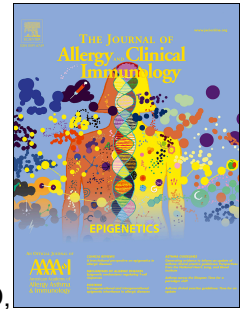


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Percutaneous sensitization is limited by *in situ* inhibition of cutaneous dendritic cell migration via skin-resident regulatory T cells

Sho Hanakawa, MSc, Akihiko Kitoh, MD, PhD, Rintaro Shibuya, MD, Teruki Dainichi, MD, PhD, Takashi Nomura, MD, PhD, Tetsuya Honda, MD, PhD, Gyohei Egawa, MD, PhD, Atsushi Otsuka, MD, PhD, Saeko Nakajima, MD, PhD, Mitsugu Fujita, MD, PhD, Kenji Kabashima, MD, PhD



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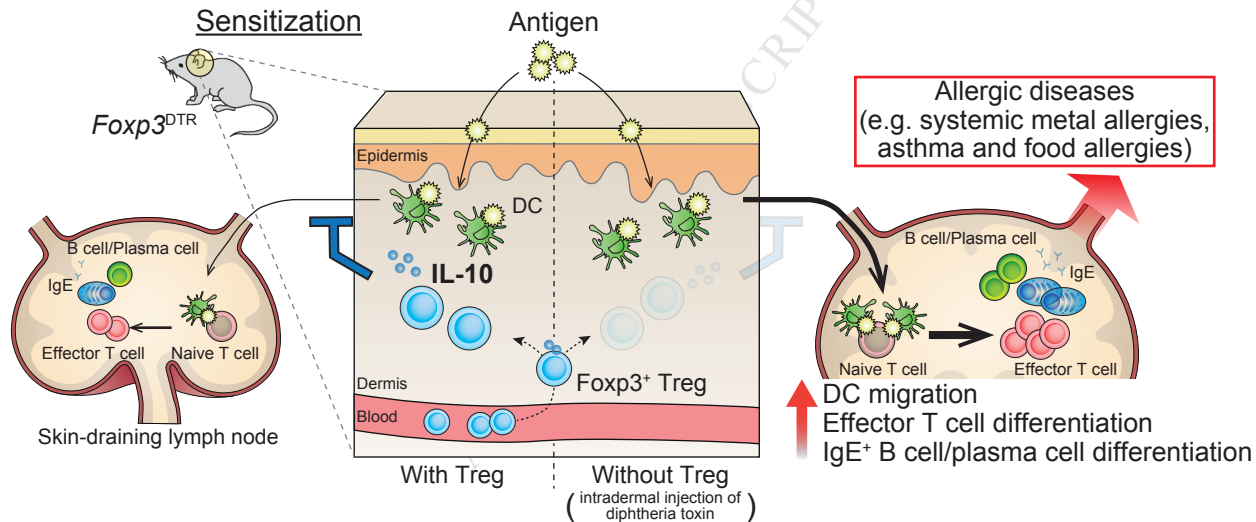
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Sho Hanakawa, MSc,^a Akihiko Kitoh,^a MD, PhD,^a Rintaro Shibuya, MD,^a Teruki Dainichi, MD, PhD,^a Takashi Nomura, MD, PhD,^a Tetsuya Honda, MD, PhD,^a Gyohei Egawa, MD, PhD,^a Atsushi Otsuka, MD, PhD,^a Saeko Nakajima, MD, PhD,^a Mitsugu Fujita, MD, PhD,^b and Kenji Kabashima, MD, PhD^{a,c},

^aDepartment of Dermatology, Kyoto University Graduate School of Medicine, Kyoto, Japan; ^bDepartment of Microbiology, Kindai University Faculty of Medicine, Osaka, Japan; and ^cSingapore Immunology Network (SiGN) and Skin Research Institute of Singapore (SRIS), Agency for Science, Technology and Research (A*STAR), Singapore.

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23 Correspondence should be addressed to Dr. Akihiko Kitoh (AK) and Dr. Kenji

24 Kabashima (KK).

25 Department of Dermatology, Kyoto University Graduate School of Medicine, 54

26 Shogoin-Kawahara, Kyoto 606-8507, Japan

27 Phone: +81-75-751-3310

28 Fax: +81-75-751-4949

29 Email: kichu@kuhp.kyoto-u.ac.jp (AK) and kaba@kuhp.kyoto-u.ac.jp (KK)

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ABSTRACT

Background: Percutaneous sensitization is associated with various allergic diseases, including asthma and food allergies. However, the immunological mechanisms underlying how the skin regulates percutaneous sensitization is still unclear.

Objective: We aimed to investigate whether and how CD4⁺Foxp3⁺ regulatory T (Treg) cells residing in the skin regulate percutaneous sensitization in the skin.

Methods: Selective reduction of cutaneous Treg cells was achieved by intradermal injection of diphtheria toxin into the ear skin of *Foxp3*^{DTR} mice, in which Treg cells specifically express the diphtheria toxin receptor fused with green fluorescent protein.

Results: Thirty to forty percent of cutaneous Treg cells were capable of interleukin (IL)-10 production in both mice and humans. Selective reduction of cutaneous Treg cells at the sensitization site promoted migration of antigen-bearing dendritic cells (DCs) to the draining lymph nodes (dLNs). Accordingly, sensitization through the skin with reduced Treg cells led to enhanced antigen-specific immune responses in the dLNs, including both effector T cell differentiation and T cell-dependent B-cell responses such as the development of germinal center B cells expressing IgG1 and IgE. Furthermore, antigen-bearing cutaneous DC migration was enhanced in mice with IL-10 deficiency restricted to the cutaneous Treg-cell compartment, suggesting an important role of cutaneous IL-10⁺ Treg cells in limiting percutaneous sensitization. Treg cells with a skin-homing phenotype in skin-dLNs expressed high levels of IL-10, suggesting that they contribute to the renewal and maintenance of the cutaneous IL-10⁺ Treg-cell

population.

Conclusion: Skin-resident Treg cells limit percutaneous sensitization by suppressing antigen-bearing DC migration through *in situ* IL-10 production.

Clinical implications

Manipulating the number and/or function of skin-resident Treg cells can be a promising strategy for preventing the development of various allergic diseases and improving the efficacy of percutaneous vaccination.

Capsule summary

Numerical or functional deficiency of Treg cells restricted to the skin promotes percutaneous sensitization via activating cutaneous dendritic cell function *in situ*, thereby leading to potentially increased risk of developing various allergic diseases.

Key Words:

regulatory T cells, IL-10, dendritic cells, skin, percutaneous sensitization

Abbreviations used:

BM: Bone marrow

CD62E/P-ligands: E- and P-selectin ligands

CHS: Contact hypersensitivity

- 73 DC: Dendritic cell
- 74 DNFB: 2,4-dinitrofluorobenzene
- 75 DT: Diphtheria toxin
- 76 dLN: Draining lymph node
- 77 FITC: Fluorescein isothiocyanate
- 78 T_{FH}: Follicular helper T
- 79 GC: Germinal center
- 80 hCD2: Human CD2
- 81 NP-OVA: 4-Hydroxy-3-nitrophenylacetyl hapten-conjugated ovalbumin
- 82 OVA: Ovalbumin
- 83 TCR: T-cell receptor
- 84 Treg: Regulatory T
- 85 WT: Wild-type
- 86

INTRODUCTION

Increasing evidence suggests that antigen sensitization through the skin is associated with both inflammatory skin diseases and various allergic diseases involving other organs, such as systemic metal allergies, asthma and food allergies.¹⁻³ Thus, understanding what increases susceptibility to percutaneous sensitization is of great importance for improving the clinical management of these diseases.

