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Prostaglandin E2 enhances Th17 responses *via* modulation of IL-17 and IFN-γ production by memory CD4$^+$ T cells

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The contribution of Th1 and Th17 cells in chronic inflammatory conditions leading to autoimmunity remains highly controversial. In inflamed tissues, production of prostaglandins by COX-2 has been proposed to favor Th17 responses indirectly by increasing IL-23 and blocking IL-12 release from APC. We report here that prostaglandin E2 (PGE2) can directly modulate cytokine production by human memory CD4$^+$ T cells. TCR triggering in the presence of PGE2 increased IL-17 and reduced IFN-γ production by freshly isolated memory T cells or T-cell clones. PGE2 triggered the EP2 and EP4 receptors expressed on T cells leading to a rapid increase of retinoic-acid-related orphan receptor-γt (ROR-γt) and decrease of T-cell-specific T-box transcription factor 21 (T-bet) mRNA. Moreover, PGE2 promoted the selective enrichment of IL-17-producing cells by differentially modulating the proliferation of memory T-cell subsets *in vitro*. Taken together our results indicate that T-cell effector function is a direct target for PGE2 modulation and suggest a novel mechanism by which inhibitors of prostaglandin synthesis, such as COX-2 inhibitors, exert their anti-inflammatory effect.

Key words: IFN-γ · IL-17 · Memory T cells · Prostaglandin · Th17

Supporting Information available online

Introduction

IL-17-producing T cells (Th17) have been recently characterized as a distinct lineage of CD4$^+$ Th cells. IL-17 and Th17 cells have been shown to mediate protection against extracellular pathogens by promoting neutrophil recruitment [1–4] but also to cause immunopathology in different models of autoimmunity [5]. Differentiation of mouse Th17 cells from uncommitted naïve T-cell precursors is controlled by the lineage-specific transcription factors retinoic-acid-related orphan receptor-γt (ROR-γt) [6] and ROR-α [7], is promoted by an IL-21-autocrine loop triggered by TGF-β and IL-6 [8, 9] and inhibited by Th1- or Th2-promoting stimuli, such as IL-12, IFN-γ and IL-4. Human Th17 cell differentiation is associated with increased expression of ROR-γ, is induced by IL-1β and enhanced by IL-6 and IL-23 [10, 11] while requirement for TGF-β is still debated and may be different depending on experimental conditions [12–14]. Both *in vitro*-differentiated human and mouse Th17 cells selectively upregulate expression of the chemokine receptor CCR6 [15, 16]. CCR6 is also expressed on circulating human memory Th17 cells and it has been shown to mediate recruitment of pathogenic Th17 cells in a mouse arthritis model [16].

The notion that Th17 cells are implicated in the onset and maintenance of autoimmune diseases is supported by compelling evidence. In mice, transfer of Th17 but not Th1 cells induce EAE

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[17] and mice lacking IL-17 or treated with IL-17-blocking antibodies have delayed disease onset or much reduced inflammation in EAE or collagen-induced arthritis (CIA) [18–22]. In humans, IL-17 and IL-17-producing cells have been identified in active lesions in the brains of patients with MS and in other chronic inflamed tissues [23–25] while Th17-associated factors such as IL-17, IL-22, ROR-γt and IL-23 are highly expressed in psoriatic lesions [11] and have been associated with ulcerative colitis and Crohn disease [26–28].

Before identification of Th17 cells, Th1 cells and the Th1-associated cytokine IFN-γ were thought to play a pathogenic role in autoimmune diseases [29]. However, the findings that IFN-γ- and IFN-R-deficient mice had higher susceptibility to EAE and CIA compared with wild-type mice [30–32] and that administration of neutralizing antibodies to IFN-γ exacerbated MS in human patients [33] were pointing to an anti-inflammatory activity of IFN-γ both in the onset and in the progression of at least some autoimmune diseases. In a recent study the increased susceptibility of IFN-γ- and IFN-R-deficient mice to autoimmune diseases correlated with an increased number of IL-17-producing T cells [34], suggesting that one possible mechanism by which IFN-γ exerts its anti-inflammatory activity is through inhibition of Th17 cell differentiation and function. Notably, in humans, high IL-17 and low IFN-γ concentration in inflammatory arthritis are linked to the development of rheumatoid arthritis (RA), while high IFN-γ and low IL-17 correlated with non-rheumatoid persistent synovitis [35]. Thus, a dampening of Th1 together with an increase in Th17 responses can contribute to the shift from a balanced self-resolving inflammatory reaction to a chronic pathological inflammation; however, what could lead to a differential modulation of Th1 and Th17 responses is poorly understood. Paradoxically high Th1 and low Th17 cell numbers are found in synovial fluid of patients with established RA [36], whereby relatively low concentrations of IFN-γ [37] and high concentrations of IL-17 are detected [38, 39].

Although the mechanisms underlying this discrepancy are unknown, it is likely that the bias towards IL-17 production in a Th1-rich environment might depend on the presence of inflammatory mediators able to locally modulate cytokine release by already differentiated Th1 and Th17 cells. Several immunomodulatory molecules are abundant in chronically inflamed tissues and may directly or indirectly influence the release of IL-17 and IFN-γ. IL-23, IL-1 and IL-6 promote differentiation and expansion of human Th17 cells [10, 11, 40] whereas high concentrations of TGF-β inhibit Th1 responses *in vivo* [41] and both Th1 and Th17 responses *in vitro* [12]. TNF, prostaglandin E2 (PGE2) and IL-15 are abundant in chronically inflamed tissues where they sustain the inflammatory process. However, their possible role in modulating the IL-17/IFN-γ balance has not been investigated. TNF neutralization [42] and inhibition of PGE2 synthesis by means of COX-2 inhibitors are standard treatments in chronic inflammatory diseases [43] while a novel strategy involving IL-15 neutralization has been proposed [44].

In this context, we set out to determine whether some of the inflammatory mediators abundant in inflamed tissues are able to enhance IL-17 secretion by locally influencing T-cell effector responses, skewing *in situ* the Th1/Th17 balance towards a pathogenic Th17 response.

Results

PGE2 modulates IL-17 and IFN-γ release acting directly on memory CD4$^+$ T cells

To address whether soluble factors found in chronically inflamed tissues are able to modulate release of effector cytokines by immune cells, we stimulated total PBMC from healthy donors with the bacterial superantigen toxic shock syndrome toxin 1 (TSST-1) in the absence or presence of different inflammatory mediators and measured IL-17 and IFN-γ release in culture supernatants. As shown in Fig. 1A, IL-6 and TNF had no effect on IL-17 or IFN-γ release by TSST-stimulated PBMC. IL-1β increased production of both IFN-γ and IL-17, whereas IL-12 and IL-15 selectively increased IFN-γ production and IL-23 selectively increased production of IL-17. Strikingly, PGE2 had opposite effects since compared with untreated controls it increased IL-17 production ($p = 0.0006$) and, at the same time, strongly reduced release of IFN-γ ($p = 0.0153$). Inhibition of endogenous PGE2 production by addition to PBMC cultures of the COX inhibitor indomethacin (INDO) also resulted in reduced release of IL-17 compared with untreated controls ($p = 0.0036$), although in this case IFN-γ production was not affected (Fig. 1B and Supporting Information Fig. S1).

