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Prostaglandin E\(_2\) promotes Th1 differentiation via synergistic amplification of IL-12 signalling by cAMP and PI3-kinase

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T helper 1 (Th1) cells have critical roles in various autoimmune and proinflammatory diseases. cAMP has long been believed to act as a suppressor of IFN-\(\gamma\) production and Th1 cell-mediated immune inflammation. Here we show that cAMP actively promotes Th1 differentiation by inducing gene expression of cytokine receptors involved in this process. PGE\(_2\) signalling through EP2/EP4 receptors mobilizes the cAMP-PKA pathway, which induces CREB- and its co-activator CRTC2-mediated transcription of IL-12R\(\beta2\) and IFN-\(\gamma\)R1. Meanwhile, cAMP-mediated suppression of T-cell receptor signalling is overcome by simultaneous activation of PI3-kinase through EP2/EP4 and/or CD28. Loss of EP4 in T cells restricts expression of IL-12R\(\beta2\) and IFN-\(\gamma\)R1, and attenuates Th1 cell-mediated inflammation \textit{in vivo}. These findings clarify the molecular mechanisms and pathological contexts of cAMP-mediated Th1 differentiation and have clinical and therapeutic implications for deployment of cAMP modulators as immunoregulatory drugs.

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T helper (Th) 1 cells have critical roles in various inflammatory immune diseases. Differentiation of naïve T cells to Th1 cells is driven by two critical cytokines, interleukin (IL-12) and interferon (IFN-γ), the former derived from antigen-presenting cells and the latter mainly from T cells, at the beginning, on T-cell receptor (TCR) stimulation. Another cytokine produced on TCR activation, IL-2, is also important in the early stage of Th1 differentiation. TCR ligation initiates activation of Lck and recruitment of ZAP-70 to the TCR complex to generate multiple downstream signals, resulting in the production of cytokines including IL-2 and IFN-γ, upregulation of cell surface molecules such as CD25, and induction of transcription factors for differentiation of Th cells such as T-bet. Prostaglandin E₂ (PGE₂)-cyclic adenosine monophosphate (cAMP) signalling has long been believed to act as a potent negative regulator of T cells, especially Th1 cells, and is thought to mediate inhibition of immune inflammation in vivo. It was reported that cAMP inhibits almost all pathways downstream of TCR stimulation and suppresses T cell activation, proliferation and cytokine production. In contrast to this long-believed prevailing view, we and others previously reported that PGE₂ facilitates IL-12-driven Th1 differentiation through its receptor EP2 and EP4, answering how this cAMP action reconciled with its inhibitory effects demonstrated by many previous studies, what is the molecular mechanism whereby cAMP promotes Th1 development and, what is the pathophysiological context in which this cAMP action is used?

cAMP activates protein kinase A (PKA) and induces phosphorylation of the transcription factor cAMP responsive element (CRE)-binding protein (CREB) at Ser133. Phosphorylated CREB binds to CRE-containing promoter and initiates gene transcription, usually with its coactivator CREB-binding protein (CBP)/p300 (ref. 17). CREB-dependent gene expression is also promoted by another family of coactivators named cAMP-regulated transcriptional coactivator (CRTC) that binds to CREB in phospho-Ser133-dependent and -independent manners. Among the three members of the CRTC family, CRTC2 is present in abundance in the liver, spleen and lymph nodes. CREB binds to CRE-containing promoter and initiates gene transcription, usually with its coactivator CREB-binding protein/p300 (ref. 17). CREB-dependent gene expression is also promoted by another family of coactivators named cAMP-regulated transcriptional coactivator (CRTC) that binds to CREB in phospho-Ser133-dependent and -independent manners. Among the three members of the CRTC family, CRTC2 is present in abundance in the liver, spleen and lymph nodes. The C-terminal regulatory domain and inhibits its CRTC kinase activity, which triggers CRTC dephosphorylation and nuclear translocation. While the SIK-CRTC pathway has been shown to be crucial for such physiological processes as gluconeogenesis, neuronal survival and melanogenesis, its function in T-cell-mediated immune response has never been reported.

IL-12 and IFN-γ act on their cognate receptors to drive differentiation of Th1 cells from naïve T cells. The IL-12 receptor is composed of two subunits, β1 and β2 chains (IL-12Rβ1 and β2), among which the latter is induced specifically during Th1 differentiation and is responsible for IL-12 signal transduction. However, its expression mechanism is not known in detail. Moreover, although naïve T cells express both subunits of IFN-γ receptor, z and β chains (IFN-γR1 and R2), IFN-γR1 is downregulated shortly after TCR engagement in vivo. Whether there is any signal counteracting this downregulation of IFN-γR1 by TCR or directly inducing IFN-γR1 expression is currently unknown. Such a signal, if present, may be important, because the downregulation of IFN-γR1 may restrict IFN-γ signalling, which is essential for induction of Th1-lineage-specific transcription factor T-bet and IL-12Rβ2 in the early stage of Th1 differentiation.

Here we demonstrate that cAMP promotes Th1 differentiation through upregulation of IL-12Rβ2 and IFN-γR1 and the amplification of these cytokine signalling pathways. PGE₂ mobilizes cAMP-PKA cascade, which directly induces gene expression of IL-12Rβ2 and IFN-γR1 through CREB and CRTC2. Simultaneous activation of PI3K by PGE₂ rescues T cells from the inhibitory effects of cAMP on TCR signalling, and promotes Th1-facilitative action of cAMP by maintaining, for example, production of IL-2 and IFN-γ and expression of CD25.

We further report that selective disruption of EP4 signalling in T cells limits expression of the above Th1 cytokine receptors and attenuates Th1-mediated inflammation in vivo.

