

CCR10 regulates balanced maintenance and function of resident regulatory and effector T cells to promote immune homeostasis in the skin

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Background: CCR10 and CCL27 make up the most skin-specific chemokine receptor/ligand pair implicated in skin allergy and inflammatory diseases, including atopic dermatitis and psoriasis. This pair is thought to regulate the migration, maintenance, or both of skin T cells and is suggested to be therapeutic targets for treatment of skin diseases. However, the functional importance of CCR10/CCL27 *in vivo* remains elusive. **Objective:** We sought to determine the expression and function of CCR10 in different subsets of skin T cells under both homeostatic and inflammatory conditions to gain a mechanistic insight into the potential roles of CCR10 during skin inflammation.

Methods: Using heterozygous and homozygous CCR10 knockout/enhanced green fluorescent protein knockin mice, we assessed the expression of CCR10 on regulatory and effector T cells of healthy and inflamed skin induced by chemicals, pathogens, and autoreactive T cells. In addition, we assessed the effect of CCR10 knockout on the maintenance and functions of different T cells and inflammatory status in the skin during different phases of the immune response.

Results: CCR10 expression is preferentially induced on memory-like skin-resident T cells and their progenitors for their maintenance in homeostatic skin but not expressed on most skin-infiltrating effector T cells during inflammation. In CCR10 knockout mice the imbalanced presence and dysregulated function of resident regulatory and effector T cells result in over-reactive and prolonged innate and memory responses in the skin, leading to increased clearance of *Leishmania* species infection in the skin.

Conclusion: CCR10 is a critical regulator of skin immune homeostasis. (J Allergy Clin Immunol 2014;■■■■:■■■-■■■.)

Key words: Chemokine receptor CCR10, skin-resident T cells, regulatory T cells, migration, maintenance, immune homeostasis, allergy, dermatitis, inflammation, skin infection, *Leishmania* species

As the external surface of a body, the skin is under frequent assault from environmental agents. Immune cells in the skin are tightly regulated to tolerate harmless antigens but respond to dangerous assaults to maintain the skin's integrity and function. Skin-resident T cells are unique populations of immune cells with memory cell-like properties.¹ Among them, resident CD4⁺ regulatory T (Treg) cells are critical to maintain the immune homeostasis of healthy skin,^{2,3} whereas resident memory effector T (Teff) cells can provide faster immune responses to skin infection than those of the circulation.⁴⁻⁶ Antigen encounter in the skin might also induce memory Treg cells to counterbalance the Teff response.⁷ Dysregulation of Treg and Teff cells in the skin is associated with allergy and other skin inflammatory diseases.

CCR10 and its ligand, CCL27, make up the most skin-specific chemokine/receptor pair.⁸ With CCL27 constitutively expressed in healthy skin and further upregulated in inflamed skin in patients with psoriasis and dermatitis and in animal models, CCR10 and CCL27 have been implicated in the migration of skin T cells under both healthy and disease conditions.⁹⁻¹² In human subjects all blood CCR10⁺ T cells display memory cell markers, coexpress the skin-homing molecule cutaneous lymphocyte antigen, and respond to chemoattraction of CCL27, suggesting a role of CCR10/CCL27 in the recruitment of memory T cells in the skin.^{9,12,13} However, immunohistochemical staining in one early study found only scattered CCR10⁺ cells in the healthy skin of human subjects.¹² Instead, most T cells of the inflamed skin of patients with psoriasis and dermatitis express CCR10. Because CCL27 was also upregulated in inflamed skin, it was suggested that CCR10 and CCL27 are involved in migration of T cells during skin inflammation.¹² However, another study found only a small percentage of CCR10⁺ T cells isolated from allergen- and bacterial chancroid-induced inflamed human skin, suggesting that CCR10 is unlikely critical for migration of most T cells during the skin inflammation.¹³ Recently, it was reported that CCR10 is coexpressed on half of human blood CCR8⁺ T cells, a population with skin-homing potential, but that T cells migrating out of the healthy human skin do not express CCR10; however, nonmigrating skin-resident T cells were not assessed.¹⁴ The CCR10⁺ CD4⁺ T-cell subset of human blood is enriched with IL-22-producing cells that also preferentially express CCR6, another chemokine receptor associated with skin

