

Hair follicle–derived IL-7 and IL-15 mediate skin-resident memory T cell homeostasis and lymphoma

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The skin harbors a variety of resident leukocyte subsets that must be tightly regulated to maintain immune homeostasis. Hair follicles are unique structures in the skin that contribute to skin dendritic cell homeostasis through chemokine production. We demonstrate that CD4⁺ and CD8⁺ skin-resident memory T cells (T_{RM} cells), which are responsible for long-term skin immunity, reside predominantly within the hair follicle epithelium of the unperturbed epidermis. T_{RM} cell tropism for the epidermis and follicles is herein termed epidermotropism. Hair follicle expression of IL-15 was required for CD8⁺ T_{RM} cells, and IL-7 for CD8⁺ and CD4⁺ T_{RM} cells, to exert epidermotropism. A lack of either cytokine in the skin led to impaired hapten-induced contact hypersensitivity responses. In a model of cutaneous T cell lymphoma, epidermotropic CD4⁺ T_{RM} lymphoma cell localization depended on the presence of hair follicle–derived IL-7. These findings implicate hair follicle–derived cytokines as regulators of malignant and non-malignant T_{RM} cell tissue residence, and they suggest that the cytokines may be targeted therapeutically in inflammatory skin diseases and lymphoma.

Hair follicles are unique structures in the mammalian skin that provide a niche for keratinocyte and melanocyte stem cells^{1,2}. Various leukocyte subsets localize to hair follicles³. We recently demonstrated that hair follicles recruit skin dendritic cells to sites of minor trauma⁴. Keratinocytes in the hair follicle infundibulum and isthmus produce the chemokines CCL2 and CCL20, recruiting myeloid cells after experiencing physical perturbation. A subset of keratinocytes in the suprabasal layer of the follicular bulge region express another chemokine, CCL8, which prevents local Langerhans cell (LC) accumulation, a mechanism that may protect bulge stem cells from excessive leukocyte infiltration⁴. These data establish that hair follicles actively promote immune homeostasis.

T cells that reside in peripheral tissues have been described in recent years, and their importance is now well established^{5–9}. Skin-resident T_{RM} cells display an effector memory phenotype, and are generated after immunological insults such as viral infection^{6,8}. In the context of infection, CD4⁺ memory T cells accumulate primarily in the dermis, whereas CD8⁺ T_{RM} cells accumulate within the epidermis⁷. Both T cell subsets exhibited tropism to the hair follicles⁷. Skin T_{RM} cells express CD69 and CD103 (ref. 10). CD69 suppresses sphingosine-1-phosphate receptor 1 expression, which prevents T cells from emigrating from lymphoid organs or other tissues into circulation^{11,12}. CD103-mediated retention of T cells in the skin probably occurs by adhesion to E-cadherin¹³. The non-migratory nature of CD8⁺ T_{RM} cells has been established in parabiotic mice⁸, and CD4⁺ T_{RM} cells in human skin have been grafted onto immunodeficient mice¹⁴.

Infiltration of T cells into the epidermis is a prominent feature in both inflammatory and neoplastic human diseases, including graft-versus-host disease, drug eruptions and cutaneous T cell lymphoma (CTCL). In fixed drug eruption, CD8⁺ T cells attack the epidermis to cause keratinocyte cell death in the presence of the drug(s)¹⁵. After clinical resolution, CD8⁺ T cells with a memory phenotype persist within the epidermis^{15,16}. The majority of CTCL is caused by CD4⁺ lymphoma cells¹⁷. In the classic form—mycosis fungoides—lymphoma cells with a T_{RM} cell phenotype infiltrate the epidermis, including the follicular epithelium^{18,19}, and they slowly accumulate and proliferate to form tumors. Such epidermis- and follicle-infiltrating T cells are termed epidermotropic T cells²⁰.

Given the importance of T_{RM} cells in conferring long-term immunological memory^{6,8} and regulatory functions that maintain immunological homeostasis^{13,21,22}, elucidation of mechanisms that support long-term persistence in the skin may provide insight into T cell homeostasis in health and disease. Here we demonstrate that the epidermotropism of T_{RM} cells is supported by the hair follicle–derived cytokines IL-7 and IL-15.

RESULTS

CD4⁺ and CD8⁺ T cells in steady-state epidermis

To characterize T_{RM} cells in the epidermis, we prepared vertical sections of frozen skin samples taken from unmanipulated adult C57BL/6J mice, and then visualized CD4⁺ and CD8⁺ T cells

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using immunofluorescence microscopy. We routinely observed small numbers of both CD4⁺ and CD8⁺ T cells in the follicular epithelium (Fig. 1a). Visualization of the basement membrane using integrin α_6 staining confirmed that both CD4⁺ and CD8⁺ T cells resided within the follicular epithelium (Supplementary Fig. 1a). Staining epidermal sheets revealed that CD8⁺ T cells were present both in hair follicles and in the interfollicular epidermis, whereas CD4⁺ T cells were localized exclusively around hair follicles (Supplementary Fig. 1b). In flow cytometry analysis of epidermal cell suspensions, exclusion of LCs and dendritic epidermal T cells enabled the identification of small numbers of CD4⁺ and CD8⁺ T cells, consistent with the immunofluorescence microscopy results (Fig. 1b). The number of CD4⁺ T cells in the epidermis was comparable to that in the dermis (Fig. 1c). CD8⁺ T cells were found exclusively in epidermal cell suspensions (Fig. 1c)⁷. Contamination of the

epidermal preparation with dermal leukocytes and vice versa seemed unlikely (Supplementary Fig. 1c,d).

Phenotypic analysis suggests that most of these CD4⁺ and CD8⁺ T cells are effector memory T cells, as assessed by their cell surface expression of CD44 and CD62L, and they are resident cells, as suggested by CD103 and CD69 co-expression (Fig. 1d). CD4⁺ T cells in the epidermis expressed slightly lower levels of CD103 than did those in the dermis (Fig. 1e). Furthermore, the epidermis contained fewer CD4⁺ FoxP3⁺ regulatory T_{RM} cells than did the dermis (Fig. 1f), suggesting that the organization of CD4⁺ T_{RM} cells in the two skin compartments may be differentially regulated. Hereafter, memory T cells that reside in the epidermis and the follicles will be referred to as epidermotropic T_{RM} cells, not to indicate their entity as a distinct T cell subset, but solely to describe their localization in the skin.

