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

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

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

2. Materials and methods

2.1. Mice

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

2.2. Chemicals and monoclonal antibodies (mAbs)

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

2.3. CHS and application or administration of KP

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

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

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

2.4. Cell purification

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

2.5. Cell transfer

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

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

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

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

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

2.8. PGE_2 assay

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

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

2.9. Statistic analysis

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

3. Results

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

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

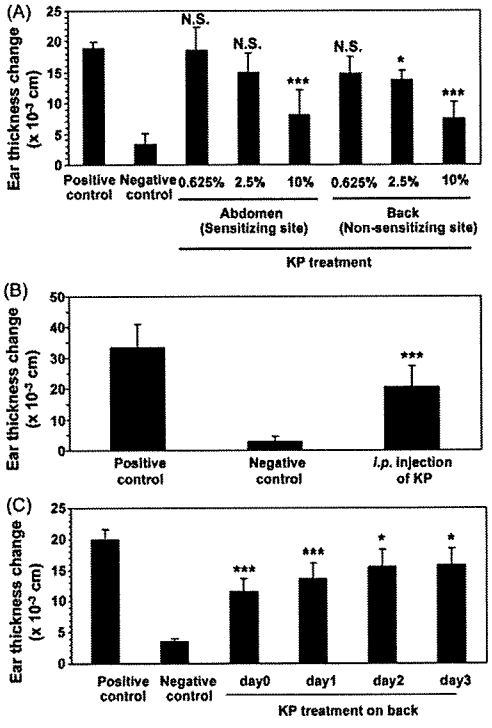

Fig. 1. Systemic suppression of CHS by KP painting on various application days. While mice were sensitized on the abdomen and challenged on the ears, KP was applied on day 0 to the sensitizing or non-sensitizing site at 0.625%, 2.5% or 10% (A). Instead of topical application of KKP, 2 mg/kg of KP was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

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

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

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

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

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

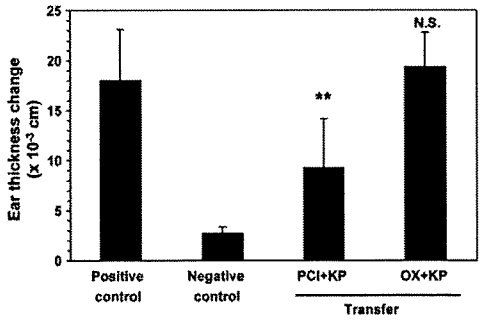

Fig. 2. Hapten-specific suppression of CHS by KP application. Twenty-four hours before PCl sensitization, mice were injected *i.v.* with 2.4×10^7 LNCs obtained from syngeneic mice that were treated with PCl + KP or OX + KP 5 days earlier. Positive control mice were sensitized and challenged without cell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

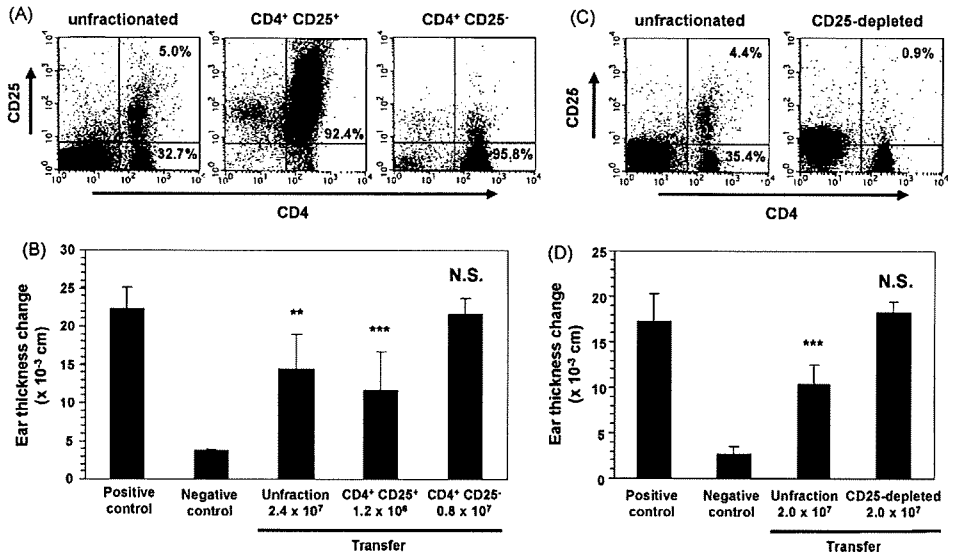

Fig. 3. Mediation of KP-induced suppression by $CD4^+$ $CD25^+$ cells. LNCs were collected from mice that were treated with PCl + KP 5 days earlier. $CD4^+$ $CD25^+$ cells or $CD4^+$ $CD25^-$ cells were purified by MACS (A). After cell separation, 2.4×10^7 unfractionated cells, 1.2×10^6 $CD4^+$ $CD25^+$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

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

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

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

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

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

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

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

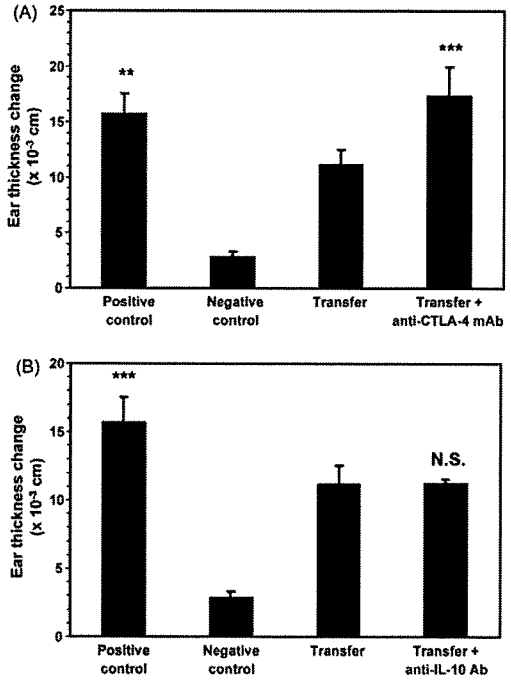

Fig. 4. Inhibition of KP-induced suppression by blockade of CTLA-4 but not IL-10. (A) Twenty-four hours before sensitization, mice were injected *i.v.* with 2.4×10^7 LNCs obtained from syngeneic mice that were treated with PCl + KP 5 days earlier (Transfer). A group of mice (Transfer + CTLA-4 mAb) were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

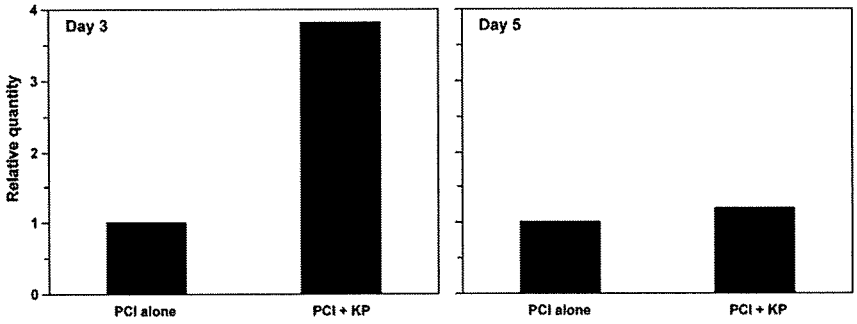

Fig. 5. Induction of Foxp3 mRNA expression in LNCs by KP. Mice were treated on the abdomen with PCl alone or PCl + KP. LNCs were collected on day 3 or day 5 after treatment. Foxp3 gene expression was quantified in a two-step real-time PCR. GAPDH gene expression was measured as an endogenous control for these PCR quantification studies. Results represent normalized mean target mRNA amounts relative to PCl-treated mice using the 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 PCl + KP-treated mice was remarkably increased compared to PCl-treated mice (Fig. 5). On day 5, such increment of Foxp3 expression was not found. Thus, the treatment with KP induced the expression of Foxp3, supporting the development of Treg cells at the induction phase.

3.6. Inhibition of PGE_2 production by KP treatment in LNCs

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

4. Discussion

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

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

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

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

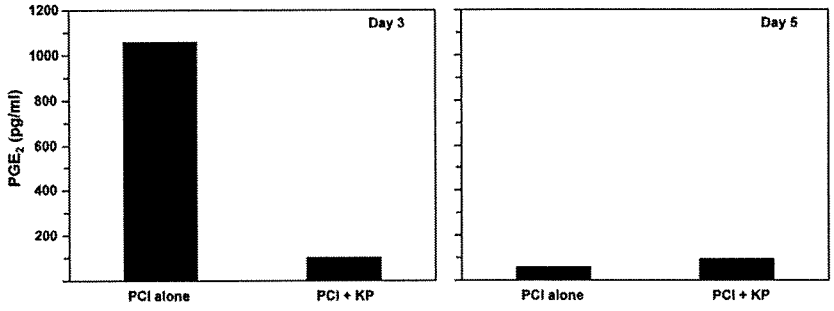

Fig. 6. Inhibition of PGE_2 production in LNCs by KP. Mice were treated on the abdomen with PCl alone or PCl + KP. LNC were collected on day 3 or 5 and cultured in cRPMI medium for 48 h. A quantitative analysis of PGE_2 release in the supernatants was performed as described in Section 2.

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

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

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

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localized disease, and photochemotherapy, isotretinoin, dapsone, or antimalarials for generalized disease. Resolution of patch-type granuloma annulare after biopsy has been reported [5].

In conclusion, we present a rare and recently described variant of granuloma annulare characterized by patches of erythema on the extremities and trunk that lack the usual clinical findings but display the classic histopathological findings of interstitial granuloma annulare. ■

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A missense mutation in exon 1 of the keratin 9 gene in a Japanese patient with "Vörner type" hereditary palmoplantar keratoderma

Epidermolytic hereditary palmoplantar keratoderma (EHPPK; OMIM: 144200) or Vörner type PPK is characterized by hyperkeratotic lesions confined to the palms and soles, histological granular degeneration and mutations in keratin 9 gene (*KRT9*; NCBI: NM000226) [1-5]. Here, we report a Japanese patient with EHPPK showing a missense mutation (R162Q) in *KRT9* located in the 1A rod domain, the highly conserved helix initiation motif of keratin 9.

