induced lymphocyte stimulation testing was tried, but it was difficult to evaluate its antigenicity, since lymphocytes were incapable of surviving during culture with cisplatin. A tentative diagnosis of drug eruption was made; she stopped taking cisplatin and her eruption was completely resolved in a week without any treatment.

A biopsy specimen from a palmar lesion revealed a perivascular mild infiltrate of lymphocytes and a few eosinophils in the dermis with mildly acanthotic epidermis. There were no epidermal necrotic cells or dermal inflammatory cells invading into the eccrine glands.

Although cisplatin is widely used for the treatment of various tumors, cisplatin-induced acral erythema has been reported in only four cases [2-4]. All patients developed symmetrical erythema of the palms and fingers and/or soles, and the eruptions were resolved by discontinuing cisplatin. However, there are clinical and histological differences among these cases. First, acral erythema occurred only one day after the initiation of chemotherapy in our case, while it appeared 3 months after the initiation in another patient [2]. Second, the lesional sites were slightly different between the cases. Third, the infiltrates consisted of lymphocytes [2], or neutrophils [4]. Finally, lichenoid or vesicular changes in the epidermis were present in two cases [2, 4] but not in our case.

The occurrence of CAE depends on various factors, which may contribute the typical localization to the palms and soles, including elevated drug concentration in eccrine glands, rapid cell proliferation, regional temperature gradient, gravitational forces, and vasculature anatomy [5]. Thus, it is likely that CAE is a toxic but not allergic reaction. Because the onset of CAE was soon after administration, our case also supports some toxic mechanism underlying CAE.

It may be noted that three out of the four reported cases are Japanese, and some ethnic genetic background might influence on the susceptibility of CAE to cisplatin. The polymorphisms for certain drug transporter genes, such as Multi drug resistance 1, might underlie the ethnic variation [6]. ■

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Successful treatment of cutaneous leishmaniasis by photodynamic therapy and cryotherapy

Cutaneous leishmaniasis (CL) sometimes brings a therapeutic problem. The treatments of Old World cutaneous leishmaniasis include cryotherapy [1], heat therapy, itraconazole [2], pentavalent antimony compounds, amphotericin B and other antimycotic drugs, topical and intralesional application of paramomycin, and in recent years also photodynamic therapy (PDT) [3-5]. Lesions of CL heal spontaneously over several months to 2-3 years, and therapy is not always essential. Nevertheless, treatment is indicated in spreading multiple lesions and when the lesion is located on the face and exposed areas of the extremities.

A 39-year-old man was referred to us with multiple lesions similar to furunculosis, which had occured 3 months previous without healing after topical steroid therapy. He was a keen diver and the lesions developed 4 weeks after his last return from vacation in Croatia. The examination revealed a healthy man with 9 slightly tender nodules and plaques of bright pink-reddish colour, sized 1 to 4 cm on the forearms, neck, and thigh. They periodically developed some blistering on the surface *(figure 1A)*. The diagnosis of cutaneous leishmaniasis was confirmed by a biopsy specimen. *Leishmania* bodies were present in the macrophages as well as extracellularily. No microorganisms were seen in PAS staining and in sections stained to show acid-fast organisms. A culture was not performed.

The following treatment was introduced: Five lesions were treated with PDT whereas the other 4 lesions were treated with cryotherapy. Before initiation of PDT, the crusts and scales were mechanically removed. Then, 20% ALA in gel (hydrochloride form) was applied on the lesions in a 1 mm thick layer. The areas were covered with an occlusive dressing for 3 hours after which the gel was washed off using 0.9 saline solution. Each lesion was illuminated with non-coherent red light with an emission spectrum of 580 to 680 nm (Medeikonos, Sweden), with a total light dose of 75 J/cm^2 and a light intensity of 88-123 mW/cm^2. The PDT procedure was repeated once a week for six-weeks.

The remaining 4 lesions were treated simultaneously with a hand-held liquid nitrogen spray unit. The liquid nitrogen was applied directly on the lesion from a distance of 2-3 cm for a freezing time of 30 seconds and a thaw of 60 seconds. Double freeze-thaw cycle treatment per session was used. The procedure was performed once a week for five weeks in total.

The lesions treated with the PDT method improved after 4 sessions and after 6 sessions they were healed with only minimal pigmentation and minor central scarring *(figure 1B)*. The lesions treated with cryotherapy healed after 5 applications with slight pigmentation and minor scar formation in the centre of the lesions, too *(figure 1C)*. No biopsies from clinically healed skin were performed after completion of the treatment. In a 12 month follow-up period no signs of reccurence occured.

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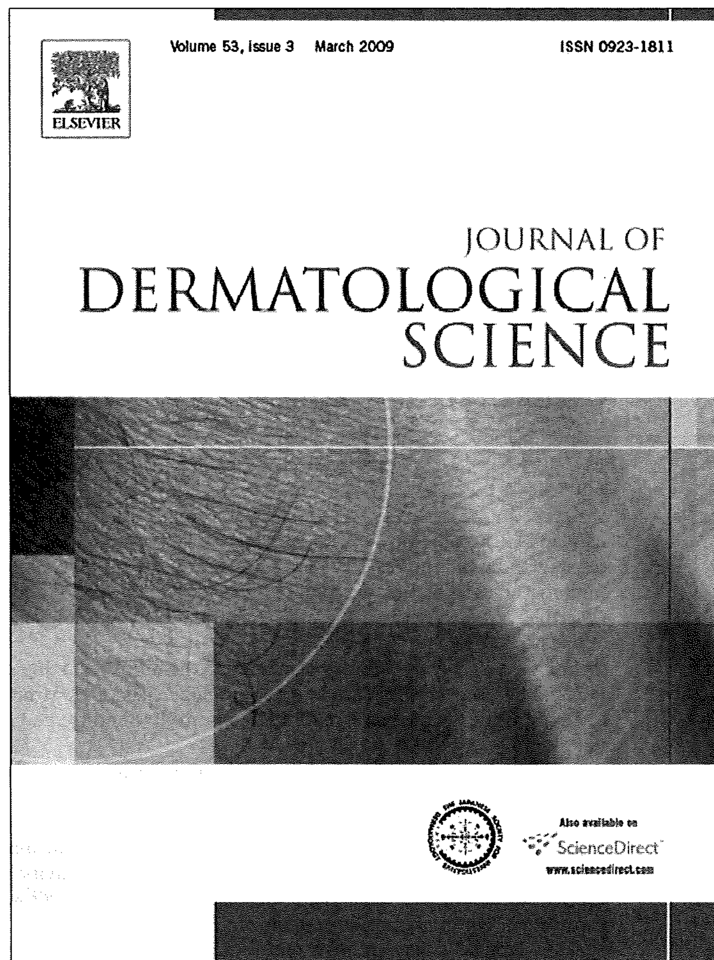

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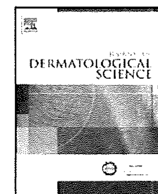

Skin application of ketoprofen systemically suppresses contact hypersensitivity by inducing CD4+ CD25+ regulatory T cells

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ABSTRACT

Background: Ketoprofen (KP) is a widely used nonsteroidal anti-inflammatory drug that inhibits prostaglandin biosynthesis. We have previously shown that topical KP treatment at the sensitizing site inhibits the development of contact hypersensitivity (CHS) to picryl chloride (PCl).
Objective: We investigated the mechanism underlying the KP-induced immunosuppression of CHS by application of KP.
Methods: We analyzed the CHS responses to the non-sensitizing site and subsequent sensitization with PCl, and by transfer of the draining lymph node cells (LNCs) from KP-tolerated mice to recipient mice. Changes in the Foxp3 expression of LNCs from KP-phototreated skin were also examined by real-time PCR.
Results: Topical application of KP to not only the sensitizing but also non-sensitizing site suppressed CHS response. The immunosuppression was transferred with LNCs from mice treated with PCl plus KP, but not from mice treated oxazolone plus KP. In this transfer study, the CD4+ CD25+ subset of LNCs exerted the suppressive effect, while CD25+ cell-depleted LNCs lost the inhibitory ability. CTLA-4 blocking with a specific antibody, but not IL-10 blocking, abrogated the activity of CD4+ CD25+ cells. Moreover, Foxp3 mRNA expression was remarkably increased in LNCs from PCl and KP-treated mice.
Conclusion: The immunosuppression of CHS by topical application of KP is systemic and hapten-specific. Treg cells play an important role in the suppressive effect by KP.
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1. Introduction

Contact hypersensitivity (CHS) is a delayed-type cutaneous reaction in which various immunocompetent cells, including epidermal Langerhans cells (LCs), dermal dendritic cells (dDCs), keratinocytes and T cells, and their cytokines and chemokines are involved [1,2]. Ketoprofen (KP) is a nonsteroidal anti-inflammatory drug (NSAID) that inhibits prostaglandin (PG) biosynthesis and widely used topical NSAID to remove pain. We have previously shown that *in vivo* application or *in vitro* addition of KP inhibits the maturation of LCs [3]. As a result, topical application of KP to the sensitizing site inhibits CHS responses to hapten. Aspirin, a representative NSAID, has an inhibitory effect on the *in vitro* maturation of LCs, but aspirin did not suppress CHS response to hapten. Thus, KP is unique in this suppressive ability, and in addition to the topical modulation of LCs, another mechanism might exist in KP-induced immunosuppression. In this respect, it remains unclear whether KP induces systemic or local immunosuppression and how KP application induces the immunological tolerance in CHS.

Regulatory T cells (Treg) prevent from harmful immune responses to self and nonself antigens in a dominant manner [4–7]. In mouse models, the responses of CHS are reduced by Treg cells-inducing treatments such as irradiation of skin with UVB, skin graft, and oral application of antigen. Treg cells can be classified into two major categories: thymus-derived natural Treg cells and those induced in the periphery [8,9]. The principal subset of natural Treg cells is CD4+ cells that constitutively express CD25 [10,11]. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is an important molecule that is expressed on the surface of Treg cells and exhibits the ability to ligate the B7 family molecules (CD80 and CD86) like CD28 [6,7]. Unlike CD28, however, cross-linking of CTLA-4 downregulates interleukin (IL)-2 production and cell cycle progression, and therefore, blockade of CTLA-4 reduces CD4+

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CD25$^+$ Treg function [12,13]. On the other hand, Foxp3, a member of the forkhead winged helix protein family of transcription factors, is an essential functional marker for Treg cells [14–16]. Retroviral transduction of Foxp3 to CD4$^+$ CD25$^-$ cells converts them into CD4$^+$ CD25$^+$ Treg-like cells with respect to phenotype and function. There are largely two kinds of suppression mechanisms by CD4$^+$ CD25$^+$ Treg cells, release of suppressive cytokines, such as IL-10, and cell-to-cell contact [11,17–20].

It has been reported that immature DCs induce Treg cells *in vivo* [21]. It was thought that the expression of indeolamine 2,3-dioxygenase activity by DCs have a broader immunological role in tolerance and immunoregulation [22]. In this mechanism, the production of IFN-γ from DCs is dependent on signaling from CTLA-4 related with STAT1, p38MAPK and NF-κB. However, DCs differentiated in the presence of a NF-κB inhibitor induce CD4$^+$ Treg cells producing IL-10 and cause antigen-specific tolerance [23]. Recently it has been reported that human DCs treated with aspirin, a major NSAID, have an inducible potential of allo-specific Treg cells. Furthermore, it is well known that salicylates including aspirin inhibit the NF-κB pathway [24,25]. The ability of KP to inhibit NF-κB [3] further predicts Treg cell induction by KP.

In this study, we demonstrate that the inhibitory effect of KP is systemic and hapten-specific. To further explore the mechanism underlying KP suppression, we highlight CD4$^+$ CD25$^+$ Treg cells, which exist in immune lymph nodes cells of mice treated with KP plus hapten.

2. Materials and methods

2.1. Mice

BALB/c mice were obtained from Kyudo Co., Ltd. (Kumamoto, Japan). Female mice, 8–11 weeks old, were used in this study.

2.2. Chemicals and monoclonal antibodies (mAbs)

KP was obtained from Hisamitsu Pharmaceutical Co., Inc. (Tokyo, Japan). Picryl chloride (PCl) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Oxazolone (OX) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Intracellular Foxp3 staining buffer set was obtained from eBioscience (San Diego, CA). Anti-mouse CTLA-4 (UC10-4F10) monoclonal antibody (mAb), phycoerythrin (PE) or PE-cy7-labeled anti-mouse CD25 (PC61) mAb, fluorescein isothiocyanate (FITC) or PE-labeled anti-mouse CD4 (L3T4) mAb (BD PharMingen, San Diego, CA), APC-labeled anti-mouse Foxp3 (NRRF-30) mAb (eBioscience) and anti-mouse IL-10 (JES5-2A5) mAb (BioSource, Ratingen, Germany) were used.

