the NO overproduction induced by 0.3 ng/ml of IL-17 led to very low inhibition of proteoglycan synthesis. In addition, a 12-fold higher concentration of IL-17 (3 ng/ml) over IL-1ß (0.25 ng/ml) was required to induce a comparable suppressive effect (20% inhibition) (Figures 4A, 4B). This different inhibitory potency on proteoglycan synthesis was more pronounced *in vivo*, since the local injection of a 10-fold excess of IL-17 (10 µg) over IL-1ß (1 µg) was observed to be 2-fold less potent in reducing the *ex vivo* sulfate incorporation into patellar cartilage (Figure 2A). However, under these experimental conditions, IL-17 and IL-1ß induced NO production to the same level in SF (Figure 2B).

To search for synergy between cytokines, rat chondrocyte beads were incubated concomitantly with low concentrations of IL-17 (0.3 and 3.0 ng/ml) and IL-1ß (0.25 and 0.5 ng/ml). The combination of both cytokines resulted in an additive effect on inhibition of proteoglycan synthesis,

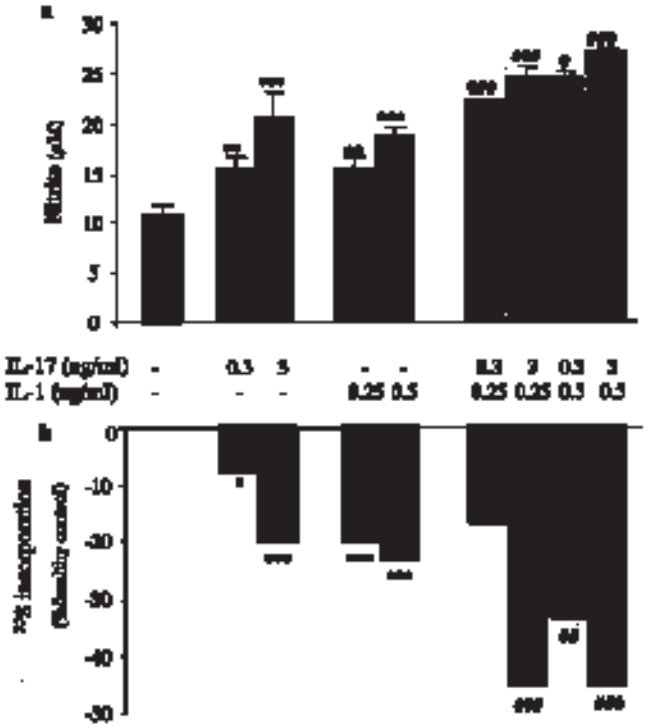

Figure 4. Effect of IL-17 (0.3 and 3.0 ng/ml) and/or IL-1ß (0.25 and 0.5 ng/ml) on nitrite release (a) and proteoglycan synthesis (b) in chondrocytes encapsulated in alginate beads. After 48 h incubation, chondrocytes beads were incubated with $Na_2{}^{35}SO_4$ for proteoglycan synthesis and culture supernatants were examined for nitrite concentration. Data are mean ± SD (a) or mean percentages (b) (n = 5). Comparisons by Fisher's exact test between IL-17 or IL-1ß stimulated chondrocytes and controls (*), or between IL-17 combined with IL-1ß and IL-1ß alone for proteoglycan synthesis, or IL-17 alone for NO production (#): */#p < 0.05, **/##p < 0.001, ***/###p < 0.0001.

except at the lowest concentration used (Figure 4B). In addition, the combination of IL-1ß and IL-17 produced more NO than was obtained with cytokines alone (Figure 4A).

Possible contribution of IL-1ß to effects of IL-17. RT-PCR analysis showed that the intraarticular injection of IL-17 stimulated the expression of IL-1ß mRNA in rat synovium (Figure 5A). However, the *in vitro* incubation of femoral head caps with IL-17 failed to induce the expression of IL-1ß mRNA (Figure 5B). Consistent with this finding, the suppressive effect of IL-17 on proteoglycan synthesis was not modified when chondrocytes were coincubated with an antibody able to neutralize rat IL-1ß (data not shown).

