# EFFECT OF INTERLEUKIN 1 AND LEUKAEMIA INHIBITORY FACTOR ON CHONDROCYTE METABOLISM IN ARTICULAR CARTILAGE FROM NORMAL AND INTERLEUKIN-6-DEFICIENT MICE: ROLE OF NITRIC OXIDE AND IL-6 IN THE SUPPRESSION OF PROTEOGLYCAN SYNTHESIS

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**We studied the role of IL-6 and nitric oxide (NO) in IL-1 and leukaemia inhibitory factor (LIF) induced suppression of proteoglycan synthesis. Cartilage explants of patellae and femoral heads were incubated with IL-1 or LIF. Conditioned media were analysed for IL-6 activity (B9-assay) and NO content (Griess). Proteoglycan synthesis was assessed using $[^{35}S]$sulfate incorporation. IL-1 dose dependently induced IL-6 synthesis and neutralizing IL-6 with antibodies did not reduce proteoglycan synthesis suppression, neither in explants nor in isolated chondrocytes. IL-6 independence was confirmed using cartilage from IL-6 deficient mice. IL-1 significantly increased NO release in normal and IL-6 deficient chondrocytes and addition of the NO synthase inhibitor, $N^G$-monomethyl-L-arginine markedly alleviated proteoglycan synthesis suppression. LIF also induced proteoglycan synthesis suppression in cartilage from normal and IL-6 deficient mice, but the suppression was neither accompanied by nor dependent on NO release. Furthermore, proteoglycan synthesis suppression during experimental arthritis was similar in both normal and IL-6 deficient mice. We concluded that IL-6 is not a necessary cofactor in IL-1 and LIF induced suppression of proteoglycan synthesis. Furthermore, only the IL-1 induced suppression was mediated by NO, suggesting that inhibition of proteoglycan synthesis may occur through different pathways.**

Cytokines are important mediators in the pathogenesis of rheumatoid arthritis (RA)[1,2] and are produced in the inflamed joint.[3,4] It has been claimed that TNF-α is driving most of the IL-1 production in the inflamed synovia of RA patients[5] suggesting a hierarchy in the dynamic interaction of cytokines. The cascade of TNF → IL-1 → LIF → IL-6 was postulated to be involved in the pathogenesis of RA.[6]

TNF-α and IL-1 share many of their biological activities,[7] and the therapeutic intervention of RA is recently directed towards antagonizing and modifying the action of these proximal cytokines.[8] Approaches with anti-TNF antibody and IL-1 receptor antagonist (IL-1ra) showed efficacy in animal models of arthritis[9–14] and recent clinical trials showed efficacy of anti-TNF treatment in RA patients.[15–17] Whether the latter treatment also prevents cartilage destruction has yet to be determined and a better understanding of mediators downstream the cytokine cascade may provide more optimal therapeutic targets.

Careful analysis of the mechanism of cartilage destruction in murine arthritis showed a pivotal role of IL-1 in chondrocyte proteoglycan synthesis inhibition.[14,18] Moreover, anti-IL-1 treatment not only abolished this suppression but also reduced the net (overall) depletion of cartilage matrix.[14,19,20] An essential costimulatory role for IL-6 was claimed in the IL-1 induced suppression of proteoglycan synthesis, using explants of human articular cartilage.[21,22] Although this dependency is not yet confirmed by other groups, it is generally accepted that IL-1 is a potent inducer of IL-6 production in cells of articular tissues, including synovial fibroblasts and chondrocytes.[23–25] Interestingly, a cytokine belonging to a family of IL-6-related proteins, leukaemia inhibitory factor (LIF), is also

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capable of suppressing chondrocyte proteoglycan synthesis.[26] LIF is a single-chain, polypeptide cytokine of approximately 20 kDa inducible in synoviocytes and articular chondrocytes by IL-1.[27,28] On chondrocytes, LIF was able to induce IL-1 and IL-6 synthesis.[27,28] However, LIF induced suppression of proteoglycan synthesis was IL-1 independent.[25]

Recent studies suggested an essential role of nitric-oxide (NO) in IL-1-induced suppression of chondrocyte proteoglycan synthesis.[29,30] Suppression could be prevented by L-arginine analogues, which are potent inhibitors of NO synthase.[29,30] Whether NO is also involved in the action of LIF has yet to be determined.

In the present study we examined the role of IL-6 and NO in IL-1 and LIF induced suppression of chondrocyte proteoglycan synthesis, both in vitro and in vivo. Since anti-IL-6 antibodies may have difficulty in penetrating intact cartilage, in vitro studies were performed with murine articular cartilage explants as well as isolated chondrocytes. Moreover, comparable experiments were done in IL-6-deficient mice. It was shown that both IL-1 and LIF action on chondrocyte proteoglycan synthesis were IL-6 independent, in vitro and in vivo. Furthermore, we confirmed the mediating role of NO in IL-1 induced suppression, but clearly showed that the LIF effect was NO independent. Our data suggest that IL-6 is not a feasible downstream target to fine tune IL-1 directed therapy, to prevent cartilage damage. Furthermore, the strategy of NO blocking will affect IL-1 action, but not that of LIF.

## RESULTS

### *Effect of IGF-1 and IL-1 on chondrocyte proteoglycan synthesis*

In order to maintain the in vivo rate of proteoglycan synthesis during culture, patellae were incubated in the presence of 0.25 μg/ml insulin-like growth factor (IGF)-1.[36] Chondrocytes in patellae from IL-6$^{+/+}$ and IL-6$^{o/o}$ mice showed an identical IGF-1 response in vitro (Fig. 1). For this, cartilage was incubated in the presence of 0.25 μg/ml IGF-1 in the next experiments.