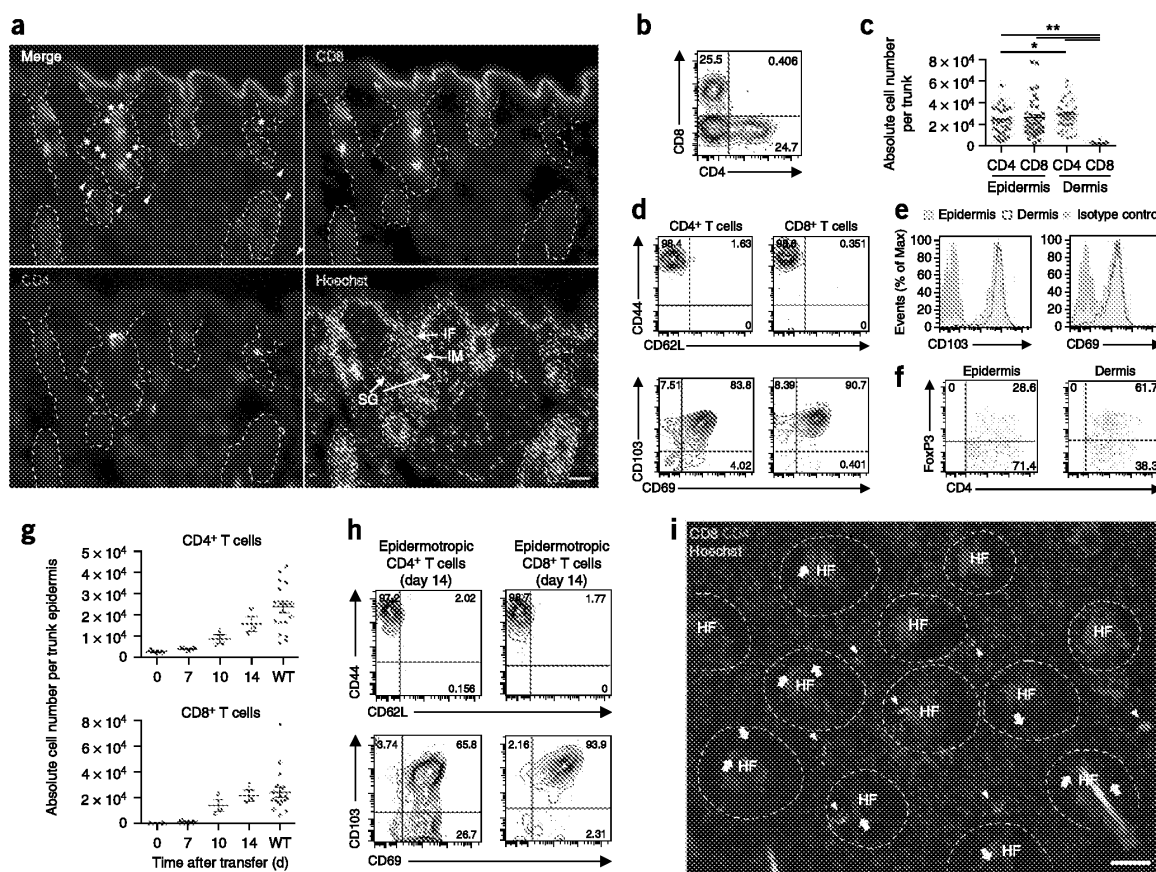


Figure 1 Epidermotropic CD4⁺ and CD8⁺ T_{RM} cells associate with hair follicles. (a) Skin sections from wild-type (WT) mice, stained as indicated. Dotted lines delineate hair follicles. Asterisks and arrowheads depict CD4⁺ T cells in epidermis and dermis, respectively. Scale bar, 50 μ m. IF: infundibulum, IM: isthmus, SG: sebaceous glands. (b) Flow cytometry analysis of CD45⁺CD3⁺MHCII⁺TCR⁺γδ⁻ epidermal cells from WT mice. Numbers represent frequencies (%) of gated subsets among the analyzed population. (c) T cell numbers in the epidermis and dermis. * P < 0.05, ** P < 0.01 (unpaired two-tailed Student's t -test). (d) CD44 and CD62L, and CD103 and CD69 expression on T cells in the epidermis. (e) CD103 and CD69 expression levels on CD4⁺ T cells in the epidermis (blue) and dermis (red) with isotype controls (gray). (f) Frequency of CD4⁺FoxP3⁺ T_{RM} cells in the epidermis and dermis. (g) Number of donor T cells in Rag2^{-/-} recipient epidermis monitored by flow cytometry at the indicated dates after adoptive transfer of WT splenocytes. (h) Epidermotropic T cells from g were analyzed using flow cytometry as in d, 14 d after transfer. (i) Epidermal sheets of a mouse from g, day 14 after transfer, were stained as indicated. Dashed lines delineate hair follicles (HF), arrows and arrowheads indicate CD4⁺ and CD8⁺ T cells, respectively. Scale bar, 100 μ m. Data are from one experiment representative of three independent experiments with three mice per group (a,b,d–i), or from ten independent experiments with a total of 42 mice (c). Each symbol represents an individual mouse; horizontal lines depict the mean; error bars indicate s.e.m. (c,g).

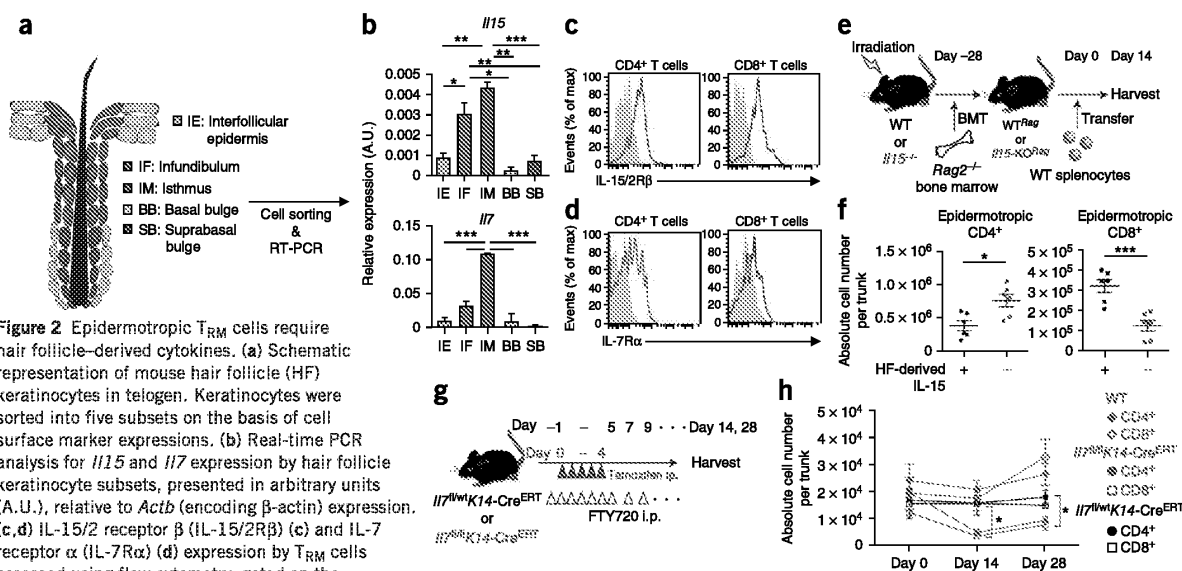


Figure 2 Epidermotropic T_{RM} cells require hair follicle-derived cytokines. (a) Schematic representation of mouse hair follicle (HF) keratinocytes in telogen. Keratinocytes were sorted into five subsets on the basis of cell surface marker expressions. (b) Real-time PCR analysis for *Il15* and *Il7* expression by hair follicle keratinocyte subsets, presented in arbitrary units (A.U.), relative to *Actb* (encoding β -actin) expression. (c, d) IL-15/2 receptor β (IL-15/2R β) (c) and IL-7 receptor α (IL-7R α) (d) expression by T_{RM} cells assessed using flow cytometry, gated on the CD45 $^{+}$ CD3 $^{+}$ MHCII $^{-}$ TCR $\gamma\delta^{-}$ population ($n = 3$). (e) Experimental scheme for the analysis of T_{RM} cell dependence on hair follicle-derived IL-15. Wild-type (WT) or *Il15* $^{-/-}$ mice were reconstituted with *Rag2* $^{-/-}$ bone marrow (WT ^{Rag} or *Il15*-KO ^{Rag}), and were adoptively transferred with WT splenocytes. BMT: bone marrow transplantation. (f) Epidermotropic T_{RM} cell numbers in recipient WT ^{Rag} or *Il15*-KO ^{Rag} mice ($n = 6$). Symbols represent individual mice. (g) Experimental scheme for analyzing T_{RM} cell dependence on hair follicle-derived IL-7. Indicated mice were treated with tamoxifen intraperitoneally (i.p.) on days 0–4. FTY720 was injected i.p. from day –1 for 7 consecutive days and every other day thereafter, until the time of tissue harvest. (h) Epidermotropic T_{RM} cell numbers after tamoxifen-induced ablation of hair follicle IL-7 ($n = 3$). Data are representative of three (b–d) or two (f, h) independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (unpaired two-tailed Student's t -test). Horizontal lines depict the mean, and error bars indicate s.e.m. in all graphs.