Cutaneous antigen exposure can induce sensitization toward both delayed- and immediate-type allergies. The former has been attributed to the differentiation and expansion of antigen-specific effector T cells, while the latter stems from T cell-dependent production of antigen-specific IgE by B cells.^{1,4} Both of these responses are initiated by the priming of antigen-specific naïve T cells in the draining lymph nodes (dLNs) of the sensitized skin, which depends on the migration of antigen-bearing dendritic cells (DCs) from the skin to the dLNs.⁵ Thus, resident cells in the skin, including both immune and non-immune cells, potentially regulate susceptibility to percutaneous sensitization by affecting the activation and migration of antigen-bearing DCs in the skin. In fact, skin barrier abnormalities—such as filaggrin deficiency—promote percutaneous sensitization and the development of allergic diseases,^{6,7} presumably due to enhanced antigen penetration. However, it is not yet clearly understood how susceptibility to percutaneous sensitization is affected by immunological conditions of the skin in the steady state, i.e., the number and functions of a specific immune cell subset residing in the skin.

FOXP3⁺ regulatory T (Treg) cells are a specialized subpopulation of CD4⁺ T cells that play a critical role in the maintenance of immunological self-tolerance, as well as in the suppression of overall immune responses against antigens.⁸ Treg cells can suppress immune responses through various mechanisms including contact-dependent inhibition of DC activation and secretion of anti-inflammatory cytokines in a context-dependent manner.⁹ In both human and murine skin, a substantial number of Treg cells are resident cells even under physiological conditions, accounting for nearly half of the CD4⁺ T cell population in the skin.¹⁰⁻¹² This number is in contrast to secondary lymphoid organs, such as the spleen and lymph nodes, where approximately 5–10% of CD4⁺ T cells are Treg cells.¹³ The distribution of Treg cells in noninflamed skin is controlled by the intrinsic expression of skin-homing receptors, such as CCR4, and adhesion molecules, such as CD103 and E- and P-selectin ligands (CD62E/P-ligands).¹⁴⁻¹⁶ Mice suffering a loss of skin-homing capabilities in the Treg cell compartment spontaneously develop severe skin inflammation with lymphocytic infiltration,^{14,17} indicating an essential role for skin-resident Treg cells in the maintenance of immunological tolerance and homeostasis in the skin. In addition, Treg cells specific for skin-associated antigens are suggested to be activated by their cognate antigens and upregulate the expression of skin-homing-associated molecules in skin-dLNs under physiological conditions.^{14,17-19} These findings imply that skin-associated antigen-specific Treg cells in skin-dLNs migrate to the skin, where they exert a suppressive effect on the development of autoimmune skin inflammation. However, it remains unclear whether and how Treg

cells distributed in the skin in the physiological steady state control percutaneous sensitization with foreign antigens.

An inhibitory role of IL-10 in percutaneous sensitization is suggested by a report that the migration of antigen-bearing cutaneous DCs to dLNs is enhanced in *Il10*^{-/-} mice.²⁰ In addition, intradermal injection of recombinant IL-10 prior to antigen exposure inhibits the differentiation of antigen-specific effector T cells in dLNs.²¹ This finding suggests that IL-10 in the skin at the site of antigen exposure can limit percutaneous sensitization. However, it is not certain whether IL-10 endogenously produced in the skin significantly contributes to the control of percutaneous sensitization. In addition, it is also unclear which type of skin-resident cell contributes to limiting percutaneous sensitization through *in situ* IL-10 production. Recent transcriptome analyses have revealed that Treg cells in murine skin highly express *Il10* transcripts compared to those in skin-dLNs under physiological conditions.²²⁻²⁴ However, the contribution of IL-10 derived from skin-resident Treg cells in the control of percutaneous sensitization has yet to be clarified.

Here, we demonstrated that the selective reduction of Treg cells in the skin at the site of antigen exposure enhances migration of antigen-bearing DCs to the dLNs, which results in the promotion of the differentiation of antigen-specific effector T cells and antigen-specific IgE-expressing cells in the dLN. Furthermore, cutaneous DC migration to the dLNs after antigen exposure was promoted by IL-10 deficiency restricted to skin-resident Treg cells. Our results indicate that percutaneous sensitization is limited

150 through the *in situ* inhibition of DC migration by skin-resident Treg cells, and this

151 inhibition is attributed to Treg cell-derived IL-10.

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METHODS

Selective depletion of cutaneous Treg cells

Diphtheria toxin (DT; Sigma-Aldrich, St Louis, Mo, USA) at a dose of 2 ng dissolved in 10 μ l of phosphate-buffered saline was injected intradermally into the ears of *Foxp3*^{DTR} mice¹³ 24 hours prior to sensitization.

Mixed bone marrow chimeras

Bone marrow (BM) cells were injected intravenously into lethally irradiated (2x 450 rads from a cesium source, 4 hours apart) *Tcrb*^{-/-} mice²⁵ at 6 to 7 weeks of age. *Tcrb*^{-/-} recipients received 4×10^6 cells of a 1:1 mixture of BM cells from *Ccr4*^{-/-} mice²⁶ and either WT or *Il10*^{-/-} mice²⁷, and were subjected to subsequent analyses or sensitization 60 days later.

RESULTS

Skin-resident Treg cells expressed IL-10 under physiological conditions in both mice and humans

First, we determined what percentage of skin-resident Treg cells expressed IL-10 using $Il10^{Venus} \times Foxp3^{hCD2}$ double-reporter mice. In $Il10^{Venus}$ mice, a cassette containing an internal ribosomal entry site and Venus was inserted immediately before the polyadenylation signal of the *Il10* gene. In $Foxp3^{hCD2}$ knock-in mice, $Foxp3^+$ cells specifically express a glycosylphosphatidylinositol (GPI)-anchored human CD2-CD52 fusion protein on their cell surface, which enabled us to detect $Foxp3^+$ cells with an anti-human CD2 antibody. Flow cytometric analysis showed that Treg cells ($hCD2^+$) comprised nearly half of the $CD4^+$ T cells in the ear skin, while less than 15% of $CD4^+$ T cells from the dLNs were Treg cells (Fig 1, A). Only a very small proportion of conventional ($hCD2^-$) $CD4^+$ T cells expressed Venus (IL-10) in both the skin and the skin-dLNs (Fig 1, A). In contrast, approximately 40% of Treg cells in the ear skin expressed Venus compared to less than 4% of Treg cells in the dLNs, suggesting that skin-resident Treg cells are characterized by IL-10 production in the steady state (Fig 1, A). In human samples, immunofluorescence staining for FOXP3 in combination with *in situ* hybridization of *IL10* mRNA showed that approximately 30% of $FOXP3^+$ cells expressed *IL10* in healthy human skin (Fig 1, B and C), suggesting that skin-resident Treg cells expressed IL-10 physiologically in humans as well.

Treg-cell reduction restricted to the skin resulted in enhanced DC migration following antigen exposure

The most important immunological event in the skin that is essential for percutaneous sensitization is the migration of antigen-bearing cutaneous DCs to the dLNs, where they prime antigen-specific T cells.⁵ Given the substantial number of Treg cells residing in naïve skin, we hypothesized that skin-resident Treg cells may regulate cutaneous DC migration following antigen exposure. To test this hypothesis, we examined whether a selective reduction of cutaneous Treg cells affected the migration of antigen-bearing DCs to the dLNs after topical hapten application. Treg cells were depleted by DT administration in *Foxp3^{DTR}* mice, in which *Foxp3⁺* Treg cells specifically express a human DT receptor (DTR) fused with green fluorescent protein (GFP).¹³ Intradermal injection of an optimal dose of DT into the ear of *Foxp3^{DTR}* mice resulted in a significant reduction of Treg cells in the DT-treated skin, but not in the dLNs, 24 hours after DT injection (Fig 2, A and B). In contrast, the number of DCs was not significantly affected in the DT-treated skin (Fig E1). DT-treated WT and *Foxp3^{DTR}* mice were treated topically with the fluorescent hapten fluorescein isothiocyanate (FITC) on the ear, followed by flow cytometric analyses of the dLNs 24 hours after FITC application. We observed that the numbers of antigen-bearing (FITC⁺) DCs in DT-treated *Foxp3^{DTR}* mice were significantly higher than those of DT-treated WT mice (Fig 2, C). We further evaluated T-cell priming activities of FITC⁺ DCs isolated from the dLNs of WT and *Foxp3^{DTR}* mice. FITC⁺ DCs were cocultured with OT-I T-cell receptor (TCR) transgenic

CD8⁺ T cells or OT-II TCR transgenic CD4⁺ T cells in the presence or absence of their respective cognate ovalbumin (OVA) peptides. FITC⁺ DCs isolated from *Foxp3*^{DTR} mice promoted OVA₂₅₇₋₂₆₄-induced IFN- γ secretion from OT-I T cells more efficiently than those from WT mice (Fig 2, D). OVA₃₂₃₋₃₃₉-induced secretion of IL-13, a central player of T_H2-type responses, from OT-II T cells was also promoted by FITC⁺ DCs from *Foxp3*^{DTR} mice more efficiently than those from WT mice, although IL-4 secretion was not observed in this experimental setting (Fig 2, E and not shown). Collectively, these results suggest that the reduction of cutaneous Treg cells results in increased migration and augments the T-cell priming activity of antigen-bearing DCs following cutaneous antigen exposure.