Peroxynitrite formation in response to cytokines. No immunoreactivity for nitrotyrosine was detected in controls and chondrocytes exposed to 3 or 30 ng/ml IL-17 (Figures 6A, 6B), whereas a limited number of cells stained positive at 300 ng/ml (data not shown). In contrast, extensive immunoreactivity for nitrotyrosine was observed in the cytoplasm of chondrocytes stimulated with IL-1ß (Figure 6C). Such immunostaining was totally prevented with either L-NMMA or CuDips (Figures 6D, 6E). The formation of nitrotyrosine and the expression of iNOS were also examined in articular cartilage removed 10 h after cytokine injection (IL-17, 10 µg/knee or IL-1ß, 1 µg/knee). Both cytokines induced the expression of iNOS in femoral condyles (Figures 7B, 7C) and tibial plateaus. However, very low immunostaining for nitrotyrosine was observed with IL-17 (1.6-fold compared to healthy control) (Figures 7E and 7D, respectively). Conversely, the number of positive cells for nitrotyrosine was about 5-fold greater in cartilage from rats injected with IL-1ß than in control animals (Figures 7F and 7D, respectively), and immunodetection for nitrotyrosine was shown to be located mainly in the middle zone of cartilage, as well as iNOS staining (Figure 7F).

Effect of L-NMMA and CuDips on cytokine effects. For both cytokines, L-NMMA was able to abolish and CuDips to slightly increase the concentrations of nitrite in culture supernatant (Table 1). The IL-17 mediated inhibition of proteoglycan synthesis was partly restored by L-NMMA (25%), but remained unchanged with CuDips. In contrast, the suppressive effect of IL-1ß on cartilage anabolism was fully reversed by both L-NMMA and CuDips.

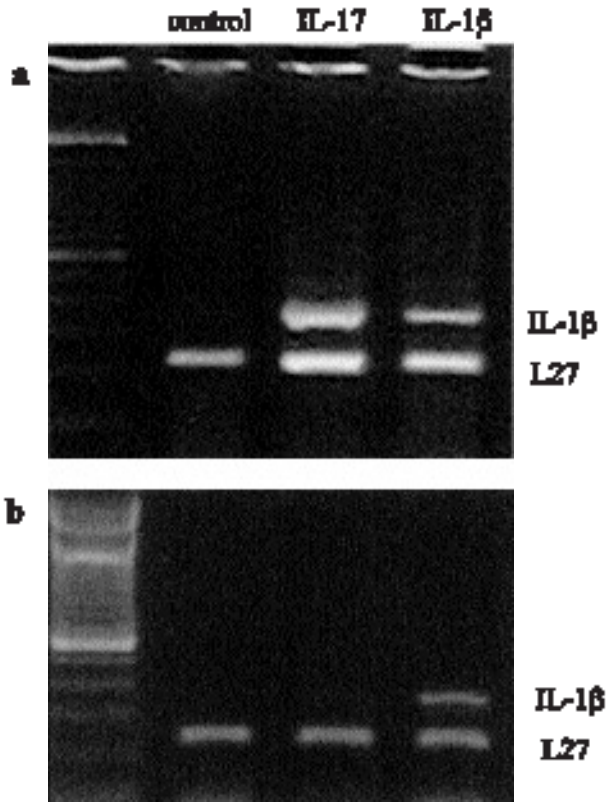

Figure 5. Expression of IL-1ß mRNA in synovium from rats injected with IL-17 or IL-1ß (a) and in rat cartilage explant stimulated with IL-17 or IL-1ß (b): 10 h after injection of IL-17 (10 µg) or IL-1ß (1.0 µg), or stimulation of cartilage with IL-17 (30 ng/ml) or IL-1ß (2.5 ng/ml), total RNA from synovium (a) and femoral head caps (b) was subjected to RT-PCR analysis (27 cycles) using specific rat IL-1ß primers that generated a 367 bp fragment. PCR amplification of L27 (generating a 225 bp fragment) was performed on the same RNA preparation to serve as an internal control for the relative amounts and integrity of template. Data are representatives of 3 or 4 experiments on different animals.

DISCUSSION

In contrast to its widely accepted effects on synoviocytes, the effect of IL-17 on cartilage anabolism remains somewhat controversial. While Lubberts, *et al*[30] demonstrated *in vitro* that IL-17 decreased proteoglycan synthesis in mouse cartilage, Dudler, *et al*[29] reported that ^{35}S incorporation into patellar cartilage was unaffected by the cytokine even after injection into mouse knee joints. We have shown for the first time that IL-17 induced in rats a suppressive effect on cartilage anabolism through a pathway distinct from that used by IL-1ß.