2.3. CHS and application or administration of KP

In CHS to PCl, mice were sensitized with 50 μl of 5% PCl in acetone/olive oil (4:1) on the clipped abdomen on day 0. Before challenge, the basal line thickness of both ears on all mice was measured with a dial thickness gauge. On day 5, all mice were challenged on both sides of each earlobe with 20 μl of 0.5% PCl in acetone/olive oil. Ear thickness was measured 24 h after challenge. The ear swelling response ($\times 10^{-3}$ cm) was expressed as the difference before and after challenge.

In CHS to OX, mice were sensitized with 50 μl of 5% OX in acetone/olive oil (4:1) on the clipped abdomen on day 0 and challenged with 20 μl of 1% OX in acetone/olive oil (4:1).

KP was applied on day 0, 1, 2 or 3 to the sensitizing or non-sensitizing site (back) at 0.625%, 2.5% or 10% as a co-solubilized form in 50 μl of the sensitizing solution (PCl or OX). Alternatively, 2 mg/kg (0.4 mg/50 μl PBS) of KP was injected intraperitoneally (*i.p.*) just before sensitization on day 0.

2.4. Cell purification

Axillary and inguinal lymph nodes were harvested from mice. They were meshed through cell strainer into RPMI-1640 (Sigma, St. Louis, MO) containing 2% fetal calf serum (FCS) to prepare single cell suspensions of lymph node cells (LNCs). To purify CD4$^+$ CD25$^+$ or CD4$^+$ CD25$^-$ cells, mouse CD4$^+$ CD25$^+$ Regulatory T Cell Isolation Kit Mouse (Miltenyi Biotech, Bergisch Gladbach, Germany) was used according to the manufacture's protocol. Non-CD4$^+$ cells were first depleted with biotin-Ab cocktail, and the flow-through fractions were magnet-separated with CD25-PE Ab followed by beads conjugated Ab to PE. After this procedure positive fraction contained CD4$^+$ CD25$^+$ cells. To purify CD25-depleted cells, LNCs were treated with CD25-PE Ab and incubated with beads conjugated Ab to PE, and flow-through fractions from magnet-columns were collected for transfer study.

2.5. Cell transfer

Donor mice were treated with 5% PCl plus 10% KP (PCl + KP) or 5% OX plus 10% KP (OX + KP) in acetone/olive oil on the clipped abdomen. Draining LNCs were taken 5 days later, and single cell suspensions were prepared. The cell number was adjusted, and 200 μl of cell suspension was injected intravenously (*i.v.*) into each recipient mouse 1 day before sensitization for recipients. Recipients were sensitized with PCl on the clipped abdomen and challenged 5 days after sensitization on the ears. The ear swelling was evaluated 24 h later.

2.6. Blocking test with anti-CTLA-4 or IL-10 mAb

Naïve recipients were injected *i.v.* with 2.4×10^7 LNCs of mice that were treated with PCl + KP 5 days before. Half of recipients were injected intraperitoneally (*i.p.*) with anti-CTLA-4 mAb (100 μg/mouse) 3 h before cell transfer or with anti-IL-10 mAb (250 μg/mouse) both 3 h before and 24 h after cell transfer. The *in vivo* function of Treg is inhibited by the application of these mAbs as reported previously [13].

2.7. Reverse transcription (RT) quantitative real-time PCR

Total mRNA was extracted from draining LNCs with the SVTotal RNA Isolation system (Promega, Madison, WI, USA) according to the manufacturer's protocol. Murine *Foxp3* gene expression (assay ID: Mm00475156_m1) 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, USA). Target gene 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, GAPDH gene expression was measured using the TaqMan rodent GAPDH control reagents (Applied Biosystems). The relative expression was calculated using the $2^{-\Delta\Delta C_T}$ method. The expression of the target gene normalized to an endogenous reference and relative to calibrator is given by the formula $2^{-\Delta\Delta C_T}$. Gene expression in PCl-treated mice was used as a calibrator expression to calculate $\Delta\Delta C_T$.

2.8. PGE_2 assay

Draining (axillary and inguinal) LNCs were cultured in cRPMI medium for 48 h. PGE_2 concentration in the supernatants was

measured using PGE_2 EIA Kit (Cayman, Ann Arbor, MI, USA) according to the manufacture's protocol.

2.9. Statistic analysis

P values were calculated using Dunnett type multiple comparison. Values <0.05 were considered significant.

3. Results

3.1. Systemic suppression of CHS to PCl by skin application of KP

Mice were sensitized with PCl alone (positive control) or PCl + KP on the clipped abdomen on day 0, and challenged on both sides of each earlobe with PCl on day 5. Negative control mice were challenged without sensitization. We have previously shown that topical application of KP at the sensitizing site suppressed the CHS response [3]. To test whether KP-induced immunosuppression is local or systemic, mice were painted with KP at 0.625%, 2.5%, or 10% simultaneously on the clipped abdomen (local effect) or on the clipped back (systemic effect) at the time of sensitization. Among these three groups, 10%, but not 2.5% or 0.625% of KP significantly suppressed CHS (Fig. 1A). When mice were painted with KP at the non-sensitizing site, KP at 10% and 2.5%, but not 0.625%, induced significant immunosuppression. Therefore, the suppressive effect of KP on CHS was irrelevant of the application site. Thus, the suppressive effect of KP on CHS is systemic. When KP was administered *i.p.* just before sensitization, CHS was also suppressed significantly (Fig. 1B).

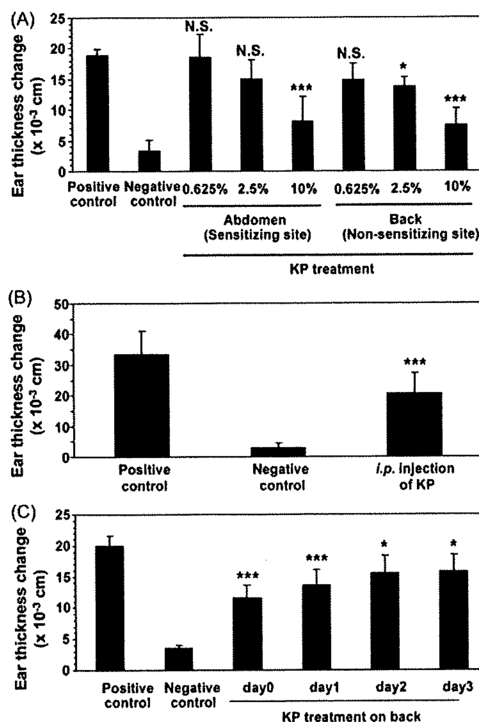

Fig. 1. Systemic suppression of CHS by KP painting on various application days. While mice were sensitized on the abdomen and challenged on the ears, KP was applied on day 0 to the sensitizing or non-sensitizing site at 0.625%, 2.5% or 10% (A). Instead of topical application of KKP, 2 mg/kg of KP was injected *i.p.* on day 0 (B). While mice were sensitized on the abdomen and challenged on the ears, KP was applied on day 0, 1, 2 or 3 to the non-sensitizing site (back) at 0.625%, 2.5% or 10% (C). Positive control mice were sensitized and challenged without KP treatment. Negative control mice were challenged without sensitization. $n = 4$, 5 or 6, error bars: SD, N.S.: not significant, $^{*}P < 0.05$, $^{***}P < 0.001$ (*vs* positive control). Similar results were obtained in three independent experiments.

To evaluate the effective timing of KP application, mice were sensitized with PCl on the abdomen and applied with 0.625%, 2.5% or 10% KP on the back on day 0, 1, 2 or 3 after sensitization. In any of the application days, significant immunosuppression was observed, but the suppression rate was the highest on day 0 (%inhibition, 51.8) and gradually decreased to day 3 (25.0%) (Fig. 1C), indicating that the suppression took place during the afferent limb.

3.2. Transfer of hapten-specific, KP-induced tolerance with immune LNCs

A transfer study with immune LNCs from systemically KP-tolerized mice was performed. Syngeneic naïve recipients were injected *i.v.* with LNCs obtained from mice that were treated with PCl + KP or OX + KP 5 days before transfer. The recipients were sensitized 24 h after transfer and challenged with PCl 5 days later. Positive control mice were sensitized and challenged without cell transfer. The recipients injected with LNCs obtained from PCl + KP-treated, but not OX + KP-treated mice, exhibited a depressed CHS response (Fig. 2). In the reciprocal experiment, donor mice were sensitized with PCl + KP or OX + KP, and LNCs were prepared 5 days after sensitization. While recipient mice were sensitized and challenged with OX, they received transfer of the 5-day LNCs from the donor mice just before sensitization. Draining LNCs from OX + KP-treated mice suppressed CHS response by 30.0% ($P < 0.0095$) as compared the non-transfer positive control mice, whereas those from PCl + KP-treated mice did not alter the CHS response. Thus, the hapten-specific suppression was confirmed by the study.

3.3. Mediation of KP-induced immunosuppression by $CD4^{+}$ $CD25^{+}$ cells

It is well known that $CD4^{+}$ $CD25^{+}$ Treg cells play an important role for dominant tolerance [4–7]. We purified $CD4^{+}$ $CD25^{+}$ T cell population from LNCs of mice that were treated with PCl + KP 5 days before. In immune LNCs, there were 5.0% $CD4^{+}$ $CD25^{+}$ and 32.7% $CD4^{+}$ $CD25^{-}$ cells (Fig. 3A). These numerical values were comparable to those of naïve mice (data not shown). The immune LNCs were purified for $CD4^{+}$ $CD25^{+}$ and $CD4^{+}$ $CD25^{-}$ cell fractions by MACS technique, with more than 90% purity (Fig. 3A). Naïve recipients were *i.v.* injected with 2.4×10^{7} of the unfractionated cells, 1.2×10^{6} (equal to 5.0% of unfractionated cells) of $CD4^{+}$ $CD25^{+}$ cells, or 0.8×10^{7} (equal to 33.3% of unfractionated cells) of $CD4^{+}$ $CD25^{-}$ cells. The recipients were sensitized with PCl 24 h after transfer, and challenged on the ears 5 days later. When mice

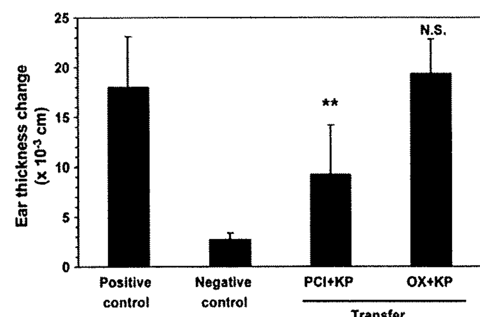

Fig. 2. Hapten-specific suppression of CHS by KP application. Twenty-four hours before PCl sensitization, mice were injected *i.v.* with 2.4×10^{7} LNCs obtained from syngeneic mice that were treated with PCl + KP or OX + KP 5 days earlier. Positive control mice were sensitized and challenged without cell transfer. Negative control mice were only challenged. $n = 6$, error bars: SD, N.S.: not significant, $^{**}P < 0.01$ (*vs* positive control). Similar results were obtained in three independent experiments.

