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

Chengcan Yao^{1,2,†}, Takako Hirata^{1,2}, Kitipong Soontrapa¹, Xiaojun Ma¹, Hiroshi Takemori³ & Shuh Narumiya^{1,2}

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- γ 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. PGE₂ signalling through EP2/EP4 receptors mobilizes the cAMP-PKA pathway, which induces CREB- and its co-activator CRTC2-mediated transcription of IL-12R β 2 and IFN- γ 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 β 2 and IFN- γ R1, and attenuates Th1 cell-mediated inflammation *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.

¹Department of Pharmacology, Kyoto University Faculty of Medicine, Kyoto 606 8501, Japan. ²Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency (JST), Tokyo 102 0075, Japan. ³Laboratory of Cell Signaling and Metabolic Disease, National Institute of Biomedical Innovation, Osaka 567 0085, Japan. [†]Present address: MRC Centre for Inflammation Research, Queen's Medical Research Institute, University of Edinburgh, Edinburgh EH16 4TJ, United Kingdom. Correspondence and requests for materials should be addressed to S.N. (email: snaru@mfour.med.kyoto-u.ac.jp).

Helper (Th) 1 cells have critical roles in various inflammatory immune diseases. Differentiation of naive 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) stimulation¹. Another cytokine produced on TCR activation, IL-2, is also important in the early stage of Th1 differentiation². 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-bet³. 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*^{4–8}. It was reported that cAMP inhibits almost all pathways downstream of TCR stimulation and suppresses T cell activation, proliferation and cytokine production⁸. 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 (refs 9,10), the major downstream signalling of which is a rise in cAMP¹¹, and that this PGE₂ action operates *in vivo* in various mouse models of immune inflammation, such as experimental allergic encephalomyelitis, contact hypersensitivity (CHS), and collagen-induced arthritis. Consistently, genome-wide association studies have revealed that the *PTGER4* (encoding human EP4) gene is associated with multiple sclerosis and Crohn's disease (CD)^{12–14}, where the association with *IL12RB2* (human IL-12R β 2), a marker of Th1 cells, was also found^{14,15}. Furthermore, a recent study shows that T cells deficient in *Gas* and, therefore, incapable of producing cAMP, display impaired Th1 differentiation and fail to induce an inflammatory response¹⁶. While these studies suggest that PGE₂-cAMP signaling promotes rather than suppresses development of Th1 cells, there are several issues remain to be answered. For example, (1) how is this cAMP action reconciled with its inhibitory effects demonstrated by many previous studies, (2) what is the molecular mechanism whereby cAMP promotes Th1 development and, (3) 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/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^{18,19}. Among the three members of the CRTC family, CRTC2 is present in abundance in the liver¹⁸, spleen and lymph nodes (<http://biogps.gnf.org/>). Under the basal conditions, CRTC2 is phosphorylated at Ser171 by salt-inducible kinase (SIK)²⁰, and sequestered in the cytoplasm. PKA phosphorylates SIK in the C-terminal regulatory domain and inhibits its CRTC kinase activity, which triggers CRTC dephosphorylation and nuclear translocation²¹. While the SIK-CRTC pathway has been shown to be crucial for such physiological processes as gluconeogenesis, neuronal survival and melanogenesis^{18,19,22}, 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 naive T cells¹. The IL-12 receptor is composed of two subunits, β 1 and β 2 chains (IL-12R β 1 and β 2), among which the latter is induced specifically during Th1 differentiation^{23,24} and is responsible for IL-12 signal

transduction²⁵. However, its expression mechanism is not known in detail. Moreover, although naive T cells express both subunits of IFN- γ receptor, α and β chains (IFN- γ R1 and R2), IFN- γ R1 is downregulated shortly after TCR engagement *in vitro*²⁶. Whether there is any signal counteracting this downregulation of IFN- γ R1 by TCR or directly inducing IFN- γ R1 expression is currently unknown. Such a signal, if present, may be important, because the downregulation of IFN- γ R1 may restrict IFN- γ signalling, which is essential for induction of Th1-lineage-specific transcription factor T-bet and IL-12R β 2 in the early stage of Th1 differentiation²⁷.

Here we demonstrate that cAMP promotes Th1 differentiation through upregulation of IL-12R β 2 and IFN- γ R1 and the amplification of these cytokine signalling pathways. PGE₂ mobilizes cAMP-PKA cascade, which directly induces gene expression of IL-12R β 2 and IFN- γ R1 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-12R β 2 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-12R β 2 (*Il12rb2*), three genes critical for Th1 differentiation, in T cells cultured under the Th1-priming conditions. PGE₂ considerably enhanced expression of *Il12rb2* and *Ifng* 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-AE1-259) or EP4 (ONO-AE1-329) but not by agonists to EP1 (ONO-DI-004) or EP3 (ONO-AE-248) (ref. 28) (Fig. 1b). Induction of IL-12R β 2 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-12R β 2 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-12R β 2 in T cells^{2,24,29}, we asked whether these cytokines signalling are involved in PGE₂-induced IL-12R β 2 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 IL-12R β 2 expression in TCR-activated T cells (Supplementary Fig. S1a). Blockade of IFN- γ (Supplementary Fig. S1b) or both IFN- γ and IL-2 (Supplementary Fig. S1c) signalling by using IFN- γ R1^{-/-} T cells³⁰ and anti-IL-2 reduced both the basal IL-12R β 2 expression and its enhancement by PGE₂. However, even without IFN- γ and IL-2 action, PGE₂ still exhibited enhancement of IL-12R β 2 expression over the basal level (Supplementary Fig. S1c). EP2 and EP4 agonists again mimicked this action of PGE₂ on expression of IL-12R β 2 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 mediate IL-12R β 2 induction by PGE₂.

Involvement of PI3K and cAMP in PGE₂-induced IL-12R β 2.

