Immunology and Cell Biology (2016) 94, 39–51

www.nature.com/icb

ORIGINAL ARTICLE

Efficient T-cell priming and activation requires signaling through prostaglandin E2 (EP) receptors

Vinatha Sreeramkumar[1,4], Miroslav Hons[2,5], Carmen Punzón[1], Jens V Stein[2], David Sancho[3], Manuel Fresno[1,6] and Natalia Cuesta[1,4,6]

Understanding the regulation of T-cell responses during inflammation and auto-immunity is fundamental for designing efficient therapeutic strategies against immune diseases. In this regard, prostaglandin E_2 (PGE_2) is mostly considered a myeloid-derived immunosuppressive molecule. We describe for the first time that T cells secrete PGE_2 during T-cell receptor stimulation. In addition, we show that autocrine PGE_2 signaling through EP receptors is essential for optimal $CD4^+$ T-cell activation *in vitro* and *in vivo,* and for T helper 1 (Th1) and regulatory T cell differentiation. PGE_2 was found to provide additive co-stimulatory signaling through AKT activation. Intravital multiphoton microscopy showed that triggering EP receptors in T cells is also essential for the stability of T cell–dendritic cell (DC) interactions and Th-cell accumulation in draining lymph nodes (LNs) during inflammation. We further demonstrated that blocking EP receptors in T cells during the initial phase of collagen-induced arthritis in mice resulted in a reduction of clinical arthritis. This could be attributable to defective T-cell activation, accompanied by a decline in activated and interferon-γ-producing $CD4^+$ Th1 cells in draining LNs. In conclusion, we prove that T lymphocytes secret picomolar concentrations of PGE_2, which in turn provide additive co-stimulatory signaling, enabling T cells to attain a favorable activation threshold. PGE_2 signaling in T cells is also required for maintaining long and stable interactions with DCs within LNs. Blockade of EP receptors *in vivo* impairs T-cell activation and development of T cell-mediated inflammatory responses. This may have implications in various pathophysiological settings.
Immunology and Cell Biology (2016) **94,** 39–51; doi:10.1038/icb.2015.62

The interaction of the T helper (Th) $CD4^+$ cells with antigen-presenting dendritic cells (DCs) during an immune response results in the productive activation of the T cell, followed by clonal expansion and differentiation into specific Th subtypes. This interaction occurs within the specialized microenvironment of secondary lymphoid organs. T-cell activation requires T-cell receptor (TCR) recognition of peptide–MHCII complexes on the DC surface as well as interaction between co-stimulatory molecules, which sets the threshold for T-cell activation. Several reports have elucidated the role of non-conventional immune molecules in the process of TCR co-stimulation. Identifying those factors is of fundamental importance to our understanding of the cellular mechanisms of inflammation.

One of the enzymes induced by pro-inflammatory stimuli is cyclooxygenase-2 (COX-2), which catalyzes the production of prostanoids that have long been considered as regulators of homeostasis and inflammation.[1] Prostaglandin E_2 (PGE_2), the most abundant and versatile prostanoid, exerts potent tissue- and cell type-selective actions.[2,3] PGE_2 binds to four high-affinity G-protein-coupled receptors, designated as EP1–4, which exhibit heterogeneity in signal transduction, tissue localization and regulation of their expression.[4,5] Among those, EP2 and EP4 have been described as the main receptor subtypes that mediate PGE_2 signaling in human and murine $CD4^+$ T cells.[6] Besides, the signaling through both of those receptors is somewhat redundant by activating cAMP-dependent signaling pathways.[5]

Myeloid cells produce high PGE_2 concentrations in response to inflammatory stimuli, which has been shown to play a suppressive role in T-cell responses.[7,8] However, recent reports have shown that lower concentrations of PGE_2 potentiate Th1 and Th17 differentiation processes.[9,10] Our main goal was to address the role of PGE_2 in T-cell activation and its implications during physiology and inflammation. Previously, we reported that Cox-2 is induced in resting T cells upon activation.[11] Here, we report for the first time that Th cells are also capable of secreting picomolar concentrations of PGE_2 upon activation. Besides, we show that $CD4^+$ T cells from EP2- or EP4-deficient mice have a partially reduced activation. To further elucidate the importance of PGE_2 in T-cell activation, we employed two previously

[1]Department of Molecular Biology, Centre for Molecular Biology Severo Ochoa, Universidad Autónoma de Madrid-CSIC, Madrid, Spain; [2]Department of Immunobiology and Lymphocyte Migration, Theodor Kocher Institute, University of Bern, Bern, Switzerland and [3]Department of Vascular Biology and Inflammation, Spanish National Centre for Cardiovascular Research 'Carlos III', CNIC, Madrid, Spain
[4]Current address: Department of Basic Biomedical Sciences, Universidad Europea de Madrid, Villaviciosa de Odón, Madrid, Spain.
[5]Current address: Institute of Science and Technology Austria, Klosterneuburg, Austria.
[6]These authors contributed equally to this work.
Correspondence: N Cuesta, Department of Basic Biomedical Sciences, Universidad Europea de Madrid, Tajo s/n, Villaviciosa de Odón, 28670, Madrid, Spain.
E-mail: natalia.cuesta@uem.es
Received 6 October 2014; revised 19 May 2015; accepted 27 May 2015; accepted article preview online 8 June 2015; advance online publication, 30 June 2015

described irreversible EP antagonists (EPAs) to block PGE_2 signaling: AH6809 and AH23848.[12,13] We found that blocking of EP2 and 4 receptors, both *in vitro* and *in vivo*, resulted in a marked defect in $CD4^+$ T-cell activation and proliferation, and decreased accumulation of $CD4^+$ T cells in draining LNs during inflammation. Moreover, functional PGE_2-EP signaling was also essential for T-cell interactions with DCs and hence important for the establishment of an efficient activation threshold in T cells.

We then proved the physiological consequences of blocking PGE_2 signaling in T cells in a collagen-induced arthritis (CIA) model in mice. In this disease, a crucial role in inflammation and disease progression has been assigned to collagen II (CII)-specific Th cells that have been activated following abnormal presentation of auto-antigens by APCs.[14] On the other hand, blocking COX-2 and EP2/EP4 have been proved to reduce arthritic responses in CIA.[9,15,16] We found that mice that received EPAs in the initial phase of CIA failed to develop chronic clinical arthritis, with a corresponding decrease in the percentage of activated interferon (IFN)-γ-producing $CD4^+$ T cells. Our study has elucidated a novel role for PGE_2 in T-cell activation in physiological and inflammatory scenarios.

RESULTS

Mouse and human $CD4^+$ T cells secrete PGE_2 and upregulate EP4 and EP2 expression during activation

T lymphocytes have not been considered important PGE_2 producers. Nevertheless, we found that both mouse and human $CD4^+$ T cells are capable of secreting PGE_2 when activated by anti-CD3 and anti-CD28 *in vitro* in a time-dependent manner. As seen in Figure 1a, mouse $CD4^+$ T cells augmented their production of PGE_2 following activation. The concentration of PGE_2 secreted by activated $CD4^+$ T cells ranged from 0.1 to 0.3 nM. Similar results were obtained from human Th cells after activation (Figure 1b).

The actions of PGE_2 in murine T cells are mediated through the EP2 and EP4 receptors.[6,17] Therefore, we analyzed the expression of total EP2 and EP4 in resting and activated $CD4^+$ T cells by flow cytometry. As seen in Figure 1c, although mouse $CD4^+$ T cells expressed considerable amounts of EP2 and EP4, its expression was augmented upon *in vitro* activation. In human $CD4^+$ T cells, although the basal levels of EP2 and EP4 are low, their expression is also increased during activation (Figure 1d).

Antagonism of EP receptors inhibits T-cell activation

To gain insight into the role of PGE_2 signaling on T-cell activation, we first used $CD4^+$ T cells from EP2 and EP4 knockout mice. T-cell activation was assessed by studying the expression of surface markers and evaluating proliferation of cells after *in vitro* activation. We chose CD69 as the marker for the early activation phase (4–16 h after T-cell activation), and CD71 and CD25 as markers for the intermediate/late phase of activation (24–48 h of activation).

Both EP2- and EP4-deficient T cells showed a partially reduced response to anti-CD3+anti-CD28 activation as compared with control littermates (Table 1). However, as EP2 and EP4 signaling are mostly redundant,[5,6] and double EP2/4 KO mice are not viable, we employed an alternative approach to block both receptors. Thus, we used two irreversible EPAs (AH6809 and AH23848), which have been found to be effective both *in vitro* and *in vivo* in blocking EP2 and EP4, and employed in numerous studies.[12,13]

To exclude the possibility that EPAs could induce cell death because of toxicity, we activated T cells with and without EPA-pre-treatment for 5 days and tested them for cell viability with a propidium iodide staining (Supplementary Figure 1). Although basal T cells contained a 20.1% of dead cells (left panel), activation for 5 days resulted in death of 50.7% of the cells (middle panel). However, cells that received EPA pre-treatment before the induction of activation showed only 41.5% of dead cells (right panel). This indicated that EPAs did not induce cell death in $CD4^+$ T cells.

As seen in Figure 2a, the antagonism of both EP receptors resulted in a decrease in the surface expression of early and intermediate/late T-cell activation markers, with the effect being seemingly most pronounced in the late/proliferative phase of T-cell activation. As shown in Figure 2b, proliferation was also markedly reduced when activated T cells had been pre-treated with EPAs.

The kinase Akt is activated upon T-cell co-stimulation and is important for T-cell activation. We therefore checked for phosphorylation of Akt in the absence and presence of EPAs. EPA-treated cells expressed much lower levels of phospho-Ser473-Akt after 24 h of activation, when compared with control activated T cells (Figures 2c and d).

Autocrine PGE_2-EP receptor signaling induces differentiation toward Th1 and regulatory T (T_{Reg}) cells

Antigen binding to the TCR stimulates the secretion of interleukin (IL)-2, an essential molecule for T-cell proliferation and differentiation. We found that the amount of IL-2 produced by T cells after activation was significantly diminished with EPAs pre-treatment (Figure 3a), which points to defective T-cell activation upon EPAs administration. Next, we checked whether blocking EP receptors alters the expression of several cytokines, characteristic of different Th subsets. We found that the levels of IFN-γ and tumor necrosis factor (TNF)-α (Th1 cytokines) secreted by activated $CD4^+$ T cells were significantly reduced on pre-treatment with EPAs (Figures 3b and c). In contrast, IL-4 (a Th2 cytokine) production was augmented by EPA treatment (Figure 3d). Pre-treatment with EPAs resulted in a decrease in IL-17 following activation (Figure 3e). These findings demonstrate that endogenous PGE_2 signaling influences cytokine production by activated T cells.

