Paracrine Upregulation of Monocyte Cyclooxygenase-2 by Mediators Produced by T Lymphocytes: Role of Interleukin 17 and Interferon-γ

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

ABSTRACT. ***Objective.*** Cyclooxygenase (COX)-2 is an inducible eicosanoid-forming enzyme that is expressed at sites of inflammation. T lymphocytes and monocytes are found in close proximity at sites of inflammation, including synovitis. We investigated whether activated T lymphocytes express COX-2 and whether activated T cells upregulate monocyte COX-2 expression.

Methods. Human T lymphocytes and monocytes were isolated from fresh buffy coats by density gradient separation followed by passage through either nylon wool columns (T lymphocytes) or counter-current elutriation (monocytes). T lymphocytes were stimulated using anti-CD3 and anti-CD28 in a co-culture system with monocytes using transwells, which prevents cell-cell contact, but allows diffusion of soluble mediators.

Results. Repeated examination of COX isotypes in resting and stimulated T cells revealed COX-1, but not COX-2. Activated T cells produced a soluble mediator(s) that upregulated monocyte COX-2. Mediator production was inhibited by cyclosporin A. Activated T cells produced interleukin 17 (IL-17) and interferon-γ (IFN-γ), and in the co-culture IL-17-neutralizing antibodies partially reduced monocyte COX-2 expression, whereas IFN-γ-neutralizing antibodies had the opposite effect. Exogenous IL-17 upregulated monocyte COX-2, although concentrations were high compared with those generated by stimulated T cells. By contrast, monocyte COX-2 expression was downregulated when monocytes were treated with IFN-γ prior to stimulation with lipopolysaccharide.

Conclusion. Soluble mediators produced by activated T cells can influence induction of monocyte COX-2, with IL-17 acting as a positive paracrine regulator and IFN-γ acting as a negative regulator. In rheumatoid joints, previously observed high concentrations of IL-17 and low concentrations of IFN-γ could contribute to T cell-driven upregulation of monocyte COX-2. (J Rheumatol 2004; 31: 1255–64)

Key Indexing Terms:

T CELLS | MONOCYTES | EICOSANOIDS
CYCLOOXYGENASE | INTERLEUKIN 17 | INTERFERON-γ

While the cause of rheumatoid arthritis (RA) remains unknown, it has been proposed that exogenous and/or endogenous autoantigens may activate T cells, which initiate an inflammatory cascade involving monocytes/macrophages and synoviocytes. Whether T cells and their secretory products are necessary to sustain synovial macrophage activation is not known.

Despite the predominance of CD4+ T cells within the synovium, there are relatively low concentrations of most T cell cytokines. While the hallmark Th1 cytokine, interferon-γ (IFN-γ), is detectable[1-4], concentrations are low compared to those in other Th1-mediated disease processes[5]. In comparison, both interleukin 1 (IL-1) and tumor necrosis factor-α (TNF-α), which are predominantly monocyte-derived, can be detected in larger amounts and have been implicated in the tissue damage seen in RA[6-8]. IL-17 is produced by CD4+ T cells and is considered a proinflammatory cytokine that stimulates macrophage production of IL-1ß, TNF-α, prostaglandin E_2 (PGE_2), and matrix metalloproteinases[9,10]. IL-17 is abundant in rheumatoid synovium and synovial fluid[11,12].

Since T cells and monocytes lie in close proximity within the rheumatoid synovium, their mutual interactions are likely to be important. We investigated whether soluble mediators from activated T cells can upregulate monocyte COX-2, the inducible COX isotype. COX activity is responsible for production of proinflammatory eicosanoids that include PGE_2 and thromboxane A_2 (TXA_2). T cells activated by T cell-specific stimuli upregulated monocyte COX-2 in the absence of direct cell-cell contact. In addition, an initial examination of T cell COX isotypes confirmed the presence

From the Rheumatology Unit, Royal Adelaide Hospital, Adelaide, Australia.

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L.K. Stamp, FRACP; M.J. James, PhD; L.G. Cleland, MD.

Address reprint requests to Prof. L. Cleland, Rheumatology Unit, Royal Adelaide Hospital, Adelaide SA 5000, Australia.

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of the constitutive isotype COX-1, but not COX-2, in activated T cells. These results provide insights into the potential activities of T cells in rheumatoid joints and could provide an explanation for the disparate findings on whether T cells can express COX-2.

MATERIALS AND METHODS

Materials were from the following sources: phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187, concanavalin A (Con-A), saponin, cyclosporin A (CSA), lipopolysaccharide (LPS), extravidin, and E-Toxa clean (Sigma Chemical, St. Louis, MO, USA); arachidonic acid (Sapphire Biosciences, Sydney, Australia); PGE_2 standard, TXB_2 standard, COX-1 monoclonal antibody (mAb), COX-2 pAb, carbocyclic TXA_2 ($cTXA_2$; Cayman Chemical, Ann Arbor, MI, USA); pyrogen-free Lymphoprep (Nycomed, Oslo, Norway); Minisorb polyethylene tubes, 24-well plates, tissue culture inserts (10 mm, 0.4 µm polycarbonate membrane; Nunc, Roskilde, Denmark); Trans-blot membrane (BioRad, North Ryde, Australia); [^{3}H]PGE_2 and [^{3}H]TXB_2 (Amersham International, Little Chafont, England). TXB_2 and PGE_2 antisera were prepared from rabbits immunized with TXB_2 and PGE_2 conjugated to thyroglobulin[13,14]. Recombinant (r) IFN-γ, IFN-γ mAb, biotin-labeled IFN-γ mAb (Endogen, Woburn, MA, USA); IFN-γ mAb, rIL–17, IL–17 mAb, biotin-labeled IL-17 mAb (R&D Systems, Minneapolis, MN, USA). The polyclonal antibody PH4 against the thromboxane receptor was a gift from Dr. P. Halushka, Charleston, SC, USA[15]. The following antibodies were obtained from the sources specified: monoclonal mouse anti-human CD3, CD14, CD16, CD19, CD28, CD69, CD3-PE, CD3-FITC, IgG1-PE or -FITC, polyclonal FITC-conjugated goat anti-mouse and polyclonal FITC-conjugated goat anti-rabbit (Becton Dickinson Pharmingen, San Diego, CA, USA); isotype-matched control antibody IB5 (IgG_1, against *Giardia intestinalis*), kindly provided by L. Spargo (Arthritis Research, Royal Adelaide Hospital, Adelaide); the antibodies 1D4.5 (IgG2a), 1A6.11 (IgG2b), and 1A6.12 (IgM) were a gift from Dr. L. Ashman, Institute of Medical and Veterinary Science, Adelaide, South Australia; and COX-2 mAb-PE conjugate, COX-1 mAb-FITC conjugate (Cayman Chemical).