Since PGE2 is able to enhance secretion of IL-23 by dendritic cells [45], we asked whether the effects observed above were indirectly mediated by IL-23. To address this, PBMC were stimulated with TSST-1 in the absence or presence of PGE2 and blocking antibodies to IL-23 or antibodies to IL-12p40 that block both IL-12 and IL-23. In the absence of PGE2, neutralization of IL-12p40 strongly inhibited IFN-γ release without affecting IL-17, indicating that reduced production of IFN-γ was not sufficient *per se* to induce increased production of IL-17 by TSST-stimulated PBMC (Fig. 1C). Addition of PGE2 increased IL-17 production even in the presence of IL-23- or IL-12p40-blocking antibodies, indicating that its effect was independent from IL-23. Blocking antibodies to IL-1β or IL-6 had also no effect on the ability of PGE2 to increase IL-17 production, although they limited accumulation of IL-17 in PBMC culture supernatants. The above results suggested the possibility that PGE2 could affect IL-17 and IFN-γ production by acting directly on T cells. To assess this possibility, highly purified CD4$^+$CD45RA$^-$CD25$^-$ memory T cells were stimulated with plate-bound CD3 and CD28 antibodies in the presence of increasing concentrations of PGE2. As shown in Fig. 1D, PGE2 increased IL-17 and decreased IFN-γ production by activated T cells over a wide range of concentrations. Interestingly, PGE2 inhibited production of IL-22, a cytokine that has been associated with Th17 responses, suggesting that these two cytokines are differentially modulated.

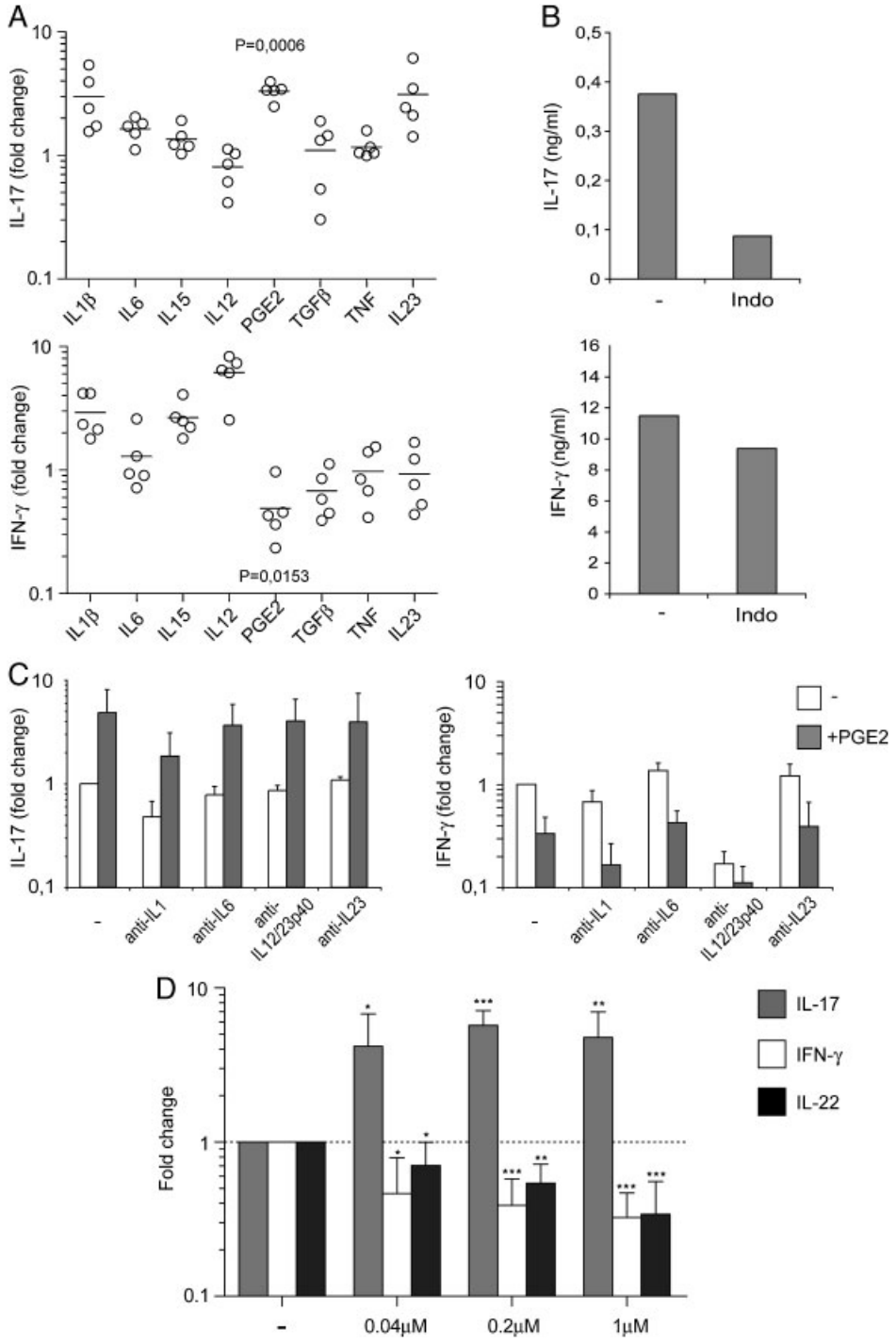

Figure 1. PGE2 distinctly affects IL-17 and IFN-γ release by PBMC and memory $CD4^+$ T cells. (A) Total PBMC were stimulated with 10 ng/mL TSST-1 with or without the indicated immunomodulatory molecules. IL-17 and IFN-γ were measured in the 36 h supernatant and cytokine levels are shown as fold change compared with TSST-1 stimulation in the absence of additional immunomodulators. Each symbol represents cells obtained from different donors. *p*-Value was calculated using one-sample *t*-test of the fold change in the presence of PGE2 with a hypothetical mean value of 1 (TSST-1alone). (B) Total PBMC were stimulated with TSST-1 in the presence or absence of 10 μM INDO. Cytokines were measured in the 36 h supernatant and results are depicted as mean of duplicate cultures. One representative donor out of four is shown. (C) Total PBMC were stimulated for 36 h with TSST-1 with or without the indicating neutralizing antibodies in the presence or absence of 0.5 μM PGE2. For each condition the fold change compared with stimulation with TSST-1 alone is plotted and data represent means+SD of five different donors. (D) $CD4^+CD45RA^-CD25^-$ total memory T cells were stimulated for 36 h with anti-CD3/anti-CD28 coated plates in the presence of the indicated concentration of PGE2. IL-17, IFN-γ and IL-22 concentrations were measured in the 36 h supernatant and data are depicted as fold change compared with cells stimulated in the absence of PGE2. Data represent mean+SD of six different donors. *p*-Values of one-sample *t*-test of the fold changes in the presence of PGE2 with a hypothetical mean value of 1 (anti-CD3, anti-CD28 alone) are $^*p<0.1$, $^{**}p<0.01$, $^{***}p<0.001$.

