Prostaglandin E\(_2\) Differentially Modulates Proinflammatory/Prodestructive Effects of TNF-\(\alpha\) on Synovial Fibroblasts via Specific E Prostanoid Receptors/cAMP

Elke Kunisch, Anne Jansen, Fumiaki Kojima, Ivonne Löffler, Mohit Kapoor, Shinichi Kawai, Ignacio Rubio, Leslie J. Crofford and Raimund W. Kinne

*J Immunol* 2009; 183:1328-1336; Prepublished online 19 June 2009; doi: 10.4049/jimmunol.0900801

http://www.jimmunol.org/content/183/2/1328

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2009/06/19/jimmunol.0900801.DC1

**Why The JI?**

- **Rapid Reviews!** 30 days* from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

**References**

This article cites 55 articles, 21 of which you can access for free at:

http://www.jimmunol.org/content/183/2/1328.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Prostaglandin E₂ Differentially Modulates Proinflammatory/Prodestructive Effects of TNF-α on Synovial Fibroblasts via Specific E Prostanoid Receptors/cAMP¹

Elke Kunisch,²* Anne Jansen,†‡ Fumiaki Kojima,‡ Ivonne Löffler,³‡ Mohit Kapoor,‡ Shinichi Kawai,§ Ignacio Rubio,‡ Leslie J. Crofford,† and Raimund W. Kinne*¹

The present study investigated the influence of PGE₂, E prostanoid (EP) receptors, and their signaling pathways on matrix metalloproteinase (MMP)-1 and IL-6 expression in synovial fibroblasts (SFs) from rheumatoid arthritis (RA) patients. RASFs expressed all four EP receptors, with selective induction of EP2 by TNF-α. TNF-α time-dependently increased intracellular cAMP/protein kinase A signaling (maximum, 6–12 h) and PGE₂ secretion (maximum, 24 h). PGE₂ and the EP2 agonists butaprost or ONO-AE1-259 (16)-deoxy-9β-chloro-15-deoxy-16-hydroxy-17,17-trimethylene-19,20-didehydro PGE₁, in turn, induced a rapid, time-dependent (maximum, 15–30 min) increase of cAMP. Additionally, cyclooxygenase-2 inhibition by NS-398 (N-(2-cyclohexyloxy-4-nitrophenyl)-methanesulfonamide) reduced the TNF-α-induced increase in IL-6 mRNA/protein, which was restored by stimulation with PGE₂ or EP2, EP3, and EP4 agonists. In contrast, TNF-α-induced MMP-1 secretion was not influenced by NS-398 and diminished by PGE₂ via EP2. Finally, 3-isobutyl-1-methylxanthine enhanced the effects of PGE₂ on MMP-1, but not on IL-6 mRNA. In conclusion, PGE₂ differentially affects TNF-α-induced mRNA expression of proinflammatory IL-6 and prodestructive MMP-1 regarding the usage of EP receptors and the dependency on cAMP. Although specific blockade of EP2 receptors is considered a promising therapeutic strategy in RA, opposite regulation of proinflammatory IL-6 and prodestructive MMP-1 by PGE₂ via EP2 may require more complex approaches to successfully inhibit the cyclooxygenase-1/2 cAMP axis. The Journal of Immunology, 2009, 183: 1328–1336.

In rheumatoid arthritis (RA),⁴ activated synovial fibroblasts (RASFs) contribute to the inflammatory/destructive potential of the aggressive synovial tissue by producing proinflammatory mediators and matrix-degrading enzymes including

*Experimental Rheumatology Unit, Department of Orthopedics, University Hospital Jena, Jena, Germany; †Department of Internal Medicine, Division of Rheumatology, Kentucky Clinic, University of Kentucky, Lexington, KY 40536; ‡Institute of Molecular Cell Biology, Center for Molecular Biomedicine, Friedrich Schiller University Jena, Jena, Germany; and §Department of Internal Medicine, Division of Rheumatology, Toho University School of Medicine, Tokyo, Japan

