Contact hypersensitivity

For contact sensitization, 25 μl of PCl solution (0.5% w/v in acetone-olive oil mixed at 4:1) was painted on the grafted or non-grafted area. For elicitation, 10 μl of 0.2% PCl solution was painted on each earlobe of the PCl-sensitized mice on day 5 after sensitization. In some experiments, 50 μl of dinitrofluorobenzene (Nacalai Tesque Co., Tokyo, Japan) solution (0.5% w/v in acetone-olive oil mixed at 4:1) was painted on the abdomen, and elicited with 10 μl of 0.3% dinitrofluorobenzene solution on each earlobe 5 days after sensitization. The thickness of each ear was measured with a micrometer 24 hours after elicitation. Swelling was expressed as the increase in ear thickness.

Culture medium

RPMI 1640 (Gibco BRL Life Technology, Grand Island, NY) was supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 10^{-5} M sodium pyruvate, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1% nonessential amino acids, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin (all from Gibco BRL Life Technology).

Immunohistochemistry

For immunofluorescence analysis, epidermal sheets were separated from the dermis with 0.5 M ammonium thiocyanate, fixed in acetone for 5 minutes at −20 °C, and stained with PE-conjugated anti-I-A^d mAb (BD Biosciences, San Diego, CA). In some experiments, the back skin of mice was frozen in Tissue-Tek OCT compound 4583 (Sakura Finetechnical Co. Ltd, Tokyo, Japan). Cryostat sections (10 μm) were fixed in acetone and stained with PE-conjugated anti-I-A^d mAb, biotin-conjugated anti RANKL mAb (R&D Systems) followed by staining with allophycocyanin-Cy7 conjugated streptavidin. Images were viewed with a Zeiss confocal microscope and processed with an LSM Image Browser (Zeiss).

Preparation of EC and dermal cell suspensions, and purification for LCs and dDCs

Skin sheets were floated in 0.2% trypsin in phosphate-buffered saline (pH 7.4) for 1 hour at 37 °C as described previously (Tokura *et al.*, 1994). The epidermis was separated from the dermis with forceps in phosphate-buffered saline supplemented with 10% fetal calf serum. EC suspensions were prepared by pipetting and filtration through nylon mesh. Dermal cells were obtained from normal or grafted skin from which the epidermis had been removed. Samples were minced and incubated for 2 hours at 37 °C in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with collagenase XI (4,830 U ml^{-1}; Sigma, Tokyo, Japan), hyaluronidase (260 U ml^{-1}; Sigma), DNase (0.1 mg ml^{-1}; ICN, Costa Mesa, CA), and 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Sigma). The obtained cells were filtered through a 40 μm filter. dDCs and other cells (mainly fibroblasts) were fractionated from the dermal cell suspensions with Ficoll-Paque (GE Healthcare UK Ltd) For enrichment of LCs and dDCs, EC suspensions and derma cell suspensions after Ficoll-Paque fractionation were purified for $CD11c^+$ and $CD11c^-$ subsets using anti-CD11c mAb and auto-MACS (Miltenyi Biotec, Gladbach, Germany). The purity of $CD11c^+$ cells was 70-80% (Supplementary information, Figure S1), containing less than 0.01% $CD3^+$ T cells as determined by flow cytometric analysis. The $CD11c^-$ cells from EC and dermal cell suspensions were mainly keratinocytes and fibroblasts, respectively, containing less than 0.05% DC subsets.

Flow cytometry

Cells were immunostained with various combinations of fluorescence-conjugated mAbs and analyzed with three-channel FACS-Canto flow cytometer (BD Biosciences) and FlowJo software (Tree Star Inc, Ashland, OH). The expression of cell-surface and intracytoplasmic cytokines were analyzed using antibodies to PE-conjugated anti-CD11c, CD80, CD86 and PE-conjugated anti-rat IgG, purified anti-mouse CD205 (DEC205) rat IgG, PerCP-conjugated anti-CD45R mAbs, PE-conjugated anti-MHC class II, biotin-conjugated anti-IL-10, and PE-Cy7-conjugated streptavidin. Antibodies were purchased from e-Bioscience (San Diego, CA). All mAbs were used at 1–5 μg 10^{-6} cells, and each incubation was performed for 30 minutes at 4 °C, followed by two washes in phosphate-buffered saline supplemented with 5% fetal calf serum and 0.02% sodium azide. Viable cells were identified by 7-AAD uptake. Intracytoplasmic IL-10 was detected in permeabilized cell suspensions using BD Cytofix/Cytoperm Plus Kit (BD Biosciences).

Apoptosis analysis

EC suspensions from control or grafted (1, 4, and 7 days after) skin were stained with FITC-conjugated MHC-class II mAb for 30 minutes on ice and stained with allophycocyanin-conjugated Annexin V and propidium iodide (Invitrogen), according to the manufacturer's protocol. Apoptosis in LCs was analyzed by a FACScant using FlowJo software (Tree Star Inc) as described earlier (Goldszmid *et al.*, 2003).

Cutaneous DC migration into draining lymph nodes

Mice were painted with 200 μl of 2% FITC (Sigma-Aldrich, St Louis, MO), and axillar and inguinal lymph nodes were taken 24 hours later. Single-cell suspensions were prepared and subjected to flow cytometric analysis.

Real-time PCR

Total RNA was extracted from axillary and inguinal lymph nodes and EC suspensions with the SVTotal RNA isolation system (Promega, Madison, WI) according to the manufacturer's protocol. Murine IL-10 gene expression was quantified in a two-step reverse transcription-PCR. cDNA was reverse transcribed from total RNA samples using the TaqMan RT reagents (Applied Biosystems, Foster, CA). Target gene expression was quantified using TaqMan Gene Expression Assay (Applied Biosystems) in the ABI PRISM 7000 sequence detection system (Applied Biosystems). The probe was synthesized with VIC as the reporter dye and Tamra as the quencher dye. The forward primer, reverse primer, and TaqMan probe were purchased from Applied Biosystems. As an endogenous control for these PCR quantification studies, glyceraldehyde-3-phosphate dehydrogenase gene expression was measured using the TaqMan rodent GAPDH control reagents (Applied Biosystems). Results represented normalized IL-10 mRNA amounts relative to skin-grafted groups using the $\Delta\Delta C_t$ method.

Measurement of cytokine amounts in culture supernatants

$CD11c^+$ and $CD11c^-$ cells were purified from EC and dermal cell suspensions. Cells of each subset (2×10^6 cells per 1.5 ml well) were

cultured in medium for 24 hours in 24-well plates (Corning Glass Works, Corning, NJ). The concentration of IL-4, IL-6, IL-10, IL-12, and TNF-α in culture supernatants was measured using a cytometric beads array system (BD Biosciences) according to the manufacturer's protocol.

Purification and adoptive transfer of Treg cells

Axillary and inguinal lymph nodes were harvested from mice. They were meshed through a cell strainer into RPMI 1640 containing 2% fetal calf serum to prepare single-cell suspensions. To purify $CD4^+CD25^+$ cells or $CD4^+CD25^-$ cells, a mouse Treg isolation kit (Miltenyi Biotec) was used according to the manufacturer's protocol. Briefly, $CD4^-$ cells were depleted with the biotin-labeled antibody cocktail. Subsequently, the $CD4^+$ fractions were magnet-separated with CD25-PE mAb. The positive fraction contained $CD4^+CD25^+$ cells with more than 95% purity, and the flow-through fractions from magnet columns were used as $CD4^+CD25^-$ fraction.

Statistic analysis

All data were statistically analyzed using Student's *t*-test. A *P*-value of less than 0.05 was considered to be significant. Bar graphs were presented as mean ± SD of the mean value.

CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Figure S1. Purification of DCs.

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Biphosphonates, although their mechanism in calciphylaxis is unknown, should be considered as an option in the management of these patients.

JB da Costa,* AG da Costa, MM Gomes
*Serviço de Dermatologica Avenida Professor Egas Moniz,1649–028 Lisboa, Portugal, *Corresponding author, fax +351217954447; E-mail: joaobc77@hotmail.com*

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Occupational contact dermatitis due to polyvinylamine

Editor

A 22-year-old man, who worked in a chemical industry and had exposure to polymer micelles, was referred to us in September 2005 for further evaluation of a pruritic erythematous and papular eruption on his face, anterior chest and all limbs (fig. 1). The dermatitis had developed 1 day after he had worked with polyvinylamine. He had no past history of atopic dermatitis.

Both patch tests with 1% and 0.1% polyvinylamine in petrolatum produced a 2+ reaction (ICDRG scale) on day 2, suggesting that polyvinylamine was the causative agent for the dermatitis. Normal healthy volunteers ($n = 4$) showed negative patch test with 1% polyvinylamine. Therefore, the patient was diagnosed as having occupational contact dermatitis caused by polyvinylamine. Treatment with oral prednisolone and avoidance of polyvinylamine improved his eruption, and he has remained symptom-free.

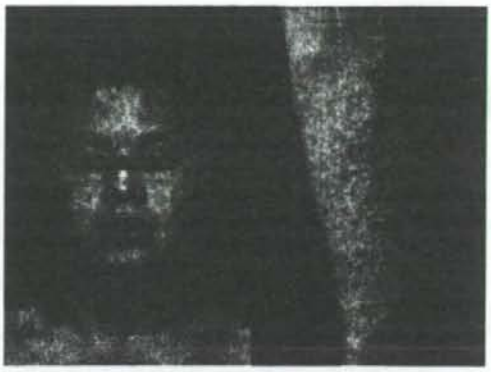

fig. 1 Erythematous papular eruption on his face and arm.

Polyvinylamine has recently become commercially available as a cationic polymer that is water absorbent.[1] Polymers are a large molecule with high molecular weight and have been rarely considered for possible sensitizing capacities.[2]

To our knowledge, this is the first report of contact dermatitis to cationic polymer, polyvinylamine. Our case strongly suggests that cationic polymers can be allergens.

