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RESEARCH PAPER

A novel antagonist of the prostaglandin E_2 EP_4 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 E_2 (PGE_2) enhances the Th17 response, exacerbates collagen-induced arthritis (CIA) and promotes inflammatory pain. The current study investigated whether selective antagonism of the PGE_2 EP_4 receptor would suppress Th1/Th17 cell development and inflammatory arthritis in animal models of RA.

Experimental approach: Effects of PGE_2 and a novel EP_4 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 EP_4 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-PGE_2 antibody. Oral administration of ER-19762 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: PGE_2 stimulates EP_4 receptors to promote Th1 differentiation and Th17 expansion and is critically involved in development of arthritis in two animal models. Selective suppression of EP_4 receptor signalling may have therapeutic value in RA both by modifying inflammatory arthritis and by relieving pain.
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Keywords: PGE_2; EP_4 receptor; EP_4 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; PGE_2, prostaglandin E_2; 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 E_2 (PGE_2) 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 ($EP_{1,\ 2,\ 3}$ and $_4$; nomenclature follows Alexander *et al.*, 2009) which selectively bind PGE_2 and mediate its effects: Activation of EP_1 receptors leads to the influx of calcium. Activation of EP_3 receptors can induce a variety of signalling events depending on the particular EP_3 splicing variant being expressed, with inhibition of adenylate cyclase activity via Gi being the most common effect. EP_2 and EP_4 receptors induce Gs-mediated activation of adenylate cyclase and a subsequent increase in intracellular cyclic AMP (cAMP). In addition, EP_4 receptors activate the phosphatidylinositol 3-kinase (PI3K) signalling pathway (Fujino *et al.*, 2003). PGE_2 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 PGE_2, 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). PGE_2 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 PGE_2. PGE_2 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 PGE_2 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, PGE_2 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 EP_2 and EP_4 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 PGE_2 stimulation of the EP_4 receptor can promote Th1 differentiation, IL-23 production in DCs, and Th17 cell expansion. These effects can be suppressed by a novel EP_4 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-PGE_2 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 PGE_2 signalling via the EP_4 receptor exerts a pro-inflammatory effect *in vivo* and is physiologically relevant to the pathology of inflammatory arthritis. EP_4 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 EP_4 receptor binding assay

The radioligand EP_4 receptor binding assay was performed using ChemiScreen recombinant human EP_4 receptor membrane preparations from Millipore, according to the manufacturer's instructions. Briefly, membranes prepared from Chem-1 cells overexpressing human EP_4 receptor cDNA (Millipore) were mixed with 1.8 $nmol \cdot L^{-1}$ [^{3}H]-PGE_2 and 5 $\mu mol \cdot L^{-1}$ unlabelled PGE_2 in the presence or absence of various concentrations of ER-819762 in binding buffer [50 $mmol \cdot L^{-1}$ HEPES, pH 7.4, 5 $mmol \cdot L^{-1}$ $MgCl_2$, 1 $mmol \cdot L^{-1}$ $CaCl_2$, 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 $mmol \cdot L^{-1}$ 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 EP_4 receptors and show induction of PLAP in response to PGE_2 and EP_4 receptor agonists, but not $EP_{1,\ 2}$ 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 × 10^4 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} PGE_2 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} $MgSO_4$ 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 $G\alpha_{15}$ protein. Endogenous EP_2 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, 1×10^5 $CD4^+$ 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 PGE_2 or EP_4 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 (5×10^5 mitomycin C-treated splenocytes in 100 μL medium + 1×10^5 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. EP_4 receptor agonists and antagonists, other cAMP-inducing agents, inhibitors of PI3K or protein kinase A (PKA) or anti-PGE_2 antibody were added during Th cell differentiation.

In order to study the effect of EP_4 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 EP_4 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 EP_4 receptor agonist/antagonists or anti-PGE_2 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 $mg \cdot kg^{-1}$ from the day of immunization. Lymph nodes were removed 6 days later, and cells were stimulated with 10 $\mu g\ mL^{-1}$ 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 $mg \cdot kg^{-1}$ 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 4×10^5 cells per 200 μL per well in complete RPMI medium and stimulated with either bCII (50 $\mu g\ mL^{-1}$) 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 IC_{50} 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 $mL \cdot kg^{-1}$ per mouse or 5 $mL \cdot kg^{-1}$ 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. PGE_2, PGE_1-alcohol (PGE_1–OH), butaprost, forskolin and anti-PGE_2 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 EP_4 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 PGE_2 and induction of cAMP was found to be mediated solely by the endogenous EP_4 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 PGE_2 binding to human EP_4 receptors (IC_{50} value of 70 ± 11 $nmol \cdot L^{-1}$; Fig. 1A), but did not displace ligand binding to several related human prostanoid GPCRs, including EP_2, DP, CRTH2 and TP receptors, and the leukotriene GPCRs LTB_4, $CysLT_1$ and $CysLT_2$ receptors (Fig. 1C). ER-819762 also suppressed human EP_4 receptor-mediated cell signalling as measured in a cAMP-dependent reporter assay (IC_{50} value of 59 ± 6 $nmol \cdot L^{-1}$) (Fig. 1B). In a larger cell signalling panel of 107 GPCRs, ER-819762 (1 $\mu mol \cdot L^{-1}$) was highly selective for EP_4 receptors, exhibiting no agonism or antagonism for any other receptor, including the related PGE_2 EP_1, EP_2 and EP_3 receptors (Table 1).

EP_4 receptor antagonism suppresses in vitro *Th1 differentiation*

As PGE_2 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

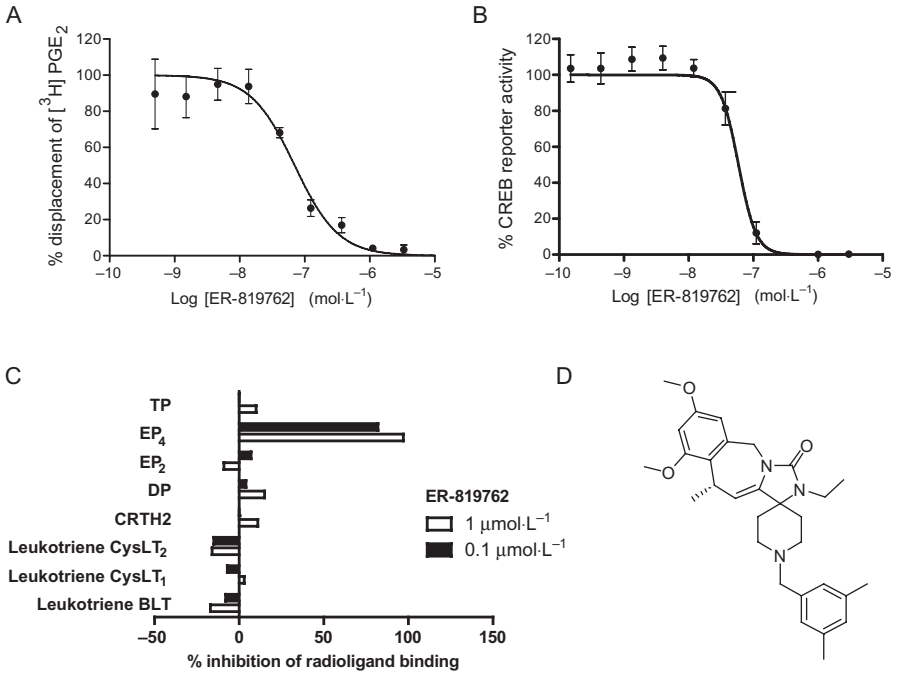

Figure 1 Activity and structure of ER-819762. (A) Competitive displacement of radiolabelled prostaglandin E_2 (PGE_2) from cell membranes overexpressing EP_4 receptors (Millipore ChemiScreen). (B) Inhibition of PGE_2-induced cAMP response element–placental-like alkaline phosphatase reporter activity in human embryonic kidney cells, which express endogenous EP_4 receptors (Supplementary Figure 2). Data are representative of mean ± 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 μmol·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 PGE_2, butaprost (an EP_2 receptor agonist) and prostaglandin E_1 alcohol (PGE_1-OH; an EP_3/EP_4 receptor agonist) significantly enhanced the differentiation of naïve $CD4^+$ T cells into Th1 cells (Fig. 2A). ER-819762 suppressed PGE_2- and PGE_1-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 PGE_2 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 μmol·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-PGE_2 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 μmol·L^{-1} (Fig. 2D).

