

# Increase of Cyclooxygenase-2 Expression by Interleukin 15 in Rheumatoid Synoviocytes

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**ABSTRACT. Objective.** To determine the effect of interleukin 15 (IL-15) on cyclooxygenase-2 (COX-2) expression in rheumatoid synoviocytes.

**Methods.** Fibroblast-like synoviocytes (FLS) were prepared from the synovial tissues of patients with rheumatoid arthritis (RA) and cultured in the presence of IL-15. Levels of COX-2 mRNA and protein expression were determined by reverse transcription-polymerase chain reaction and Western blot, respectively. ELISA was used to measure concentrations of IL-1 $\beta$ , tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) in the culture supernatants.

**Results.** IL-15 dose-dependently increased COX-2 mRNA and protein expression in FLS, but not the COX-1 mRNA level. Both IL-1 $\beta$  and TNF- $\alpha$  upregulated COX-2 mRNA comparably to IL-15, but neither IL-2 nor interferon- $\gamma$  had any effect on the COX-2 mRNA level. Treatment with anti-IL-1 $\beta$  or anti-TNF- $\alpha$  antibodies partially reduced the IL-15-stimulated COX-2 mRNA expression, suggesting that these cytokines may take part in modulating COX-2 by IL-15. Dexamethasone and pyrrolidine dithiocarbamate, but not curcumin, completely blocked the IL-15-induced upregulation of COX-2 mRNA. A gel mobility shift assay revealed that nuclear factor- $\kappa$ B (NF- $\kappa$ B) was one of the major signal molecules to mediate IL-15-induced COX-2 upregulation. The increase of COX-2 by IL-15 is PGE<sub>2</sub>-dependent because exogenous PGE<sub>2</sub> reversed the suppressive effect of NS-398, a selective COX-2 inhibitor, on COX-2 mRNA and protein expression.

**Conclusion.** This study confirms the effect of IL-15 on upregulation of COX-2 in a PGE<sub>2</sub>-dependent manner. The activation of NF- $\kappa$ B bound to the COX-2 promoter appears to be a downstream target of IL-15 stimulation in FLS, exerted either directly or through the increase in IL-1 $\beta$  and TNF- $\alpha$  production. (J Rheumatol 2004;31:875–83)

## Key Indexing Terms:

INTERLEUKIN 15 SYNOVIOCYTES CYCLOOXYGENASE-2 NUCLEAR FACTOR- $\kappa$ B

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease accompanying extensive destruction of the articular cartilage<sup>1,2</sup>. A variety of proinflammatory cytokines from macrophages or synoviocytes play crucial roles in the disease process by stimulating the proliferation of the synovium and the recruitment of inflammatory cells<sup>3</sup>. In particular, interleukin 15 (IL-15), a novel 14–15 kDa

cytokine, induces T cell proliferation and promotes the activity of cytotoxic effector cells and natural killer (NK) cells<sup>4</sup>. IL-15 is frequently found in RA synovium and is produced by both the macrophage-lineage and fibroblast-like synoviocytes (FLS)<sup>5</sup>. It has been suggested that IL-15 recruits and expands T cells in the synovial membrane, where newly employed T cells can produce tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) directly or through cell contact with macrophages<sup>6,7</sup>. Moreover, IL-15 participates in the activation and proliferation of FLS by autocrine stimulation of functional IL-15 receptor on FLS<sup>8</sup>.

Another key modulator of joint inflammation in RA is cyclooxygenases-2 (COX-2), which governs the rate-limiting steps in prostaglandin synthesis<sup>9</sup>. Enhanced COX-2 expression has been described in patients with RA and animal arthritis models and may be involved in angiogenesis, inflammation of the synovial membrane, and erosion of the cartilage and juxtaarticular bones in RA<sup>10–13</sup>. While COX-1 is constitutively expressed in various cells and tissues, COX-2 expression is readily induced by inflammatory stimuli such as lipopolysaccharide and cytokines<sup>14–16</sup>. In particular, the influence of multiple cytokines on COX-2 expression has been reported in many *in vitro* studies<sup>17–21</sup>. Proinflammatory cytokines including TNF- $\alpha$ <sup>17,18</sup>, IL-1 $\alpha$ <sup>18</sup>,

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and interferon- $\gamma$  (IFN- $\gamma$ )<sup>19</sup> have been reported to induce COX-2 expression, whereas antiinflammatory cytokines such as IL-4<sup>20</sup> and IL-10<sup>21</sup> can inhibit COX-2 induction. Thus it is possible that COX-2 expression may be finely regulated by a number of cytokines within the RA joints. Sequence analysis of the 5'-flanking region of human COX-2 gene has revealed several potential transcription regulatory sequences, including a TATA box, a C/EBP motif, 2 AP-2 sites, 3 SP1 sites, 2 nuclear factor- $\kappa$ B (NF- $\kappa$ B) sites, a cyclic-AMP response element (CRE) motif, and an Ets-1 site<sup>22</sup>. However, the activation pathway instigated by other proinflammatory factors on the synthesis of this enzyme in RA synoviocytes remains to be clarified.

We observed that IL-15, a T cell growth factor, strongly increased COX-2 expression in rheumatoid synoviocytes at the mRNA and the protein level, and this effect was in part related to the increase in IL-1 $\beta$  and TNF- $\alpha$  production by the FLS. The induction of COX-2 mRNA after an IL-15 treatment appeared to be mediated by NF- $\kappa$ B binding to its promoter. The increase of COX-2 by IL-15 is prostaglandin E<sub>2</sub> (PGE<sub>2</sub>)-dependent, because exogenous PGE<sub>2</sub> reversed the suppressive effect of NS-398, a selective COX-2 inhibitor, on COX-2 mRNA and protein expression. Collectively, our data suggest that IL-15 may play an important role in COX-2 and PGE<sub>2</sub> production, thus participating in the inflammatory cascades in RA. Our findings provide additional evidence for the combined crosstalk and the converged effect of a set of proinflammatory cytokines such as IL-15, IL-1 $\beta$ , and TNF- $\alpha$  on COX-2 expression within RA joints.

## MATERIALS AND METHODS

**Reagents and antibodies.** Human recombinant (r) IL-15 and IFN- $\gamma$  were purchased from R&D Systems (Minneapolis, MN, USA); rabbit polyclonal antibodies against human COX-2 were from Cayman Chemicals (Ann Arbor, MI, USA); peroxidase-conjugated anti-rabbit IgG was from Sigma Chemical Co. (St. Louis, MO, USA). Monoclonal antibodies (mAb) to IL-15 were obtained from R&D Systems. Dexamethasone (DEX), curcumin, and pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma. For ELISA, recombinant human IL-1 $\beta$ , IL-15, and TNF- $\alpha$ , and antibodies to these cytokines, were from R&D Systems. The [ $\gamma$ -<sup>32</sup>P]ATP was purchased from Amersham Pharmacia (Uppsala, Sweden). Reagents used for culture were found to contain < 200 pg/ml of endotoxin as determined by the Limulus amebocyte cell lysate assay.

