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

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

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

2. Materials and methods

2.1. Mice

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

2.2. Chemicals and monoclonal antibodies (mAbs)

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

2.3. CHS and application or administration of KP

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

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

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

2.4. Cell purification

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

2.5. Cell transfer

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

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

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

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

Total mRNA was extracted from draining LNCs with the SVTotal RNA Isolation system (Promega, Madison, WI, USA) according to the manufacturer's protocol. Murine *Foxp3* gene expression (assay ID: Mm00475156_m1) was quantified in a two-step RT-PCR. cDNA was reverse transcribed from total RNA samples using the TaqMan RT reagents (Applied Biosystems, Foster City, CA, USA). Target gene expression was quantified using TaqMan Gene Expression Assay (Applied Biosystems) in the ABI PRISM 7000 sequence detection system (Applied Biosystems). As an endogenous reference for these PCR quantification studies, GAPDH gene expression was measured using the TaqMan rodent GAPDH control reagents (Applied Biosystems). The relative expression was calculated using the $2^{-\Delta\Delta C_T}$ method. The expression of the target gene normalized to an endogenous reference and relative to calibrator is given by the formula $2^{-\Delta\Delta C_T}$. Gene expression in PCl-treated mice was used as a calibrator expression to calculate $\Delta\Delta C_T$.

2.8. PGE_2 assay

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

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

2.9. Statistic analysis

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

3. Results

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

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

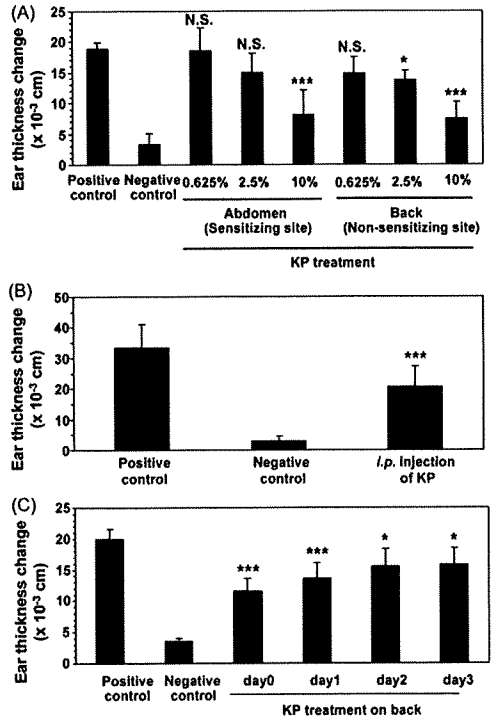

Fig. 1. Systemic suppression of CHS by KP painting on various application days. While mice were sensitized on the abdomen and challenged on the ears, KP was applied on day 0 to the sensitizing or non-sensitizing site at 0.625%, 2.5% or 10% (A). Instead of topical application of KKP, 2 mg/kg of KP was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

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

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

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

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

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

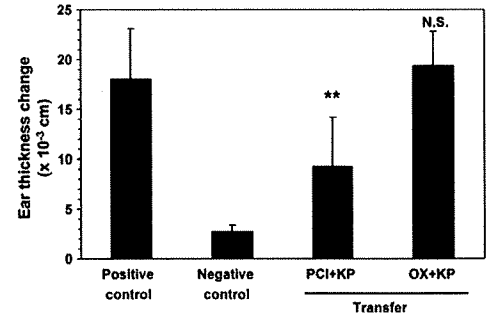

Fig. 2. Hapten-specific suppression of CHS by KP application. Twenty-four hours before PCl sensitization, mice were injected *i.v.* with 2.4×10^7 LNCs obtained from syngeneic mice that were treated with PCl + KP or OX + KP 5 days earlier. Positive control mice were sensitized and challenged without cell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

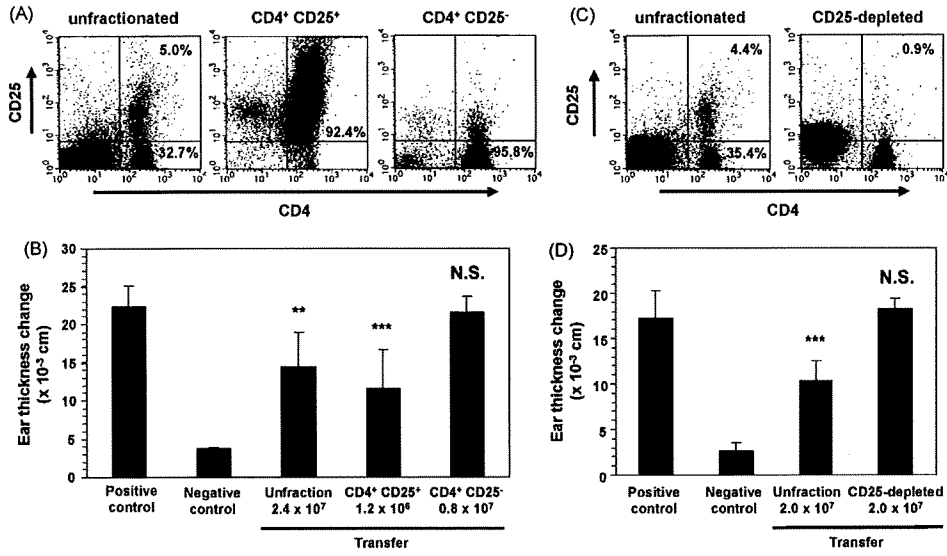

Fig. 3. Mediation of KP-induced suppression by $CD4^+$ $CD25^+$ cells. LNCs were collected from mice that were treated with PCl + KP 5 days earlier. $CD4^+$ $CD25^+$ cells or $CD4^+$ $CD25^-$ cells were purified by MACS (A). After cell separation, 2.4×10^7 unfractionated cells, 1.2×10^6 $CD4^+$ $CD25^+$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

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

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

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

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

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

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

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

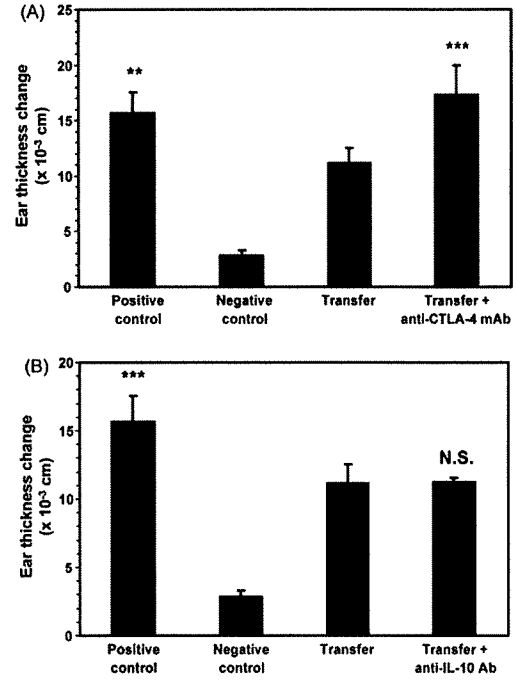

Fig. 4. Inhibition of KP-induced suppression by blockade of CTLA-4 but not IL-10. (A) Twenty-four hours before sensitization, mice were injected *i.v.* with 2.4×10^7 LNCs obtained from syngeneic mice that were treated with PCl + KP 5 days earlier (Transfer). A group of mice (Transfer + CTLA-4 mAb) were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