Results

Induction of IL-12Rβ2 in TCR-activated T cells by PGE₂. To clarify how PGE₂ facilitates Th1 differentiation, we examined effects of PGE₂ on expression of IFN-γ (Ifng), T-bet (Tbx21), and IL-12Rβ2 (Il12rb2), three genes critical for Th1 differentiation, in T cells cultured under the Th1-priming conditions. PGE₂ considerably enhanced expression of Il12rb2 mRNA from 12 and 48 h, respectively, while enhancement of Tbx21 expression was not seen until 72 h (Fig. 1a). Enhanced expression of Il12rb2 mRNA at 24 h was mimicked by agonists selective to EP2 (ONO-DI-004) or EP3 (ONO-AE1-329) but not by agonists to EP1 (ONO-AE1-259) or EP4 (ONO-AE1-329) which both inhibit IL-12 receptor expression in naive T cells (Supplementary Fig. S1f), confirming that EP2 and EP4 mediate IL-12Rβ2 protein by PGE₂, EP2 or EP4 agonist during Th1 differentiation was confirmed by flow cytometry (Fig. 1c). These data suggested that promotion of Th1 differentiation by PGE₂ is likely to be initiated through induction of IL-12Rβ2 via EP2 and EP4 receptors.

At least three cytokine signalling pathways, IL-12, IFN-γ and IL-2, are involved in Th1-priming in the culture system we used. Given that all of these cytokines have the ability to induce IL-12Rβ2 in T cells, we asked whether these cytokines signalling are involved in PGE₂-induced IL-12Rβ2 expression. We stimulated T cells with anti-CD3 and anti-CD28 without exogenous IL-12 and found that PGE₂ still upregulated Il12rb2 mRNA and protein expression (Fig. 1d,e). Furthermore, blockade of IL-12 signalling by anti-IL-12 had little effect on the basal or PGE₂-induced expression of IL-12Rβ2 protein (Supplementary Fig. S1a). Blockade of IFN-γ (Supplementary Fig. S1b) or both IFN-γ and IL-2 (Supplementary Fig. S1c) signalling by using IFN-γR1−/− and anti-IL-2 reduced both the basal IL-12Rβ2 expression and its enhancement by PGE₂. However, even without IFN-γ and IL-2 action, PGE₂ still exhibited enhancement of IL-12Rβ2 expression over the basal level (Supplementary Fig. S1c). EP2 and EP4 agonists again mimicked this action of PGE₂ on expression of IL-12Rβ2 mRNA and protein in TCR-activated T cells (Supplementary Fig. S1d,e), and the induction of Il12rb2 mRNA expression by EP2 or EP4 agonists was defective in EP2- or EP4-deficient T cells, respectively (Supplementary Fig. S1f), confirming that EP2 and EP4 mediated IL-12Rβ2 induction by PGE₂.

Involvement of PI3K and cAMP in PGE₂-induced IL-12Rβ2. Given that EP2 and EP4 stimulation activates both PI3-kinase
Figure 1 | PGE\(_2\)-cAMP signalling induces IL-12R\(\beta2\) expression in TCR-activated T cells. (a) Expression of Ifng, Tbx21 and II12rb2 mRNA by T cells activated for indicated times with antibody to CD3 and antibody to CD28 (\(x\)CD3/CD28) in the absence or presence of PGE\(_2\) under Th1-priming conditions. A portion of cells were restimulated with PMA and ionomycin for the last 4 h (72R). (b) Expression of Ifng, Tbx21 and II12rb2 mRNA by T cells activated for 24 h with \(x\)CD3/CD28 in the absence or presence of PGE\(_2\) or selective agonists to EP1 to EP4 under Th1-priming conditions. (c) Surface expression of IL-12R\(\beta2\) in T cells activated for 48 h with \(x\)CD3/CD28 in the absence or presence of PGE\(_2\) or agonists selective to EP1 to EP4 under Th1-priming conditions. Grey-filled histogram represents isotype control. ∆MFI (mean fluorescence intensity) indicates the differences between MFI of IL-12R\(\beta2\) and MFI of isotype control (right). (d) Time-course of II12rb2 mRNA expression by PGE\(_2\) in T cells activated with \(x\)CD3/CD28. (e) PGE\(_2\) induces IL-12R\(\beta2\) protein expression in T cells activated with \(x\)CD3/CD28 for 48 h. (f) Expression of II12rb2 mRNA in WT T cells (f) or IFN-\(\gamma\)/R1\(\beta\) T cells supplemented with anti-IL-2 (g), activated for 24 h with \(x\)CD3/CD28 in the absence or presence of PGE\(_2\) with or without Wortmannin, LY-294002 or H-89. (h) Expression of II12rb2 mRNA in T cells activated with \(x\)CD3/CD28 for 24 h with \(x\)CD3/CD28 in the absence or presence of PGE\(_2\) with H-89, Rp-8-Br-CAMPS or Rp-8-CPT-CAMPS. (i) Expression of IL-12R\(\beta2\) mRNA (j) and protein (k) in T cells activated with \(x\)CD3/CD28 in the presence of db-cAMP, forskolin or 3-isobutyl-1-methylxanthine (IBMX) for 12 h (i) or 48 h (j). (k) Expression of II12rb2 mRNA in IFN-\(\gamma\)/R1\(\beta\) T cells activated for 24 h with \(x\)CD3/CD28 with or without db-cAMP in the absence or presence of anti-IL-2 or LY-294002 (LY) or both. Data shown as mean ± s.e.m. are representative of two or more independent experiments with triplicates. Statistical significance was examined by unpaired two-tailed Student’s t-test, *\(p<0.05\); **\(p<0.01\); ***\(p<0.001\). NS, not significant, veh, vehicle; a.u., arbitrary units.