T cell isolation and monocyte isolation. Human mononuclear cells were purified from fresh buffy coats 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. Mononuclear cells were isolated by centrifugation for a further 20 min at 400 g. T cells were then isolated by passage of mononuclear cells through nylon fiberglass columns as described[16]. The cell composition was on average 85% CD3+ T cells as determined by flow cytometric analysis. The major contaminating cells were CD16+ cells (8%). CD14+ monocytes accounted for only 0.4% of cells and platelets up to –1 × 10^6/ml when cells were resuspended at 2 × 10^6 nucleated cells per ml.

Monocytes were isolated from mononuclear cell preparations by counter-current elutriation using a Beckman JE-5.0 elutriation rotor mounted in a Beckman J-6M/E series centrifuge (Beckman Instruments, Fullerton, CA, USA). Monocyte purity determined by flow cytometry was > 85%.

Cell stimulation. Freshly isolated T cells or monocytes were resuspended in RPMI-1640 medium supplemented with 10% heat-inactivated, low LPS fetal calf serum (FCS), L-glutamine, Hepes, penicillin (100 U/ml), and gentamycin at 2 × 10^6/ml. Cells were cultured in duplicate for each stimulation using a total volume of 1 ml (or 2.5 ml for Western immunoblots) in Minisorp nonadherent teflon tubes, or for anti-CD3/anti-CD28 stimulation in 24 or 96 well plates. Monocytes were stimulated with LPS (200 ng/ml). T cells were stimulated using Con-A (10 µg/ml) or PMA (15 ng/ml) plus calcium ionophore A23187 (1 µM). Alternatively, T cells were added to plates precoated with anti-CD3 mAb (2 µg/ml) to which soluble anti-CD28 (250 ng/ml) was added. Cells were incubated 18 h at 37°C, in 5% CO_2. Cell suspensions were then centrifuged and supernatants stored at –20°C until eicosanoid measurement.

T cell-monocyte co-culture. Freshly isolated cells were resuspended at 5 × 10^6/ml; 2.5 × 10^6 T cells in 0.5 ml were placed into 24 well plates precoated with antibodies against CD3 to which anti-CD28 (250 ng/ml) was added. A transwell (0.4 µM, 10 mm) was inserted into each well and 2.5 × 10^6 monocytes in 0.5 ml RPMI, 10% FCS placed into the transwell. The transwell prevents direct cell contact but allows diffusion of soluble mediators. After incubation for 18 h at 37°C, 5% CO_2, T cells and monocytes were collected individually for protein extraction.

Flow cytometry. To assess purity of cell populations and T cell activation, 1 × 10^6 cells were suspended in mouse monoclonal antibodies directed against CD3, CD14, CD16, CD19, or CD69 (diluted 1:50 with 10% normal human serum) and incubated on ice for 45 min. Cells were then washed twice in immunofluorescence buffer (PBS, 2% FCS, 0.01 M azide), resuspended in the secondary FITC-conjugated polyclonal goat-anti-mouse antibody diluted 1:50 with 10% normal human serum, and incubated on ice for 45 min. Cells were washed twice and resuspended in 0.5 ml FACS fix [PBS, 1% formalin (v/v), 2% glucose (w/v), 0.02% azide]. For intracellular staining, cells were fixed with 10% buffered formalin and permeabilized with saponin wash buffer (DPBS, 1% FCS, 0.1% saponin) prior to the addition of antibodies. Directly conjugated CD3-PE, CD3-FITC, or CD25-PE were used according to the manufacturer's instructions, while COX-1-FITC and COX-2-PE were used at concentrations of 100 µg/ml and 40 µg/ml, respectively. Control preparations, in which primary antibody was replaced with isotype-matched control antibodies at the same concentration, were included in each analysis. A minimum of 20,000 cells were analyzed using a Coulter® Epics® XL-MCL flow cytometer and System II v.3 software.

Determination of COX activity. COX-1 was inhibited selectively as follows. Before stimulation, T cells were treated with aspirin (55 µM) for 30 min to irreversibly inhibit COX-1. Cells were then washed twice and resuspended in fresh medium. This transient aspirin treatment followed by aspirin removal before cell stimulation allows inhibition of resident COX-1, whereas COX-2, if later upregulated following cell stimulation, is not exposed to aspirin[17]. NS398 (0.5 µM) was used to inhibit COX-2 selectively.

PGE_2 and TXB_2, the stable hydrolysis product of TXA_2, were determined in triplicate by radioimmunoassay as described[18,19]. The lower limit of detection for both these assays is 0.1 ng/ml.

Western immunoblot. A quantity of 5 × 10^6 cells were pelleted after incubation with stimuli. Cells were washed twice with PBS, followed by addition of 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 sulfate (SDS), 10% 2-mercaptoethanol]. Samples were heated at 95°C for 7 min and then stored at –20°C. Samples were separated using 9% acrylamide gel and the proteins were transferred onto Trans-Blot®. 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 COX-1 or COX-2 pAb (5 µg/ml) overnight or ß-actin mAb (1:5000 dilution) for 1 h. Membranes were washed and then exposed to horseradish peroxidase-labeled donkey-anti-rabbit or goat-anti-mouse antibody (1:20,000 dilution) for 1 h. Antibody cross-reactivity was revealed using the enhanced chemiluminescence kit, ECL™ (Amersham), according to the manufacturer's instructions.

IFN-γ and IL-17 ELISA. ELISA for IFN-γ and IL-17 were developed using commercially available antibodies listed above. The range of detection was 15–1000 pg/ml for each assay.