Received for publication March 11, 2009. Accepted for publication May 8, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ The study was supported by the German Federal Ministry of Education and Research (BMBF; Grants FKZ 01ZZ0602, 01ZZ0105, and 01ZZ0010 to R.W.K., and Interdisciplinary Center for Clinical Research (IZKF) Jena, including a grant for junior researchers to E.K.; Grants FKZ 01ZZ074B and 0313652B to R.W.K., Jena Center for Bioinformatics; Grant 01GS0413, NGFN-2 to R.W.K.), the German Research Foundation (DFG; Grants KI 439/7-1 and KI 439/6-1 to R.W.K.), National Institutes of Health/National Institute of Arthritis and Musculoskeletal and Skin Diseases Grant I R01 AR040910 to L.J.C., and a grant for the advancement of female scientists to Elke Kunisch (LUBOM Thuringia). Anne Jansen was supported by a stipend from the Friends of the Friedrich Schiller University Jena, as well as by travel allowances from JenaPharm and the Boehringer Ingelheim Foundation.

² Address correspondence and reprint requests to Dr. Elke Kunisch, Experimental Rheumatology Unit, Department of Orthopedics, University Hospital Jena, Klosterruetherether II, Strasse 1, D-07744 Eisenberg, Germany. E-mail address: elke.kunisch@med.uni-jena.de

³ Current address: Department of Internal Medicine III, University Hospital Jena, Jena, Germany.

⁴ Abbreviations used in this paper: RA, rheumatoid arthritis; RASF, rheumatoid arthritis synovial fibroblast; MMP, matrix metalloproteinase; COX, cyclooxygenase; EP, E prostanoid; NS-398, N-(2-cyclohexyloxy-4-nitrophenyl)-methanesulfonamide; IBMX, 3-isobutyl-1-methylxanthine; PKA, protein kinase A.

Copyright © 2009 by The American Association of Immunologists, Inc. 0022-1767/09/$2.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0900801

matrix metalloproteinases (MMPs) (1, 2, 3, 4, 5, 6). Under the influence of proinflammatory cytokines, for example, TNF-α or IL-1β, RASFs secrete PGE₂ (7, 8, 9). PGE₂ can raise cAMP levels in RASFs (10, 11, 12, 13, 14), and cAMP is involved in the IL-1β-induced expression of several target genes, e.g., IL-6, M-CSF, and vascular endothelial growth factor (11). PGE₂ belongs to the family of prostanoid, autocrine, and paracrine lipid mediators released by cells following mechanical injury or stimulation with cytokines or growth factors. The synthesis of the prostanois is catalyzed by the cyclooxygenase (COX) pathway (15).

PGE₂ mediates its biological functions via binding to four types of membrane-bound, G protein-coupled receptors termed E prostanoid (EP)1 to EP4 (15, 16). Following ligand binding, the EP receptors activate different signal transduction pathways. EP1 raises intracellular Ca²⁺, whereas EP3 reduces or increases cAMP by activating inhibitory G (Gi) or stimulatory G (G_s) proteins depending on the particular splice variant expressed by the cell (17). The EP2 and EP4 receptors increase intracellular cAMP by activating adenylyl cyclase via G_s proteins. However, differences in the strength of G_s coupling, activation of other signal transduction pathways, agonist-induced desensitization, and agonist-induced internalization result in a differential response of the target cell to a ligand-induced activation of the EP2 or EP4 receptors (18).

Human RASFs have consistent mRNA expression for the PGE₂ receptors EP2 and EP4, while there have been inconsistent reports of EP1 and EP3 mRNA expression (10, 19, 20, 21). Surprisingly, although EP2 and EP4 receptors are regarded as attractive pharmacological targets for RA treatment, the exact role of cAMP or other signals issued by PGE₂-challenged EP receptor subtypes, as well as their influence on the effects stimulation on TNF-α or
IL-1β, remains largely unknown. The present study sought to analyze the involvement of PGE2-dependent cAMP signaling in TNF-α-induced proinflammatory IL-6 and/or proinflammatory MMP-1 effector functions of RASFs.

Materials and Methods

Materials

Rabbit anti-human polyvalent Abs against the EP1, EP2, EP3, and EP4 receptors, PGE2, NS-398 (N-(2-Cyclohexylxoxy-4-nitrophenyl)-methane-sulphonamide), butaprost, sulprostone, and an ELISA kit for cAMP were purchased from Cayman Chemical. A competition assay for the detection of PGE2 was obtained from Biotrak (Amersham Pharmacia Biotech). Repurchased from Cayman Chemical.