Given that EP2 and EP4 stimulation activates both PI3-kinase

Th1-priming condition

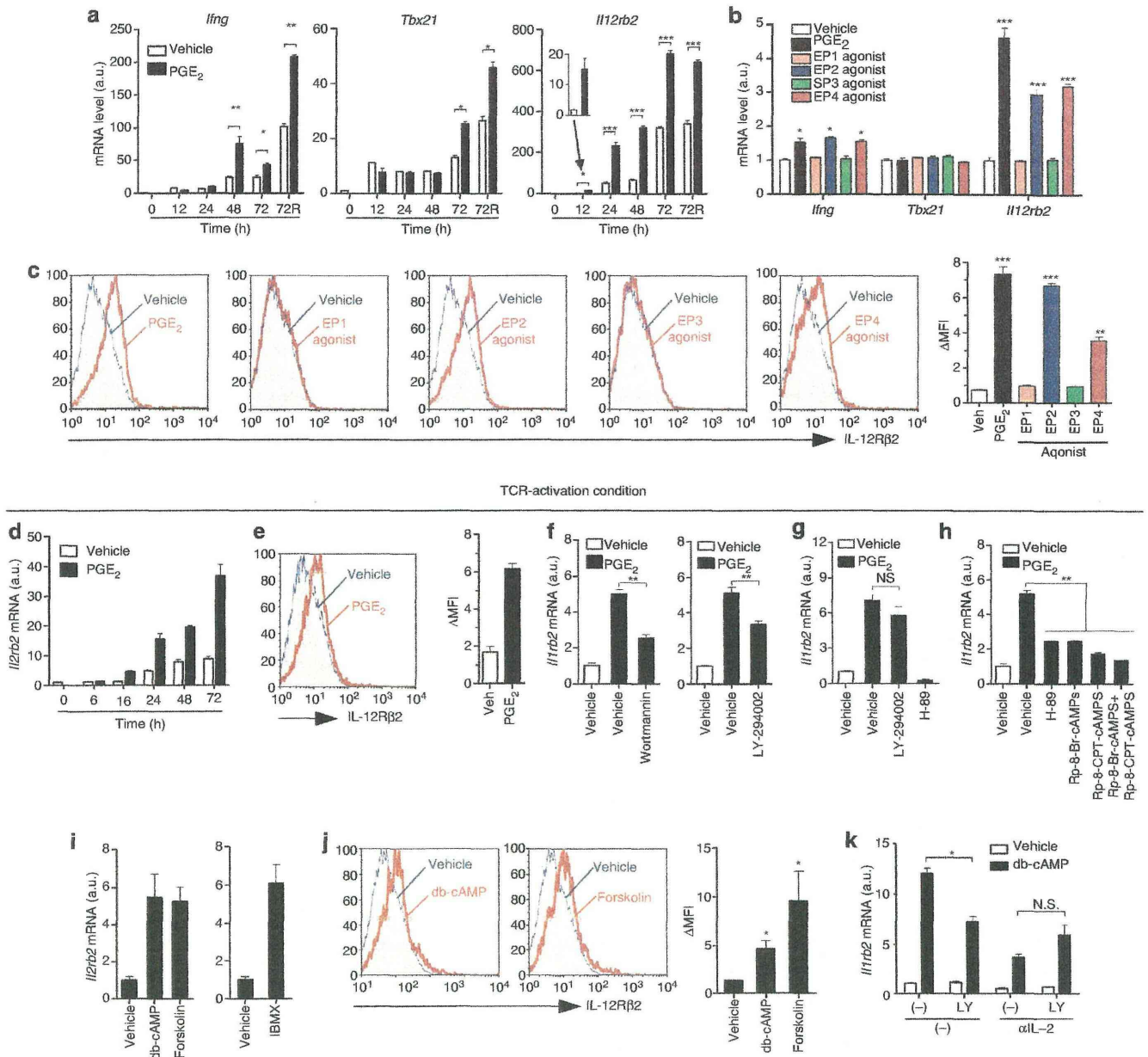


Figure 1 | PGE₂-cAMP signalling induces IL-12Rβ₂ expression in TCR-activated T cells. (a) Expression of *Ifng*, *Tbx21* and *Il12rb2* mRNA by T cells activated for indicated times with antibody to CD3 and antibody to CD28 (αCD3/CD28) in the absence or presence of PGE₂ under Th1-priming conditions. A portion of cells were restimulated with PMA and ionomycin for the last 4 h (72R). (b) Expression of *Ifng*, *Tbx21* and *Il12rb2* mRNA by T cells activated for 24 h with αCD3/CD28 in the absence or presence of PGE₂ or selective agonists to EP1 to EP4 under Th1-priming conditions. (c) Surface expression of IL-12Rβ₂ in T cells activated for 48 h with αCD3/CD28 in the absence or presence of PGE₂ or agonists selective to EP1 to EP4 under Th1-priming conditions. Grey-filled histogram represents isotype control. ΔMFI (mean fluorescence intensity) indicates the differences between MFI of IL-12Rβ₂ and MFI of isotype control (right). (d) Time-course of *Il12rb2* mRNA expression by PGE₂ in T cells activated with αCD3/CD28 for 48 h. (e) PGE₂ induces IL-12Rβ₂ protein expression in T cells activated with αCD3/CD28 for 48 h. (f,g) Expression of *Il12rb2* mRNA in WT T cells (f) or IFN-γR1^{-/-} T cells supplemented with anti-IL-2 (g), activated for 24 h with αCD3/CD28 in the absence or presence of PGE₂ with or without Wortmannin, LY-294002 or H-89. (h) Expression of *Il12rb2* mRNA in T cells activated for 24 h with αCD3/CD28 in the absence or presence of PGE₂ with H-89, Rp-8-Br-CAMPS or Rp-8-CPT-CAMPS. (i,j) Expression of IL-12Rβ₂ mRNA (i) and protein (j) in T cells activated with αCD3/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 *Il12rb2* mRNA in IFN-γR1^{-/-} CD4⁺ T cells activated for 24 h with αCD3/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.