A 28-year-old Japanese man was referred to us for evaluation of palmar and plantar hyperkeratotic lesions. The condition had developed within the first year of life and progressed until 20 years of age. He sometimes shaved the hyperkeratotic surface of the soles, but the cornified lesion recovered within a few weeks. On examination, there was a markedly thick cornified layer on the soles and palms *(figures 1A,B)*. The dorsal aspects of the hands and feet were not affected, and the borderline between the lesional and normal skin was clear. Hyperhidrosis was unremarkable. The patient was otherwise healthy. His one-year-old daughter had the same hyperkeratotic lesions on the bilateral palms and soles, but to a lesser degree. The family history was otherwise negative for similar disorders as his parents had no palmoplantar hyperkeratosis.

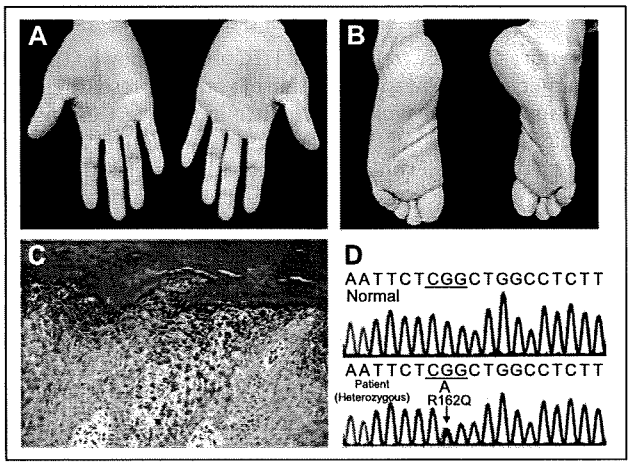

Figure 1. A, B) Clinical appearance. Bilateral palmoplantar hyperkeratotic lesions on the palms and soles. **C)** Histological findings (hematoxylin-eosin, original magnification × 20). The epidermis of the hyperkeratotic lesional skin shows epidermolytic hyperkeratosis with coarse keratohyaline granules. **D)** Sequence analysis of *KRT9* gene exon1. Sequencing of the Vörner type EHPPK patient's PCR products demonstrates heterozygous G to A substitution at nt position 551, resulting in substitution of an arginine codon (CGG) by a codon for glutamine (CAG), a mutation designated R162Q.

A skin biopsy specimen was taken from the inner aspect of his right foot. There was epidermolytic hyperkeratosis exhibiting coarse keratohyaline granules and granular degeneration *(figure 1C)*. Thus, we diagnosed the patient as having EHPPK.

Genomic DNA was extracted from peripheral blood leukocytes. As previously reported [6], the genomic regions of the *KRT9* gene exon1 were amplified via polymerase chain reaction (PCR), using a forward : K9.E1F : 5'-GGAGGTGACTCTGCTCTTGG-3' and a reverse: K9.E1R : 5'-AGGTGGATTCCCTGGCTATT-3' primer pair. A direct sequencing analysis identified a G to A transversion at nucleotide (nt) position 551, resulting in the substitution of glutamine (Q) for arginine (R), in the patient, as compared with the normal sequence *(figure 1D)*.

The R162Q missense mutation identified in our case was not novel, since the mutation was located within codon R162, in which the most common mutations, R162W and R162P, have been reported. However this is the second report of the R162Q missense mutation in Japanese patients with EHPPK. This mutation has frequently been seen in non-Japanese patients, as approximately 20% Western cases had this mutation [1-3]. The result confirms the previous reports of *KRT9* mutation underlying EHPPK and re-emphasizes the importance of codon R162 for maintenance of the intermediate keratin filament network. Since keratin 9 is confirmed to the volar skin, the abnormality of keratin 9 induces palmoplantar lesions. This dominant-negative effect on keratin network formation led to the disruption of keratin filament formation, and development of epidermolytic hyperkeratosis [4]. ■

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Large plaque type blue nevus with subcutaneous cellular nodules

Blue nevus is a benign dermal melanocytosis that includes a large spectrum of melanocytic proliferation. There are different varieties of blue nevus (BN) [1]. Among the less frequent there is the "large plaque blue nevus". We describe a case of large plaque blue nevus with subcutaneous cellular nodules.

A 31-year-old Caucasian male presented with blue-grey, non-palpable patches, darker than the surrounding skin, diffusely involving the left side of the back, the left shoulder, and the elbow *(figure 1A)*. At age 18, a cystic nodule within the lesion was biopsied, and histology showed a neurofibroma-like aspect associated with a dendritic melanocyte population. Immunohistochemistry revealed a diffuse positivity to HMB45 (gp100), NKI C3 and a focal positivity to S-100 and GFAP. An "atypical" blue nevus diagnosed. At age 19, an axillary nodule was biopsied and the pathologist diagnosed a melanocytic schwannoma. At age 25, a nodule on the arm was excised and the diagnosis was "dermal melanocytosis". Immunohistochemically, neoplastic cells expressed a strong positivity for HMB45, NKI C3, S-100 whereas CD34, neurofilaments and EMA resulted negative. At age 29, a pigmented nodule rapidly increased in size within the lesion. This nodule was surgically removed with the underlying muscular tissue. The lesion showed large nests of pale ovoid small cells containing scant melanin and areas of dendritic pigmented cells, along with many pigmented melanophages. This nodule had a zone of striking nuclear atypia: with vescicular, large, fusiform nuclei containing a prominent nucleolus; cellular and nuclear pleomorphism was also present *(figures 1B and C)*. Immunohistochemistry showed a strong reactivity of neoplastic cells for S100, CD63, MTF-1, Melan A (A103), Tyrosinase and non-reactivity for CD57 and neurofilaments. The mitotic index was 2/10HPF. Histologically the lesion was suggestive for malignant melanoma but considering the evolution of the lesion and the previous biopsies, the pathologist confirmed the diagnosis of atypical blue nevus. As the nodular lesion was not totally resected, a wide re-excision was performed.

The clinical, histological and immunohistochemical features of the lesion we describe are strikingly similar to the "large plaque-type blue nevus with subcutaneous cellular nodules" described by Busam *et al.* in 2000 [2]. The histopathological features of large plaque blue nevus are similar to ordinary blue nevus; the lesion involves the reticular dermis and subcutis. There is no melanocytic proliferation at the dermo-epidermal junction and in the papillary dermis. The reticular dermal component is characterized by some heterogeneity of melanocytes, including slender dendritic cells, fascicles of spindle cells, aggregated epithelioid melanocytes, and, occasionally, clear melanocytes. Most of them are located in the deep dermis near the subcutis and are, often, predominantly arranged in a periappendageal distribution around blood vessels and nerves. The foci of ordinary blue nevus are separated by Mongolian spot-like areas, in which pigmented or fusiform melanocytes and melanophages are widely spaced from each other as single cells without the formation of cellular aggregates. Hypercellularity areas are present in the subcutis and contain nests and fascicles of melanocytes with clear cytoplasm. Their nuclei are oval in shape with inconspicuous single nucleoli. The cellular nests are surrounded by a population of slender spindle cells, distributed loosely in a fibrous stroma, with variable numbers of heavily pigmented dendritic melanocytes or melanophages.

In conclusion, we describe a case of "large plaque-type blue nevus with subcutaneous cellular nodules". In our opinion, this lesion is characterized by an uncertain biological behaviour, thus needing close follow up. ■

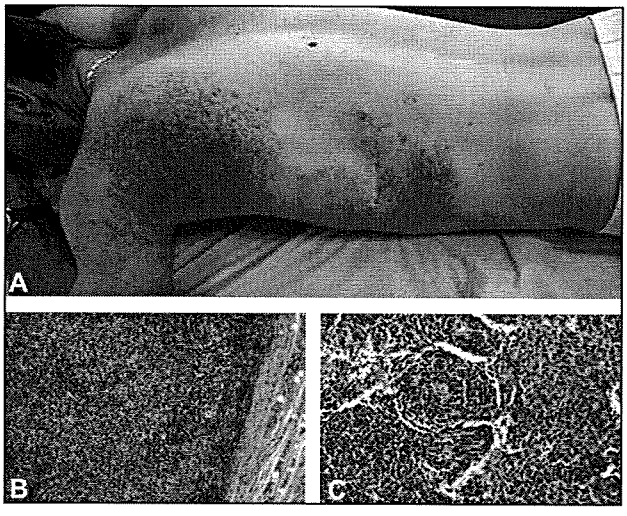

Figure 1. A) Clinical presentation of the large plaque blue nevus. **B)** Histological image of the infiltrating melanocytes of the nodule (H-E 20×). **C)** Higher magnification of the same histological specimen (H-E 40×).

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Induction of eosinophil-infiltrating drug photoallergy in mice

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ABSTRACT

Background: Drug photoallergy is one of the highly incident adverse effects. Several different histological patterns have been recognized.
Objective: To establish a murine model of the eosinophil-infiltrating type of drug photoallergy by using afloqualone (AQ), a representative photosensitive drug.
Methods: AKR/J mice were sensitized by intraperitoneal injection of afloqualone solution (2 mg/kg/mouse) and irradiation of shaved abdomen with ultraviolet A light (UVA) (12 J/cm^2). This sensitization procedure was repeated 2–12 times, and 3 days after the last immunization, mice were challenged by a subcutaneous injection of AQ solution and irradiation of the same site with UVA. The draining lymph node cells (LNCs) were used for transfer and cytokine production studies, and the challenged skin was analyzed for chemokine expression.
Results: More than 10 times of sensitization induced a massive infiltrate of eosinophils and lymphocytes at the challenged site. AKR/J mice were a high responder strain. The sensitivity was transferred with 5–8 $\times$ 10^7 immune lymph node and spleen cells into naïve mice. CD4$^+$ T cells were mainly responsible for this sensitivity, since 1 $\times$ 10^7 CD4$^+$ cells alone induced a high level of sensitivity, but CD8$^+$ T cells evoked the sensitivity to a lesser degree. Culture supernatants from AQ-photoimmuned lymph node cells contained a higher level of IL-4 and lower interferon-γ than those from mice immunized with dinitrofluorobenzene. Finally, the skin of AQ-photochallenged site exhibited high expression of CCL24/eotaxin-2, a chemokine for eosinophils.
Conclusion: It is suggested that eosinophilic drug photoallergy is mediated by sensitized Th2 cells and locally produced eosinophil-attracting chemokines.

