Decreased RORC-dependent silencing of prostaglandin receptor EP2 induces autoimmune Th17 cells

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Prostaglandin E2 (PGE2) promotes Th17 expansion while otherwise inhibiting other CD4$^+$ T cell subsets. Here, we identified a PGE2-dependent pathway that induces pathogenic Th17 cells in autoimmune disease and is regulated by the transcription factor RORC. Compared with other CD4$^+$ cell types from healthy subjects, there is a surprising lack of the prostaglandin receptor EP2 on Th17 cells; therefore, we examined the hypothesis that RORγt, which is highly expressed in Th17 cells, mediates EP2 downregulation. Chromatin immunoprecipitation followed by DNA sequencing revealed that RORγt binds directly to *Ptger2* (the gene encoding EP2 receptor) in Th17 cells isolated from WT mice. In Th17 cells isolated from humans, RORC repressed EP2 by directly silencing *PTGER2* transcription, and knock down of RORC restored EP2 expression in Th17 cells. Compared with Th17 cells from healthy individuals, Th17 cells from patients with MS exhibited reduced RORC binding to the *PTGER2* promoter region, resulting in higher EP2 levels and increased expression of IFN-γ and GM-CSF. Finally, overexpression of EP2 in Th17 cells from healthy individuals induced a specific program of inflammatory gene transcription that produced a pathogenic Th17 cell phenotype. These findings reveal that RORC directly regulates the effects of PGE2 on Th17 cells, and dysfunction of this pathway induces a pathogenic Th17 cell phenotype.

Introduction

Prostaglandin E2 (PGE2) plays an important role as an immune regulator, exerting immunosuppressive as well as immune-activating functions (1–3), and genetic variants in the prostaglandin pathway are associated with the risk of developing MS (4, 5) and other autoimmune diseases (6, 7). The influence of PGE2 on CD4$^+$ cells varies depending upon the CD4$^+$ T cell subset, PGE2 concentration, and the activation status of the cell (2). While PGE2 can suppress T cell proliferation and IFN-γ production in mature Th1 cells (8–10), it has recently been reported that PGE2 facilitates Th1 cell differentiation through EP2 and EP4 receptors when accompanied by strong T cell receptor signaling (11). Furthermore, PGE2 induces Th17 cell expansion and promotes experimental autoimmune encephalomyelitis (EAE), an animal model of MS (11–14). While there are increases in Th17 cell expansion mediated through IL-23 and IL-1 receptor upregulation (13) in Th17-polarized T cells, PGE2 inhibits IL-17 in naive T cells (15). The mechanism for these divergent effects of PGE2 on T cell function and how the prostaglandin pathways influence autoimmune diseases are not known.

PGE2 binds to the G protein–coupled receptors EP1, EP2, EP3, and EP4 (11, 16). Among these receptors, only EP2 and EP4 are significantly expressed on activated CD4$^+$ T cells (13, 17). While it has been shown that both receptors are involved in Th17 cell expansion as well as in the inhibition of Th17 cell induction (13, 15), it is unknown how EP2 and EP4 and downstream signaling events regulate CD4$^+$ T cell lineage development. Suppression of IL-10 and IFN-γ production in Th17 cells is predominantly mediated through EP4 signaling (13), and furthermore, EP4 activation is responsible for PGE2-induced immune inflammation and disease progression in EAE (11, 14). The inhibitory effect of PGE2 on Th1 cells is concentration dependent, as lower concentrations of PGE2 have been shown to facilitate Th1 differentiation (11). It has also been reported that PGE2 decreases the frequency of IFN-γ$^-$ CD4$^+$ T cells, but not the frequency of IL-17$^+$IFN-γ$^+$ double-positive CD4$^+$ T cells during Th17 cell differentiation (12, 13).

MS is an autoimmune disease that is characterized by perivenular infiltrates of CD4$^+$ and CD8$^+$ T cells in the CNS white matter and meninges, with demyelinating lesions and loss of axons in both white and gray matter (18, 19). The risk of developing MS is significantly increased in genetically susceptible subjects (5). Our recent genome-wide association studies (GWAS) have identified 2 risk alleles in *PTGER4*, the gene for the PGE2 receptor EP4 (4, 5). Previous reports have shown that PGE2 concentrations in the serum and in the cerebrospinal fluid of MS patients are elevated (20, 21). However, the role of PGE2 in the etiology of MS remains unknown. Th1 and Th17 cells play an important role in the development of EAE and are implicated in MS pathology (22–25). Moreover, we have recently shown that the most pathogenic Th17 cells are those expressing *Ifng*, *Stat4*, *Tbx21*, *Il22*, *Csf2*, and *Runx3* genes, with decreases in *Il10* and *Ahr* (26). Given the significant influence of PGE2 on Th17 cells and the occurrence of MS-associated SNPs in PGE2 receptors, we sought to investigate the role of EP2 and EP4 receptors in Th17 cells from patients with MS and in those from healthy individuals.
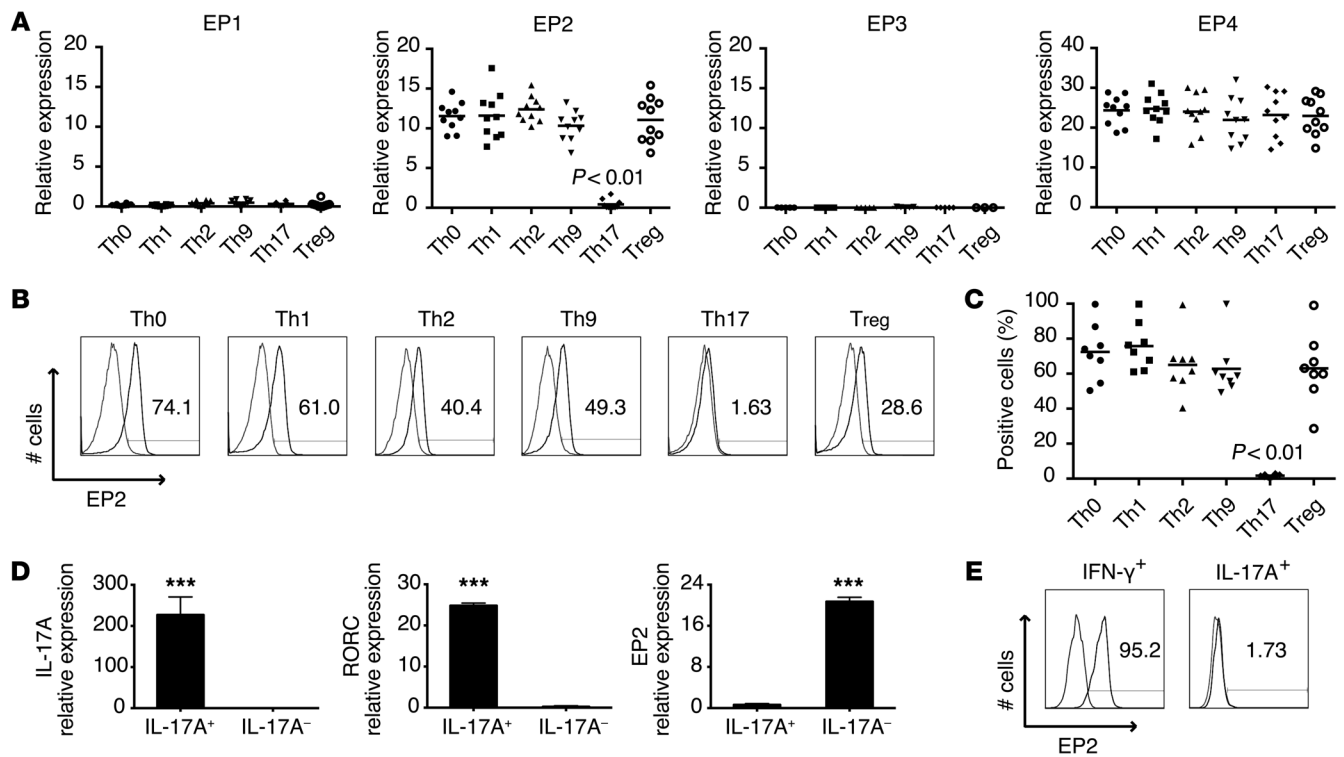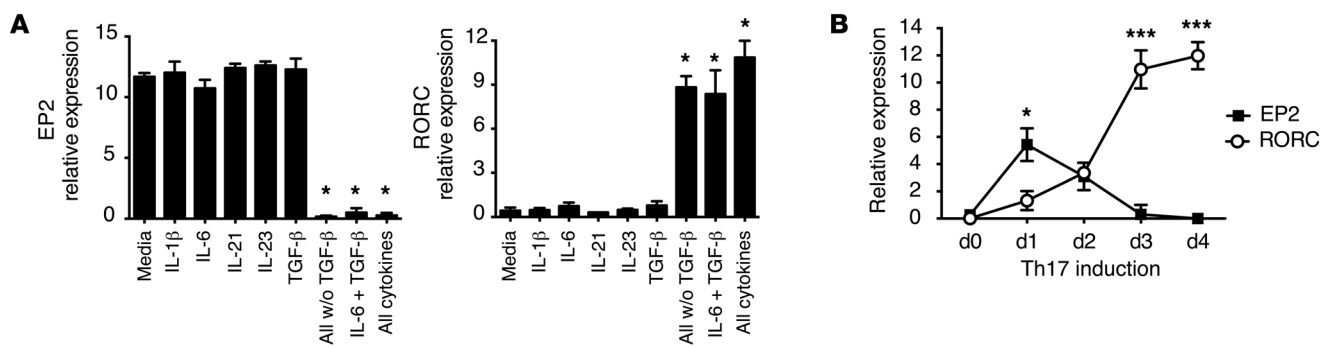
Figure 1
Prostaglandin receptor expression in human CD4+ T cell subsets. (**A**) Quantitative RT-PCR analysis of EP1, EP2, EP3, and EP4 expression in distinct CD4+ T cell subsets. *P* was calculated by 1-way ANOVA test. *n* = 10 individual donors. CD4+ subsets were differentiated from naive CD4+ cells in the presence of 2.5 μg/ml anti-CD3 antibodies, 1 μg/ml anti-CD28 antibodies, and the respective cytokines required for the induction of specific CD4+ subsets. Representative example (**B**) and cumulative results (**C**) of flow cytometric analysis of EP2 expression in Th0, Th1, Th2, Th9, Th17, and Tregs. *P* was calculated by 1-way ANOVA test. *n* = 8 individual donors. (**D**) IL-17A, RORC, and EP2 expression in ex vivo–isolated IL-17+CD4+ T cells and IL-17–CD4+ T cells. Cells were sorted by FACSAria based on cell surface expression of IL-17. ***P* < 0.001. *P* was calculated by an unpaired Student's *t* test; mean ± SEM. *n* = 10 individual donors. (**E**) Representative example of flow cytometric analysis of EP2 expression in IFN-γ+ and IL-17+ memory CD4+ T cells. Cells were analyzed for EP2 expression directly after ex vivo isolation without prior purification by FACSAria cell sorting.

