



Activated regulatory T cells are the major T cell type emigrating from the skin during a cutaneous immune response in mice

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Tregs play an important role in protecting the skin from autoimmune attack. However, the extent of Treg trafficking between the skin and draining lymph nodes (DLNs) is unknown. We set out to investigate this using mice engineered to express the photoconvertible fluorescence protein Kaede, which changes from green to red when exposed to violet light. By exposing the skin of Kaede-transgenic mice to violet light, we were able to label T cells in the periphery under physiological conditions with Kaede-red and demonstrated that both memory phenotype CD4⁺Foxp3⁻ non-Tregs and CD4⁺Foxp3⁺ Tregs migrated from the skin to DLNs in the steady state. During cutaneous immune responses, Tregs constituted the major emigrants and inhibited immune responses more robustly than did LN-resident Tregs. We consistently observed that cutaneous immune responses were prolonged by depletion of endogenous Tregs *in vivo*. In addition, the circulating Tregs specifically included activated CD25^{hi} Tregs that demonstrated a strong inhibitory function. Together, our results suggest that Tregs in circulation infiltrate the periphery, traffic to DLNs, and then recirculate back to the skin, contributing to the downregulation of cutaneous immune responses.

Introduction

Lymphocytes travel throughout the body to conduct immune surveillance. CD4⁺ helper T cells are central organizers in immune responses. Upon stimulation, naive CD4⁺ T cells differentiate into effector Th cells (1). Foxp3⁺ Tregs represent a unique subpopulation of CD4⁺ T cells that are important for maintenance of immunological homeostasis and self tolerance (2, 3). Naive T cells circulate between blood and secondary lymphoid tissues (4–7). However, it is debatable whether T cells travel through uninflamed peripheral tissues as part of their recirculation route. One type of peripheral tissue with the active afferent limb of the lymphatic system is, for example, the skin, and memory/effector T cells migrate to inflamed 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 naive 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 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 was photoconverted by exposure to violet light for 10 minutes (see Methods). Before photoconversion, all the cells in the skin of Kaede-Tg mice expressed only Kaede-green fluorescence (Kaede-green) (Figure 1, A and B). Immediately after violet light exposure to the skin, the whole skin tissue (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI40926DS1) and the skin cells of the photoconverted area showed red signal (Kaede-red), whereas virtually no draining axillary LN cells (Figure 1, A and B, and Supplemental Figure 2) or blood cells (Supplemental Figure 2)

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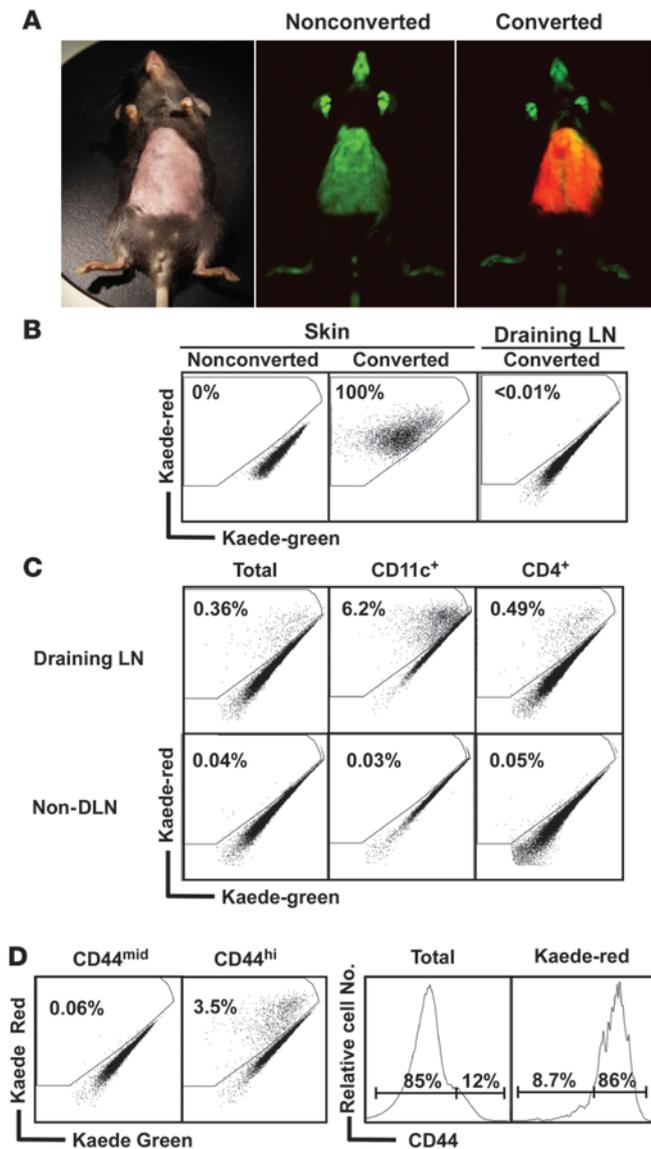


Figure 1

Cell migration from the skin to the DLN in the steady state. (A) Kaede-Tg mice were photoconverted on the clipped abdominal skin as described in Methods and observed with a fluorescence stereoscopic microscope. Nonphotoconverted clipped skin is shown as a control (middle). Note: nonclipped area remains black since light cannot reach. (B) Skin and draining axillary LN cells resected immediately after violet light exposure of the abdominal skin and resected skin cells not exposed to violet light were subjected to flow cytometric analysis to evaluate the photoconversion. (C and D) Twenty-four hours after photoconversion of the abdominal skin, cells from the draining axillary and other non-draining cervical and popliteal peripheral LNs were stained with CD11c and CD4 mAbs (C) and CD4 and CD44 mAbs (D) and subjected to flow cytometry. These data are representative of at least 5 experiments. Numbers within plots or histograms (B–D) indicate percentage of cells in the respective areas.

