RESEARCH PAPER

A novel antagonist of the prostaglandin E₂ EP₄ receptor inhibits Th1 differentiation and Th17 expansion and is orally active in arthritis models

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Background and purpose: Rheumatoid arthritis (RA) is an autoimmune disorder involving subsets of activated T cells, in particular T helper (Th) 1 and Th17 cells, which infiltrate and damage tissues and induce inflammation. Prostaglandin E₂ (PGE₂) enhances the Th17 response, exacerbates collagen-induced arthritis (CIA) and promotes inflammatory pain. The current study investigated whether selective antagonism of the PGE₂ EP₄ receptor would suppress Th1/Th17 cell development and inflammatory arthritis in animal models of RA.

Experimental approach: Effects of PGE₂ and a novel EP₄ receptor antagonist ER-819762 on Th1 differentiation, interleukin-23 (IL-23) production by dendritic cells (DCs), and Th17 development were assessed *in vitro*. The effect of ER-819762 was evaluated in CIA and glucose-6-phosphate isomerase (GPI)-induced arthritis models. In addition, the effects of ER-819762 on pain were evaluated in a model of chronic inflammatory pain induced by complete Freund's adjuvant (CFA) in the rat.

Key results: Stimulation of the EP₄ receptor enhanced Th1 differentiation via phosphatidylinositol 3 kinase signalling, selectively promoted Th17 cell expansion, and induced IL-23 secretion by activated DCs, effects suppressed by ER-819762 or anti-PGE₂ antibody. Oral administration of ER-819762 suppressed Th1 and Th17 cytokine production, suppressed disease in collagen- and GPI-induced arthritis in mice, and suppressed CFA-induced inflammatory pain in rats.

Conclusion and implications: PGE₂ stimulates EP₄ receptors to promote Th1 differentiation and Th17 expansion and is critically involved in development of arthritis in two animal models. Selective suppression of EP₄ receptor signalling may have therapeutic value in RA both by modifying inflammatory arthritis and by relieving pain.

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Keywords: PGE₂; EP₄ receptor; EP₄ antagonist; Th1; Th17; IFN- γ ; IL-17; IL-23; CIA; GPI-induced arthritis model

Abbreviations: BSA, bovine serum albumin; CIA, collagen-induced arthritis; CFA, complete Freund's adjuvant; CRE, cAMP response elements; ER-819762, (S)-1'-(3,5-dimethylbenzyl)-2-ethyl-7,9-dimethoxy-10-methyl-5,10-dihydrospiro[benzo[e]imidazo[1,5-a]azepine-1,4'-piperidin]-3(2H)-one; FBS, fetal bovine serum; GPCR, G protein-coupled receptors; GPI, glucose-6-phosphate isomerase; IBMX, isobutylmethylxanthine; imDC, immature human dendritic cell; PGE₂, prostaglandin E₂; PI3K, phosphatidylinositol 3-kinase; PLAP, placental-like alkaline phosphatase; RA, rheumatoid arthritis; Th1, T helper 1; Th17, T helper 17; TLR, Toll-like receptor

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disorder that is estimated to affect up to 1% of the population worldwide (Williams, 2006). Although not life-threatening, RA is a

painful and debilitating disease that progressively limits the ability of patients to carry on normal lives. The factors that trigger this disease are not well understood but are believed to include both genetic and environmental components. Over the last decade, novel discoveries into the regulation of the immune system have permitted a better understanding of the development of autoimmunity. Upon encountering antigen, T helper (Th) cells differentiate into several subtypes, depending on various factors such as the cytokines produced, the consequent activation of intracellular signalling pathways,

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and the expression of transcription factors. Among these T cell subtypes, Th1 and Th17 cells have been shown to be critically involved in the development of autoimmunity (Schulze-Koops and Kalden, 2001; Fouser *et al.*, 2008). While the molecular mechanisms controlling the differentiation and expansion of these T cell subsets have begun to be elucidated, specific agents to suppress the function of these critical T cell subtypes are only in early-stage development.

Prostaglandin E₂ (PGE₂) is an arachidonic acid metabolite that acts as a potent biological mediator, exerting its effects via activation of membrane G protein-coupled receptors (GPCRs). There are four receptor subtypes (EP₁, ₂, ₃ and ₄; nomenclature follows Alexander *et al.*, 2009) which selectively bind PGE₂ and mediate its effects: Activation of EP₁ receptors leads to the influx of calcium. Activation of EP₃ receptors can induce a variety of signalling events depending on the particular EP₃ splicing variant being expressed, with inhibition of adenylate cyclase activity via Gi being the most common effect. EP₂ and EP₄ receptors induce Gs-mediated activation of adenylate cyclase and a subsequent increase in intracellular cyclic AMP (cAMP). In addition, EP₄ receptors activate the phosphatidylinositol 3-kinase (PI3K) signalling pathway (Fujino *et al.*, 2003). PGE₂ is known to play important roles in mediating many inflammatory responses and is often found at increased concentrations under a variety of inflammatory conditions (Hata and Breyer, 2004). Many reports suggest that PGE₂, via the induction of intracellular cAMP, can suppress pro-inflammatory T cell function, including T cell receptor signalling and consequent production of interleukin (IL)-2 (Mustelin and Tasken, 2003; Chemnitz *et al.*, 2006). PGE₂ has also been implicated in T-cell differentiation and is reported to inhibit Th1 but not Th2 cytokines via the induction of intracellular cAMP (Betz and Fox, 1991; Gold *et al.*, 1994; Hilkens *et al.*, 1995; Okano *et al.*, 2006). However, other reports indicate a pro-inflammatory role for PGE₂. PGE₂ can induce production of IL-23 from dendritic cells (DCs), which promotes the differentiation of pro-inflammatory Th17 cells (Sheibanie *et al.*, 2004; Khayrullina *et al.*, 2008). Recent reports also suggest that PGE₂ can synergize with IL-23 to promote expansion of human Th17 cells and enhance IL-17 production (Chizzolini *et al.*, 2008; Boniface *et al.*, 2009; Napolitani *et al.*, 2009). Furthermore, PGE₂ has been shown to exacerbate symptoms in mouse models of arthritis (Sheibanie *et al.*, 2007a) and inflammatory bowel disease (Sheibanie *et al.*, 2007b), and the blockade of EP₂ and EP₄ receptor signalling in a mouse model of arthritis can alleviate the severity of the disease (Mccoy *et al.*, 2002; Honda *et al.*, 2006).

Here, we show that PGE₂ stimulation of the EP₄ receptor can promote Th1 differentiation, IL-23 production in DCs, and Th17 cell expansion. These effects can be suppressed by a novel EP₄ receptor antagonist ER-819762 ((S)-1'-(3,5-dimethylbenzyl)-2-ethyl-7,9-dimethoxy-10-methyl-5,10-dihydrospiro[benzo[e]imidazo[1,5-a]azepine-1,4'-piperidin]-3(2H)-one) (Spyvee *et al.*, 2009) or an anti-PGE₂ antibody. We also show that oral administration of ER-819762 to DBA/1 mice can effectively suppress disease in collagen-induced arthritis (CIA) or glucose-6-phosphate isomerase (GPI)-induced arthritis models. ER-819762 was also effective in treating chronic inflammatory pain in a rat model. These

results suggest that PGE₂ signalling via the EP₄ receptor exerts a pro-inflammatory effect *in vivo* and is physiologically relevant to the pathology of inflammatory arthritis. EP₄ receptors might therefore be an attractive drug target for the treatment of RA, with the potential not only to relieve pain and symptoms but also to modify the underlying aetiology of the disease

Methods

Animals

All animal studies were performed with the approval of the Animal Ethics Committee at Eisai according to Laboratory Animal Welfare guidelines. BALB/c and DO11.10 mice were purchased from Jackson Laboratory. C57BL/6 and DBA/1 mice and F344 rats were purchased from Charles River Laboratories. Mice and rats for each strain were group-housed under controlled conditions with a constant temperature (23 ± 3°C) and humidity (55 ± 5%), a 12-h light/dark cycle and *ad libitum* access to water and standard pelleted food.

Radioligand EP₄ receptor binding assay

The radioligand EP₄ receptor binding assay was performed using ChemoScreen recombinant human EP₄ receptor membrane preparations from Millipore, according to the manufacturer's instructions. Briefly, membranes prepared from Chem-1 cells overexpressing human EP₄ receptor cDNA (Millipore) were mixed with 1.8 nmol·L⁻¹ [³H]-PGE₂ and 5 µmol·L⁻¹ unlabelled PGE₂ in the presence or absence of various concentrations of ER-819762 in binding buffer [50 mmol·L⁻¹ HEPES, pH 7.4, 5 mmol·L⁻¹ MgCl₂, 1 mmol·L⁻¹ CaCl₂, 0.2% bovine serum albumin (BSA)] in a nonbinding 96-well plate, and incubated for 1–2 h at room temperature. Prior to filtration, a GF/C 96-well filter plate was coated with 0.33% polyethyleneimine for 30 min, then washed with 50 mmol·L⁻¹ HEPES, pH 7.4, 0.5% BSA. Binding reactions were transferred to the filter plate, and washed three times with wash buffer (1 mL per well per wash). The plate was dried and radioactivity counted. Binding of ER-819762 to other related prostanoid receptors was performed by MDS Pharma Services (Bothell, WA, USA) using a similar radiolabelled ligand displacement method.

