Upregulation of Synoviocyte COX-2 Through Interactions with T Lymphocytes: Role of Interleukin 17 and Tumor Necrosis Factor-α

LISA K. STAMP, LESLIE G. CLELAND, and MICHAEL J. JAMES

ABSTRACT. ***Objective.*** T lymphocytes infiltrating rheumatoid synovium may alter the function of resident synoviocytes. We investigated the influence on synoviocyte cyclooxygenase-2 (COX-2) expression and prostaglandin E_2 (PGE_2) production exerted by soluble factors released by T cells, with particular reference to interleukin 17 (IL-17).

Methods. Human peripheral blood T cells were stimulated with antibodies directed against CD3 and CD28. Harvested T cell supernatants were applied to cultured human fibroblast-like synoviocytes in culture. The effects of IL-17 alone and in combination with tumor necrosis factor-α (TNF-α) were examined using recombinant cytokines and neutralizing antibodies. Synoviocyte COX-2 expression and PGE_2 production were examined.

Results. Supernatants from stimulated T cells upregulated COX-2 expression and increased PGE_2 production by synoviocytes. The T cell supernatants were found to contain IL-17 and TNF-α. Recombinant IL-17 upregulated synoviocyte COX-2 expression and enhanced TNF-α stimulated synoviocyte COX-2 expression. The upregulation of synoviocyte COX-2 expression by supernatants from stimulated T cells was partially inhibited by addition of neutralizing antibodies against IL-17 or TNF-α or by treatment of T cells with cyclosporin A prior to stimulation.

Conclusion. Activated T cells are capable of paracrine upregulation of synoviocyte COX-2 expression and PGE_2 production through release of soluble mediators. T cell derived IL-17, especially in combination with TNF-α, may contribute to ongoing inflammation through its effects on COX-2 expression and PGE_2 production. These data provide additional evidence for the contribution of T cells in rheumatoid inflammation and highlight the potential of IL-17 as a therapeutic target. (J Rheumatol 2004;31:1246–54)

Key Indexing Terms:
SYNOVIOCYTES T LYMPHOCYTES EICOSANOIDS CYCLOOXYGENASE

In rheumatoid arthritis (RA), CD4+ T cells are prominent in the inflammatory cell infiltrate within the synovium. However, concentrations of Th1 cytokines including interferon-γ (IFN-γ), the prototypic cytokine of Th1 cells, are low[1-4], compared to those in other Th1 mediated disease processes[5,6]. Levels are also low when compared to the monocyte/macrophage derived cytokines interleukin 1ß (IL-1ß) and tumor necrosis factor-α (TNF-α), which have been implicated in the inflammatory process and joint destruction seen in RA[7,8].

Recently, high concentrations of the T cell derived cytokine IL-17 have been reported in synovial fluid from rheumatoid joints[9]. IL-17 is considered a proinflammatory cytokine, since it stimulates macrophage production of IL-1ß, TNF-α, and prostaglandin E_2 (PGE_2)[10]. IL-17 has also been shown to enhance the effects of IL-1ß on IL-6 production[11] and of TNF-α on IL-1, IL-6, and IL-8 production by synoviocytes[12]. In explants of osteoarthritic knee menisci, IL-17 synergistically enhanced IL-ß or TNF-α stimulated PGE_2 production[13].

We investigated whether T cells are capable of upregulating synoviocyte COX-2 expression and PGE_2 production, and attempted to define the role of T cell derived IL-17 in mediating these effects.

MATERIALS AND METHODS

Materials were obtained from the following sources: cyclosporin A (CSA), Extravidin (Sigma Chemical Co., St. Louis, MO, USA); aspirin (Roche, Sydney, Australia); NS398 (Cayman Chemicals, Ann Arbor, MI, USA); monoclonal antibodies (mAb) against human CD3, CD14, CD16, CD19, CD28, and polyclonal FITC-conjugated goat anti-mouse (Becton Dickinson Pharmingen, Mountain View, CA, USA); isotype matched control mAb IB5 (IgG_1, against *Giardia intestinalis*) was kindly provided by L. Spargo (Arthritis Research, Royal Adelaide Hospital, Adelaide, Australia); the isotype matched control mAb 1D4.5 (IgG_{2a}), 1A6.11 (IgG_{2b}), and 1A6.12 (IgM) were a gift from Dr. L. Ashman (Institute of Medical and Veterinary Science, Adelaide, Australia); pyrogen-free Lymphoprep™ (Nycomed, Oslo, Norway); 6 and 24 well plates (Nunc, Roskilde, Denmark); transfer-blot membrane (BioRad, North Ryde,

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Australia); PGE_2 standard, 6-keto-$PGF_{1\alpha}$ standard, thromboxane B_2 (TXB_2) standard, and anti-COX-2 polyclonal Ab (Cayman Chemicals); ^{3}[H]PGE_2, ^{3}[H]6-keto-$PGF_{1\alpha}$, ^{3}[H]TXB_2 (Amersham, Little Chalfont, England); TXB_2 and PGE_2 anti-sera were prepared from rabbits immunized with TXB_2 and PGE_2 conjugated to thyroglobulin[14,15], 6-keto-$PGF_{1\alpha}$ antisera (Sigma); recombinant (r)TNF-α, TNF-α mAb, biotin labeled TNF-α mAb (Endogen, Woburn, MA, USA); rIL-17, neutralizing IL-17 mAb, biotin labeled IL-17 Ab, neutralizing TNF-α mAb (R&D Systems, Minneapolis, MN, USA); and rIL-1ß (Boehringer Mannheim).