**Isolation of synoviocytes.** FLS cell lines were prepared from the synovial tissues of 6 RA patients undergoing total joint replacement surgery, as described<sup>23</sup>. The mean age of patients (5 women, 1 man) was 47.9 years. The mean disease duration was 62.5 months. Five out of 6 patients had a positive rheumatoid factor (RF). All had erosions on hand radiographs. The cells between the fourth and eight passages were used, during which time they made up a FLS homogenous population (< 3.5% CD14, < 1% CD3, and < 1% CD19-positive by flow cytometry analysis).

**RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) analysis.** The FLS (5  $\times$  10<sup>5</sup> cells/100 mm culture dish) were incubated with various concentrations of IL-15. After 6 h culture, which was the optimal time for COX-2 induction in the preliminary study, mRNA was extracted using RNeasy B according to the manufacturer's instruction (Biotec Laboratories, Houston, TX, USA). Reverse transcription of 5  $\mu$ g

total mRNA was carried out at 42°C using the Superscript<sup>TM</sup> reverse transcription system (Life Technologies, Gaithersburg, MD, USA). The PCR amplification of the cDNA aliquots was performed by adding 2.5 mM dNTPs, 2.5 U Taq DNA polymerase (TaKaRa Shuzo Co., Shiga, Japan), and 0.25  $\mu$ M each of the sense and antisense primers. The reaction was done in a PCR buffer (1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM Tris HCl, pH 8.3) in a total volume of 25  $\mu$ l. The following sense and antisense primers for each molecule were used (all written in 5' to 3' direction): COX-2 sense GCAGTTGTTCCAGACAAGCA, COX-2 anti-sense CAGGATACAGCTCCACAGCA; COX-1 sense ATGAGCCGGAGTCTCTTGCTCCG, COX-1 anti-sense CCCCACACCCATGGAACCAAA; GAPDH sense CGATGCTGGGCGTGAGTAC, GAPDH antisense CGTTCAGTCCAGGGATGACC. The reactions were processed in a DNA thermal cycler (Perkin-Elmer, Foster City, CA, USA). Cycling conditions were as follows: 45 s denaturation at 94°C for COX-2, 30 s denaturation at 94°C for COX-1 and GAPDH; 45 s annealing at 55°C for COX-2, 30 s annealing at 56°C for COX-1, 1 min annealing at 55°C for GAPDH; 30 s elongation at 72°C. The PCR rounds were repeated for 27 cycles for COX-2, 30 cycles for COX-1, and 25 cycles for GAPDH, which had been determined to fall within the exponential phase of amplification for each molecule. PCR products were run on a 1.5% agarose gel and stained with ethidium bromide. The mRNA expression level is presented as a ratio of the cytokine product over the GAPDH product.

**Western blotting for COX-2 protein.** The FLS (5  $\times$  10<sup>5</sup> cells/100 mm culture dish) were incubated 12 h in the presence or absence of IL-15. After incubation, the cells were harvested and lysed in 150  $\mu$ l solubilization buffer (1% Tween 20, 10 mM/l phenylmethylsulfonyl fluoride, and 50 mM/l Tris-HCl, pH 8.0). Protein extracts (25  $\mu$ g) were then separated on a 12% SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane. The membrane was blocked with phosphate buffered saline (PBS) containing 5% skim milk and 0.1% Tween 20, and then incubated with 0.25 mg/ml rabbit anti-human COX-2 polyclonal antibody in the blocking buffer at 25°C for 2 h. The membrane was subsequently incubated with peroxidase-conjugated anti-rabbit IgG (1:1000 dilution) and analyzed using an Amersham enhanced chemiluminescence system (Amersham, Arlington Heights, IL, USA). Fuji X-omat AR film (Fuji Co., Tokyo, Japan) with cassette closure times of 5 to 10 min was used to obtain adequate exposure and to visualize the bands.

**ELISA for IL-1 $\beta$ , TNF- $\alpha$ , and PGE<sub>2</sub>.** The FLS (5  $\times$  10<sup>4</sup> cells) in 24-well microtiter plates were stimulated with IL-15 and then cultured for 24 h. The amounts of IL-1 $\beta$  and TNF- $\alpha$  released into the culture supernatants were measured by ELISA, as described<sup>23</sup>, with the exception that 1% bovine serum albumin (BSA) in PBS-Tween was used for blocking and 1% BSA in PBS was used for diluting the antibodies. The standard curve was prepared with a 2-fold dilution of either rIL-1 $\beta$  or rTNF- $\alpha$  in 1% BSA-PBS-Tween. Measurement of PGE<sub>2</sub> produced by FLS was also performed in the culture supernatants by ELISA kits according to the manufacturer's instruction (R&D Systems). Detection limit for PGE<sub>2</sub> was 36 pg/ml.

**Flow cytometry for TNF- $\alpha$  production in FLS.** After stimulation of FLS with IL-15 for 12 h, 1  $\mu$ M monensin (GolgiStop; PharMingen, San Diego, CA, USA) was added to the cultures during the last 4 h. Viable cells were harvested, incubated 20 min on ice in blocking buffer (PBS with 10% normal rat serum and 0.02% 1 M sodium azide), and then stained for 20 min on ice with FITC-conjugated mouse anti-human CD90 (Dako, Glostrup, Denmark). The cells were washed twice in staining buffer [PBS containing 2% fetal calf serum (FCS) and 0.02% 1 M sodium azide] and resuspended in 100  $\mu$ l fixation buffer (Cytofix/Cytoperm; PharMingen) for 20 min on ice. The fixed cells were washed twice in permeabilization buffer, then stained with phycoerythrin (PE)-conjugated mouse anti-human TNF- $\alpha$  (PharMingen). Finally, cells were washed twice and resuspended in staining buffer. The preincubation cells were analyzed on a FACScan cytometer (Becton Dickinson, Mountain View, CA, USA). At least 5000 events were acquired from each sample and subsequently analyzed with Lysis II and cellQuest software (Becton Dickinson).

