The Journal *of* Immunology

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Supplementary Material	http://www.jimmunol.org/content/suppl/2010/08/16/jimmunol.1000906.DC1
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Nonsteroidal Anti-Inflammatory Drugs Increase TNF Production in Rheumatoid Synovial Membrane Cultures and Whole Blood

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Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit cyclooxygenase activity and hence PG production. However, the ability of NSAIDs to ameliorate pain and tenderness does not prevent disease progression in rheumatoid arthritis, a disease whose pathogenesis is linked to the presence of proinflammatory cytokines, such as TNF-α. To understand this observation, we have examined the effect of NSAIDs on the production of clinically validated proinflammatory cytokines. We show that a variety of NSAIDs superinduce production of TNF from human peripheral blood monocytes and rheumatoid synovial membrane cultures. A randomized, double-blinded, crossover, placebo-controlled trial in healthy human volunteers also revealed that the NSAID drug celecoxib increased LPS-induced TNF production in whole blood. NSAID-mediated increases in TNF are reversed by either the addition of exogenous PGE$_2$ or by a PGE$_2$ EP2 receptor agonist, revealing that PGE$_2$ signaling via its EP2 receptor provides a valuable mechanism for controlling excess TNF production. Thus, by reducing the level of PGE$_2$, NSAIDs can increase TNF production and may exacerbate the proinflammatory environment both within the rheumatoid arthritis joint and the systemic environment. *The Journal of Immunology*, 2010, 185: 3694–3701.

Rheumatoid arthritis (RA) is an autoimmune and chronic inflammatory condition causing joint destruction and significant augmented mortality that affects ~1% of the population worldwide. Central to the pathogenesis of this condition are a number of proinflammatory cytokines, such as TNF and IL-6, whose expression drives both the inflammatory and destructive processes that are characteristic of this disease (1, 2). The pivotal role of TNF in RA pathology is underscored by the success of anti-TNF biologic therapies in the clinic (3). However, economic constraints, route of administration, and potential side effects dictate that such therapeutics have not always been used early in disease progression. Rather, drugs, such as the nonsteroidal anti-inflammatory drugs (NSAIDs), are licensed for symptomatic relief of the pain associated with this condition, and they may be self administered using available over-the-counter formulations or prescribed early in the disease, with disease-modifying drugs, such as methotrexate and anti-TNF, used later.

NSAIDs exert their action by inhibiting the activity of the cyclooxygenase (COX) enzymes and thus the conversion of arachidonic acid to a number of PG derivatives (4, 5). These important immunomodulatory molecules (including PGD2, PGI2 [prostacyclin], PGF2α, thromboxane A2, and PGE2) are able to regulate such diverse processes as pain, vasodilation, cardioprotection, apoptosis, and bone remodeling (6) and are considered important in both normal homeostasis and a number of disease pathologies. Two COX enzymes are known to be important in PG production: the constitutively expressed COX1 and COX2, which can be induced by the actions of LPS and cytokines, such as TNF and IL-1β (7–9). Consequently, COX2 expression is considered to be a characteristic marker of sites of inflammation and, through the use of NSAIDs, has been a primary target of anti-inflammatory therapy for many years (10, 11). However, while the analgesic properties of NSAIDs clearly result in reduced joint pain and tenderness and improve mobility in humans, there is no evidence that NSAID therapy slows disease progression. This finding contrasts studies in the acute heterologous (bovine) type II collagen-induced mouse arthritis model, which have shown beneficial effects of NSAIDs on joint pathology and proinflammatory cytokine levels (11, 12). This discrepancy may reflect the different nature of the disease in mice, in which joint inflammation is acute but transient in comparison with the chronic human pathology.

An explanation for the lack of disease-modifying antirheumatic drug (DMARD) activity of NSAIDs in human RA has not been established. In this regard, there has been no assessment of the effects of NSAIDs on the expression of inflammatory cytokines in human disease tissue and many primary human cell populations. To address this question we have examined the effect of a range of NSAIDs on cytokine production in both primary human monocyte

and synovial membrane cell cultures. Our findings demonstrate that clinically relevant concentrations of these drugs superinduce production of the key proinflammatory cytokine TNF-α (but not IL-6 or IL-1) in both RA synovia and primary human monocytes. These data are further supported by findings from a randomized, double-blind, placebo-controlled, crossover trial in which the level of TNF produced ex vivo in whole blood from healthy volunteers after NSAID administration was significantly increased. These data may provide a mechanistic explanation for the lack of DMARD activity of NSAIDs in human disease.

Materials and Methods

Reagents

PGE2 was purchased from Sigma (Poole, Dorset, U.K.) LPS and flagellin from Alexis (Exeter, U.K.). N-palmitoyl-S-[2,3-bis (palmitoyloxy)-(2R)-propyl]-(R)-cysteine (PAM$_3$Cys) was obtained from ECM Microcollections (Tubingen, Germany). Butaprost, misoprostol, and sulprostone were obtained from Cayman Chemicals (Ann Arbor, MI). COX2-specific inhibitors were obtained from GlaxoSmithKline (Stevenage, U.K.). Diclofenac was purchased from Sigma. Drugs were dissolved in DMSO, aliquoted, and stored at −20°C before use. ELISAs were purchased from BD PharMingen (Oxford, U.K.; IL-1, IL-6, IL-8, IL-10, TNF), or R&D Systems (Minneapolis, MN; PGE$_2$). All reagents other than LPS were tested for the presence of endotoxin using a *Limulus* amebocyte assay (BioWhittaker, Walkersville, MD) and were found to be free of contaminating LPS.

Murine synovial cell culture

Synovial cell cultures were performed using a modified protocol from Malfait et al. (13). Male DBA/1 mice were immunized with 100 μg bovine type II collagen in emulsified Freund's complete adjuvant by intradermal injection at the base of the tail. At 7–10 d after onset of arthritis, the knee was opened by removal of the patella tendon, and the synovial tissue was excised. Tissue was pooled from a minimum of 10 animals. The tissue was digested in 0.5 mg/ml Liberase (Roche, Mannheim, Germany), 0.2mg/ml DNAse I (Sigma) for 30 min at 37°C with shaking. A single-cell suspension was formed by straining through a 70-μM filter, and cells were cultured at 4×10^6 cells/ml in complete DMEM for 24 h with various concentrations of celecoxib. TNF and IL-6 were assayed in the culture supernatant by ELISA according to manufacturers' instructions (R&D Systems).

Human synoviocyte cell preparation

RA synovial cells were obtained from patients undergoing elective joint replacement surgery and were prepared by mechanical and enzymatic disruption as described previously (14). Single-cell suspension cultures were prepared by mechanical disruption followed by digestion in 0.15 mg/ml type IV DNAse (Sigma) and 5 mg/ml collagenase (from *Clostridium histolyticum*; Roche) for 1–1.5 h at 37°C. Cells released were collected, counted, and plated in flat-bottom 96-well plates at a density of 1×10^5 cells/well. NSAIDs were added at the concentrations shown (Figs. 2, 5, Supplemental Figs. 1–3). All procedures received local ethics committee approval (RREC No. 1752).

Isolation of monocytes

PBMCs were prepared from buffy coat fractions of a unit of blood from a single donor using Ficoll-Hypaque. The monocytes were then isolated by centrifugal elutriation, as previously described (15). Monocyte fractions >85% purity were routinely collected in this manner. Monocytes were cultured in RPMI 1640 containing 100 U/ml penicillin-streptomycin and 5% heat-inactivated FCS at 37°C in a humidified atmosphere containing 5% CO$_2$. Cells were cultured in flat-bottom 96-well plates at 1×10^5 cells/well. Cells were preincubated with NSAIDs or agonists at the concentrations shown (Figs. 3, 4, 6) for 1 h prior to the addition of stimulus.

