

Fig. 2. Reduction of BMDC numbers by CXCR4 antagonist. (A–C) Five day cultured BMDCs were incubated for additional 2 days in the absence (CXCR4 ant-) or presence of CXCR4 antagonist (CXCR4 ant+) at 25, 250, or 2500 ng/ml and analyzed by flow cytometry. The number represents the frequency of each gated group (%) (A). Numbers of CD11c⁺ MHC class II^{high} mature BMDCs and CD11c⁺ MHC class II^{low} immature BMDCs per well were shown (B). The % inhibition by CXCR4 on each subset was shown (C). Columns show the mean ± SD from triplicated wells. Student's *t* test was performed between the indicated groups and an asterisk indicates *P* < 0.05. Data are a representative of three independent experiments.

adhesion molecules on LCs. CXCR4 antagonist decreased the number of both CD86^{high} and CD86^{low} LCs, and CD54^{high} and CD54^{low} LCs (Fig. 3B and C). The intensity of suppression was more significant in CD86^{high} and CD54^{high} LC subsets than in CD86^{low} and CD54^{low} LC subsets (Fig. 3B and C). Therefore, CXCR4 antagonist seemed to attenuate LC maturation.

Augmentation of DC-dependent hapten specific T cell proliferation by CXCL12

The relevance of the observed CXCR4-mediated regulation of DC function to immune responses is a matter to be

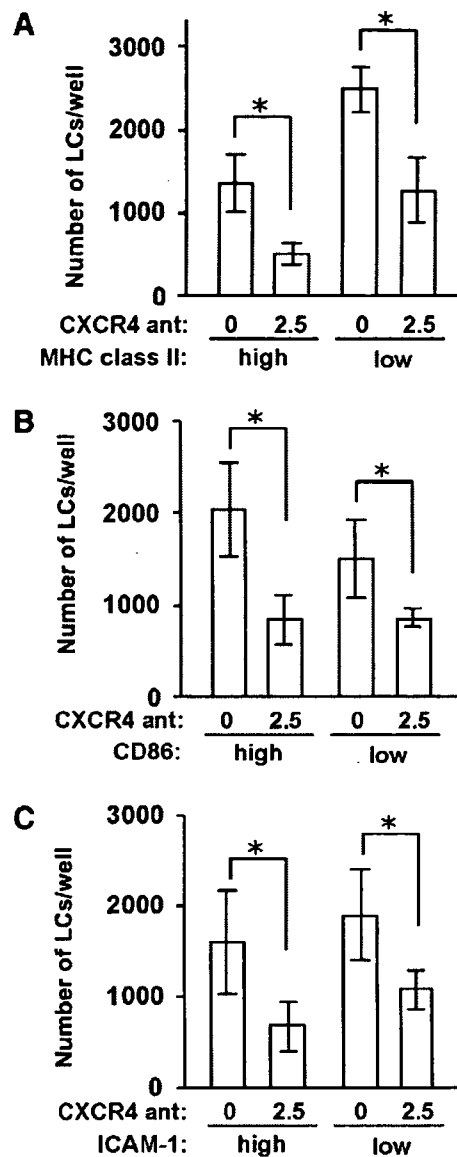


Fig. 3. Reduction of LC numbers by CXCR4 antagonist. Freshly isolated epidermal cell suspensions were incubated for 2 days in the presence or absence of 2.5 μg/ml of CXCR4 antagonist, and the numbers of CD11c⁺ MHC class II^{high} and MHC class II^{low} LC subsets (A), CD11c⁺ CD86^{high} and CD86^{low} LC subsets (B), and CD11c⁺ CD54^{high} and CD54^{low} LC subsets (C) were measured. Columns show the mean ± SD from triplicated wells. Student's *t* test was performed between the indicated groups and an asterisk indicates *P* < 0.05. Data are a representative of three independent experiments.

clarified. B6 mice were sensitized with DNFB hapten applied onto the abdomen. Five days later, the regional lymph node cells were isolated, and the responsiveness of primed T cells to DNBS, a water-soluble compound with the same antigenicity as DNFB, was tested in the presence or absence of recombinant murine CXCL12 or CXCR4 antagonist. The proliferative response of lymph node cells was enhanced by CXCL12 and suppressed by CXCR4 antagonist (Fig. 4). Such effects were not observed when 2 × 10⁵ CD4⁺ cells were stimulated with 10 ng/ml phorbol

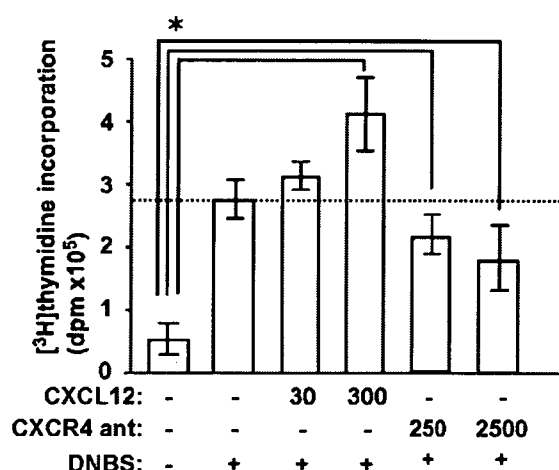


Fig. 4. Modulation of T cell-stimulatory capacity of DCs by CXCL12–CXCR4 engagements. The proliferate response of DNFB-sensitized lymphocytes to DNBS was measured in triplicate with or without CXCL12 at 30 or 300 ng/ml, or CXCR4 antagonist at 250 or 2500 ng/ml. Student's *t* test was performed an asterisk indicates $P < 0.05$. Results are a representative of three independent experiments. d.p.m; decay per minute.

myristate acetate (Sigma Chemical) and 1 μ M ionomycin (Wako, Osaka, Japan), a stimulation procedure independent of DC stimulation (data not shown). This is interpreted as an indication that CXCL12 up-modulates the antigen-presenting ability of DCs for T cells via CXCR4.

Discussion

It is a well accepted concept that chemokines are involved in not only chemotaxis but also other cellular events, such as survival, adhesion, proliferation, and differentiation [3]. For example, the roles of CCR7 on DCs have been well characterized in terms of maturation and differentiation [3]. On the other hand, the roles of CXCR4 on DCs remained largely unknown. Here, we showed that the numbers of BMDCs and LCs were decreased by CXCR4 antagonist in vitro. It is considered that LCs are incapable of proliferating in vitro when cultured as epidermal cell suspensions [16]. These results suggest that CXCR4 promotes DC survival. In fact, it was reported that CXCR4 signaling prolongs BM stromal stem cell and plasma cell survival [17,18]. In addition, CXCR4 antagonist decreased the number of the mature subsets of BMDCs and LCs than those of the immature ones. Consistently, the proliferative response of sensitized lymph node cells by restimulation in vitro was enhanced by CXCL12 and suppressed by CXCR4 antagonist. These data suggest that CXCL12–CXCR4 engagement controls DC survival and maturation.