Although Th1 differentiation was enhanced by PGE_2, 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 PGE_2 was not due to cAMP signalling. Moreover, PGE_2-stimulated Th1 differentiation was not suppressed by the PKA inhibitors H-89 (1 μmol·L^{-1}) or was only weakly suppressed by a structurally unrelated PKA inhibitor KT-5720 (10 μmol·L^{-1}), but was strongly suppressed by the PI3K inhibitor LY294002 (2 μmol·L^{-1}), as well as by ER-819762 (Fig. 3B). Higher concentrations of H-89 (10 μmol·L^{-1}) were toxic (data not shown). These results suggest that the PI3K pathway, but not the PKA-cAMP signalling pathway functioning downstream of EP_4 receptors is primarily responsible for PGE_2-enhanced Th1 differentiation. Butaprost also induced Th1 differentiation (Fig. 2A), raising the possibility that EP_2 receptors may signal via PI3K in addition to PKA-cAMP.

EP_4 receptor antagonism suppresses IL-23 secretion in human monocyte-derived DCs

It was recently reported that PGE_2 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 EP_4 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 μmol·L⁻¹)*	*GPCR Target*	*% inhibition by ER-819762 (1 μmol·L⁻¹)*	*GPCR Target*	*% inhibition by ER-819762 (1 μmol·L⁻¹)*
M1 (CHRM1)	1.1% ± 1.9%	CCK2 (CCKBR)	–8.1% ± 4.0%	NTR1	–3.7% ± 1.1%
M2 (CHRM2)	–10.5% ± 0.8%	CRF1 (CRHR1)	–16.2% ± 0.4%	FPR1	–6.6% ± 1.1%
M3 (CHRM3)	–3.2% ± 0.4%	CRF2 (CRHR2)	–9.5% ± 0.1%	FPR2/FPRL1	–7.3% ± 1.9%
M5 (CHRM5)	–4.7% ± 4.0%	D1	–8.1% ± 1.5%	NOP/ORL1	–11.2% ± 2.4%
A1 (ADORA1)	4.9% ± 6.0%	D2	0.6% ± 6.6%	δ (OPRD1)	–11.3% ± 1.0%
A3 (ADORA3)	–0.8% ± 1.4%	D5	25.2% ± 9.5%	κ (OPRK1)	8.3% ± 11.7%
A2A (ADORA2A)	13.4% ± 4.5%	ETA (EDNRA)	4.2% ± 2.4%	OX2 (HCRTR2)	–1.3% ± 0.3%
A2B (ADORA2B)	–1.4% ± 4.2%	GPR40	–3.1% ± 3.1%	PTH1 (PTHR1)	–4.7% ± 5.3%
α1A (ADRA1A)	–2.2% ± 0.1%	GPR43	5.1% ± 2.5%	PAF (PTAFR)	1.6% ± 2.3%
α2A (ADRA2A)	–13.2% ± 5.7%	GABBAb1	–9.7% ± 0.5%	PK1/GPR73	–0.4% ± 5.3%
β1 (ADRB1)	–12.1% ± 3.9%	GAL1 (GALR1)	22.9% ± 1.9%	PRP/GPR10	6.7% ± 0.7%
β2 (ADRB2)	–4.2% ± 3.4%	GCGR	–4.5% ± 2.0%	PTGDR (DP)	–13.7% ± 1.8%
C3aR	8.5% ± 0.3%	mGlu1	0.6% ± 8.2%	PTGER1 (EP1)	–4.6% ± 0.1%
C5aR	–10.2% ± 0.1%	GnRH	–12.3% ± 1.1%	PTGER2 (EP2)	(See legend)
ChemR23	3.5% ± 2.4%	H1 (HRH1)	–4.6% ± 1.0%	PTGER3 (EP3)	–2.4% ± 0.0%
AT1	–2.7% ± 1.7%	H2 (HRH2)	–4.8% ± 0.9%	PTGFR (FP)	0.1% ± 5.1%
BB2 (GRPR)	8.1% ± 0.3%	NK1 (TACR1)	–7.7% ± 6.2%	**PTGER4 (EP4)**	**90.3% ± 0.7%**
BB1	5.2% ± 4.6%	NK2	0.3% ± 2.6%	PTGIR (IP)	(See legend)
B2 (BDKRB2)	5.7% ± 1.3%	H3 (HRH3)	2.9% ± 4.1%	TBXAR2 (TP)	–9.8% ± 5.1%
AMY1	12.0% ± 0.9%	GPR54	–11.2% ± 4.4%	PAR1	–5.1% ± 9.3%
CGRP1	–7.6% ± 8.0%	BLT1 (LTB4R1)	–3.3% ± 2.9%	PAR2	–6.1% ± 8.0%
CaS	–12.6% ± 0.2%	CysLT1	–4.4% ± 4.0%	5–HT1A (HTR1A)	0.9% ± 4.5%
CB1	0.0% ± 2.5%	CysLT2	–0.7% ± 3.9%	5–HT1B	6.9% ± 15.6%
CXCR1	–5.1% ± 3.0%	S1P2 (EDG5)	–16.2% ± 0.9%	5–HT2B (HTR2B)	2.2% ± 2.9%
CXCR2	–1.7% ± 2.5%	S1P3 (EDG3)	–5.9% ± 0.6%	5–HT2C (HTR2C)	11.4% ± 3.8%
CXCR3	–14.6% ± 7.8%	LPA1 (EDG2)	–11.9% ± 5.9%	SST3	–8.9% ± 11.3%
CXCR4	–6.1% ± 2.4%	LPA3 (EDG7)	–0.2% ± 1.0%	SST4	–12.4% ± 1.8%
CCR1	–3.5% ± 1.2%	MRGX2	–8.6% ± 1.3%	GPR68(OGR1)	0.0% ± 0.0%
CCR2b	–6.7% ± 4.7%	MCHR1	–0.2% ± 0.5%	TRH	–6.9% ± 2.9%
CCR4	–10.6% ± 5.8%	MCHR2	–10.2% ± 4.0%	V1A	–0.6% ± 1.0%
CCR6	–7.5% ± 4.5%	MC4R	–3.6% ± 10.5%	V1B (AVPR1B)	–5.0% ± 1.5%
CCR7	9.6% ± 5.5%	Motilin	–12.6% ± 3.3%	V2 (AVPR2)	–17.8% ± 0.2%
CCR8	–13.2% ± 16.2%	NMUR1	–12.2% ± 4.6%	OT (OXTR)	6.5% ± 9.0%
CCR9	–15.1% ± 0.4%	Y1 (NPY1R)	15.8% ± 7.0%	PAC1 long	–7.5% ± 2.3%
CCR10	6.0% ± 2.5%	Y2 (NPY2R)	–17.7% ± 1.9%	VPAC1 (VIPR1)	–10.5% ± 1.0%
CRTH2	–2.8% ± 3.5%			VPAC2 (VIPR2)	–12.0% ± 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 EP_2 and IP receptors. No significant agonism (>15%) was found for any GPCR (data not shown), and only antagonism of EP_4 receptors was found. ER-819762 was not able to displace binding of radiolabelled PGE_2 to EP_2 receptors (Fig. 1C, MDS Pharma Profiling Service. We also used the human U2-OS cell line, which endogenously expresses EP_2 and IP but not EP_4, receptors, to assay agonism and antagonism by ER-819762 using the Corning EPIC Resonate Waveguide Biosensor system. We observed no antagonism of butaprost- (EP_2 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 PGE_1-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-PGE_2 antibody (Fig. 4B) in the absence of added prostaglandins. This suggests that IL-23 production in these activated DCs involves endogenously produced PGE_2.

