Prostacyclin-IP signaling and prostaglandin E_2-EP2/EP4 signaling both mediate joint inflammation in mouse collagen-induced arthritis

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Prostaglandin (PG)I_2 (prostacyclin [PGI]) and PGE_2 are abundantly present in the synovial fluid of rheumatoid arthritis (RA) patients. Although the role of PGE_2 in RA has been well studied, how much PGI_2 contributes to RA is little known. To examine this issue, we backcrossed mice lacking the PGI receptor (IP) to the DBA/1J strain and subjected them to collagen-induced arthritis (CIA). IP-deficient ($IP^{-/-}$) mice exhibited significant reduction in arthritic scores compared with wild-type (WT) mice, despite anti-collagen antibody production and complement activation similar to WT mice. $IP^{-/-}$ mice also showed significant reduction in contents of proinflammatory cytokines, such as interleukin (IL)-6 in arthritic paws. Consistently, the addition of an IP agonist to cultured synovial fibroblasts significantly enhanced IL-6 production and induced expression of other arthritis-related genes. On the other hand, loss or inhibition of each PGE receptor subtype alone did not affect elicitation of inflammation in CIA. However, a partial but significant suppression of CIA was achieved by the combined inhibition of EP2 and EP4. Our results show significant roles of both PGI_2-IP and PGE_2-EP2/EP4 signaling in the development of CIA, and suggest that inhibition of PGE_2 synthesis alone may not be sufficient for suppression of RA symptoms.

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Abbreviations used: CAIA, collagen antibody–induced arthritis; CIA, collagen-induced arthritis; CII, type II collagen; COX, cyclooxygenase; FGF, fibroblast growth factor; H&E, hematoxylin and eosin; IP, prostacyclin receptor; MCP-1, monocyte chemoattractant protein 1; mPGES, microsomal prostaglandin E synthase; NSAID, nonsteroidal antiinflammatory drug; PG, prostaglandin; PGI, prostacyclin; RA, rheumatoid arthritis; RANKL, receptor activator of NF-κB ligand; VEGF, vascular endothelial growth factor.

Rheumatoid arthritis (RA) is a chronic inflammatory disease of the joint characterized by inflammatory cell infiltration, synovial lining hyperplasia, and destruction of cartilage and bone. Although the etiology of RA has not yet been fully understood, recent studies have suggested the involvement of autoantibody production, immune complex formation, inflammatory cell infiltration, and tumor-like proliferation of synovium in the pathogenesis of RA (1–4). Autoantibodies, such as rheumatoid factor (anti-IgG antibody) and anti–type II collagen (CII) antibody, are detected in RA patients with high probability. These autoantibodies make immune complexes within the joint, leading to activation of the complement cascade and inflammatory cell infiltration into the joint. Particular attention is now paid to the network of cytokines, chemokines, and growth factors in the development of RA (5, 6). Infiltrated macrophages and neutrophils release IL-1β, which activates synovial cells. Activated synovial cells then produce various chemokines, cytokines, and growth factors. Chemokines such as CCL2 (monocyte chemoattractant protein 1 [MCP-1]) and CCL3 (macrophage inflammatory protein 1α) produced by synovial cells further recruit inflammatory cells into the joint (7), and IL-6 and fibroblast growth factors (FGFs) produced by synovial cells contribute to their proliferation in an autocrine manner (8). IL-6 also induces both B and T cell activation and osteoclast formation. These positive-feedback loops amplify inflammation and destruction within the joint.

Collagen-induced arthritis (CIA) and collagen antibody–induced arthritis (CAIA) are the widely used arthritis models in the mouse. CIA is induced by immunizing mice with CII, whereas CAIA is induced by the administration of a combination of monoclonal anti-CII antibodies and LPS. CAIA can be induced in

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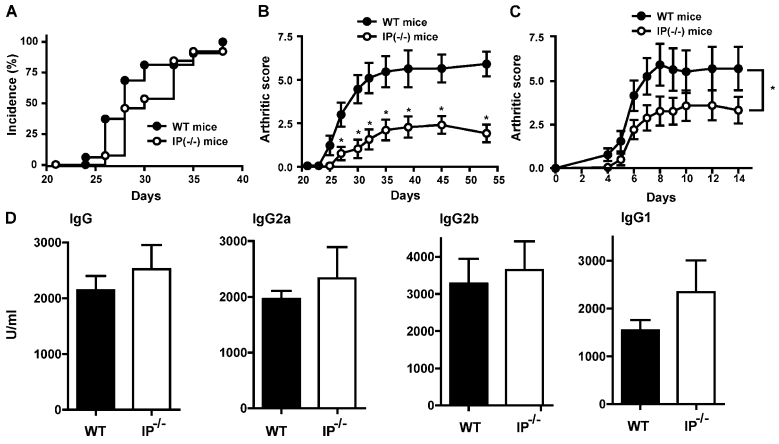

Figure 1. Effects of disruption of IP on CIA or CAIA responses, and anti-CII antibody production in $IP^{-/-}$ mice. (A) Time course of incidence of arthritis in WT and $IP^{-/-}$ mice in CIA. The cumulative number of mice showing arthritis in each group is shown as a percentage of the total number immunized with CII. ●, WT mice, $n = 15$; ○, $IP^{-/-}$ mice, $n = 13$. (B) Time course of arthritic scores in WT (●, $n = 15$) and $IP^{-/-}$ (○, $n = 13$) mice. *, $P < 0.05$ versus WT mice in CIA. Results show the mean ± SEM. (C) Time course of arthritic scores in WT and $IP^{-/-}$ mice in CAIA. *, $P < 0.05$ between WT and $IP^{-/-}$ mice (two-way ANOVA). (D) Anti-CII antibody production. Sera of WT (black bars, $n = 7$) and $IP^{-/-}$ (white bars, $n = 8$) mice in CIA were collected on day 21, and titers of anti-CII antibody in total and each IgG subclass were measured by ELISA. Results are expressed as the mean ± SEM in arbitrary units for total IgG, IgG2a, IgG2b, and IgG1.

various mouse strains with rapid onset compared with that of CIA. However, the lesions of CAIA are milder and its symptoms last for a shorter duration than CIA (9). Furthermore, an acute induction of CAIA by LPS injection may not mimic the natural course and mechanisms in RA development. On the other hand, although the induction of CIA is limited to a few mouse strains such as DBA/1J and takes about a month to develop, its lesions last for a long time and its histopathology, characterized by synovitis, pannus formation, cartilage erosion, and bone destruction in joints, is quite similar to that of human RA (10). Therefore, CIA is an arthritis model suitable for analyzing chronic joint inflammation.