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

RESULTS

Expression of COX-1 and COX-2 in T lymphocytes. COX-1 was readily detected in fresh human T cells by Western immunoblot, and the amount did not appear to change after stimulation with immobilized anti-CD3 and soluble anti-CD28 (Figure 1). However, COX-2 was not detected by Western immunoblot in untreated or stimulated T cells (Figure 1). The same null result for COX-2 was observed in T cells treated with Con-A or PMA plus A23187 (data not shown). Peripheral blood T cells from 20 different donors were used and on no occasion was COX-2 detected by Western immunoblot. Serial dilutions of platelets confirmed that COX-1 could not be detected by Western immunoblot at the levels of platelet contamination expected in the T cell preparations (data not shown). In a further attempt to exclude contaminating cells as the source of COX-1 and to identify COX-2 in T cells, flow cytometry was undertaken using intracellular staining with directly conjugated COX-1-FITC or COX-2-PE antibodies. This confirmed the presence of COX-1 in CD3+ T cells (Figure 2). COX-1 is ubiquitously expressed, accounting for the COX-1+/CD3– cells present. While a portion of CD3+ cells appear to be COX-1–, these could represent CD3+ cells with low level expression of COX-1.

While COX-2 was identified by flow cytometry in LPS-stimulated CD14+ monocytes, no COX-2 was found in T cells stimulated with PMA plus A32187, which was a potent upregulator of the T cell activation marker CD69 (Figure 2). Similar results were seen with anti-CD3/anti-CD28-stimulated T cells (Figure 2).

Production of eicosanoids by T cells. To assess COX activity and its dependence on endogenous arachidonic acid (AA) release, T cells were stimulated for 18 h, and after washing with FCS-free RPMI, cells were incubated with or without AA (10 µM) for 30 min. Without addition of AA, levels of eicosanoids observed were at or close to the limit of detection (Figure 3). Resting T cells produced detectable TXB_2 or PGE_2 with the addition of AA, but prior cell stim-

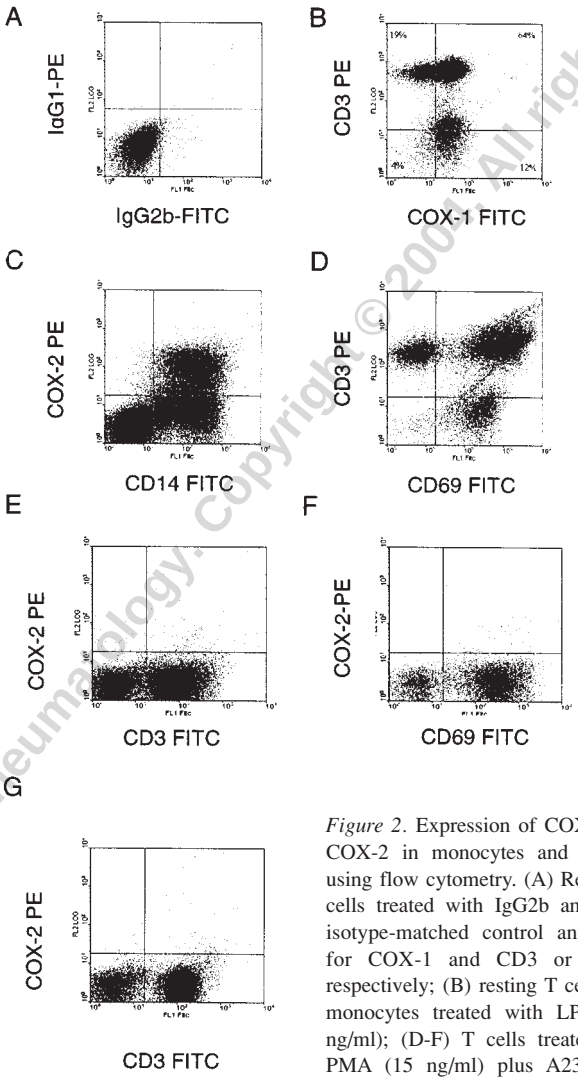

Figure 2. Expression of COX-1 and COX-2 in monocytes and T cells using flow cytometry. (A) Resting T cells treated with IgG2b and IgG1 isotype-matched control antibodies for COX-1 and CD3 or CD69, respectively; (B) resting T cells; (C) monocytes treated with LPS (200 ng/ml); (D-F) T cells treated with PMA (15 ng/ml) plus A23187 (1 µM); (G) T cells treated with anti-CD3/antiCD28. The results depicted are representative of 2 separate experiments, using cells from 2 different donors.

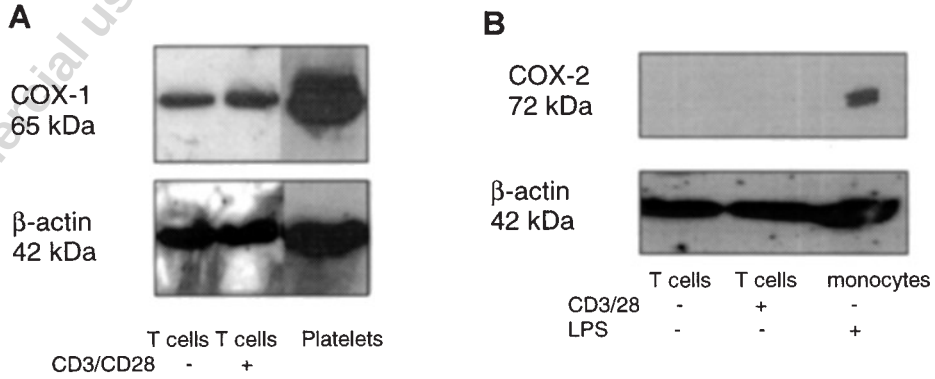

Figure 1. Western immunoblot of (A) COX-1 and (B) COX-2 in unstimulated and anti-CD3/anti-CD28-stimulated T cells. Platelets were used as a positive control for COX-1 expression and LPS-stimulated monocytes as a positive control for COX-2 expression.