EP_4 receptors antagonism suppresses IL-17 production and inhibits IL-23-induced Th17 expansion in activated $CD4^+$ T cells in vitro

We next determined whether EP_4 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 EP_4 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-TCRβ or anti-CD3/anti-CD28 antibodies. Addition of PGE_2, butaprost or PGE_1-OH (Fig. 5A) suppressed overall T cell proliferation, yet enhanced IL-17 production. Both of these PGE_2-induced effects were reversed by 0.1 or 1.0 $\mu mol \cdot L^{-1}$ ER-819762 (Fig. 5B). Further analysis by flow cytometry showed that PGE_2 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-PGE_2 antibody (Supplementary Fig. 4) could also suppress IL-23-induced Th17 expansion in the absence of exogenously added PGE_2. However, some PGE_2 could be present in the media, because we used normal 10% FBS and

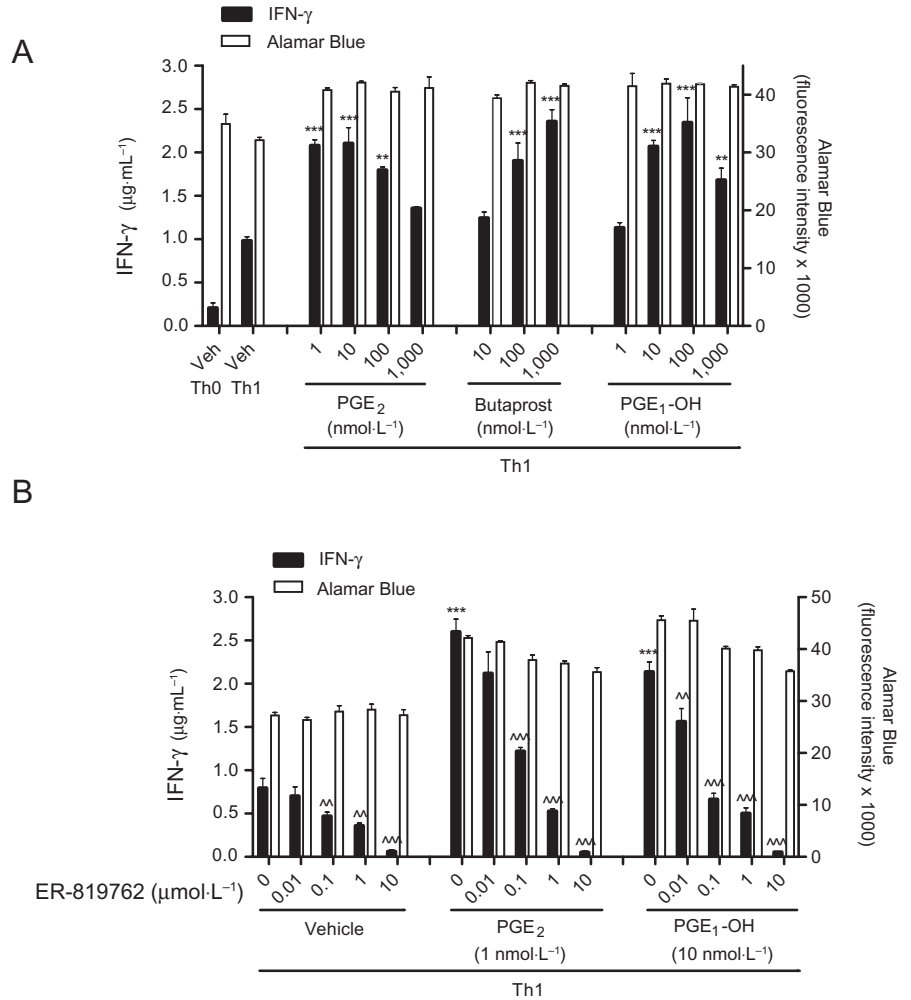

Figure 2 EP_4 receptor antagonism selectively suppresses prostaglandin E_2 (PGE_2)-induced Th1 differentiation *in vitro*. (A) Naive $CD4^+$ T cells from BALB/c mice were stimulated with α-CD3/α-CD28 under Th1-promoting conditions (see *Methods*) in media supplemented with 10% charcoal-stripped fetal bovine serum (FBS), together with PGE_2, butaprost, or PGE_1-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 PGE_2, PGE_1-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-PGE_2 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 ± 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.

not charcoal-stripped FBS for this experiment. These results suggest that PGE_2 signalling via EP_4 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 PGE_2 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 PGE_2 or PGE_1-OH enhanced expression of IL-23 receptors, the retinoic acid receptor-related orphan receptor γ-T and IL-17A mRNA (Supplementary Fig. 5A). PGE_2-induced up-regulation of IL-23 receptors and IL-17A mRNA was suppressed by ER-819762 or anti-PGE_2 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 PGE_2 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).

EP_4 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

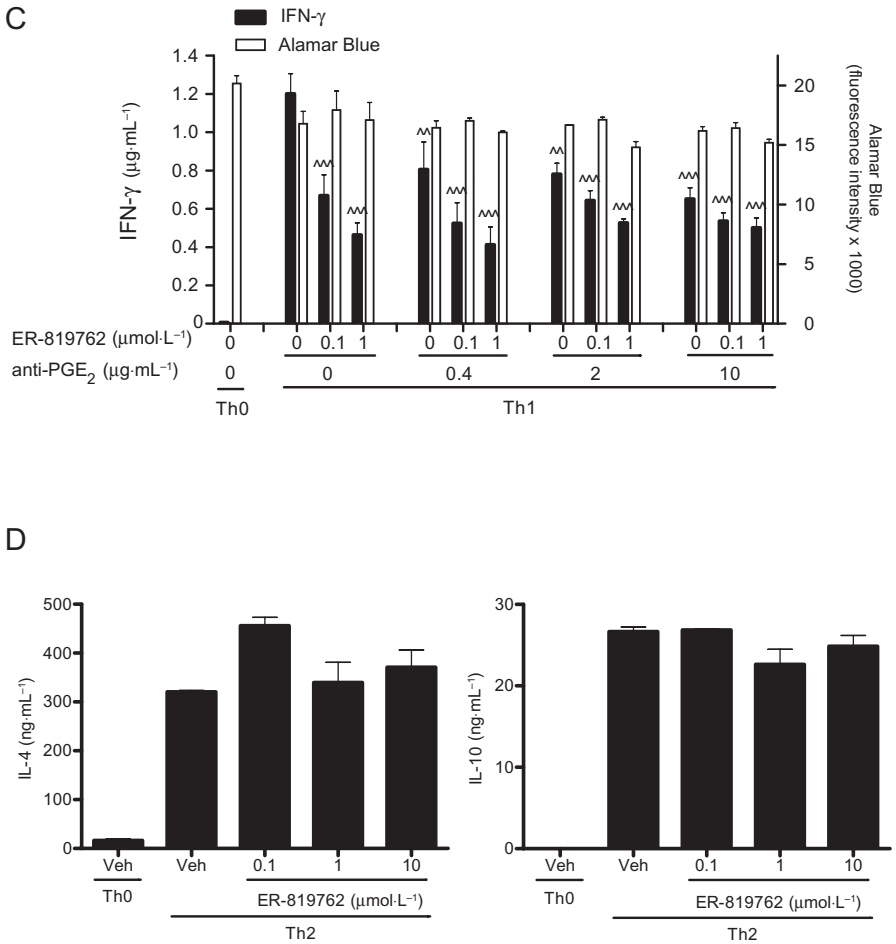

Figure 2 *Continued.*

Kalden, 2001; Fouser *et al.*, 2008), we tested whether the selective EP$_4$ 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 development 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-1α; >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