(P13K)-Akt and cAMP-PKA pathways\(^9\), we examined their involvement in IL-12R\(\beta2\) induction by PGE\(_2\). Inhibitors of P13K (LY-294002 and wortmannin) partially prevented PGE\(_2\)-induced II12rb2 expression in TCR-activated T cells (Fig. 1f). As P13K-Akt pathway mediates TCR-induced production of IFN-\(\gamma\) and IL-2 that have important roles in IL-12R\(\beta2\) expression and

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Th1 differentiation, we examined effects of LY-294002 on IL-12Rβ2 induction in TCR-activated IFN-γR1-deficient T cells supplemented with anti-IL-2. Under this condition where both IFN-γ and IL-2 signalling were blocked, LY-294002 did not inhibit PGE2-induced Il12rb2 expression (Fig. 1g). These results indicate that there are PI3K-dependent and -independent mechanisms of Il12rb2 induction and the former was dependent on IFN-γ and IL-2. Intriguingly, PKA inhibitors (H-89, Rp-8-Br-cAMPS or Rp-8-CPT-cAMPS) also attenuated PGE2-induced Il12rb2 expression in TCR-activated wild-type (WT) T cells (Fig. 1h), and H-89 completely suppressed IL-12Rβ2 induction in the absence of IFN-γ and IL-2 signalling (Fig. 1g). Conversely, expression of IL-12Rβ2 mRNA and protein was enhanced by dibutylryl cAMP (db-cAMP) and forskolin (Fig. 1i,j). Moreover, Il12rb2 mRNA was upregulated by a nonspecific phosphodiesterase (PDE) inhibitor 3-isobutyl-1- methylxanthine (Fig. 1i), suggesting that endogenous cAMP has a role in IL-12Rβ2 expression. Interestingly, cAMP-induced IL-12Rβ2 expression in TCR-activated T cells was suppressed partially by PI3K inhibitors but this suppression was not seen when both IFN-γ and IL-2 signalling were blocked (Fig. 1k).

**Direct and indirect induction of IL-12Rβ2 by cAMP.** As the above results suggest the involvement of IFN-γ and IL-2 in a part of cAMP-induced Il12rb2 expression by TCR-activated T cells, we next evaluated how and how much IFN-γ and IL-2 contribute to cAMP-induced Il12rb2 expression by stimulating T cells with or without TCR activation, with or without IFN-γ signalling, and in the presence or absence of cAMP for 12 h. In WT T cells, while db-cAMP, IFN-γ or TCR activation alone induced Il12rb2 expression at a similar level, cAMP synergistically induced Il12rb2 expression in the presence of IFN-γ or TCR or both (Fig. 2a). Addition of anti-IFN-γ did not affect the stimulation by db-cAMP in unactivated T cells, but blunted cAMP response in TCR-activated cells. Consistently, none of the above synergistic effects of IFN-γ or TCR with cAMP was seen in IFN-γR1−/− T cells at 12 h (Fig. 2a). Synergistic action of cAMP with IFN-γ or TCR activation on IL-12Rβ2 protein expression in T cells was also confirmed (Fig. 2b). We then evaluated the involvement of IL-2 signalling in cAMP-induced IL-12Rβ2 expression. We used IFN-γR1−/− T cells to exclude the effect of IFN-γ signalling. Although cAMP and TCR signalling had no synergistic action on Il12rb2 expression in IFN-γR1−/− T cells at 12 h (Fig. 2a), we noted that they synergistically induced Il12rb2 expression after 24 h and this synergistic action was eliminated by anti-IL-2 (Fig. 2c), suggesting that synergistic action on Il12rb2 expression with TCR activation and cAMP was mediated by IL-2 and this phenomenon did not appear until 24 h of stimulation. Consistently, blocking either IFN-γ or IL-2 signalling downregulated the basal, as well as the cAMP-induced Il12rb2 expression, and blocking both further reduced Il12rb2 expression in TCR-activated WT T cells at 36 h, but cAMP still enhanced Il12rb2 expression over the basal level under these conditions (Fig. 2d). These results demonstrate that there are direct and indirect mechanisms of cAMP-promoted Il12rb2 expression, and that IFN-γ and IL-2 are involved in the latter indirect mechanism.

**cAMP context-dependently regulates Th1 differentiation.** The above results that cAMP synergizes with IFN-γ and IL-2 in Il12rb2 induction in TCR-activated T cells led us to wonder how such synergistic effects are elicited by cAMP, because cAMP is known to suppress production of these cytokines by inhibiting TCR signalling. We confirmed that cAMP treatment at the beginning of TCR stimulation indeed strongly suppressed markers of T-cell activation such as CD25, IL-2 and IFN-γ.

However, this inhibitory effect of cAMP could be rescued by enhancing CD28 costimulation and, intriguingly, this rescue was repressed by LY-294002 (Fig. 2e). These results suggest that coactivation of PI3K overcomes the suppression of TCR signalling by cAMP, and allows cAMP to facilitate Il12rb2 expression. We then asked whether similar coactivation of PI3K could rescue cAMP inhibition of Th1 differentiation. To this end, we stimulated T cells with anti-CD3 and different concentrations of anti-CD28 and treated cells with db-cAMP for various periods under Th1-polarizing conditions. cAMP-mediated inhibition of Th1 differentiation was rescued or overcome by the strengthening CD28 costimulation or by the addition of cAMP at later times after TCR activation, which avoided cAMP-mediated inhibition on initiation of TCR signalling (Fig. 2f). Moreover, PGE2 did not inhibit but promoted Th1 differentiation even if it was added into cultures at the beginning of TCR ligation (0 h) at a CD28 costimulation level where cAMP showed an extremely strong inhibitory effect (Fig. 2f). This could be due to the fact that PGE2 antagonized both cAMP and PI3K pathways through EP2 and EP4 in T cells and PI3K antagonized cAMP-dependent inhibition of cAMP signalling. Similarly, cAMP-mediated inhibition of cell proliferation during Th1 differentiation was also rescued by strengthening CD28 costimulation or postponing cAMP treatment (Fig. 2g).

