Cell Sorting and Quantitative PCR

Eosinophils and $CD4^+$ T cells from the bone marrow of IL-5 transgenic mice were isolated with a cell sorter (FACSAria II; BD, Franklin Lakes, NJ). The purity of the sorted cells was typically >95%. RNA was prepared from sorted cells with RNeasy (Qiagen, Valencia, CA), and cDNA was used for quantitative PCR. Murine S1P receptor expressions were quantified using TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA) in the ABI PRISM 7000 sequence detection system (Applied Biosystems) as follows: S1P1 (Assay ID: Mm00514644_m1), S1P2 (Assay ID: Mm01177794_m1), S1P3 (Assay ID: Mm00515669_m1), S1P4 (Assay ID: Mm00468695_s1), and S1P5 (Assay ID: Mm00474763_m1). As an endogenous reference for these PCR quantification studies, β-actin gene expression was measured using the TaqMan rodent β-actin control reagents (Applied Biosystems). The relative expression was calculated using the $2_T^{-}\Delta\Delta C$ method.[20] The expression of the target gene normalized to an endogenous, β-actin, reference and relative to a calibrator was given by the formula $2_T^{-}\Delta\Delta C$. Gene expression in untreated mice was used as the calibrator to calculate $\Delta\Delta C_T$.

Chemotaxis Assay

Eosinophils in the bone marrow of IL-5 transgenic mice were tested for transmigration across uncoated 5-μm Transwell filters (Corning Costar, Corning, NY) to S1P (Sigma-Aldrich, St Louis, MO) or medium in the lower chamber over 3 hours. In some experiments, the cells were pretreated with FTY720 or SEW2871 described previously.[21] The migrated cells were enumerated by flow cytometry.[22] The medium used in this assay was RPMI 1640 with 0.5% fatty acid-free bovine serum albumin (Calbiochem, San Diego, CA).

Statistical Analysis

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

Results

Reduction of Eosinophil Infiltration into the Skin by FTY720 in a Murine Repeatedly Hapten-Challenged Dermatitis

In a murine model of contact hypersensitivity, repeated application of a hapten such as TNCB to the same skin site in BALB/c mice results in a shift from a typical delayed-type hypersensitivity to an immediate type one. As a result, the late-phase reaction and eosinophil infiltration occur, usually 4 to 8 hours after the final challenge.[18] We used this model to evaluate the effect of FTY720 on tissue eosinophilia. Skin infiltration of eosinophils was inhibited by FTY720 treatment, but infiltration of lymphocytes was not changed (Figure 1A). This was further confirmed by Papanicolou staining, which showed reduced eosinophil infiltration by FTY720 (Figure 1B).

Impairment of Cutaneous Late-Phase Reaction by FTY720 in IL-5 Transgenic Mice

A previous study has demonstrated that the late-phase reaction caused by repeated painting with hapten is po-

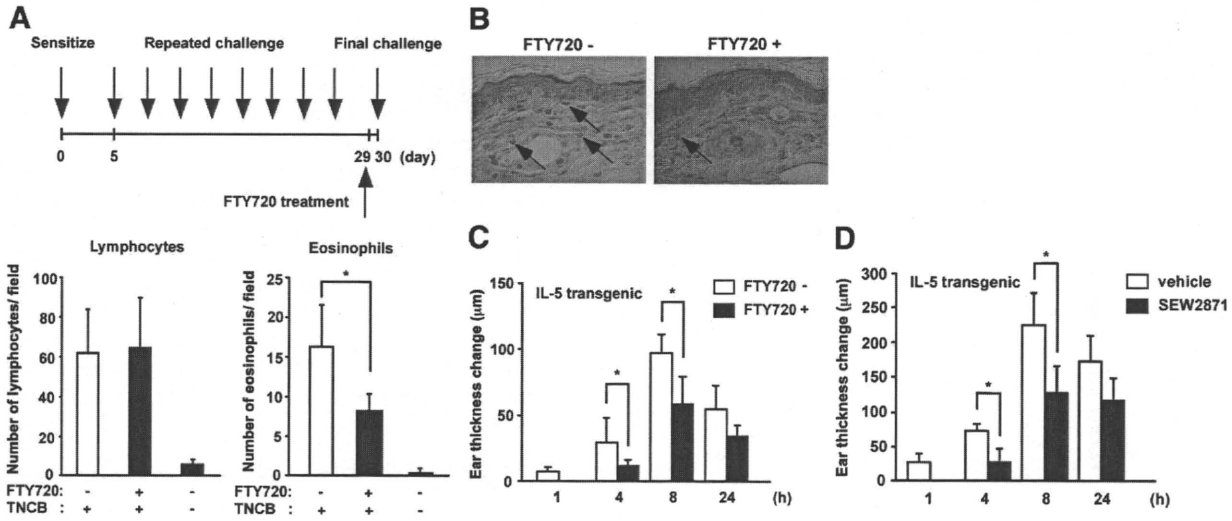

Figure 1. Effect of FTY720 on eosinophil accumulation in the skin. **A:** BALB/c mice were sensitized with TNCB on their shaved abdomens on day 0. They were then challenged on both sides of each ear on day five. The mice were repeatedly challenged on the original sensitized ears with 0.2% TNCB from days 8 to 29 at 3-day intervals. At 48 hours after the final challenge, histological sections were prepared from the skin. The total numbers of lymphocytes and eosinophils were counted in five high-power fields. $^*P < 0.05$. **B:** Representative pictures of skin sections from control and FTY720-treated mice challenged with TNCB. Data are from two independent experiments. **Arrows** indicate eosinophils. **C:** The ear swelling responses were measured before and at 1, 4, 8, and 24 hours after the final challenge. Data are from three independent experiments. $^*P < 0.05$. **D:** Similar results were obtained when mice were treated with SEW2871. Data are from two independent experiments. $^*P < 0.05$.

tentiated in IL-5 transgenic mice.[8] This enhancement may be related to tissue eosinophilia in the skin. Therefore, we investigated the significance of FTY720 on cutaneous late-phase reaction *in vivo* using IL-5 transgenic mice. IL-5 transgenic mice were sensitized with TNCB on day 0 and elicited with TNCB on the abdomen from days 5–29 at 3-day intervals. On day 30, they were challenged on both sides of each ear.

The treatment of mice with FTY720 12 hours before the challenge yielded reduced ear swelling responses at 4 and 8 hours after challenge (Figure 1C). Because the skin infiltration of eosinophils was suppressed by FTY720, the data were interpreted to result from the direct effect of FTY720 on eosinophils. Similar findings were observed when SEW2871 were administered (Figure 1D).

Numerical Alterations of Eosinophils after FTY720 Treatment

To clarify the mechanisms underlying the FTY720-induced reduction of skin eosinophils, we first evaluated the kinetics of eosinophils in the blood. It is known that FTY720 inhibits T cell egress from the secondary lymph nodes and decreases circulating T cells.[23–26] We examined the time course of decrease in the circulating Siglec-$F^+Gr\text{-}1^{int+}$ eosinophils after treatment of IL-5 transgenic mice with FTY720. The blood was drawn by intraocular venipuncture before and 1, 3, 5, and 7 days after FTY720 administration. The number of eosinophils was decreased markedly 1 to 5 days after FTY720 treatment and returned to the baseline level on day 7 (Figure 2). A similar reduction of circulating eosinophils was observed 6 hours after SEW2871 administration: vehicle, 13.6 ± 2.0 × 10/mm^3; and SEW2871 treatment, 2.2 ± 0.1 × 10/mm^3 ($P < 0.001$, mean ± SD of three mice). However, the decreased number of eosinophils in the blood returned to the normal range 48 hours after treatment with SEW2871 (12.5 ± 2.1 × 10/mm^3).

Because blood eosinophils were decreased by FTY720, we examined the localization of eosinophils after treatment with FTY720. At 24 hours after administration of

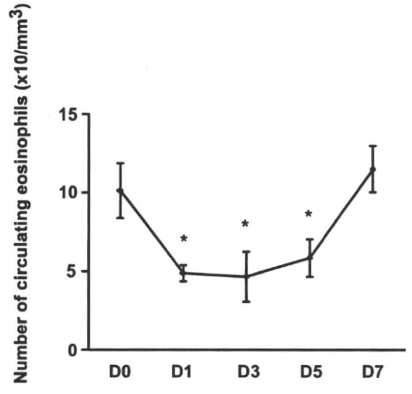

Figure 2. Numerical alterations of eosinophils in the peripheral blood following FTY720 administration. FTY720 was administered to IL-5 transgenic mice, and peripheral blood samples were periodically collected. Data are expressed as the mean ± SD. Data are from three independent experiments. *$P < 0.05$.

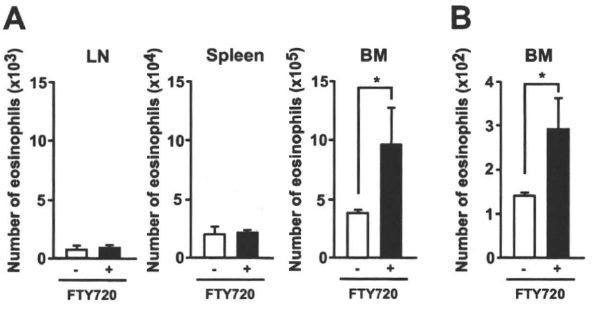

Figure 3. Accelerated accumulation of eosinophils by FTY720 in the bone marrow. **A:** FTY720 was administered to IL-5 transgenic mice. At 24 hours, lymph node cells, splenocytes, and bone marrow cells were harvested, and single-cell suspensions were analyzed by FACS for the presence of eosinophils. The homing of eosinophils to the bone marrow in the IL-5 transgenic mice was higher than that in the control mice. **B:** CFSE-labeled cells from IL-5 transgenic mice were adaptively transferred into control or FTY720-treated wild-type NZW mice. After 12 hours, the mice were killed, and eosinophils were harvested. Single-cell suspensions were analyzed by flow cytometry for the presence of CSFE-positive donor eosinophils. Error bars indicate the mean ± SEM from at least four mice per group. Data are from three independent experiments. *$P < 0.05$.