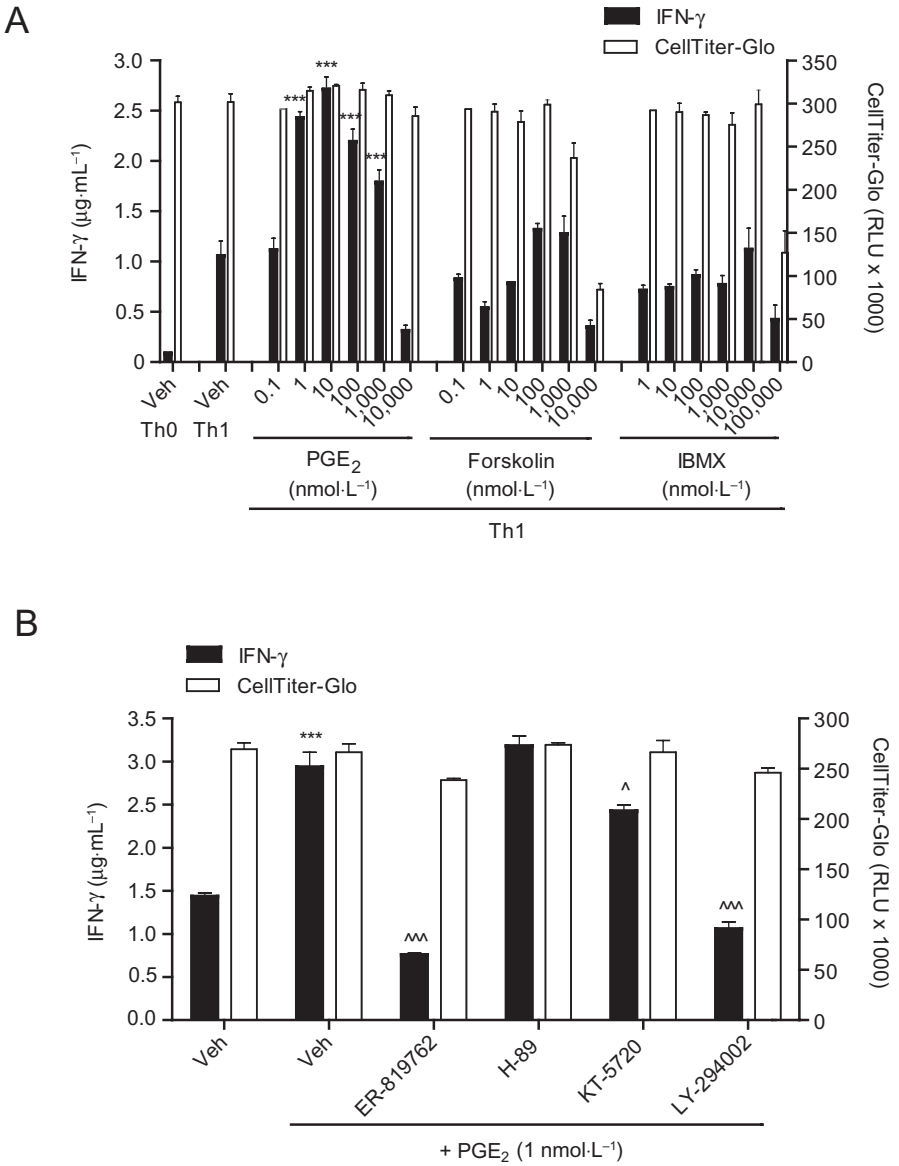

Figure 3 Enhancement of Th1 differentiation by EP_4 receptor stimulation requires phosphatidylinositol 3-kinase (PI3K) signalling. (A) The effect of cyclic AMP-inducing agents on Th1 differentiation was examined using the same methods as in Figure 2A. Cell proliferation/viability was monitored with CellTiter-Glo. (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 EP_4 receptor-mediated PGE_2 signalling is important *in vivo* for the Th1/Th17 response and the development of inflammatory arthritis.

Suppression of CFA-induced hyperalgesia in rat

PGE_2 has been reported to be a key mediator of peripheral inflammatory pain, and evidence from studies using EP_4 receptor specific antagonists and small inhibitory RNAs implicate EP_4 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).

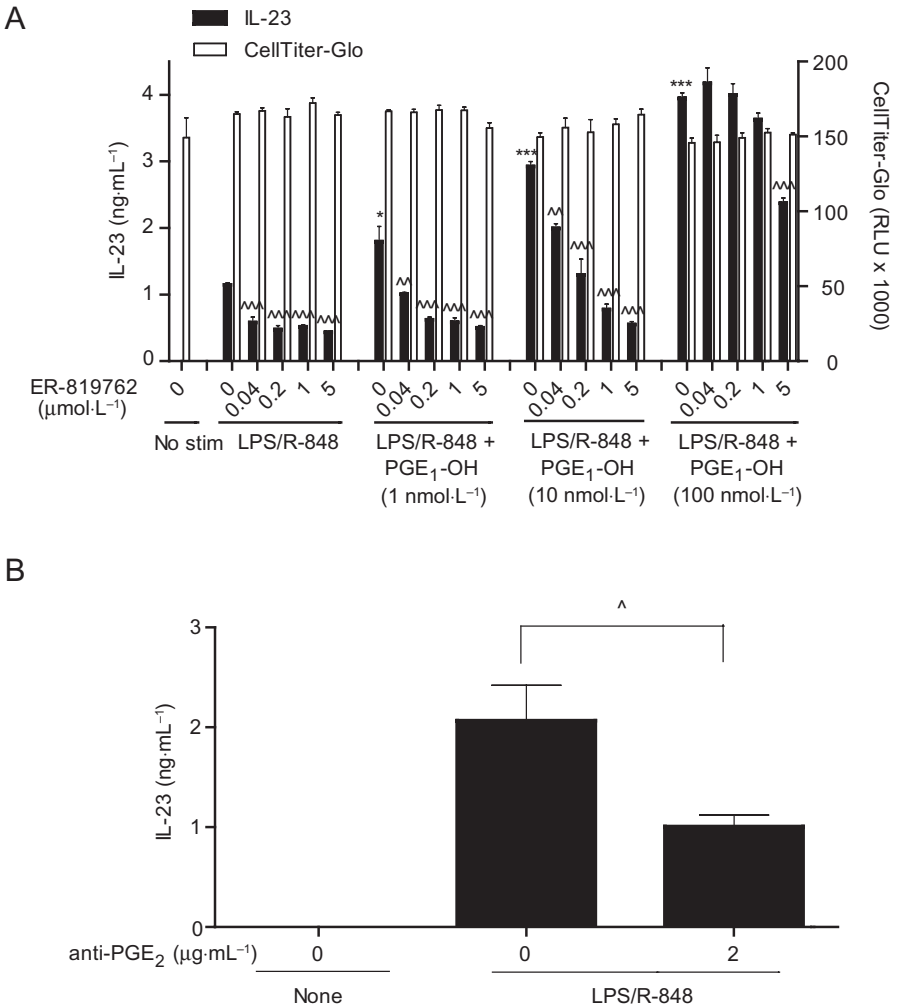

Figure 4 EP_4 receptor antagonism suppresses prostaglandin E_2 (PGE_2)-induced interleukin (IL)-23 production in human dendritic cells (DCs). (A) Human immature DCs were stimulated with 10 ng mL^{-1} lipopolysaccharide (LPS) and 2.5 μg mL^{-1} R-848 in the presence or absence of the indicated concentrations of PGE_1-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-PGE_2 antibody. 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 LPS/R-848-stimulated, no-PGE_1-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 EP_4 receptor, ER-819762, in models of inflammation. We show that antagonism of EP_4 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 EP_4 receptor signalling has other unknown pharmacological effects in these models. Nevertheless, these *in vitro* and *in vivo* results show that antagonism of EP_4 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 PGE_2 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