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Abbreviations used

BM:	Bone marrow
CHS:	Contact hypersensitivity
DNFB:	2,4-Dinitro-1-fluorobenzene
EGFP:	Enhanced green fluorescent protein
FACS:	Fluorescence-activated cell sorting
FITC:	Fluorescein 5(6)-isothiocyanate
Foxp3:	Forkhead box protein 3
OVA:	Ovalbumin
PE:	Phycoerythrin
TCR:	T-cell receptor
Teff:	Effector T
TPA:	12-O-tetradecanoyl-phorbol-13-acetate
Treg:	Regulatory T
WT:	Wild-type

localization.^{15,16} The role of CCR10 and CCL27 as homeostatic or inflammatory regulators of skin T cells in human subjects remains unclear.

Animal studies have provided complex and sometimes seemingly contradictory results on the functions of CCR10/CCL27. By using a mouse model of 2,4-dinitro-1-fluorobenzene (DNFB)-induced contact hypersensitivity (CHS), one study found that the antibody neutralization of CCL27 reduced T-cell recruitment and inflammation in the skin, suggesting a pivotal role of the CCL27/CCR10 axis in T cell-mediated skin inflammation.¹² Similarly, in the keratin 14 promoter-driven IL-4 transgenic mouse model of atopic dermatitis, subcutaneous injection of anti-CCL27 antibodies reduced inflammation.¹⁷ However, anti-CCL27 antibody treatment did not affect recruitment of transferred CD4⁺ T cells into the DNFB-inflamed skin or allergen-induced CHS responses in other studies.^{18,19} Recently, it was reported that CCR10-sufficient and CCR10 knockout CD4⁺ T cells migrate similarly to the cognate antigen-stimulated skin, demonstrating directly that CCR10 is not critical for T-cell infiltration into inflamed skin,²⁰ and the role of CCR10 in the regulation of skin T cells *in vivo* remains unknown.

We recently generated CCR10 knockout/enhanced green fluorescent protein (EGFP) knockin mice in which the CCR10 coding region was replaced with a DNA sequence coding for EGFP.^{21,22} Using heterozygous and homozygous CCR10 knockout/EGFP knockin (CCR10^{+/-} and CCR10^{-/-}) mice, we assessed the expression of CCR10 and its roles in different phases of T-cell response during skin inflammation. Here we report the first definite evidence that CCR10 is a critical regulator of skin immune homeostasis through regulating the balanced presence and function of resident Treg and Teff cells.

METHODS**Mouse models and human biosamples**

CCR10 knockout/EGFP knockin mice were generated in our laboratory.²¹ Rag1^{-/-}, Scurfy, and wild-type (WT) CD45.1⁺ congenic C57BL/6 mice were from the Jackson Laboratory (Bar Harbor, Me). CD45.1⁺CD45.2⁺ WT C57BL/6, CD45.1⁺CD45.2⁺ or CD45.1⁺CD45.2⁻CCR10^{+/-}, or CD45.1⁺CD45.2⁺Rag1^{-/-} mice were generated by proper crossing. Scurfy mice were also crossed to CCR10 knockout/EGFP knockin mice to introduce a CCR10 knockout/EGFP knockin allele for the EGFP reporter of CCR10 expression. All animal experiments were approved by the Pennsylvania State University Institutional Animal Care and Use Committee. Human healthy skin was obtained from patients undergoing the plastic surgery. Use of the

biosamples of human subjects was approved by the Institutional Review Board of Anhui Medical University.