Once foreign antigens are exposed to the skin, LCs and dermal DCs take up antigens and migrate into regional lymph nodes through lymphatic vessels. At present, it is not certain how and where these LCs and dermal DCs meet CXCL12 producing cells. CXCL12 was known to be detected in murine lymphatic vessels by our immunohisto-

chemical analysis [7]. CXCL12 from lymphatic vessels may be important for DC survival and maturation as well as DC chemotaxis. Accordingly, FITC-induced cutaneous DC migration into regional lymph nodes was impaired by CXCR4 antagonist [7]. On the other hand, it was reported that CXCR4 is expressed by human cutaneous DCs using immunohistochemical and flow cytometric analyses [15]. We detected a significant amount of CXCL12 in the culture medium. It is possible that CXCL12 produced by DCs autonomously stimulates DCs themselves through CXCR4 and thus prolongs cell survival.

Our present study suggests that CXCL12–CXCR4 engagement may play an important role for the initiation of acquired skin immune response. Consistently, CXCR4 antagonist reduced mouse hapten-induced contact hypersensitivity response [7]. Because of embryonic lethality of CXCR4 knockout mice [19–21], CXCR4 antagonist can be a useful chemical reagent to evaluate the role of CXCR4 on DCs. Understanding of factors that determine cutaneous DC functions might offer new opportunities for therapeutic intervention to suppress or stimulate the immune response.

Acknowledgments

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LETTER TO THE EDITOR

Inhibitory effects of epinastine on chemokine production and MHC class II/CD54 expression in keratinocytes

The second generation of anti-H₁ receptor antagonists or anti-allergics are used for various inflammatory skin disorders. Although they have similarities in their anti-histaminic and anti-allergic actions, each of them has different characteristic effects on immunocompetent cells and allergy-associated molecules. Epinastine belongs to this category and possesses anti-inflammatory or anti-allergic properties, including mast cell stabilization [1], suppression of costimulatory molecule expression [2], inhibition of eosinophils chemotaxis [3] and granulocytes accumulation [4], and suppression of cytokine and chemokine production [5]. In Japan, systemic preparation of epinastine has been approved for not only atopic dermatitis but also pruritic psoriasis vulgaris [6]. There is one published report on the efficacy and safety of epinastine (20 mg p.o./day) as a treatment of psoriasis vulgaris with pruritus ($n = 35$). In that report, relief of pruritus was rated as "markedly or moderately improved" in 51.1% of patient and skin eruption was improved in 21.1% [7]. In contrast to atopic dermatitis being generally accepted as a Th2 cell-polarized condition, psoriasis is mediated by Th1 cells, which produce interferon- γ (IFN- γ) and other cytokines and induce the proliferation of keratinocytes. Thus, epinastine uniquely exerts its therapeutic effectiveness for both Th1 and Th2 cutaneous diseases.

Skin is a well-organized immune organ where epidermal keratinocytes produce various cytokines and chemokines, thereby introducing T cell infiltration in the dermis and even epidermis. Keratinocytes are capable of producing Th1 chemokines, including IFN- γ -inducible protein 10 (IP-10/CXCL10), monokine induced by IFN- γ (Mig/CXCL9) and IFN- γ -inducible T-cell chemoattractant (I-TAC/CXCL11), and Th2 chemokines including thymus and activation-regulated chemokine (TARC/CCL17) and macrophage derived chemokine (MDC/CCL22). These chemokines allow these T cell populations

to migrate into the skin. To address the mechanisms underlying the dual effectiveness of epinastine for Th1- and Th2-mediated skin disorders, we investigated whether it modulates the production of Th1 and Th2 chemokines by keratinocytes. We also tested its effect on keratinocyte expression of major histocompatibility complex (MHC) class II and CD54 (intercellular adhesion molecule-1; ICAM-1) molecules.

The effect of epinastine on keratinocyte production of chemokines was examined in normal human epidermal keratinocytes (NHEK), which were purchased from Cascade BiologicsTM and were stimulated with IFN- γ and tumor necrosis factor- α (TNF- α). NHEK were obtained and grown in the serum-free keratinocyte growth medium EpiLife (Cascade Biologics, Portland, OR), and used at third passage in all experiments. Growth supplement was omitted 48 h before experiments. NHEK in 24-well plates (Corning Glass Works, Corning, NY) were stimulated with 2000 units/ml of recombinant IFN- γ (Biogamma; Maruho Pharmaceutical, Osaka, Japan) and 4000 units/ml of TNF- α (Invitrogen, Carlsbad, CA) for the first 2 h, followed by 200 units/ml IFN- γ and 400 units/ml TNF- α thereafter, as described previously [8]. Epinastine (Boehringer Ingelheim Pharmaceuticals, Ingelheim, Germany) was added at the starting of experimental culture. Three-day culture supernatants were measured for RANTES, Mig, IP-10, I-TAC and MDC using enzyme-linked immunosorbent assay (ELISA) kits (Genzyme/Techne, Minneapolis, MN) according to the manufacturer's directions. As shown in Fig. 1, the IFN- γ /TNF- α -augmented production of IP-10, Mig, I-TACK, and RANTES was significantly suppressed by the addition of epinastine to the culture in dose-dependent manners. When monitored at concentrations ranging from 10^{-9} to 10^{-6} M, the minimal concentration of epinastine to exert this suppressive effect was 10^{-8} M. Although the data were not statistically significant, MDC had a tendency to be suppressed by epinastine.

