increased production of TNF-α. Although TNF-α alone cannot stimulate NHEK cells to produce MIG, the combination of TNF-α with IFN-γ dramatically enhances MIG production in keratinocytes and other epithelial cells.[24,25]

NB-UVB and BB-UVB both alter the production of cytokines and chemokines by keratinocytes, but the extents of their modulation are different from each other. On the basis of the MED and clinically used doses, NB-UVB at an 8- to 10-fold higher dose than BB-UVB is biologically comparable with BB-UVB.[9] When compared at these doses, NB-UVB was less stimulatory than BB-UVB for the production of proinflammatory cytokines, whereas the ability of NB-UVB to downmodulate Th2 chemokine production was rather higher than that of BB-UVB. Clinically, IL-1α and TNF-α induce cutaneous inflammation, such as erythema and swelling.[26] Thus, using NB-UVB might be safer than using BB-UVB because these adverse effects could be avoided, while still retaining the suppressive effect on Th2 chemokines.

The UVB-induced suppression of Th2 chemokine production suggests that UVB exposure to the skin suppresses infiltration of Th2 cells to the epidermis. Both BB-UVB and NB-UVB are considered to be effective for the treatment of various Th2-mediated or Th2-infiltrating skin diseases, such as atopic dermatitis,[26] subacute prurigo[27] and eosinophilic pustular folliculitis.[28] However, the state of cultured monolayered keratinocytes is different from that of patients' multilayered keratinocytes and, thus, the *in vivo* outcome does not necessarily reflect the phenomenon observed in this study. In addition, the effects of NB-UVB on constituent cells of skin other than keratinocytes may participate in the total therapeutic action. Although psoriasis is a disorder mediated by Th1 cells,[29] the effectiveness of UVB is widely accepted.[30] In this disease, the inhibitory effects of UVB on keratinocyte proliferation, vascular proliferation and lymphocyte apoptosis may be involved in the underlying mechanisms.[31,32] Our study suggests that NB-UVB is more clinically beneficial than BB-UVB, even in Th2-mediated diseases.

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Prostaglandin E_2 is required for ultraviolet B-induced skin inflammation via EP2 and EP4 receptors

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Keratinocytes are the major target of sunlight, and they produce prostaglandin (PG) E_2 upon ultraviolet (UV) exposure. Although indomethacin, one of cyclooxygenase inhibitors, is known to suppress UV-induced acute skin inflammation, it remains uncertain whether endogenous PGE_2 is responsible for UV-induced skin inflammation, and which subtype of PGE_2 receptors mediates this process. UV-induced skin inflammation was investigated by using genetically and pharmacologically PGE_2 receptor-deficient mice. We applied UV-induced skin inflammation model to genetical and pharmacological PGE_2 receptor-deficient mice. We exposed UVB on these mice at 5 kJ/m^2, and examined the ear swelling and the histological findings. We also measured the blood flow using a laser doppler device to assess the intensity of UVB-induced inflammatory change. The UV-induced ear swelling at 48 h after exposure was significantly reduced in EP2$^{-/-}$, EP4$^{-/-}$ or wild-type mice treated with the EP4 antagonist compared to control mice. Consistently, inflammatory cell infiltration into the local skin, and local blood flow after UV exposure were significantly reduced by EP2 or EP4 signaling blockade. These data suggest that PGE_2-EP2/EP4 signaling is mandatory in UV-induced acute skin inflammation, presumably by enhancing blood flow in the microenvironment.
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Ultraviolet light (UV) radiation has a wide variety of actions, such as sunburn formation, immunosuppression, skin aging and cancer development. Acute skin inflammation is another effect of UV radiation and is characterized by erythema and edema. Vasodilatation at the early phase after UV exposure is a critical event, leading to skin accumulation of inflammatory cells, such as neutrophils and T cells.[1] In addition, UV exposure to the skin triggers the release of lipid mediators such as prostaglandins (PGs), which are produced via sequential pathways involving cyclooxygenase (COX) and each PG synthetase. Among PGs, PGE_2 is known to be produced abundantly by keratinocytes in the skin on UV exposure.[2,3]

PGE_2 exerts its actions by binding to four different types of G-protein-coupled receptors, known as EP1, EP2, EP3 and EP4.[4] EP2 and EP4 receptors bind to G_s and increase cAMP. EP1 receptors are coupled to G_q and EP3 receptors mostly to G_i.[4] We have previously generated mice individually deficient in each of the four subtypes of PGE_2 receptors and studied the *in vivo* significance of each PGE_2 receptor with novel findings in various aspects.[5–11]

It is well known in humans that indomethacin, one of COX inhibitors, inhibits UV-induced acute skin inflammation,[12] and an exogenous intradermal injection of PGE_2 increases skin microenvironmental blood flow.[13] Although these observations have suggested that PGE_2 serves as a mediator in the UV-induced skin alterations, it remains uncertain whether endogenous PGE_2 is responsible for UV-induced skin inflammation, and which subtype of PGE_2 receptors mediates this process. Using both

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genetical and pharmacological approaches in mice, we examined *in vivo* the potential role of PGE_2 in UV-induced skin inflammation.

Materials and methods

Animals

Mice lacking the EP1, EP2, EP3 and EP4 receptors individually ($EP1^{-/-}$, $EP2^{-/-}$, $EP3^{-/-}$ and $EP4^{-/-}$ mice, respectively) were generated as described.[5–8] With the exception of $EP4^{-/-}$ mice, each mutant was backcrossed 10 times to C57BL/6CrSlc (Japan SLC, Shizuoka, Japan), and females of the F_2 progenies of N10 mice were used with C57BL/6 female mice as their controls. Mice were maintained on a 12-h light/dark cycle under specific pathogen-free conditions. All experimental procedures were approved by the Committee on Animal Research of Kyoto University Faculty of Medicine.

Reagents

EP4 antagonist, ONO-AE3-208, 4-(4-Cyano-2-(2-(4-fluoronaphthalen-1-yl) propionylamino) phenyl) butyric acid (AE3-208) was kindly provided by Ono Pharmaceutical Co., Osaka, Japan. AE3-208 was administered (10 mg/kg/day) orally in the drinking water 2 days before UV exposure and through the experiment. A volume of 20 μl of 1% wt/vol indomethacin in acetone was topically applied on the ears of mice immediately after UV irradiation.

UVB Irradiation

Sunlamps emitting 280–360 nm with a peak emission at 312.5 nm (Toshiba FL 20SE; Toshiba Electric Co.) were used as a source of UVB. The irradiance was 5.5 J/m^2/s at a distance of 40 cm, as measured by an UVR-305/365D digital radiometer (Tokyo Kogaku Kikai KK, Tokyo, Japan). For UV-induced skin inflammation, mice were exposed to 5 kJ/m^2 of UVB. The ear swelling was measured at the indicated time points after irradiation using ear thickness gauge, and the ear thickness change was shown.[14]

Determination of PGE_2 Content in the Mouse Skin

The amounts of PGE_2 in the mouse abdominal skin at 0 and 48 h after irradiation with 5 kJ/m^2 of UVB were determined by enzyme immunoassay basically as manufacturer's protocol (Cayman Chemical). In brief, abdominal skin was excised and immediately dropped into liquid nitrogen. Frozen skin were weighted and homogenized with a polytron homogenizer in 10 ml ethanol containing 0.1 ml of 5 N HCl, which was precooled at −20°C. After centrifugation, PGE_2 in the ethanol extract were applied on SEP-PAK C18 cartridges (Waters Associates, Milford, MA, USA). The amounts of PGE_2 were measured by enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI, USA).

Histology

The skin of the ear was excised and fixed in 10% formaldehyde. Sections of 5 μm thickness were prepared and subjected to staining with hematoxylin and eosin. The numbers of neutrophils and lymphocytes per field (× 20) were counted at five randomized spots using microscopy. The diameter of randomly selected 50 blood vessels in the ear was measured.

Measurement of Skin Blood Flow

Abdominal area of mouse skin was shaved and irradiated with UVB. Blood flow was assessed using an Advance Laser (model ALF21R) Doppler flowmeter. The probe was held at six spots of abdominal area, and each reading was the mean of the six measurements.

Statistical Analysis

Data were analyzed using an unpaired two-tailed *t*-test or one-way ANOVA followed by Dunnett multiple comparisons. *P*-value of <0.05 was considered to be significant.

Results

It is known that a single exposure of UVB to mice causes marked skin inflammation, most remarkably on the ears. The extent of inflammation was evaluated by measuring the ear swelling responses. When control C57BL/6 mice were exposed to at 5 kJ/m^2 to UVB, the ear swelling became detectable within 24 h after irradiation, and the response progressed over the next 24 h as reported previously.[15] Mice receiving topical application of 1% indomethacin in acetone immediately after UV exposure showed significantly reduced ear swelling responses at each time point tested, as compared to the control mice that received acetone alone (Figure 1). As indomethacin inhibits COX, an enzyme to produce PGs, it is assumed that PGs play a pivotal role in the induction of UV-induced skin inflammation. As PGE_2 is the major PG produced by keratinocytes, we tested the magnitude of swelling responses in mice lacking each receptor subtype. The responses were significantly reduced in $EP2^{-/-}$ mice, $EP4^{-/-}$ mice and EP4 antagonist-treated C57BL/6 mice compared to the control C57BL/6 or $EP4^{+/+}$ mice at 48 h, although the response was similar in EP1 and EP3 mice (Figure 1). The baseline

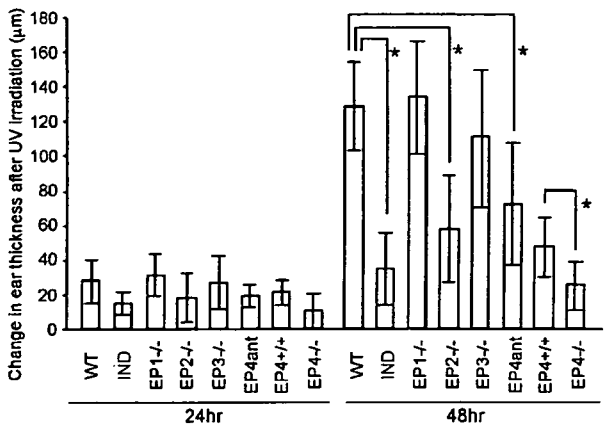

Figure 1 Impaired UV-induced ear skin swelling response by EP2 and EP4 receptor signaling blockade. C57BL/6 mice were treated without (WT, $n=15$) or with indomethacin (IND, $n=10$), or pretreated with EP4 antagonist (EP4 ant, $n=16$). Along with these mice, $EP1^{-/-}$ ($n=11$), $EP2^{-/-}$ ($n=8$), $EP3^{-/-}$ ($n=9$), $EP4^{+/+}$ ($n=6$) and $EP4^{-/-}$ ($n=6$) mice were exposed to 5 kJ/m² of UV radiation. They were examined in ear swelling responses 24 and 48 h later, and the increment in ear thickness was calculated (ear thickness after irradiation—ear thickness before irradiation). Data are a representative of three independent experiments and presented as the means ±s.d. A Student's *t*-test was performed between the indicated groups and an asterisk indicates $P<0.05$.

ear thickness of C57BL/6 with or without the EP4 antagonist, and EP-deficient mice, all without UV radiation, was comparable between these groups (data not shown). These results suggested that endogenous PGE_2 mediates UV-induced skin inflammation through EP2 and EP4 receptors. As $EP4^{-/-}$ mice were F_2 progeny and individual mice possessed mixed genetic backgrounds, we examined the response of mice administered with the selective EP4 antagonist for further evaluation of EP4 serving as critical receptors.