**Electrophoresis mobility shift assay (EMSA).** The FLS nuclear extract was prepared as described<sup>24</sup>. For induction of NF- $\kappa$ B activity, cells were pretreated with IL-15 for 1 h. A double-stranded oligonucleotide probe containing the NF- $\kappa$ B recognition site (underlined) of the human COX-2 promoter (5'-GGAGAGGGGATTCCTGCGCC-3') was generated by 5' end-labeling of the sense strand with [ $\gamma$ -<sup>32</sup>P] dATP using T4 polynucleotide kinase (TaKaRa Shuzo), and purified through NucTrap columns (Stratagene, La Jolla, CA, USA). NF- $\kappa$ B binding was performed by incubating 2  $\mu$ g of nuclear extract in 10  $\mu$ l of binding buffer containing a 400,000 cpm-labeled oligonucleotide for 30 min at room temperature. A 100 $\times$  excess of the unlabeled oligonucleotide was used for competition. The DNA-protein complex was analyzed on a 5% polyacrylamide gel in TBE buffer. The gels were then dried and exposed to BioMax-MR film (Eastman Kodak Co., Rochester, NY, USA) at -70°C for 24 h.

The supershift assay was performed to verify the identity of the bound factors using specific antibodies to p65, p50, and c-Rel proteins. Briefly, 100 $\times$  of mAb against p65, p50, and c-Rel (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to the binding reaction prior to adding the labeled probes and incubated on ice for 30 min.

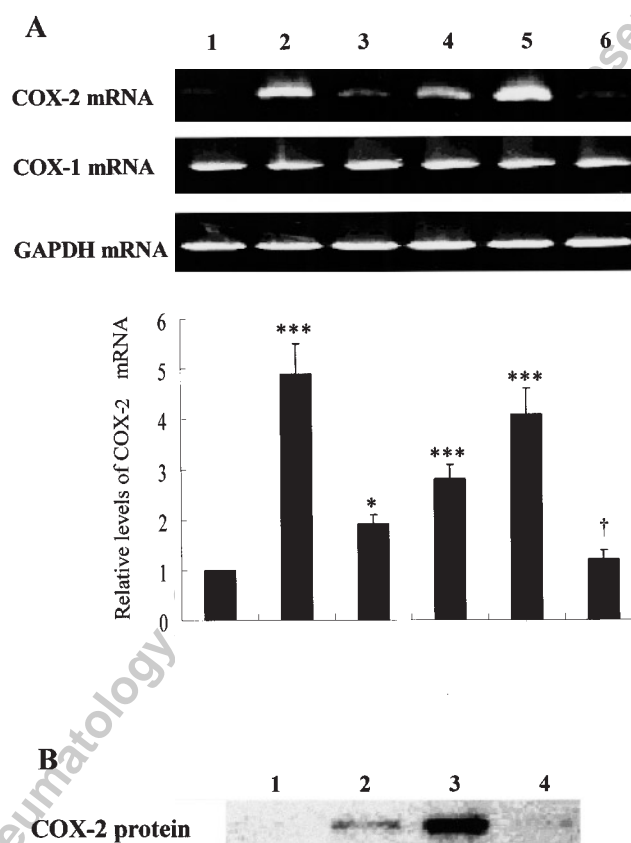
**Statistical analysis.** Data are expressed as mean  $\pm$  standard deviation (SD). Statistical analysis was performed using Student's t test for matched pairs. Differences with a confidence level of 95% or higher were considered to be statistically significant ( $p < 0.05$ ).

## RESULTS

### Induction of COX-2 by IL-15 in rheumatoid synoviocytes.

The effect of IL-15 on the synthesis of COX-2 mRNA in RA synoviocytes was examined by semiquantitative RT-PCR. As shown in Figure 1A, IL-1 $\beta$  that was used as a positive control strongly stimulated COX-2 mRNA expression (lane 2). Within the range of the concentrations tested (0.1 to 10 ng/ml), incubation with IL-15 resulted in a dose-dependent increase in COX-2 mRNA (lanes 3–5) compared to the level in the unstimulated cells (lane 1). Treatment with blocking antibodies to IL-15 canceled the effect of 10 ng/ml of IL-15 (lane 6), whereas the same concentration of isotype control antibodies (mouse anti-human IgG1; PharMingen) did not affect it (data not shown). During these experiments, COX-1 mRNA levels were unaffected by treatment with either IL-1 $\beta$  or IL-15. Results from RT-PCR analysis corresponded well with those from IL-15 immunoblotting data (Figure 1B). The FLS cells treated with 1 to 10 ng/ml of IL-15 showed a dose-dependent increase in the amount of the COX-2 protein (lane 2 and lane 3). Cotreatment with 10 ng/ml of IL-15 and its blocking antibodies brought the COX-2 level back to that of the unstimulated cells (lane 4). Overall, these results indicate that IL-15 induces COX-2 expression in RA synovial fibroblasts at both mRNA and protein level.

Time course experiments showed that IL-15-stimulated upregulation of COX-2 mRNA expression was observed as early as 1 h, peaked after 6 h, and remained high up to 12 h (Figure 2A). The COX-2 protein expression was detectable at 4 h following stimulation with IL-15, gradually increased over time, and attained steady-state at 12 h (Figure 2B). PGE<sub>2</sub> production by FLS was also instigated by IL-15 treatment in a time-dependent fashion (Figure 2C) that was quite similar to the pattern of COX-2 mRNA and COX-2 protein