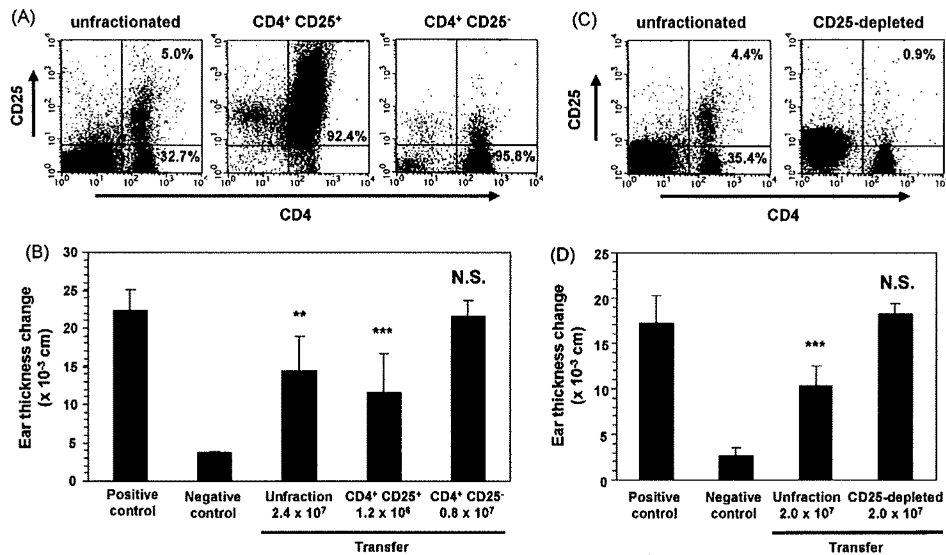

Fig. 3. Mediation of KP-induced suppression by CD4$^+$ CD25$^+$ cells. LNCs were collected from mice that were treated with PCl + KP 5 days earlier. CD4$^+$ CD25$^+$ cells or CD4$^+$ CD25$^-$ cells were purified by MACS (A). After cell separation, 2.4×10^7 unfractionated cells, 1.2×10^6 CD4$^+$ CD25$^+$ cells or 0.8×10^7 CD4$^+$ CD25$^-$ cells were injected *i.v.* into naïve recipients 24 h before PCl-sensitization (B). LNCs were collected from mice that were treated with PCl + KP 5 days earlier. CD25$^+$ cell-depleted cells were purified by MACS (C). After cell separation, 2.0×10^7 unfractionated cells or 2.0×10^7 CD25$^+$ cell-depleted cells were injected *i.v.* into naïve recipient 24 h before PCl-sensitization (D). $n = 5$, error bars: SD, N.S.: not significant, $^{**}P < 0.01$, $^{***}P < 0.001$ (*vs* positive control). Similar results were obtained in three independent experiments.

were administered with the unfractionated LNCs, their CHS response to PCl was significantly depressed (Fig. 3B). CD4$^+$ CD25$^+$ cells transferred to the recipients exerted the suppressive effect of KP at a comparable level to the unfractionated cells, while CD4$^+$ CD25$^-$ cells were not suppressive.

We also prepared CD25$^+$ cell-depleted fraction from PCl + KP-treated mice, which contained CD4$^+$ CD25$^+$ cells as low as 0.9% (Fig. 3C). Naïve recipients were injected *i.v.* with 2.0×10^7 of the unfractionated cells or 2.0×10^7 of CD25$^+$ cell-depleted cells. The immune LNCs lost the suppressive ability by depletion of CD4$^+$ CD25$^+$ cells (Fig. 3D). The results indicated that the suppressive effect of KP on CHS was mediated by CD4$^+$ CD25$^+$ cells.

3.4. Inhibition of KP-induced suppression by blockade of CTLA-4 but not IL-10

Since CTLA-4 plays an important role in the suppressive activity of Treg cells, we performed CTLA-4 blocking study as previously reported [13]. Naïve recipients were injected *i.v.* with 2.4×10^7 LNCs of mice that were treated with PCl + KP 5 days before. Half of recipients were injected *i.p.* with an anti-CTLA-4 mAb 3 h before cell transfer. The recipients were sensitized with PCl 24 h after cell transfer, and challenged 5 days later. The suppressive effect of LNCs on CHS was completely abolished when the recipients were injected with anti-CTLA-4 mAb (Fig. 4A), further supporting mediation by Treg cells of the suppressive effect of KP on CHS to PCl.

Treg cells function *via* cell-to-cell contact or release of immunosuppressive cytokines such as IL-10 [11,17–19]. Therefore, we administered the anti-IL-10 mAb 100 μg per mouse, which functioned as neutralization of IL-10 as reported previously [13]. Recipients were injected with 2.4×10^7 LNCs of mice treated with PCl + KP, and injected *i.p.* with anti-IL-10 mAbs both 3 h before and 24 h after sensitization. There was no significant difference in the response between anti-IL-10 mAb-administered and non-administered groups (Fig. 4B), suggesting no substantial role of IL-10.

3.5. High expression of Foxp3 in LNCs from PCl + KP-treated mice

Foxp3 is an important master molecule for the suppressive activity of Treg cells [14,15]. Mice were painted with PCl alone or PCl + KP on the abdomen on day 0, and we first examined the percentage of CD4$^+$ CD25$^+$ Foxp3$^+$ cells in draining LNCs by flow cytometry. On day 3 after painting, the percentage of CD4$^+$ CD25$^+$ Foxp3$^+$ cells in PCl alone group was 4.9%, and that of PCl + KP group was 4.8%. On day 5, the percentages of PCl alone and PCl + KP groups were 4.6% and 4.3%, respectively. Thus, it was difficult to detect the

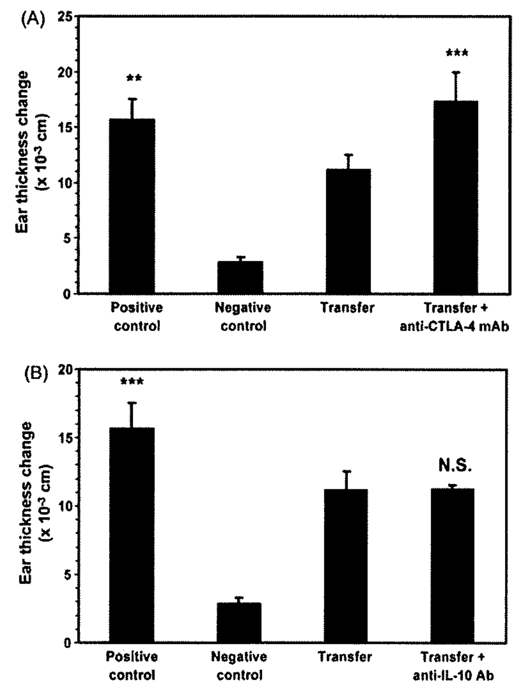

Fig. 4. Inhibition of KP-induced suppression by blockade of CTLA-4 but not IL-10. (A) Twenty-four hours before sensitization, mice were injected *i.v.* with 2.4×10^7 LNCs obtained from syngeneic mice that were treated with PCl + KP 5 days earlier (Transfer). A group of mice (Transfer + CTLA-4 mAb) were additionally injected *i.p.* with an anti-CTLA-4 mAb (100 μg/mouse) 3 h before cell transfer. (B) In the same procedure, a group of mice (Transfer + anti-IL-10 mAb) were additionally injected *i.p.* with an anti-IL-10 mAb (250 μg each/mouse) 3 h before and 24 h after PCl-sensitization. $n = 5$, error bars: SD, and N.S.: not significant. $^{**}P < 0.01$, $^{***}P < 0.001$ (*vs* transfer alone). Similar results were obtained in three independent experiments.

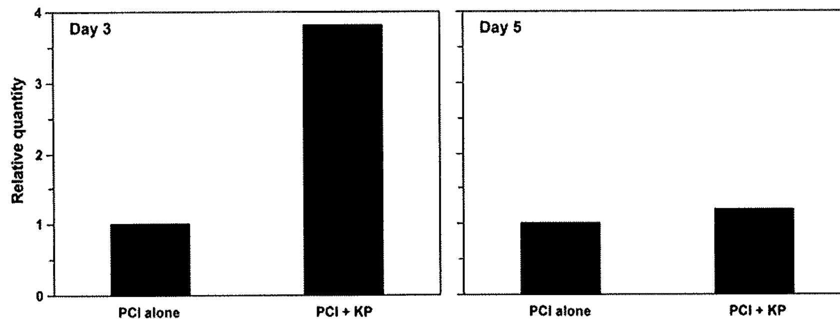

Fig. 5. Induction of Foxp3 mRNA expression in LNCs by KP. Mice were treated on the abdomen with PCl alone or PCl + KP. LNCs were collected on day 3 or day 5 after treatment. Foxp3 gene expression was quantified in a two-step real-time PCR. GAPDH gene expression was measured as an endogenous control for these PCR quantification studies. Results represent normalized mean target mRNA amounts relative to PCl-treated mice using the $\Delta\Delta$Ct method ($n = 4$ or 5).

difference by this assessment. We further evaluated the expression of Foxp3 by quantitative PCR analysis. Total RNA was extracted from LNCs on day 3 or 5 and subjected to real-time PCR analysis. On day 3, the expression of Foxp3 in LNCs from PCl + KP-treated mice was remarkably increased compared to PCl-treated mice (Fig. 5). On day 5, such increment of Foxp3 expression was not found. Thus, the treatment with KP induced the expression of Foxp3, supporting the development of Treg cells at the induction phase.

3.6. *Inhibition of PGE_2 production by KP treatment in LNCs*

To address the possibility that PGE_2 exerts an effect on Treg activity, LNCs from mice treated with PCl alone or PCl + KP were obtained on day 3 or 5 and cultured for 48 h. The supernatants were analyzed with PGE_2 EIA Kit. The concentration of PGE_2 in the supernatants of PCl alone was 10 times higher than that of PCl + KP on day 3 (Fig. 6). On day 5, the PGE_2 concentration of PCl alone was decreased to a comparable level to that of PCl + KP. These results showed that topical KP treatment inhibits PCl-induced PGE_2 release from LNCs.

4. Discussion

The present study demonstrated that skin application of KP systemically suppresses CHS *via* inducing Treg cells. In the LNC transfer study, the development of Treg cells was proven by $CD4^+$ $CD25^+$ phenotype of immunosuppressive T cells. CTLA-4 molecules on the surface play an important role in the suppressive function of Treg cells, as its blockade abolished the function. The expression of Foxp3, an important functional marker for Treg cells, was upregulated in Treg-containing LNCs. LNCs from OX + KP-treated mice did not suppress CHS response to PCl, suggesting KP-induced suppression is specific for hapten. Such antigen-specific Treg cells have been reported in several systems [13,26,27]. However, it has recently been reported that CTLA-4 inhibits Treg proliferation similar to its role on effector T cells [28]. Since T effector cells possible express CTLA-4, anti-CTLA-4 antibody also might affect another population of T cells as well as Treg cells.

Aspirin inhibits human DC maturation and immunostimulatory function by downmodulating NF-κB. Recently, it has been reported that aspirin-treated DCs have a potential to induce antigen-specific Treg cells *in vitro* [24,25]. We have reported that KP downregulates murine LCs not only in an *in vitro* culture system but also in *in vivo* application system [3]. In our study, aspirin also had the similar suppressive effect on LCs in the *in vitro* and *in vivo* tests. However, aspirin did not inhibit CHS response to PCl, whereas KP had even the systemic inhibitory effect on CHS. Therefore, KP shares Treg-inducing ability with aspirin, but it appears that topical KP treatment has a stronger potential for Treg induction. Another possibility is that the inhibitory effect of KP on CHS is independent of the NF-κB inhibition.

It has been reported that Treg cells function as suppressors *via* secreted IL-10 [29–31]. Immature DCs undergoing NF-κB inhibition induces IL-10-secreting Treg [23]. The neutralization of IL-10 did not abrogate the LNC function, suggesting that IL-10 released by Treg is not a mediator of the suppression, and the suppression might be mediated by cell-to-cell contact [32–35]. Nevertheless, IL-10 and TGF-β might play an important role in the induction of Treg cells by KP, since gene expression of both cytokines was increased in LNCs from PCl + KP treated mice (data not shown). It is possible that IL-10 is released by KP-treated LCs migrating into the draining lymph nodes and serves as Treg-inducing cytokine as we have recently shown in LCs of grafted skin [36].

Since KP was effective for the induction of Treg cells and resultant suppression of CHS when administered during the induction phase, it is possible that KP alters the function of antigen-presenting cells, or alternatively, KP might modulate T cells to become Treg cells upon antigen-presentation. We have previously reported that KP inhibits the maturation-related morphological and phenotypical alterations of LCs. However, our analysis of $FITC^+$ $I\text{-}A^+$ $B220^-$ cells in draining

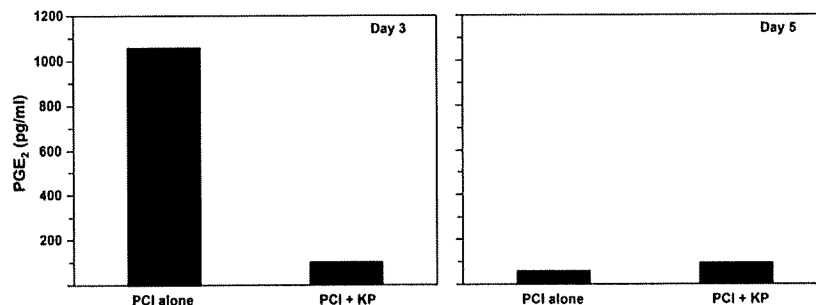

Fig. 6. Inhibition of PGE_2 production in LNCs by KP. Mice were treated on the abdomen with PCl alone or PCl + KP. LNC were collected on day 3 or 5 and cultured in cRPMI medium for 48 h. A quantitative analysis of PGE_2 release in the supernatants was performed as described in Section 2.