As the effect of PGE_2 on Th17 differentiation has already been well characterized,[6,10] we further analyzed the effect of blocking PGE_2 signaling in Th1, Th2 and T_{Reg} differentiation. Mouse $CD4^+$ T cells were differentiated in polarized cultures in the presence and absence of EPAs and the expression of distinct transcription factors for Th1 (T-bet), Th2 (GATA-3) and T_{Reg} (Foxp3) was analyzed by flow cytometry. We observed reduced T-bet levels in EPA-treated cells (around 50%) as compared with control activated T cells in the Th1-polarized cultures (Figure 3f). No changes in GATA-3 expression upon EPA treatment were seen in Th2-polarized cultures (Figure 3f). Regarding the T_{Reg} phenotype, the frequency of $Foxp3^+$ T cells was significantly reduced (60–70% on different experiments) in EPA-treated cells (Figure 3g).

Blocking T-cell EP receptors affects trafficking to regional LNs and proliferation of $CD4^+$ T cells during inflammation *in vivo*

Following an inflammatory stimulus, $CD4^+$ T cells traffic to the LNs draining the inflammatory site, where they undergo activation and proliferation. To address the *in vivo* relevance of our previous findings, EPA- or dimethyl sulfoxide (DMSO)-treated carboxyfluorescein diacetate succinimidyl ester (CFSE)-stained OT-II mouse $CD4^+$ T cells were adoptively transferred into wild-type mice, which were subsequently immunized with OVA peptide+lipopolysaccharide (LPS; to induce inflammation) or injected with physiological saline (control group). Three days later, the number of adoptively transferred Th cells in draining LNs was compared between DMSO- and EPA-treated

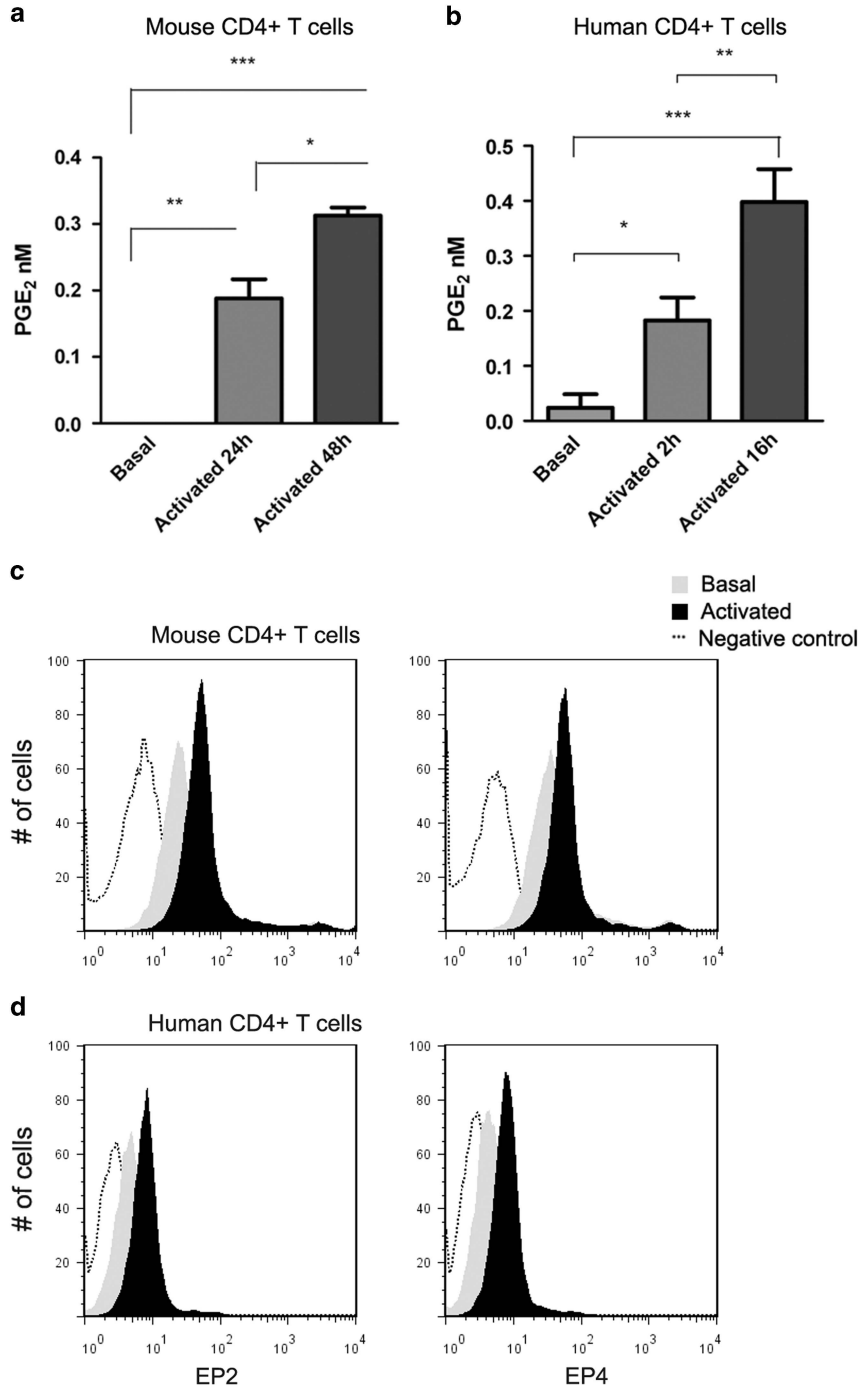

Figure 1 Activated T cell release picomolar concentrations of PGE_2, which are beneficial for proliferation. (**a**) Concentrations of PGE_2 secreted by mouse $CD4^+$ T cells after *in vitro* activation for 24 and 48 h. (**b**) Concentrations of PGE_2 secreted by human $CD4^+$ T cells after *in vitro* activation for 2 and 16 h. Data are representative of two independent experiments with values in duplicates. Values are represented as mean+s.d. One-way analysis of variance and Turkey's multiple comparison test; $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. (**c** and **d**) Histogram plots of mouse (**c**) and human (**d**) $CD4^+$ T cells, showing the expression of total EP2 and EP4 receptors in basal conditions and upon 24 h of activation. Results are representative of two independent experiments.

groups. At 72 h post transfer and immunization, although the total number of cells in draining LNs was comparable in all groups, the number of EPA-pre-treated adoptively transferred cells encountered in the draining LNs was significantly reduced as compared with the number of recovered DMSO-pre-treated cells, with a corresponding decrease in the % of $CD44^+$ activated T cells (Figures 4a and b).

To assay *in vivo* proliferation of T cells, we isolated the adoptively transferred CFSE-labeled Th cells from regional LNs of mice on day 2. CFSE dilution revealed fewer rounds of cell division and decreased cell numbers in draining LNs of mice that had received EPA-treated T cells when compared with mice that had received vehicle-treated control cells (Figure 4c). This indicates that blocking of EP receptors in T cells reduces their proliferation.

Blocking T-cell EP receptors diminishes the duration of T cell–DC interactions in the LNs

The decrease in the number of recovered adoptively transferred T cells after EPA treatment could be mainly due to three different mechanisms: defective homing to the LN; inefficient local activation/proliferation; increased egress from the LN. To discriminate among those, OVA peptide-pulsed cell tracker orange-labeled DCs were injected subcutaneously into recipient mice. $CD4^+$ T cells from OT-II mice were treated with EPAs or vehicle (DMSO), stained with CFSE and CMAC fluorescent dyes, respectively, and injected i.v. into the same recipient mouse 18 h later. The popliteal LN was excised and analyzed by two-photon intravital microscopy.

Table 1 Defective T-cell activation in EP KO mice

Mice	*Proliferation (c.p.m.)*	*CD69*		*CD25*		*CD71*	
		%	*MFI*	*%*	*MFI*	*%*	*MFI*
WT	6725	71.8	940	55.8	549	67.8	988
EP2 KO	3892**	52.3**	405**	35.9**	276**	49.7**	620**
EP4 KO	4257**	67.5*	870	47.5*	438*	63.2	865*

Abbreviations: KO, knockout; MFI, mean fluoresence intensity; WT, wild type.
Mouse $CD4^+$ T cells from the different mice were activated for 4 or 24 h and cell surface marker expression for early (CD69) and late (CD25 and CD71) activation markers was evaluated by flow cytometry (% positive cells and MFI). Proliferation was measured by (^{3}H) thymidine incorporation in cells after 72 h. Values are represented as mean of two independent experiments (three mice each). One-way analysis of variance and Turkey's multiple comparison post tests: $*P<0.05$, $**P<0.01$.

Homing was found to be unaltered when EP receptors were blocked, as the ratio of the number of adoptively transferred $CD4^+$ T cells found in draining LNs was equal when T cells had been pre-treated with EPAs or DMSO (Figure 5a).

The possibility of inefficient T-cell activation was studied by analyzing T cell–DC interactions within the draining LN using the multiphoton intravital microscopy. Supplementary Video 1 depicts a representative recording of the late phase of T-cell activation in the draining LN during an inflammatory response to OVA peptide (image stack in Figure 5b). Both subsets of treated cells were capable of contacting DCs. However, blocking of EP receptors before injection decreased the duration of the interactions between T cells and DCs in the late phase of activation (24 h after their injection into mice; $P<0.0001$; Figures 5b and c and Supplementary Video 1) and not in the early phase (within 4 h following their injection in mice; Figure 5c and Supplementary Video 2).