PGE2 affects the early transcription of IL-17, ROR-γt, IFN-γ and T-bet

We then investigated the features of PGE2-mediated modulation of IL-17 and IFN-γ production. We asked whether combination of PGE2 with other cytokines could further modulate IL-17 and IFN-γ release by memory T cells. Addition of IL-23, TGF-β and IL-4 had no effect whereas IL-1β plus IL-6 synergized with PGE2 to enhance IL-17 production without affecting IFN-γ production. In contrast, IL-12 did not influence PGE2-mediated increase of IL-17 but completely abrogated PGE2-mediated inhibition of IFN-γ (Fig. 2A and Supporting Information Fig. S2).

Next, we asked whether the continuous presence of PGE2 in the culture medium was needed for modulation of cytokine release. To address this, we stimulated total memory $CD4^+$ T cells with immobilized CD3 and CD28 antibodies in the presence of PGE2 for 2 h. Cells were then washed and re-plated in CD3 and CD28 antibody-coated plates in the presence or absence of PGE2 for the remaining time of stimulation. Evaluation of the levels of IL-17 and IFN-γ indicated that 2 h stimulation in the presence of PGE2 was sufficient to increase IL-17 production but was not sufficient to downregulate IFN-γ production (Supporting Information Fig. S3A).

Differentiation of Th1, Th2 and Th17 cells is controlled by the master transcription factors T-cell-specific T-box transcription factor 21 (T-bet), GATA-binding factor 3 (GATA-3) and ROR-γt, respectively [46]. Expression of these transcription factors is maintained in resting memory Th1, Th2 and Th17 cells and is further upregulated upon stimulation. T-bet and ROR-γt have been shown to directly control transcription of IFN-γ [47] and IL-17 [48], respectively. Thus, we asked whether PGE2 affects production of IL-17 and IFN-γ through modulation of these transcription factors. Memory T cells were stimulated in the absence or presence of PGE2 and the abundance of mRNA for ROR-γt, T-bet and GATA-3 as well as for IL-17 and IFN-γ and IL-4 was measured by quantitative PCR 4 h after stimulation (Fig. 2B and data not shown). PGE2 increased expression of ROR-γt and decreased the expression of T-bet mRNA. This effect correlated with increased amounts of IL-17 mRNA and decreased amounts of IFN-γ mRNA. GATA-3 and IL-4 transcripts were comparable to untreated controls (data not shown). In conclusion, PGE2 has a rapid effect on IL-17 and IFN-γ transcription, which correlates with early modulation of the master transcription factors ROR-γt and T-bet.

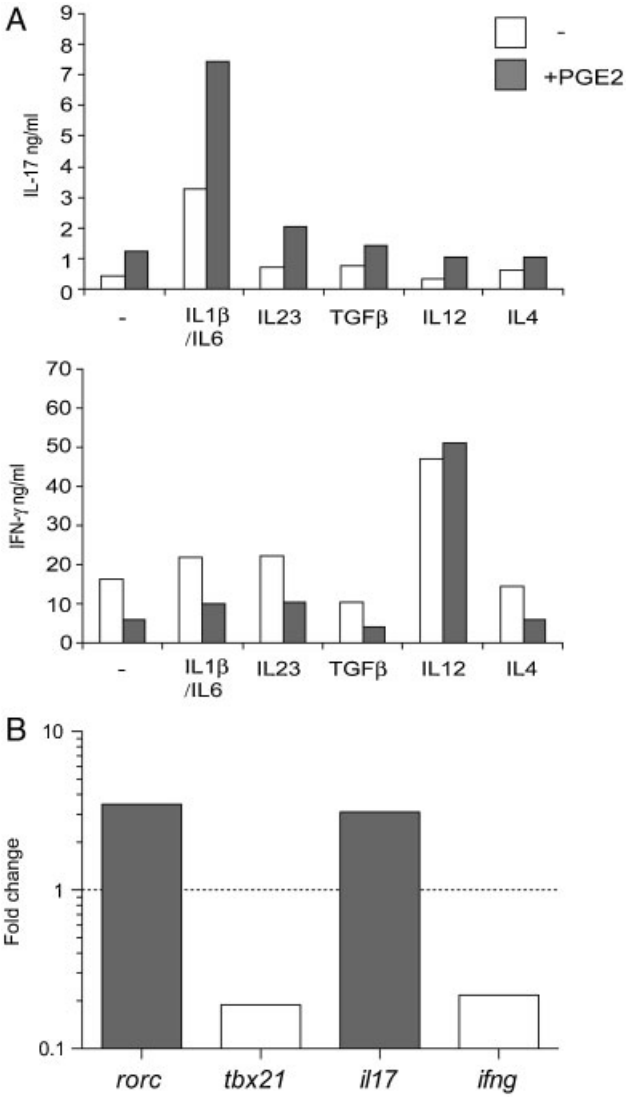

Figure 2. Modulation of cytokine production by PGE2. (A) $CD4^+$ $CD45RA^-CD25^-$ memory T cells were stimulated with anti-CD3/anti-CD28 coated plates and various cytokines in the presence or absence of PGE2. Cytokines were measured in the 36 h supernatants and results are displayed as mean of duplicate cultures. One representative donor out of four is shown. (B) $CD4^+CD45RA^-CD25^-$ memory $CD4^+$ T cells were stimulated for 4 h with anti-CD3/anti-CD28 coated plates in the presence or absence of 0.5 μM PGE2. Transcripts for *Ifng*, *Il17*, *tbx21* and *rorc* were determined by real-time PCR. The levels of transcripts in cells cultured in the presence of PGE2 are depicted as fold change compared with controls (cells cultured in the absence of PGE2). Results represent the mean of two donors.

PGE2 modulates cytokine production of Th-cell subsets

Recently, we described that functionally distinct human memory T-cell subsets can be identified by a differential expression of the chemokine receptors CCR6, CCR4 and CXCR3 [15]. Th17 cells express CCR6 together with CCR4, whereas Th2 cells express CCR4 but do not express CCR6. Th1 cells are distributed within two $CXCR3^+$ subsets: a $CCR6^+CXCR3^+$ subset that comprises also some IFN-γ/IL-17 double-producer T cells and a $CCR6^-CXCR3^+$ subset that includes some IFN-γ/IL-4 double-producers. By using chemokine receptors as surface markers to isolate functionally distinct memory T cells we asked whether PGE2 modulated cytokine production in different subsets and whether it could differentially affect cytokine production also in cells capable of producing both IL-17 and IFN-γ.