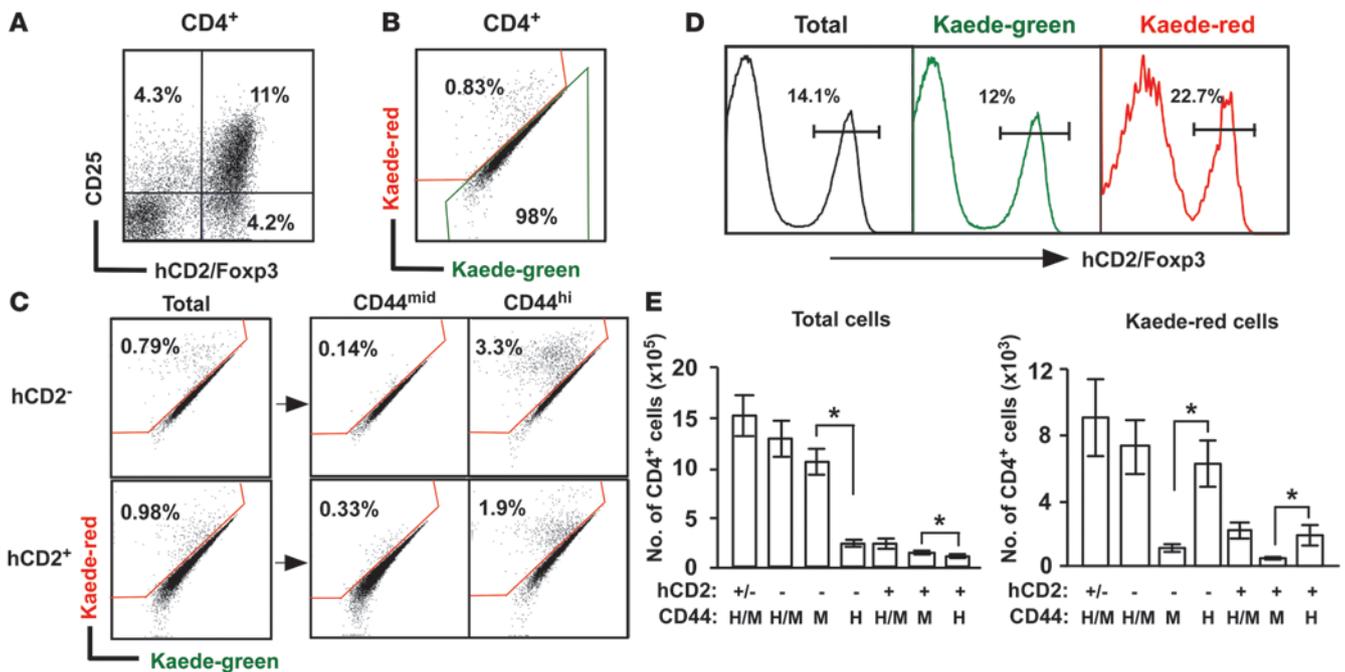
DLNs had the Kaede-red phenotype (Figure 1C). Although the frequencies of the Kaede-red positivity among dendritic cells and CD3⁺CD4⁺ T cells differed, the absolute numbers of Kaede-red dendritic cells and CD4⁺ T cells were comparable (CD4⁺ T cells vs. CD11c⁺ dendritic cells: 11621 ± 2716 cells per LN vs. 9063 ± 2333 cells per LN, n = 5 each, average ± SD). Moreover, the ratio of Kaede-red cells was higher in CD44^{hi} memory T cells than in CD44^{mid} naive T cells (Figure 1D). Consistently, the majority of Kaede-red migratory cells were of the CD44^{hi} 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/effector 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 are designated as Kaede/Foxp3^{hCD2/hCD52} mice. Since Foxp3⁺ cells coexpress 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 findings 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 hours 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 was comparable (0.79% vs. 0.98%) (Figure 2C), and the frequency of Kaede-red cells was higher in the CD44^{hi} memory subset than in the CD44^{mid} naive 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^{hi} memory cells was lower than that of CD44^{mid} naive cells in both non-Tregs and Tregs (Figure 2E). In contrast, consistent with Figures 2C and 2D, CD44^{hi} memory cells were the major Kaede-red migrants from the skin among non-Tregs and Tregs (Figure 2E).

were photoconverted. Although we found that Kaede-red proteins could be detected in the extracellular fluids when incubated for 24 hours after photoconversion of the LN cells (Supplemental Figure 3), we confirmed that the extracellular photoconverted Kaede proteins could not be transferred into T cells in vitro (Supplemental Figure 4).

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 hours 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), 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). In contrast, almost no Kaede-red CD11c⁺ dendritic cells were detected in the non-DLNs (Figure 1C). We next evaluated CD4⁺ T cell migration from the skin and found that 0.49% of CD3⁺CD4⁺ T cells in the

**Figure 2**

Migration of Tregs from the skin to DLNs. (A–E) The DLN cells of Kaede/Foxp3^{hCD2/hCD52} mice photoconverted on the abdomen 24 hours prior were stained with CD4, CD25, and hCD2 mAbs. Shown here are the flow cytometric plots for hCD2/Foxp3 and CD25 staining among CD4⁺ cells (A) and Kaede-red and Kaede-green expression on hCD2⁺CD4⁺ cells among skin DLN cells (B). (C) The DLNs and non-DLNs from the mice 24 hours after photoconversion were stained with CD4, hCD2, and CD44 mAbs and subjected to flow cytometry. (D) hCD2/Foxp3 expression in total (Kaede-red plus Kaede-green), Kaede-green, and Kaede-red CD4⁺ cells was compared by flow cytometry. (E) The numbers of CD44^{mid} naive (M), CD44^{hi} memory (H), and naive plus memory (H/M) phenotypes of hCD2⁺CD4⁺ non-Tregs (–), hCD2⁺CD4⁺ Tregs (+), and total (hCD2⁺ and hCD2[–]; +/-) CD4⁺ T cells in total CD4⁺ (Kaede-red plus Kaede-green) cells and Kaede-red cells in the DLNs were counted. Data are presented as means ± SD and are representative of 3 independent experiments. Student's *t* test was performed between the indicated groups. **P* < 0.05. Numbers within plots or histograms indicate percentage of cells in the respective areas (A–D).

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 2,4-dinitro-1-fluorobenzene (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 hours 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%; Figure 3, C and D). Again, the CD44^{hi} memory cells were major migrants from the challenged skin similarly to the steady state (Figure 3D and Figure 2E). The number of total CD4⁺ T cells in DLN increased by 3-fold during contact hypersensitivity (CHS) compared with 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 (Figure 2E and Figure 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 with 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 more Tregs than non-Tregs accumulate 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 humans, 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 comparable to that of 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 the 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 5).