Here, we examined the role of PGE2 in the development of potentially pathogenic Th17 cells and observed loss of PGE2 receptor EP2 expression on Th17 cells mediated by RORC, which directly silenced the EP2 receptor *PTGER2* gene. In contrast, expression of EP2 was partly restored on Th17 cells from patients with MS due to diminished *PTGER2* silencing. We observed increased proliferative responses with lower signal strengths induced by anti-CD3 cross-linking, and these responses correlated with both increased EP2 expression and GM-CSF production by Th17 cells in patients. Finally, the binding of RORC to *PTGER2* in Th17 cells was decreased in MS patients as compared with those from healthy controls when cells were stimulated with the same strength of T cell receptor signaling. These findings indicate that EP2 expression on MS Th17 cells is mediated in part by the lower T cell–signaling threshold observed in human autoimmune disease (27). Our results offer a mechanism by which EP2 downregulation protects normal Th17 cells from PGE2-mediated IFN-γ and GM-CSF induction and indicate a role of the PGE2 pathway in human autoimmune disease.

Results

EP2 expression is specifically suppressed in Th17 cells. In order to investigate the role of PGE2 receptors in patients with MS, we first analyzed the expression of PGE2 receptors in nor-

mal human Th17 cells and other CD4+ T cell subsets. Naive CD45RA+CD45RO–CD4+ T cells were differentiated into Th0, Th1, Th2, Th9, or Th17 cells and cultured for 4 days in the presence of anti-CD3 and CD28 mAbs and the respective cytokines. CD4+CD25hiCD127loFOXP3hi Tregs were also analyzed ex vivo for PGE2 receptor expression. Efficient differentiation into CD4+ T cell subsets was verified by transcription factor analysis. Th17 cells expressed *RORC*, *RORA*, *AHR*, *IL1R1*, *IL23R*, *CCR6*, *IL17A*, *IL17F*, and *IL22* (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI72973DS1) and produced IL-17A (Supplemental Figure 1, B–D). In contrast to all other CD4+ T cell subsets, Th17 cells from healthy individuals did not express EP2 (Figure 1, A–C), even after stimulation with high concentrations of anti-CD3 antibodies (Supplemental Figure 2). To verify our results in in vivo primed Th17 cells, we analyzed EP2 expression in highly purified Th17 cells obtained by sorting based on IL-17A cell surface expression. These IL-17A+ cells expressed high levels of RORC and almost undetectable levels of EP2 (Figure 1D). Flow cytometric analysis of ex vivo isolated IL-17A+ or IFN-γ+ memory CD4+ T cells showed high expression of EP2 in IFN-γ+ CD4+ T cells and very low expression in IL-17A+ CD4+ T cells (Figure 1E). Expression of other PGE2 receptors did not differ between

Figure 2
Expression of EP2 negatively correlates with RORC expression in CD4+ T cells. (**A**) Naive CD45RA+CD45RO−CD4+ T cells from healthy controls were cultured with the indicated cytokines. EP2 and RORC expression was analyzed by RT-PCR. *$P < 0.01$ by 1-way ANOVA (mean ± SEM; $n = 10$). (**B**) Time kinetics of EP2 and RORC expression in Th17 cells induced from naive CD45RA+CD45RO−CD4+ T cells. ***$P < 0.0001$ by an unpaired Student's t test comparing EP2 expression at 0 and 24 hours; mean ± SEM. $n = 5$.

different CD4+ T subsets (Figure 1, A–C). EP2 downregulation in Th17 cells was therefore the only variation of PGE2 receptor expression we observed in the different CD4+ T cell subsets.

EP2 negatively correlates with RORC in CD4+ T cells. Differentiation of human Th17 cells is induced by T cell activation in the presence of TGF-β and the Th17 cell–inducing cytokines IL-1β, IL-6, IL-21, or IL-23 (28) depending on whether they are naive or memory CD4+ T cells. To verify whether EP2 downregulation is caused by a specific cytokine or by a combination of Th17-inducing cytokines, we cultured naive CD4+ T cells with IL-1β, IL-6, IL-21, IL-23, or TGF-β alone or with a combination of these cytokines for 4 days. While the expression of EP2 was suppressed by a combination of Th17 cell–inducing cytokines, none of these cytokines alone was sufficient to downregulate EP2 expression (Figure 2A). The combination of TGF-β and IL-6 was also able to induce RORC and resulted in highly efficient downregulation of EP2 (Figure 2A). However, we found that TGF-β was not required for the suppression of EP2 expression in Th17 cells (Figure 2A). Efficient induction of RORC under Th17-skewing conditions highly correlated with EP2 downregulation ($P < 0.001$). The presence of IL-17A in the cell culture had no influence on EP2 expression (data not shown). After an initial increase during the first 24 hours, we observed that expression of EP2 decreased continuously during Th17 cell differentiation with a combination of TGF-β, IL-1β, IL-6, IL-21, and IL-23. After 72 hours of activation, EP2 expression was absent in Th17 cells, showing a strong negative correlation between the induction of RORC and EP2 downregulation (Figure 2B). We did not find any strong correlation between EP2 expression and other transcription factors, including T-bet, GATA3, FOXP3, and IRF4 (Supplemental Figure 3).