As shown in Fig. 3A and in Supporting Information Fig. S4, PGE2 increased IL-17 production by activated $CCR6^+CCR4^+$ Th17 cells and inhibited production of IFN-γ by $CCR6^-CXCR3^+$ Th1 cells. Moreover, PGE2 increased IL-17 and decreased IFN-γ production in the $CCR6^+CXCR3^+$ subset, which comprises IL-17/

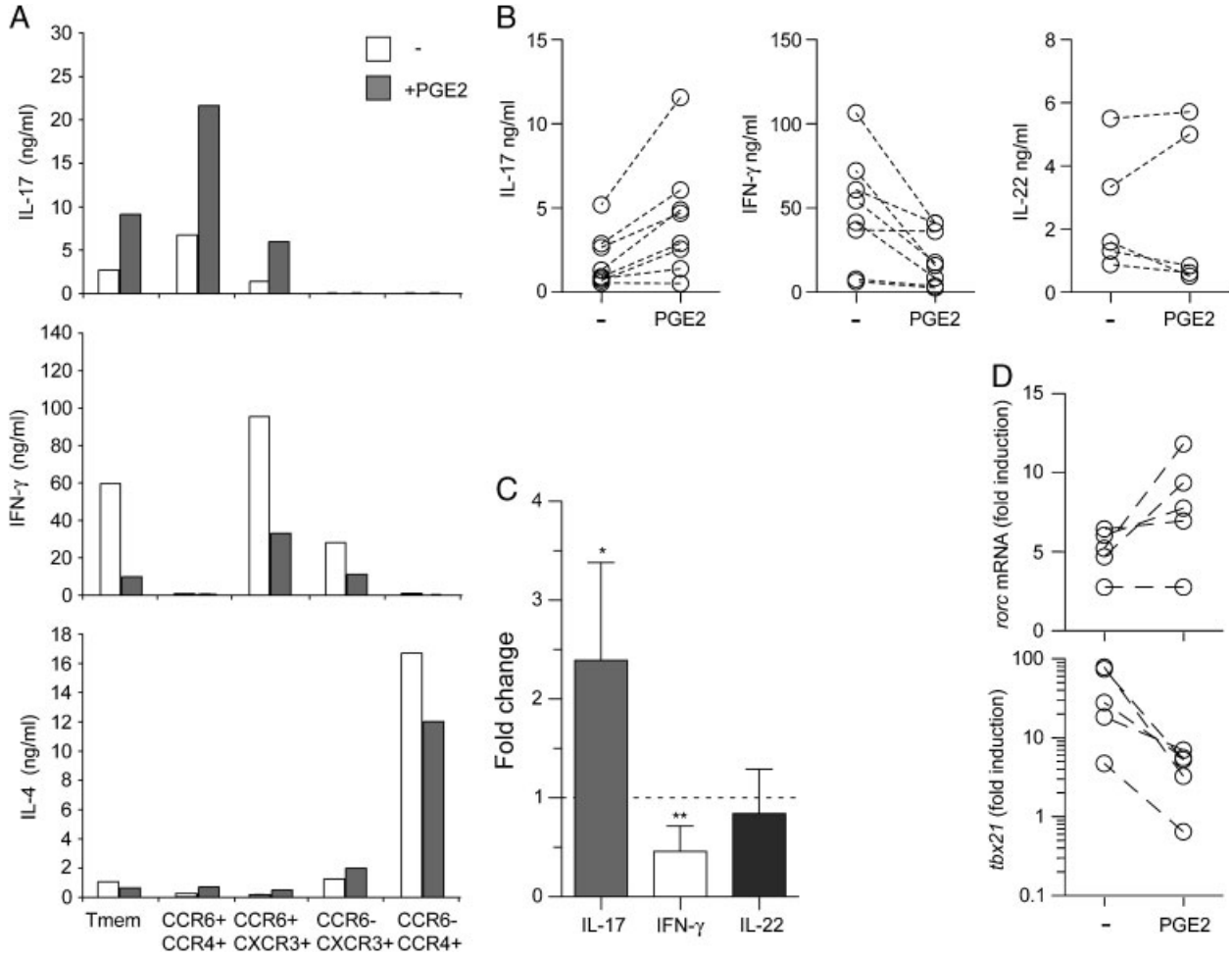

Figure 3. PGE2 directly modulates IL-17 and IFN-γ release by purified $CD4^+$ memory T-cell subsets and IL-17/IFN-γ double-producing clones. (A) Memory $CD4^+$ T-cell subsets sorted according to the expression of chemokine receptors CCR6, CCR4, CXCR3 were stimulated with anti-CD3/anti-CD28 coated plates in the presence or absence of 0.5 μM PGE2. IL-17, IFN-γ and IL-4 were determined by ELISA in the 36 h supernatant and are depicted as mean of duplicate cultures. One representative donor out of six is shown. (B) Five different IL-17, IFN-γ and IL-22 triple-producing clones and three IL-17, IFN-γ double-producing clones were stimulated with anti-CD3/anti-CD28 coated plates in the presence or absence of 0.5 μM PGE2. IL-17, IFN-γ and IL-22 concentrations were determined by ELISA in the 36 h supernatant. Each symbol represents a different clone. (C) Mean+SD of the fold changes of cytokines produced by clones stimulated in the presence of PGE2 compared with cells stimulated in the absence of PGE2 for all the clones stimulated in B. *p*-Values of one-sample *t*-test of fold changes in the presence of PGE2 with a hypothetical mean value of 1 (anti-CD3/anti-CD28 alone) are $^*p = 0.0053$, $^{**}p = 0.0006$. (D) Five different IL-17, IFN-γ double-producing clones were cultured for 4 h with anti-CD3/anti-CD28 coated plates in the presence or absence of 0.5 μM PGE2. Transcripts for *tbx21* and *rorc* were determined by real-time PCR in cultured (4 h) and non-cultured cells (0 h). Data show the fold induction of transcripts for *tbx21* and *rorc* in cultured cells in the presence or absence of PGE2 compared with non-cultured cells, each symbol represents an independent clone. *p*-Values of one-sample *t*-test of fold changes in the presence of PGE2 with a hypothetical mean value of 1 (anti-CD3/anti-CD28 alone) for *tbx21* is $p = 0.0002$, for *rorc* is $p = 0.109$.