Prostanoids, including prostaglandin $(PG)D_2$, PGE_2, $PGF_{2\alpha}$, prostacyclin $(PGI)_2$, and thromboxane A_2, are lipid mediators produced by sequential catalysis of cyclooxygenase (COX) and the respective synthase (11). They are produced in large amounts at inflammatory sites in response to various stimuli. Nonsteroidal antiinflammatory drugs (NSAIDs) that inhibit COX and suppress PG production have been long and widely used for the treatment of RA. NSAIDs reduce the extent and number of tender or swollen joints in RA patients, implicating PGs in pain and inflammation of RA. Consistently, an inducible form of COX, COX-2, is expressed in the inflammatory synovium of RA patients (12). Among PGs, PGE_2 has been suggested as a main PG species working in RA reactions because PGE_2 is detected as one of the major PGs in the synovial fluid in RA patients (13) and shows pleiotropic proinflammatory actions in vitro (for example, see reference 14). Recently, it has also been reported that mice deficient in microsomal PGE synthase (mPGES)-$1^{-/-}$ showed reduced arthritic responses in CIA (15). On the other hand, although PGI_2 is known as another major prostanoid, often detected more abundantly than PGE_2 in the synovial fluid of RA patients (13), whether PGI_2 contributes to the development of arthritis has not been tested extensively. Prostanoids exert their actions via a family of G protein–coupled receptors, (11), which include PGD receptor (DP), four subtypes of PGE receptor (EP1, EP2, EP3, and EP4), PGF receptor (FP), PGI receptor (IP), and thromboxane A receptor (TP). We have generated mice deficient in each of the PG receptor types and subtypes individually and subjected them to various disease models to define the roles of each PG (16–19). Using mice lacking the IP, we previously showed the role of PGI_2 as a mediator of acute inflammation induced by bradykinin or carrageenin, and of acute pain response to acetic acid (16). In this study, we have subjected IP-deficient mice ($IP^{-/-}$) to CIA and examined the roles of PGI_2 in this model. We have further compared the phenotype of $IP^{-/-}$ mice with those of mice lacking one of the PGE receptor subtypes (EP1, EP2, or EP3) and/or mice administered with an EP4-selective antagonist.

RESULTS

Involvement of PGI_2–IP signaling in CIA

To examine how PGI_2 contributes to the development of CIA, we backcrossed mice deficient in IP to the DBA/1J background for 10 generations. CIA was induced by intradermal injection of CII with CFA on day 0, followed by a booster injection of CII with IFA on day 21. Arthritic lesion of each paw was evaluated from day 21 for the extent of swelling, erythema, and deformity, and graded on a scale of 0–4. The sum of scores obtained from four paws was used as the arthritic score of each mouse. The arthritic lesions appeared around day 25 in WT mice, and by day 35 ~90% of the mice developed arthritis. Although the incidence of mice showing more than 1 arthritic score was not different between WT and $IP^{-/-}$ mice (Fig. 1 A), the arthritic score in $IP^{-/-}$ mice

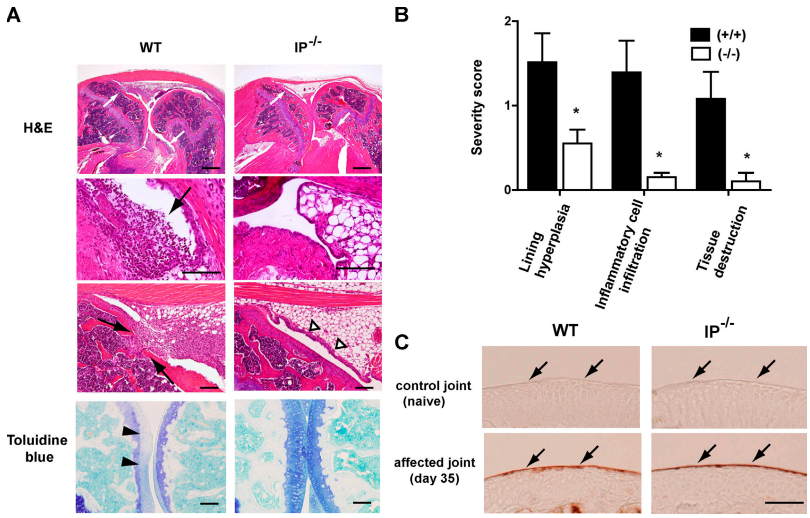

Figure 2. Histological analysis of arthritic lesions in IP$^{-/-}$ mice. (A) Representative H&E and toluidine blue sections of the knee joint of WT and IP$^{-/-}$ mice on day 35 of CIA. Top- and bottom-middle panels show higher magnification views of areas indicated by white arrows in the top panels. Arrow in the left top-middle panel indicates inflammatory cell infiltration. Arrows in the left bottom-middle panel indicate destructive pannus formation, and white arrowheads in the right bottom-middle panel indicate less proliferative synovium in IP$^{-/-}$ mice. Arrowheads in the left bottom panel indicate proteoglycan depletion in WT mice. Scale bars, 500 μm (top panels) and 100 μm (middle and bottom panels). (B) Pathological scores in WT (black bars, $n = 14$) and IP$^{-/-}$ (white bars $n = 10$) mice. Sections were blindly examined for lining hyperplasia, inflammatory cell infiltration, and tissue destruction as described in Materials and methods. Results are expressed as the mean ± SEM from two independent experiments. *, $P < 0.05$ versus WT mice. (C) Complement deposition on the cartilage surface. Sections of the knee joint from control naive WT and IP$^{-/-}$ mice (top left and right, respectively) and WT and IP$^{-/-}$ mice on day 35 of CIA (bottom left and right, respectively) were stained for C3b deposition (brown). Arrows indicate the surface of cartilages. Data shown are representative of five independent experiments. Scale bar, 100 μm.

was significantly lower than that of WT mice all through the elicitation phase (Fig. 1 B). To examine if this phenotype of IP$^{-/-}$ mice reflects the role of IP in the antibody-mediated effector mechanisms of inflammation, we applied CAIA to IP$^{-/-}$ mice. The CAIA model bypasses the immunization phase by direct injection of monoclonal anti-CII antibodies. IP$^{-/-}$ mice again showed a significant reduction in the arthritic score compared with that of WT mice, although the extent of the reduction was milder than that in CIA (Fig. 1 C). These results show that the PGI_2-IP signaling works substantially in the pathogenesis of both CIA and CAIA, and the findings in CAIA suggest the role of this signaling in the antibody-induced inflammatory responses. Next, we examined if the PGI_2-IP signaling also works in the immunization of mice in CIA. Sensitization to CII and a consecutive anti-CII antibody production are important steps in the development of CIA. We collected sera from immunized WT and IP$^{-/-}$ mice on day 21 and measured anti-CII antibody titers. The levels of anti-CII antibody in the total IgG fraction and each of the IgG2a, IgG2b, and IgG1 subclasses were similar in WT and IP$^{-/-}$ mice (Fig. 1 D). These results demonstrate that the loss of IP does not impair anti-CII antibody production in CIA.