Role of Tregs in the elicitation phase of CHS. As shown above, Tregs accumulate in the skin and they have the capacity to migrate to DLNs during the CHS response. These results prompted us to

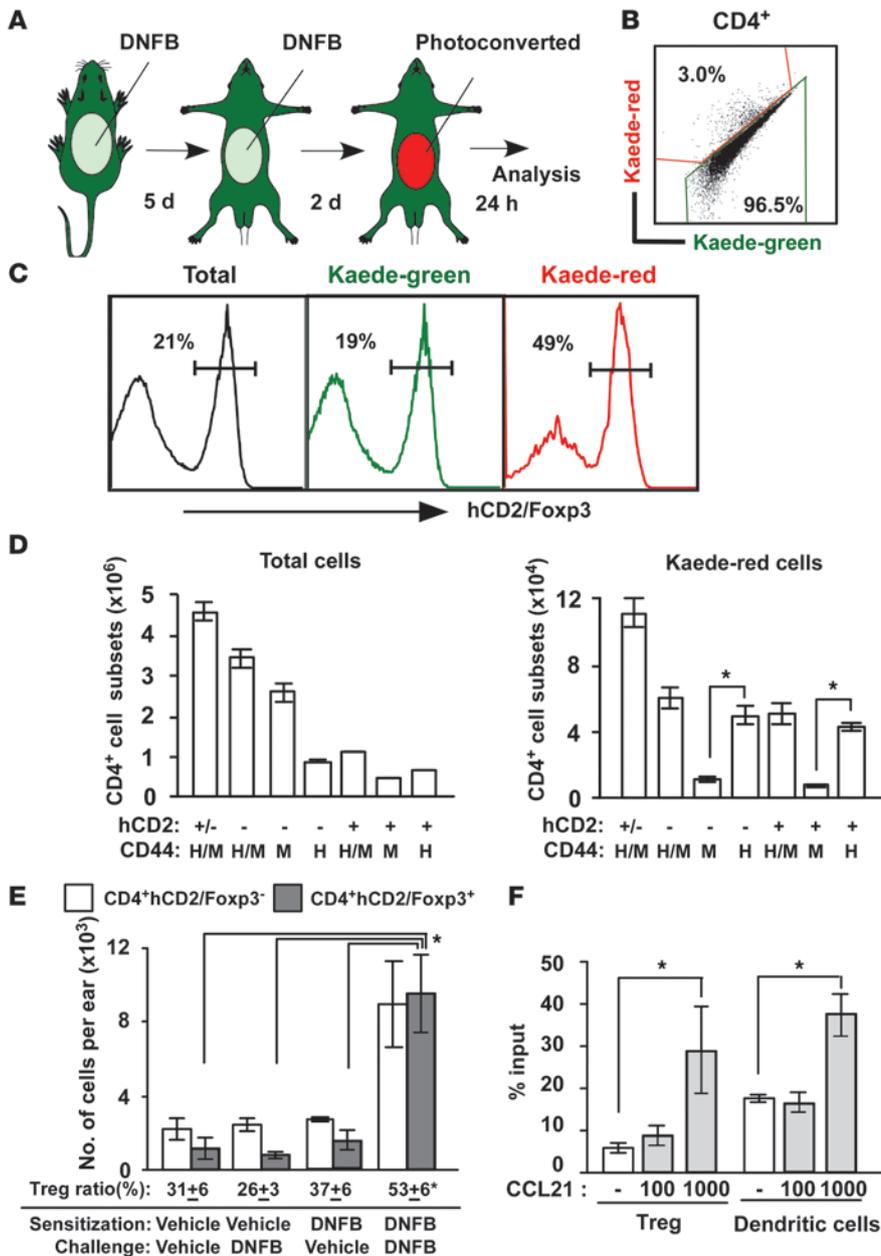


Figure 3

Cell migration from the skin to DLN during a cutaneous immune response. (A) Scheme of the experimental protocol is as follows: the dorsal skin of Kaede/Foxp3^{hCD2/hCD52} was sensitized, and 5 days thereafter the abdominal skin was challenged. 2 days after challenge, the painted areas were photoconverted, and 24 hours after photoconversion, cells from the skin DLNs were analyzed by flow cytometry. (B and C) The frequency of Kaede-red and Kaede-green cells among CD4⁺ cells, and the frequencies of hCD2/Foxp3⁺ cells in total, Kaede-green, and Kaede-red cells among CD4⁺ cells were analyzed. Numbers within plots or histograms indicate percentage of cells in the respective areas. (D) The numbers of CD44^{mid} naive (M), CD44^{hi} memory (H), and naive plus memory (H/M) phenotypes of hCD2-CD4⁺ non-Tregs (-), hCD2⁺CD4⁺ Tregs (+), and total (hCD2- and hCD2⁺; +/-) CD4⁺ T cells among total CD4⁺ cells and Kaede-red cells in the DLNs were counted. (E) Number of Tregs and non-Tregs in the skin. The mice were painted with DNFB or vehicle on the abdomen, followed by DNFB or vehicle application on the ears. The number of CD4⁺ Tregs and CD4⁺ non-Tregs and the percentage ratio of Tregs among CD4⁺ T cells in the ears were measured. (F) Transwell assay. The number of hCD2⁺CD4⁺ cells and CD11c⁺ cells of skin-cell suspensions from Foxp3^{hCD2/hCD52} mice that migrated to the lower chamber was analyzed. Data are presented as means ± SD (D–F) and are representative of 3 independent experiments. Student’s *t* test was performed between the indicated groups. **P* < 0.05 (D–F).

evaluate the role of Tregs in the cutaneous immune response. In a murine CHS model, we found that administration of Campath-1G Ab (a depleting Ab for the human CD52 antigen; ref. 22) resulted in a marked decrease in the number of Tregs in the DLNs and the skin, 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 with in control mice (Figure 4B). This enhancement of CHS response by Campath-1G Ab was not observed when C57BL/6 (B6) wild-type mice were used, which excluded the possibility of the nonspecific effect of Campath-1G Ab (Supplemental Figure 6). 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 cocultured with regional LN cells from DNFB-sensitized mice. Antigen-specific T cell proliferation induced by 2,4-dinitrobenzene sulfonic acid (DNBS), a water-soluble compound with the same antigenicity as DNFB, was significantly inhibited by addition of 6 × 10³ Kaede-red Tregs (Figure 4C). On the other hand, 8 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-