IFN-γ double-producer T cells. Interestingly, PGE2 was not able to induce production of IL-17 by any of the $CCR6^-$ subsets. We then stimulated $CD4^+$ T-cell clones capable of producing both IL-17 and IFN-γ in the absence or presence of PGE2. PGE2 consistently increased IL-17, albeit to a lower extent compared with memory cells, and inhibited IFN-γ production by stimulated T-cell clones (Fig. 3B and C), suggesting that PGE2 signaling can concomitantly increase IL-17 transcription and decrease IFN-γ transcription within the same cell. IL-22, which was produced by some of the IL-17/IFN-γ double-producer T-cell clones, was not significantly inhibited by PGE2. Finally, we asked whether the differential regulation of IL-17 and IFN-γ in double-producer T-cell clones correlated with a modulation of the expression of ROR-γt and T-bet expression. T-bet upregulation was strongly inhibited by PGE2 treatment, while expression of ROR-γ*t* was moderately increased (Fig. 3D).

PGE2 differentially influences expansion of Th cell subsets

Upon encounter with cognate antigens memory T cells are activated and proliferate to give rise to an expanded pool of effector cells. T-cell expansion is regulated by several cytokines,

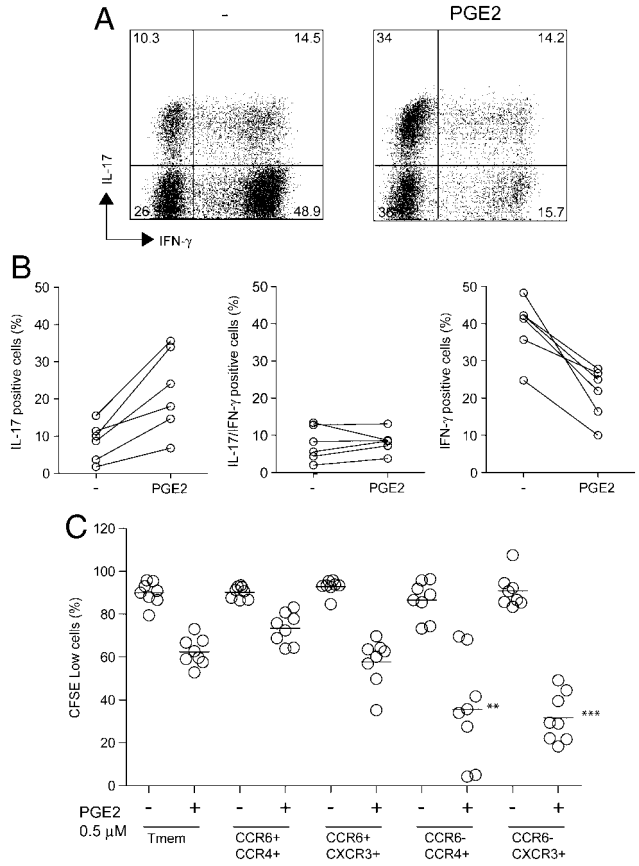

Figure 4. PGE2 favors the enrichment of Th17 cells by differentially modulating proliferation of T-cell subsets. (A) $CD4^+CD45RA^-CD25^-$ memory T cells were expanded with anti-CD3/anti-CD28 coated beads for 5 days in the presence of PGE2. Intracellular expression of IL-17 and IFN-γ was determined by flow cytometry after 5 h stimulation with PMA/ionomycin. Plots are representative of six independent experiments. (B) Percentage of cytokine-producing cells determined as in (A), from six different donors. *p*-Values calculated using a one-sample *t*-test of the fold changes of the percentage of cytokine-producing cells in the presence of PGE2 with a hypothetical mean value of 1 (anti-CD3/anti-CD28 coated beads alone) $p = 0.0034$, for IL-17-producing cells; $p = 0.0008$ for IFN-γ-producing cells . (C) CFSE-labeled memory $CD4^+$ T-cell subsets were stimulated for 5 days with anti-CD3/anti-CD28 coated plates in the presence or absence of 0.5 μM PGE2. Percentages of proliferating cells that have diluted CFSE ($CFSE^{low}$) were determined by flow cytometry. Each symbol represents cells from a different donor. Percentage of $CFSE^{low}$ cells in the $CCR4^+CCR6^-$ and $CXCR3^+CCR6^-$ cells cultured in the presence of PGE2 were significantly different from the percentage of $CFSE^{low}$ cells in the other subsets and $CD25^-CD45RA^-$ $CD4^+$ memory T cells cultured in the same condition as determined by one-way ANOVA followed by Bonferroni's multiple comparison test. $^{**}p<0.01$, $^{***}p<0.005$ compared with $CD25^-CD45RA^-$ $CD4^+$ memory T cells.

including IL-15, IL-2 and IL-7 [49]. We asked whether PGE2 had any effect on proliferation of memory T cells. First, we isolated total $CD4^+$ memory T cells and induced proliferation by polyclonal stimulation in the absence or presence of PGE2; the percentage of cytokine-producing cells was evaluated after 5 days of culture (Fig. 4A). Approximately a twofold increase in IL-17-producing cells and a twofold decrease in IFN-γ-producing cells were observed in cultures where PGE2 was added (Fig. 4B). No difference was observed in the percentage of IL-17/IFN-γ double-producing cells (Fig. 4B). The presence of PGE2 for the first 24 h of culture was sufficient to induce an increase in IL-17-producing cells and a decrease in IFN-γ-producing cells (Supporting Information Fig. S3B).

Interestingly, PGE2 markedly affected the percentage of IL-17 and IFN-γ-producing cells within the dividing population (Supporting Information Fig. S5); therefore, we next addressed whether this effect was dependent on a differential effect on the proliferation of the different memory T-cell subsets. Sorted $CCR6^+CCR4^+$, $CCR6^+CXCR3^+$, $CCR6^-CXCR3^+$ and $CCR6^-$ $CCR4^+$ memory T-cell subsets were stained with CFSE and stimulated with plate-bound CD3 and CD28 antibodies in the absence or presence of PGE2. As shown in Fig. 4C, PGE2 inhibited proliferation of all memory T-cell subsets. However, the $CCR6^-$ subsets showed higher susceptibility to PGE2 inhibition than the $CCR6^+$ IL-17-producing subsets. This behavior may explain the increase induced by PGE2 of IL-17-producing cells after *in vitro* expansion of memory $CD4^+$ T cells.

EP2 and EP4 receptors mediate cytokine modulation by PGE2

Prostaglandins bind to four distinct receptors – EP1, EP2, EP3 and EP4 – that are coupled to distinct signaling pathways and are differentially expressed in different cell types [50, 51]. To determine which of these receptors mediate the effects of PGE2 in T cells we analyzed their expression by quantitative PCR and assessed the activity of receptor-specific agonists. The results in Fig. 5A show that EP2 and EP4 were expressed in human T cells whereas EP1 and EP3 were not detected. The pattern of receptor expression was consistent with the findings that sulprostone, an EP1- and EP3-specific agonist, did not affect production IL-17 or IFN-γ by T cells (Fig. 5B), and butaprost and OH-PGE1, which are EP2 and EP4 selective agonists, respectively, increased IL-17 and decreased IFN-γ production, although not as efficiently as PGE2. We asked whether the different susceptibilities of the distinct T-cell subsets to the inhibitory effect of PGE2 on TCR-mediated proliferation (Fig. 4) correlated with a differential expression of EP2 and EP4 receptors. No relevant differences were observed in the expression of EP2 and EP4 in the $CCR6^+CCR4^+$, $CCR6^+$ $CXCR3^+$, $CCR6^-CCR4^+$ and $CCR6^-CXCR3^+$ memory T-cell subsets (Fig. 5C). This is consistent with the observation that PGE2 treatment affects either cyotkine production or cell proliferation or both in all the different subsets. Thus, the partial resistance of the $CCR6^+$ subsets to the inhibitory effects of PGE2 on proliferation might depend on factors downstream the EP receptors.