T cell isolation. Human mononuclear cells were purified from fresh buffy coat obtained from the Red Cross Blood Centre, Adelaide, South Australia. Buffy coats, diluted 1:2 with phosphate buffered saline (PBS) were layered over pyrogen-free Lymphoprep™ density separation medium (density 1.077 g/ml). Cells were centrifuged at 160 g for 10 min and the platelet-rich supernatant removed. The mononuclear cell enriched fraction was isolated by centrifugation for a further 20 minutes @ 400 × g. The mononuclear cell enriched fraction was passed through nylon fiberglass columns as described[16] to obtain a T cell enriched population. The cells were on average 85% CD3 positive T cells as determined by fluorescence activated cell sorting (FACS). The major contaminating cells were CD16 positive cells (8%). CD14 positive monocytes accounted for only 0.4% of cells, and platelets were less than 1×10^6/ml when T cells were resuspended at 2×10^6/ml.

T cell stimulation. T cells were prepared, as described above, one day prior to the experiment; 24-well tissue culture plates were precoated with anti-CD3 (2 µg/ml); then 1×10^6 T cells in 1 ml RPMI 10% fetal calf serum (FCS) were added to the plates and soluble anti-CD28 added to a final concentration of 250 ng/ml. After incubation for specified periods, T cells and supernatants were separated by centrifugation.

To inhibit T cell activation, T cells were treated with CSA (100 ng/ml) for 30 min prior to stimulation with immobilized anti-CD3 and soluble anti-CD28. Trypan blue exclusion test revealed cell survival of > 95% at 24 h.

Synoviocyte culture. Synovial fluid was collected from patients with inflammatory arthritis (RA or spondyloarthropathy) undergoing joint aspiration with their written informed consent. Fluid was centrifuged 10 min and the supernatant removed. The cells were resuspended in 6 ml warm RPMI 20% FCS with 2 µg/ml amphotericin and transferred to 50 ml tissue culture flasks. Cells were incubated at 37°C, 5% CO_2. Confluent fibroblast-like synoviocytes were released from the dish using 0.05% trypsin/0.53 M EDTA and subcultured to confluence. Experiments were undertaken on cells between the 3rd and 5th passages. FACS analysis revealed no CD14 positive cells at the 3rd passage. Culture of synoviocytes using this technique has been reported[17].

Incubation of synoviocytes with T cell supernatants. One day before the experiment, synoviocytes were trypsinized and resuspended in RPMI 20% FCS at 2.5×10^5/ml. 5×10^5 cells were added to each well of 6 well tissue culture plates. Synoviocytes were incubated overnight at 37°C, 5% CO_2. The following day the medium was removed, cells washed once with RPMI 10% FCS, and 1 ml of fresh RPMI 10% FCS added to each well. 1 ml of T cell culture supernatant was added to each well followed by culture for 18 h at 37°C, in 5% CO_2.

Inhibition of COX-1 and COX-2. Prior to test incubation, synoviocytes were treated with aspirin (55 µM) for 30 min to irreversibly inhibit COX-1. Cells were then washed twice and resuspended in fresh medium to remove any residual aspirin. The transient aspirin treatment followed by aspirin removal before cell stimulation allows inhibition of resident COX-1 with no effect on COX-2, which is not present in unstimulated cells and if upregulated by stimulation, is not exposed to aspirin[18]. NS398 (0.5 µM) was used to selectively inhibit COX-2.

Eicosanoid measurement. Thromboxane A_2 is unstable, being rapidly hydrolyzed to the more stable metabolite TXB_2, which was measured. PGE_2, 6-keto-$PGF_{1\alpha}$, and TXB_2 were determined by radioimmunoassay as described[19,20].

IL-17 and TNF-α ELISA. ELISA for TNF-α and IL-17 were developed using commercially available antibodies listed above. Cross-reactivities of the capture antibody for IL-17 are listed by the manufacturers as not significant for a range of cytokines, including TNF-α, IL-1ß, macrophage-colony stimulating factor (M-CSF), and granulocyte macrophage-colony stimulating factor (GM-CSF); and for the TNF-α assay cross-reactivities with IL-1ß, M-CSF, or GM-CSF were < 0.1%. The range of detection for each assay was 15–1000 pg/ml. Nunc plates were coated with capture antibody against IL-17 or TNF-α (5 µg/ml in 0.2 mol/l Na_2CO_3, pH 9.4) overnight at 4°C. Wells were washed with wash buffer (PBS with 0.05% Tween 20). The plate was then blocked with 0.5% bovine serum albumin (BSA) (1 h, 37°C) and then washed with wash buffer. Serial dilutions of standard cytokines or sample were added (50 µl) and then the biotin-labeled secondary antibody (50 µl) was added and incubated for 2 h at room temperature. After washing, Extravidin® peroxidase was added (in 0.5% BSA) for 15 min at 37°C, followed by the peroxidase substrate tetramethylbenzidine (TMB) in 0.5 M phosphate citrate buffer according to the manufacturer's instructions. The reaction was stopped with H_2SO_4 (2 mol/l, 100 µl). Absorbance was measured at 450 nm in a microplate reader.

Western immunoblot. Synoviocytes were trypsinized, washed twice with PBS and then equal amounts of lysis buffer (HEPES buffered Hanks' balanced salt solution, pH 7.4, 0.5% Triton X-100, 10 µg/ml leupeptin, 10 µg/ml aprotinin) and 2× sample buffer (0.125 M Trizma base, pH 6.8, 20% glycerol, 4% sodium dodecyl sulphate, 10% 2-mercaptoethanol) were added. Samples were heated at 95°C for 7 min and then stored at –20°C. The samples were subjected to electrophoresis on a 9% acrylamide gel and the proteins were transferred onto Trans-Blot® membrane. The membrane was soaked for 1 h at room temperature in Tris-buffered saline (TBS; 25 mM Tris HCl, 0.2 M NaCl, 0.15% Tween-20, pH 7.6) containing 5% dried skim milk (w/v). Membranes were exposed to specific COX-1 polyclonal Ab (10 µg/ml) or specific COX-2 pAb (5 µg/ml) overnight or ß-actin mAb (0.5 µg/ml) for 1 h. Membranes were washed and then exposed to horseradish peroxidase-labeled goat anti-mouse or donkey anti-rabbit antibody (1:20,000 dilution) for 1 h. Antibody binding was revealed using an enhanced chemiluminescence kit, ECL™ (Amersham), according to the manufacturer's instructions.