(PI3K)-Akt and cAMP-PKA pathways⁹, we examined their involvement in IL-12Rβ₂ induction by PGE₂. Inhibitors of PI3K (LY-294002 and wortmannin) partially prevented PGE₂-

induced *Il12rb2* expression in TCR-activated T cells (Fig. 1f). As PI3K-Akt pathway mediates TCR-induced production of IFN-γ and IL-2 that have important roles in IL-12Rβ₂ expression and

Th1 differentiation^{2,31}, we examined effects of LY-294002 on IL-12R β 2 induction in TCR-activated IFN- γ R1-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 *I12rb2* expression (Fig. 1g). These results indicate that there are PI3K-dependent and -independent mechanisms of *I12rb2* 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)³² also attenuated PGE₂-induced *I12rb2* expression in TCR-activated wild-type (WT) T cells (Fig. 1h), and H-89 completely suppressed IL-12R β 2 induction in the absence of IFN- γ and IL-2 signalling (Fig. 1g). Conversely, expression of IL-12R β 2 mRNA and protein was enhanced by dibutyryl cAMP (db-cAMP) and forskolin (Fig. 1i,j). Moreover, *I12rb2* mRNA was upregulated by a nonspecific phosphodiesterase (PDE) inhibitor 3-isobutyl-1-methylxanthine (Fig. 1i), suggesting that endogenous cAMP has a role in IL-12R β 2 expression. Interestingly, cAMP-induced IL-12R β 2 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-12R β 2 by cAMP. As the above results suggest the involvement of IFN- γ and IL-2 in a part of cAMP-induced *I12rb2* expression by TCR-activated T cells, we next evaluated how and how much IFN- γ and IL-2 contribute to cAMP-induced *I12rb2* 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 *I12rb2* expression at a similar level, cAMP synergistically induced *I12rb2* 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- γ R1^{-/-} T cells at 12 h (Fig. 2a). Synergistic action of cAMP with IFN- γ or TCR activation on IL-12R β 2 protein expression in T cells was also confirmed (Fig. 2b). We then evaluated the involvement of IL-2 signalling in cAMP-induced IL-12R β 2 expression. We used IFN- γ R1^{-/-} T cells to exclude the effect of IFN- γ signalling. Although cAMP and TCR signalling had no synergistic action on *I12rb2* expression in IFN- γ R1^{-/-} T cells at 12 h (Fig. 2a), we noted that they synergistically induced *I12rb2* expression after 24 h and this synergistic action was eliminated by anti-IL-2 (Fig. 2c), suggesting that synergistic action on *I12rb2* 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 *I12rb2* expression, and blocking both further reduced *I12rb2* expression in TCR-activated WT T cells at 36 h, but cAMP still enhanced *I12rb2* expression over the basal level under these conditions (Fig. 2d). These results demonstrate that there are direct and indirect mechanisms of cAMP-promoted *I12rb2* 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 *I12rb2* 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 *I12rb2* 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-priming 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 activated both cAMP and PI3K pathways through EP2 and EP4 in T cells⁹ and PI3K antagonized cAMP-dependent inhibition of TCR signalling³³. 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- γ R1 and IL-2R β by cAMP. As the above findings suggest that cAMP can induce IL-12R β 2 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 *I12rb2* 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- γ R1^{-/-} T cells stimulated with or without db-cAMP. Consistent with previous reports³, 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), *I12rb2* expression was upregulated by db-cAMP in both WT and IFN- γ R1^{-/-} T cells (Fig. 3b), verifying an IFN- γ signalling-independent induction of *I12rb2* 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- γ R1 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- γ R1 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. 3f) and upregulation of IL-2R β 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- γ R1 and IL-2R β , respectively.

Requirement of CREB for cAMP-induced IL-12R β 2 and IFN- γ R1. We next investigated the molecular mechanisms of how cAMP directly induces IL-12R β 2 and IFN- γ R1 expression. The db-cAMP-induced *I12rb2* 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 transferred