Cell-based GPCR assays

SE302 is a clone of the human embryonic kidney 293 (HEK/293) cell line containing a reporter driven by cAMP response elements (CRE) in its promoter, and producing secreted placental-like alkaline phosphatase (PLAP). HEK/293 cells express endogenous EP₄ receptors and show induction of PLAP in response to PGE₂ and EP₄ receptor agonists, but not EP₁, ₂ or ₃ receptor agonists (Supplementary Fig. 2). Cells were maintained in DMEM/F12 (50:50) (MediaTech, Inc., Manassas, VA, USA) supplemented with 10% fetal bovine serum (FBS; Tissue Culture Biologicals) plus penicillin/streptomycin. When used for assays, cells were plated in a 96-well plate at 2 × 10⁴ cells/100 µL per well in serum-free assay medium (DMEM/F12 supplemented with 0.1% BSA plus penicillin/

streptomycin) and incubated for 4–6 h. Cells were then stimulated with 3 ng mL⁻¹ PGE₂ in the presence or absence of various concentrations of ER-819762 overnight, and PLAP activity was measured by mixing 15 µL of culture supernatants with 75 µL of Lumi-phos (Lumigen, Inc.) and 60 µL of assay buffer containing 8 mmol·L⁻¹ MgSO₄ in 0.1 mol·L⁻¹ carbonate-bicarbonate buffer pH11 in a new 96-well black plate and incubated for 2 h at room temperature. Luminescence was read with an Envision 2102 Multilabel reader. Characterization of compound selectivity was performed by Millipore GPCR Profiler Service, which assays intracellular calcium mobilization in cells expressing individual GPCRs and the promiscuous Gα₁₅ protein. Endogenous EP₂ receptor activity in U2-OS cells was assayed using the EPIC Resonant Waveguide Biosensor system (Corning).

In vitro T-cell assays

Naive CD4⁺ T cells were purified from spleens of either BALB/c or DO11.10 mice by antibody-coated magnetic beads as described by the manufacturer (Robosep; StemCell Technologies). For BALB/c mice, 1 × 10⁵ CD4⁺ T cells were cultured for 3–6 days in a 96-well plate in 100 µL complete RPMI medium (CellGro) containing 10% regular FBS under: (i) neutral conditions (1 µg mL⁻¹ plate-bound anti-CD3 + 1 µg mL⁻¹ soluble anti-CD28 + 10 ng mL⁻¹ mouse IL-2), (ii) Th1-promoting conditions (neutral + 5 ng mL⁻¹ mouse IL-12 + 10 µg mL⁻¹ anti-IL-4 antibody) or (iii) Th2-promoting conditions [neutral + 10 ng mL⁻¹ of mouse IL-4 + 10 µg mL⁻¹ anti-interferon (IFN)-γ antibody]. In experiments where exogenous PGE₂ or EP₄ receptor agonists were added to the culture, charcoal-stripped FBS (Hyclone) was used, which has reduced amounts of lipids. IFN-γ or IL-4 in culture supernatants were quantified by enzyme-linked immunosorbent assay (ELISA). Cell proliferation was assayed with either Alamar Blue or CellTiter-Glo reagents according to the manufacturers' instructions. For DO11.10 mice, mitomycin C-treated splenocytes from BALB/c mice were used as antigen-presenting cells and co-cultured with naive CD4⁺ T cells in a 5:1 ratio (5 × 10⁵ mitomycin C-treated splenocytes in 100 µL medium + 1 × 10⁵ CD4 T cells in 100 µL medium). These cultures were stimulated with an ovalbumin peptide 323-339 (OVA peptide; 0.3 ng mL⁻¹) under neutral, Th1- or Th2-promoting conditions, as described above. EP₄ receptor agonists and antagonists, other cAMP-inducing agents, inhibitors of PI3K or protein kinase A (PKA) or anti-PGE₂ antibody were added during Th cell differentiation.

In order to study the effect of EP₄ receptor agonists, antagonists and cAMP-inducing agents on IL-17 production, total CD4⁺ T cells isolated from spleens of C57BL/6 mice were activated with plate-bound anti-CD3 (2 µg mL⁻¹) plus soluble anti-CD28 (2 µg mL⁻¹) in the presence or absence of IL-23 (10 ng mL⁻¹) and presence or absence of EP₄ receptor agonist/antagonists or other agents at the indicated concentrations for 3–5 days. Culture supernatants were analysed by IL-17 ELISA, and cell proliferation was measured with CellTiter-Glo.

IL-23-induced Th17 expansion

CD4⁺ T cells were isolated from C57BL/6 mice and activated with antibody against T cell receptor β chain (1 µg mL⁻¹ plate-

bound) and anti-CD28 (2 µg mL⁻¹ soluble) with or without IL-23 (30 ng mL⁻¹) for 5 days in complete RPMI medium containing 10% normal FBS. IL-17-producing cells were analysed by IL-17 intracellular staining. Briefly, cells were stimulated for 5 h with phorbol 12-myristate 13-acetate (50 ng mL⁻¹), ionomycin (500 ng mL⁻¹) and Golgistop (1 µL mL⁻¹), stained with anti-CD4 antibody, fixed and permeabilized (Cytofix/Cytoperm) and stained with anti-IL-17 antibody (all from BD Biosciences) and then analysed by flow cytometry.

In vitro human monocytes-derived DC assay

Human peripheral blood monocytes (PBMC) were isolated by Ficoll gradient from heparinized venous blood of healthy, drug-free volunteers, following written informed consent. CD14⁺ cells were purified from human PBMC using Miltenyi CD14 microbeads according to the manufacturer's instructions, and differentiated with human GM-CSF (500 U mL⁻¹) + human IL-4 (500 U mL⁻¹) in complete RPMI medium containing 10% charcoal-stripped FBS for 8 days. Detached immature DCs (imDCs) were stimulated with lipopolysaccharide (LPS) (*Escherichia coli* 0111:B4; 10 ng mL⁻¹) and the Toll-like receptor (TLR)7 ligand, R-848 (2.5 µg mL⁻¹) with or without the addition of EP₄ receptor agonist/antagonists or anti-PGE₂ at the concentrations indicated for 24 h. Concentrations of IL-23 in culture supernatants were measured by ELISA (eBioscience).

Collagen-induced arthritis model

Male DBA/1 mice were immunized by injection at the base of the tail with 0.1 mL emulsion containing 150 µg bovine type II collagen (bCII) emulsified in complete Freund's adjuvant (CFA). Three weeks after the first immunization, all mice were boosted with bCII emulsified in Freund's incomplete adjuvant. ER-819762 was given orally daily at a dose of 10, 30 or 100 mg·kg⁻¹ from day 20 after primary immunization but before disease onset (prophylactic evaluation) or after the disease induction (therapeutic evaluation). The severity of arthritic symptoms in the paws of each mouse was graded, in a double-blind manner, according to Williams (2004). At the end of the experiments, knee and ankle joints were used for radiography analysis. X-ray score was defined as the total score of a combination of osteopenia, bone erosion and new bone formation as follows: 0 – no change; 1 – slight change, 2 – moderate change; 3 – severe change (Kop *et al.*, 2006). Each treatment group consists of six to eight mice.

GPI-induced arthritis model

Male DBA/1 mice were immunized by injecting at the base of the tail 0.15 mL of emulsion containing 300 µg recombinant human GPI-glutathione-S-transferase fusion protein (hGPI) in CFA. ER-819762 was given orally daily at a dose of 10 or 30 mg·kg⁻¹ from day 6 after primary immunization but before disease onset (prophylactic evaluation) or after the disease induction (therapeutic evaluation). Each treatment group consisted of six to eight mice. Arthritic animals were clinically assessed by an arthritis scoring system as follow (Iwanami *et al.*, 2008): 0 = no evidence of inflammation, 1 = subtle

inflammation or localized oedema, 2 = easily identified swelling but localized to dorsal or ventral surface of paws, and score 3 = swelling on all aspects of paws. Serum samples were collected at the end of the study and analysed for cytokine levels by ELISA. To analyse popliteal lymph node cells, emulsified GPI was injected into the foot pad of DBA/I mice and ER-819762 was orally administered once daily at 30 mg·kg⁻¹ from the day of immunization. Lymph nodes were removed 6 days later, and cells were stimulated with 10 µg mL⁻¹ recombinant GPI and GolgiStop (BD) for 12 h (Iwanami *et al.*, 2008). IL-17- and IFN-γ-producing cells were analysed as described above.