Statistical analysis. Statistical analysis was undertaken using ANOVA followed by Neuman-Keuls tests for multiple comparisons. Results are expressed as mean ± SEM.

Ethical approval for the study was obtained from the Royal Adelaide Hospital Human Ethics Committee.

RESULTS

Expression of COX-1 and COX-2 in synoviocytes and eicosanoid production in response to IL-1β. COX-1 was readily detectable in both stimulated and unstimulated synoviocytes by Western immunoblot. Expression of COX-2 could be induced by stimulation of synoviocytes with IL-1ß (2 ng/ml; Figure 1). The induction of COX-2 by IL-1ß was associated with a significant increase in PGE_2 (Figure 1) and 6-keto-$PGF_{1\alpha}$ production (data not shown). TXB_2 was not detected (data not shown).

COX-1 and COX-2 were inhibited selectively to determine their relative contribution to prostaglandin synthesis. Resting synoviocytes, in which only COX-1 is present, were pretreated with aspirin (55 µM, 30 min) and washed before induction of COX-2 with IL-1ß. In this way, COX-1 is irreversibly inhibited in the presence of active COX-2[18]. To inhibit COX-2 activity selectively, NS398 (0.5 µM) was added to the cells. COX-2 inhibition resulted in reduction of

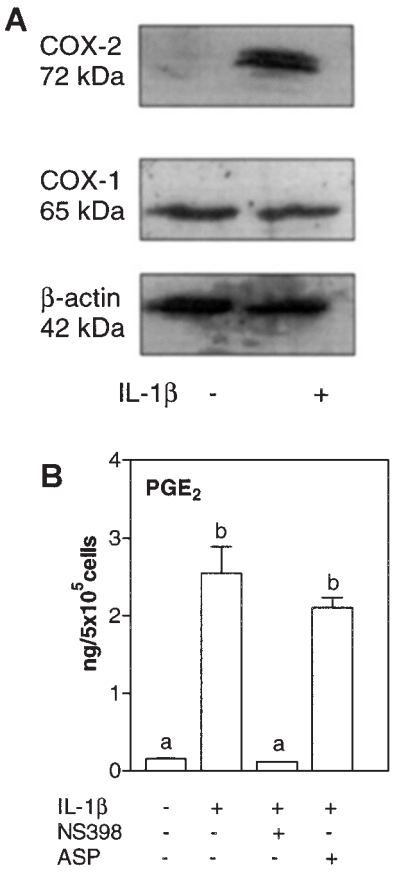

Figure 1. Cyclooxygenase expression and PGE_2 production by synoviocytes. A. Synoviocytes (5×10^5 cells/well) were treated with IL-1ß at a final concentration of 2 ng/ml for 18 h, and cells were pelleted and processed for Western immunoblot. B. Effect of COX-1 and COX-2 inhibition on synoviocyte PGE_2. Synoviocytes (5×10^5 cells/well) were treated with IL-1ß at a final concentration of 2 ng/ml for 18 h with either COX-1 inhibition (ASP: aspirin pretreatment followed by washing before the addition of IL-1ß) or COX-2 inhibition (NS398). Data shown are representative of 3 separate experiments using synoviocytes from 3 different donors. Bars with different letters are significantly different from each other, $p < 0.05$; ANOVA followed by Neuman-Keuls test for multiple comparisons.

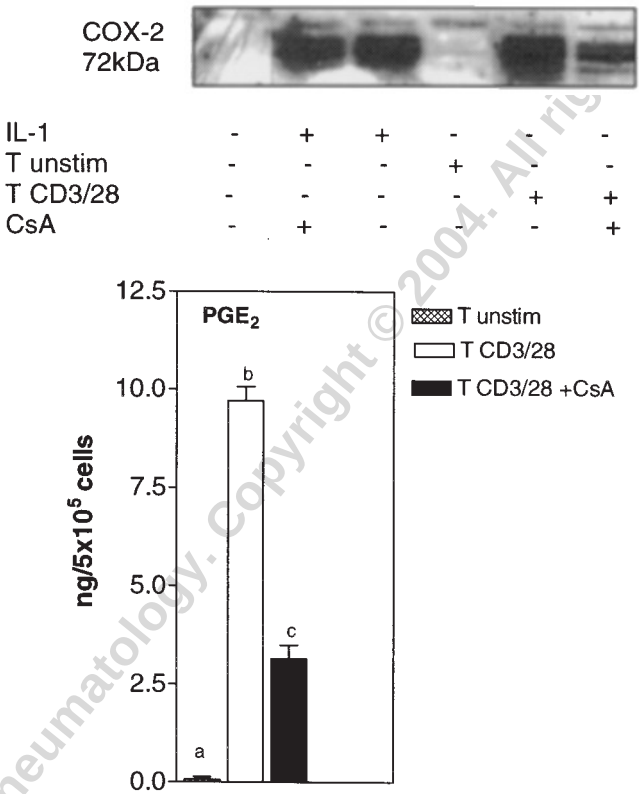

Figure 2. Effect of T cells on synoviocyte COX-2 expression and eicosanoid synthesis. Synoviocytes (5×10^5 cells/well) were incubated with either IL-1ß, T cell supernatants from unstimulated T cells (T unstim), T cell supernatants from cells stimulated with anti-CD3/anti-CD28 for 18 h (T CD3/28), or T cells treated with CSA (100 ng/ml) starting 30 min before stimulation with anti-CD3/anti-28 (T CD3/28 + CsA). Synoviocytes were also treated directly with CSA (100 ng/ml; CsA). After 18 h, cells and supernatants were collected for Western immunoblot and eicosanoid assay, respectively. Bars with different letters are significantly different from each other, $p < 0.05$; ANOVA followed by Neuman-Keuls test for multiple comparisons. Data shown are representative of 4 separate experiments using T cells from 4 different donors and fibroblast-like synoviocytes from 4 different donors.