**Upregulation of IFN-γR1 and IL-2Rβ by cAMP.** As the above findings suggest that cAMP can induce IL-12Rβ2 expression in T cells partly through IFN-γ and IL-2, the next question was how cAMP amplified IFN-γ and IL-2 signalling. We first noted that the induction of Il12rb2 by cAMP was partially reduced by treatment of the cells with cycloheximide, suggesting the involvement of new protein synthesis in this process (Fig. 3a). To identify protein(s) newly synthesized in response to cAMP, we profiled gene expression in TCR-activated WT and IFN-γR1−/− T cells stimulated with or without db-cAMP. Consistent with previous reports, cAMP inhibited gene expression of several cytokines such as Il2, Ifng, Tnf and Lta in TCR-activated T cells (Fig. 3b). In congruence with our results (Fig. 2a,b), Il12rb2 expression was upregulated by db-cAMP in both WT and IFN-γR1−/− T cells (Fig. 3b), verifying an IFN-γ signalling-independent induction of Il12rb2 by cAMP. Additionally, we noted enhanced expression of genes encoding receptors for IFN-γ and IL-2, such as Ifngr1 and Il2rb, in T cells (Fig. 3b). We confirmed by real-time PCR and flow cytometry that cAMP increased both Ifngr1 mRNA and IFN-γR1 protein expression in naive T cells (Fig. 3c,d). This action of cAMP was also observed in TCR-activated T cells, although TCR itself strongly downregulated IFN-γR1 expression (Fig. 3c,d). IFN-γ-induced STAT1 activation in naive T cells was also enhanced by pretreatment with db-cAMP, suggesting that cAMP amplifies IFN-γ signalling (Fig. 3e). Similarly, cAMP also upregulated Il2rb mRNA expression in T cells after 24 h, but not 12 h, stimulation (Fig. 3d) and upregulation of IL-2Rβ protein expression by cAMP was also confirmed using flow cytometry (Fig. 3g). These data suggested that cAMP amplifies IFN-γ and IL-2 signalling in T cells through the upregulation of IFN-γR1 and IL-2Rβ, respectively.

**Requirement of CREB for cAMP-induced IL-12Rβ2 and IFN-γR1.** We next investigated the molecular mechanisms of how cAMP directly induces IL-12Rβ2 and IFN-γR1 expression. The db-cAMP-induced Il12rb2 and Ifngr1 expression was mimicked by a PKA-specific agonist N6-Bnz-cAMP (ref. 34) (Fig. 4a) and attenuated by PKA inhibitors (Fig. 4b) in unactivated T cells, which produced neither IL-2 nor IFN-γ. We transfected...
Figure 2 | cAMP context-dependently regulates TCR activation and Th1 differentiation. (a) Expression of Il12rb2 mRNA in WT (left) and IFN-γR1–/– (right) T cells activated for 12 h without (unactivated) or with αCD3/CD28 in the presence or absence of db-cAMP, IFN-γ or anti-IFN-γ. (b) Surface expression of IL-12Rβ2 in WT or IFN-γR1–/– T cells activated for 48 h with αCD3/CD28. db-cAMP or vehicle was added from 24–48 h. (c) Expression of Il12rb2 mRNA in IFN-γR1–/– T cells activated for 24 h (unactivated) or with αCD3/CD28 in the absence or presence of db-cAMP or anti-IL-2 or both. (d) Expression of Il12rb2 mRNA in T cells activated with αCD3/CD28 for 36 h with addition of db-cAMP, anti-IFN-γ or anti-IL-2 for the last 12 h. (e) Expression of CD25 and production of IL-2 and IFN-γ by T cells activated with αCD3 and indicated concentrations of αCD28 and treated with db-cAMP or LY-294002 (LY) for 24 h. The percentages indicate the cAMP-mediated inhibition compared with each vehicle group. (f-g) T cells were activated for 48 h with αCD3 and indicated concentrations of αCD28 and treated with db-cAMP or PGE2 for indicated periods under Th1-priming conditions. Cells were then washed and reincubated for another 24 h under Th1-priming conditions followed by intracellular staining of IFN-γ (f). Effect of cell proliferation by cAMP is presented as a percentage relative to vehicle group (100%) under each αCD28 condition (g). Data shown as mean ± s.e.m. are representative of two independent experiments with triplicates. MFI, mean fluorescence intensity; a.u., arbitrary units.
Involvement of CRTC2 in cAMP-induced IL-12Rβ2 and IFN-γR1. One candidate for such factors is CRTC2 that is regulated negatively by SIK2 and functions as a CREB coactivator in many types of cells19–22. Stimulation of T cells with db-cAMP induced not only phosphorylation of CREB but also dephosphorylation of CRTC2 (Fig. 5a), decreased the amount of CRTC2 in the cytoplasm and increased the amount of dephosphorylated CRTC2 in the nucleus (Fig. 5b). This distribution is SIK2-dependent, because SIK2−/− T cells22 constitutively showed more nuclear CRTC2 than WT cells, which increased little by cAMP (Fig. 5c). As reported18,19, protein kinase inhibitor staurosporine (STS) induced dephosphorylation and nuclear translocation of CRTC2 without enhancing CREB phosphorylation (Fig. 5b). To investigate whether cAMP affects transcriptional activity of CRTC2, we performed a reporter assay with GAL4-fusion Creb1 or a scrambled siRNA and then stimulated T cells with db-cAMP. Creb1-specific siRNA reduced CREB expression in transfected cells (Fig. 4c) and suppressed cAMP-induced Il12rb2 and Ifngr1 expression compared with those in T cells transfected with scrambled siRNA (Fig. 4d). CREB is usually activated by phosphorylation at Ser133, p-CREB(S133), which promotes transcription by recruitment of the coactivator CREB-binding protein to gene loci17. However, although TCR or phorbol-12-myristate-13-acetate (PMA) plus ionomycin could induce CREB activation (Fig. 4e), they did not induce, but rather reduced Il12rb2 or Ifngr1 expression under the condition where cAMP induced both mRNAs (Fig. 4f). These results suggest requirement of other factors downstream of PKA in addition to p-CREB(S133).