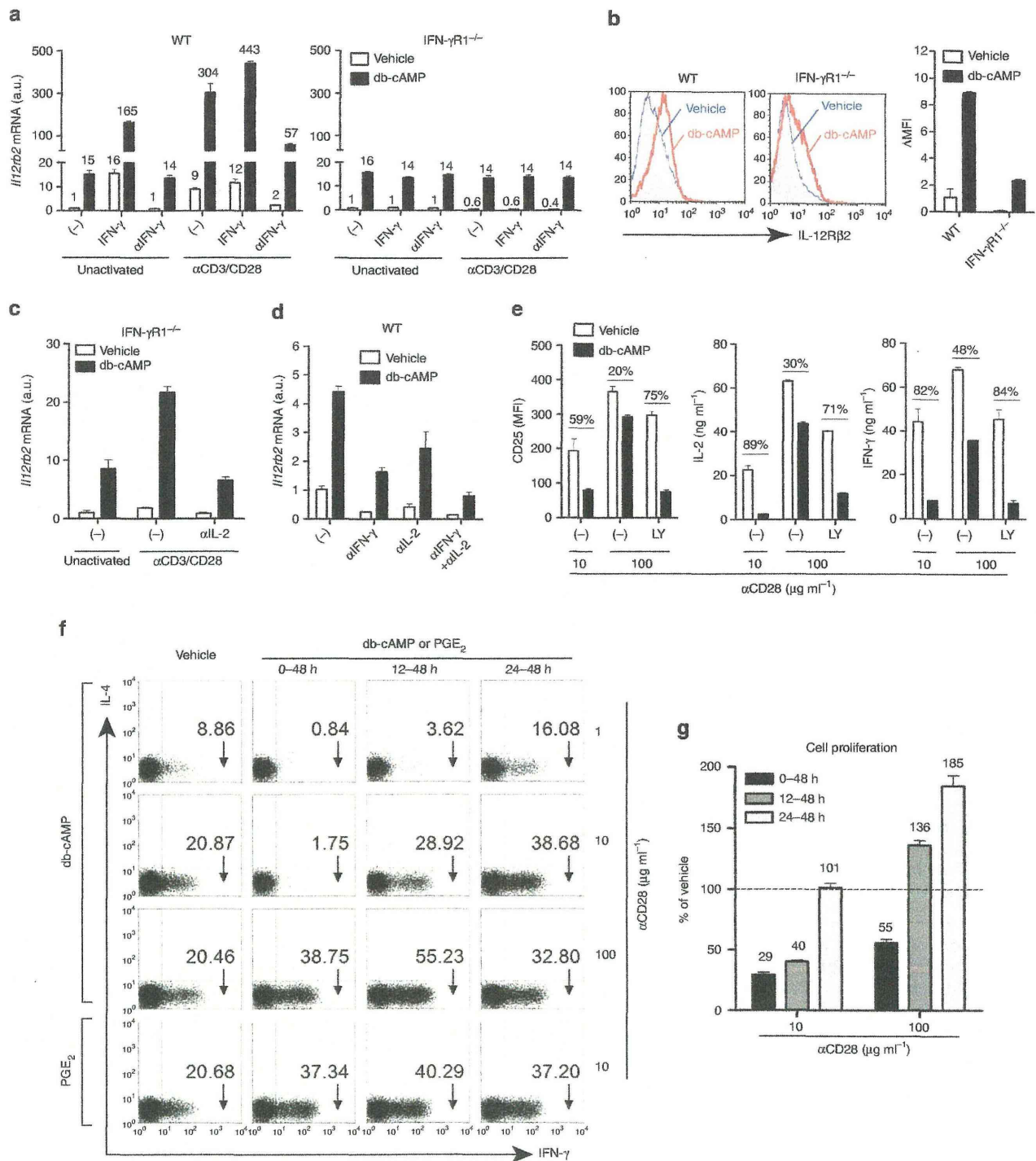


Figure 2 | cAMP context-dependently regulates TCR activation and Th1 differentiation. (a) Expression of *Il12rb2* mRNA in WT (left) and IFN-γR1^{-/-} (right) T cells activated for 12 h without (unactivated) or with αCD3/CD28 in the presence or absence of db-cAMP, IFN-γ or anti-IFN-γ. (b) Surface expression 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 without (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.

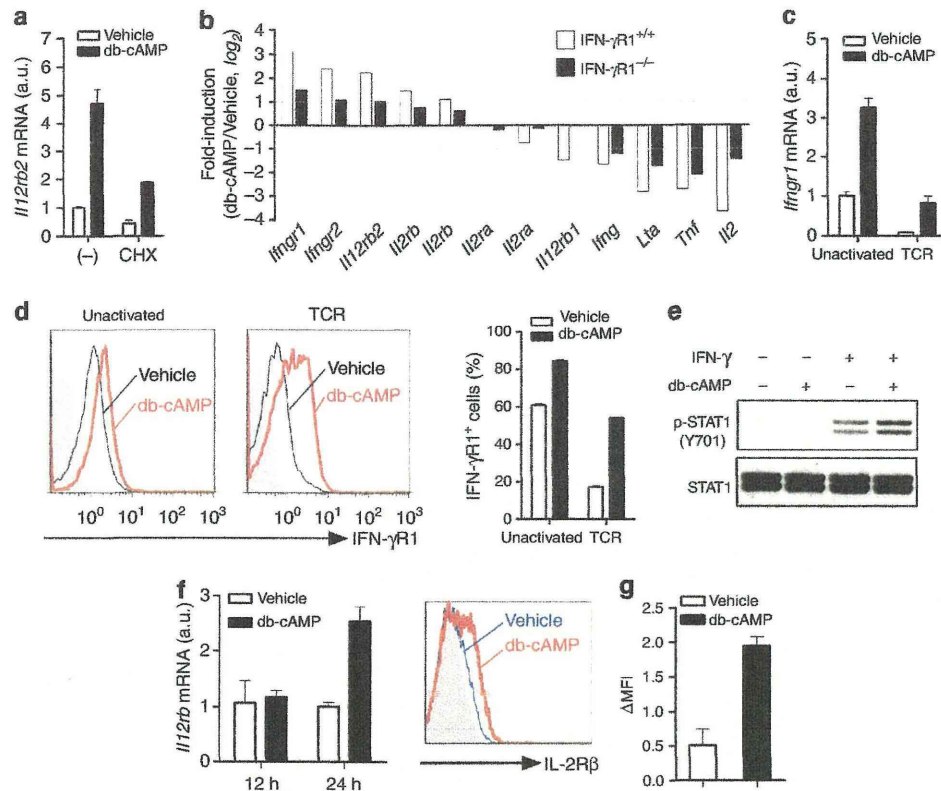


Figure 3 | cAMP amplifies IFN- γ and IL-2 signalling through induction of IFN- γ R1 and IL-2R β . (a) Expression of *Il12rb2* mRNA in T cells activated with α CD3/CD28 for 24 h with addition of db-cAMP or cycloheximide (CHX) or both for the last 12 h. (b) Profile of db-cAMP-dependent expression of cytokines and their receptors by WT or IFN- γ R1 $^{-/-}$ T cells activated for 12 h with α CD3/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- γ R1 $^{-/-}$ mouse that we used has *Ifngr1* gene disrupted by inserting the neomycin resistance gene into exon V. (c,d) Expression of IFN- γ R1 mRNA (c) and protein (d) in T cells stimulated with or without α CD3/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 *Il12rb2* mRNA in T cells treated with db-cAMP for 12 and 24 h. (g) IL-2R β protein expression in T cells activated with α CD3/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 \pm s.e.m. are representative of two independent experiments with triplicates (a,c-g) or are from one experiment (b). a.u., arbitrary units.

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).