FTY720, the levels of eosinophils homing to the lymph nodes and spleen remained unchanged, but the number of eosinophils in the bone marrow increased (Figure 3A).

To examine the involvement of S1P in the regulation of eosinophil trafficking, CFSE-labeled cells collected from IL-5 transgenic mice were adoptively transferred to wild-type naïve NZW mice treated with or without FTY720. Treatment of the mice with FTY720 increased the number of CFSE-labeled eosinophils in the bone marrow (Figure 3B). These results suggest that FTY720 regulates the localization of eosinophils by inhibiting the egress of eosinophils from the bone marrow.

Inhibition of IL-5-Induced Eosinophilia by FTY720 Treatment

It is known that i.v. administration of IL-5 induces a rapid and sustained increase in the number of circulating eosinophils by mobilization from the bone marrow in B6 mice.[19] In confirmation of this notion, i.v. administration of 500 pmol/kg IL-5 induced pronounced blood eosinophilia in B6 mice (Figure 4, A and B). This increase was attenuated by treatment with FTY720 (Figure 4, A and B). In another study, B6 mice were pretreated with or without FTY720, and IL-5 was injected i.v. 24 hours later. The number of eosinophils was significantly increased in the bone marrow of FTY720-treated mice 12 hours after injection (Figure 4C). Thus, FTY720 depresses the recruitment of eosinophils from the bone marrow pool to the blood.

S1P Receptor mRNA Expressions in Eosinophils

To measure S1P receptor abundance in eosinophils, bone marrow cells were prepared from IL-5 transgenic mice, and Siglec-$F^+Gr\text{-}1^{int+}$ eosinophils and $CD4^+$ T cells were purified by a cell sorter. We evaluated the expression levels of S1P receptor mRNAs by quantitative PCR. S1P1, S1P3, and S1P4 mRNAs were expressed in eosin-

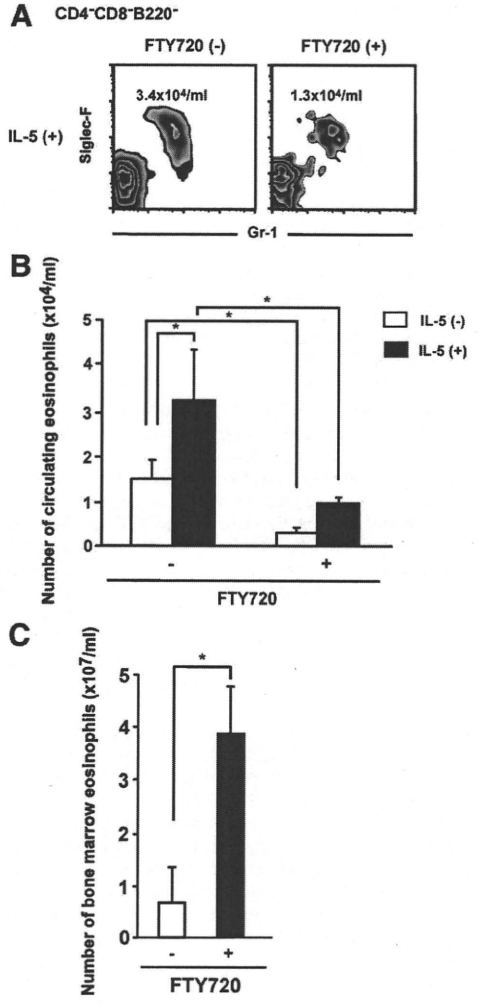

Figure 4. Effect of FTY720 after i.v. administration of IL-5 on circulating and bone marrow eosinophils. **A–C:** FTY720 was i.p. injected into B6 mice 24 hours before IL-5 injection. Mice were injected with IL-5 or vehicle alone. **A** and **B:** Circulating eosinophil numbers were determined 1 hour after injection of IL-5. **C:** After 12 hours, bone marrow samples were harvested, and single cell suspensions were analyzed by flow cytometry for the presence of eosinophils. Error bars indicate the mean ± SEM from at least four mice per group. Data are from three independent experiments. $^*P < 0.05$.

ophils, and their levels were higher than those in keratinocyte cell line PAM212. In contrast, S1P1 mRNA level was higher in T cells than eosinophils, and S1P4 level was comparable in T cells and eosinophils (Figure 5).

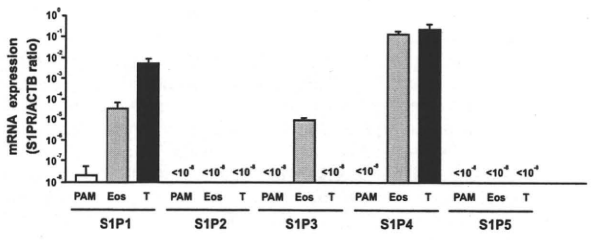

Figure 5. Quantitative PCR analysis of S1P receptor mRNAs from eosinophils. Bone marrow cells from IL-5 transgenic mice were sorted into Siglec-$F^+Gr\text{-}1^{int+}$ eosinophils and $CD4^+$ T cells. The amounts of S1P receptor mRNA were expressed as relative amounts of S1P receptor mRNA normalized to β-actin. Data are from two independent experiments.

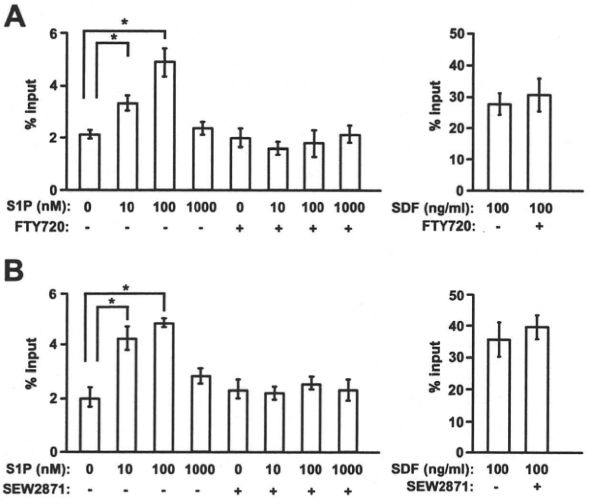

Figure 6. Chemotactic response of eosinophils to S1P. **A:** Bone marrow cells were prepared from IL-5 transgenic mice with or without FTY720. **B:** In some experiments, bone marrow cells were pretreated with 1000 nmol/L SEW2871. Chemotactic activities of eosinophils to stromal cell-derived factor 1 (SDF) were unaffected by treatment with either FTY720 or SEW2871. **A** and **B:** Input cells and cells that migrated to the lower well of the Transwell chamber in the absence of chemokine or in response to S1P were analyzed by flow cytometry to detect eosinophils. Bars represent means of triplicated Transwells, and data are from three independent experiments. $^*P < 0.05$.

Eosinophil Chemotactic Response to S1P

FTY720 inhibits T cell emigration through inhibition of S1P–S1P1 signaling.[27] Therefore, as readout of S1P1 function on eosinophils, we evaluated the chemotactic response to S1P. The cells were isolated from the bone marrow of IL-5 transgenic mice that were treated with FTY720. Chemotaxis assay using transwells showed that naive T cells had a good chemotactic response to S1P, which was ablated by FTY720 as reported previously.[12,21] Eosinophils had a significant chemotactic response to S1P but lost the ability to respond to S1P when mice were pretreated with FTY720 (Figure 6A). Similar inhibition of eosinophil chemotaxis to S1P was observed with SEW2871 (Figure 6B). The chemotactic response to stromal-cell derived factor-1 was not affected by the administration of FTY720 or SEW2871 (Figure 6). These data suggest that the migration of eosinophils toward S1P is highly dependent on S1P1.

Discussion

Our study demonstrated that the administration of FTY720 before the final challenge decreased the number of skin-infiltrating eosinophils and the cutaneous late-phase reaction. The egress of eosinophils from the bone marrow was inhibited by FTY720 treatment. This may subsequently lead to attenuate the accumulation of eosinophils in the skin.

By acting as an agonist for S1P receptors and thereby inhibiting lymphocyte egress from lymphoid organs, it has been reported that FTY720 shows a strong therapeutic potential for preventing the rejection of transplanted organs and for treating autoimmune diseases, such as autoimmune encephalomyelitis and systemic lupus ery-

thematosus.[28] In fact, some of these possible therapeutic uses are currently being clinically evaluated.[29] We have demonstrated that FTY720 impairs naive T cell circulation and markedly disrupts the acquired skin Th1 type immune response.[12] Consequently, FTY720 may have the potential to prevent the onset of disease rather than to treat memory T cell-mediated acquired skin immune responses.[12]

It remains unclear from this study how S1P-S1P receptor signaling participate in the infiltration of eosinophils from the blood to the skin. Several possible mechanisms for the regulation of eosinophil trafficking into the skin can be proposed. First, FTY720 might directly inhibit the migration of eosinophil toward the skin constituents, because eosinophils express S1P1, and S1P is induced at the epidermis of the skin.[30,31] This idea is supported by the results of our chemotaxis assay, which showed a direct suppressive effect of FTY720 on eosinophil migration toward S1P. Second, a significant reduction of the eosinophilic inflammation in the skin was observed with FTY720, suggesting that the decrease in circulating eosinophils may affect the eosinophil recruitment process. Indeed, FTY720 enhanced the accumulation of adaptively transferred eosinophils in the bone marrow, indicating that eosinophil retention in this organ is also regulated by S1P.