Next, we tested whether EPAs had an effect on the ability of effector T cells to egress or get retained in the draining LN. $CD4^+$ T cells exit the LN in response to gradients of Sphingosine 1 phosphate (S1P). This lipid is sensed by the $S1P_1$ and $S1P_3$ receptors, which are up-regulated upon T cell activation in order to exit the LN. As shown in Figure 5d, EPA treatment did not alter the inducible expression of neither $S1P_1$ nor $S1P_3$ upon T-cell activation, suggesting that EPA-treated cells are not impaired for egress. To further address this, we studied whether the cells were capable of egressing from the LN after treatment with EPAs using anti-CD62L (Mel-14) treatment to prevent further homing of T cells[18] and found that the percentages

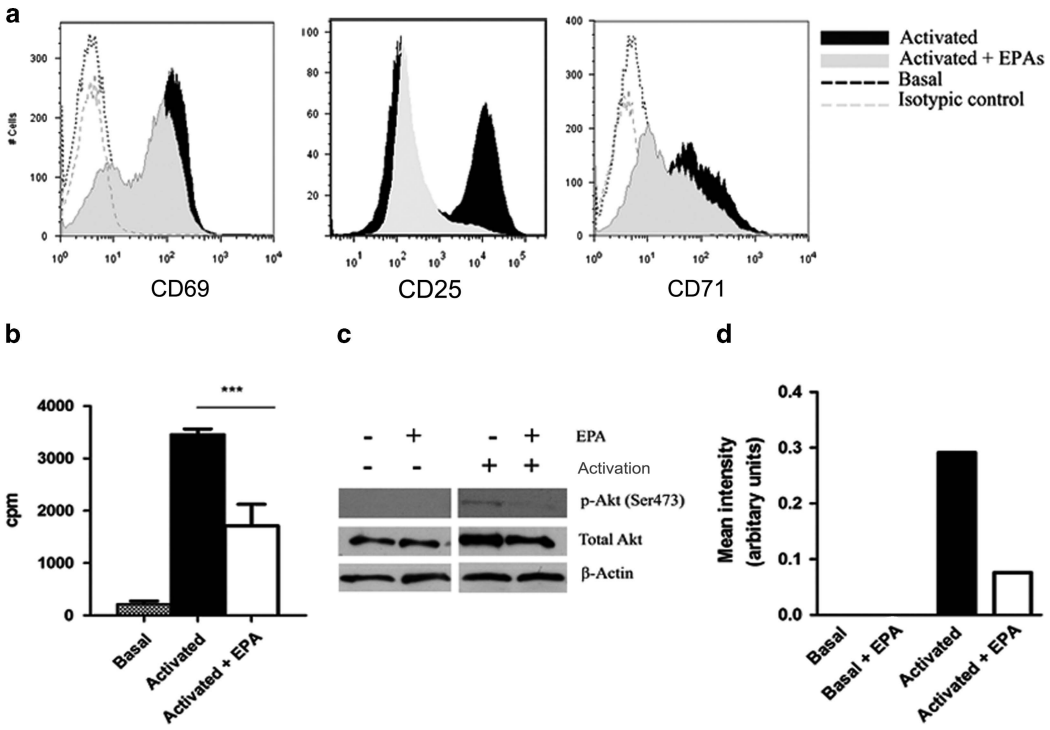

Figure 2 Antagonism of EP receptors reduces activation marker expression, proliferation, p-Akt induction and autocrine PGE_2 production in murine $CD4^+$ T cells. (**a**) Histogram plots of mouse $CD4^+$ T cells showing the expression of surface CD69, CD25 and CD71. Mouse $CD4^+$ T cells were activated for: 4 h (CD69) and 24 h (CD25 and CD71). (**b**) (^{3}H) Thymidine incorporation test in cells pre-treated with EPAs or DMSO and subject to activation. Values are represented as mean+s.d. One-way analysis of variance and Turkey's multiple comparison post tests: $*P<0.05$, $**P<0.01$, $***P<0.001$. (**c**) Western blots showing expression of phospho-Akt, total Akt and actin expression in murine $CD4^+$ T cells that were pre-treated with EPAs or DMSO, and subject to indicated treatments for 24 h. (**d**) Densitometric quantification of phospho-Akt normalized to total Akt and actin.

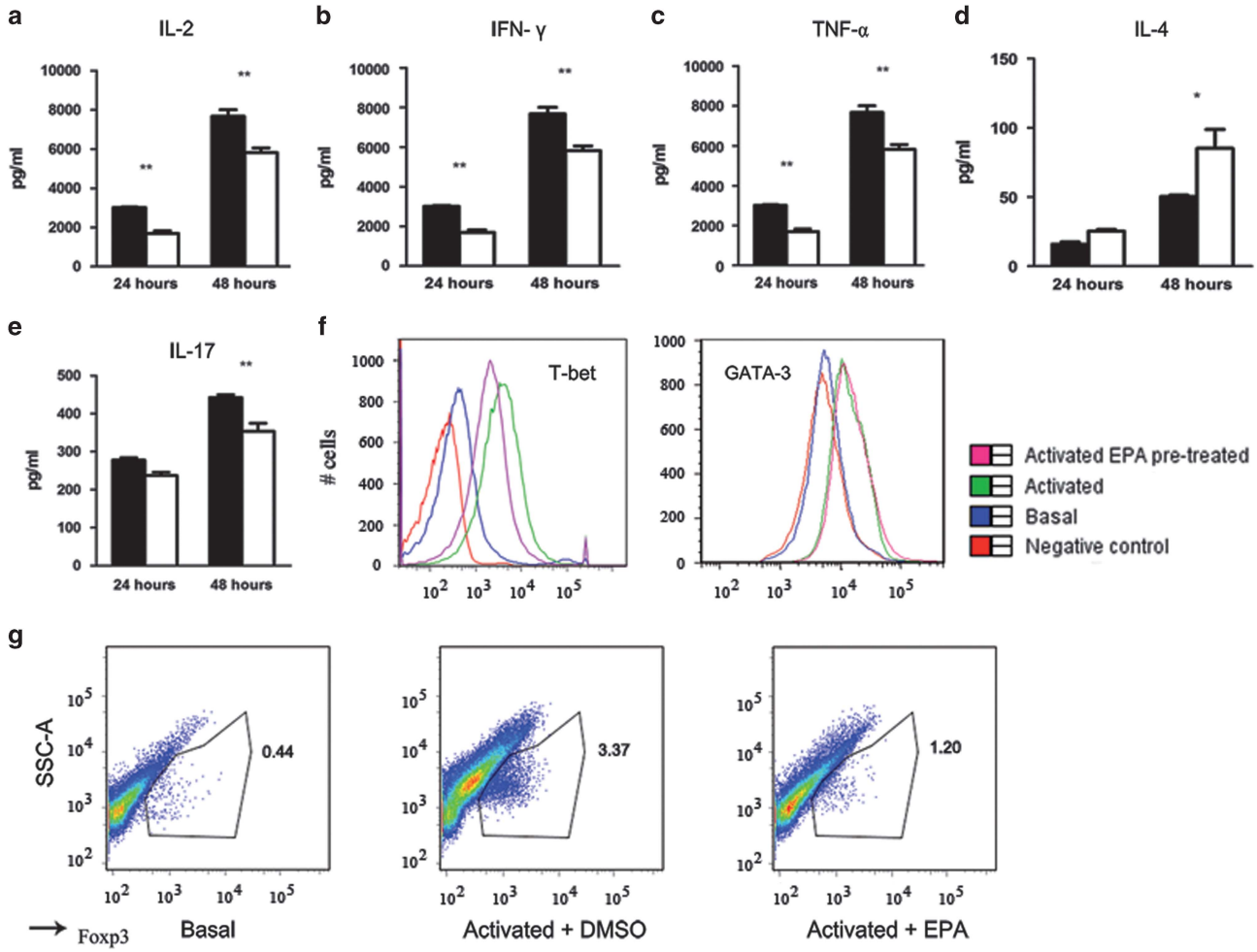

Figure 3 Antagonism of T-cell EP2/4 receptors reduces IL-2 production and differentially regulates polarization toward Th1, Th17 and T_{Reg} phenotypes. (**a**–**e**) Concentrations of cytokines secreted by mouse $CD4^+$ T cells after *in vitro* activation for 24 h: IL-2 (**a**), IFN-γ (**b**), TNF-α (**c**), IL-4 (**d**) and IL-17 (**e**). Values are represented as mean+s.d. Two-way analysis of variance and Bonferroni's post tests: $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. (**f**) Histogram plots of polarized T cells showing the expression of T-bet and GATA-3. (**g**) Dot plots of mouse $CD4^+$ T cells polarized toward a T_{Reg} phenotype and stained for intracellular Foxp3. Data are representative of three independent experiments.

of recovered Th cells in draining LNs after anti-CD62L treatment were similar when comparing EPA-treated and DMSO-treated cells (Figure 5e).

Together, the above results suggest that inefficient local Th-cell activation and not defective homing or increased egress is responsible for the decreased number of EPA-treated Th cells found in the draining LN.

Another reason why we find lesser numbers of OT-II T cells when we pre-treat them with EPAs is possibly a rapid cycling time of EPA-treated T cells in the LN, and thus reduced LN retention in the absence of antigen exposure. For this reason, we measured turning angles during T-cell encounters with OVA peptide-pulsed DCs by intravital two-photon microscopy. During the early phase of the immune response, when T cells are yet to be primed, EPA-treated cells showed a flat frequency distribution curve (Supplementary Figure 2), which indicates that EPA-treated T cells are more inclined toward a pure random, Brownian motion, in which case all turning angles would be equally probable. Wherein DMSO-treated T cells exhibited small turning angles, suggestive of directed and persistent motion, a phenotype commonly associated with chemotaxis. These data suggest that EPA-treated cells are 'always on the move' while control T cells might undergo chemotactic motion in order to be able to actively scan for antigen and hence might persist for longer time in the LN before undergoing activation.

EP antagonism prevents T cell-mediated inflammation in a mouse model of arthritis

The above findings revealed that blocking EP receptors in T cells is effective in inhibiting efficient T-cell activation *in vitro* and *in vivo*. To further analyze the pathophysiological consequences of this EP inhibition, we studied the effect of EPAs on a T-cell-mediated inflammatory disease, murine CIA, which shares many similarities with human rheumatoid arthritis. Mice that were immunized with type-II collagen (CII)+complete Freund's adjuvant (CFA) with or without DMSO treatment were considered as positive controls, and mice immunized only with CFA were the negative controls.