EP2 is directly silenced by RORC. With the strong correlation between RORC induction and EP2 suppression, we hypothesized that RORC directly interacts with *PTGER2*, the gene encoding for the EP2 receptor. We used ChIP sequencing to evaluate the binding properties of RORγt to *Ptger2* in Th17 cells from C57BL/6 WT mice or RORγt−/− knockout mice. We found that RORγt bound to the promoter region of *Ptger2* and, more strongly, to a second region at the 3′ end of the gene (Figure 3A). Th17 cells from RORγt−/− knockout mice were used as a specificity control (Supplemental Figure 4A). We observed no binding to *Ptger2* in RORγt−/− Th17 cells.

To directly investigate the mechanism for loss of the EP2 receptor in RORC-expressing Th17 cells, we used siRNA for specific knock down of RORC in human Th17 cells. We cultured naive CD45RA+CD45RO−CD4+ T cells from healthy individuals in the presence of TGF-β, IL-1β, IL-6, IL-21, and IL-23 for 3 days. Th17 cells were then transduced with either RORC-specific siRNA or mock siRNA and cultured for an additional 2 days. Efficiency of RORC knock down was controlled by Western blotting (Supplemental Figure 4B). RORC knock down restored EP2 expression, while EP2 remained repressed in mock-transduced Th17 cells (Figure 3B). To further evaluate the possibility that RORC represses transcription of EP2 by directly silencing *PTGER2*, we transfected naive CD4+ T cells with a luciferase reporter construct under the control of the *PTGER2* promoter. Transfection of the reporter plasmid, together with a construct encoding RORC, resulted in substantial and dose-dependent repression of luciferase activity, showing that EP2 expression is directly silenced by RORC (Figure 3C).

EP2 overexpression induces pathogenic gene signature in Th17 cells. EP2 is upregulated on CD4+ T cells upon TCR stimulation (29). We therefore studied the influence of TCR signaling strength on RORC-mediated EP2 downregulation. Naive CD4+ T cells transfected with a RORC expression plasmid were cultured under nonskewing (Th0) conditions with IL-2 and different concentrations of anti-CD3 antibodies. We found that strong TCR stimulation was in part able to restore EP2 expression in RORC-expressing CD4+ T cells (Figure 4A). Increased EP2 expression was not due to a loss of RORC expression (Figure 4B). These findings indicate that strong TCR signaling can partially overcome RORC-mediated downregulation of EP2 expression.

Since EP2 expression is suppressed in Th17 cells, we wished to further investigate potential effects of EP2 signaling on Th17 cells. We transfected Th17 cells with an EP2 expression plasmid, resulting in significant EP2 expression as compared with that in mock-transfected cells (Supplemental Figure 5, A and B). Transcriptional analysis of EP2-overexpressing Th17 cells showed an increase in *IFNG*, *CSF2*, *IL22*, *TBX21*, *RUNX3*, and *STAT4* expression upon activation with butaprost (Figure 5, A and B). Butaprost is a structural analog of PGE2 and a selective agonist of the EP2 receptor. The expression of IL-10 and AHR was diminished in EP2-expressing Th17 cells following EP2-specific activation. We

A

B

C
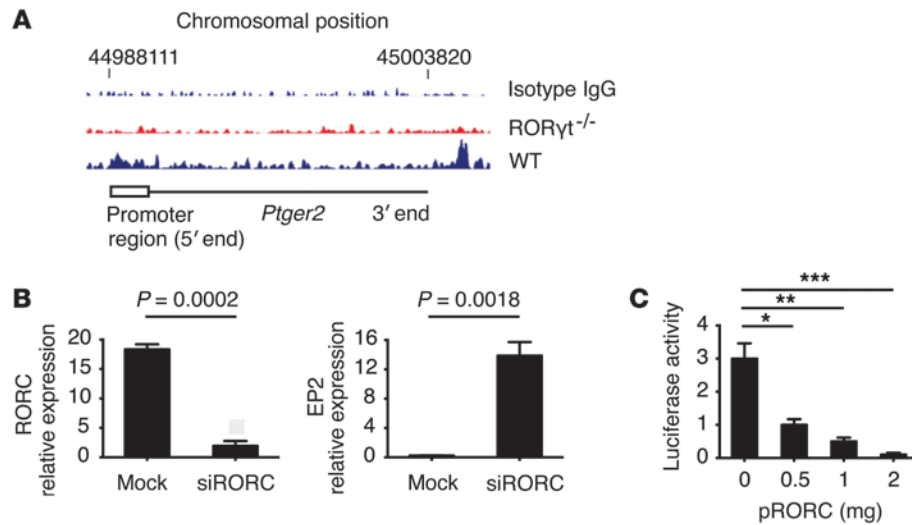
Figure 3
PTGER2 is directly silenced by RORC. (**A**) Th17 cells were induced from naive CD4+ T cells from C57BL/6 WT mice or RORγt^{−/−} knockout mice, and binding of RORγt to *Ptger2* on chromosome 14 was analyzed by ChIP-seq. Isotype IgG was used to control unspecific binding. (**B**) Knock down of RORC in Th17 cells by specific siRNA or nonsense control siRNA. Human Th17 cells were induced from naive CD4+ T cells, and RORC and EP2 expression was analyzed by RT-PCR. *P* was calculated by an unpaired Student's *t* test; mean ± SEM. *n* = 3. (**C**) Transcriptional silencing of *PTGER2* by RORC was quantified by measuring the luciferase activity in CD4+ T cells transfected with various concentrations of RORC expression plasmids and reporter constructs containing the firefly luciferase gene under the control of the *PTGER2* promoter. Results are presented relative to *Renilla* luciferase activity (cotransfected control). *P = 0.0154, **P = 0.0063, and ***P = 0.0034 by an unpaired Student's *t* test; mean ± SEM. *n* = 3.

from MS patients as compared with those from healthy individuals (Figure 6, D and E). In striking contrast, the EP4 agonist misoprostol had no influence on the frequency of IL-17A+IFN-γ+ Th17 cells in patients with MS and in healthy individuals. Moreover, EP2-specific activation during Th17 differentiation induced *IFNG* and *CSF2* gene expression (Figure 7A) and GM-CSF secretion in Th17 cells from MS patients, while having no influence on Th17 cells from healthy individuals (Figure 7B).

We wished to explore the potential mechanism for EP2 expression on CD4+ T cells from patients with MS. Since increasing T cell receptor–signaling strength led to increases in EP2 expression (Figure 4), we investigated whether there were increases in the frequency of proliferating CD4+ cells in patients with MS as compared with the frequency in control subjects. Consistent with the activated state of CD4 cells in the circulation of MS patients (31), we observed increased proliferative responses with lower signal strengths induced by anti-CD3 cross-linking in patients (Figure 8A and Supplemental Figure 6A). Finally, we observed that the strength of T cell receptor signaling correlated with both increased EP2 expression in CD4+ cells (Figure 8B) and GM-CSF production by Th17 cells from MS patients (Supplemental Figure 6B). In contrast, the expression level of RORC did not differ between Th17 cells from MS patients and those from healthy individuals (Supplemental Figure 1E).