CFA-induced hyperalgesia

Complete Freund's adjuvant, consisting of 100 µg of *Mycobacterium tuberculosis* H37 RA (Difco, Detroit, MI, USA) in 100 µL of liquid paraffin (Wako Pure Chemicals), was injected into the right hind footpad of 7-week-old male F344 rats. Three days after CFA injection, rats exhibited a lame walking reaction consisting of a three-legged gait. ER-819762 or indomethacin was orally administered at day 3 and the inhibitory effect on the lame walking reaction was evaluated, without knowledge of treatment, after 2–3 h of dosing (Higuchi *et al.*, 1986). Each treatment group consisted of seven rats.

Ex vivo lymph node studies

Male DBA/1 mice were immunized with bCII emulsified in CFA, as described above. ER-819762 was orally administered daily at a dose of 30 mg·kg⁻¹ from the day of immunization. Suspensions of single cells were prepared from draining lymph nodes from mice, 15 days after immunization. Cells were plated in a 96-well plate at 4 × 10⁵ cells per 200 µL per well in complete RPMI medium and stimulated with either bCII (50 µg mL⁻¹) or phosphate-buffered saline for 72 h. Cytokine production in culture supernatants was analysed by ELISA, and cell proliferation was measured by CellTiter-Glo.

Data analyses

All values shown in the text and figures are mean ± SD. Nonlinear regression analysis of the data and calculation of IC₅₀ values were performed using Prism 4 (GraphPad Software, San Diego, CA, USA). Statistical analysis was performed by Dunnett-type multiple comparison test or paired *t*-test; a value of *P* < 0.05 (two-sided) was considered statistically significant.

Materials

ER-819762 ((S)-1'-(3,5-dimethylbenzyl)-2-ethyl-7,9-dimethoxy -10-methyl-5,10-dihydrospiro [benzo[e]imidazo [1,5-a]azepine-1,4'-piperidin]-3(2H)-one) was synthesized at Eisai Research Institute of Boston Inc. Indomethacin was purchased from Cayman Chemicals. For *in vitro* testing, ER-819762 was dissolved in 100% dimethyl sulphoxide (DMSO) and the final concentration of DMSO in the assay was 0.1%. For *in vivo* evaluation, ER-819762 or indomethacin

was suspended in 0.5% (w/v) methylcellulose and orally administered at 10 mL·kg⁻¹ per mouse or 5 mL·kg⁻¹ per rat.

Mouse IL-2, IL-12, IL-23 and human GM-CSF were purchased from R&D Systems. Human IL-4 and GM-CSF were from Peprotec. Anti-CD3 (clone 145-2C11), anti-CD28 (clone 37.51), anti-IL-4 (clone 11B11), anti-IFN-γ (clone XMG12) and PE-anti-mouse IL-17 (clone TC11-18H10) were purchased from BD Pharmingen. Anti-TCR (clone H57-597) was purchased from eBioscience. OVA peptide, mitomycin C, H-89, KT5721, LY-294002 and isobutylmethylxanthine (IBMX) were purchased from Sigma. PGE₂, PGE₁-alcohol (PGE₁-OH), butaprost, forskolin and anti-PGE₂ IgG1 monoclonal antibody (clone 2B5) were purchased from Cayman Chemicals. LPS and R-848 were from InVivoGen. CD14⁺ cell isolation kits were from MiltenyiBiotec. CD4⁺ T cell isolation kits were from MiltenyiBiotec or StemCell Technologies. IFN-γ ELISA kits were from Pierce; IL-4 ELISA kits are from R&D Systems; IL-23 ELISA kits were from eBioscience. Intracellular IL-17 staining reagents were from BD Biosciences. Alamar Blue reagents were from Biosource International. CellTiter-Glo reagents were from Promega. CFA for mouse RA models was an emulsion form prepared by Difco (Michigan) and CFA for the lame walking model was desiccated powder from Difco, which we suspended in liquid paraffin (Wako Chemicals).

Results

Identification of selective EP₄ receptor antagonists

In the course of screening for drugs unrelated to the prostanoid receptors, we discovered a series of compounds that could suppress the expression of a stably transfected cytomegalovirus (CMV) promoter in HEK/293 cells. The CMV promoter is known to be modulated by cAMP signalling (Hunninghake *et al.*, 1989), and we found that these compounds inhibited the induction of CMV promoter activity by a factor present in FBS. The inducing factor in FBS was identified as PGE₂ and induction of cAMP was found to be mediated solely by the endogenous EP₄ receptor in HEK/293 cells (Supplementary Figs 1 and 2). A representative of this series of compounds, ER-819762 (structure shown in Fig. 1D), displaced PGE₂ binding to human EP₄ receptors (IC₅₀ value of 70 ± 11 nmol·L⁻¹; Fig. 1A), but did not displace ligand binding to several related human prostanoid GPCRs, including EP₂, DP, CRTH2 and TP receptors, and the leukotriene GPCRs LTB₄, CysLT₁ and CysLT₂ receptors (Fig. 1C). ER-819762 also suppressed human EP₄ receptor-mediated cell signalling as measured in a cAMP-dependent reporter assay (IC₅₀ value of 59 ± 6 nmol·L⁻¹) (Fig. 1B). In a larger cell signalling panel of 107 GPCRs, ER-819762 (1 µmol·L⁻¹) was highly selective for EP₄ receptors, exhibiting no agonism or antagonism for any other receptor, including the related PGE₂ EP₁, EP₂ and EP₃ receptors (Table 1).

EP₄ receptor antagonism suppresses *in vitro* Th1 differentiation

As PGE₂ has been reported to modulate T cell differentiation and function, we tested the effect of ER-819762 in Th1 and Th2 differentiation assays. Th1 differentiation was induced by activating naïve CD4⁺ T cells with anti-CD3 and anti-CD28 antibodies in 10% charcoal-stripped FBS in the presence of

Figure 1 Activity and structure of ER-819762. (A) Competitive displacement of radiolabelled prostaglandin E₂ (PGE₂) from cell membranes overexpressing EP₄ receptors (Millipore ChemoScreen). (B) Inhibition of PGE₂-induced cAMP response element-placental-like alkaline phosphatase reporter activity in human embryonic kidney cells, which express endogenous EP₄ receptors (Supplementary Figure 2). Data are representative of mean $\pm$ SD derived from three independent experiments. (C) Competitive displacement of radiolabelled ligands from cell membranes overexpressing various prostanoid and leukotriene receptors by 0.1 and 1 $\mu\text{mol}\cdot\text{L}^{-1}$ ER-819762 (data from MDS Pharma, Bothel, WA, USA). (D) Chemical structure of ER-819762: (S)-1'-((3,5-dimethylbenzyl)-2-ethyl-7,9-dimethoxy-10-methyl-5,10-dihydrospiro[benzo[e]imidazo[1,5-a]azepine-1,4'-piperidin]-3(2H)-one.

IL-2, IL-12 and anti-IL-4 antibody. Th2 differentiation was induced by IL-4 and anti-IFN- γ antibody. Addition of PGE₂, butaprost (an EP₂ receptor agonist) and prostaglandin E₁ alcohol (PGE₁-OH; an EP₃/EP₄ receptor agonist) significantly enhanced the differentiation of naïve CD4⁺ T cells into Th1 cells (Fig. 2A). ER-819762 suppressed PGE₂- and PGE₁-OH-induced IFN- γ production by Th1-differentiating cells in a concentration-dependent manner (Fig. 2B), but had no effect on cellular ATP levels (CellTiter-Glo, Promega), an indicator of cell metabolic activity. Figure 2B also shows that ER-819762 inhibited IFN- γ in the absence of added prostaglandins, suggesting that the PGE₂ produced by the T cells themselves acts in an autocrine manner to promote Th1 differentiation. ER-819762 had no effect on butaprost-stimulated IFN- γ production at up to 1 $\mu\text{mol}\cdot\text{L}^{-1}$ (Supplementary Fig. 3). Th1 and Th2 differentiation were also induced by co-culturing naïve CD4⁺ T cells isolated from DO11.10 mice with mitomycin C-treated splenocytes and activating with the OVA peptide under neutral, Th1- or Th2-polarizing conditions in normal 10% FBS as described in *Methods*. In this experiment, IFN- γ production was suppressed by either ER-819762 or a neutralizing monoclonal anti-PGE₂ antibody (clone 2B5; Fig. 2C), and these effects were non-additive. We also observed no effect of ER-819762 on Th2 differentiation at up to 10 $\mu\text{mol}\cdot\text{L}^{-1}$ (Fig. 2D).