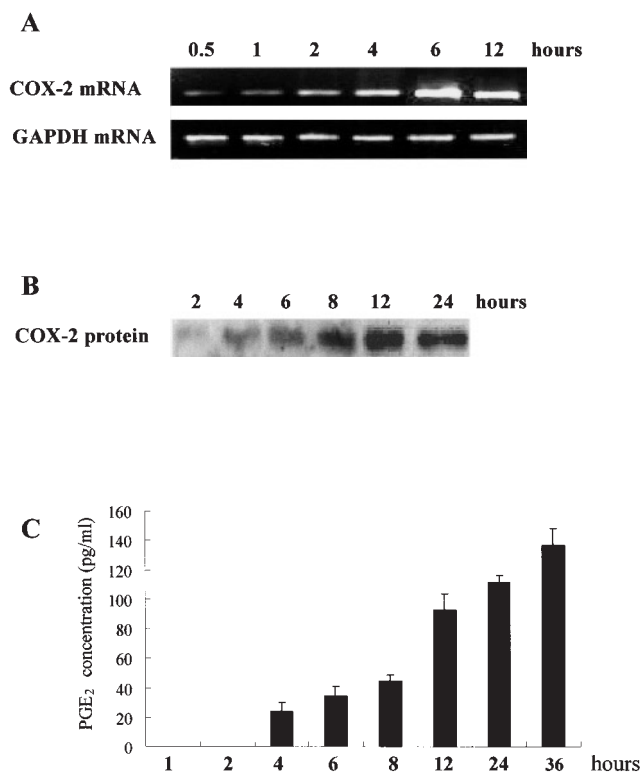


**Figure 1.** Dose-dependent activation of the COX-2 mRNA and protein by IL-15. **A.** Synovial fibroblasts ( $5 \times 10^5$  cells) were cultured 6 h in media alone (lane 1), in presence of 10 ng/ml IL-1 $\beta$  (lane 2), 0.1 ng/ml IL-15 (lane 3), 1 ng/ml IL-15 (lane 4), 10 ng/ml IL-15 (lane 5), 10 ng/ml IL-15 plus 10  $\mu$ g/ml anti-IL-15 antibodies (lane 6). Total RNA was isolated to perform RT-PCR using primers specific to COX-2, COX-1, and GAPDH. Histogram indicates COX-2 mRNA levels, expressed as fold-increase relative to COX-2 mRNA in unstimulated control cells, and corrected for levels of GAPDH mRNA signal. Data are presented as mean  $\pm$  SD of 6 to 10 independent experiments using cell lines from 10 RA patients. \* $p < 0.05$ ; \*\*\* $p < 0.001$  versus medium alone; † $p < 0.001$  versus cells treated with 10 ng/ml IL-15. **B.** Whole cell lysates of synovial fibroblasts ( $5 \times 10^5$  cells) that were treated without (lane 1) or with 1 ng/ml IL-15 (lane 2), 10 ng/ml IL-15 (lane 3), and 10 ng/ml IL-15 plus 10  $\mu$ g/ml anti-IL-15 antibodies (lane 4) were subjected to immunoblotting analysis using polyclonal antibodies against purified COX-2 proteins. Representative data from 3 independent experiments are illustrated.

synthesis. The mean concentration of PGE<sub>2</sub> released by FLS ( $5 \times 10^5$ ) was 111 pg/ml by 24 h after treatment with IL-15.

**Effect of other cytokines on COX-2 expression.** In inflamed joints, the resident synoviocytes are exposed to various proinflammatory cytokines, some of which are known to promote COX-2 synthesis. An experiment was conducted to test the effect of Th1 and the proinflammatory cytokines on COX-2 mRNA production in FLS. As shown in Figure 3, both IL-1 $\beta$  and TNF- $\alpha$  strongly induced COX-2 mRNA synthesis (lane 2 and lane 3, respectively). The amount of COX-2 mRNA stimulated with 10 ng/ml IL-15 was as high

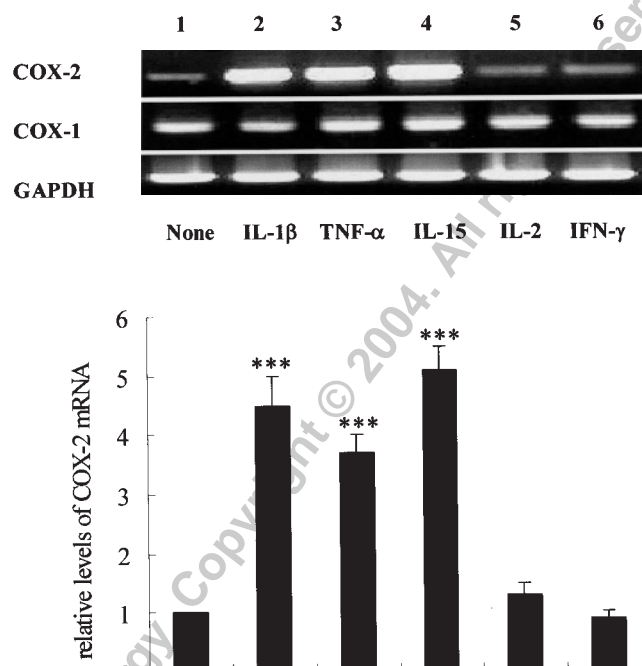




**Figure 2.** Time course of IL-15 stimulation of COX-2 mRNA, COX-2 protein, and PGE<sub>2</sub> release. Fibroblast-like synoviocytes were stimulated with 10 ng/ml IL-15 from 30 min to 24 h. At predetermined times (i.e., 30 min, 1, 2, 4, 6, 12, and 24 h), expression of COX-2 mRNA (panel A) and COX-2 protein (B) in FLS was determined by RT-PCR analysis and Western blot, respectively. PGE<sub>2</sub> levels (C) in FLS culture supernatants were measured by ELISA. A representative band from 2 independent experiments using different cell lines is shown. PGE<sub>2</sub> concentrations on the histogram indicate the mean  $\pm$  SD of 2 independent experiments in triplicate.

as that treated with the same concentration of IL-1 $\beta$  or TNF- $\alpha$  (lane 4), which indicates that the capability of IL-15 for COX-2 induction is comparable to IL-1 $\beta$  and TNF- $\alpha$ . In contrast, the cells treated with IL-2 or IFN- $\gamma$  showed no difference from the unstimulated cells (lane 5 and lane 6, respectively). Again, the amount of COX-1 mRNA in the FLS was not affected by any cytokines used in this study. This suggests that FLS may respond differentially to COX-2 synthesis on cytokine stimulation depending on the type of cytokine.

*Upregulation of COX-2 by IL-15 is partially dependent on the increase of IL-1 $\beta$  and TNF- $\alpha$  production.* Evidence suggests that IL-15 is responsible for the accumulation of TNF- $\alpha$  and IL-1 $\beta$  in RA joints<sup>5-7</sup>. In this study, IL-15 was tested to determine if it could induce TNF- $\alpha$  and IL-1 $\beta$  production in FLS. Experiments using ELISA showed that increasing IL-15 dose-proportionally raised the levels of TNF- $\alpha$  and IL-1 $\beta$  in the FLS culture supernatants (Figure 4A). To confirm the TNF- $\alpha$  production in FLS, we performed intracellular flow cytometry analysis for TNF- $\alpha$ . As shown in Figure 4B, most (> 99%) of the resting FLS at



**Figure 3.** Effect of inflammatory cytokines on COX-2 mRNA expression in synoviocytes. Total cellular RNA was extracted from untreated cells (lane 1) and cells were cultured 6 h with 10 ng/ml each of IL-1 $\beta$  (lane 2), TNF- $\alpha$  (lane 3), IL-15 (lane 4), IL-2 (lane 5), and IFN- $\gamma$  (lane 6). COX-2 mRNA expression in synovial fibroblasts was examined by RT-PCR. A representative band from 3 independent experiments is shown. Histogram illustrates the quantitative COX-2 level normalized to GAPDH level, presented as mean  $\pm$  SD of 3 independent experiments. \*\*\*p < 0.001 versus medium alone.

passage 4 had the surface marker for fibroblasts, CD90, and rarely produced TNF- $\alpha$ . When the cells were stimulated with IL-15, the proportion of TNF- $\alpha$ -producing cells was dose-dependently increased (1.4% for 0.1 ng/ml IL-15, 8.9% for 1 ng/ml IL-15, 24.0% for 10 ng/ml IL-15; Figure 4B). These results imply that the proinflammatory role of IL-15 in the joints of RA patients may be propagated in part by the accumulation of cytokines such as TNF- $\alpha$  and IL-1 $\beta$ .