Eosinophils play a key role in the development of cutaneous late-phase reactions. Circulating eosinophils are complemented from the bone marrow in an S1P-S1P1-dependent manner. Previous studies have reported that a decrease in S1P release by sphingosine kinase inhibitors controls a mouse asthmatic model. The authors demonstrated that inhaled delivery of sphingosine kinase inhibitors prevented eosinophilic inflammations and goblet hyperplasia induced by OVA administration.[32] Their observation and our finding provide a possibility that FTY720 may be a candidate of treatment modalities for eosinophilic cutaneous inflammations, such as atopic dermatitis, hypereosinophilic syndrome, and Churg-Strauss syndrome.[33,34]

There have been few studies on S1P receptor expressions on murine eosinophils and the functional role of S1P1 on eosinophils *in vivo*.[9,35] Our current study indicated that S1P1 expression was lower in eosinophils than T cells. Therefore, the S1P-S1P1 dependency of eosinophils may be lower than that of T cells, which might explain the less inhibitory effects of FTY720 on eosinophils in the blood. In the model where mice were repeatedly challenged with a hapten, FTY720 also reduced the number of skin-infiltrating eosinophils but not T cells. Consequently, FTY720 attenuated the late-phase reaction along with a reduction of eosinophil infiltration. S1P1 is known to be required for normal B cell development.[36] S1P4 signaling in migratory response of murine T cells toward S1P remains controversial.[37,38] In contrast to S1P1 and S1P4 in B and T cells, the roles of these receptors for eosinophils remain unclear. In human, the eosinophil recruitment process has been reported to depend on not only S1P1 but also S1P2 and S1P3 mRNA levels.[35] Although we cannot rule out a possibility that the localization of eosinophils is related to S1P3, our *in vivo* and *in vitro* studies using SEW2871 and FTY720 strongly suggest that S1P1 is involved in bone marrow egress and peripheral tissue localization of eosinophils.

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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

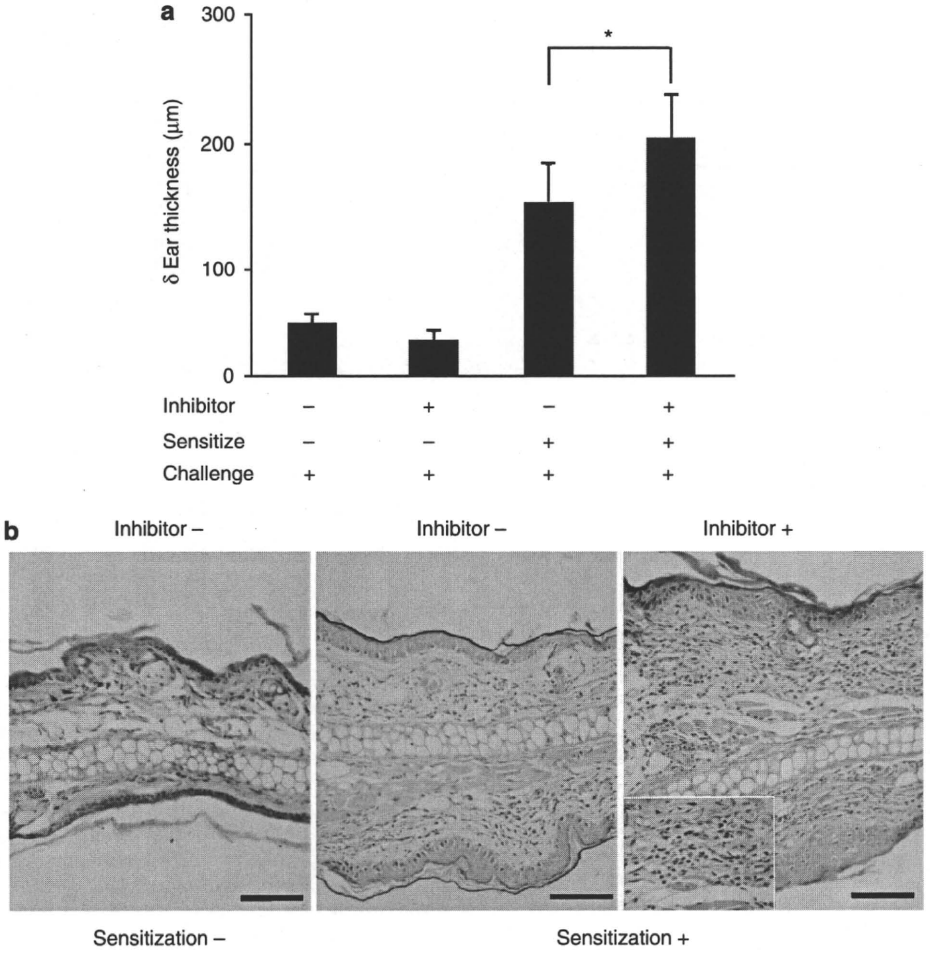

Figure 1. Increased CHS response to DNFB caused by blockade of iNOS. (**a**) For CHS model, B6 mice were immunized by the application of 0.5% DNFB to their shaved abdomens. They were challenged on both ears with 0.3% DNFB. iNOS inhibitor was applied through intraperitoneal injection (2.5 mg in 0.5 ml 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.

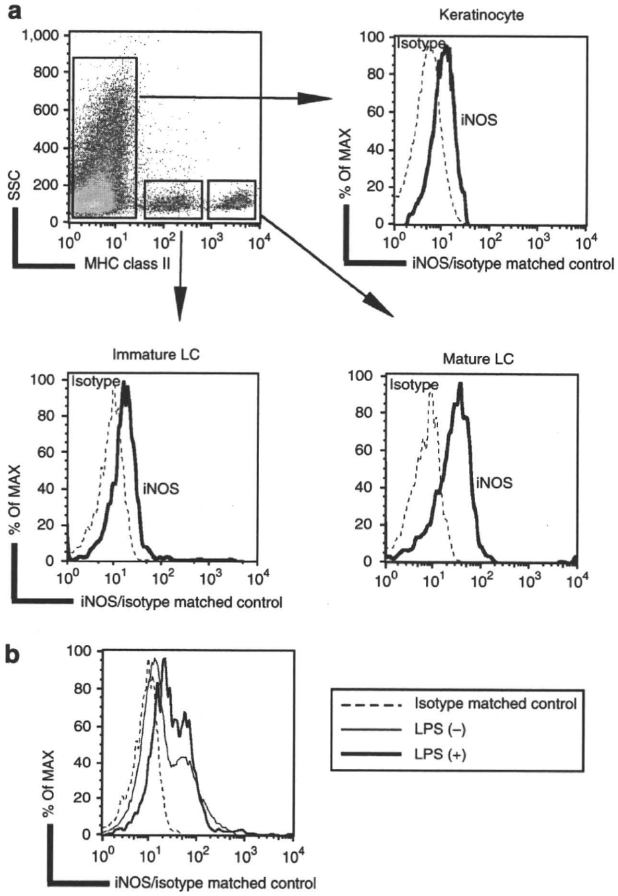

Figure 2. Expression of iNOS by both keratinocytes and LCs. (**a**) EC suspensions were analyzed for the expression of iNOS by means of flow cytometry. For intracellular detection of iNOS, cell fixation–permeabilization was performed before immunolabeling with anti-iNOS and anti-MHC class II mAbs. LCs 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.

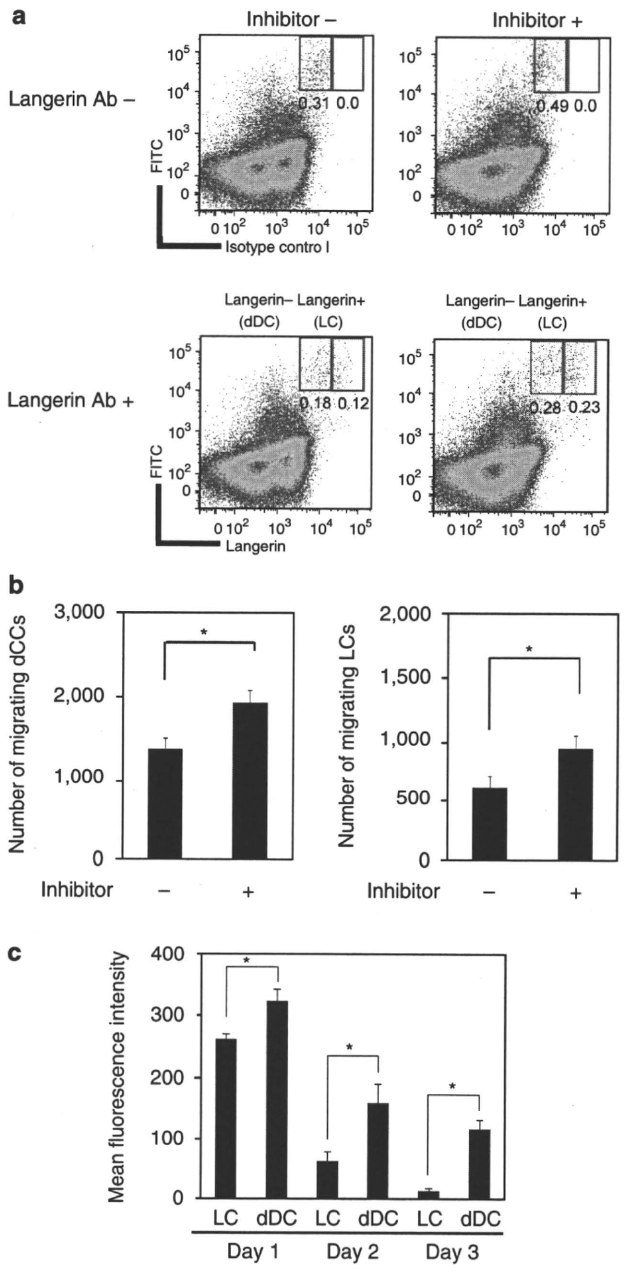

Figure 3. Augmented cutaneous DC accumulation in regional lymph nodes by iNOS blockade. (**a**) Langerin expression and FITC fluorescence in cells derived from regional lymph nodes were analyzed by means of flow cytometry 72 hours after the application of 200 μl of 2% FITC. The percentage of migrating 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). 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).