LNCs from mice topically treated with FITC plus KP showed that KP did not completely inhibit the migration of antigen-presenting cells (data not shown). It is well known that immature DCs induce immunotolerance [21], and DCs may regulate the function of Treg cells through cell-to-cell contact [37]. Such KP-modulated LCs/DCs might exist in the LNCs of KP/PCl-treated mice and induce the development of Treg cells. PGE_2 derived from bystander cells such as keratinocytes and lymph node supporter cells upregulate the maturation and migration of LCs, and potentially dDCs [38–40]. Given that KP decreases the production of PGE_2 as a cyclooxygenase inhibitor, immature LCs or DCs might present the antigen for T cells, resulting in the development of Treg cells.

On the other hand, several reports have indicated that PGE_2 promotes the expression of Foxp3 and the function of Treg cells [41,42]. A representative observation is that PGE_2 induces Foxp3 expression in even CD25-negative $CD4^+$ T cells [42]. Therefore, it appears that PGE_2 is bi-functional for antigen-presenting cells and T cells in relation to the development of Treg cells. PGE_2 is a downregulator for antigen-presenting cells to be a Treg inducer, and an upregulator for T cells to be Treg cells. In our study, PCl treatment increased the amount of PGE_2 in LNCs on day 3 after its application. KP completely inhibited PGE_2 production, and simultaneously, induced Foxp3 expression in the LNCs, indicating inverse correlation between PGE_2 production and Foxp3 expression. Therefore, it is plausible that the Treg-inducing ability of KP stems from the reduction of antigen-presenting cell maturation.

KP is not only a classical cyclooxygenase inhibitor, but also LC-downmodulator at a higher concentration. The present study demonstrates that both actions occur even when KP is delivered *via* the skin and lead to the development of Treg cells.

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Original Article

Induction of eosinophil- and Th2-attracting epidermal chemokines and cutaneous late-phase reaction in tape-stripped skin

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Abstract: Skin barrier damage induces various harmful or even protective reactions in the skin, as represented by enhancement of keratinocyte cytokine production. To investigate whether acute removal of stratum corneum modulates the production of chemokines by epidermal cells, we treated ears of BALB/c and C57BL/6 mice by tape-stripping, or acetone-rubbing as a control of acute barrier disruption procedure. There was no difference between the tape-stripped and acetone-rubbed skin sites in the increased and recovered levels of transepidermal water loss. The mRNA expression levels of all the chemokines tested, including Th1 chemokines (CXCL10, CXCL9 and CXCL11), Th2 chemokines (CCL17 and CCL22) and eosinophil chemoattractant (CCL5), were higher in the epidermal cells from BALB/c than in those of C57BL/6 mice. In particular, CCL17, CCL22 and CCL5 were remarkably elevated in BALB/c mice and augmented by tape-stripping more markedly than acetone-rubbing, whereas Th1 chemokines were enhanced by acetone-rubbing more remarkably. Tape-stripping induced dermal infiltration of eosinophils in BALB/c but not C57BL/6 mice. In a contact hypersensitivity model, where BALB/c mice were sensitized on the abdomen and challenged on the ears with fluorescein isothiocyanate, mice exhibited higher ear swelling responses at the late-phase as well as delayed-type reactions, when challenged *via* the tape-stripped skin. The challenge *via* tape-stripped skin augmented the expression of IL-4 and CCR4 in the skin homogenated samples, indicating infiltration of Th2 cells. These findings suggest that acute barrier removal induces the expression of Th2 and eosinophil chemokines by epidermal cells and easily evokes the late phase reaction upon challenge with antigen.

Key words: barrier disruption – chemokines – eosinophil – late-phase reaction – tape stripping

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Introduction

Stratum corneum, which is the outermost, cornified layer of the epidermis, serves as skin barrier and protects from external micro-organisms and chemicals and even sunlight radiation (1). When this barrier is destroyed or removed, these hazardous invaders penetrate through the skin. Upon exposure to the agents, however, the epidermis produces or expresses various protective molecules such as cytokines (2), anti-bacterial peptides (3) and cornification-promoting molecules (4).

There are both the acute and chronic disruption procedures for experimental impairment of the skin barrier in rodents (5,6). Furthermore, the acute barrier disruption has been performed by two different procedures in mice, stripping with scotch tape and rubbing with acetone cotton (2). These two treatment modalities stimulate keratinocytes to produce cytokines, such as interleukin-1α, tumor necrosis factor-α and granulocyte/macrophage colony stimulating factor and enhance contact hypersensitivity when a hapten is applied on the barrier-disrupted skin at either of the sensitization or challenge phase (2). However, the pathophysiological significance of barrier disruption in skin infiltration of inflammatory cells remains to be clarified. Given that barrier damage induces the release of chemokines from the epidermis, it is possible that certain inflammatory cells are present or prone to infiltrate in the barrier-disrupted skin.

Although both tape-stripping and acetone-rubbing have been categorized as the procedure of acute barrier disruption, it remains unknown whether these two treatments exert the same effect on the production of chemokines by epidermal cells and the resultant infiltration of leucocytes in the skin. While tape-stripping mechanically removes the cornified layer of the epidermis, acetone-rubbing chemically deletes sphingolipids, such as ceramide, existing

between the layers of corneocytes (2). Therefore, it seems that tape-stripping resembles scratching and more likely reflects clinical conditions. For example, the tape-stripped skin may share the chemokine production status with the skin of pruritic disorders such as atopic dermatitis.

This study was aimed to investigate whether tape-stripping modulates the production of chemokines by epidermal cells in a comparison with acetone-rubbing. We treated the ears of mice by tape-stripping or acetone-rubbing and examined the expression/production of chemokines by epidermal cells (ECs), the infiltration of inflammatory cells and the late-phase and delayed-type hypersensitivities. In addition, it was necessary that Th2- and Th1-preponderant mouse strains, BALB/c and C57BL/6 (B6) mice respectively, were compared in the effects of tape-stripping. Results suggest that tape-stripping induces the production of Th2 cell- and eosinophil-associated chemokines more markedly than acetone-rubbing in BALB/c mice and this skewed elaboration of chemokines determines the infiltration of Th2 cells and eosinophils and the cutaneous hypersensitivity responses.

Materials and methods

Animals and chemicals

Seven to 10 week-old female BALB/c and B6 mice were purchased from Japan SLC (Hamamatsu, Japan). These mice were maintained in the Laboratory Animal Research Center in University of Occupational and Environmental Health under specific pathogen-free conditions. All animal experiments were performed according to the guidelines for the care and use of animals approved by our university. Fluorescein isothiocyanate (FITC) was obtained from Sigma Chemical Co. (St Louis, MO).

Acute barrier disruption procedures

The procedures were reported previously (2). Mechanical barrier disruption was achieved by stripping both sides of the earlobe with cellophane tape (Nichiban, Tokyo, Japan) seven times. This manipulation effectively removed stratum corneum without hazardous haemorrhagic change. For chemical disruption, both sides of the earlobe were gently rubbed for 30 s with cotton ball dipped in absolute acetone. These two disruption procedures were performed in different mice.

Transepidermal water loss (TEWL)

Immediately (time 0) and at various times after barrier disruption with tape-stripping and acetone-rubbing, TEWL was measured in the treated and untreated earlobes with Vapa Scan AS-VT100RS (Asahi Biomed, Yokohama, Japan) in the measurement room which had a temperature of 23–25°C and a relative humidity of 49–54%.

Preparation of epidermal cells (ECs)

Skin sheets from earlobes untreated or treated with tape or acetone 6, 12 and 24 h before were floated in 0.2% trypsin (Difico Laboratories, Detroit, MI, USA), dissolved in phosphate-buffered saline (PBS; pH 7.4) for 1 h at 37°C (7). Epidermis was then separated from dermis with forceps in PBS supplemented with 10% of fetal calf serum (Gibco, Carlsbad, CA, USA). EC suspensions were prepared by pipetting and filtration through nylon mesh (pore size, 77 mm) and included 97% keratinocytes and 2% Langerhans cells as assessed using flow cytometric analysis with anti-I-A antibody (8).

Real-time quantitative PCR analysis of ECs

For detection of epidermal chemokines, EC suspensions were used as samples. Total cellular RNA of ECs was extracted with the RNA extraction kit (Promega, Madison, WI, USA) from freshly prepared skin samples. There was no remarkable difference between the acetone-rubbed and tape-stripped samples in the amounts of extracted RNA. RNA was then reverse transcribed and amplified by random hexamer in single tube assay using the TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA) with gene-specific sense and antisense primers and a detection probe labelled on the 5′ end with the reporter dye 6-FAM. Primers and probes were obtained from TaqMan Gene Expression Assays Inventories (Applied Biosystems) for CXCL10/IP-10, CXCL9/Mig, CXCL11/I-TAC, CCL17/TARC, CCL22/MDC and CCL5/RANTES. Using the ABI Prism 7000 sequence detection systems (Applied Biosystems), duplicate samples were reverse transcribed and amplified under the following consecutive steps: 2 min at 50°C, 10 min at 95°C, followed by 50 amplification cycles of 15 s at 95°C and 1 min at 60°C. Sequence-specific amplification was detected as an increased fluorescent signal of 6-FAM exceeding the threshold limit during the amplification cycle. Quantification of gene-specific message levels was determined by comparing fluorescence intensity from unknown RNA samples to the fluorescence intensity of standard curve generated from control mRNA levels. Amplification of the gene for mouse β-actin was performed on all samples to control interspecimen variations in RNA amounts. The result for each gene was normalized to the quantity of mouse β-actin detected in the sample. Levels of gene-specific message were graphed as normalized message units as determined from standard curve.

Enzyme-linked immunosorbent assay (ELISA)

Chemokine levels were studied using ELISA method. Dispersed ECs from barrier-disrupted or untreated earlobes were cultured at 1×10^6 per ml in Eagle's minimal essential medium containing 10% fetal calf serum, 1% streptomycin

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and 1% penicillin, without any additional stimulant, for 48 h at 37°C in humidified 5% CO_2 in air. When dispersed ECs are cultured for different culture periods, the amount of cytokines in the culture supernatants are markedly increased between 24 and 48 h and reached maximum at 72 h (9). As the 72-h culture possibly masks the influence of barrier disruption, we used the 48-h culture for the assessment. The Quantikine (R & D Systems, Minneapolis, MN) protocol for sandwich ELISA was used to quantify total amount of CCL17, CCL22 and CCL5 in the culture supernatants.

Histological assessment

Skin specimens were obtained from earlobes and fixed in 20% buffered formalin and embedded in paraffin. Multiple 3 mm sections were stained with haematoxylin and eosin (H&E) for eosinophil and lymphocyte counting. The numbers of eosinophils and lymphocytes in the dermis were enumerated in three high power fields of microscopy and expressed per one section (0.25 mm^2) at 400×. Each section was assessed in random order by two observers of us without the knowledge of patient identification.

Preparation of skin homogenized samples and real-time quantitative PCR analysis

To examine the cytokines and chemokine receptors of T cells, homogenized samples of whole ears were used. BALB/c mice were sensitized on the shaved abdomen with 200 μl of 1% FITC in acetone/dibutyl phthalate (1:1 ratio) three times a week for 2 weeks and earlobes were provoked by painting of 40 μl of 1% FITC 24 h after tape-stripping. At 3, 6 and 12 h after challenge, the earlobes were prepared and homogenized using a T 10 basic Ultra-Turrax (Ika-Werke, Staufen, Germany) with the Trizol reagent (Invitrogen Inc., Carlsbad, CA, USA). Total cellular RNA was extracted with the PureLink Micro-to-Midi Total RNA Purification System (Invitrogen Inc.) from Trizol samples. Primers and probes were obtained from TaqMan Gene Expression Assays Inventories (Applied Biosystems) interferon-γ (IFN-γ), interleukin-4 (IL-4), CXCR3, and CCR4. Quantitative PCR was performed as described above.