Regulatory loops between proinflammatory cytokines in SF are a key feature of RA pathogenesis. Since IL-17 was able to induce IL-1ß in synovial cells[16], chondrocytes[10], and macrophages[9], one may ask if the biological response of chondrocytes to IL-17 was mediated by IL-1ß. The direct action of IL-17 on chondrocytes was described for NO production in human cartilage using IL-1Ra[20] and soluble IL-1 receptor[19]. In our experimental conditions, mRNA for IL-1ß was detected in femoral head caps stimulated with IL-1ß, but not with IL-17. In contrast, stimulation of normal human chondrocyte monolayers with IL-17 induced upregulation of IL-1ß gene[10]. This discrepancy between human and rat chondrocytes may be explained by species specificity or by the influence of the extracellular matrix on the response of chondrocytes to IL-17. In any case, the data from our study indicate that inhibition of proteoglycan synthesis occurred through an IL-1ß-independent pathway. As a transient expression of IL-1ß mRNA was observed in the synovium of IL-17 injected rats, a possible contribution

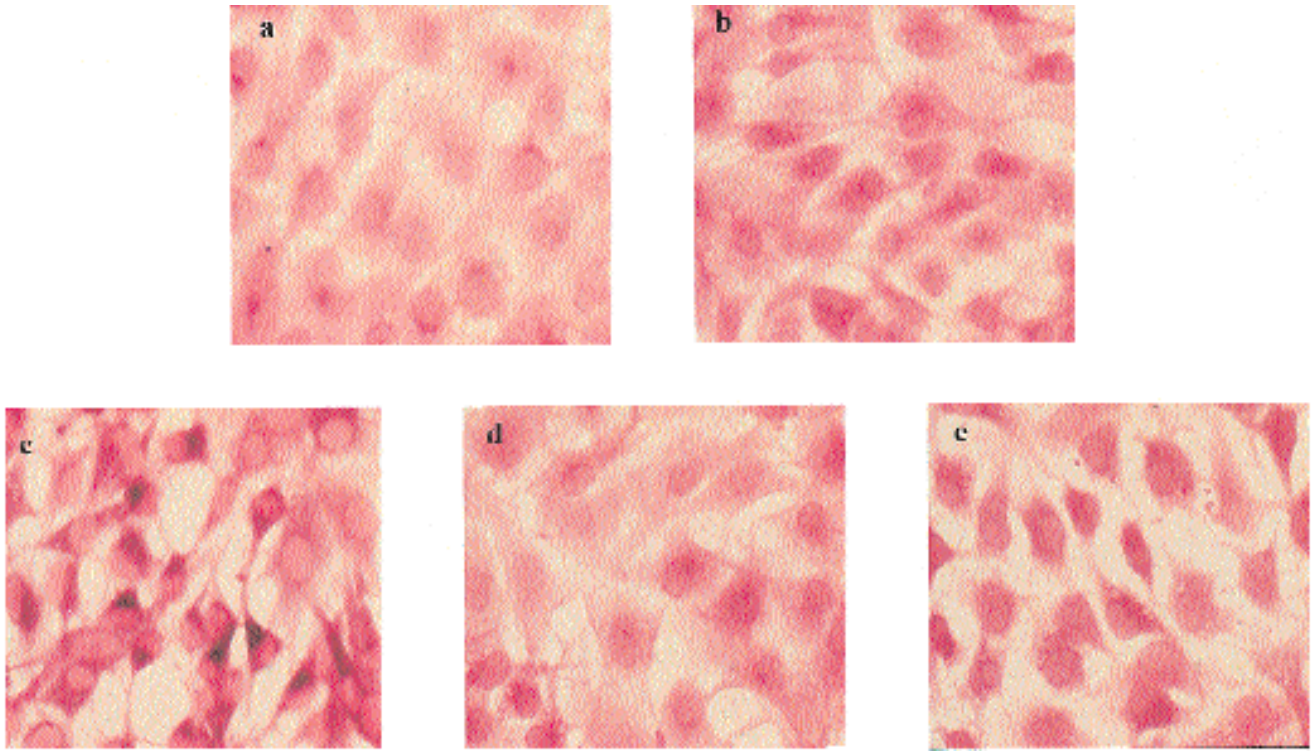

Figure 6. Representative immunostaining for nitrotyrosine in chondrocytes incubated in culture medium alone (n = 6–10) (a), or stimulated 12 h with: IL-17 (30 ng/ml) (b), IL-1ß (2.5 ng/ml) (c), IL-1ß (2.5 ng/ml) and L-NMMA (10^{-3} M) (d), or