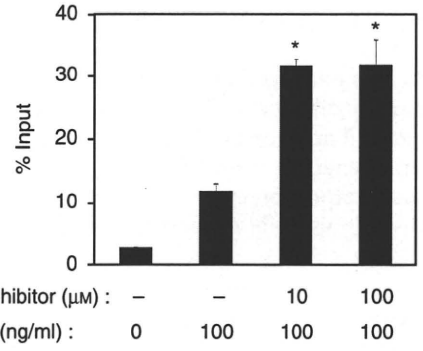

Figure 4. Chemotactic activity of epidermal LCs to CCL21. EC suspensions were incubated with or without iNOS inhibitor plus LPS in culture medium for 9 hours and applied to a transwell. CCL21 at 100 ng ml^{-1} was administered to the lower chamber. Migrating epidermal LCs in the lower chamber were identified 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.

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

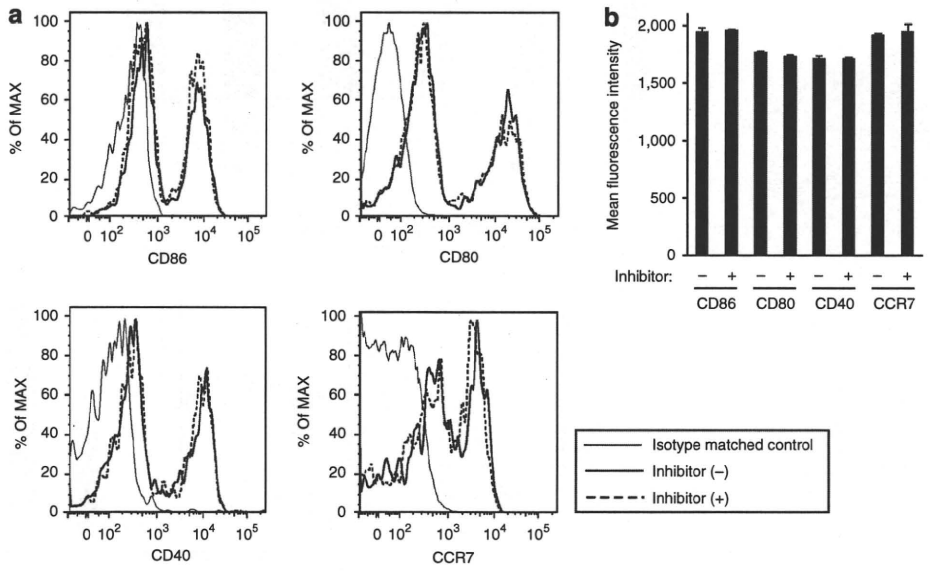

Figure 5. No modulation of CD86, CD80, CD40, or CCR7 expression in LCs by iNOS inhibitor. (**a** and **b**) EC suspensions from naive mice were cultured for 24 hours with or without iNOS inhibitor. The cultured LCs were examined for their expression levels of CD86, CD80, CD40, and CCR7. Data are representative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

CCR7 in DCs is not sufficient to guarantee its functionality (Sanchez-Sanchez *et al.*, 2006); therefore, it is possible that iNOS alters certain downstream functions of LCs without affecting CCR7 expression.

iNOS inhibitor reduces LPS-induced apoptosis of LC

We then evaluated the effect of endogenous iNOS activity on the viability of LCs. EC suspensions from the earlobes of B6 mice were cultured for 9 hours with or without LPS in the presence or absence of L-NIL, an iNOS inhibitor. LPS stimulation reduced the number of LCs, but this reduction was reversed by the addition of the iNOS inhibitor (Figure 6a). It has been reported that epidermal LCs are unable to proliferate *in vitro* when they are incubated as an EC suspension (Schuler and Steinman, 1985), suggesting that the observed effects of the iNOS inhibitor stem from a survival change.

To examine whether the iNOS inhibitor promotes the survival of LCs, cellular viability was assessed through flow cytometry after Annexin V/propidium iodide staining and 9 hours of culture (Figure 6b). This flow cytometry experiment used anti-MHC class II and anti-CD11c mAbs. The percentage of Annexin V and propidium iodide double-positive cells in samples that had been treated with 100 μM iNOS inhibitor and those that had not was as follows: 100 μM, 1.6 ± 0.4%; no addition, 2.2 ± 1.0% (mean ± SD, $n = 3$). These results suggest that the reduction of apoptotic cells that occurs through iNOS inhibitor treatment is not due to the increment of necrotic cells. We found that LPS-induced apoptosis of LCs was reduced by the addition of the iNOS inhibitor (Figure 6c), suggesting that the iNOS inhibitor promotes DC survival.

DISCUSSION

The results of this study on the effects of an iNOS inhibitor include several major findings about the involvement of NO in the sensitization phase of CHS. First, CHS as a model of acquired skin immune response was enhanced by treatment with the iNOS inhibitor. Second, the iNOS inhibitor markedly increased the number of migrating cutaneous DCs. Accordingly, the chemotactic response of LCs to CCL21 was enhanced by *in vitro* incubation with the iNOS inhibitor. Finally, the iNOS inhibitor was capable of reducing LPS-induced apoptosis of LCs.

It has generally been believed that iNOS is involved in CHS as a producer of NO and a trigger of inflammatory responses (Cals-Grierson and Ormerod, 2004). It has been

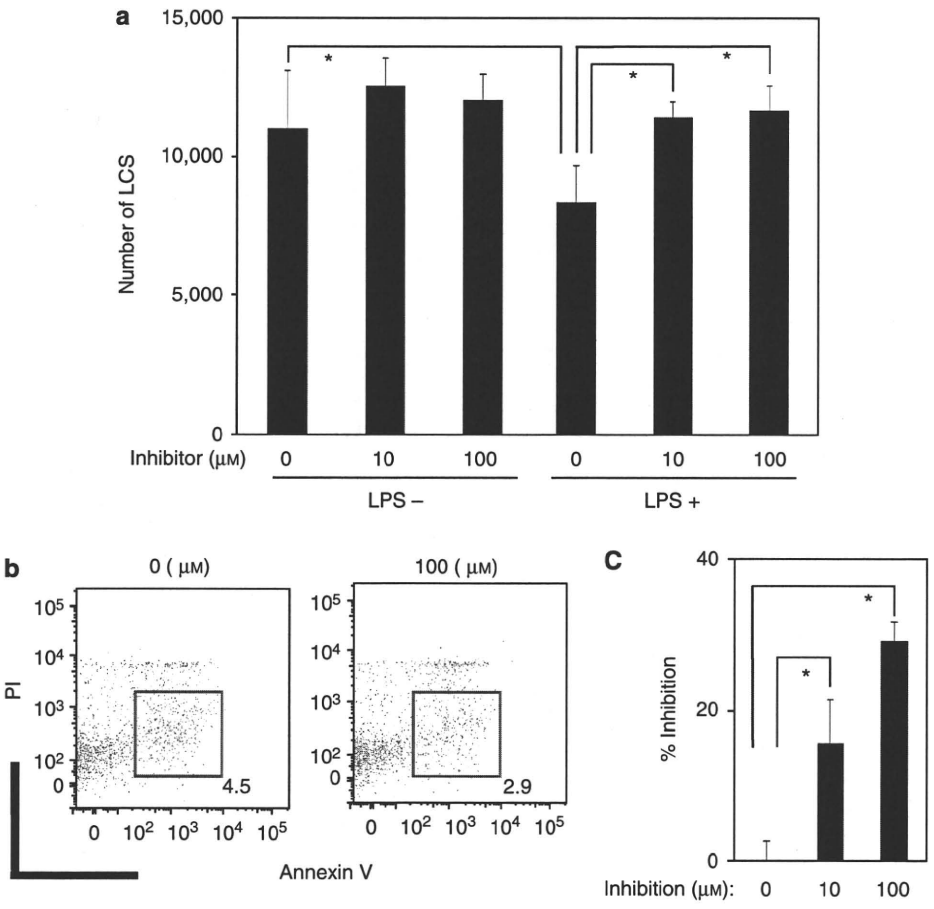

Figure 6. The effect of LPS on LC survival. (**a**) EC suspensions were cultured with or without LPS and iNOS inhibitor for 9–12 hours. iNOS inhibitor dose dependently increased the number of LCs. Columns show the mean ± SD. *$P < 0.05$. Data are representative of three independent experiments. (**b**) Apoptosis was determined using annexin V/propidium iodide double staining. (**c**) The percentage inhibition is calculated. Data represent the mean ± SD. *$P < 0.05$.

reported that iNOS and NO were produced in human skin subjected to positive patch tests to contact allergens (Cruz *et al.*, 2007; Ormerod *et al.*, 1997) and to the irritant sodium lauryl sulfate; nevertheless, it remains controversial whether iNOS is inhibitory or augmentative in CHS (Ross and Reske-Kunz, 2001). It has also been reported that an iNOS inhibitor exerted a suppressive effect on the CHS response to 2,4,6-trinitrochlorobenzene (TNCB) (Musoh *et al.*, 1998), although this effect was limited to the first few hours of the response, and neither NO production, NO-expressing cells, nor NOS isoenzymes were identified. Thus, the mode of action of iNOS in CHS remains a matter of debate.