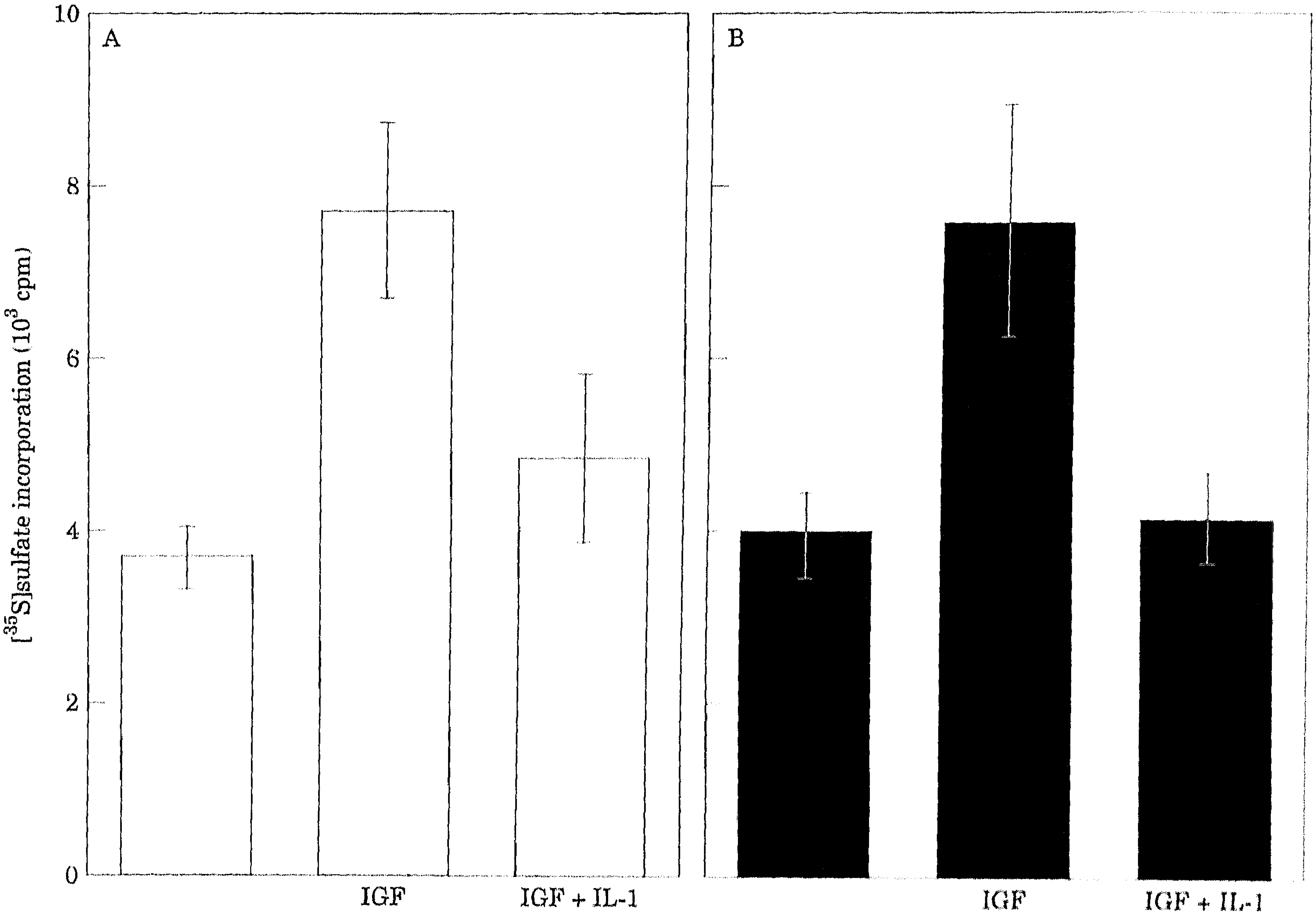

**Figure 1. IL-1-induced suppression of proteoglycan synthesis in (A) IL-6$^{+/+}$ and IL-6$^{o/o}$ mice.**

Patellae were incubated for 24 h in culture medium ($n = 6/2$ ml) followed by a 3 h pulse with [$^{35}$S]sulfate (20 μCi/ml). [$^{35}$S]sulfate incorporation is a measure of chondrocyte proteoglycan synthesis. The effect of human recombinant IGF-1 (0.25 μg/ml) and murine recombinant IL-1α (10 ng/ml) on proteoglycan synthesis was assessed. IL-6 concentrations of conditioned media of patellae from the IL-6-deficient mice (C57Bl/6x129Sv, IL-6$^{o/o}$) were below the detection limit of the B9-assay (< 0.2 pg/ml). Statistical significance was tested by using the student's *t*-test. $*P < 0.05$.

**TABLE 1. Effect of exogenous IL-1 on chondrocyte synthesis of proteoglycans and IL-6.**

| | | Patellar cartilage explants* | | Femoral head cartilage explants† | |
|---|---|---|---|---|---|
| Mice | IL-1‡ dose (ng/ml) | [$^{35}$S]Sulfate§ incorporation (cpm ± SD) | IL-6¶ concentration (ng/ml) | [$^{35}$S]sulfate incorporation (cpm ± SD) | IL-6 concentration (ng/ml) |
| C57Bl/6 (IL-6$^{+/+}$) | 0 | 2365 ± 259 | 149 | 9758 ± 1877 | 0.37 |
| | 0.1 | 1552 ± 131** | 199 | 7650 ± 2240** | 2.48 |
| | 1.0 | 1157 ± 239** | 299 | 5871 ± 642** | 4.98 |

*Patallae ($n = 6$) with a minimal amount of adjacent soft tissue.

†Femoral head cartilage explants ($n = 6$).

‡Medium changed after 24 h (200 μl of RPMI + 0.25 μg/ml IGF-1 per explant).

§Proteoglycan synthesis (mean value ± SD) as measured by [$^{35}$S]sulfate incorporation in the last 3 h of the 48-h incubation period.

¶Total IL-6 produced during the 48-h incubation period as measured with the B9-assay as described in Materials and Methods. Value of pooled samples.

Control group and IL-1-treated group were tested using the Student's $t$-test, values statistically significant: $^{**}P < 0.05$.

### *Role of endogenous IL-6 in the IL-1-induced suppression of proteoglycan synthesis*

Murine recombinant IL-1α induced a dose-related inhibition of chondrocyte proteoglycan synthesis in patellae and femoral head cartilage explants of mice (Table 1). In the culture media, IL-6 levels increased using higher IL-1 concentrations. Next, we studied the role of endogenous IL-6 by neutralizing IL-6 activity with anti-IL-6 antibodies during culture. For this, explants of femoral head cartilage were used as the IL-6 levels in the culture media was less than 2% of the values found in cultures of patellae. Addition of 10 μg/ml rat-anti mouse IL-6 monoclonal antibodies completely neutralized IL-6 activity during culture without changing normal proteoglycan synthesis rate and the IL-1 suppression (Table 2). Since the size of antibodies (150 kDa) may hamper penetration into the cartilage matrix, IL-6 neutralization studies were also performed on isolated chondrocytes. IL-1 significantly suppressed proteoglycan synthesis of isolated murine chondrocytes either in the absence (−41 ± 7%, mean of four experiments) or presence of anti-IL-6 monoclonal antibodies (−47 ± 9%, mean of four experiments) (Table 2). This argues against a major role of endogenous IL-6 in the IL-1-induced suppression of proteoglycan synthesis in IL-6$^{+/+}$ mice. Murine recombinant IL-1α (10 ng/ml) induced a pronounced suppression of proteoglycan synthesis in patellae of IL-6$^{0/0}$ mice, which was at least as high as that found in IL-6$^{+/+}$ littermates (Fig. 1). This suggest that IL-6 was not necessary for a full-blown IL-1 effect on proteoglycan synthesis.