As the strength of T cell receptor signaling correlates with increased expression of EP2, we performed ChIP-PCR to directly determine the binding activity of RORγt to *Ptger2* in Th17 cells with increasing TCR signal strengths induced by increasing con-

did not observe butaprost-induced changes in gene expression in mock-transfected Th17 cells. The expression of RORC and IL-17A was not affected by EP2 overexpression (Figure 5A), suggesting that there is no feedback loop between EP2 signaling and RORC expression. EP2-overexpressing Th17 cells produced significantly more IFN-γ and GM-CSF upon activation with an EP2-specific agonist (Figure 5C). In contrast, we found that IL-10 secretion was diminished in EP2-overexpressing Th17 cells as compared with that observed in mock-transfected cells.
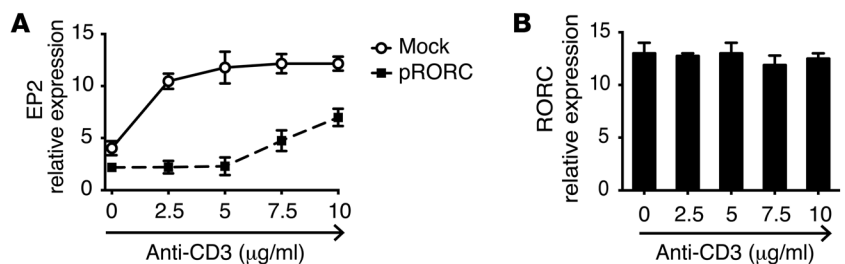
EP2 is expressed in Th17 cells from patients with MS. As increased frequencies of IL-17A+IFN-γ+ double-positive CD4+ T cells are found during EAE development and in patients with MS (30), we investigated the correlation between EP2 expression and IFN-γ induction in Th17 cells from untreated patients with relapsing-remitting MS (RRMS). Th17 cells were induced from naive CD45RA+CD4+ T cells from MS patients and healthy controls (Supplemental Figure 1, C and D). We found that, in contrast to Th17 cells from healthy subjects, Th17 cells from patients with MS expressed EP2, albeit at lower levels than did non-Th17 cells (Figure 6, A–C). Activation with the EP2-specific agonist butaprost induced expansion of Th17 cells from CD4+ CD45RO+ memory T cells obtained from MS patients. The frequency of IL-17A+IFN-γ+ cells was significantly increased in the presence of EP2 agonist during expansion of Th17 cells

A

B

Figure 4
EP2 expression in CD4+ T cells is induced by TCR stimulation. (**A**) Strong TCR stimulation partly restored EP2 expression in RORC+CD4+ T cells. Naive CD4+ T cells were transfected with a RORC expression plasmid and cultured with various concentrations of anti-CD3 antibodies under nonskewing (Th0) conditions. EP2 expression was analyzed and compared with EP2 expression in mock-transfected Th0 cells. *P* = 0.0006 comparing EP2 expression at 2.5 μg and 0 μg anti-CD3 mAbs, and *P* = 0.0057 comparing EP2 expression at 10 μg and 0 μg anti-CD3 mAb; mean ± SEM. *n* = 5. (**B**) RORC expression levels were controlled in RORC-transfected Th0 cells.
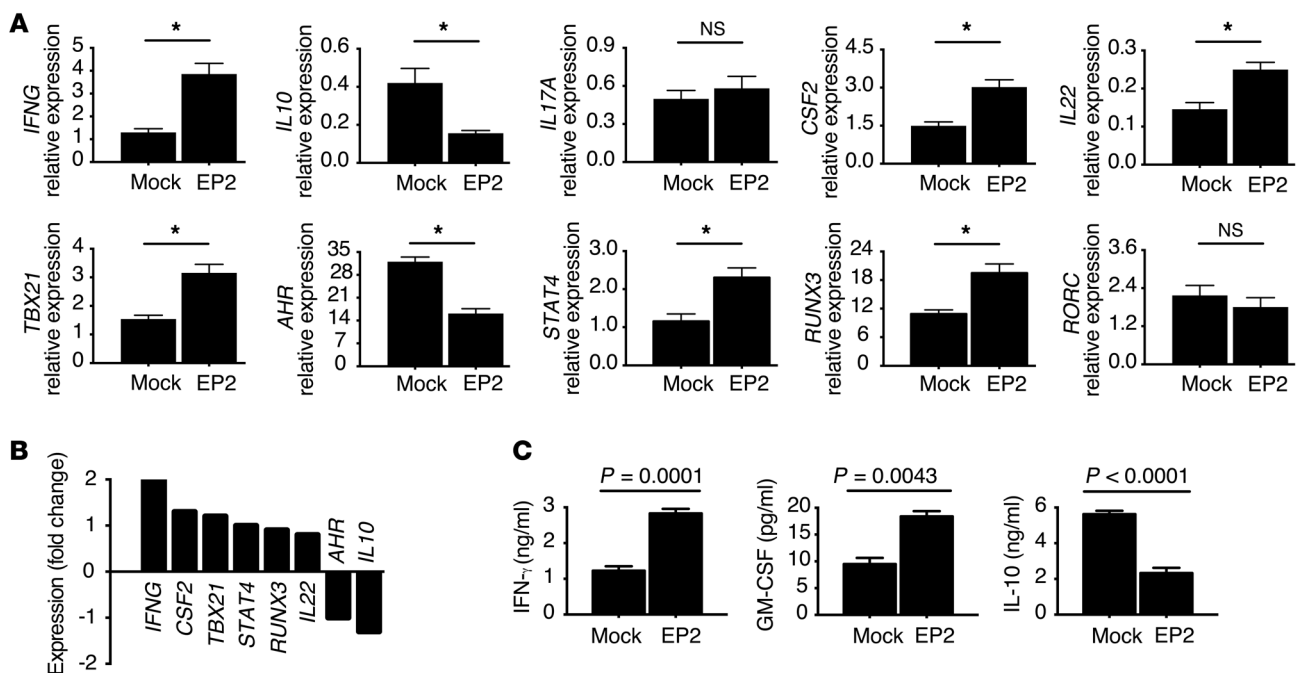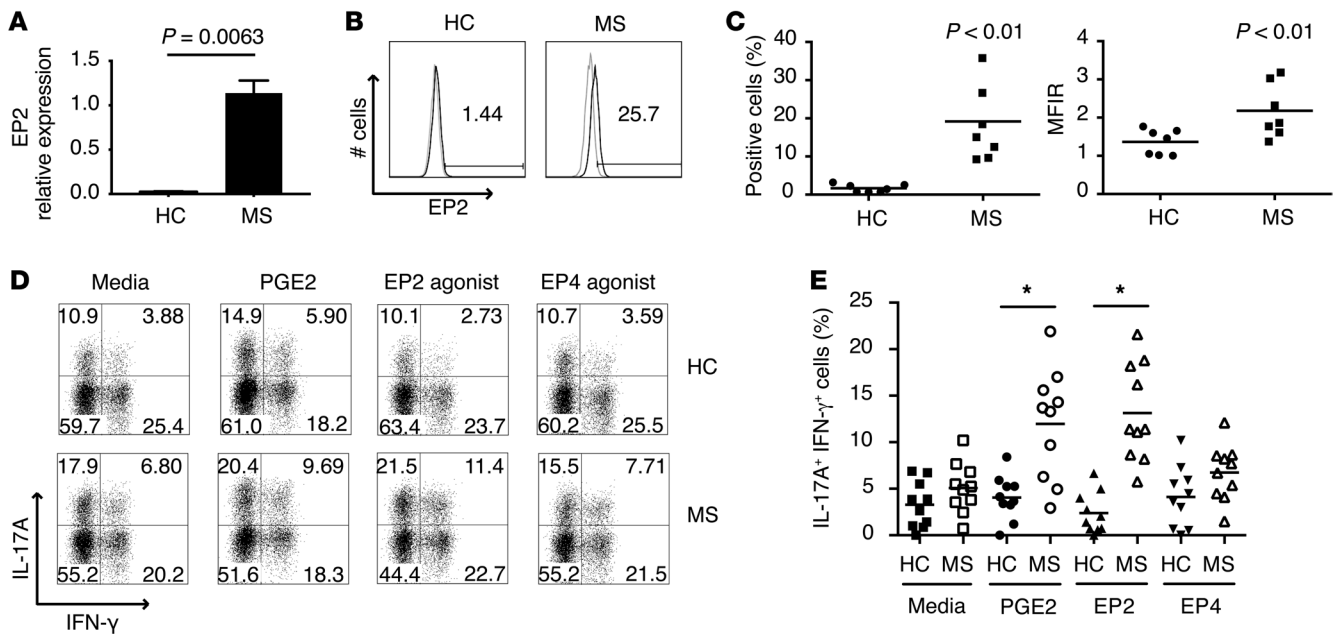
Figure 5
EP2 overexpression induces a pathogenic genotype in Th17 cells. (**A**) Quantitative RT-PCR analysis of genes in EP2-transfected Th17 cells and mock-transfected Th17 cells (mean ± SEM; *n* = 5). *P < 0.05. (**B**) Fold changes in gene expression after treatment of EP2-transfected and mock-transfected Th17 cells with butaprost and 5 μg/ml anti-CD3. (**C**) Cytokine secretion by EP2-expressing Th17 cells. EP2-overexpressing cells and mock-transduced Th17 cells were activated with an EP2 agonist in the presence of 5 μg/ml anti-CD3. Secretion of IFN-γ, GM-CSF, and IL-10 was analyzed by ELISA (mean ± SEM; *n* = 5). All *P* values were calculated by an unpaired Student's *t* test.

