Facilitation of Th1-mediated immune response by prostaglandin E receptor EP1

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Prostaglandin E_2 (PGE_2) exerts its actions via four subtypes of the PGE receptor, EP1–4. We show that mice deficient in EP1 exhibited significantly attenuated Th1 response in contact hypersensitivity induced by dinitrofluorobenzene (DNFB). This phenotype was recapitulated in wild-type mice by administration of an EP1-selective antagonist during the sensitization phase, and by adoptive transfer of T cells from sensitized $EP1^{-/-}$ mice. Conversely, an EP1-selective agonist facilitated Th1 differentiation of naive T cells in vitro. Finally, $CD11c^+$ cells containing the inducible form of PGE synthase increased in number in the draining lymph nodes after DNFB application. These results suggest that PGE_2 produced by dendritic cells in the lymph nodes acts on EP1 in naive T cells to promote Th1 differentiation.

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Abbreviations used: CHS, contact hypersensitivity; COX, cyclooxygenase; cPGES, cytosolic PGE synthase; DNBS, dinitrobenzene sulfonic acid; DNFB, dinitrofluorobenzene; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LC, Langerhans cell; mPGES-1, membrane-associated PGE synthase-1; PG, prostaglandin; PGES, PGE synthase; T-bet, T-box-expressed-in-T cells.

The immune system defends the host by exerting a wide array of responses to invading pathogens and other noxious antigens. Upon invasion, these foreign organisms and substances induce nonspecific inflammation. Concomitantly, they are ingested by APCs such as DCs and macrophages. APCs process them while they migrate toward draining LNs, and present processed antigens to naive T cells in the LNs. Engagement of the antigen complex by T cell receptor triggers clonal expansion and differentiation of T cells, which critically determines the outcome of immune responses (1, 2). $CD4^+$ T cells play a central role in orchestrating immune responses through their capacity to provide help to other cells, and can be categorized into Th1 cells characterized by secretion of IFN-γ, Th2 cells characterized by secretion of IL-4, IL-5, IL-6, and IL-13, and recently identified Th17 cells characterized by secretion of IL-17A. Similarly, $CD8^+$ T cells undergo differentiation into two subsets of cytotoxic T cells, Tc1 and Tc2 cells. In immune responses, Th1 cells are responsible for cell-mediated inflammatory reactions, such as delayed type hypersensitivity reaction, and are critical for eradication of intracellular pathogens, whereas Th2

cells are involved in optimal antibody production, particularly IgE and IgG1 subtypes, and elicit allergic/humoral immune response against extracellular pathogens, and Th17 cells mediate host immune response against extracellular bacteria, some fungi, and other microbes, which are probably not well covered by Th1 or Th2 immunity (3).

During antigen presentation, APCs produce a variety of cytokines and other substances, and the composition of cytokines to which naive T cells are exposed determines the fate of T cell differentiation (4, 5). IL-12, IL-4, and transforming growth factor-β with IL-6 are key determinants of T cell differentiation into Th1, Th2, and Th17, respectively. Although these cytokine-directed pathways make basic frameworks for T cell differentiation, and the signal transduction and transcription factors involved therein have been determined, polarization of T cell response in vivo may be influenced by other noncytokine substances in local milieu, one candidate being prostanoids.

Prostanoids, including prostaglandin (PG) D_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and thromboxane A_2, are metabolites of arachidonic acid produced by the sequential actions of cyclooxygenase (COX) and respective synthases (6). They are formed in response to various, often noxious, stimuli, and they regulate a broad range of physiological and pathological processes. Among prostanoids, PGE_2

is produced most abundantly in various phases of immune responses, and its actions on T cell development have been studied for many years. It was already known in the 1980s that PGE$_2$ is produced by APCs, inhibits production of IL-2 and IFN-γ, and suppresses proliferation of murine, as well as human, T cells in vitro (7, 8). Betz and Fox (9) examined the effect of PGE$_2$ on cytokine production from Th1, Th2, and Th0 clones, and found that PGE$_2$ inhibited production of IL-2 and IFN-γ, which are two Th1 cytokines, whereas it spared production of the Th2 cytokines IL-4 and -5. This differential action of PGE$_2$ on Th1 and Th2 cells has been confirmed by many studies (10–14). Because the best known action of PGE$_2$ is elevation of intracellular cAMP, and cAMP exerts similar Th1-selective suppression (15, 16), most, if not all, studies have assigned PGE$_2$ as a modulator of T cells raising the intracellular cAMP level. PGE$_2$ acts on a rhodopsin–type, G protein–coupled receptor to exert its actions. There are four subtypes of PGE receptor, termed EP1, EP2, EP3, and EP4, among which EP2 and EP4 are coupled to a rise in cAMP. Nataraj et al. (17) used T cells obtained from mice deficient in each EP subtype individually, and examined an immunosuppressive effect of PGE$_2$ in vitro in mixed lymphocyte reaction. They found that the immunosuppressive action of PGE$_2$ was significantly attenuated in T cells obtained either from EP2$^{-/-}$ or EP4$^{-/-}$ mice, suggesting that both EP2 and EP4 mediate suppression of PGE$_2$ on T cells. Kabashima et al. (18) additionally found that the EP4-mediated T cell suppression operates in vivo in intestinal inflammation of mice treated with dextran sodium sulfate.