We then examined the histology, which showed that the ear of C57BL/6 mice treated with or without the EP4 antagonist, and $EP2^{-/-}$ mice, all without UV exposure, were similar (Figure 1). And the histology of the ears of wild-type mice 48 h after UVB irradiation showed significant dermal edema, inflammatory cells infiltration in the upper dermis, and the marginal thickening of the epidermis. This edematous change in the dermis was milder in $EP2^{-/-}$ mice and C57BL/6 mice treated with the EP4 antagonist (Figure 2). Moreover, the infiltration of inflammatory cells was less intensive in those mice (Figure 2). Whereas neutrophils and lymphocytes in the ears of wild-type mice were increased after UVB irradiation, those numbers in $EP2^{-/-}$ mice or C57BL/6 mice treated the EP4 antagonist were lower than those in C57BL/6 mice after irradiation (Figure 3).

The edema response following UV irradiation is dependent on vascular blood flow and vascular permeability. Especially, vascular blood flow can alter fluid movement between the vascular lumen and the interstitial space by increasing the luminal hydrostatic pressure. It is known that increasing local-microenvironmental blood overflow at the early phase after UV exposure is essential to establish UV-induced skin inflammation.[1,16] We therefore measured the diameter of blood vessels in the skin 48 h after UVB irradiation in histological specimens. While blood vessels in wild-type mice were enlarged after UVB irradiation, those in $EP2^{-/-}$ mice or wild-type mice treated with the EP4 antagonist were significantly smaller (Figure 4). We then assessed the blood flow of the abdominal skin at 0, 12, 24, 36 and 48 h after UVB (5 kJ/m²) irradiation using a laser doppler device, and found that the blood flow peaked around 24 h after UV exposure, and was impaired by the EP2 or EP4 signaling blockade (Figure 5). These data suggested that PGE_2-EP2/EP4 signaling is critical in acute skin inflammation by enhancing blood flow in the microenvironment.

The above results suggested that both EP2 and EP4 are essential for the development of acute skin inflammation. However, the suppressed level of inflammation by each signaling blockade tended to be lower than the indomethacin treatment. We therefore explored the effect of simultaneous blockade of EP2 and EP4 signaling by administering $EP2^{-/-}$ mice with the EP4 antagonist. The administration of the EP4 antagonist further decreased the reduced ear swelling level of $EP2^{-/-}$ mice (Figure 6). To rule out the possibility that UV-induced PGE_2 expression is affected by EP signaling blockade, which eventually affect the skin inflammation, we measured the PGE_2 expression in the skin. PGE_2 level in the abdominal skin 48 h after irradiation from wild-type and $EP2^{-/-}$ mice with or without the EP4 antagonist were as follows (wild-type C57BL/6 mice; 90.8±10.5, $EP2^{-/-}$ mice; 80.4±12.8, $EP2^{-/-}$ mice with the EP4 antagonist; 76.3±9.0 ng/g tissue: average ±s.d., $n=4$ each). As a comparison, the baseline PGE_2 expression in the skin without UVB irradiation was 0.9±0.3 ng/g tissue: average ±s.d., $n=4$ each. These results suggest that a significant amount of PGE_2 was induced by UV exposure, and the concentration of PGE_2 in the skin was not affected by the EP signaling blockade.

Discussion

In this study, we demonstrated that UVB-induced ear swelling response, skin infiltration of neutrophils and lymphocytes, and local blood flow were reduced in $EP2^{-/-}$, $EP4^{-/-}$ or wild-type mice treated with the EP4 antagonist. These results suggest that endogenous PGE_2 mediates UVB-induced acute skin inflammation through EP2 and EP4 receptors.

The UVB-evoked acute swelling response seems to be derived mainly from vasodilatation and resultant exudates. PGE_2 exerts its physiological functions by binding to its specific receptors, EP1-EP4, which are well known to be expressed on blood vessels. Vasodilatation is performed through EP2

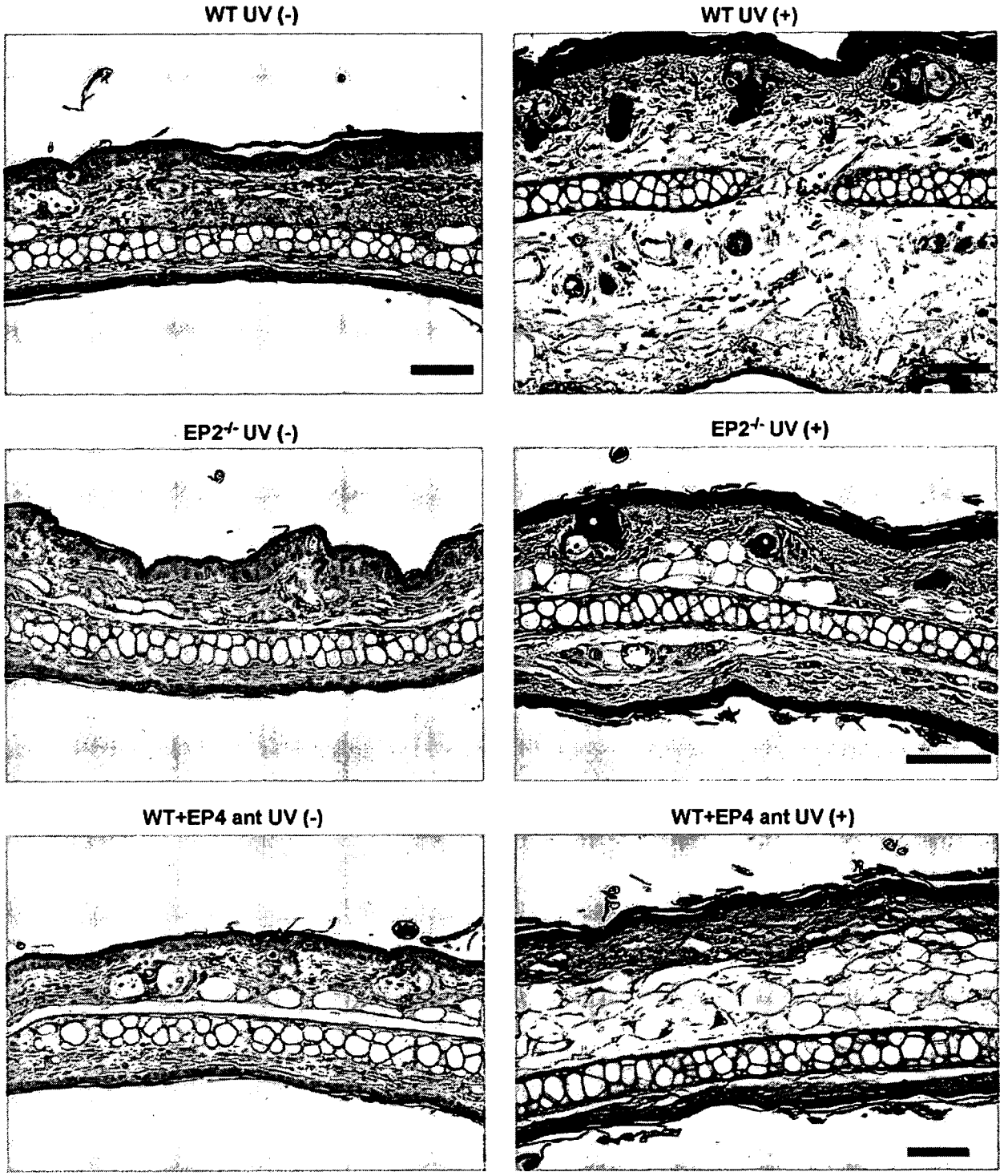

Figure 2 Histology of the skin after UV exposure. The ears of C57BL/6 mice treated with (EP4 ant) or without (WT) EP4 antagonist, and those of $EP2^{-/-}$ mice were excised before (UV (−)) and 48 h after UV irradiation (UV (+)). The edema and inflammatory cell infiltration was notified in WT mice after irradiation, which was less significant in WT mice treated with EP4 antagonist or $EP2^{-/-}$ mice. Sections of 5 μm thickness were prepared and subjected to staining with hematoxylin and eosin. Scale bars, 50 μm.

and EP4 receptors in association with increased cyclic AMP levels by coupling G_s.[17–19] Moreover, PGI_2 receptor, IP and PGD_2 receptor, DP, are known to increase cyclic AMP levels via G_s.[4] In mice, however, PGD_2 was barely detected in the skin after UVB irradiation, and PGI_2 was not examined in that study.[20] We showed that the ear swelling response in EP2 mice (58 + 12.6 μm) or C57BL/6 mice treated

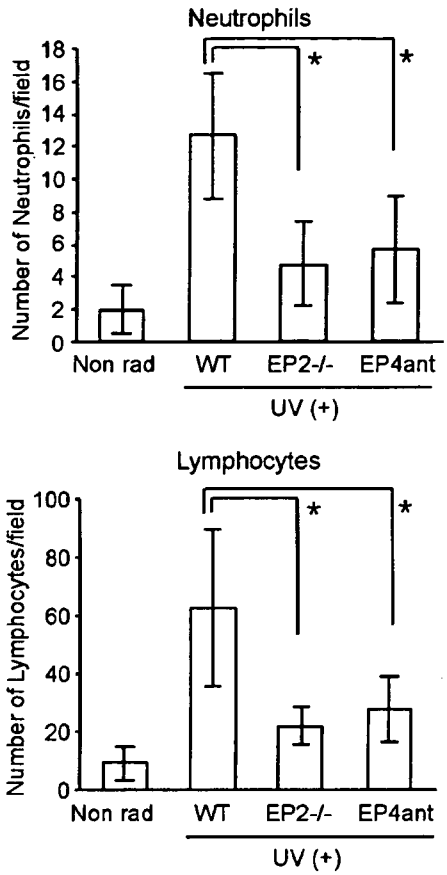

Figure 3 Number of inflammatory cells in the skin. The ears of C57BL/6 mice treated with (EP4 ant) or without (WT) EP4 antagonist, and those of $EP2^{-/-}$ mice were excised 48 h after UV irradiation (UV (+)). As comparison, the ears of C57BL/6 mice without UV exposure were used (Non-rad). The numbers of neutrophils and lymphocytes infiltrating into the skin per field (× 20) were counted at five randomized areas, and are presented as the means ± s.d. *Statistically significant differences compared with the UV exposed WT group ($P<0.05$, unpaired two-tailed *t*-test).