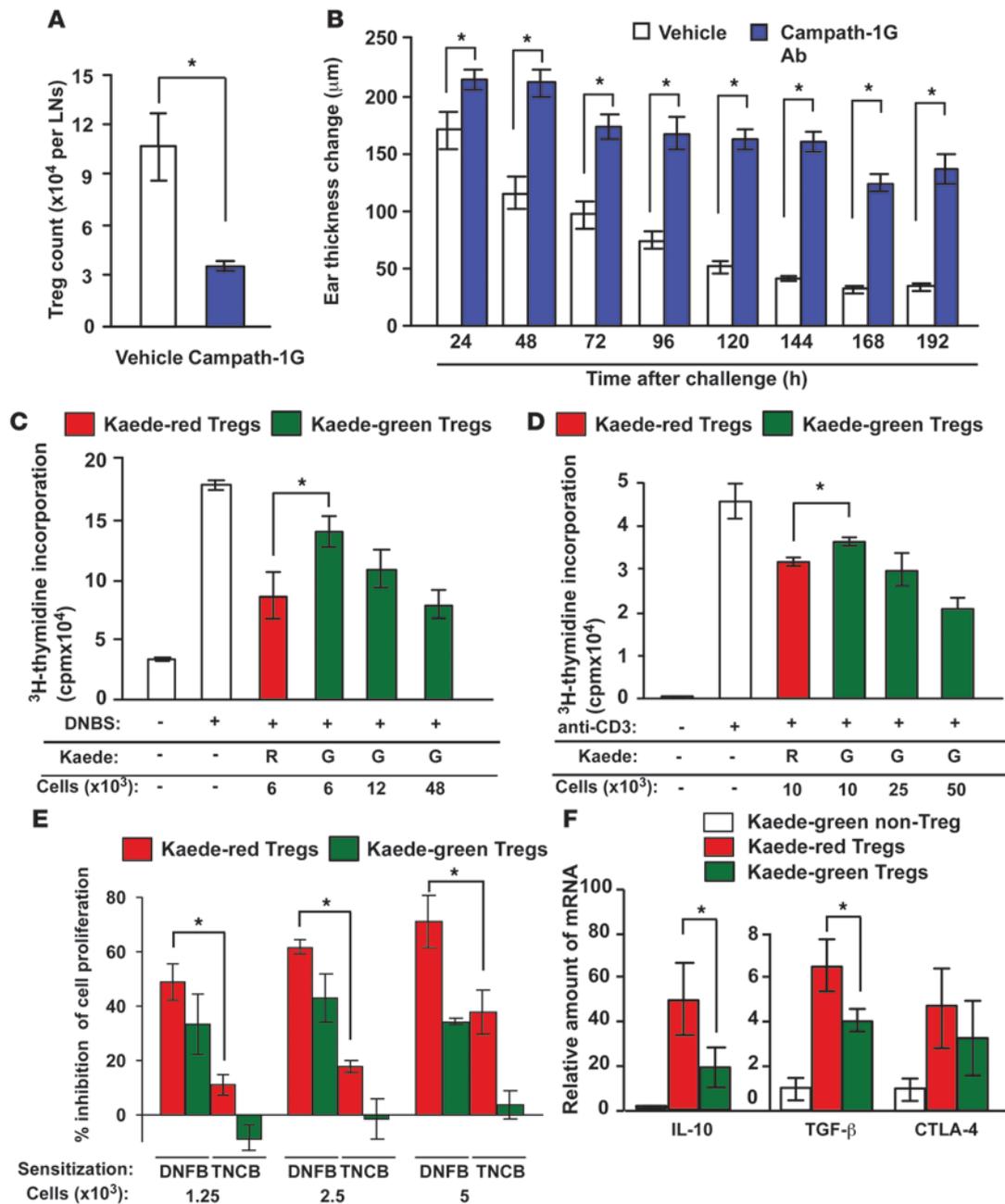


Figure 4

Enhanced ear swelling response by Treg depletion and immunosuppressive activity of Treg subsets on T cell proliferation in vitro. (A) The number of Tregs in the LNs after administration of Campath-1G Ab. (B) CHS: the Kaede/Foxp3^{hCD2/hCD52} mice were sensitized, and injected with vehicle or Campath-1G Ab before challenge ($n = 8$ for each group). (C–F) Immunosuppressive activity of Tregs. Kaede-red and Kaede-green Tregs were sorted from the Kaede/Foxp3^{hCD2/hCD52} mice, sensitized, challenged, and photoconverted. (C) Skin DLN cells of mice sensitized with DNFB were stimulated with DNBS in the presence or absence of Kaede-red Tregs or Kaede-green Tregs in vitro ($n = 3$). (D) Suppressive effect of Tregs in vitro. Kaede-red and Kaede-green Tregs were prepared as above and added to T cells stimulated with plate-bound anti-CD3 Ab. (E) Antigen specificity of Treg functions. LN cells from DNFB-sensitized or TNCB-sensitized mice were stimulated with DNBS or TNBS in vitro. Kaede-red and Kaede-green Tregs were added, and percentage inhibition of cell proliferation was evaluated as follows: (cell proliferation with DNBS or TNBS) – (cell proliferation with DNBS or TNBS in the presence of Tregs)/(cell proliferation with DNBS or TNBS) – (cell proliferation with vehicle) $\times 100$. (F) Quantitative RT-PCR analysis on mRNA for *Il10* (IL-10), *Tgfb1* (TGF- β), and *Ctla4* (CTLA-4) of Kaede-red Tregs and Kaede-green Tregs. The expression of each gene was normalized by the expression of *Gapdh*, and those in Kaede-green non-Tregs were normalized to 1 ($n = 3$). Data are representative of 3 independent experiments and presented as means \pm SD (A–F). * $P < 0.05$ between the indicated groups (Student's t test, A, B, E, and F; 1-way ANOVA followed by Dunnett multiple comparison test, C and D).