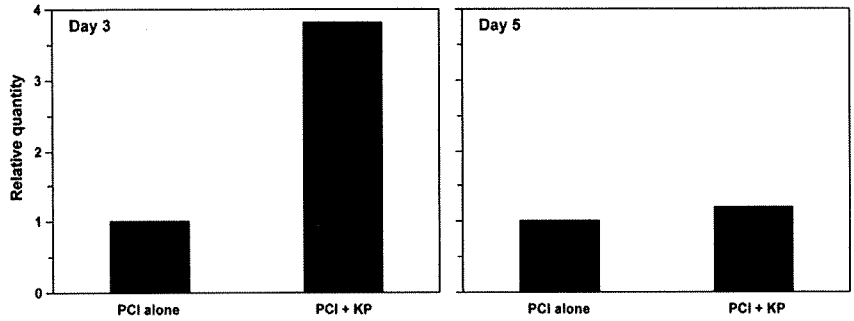

Fig. 5. Induction of Foxp3 mRNA expression in LNCs by KP. Mice were treated on the abdomen with PCI alone or PCI + KP. LNCs were collected on day 3 or day 5 after treatment. Foxp3 gene expression was quantified in a two-step real-time PCR. GAPDH gene expression was measured as an endogenous control for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

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

3.6. Inhibition of PGE_2 production by KP treatment in LNCs

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

4. Discussion

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

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

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

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

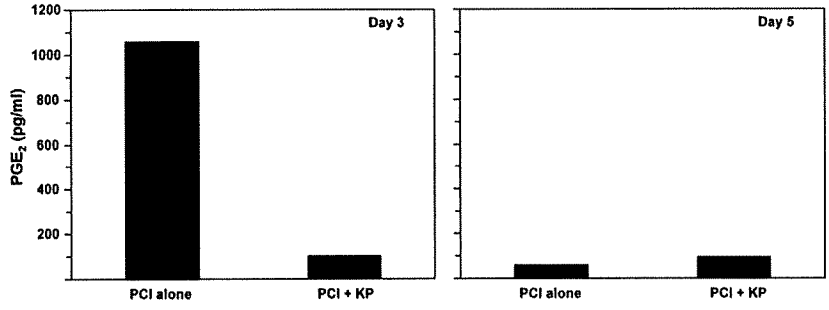

Fig. 6. Inhibition of PGE_2 production in LNCs by KP. Mice were treated on the abdomen with PCI alone or PCI + KP. LNC were collected on day 3 or 5 and cultured in cRPMI medium for 48 h. A quantitative analysis of PGE_2 release in the supernatants was performed as described in Section 2.

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

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

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

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ORIGINAL ARTICLE

IL-10-Producing Langerhans Cells and Regulatory T Cells Are Responsible for Depressed Contact Hypersensitivity in Grafted Skin

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Although skin grafting is a common surgical technique, the immunological state of grafted skin remains unelucidated. An experimental model has shown that the development of murine contact hypersensitivity (CHS) is depressed when mice are sensitized with a hapten through full-thickness grafted skin. We explored the immunological mechanisms underlying this hyposensitization, focusing on the fate of Langerhans cells (LCs). When FITC was applied to grafted skin, FITC-bearing LCs were capable of migrating to the draining lymph nodes. Epidermal cell suspensions isolated from the grafted skin produced a high amount of IL-10 as assessed by real-time PCR. Adoptive transfer of immune lymph node cells from the sensitized mice suppressed the CHS response of recipients in an antigen-specific manner. $CD4^+CD25^+$ but not $CD4^+CD25^-$ T cells purified from lymph node cells were responsible for this suppression. Finally, we detected high expression of receptor activators of nuclear factor κ-B ligand (RANKL) in the grafted skin, and found that recombinant RANKL stimulated LCs to produce IL-10. These findings suggest that the hyposensitization of CHS through the grafted skin is not attributable merely to the reduction of LC number but that IL-10-producing LCs exert a downmodulatory effect by inducing regulatory T cells.

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INTRODUCTION

Skin grafting is frequently employed in dermatology and plastic surgery. Skin grafts from the same individual can be successfully implemented 7 days after an operation, and they survive throughout the patient's life. Full-thickness skin grafts exhibit a well-matched appearance, whereas thin-thickness grafts match poorly in texture. Although there have been many cosmetic studies on skin grafting, little is known about the immunological state or the fate of immunocompetent cells in grafted skin. In particular, the function of Langerhans cells (LCs) in grafted skin is poorly understood. Clinical observations have suggested impaired immunity in grafted skin (Doiurnon *et al.*, 2001), and the lack of dermatitis on the graft is empirically known; therefore, some immunological alterations are thought to take place in the graft. Only one study has demonstrated that murine contact hypersensitivity (CHS) to a hapten cannot develop upon sensitization through full-thickness grafted skin (Yasuda *et al.*, 1996).

LCs are the major immunocompetent cells in the skin (Katz *et al.*, 1979; Stingl *et al.*, 1980) and have a positive (Silberberg-Sinakin and Thorbecke, 1980) or, in certain conditions, suppressive (Kaplan *et al.*, 2005) role in the development of CHS. They take up external antigens, migrate to draining lymph nodes, and present the antigenic determinant to naive T cells in the context of major histocompatibility complex (MHC) molecules. Thus, LCs are critical in sampling and presenting antigens in the skin. Recent studies have disclosed an immunoregulatory role of LCs. These epidermal dendritic cells (DCs) may exert a suppressive effect when they produce IL-10 (Kang *et al.*, 1998; Flacher *et al.*, 2006). This is consistent with the observation that IL-10 production by pulmonary DCs is critical for the induction of tolerance (Akbari *et al.*, 2001). Recently, it has been shown that receptor activators of nuclear factor κ-B ligand (RANKL) produced in UV light-irradiated epidermis mediate immunosuppression by modulating LCs (Loser *et al.*, 2007).

In this study, we explored the fate and functional alterations of LCs in full-thickness grafted skin using a murine CHS experimental system. Our results suggest that the immunological tolerance induced by sensitization through

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Abbreviations: CHS, contact hypersensitivity; DC, dendritic cell; dDC, dermal DC; EC, epidermal cell; LC, Langerhans cell; mAb, monoclonal antibody; MACS, magnetic cell sorting; MHC, major histocompatibility complex; PE, phycoerythrin; PCl, picryl chloride; Treg, regulatory T cell; TNF, tumor necrosis factor; RANKL, receptor activator of nuclear factor κ-B ligand

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the grafted skin is mediated by IL-10-producing LCs after the induction of $CD4^+CD25^+$ regulatory T (Treg) cells. Over-expressed RANKL in the keratinocytes of grafted skin may stimulate LCs to produce IL-10.