K Sugita,* C Koga, K Kabashima, Y Tokura
*Department of Dermatology, University of Occupational and Environmental Health, Japan, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan, *Corresponding author, tel. +81 93 6917445; fax +81 93 6910907; E-mail: k-sugita@med.uoeh-u.ac.jp*

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Impaired Initiation of Contact Hypersensitivity by FTY720

Daiki Nakashima[1], Kenji Kabashima[1], Jun-ichi Sakabe[1], Kazunari Sugita[1], Takashi Kobayashi[2], Ryutaro Yoshiki[1] and Yoshiki Tokura[1]

FTY720 inhibits lymphocyte emigration from lymphoid organs to peripheral blood by binding one of the sphingosine-1-phosphate (S1P) receptors, $S1P_1$. We investigated the effects of FTY720 in relation to murine contact hypersensitivity (CHS). CHS was impaired by FTY720 when administered during the sensitization but not the elicitation phase. Consistently, adoptive transfer of immunized lymph node cells from mice treated with FTY720 during the sensitization phase was virtually incapable of inducing CHS response in recipients. FTY720 decreased the number of blood $CD44^-$-naive T cells markedly and that of $CD44^+$ memory T cells modestly. Among memory T cells, the $CD62L^-$ effector memory subset was more resistant to FTY720 than the $CD62L^+$ central memory subset. Accordingly, the level of S1P chemotactic response was high in naive T cells, marginal in effector memory T cells, and very low in central memory T cells. Consistently, the $S1P_1$ mRNA expression level was much lower in memory T cells than in naive T cells. These findings demonstrate that S1P–$S1P_1$ signaling is essential for recirculation of naive T cells. FTY720 seems to decrease the incidence of interactions between antigen-loaded dendritic cells and circulating naive T-cell clones in the lymph nodes, thereby depressing the sensitization of naive T cells in CHS.

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INTRODUCTION

Sphingosine-1-phosphate (S1P) is recognized as a potent lipid mediator important for various physiological processes (Spiegel and Milstien, 2003; Ishii *et al.*, 2004) that binds to five subtypes of G-protein-coupled receptors, termed S1P1, 2, 3, 4, and 5 (Sanchez and Hla, 2004; Cyster, 2005). In the immune system, initial studies for evaluating the roles of S1P were done with a compound, FTY720. Following its administration *in vivo*, FTY720 is rapidly phosphorylated and binds to S1P receptors, thereby diverting S1P signaling (Brinkmann *et al.*, 2002; Mandala *et al.*, 2002; Sanchez and Hla, 2004). FTY720 inhibits lymphocyte emigration from lymphoid organs by binding to $S1P_1$, resulting in lymphopenia (Brinkmann *et al.*, 2002; Graler and Goetzl, 2004; Matloubian *et al.*, 2004).

Several studies in fetal liver chimeras or in tissue-specific knockout mice showed that T cells lacking $S1P_1$ are unable to exit the thymus and that most of the $S1P_1$-deficient B and T cells are inefficient in exiting secondary lymphoid organs, constituting one of the checkpoints controlling lymphocyte homeostasis (Allende *et al.*, 2004; Matloubian *et al.*, 2004; Cyster, 2005). S1P is a metabolite produced by many cell types, such as platelets, and is abundant in circulation and low in secondary lymphoid organs. Lymphocytes are thought to egress in chemotactic response to S1P (Schwab *et al.*, 2005).

Because FTY720-induced lymphopenia substantially impairs immune responses, FTY720 was considered to have considerable potential as a therapeutic tool (Fujita *et al.*, 1996; Suzuki *et al.*, 1996; Chiba *et al.*, 1998; Yanagawa *et al.*, 1998; Kiuchi *et al.*, 2000). Unlike other established immunomodulators, FTY720 has no inhibitory effect on cytokine production by lymphocytes (Yanagawa *et al.*, 1998). The efficacy of FTY720 has been well established in animal and human transplantation (Brinkmann *et al.*, 2002; Budde *et al.*, 2002) and is currently exploited in trials to enhance survival of incompletely major histocompatibility complex (MHC)-matched organ grafts (Budde *et al.*, 2002). In the skin immune system, it has been reported that FTY720 prevented the development of atopic dermatitis-like skin lesions in NC/Nga mice (Kohno *et al.*, 2004) and animal skin allograft rejection (Yanagawa *et al.*, 1998, 1999; Luo *et al.*, 1999). However, the precise mechanism of FTY720's action in acquired immune response remains unknown.

The skin is exposed to multiple antigens (Ags) throughout life and develops Ag-specific immune responses. Murine contact hypersensitivity (CHS) is one of the models for delayed-type hypersensitivities, consisting of two phases: sensitization and

[1] *Department of Dermatology, University of Occupational and Environmental Health, Yahatanishi-ku, Kitakyushu, Japan and* [2] *Division of Molecular and Cellular Immunology, Medical Institute of Bioregulation, Kyushu University, Higashi-ku, Fukuoka, Japan*

Correspondence: Dr Kenji Kabashima, Department of Dermatology, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan.
E-mail: kkabashi@med.uoeh-u.ac.jp

Abbreviations: Ab, antibody; Ag, antigen; B6, C57BL/6; CHS, contact hypersensitivity; DC, dendritic cell; LC, Langerhans cells; MHC, major histocompatibility complex; S1P, sphingosine-1-phosphate

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elicitation. During the sensitization phase, cutaneous dendritic cells (DCs) similar to epidermal Langerhans cells (LCs) and dermal DCs serve as professional Ag-presenting cells (Kabashima *et al.*, 2003b; Mathers and Larregina, 2006). Upon migration, cutaneous DCs undergo a process of maturation, which is represented by the expression of co-stimulatory molecules and the delivery of major MHC-Ag complexes to their surfaces. The mature cutaneous DCs then activate naive T cells in the draining lymph nodes, thereby inducing their transformation into Ag-specific memory T cells. In the elicitation phase, when the same Ag is exposed, circulating Ag-specific memory T cells migrate into the skin and are reactivated to initiate inflammation by producing mediators such as IFN-γ (Grabbe and Schwarz, 1998; Kabashima and Miyachi, 2004). Therefore, murine CHS is one of the suitable models for clarifying the role of FTY720. Here, we found that the sensitization phase, but not the elicitation phase, is the acting phase of FTY720, which presumably inhibits recirculation of naive T cells and reduces the incidence of interaction between Ag-bearing DCs and Ag-specific naive T-cell clones.

RESULTS

Impairment of CHS by FTY720 when administered during the sensitization period

We examined the significance of FTY720-mediated regulation of acquired immune responses *in vivo*. Using CHS as a model, we investigated whether FTY720 affected the immune response to an exogenous Ag. C57BL/6 (B6) mice were sensitized with DNFB as hapten on day 0. Elicitation with DNFB on the ears 5 days after sensitization induced a marked swelling response (Figure 1a). Treatment of mice with FTY720 12 hours before sensitization produced a significant decrease in ear thickness (Figure 1a). However, the administration of FTY720 on day 4.5 after sensitization did not decrease the ear swelling. The treatment of FTY720 both 12 hours before and 4.5 days after sensitization yielded a comparably reduced intensity of CHS to only the treatment with FTY720 12 hours before sensitization. Consistently, histology of the ears 24 hours after challenge showed pronounced spongiosis and extensive infiltration of lymphocytes in the edematous dermis, which were markedly reduced by the administration of FTY720 12 hours before sensitization (Figure 1b). To evaluate the effect of FTY720 on recall responses at later stages, where immune memory resides with memory T cells within secondary lymphoid tissues, we administered FTY720 to mice at later time points after sensitization. The FTY720 treatment at 9.5 and 14.5 days after sensitization slightly impaired the subsequent elicitation phase without a statistically significant difference (Figure 1c). These results suggested that FTY720 impaired the initiation but not the elicitation of CHS.

The above results indicate that the impairment by FTY720 was due to reduced generation of memory T cells during sensitization. However, this sensitization-based action of FTY720 might also affect the elicitation phase, as FTY720 is known to decrease the number of blood lymphocytes between 3 hours and 3–5 days after administration (Chiba

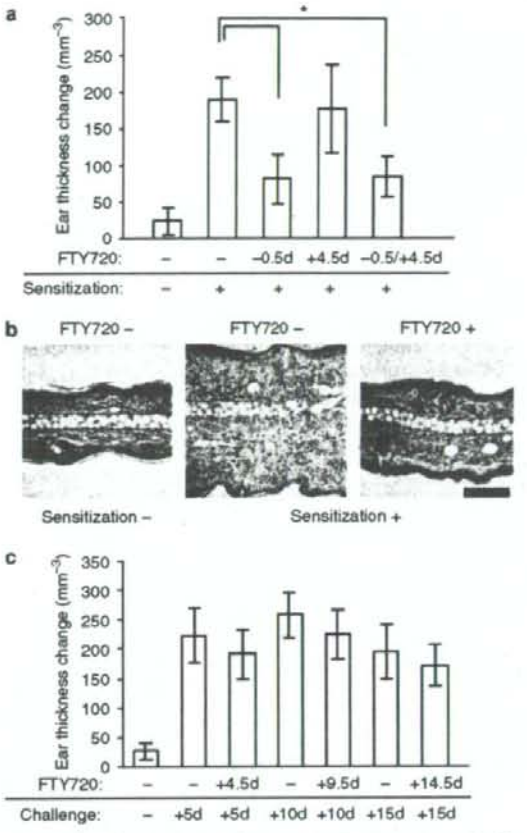

Figure 1. Effect of FTY720 on CHS response. (a) B6 mice were sensitized with DNFB on the abdominal skin and challenged with DNFB on the ears 5 days later. FTY720 was administered 12 hours before sensitization (−0.5 days (d)), elicitation (+4.5 days), or both (−0.5d/+4.5d), and the change in ear thickness after challenge was measured. Columns show the mean ± SD from at least four mice per group. Student's *t*-test was performed between the indicated groups and $^*P<0.05$. (b) Histology of the skin 24 hours after challenge. The ears of mice after sensitization and elicitation with (right panel) or without (center panel) FTY720 treatment before sensitization are shown. The ears of mice that were not sensitized are shown as a control (left panel). Scale bar = 100 μm. Data are from three independent experiments. (c) B6 mice were sensitized with DNFB on the abdominal skin, and challenged with DNFB on the ears 5, 10, and 14 days later. FTY720 was administered 12 hours before challenge (+4.5d, +9.5d, and 14.5d). Columns show the mean ± SD from at least four mice per group.

et al., 1998; Luo *et al.*, 1999), resulting in a numerical reduction of T cells participating in the elicitation. Moreover, $S1P_1$ signaling is important for endothelial cell development (Allende *et al.*, 2003), which might additionally affect the CHS response.

To further characterize the acting phase of FTY720 and to exclude its possible effect on non-hematopoietic cells, we used an adoptive transfer-induced skin inflammation model. We isolated T cells from regional lymph nodes of

DNFB-sensitized donors treated with or without FTY720 and adoptively transferred the cells into B6 naive recipients. The recipients of sensitized T cells with FTY720 treatment showed a suppressed CHS response compared with those without FTY720 treatment (Figure 2a). Conversely, when T cells from B6 donors 5 days after sensitization were transferred into B6 naive recipients pretreated with or without FTY720, subsequent challenge with DNFB elicited comparable changes in ear swelling in both experimental groups (Figure 2b). These results indicate that T cells are responsible for the FTY720-induced impairment of CHS response and that the critical time point is the sensitization but not the elicitation period.