Patients, tissue digestion, and cell culture

Synovial tissue from RA patients was obtained during open joint replacement/arthroscopic synovecctomy from the Clinic of Orthopedics, Eisenberg, Germany. All patients fulfilled the respective American Rheumatism Association criteria (22). The study was approved by the Ethics Committee of the University Hospital Jena (Jena, Germany), and patient informed consent was obtained. Immediately after synovecctomy, tissue was placed in culture medium at ambient temperature and subjected to digestion within 2 h.

RA synovial samples were digested, subsequently cultured for 7 days, and RASFs were negatively isolated as previously described (23, 24). RASFs cultured in the virtual absence of contaminating nonadherent cells and macrophages. Third-passage cells were used for all experiments. Stimulation of the cells was performed in DEMEM/0.2% lactalbumin hydrolysate. Mycoplasma contamination of the cells was excluded by 4′,6-diamino-2-phenylindole (DAPI) staining.

Cell stimulation

For kinetic analysis of the TNF-α-induced expression of EP receptors, RASFs (2.5 × 10⁵ cells/well of a 12-well plate) were allowed to adhere for 24 h in DMEM/10% FCS at 37°C and 5% CO₂. Thereafter, cells were stimulated with 10 ng/ml TNF-α (R&D Systems). After stimulation for 0, 1, 2, 4, 8, 10, and 24 h, the cells were lysed in buffer for RNA isolation.

For analysis of intracellular cAMP and protein kinase A (PKA) substrate phosphorylation, as well as mRNA and protein expression of proinflammatory/prodestructive IL-6 and MMP-1, RASFs (4 × 10⁵ cells/well of 6-well plates or 1.5–2.0 × 10⁶ cells/well of a 12-well plate) were allowed to adhere for 24 h in DMEM/10% FCS at 37°C. Thereafter, cells were pretreated with 1 μM NS-398 for 30–45 min followed by treatment with TNF-α (10 ng/ml), PGE2 (1 μM), the EP2 receptor agonist butaprost or the EP3/EP1 agonist sulprostone (each 1 μM), or selective EP agonists (EP1, EP2, EP3, and EP4; ONO; 10 μM). In selected experiments (see Figs. 2, A and B, and 4, as well as supplemental Fig. 2), 500 μM IBMX was added to each well 4 h before the end of the experiment to increase the signal for intracellular cAMP production and PKA substrate phosphorylation; in other experiments, the results were compared for cultivation with and without prior coinubcation with IBMX (see Figs. 2E and 6, 100 μM IBMX; supplemental Fig. 1, A and B, 500 μM; and supplemental Fig. 1C, 100 μM IBMX). Supernatants of the cells were collected for analysis of protein secretion.

Analysis of EP receptor, MMP-1, and IL-6 expression by real-time RT-PCR

Total RNA was isolated from RASFs using a commercially available RNA isolation kit (Macherey & Nagel) and 1 μg was reverse-transcribed using SuperScript II reagents (Invitrogen). EP1, EP2, EP3, EP4, MMP-1, and IL-6 mRNA expression was analyzed by real-time PCR in a RealPlex PCR machine (Eppendorf). PCR reactions were performed in a total volume of 20 μl in 96-well plates containing a reaction mix of HotMaster DNA poly-
Exposure of RASFs to TNF-α expression and secretion in RASFs

The data were expressed as means ± SEM. Significance was tested using the nonparametric Mann-Whitney U test. Differences were considered statistically significant for p ≤ 0.05. Analyses were performed using the SPSS 13.0 program.

Results
Role of PGs in TNF-α-induced IL-6 and MMP-1 mRNA expression and secretion in RASFs

Exposure of RASFs to TNF-α led to a marked induction of IL-6 mRNA expression and secretion (Fig. 1, A and C). To test the involvement of PGs in this process, a pharmacological approach was used to inhibit COXs. The COX-2-selective inhibitor NS-398 significantly reduced TNF-α-induced IL-6 mRNA expression and protein secretion, pointing to an enhancing role of COX-2-derived PGs in this process. In line with this notion, NS-398-blocked expression and secretion of IL-6 was restored by simultaneous administration of exogenous PGE2 (Fig. 1, A and C). Taken together, these data point to a critical role of PGs, possibly PGE2, as modulators of the proinflammatory actions of TNF-α.

