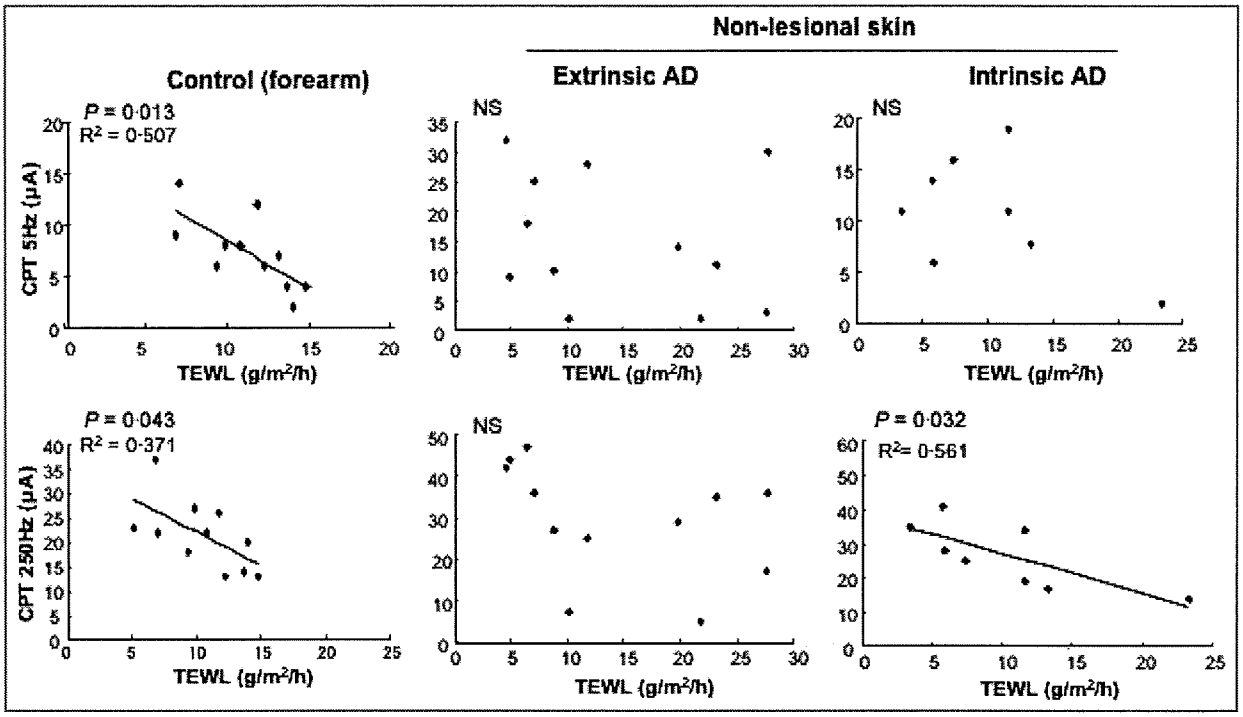

Fig 5. Relationship between transepidermal water loss (TEWL) and electric current perception threshold (CPT) in nonlesional forearm skin of patients with extrinsic and intrinsic atopic dermatitis (AD) and healthy control skin. N.S., not significant.

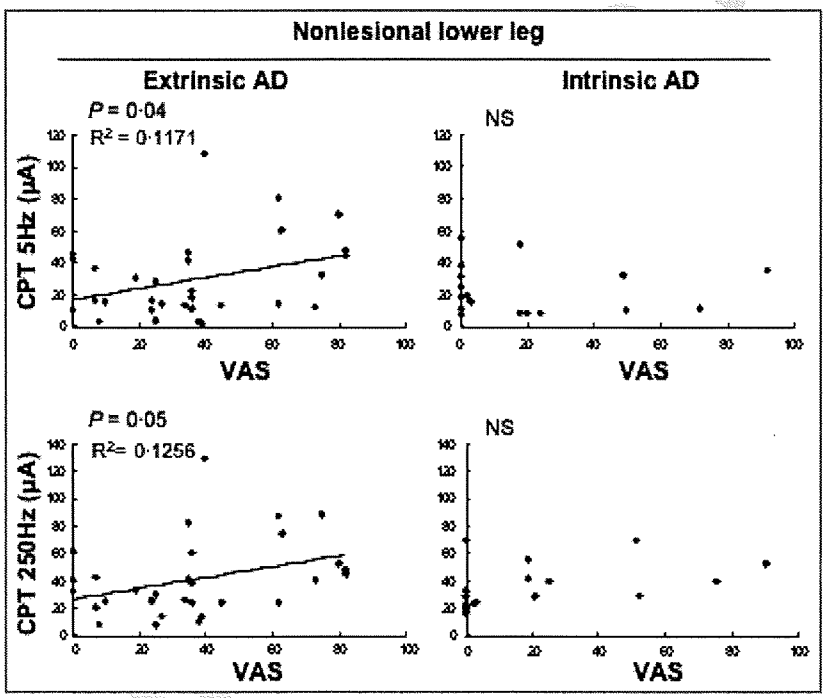

Fig 6. Relationship between visual analogue scale (VAS) score and electric current perception threshold (CPT) on nonlesional skin of patients with extrinsic and intrinsic atopic dermatitis (AD). N.S., not significant.

types accurately. Only one clear way to discriminate the two types is the serum levels of IgE,[29] but its precise cutoff value has not been determined.