To examine the effect of epinastine on the expression of MHC class II and CD54 molecules, NHEK

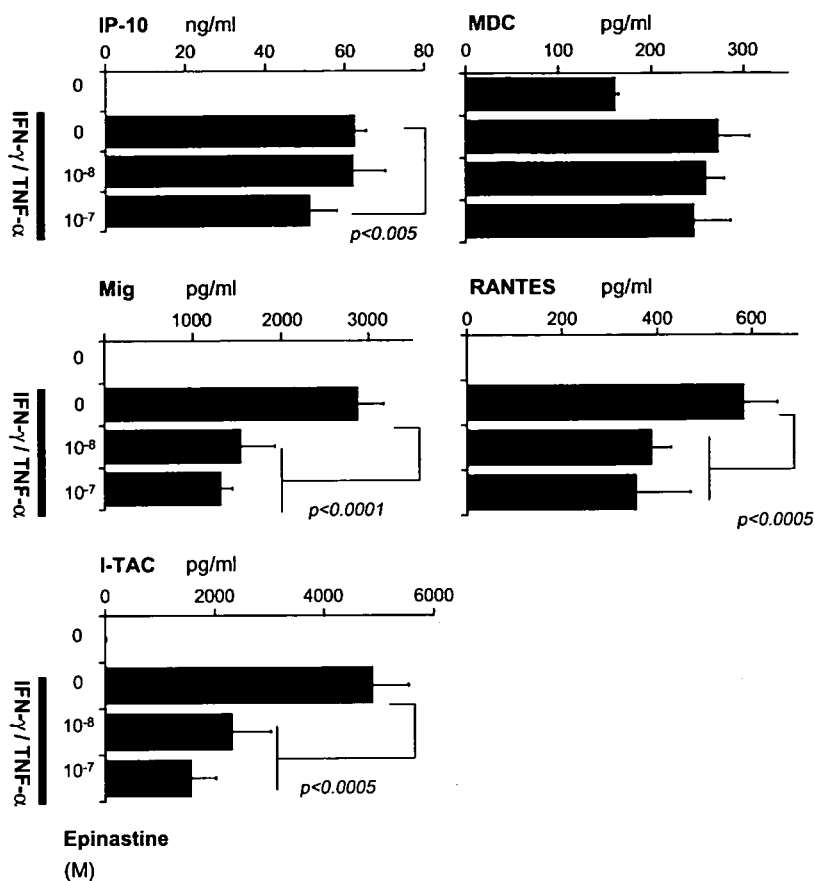


Fig. 1 Epinastine suppresses the production of IP-10, Mig, I-TAC and RANTES. Semiconfluent NHEK were stimulated with recombinant IFN- γ and TNF- α . Epinastine was added at the starting of culture at the indicated concentration in triplicate. Three-day culture supernatants were measured for IP-10, Mig, I-TAC, MDC and RANTES by ELISA. Statistical differences were determined by Student's *t*-test.

were incubated with IFN- γ (200 units/ml) in 6-well plates (Corning) with or without epinastine for 48 h. Cells were double-stained with fluorescein isothiocyanate (FITC)-conjugated anti-HLA-DR monoclonal antibody (mAb) and phycoerythrin (PE)-conjugated anti-CD54 mAb at $5 \mu\text{g}/10^6$ cells. After incubation for 60 min at 4°C with mAbs or control isotype-matched controls, cells were washed twice and analyzed on a FACS Calibur (Becton Dickinson, Mountain View, CA). All antibodies were purchased from BD Pharmingen, San Diego, CA. Mean fluorescence intensity was calculated on a log scale. As shown in Fig. 2, epinastine at 10^{-7} M profoundly downmodulated the expression of MHC class II and CD54 molecules.

This study demonstrated that epinastine downmodulates the production of chemokines in human keratinocytes. Among keratinocyte-derived chemokines, Th1 chemokines, IP-10, Mig and I-TACK, were suppressed by epinastine. Accordingly, epinastine was approved by the ministry in Japan for the treatment of psoriasis, which is a representative cuta-

neous Th1 disease. The inhibitory action was also found in RANTES, which has the ability to chemoattract both Th1 and Th2 cells. The optimally inhibitory dose of epinastine was 10^{-8} M, and the concentration of epinastine in the skin of individuals given the therapeutic dose of this drug is approximately 10^{-7} M, suggesting that the downmodulatory effect is clinically relevant.

Our previous study has shown different modulatory actions of anti-allergics on keratinocyte production of chemokines. Fexofenadine decreases Th2 chemokines, MDC and TARC, but unaffected Th1 chemokines, IP-10, Mig and I-TAC [9]. Olopatadine suppresses IP-10 production, but its level was lower than epinastine [10]. Therefore, epinastine is characterized by its wide range of suppressive capacity especially toward Th1 chemokines. In addition, epinastine reduced the expression of immunocompetent molecules, MHC class II and CD54, which may lead to blockade of the interaction between keratinocytes and T cells. Our study suggests that epinastine exerts not only an anti-histaminic action but

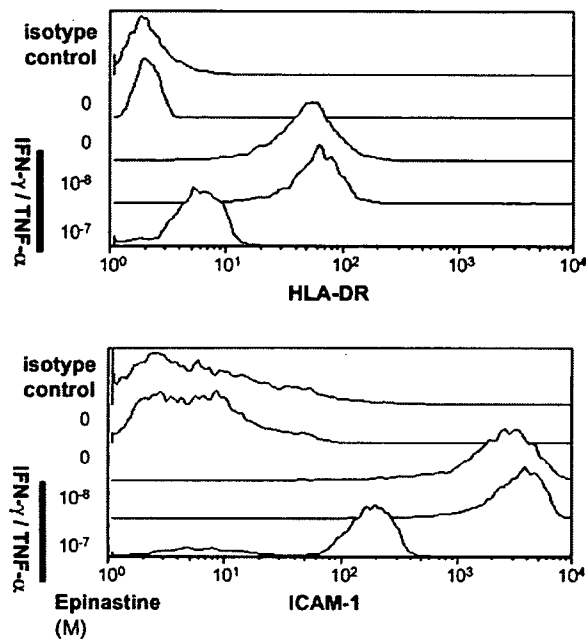


Fig. 2 Epinastine reduces the expression of MHC class II and CD54 molecules. NHEK were incubated with IFN- γ (200 units/ml) in the presence or absence of epinastine at 10^{-8} and 10^{-7} M for 48 h and the expression levels of MHC class II (HLA-DR) and CD54 (ICAM-1) were monitored by flow cytometry. Representative data from two independent experiments with similar results are shown.

also an inhibitory effect on Th1 migration toward the epidermis.

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

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Prostaglandin E₂ (PGE₂) 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 PGE₂ 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 non-cytokine substances in local milieu, one candidate being prostanoids.