RESULTS

Poor development of CHS in mice sensitized with PCl through grafted skin

To confirm the previous observation by Yasuda *et al.* (1996) and to further examine whether the suppression of CHS by sensitization through skin grafting is a local or systemic phenomenon, mice were sensitized with picryl chloride (PCl) through grafted dorsal skin or non-grafted abdominal skin after skin graft implementation (on day 7 after operation). When PCl was applied to the grafted area for sensitization, the ear swelling challenge response was significantly lower than that of the positive control without skin graft (Figure 1). In contrast, sensitization of the skin-grafted mice through the non-grafted abdominal area did not abrogate CHS response. Similar data were obtained from three independent series of experiments. Thus, the depressed CHS response occurred only when sensitization was performed through the grafted local area.

Numerical alteration and apoptosis of LCs in grafted skin

LCs are critical for CHS, as they serve as antigen-presenting cells and migrate to the draining lymph nodes (Romani *et al.*, 2003). We therefore investigated the change in number of LCs in the grafted skin along with the draining lymph nodes. Epidermal sheets were taken from the grafted skin on days 7 and 14 after operation and stained with phycoerythrin (PE)-labeled anti-I-A monoclonal antibody (mAb). The number of LCs was lower in the grafted skin (200–300 mm^{-2}) on day 7 than in the untreated control skin (800–1,000 mm^{-2}). Moreover, the number of LCs in grafted skin returned to normal on day 14 (data not shown). These results indicate that the LC number was reduced at the time of sensitization. Morphologically, LCs in the grafted skin exhibited a round appearance. This reduction in LC number was not due to the migration of LCs from the skin, as the number of DCs was not increased in the regional lymph nodes (data not shown). Epidermal cell (EC) suspensions from normal skin or grafted skin were assessed for apoptosis by flow cytometry. The fraction of apoptosis in LCs was demonstrated as the Annexin-V-positive propidium iodide–negative subset in the grafted skin (Figure 2a). The percentage of apoptotic LCs was significantly increased on day 1 after grafting, but returned to the baseline on day 4 (Figure 2b).

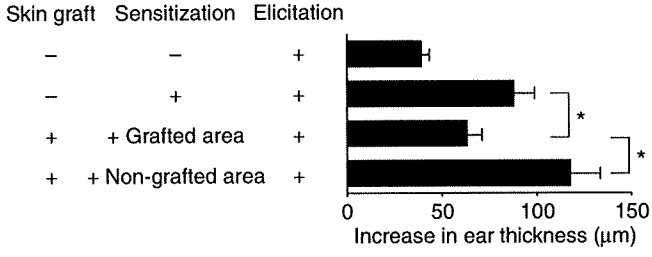

Figure 1. CHS responses in mice sensitized with PCl through grafted or non-grafted skin. Mice were sensitized with PCl on the grafted back skin or non-grafted abdominal skin 7 days after skin grafting. PCl was challenged on each earlobe 5 days after sensitization. The change in ear thickness was measured 24 hours later. Data are representative of three independent experiments. Each group consisted of more than four mice. *$P<0.05$.

Phenotypes and numbers of migrating LCs and dermal DCs in grafted skin

LCs are capable of migrating from the epidermis into the lymph nodes on sensitization (Kabashima *et al.*, 2003). The migratory ability of LCs in grafted skin was examined with FITC, which is not only a hapten but also a cell-tracking marker. On day 7 after skin grafting, FITC was applied to the grafted area. Draining lymph node cells were taken 24 hours later and labeled with allophycocyanin-labeled anti-I-A mAb, anti-mouse CD205 rat IgG, followed by PE-conjugated anti-rat IgG mAb. $FITC^+$ MHC class II^+ cells were defined as migrating DCs from the skin. CD205 is expressed by LCs as well as by dermal DCs (dDC; Henri *et al.*, 2001). In fact,

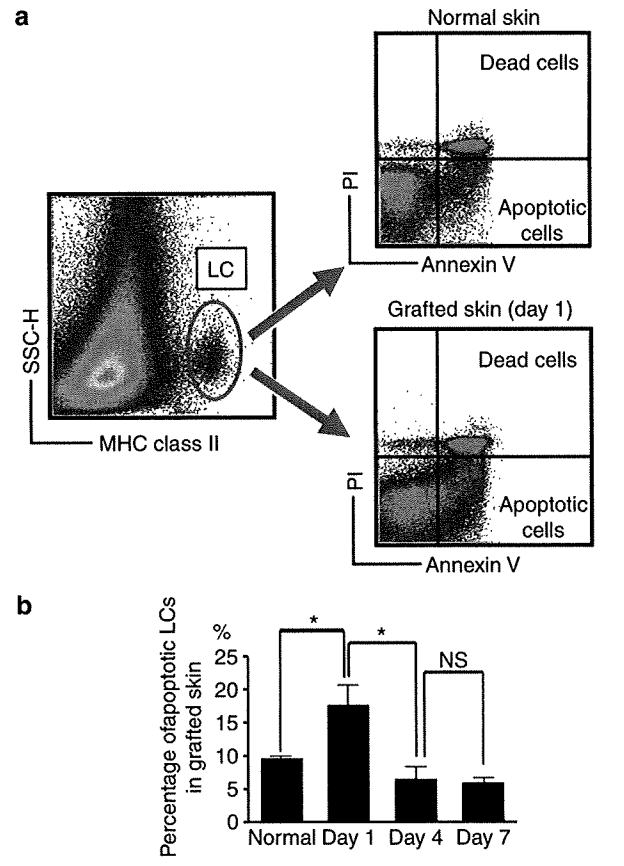

Figure 2. Numerical and morphological alterations and apoptosis of LCs in grafted skin. (**a**) Apoptosis of Langerhans cells in the grafted skin. EC suspensions from the 1-day grafted or non-grafted skin were stained with FITC-conjugated MHC class II, APC-conjugated Annexin V and PI. Apoptotic LCs were defined as MHC class II^+ Annexin V^+, but PI^-, whereas necrotic cells were double positive. (**b**) Percentage of apoptotic LCs after grafting procedure. Day 0 represents normal skin and day 1, 4, or 7 shows the day after grafting. Data are representative of three independent experiments. Each group consisted of five mice. *$P<0.05$.

migrating DCs were divided into a CD205$^+$ Langerin$^+$ subset and a CD205$^-$ Langerin$^-$ subset. Therefore, the vast majority of FITC$^+$ MHC class II^{hi} CD205$^+$ cells represent LCs, whereas FITC$^+$ MHC class II^{hi} CD205$^-$ cells are mostly dDCs (Figure 3a).

To determine whether migrating LCs/dDCs in grafted skin retained their antigen-presenting capacity, migrating DCs were stained with PE-conjugated mAbs to CD80 and CD86. CD80 and CD86 were present on both LCs and dDCs. Thus, LCs/dDCs in grafted skin retained their antigen-presenting capacity for naive T cells.

Compared to the control mice painted with FITC on normal skin, the mice sensitized at the skin-grafted site had reduced numbers of both FITC$^+$ MHC class II^{hi} CD205$^+$ and FITC$^+$ MHC class II^{hi} CD205$^-$ populations in the draining lymph nodes (Figure 3c). From three independent series of experiments, the number of LCs and/or DCs in the lymph nodes of grafted skin–sensitized mice was approximately one-fourth that of normal skin–sensitized mice (Figure 3c). Considering that the grafted skin originally had about one-fourth the LCs of normal skin, the migratory ability of LCs in grafted skin was virtually the same as that in normal skin.