Sensitization through the skin with reduced Treg cells resulted in enhanced antigen-specific immune responses

Next, we examined whether the control of DC migration and maturation by cutaneous Treg cells could possibly be associated with subsequent antigen-specific immune responses in the dLNs. WT and *Foxp3*^{DTR} mice were sensitized via exposure of the DT-pretreated ear to the hapten 2,4-dinitrofluorobenzene (DNFB) and then subjected to subsequent analyses 5 days later. Flow cytometric analysis revealed that the numbers of CD44⁺CD62L⁻ effector CD8⁺ T cells in the dLNs in *Foxp3*^{DTR} mice were significantly higher than those in WT mice (Fig 3, A). As topical application of DNFB is known to induce IFN- γ -producing CD8⁺ T cell-dominant responses,^{28,29} we next assessed the

effects of cutaneous Treg-cell reduction on the development of IFN- γ -producing effector CD8⁺ T cells in the dLNs associated with DNFB-treated skin. CD8⁺ T cells harvested from *Foxp3*^{DTR} mice secreted a significantly higher amount of IFN- γ compared to those from WT mice in response to *in vitro* stimulation with dinitrobenzene sulfonic acid (DNBS), a water-soluble analogue of DNFB (Fig 3, B). This result indicates that the development of antigen-specific IFN- γ -producing CD8⁺ T cells was enhanced by cutaneous Treg-cell reduction at the sensitization site. In fact, subsequent rechallenge with DNFB on the DT-untreated ear contralateral to the DT-treated sensitized ear elicited significantly augmented contact hypersensitivity (CHS) responses in *Foxp3*^{DTR} mice compared to WT mice, with the enhanced accumulation of IFN- γ -producing CD8⁺ T cells in DNFB-challenged skin (Fig 3, C-E, Fig E2, A). We confirmed that DT injection did not decrease Treg cells in the contralateral non-sensitized ear (Fig E2, B), excluding the possibility that direct effect of DT on the distant challenging site might contribute to the augmentation of CHS responses.

We further examined whether percutaneous sensitization with a protein antigen, ovalbumin-conjugated with 4-hydroxy-3-nitrophenylacetyl (NP-OVA), was also controlled by cutaneous Treg cells at the sensitization site. Intradermal administration of NP-OVA in alum adjuvant (NP-OVA/alum) is known to induce T_H2-related antibody responses, such as IgG1 and IgE production, in a T-cell dependent manner, with a robust development of follicular helper T (T_{FH}) cells and germinal center (GC) B cells.³⁰ WT and *Foxp3*^{DTR} mice were injected with NP-OVA/alum intradermally in the

DT-pretreated ear; 6 days later, the dLN cells were analyzed by flow cytometry. The development of T_{FH} cells and GC B cells following immunization with NP-OVA/alum was induced to a markedly greater extent in *Foxp3*^{DTR} mice compared to WT mice (Fig 3, F, Fig E3, A and B). The immunization-induced increase in the number of NP-specific IgG1- and IgE-expressing GC B cells in *Foxp3*^{DTR} mice was also more pronounced than that in WT mice (Fig 3, G, Fig E3, C). In addition, *Foxp3*^{DTR} mice showed a significantly greater number of NP-specific IgE plasma cells (PCs) compared to WT mice, although the number of NP-specific IgG1 PCs was equivalent between these two groups (Fig 3, G, Fig E3, C).

Taken together, these data suggest that sensitization through the skin in the presence of reduced Treg cells leads to enhanced antigen-specific immune responses in dLNs.

IL-10 inhibited CCR7 upregulation of DCs in human skin explants

Our results suggest that skin-resident Treg cells limit percutaneous sensitization through the inhibition of cutaneous DC migration to the dLNs. Migration of cutaneous DCs following antigen exposure has been reported to be enhanced in *Il10*^{-/-} mice,²⁰ suggesting an inhibitory role for IL-10 in cutaneous DC migration in mice. Thus, we hypothesized that IL-10 derived from Treg cells in the skin inhibits DC migration after cutaneous antigen exposure. Prior to testing this hypothesis in mice, we confirmed that IL-10 can also inhibit the migration of human cutaneous DCs using human samples. We

cultured human skin explants *ex vivo* in the presence or absence of exogenous IL-10 for 3 days and then assessed the level of CCR7 expression on cutaneous DCs, as migration of cutaneous DCs to the dLNs depends on upregulation of CCR7 expression on their surface.⁵ CCR7 expression on DCs was spontaneously induced in the cultured skin explants (Fig 4, A, Fig E4). The induction of CCR7 was attenuated by the addition of IL-10 in a dose-dependent manner (Fig 4, B and C), suggesting that IL-10 can suppress cutaneous DC migration to dLNs in humans as well as in mice.

IL-10 deficiency in cutaneous Treg cells resulted in increased DC migration after cutaneous antigen exposure

Next, we addressed the hypothesis that IL-10 production by cutaneous Treg cells has a suppressive effect on DC maturation and migration after cutaneous antigen exposure. To test this hypothesis, we aimed to generate mice in which IL-10 was specifically deficient in cutaneous Treg cells based on the previously reported observation that CCR4 is essential for the accumulation of CD4⁺ T cells, but not other types of immune cells, in the skin under homeostatic conditions.¹⁴ We first established that in T-cell-deficient (*Tcrb*^{-/-}) mice (CD45.2⁺) that were transplanted with mixed BM cells from *Ccr4*^{+/+} (CD45.1⁺) and *Ccr4*^{-/-} (CD45.2⁺) mice, CD4⁺ T cells (including Treg cells) were reconstituted predominantly with *Ccr4*^{+/+} (CD45.1⁺) BM-derived cells in the ear skin (Fig 5, A and B). In contrast, other radiosensitive cells, such as CD8⁺ T cells, dermal DCs, and macrophages, were derived from both *Ccr4*^{+/+} and *Ccr4*^{-/-} BM cells

(Fig 5, B). Radioresistant skin-resident cells, such as Langerhans cells and mast cells, were not replaced by WT BM-derived cells as expected (Fig 5, B). Therefore, in the skin of mice transplanted with mixed BM cells from *Il10*^{-/-} and *Ccr4*^{-/-} mice, CD4⁺ T cells alone should be derived predominantly from *Il10*^{-/-} BM cells which are CCR4-sufficient. This results in IL-10 deficiency restricted to CD4⁺ T cells in the skin. In addition, IL-10⁺ (Venus⁺) CD4⁺ T cells in the skin were composed exclusively of Treg cells both physiologically and 24 hours after topical DNFB application (Fig 1, A and Fig E5), suggesting that only Treg cells (among all CD4⁺ T cells in the skin) can produce IL-10 before and 24 hours after antigen exposure. Taken together, *Il10*^{-/-}/*Ccr4*^{-/-} mixed BM chimeras demonstrate IL-10 deficiency specifically in cutaneous Treg cells, at least under these conditions. Neither skin inflammation nor lymphadenopathy spontaneously developed in *Il10*^{-/-}/*Ccr4*^{-/-} mixed BM chimeras (Fig E6).