PGE_2 and 6-keto-$PGF_{1\alpha}$ synthesis, while COX-1 inhibition had no effect on synthesis of these prostaglandins (Figure 1). This preliminary experiment confirmed that resting synoviocytes produce only small amounts of eicosanoids and that stimulated cells produce more substantial amounts through induced COX-2.

Effect of T cell supernatants on COX-2 expression and eicosanoid production by synoviocytes. Supernatants from activated T cells upregulated synoviocyte COX-2 expression. This upregulation was inhibited, at least partially, by treatment of T cells with CSA (100 ng/ml; Figure 2). CSA treatment of synoviocytes had no direct effect on synoviocyte COX-2 expression induced by IL-1ß (Figure 2). The induction of synoviocyte COX-2 was associated with an increase in PGE_2 (Figure 2) and 6-keto-$PGF_{1\alpha}$ production (data not shown). Neither PGE_2 nor 6-keto-$PGF_{1\alpha}$ were detected in supernatants from activated T cells (data not shown), confirming that the eicosanoids measured in the supernatants from synoviocytes/T cell incubations were produced by the synoviocytes. Neither PGE_2 nor 6-keto-$PGF_{1\alpha}$ were detected in supernatants from synoviocytes treated with soluble anti-CD28 (data not shown).

When supernatants obtained from cultures from increasing numbers of stimulated T cells were added to synoviocytes, there was a concentration-dependent increase in synoviocyte COX-2 expression and prostaglandin production (Figure 3).

Time course for generation of synoviocyte-stimulating factors by stimulated T cells. T cells (1×10^6/ml) were stimulated with anti-CD3 plus anti-CD28 and supernatants

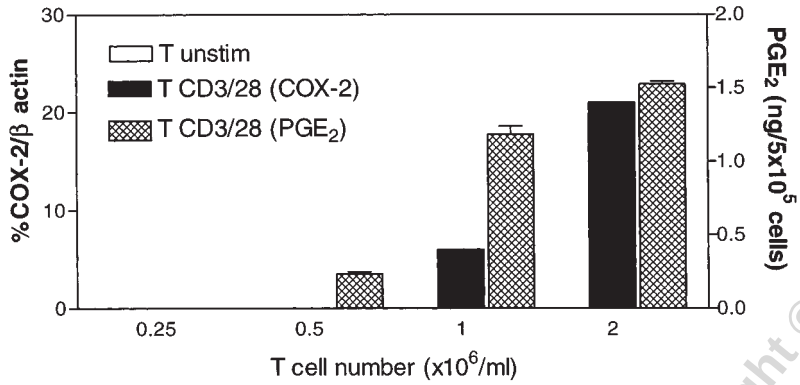

Figure 3. Effect on synoviocytes of supernatants obtained from incubating varying numbers of unstimulated T cells (T unstim) and T cells stimulated with anti-CD3/anti-CD28 (T CD3/28) for 18 h. Synoviocytes (5×10^5 cells/well) were incubated 18 h with T cell supernatants. COX-2 expressed as a percentage of intensity of ß-actin staining. PGE_2 production: mean ± SEM of triplicate determinations.

collected at 0, 2, 4, 8, and 18 h. Supernatants were stored at 4°C, and after warming to room temperature were added to synoviocytes (5×10^5), which were then incubated 18 h at 37°C. There was a time-dependent increase in T cell supernatant activity with regard to induction of synoviocyte COX-2 expression and PGE_2 production (data not shown).

Time course of synoviocyte COX-2 expression and PGE_2 synthesis: *comparison of IL-1ß with T cell supernatants*. Synoviocytes were stimulated with either IL-1ß or supernatants from activated T cells prepared by incubation of T cells in the presence of anti-CD3/anti-CD28 for 18 h. T cell supernatants were as potent as IL-1ß with regard to induction of synoviocyte COX-2 expression. While the release of PGE_2 was slightly delayed in synoviocytes stimulated with T cell supernatants, there was no significant difference at 18 h (Figure 4).

Release of IL-17 by stimulated T cells. T cells produce several proinflammatory cytokines that are potential mediators in the upregulation of synoviocyte COX-2. With stimulation of T cells by anti-CD3/anti-CD28, there was release of IL-17 and TNF-α. Synthesis of these cytokines was inhibited by treatment of T cells with CSA (100 ng/ml; Figure 5). IL-1ß was not detected in supernatants from activated T cells (data not shown). It has been reported[10] that IL-17 upregulates synoviocyte PGE_2 production and therefore we examined whether it could be responsible for upregulation of synoviocyte COX-2 induced by supernatants from stimulated T cells.

Effect of IL-17 on COX-2 activity in synoviocytes. The addition of rIL-17 to synoviocytes resulted in a concentration-dependent upregulation of COX-2 and increased PGE_2 production (Figure 6). However, at concentrations approximating the level detected in the T cell supernatants, the effect on synoviocyte COX-2 expression and PGE_2 production was small. IL-17 neutralizing antibodies were used to assess the importance of IL-17 in the stimulated T cell supernatants on the upregulation of synoviocyte COX-2. T cells were stimulated with anti-CD3/anti-CD28 for 24 h, then supernatants were collected and incubated with IL-17 neutralizing antibodies (1 µg/ml) for 4 h prior to their addition to synoviocytes. The addition of IL-17 neutralizing antibody resulted in partial inhibition of synoviocyte COX-2 expression and a more substantial reduction in PGE_2 production (Figure 7).