TNF bioassay

Levels of bioactive TNF were determined using the Walter and Eliza Hall Institute 164 cell line (clone 13) (16). Cells at 2×10^4 cells/well were incubated overnight with cell culture supernatants and actinomycin D (0.5 μg/ml) at 37°C overnight. Viability was determined by measuring absorbance at 620 nm on a spectrophotometric ELISA plate reader after the addition of MTT.

ELISA

At 24 h after stimulation of monocytes or after the addition of NSAIDs to RA, synovial cell cultures supernatants were harvested. The concentrations of TNF-α, IL-1β, IL-6, and PGE$_2$ were determined by ELISA according to the manufacturer's instructions. Absorbance was read and analyzed at 450 nm on a spectrophotometric ELISA plate reader (Labsystems Multiskan Biochromic, Helsinki, Finland) using the Ascent software program (Thermo Scientific, Surrey, U.K.).

Clinical trial

Whole blood was obtained by venepuncture from 17 healthy male volunteers (aged 27–47 y) both before and 2 h after ingestion of a single oral dose of either 200 mg celecoxib or placebo. Blood was diluted 1:1 in serum-free RPMI 1640 and stimulated with TLR ligand (100 ng/ml LPS, 10 μg/ml R848). Culture supernatants were collected after 4 h and TNF production was measured by ELISA. This trial was performed as a double-blind, crossover study and was decoded only after TNF levels were obtained. This trial was approved by the local ethics committee (Hammersmith and Queen Charlotte's and Chelsea research ethics committee, reference no. 07/QO404/44). Volunteers provided informed consent prior to their inclusion in this study.

Statistical analysis

Student paired *t* test was used to analyze the significance of changes in cytokine levels in the presence of NSAIDs. Two-tailed Wilcoxon signed rank test was used for the clinical trial data.

Results

The effect of celecoxib on cytokine production in murine synovial membranes

In animal models of arthritis, such as the heterologous type II collagen-induced arthritis (CIA) model, NSAIDs have been shown to reduce clinical disease scores and disease progression (10, 17, 18). To investigate how NSAID treatment influences the levels of proinflammatory cytokines within the joints of these animals, synovial cell membrane cultures obtained from DBA/1 mice with CIA 7–10 d after arthritis onset were examined for TNF and IL-6 expression. Fig. 1 shows that the spontaneous production of IL-6 is dramatically reduced, and the production of TNF is also decreased by the addition of the COX2-specific inhibitor celecoxib in these animals. IL-6 production is substantially decreased even at the lowest concentration tested, suggesting that IL-6 production in these cultures is more sensitive to COX inhibition than is TNF. The effect of lower doses of celebrex on IL-6 production was not investigated (Fig. 1).
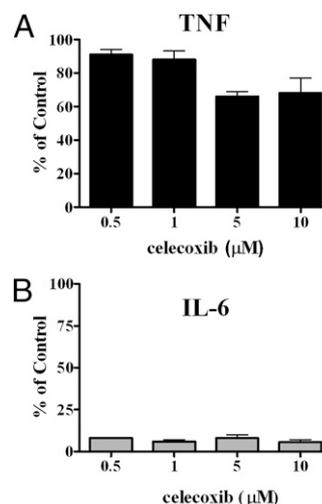
FIGURE 1. NSAIDs reduce proinflammatory cytokine production in synovial cultures from CIA mice. Spontaneous TNF (*A*) and IL-6 (*B*) release from synovial membrane cultures from CIA mice in the presence of celecoxib (0.5–10 μM). Data shown are expressed as percent change in relation to vehicle-only–treated membranes and represents mean values ± SEM. Data were generated from two separate experiments. For each experiment, tissue was pooled from at least 10 separate animals.

NSAIDs enhance TNF production in human RA synovial membranes

Whereas COX inhibitors are demonstrated to act as DMARDs in acute murine models of arthritis, this is not the case in human RA. We therefore examined the effects of celecoxib (Celebrex), another selective COX 2 inhibitor, rofecoxib (Vioxx), and the broader specificity (COX1 and COX2) inhibitor diclofenac (Voltarol) on spontaneously produced proinflammatory cytokine levels in synovial membrane cell cultures from RA patients. Fig. 2 shows that, in contrast to the murine synovial membrane cultures, the addition of celecoxib, rofecoxib, or diclofenac to cultured RA synovium does not inhibit proinflammatory cytokine production. Rather, it results in a concentration-dependent increase in the amount of TNF produced. Fig. 2A shows cells from a single donor treated with concentrations of celecoxib and rofecoxib between 41 pM and 10 μM and reveals a concentration-dependent increase in TNF production. This donor is unusual in showing little or no change in TNF level at 0.3 μM. Fig. 2B–D shows combined data generated from five to nine individual synovia at concentrations (0.3–10 μM) reported to be achieved in serum after administration of clinically relevant doses of NSAIDs recommended for symptomatic relief in RA (19, 20). All donors showed increased TNF production over this concentration range. Because absolute levels of cytokines produced in these cultures differ between patients (Supplemental Fig. 1), results are presented as mean changes in cytokine relative to untreated control cultures (Fig. 2). To confirm that the TNF produced in these cultures was biologically active, supernatants from a representative membrane were also assayed using the Walter and Eliza Hall Institute cytotoxicity assay (16). This assay shows clear increases in TNF bioactivity levels after NSAID treatment (Supplemental Fig. 2). In addition to changes in the level of TNF, NSAID treatment also alters expression of the antiinflammatory cytokine IL-10, except in this case the expression of IL-10 is reduced by as much as 50% (Fig. 2B–D).

In contrast to the effects on TNF and IL-10, and to the findings in murine synovia, IL-6 production in RA membranes is unaffected by treatment with celecoxib, rofecoxib, or diclofenac. IL-1β ex-

pression is also unaltered by NSAID treatment in RA membranes, except in those treated with celecoxib in which its production is inhibited (Fig. 2B). The reason for this finding is unclear, but as a consistent finding in all of the donors examined it is unlikely to reflect donor-to-donor variation in response to different NSAIDs, which is a well-described phenomenon between individuals. Furthermore, because this activity is not shared by the other NSAIDs examined, it is unlikely to represent an NSAID class effect. At present, the basis of this activity has not been investigated further.