Curiously, the finding by Kabashima et al. (18) is a rare example showing in vivo occurrence of the PGE$_2$-mediated immunosuppression. Treatment of animals with COX inhibitors does not necessarily enhance Th1 response. Indeed, T cell suppression by PGE$_2$ has been shown mostly in experiments using in vitro culture systems. One possible explanation for this discrepancy between the in vitro and in vivo findings is that PGE$_2$ elicits not only anti-Th1 actions but also other actions on T cells as well. Naive T cells express, in addition to EP2 and EP4, the EP1 receptor that couples to a rise in intracellular Ca^{2+} concentration, and therefore can exert actions different from EP2 and EP4 in T cells (reference 17 and this study). Because the Th1 and Th2 paradigm is well established, we focused on the role of EP1 on Th1 and Th2 immune response. Contact hypersensitivity (CHS) is a form of delayed-type hypersensitivity, a T cell–mediated immune response to reactive haptens, and it clinically manifests as contact dermatitis. Th1 lymphocytes play critical roles in CHS, although Th17 lymphocytes also, to some extent, contribute to CHS (19, 20). Dinitrofluorobenzene (DNFB)-induced CHS is an experimental model of a typical Th1-mediated CHS in which IFN-γ is a critical mediator (19, 21). We therefore used DNFB-induced CHS in this study and examined the role of EP1 in T cell–mediated immune responses by using EP1-deficient (EP1$^{-/-}$) mice, as well as an EP1-selective agonist and antagonist. We have found that PGE$_2$ acts on EP1 in naive T cells and augments their differentiation to the Th1 subset in vitro, and this signaling critically works in the sensitization phase and facilitates Th1-mediated delayed hypersensitivity
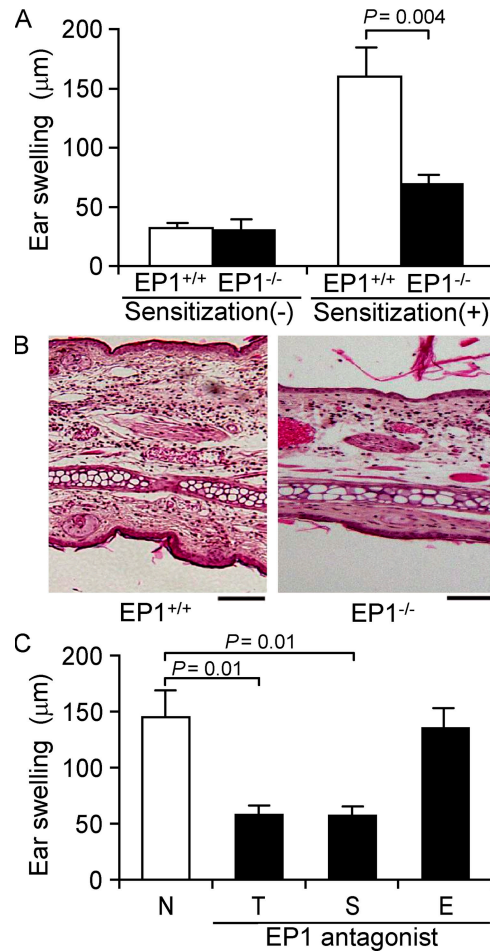
Figure 1. Impaired CHS responses in EP1$^{-/-}$ mice. (A) Ear swelling. WT mice and EP1$^{-/-}$ mice were sensitized with DNFB (Sensitization [+]) or vehicle (Sensitization [-]), challenged with DNFB 5 d later, and ear thickness was measured 24 h after the challenge (unpaired Student's t test; $n = 5$ mice per group). (B) Histology of DNFB-treated ears. Transverse sections of the ear from WT mice and EP1$^{-/-}$ mice 24 h after the challenge were stained with hematoxylin and eosin. Bar, 100 μm. (C) Effect of EP1 antagonist ONO-8713 on CHS to DNFB. WT mice were administered with either vehicle (N) or ONO-8713 all through the experimental period (T), or during sensitization (S) or elicitation (E) period, and the ear thickness was measured 24 h after challenge (unpaired Student's t test; $n = 5$ mice per group). Data are representative of at least three experiments with similar results. Data are presented as the mean $\pm$ the SEM.

reaction in vivo. Thus, this study revealed an important role of EP1 on T cells in Th1 response and raises an intriguing possibility that PGE$_2$ acts on different types of EP and can skew T cell differentiation into either Th1 or Th2 direction via EP1 or EP2/EP4, respectively, in a context-dependent manner.

RESULTS

EP1 signaling works in the sensitization phase to regulate CHS response

To investigate the role of EP1 during CHS, we sensitized WT mice and EP1$^{-/-}$ mice by painting DNFB on shaved abdomen, and we challenged the ear 5 d later by epicutaneous

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application of DNFB to elicit CHS response (22, 23). Although both WT and EP1$^{-/-}$ mice developed ear swelling after DNFB application, as measured by an increase in ear thickness, the ear swelling in EP1$^{-/-}$ mice was significantly attenuated compared with that found in WT mice (Fig. 1 A). Histological examination showed that antigen challenge induced considerable lymphocyte infiltration and edema in the dermis and spongiosis in the epidermis of the ear in sensitized (but not naive) WT mice, and that these changes were less apparent in sensitized EP1$^{-/-}$ mice than WT mice (Fig. 1 B). To confirm these findings in EP1$^{-/-}$ mice, we next examined the effect of pharmacological inhibition of EP1 on the CHS response by administering an EP1-selective antagonist ONO-8713 (24) orally at 20 mg/kg/day to the WT mice. ONO-8713 administered all through the experimental period (T) reduced the ear swelling as found in EP1$^{-/-}$ mice (Fig. 1 C). To determine the effective time window of EP1 antagonist, we then administered ONO-8713 selectively either during the sensitization period (S) or during the elicitation period (E). Treatment of ONO-8713 during the sensitization period, but not during the elicitation period, inhibited the ear swelling to the extent comparable to that found in EP1$^{-/-}$ mice or WT mice treated with ONO-8713 all through the experimental period (Fig. 1 C). These results suggest that EP1 signaling specifically works in the sensitization phase and regulates the DNFB-induced CHS response.

Impaired IFN–γ production with intact lymphocyte proliferation in the draining LNs of EP1$^{-/-}$ mice after DNFB application

To examine how the loss of EP1 affected sensitization of CHS response, we next analyzed the composition and differentiation of cells in draining LNs of EP1$^{-/-}$ mice before and after sensitization. Under the basal condition, EP1$^{-/-}$ mice showed no apparent abnormality in either the number or composition of immune cells, and, upon DNFB sensitization, they exhibited an increase in the total cell number comparable to that found in WT mice with a composition similar to that in WT mice (Fig. 2 A). Consistently, when the LN cells were collected from sensitized animals and challenged with dinitrobenzene sulfonic acid (DNBS) in vitro, [^{3}H]thymidine uptake was not significantly different between the two groups (Fig. 2 B). Intriguingly, however, production of IFN-γ induced by the DNBS challenge was markedly decreased in cells from EP1$^{-/-}$ mice as compared with those from WT mice (Fig. 2 C), whereas production of IL-4 was not suppressed in the cells from EP1$^{-/-}$ mice (Fig. 2 D). Because DNFB-induced CHS is a typical Th1–mediated, delayed-type hypersensitivity reaction and IFN-γ is a critical mediator (25, 26), these results indicate that differentiation of naive T cells to the Th1 subset is impaired in EP1$^{-/-}$ mice, and this can be a cause of impaired CHS observed in this genotype of mice.