Unaffected cutaneous DC function by FTY720

In general, the sensitization phase concerned with cutaneous DCs consists of maturation in the skin, acquisition of Ag, migration into the regional lymph nodes, and Ag presentation to naive T cells. Although FTY720 was reported to down-modulate DC functions in some animal models (Czeloth *et al.*, 2005), its action on cutaneous DCs in the acquired immune response remains unclear. Therefore, we performed an FITC-induced cutaneous DC migration assay. FITC topically applied to the skin is taken up by cutaneous DCs, which then migrate to the draining lymph nodes as FITC$^+$ MHC class II$^+$ cells (Kabashima *et al.*, 2003b). We injected FTY720 or the equivalent amount of normal saline into mice, applied FITC on the abdomen 12 hours later, and isolated axillary and inguinal draining lymph node cells 24 hours after FITC application. The number of FITC$^+$ CD11c$^+$ or FITC$^+$ MHC class II^{hi+} cutaneous DCs accumulating 24 hours after FITC application was not substantially altered by FTY720 treatment (Figure 3a-c). The only positive finding that we obtained from this series of experiments was that the number of FITC$^+$ CD86$^+$ cutaneous mature DC subsets was slightly

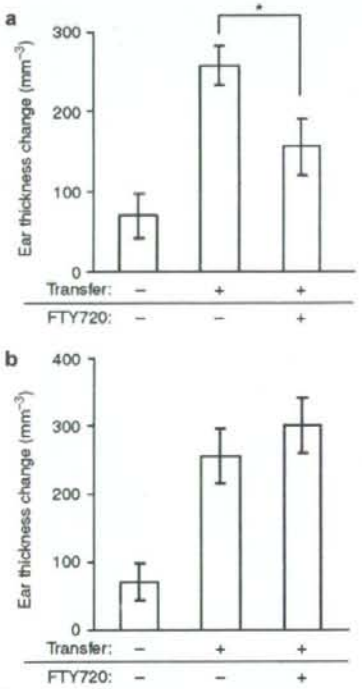

Figure 2. CHS response induced by adoptive transfer. (a) Attenuated CHS response in recipients adoptively transferred with FTY720-treated sensitized T cells. Lymphocytes were isolated and pooled from lymph nodes of DNFB-sensitized B6 donors with or without FTY720 treatment and adoptively transferred into B6 naive recipients. The recipient mice were challenged with DNFB and the change in ear thickness is shown. (b) Intact CHS response in recipients adoptively transferred with sensitized B6 T cells. Lymphocytes were isolated and pooled from lymph nodes of DNFB-sensitized B6 donors and adoptively transferred into B6 naive recipients pretreated with or without FTY720. The recipient mice were challenged with DNFB and the change in ear thickness is shown in panels **a** and **b**. Columns show the mean ± SD from five mice per group. Student's *t*-test was performed between the indicated groups. $^*P<0.05$. Data are from three independent experiments.

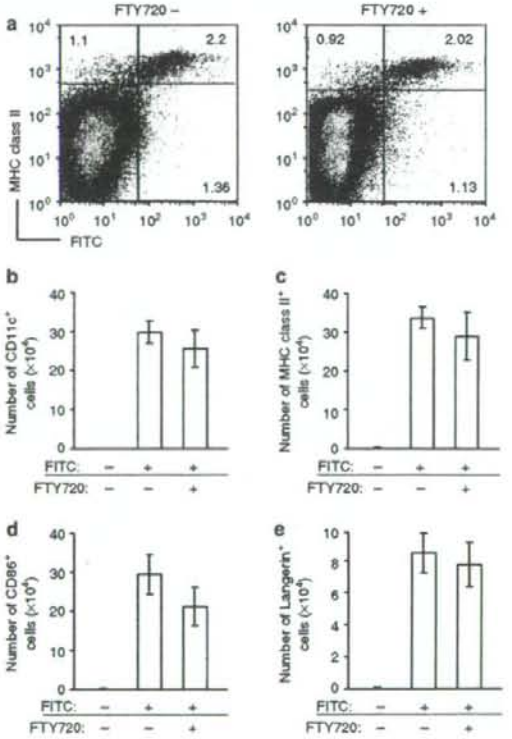

Figure 3. Influence of FTY720 on migration and maturation of cutaneous DCs. (**a-d**) B6 mice were pretreated with or without FTY720, and their abdominal skin was painted with FITC 12 hours later. The draining lymph node cells were collected 24 hours after FITC application and analyzed with flowcytometry (**a**). The FITC$^+$ CD11c$^+$ cells (**b**), FITC$^+$ MHC class II$^+$ cells (**c**), FITC$^+$ CD86$^+$ cells (**d**), and Langerin+ Langerhans cells (**e**) per mouse were enumerated (unpaired Student's *t*-test; $n=5$ per group). As a control, mice not painted with FITC were prepared (**b-d**).

decreased by FTY720, but no statistically significant difference was noted ($P=0.06$, $n=5$) (Figure 3d). Recently, the possible functional differences between LCs and dermal DCs that migrate from skin to draining LNs were highlighted (Kissenpfennig and Malissen, 2006; Schroder *et al.*, 2006; Bursch *et al.*, 2007; Ginhoux *et al.*, 2007; Poulin *et al.*, 2007). We measured the number of Langerin$^+$ LCs in the regional lymph nodes 24 hours after FITC application. FTY720 did not affect the number of Langerin$^+$ FITC$^+$ MHC class II$^+$ LCs (Figure 3e) nor that of dermal DCs, calculated as follows: (FITC$^+$ MHC class II$^+$ total migrated cutaneous DC)–(Langerin$^+$ FITC$^+$ MHC class II$^+$ migrated LCs) (data not shown).

To further evaluate the physiological significance of FTY720 on DC functions, we performed an *in vitro* assay on T-cell proliferation induced by Ag-loaded DCs. DNFB-sensitized Thy1.2$^+$ T cells were sorted from the draining lymph nodes 5 days after DNFB application and incubated with or without CD11c$^+$ DCs prepared from the draining lymph nodes 1 day after DNFB application with or without FTY720 treatment. T-cell proliferation was enhanced by the addition of Ag-loaded sorted DCs, but the extent of augmentation was comparable irrespective of FTY720 pretreatment (Figure 4).

Alteration of naive but not memory T-cell subsets in blood after FTY720 treatment

The above results suggest that FTY720 could not affect cutaneous DC migration or their Ag-presenting ability. To establish the acquired immune response, Ag-loaded DCs need to interact with Ag-specific naive T-cell clones in the draining lymph nodes for their activation and maturation. The notion that FTY720 inhibits naive T-cell circulation raises the possibility that DCs are unable to meet a sufficient number of circulating naive T cells in the lymph nodes of FTY720-treated mice. Although FTY720 is known to inhibit T-cell egress from the secondary lymph nodes (Chiba *et al.*, 1998; Sallusto *et al.*, 1999; Yanagawa *et al.*, 1999; Wherry *et al.*, 2003; Matloubian *et al.*, 2004), the inhibitory level of each T-cell subset, that is, naive, central memory, or effector memory T-cell subset, has not been evaluated quantitatively. Therefore, we examined the extent of decrease in the individual's circulating T-cell subsets after treatment with FTY720. The blood was drawn by intraocular venipuncture before and 1, 3, and 5 days after FTY720 administration. A flow cytometric analysis showed that FTY720 decreased the frequency of CD4$^+$ CD44$^-$-naive T-cell subsets more profoundly than that of CD4$^+$ CD44$^+$ memory T-cell subsets 1 day after FTY720 treatment (Figure 5a).

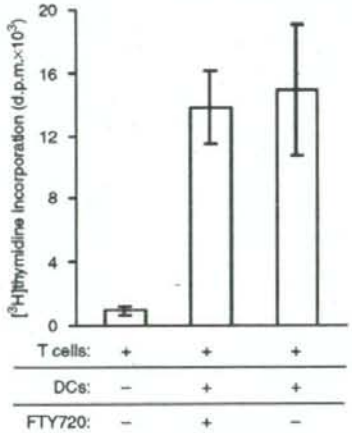

Figure 4. **Effect of FTY720 on T-cell stimulatory capacity of cutaneous DCs.** DNFB-sensitized Thy1.2$^+$ T cells were sorted from draining lymph nodes 5 days after DNFB application and incubated with or without CD11c$^+$ DCs prepared from draining lymph nodes 1 day after DNFB application with or without FTY720 treatment. Cell proliferation was measured by ^{3}H-thymidine incorporation. d.p.m., decay per minute. When DCs without hapten pretreatment were added in this assay, the enhancement of T-cell proliferation was marginal (<2000 d.p.m.). Columns show the mean ± SD from three independent experiments.

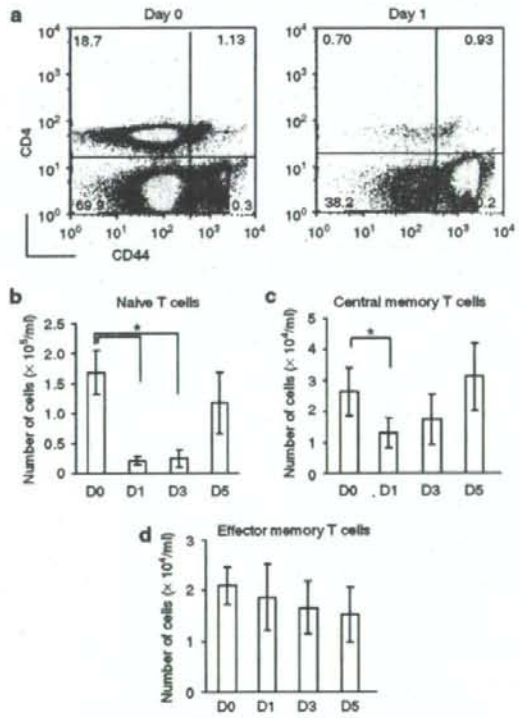

Figure 5. **Numerical alternations of naive, central, and effector memory T cells in the blood following FTY720 administration.** FTY720 was administered to B6 mice, and peripheral blood was periodically collected. Lymphocytes were stained with mAbs to CD4 and CD44 and analyzed by flow cytometry (**a**). The numbers of naive (**b**), central memory, (**c**) and effector memory T cells (**d**) per milliliter of blood were determined. Columns show the mean ± SD from four mice per group. Student's *t*-test was performed between the indicated groups. *$P<0.05$. Data are from three independent experiments.