### *Effect of exogenous IL-6 on normal and IL-1-affected proteoglycan—and NO synthesis in IL-6 deficient mice and their normal littermates*

IL-1 evoked increasing levels of nitrite, a stable end product of NO, in the culture medium of cartilage explants obtained from either normal or IL-6 deficient mice (Table 3). This showed that endogenous IL-6 was not an essential intermediate in the IL-1-induced NO release. Incubating patellae or femoral head cartilage explants with murine recombinant IL-6 did not suppress chondrocyte proteoglycan synthesis nor

**TABLE 2. Effect of IL-6 neutralization on IL-1-induced suppression of proteoglycan synthesis.**

| | | | Femoral head cartilage explants* | | Femoral head chondrocytes† | |
|---|---|---|---|---|---|---|
| | Culture medium† | | | | | |
| Mice | IL-1 | AIL-6Ab | [$^{35}$S]sulfate§ incorporation (cpm ± SD) | IL-6¶ concentration (pg/ml) | [$^{35}$S]sulfate incorporation (cpm ± SD) | IL-6 concentration (pg/ml) |
| C57Bl/6 (IL-6$^{+/+}$) | − | − | 8588 ± 816 | 412 | 1032 ± 184 | 480 |
| | − | + | 9220 ± 1104 | < 1 | 1160 ± 128 | < 1 |
| | + | − | 6468 ± 396 | 2400 | 700 ± 168 | 960 |
| | + | + | 5848 ± 892 | < 1 | 608 ± 100 | < 1 |

*Femoral head cartilage explants ($n = 6$), 1 explants per 200 μl medium supplemented with 0.25 μg/ml IGF-1. Medium changed after 24 h, and the total incubation period was 48 h.

†Isolated femoral head cartilage chondrocytes, 44 000 cells in 200 μl medium supplemented with 5% FCS, per flat-bottom well of a microtitre plate.

‡The IL-1α concentrations were, respectively, 10 ng/ml and 1 ng/ml in the explant and chondrocyte cultures. The rat-anti-IL-6 monoclonal antibody concentrations was 10 μg/ml in both explant and chondrocyte culture, enough to block 70 ng of IL-6 in the B9-assay.

§Proteoglycan synthesis (mean value ± SD) as measured by [$^{35}$S]sulfate (20 μCi/ml) uptake during the last 3 h of incubation. In chondrocyte culture, values were CPC-precipitated [$^{35}$S]sulfate incorporated proteoglycans.

¶Total IL-6 produced during the 48-h incubation period as measured with the B9-assay as decribed in Materials and Methods. Value of pooled samples.

**TABLE 3. Effect of exogenous IL-6 on chondrocyte synthesis of proteoglycans and NO.**

| | | Patellar cartilage explants | | Femoral head cartilage explants† | |
|---|---|---|---|---|---|
| Mice | Culture medium† | [$^{35}$S]sulfate‡ incorporation (cpm ± SD) | Nitrite§ concentration (μM) | [$^{35}$S]sulfate incorporation (cpm ± SD) | Nitrite concentration (μM) |
| C57Bl/6 (IL-6$^{+/+}$) | — | 1753 ± 148 | 16.4 ± 4.6 | 9836 ± 1422 | 9.5 ± 2.2 |
| | IL-1 | 832 ± 301 | 51.9 ± 16.5 | 5436 ± 874 | 28.1 ± 3.0 |
| | IL-6 | 1649 ± 74 | 9.5 ± 2.2 | 8335 ± 1413 | 3.3 ± 2.9 |
| | IL-1 + IL-6 | 759 ± 22 | 35.4 ± 2.3 | 4899 ± 985 | 22.4 ± 3.4** |
| C57Bl/6x129Sv (IL-6$^{0/0}$) | — | 1811 ± 457 | 7.8 ± 2.0 | 9262 ± 891 | 1.4 ± 2.0 |
| | IL-1 | 759 ± 94 | 50.2 ± 4.7 | 5839 ± 1302 | 29.1 ± 2.2 |
| | IL-6 | 1555 ± 141 | 8.5 ± 1.9 | 10133 ± 2546 | 4.8 ± 2.9 |
| | IL-1 + IL-6 | 694 ± 82 | 27.9 ± 3.6** | 5566 ± 1982 | 20.5 ± 6.5** |

*Femoral head cartilage explants and patellae were incubated in RPMI supplemented with 0.25 μg/ml IGF-1, 1 specimen per 200 μl medium. Medium was changed after 24 h, and the total incubation period was 48 h.

†The IL-1α concentrations were 10 ng/ml and 0.1 ng/ml in patellae and femoral head explants, respectively. The IL-6 concentration used was 100 ng/ml in cultures of patellae and femoral head explants.

‡Proteoglycan synthesis (mean value ± SD) as measured by [$^{35}$S]sulfate (20 μCi/ml) uptake during the last 3 h of incubation.

§NO was measured in pooled samples of both 24 h-incubation periods using Griess reagents and $NO_2^-$ as standard.

IL-6 significantly reduced IL-1 induced nitrite levels as tested using the Wilcoxon rank sum test, **$P < 0.05$. A dose of 10 ng/ml IL-6 had no effect on the nitrite levels (not shown).

induced NO release (Table 3). Furthermore, exogenous IL-6, upto 100 ng/ml, failed to modulate IL-1 induced suppression of proteoglycan synthesis, although the highest IL-6 dose significantly reduced IL-1-induced NO release (Table 3).