Impaired Th1 differentiation in draining LNs of EP1$^{-/-}$ mice leads to reduced Th1 response in elicitation phase of CHS

To verify the aforementioned hypothesis, we next analyzed the number of cells expressing T-box-expressed-in-T cells (T-bet),
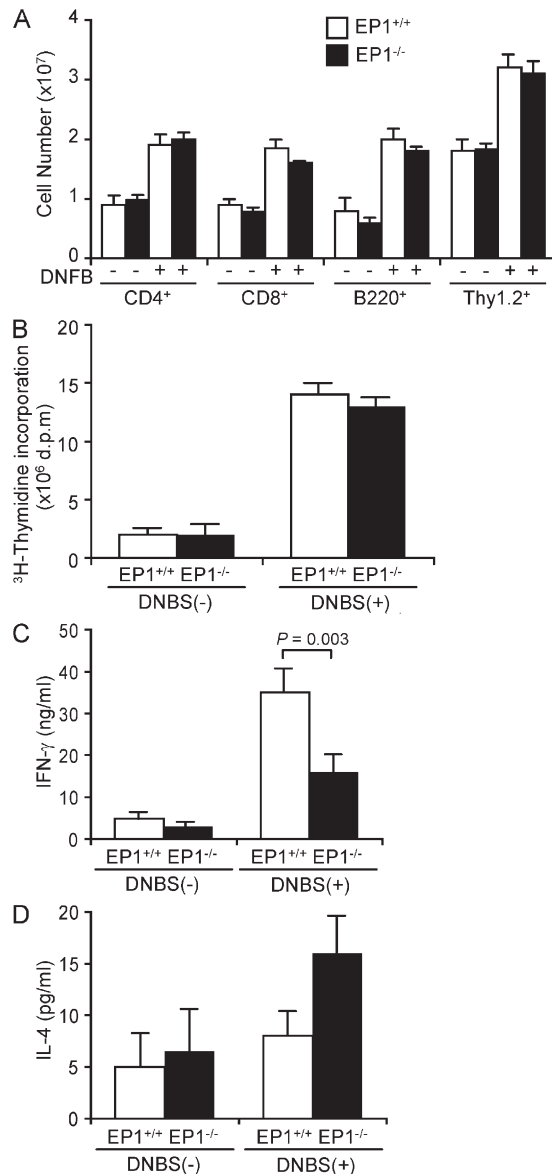
Figure 2. Impaired development of Th1 subset in CHS to DNFB in EP1$^{-/-}$ mice. (A) Number of CD4$^+$, CD8$^+$, B220$^+$, and Thy1.2$^+$ cells in draining LNs (LNs) before and after antigen application. Cells from inguinal and axillary LNs were collected before or 5 d after DNFB application, and the number of CD4$^+$, CD8$^+$, B220$^+$, and Thy1.2$^+$ cells was analyzed by flow cytometry ($n = 5$ mice per group). (B) DNBS-induced lymphocyte proliferation. Cells were collected from LNs of WT or EP1$^{-/-}$ mice 3 d after DNFB application, and cultured for 3 d with or without DNBS. Cell proliferation was measured by [^{3}H]thymidine incorporation ($n = 5$ mice per group). (C and D) DNBS-induced cytokine production. Cells were isolated from LNs of WT or EP1$^{-/-}$ mice 3 d after DNFB application, and cultured in the absence or presence of DNBS for 2 d. The amount of IFN-γ (C) or IL-4 (D) in the culture medium was measured by ELISA (unpaired Student's t test; $n = 5$ mice per group). Data are representative of at least three experiments with similar results. Data are presented as the mean ± the SEM.

which is a key transcription factor for Th1 differentiation (27), in draining LNs. Sensitization with DNFB increased the number of T-bet–expressing cells in both WT and EP1$^{-/-}$ mice. However, the number of T-bet–expressing cells in EP1$^{-/-}$ mice was significantly reduced (Fig. 3 A). Consistently, restimulation of sensitized LN cells with DNBS increased the expression levels of both T-bet and IL-12Rβ2 mRNA in WT mice, whereas the DNBS effects on cells from EP1$^{-/-}$ mice was significantly reduced (unpublished data). These results suggest that EP1$^{-/-}$ mice contain a reduced number of CD4$^+$ T cells primed to induce T-bet expression, which leads to attenuation of Th1 response. Indeed, cervical LNs after DNFB challenge to the ear in the elicitation phase showed a significant decrease in EP1$^{-/-}$ mice in the total cell number and the numbers of IFN-γ–producing cells in the CD8$^+$ cell population (Fig. 3, B and C). This IFN-γ–producing CD8$^+$ cell population consisted mainly of CD3$^+$ T cells, although CD11c$^+$ DCs were one source of IFN-γ in this population (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20070773/DC1). The number of IFN-γ–producing cells in the CD4$^+$ T cell population was also substantially decreased in EP1$^{-/-}$ mice (Fig. 3 C).

Given that, in addition to Th1 cells, Tc1 cells also function as effector cells in CHS (28–30), the aforementioned results indicate that reduced generation of Th1 and Tc1 cells underlie impaired CHS response in EP1$^{-/-}$ mice. To verify this hypothesis, we isolated and pooled T cells from regional LNs of DNFB-sensitized WT or EP1$^{-/-}$ donors and adoptively transferred the cells into naive WT recipients. The recipients of sensitized EP1$^{-/-}$ T cells demonstrated suppressed CHS responses compared with the recipients of sensitized WT T cells (Fig. 4 A). Conversely, when T cells from WT donors were transferred into naive EP1$^{-/-}$ recipients, subsequent challenge with DNFB elicited a normal CHS response (Fig. 4 B), indicating that sensitized T cells and not other cells are essential for EP1-dependent amplification of CHS response.

To examine the specificity of the impairment of immune response in EP1$^{-/-}$ mice, we analyzed the Th2 response in EP1$^{-/-}$ mice by immunizing the mice with OVA, which is a typical protein antigen for Th2 response (31). The serum concentrations of total IgE and OVA-specific IgE were measured at 12 d after OVA administration. OVA immunization increased the concentrations of total and OVA-specific IgE in WT and EP1$^{-/-}$ mice (Fig. S2, A and B, available at http://www.jem.org/cgi/content/full/jem.20070773/DC1). Although there was no significant difference, the concentrations in EP1$^{-/-}$ mice tended to be higher than those in WT mice. These results suggest that EP1$^{-/-}$ mice have impairment specifically in the development of Th1 response, but not Th2 response.

EP1 deficiency does not affect migration and maturation of DCs during the CHS

The aforementioned results showing that the EP1 deficiency impairs generation of Th1 cells in draining LNs indicates that the EP1 signaling regulates functions for Th1 differentiation of either DCs, naive T cells, or both. DCs functioning in the
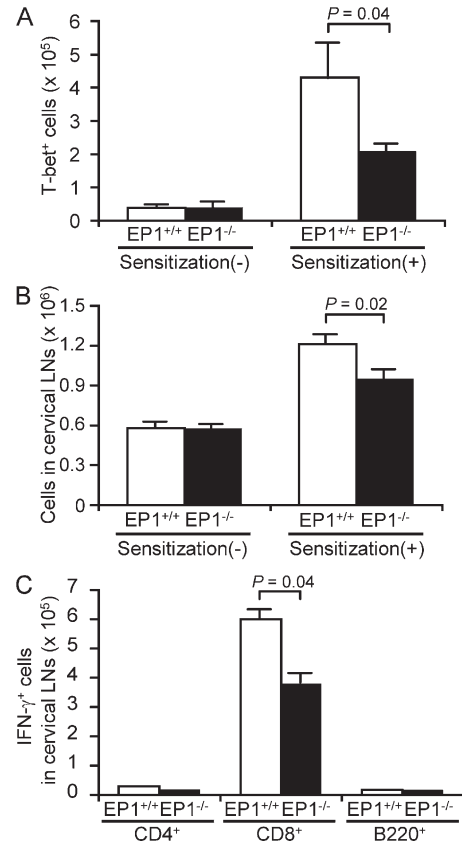