NSAIDs increase TNF production in LPS-stimulated human monocytes

Peripheral blood monocytes act as the precursor cell type for resident tissue macrophages and for those macrophage-like cells that accumulate within inflammatory sites, including the joints of patients with RA and in atherosclerotic plaques (21, 22). To determine the effect of NSAIDs on the cytokine response of human peripheral blood monocytes, cells were pretreated with celecoxib, rofecoxib, or diclofenac and were then stimulated with LPS. In common with cells from the active rheumatoid synovial cell cultures, all three NSAIDs produce concentration-dependent increases in the amount of TNF produced in response to LPS in monocyte cultures (Fig. 3A–C, Supplemental Fig. 1). At clinically relevant concentrations (0.3–10 μM) (19), celecoxib, rofecoxib, and diclofenac all cause a significant increase in TNF production (between 2- and 5.5-fold), whereas drug administration alone does not induce cytokine production (not shown). In common with their effect in RA membranes, the NSAIDs have no effect on the levels of IL-6 or IL-1β produced in response to LPS in these cells (Fig. 3D–F). In contrast to the effect on RA membranes, NSAID treatment does not inhibit the amount of IL-10 produced in these cultures. Thus, although IL-10 is able to reduce the expression of TNF in RA synovia (23), the observation that TNF levels also increase in the monocyte system, where levels of IL-10 do not concomitantly decrease, suggests that the ability of NSAIDs to enhance TNF production cannot be explained by a mechanism based on the suppression of IL-10, and other explanations are needed.
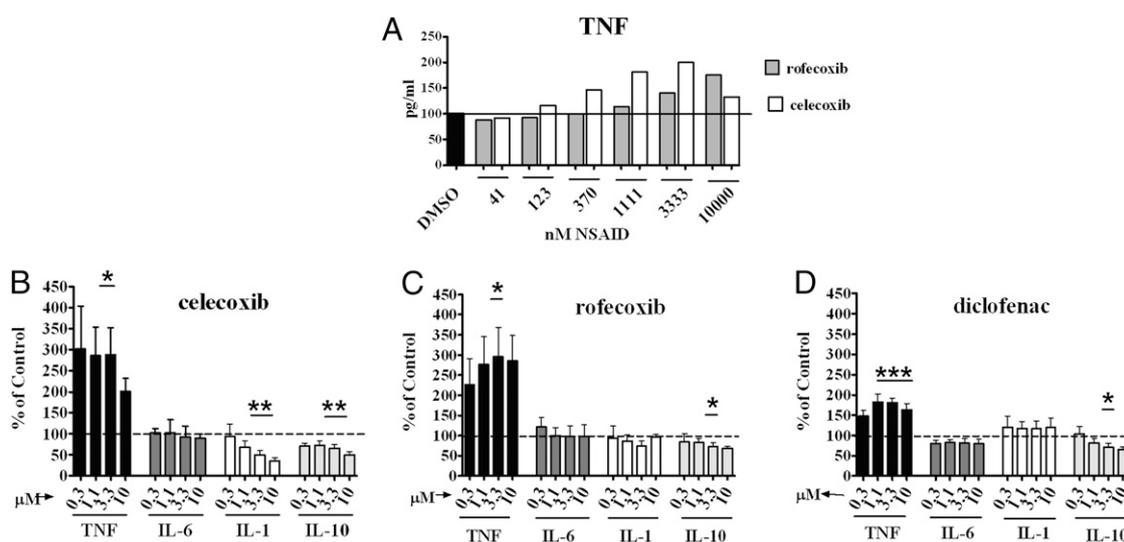
FIGURE 2. NSAIDs increase TNF expression in human RA synovial membrane cultures. A, Spontaneous TNF release by an RA synovial membrane culture from a single donor in the presence of NSAIDs (1–10,000 nM). Black bar represents vehicle control (DMSO) in the absence of NSAIDs. Spontaneous release of cytokines by RA synovial membrane cultures in the presence of clinically desirable concentrations (0.3–10 μM) of celecoxib (B), rofecoxib (C), and diclofenac (D). Data shown are expressed as percent change in relation to vehicle-only–treated membranes and represents mean values ± SEM from five to nine separate donors. The dashed line represents 100% control value (vehicle only). Statistical significance is shown for TNF at the 3.3 μM concentration. *p < 0.05; **p < 0.005; ***p ≤ 0.001.

FIGURE 3. NSAID treatment increases TNF production in human monocytes. Primary human monocytes were stimulated for 24 h with LPS in the presence or absence of increasing concentrations of celecoxib (*A*), rofecoxib (*B*), or diclofenac (*C*). Tissue culture supernatants were examined for levels of TNF by ELISA. Tissue culture supernatants from f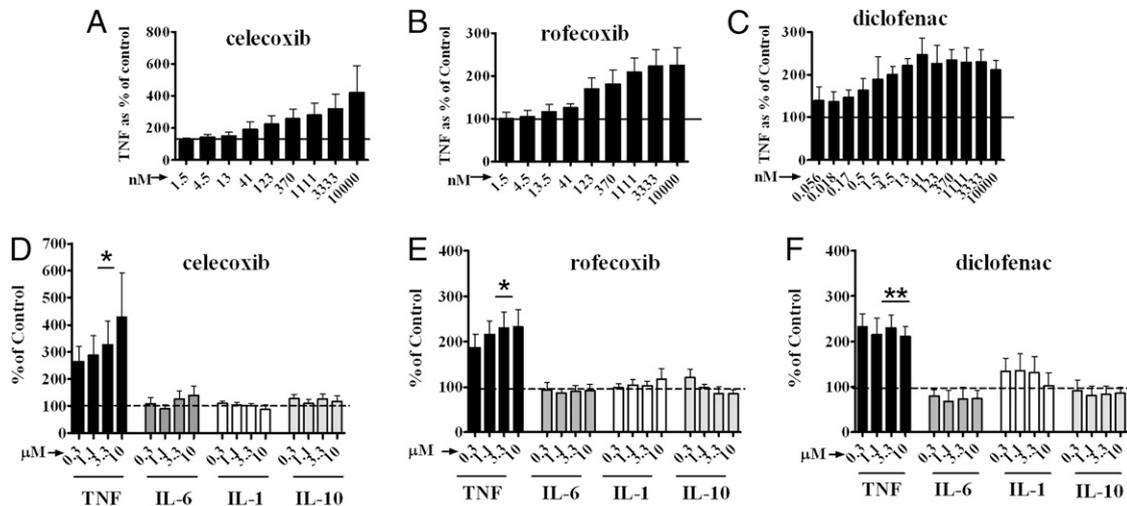ive additional donors were treated with clinically desirable (0.3–10 μM) concentrations of celecoxib (*D*), rofecoxib (*E*), or diclofenac (*F*) and were also examined for levels of TNF, IL-6, IL-1, and IL-10 by ELISA. Results shown are expressed as increases relative to cells stimulated with LPS alone and represent mean values ± SEM. The dashed line represents 100% control value (LPS only). Statistical significance is indicated for TNF at the 3.3-μM concentration. *t* Tests for other cytokines were not significant. $*p \le 0.05$; $**p \le 0.005$.

NSAID induced changes in TNF are seen in response to a variety of TLR ligands

LPS is only one of many TLR ligands that could play a role in the induction of inflammation or the response to infection or in RA (24, 25). To determine whether NSAIDs also increase TNF production induced by signaling through other TLRs, we examined the effect of NSAID treatment after stimulation with PAM_3Cys (TLR2/1) and flagellin (TLR5). Fig. 4 shows that NSAIDs also increase TNF production in monocytes stimulated with these TLR ligands, demonstrating that this function is not specific to stimulation via TLR4. These data reveal that therapeutic levels of NSAIDs increase the expression of TNF in response to a number of TLR ligands.

PGE_2 reverses the effect of NSAIDs on TNF production

PGE_2 is one of the most abundant PGs in the RA synovium and is produced as a direct result of COX activity (26). Accordingly, all NSAIDs used in this study effectively reduce PGE_2 production in RA synovial cell membrane cultures (Fig. 5*A*). PGE_2 is often regarded as a proinflammatory mediator (27). However, it may also possess potent antiinflammatory properties (28–31). To establish whether the NSAID-mediated reduction in PGE_2 was responsible for the NSAID-mediated rise in spontaneous TNF production, exogenous PGE_2 (5 μM) was added to NSAID-treated rheumatoid synovial membrane cell cultures. Levels of PGE_2 present in

FIGURE 4. NSAIDs enhance TNF production in response to other TLR ligands. Primary human monocytes were stimulated with (*A*) PAM_3Cys (10 ng/ml) or (*B*) flagellin (10 ng/ml) in the presence of 3.3 μM C, R, or D. Supernatants were harvested after 24 h, and TNF levels were measured by ELISA. Data shown represent mean values ± SEM from three or four separate donors. The dashed line represents 100% control value (TLR ligand only). $*p \le 0.05$; $**p \le 0.005$. C, celecoxib; D, diclofenac; R, rofecoxib.

synovial membrane cell preparations in the absence of NSAIDs varied among individuals (not shown); 5 μM represents at least a 6-fold excess over control levels. Fig. 5*B* shows that the addition of PGE_2 reduces the excess TNF expression produced in response to NSAIDs, resulting in TNF levels either equal to or less than those in untreated cultures. These data reveal that the increased levels of TNF in NSAID-treated RA synovial cell cultures can be attributed directly to a reduction in the level of PGE_2.