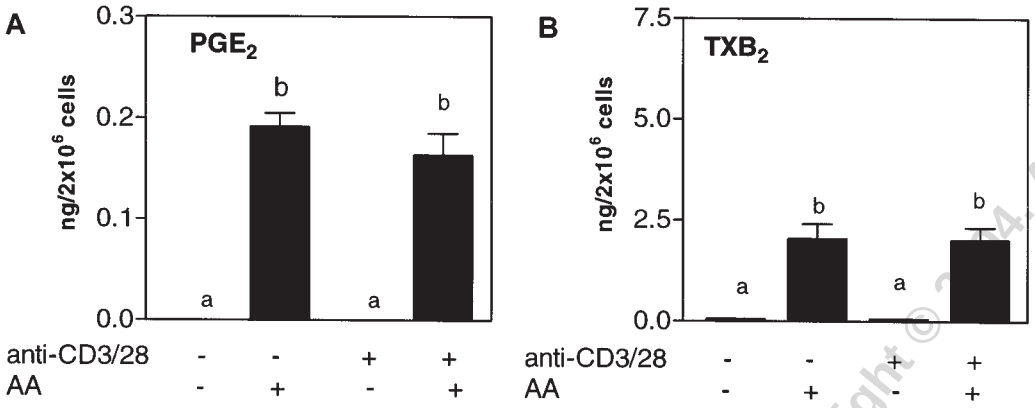

Figure 3. Production of (A) PGE_2 or (B) TXB_2 by T cells. T cells were incubated without or with immobilized anti-CD3 and soluble anti-CD28 for 18 h. After washing with RPMI, cells were incubated without or with arachidonic acid (10 µM) for 30 min. Supernatants were then collected and eicosanoids assayed. The means ± SEM for triplicate determinations are depicted. The results of one experiment that are representative of 3 are shown. Bars with different letters are significantly different from each other; $p < 0.05$, ANOVA followed by Neuman-Keuls test for multiple comparisons.

ulation with anti-CD3/anti-CD28 had no effect on the rate of eicosanoid synthesis. Thus, there is no evidence from these results of upregulated COX-2 activity in stimulated T cells (Figure 3). Similar results were observed when T cells were stimulated with PMA and A23187, in that resting T cells produced detectable PGE_2 and TXB_2 only with the addition of exogenous AA, and prior cell stimulation had no effect on the rate of eicosanoid synthesis (data not shown).

To further examine which COX isotypes are involved in T cell eicosanoid production, specific inhibitors of each isotype were used. Prior to overnight incubation, cells were either treated briefly with aspirin (55 µM), which inhibits COX-1 irreversibly, or incubated in the presence of NS398 (0.5 µM) to inhibit COX-2 selectively. Cells were stimulated for 18 h and, after washing with FCS-free RPMI, those cells already exposed to NS398 were retreated with NS398. Cells were then incubated with or without AA (10 µM) for 30 min. COX-1 inhibition significantly reduced the production of TXB_2 and PGE_2 by stimulated T cells, while COX-2 inhibition had no effect, thereby implicating COX-1 in the production of eicosanoids by T cells (Figure 4).

Role of contaminating cells on eicosanoid production attributed to T cells. Despite T cell enrichment a small number of monocytes and platelets remained in the cell preparations. Flow cytometric analysis indicated that on average ~0.4% of cells were monocytes, and therefore in an incubation of 2×10^6 cells enriched for T cells, there were on average ~8×10^3 monocytes. Platelet counts were undertaken using an auto-

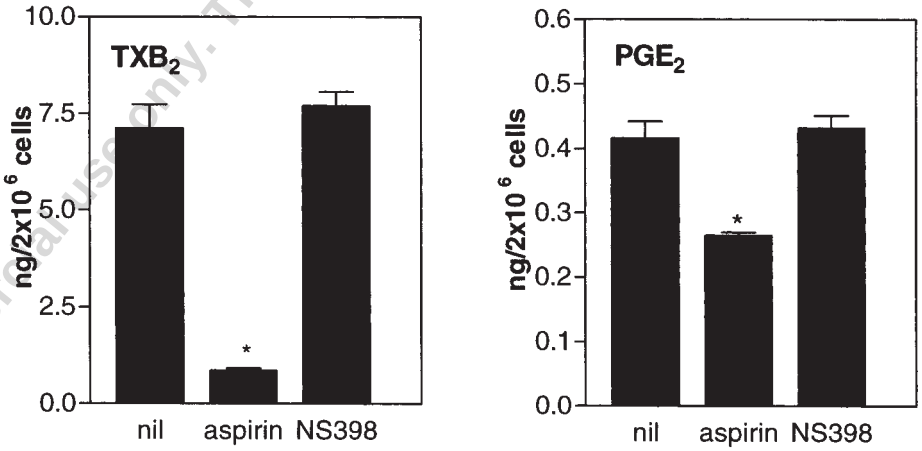

Figure 4. Effect of COX-1 and COX-2 inhibition on T cell TXB_2 and PGE_2 production. Cells were incubated with aspirin (55 µM) for 30 min and washed, or alternatively NS398 (0.5 µM) was added. Cells were then incubated with PMA (15 ng/ml) plus A23187 (1 µM). After 18 h cells were washed with FCS-free RPMI, and those previously exposed to NS398 were retreated for 15 min before addition of arachidonic acid (10 µM) for 30 min. *Significantly different from control; $p < 0.05$. Similar results were observed when T cells were stimulated with immobilized anti-CD3 and soluble anti-CD28. The results of one experiment that are representative of 3 are shown.

mated Sysmex XE-2100 (Sysmex Corp., Japan) instrument, and there were consistently fewer than 1×10^6 platelets per ml of purified T cells. To determine the significance of eicosanoid production by these contaminating cells in the T cell preparations, serial dilutions of purified monocytes and platelets were incubated for 18 h with anti-CD3/anti-CD28 or PMA plus A23187, and the supernatants were assayed for PGE_2 and TXB_2. At the concentration of monocytes or platelets expected in cell preparations, no TXB_2 or PGE_2 could be detected. In addition, LPS stimulation of the T cell preparations did not result in enhanced eicosanoid production.

Effect of T cell stimulation on monocyte COX-2 expression and eicosanoid production. T cells stimulated with immobilized anti-CD3 (2 µg/ml) and soluble anti-CD28 (250 ng/ml) upregulated COX-2 in monocytes, which were in transwells and therefore prevented from direct contact with the T cells. Treatment of T cells with CSA (100 ng/ml) commencing 30 min before stimulation with anti-CD3/anti-CD28 inhibited the upregulation of monocyte COX-2 (Figure 5). In control preparations with the monocytes or T cells individually treated with anti-CD3/anti-CD28, COX-2 was not detected. Also in control preparations with monocytes alone, CSA had no effect on LPS-induced COX-2 expression. There was a "dose-dependent" increase in monocyte COX-2 expression when the number of T cells was increased (data not shown).