Although Th1 differentiation was enhanced by PGE₂, as measured by increased IFN- γ production (Fig. 2), neither forskolin, an activator of adenylate cyclase, nor IBMX, a phosphodiesterase inhibitor, caused a statistically significant

enhancement in IFN- γ production (Fig. 3A), suggesting that the promotion of Th1 differentiation by PGE₂ was not due to cAMP signalling. Moreover, PGE₂-stimulated Th1 differentiation was not suppressed by the PKA inhibitors H-89 (1 $\mu\text{mol}\cdot\text{L}^{-1}$) or was only weakly suppressed by a structurally unrelated PKA inhibitor KT-5720 (10 $\mu\text{mol}\cdot\text{L}^{-1}$), but was strongly suppressed by the PI3K inhibitor LY294002 (2 $\mu\text{mol}\cdot\text{L}^{-1}$), as well as by ER-819762 (Fig. 3B). Higher concentrations of H-89 (10 $\mu\text{mol}\cdot\text{L}^{-1}$) were toxic (data not shown). These results suggest that the PI3K pathway, but not the PKA-cAMP signalling pathway functioning downstream of EP₄ receptors is primarily responsible for PGE₂-enhanced Th1 differentiation. Butaprost also induced Th1 differentiation (Fig. 2A), raising the possibility that EP₂ receptors may signal via PI3K in addition to PKA-cAMP.

EP₄ receptor antagonism suppresses IL-23 secretion in human monocyte-derived DCs

It was recently reported that PGE₂ can promote Th17 cell differentiation in mice by inducing DCs to preferentially produce IL-23 (Sheibanie *et al.*, 2007a; Khayrullina *et al.*, 2008). Similarly, receptors that mobilize cAMP have been reported to enhance IL-23 secretion by human DCs (Schnurr *et al.*, 2005). We therefore examined the role of EP₄ receptor signalling in immature human dendritic cells (imDCs). imDCs were generated from CD14⁺ monocytes by differentiation with GM-CSF plus IL-4 and assayed for IL-23 production in media containing charcoal-stripped FBS. IL-23 production

Table 1 Selectivity of ER-819762 against 107 G protein-coupled receptors (GPCRs)

GPCR Target	% inhibition by ER-819762 (1 $\mu\text{mol}\cdot\text{L}^{-1}$)	GPCR Target	% inhibition by ER-819762 (1 $\mu\text{mol}\cdot\text{L}^{-1}$)	GPCR Target	% inhibition by ER-819762 (1 $\mu\text{mol}\cdot\text{L}^{-1}$)
M1 (CHRM1)	1.1% $\pm$ 1.9%	CCK2 (CCKBR)	-8.1% $\pm$ 4.0%	NTR1	-3.7% $\pm$ 1.1%
M2 (CHRM2)	-10.5% $\pm$ 0.8%	CRF1 (CRHR1)	-16.2% $\pm$ 0.4%	FPR1	-6.6% $\pm$ 1.1%
M3 (CHRM3)	-3.2% $\pm$ 0.4%	CRF2 (CRHR2)	-9.5% $\pm$ 0.1%	FPR2/FPRL1	-7.3% $\pm$ 1.9%
M5 (CHRM5)	-4.7% $\pm$ 4.0%	D1	-8.1% $\pm$ 1.5%	NOP/ORL1	-11.2% $\pm$ 2.4%
A1 (ADORA1)	4.9% $\pm$ 6.0%	D2	0.6% $\pm$ 6.6%	δ (OPRD1)	-11.3% $\pm$ 1.0%
A3 (ADORA3)	-0.8% $\pm$ 1.4%	D5	25.2% $\pm$ 9.5%	κ (OPRK1)	8.3% $\pm$ 11.7%
A2A (ADORA2A)	13.4% $\pm$ 4.5%	ETA (EDNRA)	4.2% $\pm$ 2.4%	OX2 (HCRTR2)	-1.3% $\pm$ 0.3%
A2B (ADORA2B)	-1.4% $\pm$ 4.2%	GPR40	-3.1% $\pm$ 3.1%	PTH1 (PTHR1)	-4.7% $\pm$ 5.3%
α 1A (ADRA1A)	-2.2% $\pm$ 0.1%	GPR43	5.1% $\pm$ 2.5%	PAF (PTAFR)	1.6% $\pm$ 2.3%
α 2A (ADRA2A)	-13.2% $\pm$ 5.7%	GABBAb1	-9.7% $\pm$ 0.5%	PK1/GPR73	-0.4% $\pm$ 5.3%
β 1 (ADRB1)	-12.1% $\pm$ 3.9%	GAL1 (GALR1)	22.9% $\pm$ 1.9%	PRP/GPR10	6.7% $\pm$ 0.7%
β 2 (ADRB2)	-4.2% $\pm$ 3.4%	GCGR	-4.5% $\pm$ 2.0%	PTGDR (DP)	-13.7% $\pm$ 1.8%
C3aR	8.5% $\pm$ 0.3%	mGlu1	0.6% $\pm$ 8.2%	PTGER1 (EP1)	-4.6% $\pm$ 0.1%
C5aR	-10.2% $\pm$ 0.1%	GnRH	-12.3% $\pm$ 1.1%	PTGER2 (EP2)	(See legend)
ChemR23	3.5% $\pm$ 2.4%	H1 (HRH1)	-4.6% $\pm$ 1.0%	PTGER3 (EP3)	-2.4% $\pm$ 0.0%
AT1	-2.7% $\pm$ 1.7%	H2 (HRH2)	-4.8% $\pm$ 0.9%	PTGFR (FP)	0.1% $\pm$ 5.1%
BB2 (GRPR)	8.1% $\pm$ 0.3%	NK1 (TACR1)	-7.7% $\pm$ 6.2%	PTGER4 (EP4)	90.3% $\pm$ 0.7%
BB1	5.2% $\pm$ 4.6%	NK2	0.3% $\pm$ 2.6%	PTGIR (IP)	(See legend)
B2 (BDKRB2)	5.7% $\pm$ 1.3%	H3 (HRH3)	2.9% $\pm$ 4.1%	TBXAR2 (TP)	-9.8% $\pm$ 5.1%
AMY1	12.0% $\pm$ 0.9%	GPR54	-11.2% $\pm$ 4.4%	PAR1	-5.1% $\pm$ 9.3%
CGRP1	-7.6% $\pm$ 8.0%	BLT1 (LTB4R1)	-3.3% $\pm$ 2.9%	PAR2	-6.1% $\pm$ 8.0%
CaS	-12.6% $\pm$ 0.2%	CysLT1	-4.4% $\pm$ 4.0%	5-HT1A (HTR1A)	0.9% $\pm$ 4.5%
CB1	0.0% $\pm$ 2.5%	CysLT2	-0.7% $\pm$ 3.9%	5-HT1B	6.9% $\pm$ 15.6%
CXCR1	-5.1% $\pm$ 3.0%	S1P2 (EDG5)	-16.2% $\pm$ 0.9%	5-HT2B (HTR2B)	2.2% $\pm$ 2.9%
CXCR2	-1.7% $\pm$ 2.5%	S1P3 (EDG3)	-5.9% $\pm$ 0.6%	5-HT2C (HTR2C)	11.4% $\pm$ 3.8%
CXCR3	-14.6% $\pm$ 7.8%	LPA1 (EDG2)	-11.9% $\pm$ 5.9%	SST3	-8.9% $\pm$ 11.3%
CXCR4	-6.1% $\pm$ 2.4%	LPA3 (EDG7)	-0.2% $\pm$ 1.0%	SST4	-12.4% $\pm$ 1.8%
CCR1	-3.5% $\pm$ 1.2%	MRGX2	-8.6% $\pm$ 1.3%	GPR68(OGR1)	0.0% $\pm$ 0.0%
CCR2b	-6.7% $\pm$ 4.7%	MCHR1	-0.2% $\pm$ 0.5%	TRH	-6.9% $\pm$ 2.9%
CCR4	-10.6% $\pm$ 5.8%	MCHR2	-10.2% $\pm$ 4.0%	V1A	-0.6% $\pm$ 1.0%
CCR6	-7.5% $\pm$ 4.5%	MC4R	-3.6% $\pm$ 10.5%	V1B (AVPR1B)	-5.0% $\pm$ 1.5%
CCR7	9.6% $\pm$ 5.5%	Motilin	-12.6% $\pm$ 3.3%	V2 (AVPR2)	-17.8% $\pm$ 0.2%
CCR8	-13.2% $\pm$ 16.2%	NMUR1	-12.2% $\pm$ 4.6%	OT (OXTR)	6.5% $\pm$ 9.0%
CCR9	-15.1% $\pm$ 0.4%	Y1 (NPY1R)	15.8% $\pm$ 7.0%	PAC1 long	-7.5% $\pm$ 2.3%
CCR10	6.0% $\pm$ 2.5%	Y2 (NPY2R)	-17.7% $\pm$ 1.9%	VPAC1 (VIPR1)	-10.5% $\pm$ 1.0%
CRTH2	-2.8% $\pm$ 3.5%			VPAC2 (VIPR2)	-12.0% $\pm$ 1.9%