Histology of CIA in IP$^{-/-}$ mice

To evaluate arthritic lesions in WT and IP$^{-/-}$ mice histologically, we prepared sections of knee joints from WT and IP$^{-/-}$ mice on day 35 and stained them with hematoxylin and eosin (H&E) or toluidine blue (Fig. 2 A). The arthritic lesion in CIA is characterized by three parameters: cell infiltration, synovial lining hyperplasia, and tissue destruction. On day 35, joints of WT mice showed characteristic features of CIA in all three parameters: marked infiltration of inflammatory cells in the cavity, lining hyperplasia and invasive pannus formation, and bone and cartilage destruction. Compared with these findings in WT mice, IP$^{-/-}$ mice showed markedly reduced infiltration of inflammatory cells, less lining hyperplasia, and less destruction of bone and cartilage. When these parameters were graded from 0 to 3 (0, normal; 1, minimal; 2, mild; 3, severe), the score of IP$^{-/-}$ mice in each parameter was significantly lower than that of WT mice (Fig. 2 B). These results suggest that the PGI_2-IP signaling acts in promotion of joint inflammation in CIA.

In CIA, anti-CII antibody produced makes an immune complex with collagen on the cartilage surface of the joint and triggers complement activation, an essential process leading to joint inflammation in CIA (20). Large complement fragments such as C3b and C5b accumulate on the cartilage surface, and small complement fragments such as C3a and C5a promote inflammatory cell infiltration to the joint. Because the joints of IP$^{-/-}$ mice on day 35 showed markedly reduced inflammation, we next examined whether the complement activation had occurred on the cartilage surface in IP$^{-/-}$ mice. We examined deposition of C3b on the cartilage surface in the knee joints by immunohistochemistry. No C3b deposition was found on the cartilage surface of naive WT and IP$^{-/-}$ mice (Fig. 2 C, top left and top right, respectively).

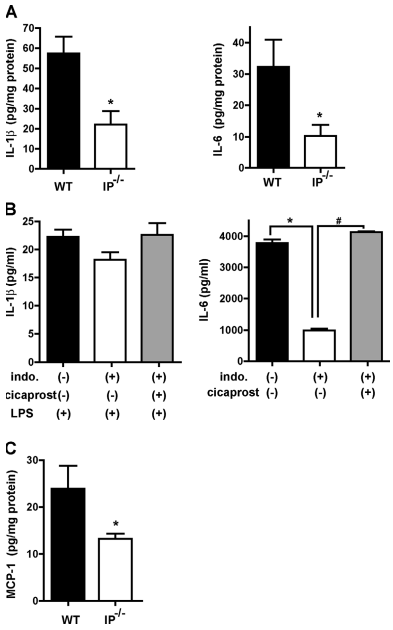

Figure 3. Concentrations of proinflammatory cytokines in arthritic paws and production by arthritic cell suspension. (A) Cytokine concentrations in arthritic paws. WT ($n = 10$) and $IP^{-/-}$ ($n = 10$) mice were killed on day 35 of CIA, and paws were isolated. The concentrations of IL-1β and IL-6 in the supernatants of the paw homogenates were measured by ELISA. The values were normalized with total protein concentration in the supernatant. Results are expressed as the mean ± SEM. *, $P < 0.05$ versus WT mice. (B) Effects of indomethacin and cicaprost on production of IL-1β and IL-6 by arthritic cell suspension. Cells were incubated in the absence or presence of 1 μM indomethacin (indo.) with or without 1 μM cicaprost as indicated. LPS was added at 1 μg/ml in the experiment for IL-1β production. After 24 h, concentrations of IL-1β and IL-6 in the supernatants were measured by ELISA. Results are expressed as the mean ± SEM ($n = 3$). *, $P < 0.05$ versus the vehicle-treated group; #, $P < 0.05$ versus the indomethacin-treated group. Data shown are representative of at least two experiments. (C) MCP-1 concentrations in arthritic paws on day 35 of CIA (WT, $n = 10$ and $IP^{-/-}$, $n = 10$). The concentration of MCP-1 was measured by ELISA as described above. Results are expressed as the mean ± SEM. *, $P < 0.05$ versus WT mice.

The deposition of C3b was clearly detected in the WT joint on day 35 (Fig. 2 C, bottom left). The joint of $IP^{-/-}$ mice also showed C3b deposition to the extent of that in the WT joint (Fig. 2 C, bottom right). These results suggest that the loss of IP suppressed the progression of the inflammatory process despite the equivalent extent of antibody production and complement activation.

Reduced concentrations of proinflammatory cytokines in the arthritic paws of $IP^{-/-}$ mice

The results described above indicate that the PGI_2-IP signaling functions in progression or enhancement of the inflammatory process in CIA. Because proinflammatory cytokines such as IL-1β, TNF-α, and IL-6 have been suggested to play important roles in RA (2), we measured the concentrations of these cytokines in the paws of arthritic mice. Paws were collected on day 35 and homogenized. The homogenates were then centrifuged and the supernatants obtained were subjected to ELISA for IL-1β, TNF-α, and IL-6. The concentrations of IL-1β and IL-6 were significantly lower in $IP^{-/-}$ mice than in WT mice (Fig. 3 A). The concentration of TNF-α was below the detection limit and could not be determined in both groups. To clarify whether the PGI_2-IP signaling can directly regulate production of these cytokines within the inflammatory joint, we next prepared arthritic cell suspension, a mixture of synovial cells and various inflammatory cells from the knee joints of arthritic WT mice on day 35. The effects of indomethacin and an IP agonist on IL-1β and IL-6 production by these cells were examined (Fig. 3 B). As the production of IL-1β by these cells was too low to detect by ELISA under the basal condition, we stimulated them with LPS. LPS induced significant production of IL-1β.However, no significant effects of either indomethacin or an IP agonist, cicaprost, were observed on the LPS-induced IL-1β production. On the other hand, the arthritic cell suspension produced a significant amount of IL-6 on a 24-h incubation. The addition of indomethacin significantly suppressed IL-6 production, and the further addition of the IP agonist completely reversed the suppressive effect of indomethacin. These results indicate that although endogenous

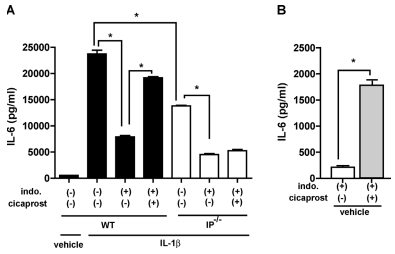

Figure 4. Potentiation of IL-1β–induced IL-6 production by IP stimulation in synovial fibroblasts. (A and B) Effects of cicaprost, an IP agonist, on IL-6 production in synovial fibroblasts stimulated by IL-1β (A) or nonstimulated synovial fibroblasts (B). Synovial fibroblasts from unimmunized WT or $IP^{-/-}$ mice were stimulated either with vehicle or 5 ng/ml IL-1β in the presence or absence of 1 μM indomethacin (indo.) and 1 μM cicaprost as indicated for 24 h. The supernatants were collected and IL-6 concentration was measured by ELISA. *, $P < 0.05$ for indicated comparison.