The expression of CCR7 and CD62L divides memory T cells into two functionally distinct subsets (Sallusto *et al.*, 1999; Masopust *et al.*, 2001; Reinhardt *et al.*, 2001; Wherry *et al.*, 2003): those that express CD62L and CCR7 and efficiently home to lymph nodes are termed central memory cells and those that lack the expression of these lymph node homing receptors and are preferentially located in non-lymphoid tissues are termed effector memory cells. Both T-cell subsets are present in the blood, lymph nodes, and spleen. Some studies have also shown that effector memory T cells acquire effector functions such as cytokine production and cytotoxicity more rapidly than central memory T cells do (Matloubian *et al.*, 2004). Therefore, we further enumerated $CD4^+$ $CD44^-$ $CD62L^+$-naive T cells, $CD4^+$ $CD44^+$ $CD62L^+$ central memory T cells, and $CD4^+$ $CD44^+$ $CD62L^-$ effector memory T cells in the blood after FTY720 administration. The number of naive T cells was decreased markedly 1 and 3 days after FTY720 treatment and returned to the baseline level on day 5 (Figure 5b). Similarly, but less remarkably, the number of central memory T cells significantly declined 1 day after FTY720 treatment and almost returned to the baseline level on day 3 (Figure 5c). In contrast, the number of effector memory T-cell subsets was not substantially affected by FTY720 treatment throughout the experimental period (Figure 5c). Similar results were obtained with $CD8^+$ T-cell subsets, and T cells isolated from mice sensitized with DNFB 5 days prior yielded virtually the same data (data not shown). These results suggest that the chemotactic response of naive T cells to S1P was more sensitive to FTY720 than that of memory T-cell subsets, and that among memory T-cell subsets, effector memory T cells were less susceptible to FTY720 than central memory T cells.

We also evaluated the kinetics of the T-cell composition in the regional lymph nodes after FTY720 treatment by enumerating T-cell subsets 1, 3, and 5 days after FTY720 treatment in comparison with FTY720 non-treatment. FTY720 did not affect the number of $CD4^+$ $CD44^-$ $CD62L^+$-naive T cells, $CD4^+$ $CD44^+$ $CD62L^+$ central memory T cells, or $CD4^+$ $CD44^+$ $CD62L^-$ effector memory T cells (Figure 6a–c).

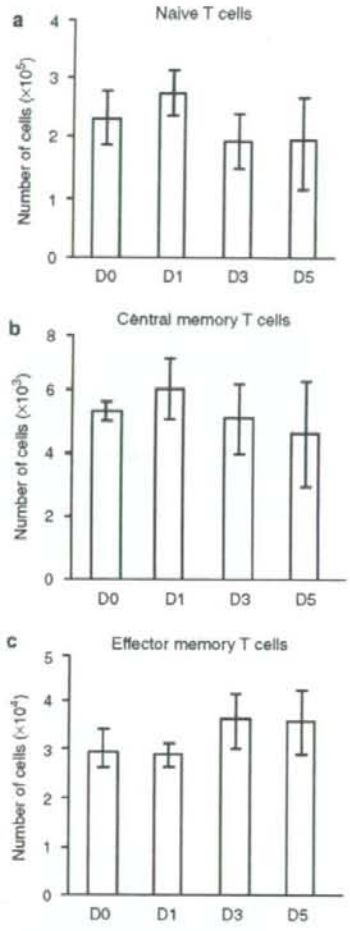

Figure 6. Numerical alternations of naive, central, and effector memory T cells in the lymph nodes following FTY720 administration. FTY720 was administered to B6 mice, and skin-draining (inguinal and axillary) lymph nodes were periodically collected. The numbers of naive (a), central memory (b), and effector memory T cells (c) per mouse were determined. Columns show the mean ± SD from four or five mice per group. Student's *t*-test was performed between the indicated groups, but no statistically significant difference ($P<0.05$) compared with the FTY720-nontreated group was detected. Data are from two independent experiments.

S1P responsiveness of T-cell subsets

FTY720 inhibits T-cell emigration through inhibition of S1P–$S1P_1$ signaling (Sawicka *et al.*, 2003). Therefore, as a readout of $S1P_1$ function on each T-cell subset, we compared the S1P chemotactic response between $CD4^+$ $CD44^-$ $CD62L^+$-naive T cells, $CD4^+$ $CD44^+$ $CD62L^+$ central memory T cells, and $CD4^+$ $CD44^+$ $CD62L^-$ effector memory T cells, which were isolated from draining lymph nodes of mice to which DNFB had been applied 5 days earlier. Chemotaxis assay using transwells showed that naive T cells had a good chemotactic response to S1P at a peak of 100 nM (Figure 7a). Consistent with the previous finding that FTY720 treatment of mice ablates S1P responsiveness in lymphocytes, T cells from mice treated with FTY720 lost the ability to respond to S1P (data not shown). In contrast, central memory T cells had a weak chemotactic activity to S1P at a peak around 1–100 nM, whereas effector memory T cells did not show any chemotactic response to S1P (Figure 7b and c). These results revealed that S1P chemotactic response was high in naive T-cell subsets, marginal in effector memory T-cell subsets, and very low in central memory T-cell subsets.

***S1P*$_1$ mRNA expression in T-cell subsets**

To measure *S1P*$_1$ abundance in each T-cell subset, regional lymph node cells were prepared from B6 mice primed with

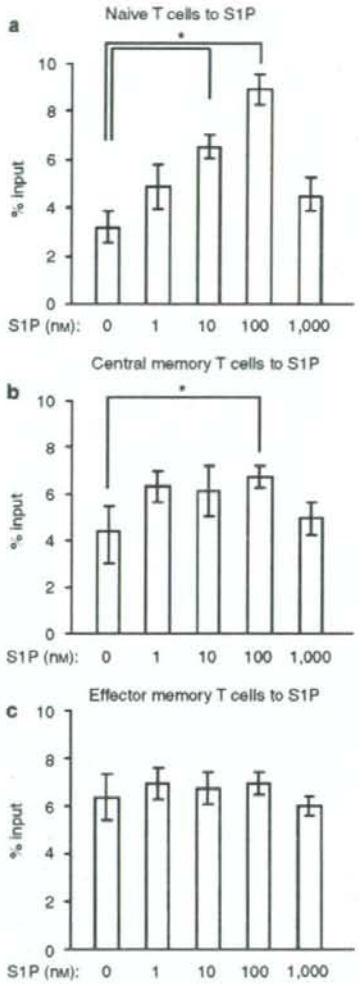

Figure 7. Chemotactic response to S1P in T-cell subsets. (a–c) Inguinal, axillary, and brachial lymph node cells were prepared from B6 mice. Input cells and cells that migrated to the lower well of a transwell chamber in the absence of S1P or in response to 1, 10, 100, or 1000 nM S1P were analyzed by flow cytometry. The percentage (%) input of naive (a), central memory (b), and effector memory (c) T cells was determined. Bars represent means ± SD of triplicated transwells, and data are from three independent experiments. Student's *t*-test was performed between the indicated groups. $^*P<0.05$.

DNFB 5 days before, and $CD4^+$ $CD44^-$ $CD62L^+$-naive T cells, $CD4^+$ $CD44^+$ $CD62L^+$ central memory, and $CD4^+$ $CD44^+$ $CD62L^-$ effector memory T cells were purified by a cell sorter. We evaluated $S1P_1$ mRNA levels by quantitative PCR and found that memory T cells had one-fifth of the $S1P_1$ transcript level of naive T cells. Among memory T-cell subsets, effector memory T cells exhibited half the level of transcript of central memory T cells (Figure 8).

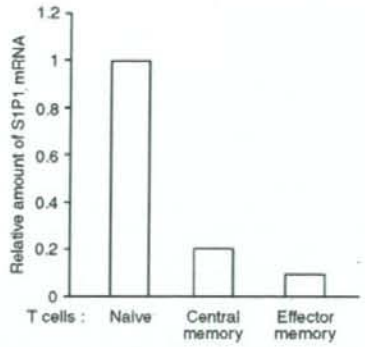

Figure 8. Quantitative PCR analysis of $S1P_1$ mRNA from T-cell subset. Lymph node cells from B6 mice 5 days after sensitization with DNFB were sorted into $CD4^+$ $CD44^-$ $CD62L^+$ naive, $CD4^+$ $CD44^+$ $CD62L^+$ central memory, and $CD4^+$ $CD44^+$ $CD62L^-$ effector memory T cells. The amounts of $S1P_1$ mRNA were expressed as relative amount of mRNA normalized to β-actin, and $S1P_1$ mRNA of naive T cells were set to 1. Data are from three independent experiments.

DISCUSSION

The results from this study support several major conclusions. First, CHS as a model of acquired skin immune response was inhibited by treatment with FTY720 during the sensitization but not the elicitation phase. Second, FTY720 did not modulate cutaneous DC functions in relation to CHS. Third, FTY720 decreased the number of circulating naive T cells more markedly than that of memory T cells. Among memory T cells, effector memory T cells were less susceptible to FTY720 than central memory T cells. Accordingly, the chemotactic response to S1P was high in the naive T-cell subset, modest in the central memory T-cell subset, and almost negligible in the effector memory T-cell subset. Finally, $S1P_1$ expression level was higher in naive T cells than in memory T cells.

It has generally been thought that FTY720 causes immunosuppression through enhancement of lymphocyte sequestration into secondary lymphoid organs, thereby preventing their Ag-activated T-cell egress to sites of inflammation. However, it remains controversial whether the effect of FTY720 is prophylactic or therapeutic. FTY720 was reported to have preventive value as observed in animal skin allograft rejection and in the experimental autoimmune encephalomyelitis model (Yanagawa *et al.*, 1998; Luo *et al.*, 1999; Yanagawa *et al.*, 1999). On the other hand, there have been two reports on the possible therapeutic role of FTY720 as seen in autoimmune and immune responses provoked by Ag-specific Th1 and Th2 (Sawicka *et al.*, 2003; Graler and Goetzl, 2004). FTY720 was shown to prevent or reduce relapse of human multiple sclerosis (Dev *et al.*, 2008) and memory T-cell-mediated colitis (Fujii *et al.*, 2006). Recently, it was reported that FTY720 affected trafficking of lymphocytes to the epithelium (Kunisawa *et al.*, 2007). An interesting issue to be pursued in the future is whether FTY720 modulates T-cell migration to the skin. In this study, we

demonstrated that the major action phase of FTY720 on CHS is the sensitization but not the elicitation limb, and therefore FTY720 functions as a prophylactic but not therapeutic modality in a mouse CHS model. Although the reason for this discrepancy remains unknown, the differences might be due to the animal models used, the type of immune or autoimmune diseases, and the extent of central/effector memory T cells involved.