Involvement of CRTC2 in cAMP-induced IL-12R β 2 and IFN- γ R1. One candidate for such factors is CRTC2 that is regulated negatively by SIK2 and functions as a CREB coactivator in many types of cells^{19–22}. 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 reported^{18,19}, 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 CRTC2 in WT and SIK2 $^{-/-}$ T cells. While db-cAMP increased GAL4-transcriptional activity in WT cells, the basal GAL4 activity was already higher in SIK2 $^{-/-}$ T cells and no further increase was observed upon cAMP addition (Fig. 5d). These findings suggest that SIK2-CRTC2 pathway functions downstream of cAMP in T cells.

To corroborate that SIK2-CRTC2 pathway mediates cAMP-induced expression of *Il12rb2* and *Ifngr1*, we depleted CRTC2 in T cells by siRNA (Fig. 5e). CRTC2 depletion considerably suppressed db-cAMP-induced *Il12rb2* and *Ifngr1* expression (Fig. 5f). Conversely, STS induced expression of *Il12rb2* and *Ifngr1* mRNA as potently as db-cAMP (Fig. 5g). Expression of *Nr4a2*, a gene known to be regulated by CRTC2 and p-CREB³⁵, was also reduced by CRTC2 siRNA and induced by STS and db-cAMP (Fig. 5f,g), while another p-CREB-target gene *Fosb* was induced only by db-cAMP (Fig. 5g), probably reflecting

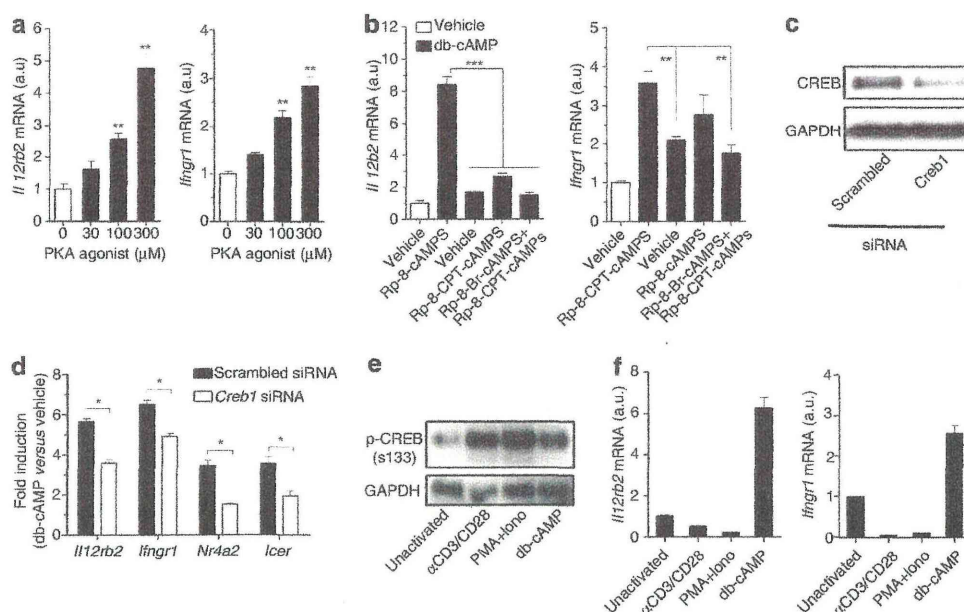


Figure 4 | Activation of PKA-CREB pathway is required but insufficient for cAMP-induced IL-12R β 2 and IFN- γ 1 expression. (a) Expression of *Il12rb2* and *Ifngr1* mRNA in T cells stimulated with indicated concentrations of a PKA agonist, N⁶-Bnz-cAMP, for 12 h. (b) Expression of *Il12rb2* and *Ifngr1* 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 (c) 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 *Nr4a2* were used as positive controls for CREB-dependent genes. (e) Immunoblot for p-CREB(S133) in IFN- γ 1^{-/-} T cells stimulated without (unactivated) or with either α CD3/CD28, PMA (20 ng ml⁻¹) plus ionomycin (500 ng ml⁻¹) (PMA + Iono), or db-cAMP for 30 min. (f) Expression of *Il12rb2* and *Ifngr1* mRNA in IFN- γ 1^{-/-} T cells stimulated as in e in the presence of anti-IL-2 for 12 h. Data shown as mean \pm 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.

less CRTC2 contribution³⁵. Consistently, overexpression of a constitutively active form of CRTC2, CRTC2(S171A), which could not be phosphorylated at the site Ser171 and, therefore, retained in the nucleus²⁰, enhanced the basal expression of *Il12rb2* and *Ifngr1*, while overexpression of a constitutively active form of SIK2, SIK2(S587A), which constitutively phosphorylated CRTC2 and thus prevented CRTC2 nuclear translocation³⁶, suppressed the db-cAMP-induced *Il12rb2* and *Ifngr1* expression (Fig. 5h). Furthermore, comparing to WT cells, SIK2^{-/-} T cells have a higher level of *Il12rb2* and *Ifngr1* mRNA, which could be suppressed by overexpression of WT SIK2 or the SIK2(S587A) mutant (Fig. 5i). These data suggest that CRTC2 mediates cAMP-PKA signalling-induced expression of *Il12rb2* and *Ifngr1*.