Effect of TNF-α blockade upon synoviocyte stimulating activity in supernatants from activated T cells. To investigate the role of T cell-derived TNF-α in the upregulation of synoviocyte COX-2 induced by supernatants from stimulated T cells, TNF-α neutralizing antibodies were added to the supernatants. Supernatants from T cells stimulated with anti-CD3 and anti-CD28 for 24 h were incubated with neutralizing antibodies against TNF-α (1 µg/ml) for 4 h prior to addition to synoviocytes. After a further 18 h incubation, cells and supernatants were collected. The presence of TNF-α neutralizing antibodies resulted in substantial inhibition of the T cell supernatant stimulating activity for synoviocyte COX-2 expression and PGE_2 production (Figure 7).

Effect of IL-17 in combination with TNF-α on synoviocyte COX-2. To examine whether there was synergy between TNF-α and IL-17, synoviocytes were incubated with TNF-α (2 ng/ml) in the presence or absence of IL-17. The addition of TNF-α at 2 ng/ml, which is in the range detected in T cell supernatants under these experimental conditions, had no effect on synoviocyte COX-2 expression or PGE_2 production. Similarly, the addition of IL-17 at 1 ng/ml, which approximates the concentration detected in T cell supernatants under these experimental conditions, had no detectable effect on synoviocyte COX-2 expression or eicosanoid production when used alone. However, the combination of IL-17 and TNF-α resulted in enhanced

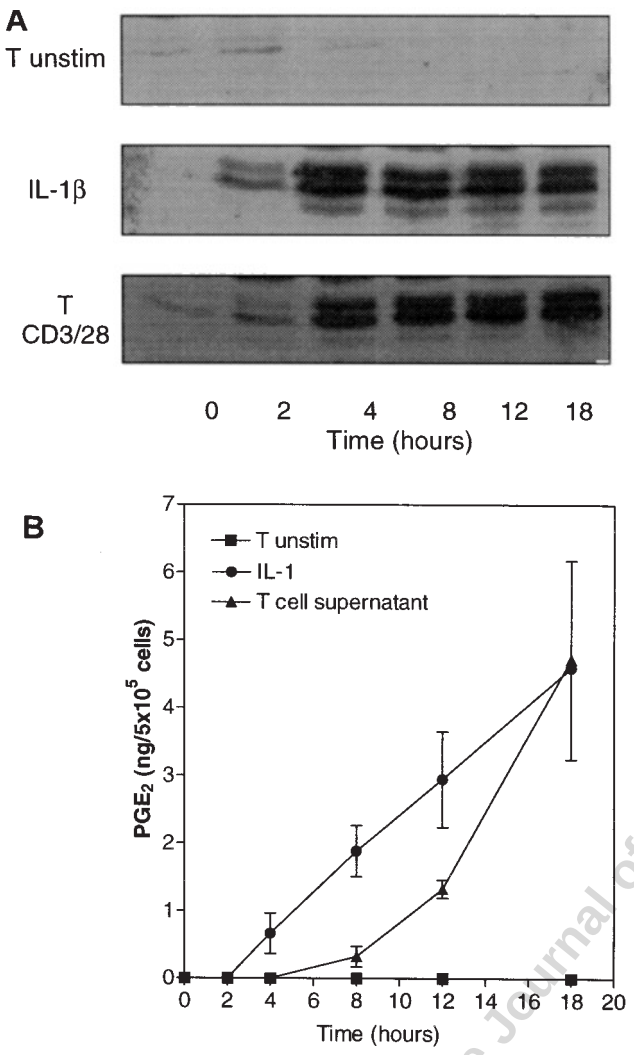

Figure 4. Time course of (A) COX-2 expression and (B) PGE_2 production (mean ± SEM of triplicate determinations) by synoviocytes stimulated with either IL-1ß (2 ng/ml) or supernatants from T lymphocytes that had been cultured in the absence (T unstim) or presence of anti-CD3/anti-CD28 (T CD3/28) for 18 h.

synoviocyte COX-2 expression and PGE_2 production (Figure 8).

DISCUSSION

The results indicate that soluble mediators produced by T cells stimulated by antibodies against CD3 and CD28 can upregulate synoviocyte COX-2 expression and PGE_2 production. Further, these effects can be at least partially inhibited by CSA.

T cells produce a number of cytokines that are candidates for the paracrine upregulation of synoviocyte COX-2 observed in this study. We demonstrated that stimulated T cells produce IL-17 and TNF-α. Within our T cell-enriched populations, there were a small number of contaminating monocytes (~0.4%), and thus the TNF-α in the T cell supernatants could have arisen from contaminating monocytes. However, IL-1ß, which is a major monocyte derived cytokine, could not be detected in the stimulated T cell preparations, suggesting that the degree of monocyte contamination in the T cell preparations was not likely to be contributing to the TNF-α in the T cell supernatants. In addition, CSA inhibited TNF-α release by the stimulated T cell preparation, providing further evidence that the TNF-α found in the supernatants was T cell derived. Because of their presence within rheumatoid synovial fluid, as well as their proinflammatory actions, IL-17 and TNF-α are candidate mediators of rheumatoid joint pathology.

IL-17 is a relatively recently described cytokine, which is produced by CD4+ T cells and has been found in substantial amounts in rheumatoid synovial fluid[9,21]. IL-17 is considered to be a proinflammatory cytokine and many of its actions are similar, although in isolation less potent, than those of IL-1ß and TNF-α. IL-17 has been shown to induce expression of IL-6, IL-8, and adhesion molecules in human fibroblasts[22]. Epithelial cells, endothelial cells, and primary cultured synovial fibroblasts respond to IL-17 by expressing IL-6 and IL-8, and producing PGE_2[23]. IL-17 induces the release of IL-1ß and TNF-α, IL-6, PGE_2, IL-10, and IL-12 by human macrophages[10]. In terms of regulation of IL-17 expression, IL-12, a monocyte-derived cytokine, has been reported to promote a switch from Th2 to Th1 phenotype and to induce production of IL-17 in a T cell line[24].