Contact hypersensitivity (CHS)

Mice were sensitized with FITC by painting of the shaved abdomen with 200 μl of 1% FITC. Five days after sensitization, the earlobes were barrier disrupted or untreated. After 24 h, mice were elicited by painting of both sides of earlobes with 40 μl of 0.5% FITC and the increase in ear thickness was measured immediately before and 1, 4, 8 and 24 h after painting using a dial thickness gauge (Ozaki Co, Tokyo, Japan). Ear swelling was calculated as (ear thickness after challenge) − (ear thickness before challenge).

Statistical analysis

Data were analysed using an unpaired two-tailed t-test. $P < 0.05$ was considered to be significant.

Results

Absence of differences in TEWL following treatment between tape-stripping and acetone-rubbing and between BALB/c and B6

In advance of testing the actions on the epidermal chemokine production and cell infiltration, we compared tape-stripping and acetone-rubbing in their effects on TEWL, a representative marker for the barrier function. We used two strains of mice, Th2-preponderant BALB/c and Th1-preponderant B6 mice. Earlobes of mice were stripped with cellophane tape or rubbed with acetone and TEWL was monitored after treatment. TEWL was elevated immediately after either of the treatments and declined thereafter at comparable levels (Fig. 1). Therefore, there was no difference between tape-stripped and acetone-rubbed skin sites in the increment and recovery of TEWL. Furthermore, BALB/c (Fig. 1a) and B6 mice (Fig. 1b) had virtually the same TEWL values following the treatments.

Higher expression of Th2 and eosinophil chemokine mRNAs by tape-stripping than acetone-rubbing in ECs of BALB/c mice

Earlobes of mice were stripped with cellophane tape or rubbed with acetone and EC suspensions were prepared from the ears at 6, 12 or 24 h after treatment and subjected to real-time PCR analysis. As shown in Fig. 2, the two barrier disruption procedures differentially induced the expression of mRNA for Th1 chemokines (CXCL10, CXCL9 and CXCL11), Th2 chemokines (CCL17 and CCL22) and eosinophil-chemoattracting chemokine (CCL5) (10,11), depending on the mouse strains and the timing after treatment. BALB/c mice exhibited higher expression levels of all the chemokines than did B6 mice. In BALB/c mice, the increased expression was discernible at 12 h and remarkable at 24 h after treatment. CCL17, CCL22 and CCL5 were more strongly induced by tape-stripping than acetone-rubbing, but inversely, CXCL10, CXCL9 and CXCL11 were expressed more remarkably by acetone-rubbing than tape-stripping. Thus, tape-stripping is capable of inducing the production of Th2 chemokines by ECs in BALB/c mice.

To confirm the expression of Th2 chemokines and CCL5 promoted by tape-stripping, EC suspensions were prepared from BALB/c mice at 6, 12 or 24 h after the treatment and cultured for 48 h. The chemokine concentration in the culture supernatants was measured using ELISA. ECs from the treated mice produced higher levels of CCL17, CCL22 and

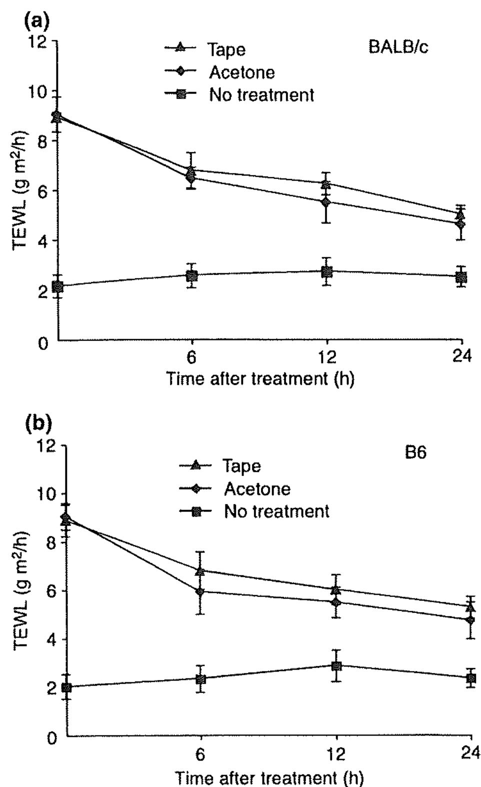

Figure 1. TEWL following tape-stripping or acetone-rubbing. Earlobes of BALB/c (a) and B6 mice (b) were stripped with tape or rubbed with acetone. Immediately after treatment (time 0), or 6, 12 or 24 h after treatment, TEWL was measured. Data are expressed as the mean ± SD of five mice.

CCL5 than those of untreated mice (Fig. 3). Again, tape-stripping induced the production of these chemokines, particularly CCL17, at significantly higher levels than acetone-rubbing.

Infiltration of eosinophils in tape-stripped ears of BALB/c mice

As tape-stripping stimulated ECs to produce chemokines, we monitored the infiltrate of inflammatory cells in the tape-stripped earlobes of BALB/c and B6 mice. Interestingly, we found that the tape-stripped ears of BALB/c mice showed infiltration of eosinophils in the dermis (Fig. 4a,b). Eosinophils appeared in the dermis at 8 h after treatment and increased in number at 24 h (Fig. 4c). Acetone-rubbing did not induce such an infiltrate of eosinophils. In B6 mice, the tape-stripping-provoked infiltrate of eosinophils was barely perceptible (Fig. 4d). Thus, the enhanced expression of CCL5 seems to be significant *in vivo*. In both strains, lymphocytes also infiltrated after either treatment with acetone or tape (Fig. 4e,f).

Augmented expression of IL-4 and CCR4 by challenge *via* tape-stripped skin in repeatedly sensitized mice

To examine the effect of tape-stripping on the induction of Th2 cells, we used the repeated sensitization method. As even the repeated sensitization with a hapten does not exclusively induce Th2 cells, Th1 cytokines are simultaneously increased by the challenge to some extent. BALB/c mice were sensitized with 1% FITC three times a week for 2 weeks on the abdomen and challenged with 1% FITC on the earlobes untreated or stripped with tape 24 h before. The ears were taken at 3, 6 or 12 h after challenge, homogenized and subjected to real-time PCR analysis for the expression of IFN-γ, IL-4, CXCR3 and CCR4. IFN-γ and IL-4 are representative Th1 and Th2 cytokines respectively, and CXCR3 and CCR4 are Th1 and Th2 chemokine receptors respectively (11). The expression of IFN-γ was increased at 3–12 h after challenge and the challenge *via* tape-stripped skin elevated its expression compared with the challenge *via* untreated skin (Fig. 5a). IL-4 expression was also enhanced by the challenge *via* tape-stripped skin at 3, 6 and 12 h (Fig. 5b). As to the chemokine receptors, while CXCR3 was not affected by challenge through the tape-stripped skin (Fig. 5c), CCR4 was augmented by the challenge (Fig. 5d). Thus, the expression of Th2 cytokine as well as IFN-γ and Th2 chemokine receptor was augmented in the tape-stripped and challenged skin, suggesting promoted accumulation of Th2 cells by tape-stripping.

Enhancement of both late-phase and delayed-type hypersensitivities in tape-stripped mice

It has been reported that the delayed-type contact hypersensitivity is enhanced through the barrier-disrupted skin (2). We further explored the *in vivo* significance of tape-stripping-augmented chemokine production in cutaneous hypersensitivities. BALB/c mice were sensitized with 1% FITC and challenged with 0.5% FITC on the tape-stripped or untreated earlobes. At 8 and 24 h after challenge, higher ear swelling responses were observed in mice challenged *via* tape-stripped ears than those challenged *via* untreated skin (Fig. 6). Acetone-rubbing, instead of tape-stripping, did not enhance the ear swelling responses at 8 h after challenge, while the treatment augmented the response 24 h after challenge (data not shown). Therefore, elicitation through the tape-stripped skin augmented not only the delayed-type but also the late-phase reactions of contact hypersensitivity where Th2 cells and eosinophils are involved (12).

Discussion

Our study showed that acute barrier disruption upregulates the production/expression of chemokines by ECs, depend-

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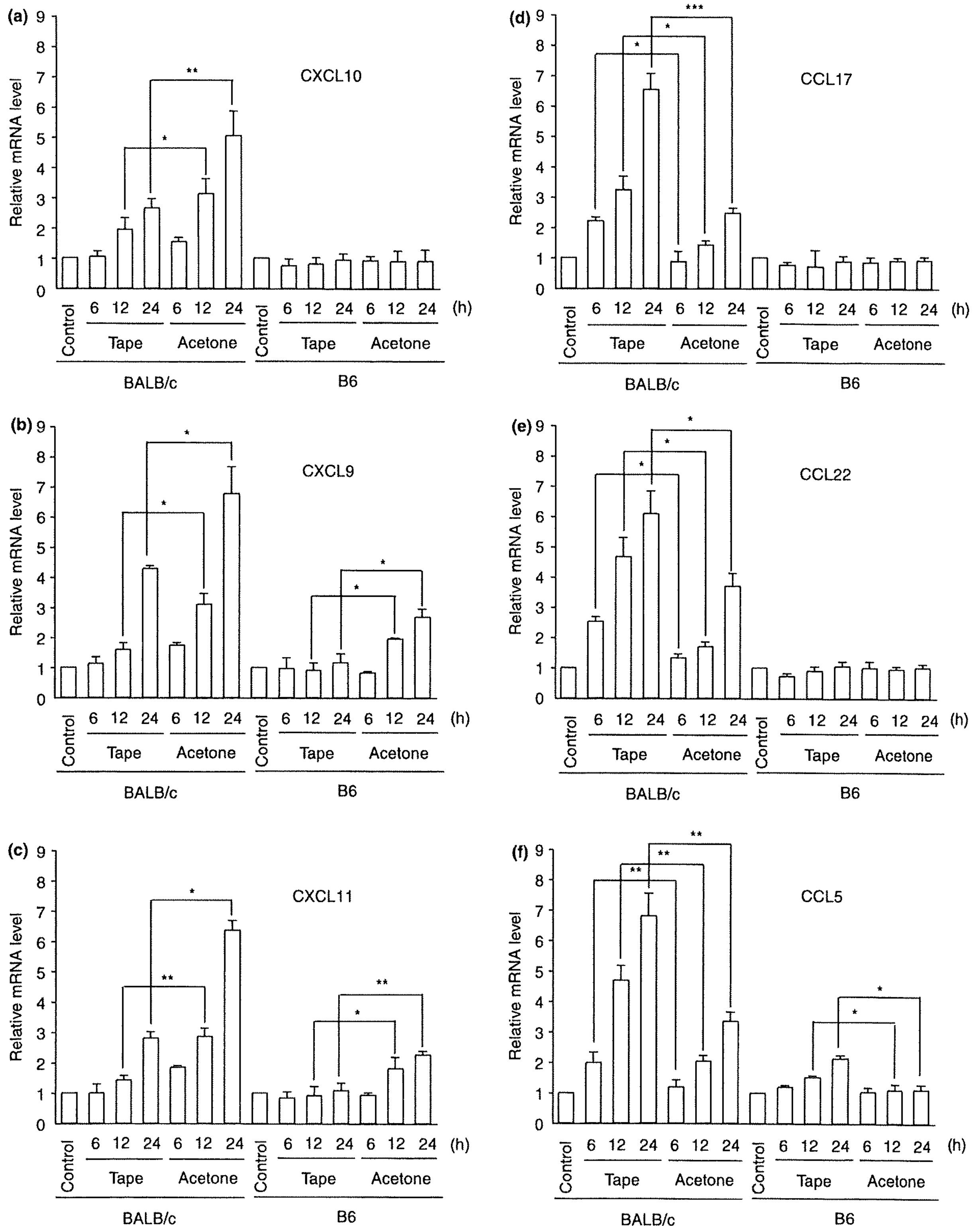

Figure 2. Real-time PCR analysis of mRNA expression for chemokines in ECs from barrier-disrupted earlobes. Earlobes of BALB/c and B6 mice were stripped with tape or rubbed with acetone. At 6, 12 and 24 h after treatment, EC suspensions were prepared and subjected to real-time PCR analysis for chemokines, including Th1 chemokines (CXCL10, CXCL9 and CXCL11), Th2 chemokine (CCL17 and CCL22) and CCL5. The expression of mRNA is represented as fold increase (2^{-DDC_t}), where DDC_t = [DC_t (sample)] – [DC_t (ECs without treatment)] and DC_t = [C_t (sample)] – [C_t (b-actin)]. Data are expressed as the mean ± SD of five mice (*$P < 0.05$, **$P < 0.005$, ***$P < 0.0001$).