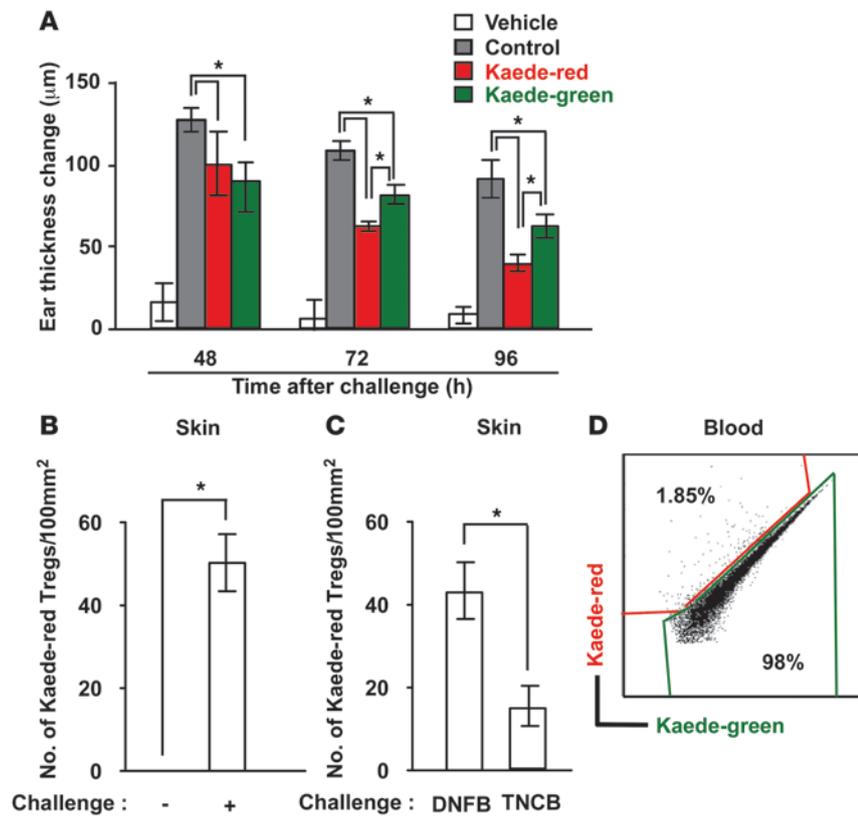


Figure 5

Immunosuppressive effect of Kaede-red Tregs in the skin. (A) Suppression of CHS response by Kaede-red or Kaede-green Tregs. Kaede-red or Kaede-green Tregs (4×10^3 cells/ear) of Kaede/Foxp3^{hCD2/hCD52} mice sensitized, challenged, and photoconverted as in Figure 3A were injected into ear skin of mice sensitized with DNFB 5 days prior. Immediately after injection, the mice were challenged, and the ear thickness change was measured at 48, 72, and 96 hours after challenge. (B–D) The mice were sensitized, challenged, and photoconverted as in Figure 3A. Twenty-four hours after photoconversion, 20 μl of 0.3% DNFB (challenge; +) or vehicle (challenge; –) (B) or 20 μl of 0.3% DNFB or 20 μl of 1% TNCB (C) was painted onto the ear. Twenty-four hours later, the ear skin and blood (D) were collected and dissociated for flow cytometry. The number of Kaede-red Tregs in the skin and the frequency of Kaede-red Tregs in CD4⁺ T cell subset of the blood were evaluated ($n = 3$, each group). Data are presented as means ± SD and representative of 3 independent experiments (A–C). Student’s *t* test was performed between the indicated groups. **P* < 0.05. Numbers within plots indicate percentage of cells in the respective areas (D).

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 cells 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-nonspecific 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 an extent of inhibition similar to that 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 2,4,6-trinitro-l-chlorobenzene (TNCB) sensitization and restimulated them with DNBS or trinitrobenzene sulfonic acid (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 antiproliferative 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 mRNA levels of *Il10* and *Tgfb1* 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 levels 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 antiproliferative 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 challenged 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 hours 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 rechallenged with DNFB and vehicle (Figure 5B) or TNCB (Figure 5C), respectively. Another 24 hours later, Kaede-red Tregs were observed in the ears challenged with DNFB, but not in those challenged with vehicle (Figure 5B). The ear rechallenged with a different hapten, TNCB, contained Kaede-red Tregs, but its number was lower than

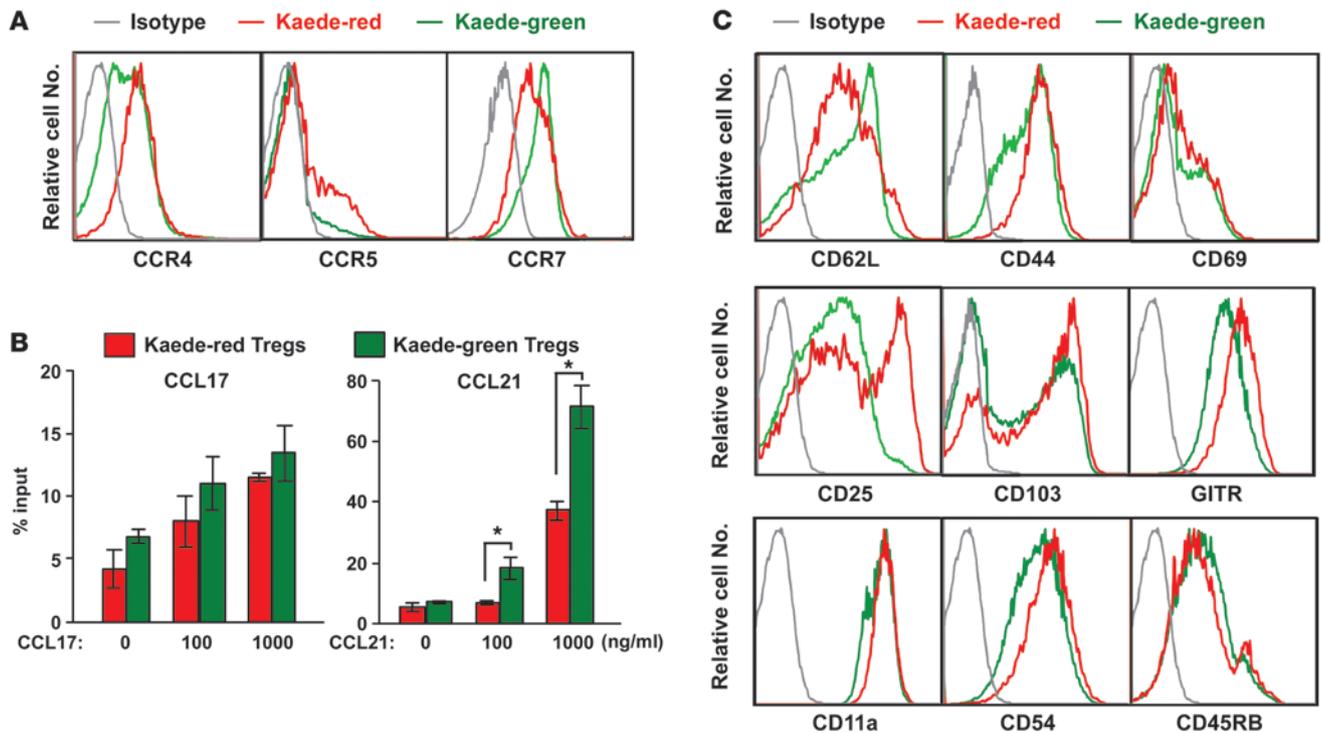


Figure 6

Surface molecule expressions on Kaede-red and Kaede-green cells. (A) Chemokine receptor expression. Skin DLN cells were prepared from the mice sensitized, challenged, and photoconverted as in Figure 3A. These LN cells were stained with isotype-matched control, CCR4, CCR5, and CCR7 mAbs, and the expression levels of Kaede-red and Kaede-green Tregs were evaluated by flow cytometry. (B) Transwell assay. DLN cells were transferred to the upper chamber of the transwell, and CCL17 or CCL21 was added to the lower chamber. The cells were incubated for 3 hours, and the numbers of Kaede-red and Kaede-green cells that migrated to lower chamber were analyzed by flow cytometry. Data are presented as means \pm SD and representative of 2 independent experiments. Student's *t* test was performed between the indicated groups. **P* < 0.05. (C) Surface molecule expression. LN cells were stained with isotype-matched control, CD62L, CD44, CD69, CD25, and CD103 mAbs, and the expression levels were evaluated by flow cytometry. These data are representative of 3 independent experiments.