Figure 3. **EP1$^{-/-}$ mice showed reduced Th1 response in the elicitation phase of CHS.** Cells were collected from LNs 5 d after the sensitization (A) or challenge (B and C) with DNFB, and the cell number was counted by flow cytometry. (A) Number of T-bet$^+$ cells from inguinal and axillary LNs during sensitization phase. Sensitization increased the number of T-bet$^+$ cells in WT and EP1$^{-/-}$ mice, but the increase was attenuated in EP1$^{-/-}$ mice (unpaired Student's t test; n = 5 mice per group). (B) Number of cells in cervical LNs during elicitation phase. EP1$^{-/-}$ mice showed decreased cell number in cervical LNs 24 h after challenge (unpaired Student's t test; n = 5 mice per group). (C) Analysis of lymphocyte subsets in regional LNs after challenge. Cells were collected from cervical LNs 24 h after the challenge, and the numbers of CD4$^+$, CD8$^+$, and B220$^+$ cells containing IFN-γ were analyzed by flow cytometry (unpaired Student's t test; n = 5 mice per group). Data are representative of at least three experiments with similar results. Data are presented as the mean ± the SEM.

CHS model are cutaneous DCs, such as Langerhans cells (LCs) and dermal DCs (25). After encountering DNFB, cutaneous DCs migrate to LNs and undergo maturation during migration, which includes increased expression of MHC and costimulatory molecules at their cell surface and the production of cytokines, one being IL-12, which is a key cytokine for Th1 differentiation of CD4$^+$ T cells (1, 5). Because EP1 is expressed, albeit weakly, on cutaneous DCs (23), we examined whether EP1 is involved in the functions of cutaneous DCs by comparing abundance, migration, and maturation of cutaneous DCs during CHS between WT and EP1$^{-/-}$ mice. Consistent with the finding that T cell proliferation, by itself, occurred normally in EP1$^{-/-}$ mice, we did not detect any difference

between the two genotypes in the morphology and the number of LCs in the skin in steady state, reduction of epidermal LCs after the antigen application and an increase of CD11c⁺MHC class II⁺ cells in the draining LNs 24 h after the application (Fig. S3, A–C, available at http://www.jem.org/cgi/content/full/jem.20070773/DC1). Antigen-induced expression of the costimulatory molecules CD80 and CD86 on DCs was unaffected in EP1$^{-/-}$ mice (Fig. S3 D). To test whether EP1 regulates production of IL-12 by DCs, we examined the number of IL-12⁺CD11c⁺ cells in the regional LNs. EP1$^{-/-}$ mice exhibited a similar time-dependent increase in the number of IL-12⁺CD11c⁺ cells in LNs compared with that in WT mice after the DNFB application (Fig. S3 E). Furthermore, the addition of the EP1 agonist DI-004 (32) to WT DCs did not enhance the anti-CD40–stimulated production of IL-12 in vitro (Fig. S3 F). These results suggest that EP1 signaling in DCs, if present, does not play a substantial role in inducing Th1 differentiation in our model of CHS.

EP1 activation facilitates Th1 differentiation, but suppresses Th2 differentiation in vitro

The aforementioned results, when taken together, strongly suggest the importance of EP1 signaling in naive T cells in Th1 and Tc1 cell generation. Therefore, we examined the level of EP1 expression in naive T cells by quantitative PCR analysis. This analysis revealed that EP1 is expressed to a level comparable to EP2 and EP4 in both CD4⁺ and CD8⁺ T cells (Fig. 5 A). EP1 expression on naive T cells was confirmed by flow cytometry using polyclonal antibody for EP1 (Fig. 5 B). Given the well-known actions of EP2 and EP4 on T cells, it is quite likely that EP1 also mediates physiological response in T cells. To dissect an EP1 action in Th1 differentiation, we exploited the in vitro culture system for Th differentiation, and exposed purified naive CD4⁺ T cells to either the Th1- or the Th2-skewing conditions (33) in the presence or absence of an EP1 agonist. We then assessed the extent of Th1/Th2 differentiation by FACS analyses with the intracellular IFN-γ and IL-4 as markers of Th1 and Th2 cells, respectively. Under the Th1-skewing condition, in which naive CD4⁺ T cells differentiated selectively to IFN-γ–producing T cells, activation of EP1 by the EP1 agonist DI-004 significantly increased the number of IFN-γ–producing cells in naive CD4⁺ T cells isolated from WT mice in a dose-dependent manner (Fig. 5 C). This effect of DI-004 on Th1 differentiation was impaired in naive CD4⁺ T cells from EP1$^{-/-}$ mice, indicating that the facilitation by DI-004 was mediated via EP1. Similar to Th1 and Th2 cells, the two subsets of CD8⁺ cytotoxic T cells, Tc1 and Tc2 cells, expressed Th1 cytokines such as IFN-γ and Th2 cytokines such as IL-4, respectively. It is also known that the Th1- and Th2-skewing conditions can differentiate naive CD8⁺ T cells into Tc1 and Tc2 cells. Therefore, we examined whether the EP1 stimulation also facilitates Tc1 differentiation. The addition of DI-004, indeed, facilitated Tc1 differentiation from naive CD8⁺ T cells under a Th1-skewing condition, which was suppressed in CD8⁺ T cells from EP1$^{-/-}$ mice (Fig. 5 D).
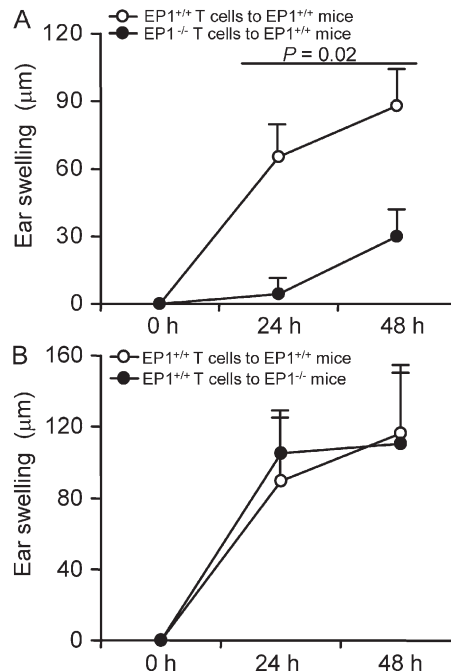
Figure 4. CHS response after adoptive transfer. (A) Attenuated CHS response in WT recipients adoptively transferred with sensitized EP1$^{-/-}$ T cells. T cells were isolated and pooled from LNs of DNFB-sensitized WT (○) or EP1$^{-/-}$ (●) donors and adoptively transferred into naive WT recipients (repeated ANOVA; $n = 5$ per group). (B) Intact CHS response in EP1$^{-/-}$ recipients adoptively transferred with sensitized WT T cells. T cells from sensitized WT donors were transferred into naive WT (○) or EP1$^{-/-}$ (●) recipients (repeated ANOVA; $n = 5$ per group). Data are representative of three experiments with similar results. Data are presented as the mean ± the SEM.