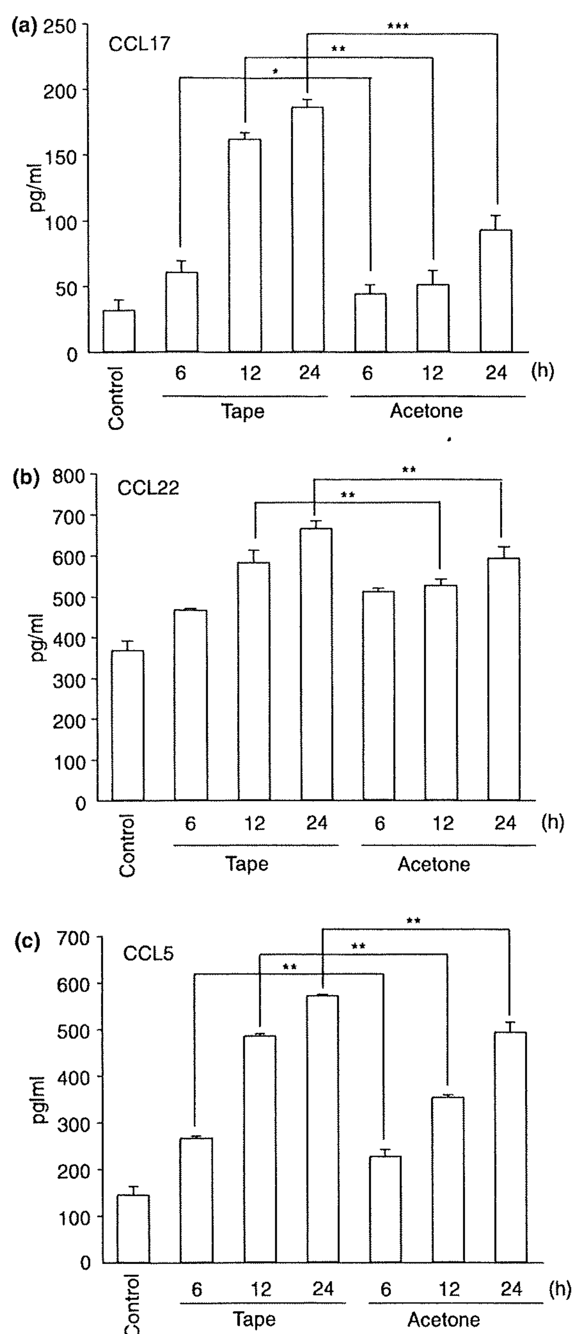

Figure 3. ELISA for chemokines in culture supernatants from ECs obtained from barrier-disrupted earlobes in BALB/c mice. Earlobes of BALB/c mice were stripped with tape or rubbed with acetone. At 6, 12 and 24 h after treatment, EC suspensions were prepared and cultured for 48 h. The concentration of CCL17, CCL22 and CCL5 in the culture supernatants was measured using ELISA. Data are expressed as the mean ± SD of five mice (* $P < 0.05$, ** $P < 0.005$, *** $P < 0.0001$).

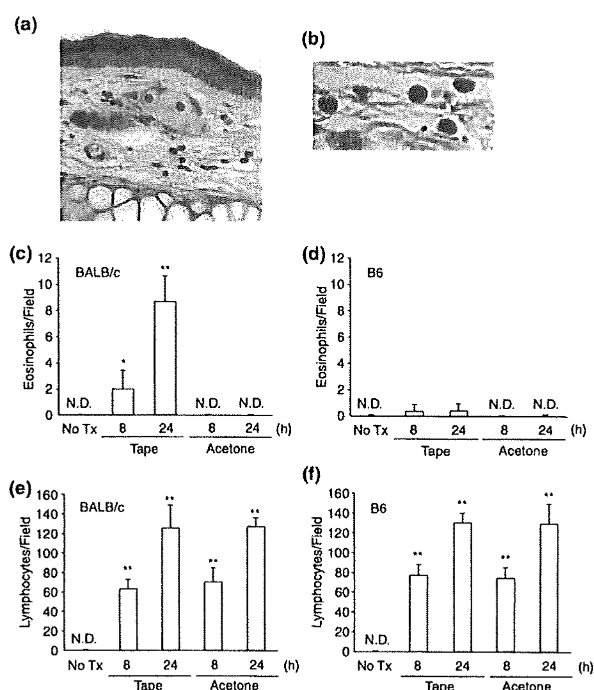

Figure 4. Histological pictures and numbers of infiltrating eosinophils and lymphocytes in tape-stripped earlobes of BALB/c mice. Earlobes of BALB/c and B6 mice were tape-stripped and acetone-rubbed and histological sections (H&E) were prepared 8 and 24 h later. Control sections were obtained from non-treated earlobes. (a, b) Histological picture of BALB/c mice at 24 h after tape-stripping (original magnification 200x and 400x). The numbers of eosinophils and lymphocytes were counted per one section (0.25 mm^2) at 400x. (c) Eosinophil counts in BALB/c mice treated with acetone-rubbing or tape-stripping. (d) Eosinophil counts in B6 mice treated with acetone-rubbing or tape-stripping. (e) Lymphocyte counts in BALB/c mice treated with acetone-rubbing or tape-stripping. (f) Lymphocyte counts in B6 mice treated with acetone-rubbing or tape-stripping. Data are expressed as the mean ± SD of five mice (* $P = 0.0061$, ** $P < 1.0 \times 10^{-6}$). No Tx, no treatment; and N.D., not detected.

ing on the procedure of disruption, type of chemokines and strain of mice. Th2 chemokines CCL17 and CCL22 and eosinophil chemoatrractant CCL5 (10,11) were augmented by tape-stripping more markedly than acetone-rubbing, while Th1 chemokines CXCL10, CXCL9 and CXCL11 (10,11) were enhanced by acetone-rubbing. The increased production of CCL17, CCL22 and CCL5 was clearly observed in Th2-polarized BALB/c mice but not in Th1-dominant B6 mice. It should be stressed that, in accordance with this observation, tape-stripping allowed eosinophils to infiltrate in the dermis of BALB/c mice. In addition, FITC challenge *via* tape-stripped ears of sensitized BALB/c mice induced the expression of IL-4 and CCR4, indicating accumulation of Th2 cells in the tape-stripped and hapten-challenged skin. Accordingly, the tape-stripped mice showed increased responses at 8 h as well as 24 h when they were challenged *via* the treated ears. These findings suggest that tape-stripping stimulates ECs to express/produce Th2 chemokines and eosinophil chemoattractant and hapten application *via* the tape-stripped skin evokes the late phase reaction.

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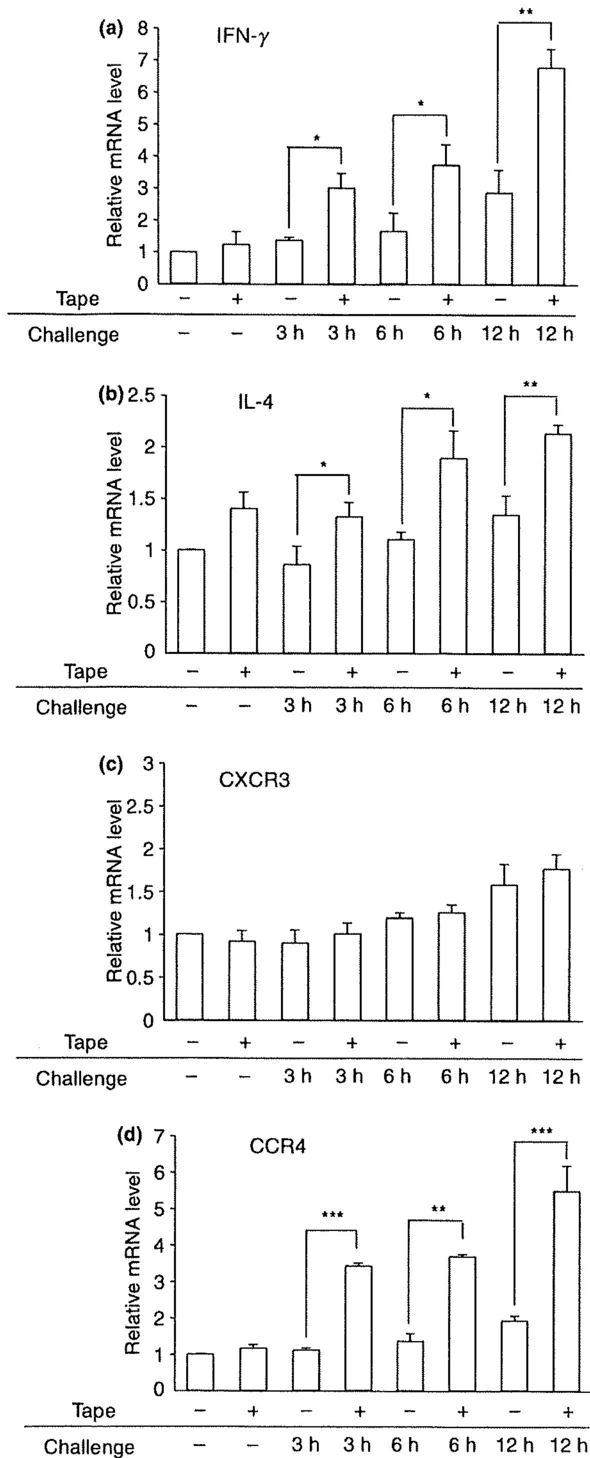

Figure 5. Real-time PCR analysis of mRNA expression for IFN-γ, IL-4 and CCR4 in the challenged skin. BALB/c mice were sensitized on the shaved abdomen with 1% FITC three times a week for 2 weeks and earlobes were provoked by painting of 1% FITC 24 h after tape stripping. At 3, 6 and 12 h after challenge, the earlobes were prepared and homogenized and subjected to real-time PCR analysis to assess the expression of IFN-γ, IL-4 and CCR4. Data are expressed as the mean ± SD of five mice (*$P < 0.05$, **$P < 0.005$, ***$P < 0.0001$).

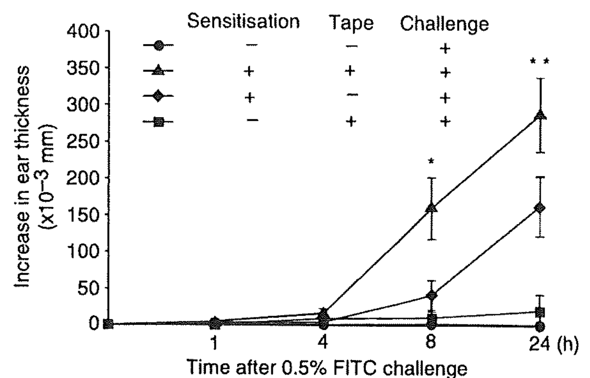

Figure 6. Augmentation of CHS to FITC n barrier-disrupted mice. BALB/c mice were repeatedly sensitized on the shaved abdomen with 1% FITC, and earlobes were provoked by painting of 0.5% FITC after tape stripping. Ear swelling responses were measured 1, 4, 8 and 24 h after challenge. Data represent Δ ear swelling from the basal ear thickness and are expressed as the mean ± SD of five mice (*$P = 0.00046$, **$P = 0.0026$).

Both tape-stripping and acetone-rubbing are known as a procedure for the acute barrier disruption, and in fact, the TEWL values of the treated skin were comparable in our study. However, the two treatments had different capacities to stimulate ECs to produce Th1 and Th2 chemokines. As keratinocytes can produce both Th1 and Th2 chemokines (11), this observation might be interpreted as an indication that tape-stripping and acetone-rubbing preferentially stimulate keratinocytes to produce Th2 and Th1 chemokines respectively. However, our recent study suggests that the main sources of Th1 (CXCL10, CXCL9 and CXCL11) and Th2 chemokines (CCL17 and CCL22) are keratinocytes and Langerhans cells respectively (13). We found that repeated application of hapten induces Th2 chemokine production by Langerhans cells. This raises an alternative possibility that tape-stripping stimulates Langerhans cells to produce Th2 chemokines and does keratinocytes to produce Th1 chemokines. In this scenario, acetone-rubbing possibly induces keratinocyte Th1 chemokine production without stimulating Langerhans cells to produce Th2 chemokines. Langerhans cells are also known to release CCL5 (14) and the observed increment of this eosinophil attractant might be derived from Langerhans cells. Tape-stripping has been shown to activate keratinocytes to produce Langerhans cell-maturating cytokines including interleukin-1α, tumor necrosis factor-α and granulocyte/macrophage colony stimulating factor (2). In parallel with this maturation, Langerhans cells might also release Th2 and eosinophil-chemoattracting chemokines.