Prostanoids, including prostaglandin (PG) D₂, PGE₂, PGF_{2 α} , PGI₂, and thromboxane A₂, 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, PGE₂

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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 PGE₂ 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 PGE₂ on cytokine production from Th1, Th2, and Th0 clones, and found that PGE₂ 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 PGE₂ on Th1 and Th2 cells has been confirmed by many studies (10–14). Because the best known action of PGE₂ is elevation of intracellular cAMP, and cAMP exerts similar Th1-selective suppression (15, 16), most, if not all, studies have assigned PGE₂ as a modulator of T cells raising the intracellular cAMP level. PGE₂ 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 PGE₂ in vitro in mixed lymphocyte reaction. They found that the immunosuppressive action of PGE₂ was significantly attenuated in T cells obtained either from EP2^{-/-} or EP4^{-/-} mice, suggesting that both EP2 and EP4 mediate suppression of PGE₂ 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 PGE₂-mediated immunosuppression. Treatment of animals with COX inhibitors does not necessarily enhance Th1 response. Indeed, T cell suppression by PGE₂ 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 PGE₂ 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 Ca²⁺ 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 PGE₂ 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 reaction

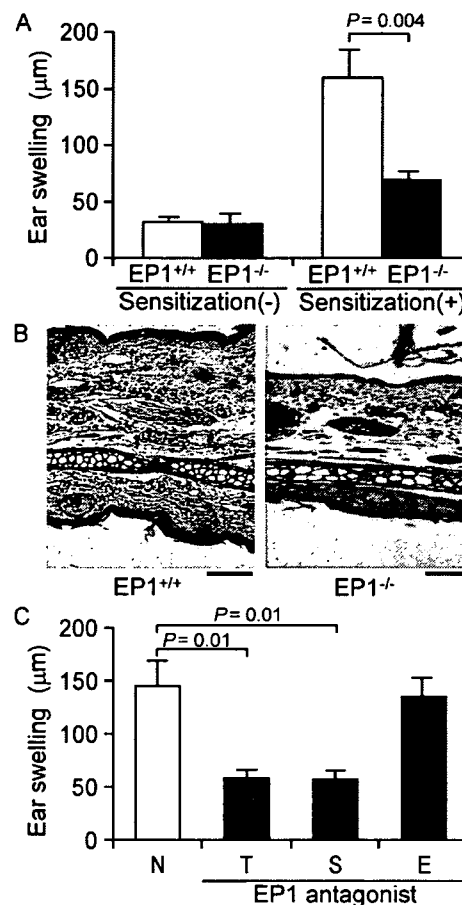


Figure 1. Impaired CHS responses in EP1^{-/-} mice. (A) Ear swelling. WT mice and EP1^{-/-} mice were sensitized with DNFB (Sensitization [+]) or vehicle (Sensitization [-]), challenged with DNFB 5 d later, and ear thickness was measured 24 h after the challenge (unpaired Student's *t* test; *n* = 5 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.

in vivo. Thus, this study revealed an important role of EP1 on T cells in Th1 response and raises an intriguing possibility that PGE₂ 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

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*, [³H]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),

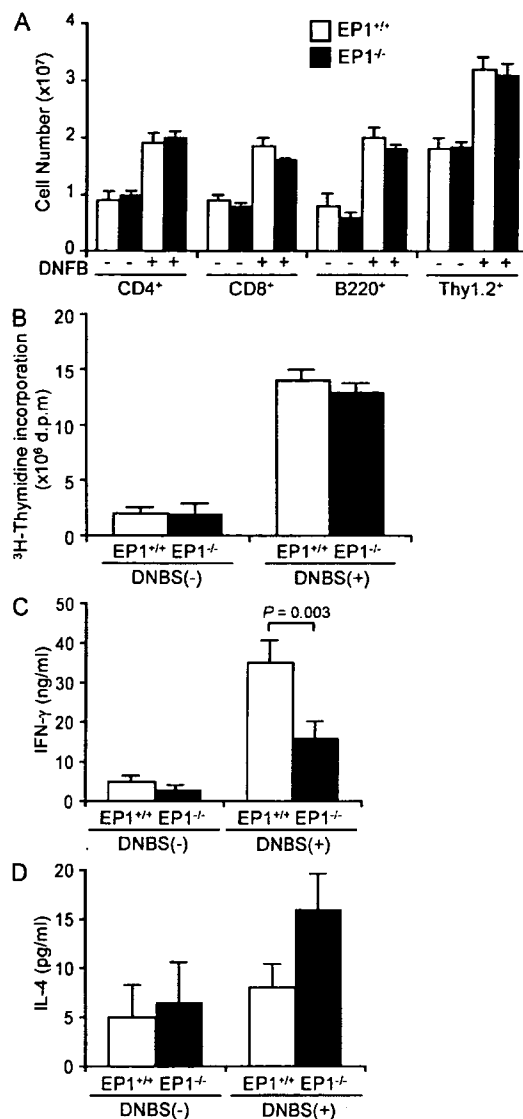


Figure 2. Impaired development of Th1 subset in CHS to DNFB in EP1^{-/-} mice. (A) Number of CD4⁺, CD8⁺, B220⁺, and Thy1.2⁺ cells in draining LNs (LNs) before and after antigen application. Cells from inguinal and axillary LNs were collected before or 5 d after DNFB application, and the number of CD4⁺, 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 [³H]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 \pm 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

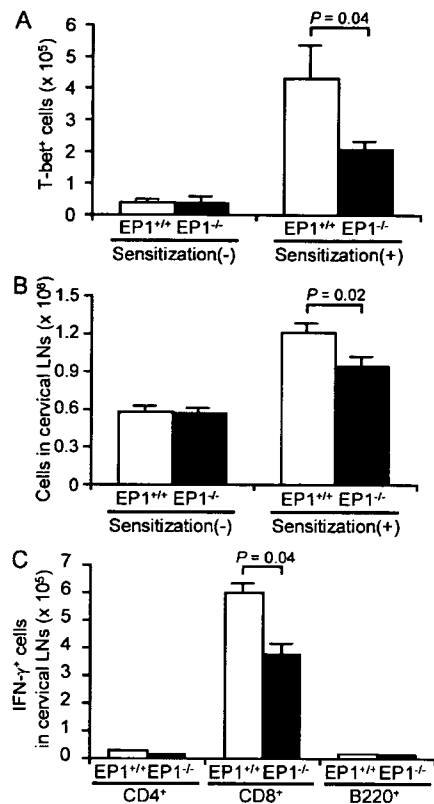


Figure 3. EP1^{-/-} mice showed reduced Th1 response in the elicitation phase of CHS. Cells were collected from LNs 5 d after the sensitization (A) or challenge (B and C) with DNFB, and the cell number was counted by flow cytometry. (A) Number of T-bet⁺ cells from inguinal and axillary LNs during sensitization 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).