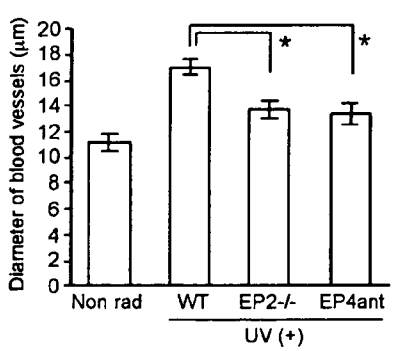

Figure 4 Diameter of blood vessels in the skin. The ears of C57BL/6 mice treated with (EP4 ant) or without (WT) EP4 antagonist, and those of $EP2^{-/-}$ mice were excised 48 h after UV irradiation (UV (+)). As comparison, the ears of C57BL/6 mice without UV exposure were used (Non-rad). The diameter of 50 randomized blood vessels in the dermis were measured, and are presented as the means ± s.e.m. *Statistically significant differences compared with the UV exposed WT group ($P<0.05$, unpaired two-tailed *t*-test).

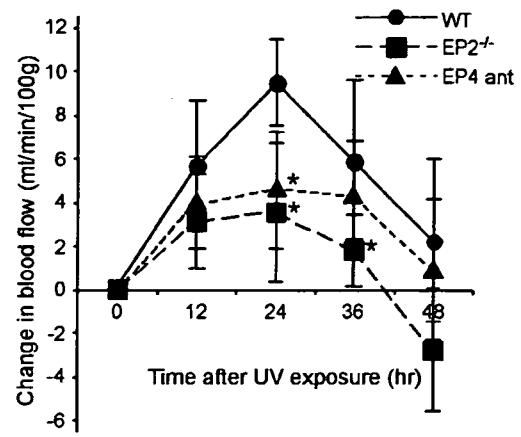

Figure 5 Blood flow of the skin after UV exposure. The blood flow of the abdomen of C57BL/6 mice treated with (EP4 ant, $n=5$) or without (WT, $n=5$) EP4 antagonist, and that of $EP2^{-/-}$ ($n=5$) mice were measured using laser Doppler flowcytometer at indicated time points. These data are a representative of three independent experiments and are presented as the means ± s.d. *Statistically significant differences compared with the UV exposed WT group ($P<0.05$, Dunnett multiple comparisons).

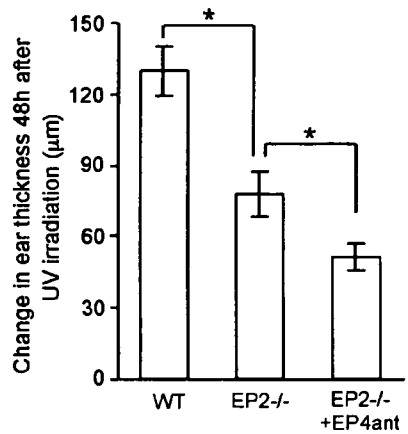

Figure 6 Enhanced impairment of UV-induced ear skin swelling response by combinational signaling blockade of EP2 and EP4 receptors. C57BL/6 (WT) mice and $EP2^{-/-}$ mice treated with or without EP4 antagonist were exposed to 5 kJ/m² of UV radiation ($n=5$, each). They were examined in the ear swelling response 48 h later. These data are a representative of two independent experiments and are presented as the means ± s.d. Student's *t*-test was performed between the indicated groups and an asterisk indicates $P<0.05$.

with the EP4 antagonist (72 + 14.3 μm) was not as low as mice treated with indomethacin (35 + 8.6 μm). Therefore, we explored the effect of simultaneous blockade of EP2 and EP4 signaling by administering $EP2^{-/-}$ mice with the EP4 antagonist and found that the administration of the EP4 antagonist further decreased the ear swelling of $EP2^{-/-}$ mice (51 + 5.6 μm). This extent of reduced ear swelling level was not as same as indomethacin-treated group, but quite similar. It might be interesting to investigate the role of other PGs, such as

PGI_2, in the development of inflammation. In human, both PGD_2 and 6-keto $PGF_{1\alpha}$, stable metabolites of PGI_2, were detected in the skin after UVB irradiation.[21] The differences may exist in the usage of PGs for the UV-induced skin response between mice and human. Nonetheless, the importance of PGE_2, as documented in this study, is particularly notable. As cAMP is the downstream signaling in both EP2 and EP4, we expected that the differences in cAMP production are involved in the synergistic effect of EP2 deficiency and the EP4 antagonist treatment. However, the cAMP level in these mice were barely detected in the skin, and not elevated by UV exposure (data not shown). Moreover, there was no significant difference in cAPM production between these groups. We presume that cAMP is difficult to be detected in the skin en bloc even though it is induced in the blood vessels after UV exposure. One possibility is that PGE2 is produced consistently after UV exposure and may not elevate cAMP levels robust enough to be detected *in vivo*. The local cAMP production around the vessels may be elevated, but it is difficult to detect this slight increase.

Keratinocytes are the major target of UV radiation and play a central role in the inflammatory and immune modulatory changes observed after UV exposure, at least partly via the UV-induced release of cytokines (IL-1, IL-6, IL-8, IL-10, GM-CSF, TNF-α)[22] and COX products.[23] IL-6 induces fever and the acute phase response and stimulates leukocytes infiltration in the skin.[24] PGE_2 plays a role in vasodilatation and in the erythemal response of the skin after solar exposure.[25] It has been reported that nitric oxide is involved in the induction of vascular dilatation after UVB exposure.[3,13,26] As indomethacin treatment did not completely inhibit the UV-induced ear swelling response, there still remains the possibility that other factors are involved in this process. IL-6 and nitric oxide are the candidate in this process at present. So far, using normal human keratinocytes, Pupe *et al*[27] demonstrated that eicosaepentaenoic acid decreased both PGE_2 and IL-6 secretion induced by UV-irradiation.[27] However, the relationship between PGE_2, IL-6 and nitric oxide *in vivo* events remains unknown and is an interesting issue to be pursued in the future. In this study, we focused on the acute phase of UV-induced skin inflammation. During the acute process of fluid exudation, the role of histamine release from mast cell is important by increasing the vascular permeability.[28] Although PGE_2 reportedly suppresses mediator release by some mast cell subtypes *in vitro*,[29] it also enhances mediator release from mouse mast cells.[30] The mechanisms and EP receptor subtypes responsible for PGE_2-mediated inhibition of mast cell activation in UV-induced skin inflammation are incompletely understood.

At cellular levels, UV radiation triggers cytokine production,[22] regulates surface expression of adhesion molecules,[31] affects cellular mitosis,[32] and induces apoptotic cell death of skin components.[33] The UV-induced keratinocyte apoptosis is an important factor for preventing from skin cancer formation. EP2 and EP4 signaling blockade can be prophylactic for the acute sunburn reaction in which vasodilatation, exudation, and inflammation participate. On the other hand, it has been already reported that PGE_2-induced skin cancer is mediated by EP1 or EP2 receptor signaling,[34,35] and there still remains the possibility that other PGE_2 receptor signaling may be involved in this process. The present study clearly demonstrated the deep relationship between PGE_2 and acute UV reactions. The involvement of PGE_2 and its receptors in more chronic UV-induced conditions, such as cancer development, is an issue to be elucidated.

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Immunopathology and Infectious Disease

CXCL12-CXCR4 Engagement Is Required for Migration of Cutaneous Dendritic Cells

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CCR7 is regarded as an essential chemokine receptor for cutaneous dendritic cell (DC) migration into the regional lymph nodes. However, complete migratory inhibition cannot be obtained in CCR7-deficient mice, suggesting that there exist other chemokine receptors involved in this process. Initially, we found that CXCR4 was highly expressed on migrated cutaneous DCs and that its ligand, CXCL12, was detected in the LYVE-1$^+$ lymphatic vessels in the skin. FITC-induced cutaneous DC migration into the draining lymph nodes was impaired by the specific CXCR4 antagonist 4-F-Benzoyl-TN14003. Among FITC$^+$ cells, Langerin$^+$ Langerhans cells and Langerin$^-$ (dermal) dDC subsets were detected as CD11c^{high+}CD11b^{int+} cells and CD11c^{high+}CD11b^{high+} plus CD11c^{low+}CD11b^{int+} cells, respectively, both of which were suppressed by CXCR4 antagonist. Moreover, *in vivo* contact hypersensitivity response was impaired by CXCR4 antagonist administered during the sensitization phase. The *in vitro* proliferative response to dinitrobenzene sulfonic acid of sensitized lymph node cells was inhibited by CXCR4 antagonist treatment. These findings demonstrated that CXCL12-CXCR4 engagement on cutaneous DCs plays a crucial role in the initiation of skin immune response by enhancing cutaneous DC migration. *(Am J Pathol 2007, 171:1249–1257; DOI: 10.2353/ajpath.2007.070225)*

It is in the lymphoid organs that T lymphocytes and antigen-presenting cells such as dendritic cells (DCs) participate to generate adaptive immune responses.[1–3] There are two subsets of DCs in the skin, dermal DCs (dDCs) and epidermal Langerhans cells (LCs). The arrival of antigen-bearing DCs into lymph nodes from peripheral sites begins several hours after antigen exposure and reaches its peak for 1 to 3 days, depending on the type of antigen and DCs. However, the precise repertoire of signals that regulate these processes is not fully elucidated.[2–6] Recently, based on *in vitro* studies of chemotaxis and chemokine receptor expression[5,7,8] and *in vivo* studies using relevant rodent models, central roles for various chemokines and their receptors in DC migration have been identified.[2–6,9] Using human monocyte-derived DCs, it was reported that immature DCs express CCR1, CCR2, CCR5, and CXCR1 and that the induction of DC maturation by lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α), or CD40L results in up-regulated expression of CCR7 and CXCR4.[8] CCR7 is a well-known chemokine receptor responsible for regulating DC function. CCR7 deficiency dramatically impairs migration of activated cutaneous DCs into draining lymph nodes 24 hours after fluorescein isothiocyanate (FITC) application, with profound morphological alterations in the architecture of secondary lymphoid organs.[10] However, it should be noted that this impairment of migration is incomplete. An another line of study using *plt* mice, which lack CCR7 ligands, has revealed that CCR7 ligand deficiency leads to an imperfect (approximately 70%) decrease in the number of FITC$^+$ migrated DCs in the draining lymph nodes.[11] These data have suggested that there should

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exist other chemokines/chemokine receptors responsible for cutaneous DCs migration into lymph nodes.