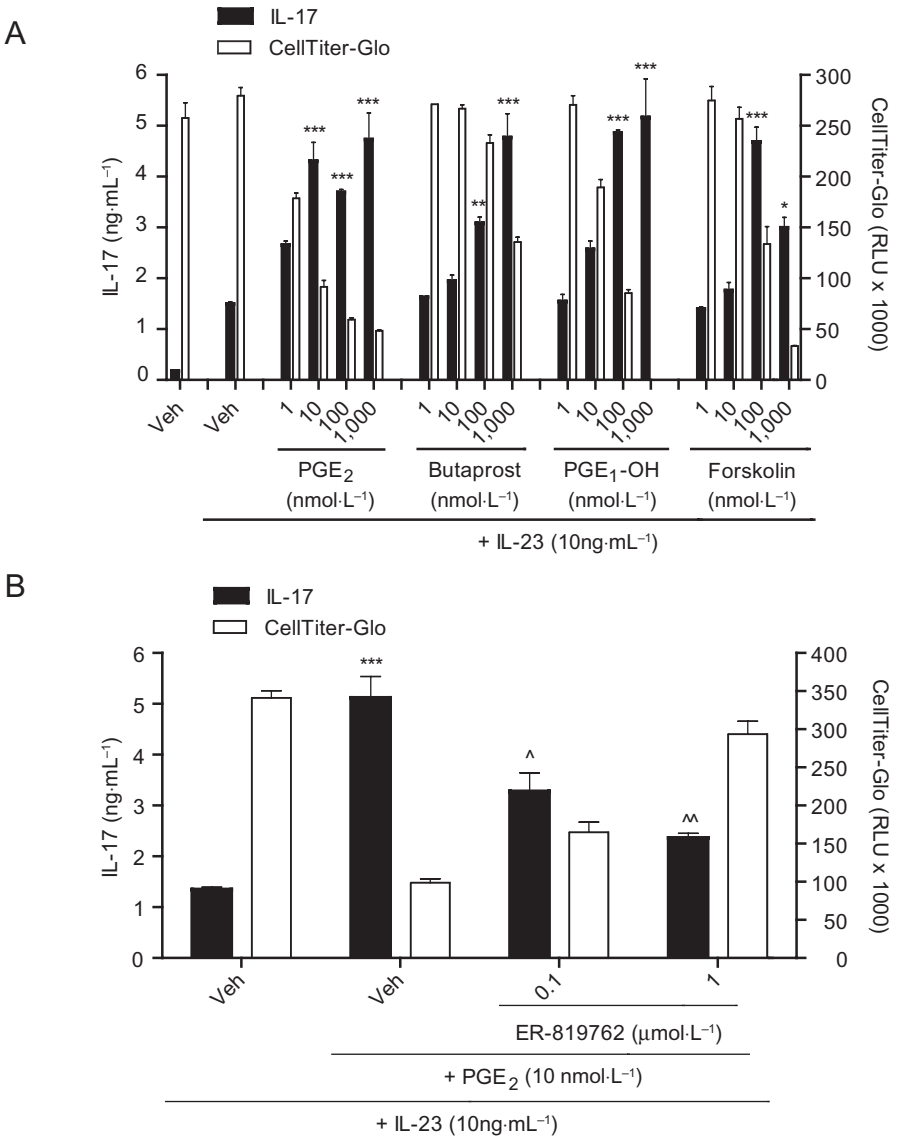

Figure 5 Prostaglandin E_2 (PGE_2)-EP_4 receptor signalling regulates Th17 cell development. (A) Total $CD4^+$ T cells isolated from mouse splenocytes were stimulated with anti-CD3/anti-CD28 plus interleukin (IL)-23 in the presence or absence of exogenous PGE_2, butaprost, PGE_1-OH, or forskolin for 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 PGE_2, butaprost, PGE_1-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-PGE_1-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.

PGE_2 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 EP_4 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 PGE_2 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 PGE_2 stimulation of EP_4 receptors (Lin *et al.*, 2006; Nakao *et al.*, 2007). Selective inhibition of EP_4 receptor signalling by several different EP_4 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

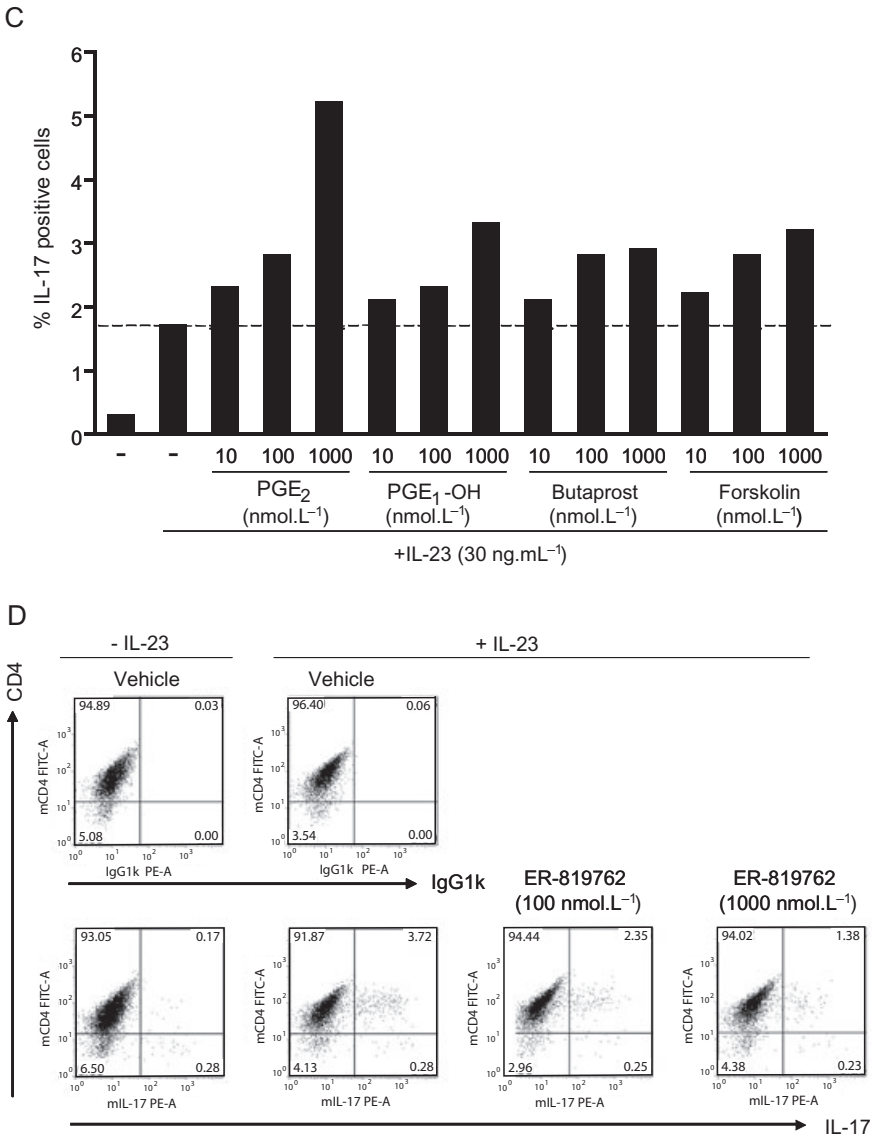

Figure 5 *Continued.*

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 PGE_2-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 EP_4 receptor antagonist may have multiple benefits in relieving both the symptoms and modifying the disease mechanisms leading to RA.