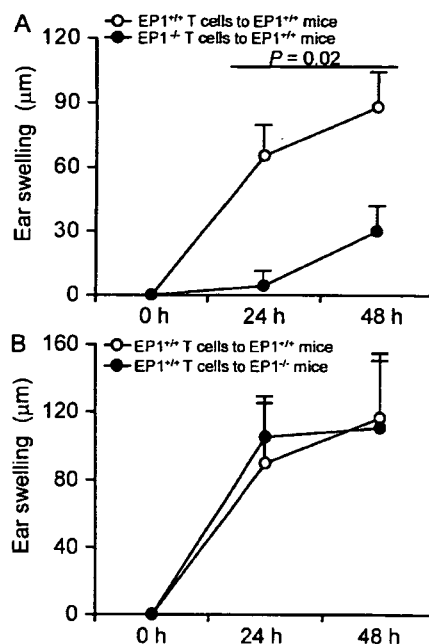


Figure 4. CHS response after adoptive transfer. (A) Attenuated CHS response in WT recipients adoptively transferred with sensitized EP1^{-/-} T cells. T cells were isolated and pooled from LNs of DNFB-sensitized WT (○) or EP1^{-/-} (●) donors and adoptively transferred into naive WT recipients (repeated 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 \pm 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 PGE₂ 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 PGE₂ 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,

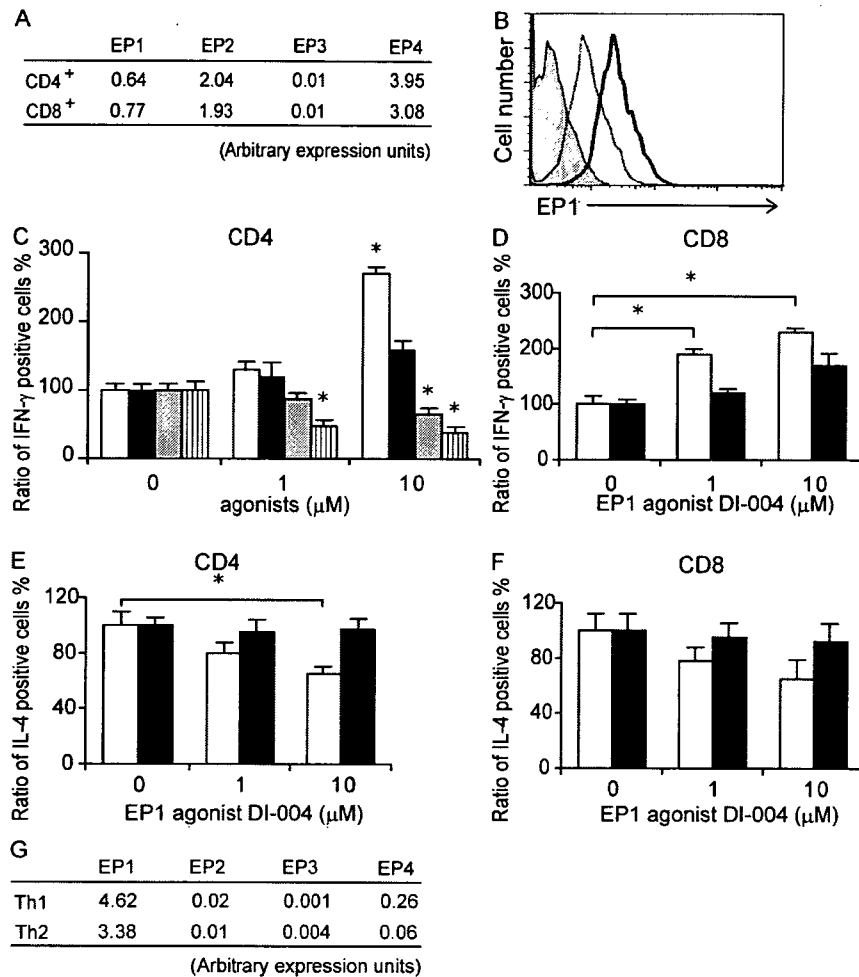


Figure 5. Effect of EP1 stimulation on T cell differentiation to Th1 and Th2 subsets in vitro. (A) Quantitative PCR analysis on expression for mRNAs of four EP subtypes in CD4⁺ and CD8⁺ T cells. Arbitrary expression units are shown. (B) Flow cytometric analysis of EP1 expression on naive CD4⁺ T cells. 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 Th1-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 Th2-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 \pm 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

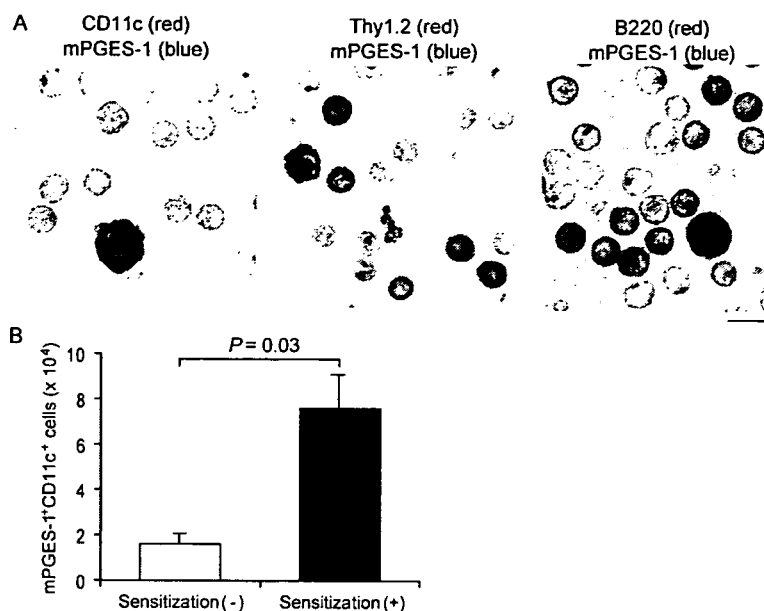


Figure 6. CD11c⁺ DCs expressing mPGES-1 increase in number in draining LNs after DNFB application. (A) Identification of LN cells expressing mPGES-1. Cells were collected from the draining LNs on day 1 after DNFB application, subjected to cytospin, and stained for mPGES-1, CD11c, Thy1.2, and B220. Bar, 10 μ m. (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 PGE₂-EP1 pathway was overcome by strong immunization stimulus.