1. Introduction

Drug photosensitivity is one of the adverse reactions and clinically recognized as photosensitivity dermatitis [1]. The action spectrum is usually ultraviolet A light (UVA) and UVB exceptionally may induce this sensitivity or augment the UVA-evoked sensitivity level [2]. Various drugs have been reported to induce photosensitivity, including quinolones [3], afloqualone (AQ) [4], piroxicam [5], non-steroidal anti-inflammatory drugs [6], and others. There are phototoxic [2] and photoallergic [3–7] mechanisms in drug sensitivity, and the incidence of photoallergy is higher than that of phototoxicity [3]. Two theories of photoantigen formation have been put forward to explain the photoallergic mechanisms, prohapten and photohapten [8]. Prohapten is converted to ordinary hapten by UVA exposure and can bind to protein. On the other hand, photohapten needs to coexist non-covalently with protein prior to UVA exposure, and UVA induces a covalent bond between them. The vast majority of photosensitizing drugs are photohapten rather than prohapten [3,4,9].

We have taken several different approaches to establish mouse models of drug photoallergy with the use of afloqualone [4], quinolones [10], and ketoprofen [6]. Drug photoallergy is successfully induced and elicited by systemic administration of a drug and subsequent UVA irradiation of the skin [4,6,10], which is mimicry of clinical drug photoallergy. In another system, photoallergy is induced by sensitization and elicitation with subcutaneous injections of epidermal cells that are *in vitro* photomodified with a drug under UVA exposure [4,9]. The involvement of T cells in drug photoallergy has been clearly demonstrated by mouse models of photoallergy to fluoroquinolone, afloqualone, and ketoprofen [6,10]. Drug photoallergy is mediated by CD4$^+$ T cells [10], and CD8$^+$ T cells may enhance CD4$^+$ T cell responses [6]. Epidermal Langerhans cells are photomodified with a given drug and capable of inducing the proliferation of primed CD4$^+$ T cells [10].

Clinically, drug photoallergy shows eythematous eruption [1] and lichenoid eruption [11,12], and rarely bullous eruption [13] and leukomelanoderma. The erythematous eruption is

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the common type of drug photoallergy and may have scaling on the surface. The lichenoid eruption is not uncommon and similar to lichen planus. This type is characterized clinically by erythematous but dark-colored papules and histologically by $CD8^+$ T cells infiltrating in the upper dermis and attacking keratinocytes [11]. Leukomelanoderma displays a unique clinical appearance of mixture of pigmentation and depigmentation and occurs in dark-colored individuals such as Japanese. Thus, drug photoallergy is heterogeneous as ordinary allergic drug eruptions, and different populations of T cells may induce different clinical and histological appearances. In some patients with the erythematous, lichenoid, and bullous eruptions, biopsied specimens exhibit infiltration of eosinophils as well as lymphocytes [12,3].

In this study, we attempted to establish a murine model of eosinophil-infiltrating photoallergy by administration of AQ in combination with UVA irradiation. Repeated sensitization with AQ + UVA successfully induced eosinophil infiltration upon challenge with subcutaneous AQ + UVA irradiation in AKR/J mice. Here we present the procedure for eosinophil-infiltrating photoallergy and characterize T cell subsets and cutaneous chemokines responsible for this sensitivity.

2. Materials and methods

2.1. Animals

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

AQ was obtained from Tanabe Pharmaceutical Co. (Osaka, Japan) and enoxacin (ENX) from Shionogi Pharmaceutical Co. (Osaka, Japan). 2,4-Dinitro-1-fluorobenzene (DNFB) was purchased from Nacalai Tesque Co. (Tokyo, Japan).

2.2. Sensitization and challenge of drug photoallergy to AQ

An AQ solution (10 mg/ml) in phosphate-buffered saline (PBS; pH 7.4) was prepared as described previously [4]. For sensitization, AKR/J and BALB/c mice received an intraperitoneal (i.p.) injection of AQ solution (2 mg/kg/mouse), and 2 h after injection, they were irradiated with UVA (12 J/cm^2) on the shaved abdomen, as reported previously in quinolone photoallergy [9]. This sensitization procedure was repeated twice a week for 2–6 weeks. Three days after the last immunization, mice were challenged by a subcutaneous (s.c.) injection of AQ solution (2 mg/kg/mouse), or ENX solution (2 mg/kg/mouse) as control, into the shaved abdomen and subsequent irradiation of the same site with UVA (12 J/cm^2).

2.3. Adoptive lymphocytes transfer

AKR/J mice were sensitized with AQ + UVA 10 times. Single cell suspensions were prepared from axillary and inguinal lymph node cells (LNCs) and spleen cells of the sensitized mice. Cells were purified for $CD4^+$ cells (purity >95%) by the AutoMACS magnetic separation system with a kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's directions. Crude immune cells (5×10^7 cells/mouse) or purified $CD4^+$ T cells (1×10^7 cells/mouse) were transferred to naïve AKR/J mice by intravenous (i.v.) injection. Two days after injection, they were challenged by a subcutaneous injection of AQ + UVA irradiation of the shaved abdomen.

2.4. Histological assessment

Two days after the challenge with AQ + UVA, mice were sacrificed, and the abdominal skin was excised. Two to three micrometers-thick sections were cut and stained with hematoxylin and eosin.

2.5. Measurement of cytokines produced by LNCs

The culture medium was RPMI-1640 (Gibco BRL Life Technology Inc., Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 10^{-5} M sodium pyruvate, 25 mM HEPES, 1% nonessential amino acids, and 100 units/ml penicillin, and 100 μg/ml streptomycin (all from Gibco). Immune LNCs from mice sensitized with AQ + UVA 10 times were cultured in 24-well plates (Corning Inc., Corning, NY; 1×10^6/ml of culture medium) at 37 °C in 5% CO_2 in air in the presence of anti-CD3 mAb (soluble form stimulatory for T cells; BD Biosciences (San Diego, CA) at 5 μg/ml and anti-CD28 mAb (Immunotech, Marseille, France) at 5 μg/ml. After 72-h culture, the supernatants were collected and were stored at −80 °C until use. The concentrations of interferon-γ (IFN-γ), IL-4, and IL-5 in the supernatants were measured by enzyme-linked immunosorbent assay (ELISA) with kits (R&D Systems, Minneapolis, MN) according to the manufacturer's directions.

2.6. Real-time quantitative PCR

Total mRNA was extracted from the mice ears with the SVTotal RNA Isolation system (Promega, Madison, WI) according to the manufacturer's protocol. Target gene expression was quantified in a two-step RT-PCR. cDNA was reverse transcribed from total RNA samples using the TaqMan RT reagents (Applied Biosystems, Foster City, CA). Murine CXCL12 (Assay ID: Mm00445552_m1) expression was quantified using TaqMan Gene Expression Assay (Applied Biosystems) in the ABI PRISM 7000 sequence detection system (Applied Biosystems). As an endogenous reference for these PCR quantification studies, glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene expression was measured using the TaqMan rodent GAPDH control reagents (Applied Biosystems). The relative expression was calculated using the $2^{-\Delta\Delta C_T}$ method [14]. 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 control mice was used as a calibrator expression to calculate $\Delta\Delta C_T$. As control, earlobes painted with 25 μl of 0.3% DNFB in 4:1 (v/v) acetone/olive oil was used.

2.7. Statistical analysis

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

3. Results

3.1. Induction of eosinophil-infiltrating photoallergy by AQ + UVA in AKR/J mice

We have previously induced photosensitivity to AQ with AQ-photomodified epidermal cells in BALB/c mice. AQ-photomodified epidermal cells were prepared *in vitro* by UVA irradiation of epidermal cell suspensions in AQ solution, and mice were sensitized and elicited by s.c. injections of those photomodified cells into lateral aspects of the back and footpads, respectively [4]. Here we attempted to sensitize mice by systemic administration of AQ and UVA irradiation of the skin. Since UVA irradiation is

performed *in vivo* in this procedure, melanin pigment may affect the degree of photosensitivity as seen in contact photosensitivity [15]. Therefore, we used albino mice, BALB/c and AKR/J mice in the present study.

The histological changes at the challenged skin were investigated in mice sensitized with AQ + UVA. BALB/c and AKR/J mice were sensitized by an i.p. injection of AQ and subsequent UVA irradiation of shaved abdomen. When this sensitization was performed 10 times and the challenge was done with the same procedure, we could not obtain substantial infiltration of inflammatory cells at the challenged site in either mouse strain (data not shown). Therefore, we immunized mice 10 times (twice a week for 5 weeks) with AQ + UVA, and 3 days after the last sensitization, they were challenged by s.c. injection of AQ on the shaved abdomen and subsequent irradiation of the same site with UVA. As shown in Fig. 1, while AKR/J mice sensitized with AQ + UVA 2 times had no induction of the sensitivity (Fig. 1A), 10-time sensitization exhibited a massive infiltrate of inflammatory cells at the challenged site (Fig. 1B). A high power view revealed that the infiltrate consisted of a high number of eosinophils with occasional lymphocytes (Fig. 1C). BALB/mice sensitized even 10 times did not show infiltration of eosinophils (data not shown), indicating strain dependency of this photoallergy. There were clinical differences in the skin between the 10-time sensitized and challenged mice and the non-sensitized and challenged mice. While the only challenged mice showed virtually no skin change, the 10-time sensitized and challenged mice exhibited scaly and crusty lesions with erythema (Fig. 2).