It is possible that iNOS first modulates keratinocytes so that they produce cytokines, thereby subsequently modifying LC function. Yet, we found that the production levels of GM-CSF and tumor necrosis factor-α in the culture supernatant of primary keratinocytes of B6 mice cultured for 72 hours were not significantly affected by the presence of the iNOS inhibitor (Supplementary Figure S2). With regard to the effect of iNOS on T cells, we cultured immune $CD4^+$ T cells for 72 hours with varying concentrations of the iNOS inhibitor in the presence of anti-CD3 mAb and found that the iNOS inhibitor was incapable of stimulating T cells *per se* (Supplementary Figure S3).

LCs have traditionally been believed to have a role in the induction of CHS, but three research groups have reported three contradictory findings after applying haptens to transgenic mice deficient in LCs: a diminished reaction (Bennett *et al.*, 2005), an enhanced reaction (Kaplan *et al.*, 2005), and an unchanged response (Kissenpfennig *et al.*, 2005). Moreover, recent findings suggest that dDCs has a critical role in initiating CHS (Fukunaga *et al.*, 2008). In our study, dDCs augmented iNOS expression in response to hapten application more than LCs did. Our findings suggest that iNOS can suppress cutaneous DC migration and survival. Given that, in CHS, dDCs and LCs have positive and regulatory capacities, respectively, our findings on cutaneous DCs seem to be consistent with the observation that iNOS inhibitor induces an enhancement of CHS.

The findings of our study are clinically relevant in two respects. First, iNOS and NO exert immunosuppressive effects on cutaneous inflammation. In this context, the *in vivo* immunosuppressive effect of NO has also been shown in human studies (Kuchel *et al.*, 2003). Second, iNOS reduces cutaneous DC function and survival in the sensitization phase of CHS. The observation that NO directly reduces the number of LCs in the human epidermis supports our conclusion (Mowbray *et al.*, 2008).

MATERIALS AND METHODS

Animals and reagents

Female B6 mice were purchased from Japan SLC (Hamamatsu, Japan). All experiments were conducted on 8-week-old mice. The mice were maintained on a 12-hour light/dark cycle under a specific pathogen-free condition. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Occupational and Environmental Health. L-NIL (a highly selective inhibitor of iNOS enzymatic activity) and LPS were obtained from Sigma-Aldrich (St Louis, MO). CCL21 was purchased from R&D Systems (Minneapolis, MN).

DNFB-induced CHS model

B6 mice were sensitized through the application of 25 μl of 0.5% (v/v) DNFB in 4:1 acetone/olive oil to their shaved abdomens on day 0. They were then challenged on both sides of each ear with 20 μl of 0.3% (v/v) DNFB. Ear thickness change was calculated as follows: (ear thickness 24 or 48 hours after challenge)−(ear thickness before challenge). iNOS was inhibited with L-NIL as described previously (Diefenbach *et al.*, 1998). Briefly, L-NIL was applied by intraperitoneal injection (2.5 mg in 0.5 ml PBS twice daily) for 6 consecutive days starting 1 day before sensitization. We chose his protocol because treatment with L-NIL at this concentration and frequency for 4-13 days is one of the most common methods of blocking *in vivo* activity of iNOS (Diefenbach *et al.*, 1998; Stallmeyer *et al.*, 1999).

EC preparation and culture

EC suspensions were prepared as described previously (Tokura *et al.*, 1994). Ears of naive mice were split along the plane of the cartilage, which was then removed together with the subcutaneous tissue. These specimens were incubated for 1 hour at 37 °C in a 0.2% solution of trypsin in PBS. After incubation, the epidermis was separated from the dermis and the separated epidermal sheets were rubbed to disperse the ECs in PBS supplemented with 10% fetal calf serum. The cells were filtered and washed twice in PBS. As a culture medium, RPMI-1640 (Sigma-Aldrich) was supplemented with 10% heat-inactivated fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin.

Preparation of dermal cell suspensions

Dermal cells were obtained from normal murine skin from which the epidermis had been removed. Samples were minced and incubated for 2 hours at 37 °C in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with collagenase (2 mg ml^{-1}; Sigma-Aldrich), hyaluronidase (260 U ml^{-1}; Sigma-Aldrich), DNase (0.1 mg ml^{-1}; ICN, Costa Mesa, CA), and 10 mM HEPES (Sigma-Aldrich). The obtained cells were filtered through a 40-μm filter.

Flow cytometry

For flow cytometry, cells were plated at a density of 1×10^6 cells per well in 96-well U-bottomed plates (Falcon, BD Biosciences, San Jose, CA). Cells were then stained for 20 minutes on ice with mAbs in 25 μl of PBS containing 2% fetal calf serum, 1 mM EDTA, and 0.1% NaN_3, and washed twice with 200 μl of this buffer. Data were collected on a FACSCanto system (BD Biosciences) and analyzed with FlowJo software (TreeStar, San Carlos, CA). The mAbs used were as follows: FITC-conjugated anti-CD86 and Annexin V mAbs, PE-conjugated anti-CD80 and CD40 mAbs, PE-Cy5-conjugated anti-MHC class II mAb, APC-conjugated anti-CD11c mAb (all from BD Biosciences), and PE-Cy7-conjugated anti-CCR7 mAb (eBioscience, San Diego, CA). For detection of Langerin and iNOS, anti-Langerin Ab (eBioscience), PE-conjugated anti-iNOS Ab (Santa Cruz Biotechnology, Santa Cruz, CA), and PE-Cy5-conjugated streptavidin were

used after fixation and permeabilization of cells using a Cytofix/Cytoperm Kit (BD Biosciences).

Histology

At 48 hours after the challenge with hapten, the ears of B6 mice were excised and fixed in 10% formaldehyde. Sections of 5-μm thickness were prepared and stained with hematoxylin and eosin.

FITC-induced cutaneous DC migration

The shaved abdomens of the mice were painted with 200 μl of 2% FITC (Sigma-Aldrich) dissolved in a 1:1 (v/v) acetone/dibutyl phthalate (Sigma-Aldrich) mixture, and the iNOS inhibitor was applied through intraperitoneal injection (2.5 mg in 0.5 ml PBS) twice daily for 4 days. Cutaneous DCs migrating into the draining inguinal and axillary lymph nodes were then counted by means of flow cytometry (Kabashima *et al.*, 2007) using Flow-Count Fluorospheres (Beckman Coulter, Fullerton, CA). The principle of Flow-Count Fluorospheres is based on the precise mixing of microparticles whose concentration and volume are known. Before flow cytometric analysis, 10 μl of Flow-Count Fluorospheres were added to each specimen. The percentages of fluorospheres and migrating DCs within each node were then determined using the FACSCanto system (BD Biosciences). To find the number of migrating DCs, the ratio of DCs to fluorospheres was counted using the following formula, based on Reimann *et al.* (2000), with some modifications: number of migrating DCs = (percentage of migrating DCs/percentage of fluorospheres) × number of fluorospheres.

Chemotaxis assay

EC suspensions were incubated for 9 hours with or without the iNOS inhibitor, and then tested for transmigration across uncoated 5-μm transwell filters (Corning Costar, Corning, NY) to CCL21 or medium in the lower chamber for 3 hours. Migrating cells were enumerated by means of flow cytometry (Ngo *et al.*, 1998). The medium used in this assay was RPMI-1640 with 0.5% fatty acid-free bovine serum albumin (Calbiochem, San Diego, CA).

Apoptosis analysis

The EC suspensions from B6 mice were stained with PE-Cy5-conjugated anti-MHC class II mAb for 20 minutes on ice, then stained with FITC-conjugated Annexin V and propidium iodide (BD Pharmingen, Franklin Lakes, NJ), according to the manufacturer's protocol. The number of LCs was assessed by means of flow cytometry with anti-MHC class II and APC-conjugated anti-CD11c mAbs. Apoptosis in LCs was analyzed using a FACSCanto system with FlowJo software.

Statistical analysis

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

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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Cholinergic Urticaria: Studies on the Muscarinic Cholinergic Receptor M3 in Anhidrotic and Hypohidrotic Skin

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TO THE EDITOR

Cholinergic urticaria (CU) is a sweating-associated, syringeal orifice-coincident wheal mediated by acetylcholine. CU is occasionally associated with depressed sweating, as reported under the name of anhidrosis (complete lack of sweating) or hypohidrosis (incomplete lack of sweating) (Itakura *et al.*, 2000). There have been reported 29 patients with CU with anhidrosis and/or hypohidrosis (CUAH) (Kay and Maibach, 1969; Itakura *et al.*, 2000; Yoshida *et al.*, 2009). One hypothesis for the relationship between the wheal formation and the depressed sweating is that the patients are hypersensitive to unknown substance(s) in their sweat and develop wheals in response to sweat leaking from the syringeal ducts to the dermis possibly by obstruction of the ducts (Adachi *et al.*, 1994; Kobayashi *et al.*, 2002). In fact, some patients with common CU exhibit wheals to intradermal injection of the patients' own diluted sweat as well as acetylcholine and histologically show sweat duct obstruction (Fukunaga *et al.*, 2005). However, none of the reported patients with CUAH were positive to the intradermally injected autologous sweat, suggesting that "sweat hypersensitivity" is not responsible for CUAH. In addition, if sweat hypersensitivity is the mechanism, the anhidrotic area should be the predilection site for wheal, but such an observation has not been reported.