PGE₂ production by CD11c⁺ DCs in draining LNs after DNFB stimulation

We have thus demonstrated the critical role of the PGE₂-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 PGE₂ in this process. It was reported that APCs, including DCs, can produce a large amount of PGE₂ in vitro (7). Consistently, WT CD11c⁺ DCs purified from the spleen produced PGE₂ 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 PGE₂, even upon anti-CD3 stimulation (Fig. S7 A, available at <http://www.jem.org/cgi/content/full/jem.20070773/DC1>). To characterize the PGE₂ production by DCs, we examined the identity of PGE synthase (PGES) involved in this process. PGE₂ 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 PGE₂ 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 PGE₂ without stimulation, and whereas stimulation with LPS induced the production of PGE₂ in a dose-dependent manner in WT cells, no LPS-induced augmentation of PGE₂ production was observed in mPGES-1^{-/-} DCs (Fig. S7 B), suggesting that mPGES-1 is responsible for PGE₂ 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 PGE₂ there during antigen-induced T cell proliferation and differentiation. PGE₂ produced in this manner in the LNs can act on T cells in a paracrine manner.

DISCUSSION

Although it has long been known that PGE₂ exerts immunosuppression in vitro, little is known how PGE₂ is involved in immune response in vivo. We used EP1^{-/-} mice and addressed this issue. Our results suggest that the PGE₂-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 PGE₂-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 PGE₂ acts through EP1 for amplification of Th1 differentiation.

The PGE₂ action we found here is opposite to the well-known PGE₂-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 PGE₂ 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 PGE₂ 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 PGE₂ 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 PGE₂ 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 PGE₂.

If so, then where in the body is PGE₂ produced, and does it exert its action in the course of sensitization? Because PG acts only in the vicinity of its synthesis, PGE₂ 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 PGE₂ 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 PGE₂ 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 PGE₂ that acts on EP1 on naive T cells to facilitate Th1 differentiation.

In conclusion, we found that the PGE₂-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 μ l 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 μ l of 0.3% DNFB

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- μ m 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 μ g/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 μ g/ml DNBS sodium for 3 d, and were pulsed with 0.5 μ Ci [³H]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 manufacturer's instructions (Endogen), as previously described (11). PGE₂ 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 Biotec) 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 μ g/ml of plate-bound anti-CD3 and in the presence of 10 ng/ml IL-12 and 10 μ g/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 μ g/ml anti-IFN- γ , and 2,500 U/ml IL-2 for Th2 differentiation with or without DI-004, butaprost, AE-1-329, or PGE₂ (1 μ M or 10 μ M). 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 Biotec) 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 \pm 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 PGE₂ 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 PGE₂ 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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T cell populations propagating in the peripheral blood of patients with drug eruptions

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Summary

Background: In the non-immediate type of drug eruptions, the populations of circulating T cells may be altered as a consequence of T cell responses to a culprit drug.

Objective: The aim of this study was to investigate differences among the types of drug eruptions in propagating T cell populations of the patients' peripheral blood.

Methods: The type of eruptions were divided into three categories: (1) generalized maculopapular eruption (MPE), (2) erythema multiforme (EM)/Stevens–Johnson syndrome (SJS), and (3) drug-induced hypersensitivity syndrome (DIHS) or drug rash with eosinophilia and systemic symptoms (DRESS). T cell populations were phenotypically analyzed by flow cytometry in the percentage of T helper (Th) 1 (CXCR3⁺CD4⁺), Th2 (CCR4⁺CD4⁺), Tc1 (CXCR3⁺CD8⁺), and Tc2 (CCR4⁺CD8⁺) subsets and their activation states as assessed by CD69, CD25 or HLA-DR positivity.

Results: Upon occurrence of both MPE and EM/SJS, Th2 cells outnumbered Th1 cells, whereas Tc1 and Tc2 cells differentially predominated in EM/SJS and MPE, respectively. An increase of HLA-DR⁺CD8⁺ cells in EM/SJS type provided another supportive evidence for Tc1 stimulation. In DIHS, during the development of the second wave of eruption and/or liver dysfunction associated with anti-HHV6 antibody elevation, CD4⁺ cells were gradually decreased, but CD8⁺ cells were inversely increased. Tc1 cells were increased as well as Th1 cells. Finally, in all the three groups, there existed a considerable number of CD25⁺CTLA-4⁻CD4⁺ T cells.

Conclusion: Our study suggests that Th2/Tc2 and Th2/Tc1 cells mediate MPE and EM/SJS, respectively, and Tc1 cells are involved in the pathogenesis of DIHS at the late stage.

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1. Introduction

Drug eruptions are one of the highly incident adverse reactions induced by drugs. There have been described various types of drug eruptions, which encompass broadly from the type 1 to 4 of the classical allergy criteria by Coombs and Gel. The delayed hypersensitivity cutaneous reactions most frequently occur with the particular culprit drug [1]. These T-cell mediated eruptions include disseminated maculopapular eruption (MPE), erythema multiforme (EM), Stevens–Johnson syndrome (SJS), toxic epidermal necrolysis (TEN), lichen planus, and fixed drug eruption [2]. Many histological and immunological studies have been performed to characterize each type of the drug eruptions. EM, SJS and TEN seem to be categorized by the same spectrum of condition, in which cytotoxic T lymphocytes (CTL) attack epidermal keratinocytes in a manner of graft-versus-host (GVH) reaction [3]. On the other hand, the maculopapular type does not show liquefaction degeneration of epidermal cells, but exhibits non-specific perivascular infiltration of T cells intermingled with various degrees of eosinophils [4].

Recent findings have indicated a novel entity of drug eruption, which is closely associated with reactivation of human herpesvirus 6 (HHV6) [5,6]. This mysterious disorder has been called several different names, such as drug-induced hypersensitivity syndrome (DIHS) and drug rash with eosinophilia and systemic symptoms (DRESS) [7]. The mechanisms underlying the sequential or concurrent development of drug reaction and virus reactivation remains unfully elucidated, but both responses to drug and HHV6 may be involved in the occurrence of two-phase or prolongation of eruption, liver dysfunction, eosinophilia, and appearance of circulating atypical lymphocytes.

It is well known that upon occurrence of T cell-mediated drug eruptions, the populations of circulating T cells may be skewed [8–10]. In a representative cascade of drug eruption, peripheral T cells react with a culprit drug, migrate to the skin, and trigger dermatitis as a consequence of immunological or inflammatory responses [1]. Given this scenario, it is likely that the alterations in the peripheral T cell subsets are detectable, depending on each type of drug eruptions.