## *Effect of LIF on chondrocyte proteoglycan synthesis*

High amounts of murine recombinant LIF (100 ng/ml) were able to decrease chondrocyte proteoglycan synthesis significantly for −28.0 ± 7.6% (mean of five experiments) in cultures of patellae (Table 4) and −26.3 ± 1.5% (mean of three experiments) in femoral head cartilage explants of IL-6$^{+/+}$ mice (not shown). IL-6 played no role in the LIF induced suppression of proteoglycan synthesis which was at least as high in explants from IL-6$^{0/0}$ mice (Table 4). However compared to IL-1, the extent of proteoglycan synthesis inhibition induced by LIF was considerably smaller (Table 4). Moreover, IL-1 induced nitric-oxide production, whereas the nitrite levels in the culture media of cartilage stimulated with LIF did not exceed the spontaneously produced nitrite levels (Table 4). This suggests that NO did not mediate the LIF-induced suppression of proteoglycan synthesis.

## *Role of NO in IL-1-, and LIF-induced suppression of proteoglycan synthesis*

We further examined the role of NO by using the inhibitor of the NO-synthase, N$^G$-monomethyl

**TABLE 4. Comparison between IL-1- and LIF-induced inhibition of proteoglycan synthesis.**

| | | Patellar cartilage explants* | |
|---|---|---|---|
| Mice | Culture medium | [$^{35}$S]sulfate‡ incorporation (cpm ± SD) | Nitrite† concentration (μM) |
| C57Bl/6 (IL-6$^{+/+}$) | | 1582 ± 304 | 21.9 ± 6.8 |
| | LIF | 1208 ± 95** | 18.6 ± 6.8 |
| | IL-1 | 433 ± 51** | 93.4 ± 12.9** |
| C57Bl/6x129Sv (IL-6$^{0/0}$) | — | 1777 ± 194 | 23.1 ± 6.5 |
| | LIF | 1184 ± 91** | 27.3 ± 8.1 |
| | IL-1 | 558 ± 126** | 119.1 ± 18.3** |
| C57Bl/6x129Sv (IL-6$^{+/+}$) | — | 1703 ± 173 | 25.2 ± 6.1 |
| | LIF | 1169 ± 92** | 21.9 ± 3.0 |
| | IL-1 | 684 ± 86** | 108.9 ± 12.9** |

*Patellae ($n = 6$) were incubated for 48 h in RPMI supplemented with 0.25 μg/ml IGF-1. The concentrations of IL-1α and LIF were 10 ng/ml and 100 ng/ml, respectively.

†Medium was changed after 24 h (200 μl/patella). NO was measured in pooled samples of both 24-h incubation periods using Griess reagents and $NO_2^-$ as a standard.

‡Proteoglycan synthesis (mean value ± SD) as measured by [$^{35}$S]sulfate incorporation in the last 3 h of the 48-h incubation period. Control group and cytokine treated group were tested using the Student's *t*-test, values statistically significant: **$P < 0.05$.

**TABLE 5. Role of nitric oxide in IL-1-, and LIF-induced inhibition of proteoglycan synthesis.**

| | | Patellar cartilage explants* | | Femoral head cartilage* | |
|---|---|---|---|---|---|
| Mice | Culture medium† | [$^{35}$S]sulfate‡ incorporation (cpm ± SD) | Nitrite§ concentration (μM) | [$^{35}$S]sulfate incorporation (cpm ± SD) | Nitrite concentration (μM) |
| C57Bl/6x129Sv (IL-6$^{+/+}$) | — | 1703 ± 173 | 25.2 ± 6.1 | 8872 ± 1237 | 13.2 ± 2.3 |
| | NMMA | 1723 ± 157 | 7.8 ± 1.5** | 8682 ± 1513 | 7.2 ± 1.6** |
| | IL-1 | 684 ± 86 | 108.6 ± 12.9 | 3827 ± 784 | 53.8 ± 13.0 |
| | IL-1 + NMMA | 1016 ± 74** | 8.9 ± 3.1** | 6328 ± 1106** | 8.0 ± 2.2** |
| | LIF | 1288 ± 115 | 25.9 ± 4.2 | 6305 ± 1791 | 20.4 ± 2.0 |
| | LIF + NMMA | 1460 ± 108** | 8.3 ± 1.7** | 5860 ± 1215 | 10.7 ± 1.7** |
| C57Bl/6x129Sv (IL-6$^{0/0}$) | — | 1777 ± 194 | 23.1 ± 6.5 | 8212 ± 1070 | 16.7 ± 1.9 |
| | NMMA | 1569 ± 231 | 8.9 ± 1.5** | 7614 ± 1345 | 7.7 ± 2.0** |
| | IL-1 | 558 ± 126 | 119.1 ± 18.3 | 4812 ± 899 | 60.4 ± 9.4 |
| | IL-1 + NMMA | 1153 ± 168** | 9.3 ± 2.9** | 6585 ± 648** | 8.9 ± 3.0** |
| | LIF | 1364 ± 186 | 31.5 ± 5.3 | 6865 ± 1185 | 20.4 ± 2.9 |
| | LIF + NMMA | 1060 ± 155** | 10.7 ± 2.6** | 6240 ± 1017 | 10.7 ± 1.7** |

*Femoral head cartilage explants ($n = 6$) and patellae ($n = 6$) were incubated in RPMI supplemented with 0.25 μg/ml IGF-1, 1 specimen per 200 μl medium. Medium was changed after 24 h, and the total incubation period was 48 h.

†The IL-1α concentrations were 10 ng/ml and 0.1 ng/ml in patellae and femoral head explants, respectively. The NMMA concentration used was 1 mg/ml. The LIF concentrations were 100 ng/ml and 25 ng/ml in patellae and femoral head explants, respectively.

‡Proteoglycan synthesis (mean value ± SD) as measured by [$^{35}$S]sulfate (20 μCi/ml) uptake during the last 3 h of incubation.

§NO was measured in pooled samples of both 24-h incubation periods using Griess reagents and $NO_2^-$ as a standard.

NMMA-treated groups and their respective control groups were tested using the Wilcoxon rank sum test, values were statistically significant: **$P < 0.05$. Note that the NMMA treatment had opposite effect on IL-1 and LIF-induced proteoglycan synthesis suppression.

L-arginine (NMMA). A concentration of 1 mg/ml of NMMA completely prevented the IL-1-inducible NO production and significantly reduced the IL-1 effect on proteoglycan synthesis in cultures of patellae and femoral head cartilage obtained from IL-6$^{+/+}$ or IL-6$^{0/0}$ mice (Table 5). Incubation of femoral head cartilage explants or patellae with LIF did not increase the NO-levels in their conditioned medium. In the presence of NMMA, as expected, LIF-induced suppression of proteoglycan synthesis was not decreased (Table 5).