Table I. Clusters of genes induced by IL-1β in synovial fibroblasts

Cluster (IL-1β)	(IL-1β, indo.)	(IL-1β, indo., IP agonist)
1		increase 0
2	increase 19	decrease 18
3		no change 1
4		increase 138
5 increase 489	decrease 183	decrease 11
6		no change 34
7		increase 45
8	no change 287	decrease 44
9		no change 198

Genes whose expression levels are increased by IL-1β stimulation are clustered first to three groups based on the effect of indomethacin (indo.) treatment (increase, decrease, and no change), and then further grouped into three based on the IP agonist (iloprost) actions.

Table II. Classification of genes whose expression level was potentiated by an IP agonist

Group	Unigene code	Gene symbol	Gene profiles	Vehicle vs. Il-1β Log_2(fold)	Il-1β vs. Il-1β+Indo Log_2(fold)	Il-1β+Indo vs. Il-1β+Indo+IP agonist Log_2(fold)
Inflammation	Mm.35814	Il11	Interleukin 11	4.1	−3	2.3
	Mm.35692	Il1rl1	Interleukin 1 receptor–like 1	2.3	−1	0.3
	Mm.896	Il1r1	Interleukin 1 receptor, type I	0.7	−0.7	0.9
	Mm.14595	Csf2rb1	Colony stimulating factor 2 receptor, β1, low-affinity	1.5	−0.6	1.3
	Mm.4186	Pla2g4a	Phospholipase A2, group IVA(cPLA2) (cytosolic, calcium-dependent)	1.1	−0.6	0.5
	Mm.1401	Cxcr4	Chemokine (C-X-C motif) receptor 4	0.7	−1.1	1.3
	Mm.157750	Cxcl7	Cheymokine (C-X-C motif) ligand 7	1.1	−0.8	1.2
Proliferation	Mm.31540	Vegfa	VEGF-A	1.8	−0.9	0.9
	Mm.57094	Fgf2	FGF-2	2.4	−2.7	2
	Mm.57177	Fgf7	FGF-7	0.9	−0.6	0.5
	Mm.20424	Fgf18	FGF-18	2.6	−4	2
	Mm.3879	Hif1a	Hypoxia inducible factor 1, α subunit	1	−0.8	0.7
	Mm.27969	Uap1	UDP-N-acetylglucosamine pyrophosphorylase 1	2.7	−3	3.2
	Mm.2580	Sdc1	Syndecan 1	0.8	−0.6	1.8
	Mm.4575	Cspg2	Chondroitin sulfate proteoglycan 2	0.8	−1	1.5
	Mm.2542	Has1	Hyaluronan synthase1	2.2	−2.7	3.3
	Mm.5148	Has2	Hyaluronan synthase 2	1.3	−1.6	1.8
Bone and tissue destruction	Mm.6426	Tnfsf11 (RANKL)	Tumor necrosis factor (ligand) superfamily, member 11	4.2	−3.1	2.9
	Mm.1421	Adamts1	A disintegrin-like and metallo-protease (reprolysin type) with thrombospondin type 1 motif, 1	2.4	−1.5	0.7
	Mm.39718	Adamts5	A disintegrin-like and metallo-protease (reprolysin type) with thrombospondin type 1 motif, 5	1.1	−0.9	0.3
	Mm.15969	Adam8	A disintegrin and metalloprotease domain 8	0.6	−0.6	1.2
	Mm.44170	Vdr	Vitamin D receptor	1.5	−3.2	3.4

22 genes are chosen from cluster 4 of Table I and are classified based on their functions. The change in expression level is indicated as a logarithm of the fold-change. Indo, indomethacin.

PGs do not directly regulate IL-1β production in arthritic cell suspension, they contribute to IL-6 production, and the PGI_2-IP signaling can mediate this process. IL-1β and IL-6 are released mainly by inflammatory cells and synovial cells, respectively (1). To access whether a reduced IL-1β concentration in $IP^{-/-}$ mice was due to impaired inflammatory cell infiltration caused by reduced chemokine production in $IP^{-/-}$ mice, we measured MCP-1 in the arthritic paws of WT and $IP^{-/-}$ mice in the same manner as described above. MCP-1 is one of the chemokines that promotes RA (7, 21). $IP^{-/-}$ mice showed a significant reduction in the MCP-1 concentration compared with that of WT mice (Fig. 3 C).

Enhancement of IL-6 production in synovial fibroblasts by PGI_2-IP signaling

The results described above suggest that the PGI_2-IP signaling facilitates the activation of synovium in inflammatory joints. Synovial fibroblasts are a major cell population in the synovium and have recently been recognized as one of the pivotal effectors in the inflamed joint because of their ability to produce various inflammatory mediators (1). To obtain mechanistic insights into the role of IP signaling in synovial activation, we prepared synovial fibroblasts from normal knee joints of WT and $IP^{-/-}$ mice and expanded them by culture. They were then activated by IL-1β for 24 h in the absence or presence of indomethacin and cicaprost, and the effects of these compounds on the IL-1β–induced IL-6 production were examined (Fig. 4 A). IL-1β stimulation significantly increased IL-6 production by synovial fibroblasts from both WT and $IP^{-/-}$ mice. However, the IL-6 production by the $IP^{-/-}$ cells was significantly less than that by the WT fibroblasts, indicating that PGI_2 was endogenously formed by IL-1β stimulation and worked for IL-6 production. This suggestion was supported by an experiment examining the effects