Signaling via EP2 receptors mediates cytokine changes in primary human monocytes

PGE_2 is known to signal via four distinct cell surface receptors, known as EP1, EP2, EP3, and EP4 (6). The EP2 and EP4 receptors both mediate increases in cAMP levels (6), and agents that raise cAMP levels are known to decrease TNF production in monocytes (31, 32). Thus, signaling by PGE_2 via the EP2 or EP4 receptors may provide the mechanism by which PGE_2 reduces levels of TNF. To investigate this hypothesis, primary human monocytes were stimulated with LPS in the presence of either PGE_2, which can interact with all four EP receptors (EP1, 2, 3, and 4), or various concentrations of selective EP receptor agonists: butaprost (EP2), misoprostol (EP2, 3, 4), or sulprostone (EP1, 3). Our data show that PGE_2 and both butaprost and misoprostol are able to inhibit TNF production in this system in a dose-dependent manner. In direct contrast, sulprostone was unable to effect a change in TNF production (Fig. 6*A*). These data demonstrate that signaling via the EP2 receptor (butaprost) and possibly also via the EP4 receptor (misoprostol) is able to mimic the effect of PGE_2 on TNF production in this system. In contrast, signaling via the EP1 and EP3 receptors is not involved in cytokine modulation.

EP2 agonists decrease spontaneous TNF production in RA membrane cultures

Because monocyte-macrophages are considered to be the major source of TNF-α in RA synovial tissue, additional studies were performed to investigate the effects of EP-receptor selective agonists on spontaneous TNF production by cultured synovial membrane cells. We show in this study that in human RA synovial membrane cultures, treatment with PGE2 itself, and with both butaprost (EP2) and misoprostol (EP2, 3, 4) reduces the spontaneous production of
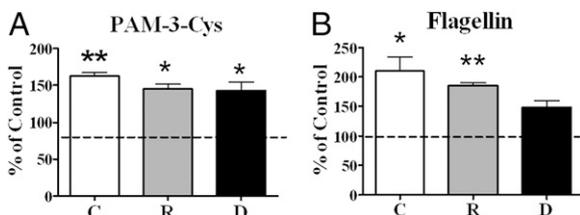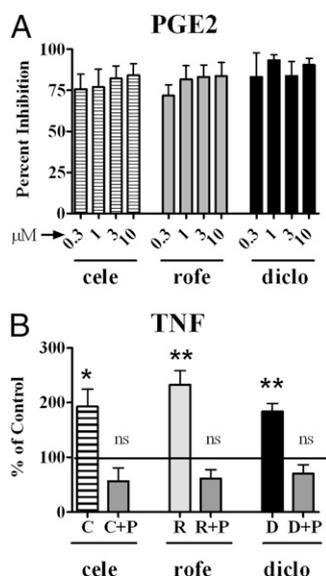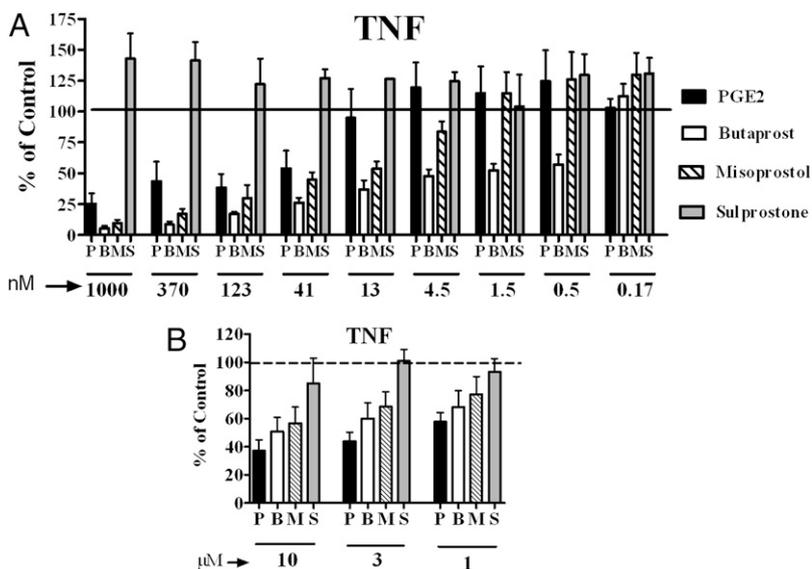
FIGURE 5. NSAID-induced changes in PGE$_2$ mediate the changes observed in spontaneous TNF expression. *A*, RA synovial membrane cultures were incubated for 24 h with the indicated concentrations of NSAIDs. Supernatants were then analyzed by ELISA for PGE$_2$ content. The graph shows percent inhibition when compared with untreated membranes. Data shown represent mean values ± SEM from three to six separate donors. *B*, Effect of addition of exogenous PGE$_2$ (5 μM; +P) on TNF levels in NSAID-treated (10 μM) synovial membrane cultures. The graph shows percent changes relative to untreated membranes (solid line). Data shown represent mean values ± SEM from six or seven separate donors. cele, celecoxib; diclof, diclofenac; ns, no significant change; rofe, rofecoxib. ∗$p \leq 0.05$; ∗∗$p \leq 0.005$.

TNF (Fig. 6*B*). As seen in monocyte cultures, sulprostone (EP1, 3) was ineffective. These results highlight the importance of PGE$_2$ signaling via its EP2 receptor in controlling expression of TNF within the human synovial membrane.

NSAIDs cause increased TNF production in whole blood of human subjects following NSAID treatment in vivo

To assess the relevance of these data to in vivo effects in human subjects, we extended this study to an in vivo environment. We conducted a randomized, double-blind, crossover, placebo-controlled trial investigating the effect of oral administration of celecoxib on

TNF production in whole blood. Seventeen normal healthy male volunteers (aged 25–47 y) received a single oral dose of either 200 mg celecoxib or placebo. Blood was taken both immediately before receiving the dose and 2 h afterward, to coincide with peak serum levels of celecoxib (33). Whole blood cell cultures were then established according to the method described by Schippers et al. (34). Basal activation of circulating monocytes in the blood of healthy individuals is extremely low, and subsequently cytokine production is below the level of detection. Consequently stimulation ex vivo with LPS (TLR4 ligand) or R848 (TLR7/8 ligand) was used to mimic cellular encounter with an inflammatory stimulus, such as an invading pathogen or endogenous damage-associated molecular pattern (DAMP). Supernatants from stimulated cells were collected at 4 h, and TNF production was assessed by ELISA. The results shown in Fig. 7 demonstrate that after a single dose of NSAID, the production of TNF in whole blood is significantly increased in response to ex vivo stimulation by either LPS ($p = 0.0342$) or R848 ($p = 0.0049$). In contrast, when the same individuals received placebo drug, there was no significant change in cytokine production. These data show that the effects of NSAIDs observed using in vitro cell cultures are reproduced in blood cells that were exposed to the drug in vivo under clinically relevant conditions, and they suggest that the changes in cytokine expression demonstrated in the rheumatoid synovial cultures are likely to be mirrored at a systemic level after ingestion of a widely prescribed therapeutic concentration of celecoxib.