### *Role of endogenous IL-6 in the suppression of proteoglycan synthesis studied in vivo using normal and IL-6$^{0/0}$ mice*

Next, we examined the role of IL-6 in the suppression of chondrocyte proteoglycan synthesis in vivo. A single injection of 10 ng murine IL-1α given intra-articularly in the right knee provoked a pronounced suppression of proteoglycan synthesis of −47.5 ± 10.0% in IL-6$^{0/0}$ mice (mean of four experiments) (Table 6). This was not significantly different from the IL-1 induced suppression in IL-6$^{+/+}$ mice: −42.5 ± 15.1%, IL-6 seems not to be essential. However, high dosages of IL-1 were needed to provoke a clear suppression of the chondrocyte proteoglycan synthesis,[14] therefore, to meet more physiological levels of IL-1, heat-inactivated zymosan was injected into the joint cavity. This elicited local cytokine production with IL-1 peak values at the 3rd hour, and IL-6 peak values at the 6th hour after zymosan injection (Fig. 2). At 48 h after zymosan injection, IL-1 and IL-6 levels returned to baseline (Fig. 2). At all time points taken, wash-out of patellae with adjacent synovial tissue demonstrated increased amounts of NO during arthritis (Fig. 2). The amounts of IL-1 and NO found

**TABLE 6. IL-1- or arthritis-induced inhibition of proteoglycan synthesis in patellae.**

| | | C57Bl/6 (IL-6$^{+/+}$) | | | C57Bl/6x129Sv (IL-6$^{0/0}$) | | |
|---|---|---|---|---|---|---|---|
| | | [$^{35}$S]sulfate incorporation* (cpm ± SD) | | | [$^{35}$S]sulfate incorporation (cpm ± SD) | | |
| Mice | Time | Left | Right | Suppression | Left | Right | Suppression |
| IL-1 injection† | Exp 1. Day 1 | 1722 ± 263 | 1018 ± 203 | 41% | 1406 ± 232 | 814 ± 131 | 42% |
| | Exp 1. Day 2 | 1880 ± 304 | 714 ± 164 | 62% | 1838 ± 488 | 749 ± 119 | 59% |
| Zymosan-induced arthritis‡ | Exp 2. Day 2 | 2456 ± 382 | 1202 ± 121 | 51% | 2264 ± 382 | 872 ± 63 | 61% |
| | Exp 3. Day 2 | 3957 ± 712 | 1483 ± 205 | 63% | 4153 ± 891 | 1137 ± 198 | 73% |

*Patellae of right and left knee joints ($n = 6$ mice per group) were dissected and ex vivo labeled with [$^{35}$S]sulfate for 3 h.

IL-1α (10 ng) was injected intra-articularly into the right knee joint.

‡Monoarticular arthritis was elicited by intra-articular injection of 180 μg of heat-inactivated zymosan into the right knee joint. No differences in joint swelling ($^{99}$technetium uptake) between the two mouse strains were found at day two of arthritis (data not shown).

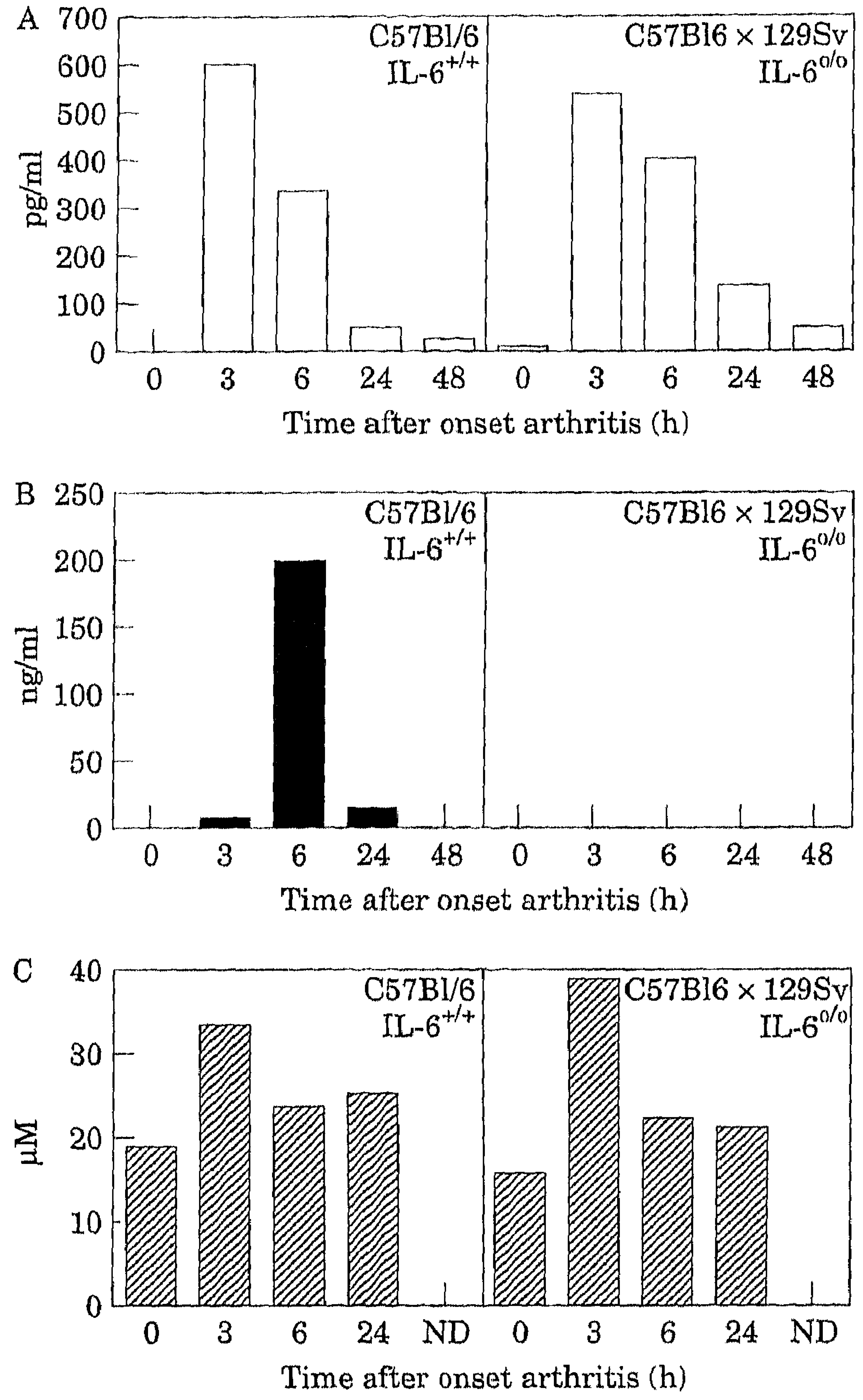

**Figure 2. IL-1, IL-6, and NO release by patellae with adjacent synovial tissue during the course of zymosan-induced arthritis.**