We then assessed the migration of antigen-bearing cutaneous DCs in WT/*Ccr4*^{-/-} or *Il10*^{-/-}/*Ccr4*^{-/-} mixed BM chimeras by an *in vivo* DC migration assay using FITC (Fig 5, C). *Il10*^{-/-}/*Ccr4*^{-/-} chimeras showed a significantly increased number of FITC⁺ DCs in the dLNs 24 hours after sensitization compared to WT/*Ccr4*^{-/-} chimeras (Fig 5, D). CD8⁺ T cells from the dLNs of *Il10*^{-/-}/*Ccr4*^{-/-} chimeras sensitized with DNFB produced IFN-γ in response to DNBS at significantly higher levels compared to those from the dLNs of DNFB-sensitized WT/*Ccr4*^{-/-} chimeras (Fig 5, E), indicating enhanced development of DNFB-specific effector CD8⁺ T cells.

Collectively, these data suggest that DC migration following cutaneous antigen

exposure is inhibited by IL-10 derived from cutaneous Treg cells.

IL-10⁺ Treg-cell population in the skin is constitutively renewed by the migration of IL-10⁺ skin-tropic Treg cells

Considering our prior results, a breakdown in maintenance of the IL-10⁺ Treg-cell population in the skin could lead to enhanced percutaneous sensitization. We therefore ultimately investigated how the IL-10⁺ Treg-cell population is maintained in noninflamed skin. Although the distribution of Treg cells in the skin requires T cell migration into the skin,^{14,17} it is unclear to what extent continuous migration of T cells into the skin contributes to the physiological maintenance of the cutaneous Treg-cell population. Thus, to assess the contribution of circulating cells to the maintenance of cutaneous Treg cells, we performed parabiosis experiments in which the blood circulation of a CD45.2 mouse was joined with that of an age- and sex-matched congenic CD45.1 mouse. In the ear skin of CD45.2 parabionts, CD4⁺ T cells (including both Treg and non-Treg CD4⁺ T cells) were composed of 25% and 50% CD45.1⁺ cells after 4 and 8 weeks of parabiosis, respectively (Fig 6, A). These results suggest that cutaneous Treg cells were replenished by circulating cells recruited to the skin, with a turnover time of approximately 8 weeks, rather than being maintained *in situ* by long-lived memory cells.

Next, we asked whether Treg cells outside the skin that were tropic to the skin (i.e., skin-tropic Treg cells) had the capacity to produce IL-10. Given that the adhesion

molecules CD103 and CD62E/P-ligands are functionally important for physiologic Treg-cell migration from the circulation to the skin, skin-tropic Treg cells are thought to express CD103 and CD62E/P-ligands in addition to CCR4.¹⁵⁻¹⁸ Previous studies have demonstrated that Treg cells undergo switching to nonlymphoid tissue-homing phenotypes in secondary lymphoid organs.^{14,17} In particular, skin-dLNs contain a substantial number of Treg cells expressing CD103 and CD62E/P-ligands, which have been shown to also express CCR4.¹⁵⁻¹⁸ Flow cytometric analysis revealed that approximately 20% of the CD103⁺CD62E/P-ligand⁺ Treg-cell population in the skin-dLNs of *Il10*^{Venus} × *Foxp3*^{hCD2} mice expressed Venus while only minimal percentages of other Treg-cell populations expressed Venus (Fig 6, B and C), suggesting that skin-tropic Treg cells in the skin-dLNs were competent for IL-10 production. Collectively, IL-10⁺ skin-tropic Treg cells likely contribute to the renewal and maintenance of the cutaneous IL-10⁺ Treg-cell population via continuous migration into the skin under homeostatic conditions.

DISCUSSION

The present study identifies an immunological mechanism how the skin regulates percutaneous sensitization. We find that percutaneous sensitization is limited through the *in situ* inhibition of cutaneous DC migration by skin-resident Treg cells, and this inhibition is attributed to their IL-10 production. Our results demonstrated that failure of this mechanism promotes the differentiation of IFN- γ -producing effector T cells and IgE-expressing GC B cells/PCs following cutaneous antigen exposure, which can be related to the development of delayed- and immediate-type allergies, respectively.

It has been generally accepted that Treg cells can exert their suppressive effects through their IL-10 production,^{31,32} but in fact, the physiological significance of Treg cell-derived IL-10 *in vivo* has not been well established. Only one previous study has addressed this issue and demonstrated that Treg cell-specific IL-10-deficient mice exhibit spontaneous inflammation in the colon, but not in the skin or lung, and an exacerbated CHS response after hapten sensitization and subsequent challenge through the skin.³³ However, it has not yet been clarified when (during sensitization or challenge) and where (in the skin or dLN) Treg cell-derived IL-10 exerts its inhibitory effects on the induction of CHS. Our study revealed that IL-10 derived from Treg cells in the skin limits percutaneous sensitization, thereby contributing to the inhibition of CHS induction. Possible mechanisms by which IL-10 derived from cutaneous Treg cells inhibits DC migration include both direct and indirect pathways. Previous *in vitro* studies have shown that IL-10 can act on murine BM- and human monocyte-derived

DC-like cells to inhibit lipopolysaccharide-induced CCR7 upregulation, thereby attenuating their migration toward CCL19 and CCL21, suggesting a direct inhibitory effect of IL-10 on DC migration to dLNs.^{34,35} In addition, IL-10 may indirectly suppress DC migration after cutaneous antigen exposure through acting on other skin-resident cells to downregulate the expression of proinflammatory cytokines that promote DC migration.^{36,37}

We show that antigen-specific immune responses in the dLNs induced by cutaneous antigen exposure are enhanced by DT-mediated reduction of cutaneous Treg cells and in *Il10^{-/-}/Ccr4^{-/-}* BM chimeras. In addition, our results suggest that increased migration of antigen-bearing cutaneous DCs contributes to the enhanced responses in the dLNs. However, there are limitations in our study. First, we cannot exclude a possibility that the enhanced responses observed in the dLNs might be in part due to marginal reduction of LN Treg cells caused by DT influx into the dLNs from DT-treated skin. In addition, in *Il10^{-/-}/Ccr4^{-/-}* chimeras, one-half of T and B cells in the dLNs are deficient in IL-10 expression. This may contribute to the enhanced development of effector T cells in the dLNs. Moreover, since IL-10 derived from T and B cells is known to promote the development of T_{FH}, GC B cells, and PCs^{38,39}, the suppressive effect of cutaneous Treg cell-derived IL-10 on the development of T_{FH} and IgE-expressing GC B cells/PCs cannot be directly addressed using this experimental model.

We show that the IL-10⁺ Treg-cell population in the skin is maintained by the constitutive renewal from circulating cells. In addition, our results suggest that the

skin-tropic Treg cells from the skin-dLNs, which are capable of IL-10 production, likely contribute to the renewal of the cutaneous IL-10⁺ Treg-cell population via continuously migrating into the skin under homeostatic conditions. However, it is possible that IL-10⁺ cutaneous Treg cells were also derived in part from non-Treg cells and IL-10⁻ Treg cells that had undergone phenotypic conversion after migration to the skin. Although the precise mechanisms regarding how the number and function of IL-10⁺ Treg cells in the skin are controlled remain to be elucidated in the future, manipulating the number and/or function of skin-resident Treg cells can be a promising strategy for preventing the development of various allergic diseases and improving the efficacy of vaccination through the skin.