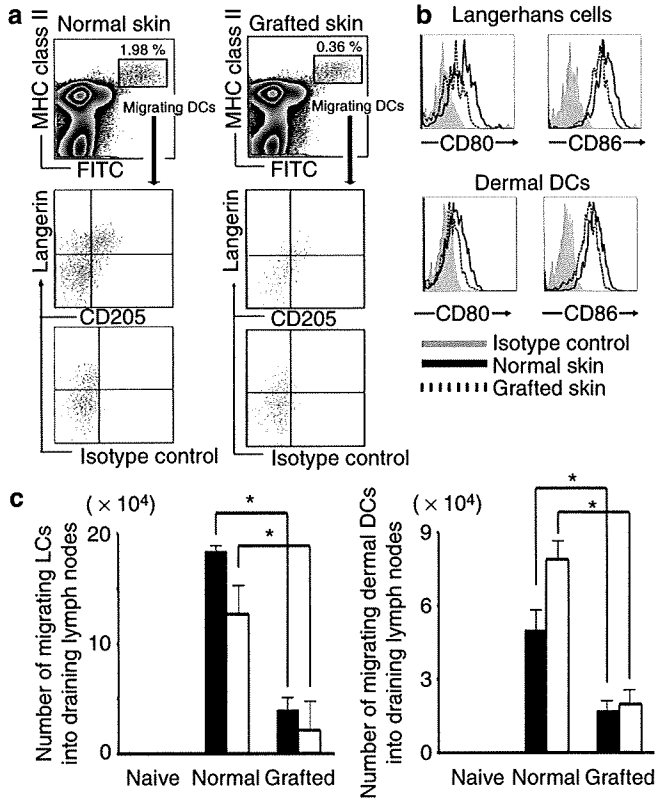

Figure 3. Expression of co-stimulatory molecules and number of migrating DCs in grafted mice. (**a**) Draining lymph node cells were taken from mice painted with FITC on the grafted or normal skin and stained with anti-MHC class II, CD205 and Langerin mAbs. CD205$^+$ cells are virtually the same population as Langerin$^+$ cells. (**b**) After being stained with anti-CD205, CD80, and CD86 mAbs, lymph node cells were subjected to flow cytometric analysis to assess the expression of co-stimulatory molecules. (**c**) Numbers of FITC$^+$ MHC class II^{hi} CD205$^+$ cells (mainly LCs) and FITC$^+$ MHC class II^{hi} CD205$^-$ cells (mainly dDCs) migrating from the skin to the draining lymph nodes. The numbers are calculated based on flow cytometric analysis gated as seen in Figure 3a. Each group consisted of more than four mice. *$P<0.05$.

Increased IL-10 expression in grafted skin

It is well known that UV B (UVB) radiation has immunosuppressive effects on normal cutaneous processes (Elmets *et al.*, 1983; Yagi *et al.*, 1996) and this UV-induced immunosuppression is associated with upregulation of anti-inflammatory Th2 cytokines, IL-4 and IL-10 (Rivas and Ullrich, 1992; Shreedhar *et al.*, 1998). Among cytokines, IL-10 is critical for CHS suppression (Simkin *et al.*, 2000), and both LCs (Takashima, 1995; Flacher *et al.*, 2006) and keratinocytes (Rivas and Ullrich, 1992) are possible candidates for the IL-10 source. To address the involvement of IL-10, mice receiving skin grafts (day 0) were either sensitized with PCl (day 7) on the dorsal grafted skin or untreated. As control, mice without skin grafts were sensitized with PCl on the dorsal skin or untreated. EC suspensions were obtained from the dorsal skin of these four experimental groups (day 8). As shown in Figure 4a, whereas the normal skin had little or no ability to elaborate IL-10 irrespective of PCl sensitization, the grafted skin expressed high levels of IL-10 mRNA, as assessed by real-time PCR. Relative amounts of mRNA were measured by the $\Delta\Delta C_t$ method (Atarashi *et al.*, 2007). Although both grafted skin samples with and without subsequent PCl painting yielded IL-10 mRNA, the nonsensitized epidermis was more productive; it may be that IL-10 produced by keratinocytes was reduced in proportion to

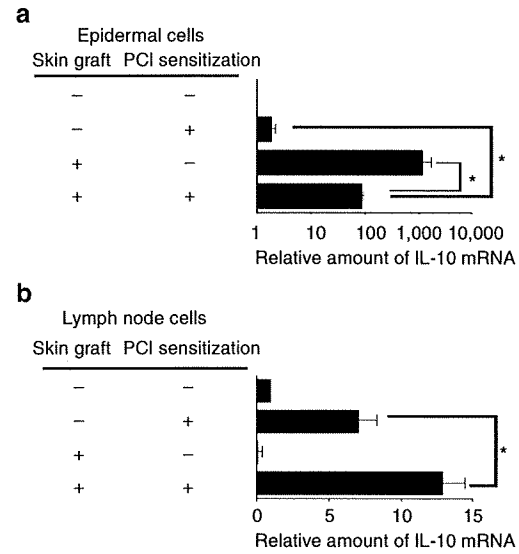

Figure 4. IL-10 expression in ECs and lymph node cells from grafted mice. (**a**) Epidermal sheets were prepared from grafted (7 days after operation) or non-grafted skin that was painted with PCl or left untreated. IL-10 mRNA expression was measured by real-time PCR. (**b**) Draining lymph node cells were obtained 5 days after sensitization of mice through grafted or non-grafted skin. IL-10 mRNA expression was measured by real-time PCR. The relative amounts of mRNA expression were calculated using the $\Delta\Delta C_t$ method. Each group consisted of more than four mice. Data are the mean ± SD of three independent experiments. *$P<0.05$.

PCl sensitization or, alternatively, IL-10-producing LCs emigrated on sensitization.

Along with ECs, we examined the expression of IL-10 mRNA in the draining lymph nodes. Lymph node cells were prepared from mice receiving skin grafts and/or subsequent PCl painting. Skin grafting alone did not augment IL-10 production by lymph node cells (Figure 4b). PCl sensitization on the grafted skin dramatically enhanced the production of IL-10. This increase of IL-10 was considered to be derived from LCs or proliferative T cells in the draining lymph nodes. As the PCl-painted grafted skin produced less IL-10 than the PCl-non-painted grafted skin (see Figure 3a), we postulate that IL-10-producing LCs emigrated from the grafted skin to the lymph nodes on sensitization with PCl.