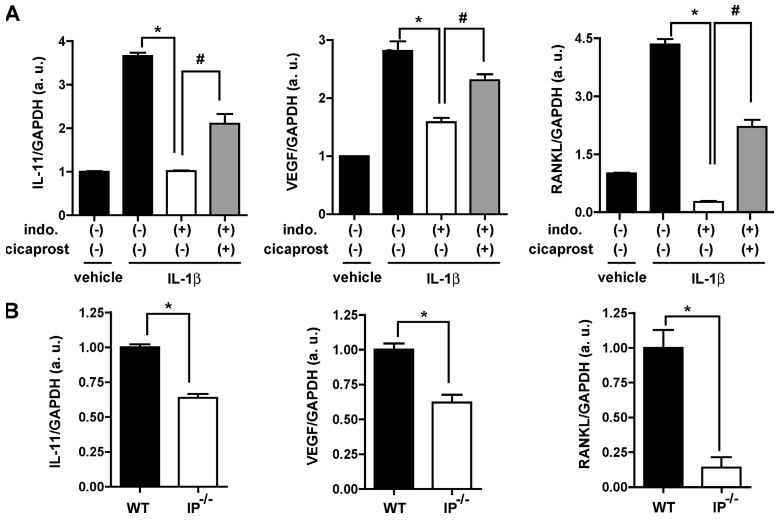

Figure 5. Real-time RT-PCR analysis of IL-11, VEGF, and RANKL mRNA in synovial fibroblasts and arthritic paws. (A) IL-11, VEGF, and RANKL mRNA in synovial fibroblasts. WT synovial fibroblasts were prepared and stimulated as indicated. After 6 h of stimulation, the cells were harvested and total RNA was extracted for quantification of IL-11, VEGF, and RANKL mRNA. The amount of each mRNA was normalized to that of GAPDH mRNA, and an arbitrary unit (a.u.) was defined with the value of the vehicle-treated synovial fibroblasts as 1. Results are expressed as the mean ± SEM. For the indicated comparison: *, $P < 0.05$; #, $P < 0.05$. Data shown are representative of at least three experiments. (B) Decreased expression of IL-11, VEGF, and RANKL genes in the arthritic paws of $IP^{-/-}$ mice. The paws were obtained from WT ($n = 8$) and $IP^{-/-}$ ($n = 8$) mice on day 35 of CIA. Total RNA was prepared and used for quantification of mRNA for the indicated genes as in A.

of indomethacin and cicaprost in WT cells. Indomethacin significantly inhibited IL-6 production in the WT fibroblasts, and the further addition of the IP agonist reversed the inhibition by indomethacin. Cicaprost, on the other hand, had no effect on IL-6 production in the $IP^{-/-}$ cells. Notably, the IL-6 production by the indomethacin-treated WT fibroblasts was significantly less than that by the $IP^{-/-}$ cells, and the production of IL-6 by the $IP^{-/-}$ cells was further attenuated by the addition of indomethatcin, suggesting that PGs other than PGI_2 also worked in this process. It was also noted that the treatment of nonactivated WT synovial fibroblasts with cicaprost alone could increase IL-6 production to some extent, and cicaprost further exerted synergistic effects with IL-1β (vehicle: 210.9 ± 32.5 pg/ml; cicaprost alone: 1,743.8 ± 127.9 pg/ml; IL-1β alone: 7,858.2 ± 305 pg/ml; cicaprost and IL-1β: 19,179 ± 196.5 pg/ml; Fig. 4, A and B).

Elevated expression of genes associated with inflammation, synovial proliferation, and bone destruction by PGI2-IP signaling

To evaluate the action of the PGI_2-IP signaling in synovial fibroblasts in more detail, we next used the microarray analysis using an Affymetrix Mouse Genome 430A 2.0 GeneChip that contains ∼14,000 genes. Synovial fibroblasts were treated with either vehicle, IL-1β, IL-1β with indomethacin, or IL-1β with indomethacin plus an IP agonist (iloprost) for 6 h and used for analysis on the gene expression profiles (see Table S1, available at http://www.jem.org/cgi/content/full/jem.20051310/DC1). We used Statistical Algorithm in the Affymetrix GeneChip Expression Analysis software and first compared the expression profile of synovial fibroblasts treated with vehicle and those treated with IL-1β. After exclusion of expressed sequence tags and overlapped genes, we detected 489 genes, the expression of which were statistically increased by the IL-1β treatment. Of the 489 genes, 183 were decreased in their expression by treatment with indomethacin, suggesting that endogenous PG(s) enhances the expression of these genes. Of these 183 genes, the expression of 138 was increased again by further addition of the IP agonist (Table I). In the 138 genes, we focused on 22, which are known or have been suggested to be involved in the pathogenesis of arthritis (Table II).

We classified these 22 genes into three groups based on their involvement in inflammation, synovial proliferation, or bone and tissue destruction. The first group consisting of seven genes is related to inflammation and includes those for IL-11 (22), IL-1 receptor, CSF receptor, CXC chemokine ligand 7 (23), CXC chemokine receptor 4 (24), and cytosolic phospholipase A_2 (25). There were 10 genes related to the synovial proliferation, i.e., FGF-2 (26, 27), FGF-7, FGF-18, vascular endothelial growth factor (VEGF; references 26 and 28), and hypoxia inducible factor 1α, syndecan 1 (29), chondroitin sulfate proteoglycan 2 (29), hyaluronan synthase 1, hyaluronan synthase 2 (30), and UDP-*N*-acetylglucosamine pyrophosphorylase 1. Five genes were identified as those related to bone and tissue destruction. They are genes for receptor activator of NF-κB ligand (RANKL; references 31 and 32), vitamin D receptor, a disintegrin and metalloprotease 8 (33), a disintegrin and metalloprotease with thrombospondin motifs 1, and a disintegrin and metalloprotease with thrombospondin motifs 5 (34). To confirm and quantify the effects of the IP signaling on the expression of these genes, we performed

real-time RT- PCR analysis on genes for IL-11, VEGF, and RANKL (Fig. 5 A). Consistent with the microarray analysis, the treatment with indomethacin significantly decreased expression of these genes and the additional treatment with the IP agonist reversed this inhibition. To examine a possibility that these actions of the IP signaling were secondary to IL-6 production, we added exogenous IL-6 to synovial fibroblasts and examined its effects on the expressions of those genes. We found that the addition of IL-6 did not change the expression level of these genes (unpublished data), suggesting that expression of the above genes is regulated by the IP signaling independent of IL-6. Next, we examined whether the absence of the IP signaling affects the expression of these genes in vivo. As expected, the expression of IL-11, VEGF, and RANKL were significantly decreased in the arthritic paws of $IP^{-/-}$ mice compared with that of WT mice (Fig. 5 B). These results suggest that the PGI_2-IP signaling promotes progression of CIA by enhancing the expression of IL-6 and other arthritis-related genes in synovial fibroblasts.

Identification of EP receptor subtypes working in the elicitation phase of CIA

We have thus clarified the role of PGI_2-IP signaling in the development of CIA. However, although the CIA score was significantly suppressed by the loss of IP, there was still a small extent of inflammation remaining in $IP^{-/-}$ mice. To examine if other PG signaling contributes to this remnant inflammation, we treated $IP^{-/-}$ mice with indomethacin from day 21. We found that administration of indomethacin abolished the arthritis in $IP^{-/-}$ mice almost completely (Fig. 6 A). These results suggest that PG signaling other than the PGI_2-IP pathway also works in progression of CIA. The attenuation of IL-6 production by $IP^{-/-}$ synovial fibroblasts with indomethacin in vitro described above (Fig. 4 A) also supports this possibility. Because plenty of evidence supports the role of PGE_2 in RA (14, 15), we suspected that PGE_2 plays a role in this process and wanted to identify PGE receptor subtypes involved. Therefore, we backcrossed mice deficient in each of the four subtypes of the PGE receptor ($EP1^{-/-}$, $EP2^{-/-}$, $EP3^{-/-}$, and $EP4^{-/-}$) individually to the DBA/1J background for 10 generations. Because $EP4^{-/-}$ mice could not survive in this background because of the patent ductus arteriosus (35), we administered an EP4-selective antagonist (ONO-AE3-208) at a dose of 10 mg/kg/day to evaluate the role of the EP4 receptor in CIA. This dose of the drug was previously shown to be sufficient in suppressing EP4 functions in vivo (19). When $EP1^{-/-}$, $EP2^{-/-}$, or $EP3^{-/-}$ DBA/1J mice were subjected to CIA, all of these strains of mice developed swelling and erythema in the paws comparable to those in WT mice (Fig. 6 B). Moreover, the administration of the EP4 antagonist during the elicitation phase elicited little effect on the arthritic symptoms of WT mice (Fig. 6 C). Thus, despite the reduced CIA responses in mPGES-$1^{-/-}$ mice (15), loss or inhibition of a single EP subtype alone does not significantly affect the progression of CIA, suggesting a redundancy among the EP subtypes.