In contrast to these results on Th1 and Tc1 cells, the addition of DI-004 decreased the number of IL-4–producing cells in a dose-dependent manner both from naive CD4⁺ cells and CD8⁺ T cells under the Th2-skewing condition (Fig. 5, E and F). This inhibitory effect of DI-004 on Th2 differentiation was again impaired in naive T cells isolated from EP1$^{-/-}$ mice. These results, thus, suggest that EP1 activation facilitates Th1 differentiation, but suppresses Th2 differentiation, at least under these in vitro conditions. To clarify the impact of different PGE$_2$ signaling on T cell differentiation, we next exposed CD4⁺ T cells to either an EP2 agonist or an EP4 agonist under the aforementioned Th1-skewing conditions. Both butaprost, which is an EP2 agonist, and AE-1-329, which is an EP4 agonist, suppressed differentiation to Th1 in a concentration-dependent manner, although AE-1-329 showed a stronger effect than butaprost (Fig. 5 C). Consistent with previous reports (9–11), the addition of PGE$_2$ itself to naive CD4⁺ T cells in this system suppressed Th1 differentiation like EP2 or EP4 agonist (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20070773/DC1). Finally, to clarify the role of EP receptor subtypes in T cell differentiation, we examined expression of EP subtypes in Th1- and Th2-differentiated T cells (Fig. 5 G). We found that EP1 is up-regulated both in Th1 and Th2 cells, whereas EP2 and EP4 are down-regulated,
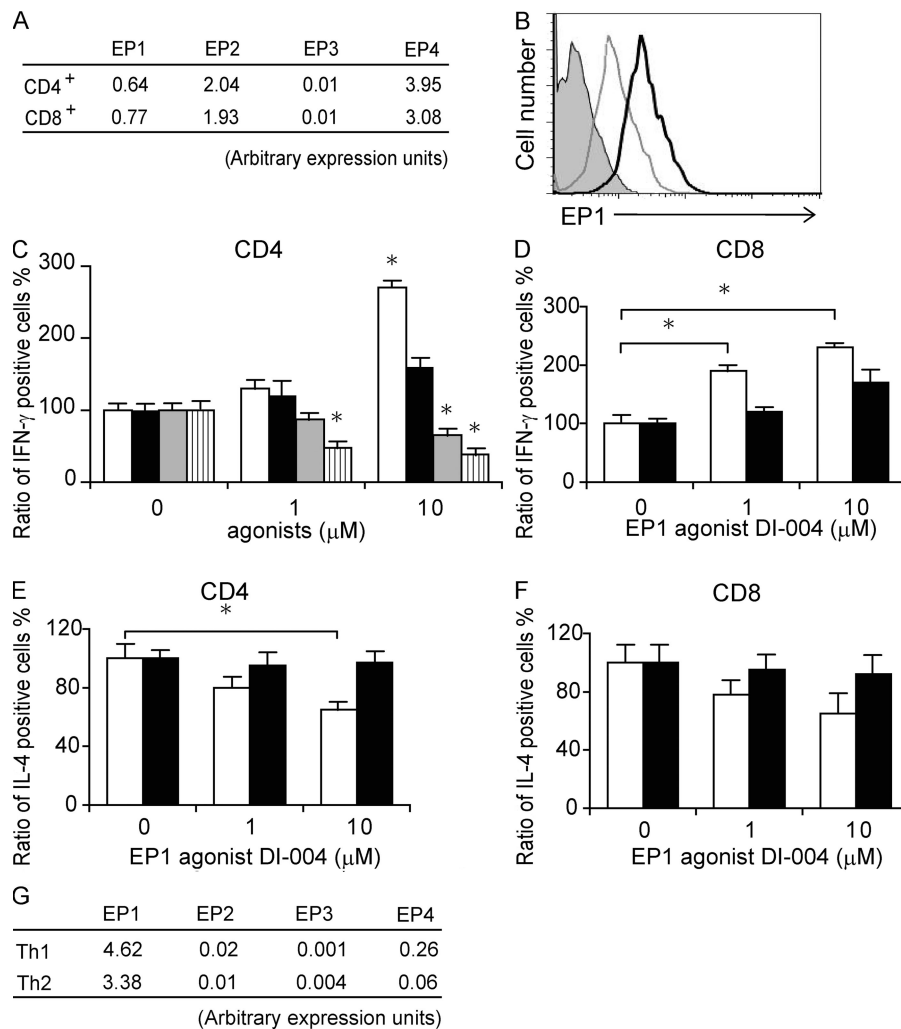
Figure 5.   Effect of EP1 stimulation on T cell differentiation to Th1 and Th2 subsets in vitro. (A) Quantitative PCR analysis on expression for mRNAs of four EP subtypes in CD4+ and CD8+ T cells. Arbitrary expression units are shown. (B) Flow cytometric analysis of EP1 expression on naive CD4+ T cells. Naive CD4+ T cells from WT mice were stained for EP1 with rabbit polyclonal anti-EP1 antibody and Alexa Fluor 488–labeled anti–rabbit IgG in the presence (dotted line) or absence (solid line) of antigenic peptide and subjected to flow cytometry. A control experiment using the second antibody alone is shown in gray. (C) Enhancement by EP1 stimulation and suppression by EP2 and EP4 stimulation of Th1 differentiation of naive CD4+ T cells. Naive CD4+ T cells from WT mice were cultured under the Th1 skewing condition with indicated concentrations of an EP1 agonist (DI-004, white columns), an EP2 agonist (butaprost, gray columns), and an EP4 agonist (AE-1-329, striped columns) or naive CD4+ T cells from EP1−/− mice were cultured in the same conditions with an EP1 agonist (black columns). (D) Naive CD8+ T cells from WT (white columns) and EP1−/− mice (black columns) were cultured under the Tc1-skewing condition with or without DI-004. (E) Naive CD4+ T cells from WT (white columns) and EP1−/− mice (black columns) were cultured under the Th2-skewing condition with or without DI-004. (F) Naive CD8+ T cells from WT (white columns) and EP1−/− mice (black columns) were cultured under the Tc2-skewing condition with or without DI-004. *, $P < 0.05$ versus respective control values without each agonist (ANOVA with Tukey-Kramer multiple-comparison test; $n = 5$ per group). Data are representative of at least three separate experiments with similar results. (G) Quantitative PCR analysis on expression for mRNAs of four EP subtypes in Th1- and Th2-differentiated T cells. Th1 and Th2 cells differentiated from naive T cells were isolated as described in the Materials and methods and subjected to quantitative PCR analysis. Data are presented as the mean ± the SEM.

suggesting a possibility that EP1 works dominantly during T cell differentiation to Th1 and Th2.