While the expression of monocyte COX-2 was increased by co-incubation of monocytes in transwells with anti-CD3/anti-CD28-stimulated T cells, there was no associated increase in PGE_2 or TXB_2 production (data not shown). Since eicosanoid production requires release of arachidonate by cytosolic phospholipase A_2 ($cPLA_2$), we investigated whether the phosphorylated (activated) form of this enzyme was present in monocytes stimulated in transwells using Western immunoblot. While no activated $cPLA_2$ was detected, the meaning of the findings is uncertain, since the antibody was found to stain variably in LPS-stimulated monocytes.

Effect of TXA_2 on monocyte COX-2 expression. It has been reported that the stable thromboxane analog carbocyclic TXA_2 ($cTXA_2$) can upregulate COX-2 in human umbilical vein endothelial cells[20]. Since TXA_2 production was observed in T cells, it is potentially a mediator in the upregulation of monocyte COX-2. However, addition of $cTXA_2$ (0.01 to 10 µg/ml) to freshly prepared human monocytes did not induce monocyte COX-2 expression (data not shown).

Production of IL-17 and IFN-γ by T cells. IL-17 and IFN-γ are both T cell-derived cytokines that are regarded as proinflammatory and which are potential mediators in the upregulation of monocyte COX-2 during co-incubation of T cells. To assess production of IL-17 and IFN-γ by stimulated T cells and the extent of inhibition by CSA, T cells were stimulated with immobilized anti-CD3 and soluble anti-CD28 with or without CSA (100 ng/ml) for 48 h. Stimulated T cells produced both IL-17 and IFN-γ in a time-dependent manner and production of both was significantly inhibited by pretreatment of cells with CSA (Figure 6).

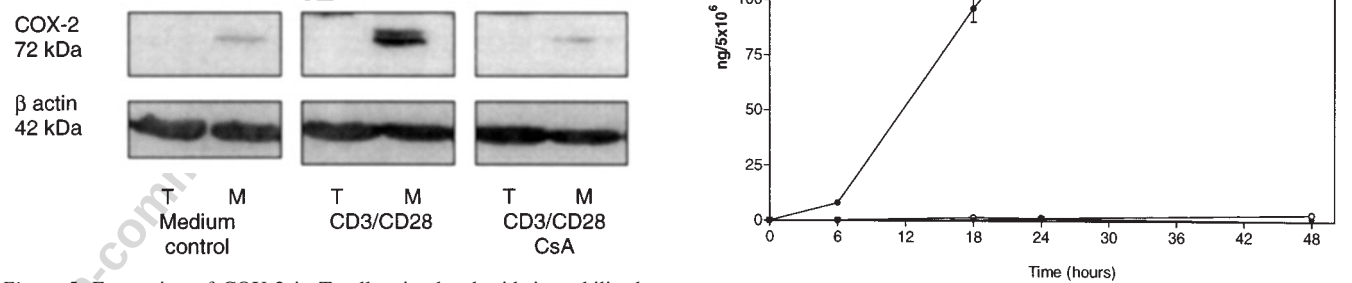

Figure 5. Expression of COX-2 in T cells stimulated with immobilized anti-CD3 and soluble anti-CD28 and COX-2 in monocytes co-incubated with the T cells in transwells. T cells (2.5×10^6 in 0.5 ml) were placed in the bottom of a 24 well plate and monocytes (2.5×10^6 in 0.5 ml) were placed in the transwell inserts. Where indicated, cyclosporin A (CsA; 100 ng/ml) was added commencing 30 min before stimulation. After 18 h, both T cells (T) and monocytes (M) were processed for Western immunoblot.

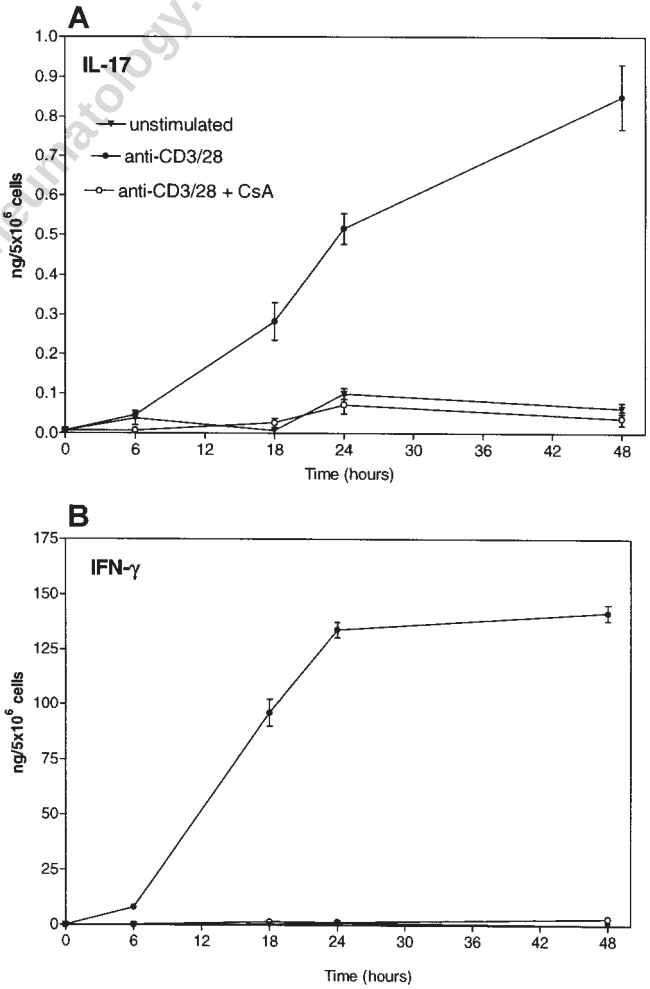

Figure 6. Production of (A) IL-17 and (B) IFN-γ by T cells over 48 h. T cells were stimulated with immobilized anti-CD3 and soluble anti-CD28 in the presence or absence of cyclosporin A (CsA; 100 ng/ml) for 48 h. Supernatants were collected at 6, 18, 24, and 48 h and assayed for IL-17 and IFN-γ by ELISA. Results are shown as the mean ± SEM, n = 3 replicates. The findings shown are from a single experiment that is representative of 3 separate experiments.