In conclusion, our results showed that skin-resident Treg cells limit percutaneous sensitization by inhibiting DC migration through *in situ* IL-10 production. These findings imply that genetic, physiological, and environmental factors impairing IL-10⁺ Treg cell numbers or function in the skin can be risk factors for both skin inflammation and other allergic diseases known to be associated with sensitization through the skin, such as systemic metal allergies, asthma and food allergies.¹⁻³

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FIGURE LEGENDS**FIG 1. Skin-resident Treg cells were capable of IL-10 production in both mice and humans**

A, Representative flow cytometric plots of $\text{TCR}\beta^+\text{CD4}^+$ T cells, and the frequency of Venus^+ cells among Treg ($\text{CD4}^+\text{hCD2}^+$) cells isolated from the skin and dLNs of $\text{Il10}^{\text{Venus}} \times \text{Foxp3}^{\text{hCD2}}$ mice. **B**, Representative images of human eyelid skin from 3 healthy subjects. White arrowheads indicate $\text{IL10}^+\text{FOXP3}^+$ cells. Scale bars = 50 μm **C**, The frequency of Il10^+ cells among all FOXP3^+ cells in 5 high-powered fields (HPF) at 20x magnification from each histological slide. Samples from 3 different donors were examined. **** $P < .0001$. Data are represented as each value (symbols) and their means $\pm$ SDs (bars). ns, Not significant.

FIG 2. Selective reduction of cutaneous Treg cells enhanced DC migration

Representative flow cytometric plots of $\text{TCR}\beta^+\text{CD4}^+$ T cells (**A**) and the number of $\text{CD4}^+\text{GFP}^+$ Treg cells (**B**) from $\text{Foxp3}^{\text{DTR}}$ mice given a single intradermal injection of DT 24 hours before analysis. **C**, Representative flow cytometric plots of $\text{CD45}^+\text{B220}^-$ cells and the number of FITC^+ cells in the dLNs of WT and $\text{Foxp3}^{\text{DTR}}$ mice 24 hours after FITC application. Purified CD8^+ T cells from OT-I mice (**D**) or CD4^+ T cells from OT-II mice (**E**) were incubated with FITC^+ DCs from WT and $\text{Foxp3}^{\text{DTR}}$ mice in the presence or absence of the indicated peptide for 72 hours. $\text{IFN-}\gamma$ (**D**) and IL-13 (**E**) in culture supernatants as measured by ELISA and the Cytometric Bead Array,

respectively. $*P < .05$, $**P < .01$, $***P < .001$. Data are represented as each value (symbols) and their means $\pm$ SDs (bars). *ns*, Not significant. *nd*, Not detected.

FIG 3. Sensitization through the skin with reduced Treg cells resulted in enhanced antigen-specific immune responses

A, Number of effector ($CD44^+CD62L^+$) $CD8^+$ T cells from dLNs of WT and $Foxp3^{DTR}$ mice 5 days after topical application of DNFB. **B**, IFN- γ production from DNBS-stimulated $CD8^+$ T cells. $CD8^+$ T cells from dLNs of mice 5 days after sensitization with DNFB were cocultured with $CD11c^+$ splenic cells from naïve mice in the presence or absence of DNBS for 72 hours. **C**, Schematic representation of CHS. WT and $Foxp3^{DTR}$ mice were sensitized with 0.5% (v/v) DNFB on DT-treated ears on day 0. Mice were challenged with 0.3% (v/v) DNFB on the contralateral ear on day 5. **D**, Kinetics of ear swelling. **E**, Representative H&E staining of ear skin from WT and $Foxp3^{DTR}$ mice 24 hours after challenge. Scale bars = 50 μ m. **F**, Number of T_{FH} cells and GC B cells from dLNs of WT and $Foxp3^{DTR}$ mice 6 days after intradermal administration of NP-OVA/alum. **G**, The number of NP-IgG1/IgE-expressing GC B cells or PCs. $*P < .05$, $**P < .01$, $***P < .001$, $****P < .0001$. Data are represented as each value (symbols) and their means $\pm$ SDs (bars). *ns*, Not significant. *nd*, Not detected.

FIG 4. IL-10 suppressed CCR7 expression on DCs from cultured human skin

A, Expression levels of CCR7 on DCs in human skin biopsy samples before and after incubation for 3 days. Representative histograms (**B**) and fold-changes of CCR7 expression (**C**) on DCs cultured with the indicated concentrations of IL-10 compared to DCs cultured in the absence of IL-10. * $P < .05$, ** $P < .01$, **** $P < .0001$. Data are represented as each value (*symbols*) and their means $\pm$ SDs (*bars*).

FIG 5. IL-10 deficiency in cutaneous Treg cells resulted in increased migration of DCs after cutaneous antigen exposure

A, Schematic representation of the mixed BM chimera experimental set-up. **B**, Chimerism (% CD45.1⁺ cells) of immune cells in skin from $Ccr4^{+/+}$ (CD45.1)/ $Ccr4^{-/-}$ (CD45.2) chimeras. **C**, Schematic representation of the mixed BM chimera experimental set-up. **D**, Number of FITC⁺ cells in the dLNs of chimeras treated topically with FITC 60 days after BM transplantation. **E**, IFN- γ levels present in culture supernatants from DNBS-stimulated T cells. CD8⁺ T cells from dLNs of mice 5 days after sensitization with DNFB were cocultured with CD11c⁺ splenic cells from naïve mice in the presence or absence of DNBS for 72 hours. * $P < .05$, **** $P < .0001$. Data are represented as each value (*symbols*) and their means $\pm$ SDs (*bars*). *ns*, Not significant.

FIG 6. Skin-tropic Treg cells in skin-dLNs expressed IL-10

A, Chimerism (% CD45.1⁺ cells) of CD4⁺Foxp3⁻ non-Treg and CD4⁺Foxp3⁺ Treg cells

591 in skin from untreated CD45.2⁺ parabionts of CD45.1/CD45.2 parabiotic pairs. Each bar
592 represents an individual mouse. Representative flow cytometric plots of TCRβ⁺CD4⁺ T
593 cells (**B**) and the frequency of Venus⁺ cells among CD4⁺hCD2⁺ cells (**C**) from dLNs of
594 *Il10*^{Venus} × *Foxp3*^{hCD2} mice. *****P* < .0001. Data are represented as each value
595 (symbols) and their means ± SDs (bars).

596

FIG 1
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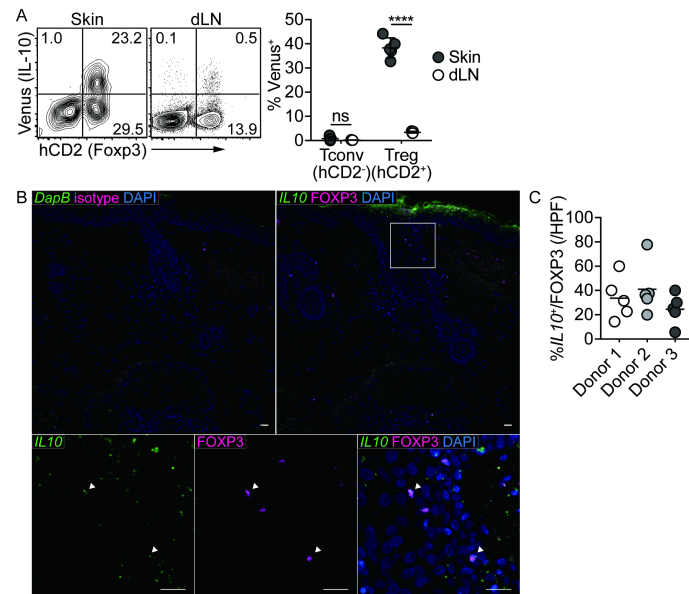


FIG 2

Hanakawa et al.

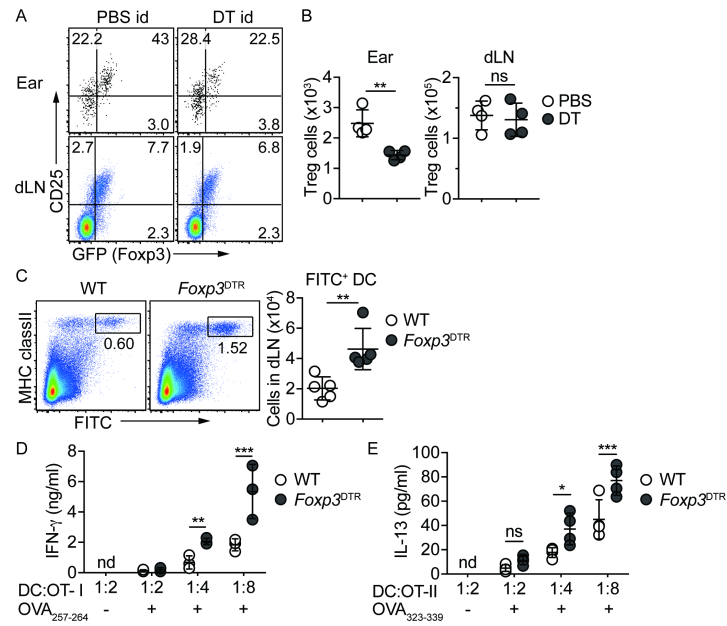


FIG 3

Hanakawa et al.