It should be noted that the condition of the in vitro differentiation in Fig. 5 was set by choosing the concentrations of cytokines and the time points of experiment to the condition in which initial rise of Th1 differentiation can be observed. The effects of EP1 stimulation were seen only in this condition and were masked in the condition in which maximum

Th1 differentiation is achieved, for example, by prolonged incubation with IL-12 (Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20070773/DC1). These results suggest that the EP1-dependent mechanism functions as a booster of suboptimal conditions. To test such a booster function of EP1 in vivo, we performed the immunization with a high dose (1%) of DNFB, and examined the DNFB-elicited ear inflammation. We found that under these conditions, EP1−/− mice exhibited a
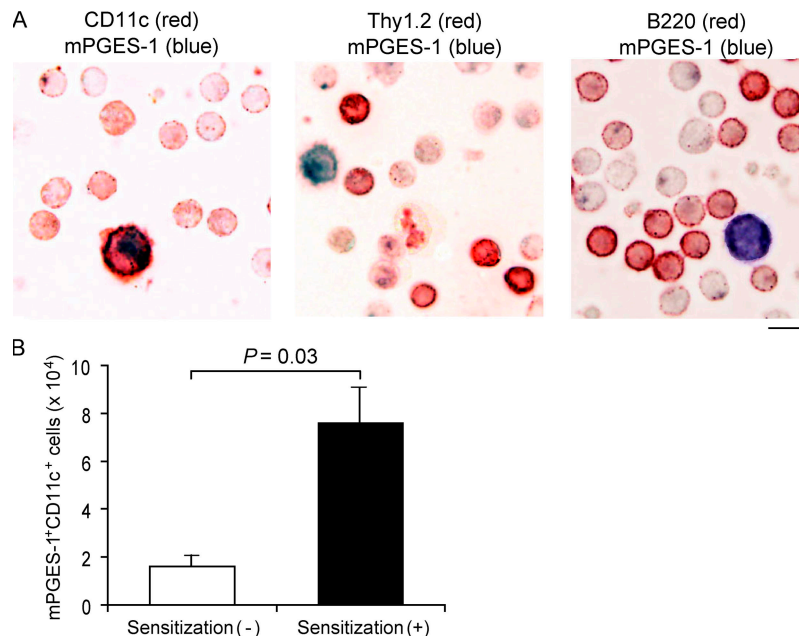
Figure 6. CD11c+ DCs expressing mPGES–1 increase in number in draining LNs after DNFB application. (A) Identification of LN cells expressing mPGES-1. Cells were collected from the draining LNs on day 1 after DNFB application, subjected to cytospin, and stained for mPGES-1, CD11c, Thy1.2, and B220. Bar, 10 μm. (B) Increased number of mPGES-1$^+$CD11c$^+$ cells in draining LNs during sensitization phase of CHS. Cells were collected from inguinal and axillary LNs 24 h after the sensitization and analyzed by flow cytometry (unpaired Student's t test; $n = 5$ per group). Data are representative of at least three experiments with similar results. Data are presented as the mean $\pm$ the SEM.

similar extent of ear swelling compared with WT mice (Fig. S6), suggesting that the requirement of the PGE$_2$–EP1 pathway was overcome by strong immunization stimulus.

PGE$_2$ production by CD11c$^+$ DCs in draining LNs after DNFB stimulation

We have thus demonstrated the critical role of the PGE$_2$–EP1 signaling in Th1 differentiation during CHS. To further characterize the mechanism responsible for these observations, we tried to identify a cell type that produces PGE$_2$ in this process. It was reported that APCs, including DCs, can produce a large amount of PGE$_2$ in vitro (7). Consistently, WT CD11c$^+$ DCs purified from the spleen produced PGE$_2$ under the unstimulated conditions, and this production was further augmented by LPS stimulation, whereas CD4$^+$ or CD8$^+$ T cells released only a negligible amount of PGE$_2$, even upon anti-CD3 stimulation (Fig. S7 A, available at http://www.jem.org/cgi/content/full/jem.20070773/DC1). To characterize the PGE$_2$ production by DCs, we examined the identity of PGE synthase (PGES) involved in this process. PGE$_2$ is produced by the sequential catalysis of COX and PGES. Each of these enzymes has two molecular isoforms, constitutive COX-1 and inducible COX-2 and constitutive cytosolic PGES (cPGES) and inducible membrane-associated PGES-1 (mPGES-1), respectively (34). In macrophages, proinflammatory stimuli induce expression of COX-2 and mPGES-1 together, which then work in concert for PGE$_2$ production (35). Therefore, we purified DCs from the spleen of mPGES-1–deficient (mPGES-1$^{-/-}$) mice (36) and cultured them with various

concentrations of LPS for 24 h. Both WT and mPGES1$^{-/-}$ DCs secreted a small amount of PGE$_2$ without stimulation, and whereas stimulation with LPS induced the production of PGE$_2$ in a dose-dependent manner in WT cells, no LPS-induced augmentation of PGE$_2$ production was observed in mPGES-1$^{-/-}$ DCs (Fig. S7 B), suggesting that mPGES-1 is responsible for PGE$_2$ production by activated DCs. Based on these findings, we wondered whether activated DCs expressing mPGES-1 migrate to draining LNs after immunization. Indeed, staining of dissociated LN cells for mPGES-1 and immune cell markers revealed that CD11c$^+$ DCs, but not by Thy1.2$^+$ T cells or B220$^+$ B cells, expressed mPGES–1 in draining LNs (Fig. 6 A), and the flow cytometry showed that the number of mPGES-1$^+$CD11c$^+$ cells significantly increased in draining LNs after DNFB sensitization (Fig. 6 B). These results indicate that the DNFB immunization induces mPGES-1 expression in CD11c$^+$ DCs, which then reach the draining LNs and actively produce PGE$_2$ there during antigen-induced T cell proliferation and differentiation. PGE$_2$ produced in this manner in the LNs can act on T cells in a paracrine manner.