CXCR4 is a G-protein-coupled receptor expressed by a wide spectrum of cells. Its physiological importance in hematopoiesis and development of the vasculature and central nervous system has been emphasized by the lethal phenotype of its knockout mice. On the other hand, CXCR4 expression on monocyte-derived DCs is enhanced along with their activation, and DCs have chemotactic response to the CXCR4 ligand CXCL12 (stromal-cell derived factor-1) *in vitro*.[12] CXCR4 is also detected in human LCs, and its expression level is increased by granulocyte macrophage–colony-stimulating factor (GM-CSF).[13] Nevertheless, there is little knowledge about the function of CXCR4 in cutaneous DCs and its contribution to directional migration of DCs on skin inflammation *in vivo*. CXCL12 is expressed by murine stromal cells in the red pulp of spleen and the medulla of lymph nodes and by human skin endothelial cells.[5,14–16] CXCL12/CXCR4 interactions are largely unique and nonpromiscuous. In mice, CXCL12 or CXCR4 gene knockouts generate a similar phenotype, characterized by deficient B lymphopoiesis and myelopoiesis and abnormal neuronal and cardiovascular development.[17–19] Embryonic lethality associated with either CXCR4 or CXCL12 gene knockouts emphasizes the critical and unique role played by these gene products during development. This chemokine also plays a critical role in lymphocytic circulation and immune surveillance in the postnatal life. *In vitro*, CXCL12 has potent chemoattractant properties for cells expressing CXCR4, such as monocytes, lymphocytes, and $CD34^+$ hematopoietic stem cells.

In light of the emerging significance of various members of the chemokine system in DC biology, we tested the hypothesis that the chemokine receptor CXCR4 and its ligand CXCL12 influence cutaneous DC function and adaptive immune responses. We found that CXCR4 is highly expressed on activated cutaneous DCs and that CXCL12 is expressed in the lymphatic vessels of the skin. Mice treated with CXCR4 antagonist exhibited significantly impaired cutaneous DC migration and reduced contact hypersensitivity (CHS) response. These findings collectively provide evidence for an important role of CXCR4 in cutaneous DC functions.

Materials and Methods

Animals and Reagent

Female C57BL/6 (B6) mice at 8 weeks of age were purchased from Japan SLC (Hamamatsu, Japan). Mice were maintained on a 12-hour light/dark cycle under specific pathogen-free conditions. Protocols were approved by the Institutional Animal Care and Use Committee of the University of Occupational and Environmental Health.

For CXCR4 antagonist treatment, Alzet osmotic pumps (7-day duration, 0.5 μl per hour pumping rate; model 1007D; Durect Corporation, Cupertino, CA) were loaded with 40 mg/ml CXCR4 antagonist, 4F-benzoyl-TN14003,[20,21] in saline and were implanted subcutaneously to the back under intraperitoneal anesthesia according to the manufacturer's instructions. The administered dose was calculated to be 0.48 mg per kg body weight per day. No toxicity of CXCR4 antagonist was observed at 5 μmol/L *in vitro* as reported previously.[22] Moreover, the selectivity of the antagonist was confirmed by the finding that there was no significant inhibition against Ca^{2+} mobilization induced by MIP-1α stimulation through CCR5 (IC_{50} = 22 μmol/L) and against Ca^{2+} mobilization induced by sphingosine-1-phosphate stimulation through EDG3 (IC_{50} > 30 μmol/L) by the treatment of CXCR4 antagonist (data not shown). To characterize its specificity further, epidermal cell suspensions were applied to transwell for chemotaxis assay (see below for method). The chemotaxis of major histocompatibility complex (MHC) class II^+ LCs to CXCL12 was inhibited by CXCR4 antagonist, but such inhibitory effect was not observed toward CCR7 ligand, CXCL21 (data not shown).

Cell Preparation and Cultures

Complete RPMI (cRPMI), RPMI 1640 medium (Sigma, St. Louis, MO) containing 10% heat-inactivated fetal calf serum (Invitrogen, Carlsbad, CA), 5×10^{-5} mol/L 2-mercaptoethanol, 2 mmol/L L-glutamine, 25 mmol/L HEPES (Cellgro, Herndon, VA), 1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin was used as culture medium. For depleting DCs, lymph node cells were dispersed and sorted to $CD11c^-$ population using CD11c microbeads with autoMACS per the manufacturers' protocol (Miltenyi Biotech, Gladbach, Germany). After depletion, the frequency of $CD11c^+$ DC fraction was less than 0.02%.

For organ culture assay, the skin of mouse ears were split along with cartilage, and the dorsal ear skin without cartilage was floated in a dermal side-down manner in 24-well tissue culture plates (Costar; Corning Life Sciences, Acton, MA) at 37°C. Twenty-four hours later, the cells in the wells were collected for analysis.[23]

Flow Cytometry and Immunohistochemistry

Cell suspensions were prepared from lymph nodes by mechanical disruption on 70-μm nylon cell strainers (BD Falcon, San Jose, CA). For flow cytometry, cells were plated at a density of 1×10^6 cells per well in 96-well U-bottomed plates (Falcon). They were stained for 20 minutes on ice with antibodies (Abs) in 25 μl of phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS), 1 mmol/L ethylenediamine tetraacetic acid (EDTA), and 0.1% NaN_3 and were washed twice with 200 μl of this buffer after each step. For staining with CXCR4, cells were preincubated with CD16/32 monoclonal Ab in 0.5% bovine serum albumin (BSA) containing RPMI 1640 medium for 30 minutes for resensitization and Fc receptor blocking and then stained as above. Data were collected on a FACSCanto or FACSCalibur (BD Biosciences, San Diego, CA) and analyzed with FlowJo software (TreeStar, San Carlos, CA).

Abs used were as follows: phycoerythrin (PE)-conjugated anti-CXCR4 (2B11; BD Biosciences) and isotype-matched control IgG2a, PE-Cy5-conjugated anti-MHC class II Ab, PE-Cy7-conjugated CD11b and B220 Ab, and allophycocyanin (APC)-conjugated anti-CD11c Ab (all from BD Biosciences). Langerin was detected using a specific Ab (929F3; kindly provided by Sem Saeland, Schering Plough) in permeabilized cell suspensions, followed by visualization with anti-rat Ig conjugated to PE.

For immunofluorescence analysis, the ears of B6 mice 24 hours after application with hapten were frozen in Tissue-Tek OCT compound 4583 (Sakura Finetechnical Co. Ltd., Tokyo, Japan). Cryostat sections (10 μm) were fixed in acetone and stained as described previously[24] with the following reagents: goat anti-mouse CXCL12 Ab (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or rat anti-mouse LYVE-1 Ab (R&D Systems, Minneapolis, MN). Goat anti-CXCL12 Ab, after incubation with CXCL12 blocking peptide (62.5 μg/1 mg of antibody; Santa Cruz Biotechnology, Inc.) for 1 hour on ice and was centrifuged at 13,000 rpm for 1 minute, and the supernatant was used for control staining. Goat and rat Abs were detected using Alexa Fluor 488 rabbit anti-goat IgG (H+L) (Invitrogen, Molecular Probes, Carlsbad, CA) and PE-conjugated donkey anti-rat IgG (H+L) (Jackson ImmunoResearch, West Grove, PA), respectively, mounted with Prolong Gold antifade reagent (Invitrogen, Molecular Probes), and viewed with a Zeiss Axioplan fluorescence microscopy. Images were acquired on a 600CL-CU cooled charge-coupled device video camera (Pixera, Los Gatos, CA) and were processed with InStudio 1.0.0 (Pixera).

Quantitative RT-PCR

Total mRNA was extracted from the mice ears with the SVTotal RNA Isolation system (Promega, Madison, WI) according to the manufacturer's protocol. Target gene expression was quantified in a two-step RT-PCR. cDNA was reverse transcribed from total RNA samples using the TaqMan RT reagents (Applied Biosystems, Foster City, CA). Murine CXCL12 (Assay ID: Mm00445552_m1) expression was quantified using TaqMan Gene Expression Assay (Applied Biosystems) in the ABI PRISM 7000 sequence detection system (Applied Biosystems). As an endogenous reference for these PCR quantification studies, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene expression was measured using the TaqMan rodent GAPDH control reagents (Applied Biosystems). The relative expression was calculated using the $2^{-\Delta\Delta CT}$ method.[25] The expression of the target gene normalized to an endogenous reference and relative to calibrator is given by the formula $2^{-\Delta\Delta CT}$. Gene expression in untreated mice was used as a calibrator expression to calculate $\Delta\Delta C_T$.

Chemotaxis Assay and FITC-Induced Cutaneous DC Migration

Cells were tested for transmigration across uncoated 5-μm Transwell filters (Corning Costar Corp., Corning, NY) for 3 hours to CXCL12, CCL21 (R&D Systems), or medium in the upper or lower chamber and were enumerated by flow cytometry.[26] For FITC-induced cutaneous DC migration, mice were painted on the shaved abdomen with 200 μl of 2% FITC (Sigma) dissolved in a 1:1 (v/v) acetone/dibutyl phthalate (Sigma) mixture, and the number of migrated cutaneous DCs into draining inguinal and axillary lymph nodes was enumerated by flow cytometry. In some experiments, mice ears were painted with 20 μl of 0.5% FITC, and draining cervical lymph nodes were analyzed as above.