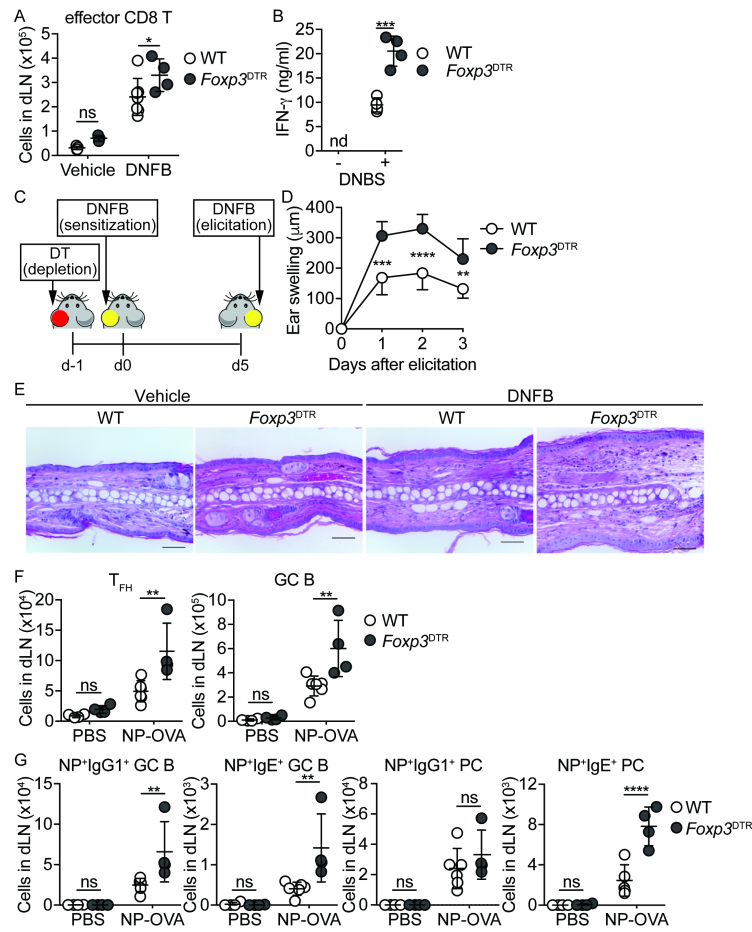


FIG 4
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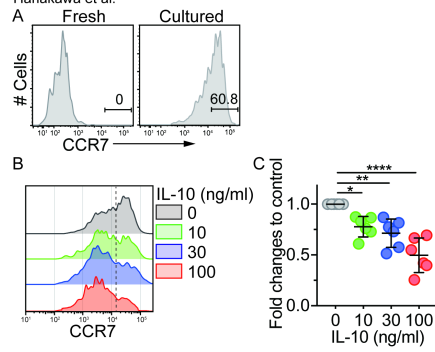


FIG 5

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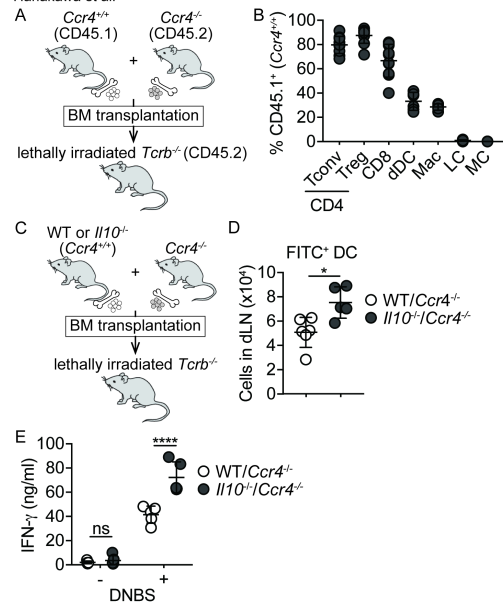
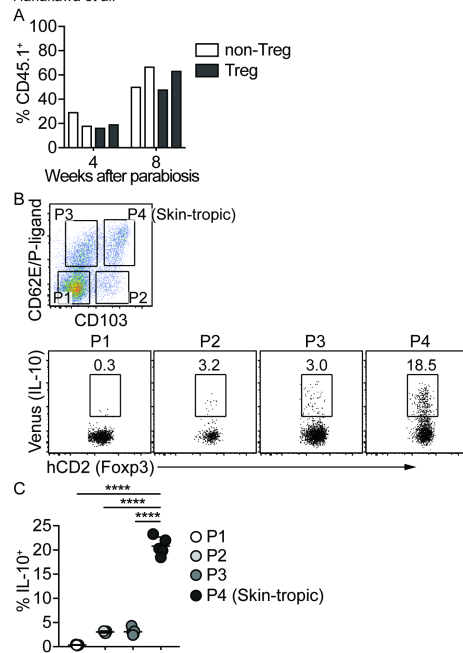


FIG 6
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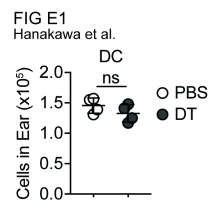


FIG E2
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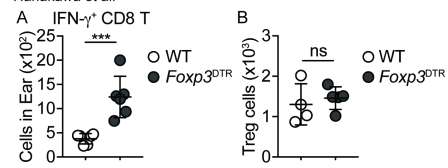


FIG E3
Hanakawa et al.

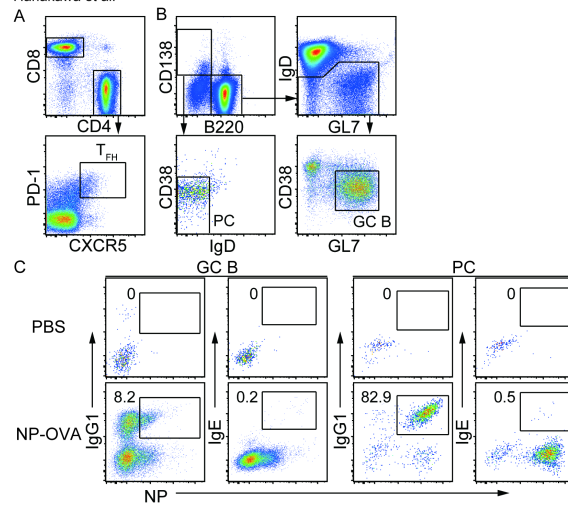


FIG E4
Hanakawa et al.

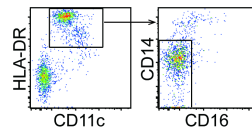


FIG E5
Hanakawa et al.

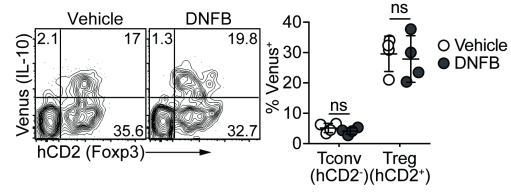
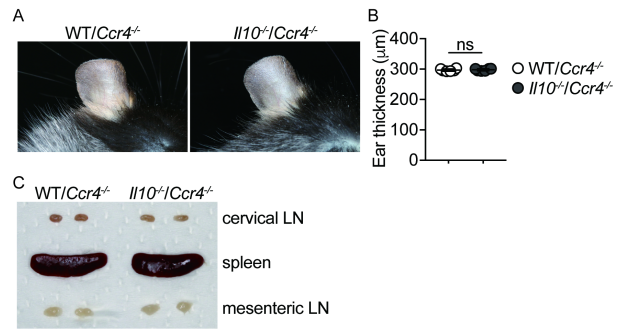


FIG E6
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ONLINE SUPPLEMENTAL INFORMATION

SUPPLEMENTAL METHODS

Mice

C57BL/6N mice were purchased from Japan SLC (Kyoto, Japan). *Foxp3*^{DTR},¹ *Foxp3*^{hCD2},² *Il10*^{Venus},³ *Ccr4*^{-/-},⁴ *Tcrb*^{-/-},⁵ and *Il10*^{-/-6} mice were obtained as described previously. Age- and sex-matched mice were used as controls at 6.5 to 9 weeks of age in this study. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Kyoto University Graduate School of Medicine.