Effect of IL-17 on monocyte COX-2. To investigate the influence of IL-17 on monocyte COX-2 expression, rIL-17 was added to freshly prepared human peripheral blood monocytes and incubated for 18 h. rIL-17 upregulated monocyte COX-2 expression and TXB_2 and PGE_2 production in a dose-dependent manner (Figure 7). While IL-17 was capable of upregulating monocyte COX-2 expression, the doses required were far in excess of those released by activated T cells.

To examine whether T cell-derived IL-17 had a paracrine role in upregulation of monocyte COX-2, anti-CD3/anti-CD28-stimulated T cells and monocytes were co-incubated using transwells in the presence or absence of IL-17 neutralizing antibody (10 µg/ml). Addition of IL-17 neutralizing antibody resulted in partial inhibition of anti-CD3/anti-28 T cell-stimulated monocyte COX-2 (Figure 8). An isotype-matched antibody to an irrelevant specificity had no effect (data not shown).

Effect of IFN-γ on monocyte COX-2. Addition of rIFN-γ (150 ng/ml) to monocytes during an 18-h incubation period did not upregulate monocyte COX-2. However, treatment of monocytes with rIFN-γ (150 ng/ml) commencing 1 or 4 h prior to the addition of LPS resulted in suppression of LPS-stimulated monocyte COX-2 (Figure 9). PGE_2 and TXB_2 synthesis also were suppressed by IFN-γ pretreatment (Figure 9).

To examine whether T cell-derived IFN-γ had a paracrine role in regulating monocyte COX-2, co-incubation of anti-CD3/anti-CD28-stimulated T cells and monocytes was undertaken using the transwell system in the presence or absence of neutralizing antibody against IFN-γ (1 µg/ml). Addition of IFN-γ-neutralizing antibody led to an increase in monocyte COX-2 expression (Figure 10). Isotype-matched control antibody had no effect (data not shown).

DISCUSSION

The results indicate that anti-CD3/anti-CD28-stimulated T cells can upregulate monocyte COX-2 and that this can be inhibited by cyclosporin A. Further, the paracrine effect of T cells on monocytes is due to soluble mediators and does not require cell-cell contact.

The history of studies on T cells and prostaglandin production has been equivocal with regard to whether T cells have COX and whether they can synthesize eicosanoids. Since the discovery of COX-2 there have been reports that COX-2 can be detected by Western immunoblot in human peripheral blood T cells and T cell lines[21-26], thymic cell lines[27], and T cells within the small intestine[28]. However, there are variable findings. For example, although Pablos, *et al* identified COX-2 in stimulated human T cells and Jurkat cells by Western immunoblot, they observed only COX-1 but not COX-2 by immunocytochemistry of peripheral blood mononuclear cells[23]. While we have identified COX-1 in human peripheral blood T cells, we did not detect

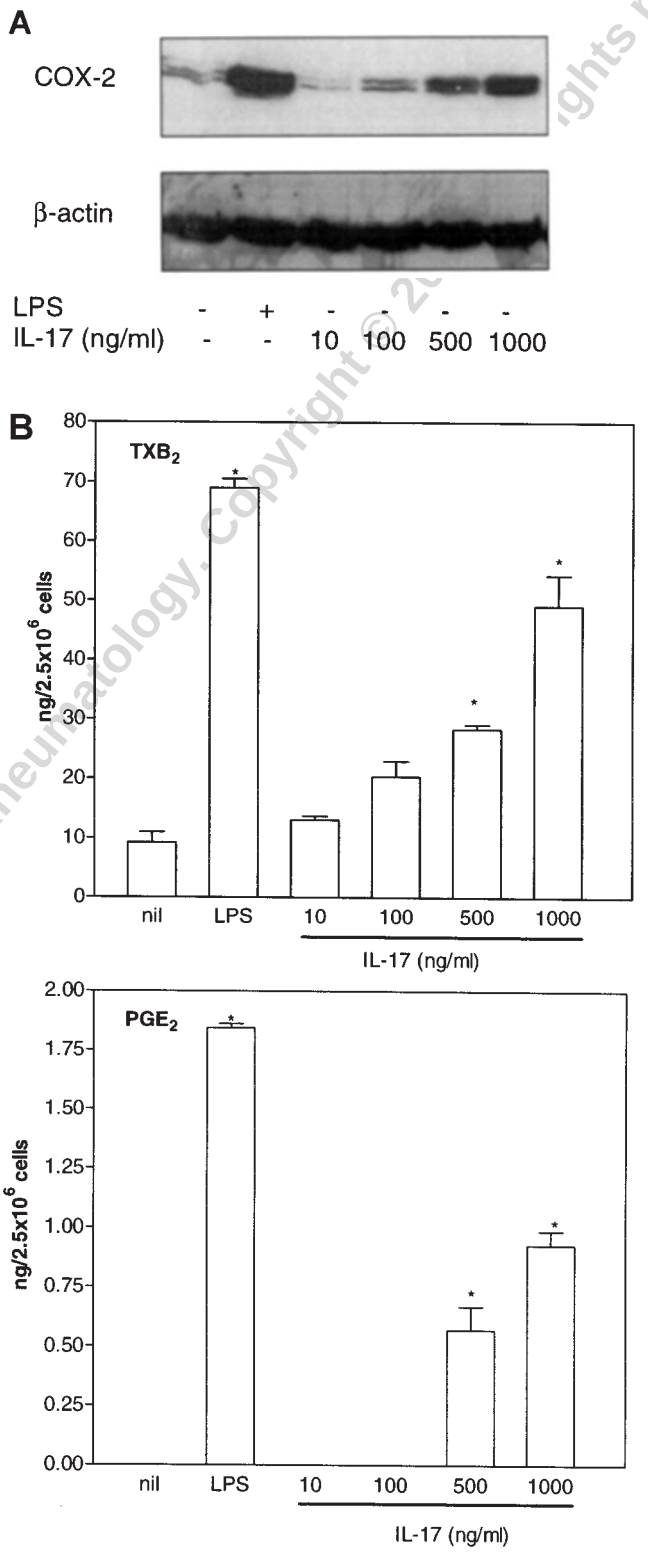

Figure 7. Effect of IL-17 on (A) monocyte COX-2 expression and (B) eicosanoid synthesis. Monocytes were incubated with LPS (200 ng/ml) or IL-17 (10–1000 ng/ml) for 18 h prior to collection of supernatants for PGE_2 and TXB_2 assay and cells for protein extraction. Error bars represent the mean ± SEM of 3 replicates (*$p < 0.05$). The results shown are representative of 4 separate experiments.