As observed for IL-6, TNF-α also significantly induced the mRNA expression and secretion of MMP-1 in RASFs (Fig. 1, B and D). However, NS-398 did not significantly reduce, but even numerically enhanced, MMP-1 mRNA expression and secretion. Concordantly, addition of exogenous PGE2 significantly reduced the NS-398-enhanced mRNA expression and secretion of MMP-1 upon TNF-α stimulation (Fig. 1, B and D). Therefore, PGE2, and possibly other PG species appear to have critical and partially opposite effects on proinflammatory and prodestructive signaling by TNF-α in RASFs.

TNF-α activates the cAMP/PKA signaling pathway in RASFs

TNF-α induced a gradual, time-dependent increase in cAMP levels that reached a maximum after 8–10 h of stimulation (Fig. 2A).
β-adrenoreceptor agonist isoproterenol, a well-known cAMP-elevating agent, was used as a positive control in this and forthcoming experiments. To confirm with an independent approach that TNF-α addressed the cAMP/PKA signaling cassette, the phosphorylation status of PKA target proteins was assessed using a phosphorylation-specific Ab that selectively detects the minimum RRXS/T consensus target sequence for PKA in its phosphorylated state. The corresponding experiment (Fig. 2B) illustrated a time-dependent increase in the phosphorylation of multiple PKA targets, in full agreement with the cAMP measurements, and thus confirming that TNF-α activates the cAMP/PKA pathway.

Since TNF-α induced both cAMP/PKA signaling and PGE2 release with similar kinetics (Fig. 2A–C) and PGE2 increased cAMP in RASFs (Fig. 2D; enhanced by IBMX, Fig. 2E), PGs may mediate the activation of the cAMP/PKA pathway by TNF-α. "TNF-α induces the expression of EP2 receptors in RASFs"

PCR of total RNA preparations from RASFs was performed and mRNA for all four EP receptors was detected in these cells (Fig. 3, A and B). Intriguingly, TNF-α induced a time-dependent increase in EP2 mRNA (maximum, 10 h), as assessed by both real-time (Fig. 3A) or conventional PCR (Fig. 3B). In contrast,
stimulation with TNF-α reduced EP4 mRNA levels and left EP1 and EP3 mRNA unchanged.

To confirm these data, Western blots of cell lysates were performed. As shown in Fig. 3C, RASF extracts contained all four EP receptor proteins (i.e., EP1 (42 kDa), EP2 (52 kDa), EP3 (53 kDa), and EP4 (65 kDa)). In agreement with the PCR data, TNF-α significantly up-regulated EP2 protein expression following stimulation for 30 h (1.4-fold; see quantification in Fig. 3D). In contrast, EP4 protein was significantly down-regulated (43% reduction), whereas EP1 or EP3 protein levels were not altered by TNF-α stimulation.

PGE2 and selective EP receptor agonists modulate cAMP/PKA pathway activation by TNF-α and TNF-α-induced secretion of IL-6 and MMP-1 in RASFs

COX-2 inhibition with NS-398 completely prevented the increase in cAMP levels induced by TNF-α (Fig. 4A), further underlining

FIGURE 3. Influence of TNF-α on the EP receptor expression in RASFs. RASFs were stimulated with 10 ng/ml TNF-α for different time points (A), 8 h (B), or 30 h (C and D). EP receptor expression was analyzed by quantitative real-time RT-PCR (A) or conventional RT-PCR (B). To analyze the influence of TNF-α on EP receptor protein levels, protein extracts were subjected to Western blot analysis using specific Abs against the EP1, EP2, EP3, and EP4 receptors (C; quantification in D); means ± SEM for five patients with RA; +, p ≤ 0.05 Mann-Whitney U test vs the 0 h time point (A) or vs culture without TNF-α (D); *, p ≤ 0.05 Mann-Whitney U test vs indicated time points (A).