To determine whether IL-1 $\beta$  and TNF- $\alpha$  released by the IL-15-stimulated FLS could exert the stimulatory effect on COX-2 mRNA expression, FLS were cultured with IL-15 in the presence or absence of blocking antibodies to IL-1 $\beta$  or TNF- $\alpha$ . As shown in Figure 5, mAb to IL-1 $\beta$  (lane 3) or TNF- $\alpha$  (lane 4) partially inhibited the IL-15 mediated induction of COX-2 mRNA expression. On the other hand, COX-2 mRNA expression was not inhibited after cell treatment with anti-IL-10 mAb (lane 5). This suggests that IL-1 $\beta$  and TNF- $\alpha$  may function, at least in part, as mediators of IL-15-stimulated COX-2 induction in the FLS. However, since IL-15 was still able to increase the COX-2 mRNA in spite of the treatment with anti-IL-1 $\beta$  mAb or anti-TNF- $\alpha$  mAb, other mechanisms such as the intrinsic regulation of transcriptional factors or other mediators may also be involved in this upregulation.

DEX and PDTC, but not curcumin, completely blocked IL-15 induced COX-2 upregulation. It has been documented that IL-15 induces activation of the transcription factor NF- $\kappa$ B in human neutrophils<sup>25</sup>, and the inhibitory effect of the glucocorticoids and antioxidant PDTC on NF- $\kappa$ B activation are well known in other types of cells<sup>26,27</sup>. To examine which effectors exist downstream of IL-15-mediated COX-2 induction in the synovial fibroblasts, we tested the effects of

the known inhibitors of the various transcription factors bound to the COX-2 promoter. The results showed that IL-15-induced COX-2 mRNA expression was suppressed by DEX and PDTC. However, curcumin, an AP-1 inhibitor, only partially blocked the IL-15-stimulated COX-2 induction (Figure 6). The inhibitory effects of DEX or PDTC were not due to a nonspecific toxicity, since the viability of the FLS determined by the MTT assay was not influenced by either DEX (0.1 nM to 2  $\mu$ M) or PDTC (10 to 400  $\mu$ M) (data not shown).

NF- $\kappa$ B was one of the major pathways to mediate COX-2 induction by IL-15. It is reported that signaling via NF- $\kappa$ B is involved in regulating the COX-2 expression induced by IL-1 $\beta$  in RA synoviocytes<sup>28</sup>. Knowing that a NF- $\kappa$ B inhibitor, PDTC, but not an AP-1 inhibitor, completely repressed COX-2 induction by IL-15, we examined the level of NF- $\kappa$ B activity for COX-2 transcription within the FLS using a gel mobility shift assay of the NF- $\kappa$ B recognition sequence in the COX-2 promoter. The result showed that the quantity of DNA-protein complex was clearly increased in cells treated with IL-1 $\beta$  and IL-15 (Figure 7A), indicating that induction of COX-2 transcription by these cytokines was interceded by NF- $\kappa$ B binding to the promoter. To examine the NF- $\kappa$ B subtypes that bind to the COX-2 promoter, we performed a supershift analysis using specific antibodies to p65, p59, and c-Rel. As shown in Figure 7B, the proteins associated with the NF- $\kappa$ B site were recognized by mAb to