LCs as a source of IL-10 in grafted skin

To dissect the cytokine-producing populations in grafted skin, EC and dermal cell suspensions were prepared from grafted (day 7) or normal skin. EC suspensions and dermal cell suspensions were fractionated to $CD11c^+$ (containing 70–80% LCs or dDCs and <0.01% T cells) and $CD11c^-$ (containing mainly keratinocytes or fibroblasts and <0.05% DCs) subsets with magnetic cell sorting (MACS). As shown in Figure 5, both $CD11c^+$ and $CD11c^-$ fractions produced a high amount of IL-10, indicating that not only keratinocytes but also LCs from the grafted skin were stimulated to produce IL-10. In contrast, $CD11c^-$ cells secreted more IL-6 in grafted skin than normal skin, suggesting that keratinocytes in the graft produced IL-6. Both $CD11c^+$ and $CD11c^-$ cells produced higher amounts of tumor necrosis factor-α (TNF-α) in the grafted skin than in the normal skin. Surgical trauma induces an early hyperinflammatory response, which is characterized by proinflammatory TNF-α, IL-1, and IL-6 cytokine release (Menger and Vollmar, 2004). IL-6 has a crucial role in the neutrophil and macrophage infiltration in the wound healing process (Lin *et al.*, 2003). Thus, this increase of IL-6 and TNF-α is considered to be a hyperinflammatory response of the wound healing process.

Induction of $CD4^+CD25^+$ regulatory T cells by sensitization through grafted skin

The presence of IL-10-producing LCs in the epidermis and lymph nodes raised the possibility that Treg cells were induced in mice sensitized with PCl through grafted skin. We performed an adoptive transfer study to evaluate this possibility. Donor mice were sensitized with PCl on the grafted skin, and lymph nodes and spleen cells were taken from the mice 5 days later (Figure 6, top). $CD4^+CD25^+$ and $CD4^+CD25^-$ subsets were purified from the pooled cells by

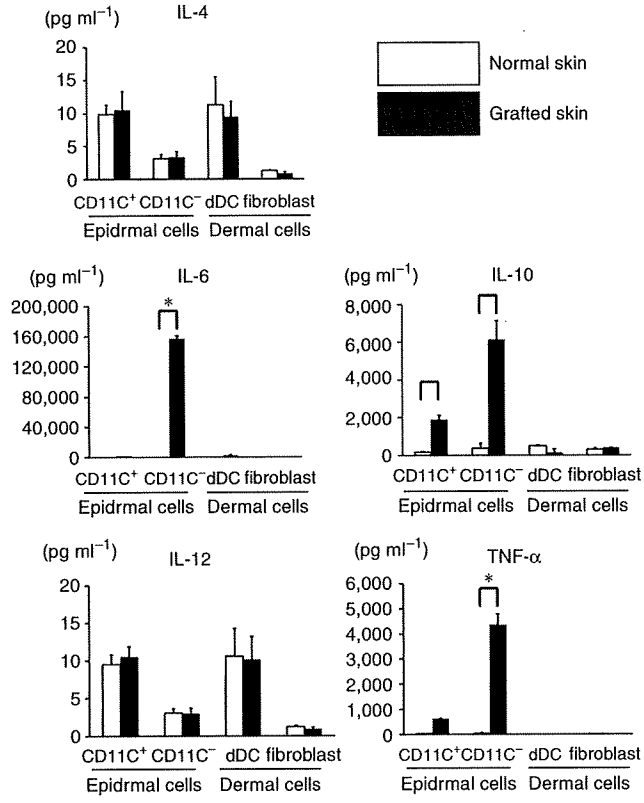

Figure 5. Cytokine production by ECs and dermal cells. EC suspensions were obtained from grafted or non-grafted skin, and fractionated to $CD11c^+$ cells (70–80% LCs) and $CD11c^-$ cells (LC-depleted cells, mainly keratinocytes) with auto-MACS using CD11c microbeads. Dermal cell suspensions were centrifuged with Ficoll-Paque, followed by auto-MACS, to fractionate dDCs and other cells (mainly fibroblasts). Each subset was cultured for 24 hours. The amounts of IL-4, IL-6, IL-10, IL-12, and TNF-α in the culture supernatants were quantified using cytometric beads array. Data are the mean ± SD of three independent experiments. Each group consisted of more than four mice. *$P<0.05$.

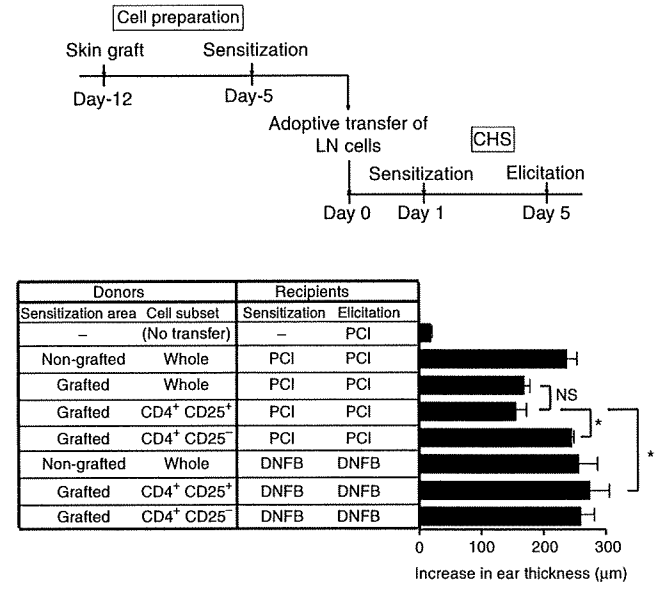

Figure 6. Transfer study of $CD4^+CD25^+$ cells from donor mice sensitized through grafted skin. Mice were sensitized with PCl on the grafted (7 days after operation) or non-grafted area. Five days after sensitization, draining lymph nodes and spleen cells were taken from the mice. Whole cells, $CD4^+CD25^+$ sorted cells, or $CD4^+CD25^-$ sorted cells were transferred into syngeneic naive mice (5×10^6 for each mouse). Then, the recipients were sensitized on the dorsum and challenged on the ears with hapten (PCl or DNFB) as indicated in the figure. Change in ear thickness was measured 24 hours later. Each group consisted of more than four mice. Data are representative of three independent experiments. *$P<0.05$.

MACS. Whole unfractionated cells or cells of each subset were transferred into syngeneic naive recipients (5×10^6 for each mouse), which were then sensitized and challenged with PCl. The transfer of $CD4^+CD25^+$ T cells decreased the CHS response of the recipients, whereas $CD4^+CD25^-$ T cells were not suppressive (Figure 6, bottom). When recipient mice were sensitized and challenged with another hapten dinitrofluorobenzene, such suppression was not observed, suggesting that the immunosuppression was antigen specific. Thus, the depressed sensitization through the grafted skin was associated with the appearance of $CD4^+CD25^+$ Treg cells.