Mice received EPAs before the booster dose of CII, and control mice received DMSO injections. The assessment of morphological changes in the paw indicated that forelimbs and hindlimbs of positive controls and DMSO-treated animals showed signs of chronic arthritis such as erythema, inflammation and/or ankylosis of the limb. On the contrary, EPA-treated mice showed normal morphology of both limbs

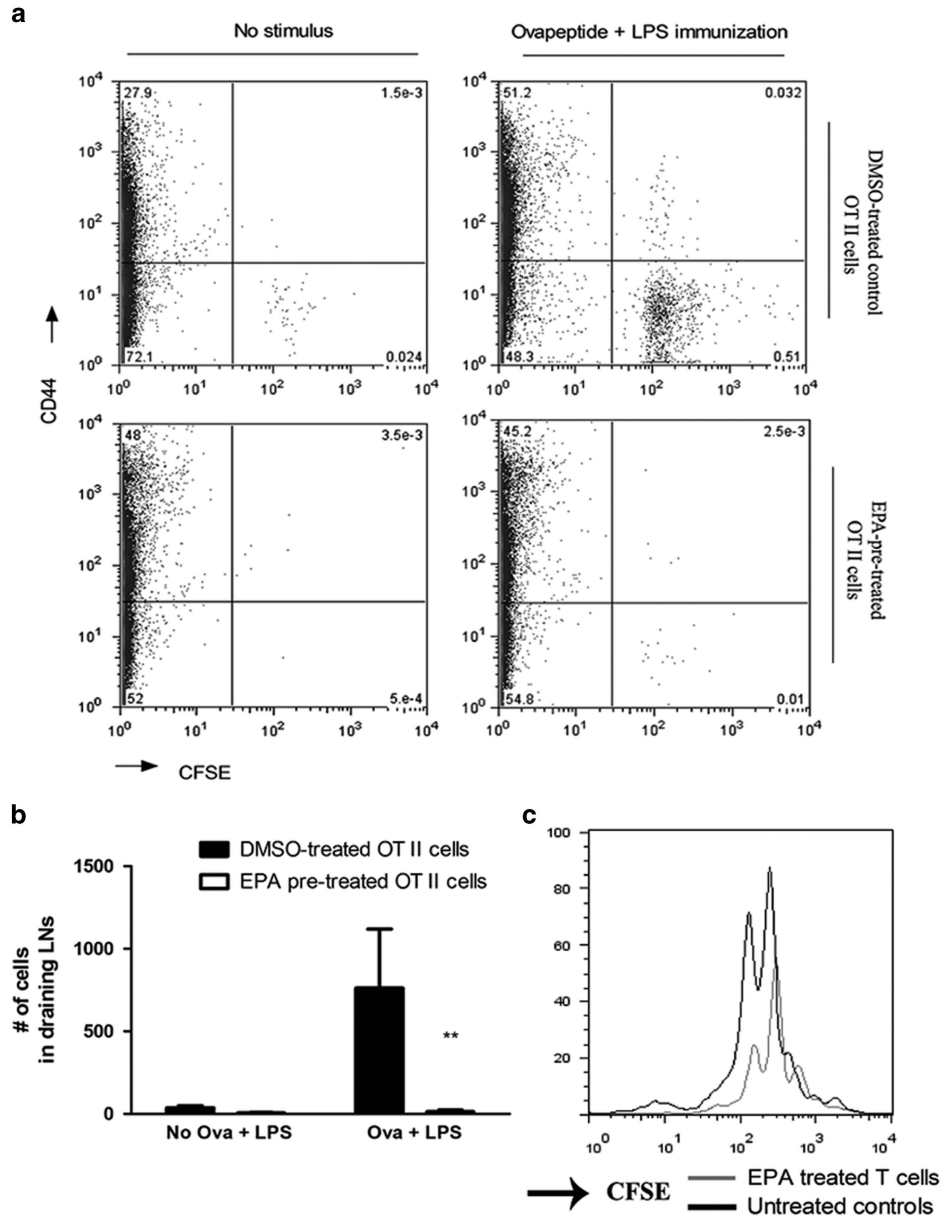

Figure 4 Blocking EP receptors in CD4+ T cells affects their accumulation and proliferation in regional LNs during inflammation *in vivo*. (**a**) Representative dot plots showing adoptively transferred DMSO- and EPA-treated cells from draining LNs of mice that received ovapeptide+LPS immunization and mice with no immunization. (**b**) Absolute numbers of CD4+ T cells from draining LN suspensions 3 days after immunization. Two-way analysis of variance and Bonferroni's post tests: $*P<0.05$, $**P<0.01$, $***P<0.001$. (**c**) Histograms showing CFSE dilution of adoptively transferred OT-II CD4+ T cells untreated or treated with EPAs and isolated from regional LNs of mice that had received OVA-pulsed DCs on day 2. Data are representative of three independent experiments.

(Figure 6a). Mice that received EPA treatment showed sharply reduced clinical scores as compared with positive controls. Interestingly, the arthritic scores were almost comparable to negative controls that received only CFA immunization (Figure 6b). To address the effects of EPAs on the local and systemic inflammatory status, we analyzed IFN-γ and anti-CII IgG levels present in serum at day 35. Serum from mice with EPA treatment contained titers of anti-CII IgG that were nearly half of that found in CII-CFA- and DMSO-treated arthritic mice (Figure 6c). In addition, EPA-treated mice presented significantly lower amounts of serum IFN-γ as compared with positive controls (Figure 6d). Both results are indicative of lower T cell-mediated inflammation, B-cell help and evolution of the disease upon EPA treatment, during the T-cell immunization phase of the disease.

The knee and elbow joints of mice from all groups were also examined histologically. Mice with CII+CFA immunization developed histopathological lesions consistent with CIA, characterized by a hyper-proliferative synovial membrane and marked infiltration of inflammatory cells within the joint spaces (Figure 6e). CFA-treated mice did not show any signs of inflammation, as expected (Figure 6f). In DMSO-treated animals, the synovial membrane showed signs of hyper-proliferation in some areas, although immune cell infiltration was lesser than in mice with CII+CFA treatment (Figure 6g). On the

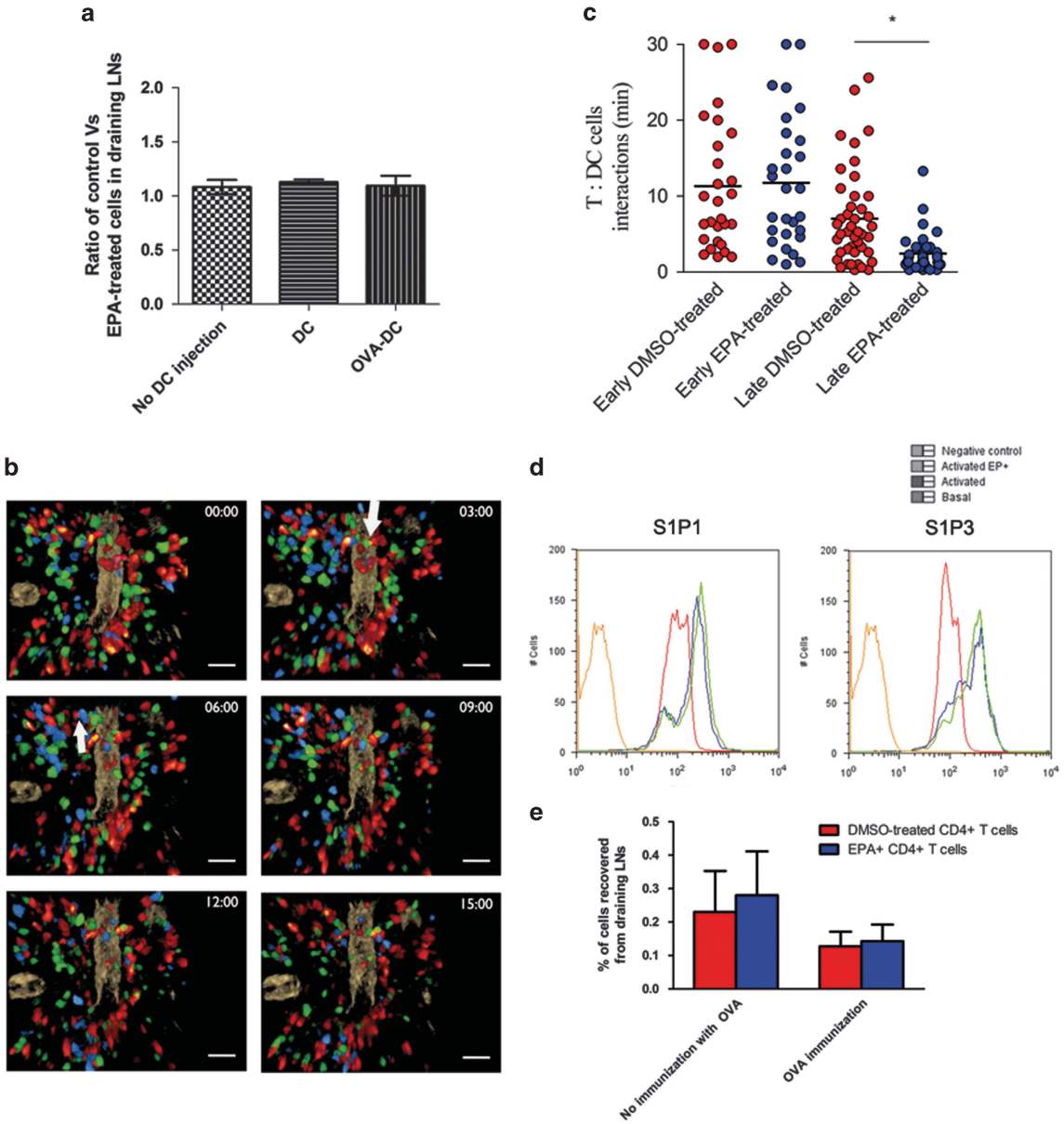

Figure 5 Blocking of T-cell EP receptors reduced the duration of T cell–DC interactions but did not alter the egress phenotype of Th cells from draining LNs during inflammation. (**a**) Representative graph from a homing assay indicating the ratio of EPA-treated and DMSO-treated $CD4^+$ T cells in draining LNs of mice, 5 h after immunization. Four mice were used per group and data are representative of three independent experiments. One-way analysis of variance (ANOVA) was used for data analysis. (**b**) Representative image stack from a 30-min video recorded during the late phase of T-cell activation using an intravital two-photon microscope. Green cells: CFSE-labeled DMSO-treated control $CD4^+$ T cells, blue cells: Cell tracker blue-EPA-treated $CD4^+$ T cells, red cells: Cell tracker orange-stained DCs, pale brown: PNAd-labeled high endothelial venules. White arrow in the top right panel shows a DMSO-treated control $CD4^+$ T cell interacting with a DC. White arrow in the second row, left panel indicates a blue EPA-treated $CD4^+$ T cell in interaction with a DC. (**c**) Quantitative analysis of T cell–DC interactions in the early and late phases of T-cell activation. A total of 137 interactions were analyzed manually in three-dimensional with at least a minimum of 30 interactions per group. Results are representative from three independent experiments using two mice each (six mice in total) for the early and late phases of T-cell activation. Values are represented as mean+s.d. One-way ANOVA and Turkey 's multiple comparison post tests: $^*P<0.05$. (**d**) Histograms showing $S1P_1$ (left panel) and $S1P_3$ (right panel) expression in mouse $CD4^+$ T cells activated *in vitro* for 72 h. Orange line: isotypic control; red line: basal cells; blue line: activated T cells; green line: activated and EPA-treated cells. Histograms are representative of two independent experiments. (**e**) Percentages of recovered EPA and DMSO-treated cells from draining LNs in the egress assays. Four mice were used per group and data are representative of three independent experiments. One-way ANOVA was used for data analysis (see also Supplementary Videos 1 and 2).

other hand, joints from mice that received EPA preventive treatment had no signs of inflammation (Figure 6h), in agreement with their clinical scores and normal limb morphology. This confirmed that mice with EPA preventive treatment were indeed resistant to development of arthritis.