IL-1ß (2.5 ng/ml) and CuDips (10^{-5} M) (e). Fixed cells were incubated with a rabbit polyclonal antibody specific for nitrotyrosine (1.5 µg/ml), then incubated with a biotinylated goat anti-rabbit IgG. Staining was carried out using an amplification system with avidin-biotinylated horseradish peroxidase complexes and 3,3'-diaminobenzidine. Cells were counterstained with eosin. All representative sections are at original magnification ×400.

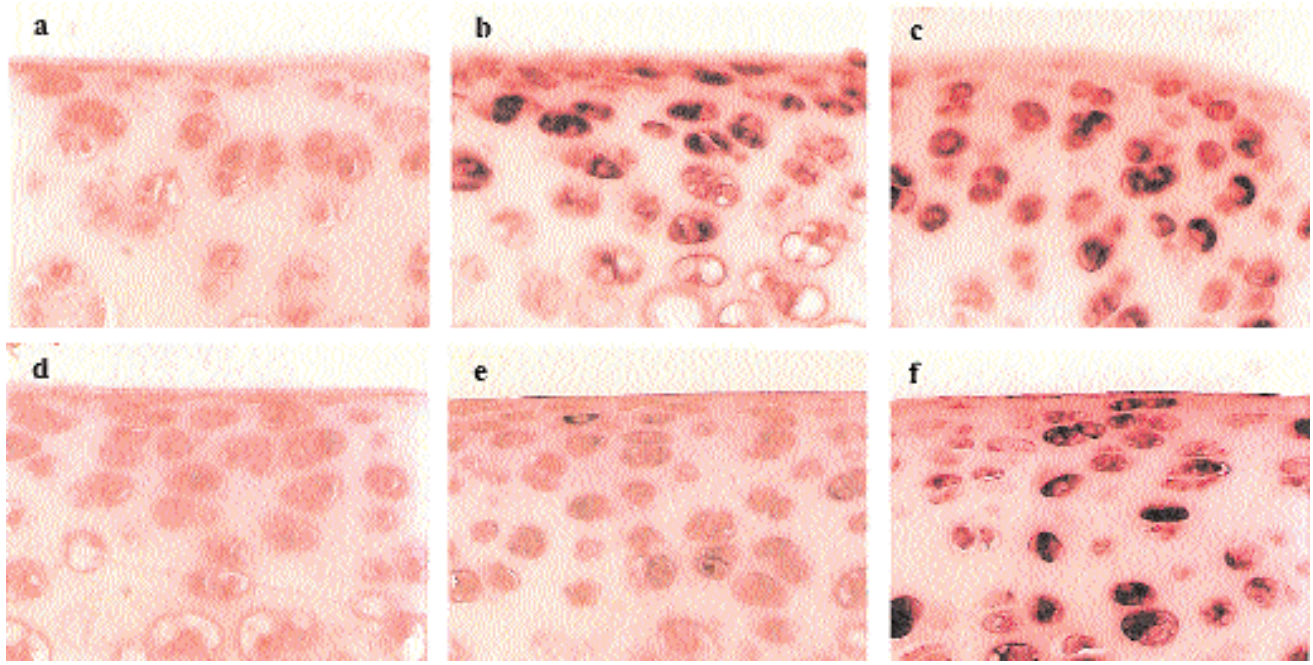

Figure 7. Representative sections (n = 8) of immunostaining for nitrotyrosine and iNOS in cartilage samples collected 10 h after IA injection of IL-1ß (1.0 µg/knee) or IL-17 (10.0 µg/knee). (a) controls for iNOS staining; (b) iNOS staining in response to IL-17; (c) iNOS staining in response to IL-1ß; (d) controls for nitrotyrosine staining; (e) nitrotyrosine staining in response to IL-17; (f) nitrotyrosine in response to IL-1ß. Paraffin sections of femoral condyles were incubated with rabbit polyclonal antibodies against nitrotyrosine (10.0 µg/ml) or iNOS (1.3 µg/ml), then incubated with a biotinylated goat anti-rabbit IgG. Staining was carried out by an amplification system with avidin-biotinylated horseradish peroxidase complexes and 3,3'-diaminobenzidine. Slides were counterstained with eosin. All representative sections are at original magnification ×400.