EP_4 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 PGE_2 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 PGE_2 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 PGE_2 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, RORγt, the chemokine CCL20 and its receptor CCR6. Boniface *et al.* (2009) suggested that EP_2 receptors may be more important than EP_4 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 EP_2 receptor-selective agonist butaprost, but our data show that antagonism of EP_4 receptors is sufficient to suppress

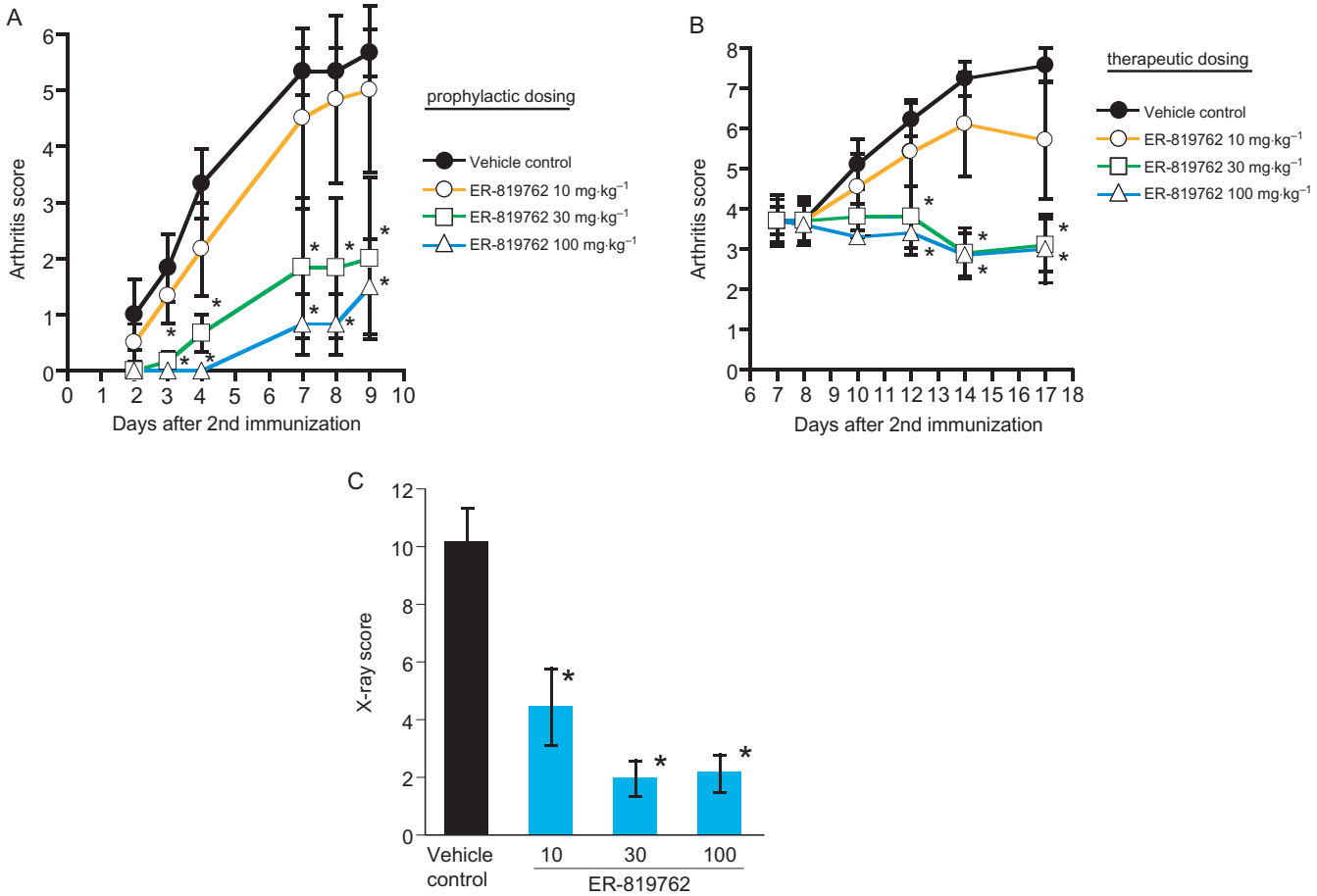

Figure 6 EP_4 receptor antagonism suppresses disease and Th1/Th17 cytokines in collagen-induced arthritis in mice. (A) DBA/1 mice were immunized with bovine type II collagen (bCII)/complete Freund's adjuvant (CFA) (primary immunization) and boosted with bCII in incomplete Freund's adjuvant at day 21 (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.

PGE_2-mediated Th17 expansion and IL-17 production in mouse cells. Our data also showed that PGE_2 did not promote Th17 differentiation *per se*, as we did not see an increase in Th17 cell frequency following PGE_2 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 PGE_2 in the presence of IL-23, indicating the expansion of pre-differentiated Th17 cells. Napolitani *et al*. (2009) suggested that PGE_2 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 PGE_2 in the absence of IL-23 co-stimulation (data not shown). In addition, we showed that EP_4 receptor stimulation can enhance IL-23 production by activated human DCs and that this activity can be inhibited by a selective EP_4 receptor antagonist or anti-PGE_2 antibody in the presence or absence of exogenously added PGE_2. Sheibanie *et al*. (2007a) have also recently reported that PGE_2 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 PGE_2 stimulation of EP_4 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 PGE_2 promoted IFN-γ production under Th1-differentiation conditions, production started to decrease at higher concentrations of PGE_2 or PGE_1-OH without loss of cell viability (Figs 2A and 3A). Similar results were seen with higher concentrations of butaprost (data not shown). Thus, PGE_2 appears to have a bimodal effect on immune stimulation; promoting inflammation at lower concentrations while attenuating inflammation