Patellae with adjacent soft tissue were dissected in a standardized manner to obtain large specimen of synovia. Patellae were incubated in 200 μl RPMI for 1 h at room temperature followed by 24 h at 37°C for assessment of IL-1/IL-6 (A,B) and NO (C), respectively. Data represent the mean value of 4 patellae per time point, IL-1 and NO levels between IL-6$^{+/+}$ and IL-6$^{o/o}$ mice were not significantly different. IL-6 in conditioned media of patellae from IL-6$^{o/o}$ mice could not be detected using the B9 assay. ND = not done.

during zymosan-induced arthritis were not significantly different between IL-6$^{o/o}$ mice and their normal littermates (Fig. 2). Furthermore, arthritis caused a marked suppression of proteoglycan synthesis in both mouse strains (Table 6). This demonstrated that IL-6 was not a necessary (co)factor in the IL-1 induced-, or arthritis related suppression of chondrocyte proteoglycan synthesis in vivo.

## DISCUSSION

The cytokines IL-1 and LIF are both capable of suppressing chondrocyte proteoglycan synthesis in murine articular cartilage. Using neutralizing antibodies and knock-out mice, we demonstrated that IL-6 did not mediate the IL-1 and LIF effect on chondrocyte synthesis. Furthermore, both cytokines have independent second mediator pathways involved in the suppression of proteoglycan synthesis, IL-1 was partly mediated by NO and LIF was not mediated by NO.

Little is known about the local role of IL-6 in cartilage pathology of arthritis. There is circumstantial evidence that IL-6 plays a costimulatory role in the IL-1-induced suppression of proteoglycan synthesis in human articular cartilage explants.[21,22] However, if in these studies IL-1 dosages of 40 pg/ml or higher were used no effect of IL-6 neutralization on the IL-1-induced proteoglycan synthesis suppression was found. The lowest IL-1 dose we used in this study was 100 pg/ml, which was in the normal range of synovial fluid IL-1 levels in rheumatoid arthritis.[37] Furthermore, we demonstrated that during murine experimental arthritis (ZIA) sufficient amounts of IL-1 were synthesized in the joint to mediate the suppression of proteoglycan synthesis.[14] Induction of arthritis in IL-6-deficient mice also resulted in local IL-1 generation, and in a pronounced inhibition, indistinguishable of that seen in IL-6$^{+/+}$ mice. For this, IL-6 was not involved in the IL-1 induced suppression of chondrocyte proteoglycan synthesis in murine articular cartilage. This was in line with a previous in vivo study in which we observed that anti-IL-6 antibody treatment had no effect on the inhibited proteoglycan synthesis during antigen-induced arthritis although in that particular study we could not exclude that the endogenous IL-6 was not completely neutralized.[14] Furthermore, addition of high amounts of IL-6 was unable to modulate the effect of low and high dosages of IL-1 on chondrocyte proteoglycan synthesis in mouse (Table 3), and in other species.[18,39] We and others found that bovine chondrocytes did not produce IL-6 spontaneously or after IL-1 exposure, still human recombinant IL-1 markedly inhibited proteoglycan-synthesis.[40] Analogous to our observations in the mouse, addition of high amounts of human recombinant IL-6 also had no effect on normal or IL-1 affected proteoglycan synthesis in the bovine system (data not shown). This also argues against an essential role for IL-6 in the IL-1-induced suppression of proteoglycan synthesis, in this case, in bovine articular cartilage. However, we can not exclude a possible bypass in bovine chondrocytes because of their impairment of IL-6 synthesis in response to IL-1 and this could also have occurred in the IL-6-deficient mice. For this, we also performed IL-6 neutralizing experiments using anti-IL-6 antibodies in cultures of isolated murine articular chondrocytes and on cartilage explants. Neutralizing IL-6 bioactivity did not prevent the IL-1-induced suppression of proteoglycan synthesis, confirming the results obtained in the IL-6-deficient mice.

LIF, a mediator downstream in the cytokine cascade in RA, could also inhibit chondrocyte proteoglycan synthesis in murine articular cartilage. In order to investigate whether IL-6 mediated the LIF effect, LIF was tested on cartilage of IL-6$^{0/0}$ mice. Since similar suppression of proteoglycan synthesis was found, IL-6 seems not to be essential. LIF belongs to a group of related cytokines including IL-6, which have overlapping functions and share the same signal-tranducing peptide (gp130) in conjunction with their non-signalling cytokine-specific receptors.[41,42] However, IL-6 could not suppress chondrocyte proteoglycan synthesis in mouse (Table 3), cow,[40] and man.[38,39] This showed that LIF and IL-6 possess differential activities as was already seen in other cell systems, e.g. macrophage differentiation and several bioassays.[43,44] If these cytokines are not always interchangeable, IL-6 may antagonize LIF by competing for the gp130 receptor. High levels of LIF (ranging from 1 to 43 ng/ml) were found in synovial fluid of patients with rheumatoid arthritis (RA).[45] The amounts of LIF used in the present study were, however, higher (10 and 100 ng/ml). On the other hand, in cultures of patellae the concentration of IL-6 reached values between 150 and 300 ng/ml, which was also at least 15 times higher than the concentrations found in synovial fluid of RA-patients, 10–25 ng/ml.[34,46,47] For this, the concentrations of IL-6 in conditioned media matched the amounts of LIF used in this study. The effect of LIF on proteoglycan synthesis was not enhanced in cartilage derived from IL-6$^{0/0}$ mice as compared to IL-6$^{+/+}$ mice, arguing against the premise of competition between IL-6 and LIF for the gp130 receptor in chondrocytes.