Table 1. Effect of L-NMMA and CuDips on IL–1 or IL–17 mediated inhibition of proteoglycan synthesis and NO production in rat chondrocyte beads. Proteoglycan synthesis was determined by ^{35}S incorporation and nitrite level was measured in culture supernatants. Chondrocytes encapsulated in alginate beads were incubated 48h with cytokine in the presence or not of L–NMMA (10^{-3}M) or CuDips (10^{-5} M). Comparisons by Fisher's exact test between cytokine alone and with L–NMMA or CuDips. Data are mean percentages.

	Proteoglycan Synthesis, %	Nitrite, µM
Control	100	7.7 ± 0.5
IL–1, 2.5 ng/ml	76	18.5 ± 1.7
IL–1 + L–NMMA, 10^{-3}M	103**	6.1 ± 1.3**
IL–1 + CuDips, 10^{-5} M	95**	22.6 ± 2*
IL–17, 30 ng/ml	78	16.7 ± 0.7
IL–17 + L–NMMA, 10^{-3} M	83*	6.8 ± 0.3 **
IL–17 + CuDips, 10^{-5} M	76	19 ± 1.8*

* $p < 0.05$; ** $p < 0.0001$.

of IL-1ß cannot be totally ruled out *in vivo*. However, this proposal may have little pathological relevance since an anti-IL-1α,ß antibody failed to reduce inflammation and joint damage after the local overexpression of IL-17 in the collagen induced arthritis model[16].

That IL-17 and IL-1β acted differently on cartilage is further supported by the difference of the febrile response between IL-17 and IL-1ß and by the costimulation experiment showing that their combination at low concentrations was more efficient than either cytokine alone. In contrast, no enhancement of NO production was observed when human osteoarthritis (OA) chondrocytes were incubated with IL-1ß and IL-17[20]. Such discrepancy may be explained by the pathological status of OA chondrocytes, which express a higher level of IL-1 receptors[35] and may respond extensively to their stimulation. Therefore, the NO pathway may be activated maximally by IL-1ß in OA chondrocytes so that no more activation would be provided by IL-17[20]. Although IL-1ß is a well known inhibitor of glycosaminoglycan synthesis in cartilage[36], the additive effect of low concentrations of IL-1ß and IL-17, which was also reported for rheumatoid synovium[8] and mouse cartilage exposed to high concentrations of both cytokines[30], may have pathophysiological relevance in the context of cartilage damage.

In our experimental conditions, IL-17 stimulated NO production and suppressed proteoglycan synthesis in a dose related manner, as for IL-1ß[32], suggesting that the inhibitory effect of IL-17 on cartilage anabolism could be mediated by NO. However, the contribution of NO to the decrease in proteoglycan synthesis was different depending on the cytokine used. L-NMMA inhibited NO synthesis and restored proteoglycan synthesis fully in IL-1ß stimulated chondrocytes but only slightly in cells treated with IL-17. These data indicate clearly that NO contributed weakly to the IL-17 induced loss of cartilage anabolism in rats. This is in contrast with studies in mice that showed that IL-17 has no effect on cartilage explants incubated with an iNOS inhibitor (L-N-[1-iminoethyl]ornithine or L-NIO) or obtained from iNOS knockout mice[30]. One may ask whether this apparent discrepancy between our data and the findings of Lubberts, *et al* can be explained by differences in the selectivity of inhibitors for iNOS or in the culture system used. However, L-NIO and L-NMMA were shown to restore cartilage anabolism in rat chondrocytes exposed to IL-1ß in 3 different culture systems[37]. A possible explanation of the stronger contribution of NO to the effects of IL-17 in mice than in rats may be due to species specificity. Indeed, such variable involvement of NO in the inhibition of proteoglycan synthesis has been suggested for IL-1ß[38].