2,4-Dinitro-1-Fluorobenzene (DNFB)-Induced CHS Model

For CHS model, B6 mice were immunized by application of 25 μl of 0.5% DNFB in 4:1 (v/v) acetone/olive oil to their shaved abdomens on day 0. They were challenged on the right ear on day 5 with 20 μl of 0.3% (w/v) DNFB.[27] Ear thickness was measured before and 24 hours after challenge to assess inflammation. For treatment with CXCR4 antagonist 4F-benzoyl-TE14003, the compound was administered during the sensitization period (from 1 day before DNFB sensitization to 3 days after DNFB sensitization), elicitation period (from 1 day before challenge to 1 day after challenge), or both periods.

For 2,4-dinitrobenzene sulfonic acid (DNBS)-dependent *in vitro* proliferation of lymph node cells, cells were prepared from draining axillary and inguinal lymph nodes 5 days after the DNFB sensitization on the abdomen. CXCR4 antagonist was implanted subcutaneously to the backside of the skin from 1 day before DNFB sensitization to 5 days after. Cells (4×10^5) were cultured for 3 days with DNBS (50 μg/ml), a water-soluble compound with the same antigenicity as DNFB, and were pulsed with 1 μCi of [^{3}H]thymidine for the last 24 hours of culture.

Statistical Analysis

Data were analyzed using an unpaired two-tailed *t*-test. A P value of less than 0.05 was considered to be significant.

Results

CXCR4 Expression in Cutaneous DCs

Initially, we evaluated the expression levels of CXCR4 on migrated cutaneous DCs and resident DCs in the regional lymph nodes of mice by flow cytometry. FITC, known to induce DC maturation and mobilization,[28,29] was painted on the shaved abdomen, and the regional lymph node cells were isolated 24 hours later. After Fc receptor blocking with CD16/32 Ab (BD Biosciences) for 30 minutes, cells were incubated with PE-labeled CXCR4 or isotype-matched control Abs. Significant amounts of CXCR4 were detected in the MHC class II$^+$ DCs, and among them, the FITC$^+$ migrated cutaneous DC subset expressed a higher level of CXCR4 than the FITC$^-$ resi-

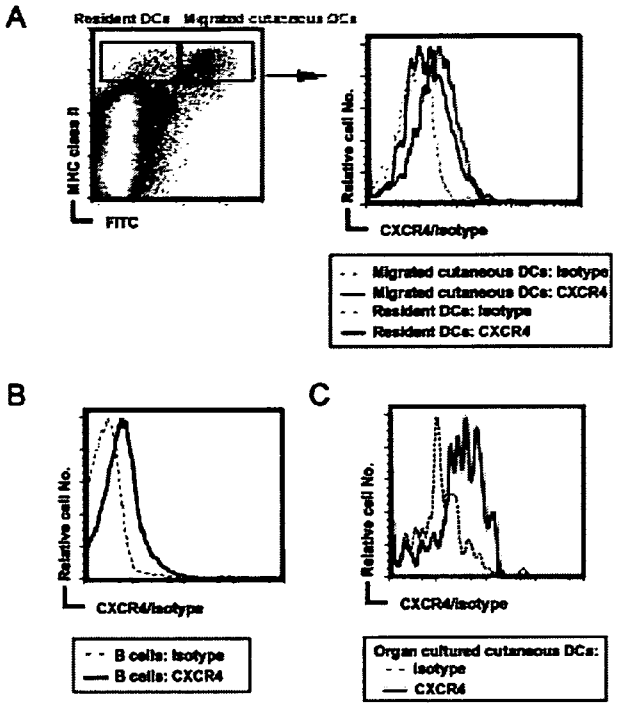

Figure 1. CXCR4 expression on resident DCs and migrated cutaneous DCs in lymph nodes. **A** and **B:** Draining lymph node cells were prepared from mice 24 hours after FITC painting on the abdomen. The profiles show flow cytometric analysis of the cells with the indicated markers. MHC class II^+ DCs were subdivided into $FITC^+$-migrated cutaneous DCs and $FITC^-$ resident DCs. The profiles show histograms of CXCR4 expression on MHC class II^+ $FITC^+$-migrated cutaneous DCs and MHC class II^+ $FITC^-$ resident DCs (**A**) and $B220^+$ B cells (**B**). Data are a representative of three independent experiments. **C:** Skin organ explants from the ears of the mice were incubated for 24 hours, and the expression of CXCR4 on the emigrated MHC class II^+ $CD11c^+$ cutaneous DCs was examined. Data are a representative of three independent experiments. As control, rat IgG2a isotype-matched control was used (**A–C**).

dent DC subset (Figure 1A). As a comparison, we monitored the expression level of CXCR4 in $B220^+$ B cells where CXCR4 was also expressed (Figure 1B). It is worth noting that the level of CXCR4 expression on the migrated cutaneous DCs was comparable or even higher than that on B cells. Then, we performed a skin explant culture assay and analyzed the cells that migrated into the culture medium 24 hours after incubation. We found that MHC class II^+ $CD11c^+$ cutaneous DCs were already positive for CXCR4 (Figure 1C), suggesting the precedent up-regulation of CXCR4 on cutaneous DCs in the skin, where DCs are ready to migrate toward lymphatic vessels.

CXCL12 Responsiveness of Resident and Migrated DCs

To assess the chemotactic activity of resident and migrated cutaneous DCs to CXCL12, we prepared draining lymph node cells 24 hours after FITC application and applied them on chemotaxis assay using transwells. Both $FITC^-$ MHC class II^+ resident DCs and $FITC^+$ MHC class II^+ migrated DCs showed chemotactic response to CXCL12 in a dose-dependent manner (Figure 2A). The response was more pronounced in $FITC^+$ MHC class

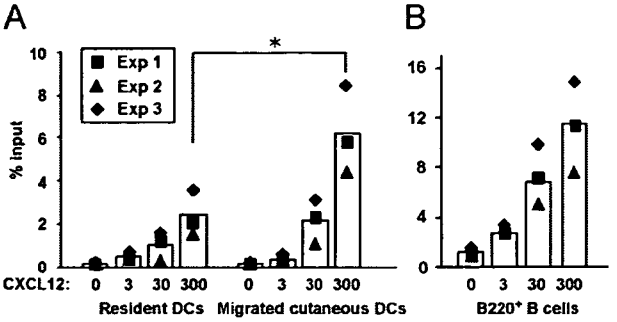

Figure 2. Chemotactic responses of resident DCs, migrated cutaneous DCs, and B cells to CXCL12. **A** and **B:** Draining lymph node cells were prepared from mice 24 hours after FITC application on the abdomen. Input cells and cells that migrated to the lower well of a transwell chamber in the absence of CXCL12 (0) or in response to 3, 30, or 300 ng/ml CXCL12 were analyzed by flow cytometry to detect MHC class II^+ $FITC^+$-migrated cutaneous DCs, MHC class II^+ $FITC^-$ resident DCs (**A**), and $B220^+$ B cells (**B**). **Filled symbols** indicate three independent experiments, and columns represent the average. A Student's *t*-test was performed between the indicated groups, and an **asterisk** indicates $P < 0.05$.

II^+-migrated DCs (Figure 2A). As a positive control, the chemotaxis test of $B220^+$ B cells to CXCL12 was simultaneously performed in parallel with DCs (Figure 2B).

CXCL12 and CCL21 Responsiveness of Epidermal LCs

To evaluate whether CXCL12-CXCR4 interactions could serve as an optional backup to CCL21-CCR7 interactions or coordinated interplay between them, we examined the chemotactic activity of LCs to CXCL12 and CCL21. Epidermal cell suspensions were incubated in cRPMI for 24 hours and applied to transwells with or without CXCL12, CCL21, or both in combination of the upper and lower chambers. The migrated epidermal LCs were identified as MHC class II^+ cells in the lower chamber. When CXCL12 or CCL21 was added to the lower chamber, LCs had a good chemotactic response to either of them, but the additional effect was not observed with CXCL12 or CCL21 combinatorially administered to the lower chamber (Figure 3). Interestingly, when CCL21 was added to the upper chamber, the chemotactic response to CXCL12 was significantly abrogated, but such an effect was not observed in the chemotaxis to CCL21 with CXCL12 added to the upper chamber (Figure 3). These data suggest that CXCL12-CXCR4 interactions can interplay coordinately with CCL21-CCR7 interactions and implicate that when CXCL12 and CCL21 coexist, LCs preferentially migrate into CCL21-producing sites.

In addition, the finding that CCL21 added to the upper chamber abrogated the chemotactic response of LCs to CXCL12 and CXCL12 added to the upper chamber unaffected the response to CCL21 raised a possibility that CCL21 down-regulates the expression of CXCR4 and CXCL12 does not affect CCR7 expression. We thus stained MHC class II^+ LC in the epidermal cell suspensions for CXCR4 and CCR7 5 hours after incubation with CCL21 or CXCL12. The treatments, however, did not alter the chemokine receptor expression levels at all (data not shown).

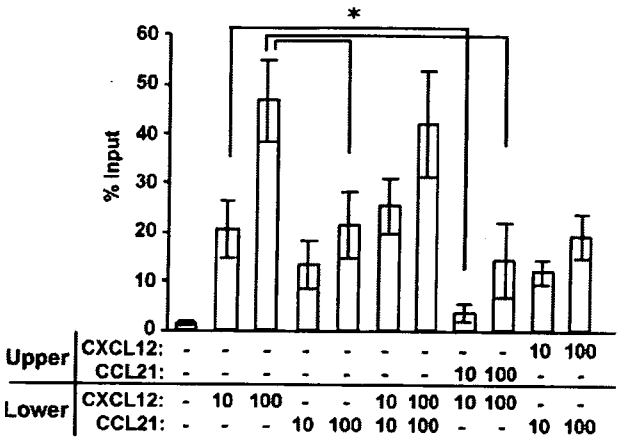

Figure 3. Epidermal LCs chemotactic activity to CXCL12 and CCL21. Epidermal cell suspensions (1×10^6) were incubated in cRPMI for 24 hours and applied to a transwell. Ten or 100 ng/ml CXCL12, CCL21, or CXCL12 and CCL21 in combination were administered to the upper or lower chamber. Migrated epidermal LCs were identified as MHC class II^+ subset in the lower chamber. The % input was calculated as follows: (the number of LCs migrated into the lower chamber)/(the number of LCs applied into the upper chamber) × 100. Columns show the mean ± SD from three independent experiments. Student's *t*-test was performed between the indicated groups, and an **asterisk** indicates $P < 0.05$.