In this study, we tentatively divided the patients with AD into two groups by IgE levels of > 400 and < 220 U mL^{-1}, because the normal range in Japanese individuals is < 220 U mL^{-1}. This division was confirmed by a high percentage and high scores of positive RAST to *D. pteronyssinus* in extrinsic AD and a low percentage in intrinsic AD. We found that more of our patients had intrinsic than extrinsic AD, and that women were more likely than men to have intrinsic AD, as already reported in previous studies.[5] In contrast to

extrinsic AD, intrinsic AD is thought to show a normal skin barrier function. We validated this general concept by measuring skin surface hydration and TEWL, and found no significant difference in these values between patients with intrinsic AD and normal individuals, while the patients with extrinsic AD had lower surface hydration levels and higher TEWL levels than the normal subjects.

C-fibres (unmyelinated fibres) are sensory nerves conducting pruritus. A transcutaneous electric current at 5 Hz can stimulate C-fibres.[25] Alternating current stimulus at 250 Hz activates Aδ-fibres (small myelinated fibres), which may also participate in itch. The condition of the stratum corneum may modify the perception by affecting the current or other factors. In our study, low levels of hydration of the stratum corneum reduced CPT. This suggests that the itch perception to external stimuli is promoted in skin with low hydration.

The difference in the barrier function between the extrinsic and intrinsic types raised the possibility that the elicitability of pruritus differs between them. In normal individuals, CPT and skin surface hydration or TEWL were correlated with each other, suggesting that the barrier-damaged skin is sensitive to external irritants. In normal individuals, Kobayashi *et al.*[23] have reported that CPT is inversely correlated with TEWL levels after tape stripping, providing further evidence that barrier damage leads to elicitability of sensation. In our study, the correlation between CPT and skin surface hydration and the inverse correlation between CPT and TEWL were also found in patients with intrinsic AD, suggesting that intrinsic AD shows a normal skin barrier and elicitability of sensation to external stimuli. In contrast, the patients with extrinsic AD showed different elicitability with individual variations presumably due to the low surface hydration. Kobayashi *et al.*[23] have also shown that the skin of patients with AD is not extremely sensitive as compared with that of normal individuals to the electric stimulation of their Aδ- and C-fibres. Their patients seem to include those with both extrinsic and intrinsic AD. Our results suggest that some patients with extrinsic AD have high CPT levels despite the impaired barrier function.

Pre-existing pruritus elevated CPT on the nonlesional skin of patients with extrinsic AD, as CPT and VAS were correlated with each other in the nonlesional sites of the extrinsic type. Accordingly, Ikoma *et al.*[30] found that when histamine prick tests are performed in nonlesional skin of patients with AD, itch rating increases more slowly and is significantly lower than in controls.[30] Our unexpected finding was not observed in intrinsic AD. It is possible that in the already itchy skin of extrinsic AD, Aδ- and C-fibres are in a stimulated state, resulting in the insensitivity to external irritants, while the steady-state interaction between the barrier and sensory fibres might be kept in intrinsic AD. The end of sensory fibres in the skin of extrinsic AD seems to be continuously stimulated by the damaged stratum corneum, leading to the elevated CPT.

Our study suggests that the two types of AD are different from each other in the mode of elicitability of pruritus, because of the different skin barrier states between them. Furthermore, it was recently found that IgE autoantibodies can target keratinocytes in AD; this might promote barrier damage and modify resultant itch elicitability in extrinsic AD.[31] As the response of sensory nerves to irritants appears to be intact in intrinsic AD, the mechanisms of pruritus underlying this type of AD are an important issue to be elucidated.

Acknowledgments

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Eruptive generalized granuloma annulare presenting with numerous micropapules

Granuloma annulare (GA) is a benign, inflammatory, and self-limited dermatosis, and its clinical variants are classified into localized, generalized (or disseminated), nodular, perforating, subcutaneous, and pustular forms.[1] In generalized GA, there have been many reports regarding its associated conditions, including diabetes mellitus, lipid metabolic disorders, lymphoma, or other malignant diseases, thyroid diseases, acquired immunodeficiency syndrome, and hepatitis C and other viral infections.[2–6] Generalized GA exhibits variously sized individual lesions from case to case, as it may consist of multiple skin-colored papules, small annular plaques, or large violaceous patches (1–6). Here, we report a case of generalized GA presenting with an extremely eruptive manifestation in association with diabetes mellitus.

A 71-year-old man had a 3-month history of an asymptomatic skin eruption that initially began on his chest and subsequently spread to his back and upper arms. He was taking no medication and his family history was unremarkable. On examination, the patient had an erythematous eruption composed of numerous tiny red papules on his chest and back (Fig. 1a–c). Since papules were very small and