ER-819762 was assayed by Millipore GPCR Profiler Service, which monitors calcium flux in cells expressing the specific GPCR and promiscuous GPCR-coupling $G\alpha_{15/16}$ proteins. Both agonism and antagonism of the GPCRs listed above were examined, except for EP₂ and IP receptors. No significant agonism (>15%) was found for any GPCR (data not shown), and only antagonism of EP₄ receptors was found. ER-819762 was not able to displace binding of radiolabelled PGE₂ to EP₂ receptors (Fig. 1C, MDS Pharma Profiling Service). We also used the human U2-OS cell line, which endogenously expresses EP₂ and IP but not EP₄ receptors, to assay agonism and antagonism by ER-819762 using the Corning EPIC Resonate Waveguide Biosensor system. We observed no antagonism of butaprost- (EP₂ receptors) or iloprost- (IP receptors) induced signalling by 10 μM ER-819762, and no signalling induced by compound alone in this cell line (data not shown). Bold texts indicate positive results.

could be induced in imDCs by stimulation with LPS, a ligand for the TLR4, and co-stimulation with the TLR7 ligand R-848 (Fig. 4A), but not by LPS or R-848 alone (data not shown). We found that the EP_{3/4} receptor agonist PGE₁-OH enhanced LPS/R-848-induced IL-23 production in imDCs and that this response was antagonized by ER-819762 (Fig. 4A). These experiments were performed using charcoal-stripped 10% FBS, yet we observed that IL-23 production was partially suppressed by either ER-819762 (Fig. 4A) or by anti-PGE₂ antibody (Fig. 4B) in the absence of added prostaglandins. This suggests that IL-23 production in these activated DCs involves endogenously produced PGE₂.

EP₄ receptors antagonism suppresses IL-17 production and inhibits IL-23-induced Th17 expansion in activated CD4⁺ T cells in vitro

We next determined whether EP₄ receptor stimulation might influence the development or function of Th17 cells, which

play a critical role in inflammation and autoimmune diseases (Fouser *et al.*, 2008). First we tested the effect of EP₄ receptor stimulation on IL-17 production, a typical cytokine of Th17 cells, in activated CD4⁺ T cells. Total CD4⁺ T cells were isolated from mouse splenocytes, which include both naïve and memory T cells, and pre-existing Th17 cells were stimulated for 3–5 days using IL-23 and either anti-TCR β or anti-CD3/anti-CD28 antibodies. Addition of PGE₂, butaprost or PGE₁-OH (Fig. 5A) suppressed overall T cell proliferation, yet enhanced IL-17 production. Both of these PGE₂-induced effects were reversed by 0.1 or 1.0 $\mu\text{mol}\cdot\text{L}^{-1}$ ER-819762 (Fig. 5B). Further analysis by flow cytometry showed that PGE₂ stimulation increased the percentage of IL-17-producing cells within this population (Fig. 5C), and that this increase was suppressed by ER-819762 (data not shown). ER-819762 (Fig. 5D) or anti-PGE₂ antibody (Supplementary Fig. 4) could also suppress IL-23-induced Th17 expansion in the absence of exogenously added PGE₂. However, some PGE₂ could be present in the media, because we used normal 10% FBS and

Figure 2 EP₄ receptor antagonism selectively suppresses prostaglandin E₂ (PGE₂)-induced Th1 differentiation *in vitro*. (A) Naive CD4⁺ T cells from BALB/c mice were stimulated with α -CD3/ α -CD28 under Th1-promoting conditions (see *Methods*) in media supplemented with 10% charcoal-stripped fetal bovine serum (FBS), together with PGE₂, butaprost, or PGE₁-OH at the indicated concentrations. After 3 days, interferon (IFN)- γ production was analysed by enzyme-linked immunosorbent assay (ELISA) and cell proliferation/viability was monitored by AlamarBlue. (B) was the same as (A), except that cells were cultured in the presence or absence of the indicated concentrations of PGE₂, PGE₁-OH and/or ER-819762. (C) Naive CD4⁺ T cells isolated from DO11.10 mice were stimulated with ovalbumin (OVA)/antigen-presenting cells (APCs) under Th1-promoting conditions in 10% normal FBS in the presence or absence of ER-819762 and/or anti-PGE₂ for 3 days. IFN- γ production in culture supernatants was analysed by ELISA and cell proliferation/viability was monitored by AlamarBlue assay. (D) Naive CD4⁺ T cells isolated from DO11.10 mice were stimulated with OVA/APCs under Th2-promoting conditions in normal 10% FBS in the presence or absence of ER-819762 for 6 days. Cells were collected at day 6 and re-stimulated with α -CD3 overnight. Cytokine [interleukin (IL)-4, left; IL-10, right] production in culture supernatants was analysed by ELISA. All data are shown as means $\pm$ SD ($n = 3$). Statistical analysis was performed by Dunnett-type multiple comparison test. *, $\wedge$ indicate $P < 0.05$; **, $\wedge\wedge$ indicate $P < 0.01$ and ***, $\wedge\wedge\wedge$ indicate $P < 0.001$ levels of significance. *, **, *** induction compared with Th1, vehicle, no ER-819762 control. $\wedge$, $\wedge\wedge$, $\wedge\wedge\wedge$ inhibition compared with untreated controls within each group. These data are representative of at least two independent experiments.

not charcoal-stripped FBS for this experiment. These results suggest that PGE₂ signalling via EP₄ receptors results in an increase in IL-17 production and/or in the fraction of IL-17-producing cells in the population while suppressing non-Th17 cell proliferation. These results also indicate that PGE₂ produced by the T cells themselves and/or present in serum is involved in IL-17 production or/and Th17 cell expansion. Stimulation of these cells in the presence of PGE₂ or PGE₁-OH enhanced expression of IL-23 receptors, the retinoic acid receptor-related orphan receptor γ -T and IL-17A mRNA (Supplementary Fig. 5A). PGE₂-induced up-regulation of IL-23 receptors and IL-17A mRNA was suppressed by ER-819762 or anti-PGE₂ antibodies (Supplementary Fig. 5B and data not

shown). IL-17 production and the percentage of IL-17-producing cells were also increased by other cAMP-inducing agents including butaprost or forskolin (Fig. 5A and C), suggesting a possible role for cAMP signalling in this process. No significant enhancement of PGE₂ on Th17 cell differentiation was observed when naïve CD4⁺ T cells were activated in the presence of transforming growth factor (TGF)- β + IL-6, and no inhibitory effect of ER-819762 was observed in this system (data not shown).

EP₄ receptor antagonist suppresses inflammatory arthritis

As Th1 and Th17 cells have been implicated in the pathogenesis of autoimmune diseases such as RA (Schulze-Koops and

Figure 2 Continued.

Kalden, 2001; Fouser *et al.*, 2008), we tested whether the selective EP₄ receptor antagonist ER-819762 might influence disease in two mouse models of RA. We first tested the effect of ER-819762 in the murine CIA model, an animal model widely used for assessing therapeutic agents for treatment of RA. When given orally prior to the onset of disease, ER-819762 dose-dependently suppressed the clinical signs of arthritis and delayed disease onset (Fig. 6A). ER-819762 also significantly suppressed disease progression when it was administered subsequent to the onset of disease (Fig. 6B). Furthermore, ER-819762 retarded bone erosion in the CIA model, as demonstrated by radiological evaluation (Fig. 6C).

Matsumoto *et al.* (2008) have suggested that the GPI-induced arthritis model is more predictive of clinical efficacy than the CIA model, as the therapeutic effects of anti-tumour necrosis factor (TNF), anti-IL-6 and CTLA-4/Ig fusion in the GPI-induced arthritis model are similar to those seen in human patients treated with the equivalent biological agents. In the GPI-induced arthritis model in DBA/1 mice, oral administration of ER-819762 significantly reduced arthritis severity and delayed disease onset when administered prior to the onset of disease (Fig. 7A). ER-819762 also significantly suppressed disease progression when administered after the establishment of arthritis (Fig. 7B). Collectively, these results demonstrate that ER-819762 not only prevents the develop-

ment of inflammatory arthritis, but is effective against the established disease by reducing both inflammation and joint destruction in animal models of RA.

Suppression of Th1 and Th17 responses in vivo

To determine whether suppression of arthritis by ER-819762 is associated with reduced Th1 and Th17 responses, we performed *ex vivo* challenge of cells obtained from mice with CIA. Cultured lymph node cells obtained from CIA mice were challenged *ex vivo* with collagen and cytokine production was determined. Cells from mice that had been treated with ER-819762 had significantly lower production of IFN- γ (>90%), IL-17 (>80%), TNF- α (>60%), IL-6 (>70%), osteopontin (>55%) and the chemokine CCL3 (MIP-1 α ; >35%) compared with lymph node cells from vehicle-treated mice (Fig. 6D). Overall lymph node cell proliferation was suppressed in ER-819762-treated mice to levels similar to those in non-immunized mice (data not shown).