unactivated T cells with a small interfering RNA (siRNA) for Creb1 or a scrambled siRNA and then stimulated T cells with db-cAMP. Creb1-specific siRNA reduced CREB expression in transfected cells (Fig. 4c) and suppressed cAMP-induced Il12rb2 and Ifngr1 expression compared with those in T cells transfected with scrambled siRNA (Fig. 4d). CREB is usually activated by phosphorylation at Ser133, p-CREB(S133), which promotes transcription by recruitment of the coactivator CREB-binding protein to gene loci17. However, although TCR or phorbol-12-myristate-13-acetate (PMA) plus ionomycin could induce CREB activation (Fig. 4e), they did not induce, but rather reduced Il12rb2 or Ifngr1 expression under the condition where cAMP induced both mRNAs (Fig. 4f). These results suggest requirement of other factors downstream of PKA in addition to p-CREB(S133).

Figure 3 | cAMP amplifies IFN-γ and IL-2 signalling through induction of IFN-γR1 and IL-2Rβ. (a) Expression of Il2rb2 mRNA in T cells activated with αCD3/CD28 for 24 h with addition of db-cAMP or cycloheximide (CHX) or both for the last 12 h. (b) Profile of db-cAMP-dependent expression of cytokines and their receptors by WT or IFN-γR1−/− T cells activated for 12 h with αCD3/CD28 followed by treatment with db-cAMP or vehicle for another 3 h. Data shown in bar graphs represents fold change (db-cAMP versus vehicle) in mean intensity from each probe in biological duplicates. The probe for Ifngr1 in this array is targeted to a segment of sequence in the exon VII, while The IFN-γR1−/− mouse that we used has Ifngr1 gene disrupted by inserting the neomycin resistance gene into exon V. (c,d) Expression of Ifngr1 mRNA (c) and protein (d) in T cells stimulated with or without αCD3/CD28 in the absence or presence of db-cAMP for 12 h. (e) Immunoblot for p-STAT1 (Y701) and STAT1 in T cells pretreated with db-cAMP for 12 h, washed and restimulated with 1 ng ml−1 IFN-γ for another 30 min. (f) Expression of Il2rb mRNA in T cells treated with db-cAMP for 12 and 24 h. (g) IL-2Rβ protein expression in T cells activated with αCD3/CD28 for 2 days, allowed to rest for another 2 days, then restimulated with db-cAMP in the presence of IL-2 for 24 h. Data shown as mean ± s.e.m. are representative of two independent experiments with triplicates (a,c-g) or are from one experiment (b), a.u., arbitrary units.

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Figure 4 | Activation of PKA-CREB pathway is required but insufficient for cAMP-induced IL-12Rb2 and IFN-γR1 expression. (a) Expression of *Il12rb2* and *Ifngr1* mRNA in T cells stimulated with indicated concentrations of a PKA agonist, N6-Bnz-cAMP, for 12 h. (b) Expression of *Il12rb2* and *Ifngr1* mRNA in T cells stimulated with db-cAMP in the presence of PKA inhibitor, Rp-8-Br-cAMPS and/or Rp-8-CPT-cAMPS for 12 h. (c,d) T cells treated with scrambled or Creb1 siRNA for 48 h, followed by immunoblot for CREB (e) or stimulated with db-cAMP for another 12 h for detection of mRNA expression. (d) mRNA expression is normalized to vehicle-treated of each siRNA-transfected cells. Icer and N4a2 were used as positive controls for CREB-dependent genes. (e) Immunoblot for p-CREB(S133) in IFN-γR1−/− T cells stimulated without (unactivated) or with either αCD3/CD28, PMA (20 ng ml−1) plus ionomycin (500 ng ml−1) (PMA + lono), or db-cAMP for 30 min. (f) Expression of *Il12rb2* and *Ifngr1* mRNA in IFN-γR1−/− T cells stimulated as in e in the presence of anti-IL-2 for 12 h. Data shown as mean ± s.e.m. are representative of two (a–c,e,f) or four (d) independent experiments with triplicates. Statistical significance was examined by unpaired two-tailed student’s t-test, *P < 0.05; **P < 0.01; ***P < 0.001. a.u., arbitrary units.

Binding of p-CREB and CRTC2 to *Il12rb2* and *Ifngr1* gene loci.