The study design was approved by the review board of University of Occupational and Environmental Health. Measurements in this study were performed after informed consent had been obtained. The study was conducted according to the Declaration of Helsinki guidelines. To address its mechanism, we investigated four nonsmoker male CUAH patients, aged 23 (case 1), 24 (case 2), 50 (case 3), and 36 years (case 4). The exercise challenge and iodine–starch assessment for sweat induction (Kobayashi *et al.*, 2002) revealed that the skin surfaces of all patients were divided into the anhidrotic and hypohidrotic areas (Figure 1a). The skin with normal sweating was not seen in any patient. As represented by case 1, the applied iodine–starch was discolored by sweat in the hypohidrotic but not anhidrotic area (Figure 1b, left), and following exercise challenge, the patients developed pinpoint wheals in only the hypohidrotic areas (Figure 1b, right). Intradermal injection of acetylcholine yielded wheal with sweating at only the hypohidrotic areas (Figure 1c). The injection of autologous sweat or serum did not produce wheal in either hypohidrotic or anhidrotic area. Histologically, a periglandular lymphocytic infiltrate was observed around eccrine glands in the anhidrotic but not hypohidrotic area of all patients (Figure 1d). There was no occlusion of sweat ducts. By immunohistochemistry, the infiltrating lymphocytes consisted of a mixture of $CD4^+$ and $CD8^+$ T cells (CD4/CD8 ratio, 1.21; Figure 1e).

Skin sections were immunohistochemically stained with anti-cholinergic receptor muscarin 3 (CHRM3) antibody (H-210, 1:50; Santa Cruz Biotechnology, Santa Cruz, CA), which is the most important CHR for sweating

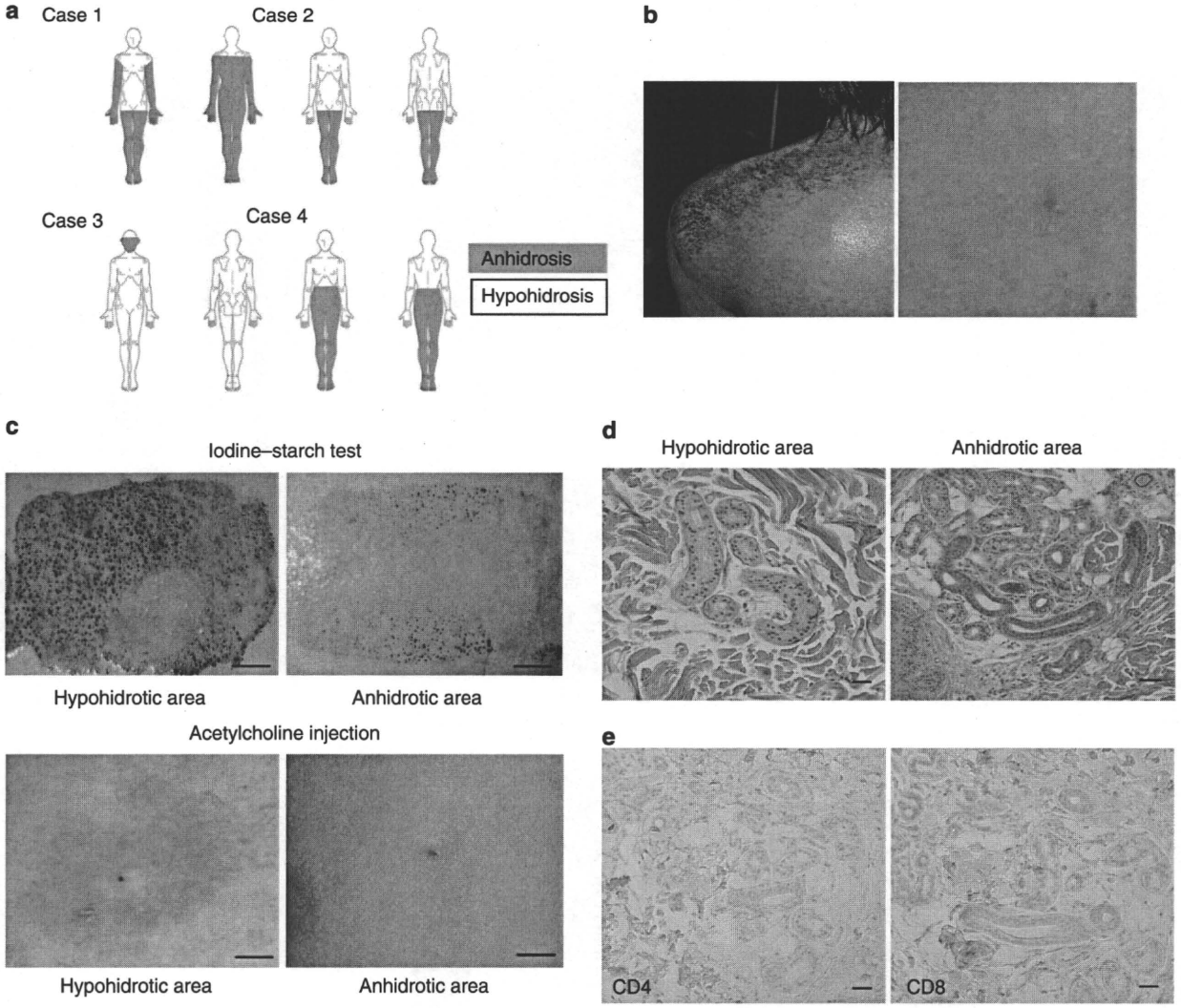

Figure 1. Clinical and histological studies. (**a**) Anhidrotic and hypohidrotic areas in each case. (**b**) Left: Iodine–starch test in case 1. Whereas the applied iodine–starch was discolored by sweat on the left shoulder (hypohidrotic area), such a black color change was not seen on the back, indicating the complete absence of sweating (anhidrotic area). Right: Exercise challenge induced wheals that coincided with sweat orifice on the hypohidrotic area. (**c**) Acethylcholine injection test in the anhidrotic and hypohidrotic areas as represented by case 1. After determination of the anhidrotic and hypohidrotic areas by exercise challenge and iodine–starch application (upper panel), acetylcholine was injected at both sites. Wheal occurred at the hypohidrotic but not anhidrotic area (lower panel). Bar = 1 cm. (**d**) Histological microphotographs of anhidrotic and hypohidrotic areas in case 1. Biopsy specimens were taken from the anhidrotic and hypohidrotic areas (hematoxyline and eosin, original magnification × 100). Note that lymphocytes infiltrate around the sweat glands in the anhidrotic but not hypohidrotic area. Bar = 30 μm. (**e**) Immunophenotype of infiltrating lymphocytes. The infiltrating lymphocytes were immunohistochemically stained for CD4 (left) and CD8 (right). Bar = 30 μm.

(Schiavone and Brambilla, 1991). Eccrine epithelial cells of a normal subject expressed CHRM3 (Figure 2a, left) as well as the epidermis and hair follicle cells. Although the cells in the hypohidrotic area bore CHRM3 at lower but moderate levels (Figure 2a, middle), no staining was seen in the anhidrotic areas (Figure 2a, right). Digitalized specimens were exported to JPG files by NDP view software (Hamamatsu Photonics, Hamamatsu, Japan), and three different areas of the cytoplasm of eccrine epithelial cells were expressed as "red density" (RD) (Hino *et al.*, 2010). CHRM3 expression was significantly lower in the anhidrotic than hypohidrotic areas (Figure 2b). However, specificity is the major issue in this immunohistochemical study. Therefore, real-time PCR was performed. Total RNA from serial sections was extracted in cases 1, 2, and 4 and reverse transcribed into complementary DNA using the Qiagen RNeasy FFPE Kit (Qiagen, Hilden, Germany) and first-strand cDNA synthesis kit RT-PCR (Roche Diagnostics, Indianapolis, IN). The expression levels of *CHRM3* and control *GAPDH* (*glyceraldehyde-3-phosphate dehydrogenase*) were examined using × 20 Assays-on-Demand Gene Expression Assay Mix (Hs00265216_s1 and Hs00266705_g1, respectively; Applied Biosystems, Foster City, CA). We performed PCR using RNAs without