We next examined the frequency of Th1 and Th17 cells found in the lymph nodes of GPI-induced arthritic mice. Popliteal lymph nodes were obtained from naïve, vehicle- or ER-819762-treated animals at 6 days post immunization and analysed by intracellular IFN- γ and IL-17 staining. The percentage of IL-17- and IFN- γ -producing CD4⁺ T cells was

Figure 3 Enhancement of Th1 differentiation by EP₄ receptor stimulation requires phosphatidylinositol 3-kinase (PI3K) signalling. (A) The effect of cyclic AMP-inducing agents on Th1 differentiation was examined using the same methods as in Figure 2A. Cell proliferation/viability was monitored with CellTiter-Glo. (B) The effect of PKA and PI3K inhibitors on Th1 differentiation was examined as in (A). Inhibitor concentrations were as follows: 1 μmol·L⁻¹ H-89, 10 μmol·L⁻¹ KT-5720, 2 μmol·L⁻¹ LY-294002 and 1 μmol·L⁻¹ ER-819762. All data are shown as means ± SD (*n* = 3). Statistical analysis was performed by Dunnett-type multiple comparison test. *, ^ indicate *P* < 0.05; **, ^^ indicate *P* < 0.01 and ***, ^^ indicate *P* < 0.001 levels of significance. *, **, *** induction compared with Th1, vehicle, no ER-819762 control. ^, ^^, ^^ inhibition compared with untreated controls within each group. These data are representative of at least two independent experiments.

significantly lower in ER-819762-treated mice compared with vehicle-treated controls (Fig. 7C). The serum levels of IL-17 and IFN-γ were also significantly decreased in ER-819762-treated animals (Fig. 7D). Collectively these results indicate that EP₄ receptor-mediated PGE₂ signalling is important *in vivo* for the Th1/Th17 response and the development of inflammatory arthritis.

Suppression of CFA-induced hyperalgesia in rat

PGE₂ has been reported to be a key mediator of peripheral inflammatory pain, and evidence from studies using EP₄ receptor specific antagonists and small inhibitory RNAs impli-

cate EP₄ receptors in this process (Lin *et al.*, 2006; Nakao *et al.*, 2007; Clark *et al.*, 2008). We therefore examined if ER-819762 might modulate the inflammatory pain response in a CFA-induced chronic inflammatory pain model in the rat. In this model, rats develop a lame walk reaction, characterized by a three-legged gait, 3 days after CFA injection. In the vehicle control group, 7 of 7 rats exhibited a positive lame walk reaction, while in the group treated with 100 mg·kg⁻¹ ER-819762, 0 of 7 rats exhibited a positive lame walk reaction (Fig. 8). As a positive control, rats were also treated with the cyclooxygenase (COX) inhibitor, indomethacin and we observed significant suppression of lame walking at doses of 1 and 3 mg·kg⁻¹ (Fig. 8).

Figure 4 EP₄ receptor antagonism suppresses prostaglandin E₂ (PGE₂)-induced interleukin (IL)-23 production in human dendritic cells (DCs). (A) Human immature DCs were stimulated with 10 ng mL⁻¹ lipopolysaccharide (LPS) and 2.5 µg mL⁻¹ R-848 in the presence or absence of the indicated concentrations of PGE₁-OH and/or ER-819762 for 24 h. IL-23 in culture supernatants was measured by enzyme-linked immunosorbent assay and cell proliferation/viability was monitored with CellTiter-Glo. (B) Same as (A) but in the presence or absence of 2 µg mL⁻¹ anti-PGE₂ antibody. All data are shown in means $\pm$ SD ($n = 3$). Statistical analysis was performed by Dunnett-type multiple comparison test: *, ^ indicate $P < 0.05$; **, ^^ indicate $P < 0.01$ and ***, ^^ indicate $P < 0.001$ levels of significance. *, **, *** induction compared with LPS/R-848-stimulated, no-PGE₁-OH, no-ER-819762 control, ^, ^^, ^^ inhibition compared with no-ER-819762 controls within each group. These data are representative of at least two independent experiments.

Discussion

In the present study, we describe the pharmacological actions of a novel and highly selective antagonist of the EP₄ receptor, ER-819762, in models of inflammation. We show that antagonism of EP₄ receptor activation can suppress Th1 differentiation, production of IL-23 in DCs, and Th17 cell expansion *in vitro*. In addition, when tested in two mouse models of RA, ER-819762 was very effective in suppressing disease symptoms *in vivo*. A significant body of research has linked Th1 and Th17 cell development and function to autoimmune disease (Schulze-Koops and Kalden, 2001; Fouser *et al.*, 2008), and we observe in the mouse RA disease models (CIA and G6PI) that treatment with ER-819762 suppresses the ability of lymph node T cells to produce IFN- γ and IL-17 *ex vivo* in response to stimulation. We also observed reduced levels of IFN- γ and IL-17 in the serum of ER-819762-treated versus control mice

in the G6PI model (Fig. 7D). However, although the suppressive effects of ER-819762 observed *in vitro* and *in vivo* are consistent, we cannot directly attribute suppression of disease in the animal models to inhibition of Th1 or Th17 development or function *in vivo*. It is possible that suppression of EP₄ receptor signalling has other unknown pharmacological effects in these models. Nevertheless, these *in vitro* and *in vivo* results show that antagonism of EP₄ receptors can suppress a broad range of pro-inflammatory responses relevant to the development of autoimmunity.

These results were initially unexpected, as earlier studies had demonstrated that PGE₂ suppresses T cell-mediated inflammation by increasing intracellular cAMP, inhibiting Th1 cytokine IFN- γ production, and inhibiting T cell activation and proliferation (Betz and Fox, 1991; Gold *et al.*, 1994; Hilkens *et al.*, 1995; Okano *et al.*, 2006). However, more recent reports have demonstrated the pro-inflammatory effects of

Figure 5 Prostaglandin E₂ (PGE₂)-EP₄ receptor signalling regulates Th17 cell development. (A) Total CD4⁺ T cells isolated from mouse splenocytes were stimulated with anti-CD3/anti-CD28 plus interleukin (IL)-23 in the presence or absence of exogenous PGE₂, butaprost, PGE₁-OH, or forskolin for 3 days. IL-17 in culture supernatants was measured by enzyme-linked immunosorbent assay. (B) Same methods as in (A), except that ER-819762 was added at the indicated concentrations. (C) Total CD4⁺ T cells were stimulated with α-TCRβ/α-CD28 ± IL-23 in the presence or absence of exogenously added PGE₂, butaprost, PGE₁-OH, or forskolin for 5 days and the percentage of Th17 cells was analysed by IL-17 intracellular staining. The horizontal broken line represents the level of IL-17 positive cells in the presence of IL-23 only. (D) Same methods as in (C), except that no PGs were added, and ER-819762 was added at the indicated concentrations. The number of Th17 cells was analysed by IL-17 intracellular staining. Upper plots show staining with control isotype-matched staining antibody, bottom plots show staining with anti-IL-17 antibody. First two columns show unstimulated and IL-23-stimulated cells. Right-hand lower two plots show IL-23-stimulated cells treated with different concentrations of ER-819762. All data are shown in means ± SD (*n* = 3). Statistical analysis was performed by Dunnett-type multiple comparison test: *, ^ indicate *P* < 0.05; **, ^^ indicate *P* < 0.01 and ***, ^^ indicate *P* < 0.001 levels of significance. *, **, *** induction compared with lipopolysaccharide/R-848-stimulated, no-PGE₁-OH, no-ER-819762 control, ^, ^^, ^^ inhibition compared with no-ER-819762 controls within each group. These data are representative of at least two independent experiments.

PGE₂ in Th17 development (Chizzolini *et al.*, 2008; Boniface *et al.*, 2009; Napolitani *et al.*, 2009) and DC activation (Sheibanie *et al.*, 2004; Khayrullina *et al.*, 2008). As antagonism of EP₄ receptor signalling suppressed Th1 differentiation, Th17 cell expansion, and the development of pathologies in mouse CIA- and GPI-induced arthritis, we propose that the immune stimulatory activities of PGE₂ are relevant to these diseases.

Another debilitating aspect of RA is the pain associated with joint inflammation. This inflammatory pain is mediated, at

least in part, by PGE₂ stimulation of EP₄ receptors (Lin *et al.*, 2006; Nakao *et al.*, 2007). Selective inhibition of EP₄ receptor signalling by several different EP₄ receptor antagonists has been shown to cause a marked reduction in joint pain, mechanical and thermal hyperalgesia and oedema in rat and in guinea pig models of pain and inflammation, often with similar efficacy to that observed with selective COX-2 inhibitors such as rofecoxib (Lin *et al.*, 2006; Nakao *et al.*, 2007; Clark *et al.*, 2008; Murase *et al.*, 2008; Jones *et al.*, 2009). Consistent with these findings, we observed that ER-819762 was

Figure 5 Continued.

effective in relieving inflammatory pain in a rat model of inflammatory pain induced by CFA injection into the paws. The analgesic effect of ER-819762 could be associated with reduced peripheral sensitization by suppression of PGE₂-mediated action on the peripheral terminals of nociceptor sensory neurons (Lin *et al.*, 2006). Alternatively, inhibition of IFN- γ and TNF- α by ER-819762 can also have an analgesic effect, because these cytokines have been shown to induce hypernociception (Verri *et al.*, 2006). Thus, an EP₄ receptor antagonist may have multiple benefits in relieving both the symptoms and modifying the disease mechanisms leading to RA.