It has been a matter of controversy whether FTY720 impairs the egress of memory T-cell subsets from the secondary lymphoid organs. Some investigators sought to evaluate the effect of FTY720 on circulating T-cell subsets, but the number of memory T cells was too low to assess precisely (Chiba *et al.*, 1998). Another group of investigators reported that activated T cells stimulated with anti-CD3 and anti-CD28 antibody (Ab) lost their chemotactic activity to S1P (Fujii *et al.*, 2006). Here we demonstrated that circulating memory T cells were resistant to FTY720 and that Ag-specific memory T cells were transferable and capable of inducing skin inflammation even in recipients treated with FTY720, suggesting that FTY720 does not affect memory T-cell egress from the secondary lymph nodes into the circulation. Accordingly, memory T cells—especially effector memory T cells—lost the chemotactic response to S1P, and circulating memory T cells were detected in the blood, indicating the possible existence of a mechanism other than S1P-$S1P_1$ signaling underlying memory T-cell egress from the regional lymph nodes.

Another controversial issue has been whether FTY720 affects lymphocytes in the regional lymph nodes. Although FTY720 was suggested to deplete naive T cells in peripheral lymphoid organs (Hofmann *et al.*, 2006), we found that the number of naive T cells in the regional lymph nodes after sensitization with DNFB was not changed by FTY720 in our experimental settings (data not shown). Interestingly, recent data showed that increased S1P in inflamed peripheral tissues induced T-cell retention and suppressed T-cell egress from those tissues (Ledgerwood *et al.*, 2008). How FTY720 affects the homeostasis of lymphocytes in the skin under skin inflammation such as CHS response is an issue to be pursued in the future.

We speculate about the mechanisms underlying the hyposensitization by FTY720 as follows: FTY720 administration during sensitization reduced the number of circulating naive T cells by inhibiting their emigration to the peripheral blood as demonstrated herein and by accelerating lymphocyte homing to lymph nodes and Peyer's patches as reported in another study (Idzko *et al.*, 2002). We hypothesize that the inhibitory effect of FTY720 on CHS stems from this dynamic change of T-cell circulation. It has been thought that there exist about 1×10^9 lymphocytes in one entire mouse, but there are only 1×10^7 lymphocytes in the draining lymph nodes of the abdominal skin. Therefore, a great number of naive T-cell clones may be supplied from the blood to the lymph nodes, where they are converted to memory T cells. Given that very few naive T cells are present in the circulation after FTY720 administration, the frequency of Ag-bearing DCs interacting with Ag-specific naive T-cell clones might be reduced to 1/100 of those in FTY720-nontreated mice. In fact, FTY720 treatment did not completely abrogate the CHS response (see Figure 1a), suggesting that naive T cells remaining in the regional lymph nodes may participate in the incomplete establishment of CHS response. We assume that this reduction in the incidence of DC–T-cell interaction may lead to impairment of the acquired skin immune response.

One of the important factors in the initiation of acquired skin immune response is the role of DCs. It is controversial how DC functions are controlled by S1P and thus are modified by FTY720. The responsiveness of DCs to S1P has been variously reported, as S1P attracts human blood monocyte-derived immature but not mature DCs (Czeloth *et al.*, 2005), murine mature but not immature DCs (Renkl *et al.*, 2004), or both immature and mature murine DCs (Idzko *et al.*, 2006). On the other hand, the local application of FTY720 by inhalation suppressed murine allergic asthma by inhibiting the migration of lung DCs to the mediastinal lymph nodes (Idzko *et al.*, 2006), and FTY720 inhibited FITC-induced DC migration in a certain condition (Czeloth *et al.*, 2005). Recent studies have highlighted differences between LCs and dermal DCs that migrate from skin to draining LNs (Kissenpfennig and Malissen, 2006; Schroder *et al.*, 2006; Bursch *et al.*, 2007; Ginhoux *et al.*, 2007; Poulin *et al.*, 2007). It would be valuable to know whether FTY720 has a measurable effect on the migration of the individual subsets. However, there was no effect of FTY720 on each subset in migration activity. In addition, we could not detect the functional modulation of DCs by FTY720 in our experimental settings.

Our present study provides a previously unidentified finding that FTY720 impairs naive T-cell circulation, thereby markedly disrupting the acquired skin Th1 type immune response. Consequently, FTY720 may have the potential to prevent the onset of disease rather than to treat memory T-cell-mediated acquired skin immune responses. Further investigations on the mode of action of FTY720 may increase its clinical usage and provide additional insights into the means of effectively manipulating immune responses *in vivo*.

MATERIALS AND METHODS

Animals and reagent

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

For treatment with FTY720 (Novartis Pharmaceuticals, Basel, Switzerland), B6 mice were injected intraperitoneally with $1.0\,mg\,kg^{-1}$ of FTY720 or with an equivalent volume of normal saline at the indicated time points.

Cell preparation and cultures

RPMI-1640 (Sigma, St Louis, MO) containing 10% heat-inactivated fetal calf serum (Invitrogen, Carlsbad, CA), 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Cellgro, Herndon, VA), 1 mM

nonessential amino acids, 1 mM sodium pyruvate, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin was used as a culture medium unless otherwise stated. Blood leukocytes were isolated by cardiac puncture and red blood cell lysis buffer (Kabashima *et al.*, 2006).

Flow cytometry and histology

Cell suspensions were prepared from lymph nodes by mechanical disruption on 70 μm nylon cell strainers (BD Falcon, San Jose, CA). For flow cytometry, cells were plated at a density of 1×10^6 cells per well in 96-well U-bottomed plates (BD Falcon). They were stained for 20 minutes on ice with Abs in 25 μl of phosphate-buffered saline containing 2% fetal calf serum, 1 mM EDTA, and 0.1% NaN_3, and were then washed twice with 200 μl of this buffer after each step (Kabashima *et al.*, 2007). Data were collected on a FACSCanto (BD Biosciences, San Diego, CA) and analyzed with FlowJo software (TreeStar, San Carlos, CA). Abs used were FITC-conjugated anti-CD62L Ab, PE-conjugated anti-CD44 and CD86 Abs, PE-Cy5-conjugated anti-MHC class II Ab, PE-Cy7-conjugated CD4 Ab, and allophycocyanin-conjugated anti-CD11c Ab (all from BD Biosciences). For detection of Langerin, anti-Langerin Ab (eBioscience, San Diego, CA) was used after fixation and permeabilization by the cytofix/cytoperm kit (BD Bioscience).

For histological analysis, the ears of B6 mice 24 hours after challenge with hapten were excised and fixed in 10% formaldehyde. Sections of 5 μm thickness were prepared and stained with hematoxylin and eosin.

Cell sorting and quantitative PCR

Naive, central memory, and effector memory T cells from lymph node cells of B6 mice sensitized with DNFB 5 days earlier were isolated with a cell sorter (EPICS Elite, Becton Dickinson, Franklin Lakes, NJ). The purity of the sorted cells was typically >90%. RNA was prepared from sorted cells with RNeasy (Qiagen, Valencia, CA), and cDNA was used for quantitative PCR. Murine *S1P$_1$* (Assay ID: Mm00514644_m1) expression was quantified using TaqMan Gene Expression Assay (Applied Biosystems) in the ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA). 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 (Livak and Schmittgen, 2001). The expression of the target gene normalized to an endogenous reference and relative to calibrator was given by the formula $2_T^{-\Delta\Delta C}$. Gene expression in untreated mice was used as a calibrator expression to calculate $\Delta\Delta C_T$.

Chemotaxis assay and FITC-induced cutaneous DC migration

Regional lymph node cells 5 days after DNFB application to the abdomen were tested for transmigration across uncoated 5-μm transwell filters (Corning Costar Corp., Corning, NY) to S1P (Sigma-Aldrich, St Louis, MO) or medium in the lower chamber for 3 hours, and the migrated cells were enumerated by flow cytometry (Kabashima *et al.*, 2006). The medium used in this assay was RPMI-1640 with 0.5% fatty acid-free BSA (Calbiochem, San Diego, CA).

For FITC-induced cutaneous DC migration, the shaved abdomens of mice were painted with 200 μl of 2% FITC (Sigma) dissolved in 1:1 (vol/vol) (Sigma), and the number of migrated cutaneous DCs into draining inguinal and axillary lymph nodes was determined by flow cytometry (Kabashima *et al.*, 2003b).

DNFB-induced CHS model and adoptive transfer

For the CHS model, B6 mice were immunized by application of 25 μl of 0.5% DNFB in 4:1 (vol/vol) acetone/olive oil to their shaved abdomens on day 0. They were challenged on the right ear on days 5, 10, or 15 with 20 μl of 0.3% (vol/vol) DNFB (Kabashima *et al.*, 2003a). Ear thickness was measured before and 24 hours after challenge, and the change in ear thickness was calculated as follows: (ear thickness 24 hours after challenge)-(ear thickness before challenge).

For adoptive transfer, regional axillary and inguinal lymph node cells were prepared from B6 mice sensitized with 25 μl of 0.5% DNFB on the abdomen 5 days earlier. Mice were pre-administered with or without FTY720 12 hours before being sensitized. Thy1.2$^+$ T cells were purified with beads conjugated with anti-Thy1.2 Ab (Miltenyi Biotec, Auburn, CA) using auto MACS (Miltenyi Biotec) according to the manufacturer's protocol. Sorted T cells (5×10^6, purity >95%) were injected intravenously into naive B6 mice treated with FTY720 or control normal saline 12 hours earlier. The ears of the mice were challenged with 20 μl of 0.3% DNFB 1 hours later, and ear thickness was measured before and 24 hours after challenge.

For the cell proliferation assay, Thy1.2$^+$ T cells were purified from axillary and inguinal lymph nodes 5 days after 25 μl of 0.5% DNFB application to the abdomen. Similarly, CD11c$^+$ DCs were sorted from regional lymph nodes 1 day after DNFB application to the abdomen of mice treated with FTY720 or control normal saline 12 hours earlier. The sorted T cells (5×10^5, purity >95%) by auto-MACS were incubated for 72 hours with or without purified Ag-loaded DCs (5×10^4, purity >70%), pulsed with 1 μCi ^{3}H-thymidine for the last 24 hours of culture, and subjected to a liquid scintillation counter.