Chemical reagents and induction of skin inflammation

DNFB, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), fluorescein 5(6)-isothiocyanate (FITC), and chicken ovalbumin (OVA) were purchased from Sigma-Aldrich (St Louis, Mo). Cholera toxin was purchased from List Biological (Campbell, Calif).

To induce classic CHS responses, mouse abdomens were shaved and sensitized with 100 μ L of 0.5% DNFB in 4:1 acetone/olive oil at days 0 and 1. At day 5, the baseline ear thicknesses of both the right and left ears were measured with a micrometer gauge. Immediately after ear measurement, 10 μ L of 0.2% DNFB solution or control solvents (20 μ L total) was topically applied to each side of the ear. Ear thickness was measured at various days after chemical challenge on the ear. The change in ear thickness (Δ T) was calculated by subtracting ear thickness before chemical treatment from ear thickness after chemical application. The memory CHS response was induced similarly to the classic CHS response except that ears were challenged with DNFB 1 month after DNFB sensitization.

For DNFB-, FITC-, or TPA-induced innate skin inflammation, 10 μ L of the chemicals (0.5% DNFB in 4:1 acetone/olive oil, 0.5% FITC in 1:1 acetone/dibutyl phthalate, or 100 μ g/mL TPA in acetone) was applied once to each side of an ear. Ear thickness was measured at various days after application.

OVA-induced skin inflammation was elicited as previously reported,²³ except that total OVA proteins instead of peptides were epicutaneously applied to mouse skin.

Skin cell isolation

Skin cells were prepared similarly, as previously described.²¹ Briefly, mouse hair was removed from the skin by using hair clippers and Nair (Church & Dwight, Princeton, NJ). Mouse skin was excised, trimmed of subcutaneous fat, and minced after a 2-hour digestion with 4 mg/mL Collagenase Type I (Worthington, Lakewood, NJ), 2 mg/mL Collagenase Type IV (Worthington), 2 mg/mL hyaluronidase type I-s (Sigma-Aldrich), and 4% BSA (Sigma-Aldrich) in Dulbecco modified Eagle medium. Thirty minutes before the end of digestion, 0.0001% DNase (Sigma-Aldrich) was added to the digestion buffer. Mononucleocytes were enriched from the cell preparations by using Percoll gradients (40%/80%). The similarly isolated human skin cells were allowed to recover in culture medium overnight before flow cytometric analysis.

Bone marrow cell reconstitution

Cell sorter-purified EGFP⁻ bone marrow (BM) cells of CCR10^{+/-} (CD45.1⁺CD45.2⁻) and CCR10^{-/-} (CD45.1⁺CD45.2⁺) mice were mixed 1:1 and injected intravenously into lethally irradiated (950 rad) WT C57BL/6 or CCR10^{+/-} (CD45.1⁺CD45.2⁺) mice (total of 10⁶ cells per mouse). The recipients were analyzed 7 to 8 weeks after transfer.

Skin *Leishmania major* infection

L major NIH Friedlin V1 strain (MHOM/IL/80/FN) was grown in M199 medium supplemented with 25 mmol/L HEPES and 20% FCS until a stationary phase. Mice were injected subcutaneously with 1 $\times$ 10⁶ stationary-phase promastigotes in the ear dermis. Infected ears were collected at various time points, and total genomic DNA was extracted with DNeasy Tissue Kit (Qiagen, Valencia, Calif). Tissue *L major* genomic DNA levels were quantified by using quantitative PCR with primers (JW11: CCTATTTTACACCAAC CCCCAGT; JW12: GGGTAGGGGCGTTCTGC GAAA) specific to *L major* 16S rDNA and normalized on levels of mouse genomic β -actin, as previously described.²⁴

***In vivo* T-cell transfer**

EGFP⁻ CD3⁺ T cells were sorter purified from splenocytes of CCR10^{+/-} or CCR10^{-/-} mice. Splenic EGFP⁻ CD3⁺ T cells of CCR10^{+/-}