3.2. Number of sensitization with AQ + UVA to induce eosinophil infiltration and requirement of both AQ and UVA for induction

The frequency of sensitization that induces eosinophilic infiltration upon challenge was tested in AKR/J mice. Mice were sensitized 2–12 times (twice a week) and challenged with AQ + UVA. A significant increase in the number of eosinophils was observed in mice sensitized 8 times (Fig. 3A). Ten-time sensitization induced a higher eosinophil number (more than 30 cells/25 μm^2), which was comparable to 12-time sensitization. Thus, we sensitized AKR/J mice 10 times with AQ + UVA in the following study. We also counted the number of lymphocytes and the found that 5.0 + 1.0 lymphocytes/25 μm^2 infiltrated in the 2-time sensitized and challenged mice and 6.7 + 1.2 lymphocytes/25 μm^2 did in the 10-time sensitized and challenged mice. Therefore, the number of lymphocytes was smaller than that of eosinophils.

AKR/J mice sensitized with AQ + UVA were challenged with AQ or ENX, another representative photosensitizing drug [9,10], in combination with UVA. AQ or UVA alone did not elicit a substantial infiltrate of eosinophils, although AQ alone slightly increased eosinophil infiltration presumably because of serving as an ordinary antigen (Fig. 3B). ENX + UVA evoked no eosinophil infiltration, indicating its photoantigenic specificity.

3.3. Transfer of AQ photoallergy with immune lymphocytes

Immune LNCs and spleen cells were obtained from AKR/J mice sensitized with AQ + UVA 10 times and were injected i.v. into naïve syngeneic recipients. Recipient mice challenged by a s.c. injection of AQ and UVA irradiation of the shaved abdominal skin exhibited an intense skin inflammatory change with a massive infiltrate of eosinophils and lymphocytes (Fig. 4A). The same histological change was obtained by transfer of $CD4^+$ T cells purified from draining LNCs (Fig. 4B), while transfer of $CD8^+$ T cells evoked the response to a lesser degree (Fig. 4C). Mice receiving transfer of LNCs and spleen cells did not show the skin response upon challenge. Thus, $CD4^+$ T cells were mainly responsible for the sensitivity, but $CD8^+$ T cells at least partly participated in the response.

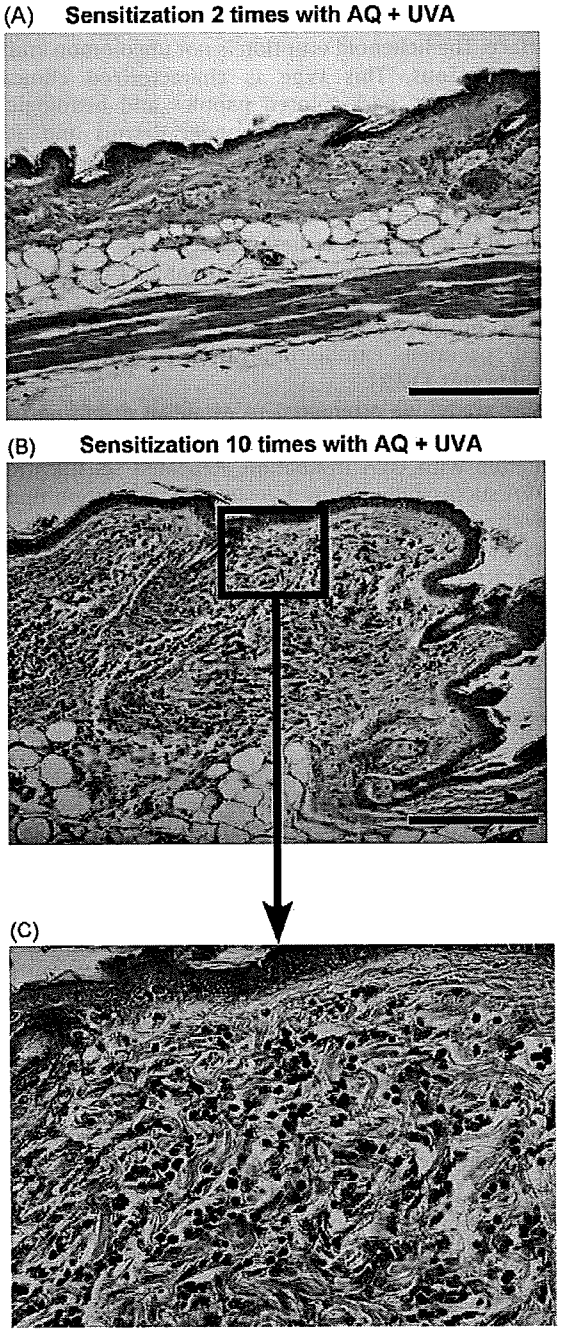

Fig. 1. Histological pictures of abdominal skin challenged with AQ + UVA in AKR/J mice sensitized 2 or 10 times with AQ + UVA. AKR/J mice were sensitized by i.p. injection of AQ (2 mg/kg/mouse) + irradiation with UVA (12 J/cm^2) 2 (A) or 10 times (B) and challenged by a s.c. injection of AQ (2 mg/kg/mous) + irradiation with UVA (12 J/cm^2). (C) High magnification of B, showing massive infiltration of eosinophils and lymphocytes. Scale bar, 100 μm.

3.4. Cytokine production pattern of draining LNCs

Draining LNCs from AKR/J mice sensitized with AQ + UVA 10 times were cultured in the presence of anti-CD3 and anti-CD28 antibodies, and 72-h culture supernatants were measured in the concentrations of IFN-γ, IL-4, and IL-5. As a control, draining LNCs from AKR/J mice sensitized by skin application of 0.5% DNFB 5 days

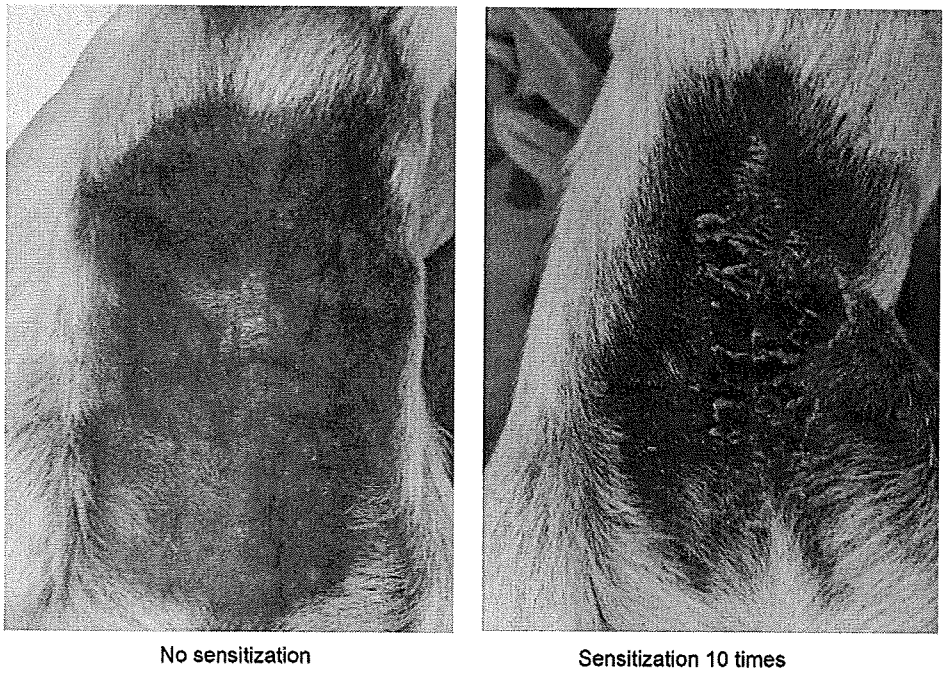

Fig. 2. Skin reaction evoked by AQ + UVA in 10-time sensitizes mice. AKR/J mice were sensitized 10 times and challenged with AQ + UVA (right), while the control mice were challenged with AQ + UVA without sensitization (left).

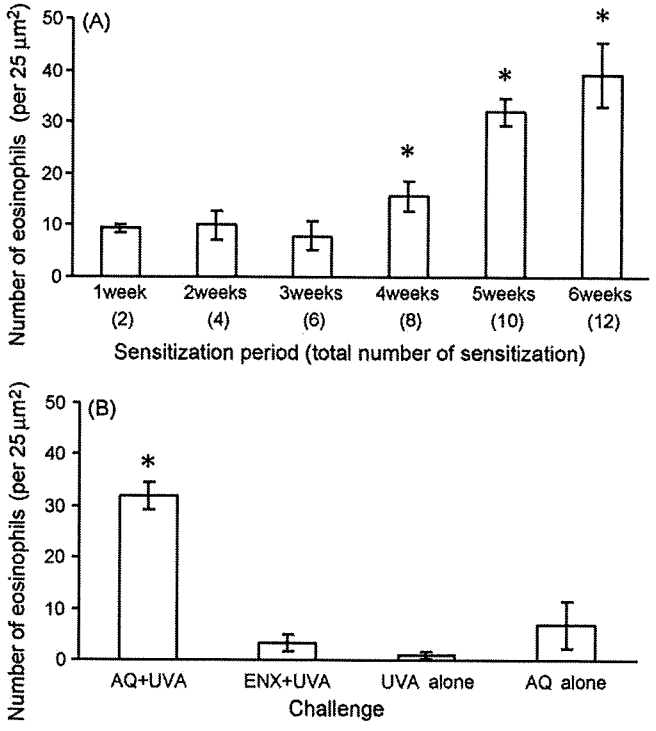

Fig. 3. Frequency of sensitization with AQ + UVA and elicitation procedure for eosinophil infiltration. (A) AKR/J mice were sensitized by i.p. injection of AQ + irradiation with UVA 2–12 times (twice a week) and challenged by a s.c. injection of AQ + irradiation with UVA. In the excised abdominal skin, eosinophils were enumerated as the number per 25 mm^2. Data are the mean + SD of four mice. *$P < 0.05$, compared with the 2 time challenged group. B: AKR/J mice were sensitized by i.p. injection of AQ + irradiation with UVA 10 times (2 times a week) and challenged by intradermal injection of AQ (or ENX as control) and/or irradiation with UVA. The number of eosinophils was counted and expressed as the number per 25 mm^2. Data are the mean + SD of four mice. *$P < 0.001$, compared with the other three groups.

before were used. LNCs from the AQ-photosensitized mice produced a higher amount of IL-4 and a lower amount of IFN-γ than those from the DNFB-sensitized mice (Fig. 5). The amounts of IL-5 was too low to discriminate the both. The results suggest that LNCs from the AQ-photosensitized mice contained a high frequency of Th2 cells.