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.

ACKNOWLEDGMENTS
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Possible Pathogenic Role of Th17 Cells for Atopic Dermatitis

Chizuko Koga[1], Kenji Kabashima[1], Noriko Shiraishi[1], Miwa Kobayashi[1] and Yoshiki Tokura[1]

The critical role of IL-17 has recently been reported in a variety of conditions. Since IL-17 deeply participates in the pathogenesis of psoriasis and keratinocyte production of certain cytokines, the involvement of T helper cell 17 (Th17) in atopic dermatitis (AD) is an issue to be elucidated. To evaluate the participation of Th17 cells in AD, we successfully detected circulating lymphocytes intracellularly positive for IL-17 by flow cytometry, and the IL-17$^+$ cell population was found exclusively in CD3$^+$CD4$^+$ T cells. The percentage of Th17 cells was increased in peripheral blood of AD patients and associated with severity of AD. There was a significant correlation between the percentages of IL-17$^+$ and IFN-γ$^+$ cells, although percentage of Th17 cells was not closely related to Th1/Th2 balance. Immunohistochemically, IL-17$^+$ cells infiltrated in the papillary dermis of atopic eczema more markedly in the acute than chronic lesions. Finally, IL-17 stimulated keratinocytes to produce GM-CSF, TNF-α, IL-8, CXCL10, and VEGF. A marked synergistic effect between IL-17 and IL-22 was observed on IL-8 production. The number of Th17 cells is increased in the peripheral blood and acute lesional skin of AD. Th17 cells may exaggerate atopic eczema.

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INTRODUCTION

IL-17-producing CD4$^+$ T helper cells (Th17 cells) have crucial functions in host defense, and dysregulated Th17 cell responses mediate a variety of autoimmune and inflammatory conditions such as rheumatoid arthritis, inflammatory bowel disease, and experimental autoimmune encephalomyelitis (McKenzie *et al.*, 2006). IL-6 and transforming growth factor-β are both required for induction of Th17 cells, whereas IL-23 is necessary for establishment of the Th17 lineage (Bettelli *et al.*, 2006; Mangan *et al.*, 2006). Th17 cells coexpress IL-22 (Liang *et al.*, 2006; Zheng *et al.*, 2007), which belongs to the IL-10 family of cytokines and its receptor is expressed on a variety of epithelial tissues (Wolk *et al.*, 2004). IL-17 and IL-22 cooperatively enhance some immunological responses (Liang *et al.*, 2006).

An important function of IL-17 is to coordinate local tissue inflammation through upregulation of proinflammatory and neutrophil-mobilizing cytokines and chemokines, including IL-6, GM-CSF, tumor-necrosis factor-α (TNF-α), IL-1β, KC/CXCL1, MCP-1/CCL2, MIP-2/CXCL2, MCP-3/CCL7, and MIP-3α/CCL20, as well as matrix metalloproteases, to enable activated T cells to migrate through the extracellular matrix (Nakae *et al.*, 2002; Kolls, 2006). A close relationship between IL-17 and the cutaneous milieu has been suggested by a number of observations. IL-17 induces production of certain cytokines, chemokines, and antimicrobial peptides by keratinocytes (Albanesi *et al.*, 1999, 2000; Liang *et al.*, 2006). Its cooperation with IL-22 has been documented in the antimicrobial peptide elaboration (Liang *et al.*, 2006).

Recent findings have suggested that Th17 cells profoundly participate in the pathogenesis of certain skin disorders, in particular, psoriasis (Albanesi *et al.*, 2000; Zheng *et al.*, 2007). In this Th1-mediated (Lew *et al.*, 2004), chronic inflammatory disease with epidermal hyperplasia, the role of IL-22 for dermal inflammation and acanthosis is stressed (Boniface *et al.*, 2005; Zheng *et al.*, 2007). On the other hand, involvement of IL-17 has also been shown in allergen-specific immune responses (Nakae *et al.*, 2002; Kolls, 2006). IL-17 mRNA has been detected in skin affected by allergic contact dermatitis (Teunissen *et al.*, 1998) as well as psoriasis (Zheng *et al.*, 2007). Considering the proinflammatory property of IL-17, it is an interesting issue whether and how Th17 cells are involved in the pathogenesis of atopic dermatitis (AD), a representative skin disease with a chronic clinical course (Homey *et al.*, 2006; Maintz and Novak, 2007). AD merits Th17 investigation because it is known as a Th2 cell-mediated disease, and one can evaluate the biased relationship between Th17 cells and Th1/Th2 balance in relation to psoriasis. Perhaps more importantly, the effects of IL-17 and resultant outcomes on keratinocytes and dermal vasculature may provide some insights to AD pathophysiology.

[1]*Department of Dermatology, University of Environmental and Occupational Health, Kitakyushu, Japan*

Correspondence: Dr Chizuko Koga, Department of Dermatology, University of Environmental and Occupational Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan.
E-mail: chizu-ko@med.uoeh-u.ac.jp or
Dr Kenji Kabashima
E-mail: kkabashi@med.uoeh-u.ac.jp

Abbreviations: AD, atopic dermatitis; HPF, high-power field; NHEK, normal human epidermal keratinocyte; PBMC, peripheral blood mononuclear cell; Th, T helper; TNF, tumor-necrosis factor; VEGF, vascular endothelial growth factor

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In this study, we investigated Th17 cells in the peripheral blood and skin lesions of AD, with a study of the effect of IL-17 on the production of cytokines/chemokines and vascular endothelial growth factor (VEGF) by keratinocytes. In particular, to enumerate Th17 cell, we successfully analyzed the cells by intracellular cytokine staining. Results suggest that Th17 cells are elevated in the blood and can function as an upmodulator in skin lesions of AD.

RESULTS

Increased percentage of IL-17$^+$CD4$^+$ T cells in PBMCs from severe AD patients

The atopic patients enrolled in this study were divided into three different severity groups (mild, moderate, and severe) according to the criteria of Rajka and Langeland (1989). The grouping was validated with a blood severity marker for AD, lactate dehydrogenase (Mukai *et al.*, 1990; Jacyk and Ungerer, 1991; Figure 1). As reported previously (Mukai *et al.*, 1990), severity of AD correlates well with lactate dehydrogenase level (Figure 1).

Circulating T cells bearing IL-17 in the cytoplasm were examined in AD patients. Since CD4 expression on T cells is downregulated during culture with the stimulants, CD3$^+$ and CD8$^+$ T cells positive for intracytoplasmic IL-17 were analyzed by flow cytometry. Figure 2 shows representative flow cytometry data from an AD patient. There was a discernible population of IL-17$^+$CD3$^+$ and IL-17$^+$CD8$^-$ T cells in the peripheral blood mononuclear cells (PBMCs), whereas the number of IL-17$^+$CD8$^+$ T cells was not substantial, indicating that CD4$^+$ T cells are the major source of IL-17.

Figure 3a summarizes the percentages of IL-17$^+$CD4$^+$ cells in the patients tested. The values differed significantly among the three groups of AD patients, with different severity. IL-17$^+$CD4$^+$ T-cell number was higher in the severe group ($0.71 + 0.09$) than the healthy control ($0.42 + 0.07$). The mean percentage of IL-17$^+$CD4$^+$ cells in psoriasis vulgaris ($1.3 + 0.2$) was slightly higher than that of severe AD but there was no statistical significance. As for expression of other cytokines, there was a tendency of both

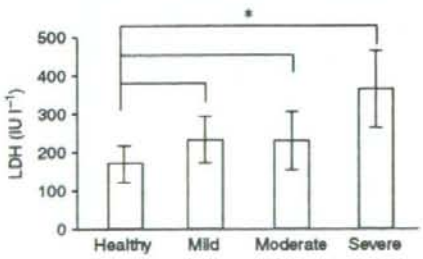

Figure 1. Lactate dehydrogenase levels in different severity groups of AD patients. The AD patients were classified as acute ($n = 23$), mild ($n = 7$), moderate ($n = 5$), and severe ($n = 11$) by clinical findings, and serum levels of lactate dehydrogenase from AD patients were compared with that in healthy donors. Student's *t*-test was performed between the indicated groups and an asterisk indicates $P < 0.05$.

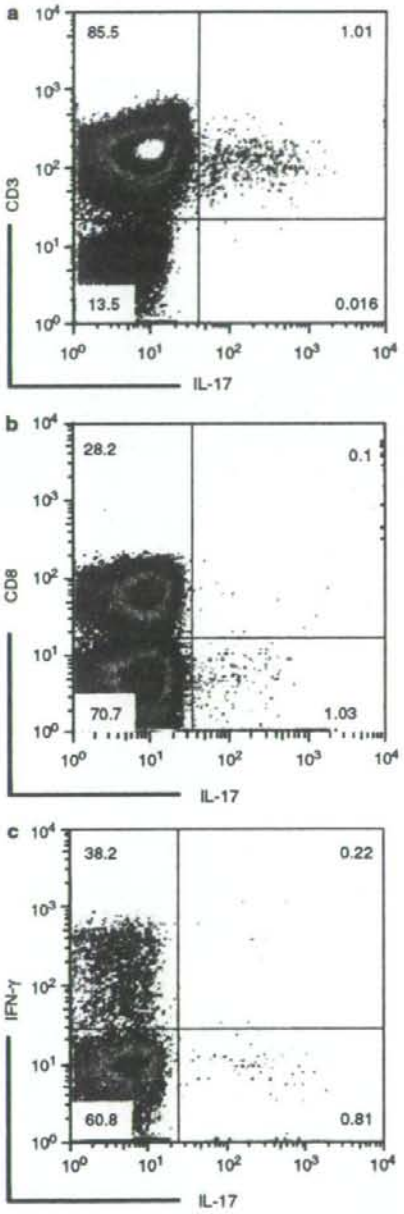

Figure 2. Flow cytometric analysis of IL-17$^+$ cells in peripheral blood lymphocytes. PBMCs isolated from a 30-year-old man with AD were stimulated with PMA and ionomycin for 8 hours and Golgistop was added. Then, production of IL-17 by PBMCs was determined at the single-cell level by intracellular cytokine staining and flow cytometric analysis. The numbers in the upper and lower right quadrants represent the percentage of IL-17$^+$ cells with or without expression of CD3$^+$ or CD8$^+$ cells in the total lymphocyte populations gated.