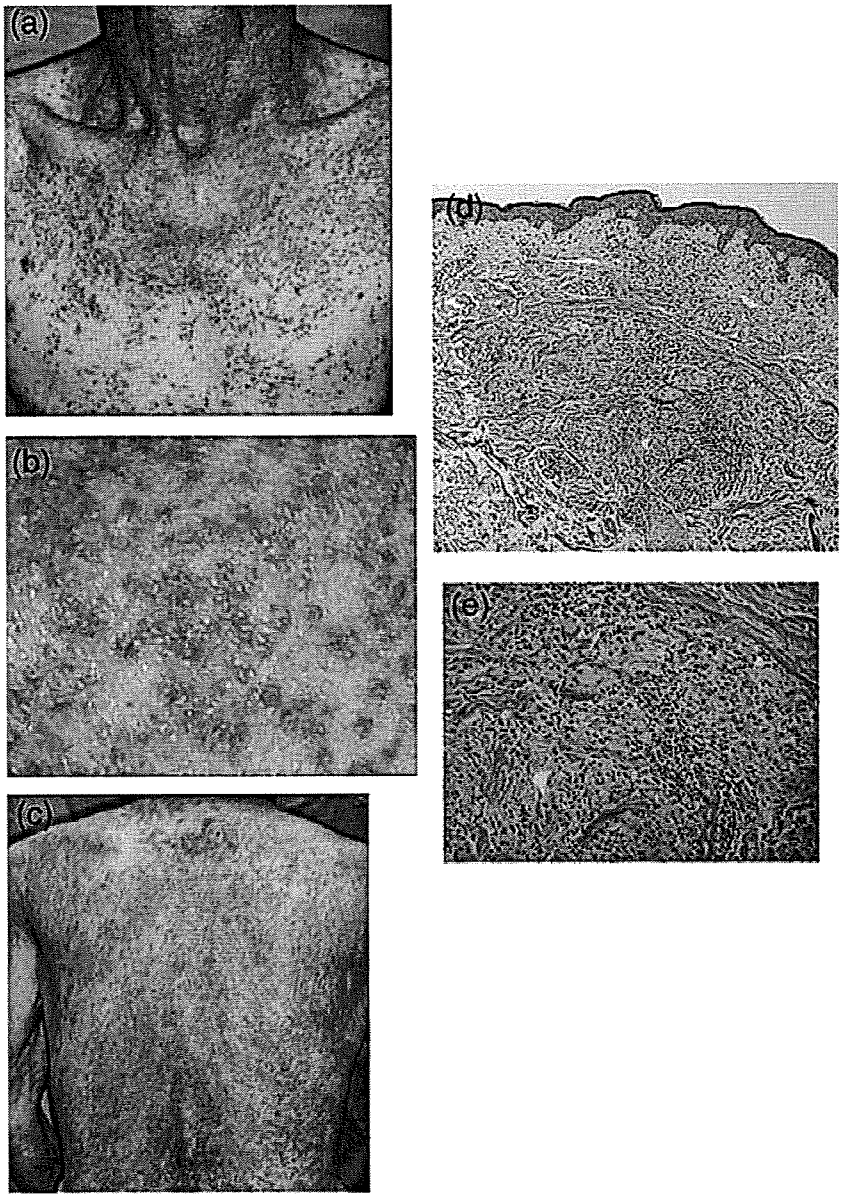

Figure 1 Clinical and histologies. (a) Eruption on the upper chest. (b) Close inspection of numerous tiny papules on the upper chest. (c) Coalesced small papules on the back. (d) Low magnification of biopsy specimen from a papule, forming a granulomatous nodule stained with H&E. (e) High magnification of granulomatous nodule

coalesced, we made the differential diagnoses of pityriasis rubra pilaris and miliaria rubra. A biopsy specimen from a papule on his chest showed nodular aggregation of histiocytes and lymphocytes in the upper dermis. There was no central necrobiotic area or histiocyte palisading. Histiocytes had homogeneous cytoplasm and partly exhibited epithelioid granuloma, and multinucleated giant cells were intermingled. Neither asteroid body, acid-fast bacilli positive cells, nor giant cell inclusions were found. Laboratory tests showed elevation of serum glucose (468 mg/dL) and HbA1c (10.6%) with marked glucosuria. We excluded sarcoidosis because of neither elevation of serum angiotensin converting enzyme nor abnormality in chest roentgenography. The eruption was diagnosed as generalized GA. The treatment of diabetes mellitus with insulin improved the skin lesions gradually for the following 2 months. He was further treated with narrowband ultraviolet B (UVB) therapy in combination with oral tranilast (200 mg daily). Two months later, the skin lesions almost disappeared with slight pigmented sequelae. Since the eruption was already in partial remission prior to the application of tranilast and UVB, the treatment of diabetes seemed to be more effective.

This case of generalized GA is characterized by numerous micropapules that were partly coalesced into plaques. Its eruptive property made the diagnosis difficult on the first inspection so that we could not estimate the eruption as even granulomatous disorder. Histologically, individual papules were featured by the nodular aggregation of histiocytes and the lack of palisading and necrobiotic moieties. As seen in our case, histiocytes in generalized GA occasionally adapt a sarcoid or epithelioid-nodular pattern without palisading granuloma, and in such cases necrobiosis is minimal. The patient was successfully treated with insulin and possibly with the subsequent narrowband UVB therapy and oral tranilast. Usually, generalized GA is slowly improved by various treatments, such as topical and oral corticosteroids, hydroxychloroquine, dapsone, cryosurgery, niacinomide, cyclosporine, chlorambucil, retinoids, and phototherapies.[4] However, the patient showed a relatively prompt therapeutic response, further suggesting the eruptive, unfully blown granulomatous nature of the present case.

It is suggested that generalized GA possibly exhibits an extremely eruptive clinical manifestation as a result of severe diabetes mellitus. The accurate diagnosis may lead to successful treatment of the disease.

Ko-ichiro Ine
Kenji Kabashima
Chizuko Koga
Miwa Kobayashi
Yoshiki Tokura
Department of Dermatology, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan

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

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Wolf's isotopic response – Furuncles at the site of healed herpes zoster in an Indian male

A 34-year-old Indian male presented with a 4-d history of multiple furuncles at the site of healed herpes zoster on his left mandibular region. He had been treated 4 weeks previous for herpes zoster of the same region in our department with oral acyclovir tablet 800 mg five times daily for 7 d, along with topical acyclovir cream and diclofenac tablet 200 mg twice daily for 10 d. The lesions healed completely within 2 weeks, leaving hyperpigmentated scars.

Cutaneous examination showed multiple, discretely arranged furuncles varying from 5 to 9 mm in size over the scars of healed herpes zoster over his left mandibular region (Fig. 1), in the distribution of sensory area innervated by the mandibular branch of the trigeminal nerve. Pus culture from the furuncles grew *Staphylococcus aureus* sensitive to cloxacillin and roxithromycin. KOH examination and culture for fungus was negative. The furuncles healed completely with Cloxacillin 500 mg was given four times daily for 8 days. His routine hematological examination and blood sugar were normal and ELISA for HIV 1 and 2 was negative.