3.5. Chemokine production in AQ-photochallenged skin

AKR/J mice were sensitized 10 times and challenged with AQ + UVA. The elicited skin was subjected to quantitative real-time PCR to measure mRNA levels for chemokines, including eosinophil-associated chemokines (CCL24/eotaxin-2 and CCL5/RANTES), Th1-associated chemokines (CXCL10/IP-10 and CXCL9/Mig), Th2-associated chemokines (CCL17/TARC and CCL22/MDC). As a control, AKR/J mice were painted with 0.2% DNFB on the abdomen, and the skin was excised 2 days later. In the level relative to β-actin, the expression of CCL24 was elevated (Fig. 6A), suggesting its involvement in eosinophil infiltration.

To further characterize the chemokine expression, we compared the AQ + UVA-challenged skin with normal abdominal skin of mice. However, since the normal skin had extremely low levels of chemokine expression, it was difficult to calculate the relative intensity of each chemokine mRNA in AQ + UVA-challenged skin. Then, we used hapten-applied skin for comparison. We compared the expression of chemokines between AQ + UVA-challenged abdominal skin and DNFB-challenged ear skin of DNFB-sensitized mice. Although we found that AQ + UVA-challenged skin was skewed to Th2 cells as observed with low CXCL10 and high CCL22 levels, a discrepancy existed with a relatively high level of CXCL9 (data not shown). It is possible that these results were influenced by the difference in the skin specimens used for AQ + UVA and DNFB, namely abdomen and ears. We therefore used the abdominal skin of DNFB single application. This was not a challenged skin, but the expression levels of chemokines were appropriate for comparison with AQ + UVA-challenged abdomen. When compared to the DNFB-painted abdominal skin of mice without sensitization, both CCL24 and CCL5 were elevated along

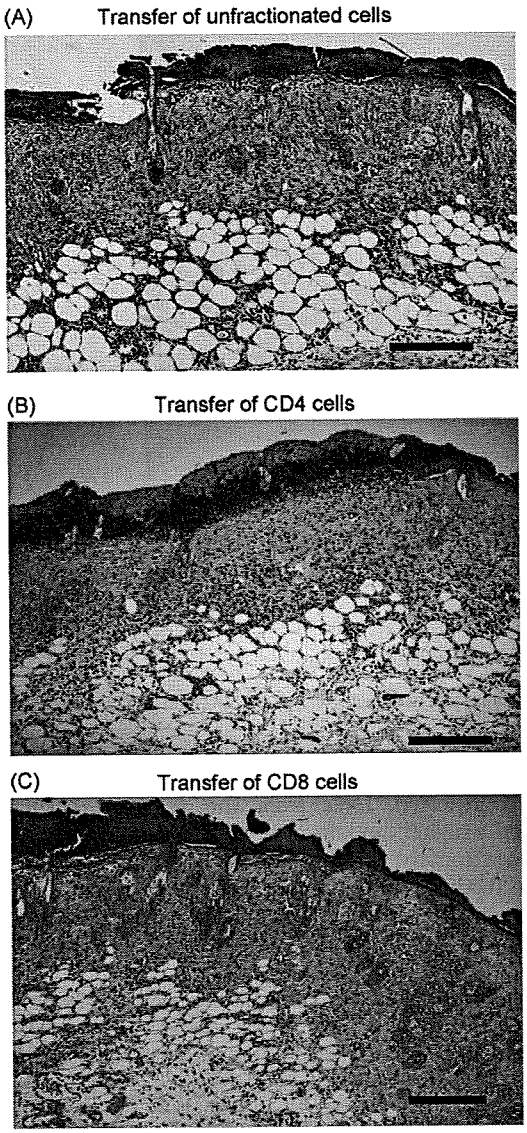

Fig. 4. Histological pictures of challenged abdominal skin of recipients transferred with AQ + UVA-immune LNCs. Donor AKR/J mice were sensitized with AQ + UVA 10 times. Draining LNCs were taken from the mice and non-purified or purified for $CD4^+$ or $CD8^+$ T cells. Recipient mice were administered i.v. with crude LNCs (A), $CD4^+$ T cells (B), or $CD8^+$ T cells (C) and challenged with AQ + UVA. Bar = 50 μm.

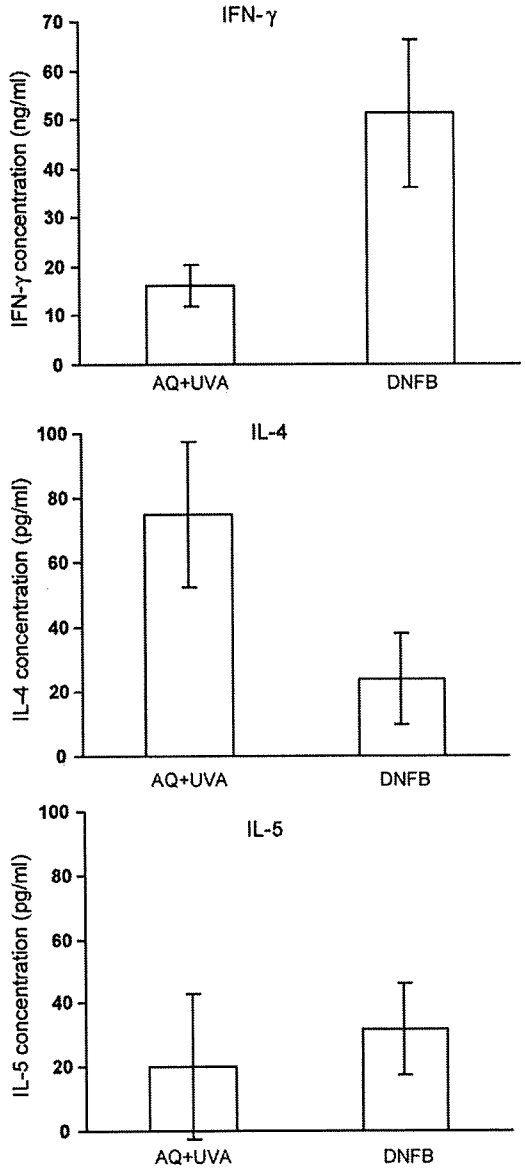

Fig. 5. Cytokine concentration in culture supernatants of immune lymph node and spleen cells. Immune lymph nodes and spleen cells were taken from mice sensitized with AQ + UVA 10 times or from mice sensitized with DNFB as control. They were cultured for 72 h in the presence of anti-CD3 mAb (soluble form stimulatory for T cells; BD Biosciences, San Diego, CA) at 5 μg/ml and anti-CD28 mAb (Immunotech, Marseille, France) at 5 μg/ml. Culture supernatants were subjected to ELISA for measurement of IFN-γ, IL-4, and IL-5. Data are the mean + SD ($n = 3$). *$P < 0.05$, compared with AQ + UVA group. These data represent one of three independent experiments.

with marked elevation of both Th2 chemokine CCL22 and CCL17 in the AQ + UVA-sensitized and challenged mice (Fig. 6B).

4. Discussion

In this study, we established a mouse model of photoallergy to AQ using AKR/J mice, which shows marked skin infiltration of eosinophils upon challenge with both AQ and UVA. This eosinophilic photoallergy is induced by repeated systemic administration of AQ and irradiation of UVA to the skin. The sensitivity was photoantigen-specific, and AKR/J strain was a high responder. The eosinophil-infiltrating histology was transferred to naïve recipients by i.v. injection of immune IL-4-producing $CD4^+$ T cells, indicating that Th2 cells mediate the tissue eosinophilia.