Computational analysis identified several putative half or full CRE sites in the promoter/enhancer regions of *Il12rb2* and *Ifngr1* gene loci (Fig. 6a). We performed chromatin immuno precipitation analysis and observed that upon cAMP stimulation, p-CREB(S133) and CRTC2 were recruited to several of these sites located from −3.6 to +0.8 kb in *Il12rb2* gene locus (Fig. 6b, sites E-L). Similarly, cAMP increased binding of p-CREB(S133) and CRTC2 to two sites around the transcription start site and one site at position +4.6 kb in the first intron of *Ifngr1* gene (Fig. 6c, sites M, N and P). These results suggest that CREB and CRTC2 activated by cAMP are recruited to the sites in the promoter/enhancer regions of *Il12rb2* or *Ifngr1* gene loci.

We cloned a −4.6 kb to +70 bp fragment of the *Il12rb2* promoter/enhancer region in the pGL4 luciferase reporter vector, transfected it into EL4 cells and stimulated with db-cAMP or forskolin. db-CAMP and forskolin induced *Il12rb2* promoter reporter activity (Fig. 6d) and this CAMP-induced *Il12rb2* promoter activity was suppressed by treatment with Creb1 siRNA, overexpression of the CREB(S133A) mutant or Crtc2 siRNA (Fig. 6e–g), indicating that both p-CREB(S133) and CRTC2 regulate CAMP-activated *Il12rb2* transcription.

EP4 signalling in T cells enhances Th1 inflammation in vivo.

To study whether PGE2-EP4-cAMP signalling in T cells regulates cytokine receptors and Th1 response in vivo, we generated mice with deletion of EP4 in T cells by crossing Lck-Cre mice with EP4-floxed mice. Loss of EP4 in CD4+ T cells was confirmed in Lck-Cre+ EP4fl/fl and Lck-Cre− EP4fl/fl mice (Fig. 7a). To investigate whether EP4 deficiency in T cells affects in vivo Th1 differentiation and expression of Th1-related cytokine receptors, we used the CHS model and immunized Lck-Cre− EP4fl/fl or Lck-Cre+ EP4fl/fl mice by painting the shaved abdomen with dinitrofluorobenzene (DNFB) on day 0 and purified CD4+ T cells in draining lymph nodes (dLNs) on day 5. CD4+ T cells from Lck-Cre− EP4fl/fl mice produced less amounts of IFN-γ and IL-2 (Fig. 7b) and exhibited less mRNA expression of Th1 cytokine receptors, for example, *Il12rb2*, *Ifngr1*, *Il2rb* and CREB/CRTC2-targeted gene Icer than T cells from littermate control Lck-Cre− EP4fl/fl mice (Fig. 7c). Consistent with the above results, the numbers of IL-12Rb2+ and IFN-γR1-expressing CD4+ T cells in dLNs from Lck-Cre− EP4fl/fl mice were also lower than those from control mice (Fig. 7d). We also found that *Cd69* gene expression and the number of CD4+ CD69+ T cells were decreased in dLNs from DNFB-sensitized Lck-Cre− EP4fl/fl mice (Fig. 7c,d). Moreover, when we adoptively transferred dLN
cells from DNFB-sensitized Lck-Cre^{+} EP4^{fl/fl} or Lck-Cre^{-} EP4^{fl/fl} mice into naive C57BL/6 mice on day 5 and immediately challenged the recipient mice by painting the same antigen on the ear, transfer of EP4-deficient T cells induced less ear swelling (Fig. 7e).

We next isolated CD45RB^{hi}CD25^{-} CD4^{+} naive T cells from the spleen of Lck-Cre^{-} EP4^{+/-} or Lck-Cre^{-} EP4^{fl/fl} mice, transfected them into mice deficient in recombination-activating gene 2 (Rag2^{-/-} mice), and monitored development and progression of colitis. Transfer of naive T cells of both genotypes induced colitis in Rag2^{-/-} mice. However, the transfer of Lck-Cre^{+} EP4^{fl/fl} T cells induced milder body weight loss (Fig. 7f) and weaker colonic inflammation than that of T cells from littermate control Lck-Cre^{+} EP4^{+/-} mice (Fig. 7g). Moreover, mesenteric lymph node CD4^{+} T cells from Rag2^{-/-} mice reconstituted with Lck-Cre^{+} EP4^{fl/fl} T cells produced less amounts of IFN-γ and IL-2 (Fig. 7h) and expressed lower levels of Il12rb2, Ifngr1, Il2rb, Cd69 and Icer mRNA.
EL4 cells stably expressing the examined by unpaired two-tailed Student’s t-test, *P<0.05; **P<0.01.

(Fig. 7i) than CD4+ T cells from Rag2−/− mice reconstituted with Lck-Cre+ EP4+/+ T cells. We also repeated this transfer experiment using CD45RBhiCD25− CD4+ naive T cells from Lck-Cre+ EP4fl/fl mice or EP2−/− mice. We have found attenuation of the colitis also in the Rag2−/− mice reconstituted with Lck-Cre+ EP4fl/fl T cells and have not found any difference of colitis development between the groups of mice (data not shown). These results confirm the importance of EP4 and indicate that EP2 has, if any, a minor role in T cells, a loss of which can be compensated for by EP4.

Discussion
Here we have dissected the molecular mechanism underlying PGE2-induced facilitation of Th1 differentiation, and revealed the role of cAMP in this process. PGE2 acts on EP2 or EP4 receptors to generate cAMP and cAMP activates PKA, which in turn directly phosphorylates CREB and induces dephosphorylation and nuclear translocation of CRTC2 through inhibiting SIK2. Activated CREB and CRTC2 together promote gene transcription of IL-12Rβ2 and IFN-γR1, receptors for two critical cytokines of Th1 differentiation. Our findings are consistent with recent reports that PGI2-IP signalling promotes Th1 differentiation.
through cAMP-PKA pathway in vitro and enhances CHS responses in vivo, and that Gαs-deficient T cells which fail to produce cAMP, show impaired differentiation into Th1 cells and that the addition of cAMP analogue restores Th1 differentiation in these cells. Our study has thus verified the facilitative role of cAMP in Th1 differentiation by clarifying its molecular mechanism.