RA is characterized by enhanced lymphatic flow and increased LN size, and different cytokines have been shown to contribute to the pathology of arthritis.[19–21] Thus, we evaluated popliteal and inguinal LN suspensions for total cell number and cytokine production. Draining LNs from mice treated with EPAs

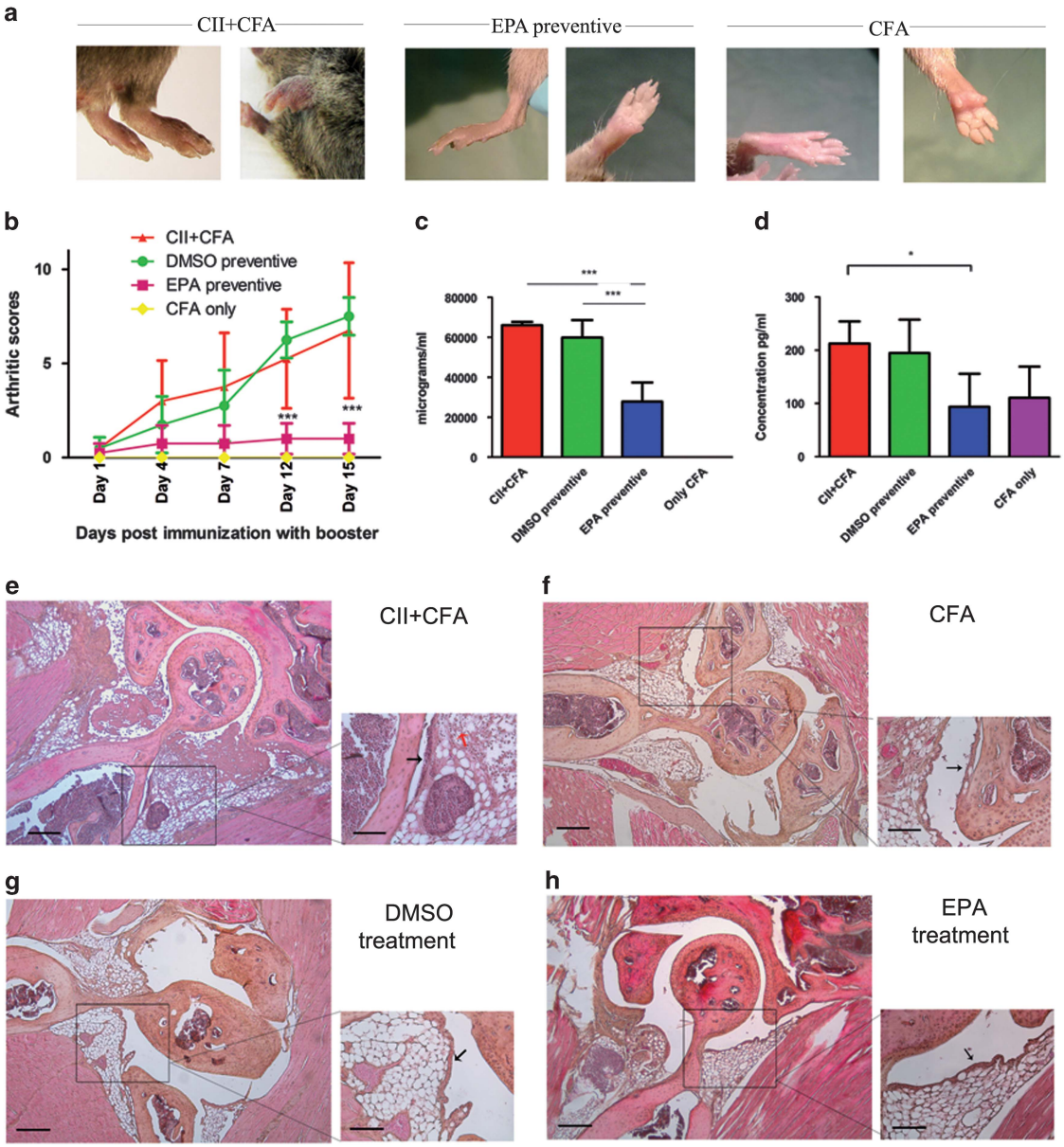

Figure 6 Mice that received EPA preventive treatment failed to develop arthritis. (**a**) Representative pictures of the hind- and forelimbs from mice that received indicated treatments, taken on day 35 of the trial. Images are representative of two different experiments with five mice per treatment group. (**b**) Clinical scores of all groups of animals. Values are calculated as follows: arthritic scores of the four limbs of each animal per group were added and the graph represents the mean+s.d. of two independent experiments using four animals per group. Mean scores were analyzed using two-way analysis of variance and Bonferroni post tests. $*P<0.05$, $**P<0.01$, $***P<0.001$. (**c**, **d**) IFN-γ (**c**) and anti-CII IgG (**d**) levels present in serum at day 35 after induction of arthritis in all groups of animals. 4-5 mice per group. One-way ANOVA was used for data analysis. (**e**–**h**) Hematoxylin and eosin staining of paraffin sections obtained from knee joints of mice belonging to the indicated treatment groups. Black arrows point to the synovial membrane; immune infiltration is indicated with a red arrow. Scale bars in the left panel of each figure correspond to 150 μm, whereas scale bars on the right panel of each figure correspond to 40 μm. Five mice per treatment group were analyzed.

showed a highly significant reduction in cellularity as opposed to DMSO-treated and untreated positive controls (Figure 7a).

In addition, LN cells were re-activated *in vitro* with CII and supernatants were collected. TNF-α, IFN-γ and IL-17 levels in the supernatants of LN cells from mice that received EPA treatment were negligible in contrast to positive controls (Figures 7b–e). As it is known that PGE_2 induces COX-2 expression to maintain a positive feedback loop through the EP4 and EP2 receptors for further induction of PGE_2 secretion,[22] we decided to also check for PGE_2 production by draining LN cells. The amount of PGE_2 secreted was also significantly lower in LN cells from mice that had received EPA treatment (Figure 7f).

Draining inguinal LN cells following re-activation were further stained for CD4 and activation markers CD71 and CD44. As shown in Figures 7g and h, the % of $CD71^+$ and $CD44^+$ activated Th cells found in draining LNs was diminished by 51.7% and 29.6%, respectively, in EPA-treated mice as compared with positive controls. Activation of $CD4^+$ T cells from LNs of DMSO-treated mice was similar to positive controls. These results indicate that EPA treatment was effective in