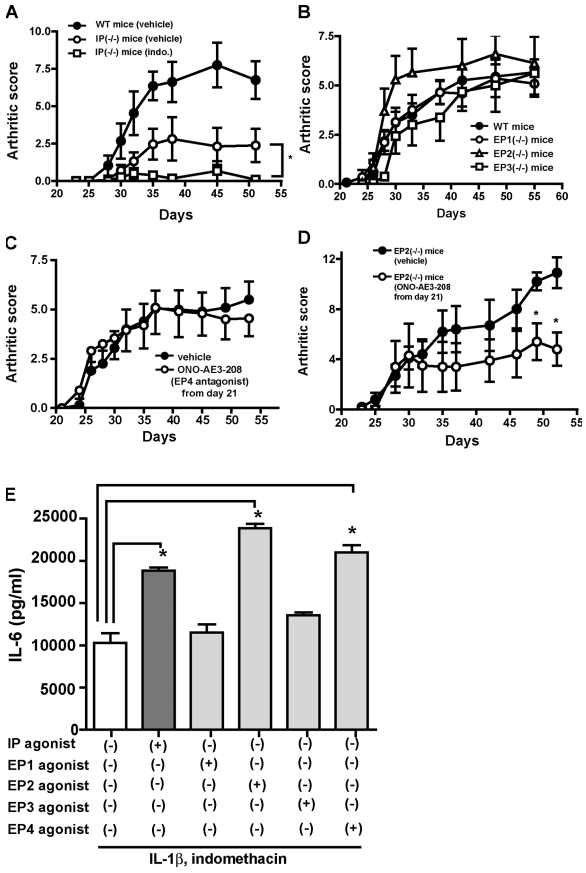

Figure 6. Effect of disruption of EP1, EP2, and EP3 or treatment with an EP4 antagonist on arthritic inflammation in CIA. (A) Time course of arthritic scores in WT mice treated with vehicle (closed circle, $n = 7$) or in $IP^{-/-}$ mice treated either with vehicle (○, $n = 7$) or indomethacin (4 mg/kg/day; □, $n = 6$) in CIA. Vehicle or indomethacin was administered from day 21. *, $P < 0.05$ between $IP^{-/-}$ mice treated with vehicle and $IP^{-/-}$ mice treated with indomethacin (two-way ANOVA). (B) Time course of arthritic scores in WT (●, $n = 37$), $EP1^{-/-}$ (○, $n = 20$), $EP2^{-/-}$ (△, $n = 10$), and $EP3^{-/-}$ (□, $n = 8$) mice in CIA. Results show the mean ± SEM. No significant difference was detected in the arthritic score at each time point between WT mice and mice deficient in each EP subtype (Student's *t* test). (C) Time course of arthritic scores in WT mice treated either with vehicle (●, $n = 10$) or an EP4 antagonist (ONO-AE3-208, 10 mg/kg/day; ○, $n = 10$) in CIA. Vehicle or the EP4 antagonist was administered from day 21. (D) Time course of arthritic scores in $EP2^{-/-}$ mice treated with vehicle (●, $n = 5$) or the EP4 antagonist (○, $n = 5$) in CIA. Vehicle or EP4 antagonist (10 mg/kg/day) was administered from day 21. *, $P < 0.05$ versus vehicle-treated mice. (E) Effects of the IP agonist (1 μM cicaprost), the EP1 agonist (1 μM ONO-DI-004), the EP2 agonist (1 μM butaprost), the EP3 agonist (1 μM AE248), and the EP4 agonist (1 μM ONO-AE1-329) on IL-6 production in synovial fibroblasts. Synovial fibroblasts were treated as indicated, and IL-6 production was measured as described above. Results are expressed as the mean ± SEM ($n = 3$). *, $P < 0.05$ for the indicated comparison. Data shown are representative of at least three experiments.

Because the PGI_2-IP pathway uses the cAMP generation as a major signaling pathway and both EP2 and EP4 share this signaling, we suspected that EP2 and EP4 work redundantly to mediate PGE_2 action in CIA and either one of the two can

compensate for the loss of the other. To test this hypothesis, we administered the EP4 antagonist to $EP2^{-/-}$ mice during the elicitation phase in CIA. Administration of the EP4 antagonist reduced the arthritic score significantly compared with that found in the vehicle-treated control $EP2^{-/-}$ mice (Fig. 6 D). These results support our hypothesis that EP2 and EP4 works redundantly for elicitation of CIA. Consistently, when agonists selective to each of the EP subtypes were added to the synovial fibroblast culture to examine their activity to enhance the IL-1β–stimulated IL-6 production, the compounds selective to EP2 and EP4, but not those to EP1 and EP3, potently enhanced the IL-6 production (Fig. 6 E). The EP2 and EP4 agonists also share the activity to enhance production of IL-11, VEGF, and RANKL in this in vitro culture system (unpublished data).

DISCUSSION

The importance of PGs in the pathogenesis of RA has long been recognized through the wide use of NSAIDs for RA treatment (36, 37). Indeed, a large amount of PGI_2 and PGE_2 was detected in the synovial fluid of arthritic joints, suggesting the actions of these PGs in inflammatory sites (13). However, how each PG, PGI_2 in particular, works in RA remains largely unknown. To examine this issue, we used CIA as a mouse model for RA and subjected $IP^{-/-}$ mice in the DBA/1J background to this model. We have found that $IP^{-/-}$ mice exhibited significantly reduced clinical and histological arthritic scores compared with control mice, demonstrating the importance of PGI_2-IP signaling in the pathogenesis of CIA. This is an interesting result because PGI_2 has been considered as a mediator of acute inflammation by causing vasodilation or enhancing vascular permeability, and less attention has been paid to the contribution of PGI_2 to chronic inflammation. It has long been thought that PGE_2 is the primary PG responsible for inflammation in RA. Our results indicate, however, that PGI_2 works as much as PGE_2 in the progression of CIA. $IP^{-/-}$ mice showed significantly reduced arthritic scores in CIA despite the level of anti-CII titers comparable with WT mice and also exhibited reduced responses in CAIA, indicating that the PGI_2-IP pathway works in the antibody-dependent effector mechanisms of joint inflammation.