IFN-β inhibits IL-17 but not IFN-γ release by purified memory $CD4^+$ T cells

PGE2 is a proinflammatory mediator involved in chronic inflammation and COX inhibitors that block PGE2 synthesis are

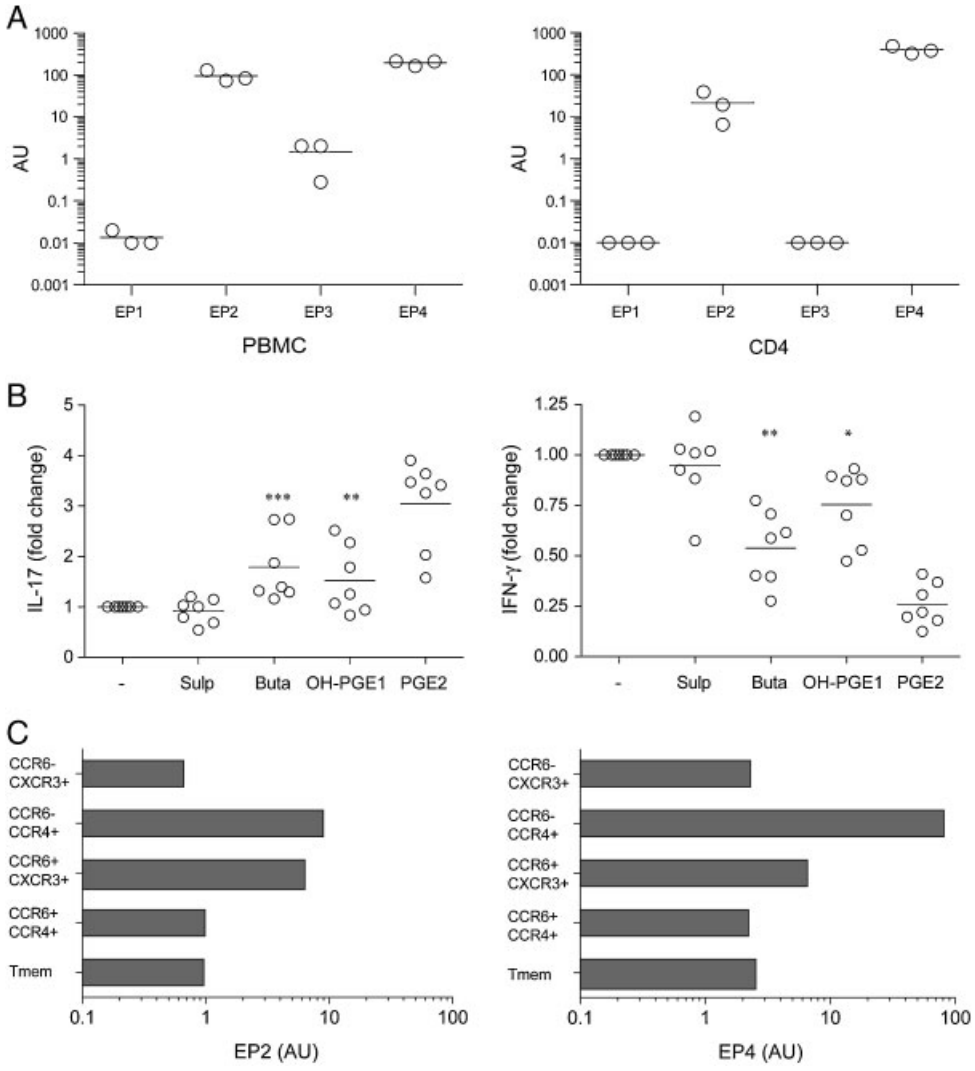

Figure 5. PGE2 modulation of T-cell activation is mediated by the EP2 and EP4 receptors. (A) The abundance of RNA encoding for EP1, EP2, EP3 and EP4 was determined by quantitative PCR in PBMC and purified memory $CD4^+$ T cells of three different donors. AU, arbitrary units. (B) Memory T cells were stimulated with anti-CD3/anti-CD28 coated plates in the presence or absence of PGE2 and EP1/EP3 agonist sulprostone, EP2 agonist butaprost and EP4 agonist OH-PGE1. Cytokines were measured in the 36 h supernatants and shown as fold change compared with cytokine concentration in vehicle-treated cultures. Each symbol represents cells from different donors. *p*-Values for cytokine production in the presence of the indicated agonists *versus* cells stimulated in the absence of any agonists of EP receptors: $^*p<0.1$, $^{**}p<0.05$, $^{***}p<0.001$. (C) The abundance of RNA encoding for EP2 and EP4 was determined by quantitative PCR in the total $CD25^-CD45RA^-CD4^+$ memory T cells and the different sorted subsets. Data are representative of four separate experiments.

currently used in the treatment of several autoimmune diseases. The above results showing that PGE2 has the unique capacity to increase IL-17 production and, at the same time, to decrease IFN-γ production by $CD4^+$ T cells may provide a possible mechanism by which inhibitors of PGE2 synthesis exert their beneficial effects in chronic inflammatory disease where IL-17 is believed to play an important role [43].

Treatment with recombinant IFN-β has been shown to reduce progression of disease in the relapsing remitting form of MS [52], but the mechanism by which IFN-β can limit central nervous system inflammation is not clear. Thus, we asked whether recombinant IFN-β was able to modulate release of IL-17 and IFN-γ by memory $CD4^+$ T cells limiting pathogenic Th17 responses and enhancing potentially beneficial Th1 responses. Purified total memory $CD4^+$ T cells were stimulated in the presence of increasing concentrations of IFN-β. A substantial inhibition of IL-17 but not of IFN-γ release was observed (Fig. 6A). However, when total PBMC were stimulated with TSST-1 in the presence of IFN-β, not only IL-17 but also IFN-γ release was strongly inhibited (Fig. 6B). These results suggest that IFN-β can differentially modulate cytokine production by $CD4^+$ memory T cell but that the anti-inflammatory effect of IFN-β might be only partially explained by its direct effect on T-cell cytokine release.