In addition, although intraarticular injection of IL-17 (10 µg/knee) or IL-1ß (1 µg/knee) provided a similar release of NO in SF, IL-17 was shown to be less potent than IL-1ß in reducing *ex vivo* ^{35}S incorporation into rat patellar cartilage, as was the case in mice[30]. In addition, a low concentration of IL-17 (0.3 ng/ml) increased NO production over the control level, while it marginally inhibited proteoglycan synthesis in chondrocyte beads. These data strengthen the concept that substantial production of NO may not be predictive of any inhibitory effect on cartilage anabolism and that other mediators are involved to provoke chondrocyte dysfunction.

Among mechanisms explaining the variable contribution of NO to the inhibitory potency of cytokines in proteoglycan synthesis, we investigated the involvement of superoxide anion. This factor was shown to participate in IL-1ß mediated cartilage injury[39], and it can combine with NO into peroxynitrite, a strong promoter of molecular and tissue oxidative damage[40]. We observed that 3-nitrotyrosine, an established biological marker of peroxynitrite generation[41], was detected in isolated chondrocytes cultured with IL-1ß, and mainly in the middle zone of cartilage from IL-1ß injected rats. The absence of immunoreactivity for nitrotyrosine in chondrocytes incubated with L-NMMA or CuDips, together with its colocalization with the staining for iNOS in cartilage, indicated that tyrosine nitrosylation after IL-1ß stimulation occurred through peroxynitrite generation. In addition, the inhibition of NO production by L-NMMA or of superoxide production by CuDips fully reversed the suppressive effect of IL-1ß on proteoglycan synthesis. These data provide further evidence for the pivotal role of peroxynitrite in the metabolic dysfunction of chondrocyte under IL-1ß stimulation, as suggested in bovine cartilage[42]. In contrast, despite high nitrite levels after IL-17 stimulation or injection, no or little immunostaining for nitrotyrosine was noted *in vitro* and *in vivo*. Further, L-NMMA slightly restored the IL-17 mediated inhibition of proteoglycan synthesis, whereas CuDips was ineffective. These agents suggest that the distinct suppressive effect of IL-1ß and IL-17 on cartilage anabolism could be explained, at least in part, by their different ability to generate peroxynitrite. Thus, superoxide anion rather than NO may be the limiting

factor for inhibition of proteoglycan synthesis from a kinetic or a spatial point of view. Further evidence that superoxide may contribute to the deleterious effect of NO during RA is indicated by the more severe cartilage lesions induced by the local overexpression of IL-17 in mice with collagen induced arthritis than in naive mice[16]. In this case, one may suggest that peroxynitrite formation could be favored in arthritic animals due to the production of superoxide by infiltrating polymorphonuclear cells in the vicinity of chondrocytes overproducing NO in response to IL-17. A recent study supports the key role of superoxide as a regulator of NO effects on chondrocyte dysfunction — combination of NO with other ROS was shown to induce chondrocyte death, while NO was not cytotoxic by itself[43]. Thus, if cartilage anabolism depends on peroxynitrite formation, one can expect that any change in NADPH oxidase activity will modulate the deleterious properties of NO-producing cytokines. Indeed, chondrocytes can spontaneously produce superoxide at a low rate mediated by a NADPH-oxidase complex, whereas higher output was reported in the presence of IL-1ß[44]. However, the inability of IL-17 to stimulate this enzymatic activity remains to be confirmed.

In conclusion, we found that IL-17 is a suppressive mediator of rat cartilage anabolism independent from IL-1ß, although an additional effect of IL-1ß cannot be ruled out *in vivo* since IL-17 stimulated its expression in synovium. We also showed for the first time that NO production is not sufficient to account for the inhibition of proteoglycan synthesis induced by IL-17, and that the greater suppressive effect of IL-1ß compared to IL-17 may be explained by its ability to generate peroxynitrite. Thus, this study supports a pivotal role of peroxynitrite in cytokine mediated decrease in proteoglycan synthesis in rheumatic diseases. In this case, since IL-17 is a NO-producing cytokine with additive effects when combined with low concentrations of IL-1, it may play a key role in cartilage destruction during RA for which infiltrating cells produce high levels of superoxide and proinflammatory cytokines. In addition, combined therapy with NOS inhibitors and antioxidants or the use of peroxynitrite decomposition catalyst may be promising strategies to protect cartilage against cytokine mediated injury.

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