Discussion

In this study we demonstrate that NSAIDs significantly increase spontaneous TNF production in synovial membrane cultures from patients with RA. These studies have used COX2-specific NSAIDs (celecoxib and rofecoxib) and a COX1- and COX2-bispecific NSAID (diclofenac). Indeed, additional studies with clinically relevant doses of aspirin, a COX1 inhibitor, also produce increases in TNF expression in RA synovial membrane cultures and primary human monocytes (Supplemental Fig. 3), revealing a role for both COX1 and COX2 in PGE$_2$ regulation in the synovial membrane. These findings, in combination with the ability of PGE$_2$ to reverse the NSAID-induced increase in TNF, suggest that it is the NSAID-induced reduction in PGE$_2$ expression that is responsible for the alterations in cytokine production seen.

The concentrations of NSAIDs used in these studies reflect those achieved in serum after administration of therapeutic doses of NSAIDs (20). Although no such data are available for the levels of

FIGURE 6. Signaling via the EP2 receptor mimics PGE$_2$-mediated changes in TNF. *A*, Primary human monocytes were stimulated with LPS (10 ng/ml) in the presence or absence of various concentrations of P, B, M, or S. After 24 h culture, supernatants were collected and levels of TNF were analyzed by ELISA. Data shown represent mean values ± SEM from three or four separate donors and represent percent change relative to diluent only (solid line). *B*, Human RA synovial membrane cultures were treated with 10, 3, or 1 μM P, B, M, or S. Spontaneous release of TNF was measured after 24 h by ELISA. Data shown are expressed as percent change in relation to diluent-only–treated membranes and represent mean values from four separate donors ± SEM. The dashed line represents 100% control value (diluent only). B, butaprost; M, misoprostol; P, PGE$_2$; S, sulprostone.

FIGURE 7. A single dose of celecoxib in vivo increases TLR-induced TNF production in whole blood. Healthy volunteers (aged 25–47 y) were given a single oral dose of either 200 mg celecoxib (*A, C*) or placebo (*B, D*). Whole blood was taken both before and 2 h after ingestion of drug or placebo and stimulated with 100 ng/nl LPS (*A, B*) or 10 µg/ml R848 (*C, D*). After 4 h culture, supernatants were harvested and examined for levels of TNF. Paired results (before and after) are shown for each individual volunteer. Statistical analysis was performed using the Wilcoxon signed rank test. ns, not significant.
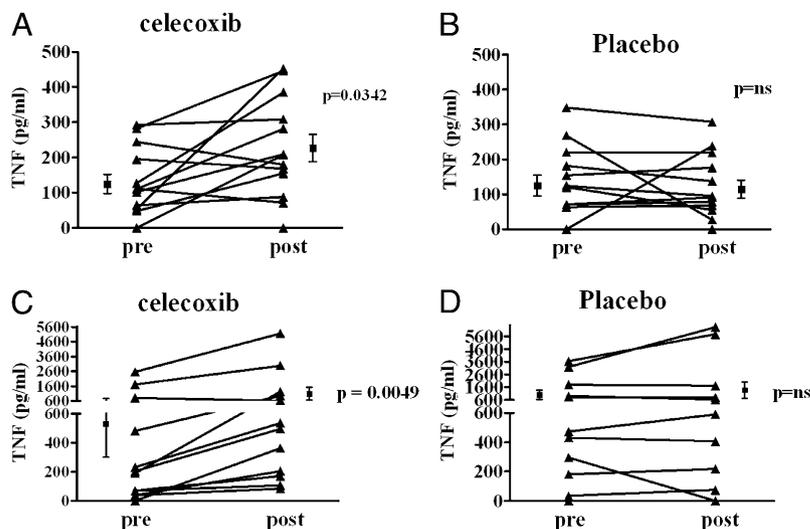
NSAIDs achieved in synovial fluid, by using a wide concentration range in the synovial membrane cell assay we have demonstrated that the compounds have pharmacologic activity in this system. Moreover, the potencies of NSAID effects seen in the in vitro cell systems occur at concentrations lower than those achieved in plasma following clinically relevant doses. It is likely that the changes in cytokine profile in the tissue explants used in this study would also be replicated locally in situ in the synovium, and these results provide a justification for further clinical investigation. Effects on cytokine levels in vivo could possibly be investigated by taking sequential synovial biopsy specimens after dosing with NSAIDs. However, this type of investigation is beyond the scope of this study.

At first our findings may appear difficult to reconcile with the effectiveness of NSAIDs in some animal models of RA where NSAIDs have DMARD-like activity. In agreement with this finding, a range of studies including selective inhibition (10) or genetic ablation (35) of COX2, PG E synthase-1 or EP2 or -4 receptor deficiency (12) have demonstrated the involvement of PGE_2 in arthritis associated joint pathology in rodents. However, whereas proinflammatory cytokines are central to the pathology of RA in both human and rodent models, the relative importance of TNF and IL-6, and the effect of PGE2 on these cytokines in rodent models of RA may differ from that in humans. Thus, in a rat adjuvant arthritis model, selective inhibition of COX2 (10) or PGE_2 (36) attenuates inflammation and inhibits both joint IL-6 expression (mRNA) and serum IL6 levels, but has no substantial effect on TNF-α mRNA expression. Evidence for the enhancement of IL-6 and suppression of TNF-α production by EP2–EP4 receptor agonists has also been reported using synovial tissue from mice with pristane arthritis (37), whereas studies using synoviocytes from CIA mice showed that IL-6 production was enhanced by EP2 and EP4 agonists and inhibited by indomethacin (12). Results of the current study showing celecoxib inhibition of spontaneous IL-6 and TNF production by mouse CIA synovial membrane cells are consistent with these earlier findings (Fig. 1) and suggest that in rodent arthritis models, endogenous PGs may promote disease through enhanced production of IL-6 (mRNA-protein). Thus, the inhibition of PGE_2-dependent IL-6 production may provide a rationale for the disease-modifying activity of NSAIDs.

Such differences may reflect the different nature of rodent disease models. In particular, the commonly used murine CIA model, induced with heterologous (bovine) type II collagen (Fig. 1), is an acute, monophasic disease. In contrast, RA in humans is a chronic, progressive condition that more closely resembles the chronic disease induced in mice using autologous type II collagen. It is interesting to note that, in common with human RA, NSAIDs do not act as DMARDs in this chronic CIA model (38). Such differences in human RA and heterologous CIA may also be reflected in the cellular composition of synovial cell membrane preparations. Immunohistologic studies of RA membranes have shown the presence of infiltrating T cells, plasma cells, monocyte-macrophage–like cells, granulocytes, and NK cells (39, 40). In agreement, studies in this laboratory show that human RA membrane preparations contain ~60% CD45+ cells, with the remainder being fibroblast-like cells (not shown). Whereas murine CIA membranes also contain T cell populations and monocyte-macrophage–like cells (41), the proportion of fibroblast-like cells is thought to be higher in murine CIA membrane preparations. Because these cells are potent producers of IL-6 (42, 43), this finding may offer an explanation for the enhanced role of IL-6 and, thus, the effectiveness of PGE_2 inhibition in CIA.