that of the ear rechallenged with DNFB (Figure 5C). In addition, Kaede-red Tregs were detected in CD4⁺ cells of the blood 24 hours after rechallenge (1.79% \pm 0.07%, average \pm SEM, *n* = 3) (Figure 5D). Moreover, a 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 the abdominal skin with DNFB 4 days later. Two days after challenge, the DLNs of the mice were photoconverted and the ears were rechallenged 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 7). These results suggest that the Tregs that egressed from the skin had a capacity to remigrate 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^{hi} 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 glucocorticoid-induced TNFR family-related gene/protein (GITR), another marker of Tregs (2, 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^{hi} 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^{hi} subset (Figure 6C) that has not, to our knowledge, been thoroughly described before. Initially, we sought to characterize the localization of CD25^{hi} Tregs and found that CD25^{hi} 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

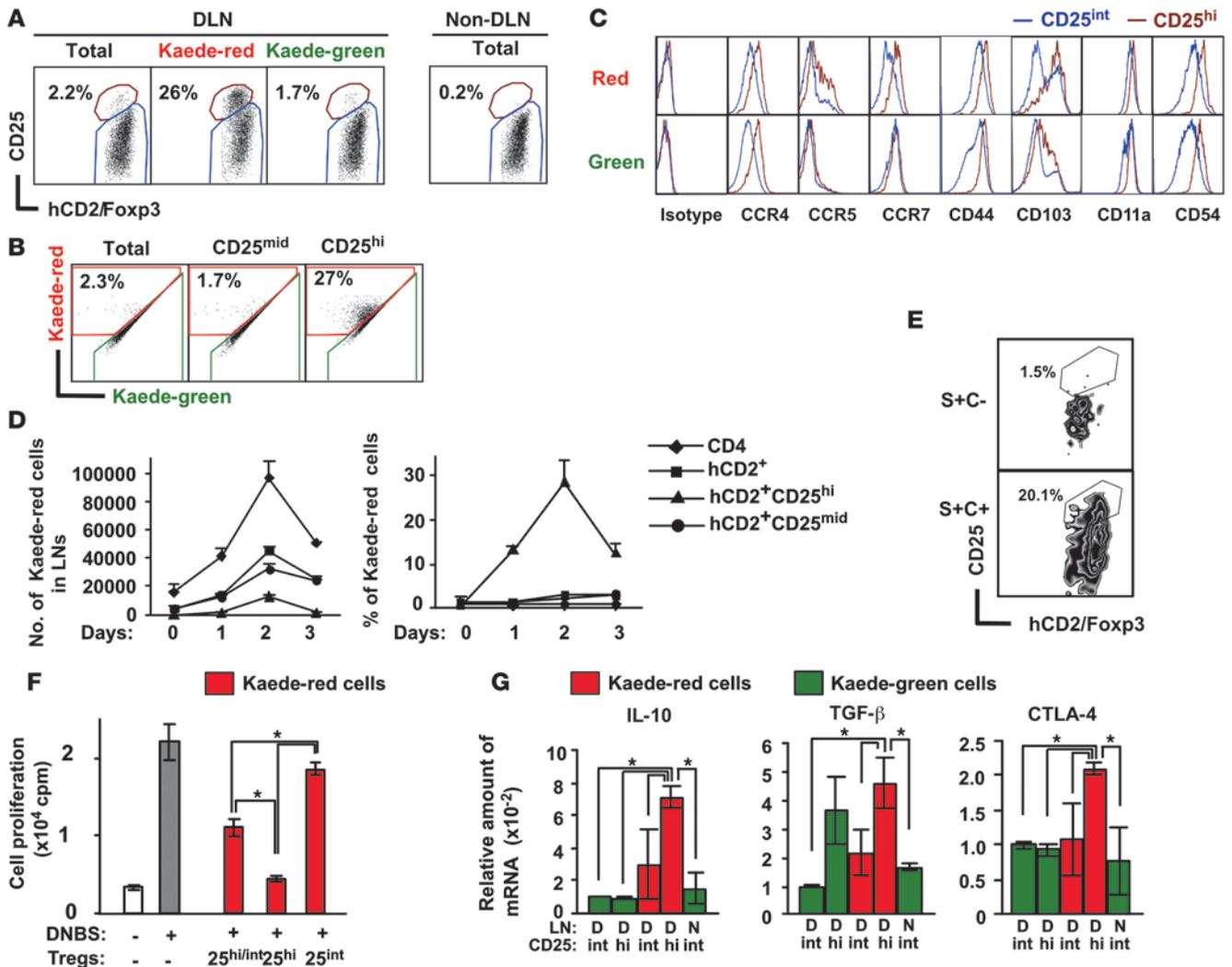


Figure 7

Kinetics and suppression activity of CD25^{hi} Kaede-red migratory Tregs. (A–C) Characterization of CD25^{hi} subset. Kaede/Foxp3^{hCD2/hCD52} mice were treated as in Figure 3A, and the expression levels of hCD2/Foxp3 and CD25 on CD4⁺hCD2/Foxp3⁺ Tregs in total, Kaede-red, and Kaede-green DLN cells and in non-DLN cells (A), the frequency of Kaede-red populations in each population (B), and the expression levels of surface markers on Kaede-red or Kaede-green Tregs in the DLNs (C) were analyzed. (D) Kinetics of T cell migration. Kaede/Foxp3^{hCD2/hCD52} mice were sensitized and challenged as in Figure 3A and photoconverted immediately (day 0), 1 (day 1), 2 (day 2), or 3 (day 3) days after challenge. The number of each subset migrating for 24 hours after photoconversion and the frequency of Kaede-red cells among each subset were measured. (E) Foxp3^{hCD2/hCD52} mice were sensitized with DNFB (S+) and challenged with DNFB (C+) or vehicle (C–). Skin suspensions were evaluated for the expression of hCD2/Foxp3 and CD25. (F) Skin DLNs cells of sensitized B6 mice were stimulated in the absence or presence of Kaede-red total hCD2⁺ Tregs (25^{hi/int}), CD25^{hi} Tregs (25^{hi}), or CD25^{int} Tregs (25^{int}). (G) mRNAs for *Il10* (IL-10), *Tgfb1* (TGF-β), and *Ctla4* (CTLA-4) of Kaede-green CD25^{int} or CD25^{hi} Tregs, Kaede-red CD25^{int} or CD25^{hi} Tregs, or Kaede-green CD25^{int} Tregs in DLNs (D) or non-DLNs (N) were evaluated. The expression level in Kaede-green CD25^{int} Tregs was normalized to 1. Data are presented as means ± SD (n = 3) (D, F, and G). *P < 0.05 between indicated groups. (F and G). Numbers within plots or histograms indicate percentage of cells (A, B, and E).