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 immunoprecipitation 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 mice³⁷ with EP4-floxed mice³⁸. Loss of EP4 in CD4⁺ T cells was confirmed in Lck-Cre⁺EP4^{fl/fl} and Lck-Cre⁺EP4^{fl/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⁻EP4^{fl/fl} or Lck-Cre⁺EP4^{fl/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⁺EP4^{fl/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/*Icer* than T cells from littermate control Lck-Cre⁻EP4^{fl/fl} mice (Fig. 7c). Consistent with the above results, the numbers of IL-12R β 2- and IFN- γ 1-expressing CD4⁺ T cells in dLNs from Lck-Cre⁺EP4^{fl/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⁺EP4^{fl/fl} mice (Fig. 7c,d). Moreover, when we adoptively transferred dLN

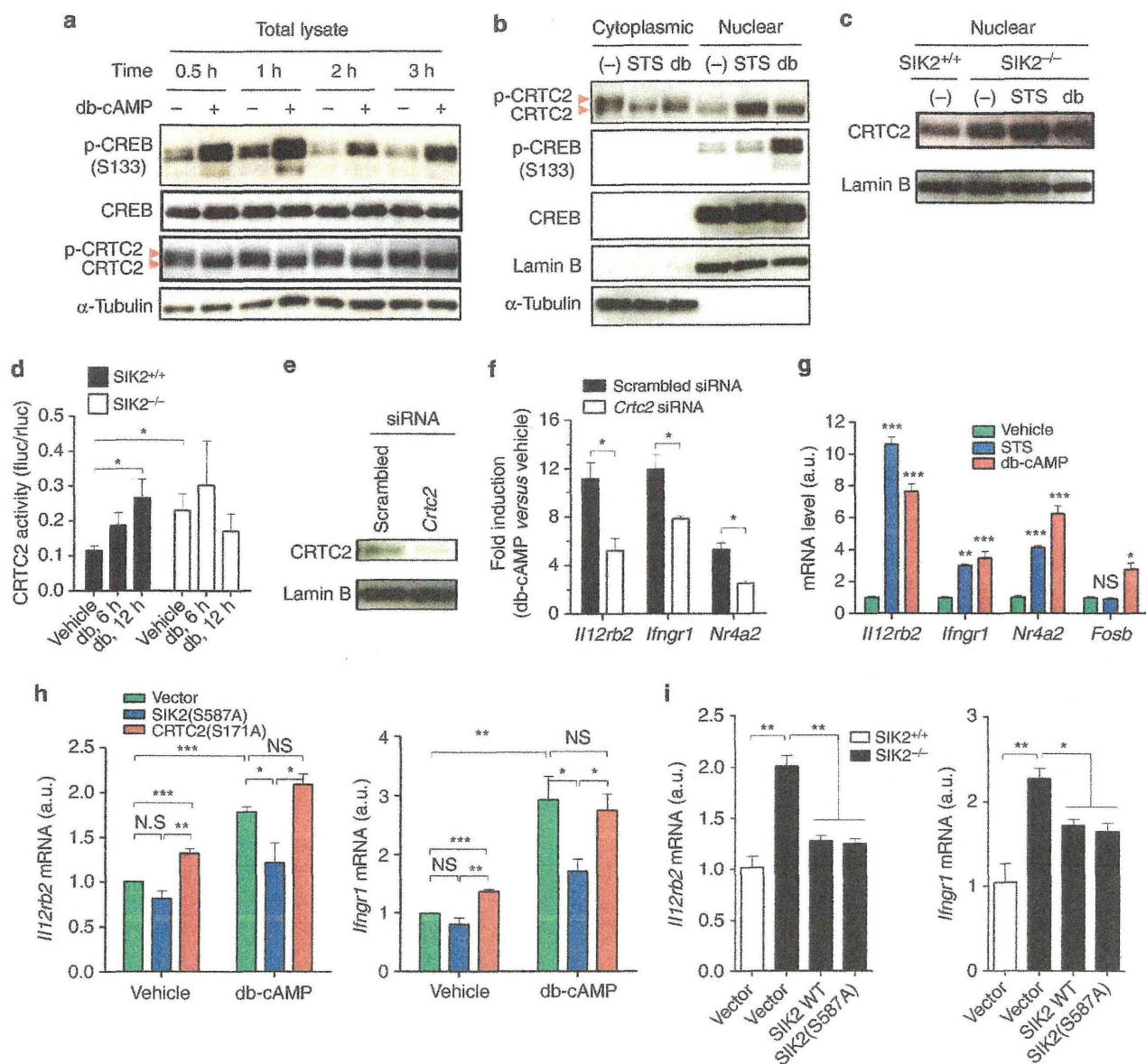


Figure 5 | SIK2-CRTC2 signalling mediates cAMP-induced expression of *Il12rb2* and *Ifngr1*. (a) cAMP induces CREB phosphorylation and CRTC2 dephosphorylation in T cells. (b) cAMP and STS reduces cytoplasmic CRTC2 and increases nuclear CRTC2 in T cells that were rested for 2 days and then stimulated for 1 h. (c) cAMP does not increase nuclear CRTC2 in SIK2^{-/-} T cells. (d) GAL4 reporter assay of CRTC2 activity of SIK2^{+/+} or SIK2^{-/-} T cells that were transfected with GAL4-fusion pM-mCRTC2 and pTAL-5xGAL4-Luciferase reporter and stimulated with db-cAMP for the indicated times. The activity is shown as the ratio of firefly luciferase activity to renilla pRL-TK Luciferase activity. (e,f) T cells were transfected with scrambled or *Crtc2* siRNA for 36 h, followed by immunoblotting of CRTC2 (e) or stimulating with db-cAMP for another 12 h for detection of mRNA expression (f). mRNA expression in db-cAMP-treated cells is normalized to vehicle-treated of each siRNA-transfected cells. (g) Expression of indicated mRNA in T cells stimulated with STS or db-cAMP for 12 h. (h) CRTC2(S171A) increases basal expression of *Il12rb2* and *Ifngr1*, while SIK2(S587A) reduces cAMP-induced expression of these genes in TCR-activated T cells. (i) SIK2 WT and S587A mutant reduce basal expression of *Il12rb2* and *Ifngr1* in SIK2-deficient T cells. Data shown as mean \pm s.e.m. are representative of two (a–e,i) or three (f,g) independent experiments with triplicates or are from four independent experiments (h). Statistical significance was examined by unpaired two-tailed student's *t*-test, **P* < 0.05; ***P* < 0.01; ****P* < 0.001. NS, not significant; a.u., arbitrary units.