The differences in the chemokine expression between the two stains were clearly seen with tape-stripping. BALB/c mice were more susceptible to tape-stripping than B6 mice in the

expression of all the chemokines examined. In particular, the expression of the Th2 chemokines and eosinophil chemoatrractant was markedly promoted in BALB/c mice. Although the increased expression of the Th2 and eosinophil chemokines in BALB/c mice is in accordance with the Th2-skewing property of this mouse strain, the exact mechanism underlying this preponderant expression remains unknown. To address this issue, we cultured keratinocytes from BALB/c and B6 mice, examined the production of Th2 chemokines after stimulation with interferon-γ and/or tumor necrosis factor-α and found no difference in the chemokine production between the two strains of mice. Therefore, the keratinocytes themselves are considered not to differ from each other. Given that interferon-γ suppresses Langerhans production of Th2 chemokines (13), the difference in the Th2 chemokine production might be attributable to the different Th1 or Th2 cytokine dominancy in each strain.

The tape-stripped skin exhibited enhanced degrees of the late-phase as well as delayed-type reactions upon challenge with hapten. This is thought to be a reflection of the infiltrates of eosinophils and Th2 cells (15). It is well known that patients with atopic dermatitis have skin barrier impaired by loss of filaggrin (16), decreased amounts of ceramide (8) and secondary damage following inflammation (17). Scratching caused by itch further exaggerates the barrier damage in atopic patients (18). Both the delayed-type and late-phase reactions have been put forward for the mechanisms underlying skin lesions of atopic dermatitis (19). The delayed-type reaction is clinically represented by eczematous dermatitis and mediated by Th1 and Tc1 cells (20). On the other hand, the late-phase reaction is clinically recognized by edematous erythema and mediated by Th2 and eosinophils (21). The late-phase reaction is prone to occur in the stratum corneum-removed skin and scratching exacerbates certain skin disorders such as atopic dermatitis by inducing Th2 and eosinophil-attracting chemokines. Moreover, it is tempting to speculate from our study that scratching could induce eosinophil infiltration in healthy skin and yield a late phase reaction without application of an allergen. Studies in human skin may clarify these important issues.

Acknowledgement

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Conflict of interest statement

The authors state no conflict of interest.

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ORIGINAL ARTICLE

Inducible Nitric Oxide Synthase Downmodulates Contact Hypersensitivity by Suppressing Dendritic Cell Migration and Survival

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Nitric oxide (NO) has several important roles in various physiological settings; one of the NO synthases, inducible NO synthase (iNOS), is induced by external stimulation of the skin. A prototypic example of external stimulation is hapten exposure, which induces the T-cell-mediated immune response known as contact hypersensitivity (CHS). We herein report on cutaneous dendritic cell (DC) function in the presence of an iNOS-specific inhibitor during the sensitization phase of CHS. First, we examined epidermal cell (EC) suspensions using flow cytometry with an iNOS antibody and confirmed that iNOS was expressed in the cytoplasm of Langerhans cells (LCs). We then studied the role of iNOS in CHS, and found that responses to DNFB were enhanced by the addition of an iNOS inhibitor during sensitization. Similarly, the iNOS inhibitor augmented FITC-induced migration of cutaneous DCs, including Langerin$^+$ LCs and Langerin$^-$ dermal DCs, to draining lymph nodes. Finally, we showed that iNOS inhibitor enhanced LC survival *in vitro*. We concluded that NO suppresses migration and survival of cutaneous DCs, resulting in a downmodulation of CHS.

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INTRODUCTION

Inducible nitric oxide (NO) synthase (iNOS) is one of the three isoenzymes that generate NO from its precursor L-arginine. In the skin, keratinocytes (Arany *et al.*, 1996), Langerhans cells (LCs) (Qureshi *et al.*, 1996), dermal fibroblasts (Wang *et al.*, 1996), and melanocytes (Rocha and Guillo, 2001) express iNOS upon stimulation with inflammatory cytokines and/or lipopolysaccharide (LPS). Although NO can be proinflammatory when produced in large amounts, it may also regulate adaptive immune responses (Kuchel *et al.*, 2003). The best characterized example is the induction of iNOS by LPS and IFN-γ in murine macrophages (Lu *et al.*, 1996), LCs (Qureshi *et al.*, 1996), and keratinocytes (Yamaoka *et al.*, 2000). Although some information has thus been accumulated regarding the *in vitro* effects of iNOS on skin immunocompetent cells, the *in vivo* actions of iNOS remain unknown.

Murine contact hypersensitivity (CHS) is an antigen-specific immune response consisting of the two phases, namely, sensitization and elicitation. The constituents involved in its pathogenesis are Th1/Tc1 cells serving as helper/effector cells (Akiba *et al.*, 2002); cutaneous dendritic cells (DCs), including epidermal LCs and dermal DCs (dDCs), as antigen-presenting cells (Kissenpfennig and Malissen, 2006); and keratinocytes as a source of IL-1α, tumor necrosis factor-α, and GM-CSF to the LCs (Sugita *et al.*, 2007). iNOS is induced in LCs and keratinocytes by contact allergens; this supports the view that iNOS has a role in CHS (Morita *et al.*, 1996). It has previously been reported that an iNOS inhibitor injected intradermally during the elicitation phase suppressed CHS responses (Ross *et al.*, 1998), but the specificity of this iNOS inhibitor is not clear; furthermore, the role of iNOS in the sensitization phase remains unknown.

In this study, we investigated the effects of an iNOS-specific inhibitor in order to determine whether iNOS functions as a positive or negative regulator in CHS. Our results show that iNOS suppresses the CHS response by downmodulating the migration and survival of DCs.

RESULTS

iNOS inhibitor enhances CHS response to DNFB

First, we tested the degree of CHS response in mice treated with L-N^6-iminoethyl-lysine (L-NIL), an iNOS inhibitor.

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Abbreviations: Ab, antibody; B6, C57BL/6; CCL21, CC chemokine ligand 21; CCR7, CC chemokine receptor 7; CHS, contact hypersensitivity; DC, dendritic cell; dDC, dermal DC; EC, epidermal cell; iNOS, inducible nitric oxide synthase; LC, Langerhans cell; L-NIL, L-N^6-iminoethyl-lysine; LPS, lipopolysaccharide; NO, nitric oxide; PBS, phosphate-buffered saline

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The mice were sensitized and challenged with DNFB, and their ear swelling responses were measured 24 hours after the challenge. A significantly higher degree of ear swelling response was observed in C57BL/6 (B6) mice treated intraperitoneally with L-NIL throughout the sensitization phase than in non-treated control mice (Figure 1a). Similar results were obtained 48 hours after the challenge (data not shown). In addition, histological analysis of the L-NIL-treated mice showed a remarkable infiltration of lymphocytes into the edematous dermis, which was not seen in untreated mice (Figure 1b). To confirm that L-NIL was biologically active in the skin when administered systemically, we measured the NOx ($NO_2^- + NO_3^-$) concentration of DNFB-sensitized skin. NOx production induced by DNFB was inhibited by an intraperitoneal injection of L-NIL (Supplementary Figure S1), suggesting that L-NIL is biologically active in lesional skin even when it is administered systemically.

iNOS expression in keratinocytes and LCs

Freshly isolated murine epidermal cells (ECs) were incubated for 24 hours in a culture medium, and the LCs and keratinocytes among them were analyzed for iNOS expression with flow cytometry. Both the keratinocytes and the LCs bore iNOS in the cytoplasm (Figure 2a). iNOS expression was greater in the mature LCs (major histocompatibility complex (MHC) class II high expression) than in the immature LCs (MHC class II intermediate expression). We carried out the same analysis on ECs that had been cultured for 24 hours in the presence of LPS, and found that LPS increased the number of LCs that highly expressed iNOS (Figure 2b).

iNOS inhibitor increases cutaneous DC accumulation in regional lymph nodes

To investigate the *in vivo* significance of iNOS for cutaneous DCs, we performed an FITC-induced cutaneous

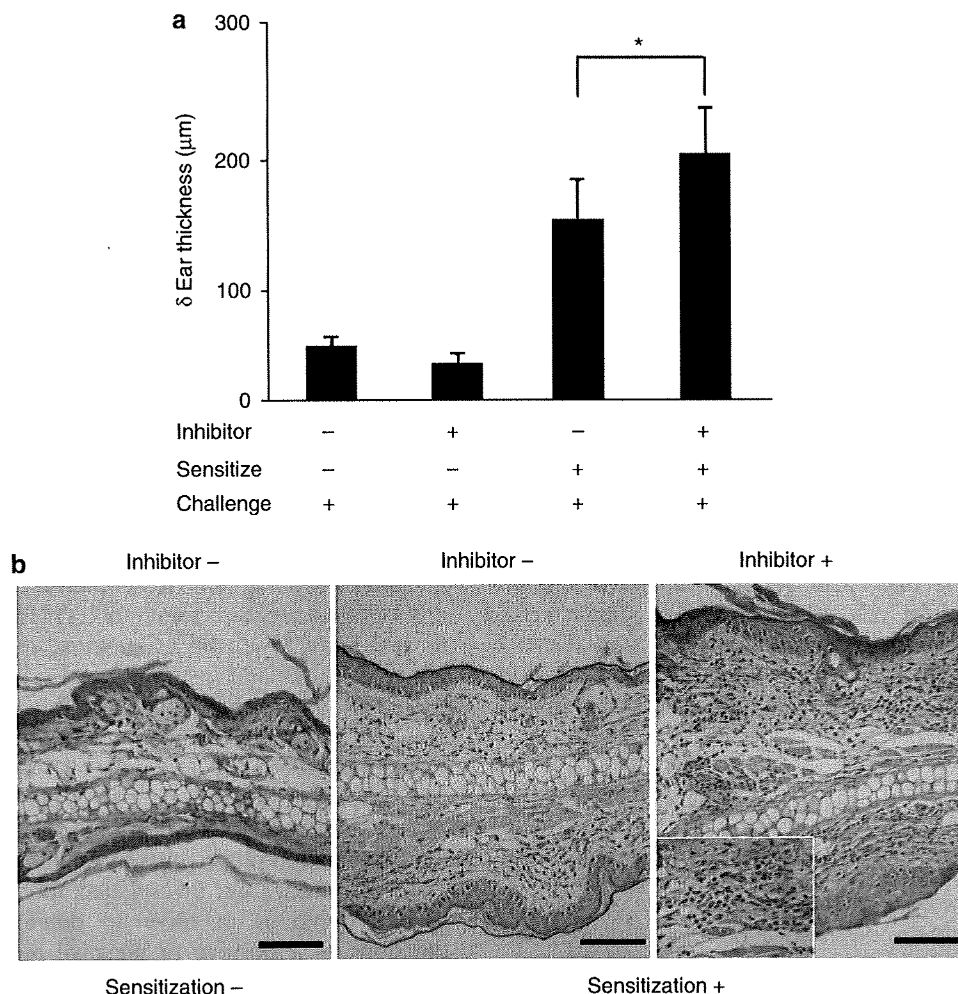

Figure 1. Increased CHS response to DNFB caused by blockade of iNOS. (**a**) For CHS model, B6 mice were immunized by the application of 0.5% DNFB to their shaved abdomens. They were challenged on both ears with 0.3% DNFB. iNOS inhibitor was applied through intraperitoneal injection (2.5 mg in 0.5 ml PBS twice daily). Ear thickness swelling was measured 24 hours later. Data are expressed as the mean ± SD of five mice. *$P<0.05$. (**b**) Non-sensitized ears, challenged ears, and challenged ears from non-treated mice (inhibitor –) were stained with hematoxylin and eosin. Inset: close-up view of hematoxylin and eosin staining of ears from mice treated with iNOS inhibitor, showing perivascular lymphocytic infiltration. Bar = 80 μm. Data are from three independent experiments.