Activated regulatory T cells are the major T cell type emigrating from sensitized skin

Michio Tomura[1], Tetsuya Honda[2], Hideaki Tanizaki[2], Atsushi Otsuka[2], Gyohei Egawa[2,3], Yoshiki Tokura[4], Herman Waldmann[5], Shohei Hori[6], Jason G Cyster[7], Takeshi Watanabe[3], Yoshiki Miyachi[2], Osami Kanagawa[1], and Kenji Kabashima[2,3]

[1]Laboratory for Autoimmune Regulation, Research Center for Allergy and Immunology, RIKEN, 1-7-22 Suehiro-cho, Tsurumi, Yokohama City, Kanagawa 230-0045, Japan

[2]Department of Dermatology, and

[3]Center for Innovation in Immunoregulative Technology and Therapeutics, Kyoto University Graduate School of Medicine, Yoshida-Konoe, Kyoto 606-8501, Japan

[4]Department of Dermatology, University of Occupational and Environmental Health, Kitakyushu 807-8555 Japan

[5]Sir William Dunn School of Pathology, South Parks Road, Oxford, OX1 3RE, UK

[6]Research Unit for Immune Homeostasis, Research Center for Allergy and Immunology, RIKEN, 1-7-22 Suehiro-cho, Tsurumi, Yokohama City, Kanagawa 230-0045, Japan

[7]Howard Hughes Medical Institute and Department of Microbiology and Immunology, University of California San Francisco, 513 Parnassus Ave., San Francisco, CA 94143-0414, USA

Authorship note: Michio Tomura and Tetsuya Honda contributed equally to this work.

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Abbreviations:

cytotoxic T lymphocyte associated molecule-4, CTLA-4; contact hypersensitivity, CHS; draining lymph node, DLN; 2,4-dinitrobenzene sulfonic acid, DNBS; 2,4-dinitro-1-fluorobenzene, DNFB; glucocorticoid-induced TNFR family-related gene/protein, GITR; trinitrobenzene sulfonic acid, TNBS; 2,4,6-trinitro-l-chlorobenzene, TNCB

Address correspondence and reprint requests to:

Dr. Kenji Kabashima

Department of Dermatology, and Center for Innovation in Immunoregulative Technology and Therapeutics, Kyoto University, Yoshida-Konoe, Kyoto, 606-8501, Japan

Email: kaba@kuhp.kyoto-u.ac.jp

Phone: +81-75-753-9502; Fax: +81-75-753-9500; Email: kaba@kuhp.kyoto-u.ac.jp

Dr. Michio Tomura

Laboratory for Autoimmune Regulation, Research Center for Allergy and Immunology, RIKEN, 1-7-22 Suehiro-cho, Tsurumi, Yokohama City, Kanagawa 230-0045, Japan

Email: tomura@rcai.riken.jp

Phone: +81-45-503-9699; Fax: +81-45-503-9697

ABSTRACT

Regulatory T cells play an important role protecting the skin from autoimmune attack. However, the extent of regulatory T cell trafficking between the skin and draining lymph nodes (DLNs) is unknown. Using photoconvertible protein Kaede-transgenic mice, we could label T cells in the periphery under physiological conditions and have demonstrated that memory phenotype of $CD4^{+}Foxp3^{-}$ non-regulatory T cells (non-Tregs) and $CD4^{+}Foxp3^{+}$ Tregs migrated from the skin to DLNs in the steady state. During cutaneous immune responses, Tregs constituted major emigrants and inhibited immune responses stronger than LN-resident Tregs. Consistently, a cutaneous immune response was prolonged by depletion of endogenous Tregs *in vivo*. In addition, the circulating Tregs specifically include activated $CD25^{hi}$ Tregs that demonstrated strong inhibitory function. These results suggest that Tregs infiltrating into the periphery traffick to DLNs and recirculate again to the periphery that contribute to down-regulate immune responses.

INTRODUCTION

Lymphocytes travel throughout the body to conduct immune surveillance. $CD4^+$ helper T cells are central organizers in immune responses. Upon stimulation, naïve $CD4^+$ T cells differentiate into effector Th cells (1). $Foxp3^+$ regulatory T cells (Tregs) represent a unique subpopulation of $CD4^+$ T cells that are important for maintenance of immunological homeostasis and self-tolerance (2, 3). Naïve T cells circulate between blood and secondary lymphoid tissues (4-7). However, it is debate as to whether T cells travel through uninflammed peripheral tissues as part of their recirculation route. Peripheral tissues with the active afferent limb of the lymphatic system are for example, the skin, and memory/effector T cells migrate to inflammed skin using CCR4 and CCR10 (8-10). Classic studies employing cannulation of afferent lymph vessels have shown that $CD4^+$ memory/effector cells make up nearly all cells in the afferent lymph of sheep (6, 11-13). On the other hand, Debes et al. have reported that $CD4^+$ cells, especially naïve subsets, migrate from the skin in a CCR7 dependent manner using subcutaneous injection of fluorescent-labeled lymphocytes (14). However, the above experiments require traumatic or artificial procedures to follow or label T cells. Therefore, it is of interest to clarify whether T cells in the peripheral organs such as the skin migrate to draining LNs (DLNs) and to identify the T cell subsets of migration and their roles under physiological conditions.

To directly assess cells migrating from the peripheral tissue, we have devised a new experimental system that involves labeling resident cells using transgenic (Tg) mice expressing the Kaede protein. Kaede is a photoconvertible green fluorescence protein cloned from stony coral (15, 16) that changes its color from green to red when exposed to violet light (16). Therefore, the Kaede-Tg mouse system is an ideal tool for monitoring precise cellular movements *in vivo* at different stages of the immune response (17).