Kaede-green Tregs of the DLNs or in non-DLNs (Figure 7A). Consistently, the frequency of the Kaede-red population in the CD25^{hi} population was greater than that in the CD25^{mid} population (Figure 7B). These CD25^{hi} 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^{hi} 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^{hi} subsets (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 hours after each photoconversion, and the number of Kaede-red CD4⁺, CD4⁺hCD2⁺, CD4⁺hCD2⁺CD25^{hi}, and CD4⁺hCD2⁺CD25^{mid} cells migrating for 24 hours after photoconversion was determined (Figure 7D). The peak response of cell migration from the skin occurred on day 2 (between 48 and 72 hours after challenge) when the frequency of Tregs among CD4⁺ T cells migrating from



the skin was high (Figure 7D). In addition, CD4⁺hCD2⁺CD25^{hi} cells were detected only at this time point (Figure 7D) and showed a high frequency of Kaede-red positivity, especially on day 2 (Figure 7D), suggesting that this subset is replaced by the skin-derived cells more readily than other subsets.

Strong immunosuppressive activity of CD25^{hi} Kaede-red migratory Tregs. To evaluate whether CD25^{hi} 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^{hi} Tregs in the challenged local skin, but few in the nonchallenged skin 48 hours after the challenge (Figure 7E), suggesting that CD4⁺hCD2⁺CD25^{hi} cells are induced in the skin and migrate into the DLNs.

To determine the role of skin-derived CD25^{hi} Tregs, Treg subsets were isolated from the DLNs of mice pretreated as in Figure 3A and cocultured with DLN cells from DNFB-sensitized mice. The CD25^{hi} 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^{hi} Treg subsets. In agreement with the above *in vitro* result, Kaede-red CD25^{hi} Tregs contained significantly higher amounts of *Il10*, *Tgfb1*, and *Ctla4* than Kaede-red CD25^{int} Tregs in the DLNs, Kaede-green CD25^{hi} 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^{hi} Tregs and Kaede-green CD25^{hi} Tregs in DLNs (Figure 7G). These results suggest that CD25^{hi} 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 with LN-resident Tregs. Moreover, depletion of endogenous Tregs *in vivo* prolonged the CHS response. Finally, these circulating Tregs specifically included the CD25^{hi} 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 constituent in the afferent lymph by cannulation of sheep (6, 11–13), and the other suggested the naive subset is dominant using subcutaneous injection of fluorescent-labeled lymphocytes (14). Recently, effector/memory phenotype of Tregs has 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 the 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 naive T cells circulate between blood and LNs, cells of the memory/effector T cell phenotype, 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 of DNFB-sensitized LN cells in a ratio as low as 1:100 (Tregs to LN cells), but such an inhibitory effect was not observed in non-antigen-specific mitogen-induced T cell proliferation systems. Therefore, Tregs circulating between the skin and LNs may inhibit not only T cells, but also antigen-presenting cells, such as dendritic cells, or antigen-presenting cell–T cell interactions. Moreover, subcutaneous injection of migratory Tregs into the skin suppressed CHS more markedly than that of LN-resident Tregs. Similar findings were observed when these Tregs were transferred intravenously (data not shown), suggesting that Tregs migrating from the skin hold a high immunosuppressive potential.

The CD25^{hi} subset that migrated from the skin seems to have an activated phenotype, indicated by the positivity of CD25 and CD103. It has been reported that transfer of preactivated CD25⁺CD103⁺ cells strongly suppressed T cell proliferation (32) and CD25⁺CD103⁺ cells are the main producer of IL-10 after TCR stimulation (29). The CD25^{hi} subset in our finding expresses high levels of CD103 and IL-10 and strong suppressive capacity and phenotype, consistent with an activated effector/memory Treg subset (28, 33). It should be noted that we demonstrate that the CD25^{hi} subset was localized in the skin and only transiently migrated from the skin after CHS elicitation. Thus, the role of skin in generation, education, and spatiotemporal regulation of this CD25^{hi} subset during immune responses needs to be elucidated in the future, which may lead us to understand the role of peripheral tissues in regulation of immune responses.

Notably, Treg cell circulation was remarkably induced during cutaneous immune responses. Therefore, we have focused on the roles of Tregs instead of effector/memory T cells migrating from the skin. In fact, the administration of migratory Tregs strongly suppressed CHS response at the later phase after a challenge (Figure 5A), and *in vivo* depletion of Tregs prolonged the CHS response, particularly during the later phase (Figure 4B). These results suggest that these circulating Tregs might be involved in the termination of immune responses. However, immune responses and homeostasis are regulated and maintained by the balance between Tregs and effector/memory T cells, and it has been thought that CHS occurs by the dominance of effector/memory T cells over Tregs. Hence, it is intriguing that the elicitation of CHS induces Tregs despite their possible antagonistic role for the development of acquired immune response. In this sense, it will be of interest to explore more the roles of effector/memory T cells and Tregs migrating from the skin in regulating immune response. Clarification of these issues will lead not only to understanding of the novel mechanism of cutaneous immune responses but also to control of systemic immune responses through modulating cutaneous immunity.



Methods

Mice and photoconversion. Tg mice carrying Kaede cDNA under the CAG promoter were established previously (17). These mice with B6 genetic background expressed photoconvertible Kaede in all of their cell types. It should be noted that the use of violet light (436 nm) rather than harmful UVA (320–400 nm) or UVB (290–320 nm) allowed us to photoconvert Kaede in the cells with no detectable damage (17).

Because of the moiety of its wavelength, violet light exposure penetrates through the skin to subcutaneous tissue, but not further (data not shown). Although the exposure of Kaede to violet light permanently changes its structure and photoconverted Kaede has a very long biological half-life in lymphocytes, cell proliferation dilutes photoconverted Kaede with newly synthesized nonphotoconverted Kaede, and after several cell divisions, the detection of red fluorescence becomes difficult (17). Moreover, exposure of the cells to violet light for 10 minutes has no effect on T and B cell proliferation (17). To exclude the immunomodulatory effect of photoconversion in vivo, we used the CHS model. Photoconversion of the abdominal skin immediately after sensitization on the abdomen did not affect CHS response (data not shown). When mRNA levels of *Il1b* were examined 6 hours after photoconversion (436 nm) or low-dose (3 kJ/m²) UVB exposure, a significant increase of mRNA levels of *Il1b* was observed by UVB but not by photoconversion (Supplemental Figure 8). Therefore, we assume that photoconversion of the skin does not provoke significant inflammation in the skin or inflammatory stimuli in keratinocytes.