Antibodies

Pacific Blue anti-I-A/I-E (M5/114.15.2), Pacific Blue anti-IL-17A (T11-18H10.1), FITC anti-CD4 (RM4-5), FITC anti-IgE (MRE-1), FITC anti-human CD11c (3.9), Peridinin-chlorophyll-protein (PerCP)-Cyanine5.5 anti-CD8a (53-6.7), PerCP-Cyanine5.5 anti-human CD45 (H130), phycoerythrin (PE) anti-CD11c (N418), PE anti-CD62L (MEL-14), PE anti-PD-1 (RMP1-30), PE anti-human CD16 (3G8), PE-Cyanine7 anti-CD38 (90), PE-Cyanine7 anti-CD45.1 (A20), PE-Cyanine7 anti-TCR β (H57-597), allophycocyanin (APC) anti-CD25 (PC61), APC anti-human HLA-DR (L243), and APC-Cyanine7 anti-IgD (11-26c.2a) antibodies were purchased from BioLegend (San Diego, Calif, USA). Biotin anti-CD138 (281-2), Biotin anti-CXCR5 (2G8), Brilliant Ultraviolet (BUV) 395 anti-CD45 (30-F11), BUV anti-B220 (RA3-6B2), BUV395 anti-human CCR7 (150503), Pacific Blue anti-human

CD2 (TS1/8), V450 anti-CD4 (RM4-5), V450 anti-CD8a (53-6.7), V450 anti-CD45 (30-F11), V450 anti-IgG1 (A85-1), Brilliant Violet (BV) 605 anti-CD4 (RM4-5), BV605 Streptavidin, FITC anti- $\gamma\delta$ TCR (GL3), PerCP-Cyanine5.5 anti-B220 (RA3-6B2), PerCP-Cyanine5.5 anti-CD3 ϵ (145-2C11), PE anti- $\gamma\delta$ TCR (GL3), APC anti-CD4 (RM4-5), APC anti-IFN- γ (XMG1.2), and rat anti-mouse CD16/CD32 (2.4G) antibodies were purchased from BD Biosciences (San Diego, Calif, USA). eFluor 450 anti-CD45.1 (A20), FITC anti-CD8b (eBioH35-17.2), PerCP-eFluor710 anti-CD8b (eBioH35-17.2), PE anti-CD4 (RM4-5), PE rat IgG2a kappa isotype control (eBR2a), PE-Cyanine7 anti-human CD14 (61D3), APC anti-CD44 (IM7), APC anti-Foxp3 (FJK-16s), eFluor 660 anti-GL7 (GL-7), APC Armenian hamster IgG isotype control (eBio299Arm), and APC-Cyanine7 anti-B220 (RA3-6B2) antibodies were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Preparation of single-cell suspensions

Skin dLNs were minced with scissors and incubated for 25 minutes at 37°C in RPMI 1640 medium containing 10% fetal calf serum (FCS), 1% Penicillin-Streptomycin, 1% Sodium pyruvate, 1% MEM NEAA, 500 U/ml collagenase type II (Worthington Biochemical., Freehold, NJ, USA) and 0.2 mg/ml DNase I (Sigma-Aldrich). Ears of mice were excised and separated into dorsal and ventral sheets. Human skin samples were collected with 6 mm biopsy punches (Kai medical, Gifu, Japan), subcutaneous fat tissue was removed with a number 20 scalpel (Kai medical), and the skin biopsies were

cut into small pieces with scissors. Ear pinnae and human skin were digested for 105 minutes at 37°C in 5% CO₂ in RPMI 1640 containing 10% FCS, 1% Penicillin-Streptomycin, 1% Sodium pyruvate, 1% MEM NEAA, 0.25 mg/ml Liberase TL (Roche, Basel, Switzerland) and 0.3 mg/ml DNase I. For the intracellular staining of cytokines, ear pinnae were incubated in digestion solution with 10 µg/ml Brefeldin A (Sigma-Aldrich). Digested skin sheets were placed in a Medicon (BD Biosciences) and homogenized with a Medimachine for 7 minutes. Collected cell suspensions from ears and dLNs were passed through a 40-µm pore-size cell strainer to remove cell clumps and obtain single-cell suspensions. Human skin was mashed through a 40-µm pore-size cell strainer to generate single cell suspensions. Bone marrow (BM) cells were obtained from donor mice (8 to 10 weeks old) by flushing the femurs and tibias with cold sterile RPMI 1640 medium containing 2% FCS. Cell suspensions were passed through a 40-µm pore-size cell strainer and incubated in hemolytic buffer for 3 minutes at room temperature, then washed and resuspended in cold sterile RPMI 1640 medium containing 2% FCS.

Flow cytometry

Cells were stained with fixable viability dye eFluor 506 or 780 (Thermo Fisher Scientific) according to the manufacturer's instructions for the exclusion of dead cells. Next, nonspecific antibody binding was blocked with an anti-CD16/32 antibody (BD Biosciences), and then cells were stained for surface antigens. After surface staining,

cells were fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences) and stained intracellularly. NP-specific immunoglobulin was intracellularly stained with NP-PE purchased from Biosearch Technologies (Petaluma, CA, USA) as described previously.^{26,51} For CD62E/P-ligand staining, cells were incubated with E-selectin Fc Chimera and P-selectin Fc Chimera (R&D Systems, Minneapolis, MN, USA) in Hanks' Balanced Salt Solution(+) containing 2% FCS and counter-stained with PE-conjugated human IgG1 antibody (eBioscience). Samples were assessed using a BD LSRFortessa cell analyzer (BD Biosciences), and data were analyzed using FlowJo software (TreeStar, San Carlos, Calif, USA). The numbers of each cell subset were evaluated by Flow-Count Fluorospheres (Beckman Coulter, Brea, Calif, USA) and presented as numbers per tissue.

Human skin samples

Human skin was obtained from healthy donors with no history of diseases undergoing plastic surgery at Kyoto University Hospital and other collaborating hospitals in Japan. This study was approved by the ethics committee of the Kyoto University Graduate School of Medicine (R0743).

RNA *in situ* hybridization and immunofluorescence

Human healthy skin from the upper eyelid was fixed in 10% (vol/vol) neutral-buffered formalin at room temperature for 24-36 hours, dehydrated, and embedded in paraffin.

Tissue sections cut at 5- μ m thicknesses were processed for RNA *in situ* detection using an RNAscope 2.5 HD Assay-RED Kit (Advanced Cell Diagnostics, Newark, Calif, USA) according to the manufacturer's instructions with slight modifications. Target retrieval was performed at 95°C for 5 minutes using an electric pot instead of boiling for 15 minutes, which causes nonspecific signals in our environment. Slides were incubated with protease plus at 40°C for 15 minutes. Sequences of the probes used in the study were as follows: Hs-IL10 (NM_000572.2) and Negative Control Probe DapB. For immunofluorescence, sections were blocked with Image-iT FX Signal Enhancer (Thermo Fisher Scientific) for 30 minutes at room temperature, followed by incubation with an anti-human FOXP3 antibody (236A/E7; Thermo Fisher Scientific) or mouse IgG1 isotype control antibody (BioLegend) overnight at 4°C. Samples were washed and incubated for 2 hours with Alexa Fluor 647-labeled goat anti-mouse IgG antibodies (Thermo Fisher Scientific) and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich). Finally, the sections were embedded with EcoMount (Biocare Medical, Pacheco, Calif, USA). Images were captured using a Nikon A1 confocal microscope (Nikon, Tokyo, Japan) equipped with a 20 $\times$ /0.75 objective lens. Stained cells were shown in pseudo colors processed with Imaris software (Bitplane, Zurich, Switzerland) and quantified by manual counts per power field.