Here, we used the skin as a representative of the peripheral organs, and observed the

movement of cells from the skin using Kaede-Tg mice (17). A high proportion of the migrating cells into the DLNs were Tregs that had a stronger capacity to suppress acquired immune responses than LN-resident Tregs. Moreover, these migrating T cells recirculated into the skin upon elicitation to terminate immune responses.

RESULTS

Detection of cell migration from the skin in the steady state using Kaede-Tg mice. To monitor cell migration from the skin *in vivo*, the abdominal skin of Kaede-Tg mice were photoconverted by exposure to violet light for 10 min (see **Methods**). Before photoconversion, all the cells in the skin of Kaede-Tg mice expressed only Kaede-green fluorescence (Kaede-green) (Figures 1A and 1B). Immediately after violet light exposure to the skin, the whole skin tissue (Supplemental Figure S1) and the skin cells of the photoconverted area showed red signal (Kaede-red), whereas virtually no draining axillary LN cells (Figures 1A and 1B, Supplemenal Figure S2) or blood cells (Supplemental Figure S2) were photoconverted. Although we found that Kaede-red proteins could be detected in the extracellular fluids when incubated for 24 h after photoconversion of the LN cells (Supplemental Figure S3), we confirmed that the extracellular photoconverted Kaede proteins could not be transferred into T cells *in vitro* (Supplemental Figure S4).

To evaluate cell migration from the skin in the steady state, the clipped abdominal skin of Kaede-Tg mice was exposed to violet light as in Figure 1A, and 24 h later, the draining axillary and non-draining cervical and popliteal LN cells were subjected to flow cytometry. We found that 0.36% of the DLN cells showed the Kaede-red phenotype (Figure 1C, upper left), suggesting a fraction of cells in the skin migrate to the DLNs. It is generally thought that dendritic cells are the major migrants from the skin in the steady state, and in fact 6.2% of $CD11c^+$ dendritic cells were of the Kaede-red phenotype in the DLNs (Figure 1C, upper middle). In contrast, almost no

Kaede-red $CD11c^+$ dendritic cells were detected in the non-DLNs (Figure 1C, lower middle). We next evaluated $CD4^+$ T cell migration from the skin, and found that 0.49% of $CD3^+CD4^+$ T cells in the DLNs had the Kaede-red phenotype (Figure 1C, upper right). Although the frequencies of the Kaede-red positivity among dendritic cells and $CD3^+CD4^+$ T cells differed, the absolute numbers of Kaede-red dendritic cells and $CD4^+$ T cells were comparable ($CD4^+$ T cells vs. $CD11c^+$ dendritic cells: 11621 $\pm$ 2716 cells per LN vs. 9063 $\pm$ 2333 cells per LN, n=5 each, average $\pm$ SD). Moreover, the ratio of Kaede-red cells was higher in $CD44^{high}$ memory T cells than in $CD44^{mid}$ naïve T cells (Figure 1D). Consistently, the majority of Kaede-red migratory cells were of $CD44^{high}$ memory phenotype (Figure 1D). These results suggest that predominantly T cells with the memory surface phenotype migrate from the skin into DLNs even in the steady state.

Migration of Tregs from the skin to the DLNs.

Immune responses and homeostasis are regulated by the functions of memory/effect T cells and Tregs. To determine the behaviors of these populations, we intercrossed Kaede-Tg mice with Foxp3 reporter mice expressing human CD2 and human CD52 chimeric protein, which is designated as Kaede/$Foxp3^{hCD2/hCD52}$ mice. Since $Foxp3^+$ cells co-express hCD2 on the cell surface, live $Foxp3^+$ Tregs could be labeled and sorted with anti-hCD2 monoclonal Ab. The DLN cells from Kaede/$Foxp3^{hCD2/hCD52}$ mice in the steady state were analyzed by flow cytometry. A majority of $CD25^+$ cells were hCD2 positive, but a substantial number of $hCD2^+$ cells were detected even in $CD25^-$ cells (18) (Figure 2A), which is consistent with the previous finding by the other group (19). Therefore, the following studies were performed using Kaede/$Foxp3^{hCD2/hCD52}$ mice and $hCD2^+$ cells were considered to be Tregs.

To evaluate T cell migration from the skin in the steady state, the clipped abdominal skin of Kaede/$Foxp3^{hCD2/hCD52}$ mice was exposed to violet light as in Figure 1A, and 24

h later, the draining axillary LN cells were subjected to flow cytometry. Consistent with the previous results (Figure 1D), a substantial percentage (0.83%) of photoconverted $CD4^+$ T cells were observed in the DLNs (Figure 2B). Among $hCD2^-$ non-Tregs and $hCD2^+$ Tregs, the frequency of Kaede-red cells were comparable (0.79% vs. 0.98%) (Figure 2C), and the frequency of Kaede-red cells was higher in the $CD44^{high}$ memory subset than in the $CD44^{mid}$ naïve subset (Figure 2C). In addition, Kaede-Red $CD4^+$ cells included a higher percentage of Tregs (22.7%) than total $CD4^+$ cells (14.1%) (Figure 2D). In, total $CD4^+$ populations, the number of $CD44^{high}$ memory cells was lower than that of $CD44^{mid}$ naïve cells in both non-Tregs and Tregs (Figure 2E left). In contrast, consistent with Figures 2C and 2D, $CD44^{high}$ memory cells were the major Kaede-red migrants from the skin among non-Tregs and Tregs (Figure 2E right).

Treg migration from the skin during a cutaneous immune reaction.