T_{RM} cells associate with hair follicles during epidermal entry

T_{RM} cells are generated during skin infection and accumulate not only at sites of primary inoculation, but also at distant sites^{9,23}. To model this distribution of T_{RM} cells, we adoptively transferred wild-type (WT) splenocytes into *Rag2* $^{-/-}$ mice, which lack lymphocytes. After being transferred into a lymphopenic environment, donor T cells undergo homeostatic proliferation, and we hypothesized that such T cells might distribute to peripheral tissues such as the epidermis and skin. Indeed, CD4 $^{+}$ and CD8 $^{+}$ T cells appeared in the epidermis of *Rag2* $^{-/-}$ recipients 10 d after transfer, and by day 14, their numbers had reached levels detectable in WT mice (Fig. 1g). Donor T cells displayed effector memory and resident cell phenotypes (Fig. 1h), and thus represent epidermotropic T_{RM} cells.

Visualization of CD4 $^{+}$ and CD8 $^{+}$ T_{RM} cells during active epidermal repopulation revealed close association with hair follicles (Fig. 1i). CD4 $^{+}$ T_{RM} cells initially appeared within the dermis, accumulated around hair follicles in frozen sections on day 7 after transfer, and then distributed within the epidermis and dermis thereafter (Supplementary Fig. 1e). In contrast to CD4 $^{+}$ T_{RM} cells, CD8 $^{+}$ T_{RM} cells appeared directly within the interfollicular epidermis, and then accumulated around the hair follicles (Supplementary Fig. 1e, f). Thus, the anatomical modes of entry for CD4 $^{+}$ and CD8 $^{+}$ T_{RM} cells seem to be distinct.

Hair follicle keratinocytes express *Il15* and *Il7*

IL-15 and IL-7 are important cytokines that enable the generation and maintenance of memory T cells^{24,25}. Our previous study⁴ revealed that hair follicle keratinocyte subsets exhibit distinct chemokine expression profiles. To determine whether hair follicle keratinocytes expressed mRNA encoding IL-15 and IL-7, we sorted epidermal keratinocytes

into those from the interfollicular epidermis, infundibulum, isthmus, basal layer bulge or suprabasal layer bulge⁴ (Fig. 2a). Similarly to previously described patterns of chemokine expression⁴, real-time PCR analysis revealed that both *Il15* and *Il7* mRNA were predominantly expressed by keratinocytes in the infundibulum and isthmus (Fig. 2b). Because most of the mouse pelage hair follicles are in telogen, we studied the vibrissae to determine whether cytokines were expressed during anagen. The transient portions of anagen hair follicles did not express *Il7*, but the bulb expressed low levels of *Il15* (Supplementary Fig. 2a). We also analyzed cytokine mRNA expression in major leukocyte subsets in the epidermis and found that LCs, but not dendritic epidermal T cells, expressed *Il15* (Supplementary Fig. 2b). Both leukocyte subsets lacked *Il7* expression (data not shown).

Epidermotropic T_{RM} cells require hair follicle-derived cytokines

Analysis of CD4 $^{+}$ and CD8 $^{+}$ T_{RM} cells using flow cytometry revealed that both T cell subsets expressed IL-15/2R β and IL-7R α (Fig. 2c, d). Given the unavailability of an animal model enabling conditional ablation of IL-15, we generated bone marrow (BM) chimeric mice to determine whether epidermotropic T_{RM} cells were influenced by hair follicle-derived cytokines. Reconstitution of WT or *Il15* $^{-/-}$ mice with BM from *Rag2* $^{-/-}$ mice led to the generation of lymphopenic mice that either expressed (WT ^{Rag}) or lacked (*Il15*-knockout(KO) ^{Rag}) IL-15 in peripheral tissues, including the skin (Fig. 2e). We then transferred WT splenocytes into these BM chimeric mice and analyzed the numbers of epidermotropic T_{RM} cells at day 14 after the transfer (Fig. 2e). The numbers of epidermotropic CD4 $^{+}$ T_{RM} cells had slightly increased in recipient *Il15*-KO ^{Rag} mice, whereas epidermotropic CD8 $^{+}$ T_{RM} numbers had been reduced (Fig. 2f). The numbers of CD4 $^{+}$ and

CD8⁺ T cells in the spleen were comparable in recipient *Il15-KO^{Rag}* and *WT^{Rag}* mice (Supplementary Fig. 2c), indicating that the difference in the numbers of epidermotropic CD8⁺ T_{RM} cells was due to the loss of IL-15 in the skin. The numbers of epidermotropic CD8⁺ T_{RM} cells were tenfold higher than those of unmanipulated WT mice. Upregulation of IL-15 mRNA expression in lethally irradiated mice may have contributed to the enhanced population of CD8⁺ T_{RM} cells in the epidermis (Supplementary Fig. 2d).

Because LCs also expressed *Il15* mRNA, we studied the effect of LC depletion on epidermotropic T_{RM} cells using two different models. Neither constitutive loss nor depletion of LCs affected the numbers of epidermotropic T_{RM} cells (Supplementary Fig. 3a–c). Thus, hair follicle-derived IL-15 is crucial for the maintenance of epidermotropic CD8⁺ T_{RM} cells.

A different approach was taken to evaluate the contribution of hair follicle-derived IL-7, because T cells failed to undergo homeostatic proliferation when transferred into *Il7-KO^{Rag}* mice (data not shown). We crossed *K14-Cre^{ERT}* mice²⁶ with *Il7*-floxed mice to generate mice in which ablation of IL-7 in the epidermis could be specifically induced in the skin of adult mice by administering tamoxifen injections (Supplementary Fig. 2e). Because of the potential for a continuous influx of newly generated T_{RM} cells, which could alter total cell numbers during and after IL-7 ablation, we treated mice with FTY720, a sphingosine-1-phosphate receptor 1 inhibitor that inhibits lymphocyte egress from lymph nodes, thereby preventing the influx of endogenous T_{RM} cells into the epidermis (Fig. 2g). Epidermal ablation of IL-7 reduced the numbers of both CD4⁺ and CD8⁺ T_{RM} cells 14 d after the loss of IL-7, an effect that persisted for at least 28 d (Fig. 2h). The numbers of splenic T cells remained unaffected by the loss of IL-7 in the skin (Supplementary Fig. 2f). Therefore, hair

follicle-derived IL-7 is required for both CD4⁺ and CD8⁺ T_{RM} cells to persist in the epidermis.

Impaired contact hypersensitivity responses in the absence of IL-7 and IL-15

Impaired anatomical localization of T_{RM} cells during the steady state in the absence of hair follicle-derived IL-15 and IL-7 might affect subsequent immune responses in the skin. To address this, we induced contact hypersensitivity (CHS) with a hapten in mice that lacked hair follicle-derived cytokines. To analyze the effect of IL-15 deficiency in the context of CD8⁺ T_{RM} cells, we generated *WT^{Rag}* and *Il15-KO^{Rag}* mice using BM transplantation (Fig. 2e). These BM chimeric mice were injected with CD8⁺ T cells obtained from skin-draining lymph nodes of 1-fluoro-2,4-dinitrobenzene (DNFB)-sensitized mice (Fig. 3a). Transferred CD8⁺ T cells are expected to undergo homeostatic proliferation and distribute to peripheral tissues, including the skin, in *WT^{Rag}*, but not in *Il15-KO^{Rag}* mice (Figs. 1g and 2f). Recipient mice were then challenged with DNFB, and ear-swelling responses were monitored. Both ear swelling and lymphocytic infiltration were reduced in the absence of tissue-derived IL-15 (Fig. 3b,c).