centrations of anti-CD3 antibodies. Th17 cells from RORγt^{-/-} knockout mice were used as a specificity control (Supplemental Figure 4A). Strong T cell receptor stimulation diminished RORγt binding to *Ptger2* both in the promoter region (5′ end) and at the 3′ end of the gene (Figure 8C). Thus, T cell receptor signaling had a dose-dependent inhibitory influence on RORγt binding to *Ptger2*. Importantly, even with higher concentrations of anti-CD3 antibodies, we observed significant binding of RORC to *PTGER2* in Th17 cells from healthy subjects (Figure 8D). To compare the influence of T cell receptor–signaling strength on RORC binding to *PTGER2* in MS patients and healthy controls, Th17 cells were stimulated with the same concentration of anti-CD3 antibodies and analyzed by ChIP-PCR. RORC binding to *PTGER2* was significantly more attenuated in Th17 cells from patients with MS as compared with Th17 cells from healthy control subjects (Figure 8D). Thus, the threshold of T cell receptor–mediated inhibition of the interaction between RORC and *PTGER2* was lower in Th17 cells from MS patients. Finally, we found that EP2 expression was significantly higher in autoimmune pathogenic Th17 cells from patients with MS as compared with Th17 cells from healthy controls and that the upregulation of EP2 was dependent on the strength of T cell receptor stimulation (Figure 8E). Taken together, RORC binding to *PTGER2* and RORC-mediated suppression of EP2 were diminished, while EP2 expression was higher in Th17 cells from MS patients. As a consequence, EP2 expression promoted the upregulation of IFN-γ and GM-CSF and induced a potentially pathogenic Th17 cell phenotype that was associated with a lower signaling threshold in CD4 T cells from MS patients.

Discussion
PGE2 plays different roles during inflammatory responses, showing either proinflammatory or immune-suppressive properties. Here, we show striking differences in expression of PGE2 receptors on different T cell subsets with loss of the EP2 receptor on Th17 cells. Mechanistically, loss of EP2 expression was mediated by RORC, which directly silenced the EP2 receptor *PTGER2* gene. The repression of EP2 expression on CD4^{+} T cells mediated by RORC could in part be overcome by strong TCR signaling, which inhibited the binding of RORC to *PTGER2*. In Th17 cells from patients with MS, binding of RORC to *PTGER2* was significantly reduced as compared with that in Th17 cells from healthy individuals when cells were stimulated with the same T cell receptor–signaling strength, suggesting that the lower T cell–signaling threshold observed in human autoimmune disease was responsible for the disturbed interaction between RORC and *PTGER2*. Overexpression of EP2 in Th17 cells from healthy individuals induced a potentially pathogenic Th17 cell phenotype with increased expression of *IFNG*, *STAT4*, *TBX21*, *IL22*, *CSF2*, and *RUNX3* and decreases in *IL10* and *AHR* genes. Thus, prostaglandins may mediate induction of pathogenic CD4^{+} cells in patients with autoimmune disease.

Overexpression of EP2 in Th17 cells induces a pathogenic phenotype similar to that previously described in human Th17 cells during fungal infection (32) and in mice with EAE (30). These pathogenic Th17 cells produce IL-17, IFN-γ, and GM-CSF, but no IL-10 (30). In mice, T-bet^{-/-} Th17 cells lose their ability to induce autoimmunity, further suggesting a key role for T-bet in Th17 cells in mediating a pathogenic CD4 cell phenotype (26). In this regard, our

Figure 6

EP2 expression is increased in Th17 cells from MS patients. (**A**) EP2 expression in Th17 cells from healthy controls (HC) and Th17 cells from MS patients were compared using RT-PCR (mean ± SEM; $n = 20$). Representative example (**B**) and cumulative results (**C**) of flow cytometric analysis of EP2 in Th17 cells from MS patients and healthy controls. $n = 7$. Percentage of positive cells and mean fluorescence intensity ratio (MFIR) = MFI (EP2) – MFI (isotype) are shown. Representative example (**D**) and cumulative results (**E**) of flow cytometric analysis of IL-17A and IFN-γ in CD4+ T cells from MS patients and healthy controls. CD45RO+ memory CD4+ T cells from healthy controls and MS patients were cultured for 4 days with IL-23. Where indicated, cells were cultured in the presence of PGE2, an EP2 agonist (butaprost), or an EP4 agonist (misoprostol). Percentage of IL-17A+IFN-γ+ Th17 cells in MS patients and healthy controls are shown. *$P < 0.01$. $n = 10$.

transcriptional analysis of EP2-overexpressing Th17 cells showed an upregulation of genes encoding for IFN-γ, T-bet, RUNX3, and STAT4. The transcription factors T-bet and RUNX3 cooperate to differentiate Th1 cells and induce IFN-γ production (33, 34). Furthermore, T-bet and STAT4 are critical for IFN-γ production in Th1 cells (35). The changes we observed in gene transcription that resulted in increased expression of T-bet, RUNX3, STAT4, and IFN-γ in EP2-overexpressing Th17 cells, without loss of RORC and IL-17A, indicate that these cells shifted to IL-17+IFN-γ+ double-producing cells. In addition to IFN-γ, we found that EP2-overexpressing Th17 cells expressed increased levels of GM-CSF and IL-22 but decreased levels of IL-10 and of the transcription factor AHR. Taken together, the EP2 overexpression in Th17 cells induces a gene expression profile that is similar to the gene signature found in autoimmune, pathogenic Th17 cells (26, 36).

Interestingly, EP2 is temporarily upregulated during early Th17 cell differentiation. While EP2 and EP4 signaling often acts synergistically, a number of different receptor-specific effects have been described. Activation of EP2 and EP4 induces PI3K signaling and enhances intracellular cAMP concentrations (11, 37). Suppression of IL-10 and IFN-γ production in Th17 cells is predominantly mediated through EP4 signaling (13). Furthermore, EP4 signaling is responsible for PGE2-induced immune inflammation and disease progression in EAE (11, 14). Our findings may, in part, explain the different immunological effects of PGE2 on distinct CD4+ T cell subsets, as we show that Th17 cells are activated by PGE2 through the EP4 receptor alone, while Th1, Th2, Th9, and Tregs are activated through EP2 and EP4 recep-

tors. Whether genetic variation in the EP4 receptor region (4) influences susceptibility to autoimmune disease through interactions with the EP2 receptor is unknown.