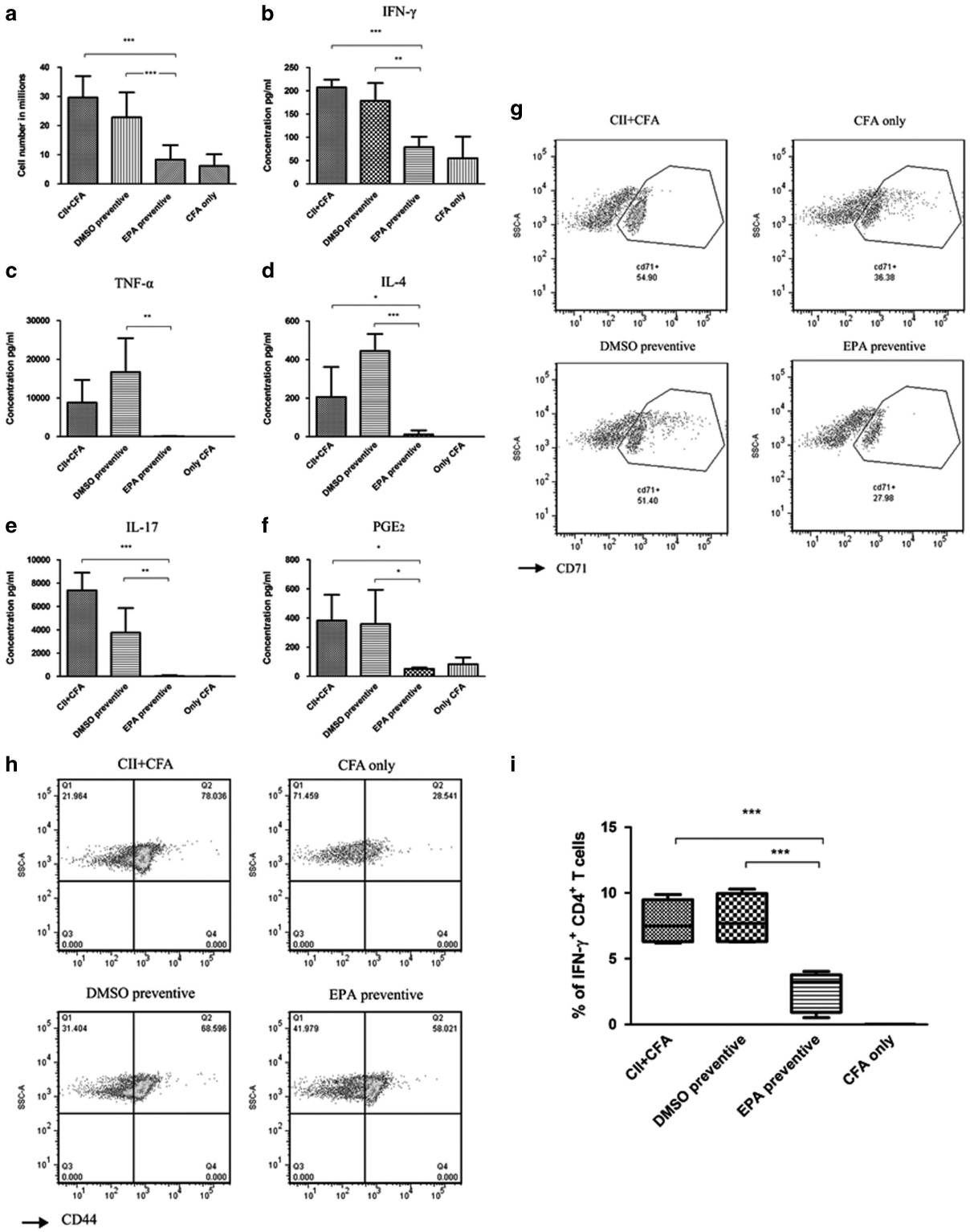

Figure 7 EPA preventive treatment reduced cellularity, inflammatory cytokine production, activated ($CD44^+$ and $CD71^+$) and IFN-γ^+ T cells in the draining LN of arthritic mice. (**a**) Total cell number of draining LN cells isolated from mice on the final day of the arthritis trial. (**d**–**h**) Concentrations of IFN-γ (**d**), TNF-α (**e**), IL-4 (**f**), IL-17 (**g**) and PGE_2 (**h**) in the supernatants of LN cells re-activated *ex vivo* with CII. (**g** and **h**) LN cells from different treatment groups of mice were obtained and activated *ex vivo* with CII. Cells were pooled for each treatment group and stained for CD4, CD71 and CD44. The $CD4^+$ population was gated and analyzed for staining of the activation markers CD71 (**g**) and CD44 (**h**). Representative dot plots of pools from five mice in each treatment group. (**i**) Bar graph showing % of IFN-γ^+ cells. LN cells from different treatment groups of mice were obtained and activated *in vitro*, permeabilized and stained for IFN-γ. Data are from five mice per group. Data are representative of two independent experiments. Results were analyzed using one-way analysis of variance and Turkey's multiple comparison tests. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

crippling the activation process of T cells within draining LNs *in vivo* during chronic inflammation in CIA.

The function of IFN-γ-secreting Th1 cells in mediating the pathogenesis of CIA has been fully documented. Therefore, we compared the amount of IFN-γ-producing Th1 cells between mice that received EPA treatment and untreated controls. In accordance with the lower percentage of activated $CD4^+$ T cells encountered in LNs from EPA-treated mice, we also found that there were correspondingly much lower IFN-γ-producing Th cells (Figure 7i). This suggested that not only did the preventive treatment with EPAs modify T-cell activation profiles but also resulted in an inhibition of Th1 differentiation.

DISCUSSION

Despite the 'classical' view of PGE_2 as a myeloid-derived pro-inflammatory and immunosuppressive molecule, regulating several functions in T-cell activation and differentiation, a new perspective has emerged owing to recent reports on the role of PGE_2 in promoting Th1 and Th17 differentiation.[6,10,17,23–25] Here, we have broadened our understanding about the role of PGE_2 in T lymphocyte patho-physiology showing for the first time that Th cells are capable of secreting PGE_2 during activation. Correspondingly, T cells increase their expression of EP2 and EP4 upon activation.

This is in correlation with our previous studies describing the induction of the limiting enzyme for PGE_2 synthesis, COX-2, after TCR stimulation of human T cells.[11] Regarding EP receptor expression, although it has been demonstrated earlier that mouse T cells express EP2 and EP4,[6,26] we are the first to our knowledge to report upregulation of protein expression of these receptors following T-cell activation. Thus, our findings clearly demonstrate that T cells augment PGE_2 secretion during activation and correspondingly increase the expression of EP2 and EP4 receptors likely to amplify signal response. We hypothesize that this increase in total EP2 and EP4 expression during T-cell activation could contribute to increment signaling pathways necessary for achieving a favorable activation threshold.

Supporting this hypothesis, EP2 or EP4 deficiency in $CD4^+$ T cells results in significant, although incomplete, reduction of their activation as compared with control littermates. This could be due to the fact that the signaling through both of those receptors is somewhat redundant by activating cAMP-dependent signaling pathways.[5] More importantly, blocking both EP receptors with irreversible inhibitors AH6809 and AH23848 (a strategy found to be effective both *in vitro* and *in vivo* and employed in numerous studies[12,13]), more severely decreased T-cell activation, as seen by a reduction in CD25 and CD71 surface expression, and proliferation. Moreover, this strategy avoids the effects of receptor compensation, with the advantage of irreversibly blocking EP receptor signaling specifically in T cells without the need of double conditional KO mice.

PGE_2 signaling has already been shown to indirectly enhance T-cell proliferation by inducing co-stimulatory molecules on DCs, but not by acting directly in T cells. The blockade of Akt activation by EPAs suggests that co-stimulatory signaling provided by autocrine PGE_2 could cooperate in promoting T cells to attain an optimal activation threshold through an Akt-dependent pathway. Interestingly, CD28 co-stimulation takes place through Akt activation,[27] so in the presence of PGE_2, co-stimulation could be less necessary.

Blocking EP receptors before T-cell activation also results in a diminished production of Th1 cytokines. A recent study reports the importance of physiological nanomolar concentrations of PGE_2 in the activation of the phosphatidylinositol-3-kinase (PI3K) pathway and its subsequent role in the promotion of Th1 differentiation.[10] This is in agreement with our findings, as PGE_2 signaling controls AKT (Protein kinase B) phosphorylation and seems to be essential for production of IFN-γ and TNF-α, and more importantly, for T-bet expression, a marker for Th1 cells.

GATA-3 expression, on the other hand, was found to be unaltered after EP antagonism, thereby suggesting that blocking of EP receptors did not modify Th2 differentiation. With respect to Th17, EP antagonism inhibits IL-17 production during T-cell activation, which is in agreement with the proposed role for PGE_2 in promoting Th17 expansion.[6]

Interestingly, blocking EP receptors also resulted in a striking reduction of the percentage of $CD4^+$ $Foxp3^+$ cells, which indicates that autocrine PGE_2 signaling is necessary for T_{Reg} polarization. Besides, the low concentrations of IL-2 secreted by $CD4^+$ T cells in response to EP antagonism serve to explain the reduced T_{Reg} expansion, as IL-2 is required by T_{Regs} to proliferate. Some reports substantiate the importance of high concentrations of PGE_2 (μM range) in induction of differentiation toward the T_{Reg} phenotype in human[28] and mouse.[29] Thus, we suggest that autocrine EP2/EP4 signaling would be required for induction of T_{Reg} differentiation following activation. In this regard, EP2 signaling impairs proliferative response of double negative T_{Reg}.[30]

More importantly, our results show for the first time a role of PGE_2/EP-mediated signaling in Th-cell trafficking to regional LNs and proliferation of Th cells during T cell-mediated inflammatory responses *in vivo*, revealing an unexpected pathophysiological role of PGE_2 in inflammation. Following an inflammatory stimulus, $CD4^+$ T cells traffic to the LNs draining the inflammatory site, where they undergo activation and proliferation. It is known that T-cell trapping within antigen-stimulated LNs is important for their productive interaction with DCs. A consequent exchange of information between these cells results in efficient T-cell priming and is necessary for establishing a full-fledged activation threshold. In our studies with two-photon intravital microscopy, we observe that EPA-treated cells exhibited a severe defect in maintenance of long, stable interactions in the late phase. This substantiates the hypothesis that blockade of EP receptors on T cells before their interaction with DCs could potentially hinder them from reaching an optimal activation threshold, thus preventing them from progressing to the long-stable interaction phase. This correlates with our *in vitro* findings showing that expression of late activation markers is decreased to a considerable extent in EPA-treated T lymphocytes. In summary, inability of summing sequential signals provided by autocrine PGE_2 could prevent EPA-treated T cells from attaining optimal activation. One of the missing sequential signals in EPA-treated cells could be the additive, co-stimulatory PI3K-Akt signaling provided by EP receptors, the lack of which results in a faulty activation threshold and obstruction of proliferative cycles. Stable DC contacts by T cells are required for IL-2 and IFN-γ production by T cells, the lack of which could be an additional mechanism by which T-cell activation is significantly impaired by EPAs *in vivo*, impeding the evolution of T cells into the proliferative phase. This would be in agreement with a decrease in cell number corresponding to the final round of cell division in EPA-treated cells found in the draining LN *in vivo* as compared with control cells, during an inflammatory response. Thus, our studies demonstrated that PGE_2-EP signaling in T cells is necessary for the progression of T cell–DC interactions in the in the LN from the initial, short-contact phase to the subsequent stable, long-contact phase during an inflammatory stimulus. This adds a new dimension to the effect of this lipid mediator in the dynamic and vital process of T-cell priming.

We have also analyzed the importance of this EP signaling in T cells during a T cell-mediated inflammatory process *in vivo*, such as CIA. We have demonstrated that blocking EP is effective in prevention of disease development, being this dependent on T-cell activation. This is in agreement with the reduction of CIA was found in $mPGES^{-/-}$ mice (26) and in $EP4^{-/-}$ mice.[31] Furthermore, administration of an EP4 antagonist to DBA/1J mice[9] reduced severity of arthritis significantly. In contrast, a previous report has pointed out the requirement of inhibiting both EP2 and EP4 in order to achieve a significant reduction in the arthritic score.[15] Together, these results support that EP2 and EP4 work redundantly for elicitation of CIA and suggest that the PGE_2-mediated EP2/EP4 signaling in the initial phase of arthritis acts to promote joint inflammation in CIA. However, an *in vivo* mechanistic explanation was not provided in those studies.

Moreover, the effect of the EPA preventive treatment in mice is compatible with a blockade of Th-cell activation and migration to LN. The reduction in IFN-γ release observed in EPA-treated mice explains the diminished inflammation encountered in EPA-treated animals, and correlates with the impairment of Th1 responses caused by EP antagonism that we have observed *in vitro*. But the most striking feature of EPA-treated mice was a failure to increase LN cellularity, and a pronounced decrease in inflammatory cytokine production by LN cells. A reduction in IFN-γ, TNF-α and IL-17 further substantiates a protective role for EPA treatment, as Th1 and Th17 cells have been shown to contribute to the immunopathogenesis of arthritis,[9] whereas TNF is one of the major cytokines that promotes inflammation in arthritis.[32]

Accordingly, T cells from mice in the EPA preventive setting showed an impaired activation profile following *in vitro* re-stimulation with CII. In addition, the amount of CII-specific IFN-γ-positive Th1 cells encountered in draining LNs of these mice was consequently reduced. This is in accordance with the observed *in vitro* reduction in Th1 polarization of EPA-treated $CD4^+$ T cells.