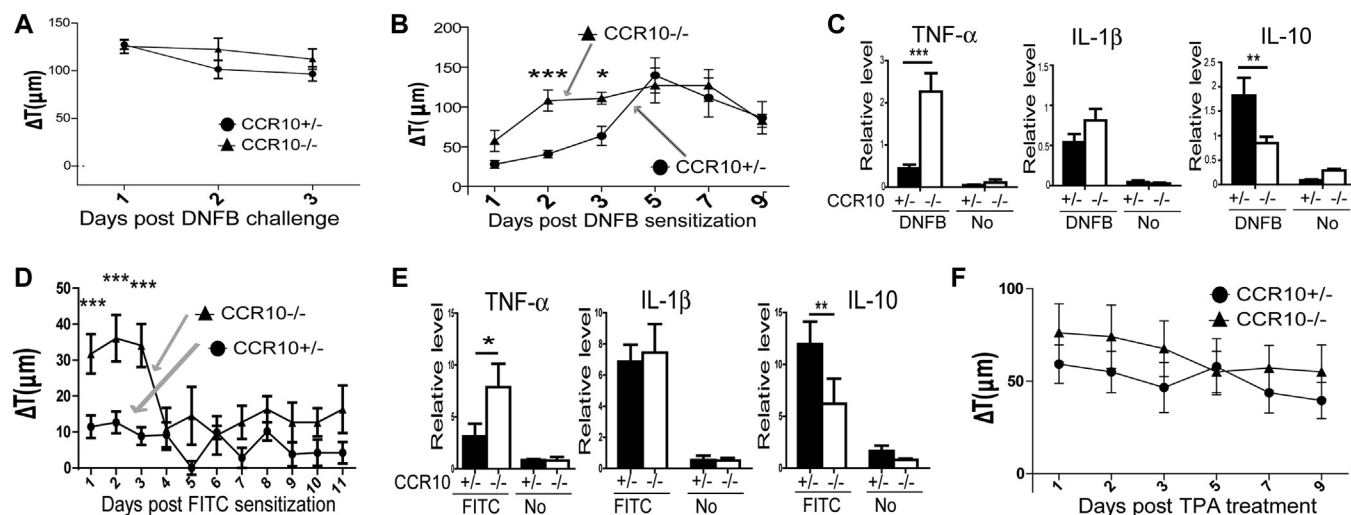


FIG 1. Overreactive innate response to stimulation in the skin of CCR10^{-/-} mice. **A**, Ear thickness changes (ΔT) of CCR10^{-/-} and CCR10^{+/+} mice in a DNFB-induced CHS assay ($n \geq 6$). **B**, Ear thickness changes of CCR10^{-/-} and CCR10^{+/+} mice after 1-time topical application of DNFB on the ear ($n \geq 10$). * $P < .05$, ** $P < .005$, and *** $P < .001$. NS, No significant difference (applied to all figures). **C**, Quantitative RT-PCR analysis of RNA isolated from treated ears 3 days after 1-time DNFB application for TNF- α , IL-1 β , and IL-10 ($n = 5$ each). No indicates untreated skin samples ($n = 3$). The values are relative levels normalized to β -actin. **D**, Ear thickness changes of CCR10^{-/-} and CCR10^{+/+} mice after 1-time topical application of FITC (0.5%; $n = 21$ for CCR10^{+/+} mice and $n = 19$ for CCR10^{-/-} mice). **E**, Quantitative RT-PCR analysis of RNA isolated from treated skin 3 days after 1-time FITC application for TNF- α , IL-1 β , and IL-10 as in Fig 1, C ($n = 4$ each). **F**, Ear thickness changes in CCR10^{-/-} and CCR10^{+/+} mice after 1-time topical application of TPA ($n \geq 10$ for each genotype).