PGE2 has paradoxical effects on Th17 cells, inhibiting the induction of Th17 cells from naive CD4+ T cells (15), but promoting expansion of mature Th17 cells (11–13). The suppression of IL-17 production during Th17 cell differentiation is mediated by inhibition of the transcription factor IRF4 and can be reversed by EP2 antagonists, but not by EP4 antagonists (15). The paradoxical effect of PGE2 on IL-17 production could be explained by differences in EP2 expression during Th17 cell induction and expansion. While activation of naive CD4+ T cells induced a temporary increase in EP2 expression during Th17 cell differentiation, EP2 was substantially suppressed in mature Th17 cells. This is consistent with the observation that EP4, and not EP2, is predominantly responsible for Th17 cell expansion and increased IL-17 production (11). Loss of EP2 on mature Th17 cells could thus explain the paradoxical effect of PGE2 on Th17 induction from naive CD4+ T cells and on the expansion of mature Th17 cells.

PGE2-mediated Th17 cell expansion induces disease progression in EAE. In patients with MS, PGE2 levels are elevated in the serum and in the cerebrospinal fluid (20, 21). However, the role of PGE2 in the pathogenesis of MS and other autoimmune diseases remains unknown. PGE2 has been shown to promote Th17 cell expansion by upregulation of IL-23 and IL-1β receptor (13). Our results showed a significant increase in the frequency of IL-17A+IFN-γ+ cells mediated through EP2 signaling in Th17 cells from patients with MS. In marked contrast, we did not observe expansion of
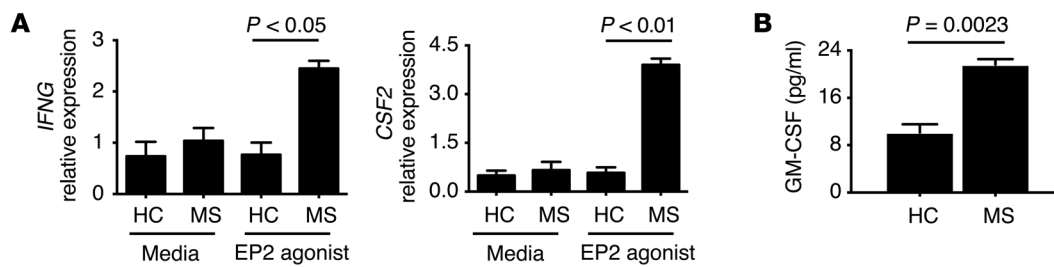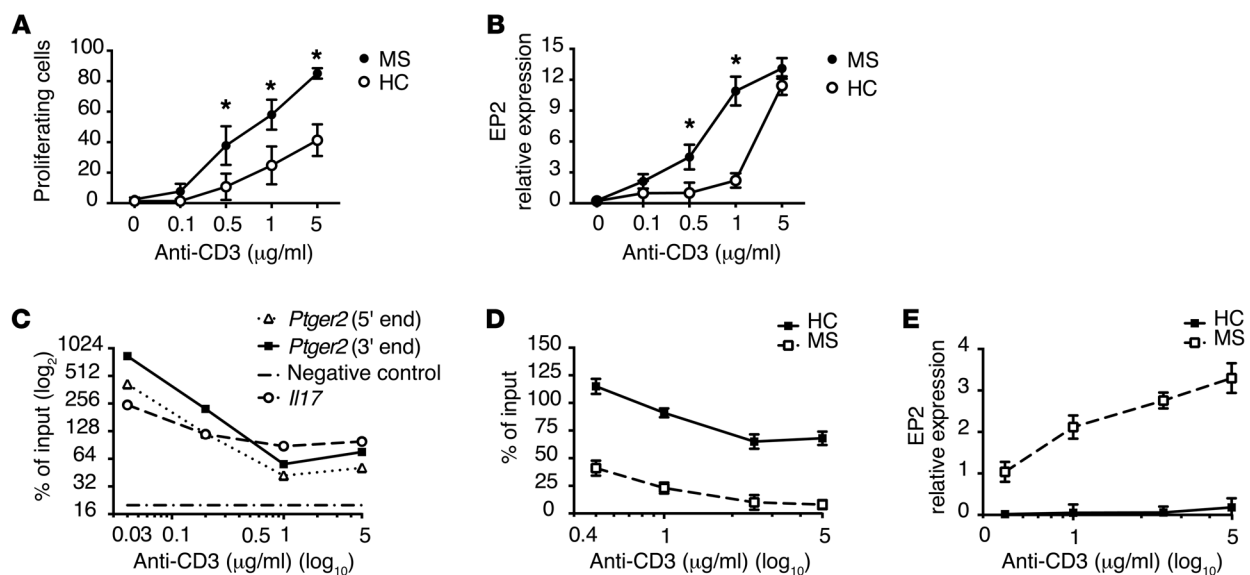
Figure 7
EP2 activation drives cytokine production in Th17 cells from MS patients. (**A**) RT-PCR analysis of *IFNG* and *CSF2* mRNA expression in Th17 cells from MS patients and healthy controls (mean ± SEM; $n = 5$). Th17 cells were differentiated for 4 days from naive CD4$^+$ T cells. Where indicated, cells were cultured in the presence of the EP2 agonist butaprost. (**B**) Secretion of GM-CSF by Th17 cells from MS patients and healthy controls was analyzed by ELISA; mean ± SEM. $n = 5$. All P values were calculated by an unpaired Student's t test.

IL-17A$^+$IFN-γ$^+$ cells by EP2 agonists in Th17 cells from healthy individuals, as EP2 expression was silenced in these cells. Nonphysiological EP2 signaling in Th17 cells therefore favored the expansion of IFN-γ$^+$GM-CSF$^+$ Th17 cells (Figure 9). We know from previous studies that all IL-17A$^+$IFN-γ$^+$ double-producing CD4$^+$ T cells are derived from Th17 cells (30). This excludes the possibility that Th1 cells are responsible for the increase in IL-17A$^+$IFN-γ$^+$ double-producing cells we observed in MS patients.

The level of EP2 expression in CD4$^+$ T cells depends on the strength of the TCR stimulation (Figure 4A). We have shown that RORC-mediated *PTGER2* silencing can be partly overcome by a strong TCR signal (Figure 4A). This is consistent with previous reports showing that strong TCR stimulation leads to transient downregulation of

IL-17 in Th17 cells mediated by decreased expression of RORγt (32). RORC expression remained stable after strong TCR stimulation of RORC-overexpressing CD4$^+$ T cells (Figure 4B). The signaling threshold of CD4$^+$ T cells is genetically decreased in autoimmune disease (27). Thus, it is likely that the enhanced TCR signaling in T cells from patients with MS and other autoimmune diseases drives the increased EP2 expression. Reduced *PTGER2* silencing may biologically reflect the consequences of an increased TCR signal strength.