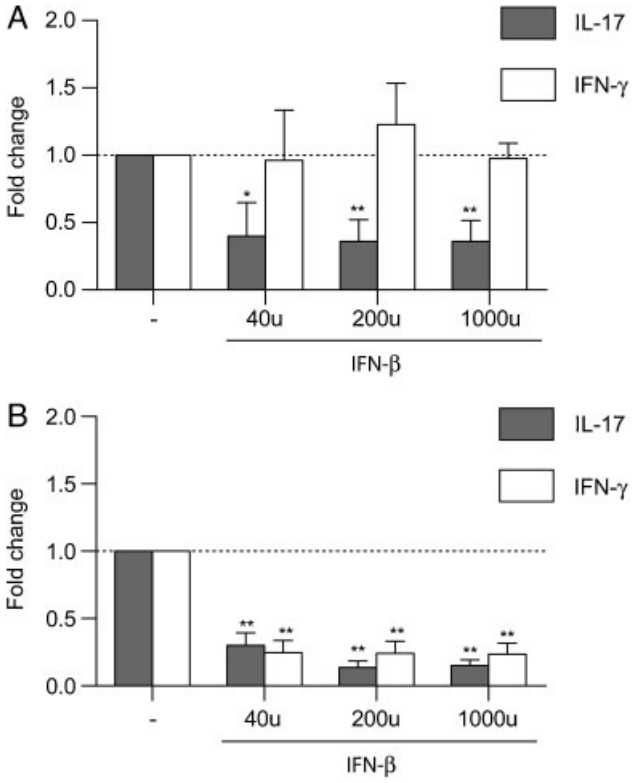

Figure 6. IFN-β differentially modulates IL-17 and IFN-γ release by memory T cells. (A) $CD4^+CD45RA^-CD25^-$ total memory T cells were stimulated with anti-CD3/anti-CD28 coated plates in the presence of different concentrations of IFN-β. IL-17 and IFN-γ concentrations were measured in the 36 h supernatant and are shown as fold change compared with the concentration of 0 U/mL. Data represent mean+SD of six different donors. (B) Total PBMC were stimulated with TSST-1 in the presence of different concentrations of IFN-β. IL-17 and IFN-γ concentrations were measured in the 36 h supernatant and are shown as fold change compared with the concentration of 0 U/mL. Data represent mean+SD of four different donors. *p*-Values of one-sample *t*-test of fold changes in the presence of PGE2 with a hypothetical mean value of 1 (anti-CD3/anti-CD28 alone for (A), TSST-1 alone for (B)). $^*p<0.01$, $^{**}p<0.001$.

Discussion

We describe here that PGE2, an inflammatory mediator that is targeted by common anti-inflammatory drugs, can act directly on effector T cells leading to an increase in IL-17 production and at the same time to an inhibition of IFN-γ production. Thus, in chronically inflamed tissues PGE2 may dramatically influence the balance between the highly inflammatory cytokine IL-17 and the negative feedback loop exerted by IFN-γ.

The modulatory effects of PGE2 are mimicked by specific agonists acting on either the prostaglandin receptors EP2 or EP4, suggesting a redundant role for these two receptors in the response of T cells to PGE2. This is consistent with recent reports showing that mice deficient in each of the different EP receptors were as susceptible as wild-type mice to the development of CIA, while partial but significant suppression was achieved by combined inhibition of the EP2 and EP4 receptors [53].

While this manuscript was in preparation Chizzolini *et al.* reported that PGE2 favored Th17 expansion *in vitro* leading to an increased accumulation of IL-17 in the culture supernatant [54]. Our data are in agreement with these observations and provide evidence that PGE2 acts by inhibiting expansion of $CCR6^-$ T cells rather than increasing proliferation of $CCR6^+$ Th17 cells. In addition to its effect on T-cell proliferation, our results also demonstrate that PGE2 can directly act on T cells by rapidly enhancing IL-17 production and, at the same time, inhibiting IFN-γ production. By modulating effector function of memory T cells, PGE2 would enhance IL-17 production even in the presence of effector cells capable of producing IFN-γ, providing a mechanism to explain why Th17 responses prevail in chronic inflammation sites, despite the presence of high numbers of Th1 cells. Moreover, the finding that PGE2 had a rapid effect on already differentiated effector T cells is in line with the fact that treatment with non-steroidal anti-inflammatory drugs (NSAID) is effective shortly after administration even when the disease is already established, and that it is able to relieve the symptoms but does not prevent disease progression [55]. Interestingly, PGE2 was able to increase IL-17 but inhibited IL-22, a cytokine that is also produced by Th17 cells and has been implicated in skin inflammatory processes such as psoriasis [56]. This finding may provide a possible explanation for the reported worsening of skin diseases in psoriatic arthritis patients treated with NSAID or COX-2 inhibitors [57].

Among different immunomodulators that are known to be produced at sites of inflammation, PGE2 was unique in its ability to enhance IL-17 production and, at the same time, inhibit IFN-γ production by human T cells. PGE2 had been already shown to increase Th17 responses by favoring IL-23 and dampening IL-12 release by APC [58, 59]. In addition, PGE2 is known to inhibit T-cell responses by blocking T-cell proliferation and IFN-γ production [60]. Our results indicate that PGE2 can directly act on T cells and promote Th17 responses independently from the presence of IL-23 and IL-12. Interestingly however, we observed that different cytokines were able to modulate the effect of PGE2. Thus, IL-12 abrogated the ability of PGE2 to inhibit IFN-γ production, while IL-1β and IL-6 synergized with PGE2 to favor IL-17 release. These results highlight that the crosstalk among different modulators present at the inflammatory site may be critical to determine the type of effector response and the outcome of the inflammatory reaction.

The mechanism by which PGE2 enhanced IL-17 production and inhibited IFN-γ production was likely mediated by the rapid increase of the Th17 transcription factor ROR-γt and a decrease in T-bet, the Th1-specific transcription factor, although we cannot exclude that other mechanisms could contribute to the modulation of these two cytokines. In addition, PGE2 could favor the enrichment of IL-17-producing cells at inflammatory sites by preferentially inhibiting proliferation of $CCR6^-$ T cells, which include Th1 cells, but not of $CCR6^+$ Th17 cells.

Recombinant IFN-β has pleiotropic anti-inflammatory effects [61] and is currently used as treatment of relapsing remitting MS [52]. We found that IFN-β had a reverse effect compared with PGE2 since it was able to inhibit production of IL-17 by purified $CD4^+$ T cells without affecting IFN-γ, leading to a skewing of the

IFN-γ/IL-17 balance in favor of IFN-γ. However, while the effect of PGE2 was the same in total PBMC or purified T cells, IFN-β inhibited both IL-17 and IFN-γ production by stimulated PBMC, suggesting that IFN-β may affect IFN-γ release indirectly acting on myeloid cells, which had been recently proposed to mediate the immunosuppresive function of IFN-β [62].