or CCR10^{-/-} mice (4×10^5) were intraperitoneally injected into Rag1^{-/-} mice separately, which were then tested for the DNFB-induced classic or memory CHS responses after cell transfer. For adoptive transfer of OVA-specific T cells, approximately 0.5×10^6 purified splenic transgenic T cells of CCR10^{+/+} or CCR10^{-/-} OT-I or OT-II mice were intraperitoneally injected into WT mice with a different CD45 polymorphism, as indicated in the relevant figures, which were then epicutaneously challenged with OVA proteins.²³

Naive T cell transfer-induced skin inflammation

EGFP⁻ naive T cells (CD3⁺CD4⁺CD25⁻CD45RB^{high}, CD45.1⁺) and Treg cells (CD3⁺CD4⁺CD25⁺CD45RB⁻, CD45.2⁺) were sorted from splenocytes of CCR10^{+/+} mice. Naive T cells alone (4×10^5) or naive T cells (2×10^5) with Treg cells (2×10^5) were intravenously injected into Rag1^{-/-} mice (CD45.1⁺CD45.2⁺). The recipients were analyzed 7 to 8 weeks after transfer.

Real-time RT-PCR analysis of cytokine transcripts

Total RNA of mouse ears was isolated and reverse transcribed to cDNA and then subjected to Sybr green real-time PCR with the following primers: TNF- α forward, TTCTATGGCCAGACCC; TNF- α reverse, GGCACCAC TAGTTGGTTGTC; IL-1 β forward, TCTCGCAGCAGCACATCA; IL-1 β reverse, CACACCAGCAGTTATCATCAT; IL-10 forward, ACCAA AGCCACAAAGCAGCC; IL-10 reverse, CCGACTGGGAAGTGGGTGC; β -actin forward, CCCATCTACGAGGGCTAT; and β -actin reverse, TGTCACGCACGATTCC. Relative levels of transcripts were calculated with the $\Delta\Delta$ cycle threshold method.

Antibodies and flow cytometry

Flow cytometry, antibodies, and analysis strategies are detailed in the **Methods** section in this article's Online Repository at www.jacionline.org. Cell gating is indicated in the figures.

In vitro T-cell stimulation

Skin EGFP⁺CD4⁺CD25⁻ T cells purified from CCR10^{+/+} and CCR10^{-/-} mice were cultured in the presence of IL-2 and coated anti-CD28/anti-T-cell receptor (TCR) β antibodies for 2 days. Supernatants were collected and analyzed with the mouse IL-17A ELISA Ready-SET-Go! Set (eBioscience, San Diego, Calif).

Statistical analyses

Data are expressed as means $\pm$ SEMs and analyzed by using the 2-tailed student *t* test or Fisher test to determine statistical significance for 2-group comparisons. ANOVA with Tukey adjustment was used for multiple-group comparisons. A *P* value of less than .05 is considered significant.

RESULTS

Overreactive innate immune response to allergen stimulation in skin of CCR10^{-/-} mice

To assess roles of CCR10 in skin inflammation, we tested CCR10^{-/-} mice in several models. In a DNFB-induced CHS assay, CCR10^{-/-} and CCR10^{+/+} mice had similar ear thickness increases (Fig 1, A), indicating that CCR10 is not critical for the T cell-mediated CHS response and consistent with the report that CCR10 knockout does not affect T-cell migration.²⁰ Surprisingly, CCR10^{-/-} mice had significantly larger ear thickness increases than CCR10^{+/+} mice early after the 1-time DNFB application (Fig 1, B), suggesting an enhanced innate response. Supporting this notion, the treated ears of CCR10^{-/-} mice exhibited much higher levels of gene expression of TNF- α and lower IL-10 levels than CCR10^{+/+} control animals (Fig 1, C). CCR10^{-/-} mice also had an enhanced innate response to 1-time treatment with another irritant/allergen, FITC (Fig 1, D and E). On the other hand,