In this photoallergy to AQ, sensitization of more than 8 times was required for the induction of Th2 cells and the resultant eosinophilia. This is in accordance with the observation found in contact hypersensitivity, a repeated hapten application leads to Th2-mediated cutaneous sensitivity upon epicutaneous challenge [16]. However, since the infiltrate consists largely of neutrophils and eosinophils with occasional lymphocytes [17], the level of eosinophil infiltration is higher in our system. By using AQ, we have previously found that s.c. injection of AQ-photomodified epidermal cells sensitize and elicit a delayed-type hypersensitivity reaction [4]. In that study, the photocoupling of cells with AQ was efficiently but artificially performed *in vitro*, and thus the AQ-photomodified cells seem to have strong photoantigenicity, thereby immunizing rodents by only one s.c. sensitization procedure. In contrast, the combination of systemic administration of AQ and UVA exposure of the skin is a clinically mimic but weaker sensitization procedure, and therefore, repeating of immunization is required for sensitizing mice. The repeated treatments, however, may result in polarization of the induced immunological state to a

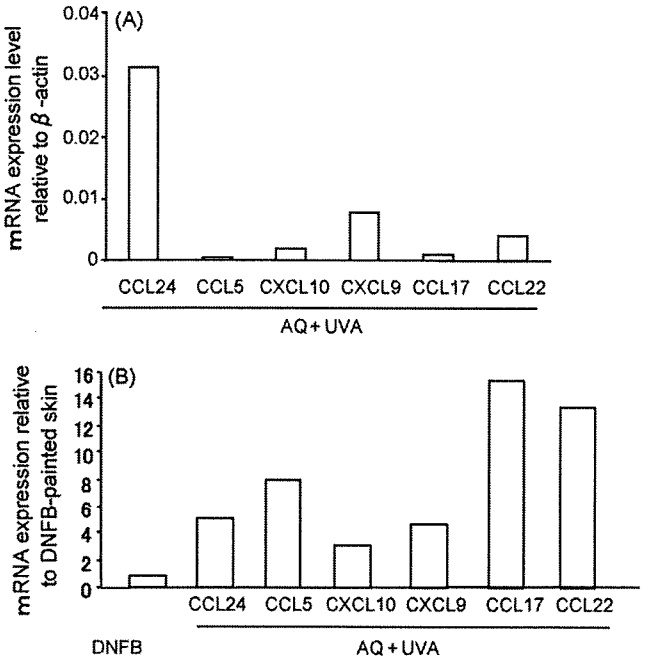

Fig. 6. Chemokine production pattern in AQ-photochallenged skin. AKR/J mice were sensitized 10 times and challenged with AQ + UVA. The elicited skin was subjected to real-time quantitative PCR to measure mRNA levels for chemokines, including CCL24, CCL5, CXCL10, CXCL9, CCL17, and CCL22. As control, AKR/L mice were painted with 0.2% DNFB on the abdomen, and the skin was excised 2 days later. Data are expressed as mRNA expression level relative to β-actin (A) or relative to each level of DNFB-painted skin (B). These data represent one of three independent experiments.

Th2-dominant condition [16]. The necessity of long duration of drug administration and UVA exposure necessary for the development of eosinophilic photoallergy seems to be in accordance with our observation.

In our mouse model of AQ photoallergy, Th2 induction, chemokine production, and eosinohpil infiltration are closely related, but the precise mechanism involving these events is still speculative. During sensitization with AQ + UVA, Th2 cell population(s) specific for AQ photoantigen is duly induced, and additionally, IgE specific for photodegradated AQ possibly develops. In the process of elicitation with AQ + UVA, the initial event that evokes the sensitivity is photomodification of antigen-presenting cells with AQ. Since AQ is injected s.c. for the challenge, the candidates for those modified cells include dermal dendritic cells, macrophages, and others. Then, cells bearing AQ photoantigen may restimulate specific Th2 cells, which directly release IL-5 and indirectly elaborate CCL24 and/or CCL5 by stimulating fibroblasts [18-20] or macrophages [21], leading to tissue eosinophilia [19,20].

There are differences between drug-specific T cell clones established from individual patients with drug eruption, as they can be Th1, Th2 or Tc1 cells [22]. In addition, some but not all patients exhibit blood and/or tissue eosinophilia [23]. In photoallergic drug eruption to AQ, the patients may show an eythematous eruption or lichenoid eruption. Whereas the latter type is mediated by Th1 and Tc1 cells [24,25], the former may be caused by Th2 cells and possibly associated with tissue eosinophilia. The present model may be a mimicry of the former type. Our study suggests that even photohapten can sensitize Th2 cells and induce tissue eosinophilia, when weak sensitization is prolonged.

Acknowledgement

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Possible involvement of T lymphocytes in the pathogenesis of Nagashima-type keratosis palmoplantaris

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Nagashima-type keratosis palmoplantaris (PPK) is an autosomal recessive, transgressive and nonprogressive form of PPK.[1] We have recently reviewed this condition and proposed it as a condition separate from classic PPK.[1] However, its pathogenesis remains unknown.

We report a patient with Nagashima-type PPK.[1] Histological examination of a biopsy from the erythematous dorsum of the hand showed a mild to moderate infiltrate of mononuclear cells in the dermal papillae (Fig. 1a,b). This specimen was stained with anti-CD4, CD8, and CD20 monoclonal antibodies, which showed that CD4+ T cells were present in greater numbers than CD8+ T cells (Fig. 1c,d), and CD20+ B cells were barely detectable (data not shown). Although we cannot exclude the possibility that CD4 T-cell infiltration may only be reactive, these

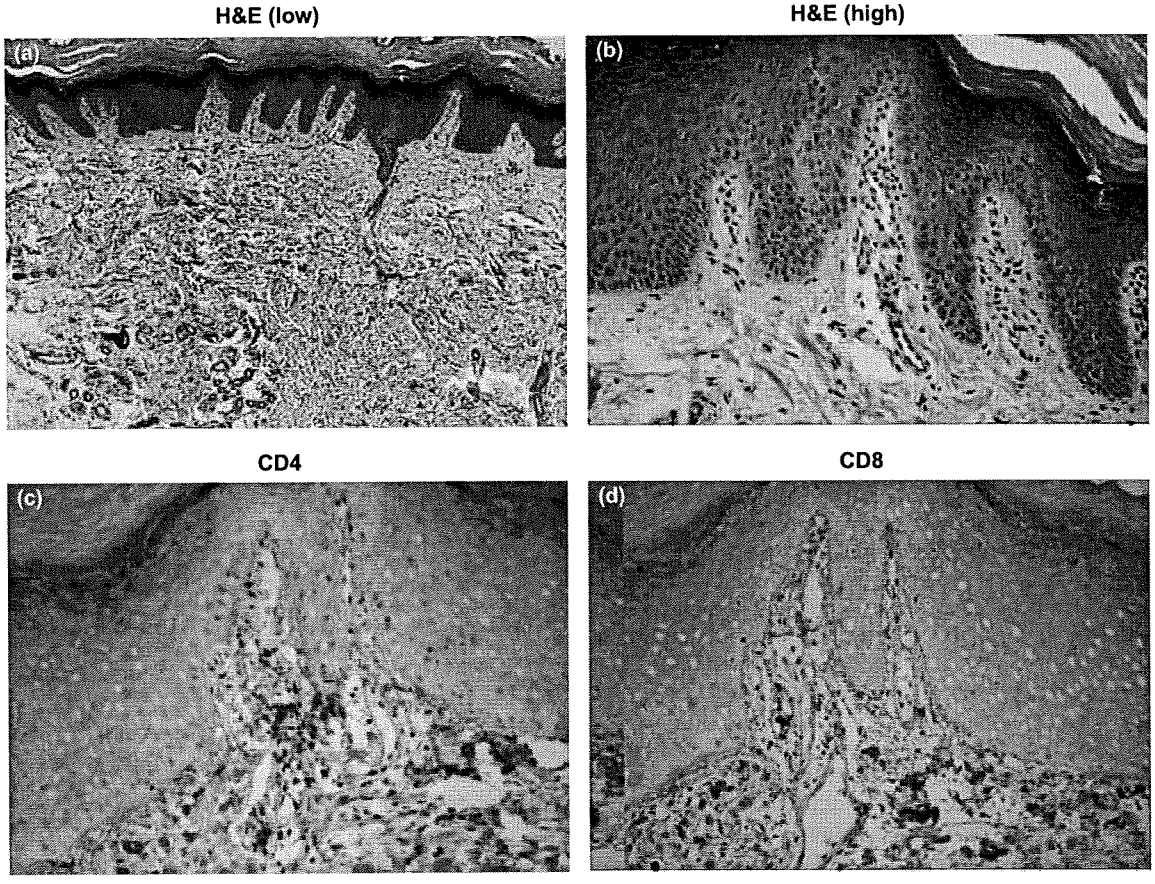

Figure 1 (a,b) A biopsy specimen taken from an erythematous hyperkeratotic lesion of the dorsum of the hand shows orthokeratotic hyperkeratosis with a mild to moderate infiltrate of mononuclear cells in the dermal papilla. Haematoxylin and eosin, original magnification (a) × 10; × 40. (c,d) Immunohistochemistry with anti-CD4 and CD8 monoclonal antibodies showed that the majority of lymphocytes were CD4+ T cells. Original magnification (c,d) × 40.

Table 1 Primers for PCR and DNA sequencing *SLURP2* (*LYNX1*).

	Sequence (5′ → 3′)	
Primers	Forward	Reverse
LYNX1, isoform b	GTGACTCAGACCCGCAGAG	AGCTGCACCTGGTAGATGGT
LYNX1, isoform a, b	AGCTCCTGAGAGGGCTGGAT	CAAGACTCCACGCAGGACTT
Sequence 1	TGCTGGGTGGAGATGAGTG	CCTGGATCTGGCTCTGTCAC
Sequence 2	CAGGGAAGGGTATGAGCAAA	

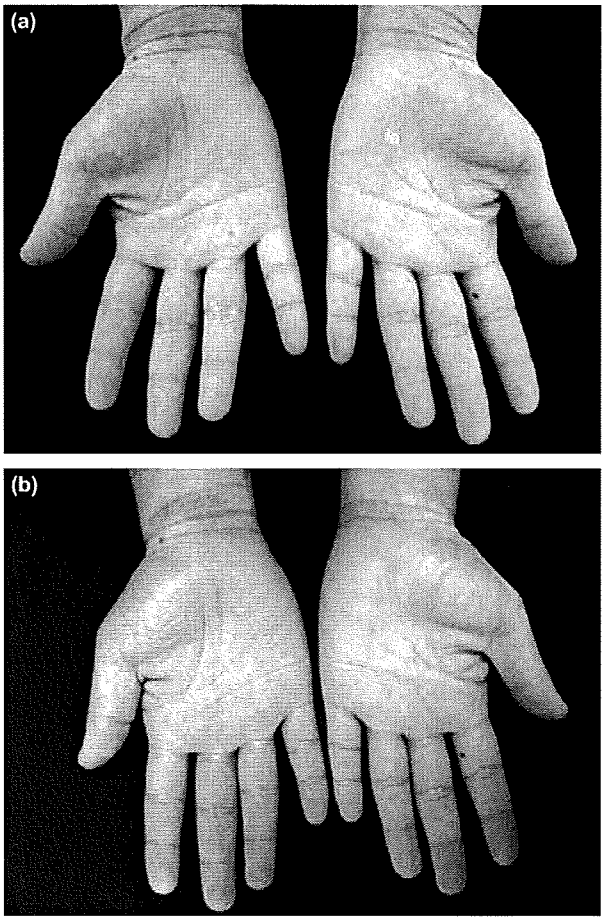

Figure 2 Effect of topical treatment with steroid and tacrolimus ointment. The patient had erythematous, hyperkeratotic palms and was treated topically for 1 week with (a) betamethasone butyrate propionate to the right hand and (b) tacrolimus ointment to the left hand.