The roles of hair follicle-derived IL-7 and T_{RM} cells were assessed in the context of CD4⁺ T cell-mediated CHS. To generate lymphopenic mice that constitutively lack IL-7 in the epidermis, *K5-Cre* mice and *Il7*-floxed mice²⁷ were each crossed to the *Rag2^{-/-}* background (*Il7^{fl/fl}K5-Cre* × *Rag2^{-/-}* mice). CD4⁺ T cells isolated from skin-draining lymph nodes of DNFB-sensitized WT mice were transferred into *Il7^{fl/fl}K5-Cre* × *Rag2^{-/-}* mice or control *Rag2^{-/-}* mice. Recipient mice were challenged with DNFB 14 d after transfer, and ear-swelling responses were assessed (Fig. 3d). Ear swelling and

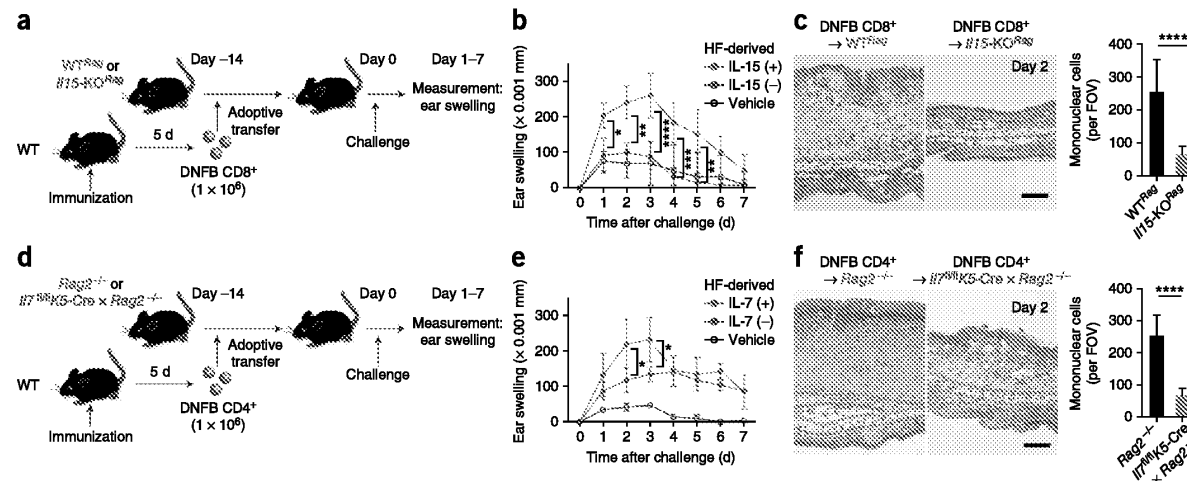


Figure 3 Impaired CHS responses in the absence of hair follicle-derived cytokines. (a) Experimental scheme for the analysis of CD8⁺ T_{RM} cell-mediated CHS in the absence of peripheral tissue (hair follicle)-derived IL-15. CD8⁺ T cells from DNFB (DNFB CD8⁺) or vehicle-immunized (vehicle CD8⁺) WT mice were transferred into indicated lymphopenic mice. 14 d later, recipients were challenged with DNFB on their ears, and ear-swelling responses were measured. (b) Ear thickness (Δmm) of DNFB CD8⁺-transferred *WT^{Rag}* (red), *Il15-KO^{Rag}* (blue) and vehicle CD8⁺-transferred *WT^{Rag}* (black) (*n* = 3). (c) H&E staining of ear sections from indicated mice, day 2 after challenge and quantification of skin-infiltrating mononuclear cells. (d) Experimental scheme for analyzing CD4⁺ T_{RM} cell-mediated CHS in the absence of hair follicle-derived IL-7. DNFB CD4⁺ or vehicle CD4⁺ were adoptively transferred into indicated lymphopenic mice. (e) Ear thickness (Δmm) of DNFB CD4⁺-transferred *Rag2^{-/-}* (red), *Il7^{fl/fl}K5-Cre* × *Rag2^{-/-}* (blue) or vehicle CD4⁺-transferred *Rag2^{-/-}* (black) (*n* = 3). (f) H&E staining of ear sections from indicated mice, day 2 after challenge and quantification of skin-infiltrating mononuclear cells. Data are from one experiment, representative of two independent experiments with three mice per group in each (b,c,e,f). Cell counts represent pools of three fields of view per section (original magnification: 100×) from three mice per group (c,f). Scale bars, 100 μm (c,f). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 (two-way ANOVA). Error bars are s.e.m.

lymphocytic infiltration were both reduced in $Il7^{fl/fl}K5-Cre \times Rag2^{-/-}$ mice on days 2 and 3 after the challenge (Fig. 3e,f). This transient effect might reflect the generation and influx of newly generated recipient T_{RM} cells after DNFB challenge.

A CD4⁺ T cell lymphoma model with skin involvement

Epidermotropism of T cells is a histological hallmark in human CTCL. In particular, lymphoma cells in mycosis fungoides exhibit a T_{RM} cell phenotype^{18,19,28}. *IL7* expression is increased in CTCL skin²⁹. Whether lymphoma cells also require hair follicle-derived cytokines remains unclear. Thus, we extended our T_{RM} cell repopulation model in $Rag2^{-/-}$ mice by generating a novel lymphoma model.

Mutations in, or upregulation of, the oncogene *MYC*^{30,31}, as well as mutations in the tumor-suppressor gene *INK4A/ARF* (officially known as *CDKN2A*), have been implicated^{17,30} in human lymphoma and CTCL. *Cdkn2a*^{-/-} mice are prone to tumor development, including T cell lymphoma^{32,33}. *Cdkn2a*^{-/-} progenitor B cells transduced with *Myc* generate B cell lymphoma³⁴. Taking advantage of these previous findings, we isolated CD4⁺ T cells from *Cdkn2a*^{-/-} mice and retrovirally transduced them with *Myc* (Fig. 4a). Approximately 50% of T cells were transduced, as determined by GFP expression (*Myc*⁺*Cdkn2a*^{-/-} CD4⁺ T cells) (Fig. 4b). Recipient $Rag2^{-/-}$ mice developed erythroderma (redness and fine scaling of the entire skin surface) approximately 3 weeks after the transfer of *Myc*⁺*Cdkn2a*^{-/-} CD4⁺ T cells (Fig. 4c). Flow cytometry analysis revealed increased numbers of epidermotropic CD4⁺ T cells in $Rag2^{-/-}$ mice that had been transferred with *Myc*⁺*Cdkn2a*^{-/-} CD4⁺ T cells as compared to those transferred with WT CD4⁺ T cells (Fig. 4d). The majority of

epidermotropic CD4⁺ T cells in *Myc*⁺*Cdkn2a*^{-/-} CD4⁺ T cell-transferred mice expressed *Myc*-GFP (Fig. 4e) with increased IL-7R α expression (Fig. 4e).

Histology revealed epidermotropism of lymphocytes with large, atypical nuclei, recapitulating that of human CTCL (Fig. 4f)¹⁷. Consistently, flow cytometry analysis revealed that *Myc*⁺*Cdkn2a*^{-/-} CD4⁺ T cells were enlarged in size (Fig. 4g). Infiltrating lymphocytes expressed *Ki-67*, demonstrating that they were proliferative (Fig. 4h). Epidermotropic CD4⁺ T cells were of the T_{RM} cell phenotype (Fig. 4i), and they accumulated around the hair follicles (Fig. 4f,j). Collectively, *Myc*⁺*Cdkn2a*^{-/-} CD4⁺ T cells that had been transferred into $Rag2^{-/-}$ mice infiltrated the skin and epidermis, exhibiting histologic features of human CTCL. These mice also exhibited lymphoma in the lymph nodes and spleen, and they died within 10 weeks after transfer (Supplementary Fig. 4), thereby also recapitulating an aspect of Sezary's syndrome, a leukemic subtype of CTCL³⁰.