FIGURE 4. Role of EP2 and EP3 receptors in TNF-α-induced cAMP production, PKA substrate phosphorylation, and IL-6 or MMP-1 secretion in RASFs. Cells were stimulated with TNF-α (10 ng/ml) in the absence or presence of NS-398, PGE2, butaprost, or sulprostone (Sulprost.; 1 μM each) with IBMX for 10 h. Intracellular cAMP was determined by RIA (A) and PKA substrate phosphorylation by Western blot (B); IL-6 secretion was analyzed by ELISA (C) and MMP-1 secretion by Western blot (D); means ± SEM for three patients with RA; +, p ≤ 0.05 Mann-Whitney U test vs control; *, p ≤ 0.05 Mann-Whitney U test vs TNF-α; ○, p ≤ 0.05 Mann-Whitney U test vs TNF-α/NS-398.
that PG release by TNF-α was involved in the up-regulation of cAMP levels (see also Fig. 2A). Accordingly, addition of PGE2, or the EP2 selective agonist butaprost restored the increase in cAMP levels to a level above that in TNF-α/NS-398-treated cells. In contrast, the EP1/3-specific agonist sulprostone did not revert the blockade exerted by NS-398. All cAMP measurement data were also confirmed by phospho-(PKA-substrate) Western blots (Fig. 4B). The effects of PGE2 were dose-dependent, both in the presence and the absence of NS-398 (supplemental Fig. 2).

To test whether the TNF-α/PGE2/cAMP axis was physiologically relevant in RASFs, the IL-6 and MMP-1 secretion was examined under the same experimental conditions (Fig. 4, C and D). Except for the induction of IL-6 by TNF-α in the presence of NS-398, IL-6 production showed a pattern identical to that of cAMP levels and PKA activity (Fig. 4, A–C). EP3 signaling induced a marginal, nonsignificant raise of the mean IL-6 concentration compared with that of the TNF-α/NS-398 treatment group, however, with a consistent increase in the paired comparison for all three individual patients (supplemental Fig. 3). In contrast, PGE2-elicited signaling and, more specifically, EP2-dependent signals, diminished the MMP-1 secretion induced by TNF-α (Fig. 4D), demonstrating that PGE2/EP2 signals have directly opposite effects on IL-6 and MMP-1 release by RASFs (for PGE2 effects, see also Fig. 1).

**Effects of PGE2 and selective EP receptor agonists on TNF-α-induced expression of mRNAs for IL-6 and MMP-1 in RASFs**

In the case of IL-6, the mRNA expression in comparison with TNF-α-stimulated RASFs in the presence of NS-398 was significantly increased by PGE2 and selective receptor agonists for EP2 (3.2-fold and 2.7-fold, respectively), but also for EP3 and EP4 (1.7-fold and 1.9-fold, respectively; Fig. 5, A and B). The above results for MMP-1 were confirmed with selective EP receptor agonists; that is, in the presence of COX-2 inhibitors only PGE2 and the EP2 receptor agonist significantly suppressed the mRNA expression in TNF-α-stimulated RASFs (Fig. 5, A and C).

**FIGURE 5.** Effect of selective EP receptor agonists on the regulation of MMP-1 and IL-6 (RT-PCR). A, RASFs were harvested at 24 h after TNF-α (10 ng/ml) stimulation with or without NS-398 (1 μM), PGE2 (1 μM), and/or selective agonists of the EP receptors 1–4 (10 μM each). mRNA expression of (B) IL-6 and (C) MMP-1 was detected by conventional RT-PCR. For each experiment, a value of 1 was assigned to the stimulation with TNF-α. Results are expressed as means ± SEM for three patients with RA; +, p ≤ 0.05 Mann-Whitney U test vs control; *, p ≤ 0.05 Mann-Whitney U test vs TNF-α; ○, p ≤ 0.05 Mann-Whitney U test vs TNF-α/NS-398.

**FIGURE 6.** Effect of the phosphodiesterase inhibitor IBMX on the regulation of MMP-1 and IL-6 by PGE2 (RT-PCR). RASFs were harvested at 24 h after TNF-α (10 ng/ml) stimulation with or without NS-398 (1 μM), PGE2 (1 μM), and/or IBMX (100 μM). mRNA expression of IL-6 and MMP-1 was detected by real-time RT-PCR (A and B). IL-6 secretion was analyzed by ELISA (C), and MMP-1 secretion was analyzed by Western blot (D). For each experiment, a value of 100% was assigned to TNF-α. Results are expressed as means ± SEM for 3 patients with RA; +, p ≤ 0.05 Mann-Whitney U test vs control; *, p ≤ 0.05 Mann-Whitney U test vs TNF-α; ○, p ≤ 0.05 Mann-Whitney U test vs TNF-α/NS-398.
Effect of the phosphodiesterase inhibitor IBMX on TNF-α-induced expression of mRNAs for IL-6 and MMP-1 in RASFs

To directly assess the relevance of cAMP for the effects of PGE₂ on the TNF-α-induced gene expression, the degradation of cAMP was inhibited by addition of IBMX. Strikingly, the mRNA expression and secretion of IL-6 remained unaffected (Fig. 6, A and C), whereas the mRNA expression and secretion of MMP-1 was further suppressed in the presence of IBMX (Fig. 6, B and D).