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, transferred 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

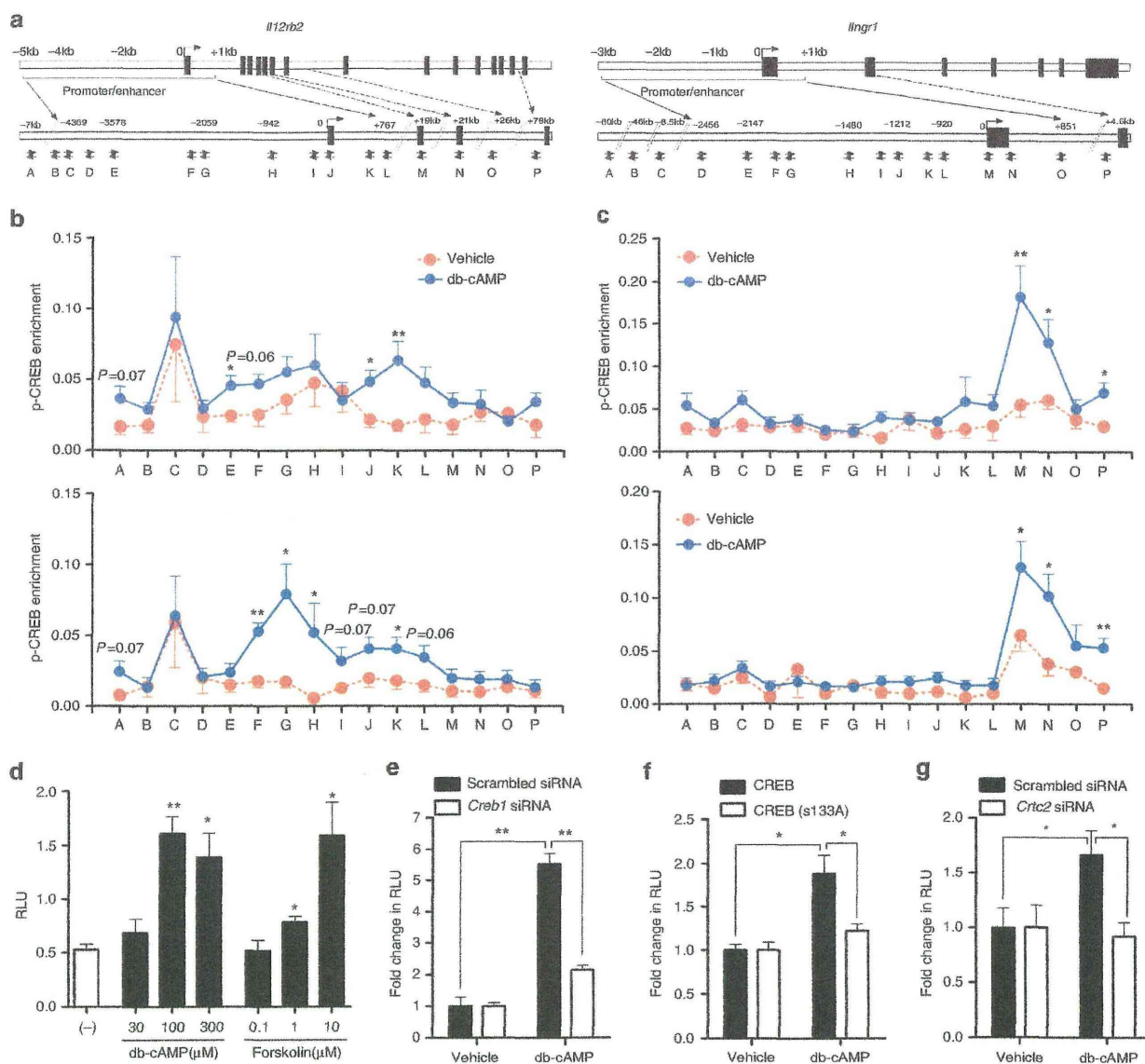


Figure 6 | Recruitment of p-CREB and CRT2 to *Il12rb2* and *Ifngr1* gene loci upon cAMP stimulation. (a) Schematic diagram of the mouse *Il12rb2* (left) and *Ifngr1* (right) genes and their promoter/enhancer regions illustrating potential CRE sites and the positions of the primers. (b,c) Enrichment of p-CREB(S133) (upper) and CRT2 (lower) at the *Il12rb2* (b) and *Ifngr1* (c) gene loci. T cells were rested for 2 days followed by stimulation with db-cAMP or vehicle for 1 h. Cells were fixed, and chromatin immunoprecipitation was performed using anti-p-CREB (S133) and anti-CRT2. Data are normalized to the input DNA and represent eight individual chromatin immunoprecipitations from four independent experiments (mean \pm s.e.m.). (d-g) Luciferase activity of EL4 cells stably expressing the *Il12rb2* luciferase reporter, stimulated for 24 h with indicated concentrations of db-cAMP, forskolin or vehicle (d), or stimulated for 24 h with db-cAMP after 40 h treatment with scrambled or *Creb1* siRNA (e), after 24 h transfection with WT CREB or the CREB(S133A) mutant (f) or after 40 h treatment with scrambled or *Crtc2* siRNA (g). Relative light unit (RLU) is normalized to vehicle control of each transfection condition (e-g). Data shown as mean \pm s.e.m. are representative of two (d-g) independent experiments with triplicates. Statistical significance was 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 *CD45RB*^{hi}*CD25*⁻*CD4*⁺ naive T cells from *Lck-Cre*⁺*EP4*^{fl/fl} mice or *EP2*^{-/-} mice. We have found attenuation of the colitis also in the *Rag2*^{-/-} mice reconstituted with *Lck-Cre*⁺*EP4*^{fl/fl} T cells and have not found any difference of colitis development between the groups of mice that received WT and *EP2*^{-/-} T cells (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 *PGE*₂-induced facilitation of Th1 differentiation, and revealed the role of cAMP in this process. *PGE*₂ 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 CRT2 through inhibiting SIK2. Activated CREB and CRT2 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 *PGI*₂-IP signalling promotes Th1 differentiation