Epidermotropism in CTCL depends on IL-7

To determine whether CD4⁺ lymphoma cells also relied on hair follicle-derived IL-7, we transferred *Myc*⁺*Cdkn2a*^{-/-} CD4⁺ T cells into $Rag2^{-/-}$ or $Il7^{fl/fl}K5-Cre \times Rag2^{-/-}$ mice (Fig. 5a). In contrast to $Rag2^{-/-}$ mice, $Il7^{fl/fl}K5-Cre \times Rag2^{-/-}$ mice did not develop erythroderma (Fig. 5b). Furthermore, the absolute numbers of epidermotropic lymphoma cells were reduced in the absence of hair follicle-derived IL-7 (Fig. 5c), whereas significant differences were not observed in the lymph nodes and spleen (Fig. 5d). Histologic analysis of control $Rag2^{-/-}$ mice revealed abundant epidermotropic

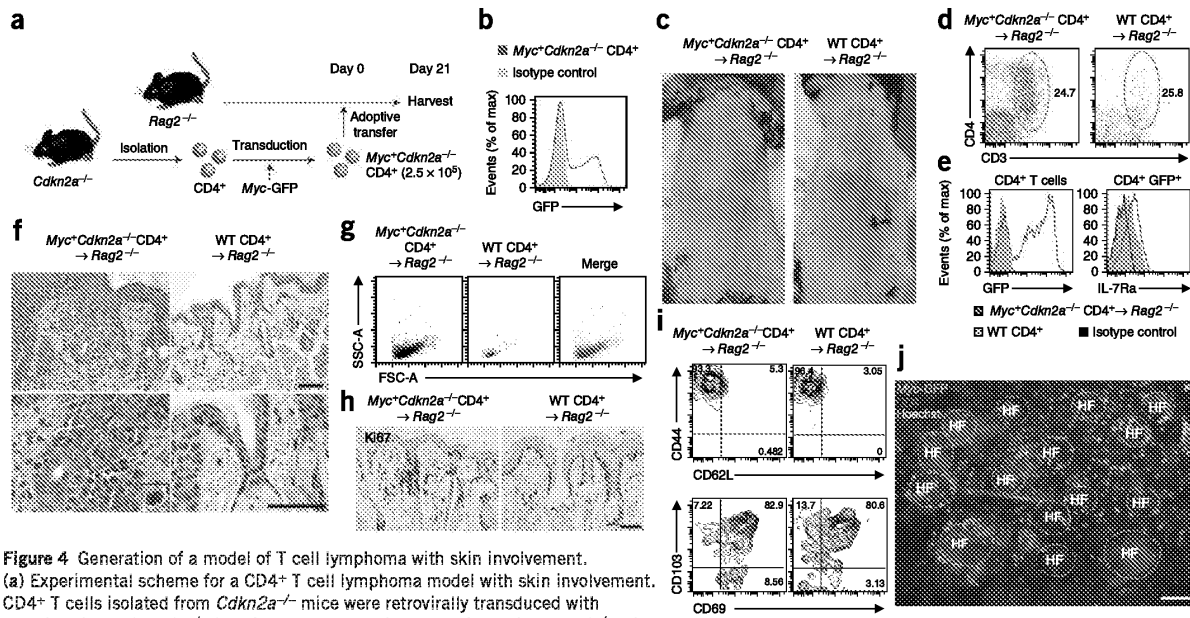
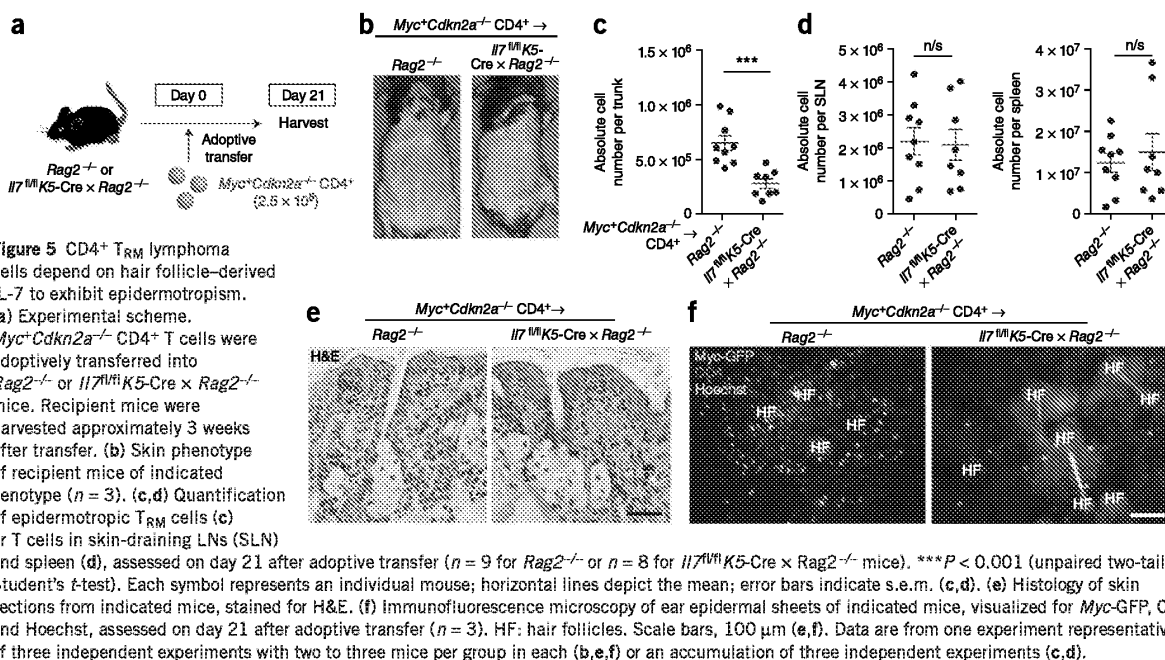


Figure 4 Generation of a model of T cell lymphoma with skin involvement. (a) Experimental scheme for a CD4⁺ T cell lymphoma model with skin involvement. CD4⁺ T cells isolated from *Cdkn2a*^{-/-} mice were retrovirally transduced with *Myc*-GFP (*Myc*⁺*Cdkn2a*^{-/-} CD4⁺) and were adoptively transferred into $Rag2^{-/-}$ mice. (b) Flow cytometry analysis of *Myc*-GFP expression on *Myc*⁺*Cdkn2a*^{-/-} CD4⁺ T cells before transfer. (c) Skin phenotype of *Myc*⁺*Cdkn2a*^{-/-} CD4⁺-transferred (left) or wild-type (WT) CD4⁺-transferred $Rag2^{-/-}$ mice (right). (d) Flow cytometry analysis of CD3⁺CD4⁺ epidermotropic cells in recipient mice, as indicated by the purple gate. CD45⁺MHCII⁺TCR $\gamma\delta$ -cells are shown. (e) Expression of GFP and IL-7R α on cells within the purple gate in d. (f) H&E staining of skin sections from indicated mice. (g) Forward scatter (FSC) and side scatter (SSC) profiles of cells within the purple gate in d. (h) Immunohistochemical staining for *Ki-67*. (i) CD44 versus CD62L, and CD103 versus CD69 expression on CD45⁺MHCII⁺TCR $\gamma\delta$ -cells are shown. (j) Immunofluorescence microscopy of epidermal sheets, visualized for *Myc*-GFP, CD4 and Hoechst. Dashed lines delineate the hair follicles (HF). Scale bar, 100 μ m (f,h,j). All data are from one experiment representative of three independent experiments with three mice per group in each.



lymphocytes with epidermal thickening, but histological changes were minimal in *Il7^{fl/fl}/K5-Cre x Rag2^{-/-}* mice (Fig. 5e). Visualization of CD4⁺ T cells in epidermal sheets confirmed these findings (Fig. 5f). Thus, epidermotropic CD4⁺ T_{RM} cells continued to require hair follicle-derived IL-7 after neoplastic transformation.