In this study, antibody neutralization of T cell derived IL-17 partially reduced the ability of T cell supernatants to upregulate synoviocyte COX-2 expression and PGE_2 production. However, there appeared to be a discrepancy between the extent of inhibition of COX-2 expression and reduction of PGE_2 production in the presence of IL-17 neutralizing antibodies. There are many potential explanations for this. PGE_2 is not the immediate product of COX-2, which synthesizes PGH_2. PGE_2 is synthesized by PGE synthase, using PGH_2 as substrate. Depending on the cellular concentration of PGH_2 in relation to the dose-response curve of PGE synthase, COX-2 activity and consequent PGH_2 production could decrease by a certain amount, and PGE_2 synthesis could decrease by a different amount. We have examined the Michaelis constants (Km) of PGE synthase and TX synthase in monocytes and reported that they can explain disproportionate changes in COX activity and eicosanoid synthesis[25]. Another possible explanation is that IL-17 can regulate the expression of PGE synthase, thereby resulting in more efficient or preferential conversion of PGH_2 produced by COX-2 to PGE_2. Unfortunately, PGE synthase could not be detected on Western immunoblot using the available antibody, and the antibody was not suitable for flow cytometry.

The addition of rIL-17 to synoviocytes, at concentrations detected in stimulated T cell supernatants, had minimal effect on synoviocyte COX-2 expression and PGE_2 produc-

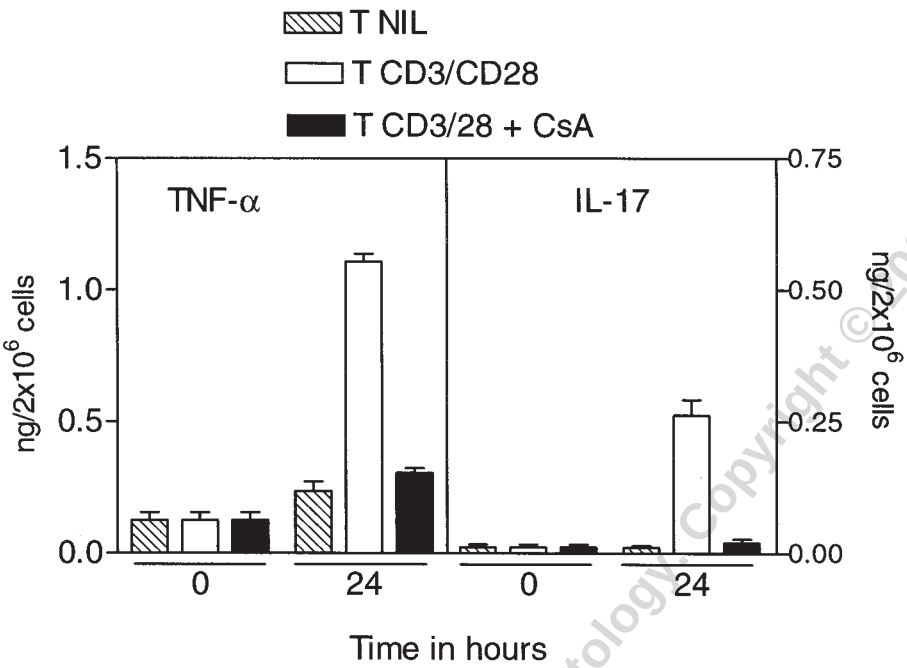

Figure 5. Release of IL-17 and TNF-α by T lymphocytes during 24 h in culture. T cells were unstimulated (T unstim) or stimulated with anti-CD3/anti-CD28 (T CD3/28). Where indicated, cells were treated with CSA (100 ng/ml) starting 30 min before stimulation with anti-CD3/anti-CD28 (T CD3/28 + CsA). Data shown are representative of 3 experiments using cells from 3 different donors.

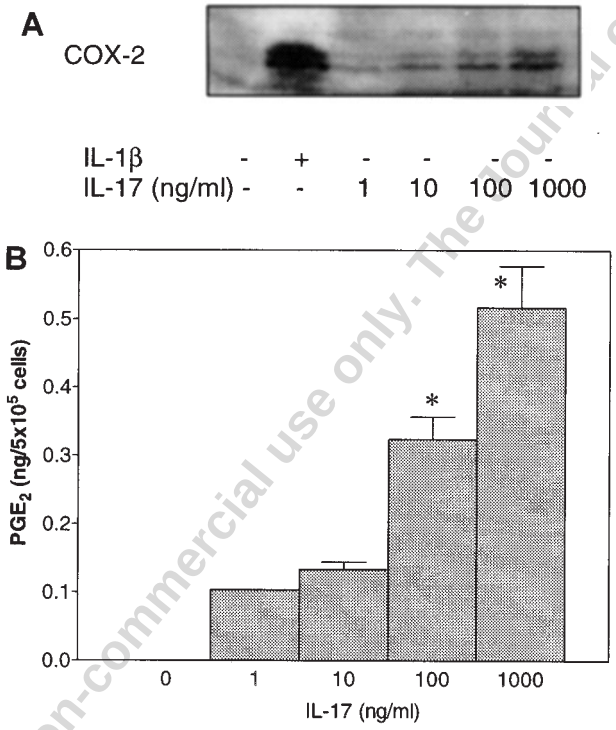

Figure 6. Effect of rIL-17 on synoviocyte (A) COX-2 expression and (B) PGE_2 production; 5×10^5 synoviocytes were incubated with increasing doses of rIL-17 for 18 h when (A) cell pellets were taken for Western immunoblot and (B) supernatants were harvested for PGE_2 assay. Data shown are representative of 4 separate experiments using cells from different donors. *$p < 0.05$, ANOVA followed by Neuman-Keuls tests for multiple comparisons.

tion. However, the inhibition observed in the presence of IL-17 neutralizing antibodies suggested that IL-17 may be affecting synoviocyte COX-2 and PGE_2 production in concert with another factor present in the supernatants. Therefore, possible IL-17 interactions with TNF-α were examined.