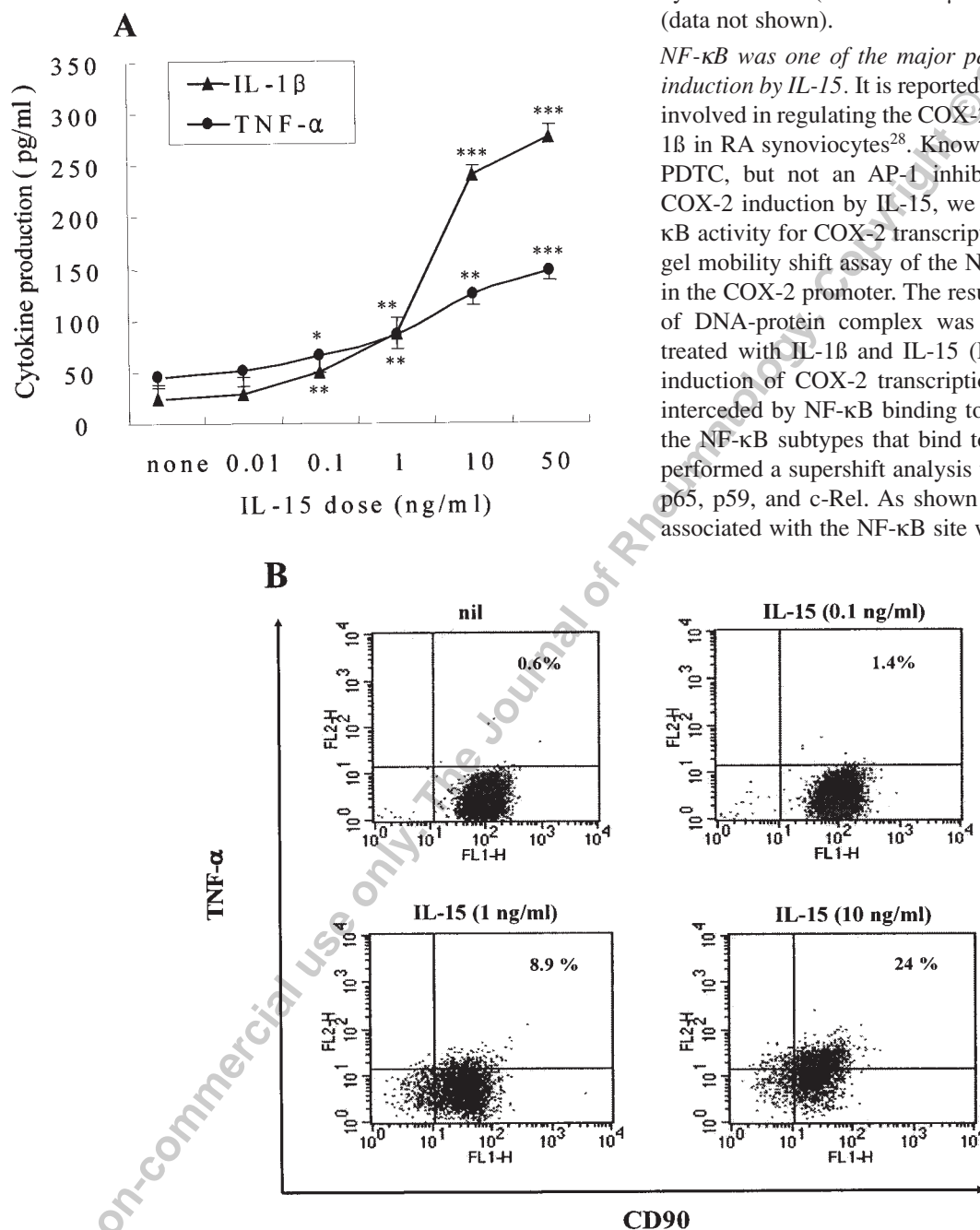
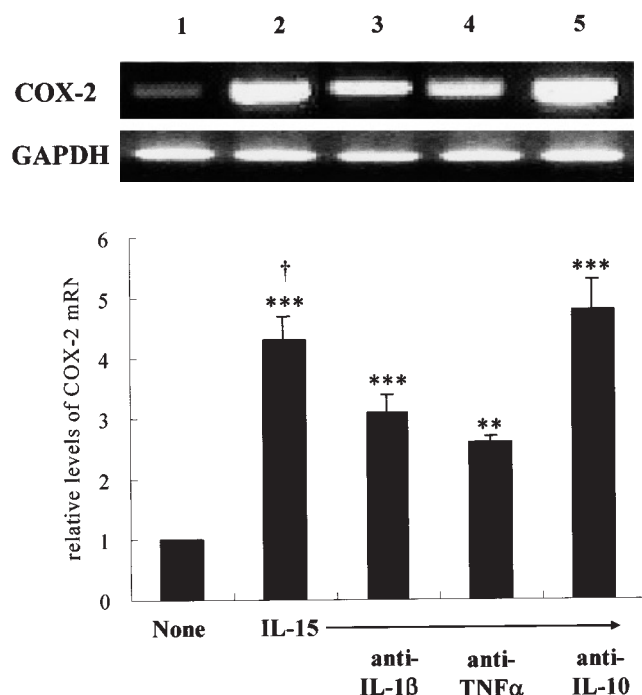
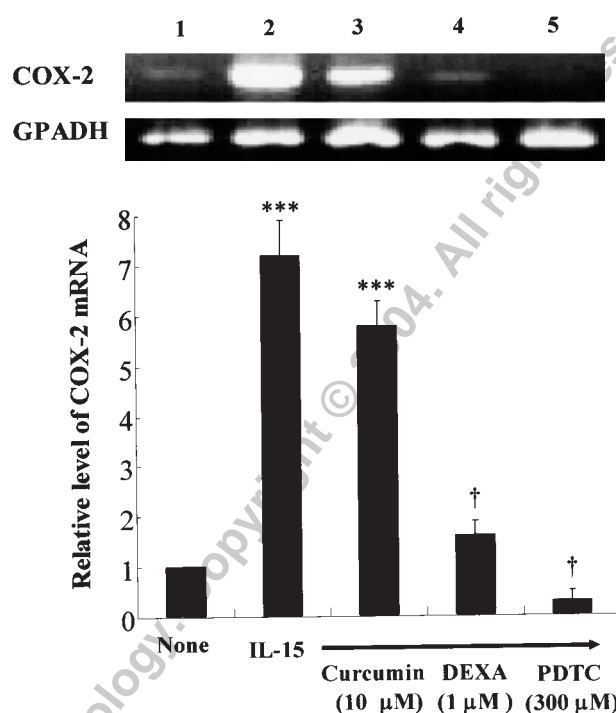


Figure 4. Production of IL-1 $\beta$  and TNF- $\alpha$  by FLS stimulated with IL-15. A. Kinetics of IL-1 $\beta$  and TNF- $\alpha$  protein were determined by ELISA in supernatants of FLS ( $5 \times 10^4$  cells) stimulated with different doses (0.1 to 50 ng/ml) of IL-15 for 24 h. Data are expressed as mean  $\pm$  SD of 5 independent experiments. \* $p$  < 0.05; \*\* $p$  < 0.01; \*\*\* $p$  < 0.001 versus medium alone. B. Flow cytometry analysis for TNF- $\alpha$  production in FLS. At passage 4, FLS were stained with FITC-conjugated anti-CD90 and PE-conjugated anti-TNF- $\alpha$  antibodies. Representative result from 2 independent experiments using different cell lines is shown.



**Figure 5.** Antibodies to IL-1β or TNF-α partially inhibited COX-2 induction by IL-15. RA synovial fibroblasts were treated with 10 ng/ml IL-15 for 6 h without (lane 2) or with neutralizing antibodies against 10 μg/ml IL-1β (lane 3), TNF-α (lane 4), and IL-10 (lane 5). RNA extracts were prepared as described and analyzed by RT-PCR with primers specific to COX-2 and GAPDH. A representative band from 3 independent experiments is shown. Histogram represents the relative amount of COX-2 transcripts normalized to GAPDH level. Data are presented as mean ± SD of 3 independent experiments. \*\*p < 0.01, \*\*\*p < 0.001 versus medium alone; †p < 0.05 versus cells treated with 10 ng/ml IL-15 + anti-IL-1β or anti-TNF-α neutralizing antibodies.



**Figure 6.** PDTC and DEX, but not curcumin, abolished the increase of COX-2 mRNA induced by IL-15. COX-2 mRNA expression after treatment without (lane 1) or with 10 ng/ml IL-15 (lane 2), IL-15 10 ng/ml plus curcumin 10 μM (lane 3), IL-15 plus DEX 1 μM (lane 4), IL-15 plus PDTC 300 μM (lane 5). The mRNA levels are expressed as the fold-increase relative to mRNA level in unstimulated control cells, and corrected for GAPDH mRNA levels. A representative band from 3 independent experiments is shown. Data on the histogram indicate the mean ± SD of 3 independent experiments. \*\*\*p < 0.001 versus medium alone; †p < 0.001 versus cells cultured with 10 ng/ml IL-15.

p65 and p50, but not by anti-c-Rel. Together, these data clearly show that NF-κB is one of the major pathways mediating IL-15-induced COX-2 upregulation.