Our findings reveal EP2 to be a potential drug target in autoimmune disease. The application of EP2 antagonists could help to reduce the frequency of pathogenic IL-17A$^+$IFN-γ$^+$ Th17 cells in patients with MS. Various prostanoid receptor antagonists have been developed in recent years, including a highly selective and potent EP2

Figure 8
Influence of TCR signaling strength on EP2 expression and RORC binding to *PTGER2* in Th17 cells. (**A**) Proliferative response of CD4$^+$ T cells from healthy controls and MS patients. Cells were cultured for 3 days in the presence of different anti-CD3 antibody concentrations, and proliferation was measured by CFSE dilution using flow cytometry. *$P < 0.05$. (**B**) CD4$^+$ T cells from MS patients upregulated EP2 at lower anti-CD3 concentrations as compared with those in healthy controls. *$P < 0.05$. (**C**) Murine Th17 cells were stimulated with different concentrations of anti-CD3 antibodies, and binding of RORγt to the promoter region (5′ end) and the 3′ end of *Ptger2* was analyzed by quantitative real-time PCR of ChIP-PCR. Dashed line indicates background using cells from RORγt$^{-/-}$ mice. Binding of RORγt to the *Il17* locus was used as a control. (**D**) ChIP-PCR analysis of RORC binding to the *PTGER2* promoter region (5′ end) in Th17 cells from MS patients and healthy controls. Th17 cells were stimulated with different concentrations of anti-CD3 antibodies (mean ± SEM; $n = 3$). (**E**) TCR signaling–dependent EP2 expression in Th17 cells from MS patients and healthy controls was analyzed by RT-PCR (mean ± SEM; $n = 3$).
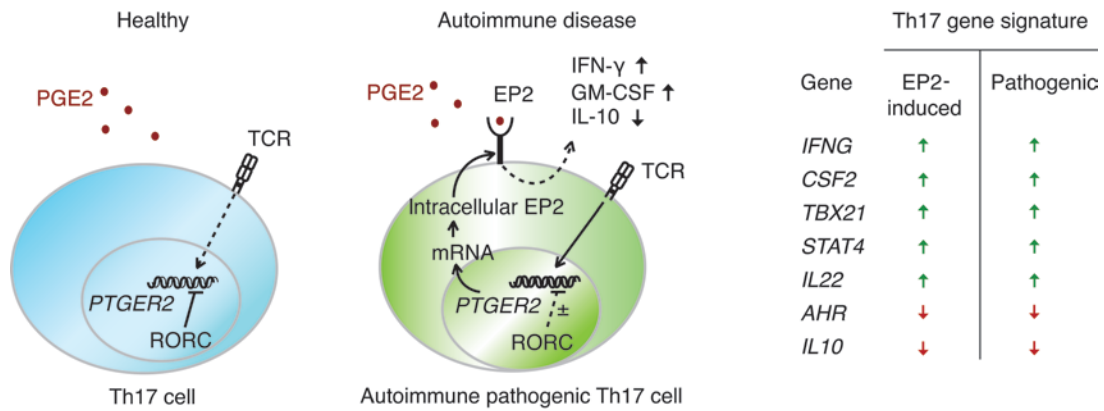
Figure 9
Model for PGE2 effects on Th17 cells in healthy individuals and autoimmune disease. TCR-induced EP2 upregulation is inhibited by RORC in Th17 cells from healthy individuals. In Th17 cells from patients with autoimmune disease, RORC-mediated *PTGER2* silencing is diminished and EP2 is expressed. EP2 signaling in pathogenic Th17 cells promotes the induction of IL-17A$^+$IFN-γ^+ cells and induces a potentially pathogenic Th17 cell phenotype (26).

receptor antagonist that is currently under clinical investigation to assess safety and tolerability in humans (38). While the specific role of IFN-γ^+GM-CSF$^+$ Th17 cells in MS pathophysiology is unknown, recent findings provide evidence that these cells are implicated in the pathogenesis of autoimmune disease. GM-CSF production in helper T cells is driven by RORγt (39), and the encephalitogenicity of Th17 cells in EAE is dependent on their production of GM-CSF (40). High frequencies of IFN-γ^+GM-CSF$^+$ Th17 cells have been found during the acute phase of EAE (30). Furthermore, IL-17$^+$IFN-γ^+ cells have been found in the CNS of MS patients (41). Th17 cells from MS patients are more likely to develop into IL-17$^+$IFN-γ^+ cells than are Th17 cells from healthy individuals (41). Thus, reduction of IL-17$^+$IFN-γ^+ Th17 cell numbers is a potential strategy for the treatment of autoimmune disease.

In summary, we describe a specific mechanism by which RORC downregulates EP2 in normal Th17 cells by directly silencing *PTGER2* and prevents induction of potentially pathogenic Th17 cells. The striking differences in EP2 expression between distinct CD4$^+$ T cell subsets provide an explanation for the opposing effects of PGE2 on Th1 and Th17 cells. Furthermore, the short-term upregulation of EP2 during early Th17 cell differentiation from naive CD4$^+$ T cells likely explains the paradoxical effects of PGE2 on Th17 cells during induction and expansion. Finally, binding of RORC to *PTGER2* and downregulation of EP2 are disturbed in Th17 cells from MS patients, leading to increased frequencies of pathogenic IFN-γ^+GM-CSF$^+$ Th17 cells. These findings identify EP2 as a potential target for the treatment of autoimmune diseases.

Methods

Study subjects. Peripheral blood was drawn from healthy individuals and patients with MS. All patients had RRMS with Kurtzke Expanded Disability Status Scale scores between 0 and 2.5. The patients were 43.5 ± 12.3 years of age. All patients were untreated or had not received treatment for at least 12 months. Healthy controls were age and sex matched with the MS patients and had no history of autoimmune disease or malignancies and no acute or chronic infections.

Human T cell isolation and cell culture. Primary human lymphocytes were isolated from peripheral blood of healthy subjects or untreated patients with RRMS by Ficoll gradient centrifugation (GE Healthcare). Untouched CD4$^+$ T cells were isolated from PBMCs by negative selection via the CD4$^+$ T cell isolation kit II (Miltenyi Biotec). Naive (CD45RA$^+$CD45RO$^-$CD25$^-$CD127$^+$) and memory (CD45RA$^-$CD45RO$^+$CD25$^-$CD127$^+$) CD4$^+$ T cells were then sorted by high-speed flow cytometry with a FACSAria (BD Biosciences) to a purity of greater 98% as verified by post-sort analysis. Dead cells were excluded by propidium iodide (BD Biosciences). Cells were cultured in 96-well, round-bottom plates (Costar) at 5×10^4 cells per well in RPMI 1640 medium, 10% (v/v) FCS (Life Technologies) and stimulated with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (1 μg/ml) Abs.

Isolation and purification of Th17 cells. Ex vivo–isolated CD4$^+$ T cells were purified from the peripheral blood of healthy donors and MS patients using the magnetic cell-sorting (MACS) technique. CD4$^+$ T cells were activated with PMA-ionomycin for 2 hours, and IL-17 expression on the cell surface was stained with anti-IL17A antibodies without prior permeabilization of the cells (42, 43). IL-17$^+$ cells and IL-17$^-$ cells were then sorted using FACSAria. *RORC* and *IL17A* mRNA expression was analyzed in both subgroups.

Differentiation of T cell subsets. For differentiation of human Th17 cells, naive CD4$^+$ T cells were cultured for 4 days with recombinant human (rh) TGF-β (5 ng/ml), IL-1β (12.5 ng/ml), IL-6 (25 ng/ml), IL-21 (25 ng/ml), and IL-23 (25 ng/ml). rhIL-2 was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID), NIH, and was used for restimulation experiments at 20 U/ml. Cells were cultured for the periods of time indicated below. For differentiation of CD4$^+$ T cell subsets, the following cytokines or mAbs were used: Th0 cells were cultured with IL-2 (20 U/ml); Th1 cells were cultured with rhIL-12 (10 ng/ml) and anti–IL-4 mAb (10 μg/ml); Th2 cells were cultured with rhIL-4 (10 ng/ml) and anti–IFN-γ mAb (10 μg/ml); and Th9 cells were cultured with rhIL-4 (10 ng/ml), rhTGF-β (5 ng/ml), and anti–IFN-γ mAbs (10 μg/ml). All cytokines and antibodies were obtained from R&D Systems. CD4$^+$CD25^{hi}CD127^{lo} Tregs were sorted by FACSAria. CD45RA$^-$CD45RO$^+$ memory CD4$^+$ T cells were cultured with 5 μg/ml anti-CD3 and 1 μg/ml anti-CD28 in the presence of rhIL-1β, rhIL-6, rhIL-21, rhIL23, and rhTGF-β for 4 days and then expanded for an additional 5 days with IL-23. Where indicated, cells were incubated with 0.1 μM PGE2, 0.1 μM butaprost, or 0.1 μM misoprostol (Cayman Chemical).