TNF-α appears to be important in the pathogenesis of RA. In the rheumatoid joint, monocytes/macrophages are the major source of TNF-α, although as we have shown, activated T cells also produce TNF-α. The addition of TNF-α neutralizing antibodies to anti-CD3/anti-CD28 stimulated T cell supernatants suppressed the ability of the T cell supernatants to stimulate synoviocyte COX-2 induction and PGE_2 synthesis. Despite this inhibition by TNF-α neutralizing antibodies, COX-2 was not induced nor PGE_2 produced in response to 2 ng/ml recombinant human TNF-α. The reported effects of TNF-α alone on the induction of synoviocyte COX-2 expression and PGE_2 production suggest that the responses are variable. It was reported that 20 ng/ml TNF-α alone had no effect on production of PGE_2 by cultured human rheumatoid synoviocytes[26], but that 5 ng/ml TNF-α stimulated PGE_2 synthesis by cultured human osteoarthritic synovial fibroblasts[27]. Since the concentrations of both IL-17 and TNF-α found in supernatants from the stimulated T cells had no measurable effect on COX-2 expression and PGE_2 synthesis, a synergistic effect of these 2 cytokines on synoviocyte COX-2 expression and PGE_2 production was considered.

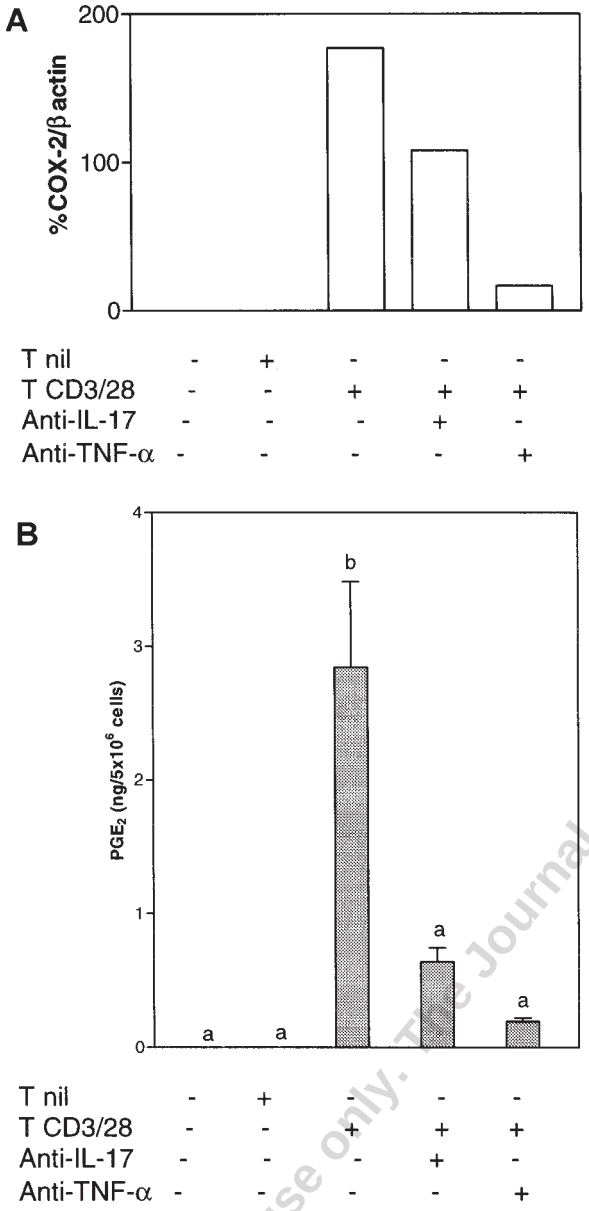

Figure 7. Effect of IL-17 or TNF-α neutralizing antibodies on synoviocyte (A) COX-2 expression and (B) PGE_2 release during incubation with supernatants from T cells that had been stimulated with anti-CD3/anti-CD28 for 24 h. IL-17 neutralizing antibodies (1 µg/ml) or TNF-α neutralizing antibodies (1 µg/ml) were added to T cell supernatants, which were incubated 4 h at room temperature before addition to synoviocytes. Synoviocytes were then cultured for 18 h at 37°C. T nil: supernatants from unstimulated T cells; T CD3/28: supernatants from anti-CD3/anti-CD28 stimulated T cells; T CD3/28 + anti-IL-17: supernatants from anti-CD3/anti-CD28 stimulated T cells treated with anti-IL-17 neutralizing antibodies; T CD3/28 + anti-TNF-α: supernatants from anti-CD3/anti-CD28 stimulated T cells treated with anti-TNF-α neutralizing antibodies. Bars with different letters are significantly different from each other, ANOVA followed by Neuman-Keuls test for multiple comparisons ($p < 0.05$). Data shown are representative of 2 separate experiments using T cells from 2 different donors and fibroblast-like synoviocytes from 2 different donors.