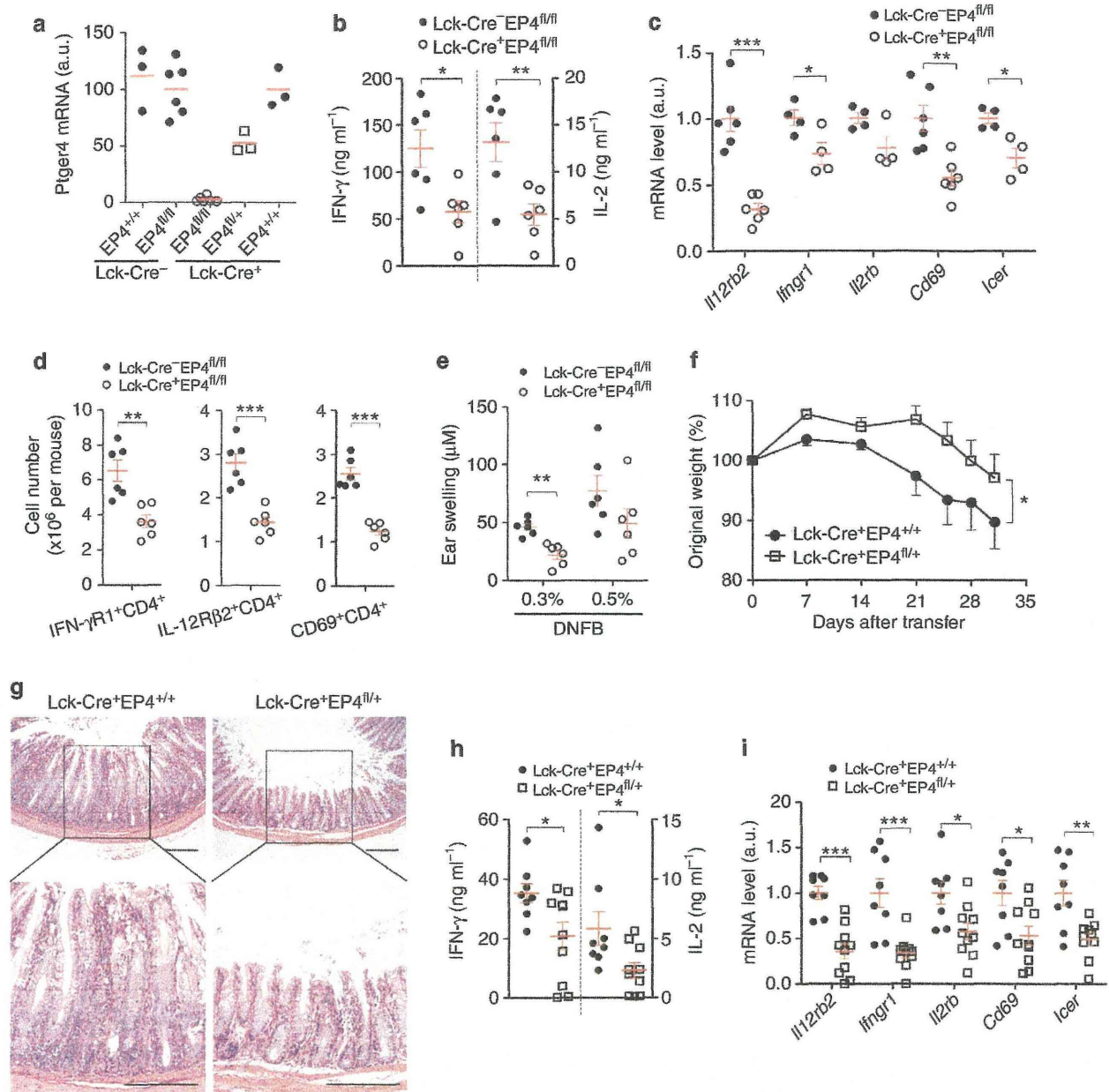


Figure 7 | EP4-cAMP signalling in T cells modulates expression of IL-12R β 2 and IFN- γ R1, and Th1 response *in vivo*. (a) Expression of *Ptger4* mRNA in CD4⁺ T cells of mice of indicated genotypes. (b) IFN- γ and IL-2 production by dLN CD4⁺ T cells isolated from DNFB-sensitized mice on day 5 and then stimulated with α CD3/CD28 for 24 h. (c) mRNA expression of Th1-related cytokine receptor genes in dLN CD4⁺ T cells isolated from DNFB-sensitized mice on day 5. *I12rb2* and *Cd69* mRNA are shown in dLN CD4⁺ T cells stimulated with α CD3/CD28 for 24 h. (d) Numbers of IL-12R β 2-, IFN- γ R1-, or CD69-positive CD4⁺ T cells in dLNs of DNFB-sensitized mice on day 5. (e) Ear swelling of WT recipient mice given intravenous transfer of dLN cells from DNFB-sensitized Lck-Cre⁻EP4^{fl/fl} or Lck-Cre⁺EP4^{fl/fl} mice. (f) Change in body weight of *Rag2*^{-/-} mice (8–10 mice per group) given intravenous transfer of Lck-Cre⁺EP4^{+/+} or Lck-Cre⁺EP4^{fl/+} naive T cells. (g) Hematoxylin and eosin staining of the colon of *Rag2*^{-/-} mice 42 days after transfer. Scale bar, 200 μ m. (h,i) IFN- γ and IL-2 production (h) and mRNA expression of indicated genes (i) in CD4⁺ T cells isolated from the mLNs of *Rag2*^{-/-} mice 42 days after transfer and stimulated with α CD3 for 3 days. Horizontal and error bars represent the mean and s.e.m., respectively. Statistical significance in e was examined by two-way ANOVA. * P < 0.05; ** P < 0.01; *** P < 0.001. a.u., arbitrary units.

through cAMP-PKA pathway *in vitro* and enhances CHS responses *in vivo*³⁹, and that *G α s*-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 cells¹⁶. 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 differentiation⁸. 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