Discussion

This study demonstrates for the first time that PGE₂ has opposite effects on MMP-1 and IL-6 synthesis, uses different PGE₂ receptors for these effects, and differentially applies the postreceptor signaling molecule cAMP. Thus, PGE₂ is a differential key mediator of inflammatory/destructive functions in TNF-α-stimulated RASFs and may exhibit both proinflammatory (10, 11, 12, 25) and antidestructive capacities (Ref. 18 and the present study). These biphasic effects of PGE₂ in RASFs may also be the reason for the inefficacy of COX-2 inhibitors to arrest joint destruction and should be considered in future studies focused on the therapeutic inhibition of COX-1/2 in RA.

PGE₂ has opposite effects on the TNF-α-induced protein expression of MMP-1 and IL-6

In RASFs, TNF-α induces the secretion of proinflammatory and prodestructive mediators, for example, IL-6, PGE₂, and MMP-1 (present study and Refs. 9, 26). The stimulatory effect of PGE₂ on the TNF-α-induced IL-6 secretion in RASFs is in agreement with previously published data in IL-1β-stimulated RASFs (11, 14). However, the molecular mechanisms involved in the interplay between PGE₂ and TNF-α for the control of IL-6 secretion in RASFs have so far remained largely undeciphered.

In marked contrast to the effects on IL-6, TNF-α-induced MMP-1 secretion was significantly reduced by PGE₂, a finding also reported in IL-1β-stimulated RASFs (13, 27, 28). This clearly implicates PGE₂ as a negative feedback molecule in the signaling pathway linking TNF-α to MMP-1 production. Whether this involves phosphorylation of p38 or expression of NURR1, or else inhibition of Erk and NF-κB activation by PGE₂, as previously reported in the context of IL-1β/TNF-α signaling, remains to be investigated (13, 29, 31).

Interestingly, in TNF-α-stimulated periodontal ligament fibroblasts PGE₂ down-regulates MMP-13 (but not MMP-1 or MMP-3) (32, 33). This emphasizes the antidestructive properties of PGE₂, but also shows clear differences between fibroblasts of different origin. Contrasting results for IL-6 in periodontal ligament fibroblasts may also indicate cell-specific differences (34).

TNF-α induces an increase of intracellular cAMP and activation of PKA

The present study shows the novel finding that stimulation of RASFs with TNF-α induces an increased production of intracellular cAMP and PKA activity. Similar findings have been previously reported for IL-1β (35), suggesting that the cAMP system represents a critical regulatory pathway in RASFs.

With regard to the increased levels of intracellular cAMP induced by TNF-α, the participation of PGs appears plausible because previous data show an increase of intracellular cAMP in RASFs following PGE₂ stimulation (35, 36). In contrast to the slow effects of TNF-α, the increase of cAMP induced by PGE₂ occurred as early as 15 min after the start of stimulation (see Fig. 2D). This difference can be explained by the delayed synthesis of PGE₂ following TNF-α stimulation (Fig. 2C) (35). In turn, cAMP may directly contribute to a further increase of PGE₂ synthesis in RASFs (37). Concurrent with the increase of intracellular cAMP, TNF-α induced a phosphorylation of PKA substrates in RASFs in a strictly COX-2-dependent manner (see Fig. 4B), showing that the increase in cAMP translated into downstream PKA signaling (supplemental Fig. 4). This establishes PKA as a target of TNF-α (and PGE₂) in RASFs, a notion previously only inferred from the use of pharmacological inhibitors (H89) or activators (Rp-cAMP) (27, 28, 38).

Only the effects of PGE₂ on TNF-α-induced MMP-1 production are cAMP-dependent (but not those on IL-6 production)

Down-regulation of TNF-α-induced MMP-1 expression by PGE₂ was mediated via cAMP. Therefore, the cAMP increase may have partial specificity for the antidestructive properties of PGE₂, because phosphodiesterase IV inhibitors reduce joint damage in arthritis models (39, 40) or RA (41, 42) by further increasing cAMP levels. Also, cAMP-dependent regulation of MMP-1 has been shown after stimulation of RASFs with IL-1β (27), indicating partially common mechanisms for postreceptor signaling of these two pivotal proinflammatory cytokines.