Augmentation of IL-10 production by LCs exposed to RANKL

It has been recently reported that LCs express receptor activators of nuclear factor κ-B, that UVB irradiation upregulates cutaneous RANKL, and that RANKL activates DCs as well as Tregs in the skin (Loser *et al.*, 2006). We hypothesized that the grafted skin expresses RANKL and activates LCs to produce IL-10. When the grafted skin was stained with anti-RANKL and MHC class II antibodies, we found that ECs of the grafted skin expressed RANKL (Figure 7a, RANKL, blue; LCs, red). Notably, RANKL was strongly expressed around LCs (merged purple), suggesting that receptor activators of nuclear factor κ-B/RANKL interactions might be important for the induction of hyposensitization in grafted skin. To identify the function of receptor activator of nuclear factor κ-B/RANKL in the skin immune system, we tested the ability of LCs to produce IL-10 when they were exposed to recombinant RANKL. EC suspensions were cultured with or without recombinant RANKL (R&D Systems, McKinley, MN) for 24 hours, and stained with anti-MHC class II antibody. Then, the cells were permeabilized and stained with anti-IL-10 antibody. We found that the addition of RANKL increased the fraction of IL-10-positive LCs (Figure 6b) and the mean fluorescence intensity of IL-10 in LCs (Figure 6c). These findings indicated that RANKL expressed by keratinocytes of grafted skin stimulates LCs to produce IL-10.

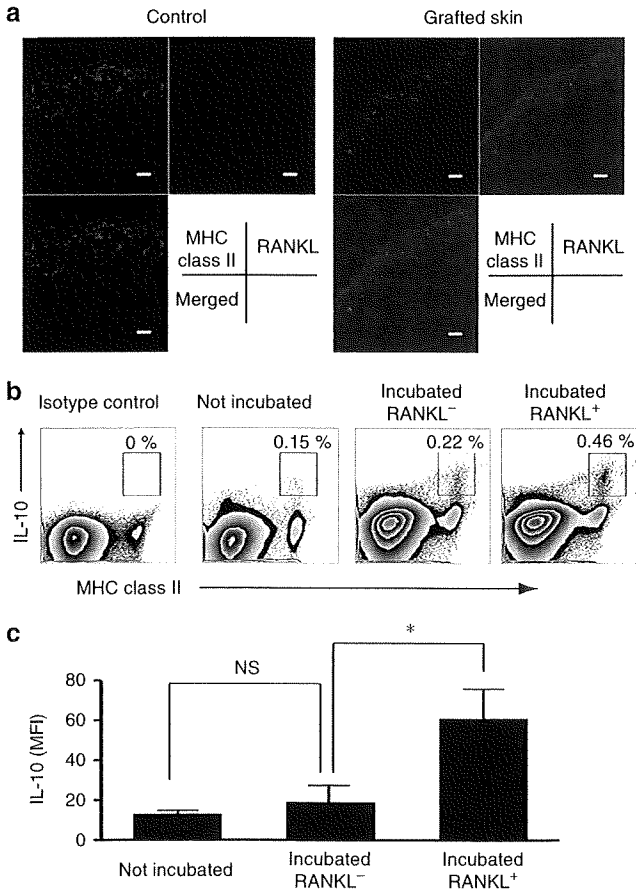

Figure 7. Induction of RANKL in grafted skin and stimulation of LCs to produce IL-10 by RANKL. (**a**) The grafted or non-grafted skin was stained with antibodies to MHC class II and RANKL. Red, MHC-class II; blue, RANKL. Merged image is shown in purple. (**b**, **c**) EC suspensions were cultured with or without recombinant RANKL for 2 days. Cells were fixed, permeabilized, stained with IL-10 and MHC class II antibodies, and analyzed by flow cytometry (**b**). The mean fluorescence intensity of IL-10 in MHC class II + LCs existing in EC suspensions. Bars indicate the mean + SD. Data are representative of three independent experiments. Each group consisted of more than four mice. Scale bar = 50 μm $^*P<0.05$.

DISCUSSION

This study addressed the immunological mechanism underlying impaired sensitization through grafted skin. CHS was depressed only when mice were immunized with hapten through grafted skin, and even skin graft-bearing mice fully developed CHS when sensitized through non-grafted skin. Therefore, the induction of immunosuppression is local, whereas its effects are specific. The local immunological condition of the grafted skin is responsible for impaired induction. The fate of LCs in the graft seems to be a key to resolving the mechanism. Mainly because of the apoptotic death of LCs, the number of LCs in freshly implemented skin was up to one-fourth that of normal skin. However, when FITC was applied to the grafted skin, FITC-bearing LCs were present in the draining lymph nodes, again at a cell number one-fourth that of normal skin-sensitized mice. Thus, LCs were capable of migrating efficiently from the grafted skin to the draining lymph nodes. As LCs can serve as both positive and negative antigen-presenting cells depending on the surrounding milieu (Silberberg-Sinakin and Thorbecke, 1980; Kaplan *et al.*, 2005), not only the numerical but also functional changes of LCs determine CHS development. We further investigated whether the hyposensitization of CHS through grafted skin was attributable merely to the reduction of LC number, or whether some function of LCs was altered.

DCs in peripheral tissues, such as epidermal LCs, remain immature in the steady state, and express small quantities of MHC class II and co-stimulatory molecules and produce low levels of immunostimulatory cytokines. During the process of antigen capture/presentation and migration into T-cell areas of regional lymph nodes, maturation of DCs simultaneously occurs, as they express high amounts of these surface molecules and cytokines (Inaba *et al.*, 1997; Huang *et al.*, 2000; Lutz and Schuler, 2002). Recent studies have revealed that peripheral tolerance is induced by immature DCs (Steinman *et al.*, 2000; Lutz and Schuler, 2002) or partially

by mature DCs that express MHC class II, CD80 and CD86 molecules but lack secretion of IL-12, IL-6, and TNF-α (Groux, 2003). On the basis of their migrating ability, it seemed that nonapoptotic, live LCs in the grafted skin were functionally mature.

Given that surgical trauma is one of the injuries that induce a hyperinflammatory response, the skin graft employed in this study likely leads to the production of proinflammatory cytokines such as TNF-α and IL-6. However, the skin graft is not merely surgical trauma, because living skin is applied to the raw surface. More specifically, the intact ECs produce high amounts of IL-6 and TNF-α (see Figure 5), suggesting that skin grafting induces inflammatory cytokine production more vigorously than simple trauma. In wound healing after skin grafting, the exaggerated inflammatory response should be downregulated in due course. Among various factors that suppress the inflammatory response, IL-10 is one of the most important candidates, because it is a potent inhibitor of the activation of monocytes/macrophages and of the expression of TNF-α and other proinflammatory mediators (Denys *et al.*, 2002). In fact, IL-10 was produced at a high level by keratinocytes in grafted skin. We explored the possibility that LCs have a polarized cytokine production pattern in the graft, which leads to peripheral tolerance. In this scenario, IL-10 is a strong candidate as a skewing cytokine.

LCs in grafted skin exhibited a rounder and less dendritic appearance than those in normal skin. Along with this morphological change, LCs in grafted skin expressed IL-10 at a higher level than those in non-grafted skin, suggesting that IL-10-producing LCs have an important role in depressed CHS. Many studies have shown that IL-10 is an essential cytokine in depression of CHS (Annacker *et al.*, 2001; Girolomoni *et al.*, 2004; Ghoreishi and Dutz, 2006). Similarly, IL-10 production by pulmonary DCs is critical for the induction of tolerance (Akbari *et al.*, 2001). Besides LCs, keratinocytes also secrete IL-10, which causes CHS suppression when overexpressed by certain stimuli such as UVB radiation (Schwarz *et al.*, 2004; Ghoreishi and Dutz, 2006). Keratinocyte-derived IL-10 might further condition LCs to be regulatory as well as suppress the injury-associated inflammation.