DISCUSSION

Although it has long been known that PGE$_2$ exerts immunosuppression in vitro, little is known how PGE$_2$ is involved in immune response in vivo. We used EP1$^{-/-}$ mice and addressed this issue. Our results suggest that the PGE$_2$–EP1 pathway in T cells facilitates T cell differentiation to the Th1 subset and Th1-mediated immune response in vivo. This suggestion is supported by several lines of evidence. First, EP1$^{-/-}$

mice exhibited a significant decrease in DNFB-induced, Th1-mediated CHS response compared with that in WT mice. This phenotype was recapitulated by administration of the EP1-specific antagonist during the sensitization period, suggesting the involvement of EP1 in the immunization step. On the other hand, production of IgE to OVA was not impaired in EP1$^{-/-}$ mice. Thus, impairment in EP1$^{-/-}$ mice was restricted to the Th1 response. Second, analysis of the LNs in the sensitization period revealed that, whereas cutaneous DCs migrated to the regional LNs and T cells proliferated in EP1$^{-/-}$ mice as seen in WT mice, differentiation of T cells to Th1 cells was significantly impaired. Furthermore, adoptive transfer experiments demonstrated the critical role of T cells in mediating the EP1 action in CHS. Third, incubation of naive T cells with the EP1-selective agonist in vitro enhanced their differentiation to either Th1 or Tc1 subset, and suppressed differentiation to either Th2 or Tc2 subset. This suppression of Th2/Tc2 subset may be caused by enhancement of Th1/Tc1 action that potentially antagonizes Th2/Tc2 differentiation. Consistent with the latter finding, EP1 deficiency tends to enhance Th2 response in vivo. Notably, the EP1 agonist failed to induce Th1 differentiation without IL-12, indicating that the EP1 stimulation itself cannot induce Th1 differentiation, but enhances the action of IL-12 (Fig. S5). Furthermore, the findings that the action of the PGE$_2$–EP1 signaling that was seen in the submaximal condition (Fig. S5 and S6) functions significantly in vivo suggests the importance of immunomodulatory substances acting in concert with cytokine signaling in determining the extent of physiological immune response. Thus, our study has revealed that PGE$_2$ acts through EP1 for amplification of Th1 differentiation.

The PGE$_2$ action we found here is opposite to the well-known PGE$_2$-induced suppression of Th1 cells found in previous studies (9, 37, 38). Interestingly, the latter action is mediated by EP2 and EP4 receptors that couple to an increase in the cAMP level, whereas stimulation of EP1 induces a rise in intracellular concentration of free calcium ion (6), which may contribute to up-regulation of T-bet (27). The impact of such differential action of PGE$_2$ mediated by different receptors is illustrated in Fig. 5 C, which shows that the addition of the EP1 agonist or the EP2 and EP4 agonists to conditions that are otherwise the same induces significant enhancement or significant suppression of Th1 differentiation, respectively. These results indicate that PGE$_2$ can exhibit different actions in the immune system dependent on the receptor subtypes differing in signal transduction pathways. If so, what determines the final outcome of PGE$_2$ action in Th cell differentiation? We observed here that EP1$^{-/-}$ mice showed decreased CHS response to DNFB because of impaired differentiation to Th1 cells. On the other hand, our previous study showed that in the absence of EP4, T cells proliferates in the lamina propria of the intestine of mice subjected to dextran sodium sulfate–induced colitis (18). Such difference may reflect the expression profile of the prostanoid receptors of T cells in each situation. We now know that expression of prostanoid receptors are dynamically modulated by various physiological and pathological stimuli. For example, expression of EP2 and EP4 in macrophages is up-regulated by bacterial endotoxin (39). Expression of the thromboxane receptor in T cells is down-regulated during T cell activation (22). In this study, we found that EP1 is up-regulated, whereas EP2 and EP4 are down-regulated, in differentiated Th1 and Th2 cells (Fig. 5 G). Furthermore, a previous study reported that PGE$_2$ acts on EP3 and promotes production of IFN-γ from established Th1 cells and Th1 clones (40). These observations indicate that the EP expression profile may vary depending on the pathogens and antigens they are exposed to, innate immune responses, and stage of T cell differentiation, and they determine the final outcome of T cells in response to PGE$_2$.

If so, then where in the body is PGE$_2$ produced, and does it exert its action in the course of sensitization? Because PG acts only in the vicinity of its synthesis, PGE$_2$ acting on naive T cells should be produced in situ in draining LNs close to the T cells. In this study, we found that CD11c$^+$ cells produce a large amount of PGE$_2$ upon inflammatory stimuli in a mPGES-1–dependent manner, and that CD11c$^+$mPGES-1$^+$ cells in the draining LNs increased in number after the DNFB application to the skin. Given that mPGES-1 and COX-2 are induced simultaneously and work in concert to produce PGE$_2$ in macrophages (41), these findings indicate that mPGES-1 is induced in DCs upon the DNFB application, and that activated DCs expressing mPGES-1 migrate to draining LNs, where they produce PGE$_2$ that acts on EP1 on naive T cells to facilitate Th1 differentiation.

In conclusion, we found that the PGE$_2$–EP1 pathway is critically involved in Th1-mediated immune response in vivo by shifting the Th1/Th2 balance to Th1 dominance. Given that the EP1 antagonist mimics the EP1$^{-/-}$ phenotype and that the EP1 agonist can facilitate Th1 differentiation in vitro, this signaling may be a step at which the Th1/Th2 balance can be manipulated pharmacologically.

MATERIALS AND METHODS

Mice. EP1$^{-/-}$ mice (42), backcrossed >10 times onto C57BL/6CrSlc (Japan SLC), and WT control littermates were bred in specific pathogen–free facilities at Kyoto University. mPGES-1$^{-/-}$ mice were provided by S. Uematsu and S. Akira (Osaka University, Osaka, Japan) (36). All experimental procedures were approved by the institutional animal care and use committee of Kyoto University Faculty of Medicine (Kyoto, Japan).

Reagents and antibodies. DNFB and DNBS were purchased from Sigma-Aldrich. An EP1 antagonist (ONO-8713), an EP1 agonist (ONO-DI-004), an EP2 agonist (butaprost), and an EP4 agonist (ONO-AE-1-329) were provided by Ono Pharmaceutical Co. FITC- or PE-conjugated RM4-5 (anti-CD4), 53–6.7 (anti-CD8), RA3-6B2 (anti-B220), HL3 (anti-CD11c), 16-10A1 (anti-CD80), GL1 (anti-CD86), M5/114.15.2 (anti-MHC class II), or IM7 (anti-CD44) mAbs were purchased from BD PharMingen. FITC- or PE-conjugated 11B11 (anti-IL-4), XMG1.2 (anti-IFN-γ), and N418 (anti-CD11c) mAbs were purchased from eBioscience. FITC-conjugated 4B10 (anti–T-bet) mAb was purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti–mPGES-1 and anti-EP1 antibodies were purchased from Cayman Chemicals.

CHS protocol. On day 0, female mice were sensitized with 25 µl of 0.5 or 1% (wt/vol) DNFB in acetone/olive oil (4/1, vol/vol) on shaved abdominal skin. On day 5, ears were challenged by application of 20 µl of 0.3% DNFB

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to their dorsal and ventral surfaces. Ear thickness was measured for each mouse before and 24 h after elicitation at a predetermined site with a micrometer, and the difference is expressed as ear swelling. For treatment with an EP1 antagonist, ONO-8713 was administered orally with food (20 mg/kg/d) during sensitization phase (day −2 to 3), during elicitation phase (day 4 to 6) or throughout the experiment (day −2 to 6). For histological examination, tissues were fixed with 10% formalin in PBS and embedded in paraffin. 5-μm thick sections were prepared and subjected to staining with hematoxylin and eosin.