***In vivo* DC migration assay**

Mice were painted on the ear with 20 μ l of 2% (w/v) FITC (Sigma-Aldrich) dissolved

in acetone/dibutyl phthalate (1:1). Samples of cervical LNs were taken for flow cytometric analysis 24 hours after FITC application.

Assessment of T-cell priming activities of DCs

CD11c⁺ cells isolated from dLNs of WT or *Foxp3*^{DTR} mice 24 hours after sensitization with FITC and CD8⁺ T cells from dLNs and spleens of naïve OT-I mice were enriched using MHC class II- and CD8a-specific MACS beads (Miltenyi Biotec), respectively, according to the manufacturer's protocols. CD4⁺ T cells from dLNs and spleens of naïve OT-II mice were enriched using CD4 T cell Isolation Kit (Miltenyi Biotec). MHC class II⁺ cells were further sorted into CD11c^{mid} MHC class II^{hi} FITC⁺ migratory DCs with BD FACS Aria II (BD Biosciences). FITC⁺ cells (5x10³ cells/well) and each listed ratio of CD8⁺ T cells was cocultured with or without 1 μM OVA₂₅₇₋₂₆₄ (H-2K^b OVA peptide; MBL, Nagoya, Japan). FITC⁺ cells (3x10³ cells/well) and each listed ratio of CD4⁺ T cells was cocultured with or without 1 μM OVA₃₂₃₋₃₃₉ (I-A^d OVA peptide; AnaSpec, Fremont, Calif, USA). For the measurement of cytokine production, culture supernatants were collected 72 hours after incubation.

Percutaneous sensitization

For DNFB sensitization, mice were treated with 20 μl of 0.5%(v/v) DNFB (Nacalai Tesque) in acetone/olive oil (4:1) on the DT-injected ear skin 24 hours after injection. For OVA immunization, mice were intradermally immunized with the antigen NP-OVA

(Biosearch Technologies) in alum adjuvant (Cosmo Bio, Tokyo, Japan) 24 hours after DT-injection. The antigens were brought to a concentration of 5 mg/ml in PBS and then mixed with an equal volume of 20 mg/ml alum adjuvant. In the experiments, 10 μ l per site were injected into the ventral side of the ear, generating immune responses in the draining cervical LNs.

Contact hypersensitivity

Five days after DNFB sensitization, the DT-noninjected contralateral ears of sensitized mice were challenged with 20 μ l of 0.3% (v/v) DNFB or vehicle alone. Ear swelling was measured daily after challenge, and data were expressed as the ear thickness at each time point minus the baseline thickness before challenge.

***In vitro* DNBS stimulation**

CD8⁺ T cells from dLNs of mice 5 days after sensitization with DNFB and CD11c⁺ splenic cells from naïve mice were enriched by CD8a- and CD11c-specific MACS beads (Miltenyi Biotec), respectively, according to the manufacturer's protocols. CD8⁺ T cells (5×10^5 cells/well) and CD11c⁺ cells (1×10^5 cells/well) were cocultured with or without 100 μ g/ml DNBS (Alfa Aesar, Haverhill, MA, USA). For the measurement of cytokine production, culture supernatants were collected 72 hours after incubation.

Cytokine measurement

The amount of IFN- γ and IL-13 present in cultured supernatants was measured by ELISA (BD) and Cytokine Beads Array (BD) according to the manufacturer's instructions, respectively.

Histology

For histological examination, ear skin samples were fixed with 10% formalin overnight and then embedded in paraffin. Sections along the median plane of the ear with a thickness of 5 μ m were prepared and subjected to staining with hematoxylin and eosin (H&E). H&E-stained sections were imaged using a digital microscope (BIOREVO BZ-9000) with a 40 $\times$ magnification lens.

Organ culture of human skin

Human skin from the upper eyelid, cheek, forearm or inguinal region was collected with a 6 mm biopsy punch (Kai medical), and subcutaneous fat tissue was removed with a number 20 scalpel (Kai medical). Skin biopsy was floated, with the dermal side in contact with complete RPMI 1640 medium supplemented with 10% FCS, 1% Penicillin-Streptomycin, 1% Sodium pyruvate and 1% MEM NEAA (GIBCO) for 3 days. For suppression, the indicated concentrations of human recombinant IL-10 (Peprotech) were added.

Parabiosis

Pairs of parabiotic mice consisting of CD45.1 and CD45.2 mice were generated as described previously.⁷ The CD45.1/CD45.2 parabiotic pairs were analyzed 4 and 8 weeks after the establishment of parabiosis. To confirm efficient blood mixing in parabiotic mice, the percentages of CD45.1⁺ and CD45.2⁺ cells among the blood leukocytes were analyzed in each mouse.

Statistical analysis

Unless otherwise indicated, the results are presented as the means $\pm$ standard deviation value and are representative of at least three independent experiments. Statistical differences between two groups were evaluated using the Mann-Whitney U test. Comparisons among groups were performed using the Tukey-Kramer multiple comparisons test. *P* values $< .05$ were considered significantly different. All statistical tests were performed using GraphPad Prism 7.0b software.

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SUPPLEMENTAL FIGURE LEGENDS**FIG E1. Cutaneous DCs were not directly affected by intradermal injection of DT**

The number of CD45⁺CD64⁻CD11c⁺MHC class II⁺ DCs in the treated ear 24 hours after DT-injection. Data are represented as each value (*symbols*) and their means $\pm$ SDs (*bars*). *ns*, Not significant.

FIG E2. Selective reduction of skin Treg cells augmented the CHS response

A, Quantification by flow cytometry of IFN- γ ⁺CD8b⁺ T cells in the skin of mice 12 hours after challenge. **B**, The number of Foxp3⁺ Treg cells in contralateral non-sensitized ear from WT and *Foxp3*^{DTR} mice 5 days after sensitization with DT injection. ****P* < .001. Data are represented as each value (*symbols*) and their means $\pm$ SDs (*bars*). *ns*, Not significant.

FIG E3. Identification of T and B cell subsets

Representative flow cytometric plots of TCR β ⁺CD4⁺ T_{FH} cells (**A**) and B cell subsets gated on live CD3e⁻ cells (**B**) in dLNs 6 days after intradermal administration of NP-OVA/alum. Cells are subdivided into PCs (B220⁻CD138⁺CD38⁻IgD⁻) and GC B cells (B220⁺IgD⁻CD38⁻GL7⁺). **C**, Representative flow cytometric plots of NP-IgG1/IgE-expressing GC B cells or PCs in dLNs 6 days after immunization.

FIG E4. Identification of DCs in cultured human skin

Identification of human DCs in skin biopsy samples. DCs were defined as CD45⁺CD11c⁺HLA-DR⁺CD14⁻CD16⁻ cells.

FIG E5. IL-10 expression levels of cutaneous Treg cells were not changed by topical DNFB application

Representative flow cytometric plots of TCRβ⁺CD4⁺ cells, and the frequency of Venus⁺ cells among Treg (CD4⁺hCD2⁺) cells isolated from sensitized skin of *Il10*^{Venus} × *Foxp3*^{hCD2} mice 24 hours after vehicle or DNFB application. Data are represented as each value (*symbols*) and their means ± SDs (*bars*).

FIG E6. *Il10*^{-/-}/*Ccr4*^{-/-} mixed BM chimeras did not develop spontaneous inflammation

A, Phenotypic appearances 116 days after BM transplantation. **B**, Ear thicknesses. **C**, Phenotypic appearances of LNs and spleens at the same date of (**A**). Data are represented as each value (*symbols*) and their means ± SDs (*bars*). *ns*, Not significant.