There are two arms of cell populations possibly mediating the IP action in the effector mechanisms: the bone marrow–derived cell population, such as macrophages and neutrophils, and the mesenchymal cell population, such as synovial fibroblasts. Here, we have focused on the action of IP in synovial fibroblasts. We have found that the IP signaling working with IL-1β enhances IL-6 production from activated synovial fibroblasts. IL-6 has been suggested to exert many actions in the pathogenesis of RA, such as B cell maturation, synovial proliferation, and osteoclast formation (8, 38). $IL\text{-}6^{-/-}$ mice showed reduced arthritic lesions in CIA (39), and the administration of anti–IL-6 receptor monoclonal antibody to RA patients resulted in a significant improvement of both clinical symptoms and laboratory findings (40). Other than IL-6, PGI_2-IP signaling induces a variety of arthritis-related genes, including IL-11, VEGF, FGF-2, and RANKL. IL-11 is a member of the IL-6 family and exerts proinflammatory effects in an acute methylated BSA/IL-1–induced arthritis model (22). VEGF and FGF-2 stimulate angiogenesis, and the blockade of their signals results in reduced arthritis severity (27, 28). RANKL is essential for osteoclast formation and mediates bone destruction (31). These findings strengthen our hypothesis that the IP-dependent activation of synovial fibroblasts plays a significant role in the effector mechanisms of inflammation in the joint. It should be mentioned that IP signaling can induce expression of these genes only in combination with IL-1β, and the stimulation of IP alone induces a significant but marginal effect. The requirement of such synergism with IL-1β well explains the lack of arthritic symptoms in patients treated with an IP agonist, such as iloprost as a vasodilator. The lack of IP signaling and consequent impairment in synovial lining hyperplasia may affect the amount of chemokine production, such as MCP-1, from synovial fibroblasts and reduce the further infiltration of inflammatory cells. The significantly lower concentration of IL-1β in the paws of arthritic $IP^{-/-}$ mice might be due to the impaired network of cytokine and chemokine signaling between synovial fibroblasts and inflammatory cells. Collectively, our current findings suggest that the PGI_2-IP signaling in synovial fibroblasts works as an amplifier of the inflammatory processes in the joint through the induction of various arthritis-related genes. Whether and how the IP signaling in the bone marrow–derived cell population contributes to this process will be examined in bone marrow chimera in our future study.

Our study also points to the role of both PGE_2 and PGI_2 in the progression of CIA. For example, although the CIA score was significantly suppressed by the loss of IP, there was still a small extent of inflammation remaining in $IP^{-/-}$ mice. Our analysis for EP signaling indicates that this remaining inflammation is mediated by PGE_2 via EP2 and EP4. Using mice deficient in the EP subtypes individually as well as the EP4-selective antagonist, we found that although loss or inhibition of a single EP does not affect the extent of CIA, simultaneous inhibition of EP2 and EP4 significantly reduced the arthritic score in CIA. These results suggest that EP2 and EP4 redundantly mediate the PGE_2 action in joint inflammation and that these PGE_2 signaling pathways work together with the IP signaling to induce a full extent of CIA. It is quite likely that EP2 and EP4 also mediate inflammation by activating synovial fibroblasts and promoting expression of arthritis-associated genes because these EP receptors share the cAMP signaling pathway with IP, and the agonists selective to EP2 or EP4 potently stimulate the expression of genes for IL-6, IL-11, RANKL, and VEGF in synovial fibroblasts in vitro. We also observed that $IP^{-/-}$ mice showed a significantly reduced arthritic score in CAIA. Involvement of EP4 in CAIA was reported previously (41, 42). Therefore, evidence now exists for a significant role for both PGE_2 and PGI_2 in both models of arthritis. Which PG or PG receptor plays a predominant role in vivo may depend on the context of

arthritis, which can determine the species of the PG(s) produced in arthritic joints.

The findings described above naturally have an impact on the strategy for the development of anti-RA drugs. Although NSAIDs have been widely used for RA patients, gastrointestinal toxicity associated with the inhibition of the COX-1 isoform limits their use. To circumvent this problem, selective COX-2 inhibitors have been introduced and rapidly gained popularity (43). However, one of the selective COX-2 inhibitors, rofecoxib, has recently been withdrawn from clinical use because a placebo-controlled trial revealed a significant rise in the rate of cardiovascular accidents in rofecoxib users (44). A major cause for such accidents is proposed to be the inhibition of COX-2–dependent PGI_2 production from endothelial cells. Indeed, it was shown experimentally that selective inhibition of the PGI_2-IP signaling led to the acceleration of atherosclerosis and thrombosis (16, 45). Therefore, this study suggests a possibility that both beneficial and adverse effects of COX-2 inhibitors in RA come at least in part from the same mechanism; i.e., inhibition of PGI_2 synthesis. Given the clinical efficacy of COX-2 inhibitors in RA patients and the current belief that PGE_2 is the major PG working in the pathogenesis of RA, several attempts are now in progress to develop selective inhibitors of mPGES, which is regulated similarly to COX-2 and, hence, is believed to work preferentially in inflammation. This study on CIA, however, suggests that PGI_2 also works significantly in RA, providing a cautious note that mPGES inhibitors may not be so effective in RA as COX-2 inhibitors.

MATERIALS AND METHODS

Materials. Mice lacking the IP, EP1, EP2, EP3, and EP4 receptor individually were generated as described previously (16, 35, 46, 47) and backcrossed 10 times to DBA/1JNcr (Japan Charles River). Mice were bred at the Institute of Laboratory Animals of Kyoto University on a 12-h light/dark cycle under specific pathogen-free conditions. All experimental procedures were approved by the Committee on Animal Research of Kyoto University Faculty of Medicine. An EP4 antagonist, ONO-AE3-208 (AE3-208), was provided by Ono Pharmaceutical Co. The structures, binding affinities, selectivities, and pharmacokinetic properties of AE3-208 have been described (19). Indomethacin was purchased from Sigma-Aldrich.

CIA. CIA was induced in 5–6-wk-old male mice. Bovine CII (Cosmo-Bio) was emulsified with CFA (Difco). Mice were immunized with the emulsion containing 100 μg CII by intradermal injection at the base of the tail on day 0 and boosted with 100 μg bovine CII emulsified with IFA (Difco) on day 21. The extent of arthritis was evaluated by eye and scored according to the method as essentially described (48). In brief, the arthritic lesion of each paw was graded on a scale of 0–4: 0, no change; 0.5, swelling and erythema of one digit; 1, swelling and erythema of two or more digits or slight swelling of the ankle or wrist joint; 2, mild swelling and erythema of the ankle or wrist joint; 3, severe swelling and erythema of the entire paw; 4, severe swelling and erythema with deformity of the entire paw.