B6 Foxp3^{hCD2/hCD52} mice were generated by homologous recombination in a B6-derived ES cell line using a targeting construct in which cDNA encoding a human CD2 and human CD52 fusion protein along with an intraribosomal entry site was inserted into the 3' untranslated region of the endogenous Foxp3 locus (18). All CD4⁺Foxp3⁺ cells expressed hCD2, but CD4⁺Foxp3⁻ cells did not (data not shown), indicating that the expression of the human CD2 reporter faithfully reflects the intracellular expression of Foxp3 in Foxp3^{hCD2/hCD52} mice. Foxp3^{hCD2/hCD52} mice (18) were intercrossed with Kaede-Tg mice to generate Kaede/Foxp3^{hCD2/hCD52} mice for further evaluation. These mice were bred in specific pathogen-free facilities at Kyoto University or RIKEN. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Kyoto University Faculty of Medicine and RIKEN.

Antibodies and flow cytometry. Fluorochrome-conjugated or biotinylated anti-human CD2, anti-mouse CD4, CD11a, CD11c, CD25, CD44, CD45RB, CD62L, CD69, CD103, GITR, CCR4, CCR5, and CCR7 mAbs were obtained from BD Biosciences, eBioscience, or Biolegend. Data were acquired using the JSAN system (Bay bioscience) or FACSCanto II flow cytometry system (BD Biosciences) and analyzed with FlowJo (TreeStar).

Cell preparation from the skin and cell sorting. Briefly, the ears were removed and split into dorsal and ventral halves, and cartilage was removed. The skin of the ears was floated on 0.25% trypsin/EDTA for 30 minutes at 37°C. Then the epidermis was peeled from the dermis, and both epidermis and dermis were minced with forceps. The minced tissues were incubated for 1 hour in collagenase II (Worthington Biochemical) containing hyaluronidase and DNaseI (Sigma-Aldrich). The cell suspensions were filtered with 40 µm of cell strainer.

For cell sorting, Kaede-red Tregs or Kaede-green Tregs were purified from inguinal and axillary LNs of Kaede/Foxp3^{hCD2/hCD52} mice. Briefly, the mice were sensitized and challenged with DNFB in the same way as the B6 mice for DNBS-induced cell proliferation. Two days after the challenge, cells of abdominal skin were photoconverted, and single-cell suspensions were prepared from inguinal and axillary LNs 24 hours after photoconversion. The cells of each population were sorted by the FACSAria II flow cytometry system (BD Bioscience).

Photoconversion, CHS model, in vivo Treg depletion, and cell proliferation assay. Photoconversion of the skin was performed as described previously (17). Briefly, mice were anesthetized, shaved, and exposed to violet light at 95 mW/cm² with a 436-nm bandpass filter using Spot UV curing equipment (SP500; USHIO).

For the CHS model, mice were immunized by application of 25 µl of 0.5% DNFB (Nacalai Tesque) in 4:1 (wt/vol) acetone/olive oil to their shaved abdomens on day 0 and challenged on the right ear on day 5 with 20 µl of 0.3% (wt/vol) DNFB (34). Ear thickness was measured before and after challenge, and ear-thickness change was calculated.

For Treg depletion in vivo, mice were injected with Campath-1G Ab through the tail vein (0.5 mg/body) 1 day before the CHS challenge (22). The injection was repeated every 4 days throughout the experiment. The same amount of vehicle or rat IgG (0.5 mg/body; Sigma-Aldrich) was used as a control.

For DNBS- or TNBS-dependent cell proliferation, mice were sensitized with 50 µl of 0.5% DNFB (wt/vol) or 50 µl of 5% TNCB (Tokyo Kasei) (wt/vol) in acetone/olive oil (4/1; vol/vol) on the dorsal skin, and 5 days later, single-cell suspensions were prepared from inguinal and axillary LNs. CD25-positive cells were depleted from the cells by Auto-MACS (Miltenyi Biotec) using PE-labeled anti-mouse CD25 antibody (eBioscience) and magnetic microbeads coated with anti-PE (Miltenyi Biotec). Less than 1% of Foxp3⁺ cells were present in the remaining LN cells. 7 × 10⁵ LN cells/well in a 96-well plate were cultured in RPMI 1640 containing 10% FBS with or without 50 µg/ml DNBS (Alfa Aesar) for 3 days. For TNBS stimulation, the LN cells were incubated in 2.5 mM TNBS (Tokyo Kasei) in PBS for 20 minutes at 37°C and subsequently washed 3 times in PBS, and 7 × 10⁵ cells/well in a 96-well plate were cultured in RPMI 1640 containing 10% FBS for 3 days. Cells were pulsed with 0.5 µCi ³H-thymidine for the last 24 hours of culture and subjected to liquid scintillation counting.

For the proliferation assay of anti-CD3 stimulation, spleen CD4⁺ cells deprived of CD25⁺ cells were sorted by auto-MACS. Then, 5 × 10⁴ cells/well were cultured in a 96-well plate coated with 1 µg/ml of anti-CD3 antibody for 72 hours. For the last 24 hours, cells were pulsed with 0.5 µCi ³H-thymidine, and its incorporation was measured.

Quantitative RT-PCR analysis. Total RNA from purified cells was isolated with the RNeasy Mini Kit (QIAGEN). Quantitative RT-PCR with the Light Cycler real-time PCR apparatus was performed according to the instructions of the manufacturer (Roche) by monitoring the synthesis of double-stranded DNA during the various PCR cycles using SYBR Green I (Roche). For each sample, duplicate test reactions were analyzed for expression of the gene of interest, and results were normalized to those of the *Gapdh* mRNA.

In vivo immunosuppression assay. A total of 4 × 10³ cells of isolated Kaede-red Tregs or Kaede-green Tregs in 20 µl PBS were subcutaneously injected into the ventral surface of each ear. Ear thickness was measured for each mouse before and at the indicated time point after elicitation with a micrometer, and the difference was expressed as ear swelling (*n* = 4–6 in each group).

Chemotaxis assay. Skin cell suspensions of Foxp3^{hCD2/hCD52} mice were tested for transmigration across uncoated 5-µm transwell filters (Corning Costar Corp.) for 3 hours to CCL21 (R&D Systems) or medium in the lower chamber, and the numbers of cells that migrated to the lower chamber were determined by flow cytometry (35). The migration index was shown as a percentage of input by dividing with total input cells in upper chamber.

Statistics. Data were analyzed with the unpaired Student's 2-tailed *t* test unless otherwise stated. A *P* value of less than 0.05 was considered to be significant.

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