We tracked the extent of $CD4^+$ T cell migration from the skin during an immune response and sought to evaluate the role of $CD4^+$ T cells migrating from the skin. The dorsal skin of Kaede/$Foxp3^{hCD2/hCD52}$ mice was sensitized with DNFB, and 5 days later, the abdominal skin was challenged with DNFB. Two days after challenge, the abdominal skin was exposed to violet light for photoconversion, and another 24 h later, the draining axillary LN cells were analyzed by flow cytometry (Figure 3A). The frequency of Kaede-red cells among $CD4^+$ T cells in the DLNs was increased up to 3% (Figure 3B) from that in the steady state (0.83%, Figure 2B). In addition, although 21% of total $CD4^+$ cells were Tregs, the number of $hCD2^+$Tregs became comparable to that of non-Tregs in Kaede-Red phenotype (49%; Figures 3C and 3D right). Again, the $CD44^{high}$ memory cells were major migrants from the challenged skin as similar to the steady state (Figures 3D right and 2E right). The number of total $CD4^+$ T cells in DLN increased by 3-fold during CHS compared to that in the steady state. However, the

number of Kaede-Red migratory non-Tregs and Tregs during CHS increased more drastically, by about 10- and 20-fold, respectively (Figures 2E and 3D).

Consistent with increase of $CD4^+$ T cells migrating from the challenged skin into DLN, the numbers of both $CD4^+$ Tregs and $CD4^+$ non-Tregs were elevated when mice were sensitized and challenged compared to the steady state and the ratio of Tregs to $CD4^+$ T cells during the immune response became higher than that in the steady state (Figure 3E). These results suggest that Tregs are more accumulated than non-Tregs in the skin during the cutaneous immune response.

It is known that cutaneous dendritic cells migrate into the DLNs in a CCR7-dependent manner (20) and that in human most circulating Tregs express skin homing receptors and CCR7 (21). To address whether skin T cells have the potential to migrate into the regional LNs, skin cell suspensions were obtained from the ears of mice sensitized on the abdomen and challenged on the ear with DNFB, and applied to a transwell assay. The Tregs showed good chemotactic responses to CCL21 comparably to MHC class II^+ cutaneous dendritic cells (Figure 3F). Similar chemotactic activity to CCL21 was seen in $CD4^+$ non-Tregs (data not shown). Since the ratio of Tregs and non-Tregs in Kaede-red $CD4^+$ T cells in LNs was comparable to that in the skin at time of photoconversion, Tregs and non-Tregs in the skin seem to have equivalent propensity to migrate to the DLN. In addition, we evaluated the CCR7 expression of Tregs in the skin before and after challenge, and found that Tregs in the skin expressed CCR7 both before and after challenge, and that the expression level of CCR7 of Tregs after challenge was slightly lower than that before challenge (Supplemental Figure S5).

Role of Tregs in the elicitation phase of contact hypersensitivity.

As above, Tregs accumulate in the skin and they have the capacity to migrate to DLNs during the CHS response. These results prompt us to evaluate the role of Tregs in the

cutaneous immune response. We used CHS as a model in combination with Campath-1G Ab, a depleting Ab for the human CD52 antigen (22), which decreased Tregs in the DLNs and the skin incompletely, but mostly, 1-3 days after injection (Figure 4A, and data not shown). Kaede/Foxp3$^{hCD2/hCD52}$ mice were sensitized with DNFB on the abdomen, and treated in the presence or absence of Campath-1G Ab. The ear thickness changes after the challenge on the ears were significantly prolonged by the treatment with Campath-1G Ab at each time point compared to in control mice (Figure 4B). This enhancement of CHS response by Campath-1G Ab was not observed when B6 wild-type mice were used, which excluded the possibility of the non-specific effect of Campath-1G Ab (Supplemental Figure S6). In addition, the ear thickness changes of mice treated with control rat IgG were comparable to those treated without Campath-1G Ab (data not shown). These results demonstrate that Tregs play an important role in the challenge phase in terminating the CHS response.

Suppressive activity of Kaede-red and Kaede-green Tregs on T cell proliferation.

To further determine the suppressive function of the Tregs migrating from the skin during the cutaneous immune response, Kaede-red and Kaede-green CD4$^+$ Tregs in the skin DLN were prepared as in Figure 3A, and co-cultured with regional LN cells from DNFB-sensitized mice. Antigen-specific T cell proliferation induced by DNBS, a water-soluble compound with the same antigenicity as DNFB, was significantly inhibited by addition of 6 x 10^3 Kaede-red Tregs (Figure 4C). On the other hand, eight times the number of Kaede-green Tregs was required to achieve a similar magnitude of inhibitory effect of the Kaede-red Tregs (Figure 4C). These data indicate that the skin-derived Tregs have a stronger inhibitory effect on hapten-specific T cell proliferation than LN-resident Tregs. It should be noted that we might underestimate the inhibitory capacity of skin-migratory T cells relative to resident Tregs, since Kaede-green cell should have included the cells migrated from the skin before

photoconversion and the cells that infiltrated to the skin after photoconversion and migrated to DLN.

We tested the effect of the Tregs on antigen non-specific T cell proliferation stimulated with membrane-bound anti-CD3 Ab. Kaede-red Tregs inhibited T cell proliferation more potently than did Kaede-green Tregs, and again a higher number of Tregs were required (Figure 4D) to obtain a similar extent of inhibition seen in Figure 4C.