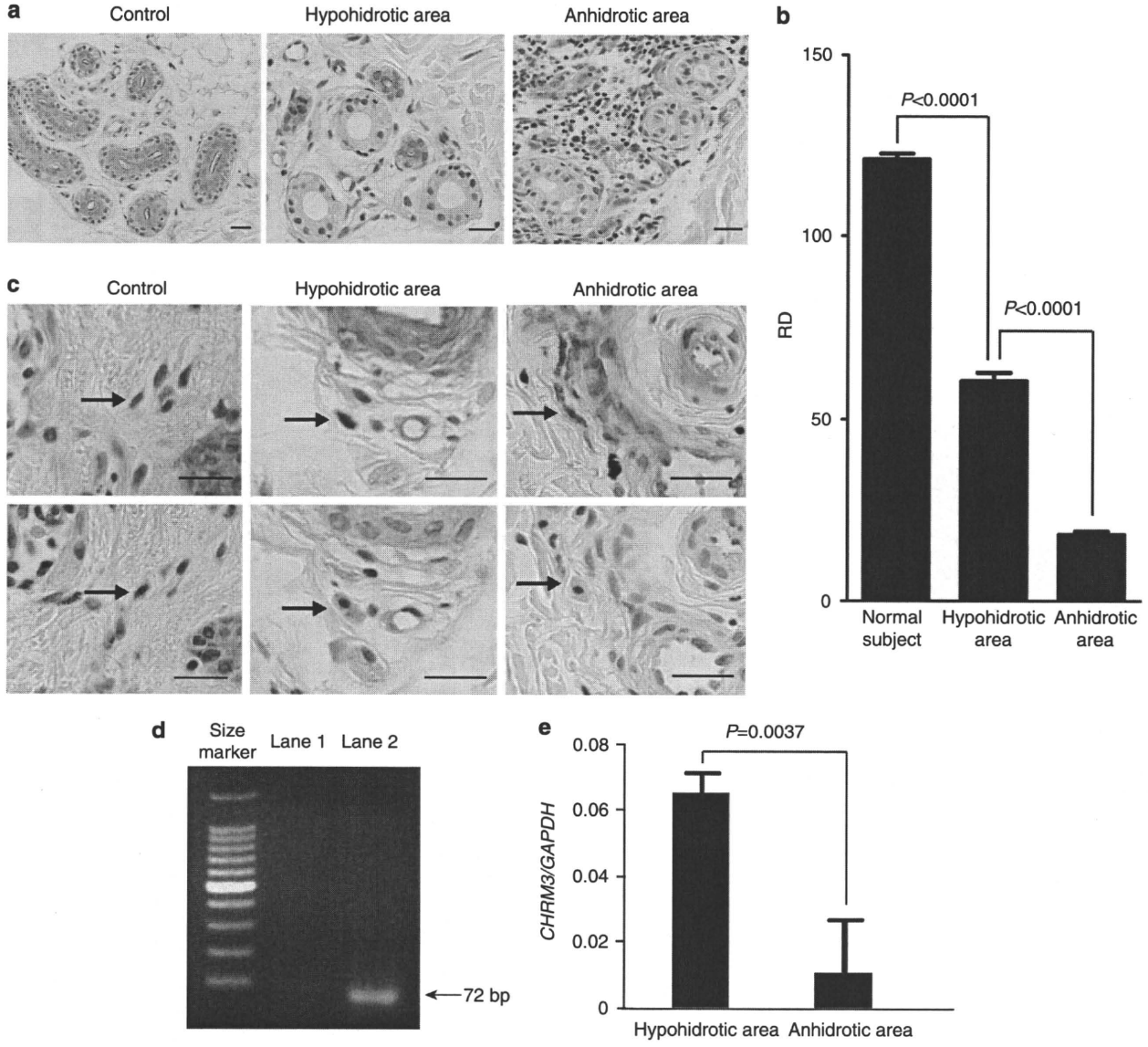

Figure 2. Expression of anti-cholinergic receptor muscarin 3 (CHRM3) in eccrine gland epithelial cells and mast cells. (**a**) CHRM3 expression in eccrine gland epithelial cells. Sections from normal subject's skin (left), and the hypohidrotic skin (middle) and anhidrotic skin (right) of case 1 were immunohistochemically stained with anti-CHRM3 antibody. Bar = 30 μm. (**b**) Staining intensity of CHRM3 expressed as "red density" (RD) in the anhidrotic and hypohidrotic areas of the four cases and in the skin of a normal subject. Three areas of eccrine epithelial cells at the anhidrotic and hypohidrotic areas in each case were subjected to RD analysis, and the mean RD intensity in each case was obtained. Then, the mean ± SD of the four cases was calculated. (**c**) CHRM3 expression in mast cells. Skin sections of a normal subject (control) and the hypohidrotic and anhidrotic skin of the patients were stained with toluidine blue (upper panel) or immunohistochemically with CHRM3 (lower panel). Serial sections of each specimen were alternatively stained for CHRM3 and toluidine blue. Mast cells were identified by toluidine blue (arrows). The corresponding sections for toluidine blue and CHRM3 are adjacent. Bar = 30 μm. (**d**) mRNA expression for CHRM3. The end products of real-time PCR using RNA without reverse transcriptase reaction (lane 1) and complementary DNA (cDNA) (lane 2) from hypohidrotic skin of case 1 were electrophoresed in 1.8% agarose gel along with 100 bp DNA ladder (Takara, Kyoto, Japan). *CHRM3* gene products were recognized at an expected size, 72 bp, only in lane 2. (**e**) The expression levels of *CHRM3* and control *GAPDH* (*glyceraldehyde-3-phosphate dehydrogenase*) in the hypohidrotic and anhidrotic areas by real-time PCR analysis. Total RNA from serial sections was extracted in cases 1, 2, and 4 and reverse transcribed into cDNA using first-strand cDNA synthesis kit RT-PCR.

reverse transcriptase step for each sample. As represented in Figure 2d, we did not amplify the *CHRM3* gene from any RNA without reverse transcriptase (lane 1), but reverse transcriptase step yielded PCR products at an expected size 72 bp (lane 2), revealing that positive *CHRM3* PCR results of real-time PCR were not derived from contaminated genomic DNA. The levels of *CHRM3* expression were higher in the hypohidrotic than anhidrotic areas (Figure 2e).

As mast cells produce histamine in response to acetylcholine (Fantozzi

et al., 1978; Blandina *et al.*, 1980), we investigated CHR expression in mast cells just in vicinity of eccrine glands. Serial sections were alternatively stained with toluidine blue and with anti-CHRM3 antibody. Mast cells were identified by positive toluidine blue staining (Figure 2c, upper panel), and CHRM3 expression by mast cells was examined in the antibody-stained adjacent sections (Figure 2c, lower panel). In a normal control, mast cells expressed CHRM3 at high levels. Mast cells in conjunction with the secretory portion expressed CHRM3 in the hypohidrotic but not anhidrotic areas. There was no significant difference in the number of mast cells between the anhidrotic and hypohidrotic areas.

Our study revealed that the skin of patients with CUAH is divided into the wheal-non-occuring anhidrotic and wheal-occuring hypohidrotic areas. Even in the hypohidrotic areas, the intradermal injection of autologous sweat did not yield wheal, suggesting the absence of sweat allergy (Tsuchiya *et al.*, 2004). We found the lack of CHRM3 expression in the anhidrotic skin, which may lead to the lack of sensitivity to acetylcholine. Mast cells are responsible for wheal formation and present just in the vicinity of eccrine glands. Neither eccrine gland cells nor mast cells expressed CHRM3 in the anhidrotic area, and it is thus reasonable that sweating and wheal formation were absent in this area. CHR mediates wheal development (Tong *et al.*, 1997), and acetylcholine can induce degranulation of mast cells (Fantozzi *et al.*, 1978; Blandina *et al.*, 1980). In the hypohidrotic area, we are tempting to speculate that acetylcholine released from nerves upon exercise cannot be completely trapped by CHR of eccrine glands and overflows to the adjacent mast cells. In this scenario, mast cells may be capable of producing histamine and resultant wheal in response to acetylcholine because of the expression of some degree of CHRM3.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Squamous Cell Carcinoma of the Skin Shows a Distinct MicroRNA Profile Modulated by UV Radiation

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TO THE EDITOR

Cutaneous squamous cell carcinoma (SCC) is the second most common skin malignancy in the general population. There are many risk factors for SCC, the most important one being solar radiation. The incidence of SCC is increased by 60–100-fold in organ transplant recipients (OTRs), making it the most common malignancy in these patients (Berg and Otley, 2002; Euvrard *et al.*, 2003). SCC in OTRs is characterized by a higher risk of metastasis in up to 20% of the patients and shows a more aggressive course than SCC in the general population (Euvrard *et al.*, 2003).

Recent work has delineated a class of small non-coding RNA species known as microRNAs. Owing to their association with the 3′-untranslated region region of target mRNAs, microRNAs have important regulatory roles in diverse cellular pathways, including

Abbreviations: OTR, organ transplant recepient; SCC, squamous cell carcinoma; UTR, untranslated region

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'hondrodermatitis of the auricle in patients with atopic dermatitis

Atopic dermatitis (AD) manifests as acute and chronic eczematous lesions, but there may be as yet uncharacterized manifestations in this highly prevalent disease. Conditions affecting both skin and cartilage of the auricle include chondrodermatitis nodularis chronica helices (CNH) [1], pseudocyst of the auricle [2], and relapsing polychondritis. However, other types of chondrodermatitis have also been reported as incidental cases [3]. We report 5 cases of chondrodermatitis in patients with AD *(table 1)*.

Case 1: A 25-year-old woman suffering from AD presented with a painful nodule on the upper helix of the right ear. She and her mother had corticophobia and refrained from using topical corticosteroids and tacrolimus, and she was treated with Chinese herbal medicines. The whole helix and anthelix were nodulous and fibrotic *(figure 1, case 1)*. In particular, an ill-shaped, tender nodule, 3 cm in diameter, was observed in the upper part of helix. Histological examination showed epidermal acanthosis and perivascular infiltration of lymphocytes. Beneath the fibrotic dermis, there was ill-defined cartilage, which was degenerated and gradually disappeared into the overlying fibrotic tissue *(figure 1, histopathology)*.