In contrast to studies in acute rodent models of RA, the role that PGE_2 plays in the destructive pathogenesis of human RA is still unclear despite the long-standing clinical use of NSAIDs in RA therapy and their undisputed ability to reduce pain and tenderness in affected joints. Such analgesic and antiswelling properties of NSAIDs in humans can be explained by the fact that, by inhibiting COX activity, NSAIDs reduce the production of a number of different prostanoids in addition to PGE_2. It is likely that a reduction in the levels of prostacyclin (a potent vasodilator and one of the dominant prostanoids in the RA membrane), is responsible for NSAID-mediated reductions in edema (26, 44). Prostacyclin and PGE_2, the other dominant prostanoid in synovial fluid are also important in mediating pain (27, 45). NSAID-mediated decreases in these prostanoids therefore provide a reasonable explanation for the pain relief provided by these drugs. However, whereas NSAIDs clearly offer symptomatic relief of inflammation, our data suggest that this may be achieved in the presence of increased levels of TNF. TNF has been implicated in bone erosion, angiogenesis, and a wide range of inflammatory conditions, including cardiovascular disease. Clinical data linking NSAID use and exacerbated RA disease scores have not been reported and might be difficult to control, given the wide availability of this class of compounds and their self administration. However, these findings may offer some explanation for the adverse effects of long-term NSAID use and increased cardiovascular events (4, 46).

The NSAID-driven increase in TNF production could potentially be counteracted by any concomitant increases in IL-10, soluble TNFR expression, or both (23). However, the observation that bioactive TNF

also increases in response to NSAID treatment would argue against the presence of effective, TNF neutralizing quantities of soluble TNFRs. Furthermore, we observed that levels of IL-10 tend to decrease in RA membranes in response to NSAID treatment. This decrease can be reversed by the addition of exogenous PGE_2 (Supplemental Fig. 4), a finding that agrees with recent data reported by Nemeth et al. (47), who showed a PGE_2-dependent increase in IL-10 production in lung monocytes and macrophages. Such additional changes in the cytokine milieu of an RA synovia may further skew its profile toward that of inflammation. However, we consider that the decrease in IL-10 in RA synovial cultures is unlikely to be responsible for the observed changes in TNF, because similar reductions in IL-10 production are not seen in primary human monocytes where comparable increases in TNF are observed. In human RA synovium we show that PGE_2 can act as an important regulator of TNF production and that this effect can be mimicked by EP2, EP4 receptor agonists, or both. These findings suggest a scenario whereby TNF produced within the synovium acts to induce the expression of COX2 and hence PGE_2. In turn, the PGE_2 is able to signal via its EP2 (and possibly also its EP4) receptors to increase cAMP levels within the cell. Increases in cAMP levels are reported to reduce TNF expression in human cells (48). Indeed, inhibitors of phosphodiesterase 4, which also act to increase intracellular levels of cAMP, are known to reduce TNF expression (31, 49, 50) and have been shown recently to do so in human RA synovial cell cultures (51). Phosphodiesterase 4 inhibitors are being actively pursued by a number of drug companies as potential therapeutics in RA. Thus, by their ability to inhibit PGE2 production, NSAIDs may allow the already excessive production of TNF in the RA joint to increase further. In this context it would be interesting to determine whether formulations that combine NSAIDs and EP receptor agonists, such as Arthrotec (i.e., diclofenac, misoprostol), would nullify the effect of NSAIDs on TNF production.

The apparently selective effect of NSAIDs on TNF production from both monocytes and RA membranes may be considered surprising given that TNF is itself able to modulate production of both IL-1 and IL-6 (52). However, although TNF contributes to IL-1 and IL-6 expression, it is unlikely to be the only stimulus within the RA joint, and there is also mounting evidence that the mechanisms, signaling pathways, and transcription factors controlling IL-1 and IL-6 expression differ from those of TNF (15, 53, 54). In particular, with relevance to the role of cAMP in EP2 receptor signaling, the TNF promoter is known to harbor a CREB site (55), whereas similar sequences have not been identified on the IL-6 and IL-1 promoters.

Having shown that NSAIDS can enhance TNF production in human RA synovial cultures and in human monocytes in vitro, we wanted to investigate whether an effect on systemic cytokine responsiveness could be detected in humans after a clinically relevant dose of the oral COX2 inhibitor celecoxib. We found that when healthy volunteers were given a single dose of this NSAID, the propensity of their peripheral blood leukocytes in freshly drawn whole blood to produce TNF in response to TLR4 and TLR8 ligands was significantly enhanced (Fig. 7). These data raise three important points. First, the systemic exposure levels of celecoxib achieved after a clinically relevant dose of this drug are sufficient to produce TNF-enhancing effects in vivo. Second, these ex vivo effects are seen when the cells are also stimulated with the TLR 8 ligand R848. Given recent evidence implicating TLR4 and TLR8 signaling in RA pathogenesis (56, 57), this finding increases the potential relevance of these observations to the lack of DMARD activity seen with NSAIDs in human RA. Third, as the process of atherosclerotic vascular disease, plaque formation, and rupture is increasingly viewed as an inflammatory process, and TNF is known to play an important role in these processes (22, 58, 59), the observation that

increases in TNF are seen when whole blood is stimulated with TLR ligands may also shed light on the relationship between these drugs and increased cardiovascular event rates (4, 46).

In summary, the data presented in this study reveal that the relatively COX2 specific (as well as the bispecific) NSAIDs, by reducing levels of PGE^2, are able to increase expression of the key proinflammatory cytokine TNF in rheumatoid synovia, in isolated peripheral blood monocyte populations, and at the systemic level in whole blood. Locally, NSAID enhancement of TNF-α production might be expected to exacerbate aspects of synovial inflammation and associated pathologic processes within the RA joint. While systemically, a consequence of NSAID treatment of patients who may already be at risk for cardiovascular disease may be to further skew their cytokine profile in favor of TNF production, and thus adversely affect vascular or cardiopathology.