To further evaluate the antigen-specificity of Tregs in T cell proliferation, we isolated the DLN cells 5 days after DNFB or TNCB sensitization, and re-stimulated them with DNBS or TNBS respectively, and added Kaede-red Tregs or Kaede-green Tregs prepared from the DLNs as in Figure 3A. Kaede-red Tregs inhibited DNBS-induced T cell proliferation more than Kaede-green Tregs (Figure 4E) as shown in Figure 4C. However, this anti-proliferative effect was not seen when these Kaede-red or Kaede-green Tregs were added to TNBS-stimulated LN cells from the mice sensitized with TNCB (Figure 4E). In addition, in the criss-cross comparison, similar antigen-specificity was observed on TNCB-immunized Kaede-red Tregs (data not shown). We also analyzed mRNA expressions of inhibitory cytokines and surface molecules by quantitative RT-PCR. Kaede-red Tregs expressed higher mRNAs levels of *Il10*, and transforming growth factor-β (*Tgfβ1*) than Kaede-green Tregs (2, 3, 23) (Figure 4F). On the other hand, although there was no significant difference, Kaede-red Tregs tended to express higher mRNA level of cytotoxic T lymphocyte associated molecule-4 (*Ctla4*) than did Kaede-green Tregs (2, 3, 23) (Figure 4F). These results suggest that Tregs migrating from the skin have a more efficient suppressive potency on T cell proliferation with abundant inhibitory mediators, and that this anti-proliferative effect shows some antigen-specificity.

Tregs recirculating from the skin inhibit local cutaneous immune response *in situ.*

The strong ability of Kaede-red Tregs to suppress *in vitro* T cell proliferation prompted us to determine whether Kaede-red Tregs can inhibit a local cutaneous immune response *in situ*. Kaede-red or Kaede-green Tregs prepared as described (Figure 3A) were injected subcutaneously into the ears of mice sensitized with DNFB 5 days before, and the ears were elicited with DNFB. The DNFB-induced ear thickness change was suppressed by the injection of Kaede-red and Kaede-green Tregs at all time points (Figure 5A). It was noted, however, that Kaede-red Tregs suppressed CHS more than Kaede-green Tregs at 72 and 96 h after challenge (Figure 5A).

Considering that Tregs function as a regulator for primed T cells, they should serve as suppressors at the challenged site. The above late-phase inhibitory action of Kaede-red Tregs raised the possibility that Tregs migrating from the skin can return to the skin and exert suppressive activity. Kaede/Foxp3$^{hCD2/hCD52}$ mice were sensitized, challenged and photoconverted as in Figure 3A. Twenty-four hours after photoconversion, the left and right ears were re-challenged with DNFB, and vehicle (Figure 5B) or TNCB (Figure 5C), respectively. Another 24 h later, Kaede-red Tregs were observed in the ears challenged with DNFB, but not in those challenged with vehicle (Figure 5B). The ear re-challenged with different hapten, TNCB, contained Kaede-red Tregs, but its number was lower than that of the ear re-challenged with DNFB (Figure 5C). In addition, Kaede-red Tregs were detected in $CD4^+$ cells of the blood 24 h after re-challenge (1.79 $\pm$ 0.07%, average $\pm$ SEM, n=3) (Figure 5D). Moreover, previous report has suggested that LN cells migrate to the skin (24). We conducted an evaluation of this report by photoconverting DLNs. We sensitized the dorsal skin of mice with DNFB, and challenged on the abdominal skin with DNFB 4 days later. Two days after challenge, the draining lymph nodes of the mice were photoconverted and re-challenged on the ears with DNFB. Twenty-four hours later, the ears of the skin were analyzed by flow cytometric analysis. We found that a substantial fraction of $CD4^+$ $hCD2^-$ non-Tregs and $CD4^+$ $hCD2^+$ Tregs were Kaede-red positive

(Supplemental Figure S7). These results suggest that the Tregs that egressed from the skin had a capacity to re-migrate to the skin upon challenge.

It has been reported that the representative chemokine receptors essential for migration of lymphocytes into the skin and LNs are CCR4 and CCR7, respectively (9, 14, 25). In addition, CCR5 may be an important chemokine receptor for Tregs to migrate into the skin (26). Kaede-red Tregs expressed higher levels of CCR4 and CCR5 and a lower level of CCR7 than Kaede-green Tregs (Figure 6A). When the skin DLN cells prepared as in Figure 3A were applied to a transwell assay, Kaede-red Tregs showed good chemotactic responses to both CCL17, a ligand for CCR4, and CCL21, a ligand for CCR7, but the chemotaxis of Kaede-red Tregs to CCL21 was weaker than that of Kaede-green Tregs (Figure 6B).

We further analyzed the surface molecules of Kaede-red Tregs in the DLNs of Kaede/Foxp3$^{hCD2/hCD52}$ mice treated as in Figure 3A. Kaede-red Tregs expressed a lower level of CD62L, but higher levels of CD44 and CD69 than Kaede-green Tregs (Figure 6C), suggesting that the skin-derived Tregs show a more memory-related T cell phenotype. Interestingly, Kaede-red Tregs contained a CD25^{high} fraction, which was barely perceptible in Kaede-green Tregs. In addition, Kaede-red Tregs expressed higher levels of CD103, an integrin important for T cell migration into the skin, as well as CD11a and CD54, integrins induced upon activation, and a GITR, another marker of Tregs (27, 28) (2). However, the expression level of CD45RB was comparable between the Kaede-red and Kaede-green Tregs. These results suggest that Kaede-red Tregs are of the memory/effector phenotype (29), and have a higher potential to migrate to the skin than LN-resident Tregs.

Kinetics and surface phenotype of CD25^{high} Kaede-red Tregs.

The above data (Figure 5A) suggest that Tregs migrating from the skin have a highly potent immunosuppressive capacity even *in situ*. One of the features of these

skin-derived Tregs is the presence of a $CD25^{high}$ subset (Figure 6C) that has not been thoroughly described before. Initially we sought to characterize the localization of $CD25^{high}$ Tregs, and found that $CD25^{high}$ cells were substantially detected in Kaede-red Tregs of the DLNs of mice pretreated as in Figure 3A, but were only somewhat or marginally detected in Kaede-green Tregs of the DLNs or in non-DLNs (Figure 7A). Consistently, the frequency of the Kaede-red population in the $CD25^{high}$ population was greater than that in the $CD25^{mid}$ population (Figure 7B). These $CD25^{high}$ Tregs showed higher levels of CCR4, CCR5, CCR7, CD44, CD103, CD11a, and CD54 than $CD25^{int}$ Tregs in the Kaede-red subset (Figure 7C). On the other hand, the expression levels of CCR5 and CD103 of the $CD25^{high}$ subset in the Kaede-green cells tended to be lower than that in the Kaede-red cells, and the expression of CCR7 in Kaede-green Tregs was similar between $CD25^{int}$ and $CD25^{high}$ subset (Figure 7C).