In CTCL, IL-7 is upregulated in human hair follicles

To examine whether our findings could be extended to humans, we studied human hair follicles. Whereas hair follicles in the trunk skin

of mice and humans are mostly found in telogen, the majority of those in human scalp are in anagen. The terminus end is called the bulb, and the portion between the bulb and the stem cell-containing bulge is referred to as the suprabulb (Fig. 6a and Supplementary Fig. 5). We dissected hair follicles from normal human scalp to isolate the interfollicular epidermis, infundibulum, bulge, suprabulb and bulb, and we obtained RNA from each of these sites (Fig. 6a)⁴. We then performed real-time PCR for *IL15* and *IL7*. *IL15* was predominantly expressed by hair follicle keratinocytes in the

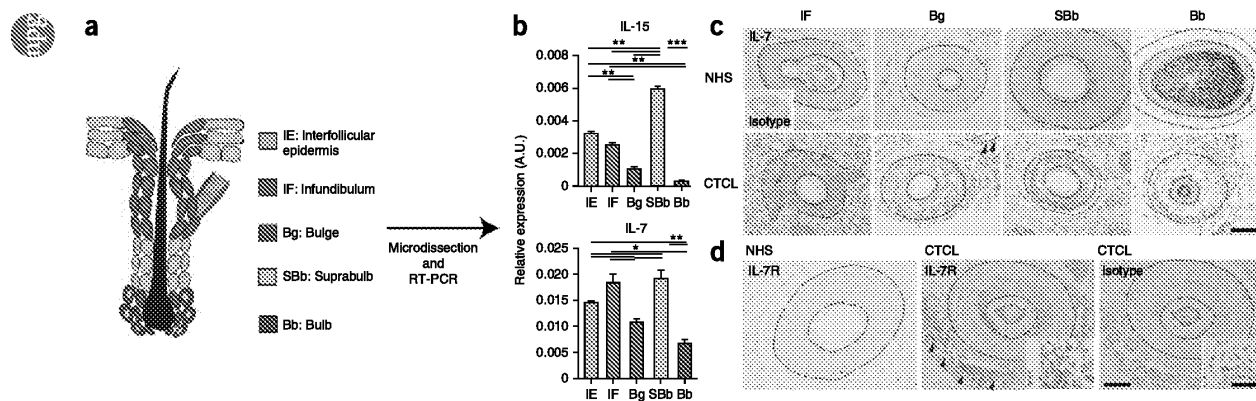


Figure 6 IL-7 and IL-15 expression in human hair follicles from normal scalp and cutaneous T cell lymphoma. (a) Schematic representation of human hair follicle keratinocytes. (b) Real-time PCR analysis for *IL15* and *IL7* expression at distinct sites in the hair follicle. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (unpaired two-tailed Student's *t*-test). Horizontal lines depict the mean, and error bars indicate s.e.m. (c) Immunohistochemistry of normal scalp sections from humans (NHS) ($n = 3$ samples from three subjects) and from subjects with CTCL ($n = 6$ samples from three subjects), stained with an IL-7-specific antibody or with an isotype control. Scale bar, 100 μ m. Arrowheads indicate arrector pili muscle (reflecting the bulge region). (d) Immunohistochemistry of scalp sections from patients with CTCL, stained with an IL-7R-specific antibody or with an isotype control ($n = 6$ samples from three subjects). Scale bar, 100 μ m in low-power field, 50 μ m in high-power field. Dashed lines delineate hair follicles. Arrowheads indicate IL-7R-positive lymphoma cells. Data are from one experiment representative of three (b,c) or two (d) independent experiments.

suprabulb, and IL7 expression was highest in the infundibulum and suprabulb (Fig. 6b).

We additionally evaluated IL-7 expression in hair follicles from both normal human scalp and lesional scalp skin of patients with CTCL. Faint immunohistochemical staining for IL-7 was detected in normal human hair follicles. IL-7 expression was apparent in a CTCL-affected subject, in which the staining was detected in the infundibulum and the suprabulbar area (Fig. 6c), consistent with the real-time PCR data from normal human scalp. In other patients, IL-7 staining was increased not only in hair follicle keratinocytes, but also in keratinocytes in the interfollicular epidermis (Supplementary Fig. 6).

Consistent with our observations in mice, IL-7R expression by T cells was increased in lesional CTCL epidermis in comparison to that on T cells in normal human scalp skin (Fig. 6d and Supplementary Fig. 5). IL-7R expression in lesional keratinocytes in CTCL seems to be increased relative to keratinocytes in normal scalp skin, although the significance of this has yet to be determined.

DISCUSSION

Epidermotropism of T_{RM} cells, both non-malignant and malignant, is regulated by the hair follicle-derived cytokines IL-7 and IL-15. Previous studies^{6–8} have focused on T_{RM} cell biology in the context of viral infections. We studied the requirement of epidermotropic T_{RM} cells during the steady state by using non-malignant and malignant models. The lack of hair follicle-derived cytokines led to the failure of both CD4⁺ and CD8⁺ T_{RM} cells to persist in skin. Impaired CHS responses in the absence of hair follicle-derived cytokines highlight the importance of homeostatic organization of T_{RM} cells before inflammation.

The requirement of IL-7 and IL-15 for the generation and maintenance of memory T cells is well established^{24,25}, but the fate of T_{RM} cells in peripheral tissues that are deprived of these cytokines remains unclear. In particular, whether the cells undergo cell death or migration deserves further attention in the context of lymphoma.

The tropism that CD4⁺ T_{RM} cells exhibit to the hair follicles demonstrated in this study is compatible with and complementary to a previous report⁷ in which approximately 30% of virus-specific CD4⁺ memory T cells were found to associate with the hair follicles after herpes simplex virus skin infection. To date, T_{RM} cells in the skin have mainly been characterized in the context of CD8⁺ T cells. Although CD4⁺ memory T cells may well be comparable to CD8⁺ T_{RM} cells as skin residents on the basis of surface marker expression and past reports^{13,22}, thorough studies on CD4⁺ memory T cells as bona fide T_{RM} cells have yet to be performed. Our observation that epidermal and dermal T_{RM} cells have subtle differences in CD103 expression, in conjunction with the higher observed ratio of CD4⁺ FoxP3⁺ T_{RM} cells in the dermis, suggests that CD4⁺ T_{RM} cells in these skin compartments might be regulated through distinct mechanisms.

In conclusion, we have demonstrated the importance of hair follicle-derived IL-7 and IL-15 in T_{RM} cell homeostasis in the epidermis, which represents an attractive therapeutic target in inflammatory skin diseases and malignant lymphoma, and which may also be relevant in other peripheral tissues.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

T.A. and K.N. conceived of and designed all experiments. Experiments were performed by T.A. with the assistance of T.K.; E.S. and H.S. provided *Cdkn2a*^{-/-} mice and assisted with retroviral transduction; T.Y. assisted with immunohistochemical staining; K.I. provided IL-7 floxed mice; S.P. provided human CTCL samples; M.A. discussed the data and provided administrative support; K.N. guided the project; and T.A. and K.N. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. C57BL/6J (CD45.2) mice and *Il15*^{-/-} mice were purchased from CLEA Japan. C57BL/6J *Rag2*^{-/-} mice were purchased from the Central Institute for Experimental Animals (Tokyo, Japan). *K14-Cre*^{ERT} mice were purchased from Jackson Laboratory (ME, USA)²⁶. *Cdkn2a*^{-/-} mice (B6.129-Cdkn2atm1Rdp) were from Mouse Models of Human Cancers Consortium (NCI-Frederick)³³. *Il7*-floxed mice, *K5-Cre* mice, *Langerin-DTA* mice and *Langerin-DTR* mice were generated as previously described^{27,33–37}. Only female mice 6–12 weeks of age were used for experiments. All mice were bred and housed in specific-pathogen-free conditions. All animal procedures and study protocols were approved by the Keio University Ethics Committee for Animal Experiments.