A question remains as to how these findings are reconciled with the long-held, traditional view that cAMP suppresses Th1 differentiation. Here we found that cAMP-mediated inhibition of TCR-induced expression of CD25, production of IL-2 and IFN-γ, Th1 differentiation and cell proliferation could all be rescued or even overcome by strengthening CD28 costimulation in a PI3K-dependent manner. Simultaneous activation of PI3K at
cAMP generation by PGE₂-EP2/EP4 signalling combined with that by CD28 costimulation can cancel out the cAMP-mediated inhibition of TCR signalling, and preserve, for example, expression of critical genes such as CD25, IL-2 and IFN-γ. The primary inhibitory site by cAMP may be Lck. Tasken and his collaborators suggested that PKA interferes with LCK activation, and this action can be antagonized by PI3K activation following CD28 costimulation.33,40

Then, how important is this PGE₂-cAMP-dependent mechanism in Th1-mediated immune response in vivo and in human immune diseases? Here we have used two disease models and demonstrated that EP₄-cAMP signalling in T cells facilitated expression of Th1 cytokine receptors and Th1 response in vivo. We have generated mice with selective deletion of EP4 in T cells, and revealed that the loss of EP4 in T cells considerably attenuates CHS response and the adoptive transfer colitis. In both models, selective blockade of EP4 in T cells prevented in vivo Th1 differentiation with downregulated expression of Il12rb2, Ilfnr1 and Il2rb genes and other CREB/CRTC2-dependent genes in lymph nodes (LN) CD4⁺ T cells. CHS is a mouse model of allergic contact dermatitis in humans, in which IFN-γ produced by CD4⁺ Th1 and CD8⁺ type 1 cytokotic T cells has an important role.41 The adoptive transfer colitis is a model of inflammatory bowel disease, particularly CD42.

Figure 8 | A model for synergistic action of cAMP and PI3K in Th1 differentiation. cAMP generated in response to PGE₂ binding to EP2/EP4 activates PKA, which phosphorylates CREB and activates CRTC2 through phosphorylation of SIK. Activated CREB and CRTC2 translocate to the nucleus and induce expression of Il12rb2, Ilfnr1 and possibly Il2rb, thus facilitating Th1 differentiation synergistically with IL-12, IFN-γ and IL-2. While cAMP-PKA inhibits T-cell activation by suppressing TCR signalling, coactivation of PI3K by EP2/EP4 and CD28 cancels this inhibition and promotes the Th1-facilitative action of cAMP.

Methods
Mice. C57BL/6 mice were obtained from Japan SLC (Shizuoka, Japan). EP2- and EP4-deficient and littermate control WT mice have been previously described.43 Mice defective in IFN-γR1 on a C57BL/6 background were kind gifts from M. Aguet. To generate EP4-deficient T cells, Lck-Cre mice were crossed to lox-flanked Ptgdr mice.38 SIK2 mice have been described elsewhere and were housed in the National Institute of Biomedical Innovation. All mice, except the SIK2 knockout mice, were housed at the Institute of Laboratory Animals of Kyoto University on a 12-h light/dark cycle under specific pathogen-free conditions. All experimental procedures were approved by the Committee on Animal Research of Kyoto University Faculty of Medicine and National Institute of Biomedical Innovation.

Plasmids. WT CREB and the CREB(S133A) mutant plasmids were kindly provided by H. Bito, pGFP-nsIK2, pGFP-nIL2RB 5878A, pGFP-mCRTC2 S171A and GAL4 fused pM-mCRTC2 and pTAL-5x GAL4 have been described.

Reagents. PGE₂ (100 nM) was obtained from Cayman Chemical. Agonists selective to each EP subtype (ONO-1080 0.4 µM, ONO-AE1-239, ONO-AE-248 and ONO-AE1-329 for EP1, EP2, EP3 and EP4, respectively) were kindly gifts of Ono Pharmaceutical Co., Japan. Dibutyryl cAMP (db-cAMP, 100 µM), N6-Bnz-cAMP (300-300 µM), wortmannin (100 nM), LY-294002 (1 µM), H-89 (10 µM), Rp-8-CPT-cAMPS (300 µM), Rp-8-Br-cAMPS (300 µM), Forskolin (1 µM) and 3-isobutyl-1-methylxanthine (100 µM) were purchased from Sigma. Cycloheximide (10 µM) and STS (5-50 nM) were purchased from Calbiochem.

Preparation and culture of CD4⁺ T cells. Naïve CD4⁺ CD45RB⁺ CD25⁻ and CD4⁺ CD25⁺ T cells were purified from spleens and LNs by using FACs Aria II (Becton Dickinson) and auto-MACS (Miltenyi), respectively. Cells were cultured in complete RPMI1640 medium containing 10% FBS. For in vitro activation of T cells, plate-bound antibodies to CD3 (10 µg ml⁻¹, clone 145-2C11, ebioscience) and CD28 (10 µg ml⁻¹ or indicated concentrations, clone 37.51, eBioscience) were used. For T₄₁ differentiation, cells were stimulated with anti-CD3 and anti-CD28 for the first 2 days, and then without anti-CD3 and anti-CD28 antibodies for
another 24 h. Cells were cultured with 2,500 U ml\(^{-1}\) rmIL-2, 10 ng ml\(^{-1}\) rmIL-12 (R&D Systems) and 5 ng ml\(^{-1}\) anti-IL-4 (clone 11B11, eBioscience) for 3 days. To reduce the background of non-phosphorylated CREB, freshly isolated CD\(^+\) T cells were cultured for 2 days in RPMI1640 medium and IL-7 (10 ng ml\(^{-1}\), R&D Systems) that was added to maintain the survival of naive T cells\(^{22}\). In some culture conditions, 10 ng ml\(^{-1}\) of IFN-γ (R&D Systems) or 10 μg ml\(^{-1}\) of anti-mouse IFN-γ (clone XM1G2, eBioscience), anti-mouse IL-12/IL-23 p40 (clone C17/8, eBioscience) or anti-mouse IL-2 II-2 (clone JES6-1A12, eBioscience) were added.