Recently, several groups observed that the IL-1-induced suppression of proteoglycan-synthesis was mediated by autocrine NO in rabbit,[29] rat,[30] and human chondrocytes.[48] LIF suppressed proteoglycan synthesis in porcine and caprine cartilage.[26] Whether NO is also involved in the action of LIF has yet to be determined. In this study we investigated the role of NO in the IL-1- and LIF-induced inhibition of proteoglycan synthesis. Murine chondrocytes spontaneously released NO during culture and IL-1, but not LIF, increased NO synthesis. In contrast, human recombinant IL-1 and LIF both were capable of inducing NO-synthesis in cultured human articular chondrocytes.[48,49] Besides the obvious species difference, isolated chondrocytes may well react differently on LIF as compared to chondrocytes maintained in their extracellular matrix. Blocking NO-synthesis using the NO antagonist, NMMA, significantly alleviated the IL-1-induced suppression but not the LIF-induced suppression in mice, and it remains to be seen whether inhibitors of the NO-synthase prevent the LIF effect on human cartilage.

The NMMA treatment reduced NO-levels below the background values, still a significant suppression of proteoglycan synthesis was evident using IL-1, suggesting that inhibition by IL-1 was not fully NO-dependent in murine articular cartilage. Furthermore, LIF inhibited proteoglycan synthesis independent of NO, suggesting that there are at least two distinct, independent intracellular pathways which lead to proteoglycan synthesis suppression. It must be noticed that the proteoglycan synthesis in porcine and caprine cartilage[26] was considerably more suppressed by LIF than in murine cartilage (Table 4). It is possible that also LIF operates via different pathways in the various species.

Conflicting observations were obtained in the bovine system. The inhibitor of iNOS, NMMA had no effect on human recombinant IL-1β-induced suppression of proteoglycan synthesis in bovine articular cartilage explants.[50] However, we (data not published) and another group[51] could completely block the IL-1-induced proteoglycan synthesis suppression with the addition of NMMA in primary cultures of bovine chondrocytes, suggesting that culture conditions may determine which pathway is taken.

Experimental arthritis in mouse caused a sharp increase of IL-1, IL-6 and NO levels in wash-outs of patellae with adjacent synovial tissue obtained within the first 24 h after onset. NO-production was not impaired in cartilage isolated from IL-6$^{0/0}$ mice, as was already demonstrated for peritoneal macrophages in these mice.[31] Therefore, it is highly unlikely that IL-6 was involved in IL-1 and NO synthesis during arthritis. Preliminary data of the cartilage destruction in zymosan-induced arthritis, a non-immunologically mediated inflammation,[52] revealed an enhanced proteoglycan loss in IL-6-deficient mice as compared to normal (IL-6$^{+/+}$) mice. Experiments are in progress to examine the role of IL-6 in the process of cartilage destruction during experimental arthritis. Anti-IL-1/TNF treatment may reduce IL-6 levels in synovial fluid of RA-patients and one may consider to replenish IL-6 for its beneficial effects on cartilage.

High levels of IL-6 are found in the synovial fluid of inflamed joints in rheumatoid arthritis and besides its systemic effect, e.g. on acute phase proteins and fever, the local effects on cartilage remain to be examined. We demonstrated that both IL-1 and LIF action on chondrocyte proteoglycan synthesis were IL-6 independent, in vitro and in vivo. Furthermore, we confirmed the mediating role of NO in IL-1-induced suppression, but clearly showed that the LIF effect was NO independent. This argues against the existence of a IL-1 → LIF → IL-6 → NO cascade in the IL-1-induced suppression of proteoglycan synthesis in murine articular cartilage. Our data suggest that IL-6 is not a feasible downstream target to fine tune IL-1-directed therapy, to prevent cartilage damage. Furthermore, the strategy of NO blocking in RA will affect IL-1 action, but probably not that of LIF.

## MATERIALS AND METHODS

### *Animals*

Homozygous IL-$6^{0/0}$ and wild-type (C57Bl/6x129/Sv)F2 mice were obtained from M. Kopf (Germany) and bred in our own animal facilities, as were the C57Bl/6 mice. Mice were housed in filter top cages under standard pathogen free conditions and fed a standard diet and tapwater *ad libitum*. At the age of 8–10 weeks they were used in the experiments.

### *Recombinant cytokines and antibodies*

Purified and biologically active mature murine recombinant IL-1α, cloned in *Escherichia coli*, was generously donated by I.G. Otterness (Pfizer Central Research, Groton CT, USA) and bioactivity was checked in a bioassay. Purified murine recombinant IL-6 was a gift from G. Ciliberto (I.R.B.M., Rome Italy), and murine recombinant LIF (carrier free) was purchased from R&D Systems Ltd (Europe). Rat anti-mouse IL-6 monoclonal antibody (IgG1) was purchased by Genzyme Corp (Cambridge MA, USA). Neutralizing capacity was verified in the IL-6 bioassay.

### *Control of IL-6-deficiency*

The IL-6 gene was disrupted in the second exon by insertion of a *neo* cassette.[31,32] Loss of wild-type IL-6 messenger RNA was confirmed by reverse transcriptase (RT)-PCR using primers bridging the insertion: 5′, TCT GCA AGA GAC TTC CAT CCA; 3′, GCA AGT GCA TCA TCG TTG TTC,[33] purchased from Pharmacia Biotech (Roosedaal, The Netherlands). Controls were included for a possible bypass of IL-6 by IL-11. Sequences of the primers for IL-11 were upstream (5′) (5′)CTG TGG GGA CAT GAA CTG TG(3′) and downstream (3′) (5′)AGC CTT GTC AGC ACA CCA G(3′). Messenger RNA from cartilage or synovial tissue, isolated with TRIzol reagent according to the protocol of the manufacturer (Life Technology, Breda, The Netherlands), was reverse-transcribed to complementary DNA (cDNA) using oligi-dT primers by standard protocol. One twentieth of the cDNA was used for one PCR reaction of 35 cycles; denaturing at 92°C for 1 min, annealing at 55°C for 1 min, followed by elongation with *Taq* DNA polymerase (Life Technologies) at 72°C for 1 min. The expected PCR products of IL-6 and IL-11 were 239 and 300 base pairs, respectively. This control was performed regularly during breeding of the animals. In cartilage obtained from IL-$6^{0/0}$ mice, no IL-6 mRNA was detected, whereas IL-6 mRNA was present in cartilage from IL-$6^{+/+}$ mice, and marked enhancement was found after IL-1 challenge. There was no evidence of enhanced IL-11 expression in cartilage from IL-$6^{0/0}$ mice. Second, culture supernatant of articular cartilage was checked for IL-6 bioactive using the B9-assay, and no bioactive IL-6 was found in conditioned medium of cartilage cultures from IL-$6^{0/0}$ mice.