CAIA. Mice were injected i.p. with a mixture of anti-CII monoclonal antibodies (2 mg/500 μl/mouse; Chondrex) on day 0, and 3 d later, LPS (50 μg/100 μl/body; *Escherichia coli*. 0111:B4; Chondrex) was injected i.p. Arthritis was scored as described for CIA.

Histological examination. Hind paws were fixed in 10% neutral-buffered formalin, decalcified in 10% EDTA in PBS, and embedded in paraffin. Sections of 7-μm thickness of the knee joint were stained with either H&E or toluidine blue at pH 4.5. Two observers blinded to the genotypes of the preparations examined the sections and scored them. Scoring was based on the degree of lining hyperplasia, inflammatory cell infiltration in the joint cavity, and tissue destruction on a scale of 0–3 (0, within normal limits; 1, minimal; 2, mild; 3, severe) essentially as described previously (25). As for tissue destruction, proteoglycan depletion, cartilage destruction, and bone destruction were scored separately, and the average of the scores of the three parameters was used to represent tissue destruction.

To examine complement deposition on the bone surface, the decalcified hind paw preparation was soaked with 7.5% polyvinyl pyrrolidone in PBS and frozen at −80°C. 10-μm thick sections of the knee joint treated with Peroxo-Block (Zymed Laboratories) were incubated with rabbit anti–mouse C3 antibody (Hycult Biotechnology) in PBS with 10% goat serum (Sigma-Aldrich) at 4°C overnight, and then with horseradish peroxidase–conjugated polyclonal goat anti–rabbit IgG antibody (DakoCytomation) at room temperature for 2 h. The color was developed with diaminobenzidine tetrahydrochloride (Wako).

ELISA for anti-CII antibodies. A 96-well plate for ELISA (Sumitomo Bakelite) was coated with 5 μg/ml bovine CII in PBS overnight at 4°C. After nonspecific binding was blocked with 1% BSA (Sigma-Aldrich) in PBS for 2 h at room temperature, the diluted serum was added and incubated overnight at 4°C. After washing with 0.05% Tween 20 in PBS, either goat anti–mouse IgG, IgG1, IgG2a, or IgG2b antibody conjugated with alkaline phosphatase (SouthernBiotech) was added and incubated for 1 h at room temperature. The wells were washed and *p*-nitro-phenyl phosphate (Sigma-Aldrich) was added as a substrate. The color developed was determined by absorbance at 405 nm. Standard curves (in arbitrary units) for anti-CII IgGs were constructed from the pooled serum of a DBA/1J mouse with a high score of CIA. The titer of the standard serum was defined as 10,000 units/ml.

Isolation and culture of synovial fibroblasts. Synovial fibroblasts were isolated and cultured essentially as described previously (49). In brief, the synovium of the knee joint from unimmunized control mice was dissected and then digested with 1 mg/ml collagenase A (Roche Diagnostics) in serum-free RPMI 1640 medium at 37°C for 2 h. The tissue digests were suspended and passed through nylon mesh. Dissociated cells were washed three times in RPMI 1640 medium containing 50 μM 2-mercaptoehtanol, 5 mM sodium pyruvate, and 10% heat-inactivated FCS, and cultured. After overnight culture, nonadherent cells were removed. At confluence, cells were trypsinized, divided into three, and replated. The passage was repeated three to four times before use.

Synovial fibroblasts were plated at a density of 2×10^4 cells/well in a 96-well plate in RPMI 1640 medium with 1% FCS and cultured with or without mouse rIL-1β (R&D Systems). Indomethacin (Sigma-Aldrich) and either cicaprost, iloprost (Cayman), ONO-DI-004, butaprost (Cayman), ONO-AE-248, or ONO-AE1-329 was further added at the indicated concentration either alone or in combination. The ONO-DI-004, ONO-AE-248, and ONO-AE1-329 were supplied by ONO Pharmaceutical. The ligand-binding specificities of these compounds for each PGE receptor subtype have been described (14). Supernatants were collected after 24 h of incubation. For RNA extraction, cells were plated at a concentration of 10^6 cells/dish in a 60-mm dish. Cells were isolated after 6 h. For some experiments, 10 ng/ml IL-6 (Calbiochem) was added instead of IL-1β.

Arthritic cell suspension was also prepared from the knee joints from mice on day 35 of CIA in the same way as described for synovial fibroblasts. The arthritic cell suspension was used without passage. The cells were plated at a concentration of 2×10^4 cells/well in a 96-well plate, and supernatants were collected after 24 h of incubation. For IL-1β measurement, 1 μg/ml LPS (*E. coli* S16B5; Sigma-Aldrich) was added.

Cytokine assays. The amount of IL-6, IL-1β, and TNF-α in culture supernatants and tissue homogenates was measured with ELISA kits (Endogen). The amount of MCP-1 in tissue homogenates was measured with ELISA kits (Biosource). For assays of cytokines in arthritic paws, samples were prepared as described previously (50).

DNA microarray analysis. Total RNA was prepared from synovial fibroblasts by using an RNeasy Mini kit (QIAGEN) and subjected to microarray analysis using a Mouse Genome 430A 2.0 Array (Affymetrix). Data were analyzed by Statistical Algorithm with the Affymetrix GeneChip Expression Analysis software (Microarray Suite 5.0). All microarray data are deposited in the Gene Expression Omnibus (GSE2676).

Quantitative real-time RT-PCR. Total RNA was obtained from arthritic paws using TRIzol reagent (Invitrogen). Complementary DNA was synthesized using Superscript II (Invitrogen). The amount of each mRNA for RANKL, IL-11, VEGF, and GAPDH was quantified by real-time RT-PCR using LightCycler 2.0 (Roche Diagnostics). The primer sequences RANKL, VEGF, and GAPDH were described previously (51–53). Primers used for IL-11 were 5′-GACTCTGGAGCCAGAGCTG-3′ (forward) and 5′-GGGATCACAGGTTGGTCTG-3′ (reverse). Data were analyzed by LightCycler Software Version 4.0.

Statistics. Data are presented as the mean ± SEM. The comparison of two groups was analyzed using Student's unpaired two-tailed *t* test or the Mann-Whitney U test. For comparison of more than two groups, one-way ANOVA was performed first, and either Dunnett's or Tukey's test was used to evaluate pairwise group difference. Two-way ANOVA was performed for comparison including two factors. A p-value of <0.05 was considered statistically significant. The analyses were performed with the use of PRISM 4.0 software (GraphPad).

Online supplemental material. Table S1 shows the number of genes in each cluster of microarray analysis and is available at http://www.jem.org/cgi/content/full/jem.20051310/DC1.

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