Human tissue. All samples for this study were obtained according to protocols approved by the Institutional Review Board of Keio University (protocol number 2003-0057, 2005-0075) or NCI, NIH (protocol number 96-C-0102). Informed consent was obtained from all subjects before the acquisition of the skin tissues. Two sets of normal human-scalp samples for gene analysis and formalin-fixed scalp samples for histopathological examination were obtained from excess normal skin that resulted from the surgical removal of benign, subcutaneous skin tumors. Formalin-fixed scalp samples from subjects with CTCL were prepared from the original biopsy specimens that were obtained from lesional skin for diagnosis.

Preparation of epidermal sheets. Epidermal sheets were prepared as before⁴ with a slight modification. Briefly, ears were split into dorsal and ventral halves using forceps. Thioglycolic acid-containing Hair Removing Body Cream Epilat (Kracie) was used to remove hair in some experiments. Ear halves were incubated for 15 min at 37 °C on 3.8% ammonium thiocyanate (Wako Pure Chemical Industries) in phosphate buffer (pH 7.0). Epidermal sheets were manually detached from the dermis under a dissecting microscope (Olympus).

Preparation of epidermal and dermal cell suspensions. Epidermal and dermal cell suspensions were prepared as before⁴ with a slight modification. Briefly, shaved whole trunk skin was harvested from the appropriate animals and, after removal of subcutaneous tissues using forceps, was floated with the epidermal side up onto 10 ml of Trypsin-EDTA solution containing 5 mL of 0.25% Trypsin (Nacalai Tesque) and 5 mL of 0.05% Trypsin-0.53 mM EDTA-4Na (Nacalai Tesque) at 37 °C for less than 30 min in a 10-cm dish. The epidermis and dermis were separated manually using forceps. The dermis was cut into small chips manually using scissors, and further incubated in 4 mL of RPMI containing 0.03% Liberase TL Research grade (Roche Applied Science) and 200 U/ml of DNase (Wako Pure Chemical Industries) for 60 min at 37 °C shaking with rotation at 200 rpm (Bioshaker, Taitec). Epidermal and dermal cells were then suspended in 5% FCS in PBS, washed and filtered through Cell strainer (BD Falcon).

Antibodies for immunofluorescence microscopy and flow cytometry analysis for mouse samples. Anti-mouse CD4 monoclonal antibody (clone GK1.5, BioLegend) was used in conjugated forms pre-labeled with PE/Cy7 or APC or labeled in house with Alexa Fluor 568 (Invitrogen). Anti-mouse CD8 monoclonal antibody (clone 53.6.7, BioLegend) was used in conjugated forms pre-labeled with FITC, APC, APC/Cy7 or AmCyan or labeled in house with Alexa Fluor 647 (Invitrogen). The following pre-labeled monoclonal antibodies and polyclonal antibodies, all obtained from BioLegend unless otherwise stated, were used for immunofluorescence microscopy and flow cytometry: CD3e (clone 145-2c11), TCR $\gamma\delta$ (clone GL3), MHC II (anti-IA/IE; clone M5/114.15.2), CD34 (clone RAM34, eBioscience), CD44 (clone IMF7), CD45 (clone 30F-11), CD49f (anti-integrin α_6 ; clone GoH3), CD62L (clone MEL-14), CD69 (clone H1.2F3), CD103 (clone 2E7), CD122 (anti-IL-15/2R β ; clone 5H4), CD127 (anti-IL-7R α ; clone SB/199), EpCAM (clone G8.8), Ly-6A/E (anti-Sca-1; clone E13-161.7). Antibodies that were labeled in-house were stored at 0.5 mg per mL concentration. All primary antibodies for flow cytometry were diluted 1:200 with the exception of clone G8.8, which was used at 1:800 dilution. For immunofluorescence microscopy, primary antibodies were used at 1:100 dilution.

Primary antibodies were detected, if needed, with Alexa Fluor labeled secondary antibodies (anti-Fluorescein/Oregon Green, A11096 or anti-GFP,

A21311; both from Life Technologies) at 1:200 dilution. Anti-mouse CD16/32 (clone 93, BioLegend) was routinely used (1:100 dilution) to block Fc γ receptors before staining.

Immunofluorescence microscopy. Staining of epidermal sheets and frozen skin sections was performed as before⁴ with slight modification. Briefly, sheets and sections were fixed in acetone for 5 min at -20 °C or in 4% paraformaldehyde (Wako Pure Chemical Industries) in PBS for 15 min at room temperature, and were rehydrated or washed in PBS for 5 min. They were blocked in 3% dry milk (Morinaga) in PBS with 5% goat serum for at least 1 h at room temperature. For intracellular staining, 0.2% Triton X-100 (Sigma-Aldrich) was added in blocking buffer. Primary antibodies were diluted in blocking buffer and incubated overnight at 4 °C. After washing, primary antibodies were detected with the appropriate secondary antibodies, and nuclei were visualized with Hoechst 33258 (Invitrogen). Mouse lips were routinely used for vertical sections because this provided a wide view of vertical and horizontal sections of both pelage hair and vibrissae. Images were mostly observed using the Zeiss Axio Observer. Z1 with or without Apotome (Carl Zeiss), collected with the Axiovision software (ver. 4.8). Adjustments of levels, if needed, were performed in Photoshop CS 5.1 (Adobe), and controls were also treated identically.

Flow cytometry analysis and cell sorting. Data were collected using FACS Canto II (BD Biosciences) and analyzed with Flow Jo (Tree Star). Non-viable cells were omitted using propidium iodide (Sigma-Aldrich) staining or Live/Dead cell (Invitrogen) pre-staining for fixed and permeabilized cells before primary antibody staining. Fluorescence activated cell sorting of hair follicle keratinocyte subsets was performed with FACSARIA II (BD Biosciences) or MoFlow (Beckman Coulter), during which cells were directly sorted into TRIzol LS (Invitrogen) and divided into five subsets on the basis of the expression of cell-surface markers as described before⁴: interfollicular epidermis (MHCII⁺CD45⁻Sca-1⁺EpCAM^{low}), infundibulum (MHCII⁺CD45⁻Sca-1⁺EpCAM^{int}), isthmus (MHCII⁺CD45⁻Sca-1⁺EpCAM^{hi}), basal bulge (MHCII⁺CD45⁻Sca-1⁺CD34⁺integrin α_6 ⁺) or supra-basal layer bulge (MHCII⁺CD45⁻Sca-1⁺CD34⁺integrin α_6 ⁻). Sorted cells were further processed for RNA extraction.

Isolation of human hair follicles for gene analysis. Samples taken from human scalp were dissected as previously described⁴. Briefly, scalp samples were dissected into five anatomical areas (interfollicular epidermis, infundibulum, bulge, suprabulb and bulb) under a dissecting microscope, and were then incubated overnight at 4 °C with 1,500 U/ml of Dispase II (Godo Syussei) in DMEM to remove non-epidermal components.

Real-time PCR. Cytokine expressions of hair follicle keratinocyte subsets were analyzed as previously described⁴. Briefly, total mRNA was purified from FACS-sorted epidermal keratinocyte population, using an RNeasy Micro Kit (Qiagen). cDNA was synthesized using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen), and then real-time PCR analysis was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and the StepOne Real-Time PCR system (Applied Biosystems), according to the manufacturer's protocol. All primers (Supplementary Table 1) were designed using Primer Express software (Applied Biosystems), and reactions were conducted under the following cycling conditions: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Normalization of mRNA expression was performed on the basis of the expression of β -actin, using the cycling threshold (ΔC_T) method, and the amount of PCR product was calculated using $2^{-\Delta C_T}$ (ref. 38). Results were presented as $\pm$ s.d.