Acknowledgments

Disclosures

References

1. Feldmann, M., F. M. Brennan, and R. N. Maini. 1996. Role of cytokines in rheumatoid arthritis. *Annu. Rev. Immunol.* 14: 397–440.
2. Smolen, J. S., and R. N. Maini. 2006. Interleukin-6: a new therapeutic target. *Arthritis Res. Ther.* 8(Suppl 2): S5.
3. Feldmann, M., and R. N. Maini. 2003. Lasker Clinical Medical Research Award. TNF defined as a therapeutic target for rheumatoid arthritis and other autoimmune diseases. *Nat. Med.* 9: 1245–1250.
4. Mitchell, J. A., and T. D. Warner. 2006. COX isoforms in the cardiovascular system: understanding the activities of non-steroidal anti-inflammatory drugs. *Nat. Rev. Drug Discov.* 5: 75–86.
5. Smith, W. L., D. L. DeWitt, and R. M. Garavito. 2000. Cyclooxygenases: structural, cellular, and molecular biology. *Annu. Rev. Biochem.* 69: 145–182.
6. Narumiya, S., Y. Sugimoto, and F. Ushikubi. 1999. Prostanoid receptors: structures, properties, and functions. *Physiol. Rev.* 79: 1193–1226.
7. Hla, T., and K. Neilson. 1992. Human cyclooxygenase-2 cDNA. *Proc. Natl. Acad. Sci. USA* 89: 7384–7388.
8. Ristimäki, A., S. Garfinkel, J. Wessendorf, T. Maciag, and T. Hla. 1994. Induction of cyclooxygenase-2 by interleukin-1 alpha. Evidence for posttranscriptional regulation. *J. Biol. Chem.* 269: 11769–11775.
9. Barrios-Rodiles, M., G. Tiraloche, and K. Chadee. 1999. Lipopolysaccharide modulates cyclooxygenase-2 transcriptionally and posttranscriptionally in human macrophages independently from endogenous IL-1 beta and TNF-alpha. *J. Immunol.* 163: 963–969.
10. Anderson, G. D., S. D. Hauser, K. L. McGarity, M. E. Bremer, P. C. Isakson, and S. A. Gregory. 1996. Selective inhibition of cyclooxygenase (COX)-2 reverses inflammation and expression of COX-2 and interleukin 6 in rat adjuvant arthritis. *J. Clin. Invest.* 97: 2672–2679.
11. Chan, C. C., S. Boyce, C. Brideau, S. Charleson, W. Cromlish, D. Ethier, J. Evans, A. W. Ford-Hutchinson, M. J. Forrest, J. Y. Gauthier, et al. 1999. Rofecoxib [Vioxx, MK-0966; 4-(4′-methylsulfonylphenyl)-3-phenyl-2-(5H)-furanone]: a potent and orally active cyclooxygenase-2 inhibitor. Pharmacological and biochemical profiles. *J. Pharmacol. Exp. Ther.* 290: 551–560.
12. Honda, T., E. Segi-Nishida, Y. Miyachi, and S. Narumiya. 2006. Prostacyclin-IP signaling and prostaglandin E2-EP2/EP4 signaling both mediate joint inflammation in mouse collagen-induced arthritis. *J. Exp. Med.* 203: 325–335.
13. Malfait, A. M., D. M. Butler, D. H. Presky, R. N. Maini, F. M. Brennan, and M. Feldmann. 1998. Blockade of IL-12 during the induction of collagen-induced arthritis (CIA) markedly attenuates the severity of the arthritis. *Clin. Exp. Immunol.* 111: 377–383.
14. Foxwell, B., K. Browne, J. Bondeson, C. Clarke, R. de Martin, F. Brennan, and M. Feldmann. 1998. Efficient adenoviral infection with IkappaB alpha reveals that macrophage tumor necrosis factor alpha production in rheumatoid arthritis is NF-kappaB dependent. *Proc. Natl. Acad. Sci. USA* 95: 8211–8215.
15. Horwood, N. J., T. H. Page, J. P. McDaid, C. D. Palmer, J. Campbell, T. Mahon, F. M. Brennan, D. Webster, and B. M. Foxwell. 2006. Bruton's tyrosine kinase is required for TLR2 and TLR4-induced TNF, but not IL-6, production. *J. Immunol.* 176: 3635–3641.
16. Espevik, T., and J. Nissen-Meyer. 1986. A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. *J. Immunol. Methods* 95: 99–105.
17. Esser, R., C. Berry, Z. Du, J. Dawson, A. Fox, R. A. Fujimoto, W. Haston, E. F. Kimble, J. Koehler, J. Peppard, et al. 2005. Preclinical pharmacology of lumiracoxib: a novel selective inhibitor of cyclooxygenase-2. *Br. J. Pharmacol.* 144: 538–550.
18. Inglis, J. J., C. A. Notley, D. Essex, A. W. Wilson, M. Feldmann, P. Anand, and R. Williams. 2007. Collagen-induced arthritis as a model of hyperalgesia:

functional and cellular analysis of the analgesic actions of tumor necrosis factor blockade. *Arthritis Rheum.* 56: 4015–4023.

19. Insel, P. 1996. *The pharmacological basis of therapeutics.* McGraw-Hill, New York.

20. Huntjens, D. R., M. Danhof, and O. E. Della Pasqua. 2005. Pharmacokinetic-pharmacodynamic correlations and biomarkers in the development of COX-2 inhibitors. *Rheumatology (Oxford)* 44: 846–859.

21. Janossy, G., G. Panayi, O. Duke, M. Bofill, L. W. Poulter, and G. Goldstein. 1981. Rheumatoid arthritis: a disease of T-lymphocyte/macrophage immunoregulation. *Lancet* 2: 839–842.

22. Monaco, C., E. Andreakos, S. Kiriakidis, C. Mauri, C. Bicknell, B. Foxwell, N. Cheshire, E. Paleolog, and M. Feldmann. 2004. Canonical pathway of nuclear factor kappa B activation selectively regulates proinflammatory and prothrombotic responses in human atherosclerosis. *Proc. Natl. Acad. Sci. USA* 101: 5634–5639.

23. Katsikis, P. D., C. Q. Chu, F. M. Brennan, R. N. Maini, and M. Feldmann. 1994. Immunoregulatory role of interleukin 10 in rheumatoid arthritis. *J. Exp. Med.* 179: 1517–1527.

24. Kaisho, T., and S. Akira. 2006. Toll-like receptor function and signaling. *J. Allergy Clin. Immunol.* 117: 979–987, quiz 988.

25. Sacre, S. M., E. Andreakos, S. Kiriakidis, P. Amjadi, M. Feldmann, F. Brennan, and B. M. Foxwell. 2007. The Toll-like receptor adaptor proteins MyD88 and Mal/TIRAP contribute to the inflammatory and destructive processes in a human model of rheumatoid arthritis. *Am. J. Pathol.* 170: 518–525.

26. Brodie, M. J., C. N. Hensby, A. Parke, and D. Gordon. 1980. Is prostacyclin in the major pro-inflammatory prostanoid in joint fluid? *Life Sci.* 27: 603–608.

27. Sugimoto, Y., and S. Narumiya. 2007. Prostaglandin E receptors. *J. Biol. Chem.* 282: 11613–11617.

28. Gomez, P. F., M. H. Pillinger, M. Attur, N. Marjanovic, M. Dave, J. Park, C. O. Bingham, III, H. Al-Mussawir, and S. B. Abramson. 2005. Resolution of inflammation: prostaglandin E2 dissociates nuclear trafficking of individual NF-kappaB subunits (p65, p50) in stimulated rheumatoid synovial fibroblasts. *J. Immunol.* 175: 6924–6930.

29. Dooper, M. M., L. Wassink, L. M'Rabet, and Y. M. Graus. 2002. The modulatory effects of prostaglandin-E on cytokine production by human peripheral blood mononuclear cells are independent of the prostaglandin subtype. *Immunology* 107: 152–159.

30. Takahashi, H. K., H. Iwagaki, R. Tamura, G. Katsuno, D. Xue, S. Sugita, S. Mori, T. Yoshino, N. Tanaka, and M. Nishibori. 2005. Differential effect of prostaglandins E1 and E2 on lipopolysaccharide-induced adhesion molecule expression on human monocytes. *Eur. J. Pharmacol.* 512: 223–230.

31. Seldon, P. M., P. J. Barnes, K. Meja, and M. A. Giembycz. 1995. Suppression of lipopolysaccharide-induced tumor necrosis factor-alpha generation from human peripheral blood monocytes by inhibitors of phosphodiesterase 4: interaction with stimulants of adenylyl cyclase. *Mol. Pharmacol.* 48: 747–757.

32. Janciauskiene, S. M., I. M. Nita, and T. Stevens. 2007. Alpha1-antitrypsin, old dog, new tricks. Alpha1-antitrypsin exerts in vitro anti-inflammatory activity in human monocytes by elevating cAMP. *J. Biol. Chem.* 282: 8573–8582.

33. Davies, N. M., A. J. McLachlan, R. O. Day, and K. M. Williams. 2000. Clinical pharmacokinetics and pharmacodynamics of celecoxib: a selective cyclo-oxygenase-2 inhibitor. *Clin. Pharmacokinet.* 38: 225–242.

34. Schippers, E. F., C. van't Veer, S. van Voorden, C. A. Martina, T. W. Huizinga, S. le Cessie, and J. T. van Dissel. 2005. IL-10 and toll-like receptor-4 polymorphisms and the in vivo and ex vivo response to endotoxin. *Cytokine* 29: 215–228.

35. Myers, L. K., A. H. Kang, A. E. Postlethwaite, E. F. Rosloniec, S. G. Morham, B. V. Shlopov, S. Goorha, and L. R. Ballou. 2000. The genetic ablation of cyclooxygenase 2 prevents the development of autoimmune arthritis. *Arthritis Rheum.* 43: 2687–2693.