Localization of CXCL12 in the Skin

It has been demonstrated that CXCL12 is expressed in the medullary cords of regional lymph nodes and human skin endothelial cells,[14–16] and its expression level is increased by skin wounding.[16] However, the role of CXCL12 in the context of antigen exposure remains unknown. We performed an immunohistochemical analysis on CXCL12 expression in the mouse skin and detected a significant amount of CXCL12 signal in the dermis 24 hours after epicutaneous immunization with DNFB (Figure 4A, right top). In addition, we observed that the CXCL12-expressing cells were tightly associated with LYVE-1^+ lymphatic vessels, whereas CXCL12-expressing cells were sparse in other areas of the skin (Figure 4A, right middle and bottom). The specificity of this staining was confirmed by the blocking peptide treatment or isotype-matched Ab staining (Figure 4A, left). On the other hand, the expression level of CXCL12 was less significant in the steady state (data not shown).

We then examined CXCL12 mRNA levels in the skin of ears treated with 20 μl of 0.2% DNFB ears for 6, 12, 24, and 48 hours. The intensities of CXCL12 probes were normalized against GAPDH as an endogenous control. The amount of CXCL12 mRNA in the DNFB-treated skin was expressed as the mean relative to that in non-DNFB-treated skin using the ΔΔCT method. Its expression was induced 6, 12, 24, and 48 hours after hapten application with peak expression at 12 to 24 hours (Figure 4B).

Impairment of Cutaneous DC Accumulation in Regional Lymph Nodes by CXCR4 Antagonist Treatment

To investigate the functions and *in vivo* significance of CXCR4 in cutaneous DCs, we performed FITC-induced

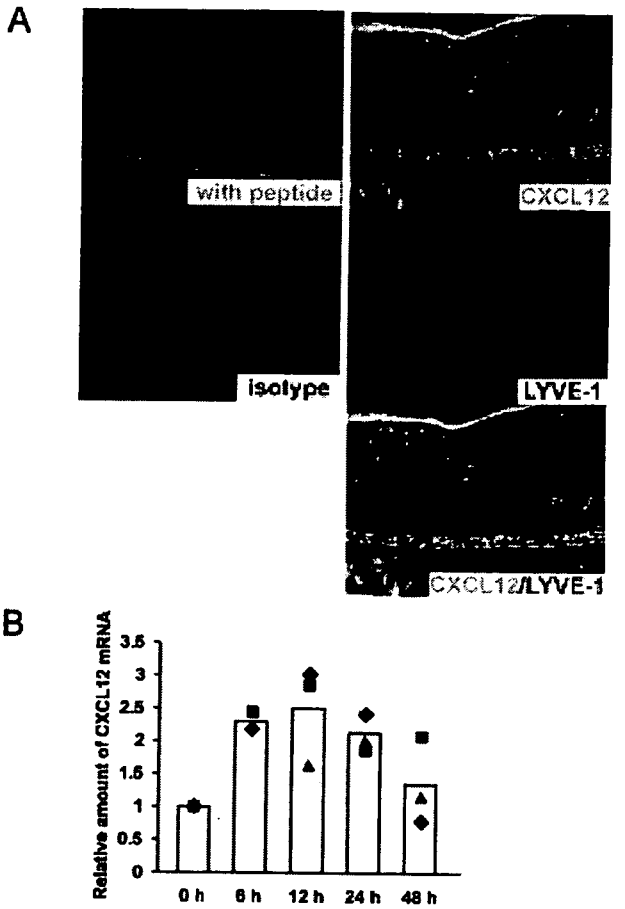

Figure 4. CXCL12 expression in lymphatic vessels of mouse skin. **A:** Skin sections from ears of mice treated with DNFB 24 hours prior were stained with goat anti-CXCL12 Ab with or without blocking peptide, and rat anti-LYVE-1 Ab or isotype control Ab, and sequentially immersed with Alexa Fluor 488 rabbit anti-goat IgG and PE-conjugated donkey anti-rat IgG, respectively (the labels are the same color as the reaction product). **B:** The ears of mice treated with 20 μl of 0.3% DNFB for 6, 12, 24, and 48 hours were isolated. The levels of CXCL12 mRNA were normalized against GAPDH as an endogenous control. The CXCL12 mRNA amounts in the skin from DNFB-treated mice relative to that from non-DNFB-treated mice (0 hours) were induced 6, 12, and 24 hours after hapten application. **Filled symbols** indicate three independent experiments, and columns represent the average.

cutaneous DC migration assay. FITC applied to the skin is taken up by cutaneous DCs, which then migrate to the draining lymph nodes as FITC$^+$ MHC class II^+ cells.[23] We isolated cervical lymph node cells 24 hours after FITC application to ears and characterized FITC$^+$ MHC class II^+ cutaneous DCs (Figure 5A) therein by flow cytometry. Among FITC$^+$ MHC class II^+ cutaneous DCs, two subsets, R1 ($CD11c^{high+}$ $CD11b^{high+}$ and $CD11c^{low+}$ $CD11b^{int+}$) and R2 ($CD11c^{high+}$ $CD11b^{int+}$), were detected when they were stained with CD11c and CD11b (Figure 5B). In these populations, only the R2 subset expressed Langerin, a marker for LCs (Figure 5C). Therefore, the R2 subset originates from LCs, and the R1 subset is from dDCs. It has been shown that after FITC painting on the skin, a rapid influx of FITC$^+$ cutaneous DCs into the draining lymph nodes occurs at a peak time of 24 hours, and FITC-labeled DCs remain elevated in number for another 2 days and then decline to the normal level by day 6.[30] We injected CXCR4 antagonist subcu-

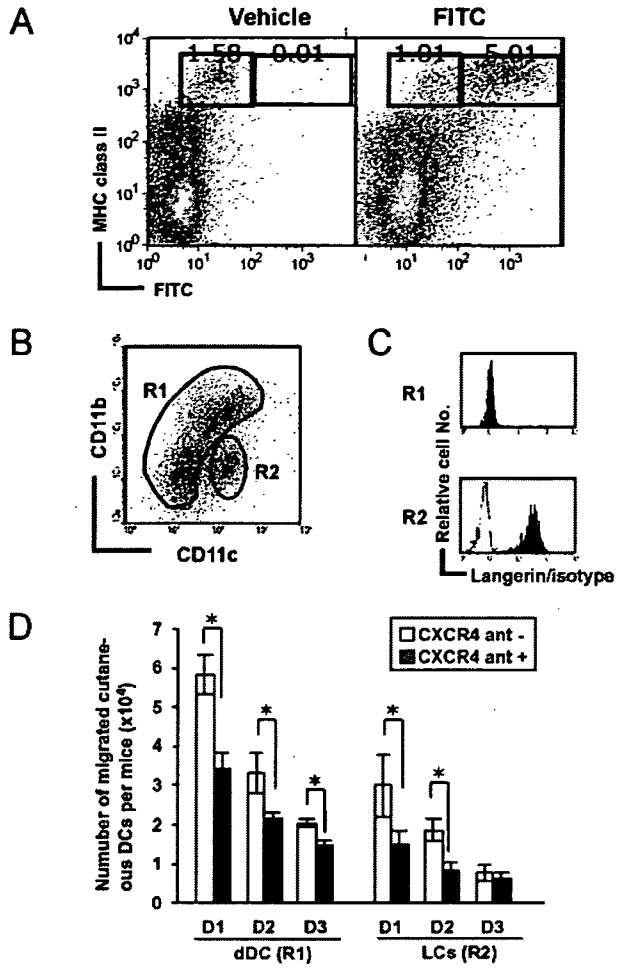

Figure 5. Impaired cutaneous DC accumulation in regional lymph nodes by CXCR4 antagonist. **A:** Flow cytometric analysis of MHC class II expression and FITC fluorescence in cells derived from the regional lymph nodes 24 hours after the application of 200 μl of 2% FITC or vehicle. **B:** Cervical lymph node cells were prepared from mice 24 hours after 10 μl of 0.5% FITC painting on the ears. Among $FITC^+$ cutaneous DCs, two subsets, R1 ($CD11c^{high+}$ $CD11b^{high+}$ and $CD11c^{low+}CD11b^{int+}$) and R2 ($CD11c^{high+}$ $CD11b^{int+}$) were identified. **C:** The histogram of Langerin is shown in each subset. Note that only R2 subset expresses LC marker Langerin. **D:** The numbers of migrated dermal DCs (dDCs) and LCs 24, 48, and 72 hours after FITC painting are calculated. Columns show the mean ± SD from at least four mice per group. 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.