In summary, we have proved that signaling through EP receptors in T cells provides co-stimulatory signals required for their complete activation, and more importantly, they are necessary for maintaining stable interactions between T cells and DCs, thereby favouring efficient T-cell priming and activation with pathophysiological consequences. This observation further emphasizes that signaling through EP receptors in the initial phase of arthritis might be important for optimal T-cell activation and, hence, disease pathogenesis.

METHODS

Ethics statement

The animal research described in this manuscript complied with Spanish (Law 32/2007) and European Union legislation (2010/63/UE). The animal experiments reported were approved by the Institutional Animal Care and Use Committee of the Centre for Molecular Biology Severo Ochoa.

Animals

B6.129/J and DBA1/J mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). C57BL/6-Tg(TcraTcrb)425Cbn/J (OT-II) mice were a kind gift from Dr Francisco Sánchez-Madrid (Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain). C57BL/6-Tg(Ptger2)Tm1Sna (EP2 knockout) mice and B6.129-Tg(Ptger4)Tm1Sna (EP4 knockout) mice were a kind gift from Dr Shu Narumiya (Kyoto University, Kyoto, Japan). Because EP4 knockout mice do not survive on a C57BL/6 background, they were maintained on a mixed background of 129/Ola × C57BL/6. All animals were fed standard rodent chow and water *ad libitum* in a pathogen-free environment.

Antibodies and reagents

FITC-anti-mouse CD69, PE-anti-mouse CD71, APC-anti-mouse CD44, PE-anti-MHC-II, FITC-anti-CD86, APC-anti-IFN-γ, APC-anti-CD4, anti-EEA-1, anti-human CD28, anti-mouse CD3ε, anti-mouse CD28 and heat-killed *Mycobacterium tuberculosis* were obtained from BD Pharmingen (San Diego, CA, USA). Anti-human CD3, biotin-anti-CD11c, APC-anti-CD25, PE-anti-Foxp3 and anti-CD62L were from eBioscience (San Diego, CA, USA). Anti-β-actin, anti-GATA-3 and anti-T-bet were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-phosphoSer473-Akt and anti-AKT were from Cell Signaling Technology (Danvers, MA, USA). Anti-human/mouse EP2 and EP4, anti-S1P1 and S1P3 and PGE_2 were from Cayman Chemicals (Ann Arbor, MI, USA).

EPAs AH6809 and AH23848, LPS from *Escherichia coli* (serotype O26:B6), propidium iodide, complete and incomplete Freund's adjuvant were purchased from Sigma-Aldrich (St Louis, MO, USA). OVA323–339 peptide was obtained from Genscript (Piscataway, NJ, USA). Recombinant mouse TNF-α was from R&D systems (Minneapolis, MN, USA). Recombinant mouse IFN-γ, IL-12, IL-2, TGF-β1 and IL-4 were from Biosource International (New York, NY, USA). CFSE, CellTracker Blue CMAC (7-amino-4-chloromethylcoumarin) and CellTracker Orange CMTMR were purchased from Invitrogen, Life technologies (Grand Island, NY, USA).

The anti-collagen II mouse IgG ELISA detection kit was purchased from MD Bioproducts (Zurich, Switzerland). The PGE_2 Enzyme immuno assay kit (monoclonal) was purchased from Cayman Chemicals. IL-2, IL-12, IFN-γ, TNF-α IL-6, IL-17 and IL-4 in cell culture supernatants or in serum samples were evaluated using ELISA kits from R&D systems.

Isolation of naïve $CD4^+$ $CD62L^+$ T cells from mouse spleens and human peripheral blood

Naïve mouse and human $CD4^+$ T cells were purified from spleens of B6.129/J mice and human peripheral blood mononuclear cells, respectively, by negative selection in an autoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany), using the $CD4^+$ $CD62L^+$ naïve T-cell isolation kit II for mouse or human cells (Miltenyi Biotec), according to the manufacturer's instructions. T cells were maintained in RPMI 1640 complete medium with 5% fetal bovine serum.

In vitro activation and treatment of T cells

$2–5 \times 10^6$ $CD4^+$ T cells were plated in six-well culture plates previously treated with 5 μg ml^{-1} anti-CD3 antibody. Anti-CD28 (1 μg ml^{-1}) was directly added to the cell suspension for indicated periods of time. Treatment of T cells with EPAs was done by adding them directly to the culture medium at a final concentration of 10 μM, incubating for 30 min at 37 °C, washing in PBS and resuspending the cells in fresh medium. This concentration was found to be sufficient to block PGE_2 signaling through EP receptors.

T-cell polarization cultures

5×10^6 mouse naïve $CD4^+$ T cells isolated from spleens of mice were activated with anti-CD3 and anti-CD28 antibodies as described before for 3 days under polarizing conditions. For polarization toward Th1 cells, recombinant mouse IL-12 (10 ng ml^{-1}), IL-2 (20 ng ml^{-1}) and IFN-γ (4 ng ml^{-1}) were added to the medium. Recombinant IL-4 at a concentration of (10 ng ml^{-1}) was used for polarization to Th2 cells. For Th17 cells, recombinant mouse TGF-1β (1 ng ml^{-1}) and recombinant mouse IL-6 (50 ng ml^{-1}) were added to the medium. For T_{Regs}, cells were activated for a period of 5 days in the presence of TGF-β1 at 2 ng ml^{-1}. Th1, Th2, Th17 cells and T_{Regs} were further stimulated for another 24 h with anti-CD3 and anti-CD28 antibodies. Only in the case of Th17 cells, IL-23 was added to the medium at a 10 ng ml^{-1} concentration, whereas Th1, Th2 and T_{Reg} cells did not receive any additional cytokine stimulation during this second stimulation period.

Proliferation assays

For radio-active (3H) thymidine incorporation assays, purified mouse $CD4^+$ naïve T cells were subject to activation in 96-well plates. After incubation for 24 h, 1 μCi (3H) thymidine (Amersham, Biosciences, Piscataway, NJ, USA) was added to each well. The cultures were harvested 18 h later and then processed

for measurement of incorporated radioactivity in a liquid scintillation counter. For CFSE dilution experiments, $CD4^+$ T cells were stained with 1 μM CFSE for 15 min, washed and subject to activation. Seventy-two hours later, fluorescence dilution corresponding to cell division was observed in FACSCanto II (Becton Dickinson, Franklin lakes, NJ, USA) and analyzed using FlowJo software (Version 6.4.1, Tree Star Inc., Ashland, OR, USA, 2005).

Flow cytometry

$CD4^+$ T cells were subject to the corresponding treatments and resuspended in PBS with 1% BSA and 1% fetal bovine serum, followed by incubation with the respective antibodies and isotype controls for 20 min at 4 °C. For intracellular markers, cells were fixed in 2% paraformaldehyde and permeabilized with 0.5% saponin for 15 min at 4 °C, before staining. T-bet and GATA-3 staining was done as described.[33] Intracellular IFN-γ staining was performed as explained before.[10] Data were collected in a FACSCanto II (Becton Dickinson) and analyzed using FlowJo software (Version 6.4.1, Tree Star Inc, 2005).

Analysis of protein expression and signaling by Immunoblotting

Total protein from cell lysates (15–20 μg) was separated in a 10% SDS-polyacrilamide gel and transferred to a nitrocellulose membrane. The membranes were blocked with 5% nonfat milk followed by incubation with corresponding antibodies overnight at 4 °C. Immuno-reactive bands were visualized using horseradish peroxidase–conjugated respective secondary antibodies and the chemiluminescent system ECL (Amersham Pharmacia Biotech). Blots were then washed and stripped for 20 min at 37 °C using Restore western blot stripping buffer (Thermo Scientific, Life Technologies, Grand Island, NY, USA) and probed using β-actin primary antibodies to confirm equal protein loading. Densitometric quantification of visualized bands was performed using the Quantity One software (Bio-Rad, Hercules, CA, USA).

Adoptive transfer experiments, homing and egress assay

5×10^6 purified $CD4^+$ $CD62L^+$ OT-II cells subject to different treatments were stained with 5 μM CFSE and were injected intravenously (i.v) into C57BL/6J mice. Recipient mice were immunized by subcutaneous injection of OVA323–339 (100 μg) and LPS (20 μg) in their footpads. Seventy-two hours later, draining LNs were obtained and cells were stained for CD44. The number of recovered $CFSE^+$ cells was evaluated by flow cytometry.

In the homing assays, EPA-treated and DMSO-treated $CD4^+$ OT-II T cells were stained with intravital dyes and injected into mice that received no stimulus, DC or OVA-pulsed DC immunization in the footpad. Five hours later, draining LNs were collected and processed. Relative number of adoptively transferred EPA-treated vs vehicle-treated control Th cells encountered in LN suspensions was analyzed by flow cytometry and expressed as a ratio. For the egress assays, recipient mice received i.v. injections of anti-CD62L (100 μg per mouse) to prevent further homing of T cells as described.[18] Twenty-four hours later, draining LNs were isolated and relative percentages of adoptively transferred T cells were determined using flow cytometry.

Two-photon intravital microscopy of popliteal LNs

BM-DCs were matured, pulsed with OVA323–339 peptide, labeled with 5 μM CellTracker Orange and injected subcutaneously in hind footpads (10^6 cells) of C57BL/6J mice. Eighteen hours later, purified $CD4^+$ T cells from OT-II mice were fluorescently labeled with 5 μM cell tracker green CFSE (EPA-treated) or 20 μM cell tracker blue CMAC (DMSO treated) and injected i.v. into recipient mice (2.5×10^6 cells from each subset). Mice were anesthetized and the popliteal LN was surgically exposed. Ten micrograms of Alexa 633-MECA79 were injected i.v. to label high endothelial venules. Two-photon laser scanning microscopy (2PM) was performed with an Olympus BX50WI fluorescence microscope equipped with a TrimScope 2PM system controlled by ImSpector software (LaVision Biotec GmbH, Bielefeld, Germany). For four-dimensional analysis of cell interactions, 11 to 16 *x–y* sections with z-spacing of 4 μm were acquired every 20 s for 30 min. Emitted light and second harmonic signals were detected through 447/55-nm, 525/50-nm, 593/40-nm and 655/40-nm bandpass filters with nondescanned detectors to generate four-color images. Sequences of image stacks were transformed into volume-rendered four-dimensional movies with Volocity software (PerkinElmer, Waltham, MA, USA), which was also used for tracking of cell interactions in four dimensions. T cell–DC interactions were analyzed within the first 4 h after T-cell transfer (early interactions) and 24 h after adoptive transfer of T cells (late interactions). Interactions between T cells and DCs were defined as the physical contacts observed in the three-dimensional grid of the software. Time of contact was also calculated.