T_{RM} cell repopulation model. Spleens from 8-week-old C57BL/6J mice were harvested and homogenized on sterile silicone meshes and were then filtered through Cell Strainer (BD Falcon). Cells were washed in 5% FCS in PBS, centrifuged and resuspended in PBS to obtain whole splenocyte single cell suspension. 5×10^6 splenocytes in 200 μ l PBS were adoptively transferred intravenously (i.v.) into 6- to 10-week-old lymphopenic recipients.

Generation of bone marrow chimeras. C57BL/6J WT or *Il15*^{-/-} mice were lethally irradiated (950 rad) and were transferred i.v. with 2×10^6 total bone

marrow cells from *Rag2*^{-/-} mice on the following day to generate lymphopenic bone marrow chimeric mice (*WT*^{Rag} or *IL15-KO*^{Rag}), which were then used as recipients in T_{RM} cell repopulation experiments.

FTY720 and tamoxifen treatment. *Il7^{fl/K14-CreERT}* or *Il7^{fl/w^{fl}/K14-CreERT}* mice were injected intraperitoneally (i.p.) with 1 mg of tamoxifen (Cayman) in 100 μ l of sunflower oil (Nacalai Tesque) per day for 5 consecutive days (days 0–4)³⁹. 30 μ g of FTY720 (Cayman) was injected i.p. from day –1 for 7 consecutive days (ref. 8) and every other day thereafter, until tissues were harvested.

FTY720 and diphtheria toxin (DT) treatment. For depletion of LCs *in vivo*, Langerin-DTR mice (or control WT mice) were injected i.p. with 500 ng of DT⁴⁰ (Sigma, USA) in 200 μ l of sterile PBS at days 0 and 7. 30 μ g of FTY720 (Cayman) was injected i.p. from day –1 for 7 consecutive days and every other day thereafter, until tissues were harvested.

Hapten-induced contact hypersensitivity. WT donor mice were sensitized as described⁴¹ with slight modification. Briefly, either 60 μ l of 0.5% DNFB in vehicle (olive oil: acetone = 1:4) or vehicle alone was topically applied to the shaved-trunk skin, ears and paws of WT mice. 5 d after sensitization, 1×10^6 CD4⁺ or CD8⁺ T cells, which were purified using a magnetic-activated cell-sorting (MACS) cell-separation system (Miltenyi Biotec) from skin-draining lymph nodes, were adoptively transferred into lymphopenic recipients. 2 weeks after transfer, the ears were challenged with 10 μ l of 0.3% DNFB. Ear swelling, calculated as (ear thickness on day *x*) – (basal ear thickness on day 0), was measured in a blinded fashion by an investigator who was not involved in the study.

T cell lymphoma model with skin involvement. Mouse *c-Myc* was cloned into the retroviral vector pMXs-IG, which was transfected and transduced as described with slight modification^{34,42}. Briefly, the pMX-based vector (*Myc-GFP*) was transfected into a packaging cell line, Plat-E⁴³, using FuGENE HD Transfection Reagent (Roche Applied Science), and then the viral supernatant was collected after 48 h of incubation. CD4⁺ T cells, which were collected and enriched from spleen and skin-draining lymph nodes of *Cd4⁺2a^{-/-}* mice by positive selection using the MACS cell-separation system, were cultured in complete medium (RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 1 mM pyruvate, 50 U/ml penicillin, 50 μ g/ml streptomycin, 0.05 mM 2-ME) supplemented with 100 U/ml hIL-2 and 2.5 μ g/ml Con A at 37 °C for 24 h. Flow-through cells that were collected after CD4⁺ T cell enrichment were irradiated (3,000 rad) and used as feeder cells for co-culture with CD4⁺ T cells. The activated CD4⁺ T cells were then resuspended in retroviral supernatant and centrifuged (3,000 r.p.m., 1 h, 30 °C). After incubation at 37 °C for 4 hours, the retroviral supernatant was removed, and the cells were further cultured in complete medium supplemented with 100 U/ml hIL-2 at 37 °C for 44 h. The whole bulk of cultured T cells were transferred i.v. into *Rag2*^{-/-} or *Il7^{fl/K5-Cre} × Rag2*^{-/-} mice. To avoid inter-experimental variability due to subtle differences in transduction efficiency, the number of transferred cells was normalized to contain 2.5×10^5 *Myc-GFP*⁺ CD4⁺ cells. Equivalent numbers of CD4⁺ T cells from WT splenocytes were transferred i.v. into *Rag2*^{-/-} mice. To address the contribution of hair follicle-derived IL-7, the bulk of transduced T cells, numbers normalized as above, were transferred into *Rag2*^{-/-} or *Il7^{fl/K5-Cre} × Rag2*^{-/-} mice.

Preparation of transverse sections. 4-mm punch biopsy specimens were sliced at four levels⁴⁴: just beneath the epidermis (infundibulum), sebaceous glands attachment (bulge), between sebaceous glands attachment and bulb (suprabulb) and bulb. Tissues were sectioned, and a single slide containing 5- μ m disks from four different layers were analyzed for histology or immunohistochemistry (Supplementary Fig. 4).

Histopathological and immunohistochemical analysis of human and mouse skin sections. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned at a thickness of 5 μ m. Sections were paraffin-depleted and rehydrated in a graded series of ethanol solutions. For histology, sections were stained with H&E. For immunohistochemistry staining for human IL-7 expression, sections were washed with PBS and treated with 3% H₂O₂, before incubation with the following primary antibody: rabbit anti-IL-7 polyclonal antibody (clone sc-7921, Santa Cruz Biotechnology) (1:100). For IL-7R expression, sections were autoclaved at 120 °C for 1 min in 5 mM EDTA buffer (pH 8.0), and were allowed to cool at room temperature before incubation with the following primary antibody: rabbit anti-IL-7R polyclonal antibody (clone sc-25475, Santa Cruz Biotechnology) (1:100). Immune complexes were detected by using the ImmPRESS reagent kit (Vector Laboratories) with 3,3'-diaminobenzidine, and sections were counterstained with hematoxylin. For immunohistochemistry of mouse samples for Ki-67 expression, sections were subjected to 20 min of microwave treatment in citrate buffer (pH 7.0) and were allowed to cool at room temperature. Non-specific binding was blocked in 3% dry milk PBS with 5% goat serum for 1 h at room temperature, before incubation with the following primary antibody: rabbit anti-Ki67 monoclonal antibody (clone SP6, Thermo Scientific Lab Vision) (1:100), and then washed, and bound antibodies were detected with Histofine Simple Stain MAX PO (Nichirei Corporation). Secondary antibodies were further visualized with ImmPACT DAB (Vector Laboratories), and sections were counterstained with hematoxylin. Images were collected via an inverted microscope (BX41, Olympus), equipped with a digital camera (DP20, Olympus).

Statistics. Statistical significance was calculated with an unpaired two-tailed Student's *t* test or a two-way ANOVA using GraphPad Prism 6 (GraphPad software). The values presented are expressed as the means $\pm$ s.e.m. Variances were similar between groups in all experiments, as determined by the *F* test using GraphPad Prism 6. For all statistical analyses, data were considered significant when *P* $\leq$ 0.05 (*), *P* $\leq$ 0.01 (**), *P* $\leq$ 0.001 (***) or *P* $\leq$ 0.0001 (****). The animal experiments were not randomized. The investigators were not blinded to allocation during experiments and analyses unless otherwise indicated.

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