### *Cartilage explant culture*

Patellae were dissected with a minimal of surrounding soft tissue (ligament, muscle and synovium), and femoral head explants were obtained by detaching the cartilage layer from the femur by a firm twist using forceps. Explants were incubated in 200 µl RPMI-1640 (Dutch modification) medium with Glutamax-1 (Gibco BRL, Life Technologies, Scotland, UK) supplemented with 0.25 µg/ml human recombinant insulin-like growth factor (IGF)-1, and gentamicin (50 mg/l) at 37°C in a humidified 5% $CO_2$ atmosphere.

### *Chondrocyte isolation, culture and proteoglycan synthesis*

Femoral head cartilage (30 explants) was digested overnight with 120 U collagenase IA (*Clostridium histolyticum*, Worthington, UK) in 2 ml of RPMI-1640 culture medium supplemented with 2% BSA. Chondrocytes were centrifugated at 250 × *g* for 10 min and resuspended in RPMI 1640 + 5% fetal calf serum. Viability was checked by trypan-blue exclusion and cell-number was determined. Around $5 \times 10^4$ chondrocytes were plated per well in a flat-bottom 96-well plate and incubated in 200 µl medium. After 24 h, 50-µl samples per well were taken for IL-6 determination, thereafter, 1 µCi of [$^{35}$S]sulfate/50 µl medium was added to each well and incubation was continued for another 4 h. Incubation was terminated by storing the plate at −20°C. After thawing, medium was vigorously pipetted a few times to disrupt the cells and the [$^{35}$S]sulfate-labelled proteoglycans were precipitated with cetylpyridinium chloride as previously described before.[34]

### *Assessment of proteoglycan synthesis in cartilage explants*

Cartilage specimens (patellae or femoral heads) were placed in RPMI 1640 supplemented with gentamicin (50 mg/l), L-glutamine (2 mM) and 40 µCi [$^{35}$S]sulfate. At the end of the 3-h incubation period, patellae were fixed in 10% formalin and subsequently decalcified in formic acid (5%), dissected and dissolved in 0.5 ml Lumasolve (Lumac, Groningen, The Netherlands). The $^{35}$S-content of each patella was measured by liquid scintillation counting and expressed as counts per minute (cpm). Data are represented as total incorporation of [$^{35}$S]sulfate or as a ratio of right over left knee (within animal control value) as paired values.

### *Assessment of cytokine bioactivities*

IL-1 activity was measured in the one-stage proliferation assay as described by Gearing *et al.*[35] The murine thymoma cell line EL-4 NOB-1 (ECACC, Porton Down, Salisbury, Wilts, UK) was used as an IL-1 specific cell producing IL-2 in response, in combination with the IL-2 sensitive CTLL2-cells (ECACC). The cells were plated out in concentrations of $1 \times 10^5$/well NOB1-cells and $4 \times 10^4$/well CTLL-cells in RPMI supplemented with 5% fetal calf serum (FCS) for 21 h.

IL-6 activity was determined by a proliferative assay using B9 cells. Briefly, $5 \times 10^3$ B9-cells in 200 µl 5%

FCS-RPMI 1640 per well were plated in a round-bottom microtitre plate and incubated for 3 days using human recombinant IL-6 as standards.

At the end of the incubation (both IL-1 and IL-6 assay), 0.5 μCi of $[^3H]$thymidine (specific activity 20 Ci/mmol, Dupont, NEN products, Boston, MA) was added per well. Three hours later, cells were harvested and thymidine incorporation (NOB1 cell are thymidine kinase deficient) was determined. Detection limit of the IL-1 assay was 0.1 pg/ml murine recombinant IL-1 and for the IL-6 assay was 1 pg/ml.

### *Nitrite determination*

The medium concentration of $NO_2^-$ (a stable breakdown product of NO) was determined by Griess reaction using $NaNO_2$ standards. Griess reagent; 0.1% naphthylethylene diamine dihydrochloride, 1:1 diluted with 1.0% sulfanilamide in 5% $H_3PO_4$. Briefly, 100 μl of conditioned medium was mixed with 100 μl of Griess reagent in a flat-bottom microtitre plate and adsorbance read at 545 nm using an ELISA plate reader.

### *Zymosan-induced arthritis*

A homogeneous suspension of 30 mg zymosan A (*Saccharomyces cerevisiae*), dissolved in 1 ml endotoxin-free saline, was obtained by boiling twice, and sonic emulsification. Arthritis was induced by intra-articular injection of 180 μg zymosan along the suprapatellar ligament into the joint cavity. The contralateral knee joint received an equal amount of saline (6 μl) and served as a within animal control. At day 2 of arthritis, joint swelling was measured, thereafter, patellae were isolated to assess cytokine levels and proteoglycan synthesis.

### *Assessment of joint swelling*

Animals were injected subcutaneously with 10 μCi $^{99}$Technetium pertechnetate ($^{99}$Tc) in 0.2 ml saline in the neck region. After 15 min, mice were sedated by intraperitoneal injection of 4.5% chloral hydrate, 0.1 ml/10 mg of body weight. The accumulation of the isotope due to the increased blood flow and edema in the knee was determined by external gamma counting and expressed as the ratio of the $^{99}$Tc uptake in inflamed over contralateral knee joint. A ratio higher than 1.1 indicates joint swelling.

### *Assessment of cytokine production by arthritic joints*

Patellae were dissected with surrounding soft-tissue consisting out of the tendon, and synovium in a standardized manner. Each patella was incubated in 200 μl serum free RPMI 1640 after 1 h at 37°C temperature, culture medium was sampled and stored at −70°C preceeding cytokine measurements, patellae were provided with new medium and incubated for another 24 h, thereafter, medium was stored at 20°C preceeding nitrite measurements.

### *Statistical analysis*

Data are expressed as mean values ± standard deviation (SD) unless stated otherwise. Statistical significance was tested using the Wilcoxon's rank sum test or with the Student's *t*-test as stated in the legends.

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