Induction of CIA in mice

DBA1/J female mice received 100 μg of chicken CII emulsified in CFA intradermally at the base of the tail. Twenty-one days after the first immunization, mice were boosted with chicken CII emulsified in Freund's incomplete adjuvant (IFA). EPAs were administered i.p. at 10 mg kg^{-1} per mouse starting from day 14, every 48 h until day 20. The evolution of arthritis was graded according to a subjective scoring system applied to each limb, using a scale of 0–4, with 4 being the most severe inflammation. The clinical scores of all limbs were summed to represent the total arthritic score in each case. The day after immunization with the booster dosage of CII+IFA was considered as day 1 and scores were recorded periodically until day 15 after booster dosage. On the last day of the experiment, mice were killed and hindlimbs and forelimbs were fixed for histological processing. Serum samples and draining inguinal LNs were also collected at this time.

In vitro stimulation of cells from draining inguinal LNs in the CIA setting

Cells were cultured in plates coated with 1 μg ml^{-1} of anti-CD3 antibody and mixed with 1 μg ml^{-1} of soluble anti-CD28 antibody and 100 μg ml^{-1} of boiled CII (denatured at 100 °C for 10 min) for 3 days. Supernatants were collected and analyzed for cytokine expression. To achieve the intracellular staining of IFN-γ, cells were treated with 1 μl ml^{-1} of Brefeldin A for the last 4 h of activation.

Statistical analysis

Data were analyzed using Prism software (Graph Pad, Inc, Version 5.0, La Jolla, CA, USA). One-way or two-way analysis of variance was used for statistical analysis, unless indicated otherwise. Turkey's multiple comparison post tests and Bonferroni's post tests were utilized, respectively. *P* values were calculated in order to determine significance: $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This manuscript has been supported by grants SAF2007-61716 and S-SAL-0159/2006 awarded by the Spanish Ministry of Science and Education and the Community of Madrid to Dr M Fresno. We thank María Chorro and Beatriz Berrocal for their excellent technical assistance.

1 Tilley SL, Coffman TM, Koller BH. Mixed messages: Modulation of inflammation and immune responses by prostaglandins and thromboxanes. *J Clin Invest* 2001; **108**: 15–23.
2 Harris SG, Padilla J, Koumas L, Ray D, Phipps RP. Prostaglandins as modulators of immunity. *Trends Immunol* 2002; **23**: 144–150.
3 Lawrence T, Willoughby DA, Gilroy DW. Anti-inflammatory lipid mediators and insights into the resolution of inflammation. *Nat Rev Immunol* 2002; **2**: 787–795.
4 Sugimoto Y, Narumiya S. Prostaglandin E receptors. *J Biol Chem* 2007; **282**: 11613–11617.
5 Alfranca A, Iniguez MA, Fresno M, Redondo JM. Prostanoid signal transduction and gene expression in the endothelium: role in cardiovascular diseases. *Cardiovasc Res* 2006; **70**: 446–456.
6 Boniface K, Bak-Jensen KS, Li Y, Blumenschein WM, McGeachy MJ, McClanahan TK *et al*. Prostaglandin E2 regulates Th17 cell differentiation and function through cyclic AMP and EP2/EP4 receptor signaling. *J Exp Med* 2009; **206**: 535–548.
7 Chemnitz JM, Driesen J, Classen S, Riley JL, Debey S, Beyer M *et al*. Prostaglandin E2 impairs CD4+ T cell activation by inhibition of lck: implications in hodgkin's lymphoma. *Cancer Res* 2006; **66**: 1114–1122.

8 Naito Y, Endo H, Arai K, Coffman RL, Arai N. Signal transduction in th clones: Target of differential modulation by PGE2 may reside downstream of the PKC-dependent pathway. *Cytokine* 1996; **8**: 346–356.
9 Chen Q, Muramoto K, Masaaki N, Ding Y, Yang H, Mackey M *et al.* A novel antagonist of the prostaglandin E(2) EP(4) receptor inhibits Th1 differentiation and Th17 expansion and is orally active in arthritis models. *Br J Pharmacol* 2010; **160**: 292–310.
10 Yao C, Sakata D, Esaki Y, Li Y, Matsuoka T, Kuroiwa K *et al.* Prostaglandin E2-EP4 signaling promotes immune inflammation through Th1 cell differentiation and Th17 cell expansion. *Nat Med* 2009; **15**: 633–640.
11 Iniguez MA, Punzon C, Fresno M. Induction of cyclooxygenase-2 on activated T lymphocytes: Regulation of T cell activation by cyclooxygenase-2 inhibitors. *J Immunol* 1999; **163**: 111–119.
12 Kundu N, Ma X, Holt D, Goloubeva O, Ostrand-Rosenberg S, Fulton AM. Antagonism of the prostaglandin E receptor EP4 inhibits metastasis and enhances NK function. *Breast Cancer Res Treat* 2009; **117**: 235–242.
13 Sinha P, Clements VK, Fulton AM, Ostrand-Rosenberg S. Prostaglandin E2 promotes tumor progression by inducing myeloid-derived suppressor cells. *Cancer Res* 2007; **67**: 4507–4513.
14 Cope AP. T cells in rheumatoid arthritis. *Arthritis Res Ther* 2008; **10** (Suppl 1): S1.
15 Honda T, Segi-Nishida E, Miyachi Y, Narumiya S. Prostacyclin-IP signaling and prostaglandin E2-EP2/EP4 signaling both mediate joint inflammation in mouse collagen-induced arthritis. *J Exp Med* 2006; **203**: 325–335.
16 Myers LK, Kang AH, Postlethwaite AE, Rosloniec EF, Morham SG, Shlopov BV *et al.* The genetic ablation of cyclooxygenase 2 prevents the development of autoimmune arthritis. *Arthritis Rheum* 2000; **43**: 2687–2693.
17 Sreeramkumar V, Fresno M, Cuesta N. Prostaglandin E2 and T cells: Friends or foes? *Immunol Cell Biol* 2012; **90**: 579–586.
18 Nombela-Arrieta C, Lacalle RA, Montoya MC, Kunisaki Y, Megías D, Marqués M *et al.* Differential requirements for DOCK2 and phosphoinositide-3-kinase gamma during T and B lymphocyte homing. *Immunity* 2004; **21**: 429–441.
19 Mauri C, Williams RO, Walmsley M, Feldmann M. Relationship between Th1/Th2 cytokine patterns and the arthritogenic response in collagen-induced arthritis. *Eur J Immunol* 1996; **26**: 1511–1518.
20 Nakae S, Nambu A, Sudo K, Iwakura Y. Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice. *J Immunol* 2003; **171**: 6173–6177.
21 Marinova-Mutafchieva L, Williams RO, Mason LJ, Mauri C, Feldmann M, Maini RN. Dynamics of proinflammatory cytokine expression in the joints of mice with collagen-induced arthritis (CIA). *Clin Exp Immunol* 1997; **107**: 507–512.
22 Diaz-Munoz MD, Osma-Garcia IC, Fresno M, Iniguez MA. Involvement of PGE2 and the cAMP signalling pathway in the up-regulation of COX-2 and mPGES-1 expression in LPS-activated macrophages. *Biochem J* 2012; **443**: 451–461.
23 Chizzolini C, Chicheportiche R, Alvarez M, de Rham C, Roux-Lombard P, Ferrari-Lacraz S *et al.* Prostaglandin E2 synergistically with interleukin-23 favors human Th17 expansion. *Blood* 2008; **112**: 3696–3703.
24 Sakata D, Yao C, Narumiya S. Prostaglandin E2, an immunoactivator. *J Pharmacol Sci* 2010; **112**: 1–5.
25 Li H, Edin ML, Gruzdev A, Cheng J, Bradbury JA, Graves JP *et al.* Regulation of T helper cell subsets by cyclooxygenases and their metabolites. *Prostaglandins Other Lipid Mediat* 2013; **104-105**: 74–83.
26 Mahic M, Yaqub S, Johansson CC, Tasken K, Aandahl EM. FOXP3+CD4+CD25+ adaptive regulatory T cells express cyclooxygenase-2 and suppress effector T cells by a prostaglandin E2-dependent mechanism. *J Immunol* 2006; **177**: 246–254.
27 Rudd CE, Taylor A, Schneider H. CD28 and CTLA-4 coreceptor expression and signal transduction. *Immunol Rev* 2009; **229**: 12–26.
28 Baratelli F, Lin Y, Zhu L, Yang SC, Heuzé-Vourc'h N, Zeng G *et al.* Prostaglandin E2 induces FOXP3 gene expression and T regulatory cell function in human CD4+ T cells. *J Immunol* 2005; **175**: 1483–1490.
29 Sharma S, Yang SC, Zhu L, Reckamp K, Gardner B, Baratelli F *et al.* Tumor cyclooxygenase-2/prostaglandin E2-dependent promotion of FOXP3 expression and CD4+ CD25+ T regulatory cell activities in lung cancer. *Cancer Res* 2005; **65**: 5211–5220.
30 Lee BP, Juvet SC, Zhang L. Prostaglandin E2 signaling through E prostanoid receptor 2 impairs proliferative response of double negative regulatory T cells. *Int Immunopharmacol* 2009; **9**: 534–539.
31 McCoy JM, Wicks JR, Audoly LP. The role of prostaglandin E2 receptors in the pathogenesis of rheumatoid arthritis. *J Clin Invest* 2002; **110**: 651–658.
32 Feldmann M, Elliott MJ, Woody JN, Maini RN. Anti-tumor necrosis factor-alpha therapy of rheumatoid arthritis. *Adv Immunol* 1997; **64**: 283–350.
33 Martin P, Villares R, Rodriguez-Mascarenhas S, Zaballos A, Leitges M, Kovac J *et al.* Control of T helper 2 cell function and allergic airway inflammation by PKCzeta. *Proc Natl Acad Sci USA* 2005; **102**: 9866–9871.

The Supplementary Information that accompanies this paper is available on the Immunology and Cell Biology website (http://www.nature.com/icb)