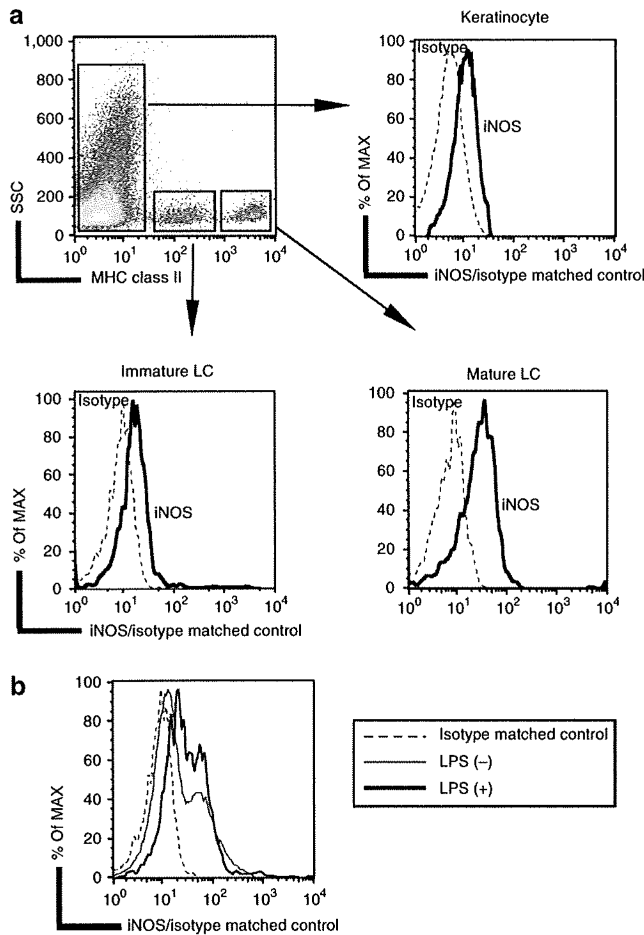

Figure 2. **Expression of iNOS by both keratinocytes and LCs.** (**a**) EC suspensions were analyzed for the expression of iNOS by means of flow cytometry. For intracellular detection of iNOS, cell fixation-permeabilization was performed before immunolabeling with anti-iNOS and anti-MHC class II mAbs. LCs or keratinocytes were gated by MHC class II positivity. (**b**) EC suspensions from naive mice were cultured with or without LPS (1 μg ml^{-1}) for 24 hours. The cultured cells were subjected to a flow cytometric analysis, which allowed us to measure the expression of iNOS. Data are from three independent experiments.

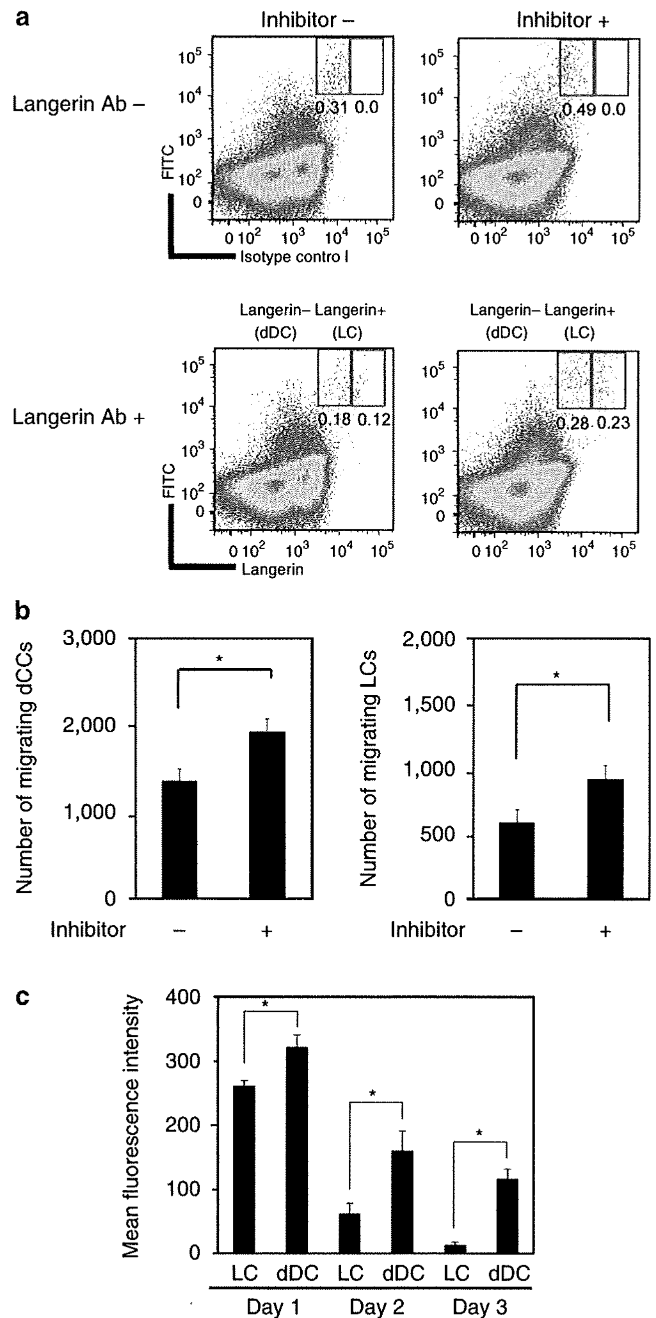

Figure 3. **Augmented cutaneous DC accumulation in regional lymph nodes by iNOS blockade.** (**a**) Langerin expression and FITC fluorescence in cells derived from regional lymph nodes were analyzed by means of flow cytometry 72 hours after the application of 200 μl of 2% FITC. The percentage of migrating LCs is indicated. (**b**) Migrating dDCs or LCs were counted 72 hours after FITC painting. Columns show the mean ± SD from at least four mice per group. *$P<0.05$. (**c**) Expression of iNOS in migrating LCs and dDCs. Draining lymph node cells were taken from mice painted with FITC on the abdomen and stained with anti-MHC class II, Langerin, and iNOS mAbs. Days 1, 2, and 3 indicate the number of days since FITC painting. Data are expressed as mean fluorescence intensity (MFI) for iNOS. MFI was the value of LCs or dDCs subtracted from that of the isotype-matched control. Columns show the mean ± SD. *$P<0.01$. Results are representative of three independent experiments.

DC migration assay. FITC applied to the skin is taken up by cutaneous DCs, which subsequently migrate to the draining lymph nodes as FITC$^+$ MHC class II$^+$ cells. We intraperitoneally injected L-NIL, an iNOS inhibitor (2.5 mg in 0.5 ml phosphate-buffered saline (PBS) twice daily for 4 consecutive days) or the equivalent amount of PBS into mice; 24 hours after the last injection, we applied FITC to the abdomen. We then isolated axillary and inguinal draining lymph node cells 72 hours after FITC application and characterized the FITC$^+$MHC class II$^+$ cutaneous DCs therein by flow cytometry. Staining for Langerin showed that two subsets of the FITC$^+$ MHC class II$^+$ cutaneous DCs, the dDCs and LCs, were present in significantly greater numbers because of treatment with iNOS inhibitor (Figure 3a and b). Therefore, the blockade of iNOS promoted lymph node accumulation of cutaneous DCs in response to skin exposure to an antigen.

iNOS expression in migrating LCs and dDCs
We examined iNOS expression in freshly isolated LCs and dDCs, both of which are capable of migrating into the lymph nodes on sensitization. The expression of iNOS in these cells was examined with FITC and anti-Langerin mAb. FITC was applied to the abdomen, and draining lymph node cells were sampled 24, 48, and 72 hours later. These cells were then labeled with anti-MHC class II mAb, anti-Langerin Ab, and anti-iNOS Ab. Although LCs are positive for Langerin, most dermal DCs are negative for Langerin (Nagao *et al.*, 2009), iNOS was present in both LCs and dDCs. The mean fluorescence intensity for iNOS was as follows: LC, 11.9±4.1; dDC, 36.6±20.5 (mean±SD of three mice).

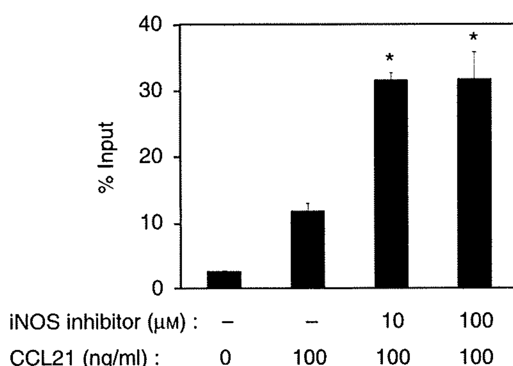

Figure 4. Chemotactic activity of epidermal LCs to CCL21. EC suspensions were incubated with or without iNOS inhibitor plus LPS in culture medium for 9 hours and applied to a transwell. CCL21 at 100 ng ml^{-1} was administered to the lower chamber. Migrating epidermal LCs in the lower chamber were identified as belonging to the MHC class II$^+$ subset. The number of migrating LCs was calculated. Columns represent the mean ± SD of triplicated transwells, and data are from three independent experiments. *$P<0.01$.

These data suggested that iNOS was weakly expressed only in freshly isolated LCs and dDCs, in amounts too small to be statistically significant. Nevertheless, we were able to observe that the dDCs showed a higher mean fluorescence intensity of iNOS expression than the LCs did (Figure 3c).

Chemotactic activity of LCs to CCL21
EC suspensions were incubated with LPS in a culture medium for 9 hours and applied to transwells in the presence or absence of L-NIL, an iNOS inhibitor. The migrating LCs in the lower chamber were identified as MHC class II$^+$ cells. CCL21 (CC chemokine ligand 21), a cytokine expressed in secondary lymphoid organs that mediates the chemotaxis of lymphocytes and DCs through its receptor, CCR7 (CC chemokine receptor 7; Saeki *et al.*, 1999), was then added to the lower chamber. All LCs exhibited a strong chemotactic response to this chemokine, but this response was significantly increased by the iNOS inhibitor (Figure 4).

iNOS inhibitor caused no alteration of the expression of co-stimulatory molecules or CCR7
The chemotaxis-promoting activity of the iNOS inhibitor, described above, raised the possibility that the iNOS inhibitor upregulates the expression of co-stimulatory molecules and CCR7. To determine whether this is the case, freshly isolated ECs were cultured for 24 hours in the presence or absence of the iNOS inhibitor, and the expression levels of these molecules were monitored by gating for MHC class II$^+$ LCs. After 24 hours of culture, a single population of LCs usually divides into two populations, with different expression levels of co-stimulatory molecules and CCR7 (Sugita *et al.*, 2007) (Figure 5a). The addition of the iNOS inhibitor did not alter the expression of CD86, CD80, CD40, or CCR7 (Figure 5a and b). In chemotaxis, however, the expression of

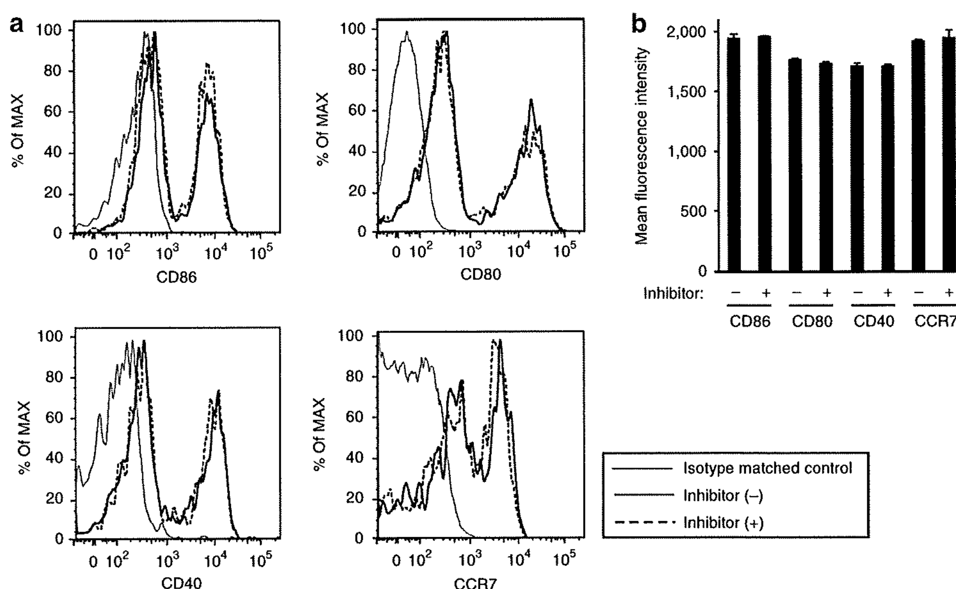

Figure 5. No modulation of CD86, CD80, CD40, or CCR7 expression in LCs by iNOS inhibitor. (**a** and **b**) EC suspensions from naive mice were cultured for 24 hours with or without iNOS inhibitor. The cultured LCs were examined for their expression levels of CD86, CD80, CD40, and CCR7. Data are representative of three independent experiments.