Antibodies, recombinant cytokines, and reagents. The following mAbs were used: anti-CD4 (RPA-T4), anti-CD45RO (UCHL1), anti-CD45RA (HI100), anti-CD25 (M-A251), anti-CD127 (hIL-7R-M21), anti-CCR6 (11A9) (all from BD Biosciences) — for surface staining; anti-IL-17A (eBio64DEC17;

eBioscience) and anti–IFN-γ (B27; BD Biosciences) — for intracellular staining; and anti-CD3 (UCHT1) and anti-CD28 (28.2) from BD Biosciences — for T cell stimulation. Anti-EP2 and anti-EP4 antibodies were purchased from Cayman Chemical.

EP2 overexpression in Th17 cells. Naive human CD45RA+CD45RO−CD4+ T cells were cultured for 2 days with 10 μg/ml anti-CD3 and 1 μg/ml anti-CD28 antibodies in the presence of rhIL-1β, rhIL-6, rhIL-21, rhIL23, and rhTGF-β. For gene overexpression, an EP2 gene expression construct (OriGene Technologies) or a mock control construct was delivered into Th17 cells by electroporation nucleofection (Amaxa) according to the manufacturer's instructions. After gene transfer, the cells were cultured for an additional 2 days under Th17-skewing conditions.

Flow cytometry. Cells were analyzed by flow cytometry after a culture period of 7 to 8 days, unless otherwise specified. For surface staining, cells were stained with the respective antibodies for 20 minutes in PBS containing 0.5% FCS and 2 mM EDTA before analysis. For intracellular staining, cells were stimulated for 4 to 5 hours with PMA (50 ng/ml) and ionomycin (250 ng/ml; both from Sigma-Aldrich) in the presence of GolgiPlug (BD Biosciences), fixed and made permeable (Fix/Perm; eBioscience) according to the manufacturer's instructions, and stained with the respective antibodies for intracellular cytokine detection for 30 to 45 minutes. Prior to fixation, cells were stained with the LIVE/DEAD cell kit (Invitrogen) to exclude dead cells. Data were acquired on an LSR II (BD Biosciences) and analyzed with FlowJo software (Tree Star Inc.).

ELISAs. CD4+ T cells were cultured with anti-CD3 (10 μg/ml) and anti-CD28 (1 μg/ml) antibodies in the presence of IL-1β, IL-6, IL-21, IL23, and TGF-β for 4 days. Supernatants were collected, and IL-17, IFN-γ, GM-CSF, and IL-10 measurement was performed according to the manufacturer's recommendations (BD Biosciences). rhIFN, rhGM-CSF, rhIL-17, and rhIL-10 were purchased from Chiron.

Real-time PCR. Cells for RNA isolation were harvested after 4 days of cell culture, unless otherwise specified, and RNA was isolated using the Absolutely RNA 96 Microprep Kit (Agilent Technologies) or the RNeasy micro kit (QIAGEN) and converted to cDNA via RT by random hexamers and Multiscribe RT (TaqMan Gold RT-PCR kit; Applied Biosystems). All primers were purchased from Applied Biosystems. All reactions were performed on a StepOnePlus Real-Time PCR System (Applied Biosystems). The values are represented as the difference in Ct values normalized to β-microglobulin for each sample using the following formula: relative RNA expression = $(2^{-dCt}) \times 10^3$.

Mice and murine Th17 cell differentiation. C57BL/6 WT mice were housed and maintained in a conventional pathogen-free facility at the Harvard Institute of Medicine.

CD4+ T cells were purified from spleen and lymph nodes from C57BL/6 WT mice with anti-CD4 microbeads (Miltenyi Biotec), then were further sorted as naive CD4+CD62LhiCD44lo T cells. Sorted cells were activated with plate-bound anti-CD3 (2 μg/ml; 1452C11; Bio X Cell) and anti-CD28 (2 μg/ml; PV1; Bio X Cell) in the presence of cytokines (recombinant mouse IL-23 from R&D Systems; all other cytokines were from Miltenyi Biotec). For Th17 differentiation, the following reagents were used: 2 ng/ml recombinant mouse TGF-β (Miltenyi Biotec), 25 ng/ml recombinant mouse IL-6 (Miltenyi Biotec), 20 ng/ml recombinant mouse IL-23 (R&D Systems), and 20 ng/ml recombinant mouse IL-1β (Miltenyi Biotec). Cells were cultured for 4 days and collected for RNA, intracellular cytokine staining, and flow cytometry.

ChIP-PCR and sequencing. ChIP was performed as described previously (44). Briefly, Th17 cells from *Rorc*+/+ C57BL/6 WT mice and CD4-Cre+ *Rorc*fl/fl mice (45) were lysed and sonicated to solubilize and shear cross-linked DNA. Purified DNA was blunted and amplified using a 2-stage PCR protocol.

ChIP-sequencing (ChIP-seq) was performed as described previously (45). reads were aligned with the NCBI Build 37 (UCSC mm9) of the mouse genome using Bowtie9. Enriched binding regions (peaks) were detected using MACS10 and QuEST, with a *P* value cutoff of 10^{-8}. Unspecific binding to RORγt was controlled by isotype control immunoglobulin (IgG) and by an anti-RORγt antibody in a RORγt-deficient cell population.

The ChIP-seq data are available in the NCBI's Gene Expression Omnibus (GEO) database (GEO GSE56020; http://www.ncbi.nlm.nih.gov/gds).

shRNA-mediated gene silencing. Lentiviral particles expressing siRNAs were obtained from Sigma-Aldrich. Lentiviral transduction of human T cells was carried out as described before (17). In brief, 5×10^4 human naive CD4+ T cells per well were stimulated for 24 hours prior to infection with anti-CD3 and anti-CD28. Cells were then transduced with viral particles containing a vector expressing RORC-specific siRNA or unspecific siRNA. Cells were transduced using an MOI of 5 and centrifugation at 800 *g* for 30 minutes at room temperature in the presence of 3 μg/ml polybrene (Millipore). The efficiency of RORC knock down was verified by Western blotting. The specific RNAi Consortium clone used for RORC knock down was TRCN0000033654. Cells were analyzed for EP2 expression 48 hours after transduction with siRNA.

Luciferase activity assay. Naive human CD4+CD45RA+CD45RO− T cells were transfected by electroporation with various concentrations of a RORC expression plasmid and with a luciferase reporter construct (SwitchGear Genomics) containing the first 104 base pairs of the *PTGER2* promoter region. Luciferase activity was analyzed 48 hours after transfection and 24 hours after activation with PMA and ionomycin using a dual luciferase assay kit (Promega).

Statistics. Statistical analysis was performed using GraphPad Prism (GraphPad Software). Data were analyzed by 1-way ANOVA using Tukey's post-hoc test in multiple groups and by a 2-tailed unpaired Student's *t* test in cases in which 2 groups were compared. Data are presented as the mean ± SEM, and *P* < 0.05 was considered statistically significant.

Study approval. The study was conducted in compliance with the Declaration of Helsinki. Before study initiation, approval was obtained from the ethics committee of Yale-New Haven Hospital (New Haven, Connecticut, USA). Written informed consent was obtained from all patients and healthy donors. All animal experiments were conducted in accordance with the guidelines outlined by the Harvard Medical Area Standing Committee on Animals at Harvard Medical School. Approval of the Harvard Medical Area Standing Committee on Animals was obtained for all animal experiments.

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