Conversely, the adoptive transfer of $CD4^+CD25^+$ T cells from the tolerant mice induced CHS suppression, whereas $CD4^+CD25^-$ T cells had no effect. This suggests that $CD4^+CD25^+$ Treg cells as well as IL-10-producing LCs participate in the skin graft-induced suppression of CHS. At present, at least four types of Treg cells can be identified based on the expression of cell-surface markers, secretion of cytokines, and suppression mechanisms (Groux, 2003). Recent accumulating evidence has indicated that Treg cells have a key role in peripheral tolerance (Takahashi *et al.*, 1998; Shevach, 2001; Taylor *et al.*, 2001; Thorstenson and Khoruts, 2001; Zhang *et al.*, 2001) under the influence of immature or maturing DCs (Min *et al.*, 2003; Roelofs-Haarhuis *et al.*, 2003).

The relationship between IL-10-producing LCs and Treg cells remains partly elucidated. In UVB-induced tolerance, intravenous transfer of Treg cells suppresses CHS, and this phenomenon is dependent on host-derived IL-10 (Ghoreishi and Dutz, 2006). Other groups have reported that Treg cells regulate the expansion of peripheral $CD4^+$ T cells with IL-10 (Annacker *et al.*, 2001). These findings have suggested that IL-10 is essential for Treg cells for successful downmodulation, and the source of IL-10 is a constituent of the immunological milieu, such as epithelial cells, or Treg cells *per se*. However, we found that LCs are the IL-10 producer, raising the possibility that IL-10-producing LCs are an inducer of Treg cells. In addition, IL-10 released from LCs also might inhibit effector T cells concerned with CHS.

A group of investigators have found that RANKL, which is expressed in the keratinocytes of inflamed skin, controls Treg cell numbers by activation of DCs (Loser *et al.*, 2007). In this study, we showed that both epidermal $CD11c^+$ LCs and $CD11c^-$ cells (keratinocytes) produce high amounts of representative proinflammatory cytokine TNF-α in the grafted area, which might lead to the expression of RANKL on keratinocytes. Our finding that LCs exposed to recombinant RANKL produced a high level of IL-10 suggests that RANKL from keratinocytes in the grafted skin can induce IL-10-producing LCs at the initiation stage of immunosuppression. The reduction in LC number may cause impaired sensitization, but in accordance with recent observations (Kaplan *et al.*, 2005; Kissenpfennig *et al.*, 2005), the altered function of LCs is more likely involved in the depression of CHS.

Our study is clinically relevant in two aspects. First, grafted skin provides a specialized immunological status, in which T cells do not respond well to external stimuli such as contactants, as a result of skewed function and a reduced number of LCs. In this context, contact dermatitis cannot easily develop in the grafted skin. Second, the skin graft may be used for the induction of antigen-specific peripheral tolerance by application of antigen through the grafted skin. This strategy may have great potential for controlling allergic diseases and autoimmune disorders. Further investigation of this skin graft-associated immunosuppression may develop safe and effective methodologies for tolerance induction.

MATERIALS AND METHODS

Mice

BALB/c (7- to 10-week-old) female mice were obtained from Kyudo Co. Ltd (Kumamoto, 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.

Preparation of skin graft

One day before skin grafting, the back of each mouse was clipped and hairs were removed with depilatory cream (Shiseido Cosmetic Co., Tokyo, Japan). A 25×20 mm area of full-thickness back skin was resected under intraperitoneal anesthesia with ketamine and dolmicam. The same skin was grafted onto the back of each mouse. The graft was sutured and fixed with a tie-over dressing. The dressing was removed on day 7 after operation.

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 FACSCanto 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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See related commentary on pg 2569

ORIGINAL ARTICLE

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.

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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

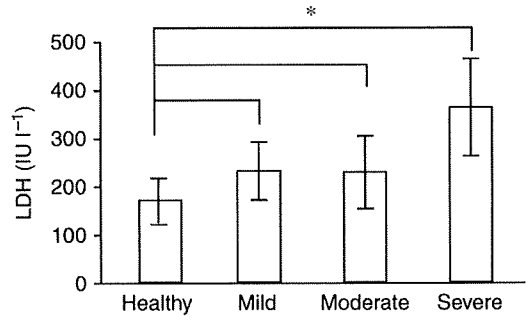

Figure 1. Lactate dehydrogenase levels in different severity groups of AD patients. The AD patients were classified as acute (n = 23), mild (n = 7), moderate (n = 5), and severe (n = 11) by clinical findings, and serum levels of lactate dehydrogenase from AD patients were compared with that in healthy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

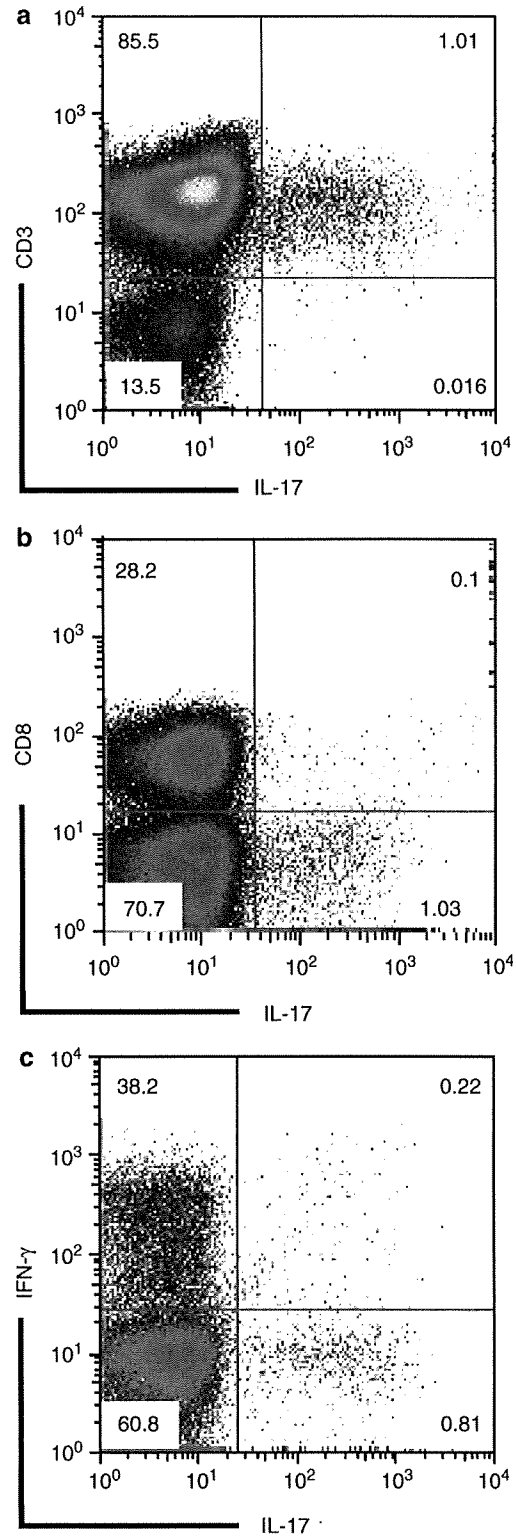

Figure 2. Flow cytometric analysis of IL-17$^+$ cells in peripheral blood lymphocytes. PBMCs isolated from a 30-year-old man with AD were stimulated with PMA and ionomycin for 8 hours and Golgistop was added. Then, production of IL-17 by PBMCs was determined at the single-cell level by intracellular 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.