We then examined the kinetics of T cell migration from the skin. Kaede/$Foxp3^{hCD2/hCD52}$ mice were sensitized and challenged as in Figure 3A, and photoconverted immediately, 1, 2, or 3 days after challenge. The DLN cells were collected 24 h after each photoconversion, and the number of Kaede-red $CD4^+$, $CD4^+hCD2^+$, $CD4^+hCD2^+CD25^{high}$, and $CD4^+hCD2^+CD25^{mid}$ cells migrating for 24 h after photoconversion was determined (Figure 7D, left). The peak response of cell migration from the skin occurred on day 2 (between 48 and 72 h after challenge) when the frequency of Tregs among $CD4^+$ T cells migrating from the skin was high (Figure 7D, left). In addition, $CD4^+hCD2^+CD25^{high}$ cells were detected only at this time point (Figure 7D, left) and showed a high frequency of Kaede-red positivity especially on day 2 (Figure 7D, right), suggesting that this subset is replaced by the skin-derived cells more readily than other subsets.

Strong immunosuppressive activity of $CD25^{high}$ Kaede-red migratory Tregs.

To evaluate whether $CD25^{high}$ Tregs are localized in the skin during immune responses,

Kaede/Foxp3$^{hCD2/hCD52}$ mice were sensitized and challenged as in Figure 3A. We detected a significant number of CD25^{high} Tregs in the challenged local skin, but few in the non-challenged skin 48 h after the challenge (Figure 7E), suggesting that CD4^{+}hCD2^{+}CD25^{high} cells are induced in the skin and migrate into the DLNs.

To determine the role of skin-derived CD25^{high} Tregs, Treg subsets were isolated from the DLNs of mice pretreated as in Figure 3A and co-cultured with DLN cells from DNFB-sensitized mice. The CD25^{high} Tregs showed much stronger suppressive activity on T cell proliferation than the CD25^{int} subset (Figure 7F).

We further examined the mRNA expression profiles of cytokines in the CD25^{high} Treg subsets. In agreement with the above *in vitro* result, Kaede-red CD25^{high} Tregs contained significantly higher amounts of *Il10*, *Tgfb1*, and *Ctla4* than Kaede-red CD25^{int} Tregs in the DLNs, Kaede-green CD25^{high} or CD25^{int} Tregs in the DLNs, or Kaede-green CD25^{int} Tregs in the non-DLNs, except in the case of *Tgfb1* expression level between Kaede-red CD25^{high} Tregs and Kaede-green CD25^{high} Tregs in DLNs (Figure 7G). These results suggest that CD25^{high} Tregs migrating from the skin play a major suppressive role in cutaneous immune response.

DISCUSSION

In this study, we found that memory/effector phenotype Foxp3^{+} Tregs, as well as Foxp3^{-} non-Tregs, migrated from the skin to DLNs in the steady state. The number of CD4^{+} T cells in the skin and their migration to DLNs were prominently increased during a cutaneous immune response. Among the migrating T cells, Foxp3^{+} Tregs constituted one of the major populations. Notably, the Tregs that migrated from the skin returned to the skin upon exposure to an antigen. The migrating Tregs held strong immunosuppressive effect and expressed high levels of mRNA for inhibitory mediators compared to LN-resident Tregs. Moreover, depletion of endogenous Tregs *in vivo* prolonged the CHS response. Finally, these circulating Tregs specifically

included the $CD25^{high}$ subset that showed an activated phenotype and a very strong inhibitory function on T cell proliferation with high levels of mRNA for inhibitory mediators. These data suggest that Tregs circulate between blood, skin and lymphoid tissues to regulate peripheral immune responses.

There have been a few studies that sought to address the possibility of T cell migration from the periphery to LNs. In their experiments, one report suggested that the memory/effector subset of $CD4^+$ T cells is the major constituents in the afferent lymph by cannulation of sheep (6, 11-13), and the other suggested the naïve subset is dominant using subcutaneous injection of fluorescent-labeled lymphocytes (14). Recently effector/memory phenotype of Tregs have been reported to migrate from blood to islet and to DLNs sequentially using an islet allograft model with transfer of *in vitro* induced Tregs (30). However, since all the above experiments require traumatic or artificial procedures to label T cells in the periphery, it remains unknown whether endogenous T cells egress from the periphery into DLNs under pathophysiological conditions. In this study, using Kaede-Tg system, we have clearly demonstrated that a subset of T cells with memory/effector phenotype migrates to DLNs in the steady state and during a cutaneous immune response. During the immune response Tregs are the major constituents and they return to the skin upon exposure to an antigen. Therefore, as naïve T cells circulate between blood and LNs, memory/effector phenotype of T cells, especially Tregs, seem to circulate between blood and the skin. In this study, we used the skin as a representative of the peripheral tissues, but it would be of interest to explore this issue in other peripheral tissues, such as lungs and intestines.

To date the roles of externally transferred Tregs in CHS have been reported (31), however, the regulatory activity of endogenous Tregs has not been fully assessed. In this study, we found that depletion of Tregs during the elicitation phase prolonged the CHS response. In addition, CHS-induced migratory Tregs suppressed the proliferation