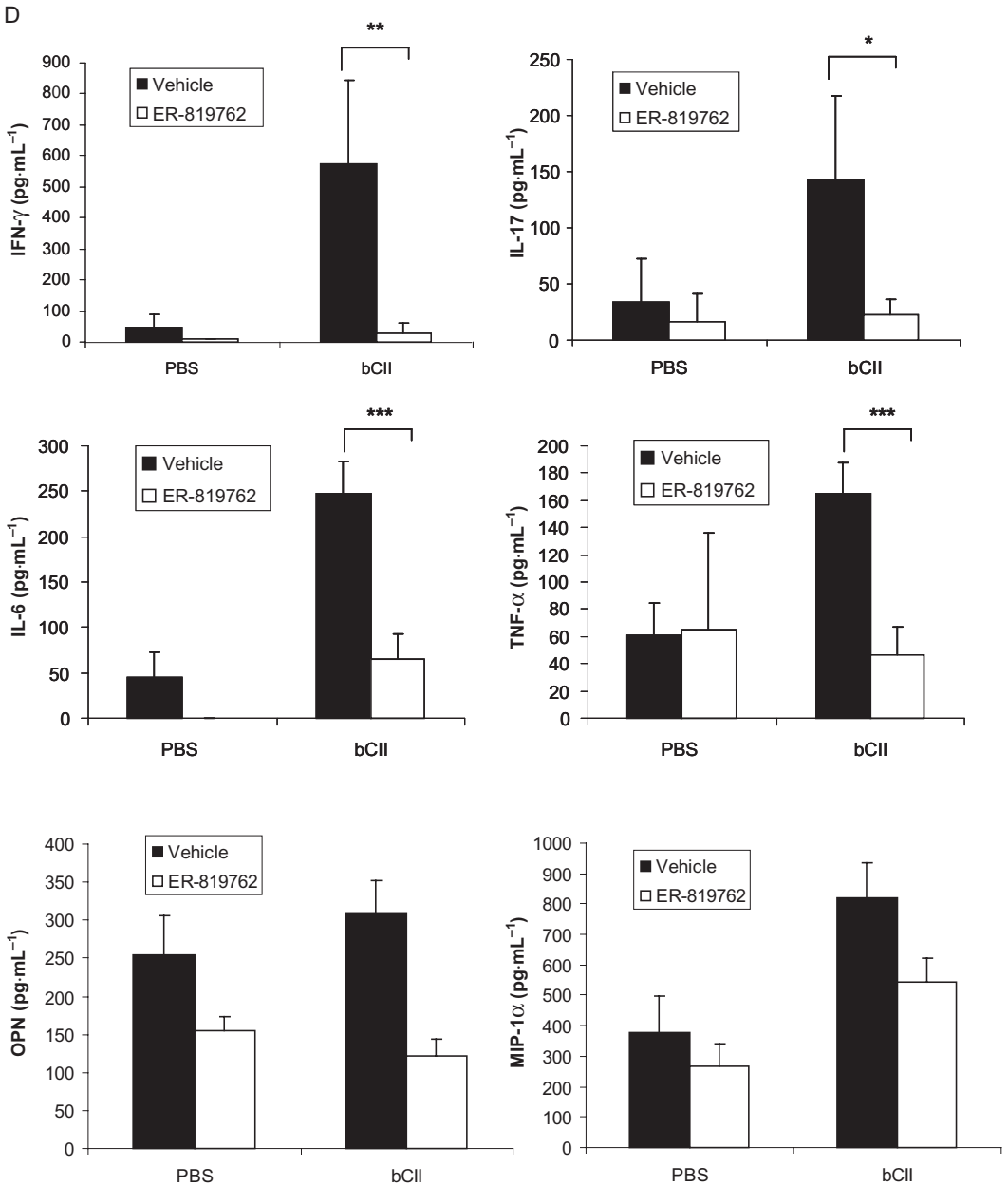

Figure 6 *Continued.*

at higher concentrations, possibly in concert with other factors that contribute to PI3K and/or cAMP signalling. This bimodal action may explain why PGE_2 exerts a pro-inflammatory effect in some systems and anti-inflammatory in others. For example, Betz and Fox (1991) have reported that PGE_2 can inhibit the production of Th1 cytokines, which is contrary to our results; however, these researchers used micromolar concentrations of PGE_2 for many of their experiments. There are also a number of potential sources of PGE_2 in cell culture systems that could contribute to higher PGE_2 levels. For example, our data suggest that autocrine production of PGE_2 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 PGE_2-immunodepleted serum stimulated EP_4 receptors in HEK/293 cells (Supplementary Fig. 1). Thus, there may be significant basal stimulation of EP_2 and EP_4 receptors in many cell culture systems, in which case further addition of exogenous PGE_2 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, EP_4 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

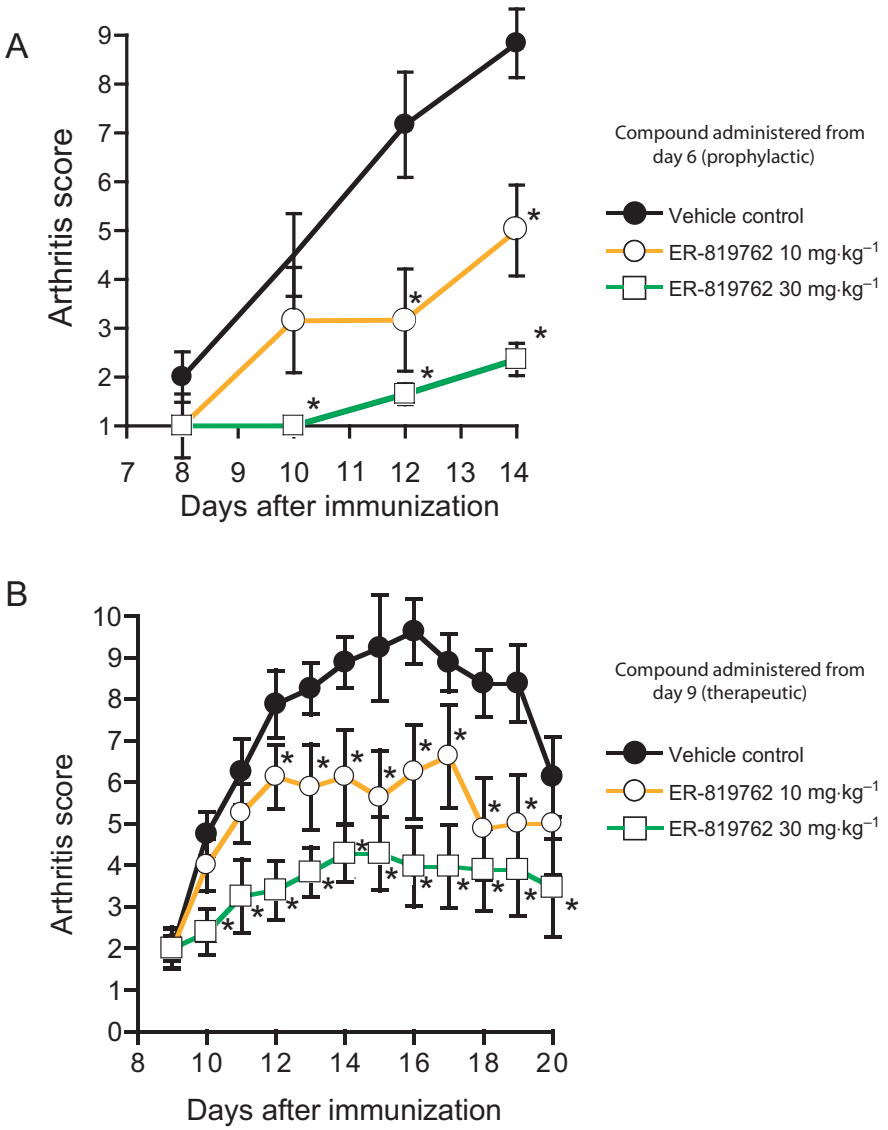

Figure 7 EP_4 receptor antagonism suppresses disease and Th1/Th17 cytokines in glucose-6-phosphate isomerase (GPI)-induced arthritis in mice. (A) DBA/1 mice were immunized with GPI/complete Freund's adjuvant to induce arthritis as described in *Methods*. ER-819762 was orally administered daily from day 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 PGD_2 produced downstream of the COX enzymes stimulates the DP receptor, which promotes allergic inflammation, while PGE_2 stimulates the EP_3 receptor, which suppresses allergic inflammation (Kunikata *et al.*, 2005). In addition, the more targeted approach of antagonizing EP_4 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 EP_4 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 PGE_2. IL-17 stimulates osteoblasts to synthesize PGE_2