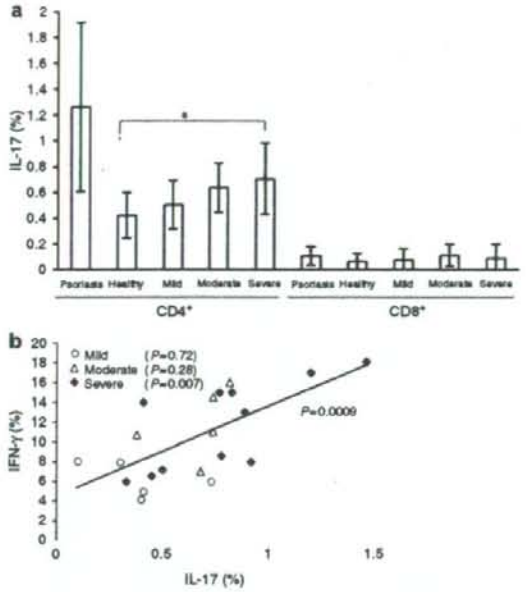

Figure 3. Percentage of circulating IL-17$^+$ T cells in relation to AD severity and IFN-γ^+ T cells. AD patients were divided into three different severity groups (mild, $n=7$; moderate, $n=5$; and severe, $n=11$). (a) Healthy subjects ($n=9$), three groups of AD, psoriatic patients ($n=16$) were compared for percentage of IL-17$^+$ and CD4$^+$ or CD8$^+$ T cells, and a significant correlation between healthy donors and severe AD patients was found ($P=0.03$). (b) IFN-γ^+ T cells of all AD patients were also analyzed. A significant correlation between percentage of IL-17$^+$ CD4$^+$ T cells and IFN-γ^+ T cells was found ($P=0.0009$). In addition, the correlation (P-value) between the percentage of IL-17$^+$ CD4$^+$ T cells and IFN-γ^+ T cells among acute, moderate, and severe AD patients was depicted.

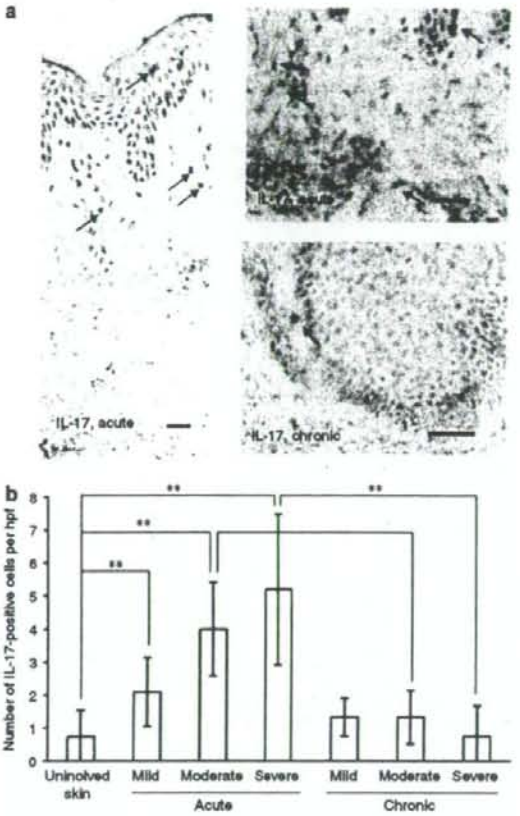

Figure 4. Immunohistochemistry of IL-17$^+$ cells in skin lesions of AD. (a) A skin specimen from an AD patient was immunohistochemically stained for IL-17. Left, low-magnification image of acute lesion; top right, high-magnification image of acute lesion; and bottom right, high-magnification image of chronic lesion. Bar = 30 μm. (b) The number of IL-17$^+$ cells was enumerated in acute and chronic lesions from mild, moderate, and severe cases of AD. Columns show mean ± SD. Student's t-test was performed between the indicated groups and an the asterisk indicates $P<0.05$.

IFN-γ^+ T cells (11.4 + 1.4) and IL-4$^+$CD4$^+$ cells (0.49 + 0.07) to be elevated in AD patients compared with in healthy subjects (IFN-γ, 9.2 + 1.5; IL-4, 0.35 + 0.07). IFN-γ^+ T cells were also elevated markedly in psoriasis (41.7 + 10.6). In AD patients, a significant correlation ($R=0.67$, $P=0.0037$) between the percentage of IL-17$^+$CD4$^+$ cells and IFN-γ^+ T cells was found (Figure 3b), but not between IL-17$^+$CD4$^+$ cells and IL-4$^+$CD4$^+$ cells (data not shown). We also examined whether IL-17$^+$CD4$^+$ T cells are related to Th1/Th2 balance. No significant correlation was found between the number of IL-17$^+$CD4$^+$ T cells and the ratio of IFN-γ^+ T cells/IL-4$^+$CD4$^+$ cells. These data suggest that AD patients have a high percentage of Th17 cells depending on the severity of atopic eczema, and the level of Th17 cells is not closely associated with Th balance.

Infiltration of IL-17$^+$ T cells in skin lesions of AD

Skin biopsy specimens were taken from acute and chronic skin lesions of patients with mild, moderate, and severe AD. The chronic form was defined by the presence of lichenified or pruriginous eruptions, whereas scaly erythematous lesions were categorized as the acute form. They were subjected to immunohistochemical staining for IL-17, IFN-γ, and IL-4. IL-17-positive lymphocytes were found in the papillary areas in the upper dermis, and some epidermotropic lymphocytes also bore IL-17 (Figure 4a). The percentage of IL-17-bearing lymphocytes was higher in the acute than in chronic lesions. In acute lesions, the number of IL-17$^+$ lymphocytes per high-power field (HPF) depended on the severity of AD (Figure 4b). There was no significant difference in the number of IFN-γ^+ cells/HPF between the acute and chronic lesions, and IFN-γ^+ cells/HPF tended to infiltrate at a higher percentage in the severe form (1.18 + 0.20) than in the mild form (0.70 + 0.01). As to IL-4$^+$ cells/HPF, no significant difference existed between acute and chronic lesions, but again, severity

dependency was observed in acute lesions (severe, 0.99 + 0.20; mild, 0.68 + 0.16).

Augmentation of keratinocyte production of GM-CSF, TNF-α, IL-8, and VEGF by IL-17

The effect of IL-17 on keratinocyte production of GM-CSF, TNF-α, IL-8, CCL5, CXCL10, CCL22, and VEGF was examined in normal human epidermal keratinocytes (NHEKs). GM-CSF and TNF-α are proinflammatory cytokines and induce maturation of Langerhans cell and dermal dendritic cells (Bechetoille *et al.*, 2006). IL-8/CXCL8 attracts neutrophils (Keller *et al.*, 2005). VEGF stimulates vascular endothelial cells to proliferate (Breier *et al.*, 1992). Since IL-22 cooperates with IL-17 for Th17 to function as an immunomodulator in certain conditions (Boniface *et al.*, 2007; Zheng *et al.*, 2007), the synergistic effects of these two cytokines were also tested. IL-17 and/or IL-22 was added at the starting of experimental NHEK culture. Three-day culture supernatants were measured for the above-mentioned cytokines, chemokines, and VEGF. As compared with the non-addition control, IL-17 markedly augmented production of proinflammatory cytokine GM-CSF (Figure 5) and TNF-α (data not shown), whereas IL-22 enhanced TNF-α but not GM-CSF. As for chemokines, IL-17 highly increased IL-8 production (Figure 5), but decreased CCL5 that of (data not shown), and IL-22 virtually shared these effects with IL-17, but to a lesser extent. CXCL10 production was augmented by both IL-17 and IL-22, with the latter being stronger, whereas CCL22 level was not changed by either IL-17 or IL-22 (data not shown). The production of VEGF was upmodulated by IL-17 but not IL-22. The results suggest that IL-17 is a powerful stimulator for keratinocytes to produce skin inflammation-associated molecules. Synergistic effects of IL-17 and IL-22 were remarkably found on IL-8 production (Figure 5), whereas they did not exhibit additive or synergistic effects on the other products.

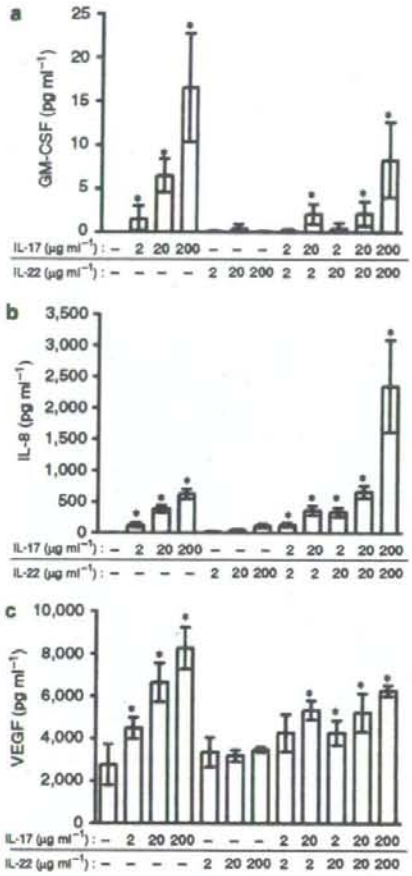

Figure 5. Effects of IL-17 and/or IL-22 on keratinocyte production of cytokines/chemokines and VEGF. NHEKs were cultured with or without IL-17 and/or IL-22 at the indicated doses. Three-day culture supernatants were subjected to analysis with the cytometric beads array system or ELISA. Columns show mean ± SD of data using triplicated wells. The asterisk indicates statistically significant differences compared with the untreated group ($P<0.05$, unpaired two-tailed *t*-test). Data are representative of those from three independent experiments.

DISCUSSION

Intracellular detection of IL-17 is necessary for enumeration of Th17 cells in PBMCs. In this study, we successfully counted their percentage by flow cytometry and investigated whether this particular T-cell population was changed in number in AD patients. Our study demonstrated that Th17 cells were increased in percentage in PBMCs of AD patients and infiltrated in the eczematous lesions of AD, and IL-17 stimulated keratinocytes to produce cytokines/chemokines and VEGF. It is noteworthy that percentage of IL-17$^+$CD4$^+$ cells in AD patients was slightly lower than that of psoriatic patients, a well-known disease whose pathogenesis involves Th17 (Zheng *et al.*, 2007).