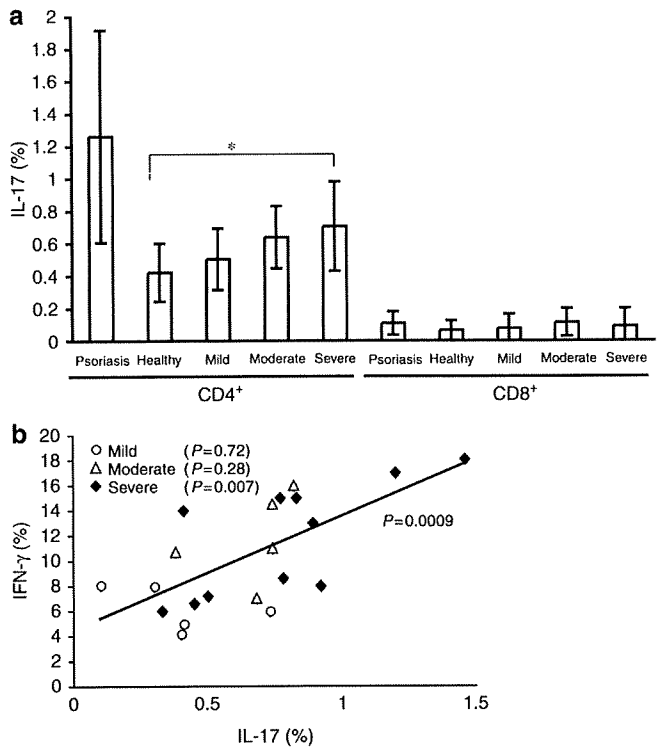

Figure 3. Percentage of circulating IL-17$^+$ T cells in relation to AD severity and IFN-γ$^+$ T cells. AD patients were divided into three different severity groups (mild, $n=7$; moderate, $n=5$; and severe, $n=11$). (**a**) Healthy subjects ($n=9$), three groups of AD, psoriatic patients ($n=16$) were 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.

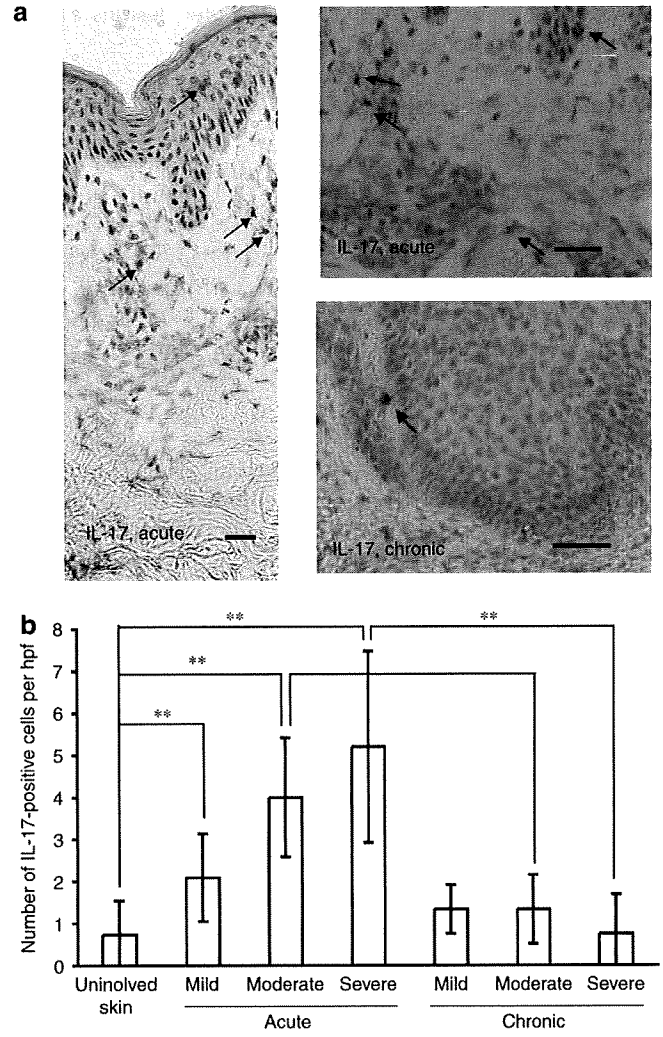

Figure 4. Immunohistochemistry of IL-17$^+$ cells in skin lesions of AD. (**a**) A skin specimen from an AD patient was immunohistochemically stained for IL-17. Left, low-magnification image of acute lesion; top right, high-magnification image of acute lesion; and bottom right, high-magnification image 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.

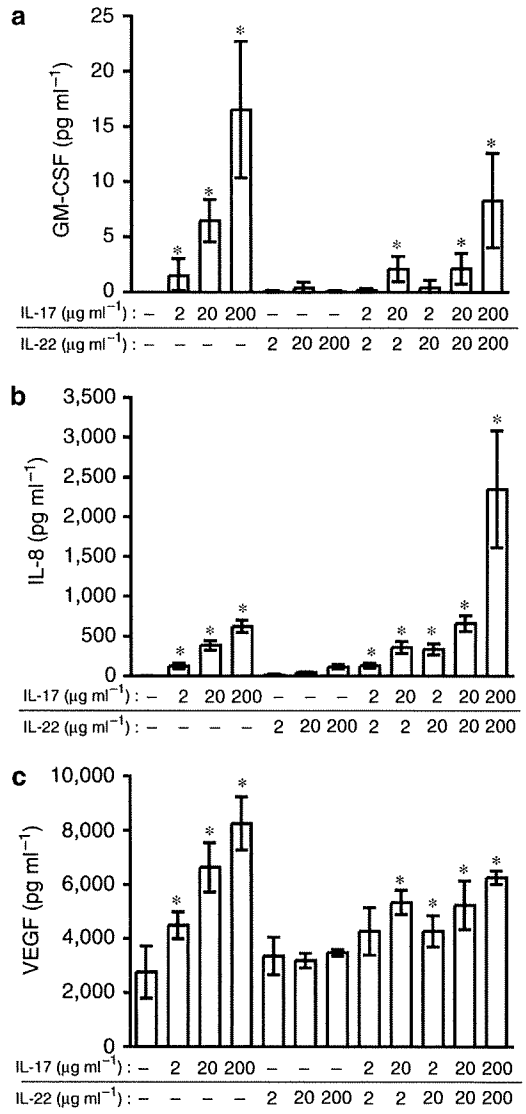

Figure 5. Effects of IL-17 and/or IL-22 on keratinocyte production of cytokines/chemokines and VEGF. NHEKs were cultured with or without IL-17 and/or IL-22 at the indicated doses. Three-day culture supernatants were subjected to analysis with the cytometric beads array system or ELISA. Columns show mean ± 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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