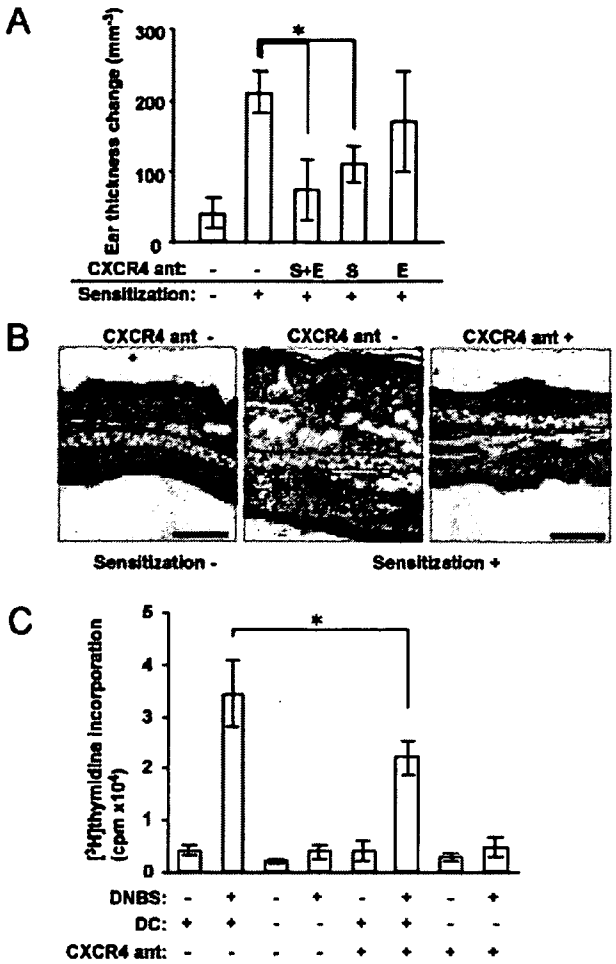

Figure 6. Perturbed initiation of skin immune response by blockade of CXCR4 engagement. **A:** Effect of CXCR4 antagonist on CHS response to DNFB. The ear thickness of DNFB-sensitized mice treated with either vehicle alone (−) or CXCR4 antagonist during sensitization (S), elicitation (E), or both (S+E) was measured after challenge with DNFB. *$P < 0.05$ versus vehicle-treated mice ($n = 6$ per group). **B:** H&E staining of ears from mice treated with ($CXCR4$ ant^+) or without (CXCR4 ant-) CXCR4 antagonist 24 hours after challenge with DNFB. Scale bar = 100 μm. **C:** The proliferative response of DNFB-immune lymphocytes to DNBS. The lymph node cells from mice sensitized by DNFB with or without CXCR4 antagonist (CXCR4 ant) treatment were stimulated with DNBS for 72 hours. Lymph node cells eliminated by $CD11c^+$ DCs were also prepared using autoMACS. The proliferative response was measured in triplicate. Columns show the mean ± SD from triplicate wells. Student's *t*-test was performed between the indicated groups, and an **asterisk** indicates $P < 0.05$. Results are representative of three independent experiments. cpm, counts per minute.

taneously into FITC-treated mice. The numbers of accumulated LCs and dDCs (represented by R2 and R1, respectively, in Figure 5B) 24 and 48 hours after FITC application was significantly reduced (Figure 5D). Therefore, loss of CXCL12-CXCR4 signaling resulted in impaired lymph node accumulation of cutaneous DCs in response to antigen exposure to the skin.

Perturbed Initiation of CHS by CXCR4 Antagonist

The relevance of the observed CXCR4-mediated regulation of cutaneous DC function to *in vivo* immune responses is a matter to be clarified. With CHS as an *in vivo* model, we investigated whether inhibition of CXCR4 signaling affected an immune response to an exogenous antigen. B6 mice were sensitized by DNFB as a hapten to the abdomen. In mice treated with CXCR4 antagonist, challenge of ears 5 days later disclosed a significant ($P < 0.05$) decrease in ear swelling compared with the nontreated control mice (Figure 6A). A histological analysis of the nontreated mice revealed pronounced spongiosis and extensive infiltration of lymphocytes in the edematous dermis, whereas the extent of such changes was markedly reduced in mice treated with CXCR4 antagonist (Figure 6B). We next administered CXCR4 antagonist either throughout the experimental protocol or selectively during the sensitization or elicitation period. Treatment with CXCR4 antagonist during the sensitization phase,

but not the elicitation phase, resulted in a significant ($P < 0.05$) decrease of ear swelling. The extent of the former's inhibition was comparable to that of the administration of the antagonist throughout CHS period (Figure 6A). These results indicated that CXCL12-CXCR4 signaling is important in the priming of T cells, which is in accordance with the notion that cutaneous DCs play a pivotal role in sensitization. We also isolated the regional lymph node cells 5 days after DNFB sensitization and examined the responsiveness of immune T cells to DNBS, a water-soluble compound with the same antigenicity as DNFB, in the presence or absence of recombinant murine CXCL12. The proliferative response of cells was significantly enhanced by the addition of DNBS, but when $CD11c^+$ cells were depleted, this proliferative response was markedly attenuated (Figure 6C), suggesting that this response is dependent on DCs. Moreover, this DNBS-induced proliferative response was inhibited by CXCR4 antagonist treatment (Figure 6C). However, such an antiproliferative effect by CXCR4 antagonist was not shown when 2×10^5 $CD4^+$ cells were stimulated with 10 ng/ml phorbol myristate acetate (Sigma Chemical) in combination with 1 μmol/L ionomycin (Wako, Osaka, Japan), which are independent of DC function (data not shown). This is interpreted as an indication that CXCL12-CXCR4 interactions are important for initiation of skin immune response by acting cutaneous DC.

Discussion

The above findings demonstrated that CXCR4 was highly expressed on cutaneous DCs, and its ligand CXCL12 was produced by the lymphatic vessels of the skin after antigen exposure. Cutaneous DCs in the regional lymph nodes had a stronger chemotactic response to CXCL12 than resident DCs, suggesting that activated DCs are attracted to the lymphatic vessels by virtue of CXCR4 and CXCL12. Consistently, FITC-induced DC migration into the lymph nodes was partially but substantially impaired by CXCR4 signaling blockade. These results suggest that CXCL12-CXCR4 interaction is important for the migration of cutaneous DCs.

Accumulating evidence has shown an essential role for chemokine system in the migration of cutaneous DCs and the maintenance of the microanatomic environment of secondary lymphoid organs.[10,11] It is well known that the migration of DCs from peripheral tissues into lymphatic vessels requires CCR7 and most likely occurs in response to CCL21, which is released from lymphatic endothelium and lymph nodes.[3,6] In contrast to CCL21 expression in lymphoid organs, its expression in lymphatic vessels is largely independent of lymphotoxin $\alpha 1\beta 2$, which is also important for DC homeostasis in the secondary lymphoid organs.[24,31,32] Thus, the lymphatic vessel seems to have some specialized system of chemokine production. In addition, complete migratory inhibition has not been achieved in CCR7-deficient mice, suggesting that there exist other factors than CCR7. One candidate is CCR2, which has already been implicated in DC homing from the skin to the lymph nodes, although the precise mechanism is unclear.[33] On the other hand, DCs have a chemotactic response to CXCL12, but its *in vivo* significance in cutaneous DC migration has not been elucidated.[12] The present study revealed that CXCL12 and CXCR4 play a key role for cutaneous DC migration into the draining lymph nodes *in vivo*.

The present study cannot clarify the respective characteristic role of CCR7 and CXCR4 in the *in vivo* migration of dDCs and LCs. This should be addressed in future studies using CCR7-deficient mice treated with or without CXCR4 antagonist. From our *in vivo* study, the extent of impairment of cutaneous DC migration into regional lymph nodes is more significant in CCR7-deficient mice[10] than in mice treated with CXCR4 antagonist. However, epidermal LCs had better chemotactic activity to CXCL12 rather than CCL21. Moreover, no additional effect was observed when both chemokines were added to the lower chamber. These data suggest that CXCL12-CXCR4 interactions interplay coordinately with CCL21-CCR7 interactions rather than backup optionally for CCL21-CCR7 signaling. Moreover, the chemotactic activity to CXCL12 was completely abrogated with CCL21 added to the upper chamber, whereas the activity to CXCL12 retained when CCL21 was added to the upper chamber. These results suggest that when both chemokines coexist, LCs preferentially migrate to CCL21-producing sites.

On the other hand, recent studies have revealed that CCR7 expression level or signaling sensitivity can be modulated by several factors,[34,35] raising a possibility that signaling via one chemokine receptor affects another receptor signaling. However, the level of CCR7 expression on LCs was not changed by CXCR4 antagonist when epidermal cell suspensions were incubated with this blocker for 24 hours in the setting of culturing without FCS as previously reported[36] (Supplemental Figure 1; see *http//ajp.amjpathol.org*).

Because of their location in the epidermis, LCs have previously been considered to initiate and control skin immune responses. For example, in a model of allogeneic graft-*versus*-host disease, LCs are sufficient for the development of cutaneous immune response.[37] However, this concept has been challenged by the findings that dDCs, but not LCs, initiate protective T-cell responses to certain epidermal viral antigens.[38] Recently, three groups of investigators have independently generated LC-deficient mice, and each type of the modified mice showed different manifestations, as they exhibited impaired,[39] not affected,[40] and enhanced CHS responses.[41] Different cutaneous DC subsets may play their own roles in the generation and regulation of immune responses.[42] In our experiments, both the cutaneous DC migration and the CHS response were partially but significantly impaired by CXCR4 blockade. Moreover, there was no difference in the antagonist inhibition of migratory activity between LCs and dDCs. Therefore, CXCR4 usage in these cell types seems to be virtually the same, and we could not further address the respective roles of LCs and dDCs in our system.

In this investigation, it was suggested that CXCL12 was expressed in the lymphatic vessels and engaged with CXCR4 on cutaneous DCs to change their function.

It should be noted that CXCR4 was already expressed in the skin when we did the organ skin culture assay and examined the expression level of CXCR4 on migrated cutaneous DCs in the culture medium 24 hours after incubation (data not shown), suggesting that CXCR4 on skin DCs is already up-regulated in the skin, where it is needed to migrate toward lymphatic vessels. On the other hand, it is known that human DCs themselves express CXCL12, suggesting that DCs attract naive T cells.[1,43,44] Rolling B cells can be induced to arrest in high endothelial venules either by CCR7 agonists or by CXCL12.[45] These findings suggest that the CXCL12-CXCR4 interaction may play critical roles in lymphocytic circulation and immune surveillance in the postnatal life. Although the number and localization of T and B cells in the regional lymph nodes were not apparently affected by CXCR4 antagonist (data not shown), this cannot negate the possibility that cells other than DCs are involved in this process. A study using mice with conditional CXCR4 depletion on DCs might clarify this issue in future.

Interactions between CXCL12 and CXCR4 are largely unique and nonpromiscuous. In mice, CXCL12 or CXCR4 gene knockouts generate a similar phenotype, and embryonic lethality associated with either CXCR4 or CXCL12 gene knockouts emphasizes the critical and unique role played by their products during development.[17–19] Because it has not been established that LCs are reconstituted efficiently in fetal liver chimeric mice, the CXCR4 antagonist is a useful chemical probe to evaluate the role of CXCR4 on cutaneous DC function. CXCR4 was considered to be the single receptor for CXCL12, but another chemokine receptor, CXCR7, has recently been identified.[46] CXCR7 is expressed in tumor cell lines, activated endothelial cells, and fetal liver cells. At present, CXCR7 was not found in immune cells, but we cannot exclude the possibility that CXCR7 may be involved in DC function. In human skin inflammatory diseases, CXCL12-positive cells, including endothelial cells and pericytes of adult small capillary blood vessels, are colocalized with CXCR4-positive inflammatory cells, such as DCs,[14] suggesting that CXCL12-CXCR4 interaction may be involved not only in mice but also in the formation of human skin immune response. Understanding of factors that determine cutaneous DC trafficking and function might offer new opportunities for therapeutic intervention to suppress or stimulate the immune response.