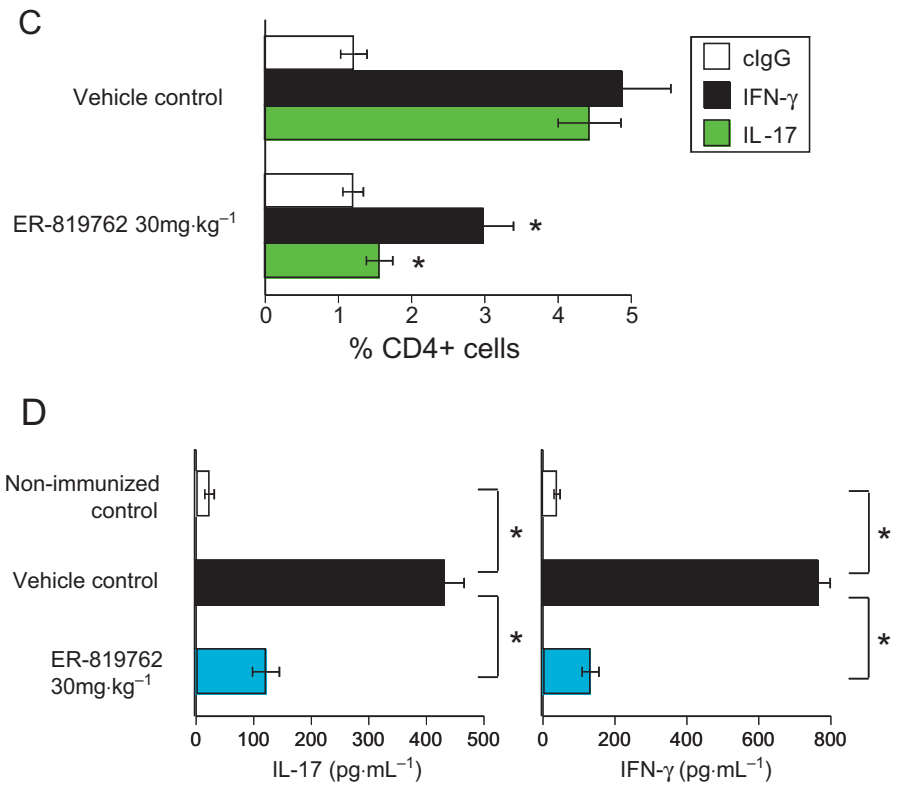

Figure 7 *Continued.*

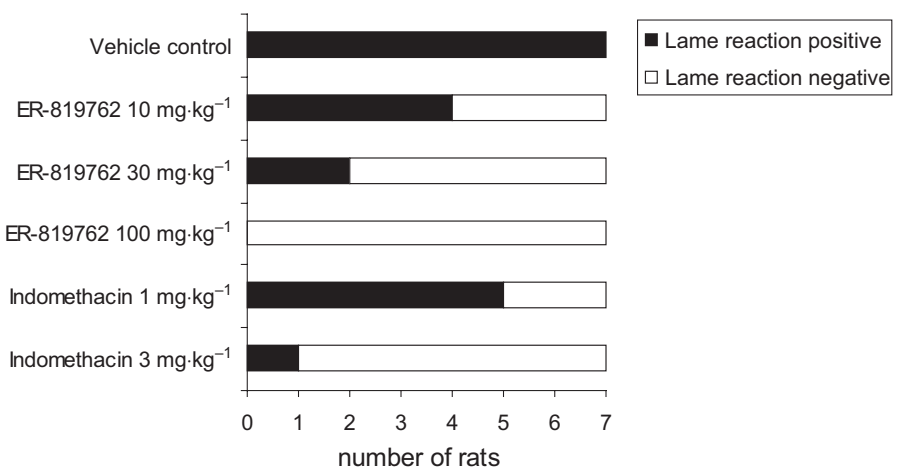

Figure 8 The analgesic effect of ER-819762 on the lame walk response in complete Freund's adjuvant (CFA) injected rats. CFA was injected into the right hind footpad of each rat (seven rats per treatment group). Three days after CFA injection, the rats exhibited a lame walking reaction as described in *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-κB (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 EP$_4$ receptor antagonist strategy may be beneficial to RA patients, including those who are insensitive to anti-TNF therapy.

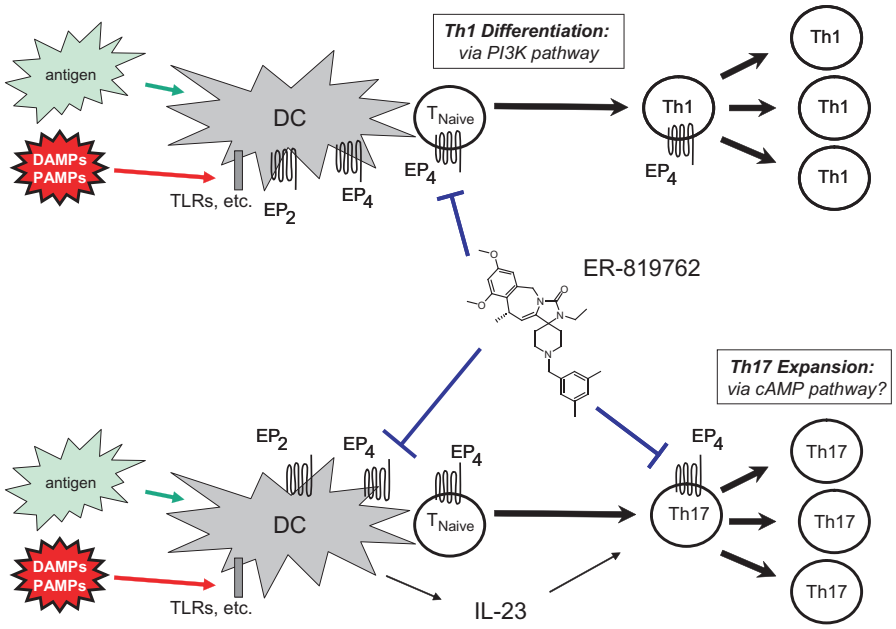

Figure 9 Multiple effects of ER-819762 on pro-inflammatory responses. Blue lines indicate the multiple steps at which ER-819762 was observed to exert an immunosuppressive effect in our studies. During infection or under conditions of chronic autoimmune inflammation, exogenous pathogen-associated molecular 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 E_2 (PGE_2)-activated EP_4 receptor signalling pathway to enhance IL-23 production by dendritic cells (DCs). EP_4 receptor signalling in naïve T cells promotes their differentiation into Th1 effector cells via the phosphatidylinositol 3-kinase (PI3K) pathway, whereas EP_4 receptor signalling promotes the expansion of Th17 effector cells via the cyclic AMP pathway. ER-819762 blocks EP_4 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 EP_4 receptor-enhanced Th17 expansion and/or IL-17 production. However, it is unknown if these actions of the EP_4 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 PGE_2-EP_4 receptor signalling promotes Th1 cell differentiation, IL-23 production by DCs and Th17 cell expansion (Yao *et al.*, 2009). This group also tested an EP_4 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 EP_4 receptor antagonism and not due to action on another, unidentified target of the compound.

In summary, we show that an antagonist of EP_4 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 EP_4 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 prostanoid 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 $\mu mol \cdot L^{-1}$ 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 E_2 (PGE_2) 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-PGE_2 antibodies (ΔP-FBS: Cayman Chemicals, clone 2B5) was not able to induce CMV activity, indicating that the CMV-inducing activity present in FBS is PGE_2 or a PGE_2-related molecule.

Figure S2 PGE_2 induction of cAMP signalling in HEK/293 cells is mediated by EP_4. 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 PGE_2 ($EP_{1,\ 2,\ 3}$, and $_4$ agonist), butaprost (EP_2 agonist) or PGE_1-OH (EP_3 and $_4$ agonist). We observed induction of PLAP activity in response to forskolin, PGE_2 or PGE_1-OH, but not to butaprost. ER-819762 could suppress induction by PGE_2 or PGE_1-OH (data not shown). We also saw no induction of CRE-PLAP by up to 100 $nmol \cdot L^{-1}$ sulprostone, an agonist of EP_3 and EP_1 (data not shown). These data indicate that of the four PGE_2 receptors, only EP_4 is able to induce cAMP signalling in HEK/293 cells.

Figure S3 Effect of ER-819762 on Th1 cell differentiation. Naive $CD4^+$ T cells from BALB/c mice were stimulated with α-CD3/α-CD28 under Th1-promoting conditions in the presence or absence of exogenous PGE_2, butaprost, PGE_1-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-PGE_2 antibody on IL-23 mediated Th17 cell expansion. Total $CD4^+$ T cells were stimulated with α-TCRβ/α-CD28 ± 30 $ng \cdot mL^{-1}$ IL-23 in the presence or absence of α-PGE_2 antibody for 5 days. No exogenous PGE_2 was added in these experiments. The number of IL-17 cells was analyzed by IL-17 intracellular staining and showed that treatment with α-PGE_2 antibody results in a striking decrease in the proportion of IL-17-producing cells induced by IL-23.

Figure S5 PGE_2 treatment induces mRNA expression of IL-23R, RORγt and IL-17A during Th17 cell development. Total $CD4^+$ T cells were stimulated with α-TCRβ/α-CD28 ± 30 $ng \cdot mL^{-1}$ IL-23 in the presence or absence of 10 $nmol \cdot L^{-1}$ PGE_2 or 100 $nmol \cdot L^{-1}$ PGE_1-OH (a) or in the presence or absence of 1 $\mu mol \cdot L^{-1}$ ER-819762 or 10 $\mu g \cdot mL^{-1}$ α-PGE_2 Ab (b) for 5 days. Total RNA was isolated and analysed by real-time PCR for the expression of IL-23R, RORγt and IL-17A mRNA.

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