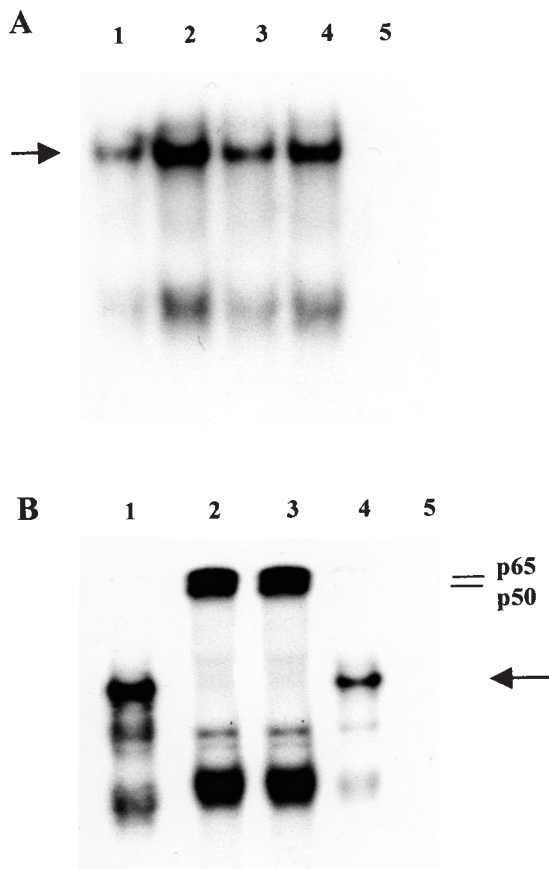
**Suppression of IL-15-induced COX-2 upregulation by NS-398 — role of PGE<sub>2</sub>.** COX-2 is the rate-limiting enzyme of PGE<sub>2</sub> synthesis. As shown in Figure 2C, IL-15 time-dependently increased PGE<sub>2</sub> production from FLS. Thus, it was of great interest to investigate the role of PGE<sub>2</sub> in COX-2 production by FLS stimulated with IL-15. Assays performed in the presence of the COX-2 selective inhibitor NS-398 (10 to 1000 nM) showed that this agent dose-dependently decreased COX-2 mRNA and protein expression (Figure 8). Moreover, when PGE<sub>2</sub> was added to the medium in increasing concentrations along with NS-398 and 10 ng/ml IL-15, the inhibitory effect of NS-398 on COX-2 mRNA and protein production was dose-dependently reversed. These results suggest that IL-15-induced COX-2 upregulation is PGE<sub>2</sub>-dependent.

## DISCUSSION

The inducible COX-2 isozyme is responsible for the prompt

upregulation of prostaglandins during the inflammatory response, which has made it the target enzyme of nonsteroidal antiinflammatory drugs (NSAID)<sup>29</sup>. In the RA synovium, COX-2 expression appears to be induced by a variety of stimuli including cytokines, immune complexes, and hypoxic stress<sup>30</sup>. However, it is not known if IL-15 accumulation in the patient's joints also affects COX-2 production. The general belief has been that IL-15 primarily activates the infiltrated T lymphocytes, but also stimulates the synovial fibroblasts by binding to functional IL-15 receptor<sup>8</sup> or through the induction of IL-17 production<sup>31</sup>. Our study demonstrates that IL-15 strongly activates COX-2 synthesis in the FLS, providing additional information on the inflammatory network of T cells, macrophages, and synovial fibroblasts in the RA synovium.

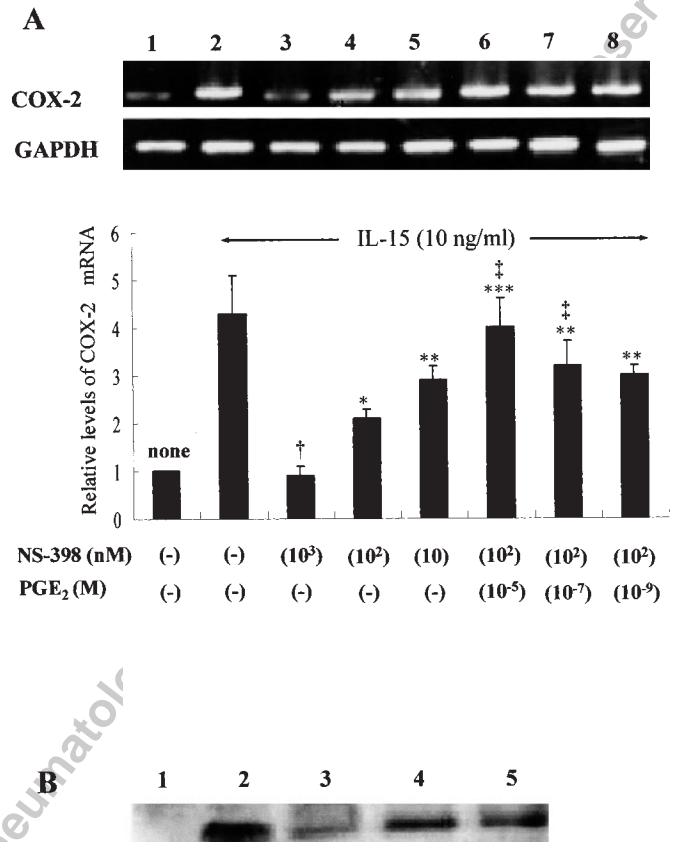
In this study, IL-15 was found to dose-dependently increase IL-1β and TNF-α production from the FLS. A similar observation was reported from a T cell study, where it was observed that IL-15 stimulation directly upregulated TNF-α synthesis and amplified the inflammatory responses<sup>6,7</sup>. Moreover, this study found that the IL-15-



**Figure 7.** Gel mobility shift assay of NF- $\kappa$ B binding activity to COX-2 promoter. **A.**  $^{32}$ P-labeled oligonucleotide representing the NF- $\kappa$ B recognition site was incubated with nuclear extract of untreated FLS (lane 1), and extracts prepared from cells treated with 10 ng/ml IL-1 $\beta$  (lane 2), 1 ng/ml IL-15 (lane 3), and 10 ng/ml IL-15 (lane 4). In lane 5, binding of nuclear extract was chased with 100-fold excess of unlabeled oligonucleotide probe. **B.** Supershift assay of the NF- $\kappa$ B site using antibodies against p65 (lane 2), p50 (lane 3), and c-Rel (lane 4). In lane 5, binding of nuclear extract was chased with 100-fold excess of unlabeled oligonucleotide probe. Cells in all lanes were prestimulated with IL-15. Arrows denote labeled oligonucleotide band shifted upon NF- $\kappa$ B binding. Representative results from 3 independent experiments using cell lines from 3 RA patients are shown.

induced activation of COX-2 expression appears to be mediated by the increase in IL-1 $\beta$  and TNF- $\alpha$  levels, at least in part. Overall, these findings suggest interdependent associations among TNF- $\alpha$ , IL-1 $\beta$ , and IL-15 that promote and maintain the proinflammatory environments in RA joints. In this respect, it is hypothesized that IL-15 could act upstream of TNF- $\alpha$  and IL-1 $\beta$  and trigger the production of inflammatory mediators including prostaglandins in chronically inflamed RA synovium, either directly or through the increase in IL-1 $\beta$  and TNF- $\alpha$  production.