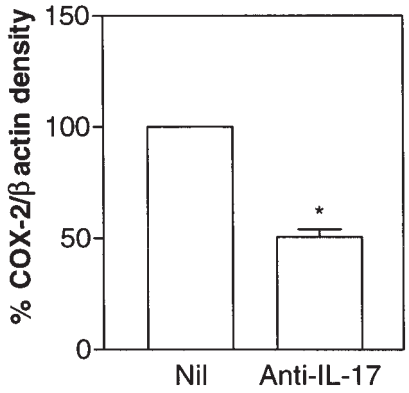

Figure 8. Effect of IL-17-neutralizing antibody on upregulation of monocyte COX-2 by anti-CD3/anti-CD28-stimulated T cells. T cells and monocytes were co-cultured using transwells in the presence or absence of IL-17-neutralizing antibody (10 µg/ml). Cells were incubated 18 h and then protein extracted from the monocytes for Western immunoblot. Shown is the density of COX-2/ß-actin expressed as percentage of the control values obtained without anti-IL-17 antibody. Data from 2 experiments using cells from different donors were pooled for analysis (*$p < 0.05$).

COX-2 by either Western immunoblot or flow cytometry after exposure to anti-CD3/anti-CD28, PMA plus A23187, or Con-A. By contrast, Iniguez, *et al* reported the presence of COX-2 in the Jurkat T cell line and anti-CD3/anti-CD28-stimulated human peripheral blood T cells by Western immunoblot. They reported that upregulation of COX-2 could be inhibited by CSA[22]. The argument by Iniguez, *et al* that the COX-2 detected was from T cells appeared to be strengthened by the use of T cell-specific stimuli, namely anti-CD3/anti-CD28 and inhibition by CSA[22].

However, our results provide an alternative explanation for these disparate findings. COX-2 detected on Western immunoblot could result from contaminating monocytes in which COX-2 has been upregulated by stimulated T cells. Monocytes express large amounts of COX-2 when exposed to inflammatory stimuli, and a relatively small number of contaminating monocytes could create a COX-2 signal on Western immunoblot of T cell preparations. Our results indicate this could occur even when T cell-specific stimuli are used. In our T cell isolates there were on average 0.4% CD14+ monocytes. In one of the reports of COX-2 in T cells, the cells were isolated by density gradient centrifugation only, a technique that is likely to result in significant monocyte contamination, and there is no comment on the percentage of T cells in their preparations[21]. Pablos, *et al*[23]

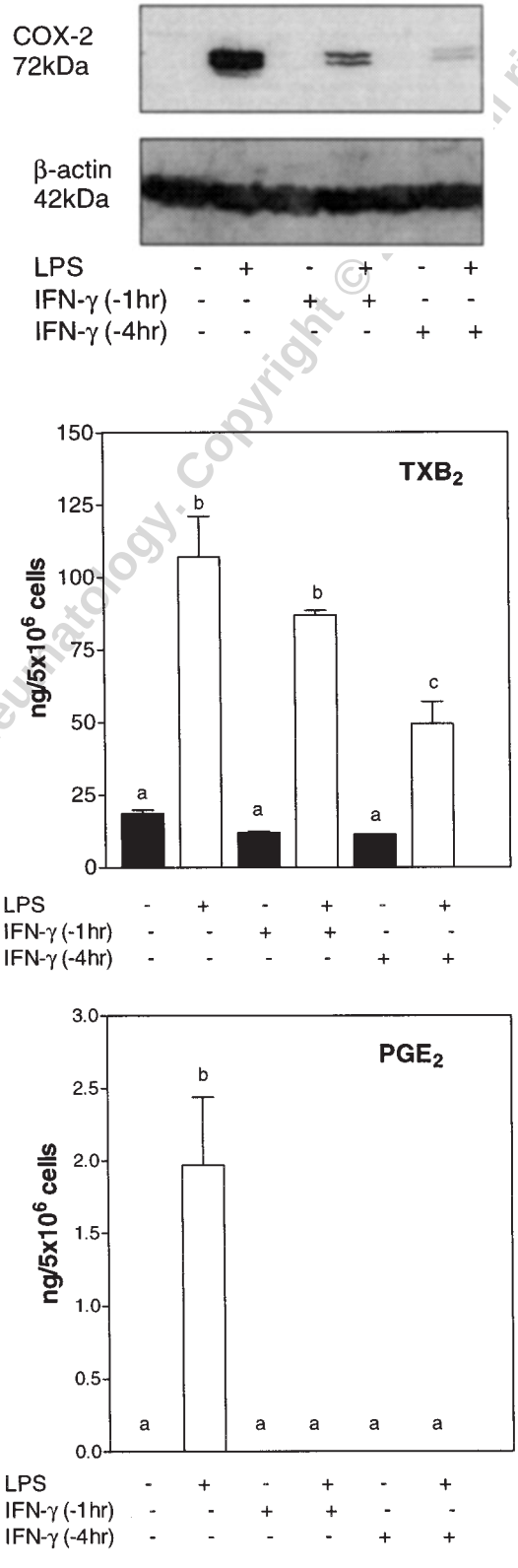

Figure 9. Effect of IFN-γ on monocyte COX-2 expression and eicosanoid synthesis. Monocytes were treated with IFN-γ (150 ng/ml) commencing 1 or 4 h before addition of LPS (200 ng/ml). After incubation for 18 h cells were processed for Western immunoblot, while supernatants were used to measure TXB_2 and PGE_2 release. Data shown are from one experiment, representative of 4 separate experiments using monocytes from different donors. Shown are the mean ± SEM of triplicate determinations, and bars with different letters are significantly different from each other; $p < 0.05$, ANOVA followed by Neuman-Keuls test for multiple comparisons.