36. Portanova, J. P., Y. Zhang, G. D. Anderson, S. D. Hauser, J. L. Masferrer, K. Seibert, S. A. Gregory, and P. C. Isakson. 1996. Selective neutralization of prostaglandin E2 blocks inflammation, hyperalgesia, and interleukin 6 production in vivo. *J. Exp. Med.* 184: 883–891.

37. Akaogi, J., H. Yamada, Y. Kuroda, D. C. Nacionales, W. H. Reeves, and M. Satoh. 2004. Prostaglandin E2 receptors EP2 and EP4 are up-regulated in peritoneal macrophages and joints of pristane-treated mice and modulate TNF-alpha and IL-6 production. *J. Leukoc. Biol.* 76: 227–236.

38. Malfait, A. M., R. O. Williams, A. S. Malik, R. N. Maini, and M. Feldmann. 2001. Chronic relapsing homologous collagen-induced arthritis in DBA/1 mice as a model for testing disease-modifying and remission-inducing therapies. *Arthritis Rheum.* 44: 1215–1224.

39. Førre, O., J. Thoen, T. Lea, J. H. Dobloug, O. J. Mellbye, J. B. Natvig, J. Pahle, and B. G. Solheim. 1982. In situ characterization of mononuclear cells in rheumatoid tissues, using monoclonal antibodies. No reduction of T8-positive cells or augmentation in T4-positive cells. *Scand. J. Immunol.* 16: 315–319.

40. Konttinen, Y. T., V. Bergroth, D. Nordström, K. Koota, B. Skrifvars, G. Hagman, C. Friman, M. Hämäläinen, and P. Slätis. 1985. Cellular immunohistopathology of acute, subacute, and chronic synovitis in rheumatoid arthritis. *Ann. Rheum. Dis.* 44: 549–555.

41. Holmdahl, R., M. E. Andersson, T. J. Goldschmidt, L. Jansson, M. Karlsson, V. Malmström, and J. Mo. 1989. Collagen induced arthritis as an experimental model for rheumatoid arthritis. Immunogenetics, pathogenesis and autoimmunity. *APMIS* 97: 575–584.

42. Butler, D. M., M. Feldmann, F. Di Padova, and F. M. Brennan. 1994. p55 and p75 tumor necrosis factor receptors are expressed and mediate common functions in synovial fibroblasts and other fibroblasts. *Eur. Cytokine Netw.* 5: 441–448.

43. Andreas, K., C. Lübke, T. Häupl, T. Dehne, L. Morawietz, C. Ringe, C. Kaps, and M. Sittinger. 2008. Key regulatory molecules of cartilage destruction in rheumatoid arthritis: an in vitro study. *Arthritis Res. Ther.* 10: R9.

44. Davies, P., P. J. Bailey, M. M. Goldenberg, and A. W. Ford-Hutchinson. 1984. The role of arachidonic acid oxygenation products in pain and inflammation. *Annu. Rev. Immunol.* 2: 335–357.

45. Murata, T., F. Ushikubi, T. Matsuoka, M. Hirata, A. Yamasaki, Y. Sugimoto, A. Ichikawa, Y. Aze, T. Tanaka, N. Yoshida, et al. 1997. Altered pain perception and inflammatory response in mice lacking prostacyclin receptor. *Nature* 388: 678–682.

46. Kearney, P. M., C. Baigent, J. Godwin, H. Halls, J. R. Emberson, and C. Patrono. 2006. Do selective cyclo-oxygenase-2 inhibitors and traditional non-steroidal anti-inflammatory drugs increase the risk of atherothrombosis? Meta-analysis of randomised trials. *BMJ* 332: 1302–1308.

47. Németh, K., A. Leelahavanichkul, P. S. Yuen, B. Mayer, A. Parmelee, K. Doi, P. G. Robey, K. Leelahavanichkul, B. H. Koller, J. M. Brown, et al. 2009. Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat. Med.* 15: 42–49.

48. Ratcliffe, M. J., A. Walding, P. A. Shelton, A. Flaherty, and I. G. Dougall. 2007. Activation of E-prostanoid4 and E-prostanoid2 receptors inhibits TNF-alpha release from human alveolar macrophages. *Eur. Respir. J.* 29: 986–994.

49. Foey, A. D., S. Field, S. Ahmed, A. Jain, M. Feldmann, F. M. Brennan, and R. Williams. 2003. Impact of VIP and cAMP on the regulation of TNF-alpha and IL-10 production: implications for rheumatoid arthritis. *Arthritis Res. Ther.* 5: R317–R328.

50. Sekut, L., D. Yarnall, S. A. Stimpson, L. S. Noel, R. Bateman-Fite, R. L. Clark, M. F. Brackeen, J. A. Menius, Jr., and K. M. Connolly. 1995. Anti-inflammatory activity of phosphodiesterase (PDE)-IV inhibitors in acute and chronic models of inflammation. *Clin. Exp. Immunol.* 100: 126–132.

51. McCann, F. E., A. C. Palfreeman, M. Andrews, D. P. Perocheau, J. J. Inglis, P. Schafer, M. Feldmann, R. O. Williams, and F. M. Brennan. 2010. Apremilast, a novel PDE4 inhibitor, inhibits spontaneous production of tumour necrosis factor-alpha from human rheumatoid synovial cells and ameliorates experimental arthritis. *Arthritis Res. Ther.* 12: R107.

52. Butler, D. M., R. N. Maini, M. Feldmann, and F. M. Brennan. 1995. Modulation of proinflammatory cytokine release in rheumatoid synovial membrane cell cultures. Comparison of monoclonal anti TNF-alpha antibody with the interleukin-1 receptor antagonist. *Eur. Cytokine Netw.* 6: 225–230.

53. Mukaida, N., Y. Mahe, and K. Matsushima. 1990. Cooperative interaction of nuclear factor-kappa B- and cis-regulatory enhancer binding protein-like factor binding elements in activating the interleukin-8 gene by pro-inflammatory cytokines. *J. Biol. Chem.* 265: 21128–21133.

54. Akira, S., H. Isshiki, T. Sugita, O. Tanabe, S. Kinoshita, Y. Nishio, T. Nakajima, T. Hirano, and T. Kishimoto. 1990. A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. *EMBO J.* 9: 1897–1906.

55. O'Donnell, P. M., and S. M. Taffet. 2002. The proximal promoter region is essential for lipopolysaccharide induction and cyclic AMP inhibition of mouse tumor necrosis factor-alpha. *J. Interferon Cytokine Res.* 22: 539–548.

56. Sacre, S. M., A. Lo, B. Gregory, R. E. Simmonds, L. Williams, M. Feldmann, F. M. Brennan, and B. M. Foxwell. 2008. Inhibitors of TLR8 reduce TNF production from human rheumatoid synovial membrane cultures. *J. Immunol.* 181: 8002–8009.

57. Midwood, K., S. Sacre, A. M. Piccinini, J. Inglis, A. Trebaul, E. Chan, S. Drexler, N. Sofat, M. Kashiwagi, G. Orend, et al. 2009. Tenascin-C is an endogenous activator of Toll-like receptor 4 that is essential for maintaining inflammation in arthritic joint disease. *Nat. Med.* 15: 774–780.

58. Dixon, W. G., and D. P. Symmons. 2007. What effects might anti-TNFalpha treatment be expected to have on cardiovascular morbidity and mortality in rheumatoid arthritis? A review of the role of TNFalpha in cardiovascular pathophysiology. *Ann. Rheum. Dis.* 66: 1132–1136.

59. Hansson, G. K. 2005. Inflammation, atherosclerosis, and coronary artery disease. *N. Engl. J. Med.* 352: 1685–1695.