The insensitivity of the augmentation of TNF-α-induced IL-6 expression by PGE₂ to an increase of cAMP suggests a relevance of other pathways. Indeed, cAMP-independent pathways (PI3K/ERK) are involved in the signaling of the EP4 receptor (43). Alternatively, IL-6 expression may only depend on cAMP at very high intracellular concentrations (44).

In contrast to our findings with TNF-α, Inoue et al. have reported that the regulation of IL-1β-induced IL-6 expression involves cAMP-dependent pathways (11) (11) (11) (11). This may indicate specific and differential regulation of IL-6 expression by different proinflammatory cytokines well below the receptor level.

TNF-α differentially regulates the expression of EP receptors

The biological function of PGE₂ is mediated by four membrane-bound receptors (15, 16), all of which are expressed in RASFs (present study and Refs. 10, 11, 12, 45). In agreement with previously published data following IL-1β stimulation, TNF-α up-regulates the expression of the EP2 (and to some degree the EP3) (11, 45). Thus, up-regulation of EP2 and/or EP3 may be a widespread response to proinflammatory signals in RASFs. In contrast to previous reports (11), TNF-α down-regulated EP4. This difference may be explained by the different cytokines used for stimulation or by different culture conditions. The induction of the enzymes involved in the synthesis of PGE₂ (8, 46, 47), in conjunction with the up-regulation of certain EP receptors by proinflammatory cytokines (present study and Refs. 11, 45), suggests that the PGE₂ signaling cascade is tightly controlled by proinflammatory cytokines, not only at the level of PGE₂ synthesis, but also at the level of expression of particular prostanooid receptor subclasses.

Individual PGE₂ receptors differentially modulate the functional effects of TNF-α

Up-regulation of the EP2 receptor by TNF-α points to a prominent role of this receptor for the TNF-α/PGE₂-elicted signal in RASFs. This was confirmed by the PGE₂/TNF-α-induced increase of intracellular cAMP and phosphorylation of PKA substrates via EP2 (butaprost), but not via EP3 (Fig. 4, A and B), and, to a minor degree, by EP4 (under IBMX; supplemental Fig. 1). The unresponsiveness of intracellular cAMP levels to sulprostone/EP3 stimulation has been reported before for other cell types (48) and likely reflects the fact that EP3 receptors are mostly coupled to Gi proteins. The weaker ability of the EP4 receptor to stimulate cAMP formation compared with EP2 has also been described (45,
The Journal of Immunology

50). This may be due to rapid desensitization of the EP4 receptor via internalization (50, 51).

Analysis of the proinflammatory IL-6 and prodestructive MMP-1 further underlined the dominant role of the EP2 receptor. Although the IL-6 mRNA expression was significantly increased by stimulation of EP2, EP3, and EP4, the magnitude of IL-6 induction via EP2 (>2.5-fold) was larger than via EP3/EP4 (<2.0-fold). This is somewhat in contrast to the results of Inoue et al. (11), who reported that the IL-6 secretion in IL-1α-stimulated RASFs was only induced by agonists for the EP receptors 2 and 4. Explanations include the usage of different proinflammatory cytokines (IL-1β vs TNF-α), different agonist concentrations (20 nM vs 10 μM), and variable EP3 mRNA/protein expression (11). The selective regulation of MMP-1 via the EP2 receptor is a novel observation.

The dominant role of EP2 in the regulation of TNF-α-induced functions of RASFs suggests that the EP2 receptor is a potential therapeutic target in RA. However, the findings presented herein indicate that this point needs to be regarded with caution. In particular, the radically opposite consequences of PGE2/EP2 signaling on TNF-α-induced IL-6 and MMP-1 secretion suggest that a blockade of EP2 activity, while being beneficial in reducing inflammatory parameters, may on the other hand exacerbate tissue destruction. Also, proinflammatory IL-6 is induced by PGE2 predominantly via EP2, but to some degree also by EP3 and EP4, making difficult the exclusive targeting of just one EP receptor.

In agreement with the present data, previous reports have also shown an influence of both EP2 and EP4 on the secretion of IL-6 and MMP-1 vs 10