Chronic inflammation is a multifaceted process associated with tissue infiltration of different inflammatory cell types and accumulation of proinflammatory mediators. PGE2 has been identified as a key player in this process based on the findings that it is present in large amounts in chronic inflamed tissues, such as the synovium of RA patients, and has pleiotropic proinflammatory activities *in vitro* [60]. Inhibition of prostaglandin synthesis by NSAID that target COX activity is a widely used treatment for RA patients, reducing the extent and number of swollen joints. However, the use of NSAID as well as of the more recently developed COX-2 inhibitors is associated with gastrointestinal and cardiovascular toxicity that limit their use at least for long-term treatments [43]. Understanding the mechanisms by which PGE2 exerts its inflammatory function may lead to the identification of new targets for safer and less-toxic anti-inflammatory treatments.

Recently, some reports demonstrated that encephalitogenic Th1 and Th17 cells are both capable of triggering EAE syndromes that, despite similarities, have distinct histopathological and immunological features mediated by distinct proinflammatory pathways [63]. Importantly, the Th1-mediated and the Th17-mediated forms of EAE differ in responsiveness to specific immunomodulatory interventions. In this context, our findings that PGE2 has opposite effects on IL-17 and IFN-γ may be of great therapeutic relevance and may provide a rationale for the use of NSAID in the treatment of Th17-mediated inflammatory diseases.

Materials and methods

Cell purification

Blood samples were obtained from the Basel Swiss Blood Center. Permission to do experiments on human primary cells was obtained from the Federal Office of Public Health (A000197/2 to F.S.). PBMC were obtained by density gradient centrifugation on Ficoll-Paque PLUS (GE Healthcare). $CD4^+$ T cells were isolated by positive selection with anti-CD4 microbeads (Miltenyi Biotec). $CD4^+CD45RA^-CD25^-$ memory T-cell subpopulations were isolated to >95% purity by FACSAria (BD Bioscience) cell sorting after five-color staining using the following antibodies: anti-CD25 FITC, anti-CD45RA FITC (both from Beckmann Coulter), anti-CCR6 PE, anti-CXCR3 APC and anti-CCR4 PE-Cy7 (all from BD Biosciences).

Cell culture

Cells were cultured in RPMI 1640 medium containing 10% v/v FBS (GIBCO BRL) and supplemented with 2 mM glutamine, 1% v/v non-essential amino acids, 1% v/v sodium pyruvate, 50 μg/mL kanamycin, 50 U/mL penicillin and 50 μg/mL streptomycin. PBMC (2×10^5) were cultured for 36 h with or without 10 ng/mL TSST-1 (Toxin Technology). $CD4^+CD45RA^-CD25^-$ memory $CD4^+$ T cells or T-cell clones (5×10^4/200 μL) were cultured for 1, 4 or 36 h in plates coated with 2 μg/mL CD3 antibodies (clone TR66) and 2 μg/mL CD28 antibodies (BD Biosciences). $CD4^+$ T cells were expanded for 5 days with beads coated with CD3 and CD28 antibodies (Miltenyi Biotec) following the manufacturer's instructions. When indicated, cultures were supplemented with 5 ng/mL TGF-β, 10 ng/mL IL-1β, 10 ng/mL IL-12, 10 ng/mL IL-15, 10 ng/mL TNF, 10 ng/mL IL-23 (all from R&D Systems), 20 ng/mL IL-6 (BD Biosciences) or 0.5 μM PGE2 (Cayman Chemical). The following neutralizing antibodies to IL-1β (8516.311, R&D Systems), IL-6R (17506, R&D Systems), IL-12/23p40 (C8.6, BD Biosciences) or IL-23 (253810, R&D Systems) were used at a concentration of 10 μg/mL. The EP1/EP3 agonist sulprostone, the EP2 agonist butaprost and the EP4 agonist prostaglandin E1 alcohol (Cayman Chemical) were used when indicated at concentrations of 1 μM. INDO (Alexis) was used at the concentration of 10 μM). Labeling of cells with CFSE was performed as described previously [64]. T-cell clones were generated from sorted $CD45RA^-CD25^-CCR6^+CXCR3^+$ memory $CD4^+$ T cells in non-polarizing conditions by single-cell deposition as described [65].

ELISA and intracellular cytokine staining

Cytokine concentrations in culture supernatants were assessed by ELISA using reagents from R&D Systems according to standard protocols and analyzed with the Softmax program. Intracellular IFN-γ and IL-17 were detected after stimulating cells with PMA and ionomycin in the presence of 10 μg/mL brefeldin A (Sigma-Aldrich). Cells were fixed and permeabilized with BD Cytofix/Cytoperm Plus (BD Bioscience) according to the manufacturer's instruction. Cells were incubated with FITC-labeled antibody to IFN-γ and APC-labeled antibody to IL-17 (eBioscience), washed and acquired on FACSCanto (BD Bioscience) or FACSCalibur (BD Biosience) and analyzed using the FlowJo software (Tree Star).

Real-time quantitative PCR

Total RNA was extracted using the ABI PRISM 6100 Nucleid Acid PrepStation (Perkin-Elmer Applied Biosystems) according to the manufacturer's instructions. Random hexamer and an MMLV reverse transcriptase kit (Stratagene) were used for cDNA synthesis. Transcripts were quantified by real-time quantitative PCR on an ABI PRISM 7700 Sequence Detector (Perkin-Elmer Applied Biosystems) with Applied Biosystems predesigned TaqMan Gene Expression Assays and reagents according to the manufacturer's instructions. The following probes were used: *RORC* Hs01076112_m1, *TBX21* Hs00203436_m1, *GATA3* Hs00231122_m1, *Il17a* Hs99999082_m1, *Ifng* Hs99999041_m1,

Il4 HS00174122_m1, *EP1* Hs00168752_m1, *EP2* Hs00168754_m1, *EP3* Hs00168755_m1, *EP4* Hs00168761_m1. For each sample, the mRNA abundance was normalized to the amount of 18S rRNA and was expressed as arbitrary units.

Statistical analysis

Statistical significance was analyzed using various statistical tests as indicated in the legend of each figure. A one-sample *t*-test compared with a hypothetical mean value of 1 was used to analyze the fold change in cytokine release in cells cultured in the presence or absence of PGE2. One-way ANOVA followed by Dunnett post-test was used to compare the effect in IL-17 and IFN-γ release of the combination of PGE2 with different modulatory cytokines. One-way ANOVA followed by Bonferroni's multiple comparison test was used to determine statistically significant differences in the percentage of $CFSE^{low}$ cells in memory T-cell subsets stimulated in the presence of PGE2.

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Abbreviations: **CIA:** collagen-induced arthritis · **GATA-3:** GATA-binding factor 3 · **INDO:** Indomethacin · **NSAID:** non-steroidal anti-inflammatory drugs · **PGE2:** prostaglandin E2 · **RA:** rheumatoid arthritis · **ROR-γt:** retinoic-acid-related orphan receptor-γt · **T-bet:** T-cell-specific T-box transcription factor 21 · **TSST-1:** toxic shock syndrome toxin 1

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