Flow cytometry. Anti-CD11c, -MHC class II, -FITC, -CD80, -CD86, -CD4, -CD8, and -B220 antibodies were used for surface staining of cells. For detection of EP1, CD4$^+$ T cells were isolated from spleen of WT mice by using auto-MACS. Cells were fixed with 4% paraformaldehyde, permeabilized and blocked with 0.25% Triton X-100/10% normal goat serum/1% BSA/0.25% carrageenan type IV, and stained with antibody to EP1. EP1 was visualized by Alexa Fluor 488–labeled anti–rabbit IgG monoclonal antibody. For intracellular cytokine staining, cells were first restimulated for 6 h with 50 ng/ml phorbol 12-myristate 13-acetate and 500 ng/ml ionomycin in the presence of 5 μg/ml brefeldin A (Sigma-Aldrich), and then fixed in 1% (wt/vol) paraformaldehyde and PBS, made permeable with 0.5% (wt/vol) saponin in flow cytometry buffer (1% [wt/vol] BSA and 0.05% [wt/vol] sodium azide in PBS) and stained intracellularly with PE-conjugated anti–mouse IFN-γ and FITC-conjugated anti–mouse IL-4. For T-bet staining, single-cell suspensions were prepared from draining LNs of WT or EP1$^{-/-}$ mice sensitized with vehicle or DNFB on day 5. The cells were fixed, permeabilized, stained with anti–T-bet antibody, and subjected to flow cytometry.

Lymphocyte proliferation assay. For DNBS-dependent proliferation, single-cell suspensions were prepared from inguinal and axillary LNs of mice sensitized with DNFB on day 3. 4 × 10^5 cells were cultured in RPMI 1640 containing 10% FBS with or without 50 μg/ml DNBS sodium for 3 d, and were pulsed with 0.5 μCi [^{3}H]thymidine for the last 24 h of culture, and subjected to liquid scintillation counting. For measurement of cytokine production, the culture supernatants at 48 h were collected.

ELISA. The amount of IFN-γ and IL-4 in culture supernatants was measured by ELISA according to the manufacture's instructions (Endogen), as previously described (11). PGE$_2$ in the culture medium was measured by an EIA kit (GE Healthcare).

Adoptive transfer. For adoptive transfer, T cells were prepared from the LNs of sensitized WT and EP1$^{-/-}$ mice on day 5, and 5 × 10^6 cells were injected i.v. into naive WT or EP1$^{-/-}$ mice. The ears of these animals were challenged with DNFB 1 h later, and the ear thickness was measured 24 h after challenge.

Immunostaining. For immunological detections of cells in LNs, cells were dissociated with 2.5 mg/ml collagenase A and 10 mg/ml DNase I in RPMI 1640. After fixation with 2% paraformaldehyde, the samples were treated with antibodies against cell markers; biotinylated B220 (1:100), biotinylated Thy1.2 (1:100), or CD11c (N418, 1:20) combined with biotinylated anti–hamster IgG. Signals were detected by Vector Stain ABC Elite kit. They were then subjected to staining with the rabbit anti–mPGES-1 antibody (1:500 dilution) combined with secondary horseradish peroxidase–conjugated anti–rabbit IgG, and signals were detected with Vector SG as a substrate.

Th1/Th2 differentiation. C57BL/6 and BALB/c mice were used for the Th1 and Th2 differentiation assay, respectively. Auto-MACS (Miltenyi Biotec) were used to purify the subpopulations of splenocytes. The purity of each subpopulation was confirmed as >90% by flow cytometry on the EPICS XL (Beckman Coulter). For enrichment of naive CD4$^+$ or CD8$^+$ T cells, spleen cells were stained with FITC-labeled anti-CD44. CD44$^+$ cells were depleted with magnetic microbeads coated with anti-FITC by auto-MACS. Collected CD44$^-$ fractions were stained with anti-CD4– or anti-CD8–conjugated microbeads (Miltenyi Biotech) for positive purification of CD4$^+$ or CD8$^+$ T cells, respectively. The purity of fractionated CD4$^+$ or CD8$^+$ T cells was

consistently >90%. For in vitro differentiation assays (33), CD4$^+$ or CD8$^+$ T cells were stimulated for 2 d with 10 μg/ml of plate-bound anti-CD3 and in the presence of 10 ng/ml IL-12 and 10 μg/ml anti–IL-4, and 2,500 U/ml IL-2 for Th1 differentiation, or in the presence of 10 ng/ml IL-4, 10 μg/ml anti-IFN-γ, and 2,500 U/ml IL-2 for Th2 differentiation with or without DI-004, butaprost, AE-1-329, or PGE$_2$ (1 μM or 10 μM). 40 or 48 h after primary stimulation, cells were washed and further cultured under the Th1 or Th2 condition without anti-CD3. 18 h after incubation, cells were stimulated by PMA and ionomycin for 6 h, and cytokine production was determined by intracellular cytokine staining, as previously described (22). For detection of EP receptors on Th1 and Th2 cells, after primary stimulation for 48 h, cells were washed and further cultured under the Th1 or Th2 condition without anti-CD3. 6 d later, IFN-γ–producing Th1 cells and IL-4–producing Th2 cells were isolated by using the cytokine secretion assays kits (Miltenyi Biotech) and subjected to quantitative PCR.

Quantitative PCR analysis. Total RNA from purified cells was isolated with the RNeasy kit using on-column DNase I digestion (QIAGEN). Quantitative RT-PCR with the Light Cycler real-time PCR apparatus was performed according to the instructions of the manufacturer (Roche) by monitoring the synthesis of double-stranded DNA during the various PCR cycles using SYBR Green I (Roche). For each sample, triplicate test reactions and a control reaction lacking reverse transcriptase were analyzed for expression of the gene of interest, and results were normalized to those of the "housekeeping" GAPDH mRNA. Arbitrary expression units were calculated by division of expression of the gene of interest by GAPDH mRNA expression and multiplication of the result by 1,000.

Statistical analysis. Data are presented as the mean ± the SEM. Unless otherwise indicated, all P values were calculated with the two-tailed Student's *t* test.

Online supplemental material. Fig. S1 shows analysis of the IFN-γ–producing CD8$^+$ cell population in cervical LNs after challenge. Fig. S2 shows serum IgE levels after OVA immunization in EP1$^{-/-}$ mice. Fig. S3 includes supporting data for intact migration and maturation of DCs in EP1$^{-/-}$ mice. Fig. S4 shows the effect of PGE$_2$ on T cell differentiation to Th1. Fig. S5 shows enhancing effect of EP1 agonist on IL-12–induced T cell differentiation to Th1. Fig. S6 shows CHS response of EP1$^{-/-}$ mice and WT mice to sensitization with a higher dose of DNFB. Fig. S7 shows mPGES-1–dependent PGE$_2$ production by CD11c$^+$ cells. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20070773/DC1.

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