EP₄ receptors have been reported to signal by at least two pathways (Regan, 2003): (i) activation of adenylate cyclase via the G_s protein to increase cAMP, and (ii) activation of PI3K via a G protein-independent signalling process. The suppression of T-cell activation by PGE₂ and other cAMP-elevating agents was proposed to be mediated by the activation of PKA, activation of C-terminal src kinase (Csk) and repression of

leukocyte-specific protein tyrosine kinase (Lck)-dependent signalling through the T cell receptor (Mustelin and Tasken, 2003; Chemnitz *et al.*, 2006). In this study, however, we show that PGE₂ utilizes the PI3K pathway to promote Th1 differentiation (Fig. 3). Our data also suggest that the cAMP signalling pathway may promote Th17 expansion (Fig. 5), although our results are not definitive.

Recently, Chizzolini *et al.* (2008), Boniface *et al.* (2009) and Napolitani *et al.* (2009) have reported that PGE₂ can enhance the expansion and/or production of Th17 cells via cAMP signalling, and that this is accompanied by enhanced expression of IL-23R, IL-1R1, ROR γ t, the chemokine CCL20 and its receptor CCR6. Boniface *et al.* (2009) suggested that EP₂ receptors may be more important than EP₄ receptors for Th17 cell development and/or expansion, at least in human cells. We also observed enhanced IL-17 production and modest expansion of Th17 cells by incubation with the EP₂ receptor-selective agonist butaprost, but our data show that antagonism of EP₄ receptors is sufficient to suppress

Figure 6 EP₄ receptor antagonism suppresses disease and Th1/Th17 cytokines in collagen-induced arthritis in mice. (A) DBA/1 mice were immunized with bovine type II collagen (bCII)/complete Freund's adjuvant (CFA) (primary immunization) and boosted with bCII in incomplete Freund's adjuvant at day 21 (second immunization) to induce arthritis as described in *Methods*. ER-819762 was orally administered daily from day 20 after primary immunization but before the onset of disease, and arthritis scores were monitored over the course of the study as described in *Methods*. (B) Same methods as in (A), but ER-819762 was administered after induction of disease on day 7 after second immunization. (C) Radiological analysis of inflamed paws at the end of the therapeutic collagen-induced arthritis study shown in 6B. The X-ray score is defined in *Methods*. (D) *Ex vivo* cytokine analysis. Mice were immunized with bCII/CFA or vehicle, similar to (A), except that ER-819762 was administered from the day of primary immunization (day 0). Lymph node cells were purified at day 15 and cultured in the presence of bCII (50 µg mL⁻¹) or phosphate-buffered saline for 72 h, and cytokine production was analysed. Statistical analysis was performed by Dunnett-type multiple comparison test compared with vehicle control (A–C) or paired *t*-test (D). Levels of significance: *P < 0.05; **P < 0.01; ***P < 0.001. These data are representative of at least two independent experiments.

PGE₂-mediated Th17 expansion and IL-17 production in mouse cells. Our data also showed that PGE₂ did not promote Th17 differentiation *per se*, as we did not see an increase in Th17 cell frequency following PGE₂ stimulation of purified naïve CD4⁺ T cells in the presence of TGF-β and IL-6. Rather, we observed an increase in Th17 cells when total CD4⁺ T cells were stimulated with PGE₂ in the presence of IL-23, indicating the expansion of pre-differentiated Th17 cells. Napolitani *et al.* (2009) suggested that PGE₂ acts by inhibiting expansion of CCR6⁺ T cells rather than increasing the proliferation of CCR6⁺ Th17 cells, independent of IL-23. In agreement with this report, we also observed enhanced IL-17 production by PGE₂ in the absence of IL-23 co-stimulation (data not shown). In addition, we showed that EP₄ receptor stimulation can enhance IL-23 production by activated human DCs and that this activity can be inhibited by a selective EP₄ receptor antagonist or anti-PGE₂ antibody in the presence or absence of exogenously added PGE₂. Sheibanie *et al.* (2007a) have also

recently reported that PGE₂ exacerbates disease in the CIA mouse by enhancing DC IL-6 and IL-23 production, the latter of which maintains Th17 cell survival and proliferation and consequently promotes IL-17 production. Collectively, these results support the idea that PGE₂ stimulation of EP₄ receptors promotes Th17 cell expansion at two stages by: (i) enhancing IL-23 production by DCs, and (ii) directly acting on memory T cells to promote IL-17 production and Th17 cell expansion (Sheibanie *et al.*, 2004; Chizzolini *et al.*, 2008; Khayrullina *et al.*, 2008; Boniface *et al.*, 2009).

We observed that while low concentrations of PGE₂ promoted IFN-γ production under Th1-differentiation conditions, production started to decrease at higher concentrations of PGE₂ or PGE₁-OH without loss of cell viability (Figs 2A and 3A). Similar results were seen with higher concentrations of butaprost (data not shown). Thus, PGE₂ appears to have a bimodal effect on immune stimulation; promoting inflammation at lower concentrations while attenuating inflammation

Figure 6 Continued.

at higher concentrations, possibly in concert with other factors that contribute to PI3K and/or cAMP signalling. This bimodal action may explain why PGE₂ exerts a pro-inflammatory effect in some systems and anti-inflammatory in others. For example, Betz and Fox (1991) have reported that PGE₂ can inhibit the production of Th1 cytokines, which is contrary to our results; however, these researchers used micromolar concentrations of PGE₂ for many of their experiments. There are also a number of potential sources of PGE₂ in cell culture systems that could contribute to higher PGE₂ levels. For example, our data suggest that autocrine production of PGE₂ can significantly contribute to Th1 differentiation, Th17 expansion and IL-23 production by DCs. We also observed that normal FBS, but not charcoal-stripped or PGE₂-immunodepleted serum stimulated EP₄ receptors in HEK/293

cells (Supplementary Fig. 1). Thus, there may be significant basal stimulation of EP₂ and EP₄ receptors in many cell culture systems, in which case further addition of exogenous PGE₂ could reduce inflammation.

COX inhibitors have also shown some efficacy in animal models of RA (Ochi *et al.*, 2003). Our results suggest that among the downstream effectors of the COX pathway, EP₄ receptors may play a particularly important role in the pathology of RA. In our own experiments, we saw only limited efficacy of the COX inhibitor indomethacin in suppressing arthritis in the mouse CIA model, and higher dosing was limited by toxicity (data not shown). Thus, a selective antagonist(s) of one or more critical downstream prostaglandin receptors may be more effective than broad inhibition of COX activity. Prostaglandins play a variety of

Figure 7 EP₄ receptor antagonism suppresses disease and Th1/Th17 cytokines in glucose-6-phosphate isomerase (GPI)-induced arthritis in mice. (A) DBA/1 mice were immunized with GPI/complete Freund's adjuvant to induce arthritis as described in *Methods*. ER-819762 was orally administered daily from day 6 after immunization, but before the onset of disease. Clinical scores were monitored over the course of the study. (B) Same methods as in (A), but ER-819762 was administered after disease induction (day 9). (C) Same methods as in (A), but ER-819762 was administered from the day of immunization. Popliteal lymph node cells were removed from mice at day 6 and re-stimulated with GPI in culture. interleukin (IL)-17- and interferon (IFN)- γ -producing cells were quantified by intracellular staining and flow cytometry. Experiments with isotype control IgG are shown as cIgG. (D) Serum was collected at the end of the GPI study shown in (A), and analysed by IL-17 and IFN- γ enzyme-linked immunosorbent assay. Statistical analysis was performed by Dunnett-type multiple comparison test compared with vehicle control (A and B) or paired *t*-test (C and D). Levels of significance: *P < 0.05; **P < 0.01; ***P < 0.001. These data are representative of at least two independent experiments.

roles in modulating inflammation and can exert both anti- and pro-inflammatory effects. For example, one proposed explanation for why aspirin and other COX inhibitors are ineffective in treating allergic inflammation is that PGD₂ produced downstream of the COX enzymes stimulates the DP receptor, which promotes allergic inflammation, while PGE₂ stimulates the EP₃ receptor, which suppresses allergic inflammation (Kunikata *et al.*, 2005). In addition, the more targeted approach of antagonizing EP₄ receptors might suppress inflammation without the side-effects associated with some non-steroidal anti-inflammatory drugs and COX inhibitors, including increased gastrointestinal and

cardiovascular risks. Consistent with this, Takeuchi *et al.* (2007) showed that the EP₄ receptor antagonist CJ-042794 did not produce any damaging effects in the gastrointestinal mucosa of control or adjuvant-induced arthritic rats, whereas indomethacin caused gross lesions. More importantly, we found that ER-819762 not only could prevent, but could suppress established disease in the CIA and GPI-induced arthritis models. Bone destruction in CIA was also significantly reduced by ER-819762. The effects of ER-819762 in suppressing bone destruction may be due in part to suppression of osteoclastogenesis promoted by IL-17 and PGE₂. IL-17 stimulates osteoblasts to synthesize PGE₂

Figure 7 Continued.