findings indicate that the pathogenesis may be related to the effects of T cells infiltrating into the skin. The patient had been treated topically with high-potency 0.12% betamethasone valerate ointment with limited mild improvement before his visit to our clinic. We then applied 0.1% tacrolimus ointment (Astellas, Tokyo, Japan) to the left hand and 0.05% betamethasone butyrate propionate ointment (GlaxoSmithKline, Tokyo, Japan) to the right hand, and compared the therapeutic effect between the immunosuppressant and the steroid treatment. The erythematous hue and hyperkeratosis of both hands were improved with both treatments, but improvement was greater on the left than on the right hand (Fig. 2a,b), suggesting that Nagashima-type PPK can, at least in part, be induced or exacerbated by T cells infiltrating into the skin.

In contrast, it has been reported that the pathogenesis of several PPKs is attributable to disturbed gene function, such as those involving structural proteins, the cornified envelope, cell–cell communication, transmembrane signal transduction, and cholinergic signalling molecules.[2] Nagashima-type PPK shares many clinical signs with mal de Meleda (MDM) induced by *SLURP1* mutations.[3] In addition, several nicotinic receptors are expressed in the skin and skin appendages. Moreover, *SLURP1* [National Center for Biotechnology information (NCBI) NM_020427] and *SLURP2 (LYNX1)* (NCBI: NM_177458) genes were found in the immune system as well as the skin.[4] We have reported previously that no mutation existed in either exons or introns of *SLURP1*.[1] Therefore, we sequenced the DNA of the *SLURP2* gene, whose role remains unknown. After approval by the local ethics board, genomic DNA was isolated from peripheral blood leucocytes of the patient, and was amplified and sequenced as reported previously,[1] using primers for PCR and DNA sequencing (Table 1). The results showed no mutation in *SLURP2*.

Further clinical and genetic studies may reveal the pathogenesis underlying PPK. We believe it is important to draw attention to a possible role for T cell-mediated inflammation in the pathogenesis of this apparently genetic disorder.

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'Nagashima-type' keratosis palmoplantaris in two siblings

Editor

'Nagashima-type' keratosis palmoplantaris is characterized by transgressive and non-progressive palmoplantar keratosis (PPK) with autosomal-recessive trait. It was first described in 1977[1] and then followed by about 20 cases in Japanese literature. [2] Recently, we have reported the first case of 'Nagashima-type' PPK in English literature.[2] Its clinical manifestations were improved by topical treatment with 0.1% tacrolimus ointment, suggesting that Nagashima-type PPK can be induced or exacerbated by T cells infiltrating into the skin.[3] Here, we experienced two siblings with this type of PPK.

A 31-year-old woman and her 29-year-old younger sister presented with bilateral reddish, palmoplantar hyperkeratotic lesions (Fig. 1). These manifestations started within the first 3 years of their lives and gradually progressed until their late teens. Their conditions were associated with hyperhidrosis on the palms and soles with distinct odour and maceration. Neither the elbows, knees, hair, nails, nor teeth were involved. These skin lesions were

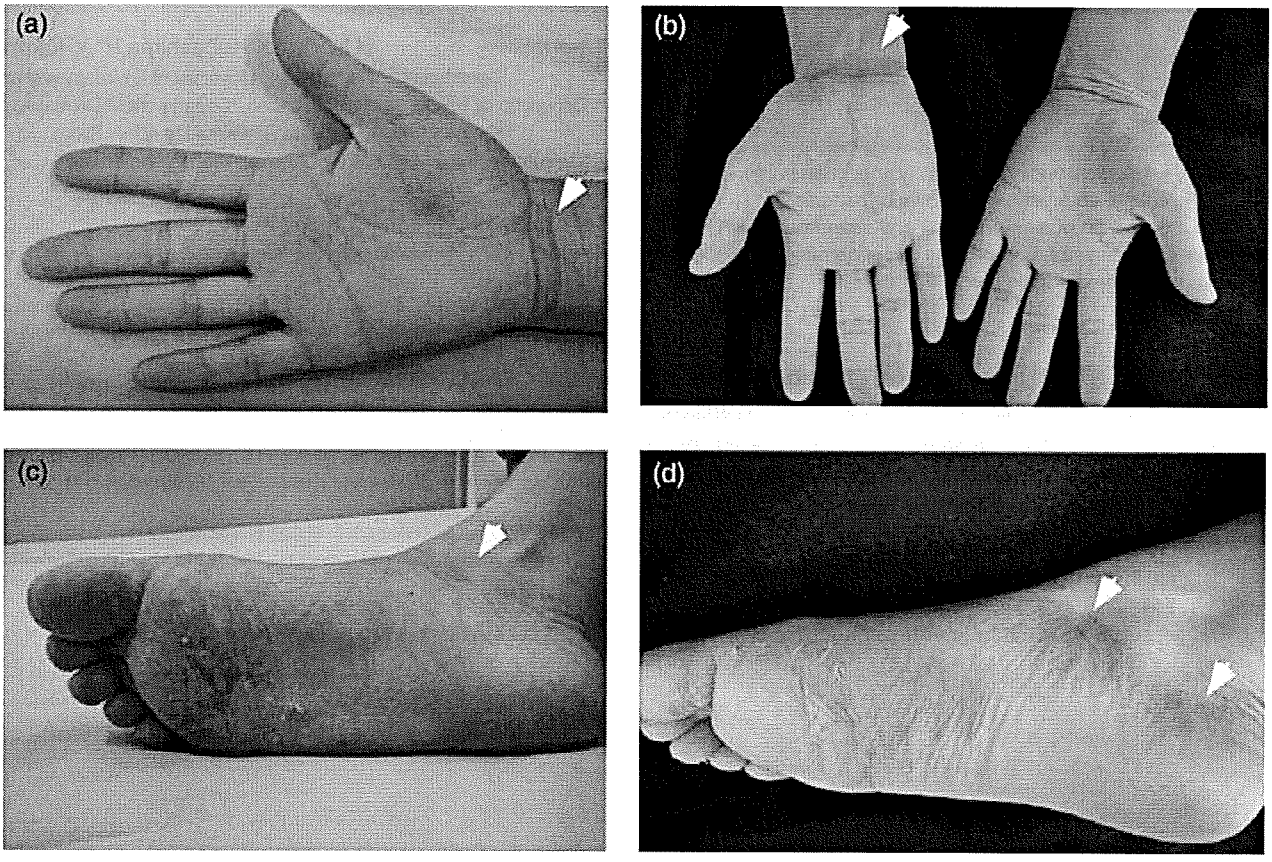

Figure 1 Clinical appearance. The pictures show bilateral reddish, palmoplantar hyperkeratotic lesions on the palms and soles (a, c; older sister, b, d; younger sister). Arrow heads depict borders of well-demarcated hyperkeratotic erythema.

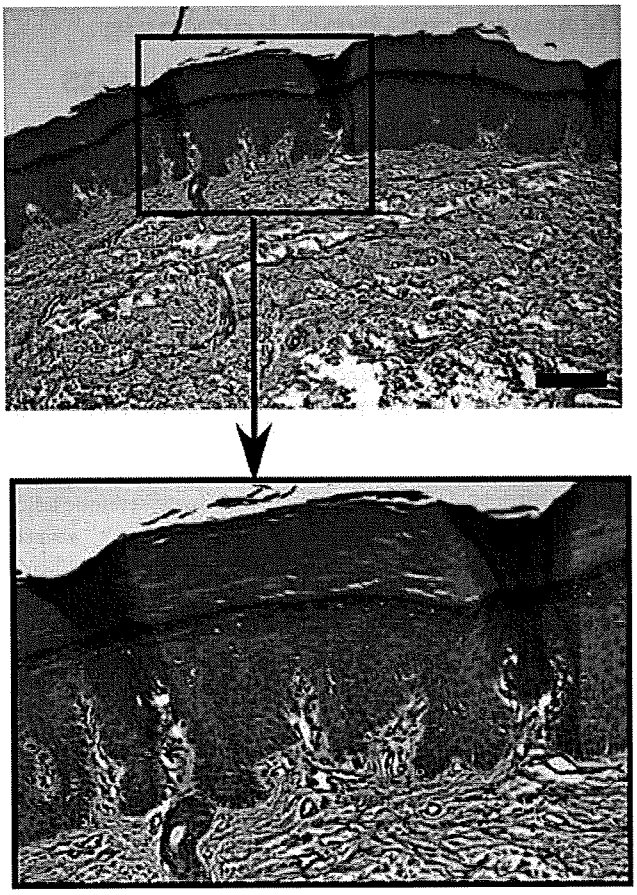

Figure 2 Histological findings. A biopsy specimen from the medial part of the sole of younger sister shows orthokeratotic hyperkeratosis, mild hypergranulosis, and acanthosis (a). A mild sparse infiltrate of lymphocytes in the upper dermis is also noted in a high power view (b). Scale bar, 200 mm.

resistant to a conventional treatment with topical steroid ointments. The younger sister had a past medical history of asthma and congenital ptosis. Both of them had past medical histories of cervical uterine cancer, but were otherwise healthy. The patients were isolated cases in the family and their parents were not consanguinous couples. KOH tests for detecting fungus on the palms and soles were negative. The patients declined to perform the genetic analysis.