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CXCR4 engagement promotes dendritic cell survival and maturation

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Abstract

It has been reported that human monocyte derived-dendritic cells (DCs) express CXCR4, responsible for chemotaxis to CXCL12. However, it remains unknown whether CXCR4 is involved in other functions of DCs. Initially, we found that CXCR4 was expressed on bone marrow-derived DCs (BMDCs). The addition of specific CXCR4 antagonist, 4-F-Benzoyl-TN14003, to the culture of mouse BMDCs decreased their number, especially the mature subset of them. The similar effect was found on the number of Langerhans cells (LCs) but not keratinocytes among epidermal cell suspensions. Since LCs are incapable of proliferating in vitro, these results indicate that CXCR4 engagement is important for not only maturation but also survival of DCs. Consistently, the dinitrobenzene sulfonic acid-induced, antigen-specific in vitro proliferation of previously sensitized lymph node cells was enhanced by CXCL12, and suppressed by CXCR4 antagonist. These findings suggest that CXCL12–CXCR4 engagement enhances DC maturation and survival to initiate acquired immune response.

Keywords: Langerhans cell; Dendritic cell; Maturation; Survival; Antagonist; CXCR4; CXCL12; Proliferation

Dendritic cells (DCs) are potent antigen-presenting cells in the immune system especially for T cell activation and maturation [1,2]. It is well established that DC maturation is induced by cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, pathogens, lipopolysaccharide (LPS), and CD40 ligand [1]. Chemokines were originally known as chemoattractant, but they have currently been evaluated as one of the important candidates for modulators of DC functions [3].

It was reported that the maturation of human monocyte-derived DCs by LPS, TNF-α or CD40L resulted in enhanced expression of CCR7 and CXCR4 [4]. Although the roles of CCR7 on DCs have been well characterized [3], those of CXCR4 remain largely unknown except that CXCR4 signaling promotes chemotaxis to its ligand, CXCL12 (stromal-cell derived factor-1; SDF-1α) in vitro and in vivo [5–7].

In light of the emerging significance of chemokine systems in DC biology, we examined the hypothesis that the CXCL12–CXCR4 engagement influences DC functions as well as chemotaxis. We found that CXCR4 was expressed on murine bone marrow-derived DCs (BMDCs) and epidermal Langerhans cells (LCs), that CXCL12 was produced by BMDCs, and that CXCR4 signaling promotes DC maturation and survival.

Materials and methods

Animals and reagent. Eight weeks old female C57BL/6 (B6) mice were purchased from Japan SLC (Hamamatsu, Japan) and maintained on a 12-h light/dark cycle under specific pathogen-free conditions. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Occupational and Environmental Health.

For CXCR4 antagonist treatment, 4F-Benzoyl-TN14003 was used as CXCR4 antagonist [8,9]. No toxicity of CXCR4 antagonist was observed at 5 μM as reported previously [10], and the selectivity of the antagonist

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was confirmed by the absence of significant inhibition against Ca^{2+} mobilization induced by MIP-1α stimulation through CCR5 ($IC_{50} = 22$ μM) and against Ca^{2+} mobilization induced by sphingosine-1-phosphate stimulation through EDG3 ($IC_{50} > 30$ μM) by the treatment of CXCR4 antagonist (data not shown).

Cell preparation and cultures. RPMI-1640 (Sigma, St. Louis, MO) containing 10% heat-inactivated fetal calf serum (Invitrogen, Carlsbad, CA), 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 25 mM Hepes (Cellgro, Herndon, VA), 1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin was used as culture medium otherwise stated. BMDC culture was performed as described [11]. In brief, 5×10^6 BM cells were cultured in 10 cm tissue culture dishes in 10 ml of medium supplemented with 10 ng/ml recombinant murine GM-CSF (PeproTech, Rocky Hill, NJ) for 5 days. Loosely adherent cells were harvested at day 5 and incubated at 1×10^6/ml with or without CXCR4 antagonist in the presence or absence of GM-CSF for another 2 days. The CXCL12 amounts in the culture supernatants were measured with an ELISA kit (R&D systems, Minneapolis, MN) as manufacturer's protocol. Epidermal cell suspensions were obtained from the earlobes of mice with trypsin treatment and cultured without FCS for 48 h [12,13].

Flow cytometry. For flow cytometry, cells were prepared and analyzed with FACSCanto (BD Biosciences) and FlowJo software (TreeStar, San Carlos, CA) [14]. Antibodies (Abs) used were: phycoerythrin (PE)-conjugated anti-CXCR4 (2B11; BD Biosciences) and isotype matched control IgGs, FITC-conjugated anti CD54 Ab, PE-conjugated anti C86 Ab, PE-Cy5-conjugated anti-MHC class II Ab, and allophycocyanin (APC)-conjugated anti-CD11c Ab (all from BD Biosciences).

Hapten specific T cell proliferation model. For 2,4-dinitrobenzene sulfonic acid (DNBS)-dependent in vitro T cell proliferation, cells were prepared from draining axillary and inguinal lymph nodes of mice 5 days after sensitization on abdomen with 25 μl of 0.5% 2,4-dinitro-1-fluorobenzene (DNFB) in 4:1 (v/v) acetone/olive oil. Cells (4×10^5/well of 96 well plates) were cultured for 3 days with DNBS (50 μg/ml), a water-soluble compound with the same antigenicity as DNFB, in the presence or absence of CXCL12 (R&D systems) and were pulsed with 1 μCi ^3H thymidine for the last 24 h of culture.

Statistical analysis. Data were analyzed using an unpaired two-tailed *t*-test. *P* value of less than 0.05 was considered to be significant.

Results

CXCR4 expression in BMDCs and LCs

Initially, we evaluated the expression levels of CXCR4 on BMDCs by flow cytometry. BM cells were incubated in the culture medium with GM-CSF for 5 days. Significant amounts of CXCR4 were detected in the $CD11c^+$ BMDCs, but $CD11c^-$ fraction expressed CXCR4 to a much lesser degree (Fig. 1A). We then compared the expression level of LCs. Among epidermal cell suspensions, CXCR4 was expressed on MHC class II^+ epidermal LCs but merely barely detected on MHC class II^- KCs (Fig. 1B).

Reduction of BMDC and LC numbers by CXCR4 antagonist treatment

To address whether CXCR4 signaling is involved in the functions of DCs, we added CXCR4 antagonist to BMDC cultures. Two-day treatment with this antagonist significantly decreased the numbers of both mature $CD11c^+$ MHC class II^{high+} DCs and immature $CD11c^+$ MHC class II^{low+} DCs (Fig. 2A and B). Moreover, CXCR4 antagonist suppressed the number of the mature subset of DCs more markedly than that of the immature subset (Fig. 2C). These

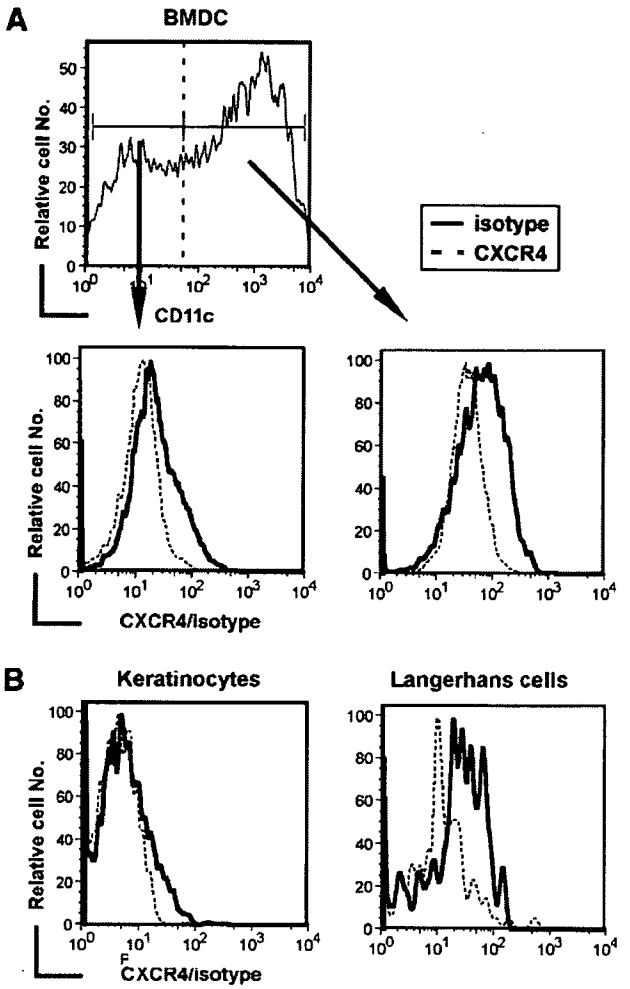

Fig. 1. CXCR4 expression on BMDCs and LCs. (A) BMDCs were prepared after 5 day culture of BM cells with GM-CSF. The expression of CXCR4 on $CD11c^+$ BMDCs and $CD11c^-$ non-DC fraction was evaluated. (B) Epidermal cell suspensions were prepared, and MHC class II^+ LCs and MHC class II^- keratinocytes were evaluated for CXCR4 expression. The profiles show flow cytometric analysis of the cells with the indicated markers, and as a control, rat IgG2a isotype-matched control was used.

results indicated that CXCR4 engagement promoted the maturation, and survival and/or proliferation of DCs. 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 (8.8 ± 3.6 ng/ml, $n = 3$) after BMDC incubation.

We then prepared epidermal cell suspensions from mouse earlobes and cultured them for 2 days. CXCR4 antagonist reduced the number of both $CD11c^+$ MHC class II^{high+} mature LCs and $CD11c^+$ MHC class II^{int+} immature LCs (Fig. 3A). It was reported that epidermal LCs are unable to proliferate in vitro when they are incubated as epidermal cell suspension [16]. Our results together with the previous report suggest that CXCR4 signaling promotes the survival of LCs. Moreover, we examined the expression of other co-stimulatory molecules and