Figure 8 The analgesic effect of ER-819762 on the lame walk response in complete Freund's adjuvant (CFA) injected rats. CFA was injected into the right hind footpad of each rat (seven rats per treatment group). Three days after CFA injection, the rats exhibited a lame walking reaction as described in *Methods*. Compounds were given orally 3 days after CFA and the lame reaction was evaluated at 3 h after drug administration. These data are representative of at least two independent experiments.

and express receptor activator of NF- κ B (RANK), which induces osteoclastogenesis (Kuligowska and Odrowaz-Sypniewska, 2004). We have observed that RANK-ligand mRNA levels in arthritic joints were lower in mice treated with an ER-819762 analogue in both the CIA and GPI-induced arthritis mouse models (unpublished results). Previous studies have also reported that anti-TNF- α therapy was

effective in the GPI-induced arthritis model (Matsumoto *et al.*, 2008), but had little effect treating disease in the CIA model (Joosten *et al.*, 1996; Williams *et al.*, 2000). In contrast, ER-819762 was effective in both models, suggesting that an EP₄ receptor antagonist strategy may be beneficial to RA patients, including those who are insensitive to anti-TNF therapy.

Figure 9 Multiple effects of ER-819762 on pro-inflammatory responses. Blue lines indicate the multiple steps at which ER-819762 was observed to exert an immunosuppressive effect in our studies. During infection or under conditions of chronic autoimmune inflammation, exogenous pathogen-associated molecular pattern stimuli (PAMPs) and/or endogenous danger-associated molecular pattern stimuli (DAMPs) drive immune cell activation in conjunction with antigen. In the case of Toll-like receptors, this signalling synergizes with the prostaglandin E₂ (PGE₂)-activated EP₄ receptor signalling pathway to enhance IL-23 production by dendritic cells (DCs). EP₄ receptor signalling in naive T cells promotes their differentiation into Th1 effector cells via the phosphatidylinositol 3-kinase (PI3K) pathway, whereas EP₄ receptor signalling promotes the expansion of Th17 effector cells via the cyclic AMP pathway. ER-819762 blocks EP₄ receptor-enhanced Th1 differentiation and suppresses Th17 function both indirectly, by reducing DC IL-23 production and, as a consequence, Th17 survival, and directly by suppressing EP₄ receptor-enhanced Th17 expansion and/or IL-17 production. However, it is unknown if these actions of the EP₄ receptor antagonist can completely account for suppression of disease in the animal models, and other mechanisms are possible in addition.

These results and methodologies have been shared earlier with colleagues in another laboratory, and they have recently confirmed that PGE₂-EP₄ receptor signalling promotes Th1 cell differentiation, IL-23 production by DCs and Th17 cell expansion (Yao *et al.*, 2009). This group also tested an EP₄ receptor antagonist with a very different molecular structure from ER-819762, supporting the idea that the anti-inflammatory effects of ER-819762 are indeed due to EP₄ receptor antagonism and not due to action on another, unidentified target of the compound.

In summary, we show that an antagonist of EP₄ receptors, ER-819762, can suppress inflammation at multiple stages, as summarized in Fig. 9, as well as moderating inflammatory pain. Our results suggest that selective antagonism of EP₄ receptors could have therapeutic benefit in modifying both the underlying pathology of RA and alleviating pain, thus providing potential total management for RA patients.

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Conflict of Interest

All authors were employed by Eisai Inc. (USA) or Eisai Co., Ltd. (Japan) at the time of these studies. The authors have no further conflicting financial interests.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Modulation of CMV promoter activity by ER-819762. Our initial drug screen utilized the cytomegalovirus immediate-early (CMV) promoter driving expression of a protein unrelated to the prostanoïd receptors in HEK/293 cells. After we observed down-regulation of protein expression by ER-819762, we examined activity of the CMV promoter by stably transfecting a plasmid containing the Renilla luciferase reporter driven by the CMV promoter (pRL-CMV; Promega) into HEK/293 cells. Cells were cultured overnight in DMEM media supplemented with 0.1% fatty-acid free bovine serum albumin (Sigma A0281), and Renilla luciferase activity was assayed the next day (Promega Renilla Luciferase assay kit). Addition of 10% fetal bovine serum (FBS) to the cultures up-regulated CMV activity, and this induction was suppressed by 1 $\mu\text{mol}\cdot\text{L}^{-1}$ ER-819762. Pre-treatment of FBS by incubation with activated charcoal (CSFBS), which removes a variety of lipids, abolished induction of the CMV promoter. Activity could be restored by addition of prostaglandin E₂ (PGE₂) to CS-FBS, and this activity was inhibited by ER-819762. Addition of the cAMP-inducing agent forskolin (FSK) could also induce CMV activity, but this induction was not suppressed by ER-819762. FBS that had been immunodepleted using anti-PGE₂ antibodies ($\Delta\text{P-FBS}$; Cayman Chemicals, clone 2B5) was not able to induce CMV activity, indicating that the CMV-inducing activity present in FBS is PGE₂ or a PGE₂-related molecule.

Figure S2 PGE₂ induction of cAMP signalling in HEK/293 cells is mediated by EP₄. HEK/293 cells were stably transfected with a vector containing response elements for the CREB transcription factor driving expression of a secreted alkaline phosphatase reporter (CRE-PLAP). This reporter construct can be up-regulated by stimuli that induce intracellular cAMP, as shown here for forskolin. We also stimulated these cells with

PGE₂ (EP₁, ₂, ₃, and ₄ agonist), butaprost (EP₂ agonist) or PGE₁-OH (EP₃ and ₄ agonist). We observed induction of PLAP activity in response to forskolin, PGE₂ or PGE₁-OH, but not to butaprost. ER-819762 could suppress induction by PGE₂ or PGE₁-OH (data not shown). We also saw no induction of CRE-PLAP by up to 100 $\text{nmol}\cdot\text{L}^{-1}$ sulprostone, an agonist of EP₃ and EP₁ (data not shown). These data indicate that of the four PGE₂ receptors, only EP₄ is able to induce cAMP signalling in HEK/293 cells.

Figure S3 Effect of ER-819762 on Th1 cell differentiation. Naïve CD4⁺ T cells from BALB/c mice were stimulated with $\alpha\text{-CD3}/\alpha\text{-CD28}$ under Th1-promoting conditions in the presence or absence of exogenous PGE₂, butaprost, PGE₁-OH plus increasing amount of ER-819762 for 3 days. IFN- γ production (solid bars) was analyzed by ELISA and cell proliferation/viability (open bars) was monitored by AlamarBlue assay.

Figure S4 Effect of anti-PGE₂ antibody on IL-23 mediated Th17 cell expansion. Total CD4⁺ T cells were stimulated with $\alpha\text{-TCR}\beta/\alpha\text{-CD28} \pm 30 \text{ ng}\cdot\text{mL}^{-1}$ IL-23 in the presence or absence of $\alpha\text{-PGE}_2$ antibody for 5 days. No exogenous PGE₂ was added in these experiments. The number of IL-17 cells was analyzed by IL-17 intracellular staining and showed that treatment with $\alpha\text{-PGE}_2$ antibody results in a striking decrease in the proportion of IL-17-producing cells induced by IL-23.

Figure S5 PGE₂ treatment induces mRNA expression of IL-23R, ROR γt and IL-17A during Th17 cell development. Total CD4⁺ T cells were stimulated with $\alpha\text{-TCR}\beta/\alpha\text{-CD28} \pm 30 \text{ ng}\cdot\text{mL}^{-1}$ IL-23 in the presence or absence of 10 $\text{nmol}\cdot\text{L}^{-1}$ PGE₂ or 100 $\text{nmol}\cdot\text{L}^{-1}$ PGE₁-OH (a) or in the presence or absence of 1 $\mu\text{mol}\cdot\text{L}^{-1}$ ER-819762 or 10 $\mu\text{g}\cdot\text{mL}^{-1}$ $\alpha\text{-PGE}_2$ Ab (b) for 5 days. Total RNA was isolated and analysed by real-time PCR for the expression of IL-23R, ROR γt and IL-17A mRNA.

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