CD4⁺ T cells are divided into Th1 and Th2 subsets on the basis of their cytokine production pattern. Recent accumulated evidence indicates that Th1 and Th2 cells express chemokine receptor CXCR3 and CCR4, respectively [11,12]. Similarly, T cytotoxic (Tc) cells are thought to consist of Tc1 and Tc2 cells bearing CXCR3 and CCR4, respectively [13], and increment of Tc2 has been observed in some skin

diseases [14]. On the other hands, a subset of CD4⁺ T cells bearing CD25 has been well characterized as regulatory T cells (Treg) that suppress the effector functions of CD4⁺ and CD8⁺ cells [15]. These markers allow T cell populations to be analyzed by flow cytometry.

In this study, we investigated circulating T cell populations in patients with drug eruptions, focusing on Th1/Tc1 or Th2/Tc2 polarization and their activation state. To clarify the relationship between the eruption type and the T cell subset, the eruptions were categorized into (1) MPE, (2) EM/SJS, and (3) DIHS/DRESS. Results suggest that the numerical changes of peripheral T cell subsets reflect the eruption type and immunological reactions to a culprit drug.

2. Materials and methods

2.1. Subjects

Seventeen patients with MPE, 5 with EM, 2 with SJS, and 3 with DIHS were enrolled in this study (Table 1). The diagnosis of drug eruption was made on the basis of clinical features and courses, lymphocyte transformation test, and provocation test. In cases 1, 2, 10, 15, 22, 25, 26 and 27, the diagnosis of drug eruption was made by positive lymphocyte transformation test. In cases 7 and 20, provocation test was performed with positive results. In the other patients, the diagnosis was made on the basis of clinical courses. MPE consisted of maculopapules of various sizes on different parts of the body. EM was composed of target or "bull's eye" erythematous edematous macules in varied distribution. SJS had erythema and maculopapules followed by vesicles and bullae with mucosal involvement. All the patients with EM or SJS showed serum titers indicating past infection of herpes simplex virus and no infection of mycoplasma pneumoniae. None of EM patients had a recurrent episode of this type of eruption. DIHS/DRESS is characterized by high fever, generalized skin rash, lymphadenopathy, circulating atypical lymphocytes, blood eosinophilia and multiple organ dysfunction [5–7]. DIHS was diagnosed by fulfilling these criteria and verifying seroconversion of anti-HHV6 IgG antibody transiently during the course. All patients were informed about the purpose of this study and agreed to participate.

2.2. Histology of skin lesions

Ten patients with MPE, 4 with EM, 1 of SJS, and 3 with DIHS were examined histologically. Skin biopsy specimens from acute skin lesions were fixed in 4%

Table 1 Patients in the study

| Case | Age/sex | Causative drug | Type of reaction | Time to reaction (days) | Histology | Number of eosinophils ^a |
|------|---------|-------------------------|------------------|--------------------------|-----------|------------------------------------|
| 1 | 69/F | Pravastatin | MPE | Intermittently (5 years) | PVL | 2 |
| 2 | 55/F | Carbamazepine | MPE | No information | ND | ND |
| 3 | 79/F | Amlodipine | MPE | No information | SP, PVL | 2 |
| 4 | 71/M | Theophylline | MPE | 2 | ND | ND |
| 5 | 83/M | Imipenem | MPE | 14 | ND | ND |
| 6 | 48/M | Iohexol | MPE | 1 | ND | ND |
| 7 | 57/M | Ibuprofen | MPE | 2 | ND | ND |
| 8 | 54/M | Iohexol | MPE | 1 | SP, PVL | 37 |
| 9 | 74/M | Ticlopidine | MPE | 9 | PVL | 3 |
| 10 | 78/F | Sulpiride | MPE | 2 | SP, PVL | 8 |
| 11 | 47/M | Cefpirome | MPE | 2 | ND | ND |
| 12 | 80/F | Allopurinol | MPE | 50 | PVL | 16 |
| 13 | 80/M | Loxoprofen | MPE | 2 | SP, PVL | 4 |
| 14 | 67/F | Clarithromycin | MPE | 8 | SP, PVL | 5 |
| 15 | 70/M | Carbamazepine | MPE | 20 | ND | ND |
| 16 | 31/F | Azithromycin | MPE | 17 | ND | ND |
| 17 | 66/M | Supplement (Turmeric) | MPE | Intermittently (7 years) | PVL | 2 |
| 18 | 70/M | Allopurinol | EM | No information | LTR | 38 |
| 19 | 63/M | Levofloxacin | EM | 2 | ECN, LTR | 5 |
| 20 | 54/M | Supplement (Turmeric) | EM | Intermittently (5 years) | ECN, LTR | 0 |
| 21 | 27/F | Risperidone | EM | 7 | ND | ND |
| 22 | 39/F | Carbamazepine | EM | 90 | ECN, LTR | 74 |
| 23 | 74/M | Cefcapene | SJS | 2 | ND | ND |
| 24 | 57/M | Thalidomide | SJS | 68 | ECN, LTR | 0 |
| 25 | 61/M | Carbamazepine | DIHS | 37 | ECN, LTR | 0 |
| 26 | 76/F | Supplement (Supirurina) | DIHS | 30 | PVL | 5 |
| 27 | 46/F | Carbamazepine | DIHS | 40 | SP, PVL | 0 |

PVL, perivascular lymphocyte; SP, spongiosis; ECN, epidermal cell necrosis; LTR, lichenoid tissue reaction; ND, not done. In cases 15, 18, and 22, prednisolone 10–20 mg daily was orally administered for 1–2 days, when the blood samples were taken.

^a The number of tissue eosinophils per high power view (400×).

formalin and stained with H&E for histology. We observed histological changes of skin lesions and counted the number of tissue eosinophils per high power view (400×) of inflammatory skin lesions.

2.3. Flow cytometric analysis

Blood samples were taken from patients with MPE, EM and SJS 2 to 5 days after the onset of eruption. In DIHS, blood samples were obtained 2–3 times from each patient, and the timing was described below. Peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized venous blood of patients by means of Ficoll–Paque (Sigma Chemical Co., St. Luis, MO) density gradient centrifugation. Cells were double-stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD4, anti-CD8 or anti-HLA-DR monoclonal antibody (mAb) and phycoerythrin (PE)-conjugated anti-CD4, anti-CD8, anti-CD69, anti-CD25, anti-CXCR3, anti-CCR4 mAb, or anti-CTLA-4 (all from PharMingen, San Diego, CA) at 2 µg/10⁶ cells. Hanks' balanced salt solution containing 0.1% NaN₃ and 1% fetal calf serum was used as

the staining buffer. After incubation for 30 min at 4 °C with mAbs or control isotype-matched controls, cells were washed twice and analyzed on a FACS Calibur (Becton Dickinson, Mountain view, CA).