A biopsy sample was taken from the medial part of the sole from the younger sister, which showed orthokeratotic hyperkeratosis, mild hypergranulosis, acanthosis, and a mild sparse lymphocytic infiltrate in the upper dermis (Fig. 2). There was no viral inclusion , granular degeneration, or atypical epithelial cell. The clinical appearances and histology of these patients were consistent with 'Nagashima-type' PPK.

Since its clinical findings were similar to but milder than those of mal de Meleda (MDM), 'Nagashima-type' PPK was originally described as a mild form of MDM. However, MDM in general follows a progressive course throughout their lives, usually accompanied with perioral erythema and occasionally brachydactyly, nail abnormalities, and lichenoid plaques[4] which were not seen in 'Nagashima-type' PPK. In addition, no mutation in *SLURP1*, responsible for MDM,[5] was found in 'Nagashima-type' PPK.[3] Therefore, we have proposed 'Nagashima-type' PPK as the novel entity of PPK.

We showed herein two cases of siblings from their non-affected parents, suggesting that the mode of inheritance is autosomal recessive. These patients are the second report of 'Nagashima-type' PPK in English literature. Until recently, the clinical features of 'Nagashima-type' PPK has not well been appreciated in Western countries. With more reports and concise clinical observations including genetic approach, we can address the pathomechanism underlying PPK.

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via nicotinic acetylcholine receptor–mediated pathways.[4]

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Loss of tumor cell CCR4 expression upon leukemic change in adult T-cell leukemia/lymphoma

To the Editor: A 77-year-old Japanese male was referred to our department in January 2006 for further evaluation of a 1-year history of skin tumors. The initial examination revealed several tumors/nodules on the trunk, right upper arm (Fig 1, *A*), and right buttock. Laboratory studies revealed a leukocyte count of 6400/μL (normal, 3500-9100/μL), with 22% normal lymphocytes (normal, 15.6-49.4%) and 1% atypical lymphoid cells. Flow cytometric analysis of peripheral blood mononuclear cells (PBMCs) showed that approximately 2% of lymphocytes were positive for CD4, CD25, and CCR4 (Fig 2, *A*). A biopsy specimen taken from a tumor disclosed massive dermal infiltration of large atypical lymphoid cells with epidermotropism (Fig 1, *B*). Immunohistochemical staining showed that the tumor cells were positive for CD4 (Fig 1, C). Flow cytometric analysis of skin tumor–infiltrating cells revealed that 38.6% of lymphocytes were positive for both CD25 and CCR4 (Fig 2, *B*). Human T-cell lymphotropic virus-1 monoclonal integration was confirmed by a Southern blot analysis. Computed tomographic examinations showed no abnormality in the internal organs. Based on these findings, the condition was diagnosed as the smoldering type of adult T-cell leukemia/lymphoma (ATLL).[1]

During the following year, the patient was treated intermittently with electron beam radiation (30 Gy per tumor) or systemic or intralesional interferon-γ (IFN-γ) injections (1×10^6 international unit [IU]/d). While the tumors disappeared with the treatments or occasionally regressed spontaneously, new lesions reappeared on the lower back and extremities. In May 2007, after the tumors enlarged and spread, he was treated with a combination of etoposide (25-75 mg/d intermittently for 1-2 weeks) and prednisolone (10-20 mg/d). The peripheral lymphocyte counts from May 2007 to August 2007 were within the normal range. In August 2007, however, he developed many tumors on the trunk (Fig 1, *D*). He was treated with elecron beam radiation (30 Gy), prednisolone (20 mg/d), and intralesional IFN-γ injections with (1×10^6 IU/d), followed by methylprednisolone pulse therapy. Although his skin lesions

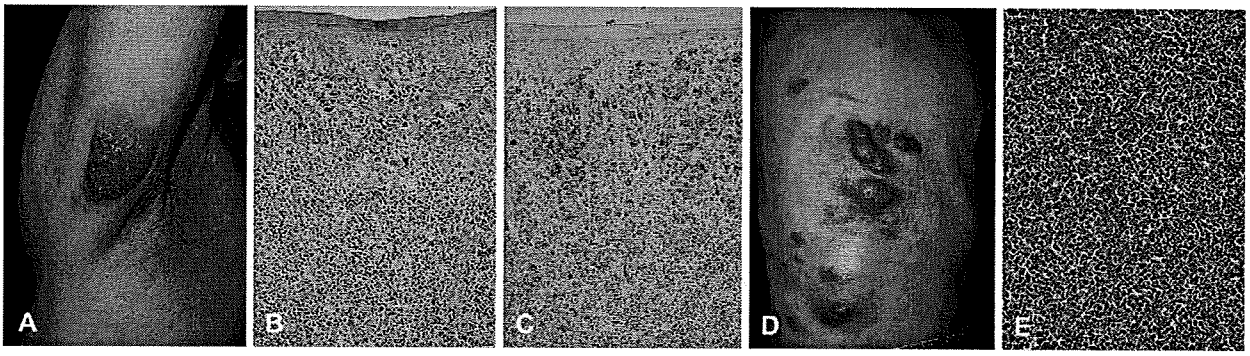

Fig 1. **A**, Initial presentation in January 2006. **B**, Histology of the skin tumor. **C**, Immunohistochemical staining for CD4. **D**, Recurrent tumors in August 2007. **E**, Splenic involvement with tumor cells (September 2007). (**B** and **E**, Hematoxylin–eosin stain; original magnification: **B**, **C**, and **E**, ×200.)

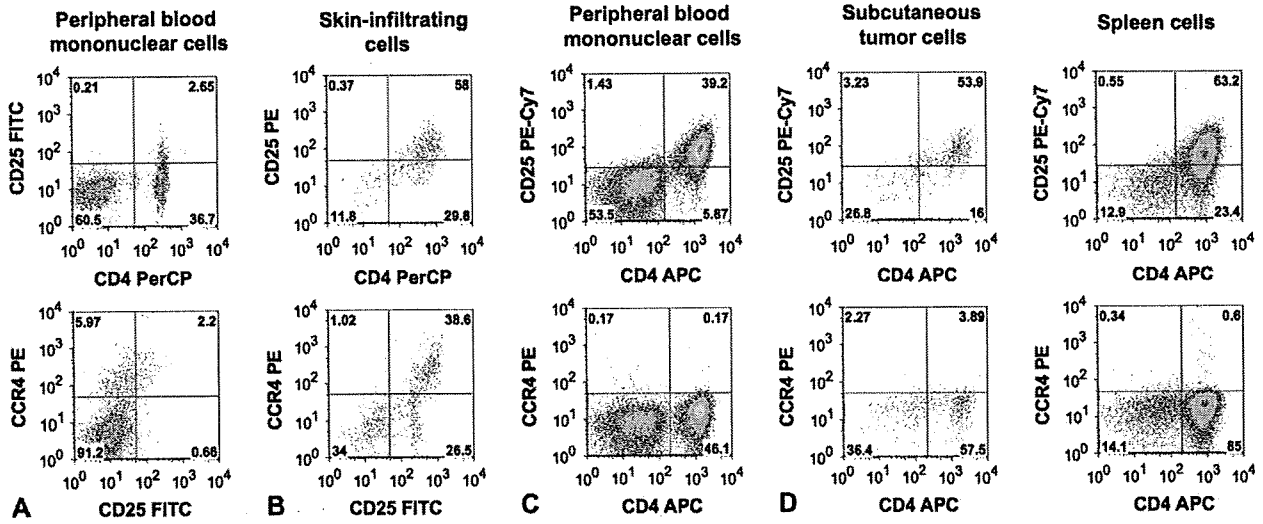

Fig 2. **A** and **B**, Flow cytometry of peripheral blood mononuclear cells and tumor-infiltrating cells during the nonleukemic phase in January 2006. **C**, Peripheral blood mononuclear cells and subcutaneous and spleen cells (**D**) after the leukemic conversion in September 2007. They were double-stained with the indicated monoclonal antibodies.

were markedly improved, the serum lactate dehydrogenase level subsequently increased from 720 IU/L (normal, 119-229) on September 20, 2007 to 1914 IU/L on September 25, 2007. At this time, the leukocyte count increased to 16,200/μL; approximately 39.2% of lymphocytes were positive for CD4 and CD25 but only 0.17% of lymphocytes were positive for CD4 and CCR4 (Fig 2, *C*). The patient died of respiratory dysfunction on September 25, 2007. Autopsy examination revealed lymphoma cell invasion into the subcutis, lung, liver, spleen (Fig 1, *E*), kidney, prostate, rectum, and bone marrow. The subcutaneous and splenic tumor cells contained only 3.89% and 0.60% $CCR4^+$ $CD4^+$ cells, respectively (Fig 2, *D*).

There has been no report of leukemic change in ATLL associated with loss of CCR4. The concomitant invasion of CCR4-negative tumor cells into the spleen and other organs is also particularly remarkable. We also observed that a patient with primary cutaneous anaplastic large cell lymphoma with fatal leukemic outcome showed a loss of CCR4 as well as cutaneous lymphocyte-associated antigen.[2] These cases suggest that the change of skin homing receptor expression in cutaneous T-cell lymphoma dramatically alters the clinical behavior of the tumor cells.

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Antiretroviral-induced genital ulceration

To the Editor: We report a 45-year-old male with HIV on highly active antiretroviral therapy (HAART), with a viral load of 300, CD4 count of 550, and penile ulcers since 1999. A biopsy of one of the ulcers in 2005 reported changes consistent with herpes simplex virus (HSV) infection; however, the ulcers were unresponsive to multiple courses of acyclovir and intravenous Foscarnet (AstraZeneca, London, UK). His bloodwork argued against rheumatologic causes for the ulcerations, including a negative antinuclear antibody, negative rheumatoid factor, and an erythrocyte sedimentation rate within normal limits. The patient also tested negative for syphilis, hepatitis B,