**Surface and intracellular staining.** For surface staining, CD\(^+\) T cells were directly stained with phycoerythrin (PE)-conjugated anti-mouse CD119 (IFN-γR1, clone 2E2, eBioscience), PE-conjugated rat anti-mouse CD122 (IL-2Rβ, clone TM-P1, BD Pharmingen) or PE-conjugated anti-mouse CD69 (clone H1.2F3, eBioscience), or stained firstly with hamster anti-mouse IL-12Rβ2 (BD Pharmingen) and then with PE-conjugated mouse anti-Armenian and Syrian hamster IgG cocktail (BD Pharmingen). For intracellular staining, CD\(^+\) T cells were restimulated with 50 ng ml\(^{-1}\) PMA (Sigma) and 500 ng ml\(^{-1}\) ionomycin (Sigma) in the presence of GolgiPlug (BD Pharmingen) for the last 4–5 h of incubation. Cells were fixed and permeabilized with Cytofix/Cytoperm (BD Pharmingen) and stained with fluorescein isothiocyanate (FITC)-conjugated antibody to IFN-γ (clone XM1G2, eBioscience), and PE-conjugated antibody to IL-4 (clone 11B11, eBioscience). Quantitative flow cytometry was performed on an Epics XL-MCL (Beckman Coulter) or FACS Calibur (BD Bioscience).

**Fractionation and western blot.** Cytoplasmic and nuclear cell fractions were prepared using ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem). Western blotting was performed according to a protocol from Cellex (Epsom, UK). Anti-mouse CREB, p-CREB (S133), STAT1, and p-STAT1 (Y701) antibodies were obtained from Cell Signaling. Anti-mouse GAPDH (6C5, Ambion), anti-mouse β-tubulin (DM 1A, Sigma) and anti-goat lamin B (C-20, Santa Cruz) were used as internal control. Anti-rabbit CRTC2 anti-serum has been described\(^{61}\). Immunoreactive proteins were visualized by using the enhanced chemiluminescence system from Fuji Film (LAS-2000).

**Enzyme-linked immunosorbent assay.** For the detection of IL-2 and IFNγ production, the supernatants of cell cultures were collected, and the manufacturer’s instructions were followed (Pierce).

**Real-time PCR.** RNA purification and reverse transcription were performed by using Rneasy Mini Kit (Qiagen) and High-capacity cDNA Reverse Transcription kit (Applied Biosystems). Enzymatic Chromatin IP Kits (Cell signaling) according to the manufacturer’s instructions were followed (Pierce).

**Enzyme-linked immunosorbent assay.** We sensitized Lck-Cre\(^+\) EP4\(^{-}\) and Lck-Cre\(^-\) EP4\(^{+}\) mice with 25 μl of 1% (w/v) DNFB in acetone/olive oil (4/1, v/v) on shaved abdominal skin on day 0. dLN cells of one mouse were collected on day 5, and then transferred into one naive B6 mouse. The recipient mice were immediately challenged by application of 20 μl of 0.3 or 0.5% DNFB to their ear, and ear thickness was measured with a micrometre for each mouse before and 24 h after elicitation, and the difference is expressed as ear swelling. Alternatively, the dLN cells were harvested from DNFB-sensitized mice on day 5 to detect mRNA or protein expression of cytokine receptors or were subjected to isolation of CD4\(^+\) T cells by anto-MACS, and in vitro restimulated with anti-CD3 and anti-CD28 for 24 h for measuring cytokine production.

**Colitis model.** Adoptive transfer colitis model has been described\(^{43}\). Briefly, naive CD4\(^+\) CD25\(^-\) CD45RB\(^-\) T cells were prepared from Lck-Cre\(^+\) EP4\(^{-}\) or Lck-Cre\(^-\) EP4\(^{+}\) mice by cell sorting. Cells (5 × 10\(^5\) cells per mouse) were transferred intravenously into mice deficient in recombination-activating gene 2 (Rag2). CD4\(^+\) T cells from mesenteric lymph nodes of each recipient mouse were purified by auto-MACS on day 42 after T-cell transfer and stimulated with anti-CD3 for 72 h. IFN-γ and IL-2 production in supernatants and gene expression in CD4\(^+\) T cells were determined by enzyme-linked immunosorbent assay and real-time PCR, respectively.

**Data analysis.** All data were expressed as mean ± s.e.m., and statistical significance was examined by the unpaired two-tailed Student’s t-test except where indicated, using GraphPad (Prism) or Excel (Microsoft).

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Erratum: Prostaglandin E₂ promotes Th1 differentiation via synergistic amplification of IL-12 signalling by cAMP and PI3-kinase

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This Article contains errors in Fig. 6 that were introduced during the production process. In panels b and c, the y axes of the lower graphs should have been labelled ‘CRTC2 enrichment’. The correct version of the figure appears below.
Figure 6