2.4. Statistical analysis

Wilcoxon test employed to evaluate statistical significance between the means. $P < 0.05$ was considered significant.

3. Results

The patients enrolled in this study were listed in Table 1. EM and SJS were categorized into the same group, because of the clinical and pathophysiological similarities between these two epidermis-attacking diseases [3]. Thus, T cell populations were analyzed in the following three groups: MPE, EM/SJS, and DIHS. We first show data from the patients with MPE and EM/SJS by comparing these two groups, and separately present data of DIHS because

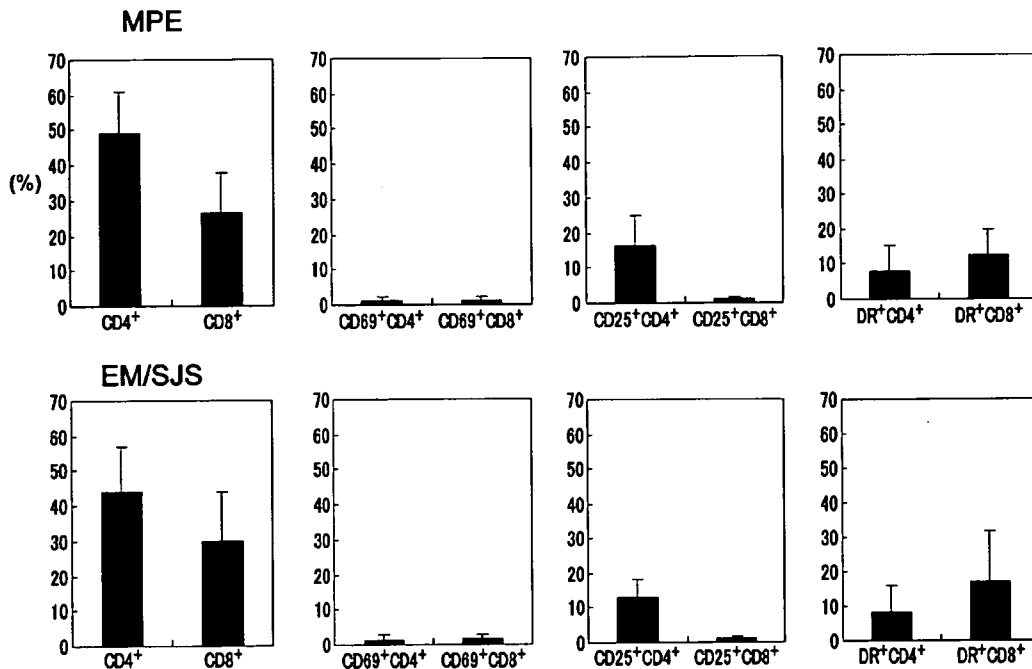


Fig. 1 Percentages of activated CD4⁺ and CD8⁺ T cells in the peripheral blood of patients with MPE and EM/SJS. Data are expressed as the mean \pm S.D.

of the presence of dynamic phenotypic changes during the clinical course.

3.1. MPE versus EM/SJS

PBMCs obtained at the active stage of MPE and EM/SJS were examined in the percentages of CD4⁺ and CD8⁺ cells bearing either of the activation markers, CD69, CD25 or HLA-DR. Data are summarized in Fig. 1. The mean percentages of CD4⁺ and CD8⁺ cells in both MPE and EM/SJS were within normal ranges (CD4⁺ cells, 25–56%; CD8⁺ cells, 17–44%), and there was no significant difference between them in these values. The percentages of CD4⁺ and CD8⁺ T cells bearing an early activation marker CD69 were low. A considerable number (mean, ~15%) of CD25⁺CD4⁺ cells were present in both groups, whereas CD25⁺CD8⁺ cells were negligible. In the three cases examined (cases 2, 3 and 20), CTLA-4, a marker of Treg [16], was not expressed on CD4⁺ cells. Both CD4⁺ and CD8⁺ cells bearing HLA-DR were high in percentage as compared to normal healthy subjects (HLA-DR⁺ T cells, <2%). There was a tendency that CD8⁺ T cells expressed late activation marker HLA-DR at a higher percentage in EM/SJS than MPE, while the numbers of HLA-DR⁺CD4⁺ cells were comparable.

We found an intriguing difference between MPE and EM/SJS in CXCR3⁺CD4⁺, CCR4⁺CD4⁺, CXCR3⁺CD8⁺, and CCR4⁺CD8⁺ subsets, representing Th1, Th2, Tc1, and Tc2 populations, respectively. In 6 normal healthy subjects, the percentages (mean \pm S.D.) of these

populations were as follows: CXCR3⁺CD4⁺, 1.52 ± 0.55 ; CCR4⁺CD4⁺, 4.22 ± 1.07 ; CXCR3⁺CD8⁺, 1.39 ± 0.68 ; CCR4⁺CD8⁺, 0.37 ± 0.16 . Fig. 2 shows data from each representative patient with MPE and EM/SJS. In MPE, Th2 and Tc2 cells outnumbered Th1 and Tc1 cells, respectively. Despite possessing Th2 dominance, a patient categorized as EM/SJS had a higher percentage of Tc1 cells than Tc2 cells. As summarized in Fig. 3, similar results were obtained from all patients with MPE and EM/SJS. Whereas Th2 cells overcame Th1 cells in all cases of both groups, Tc2 dominance in MPE and Tc1 dominance in EM/SJS provided a striking contrast. Despite the GVHD nature of EM, 2 patients with EM (cases 18 and 22) had high numbers of tissue eosinophils (Table 1), seemingly consistent with the Th2 elevation.

3.2. DIHS

In each patient with DIHS, we monitored alterations in T cell subsets at two or three time points during the disease course. The patients had virtually two peaks of eruptions and associated laboratory abnormalities. In Fig. 4, the first peak was expressed as "onset", when the patients were admitted to our hospital within 5 days after the occurrence of eruptions, and the second one was designated "recurrence", when the eruptions recurred or deteriorated with the occurrence of liver dysfunction at 2–4 weeks after the onset. Anti-HHV6 antibody was elevated in all patients 4–5 weeks after the onset. The