Case 2: A 34-year-old man presented with painful nodules and indurations on his bilateral ears. He first noticed the lesions in his childhood. Although his AD was treated with topical corticosteroids and tacrolimus, an eczematous eruption persisted for a long time on his whole body. On examination, the eruption had spread to the whole body surface with severe scratch marks. A large elastic-hard nodule was present on the right upper helix, and the left helix

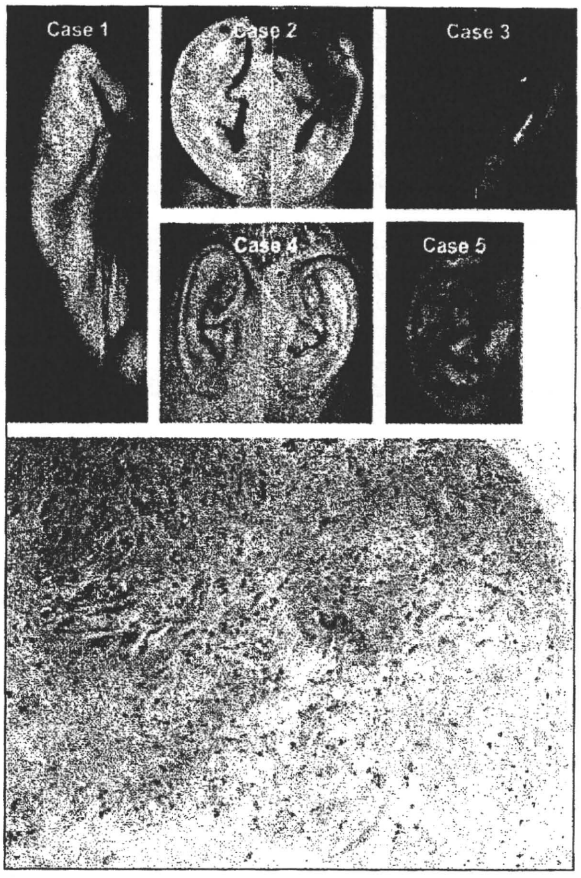

Figure 1. Clinical appearances of cases 1-5 and histopathological changes in case 1. Case 1: The whole helix and anthelix of the right ear are nodulous and fibrotic with a scaly erythematous surface. Case 2: A large elastic-hard nodule is present on the right upper helix, and the left helix is deformed with an eczematous and crusty, overlying skin. Case 3: Multiple, subcutaneous nodules are observed on the posterior aspect of both auricles. Case 4: Multiple, small, subcutaneous nodules with elastic-hard consistency are observed on both helices. Case 5: The right auricle is deformed with elastic hard, swollen cartilage. Histopathology of Case 1: Beneath the fibrotic dermis, degenerated cartilage is present (hematoxylin-eosin stain; original magnification: ×100.).

Table 1. Summary of five patients with chondrodermatitis of the auricle associated with AD

Case	Age	Sex	Sites	Duration of AD (years)	SCORAD	Blood Eosinophils (/μL)	IgE (U/mL)	LDH (IU/L)
1	25	F	Right	20	66.4	756	8,800	348
2	34	M	Bilateral	31	85.5	1,108	32,000	232
3	30	M	Bilateral	10	77.0	1,409	7,800	359
4	46	M	Bilateral	41	64.0	5,106	110,000	512
5	46	F	Right	36	70.5	2,258	13,000	266
Mean	36.2			27.6	72.7	21,27.4	34,320	343.4
± SD				± 12.5	± 10.8	± 1,755.4	± 43,422.3	± 108.5
Normal range						70-450	119-229	0-170

was deformed with an eczematous overlying skin *(figure 1, case 2)*. There was no fluctuance or fluid retention.

Case 3: A 30-year-old man had painful nodules on the helices of both ears. Long-term AD eruptions persisted on his whole body. Ill-defined, multiple, tender, subcutaneous nodules and indurations were observed on the posterior aspect of both auricles *(figure 1, case 3)*.

Case 4: A 46-year-old man had multiple indurations on both ears. He was treated with Chinese herbal medicines, but his AD lesions did not improve. Multiple, tender, subcutaneous nodules and indurations with elastic-hard consistency were observed on both helices *(figure 1, case 4)*.

Case 5: A 46-year-old woman presented with tender, subcutaneous, elastic-hard lesions on the peripheral helix of her right ear. Her AD was treated with topical corticosteroids and Chinese herbal medicines. Her right auricle was deformed with elastic-hard, swollen cartilage *(figure 1, case 5)*.

In these five patients, the clinical changes are derived mainly from cartilage degeneration and dermal fibrosis. CNH and pseudocyst of the auricle may be differential diagnoses. CNH is characterized by a painful, dome-shaped, firm, inflammatory nodule, often covered with a crust [4, 5]. Cartilage is often disrupted, and transepidermal elimination of necrotic dermal debris may also be observed. The lesions in our cases were not single dome-shaped nodules. Pseudocyst of the auricle is an asymptomatic, fluctuant swelling on the upper portion of the anterior aspect of the ear [6]. Histologically, there is an intracartilagenous cavity in pseudocyst of the auricle, which differs from the lesions in our patients.

Thus, we diagnosed the lesions as chondrodermatitis associated with AD. The onset of AD was in their first decade in almost all cases. All patients had high circulating eosinophil counts and IgE levels. There has been no case of less severe atopic dermatitis associated with chondrodermatitis nodularis helicis in the literature. The long duration of AD and inadequate control of AD eruptions seem to contribute to the development of chondrodermatitis. The association of chondrodermatitis with AD is probably not merely coincidence and AD may provide a background for the forming of chondrodermatitis. ■

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Verrucous epidermal nevus successfully treated with photodynamic therapy

Verrucous epidermal nevus (VEN), also called linear epidermal nevus, is a skin disorder that presents commonly at birth. VEN is frequently resistant to multiple treatment modalities [1, 2]. We report a case of VEN successfully treated with photodynamic therapy (PDT).

A 9-year-old girl presented with asymptomatic linear papillomatous and hyperkeratotic papules on the right thigh which developed at birth. The family history was noncontributory and the patient had never had any treatment before visiting our clinic. Physical examination revealed multiple linearly-arranged papillomatous and hyperkeratotic papules on the right thigh *(figure 1A)*. A skin biopsy performed on a lesion showed hyperkeratosis, papillomatosis, and acanthosis with elongation of the rete ridges. Focal epidermolytic hyperkeratosis was also observed *(figure 1B)*. Treatment with methyl-aminolevulinate (MAL) (Metvix®; Galderma, France)-PDT was attempted. The treatment area was covered with an occlusive polyurethane film (Tegaderm®; 3M, St Paul, MN, USA) plus a light-opaque

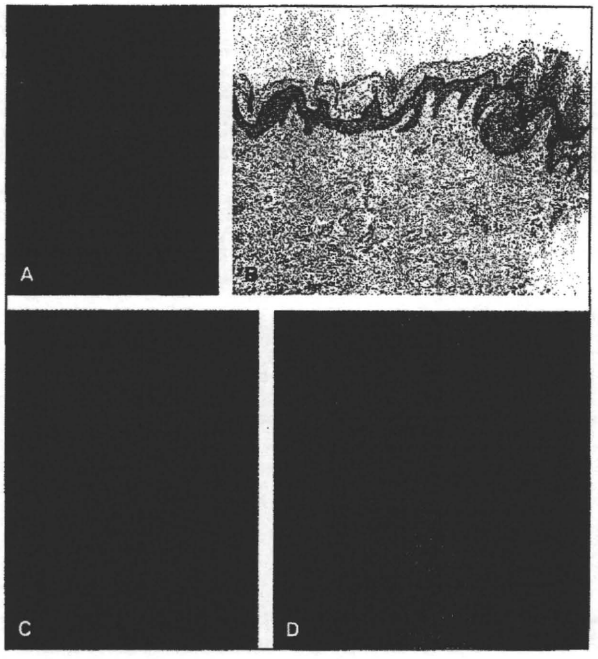

Figure 1. A) Before treatment, multiple linear arranged papillomatous and hyperkeratotic papules on the right thigh. **B)** Hyperkeratosis, papillomatosis and acanthosis with elongation of the rete ridges. Focal epidermolytic hyperkeratosis was seen on the right side (H&E, ×100). **C)** Improved skin lesion with hyperpigmentation after 4 sessions of treatment. **D)** Hyperpigmentation resolved and the good clinical outcome was maintained after 3 months of follow up; however, a hypertrophic scar remained at the biopsy site.

this phenomenon really exists in physiologic conditions, some secretory factors from ADSCs might have more or less therapeutic potential for hair growth. The results presented here suggest that ADSCs promote hair growth by increasing the proliferation of DPCs, and possibly epithelial cells, through modulation of cell cycle, and activating anagen phase in hair cycles. Therefore, the rational manipulation of ADSCs might be a promising tool for hair growth promotion.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jdermsci.2009.10.013.

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Letter to the Editor

Food-dependent anaphylaxis with serum IgE immunoreactive to dairy products containing high-molecular-weight proteins☆

Food-induced anaphylaxis is one of the most common causes of anaphylaxis [1].

Establishing the cause of recurrent anaphylaxis is one of the most important goals of management because identification of the responsible allergens allows avoidance of further exposure.

Food-dependent exercise-induced anaphylaxis (FDEIA) is a distinct form of food allergy characterized by wheals, angioedema, wheezing, dyspnea and occasional loss of consciousness, induced by a combination of causative food ingestion and physical exercise [2]. Recent investigations have documented that aspirin or non-steroidal anti-inflammatory drugs (NSAIDs), in place of exercise, can also evoke allergic reactions to food products [3,4]. Furthermore, it has been found that ingestion of aspirin combined with exercise increases gastrointestinal permeability in humans [5], and that some food additives, such as preservatives, contain aspirin-like substances [6]. Herein we report a case of anaphylaxis induced by ingestion of processed cheese following aspirin intake, but not

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