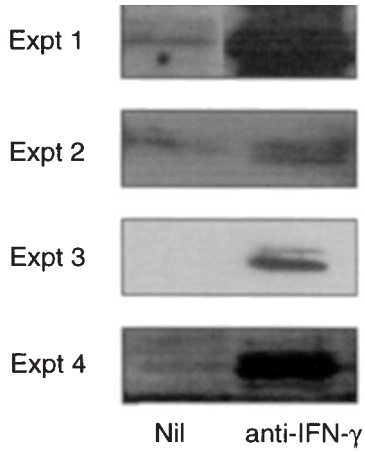

Figure 10. Effect of IFN-γ antibody on monocyte COX-2 expression. T cells and monocytes were co-incubated in 24 well plates, with separation by transwells in the absence (nil) or presence (anti-IFN-γ) of IFN-γ-neutralizing antibody (1 µg/ml). After incubation for 18 h monocytes and T cells were collected and processed for Western immunoblot. Data from 4 separate experiments using 4 different donors are shown.

report < 2% My4+ monocytes in their isolates. T cell preparations with even partial monocyte contamination may display monocyte COX-2 on Western immunoblot in response to specific T cell activation. However, COX-2 would not be detected when T cells are specifically examined by flow cytometry, for example. This proposition could explain the discordance between our findings and those reported previously[21-24,26].

While some studies have demonstrated prostaglandin production by murine and human T cells[29-34], others have not[35-37]. The purity of T cells has been questioned, with contaminating monocytes and platelets thought to be responsible for prostaglandins detected[38]. Our studies have shown that human T cells are capable of producing TXA_2 and small amounts of PGE_2 via COX-1. While monocytes cannot be definitively excluded as contributing to the eicosanoids detected, the lack of COX-2 in the stimulated T cell preparations would suggest that there is minimal monocyte contamination, and that their potential contribution to the detected eicosanoids is not likely to be significant. With regard to contaminating platelets, it is possible that they may have contributed to TXB_2 detected in our T cell preparations. However, we have demonstrated the presence of COX-1 by both Western immunoblot and flow cytometry in CD3+ T cells. Further, since platelets do not produce PGE_2, at least some of the detected eicosanoids must be produced by T cells.

Whether the small amounts of eicosanoids produced by T cells contribute significantly to the inflammatory response in the rheumatoid synovium is unclear. While TXA_2 has been reported to upregulate COX-2 in human umbilical vein endothelial cells[20], it was not responsible for the upregulation of monocyte COX-2 by activated T cells. However, TXA_2 has been reported to facilitate monocyte IL-1ß and TNF-α production[18], whereas PGE_2 has the opposite effects. Thus the higher levels of TXA_2 relative to PGE_2 produced by T cells may contribute to TNF-α and IL-1ß production by monocytes, although this is speculative.

T cells produce a number of cytokines that are candidates for the paracrine upregulation of monocyte COX-2 observed in our study. IFN-γ is generally regarded as proinflammatory and its influence on monocyte COX-2 expression is therefore of special interest. It has been reported that IFN-γ upregulated COX-2 in mouse peritoneal macrophages[39], but had no effect on COX-2 mRNA in U937 monocytic cells[40] or on COX-2 protein expression in human monocytes[41]. However, pretreatment of U937 cells with IFN-γ augmented COX-2 expression in response to subsequent LPS stimulation[40]. In contrast, it has been reported that pretreatment of U937 cells with IFN-γ inhibited COX-2 expression in IL-1ß-stimulated, but not LPS-stimulated cells[42].

Our results indicate that pretreatment of monocytes with IFN-γ inhibited LPS-stimulated monocyte COX-2 expression and eicosanoid production. Further, inhibition of T cell-derived IFN-γ with IFN-γ-neutralizing antibody enhanced the expression of COX-2 in monocytes co-incubated with anti-CD3/anti-CD28-stimulated T cells. The results suggest that IFN-γ is a paracrine negative regulator of monocyte COX-2 expression in T cell-monocyte interactions.

While levels of IFN-γ are relatively low in rheumatoid synovium, IL-17 is abundant in rheumatoid synovium and synovial fluid (5–5000 pg/ml)[11,12]. IL-17 has been shown to induce PGE_2 production in epithelial cells, endothelial cells, and primary cultured synovial fibroblasts[43]. In human chondrocytes and adherent human macrophages, IL-17 induced an increase in COX-2 expression[10,44]. However, IL-17 has been reported to have no direct effect on cytokine secretion by human blood monocytes, leading the authors to suggest a limited role of IL-17 in T cell-driven inflammatory processes[43]. Our results show that at high concentration IL-17 can induce monocyte COX-2 expression and eicosanoid production. At lower concentrations (≤ 10 µg/ml), which are still above that produced by activated T cells, there is minimal effect on monocyte COX-2 expression. However, IL-17-neutralizing antibody at least partially reduced the upregulation of monocyte COX-2 in interactions between stimulated T cells and monocytes. These results suggest that IL-17 is acting in combination with other soluble mediators produced by activated T cells to induce monocyte COX-2 expression. A possible contributor to IL-17 action is TNF-α, because we have observed that IL-17 synergizes with TNF-α to induce COX-2 in synovial fibroblasts (unpublished results). Synergy between IL-17 and TNF-α may have special functional importance since these cytokines appear to be coordinately expressed in antigen-stimulated T cells[45].

Thus, low levels of IFN-γ and high levels of IL-17 may be important in determining expression of monocyte COX-2 in the rheumatoid joint. COX-2 upregulation would

increase the synthesis of PGE_2, which mediates joint swelling and tenderness, and would increase the synthesis of TXB_2, which can act in an autocrine manner to facilitate monocyte IL-1ß and TNF-α production[18].

In summary, there was no evidence for COX-2 expression in T cells, but the findings provide a potential explanation for the reports of T cell COX-2 expression. The results indicate that soluble mediators produced by activated T cells can influence the expression of monocyte COX-2 and eicosanoid production. Both IL-17 and IFN-γ may be important in determining the paracrine effects of T cells on monocyte COX-2 expression as positive and negative regulators, respectively. The results suggest that the high levels of IL-17 and relatively low levels of IFN-γ that have been reported in rheumatoid joints may be responsible for T cell-driven upregulation of monocyte COX-2. The effects of IL-17 on monocyte COX-2 expression, in addition to its effect on joint damage in animal models of arthritis[46,47], add to the validation of IL-17 as a therapeutic target in rheumatoid arthritis and other inflammatory diseases.

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