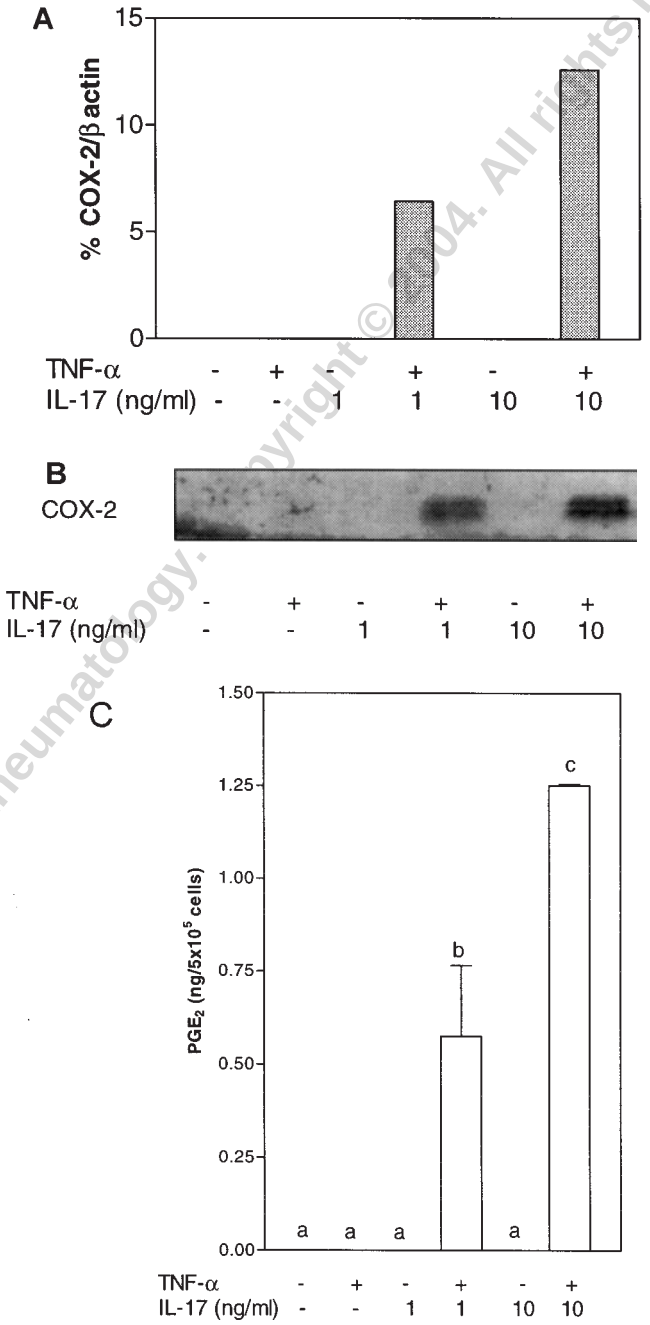

Figure 8. Effect of IL-17 on TNF-α stimulated synoviocytes (A) COX-2 expression expressed as the density of COX-2 staining as a percentage of the housekeeping protein ß-actin; (B) a representative Western immunoblot; and (C) PGE_2 production. 5×10^5 synoviocytes were stimulated with TNF-α (2 ng/ml) or IL-17 at the specified doses in the presence or absence of TNF-α (2 ng/ml) for 18 h. Bars with different letters are significantly different (ANOVA followed by Neuman-Keuls test for multiple comparisons; $p < 0.05$). Data shown are representative of 4 separate experiments.

IL-17 has been reported to enhance many of the effects of IL-1ß and TNF-α on monocytes and synoviocytes[11-13,28]. In this study, addition of IL-17 and TNF-α (at the low levels detected in our T cell supernatants) together produced

substantial expression of synoviocyte COX-2 and PGE_2, whereas neither cytokine alone at these concentrations produced much effect. The observation that anti-IL-17 treatment of the T cell supernatants did not completely remove all synoviocyte stimulating activity could be explained by residual free IL-17 acting in synergy with the TNF-α in the T cell supernatants, since it is not possible to know whether the antibody neutralized all of the IL-17. Yamamura, *et al* demonstrated that resting T cells induced synoviocyte PGE_2 production, and the effect was enhanced substantially by the presence of exogenous IL-17[29]. While IL-2 and IL-17 could not be detected in their resting T cells, TNF-α was not examined. Therefore it is possible that small amounts of TNF-α were secreted from the T cells, which then synergized with the exogenous IL-17, resulting in enhanced PGE_2 production. These data combined suggest that IL-17 and TNF-α act together in T cell supernatants to upregulate synoviocyte COX-2 expression and PGE_2 production.

In animal models of arthritis, IL-17 has been implicated as a stimulus for inflammatory tissue damage. In a murine model, repeated injection of IL-17 into the knee joint resulted in joint inflammation similar to that seen with repeated injection of IL-1ß[30]. Treatment of rats with adjuvant arthritis with an IL-17 receptor IgG1 Fc fusion protein resulted in a dose-dependent reduction in paw volume and reduced radiographic and histology scores in arthritic rats compared with controls[31]. IL-17 has been reported to contribute to bone destruction through induction of matrix metalloproteinases[32], induction of osteoclasts[33], and inhibition of proteoglycan synthesis and increasing proteoglycan degradation[34]. While some of the effects of IL-17 may be enhanced by the presence of IL-1ß and TNF-α, IL-17 can act in an independent manner to contribute to tissue destruction[35].

Following insights into the role of cytokines in RA, biological agents that block the effects of specific cytokines have been developed. Both neutralizing TNF-α monoclonal antibodies and a soluble TNF-α receptor fusion protein decoy receptor have been shown to reduce disease activity and joint damage in RA[36-38]. The observed effects of TNF-α blockade may, at least in part, involve inhibition of effects in which IL-17 is acting as a synergistic agent. Recombinant human IL-1 receptor antagonists may also reduce joint erosion in RA[39,40]. While these agents have proven efficacy, their lack of efficacy in some patients indicates that no single cytokine is invariably responsible for the inflammatory process seen in RA. It may be that combination anticytokine therapy may produce a better response than inhibition of a single cytokine in some patients. This proposition is supported by observations of additive interactive effects of cytokine blockers with different specificities in experimental models of arthritis and inflammatory tissue damage[21,41].

In summary, soluble mediators produced by activated T cells can influence the expression of synoviocyte COX-2 and eicosanoid production. The results indicate that T cell derived IL-17, in combination with TNF-α, contributes substantially to T cell driven upregulation of synoviocyte COX-2. The effects of IL-17 on synoviocyte COX-2 expression, along with early observations that IL-17 is involved in joint damage in animal models of arthritis[31,35], add to the validation of IL-17 as a therapeutic target in RA and other inflammatory diseases. The potential of IL-17 blockade as a component of combination cytokine blockade remains to be determined.

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