The important role of Th17 cells for AD is suggested by the finding that percentage of Th17 was associated with severity of AD. The percentage of Th17 was significantly correlated with the percentage of IFN-γ-producing Th1 cells, but not with that of IL-4-producing Th2 cells. Moreover, there was no strong association of Th17 cells with Th1/Th2 balance. These results suggest that Th17 cells participate in the development of AD as an enhancer, but not an immune-polarizer, of AD. While IFN-γ-producing T cells are involved in the chronic lesions of AD (Grewe *et al.*, 1994), Th17 cells possibly play a role for prolonged exaggeration of AD lesions.

An immunohistochemical study of atopic eczema revealed infiltration of IL-17-secreting cells in the lesional skin. In accordance with the previous study (Toda *et al.*, 2003), Th17 infiltrated more markedly in acute eczematous lesions than in chronic ones in a severity-dependent manner. Thus, Th17 is considered to serve as an initial cytokine source for development of the skin lesions. T-cell-driven keratinocyte activation plays a relevant role in the pathogenesis of prolonged inflammatory skin disorders, including AD. Our

evaluation of the effects of IL-17 on keratinocyte production of cytokines, chemokines, and VEGF demonstrated that IL-17 stimulates NHEKs to produce GM-CSF, TNF-α, IL-8, CXCL10, and VEGF. IL-17 increases production of GM-CSF, IL-6 (Albanesi *et al.*, 2000), and IL-8 (Albanesi *et al.*, 1999) directly and in synergism with IFN-γ, IL-4, and/or TNF-α, whereas CCL5 production is decreased (Albanesi *et al.*, 1999). Their observations are virtually the same as ours, except for CXCL10, which we found to be upregulated by IL-17 and more remarkable by IL-22. IL-17 also stimulates keratinocytes to express CD54 in the presence of IFN-γ (Albanesi *et al.*, 1999). Thus, keratinocyte production of proinflammatory cytokines is augmented in Th17-infiltrating atopic skin lesions. Th17 also may promote proliferation of dermal vessels by VEGF, a sequential event for exacerbation of AD (Wakita *et al.*, 1994).

In previous studies, the synergistic effect of IL-17 and IL-22 has not been studied fully. Since IL-17 and IL-22 are coexpressed by Th17 cells and expression of both cytokines is initiated by transforming growth factor-β (Liang *et al.*, 2006; Zheng *et al.*, 2007), their synergistic effects on keratinocytes are an issue to be clarified, but has been reported in only one study, which demonstrated synergism in keratinocyte expression of antimicrobial peptides (Zheng *et al.*, 2007). We found significant synergism in IL-8 production, but not for any other products.

Psoriasis and AD are two representative inflammatory skin diseases. Although mediated by Th1 (Gudjonsson *et al.*, 2004) and Th2 cells (Leung and Soter, 2001), respectively, there are exacerbating factors common to these disorders, as exemplified by keratinocyte-derived proinflammatory cytokines and VEGF (Wakita *et al.*, 1994; Bhushan *et al.*, 1999). It is suggested that IL-17 is involved in this common process of skin inflammation as a proinflammatory cytokine.

MATERIALS AND METHODS

All experiments were conducted in accordance with the Declaration of Helsinki Principles.

Subjects

A total of 23 patients with AD (aged 9–51 years; 13 men and 10 women), and nine healthy non-AD volunteers with low serum IgE levels (aged 24–38 years; 5 men and 4 women) were enrolled in this study. AD was diagnosed according to the criteria of Hanifin and Rajka (1980). Severity of AD was evaluated by the criteria of Rajka and Langeland (1989) and AD was classified into mild, moderate, or severe. Serum IgE levels of the patients ranged from 43 to 160,000 IU ml^{-1} and averaged 14,149 IU ml^{-1}. None of the patients had oral steroids or immunosuppressants. Sixteen patients with psoriasis vulgaris (aged 26–77 years all men; average PASI score, 11.3 + 2.9) were also tested as a disease control. 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.

Intracellular cytokine staining of PBMCs

PBMCs were isolated from patients and control subjects by standard Ficoll-Paque method (Phamacia, Uppsala, Sweden). Intracellular cytokines were stained according to the protocol of Cytostain (Immunotech, Marseille, France), with a few modifications. Briefly, cells (2×10^6 cells ml^{-1}) were incubated in complete RPMI (RPMI-1640 (Sigma Chemical Co., St Louis, MO) containing 10% heat-inactivated fetal calf serum (Invitrogen, Carlsbad, CA), 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 25 mM HEPES (Cellgro, Herndon, VA), 1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin) in a 24-well plate with 10 ng ml^{-1} of phorbol-12-myristate 13-acetate (Sigma Chemical Co.), 10^{-6} M of ionomycin (Wako, Osaka, Japan), and 0.7 μl of Golgistop (BD Biosciences, San Diego, CA) for 8 hours. Then, cells were washed and directly stained with PerCP-conjugated anti-CD8 mAb (BD Biosciences) and subsequently with APC-conjugated anti-CD3 mAb (BD Biosciences) for 20 minutes at 4 °C. After washing, 100 μl of Cytofix/Cytoperm buffer (BD Biosciences) was added to each well and incubated for 20 minutes at room temperature, and washed with Perm/Wash solution as per manufacture's protocol (BD Biosciences). They were stained with phycoerythrin-labeled anti-IL-17, IL-4, or IL-5, and FITC-labeled anti-IFN-γ mAb, for 20 minutes at 4 °C. Fluorescence profiles were analyzed by flow cytometry in FACSCanto (BD Biosciences).

Immunohistochemical staining

We obtained 27 biopsy specimens from 13 atopic patients. The specimens were obtained from acute and chronic lesions of AD and frozen in Tissue-Tek OCT compound. Chronic lesions were defined as lichenified or pruriginous eruptions, and scaly erythematous lesions were categorized as acute lesions. Cryostat sections (7 μm) were fixed in acetone and stained as described previously (Kabashima *et al.*, 2005) with rat anti-human IFN-γ, IL-4, and IgG1 (BD Biosciences), and mouse anti-human IL-17 and IgG1 (eBiosciences, San Diego, CA). Rat and mouse antibodies were detected using biotinylated anti-rat and mouse IgG (Dako Cytomation, Kyoto, Japan), followed by treatment with horseradish peroxidase-conjugated streptoavidin (KPL, Gaithersburg, MD). Peroxidase enzyme reactions were developed with conventional substrates, diaminobenzidine (Sigma Chemical Co.). The sections were lightly counterstained with hematoxylin. The number of immunoreactive lymphocytes was enumerated from five HPFs in the dermis of each immunostained section. Data were expressed as the number of IL-17 cells/HPF.

Keratinocyte culture and measurements of cytokines and VEGF

NHEKs were purchased from Cascade Biologics (Portland, OR). They were grown in the serum-free keratinocyte Epilife growth medium (Cascade Biologics) and used at the third passage in all experiments (Kobayashi *et al.*, 2007). Growth supplement was omitted 48 hours before experiments. IL-17 and/or IL-22 (R&D Systems, Minneapolis, MN) were added at the beginning of experimental culture using 24-well plates (Corning Glass Works, Corning, NY). Three-day culture supernatants were examined for GM-CSF, TNF-α, IL-8, RANTES/CCL5, IP-10/CXCL10, MDC/CCL22, and VEGF levels. The concentration of these substances, except for that of CCL22, was measured using cytometric beads array system (BD Bioscience) according to the manufacturer's protocol. The concentration of CCL22 was measured using ELISA kits (BD Bioscience) according to the manufacturer's directions.

Statistical analysis

Student's *t*-test (impaired) was employed to determine statistical differences between means. Correlations were studied by Pearson product-moment correlation coefficient.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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Table 1 *Ex vivo* skin penetration and permeation of eucalyptol (μg/cm²; mean ± S.D.; $n = 4$)

Amount of eucalyptol	Child skin			Adult skin		
	15 min	30 min	60 min	15 min	30 min	60 min
Stratum corneum total	712 ± 43	763 ± 40	698 ± 78	564 ± 46	549 ± 59	575 ± 127
Epidermis with *dermis*	460 ± 184	1248 ± 281	1357 ± 158	319 ± 120	543 ± 54	845 ± 98
Skin total	1172 ± 98	2011 ± 170	2055 ± 193	883 ± 138	1092 ± 108	1420 ± 161
Acceptor fluid	0	45 ± 16	73 ± 17	0	0	0

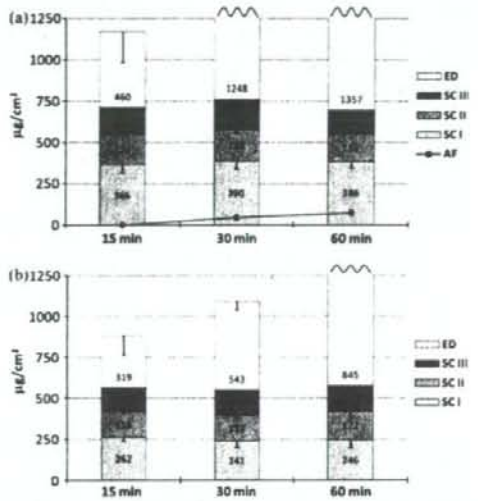

Fig. 1 Absorption of eucalyptol (mean ± S.D., $n = 4$). (a) In child skin layers. (b) In adult skin layers. *Abbreviations*: ED, *epidermis* with *dermis*; SC III, inner *stratum corneum*; SC II, middle *stratum corneum*; SC I, outer *stratum corneum*.

Even if side effects for eucalyptol are sporadically reported, the possibility of their appearance when using eucalyptol containing drug products should be appreciated. The molecular mass of eucalyptol (<500 Da) predicts it to have a possible allergenic factor [8]. This seems to be essential for the application of eucalyptol containing preparations onto the skin of young children because their skin can be outright "open" for exogenous substances.

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Krzysztof Cal*
Monika Sopala
Medical University of Gdansk,
Department of Pharmaceutical Technology,
Hallera 107, 80-416 Gdansk, Poland

*Corresponding author. Tel.: +48 58 349 3183;
fax: +48 58 349 3190

E-mail address: kcal@wp.pl (K. Cal)

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LETTER TO THE EDITOR

Evaluation of photoallergic potential of chemicals using THP-1 cells

KEYWORDS
Photoallergy; Photohapten; Prohapten; THP-1

Both systemic and topical exogenous photosensitizers evoke cutaneous photosensitivity and clinically recognized drug photosensitivity and photocontact dermatitis, respectively. The action spectrum of these two types of photosensitivity is mainly ultraviolet A (UVA) [1]. Photosensitive chemicals have both phototoxic and photoallergic potentials. Phototoxic reaction is mainly mediated by free