It is well established that macrophage-like synoviocytes are the main cell type to produce IL-1 $\beta$  and TNF- $\alpha$ <sup>32</sup>. We found that IL-15 had a similar effect on the production of COX-2 by cultured macrophages of healthy controls or



**Figure 8.** Role of PGE<sub>2</sub> on IL-15-induced COX-2 upregulation. FLS were stimulated with 10 ng/ml IL-15, and then analyzed for COX-2 mRNA and protein expression by RT-PCR and Western blot, respectively. **A.** COX-2 mRNA expression after treatment without (lane 1) or with IL-15 (lane 2), IL-15 + NS-398 1000 nM/l (lane 3), IL-15 + NS-398 100 nM/l (lane 4), IL-15 + NS-398 10 nM/l (lane 5), IL-15 + NS-398 (100 nM/l) + PGE<sub>2</sub> (10<sup>-5</sup> M) (lane 6), IL-15 + NS-398 (100 nM/l) + PGE<sub>2</sub> (10<sup>-7</sup> M) (lane 7), and IL-15 + NS-398 (100 nM/l) + PGE<sub>2</sub> (10<sup>-9</sup> M) (lane 8). The mRNA levels are expressed as fold-increase relative to mRNA level in unstimulated control cells, and corrected for GAPDH mRNA levels. **B.** COX-2 protein expression after treatment without (lane 1) or with IL-15 (lane 2), IL-15 + NS-398 100 nM/l (lane 3), IL-15 + NS-398 (100 nM/l) + PGE<sub>2</sub> (10<sup>-5</sup> M) (lane 4), and IL-15 + NS-398 (100 nM/l) + PGE<sub>2</sub> (10<sup>-7</sup> M) (lane 5). Representative band from 3 independent experiments is shown. Data on the histogram indicate mean  $\pm$  SD of 3 independent experiments. \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001 versus medium alone; † $p$  < 0.001 versus cells stimulated with 10 ng/ml IL-15; ‡ $p$  < 0.01 versus cells treated with 10 ng/ml IL-15 + NS-398 100 nM/l.

patients with RA (data not shown). Thus, it is possible that the increased number of contaminating CD14(+) macrophage-like synoviocytes stimulated by IL-15 may relate directly to cytokine induction, particularly TNF- $\alpha$ . However, there is also ample evidence of an important role of FLS in the production of TNF- $\alpha$ <sup>23,33,34</sup>. We clearly showed that synoviocytes expressing the surface marker for fibroblasts, CD90, produced large amount of TNF- $\alpha$  when they were stimulated by IL-15. Our data support the notion that, similarly to macrophage-like synoviocytes, FLS may



be actively involved in the amplification of inflammatory cascades in RA joints by secreting proinflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$ .

That IL-15 exerts biological properties similar to IL-2 is not surprising, considering that these cytokines share 2 out of 3 subunits of their cognate receptors<sup>35</sup>. Interestingly, this study showed that IL-2 did not affect the synthesis of COX-2 mRNA in the FLS, in contrast to the strong induction upon IL-15 treatment. Such difference may be due to exclusive signaling through the  $\alpha$ -subunit of the IL-15 receptor complex that is unique from its counterpart in the IL-2 receptor. In this regard, the IL-15-mediated induction of COX-2 expression is most likely to be propagated through  $\alpha$ -chain-associated signaling factors such as Syk<sup>36</sup>, rather than via the Jak/Stat pathway, which is shared by IL-2 and IL-15 receptors.

A promoter of the COX-2 gene contains binding sites for NF- $\kappa$ B and AP-1<sup>22,28,37</sup>, which are universal downstream effectors of the various inflammatory signals. Since IL-15 has been shown to induce both NF- $\kappa$ B and AP-1 activation in human neutrophils<sup>25</sup>, we tested whether chemical inhibitors of these transcription factors influence IL-15-mediated COX-2 induction in FLS. The results showed that curcumin, an AP-1 blocker, did not greatly if at all affect the IL-15-induced increase in COX-2 transcription. In contrast, treating the FLS with PDTC reduced the quantity of COX-2 mRNA, indicating that the signal from IL-15 is mainly transferred to the COX-2 promoter via NF- $\kappa$ B, which was confirmed by EMSA for NF- $\kappa$ B activity. However, other factors in the supernatant such as IL-1 $\beta$  and TNF- $\alpha$  might be implicated, because they could be instigated by IL-15 stimulation. As well, the stimulatory effect of IL-15 on COX-2 expression involves additional mediators that remain to be identified. Further experiments including a promoter study with an appropriate mutant are required to dissect the signaling pathway implicated solely in IL-15 induction of COX-2 mRNA.

It has been reported that COX-2 is mostly regulated at the posttranscriptional level, and this regulation is p38 and PGE<sub>2</sub>-dependent<sup>38,39</sup>. Again, posttranscriptional mRNA destabilization may be an important mechanism in the downregulation of COX-2 mRNA by DEX<sup>40</sup>. In our study, IL-15 time-dependently increased PGE<sub>2</sub> production by FLS (Figure 2C). Moreover, NS-398, which is known to selectively inhibit endogenous PGE<sub>2</sub> production, mitigated the IL-15-induced COX-2 upregulation, and the inhibitory effect of NS-398 on COX-2 expression was reversed by the addition of PGE<sub>2</sub> (Figure 8). These results, together with previous findings<sup>38,40</sup>, suggest that IL-15-stimulated PGE<sub>2</sub> may augment COX-2 expression in FLS by the modification of COX-2 mRNA stability at the posttranscriptional level. The data are also consistent with reports describing the positive feedback role of PGE<sub>2</sub> in COX-2 regulation<sup>39,41,42</sup>. In this case, the cycle of RA inflammation could be broken by

treatment with NSAID or DEX through the downregulatory effect of these drugs on COX-2 expression (Figure 8 and Figure 6, respectively).

Our study describes for the first time the effect of IL-15 on the upregulation of COX-2 in a PGE<sub>2</sub>-dependent manner. The activation of NF- $\kappa$ B bound to the COX-2 promoter appears to be a downstream target of IL-15 stimulation in fibroblast-like synoviocytes, exerted either directly or through the increase in IL-1 $\beta$  and TNF- $\alpha$  production. These results may provide an additional clue to the processes of the inflammatory network of T cells and synoviocytes in rheumatoid synovium.

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