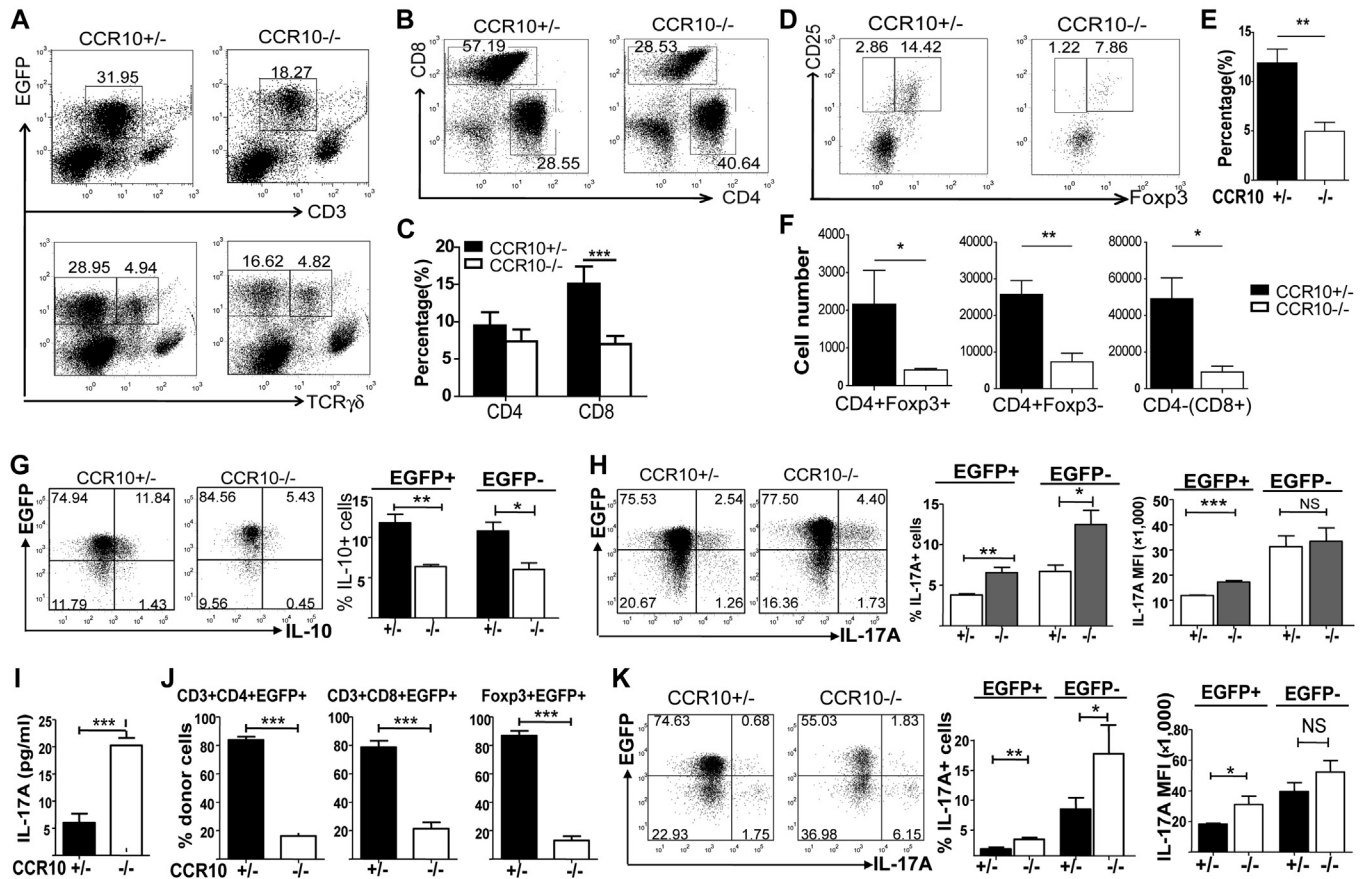


FIG 2. Imbalanced maintenance and dysregulated functions of Treg and Teff cells in the skin of CCR10^{-/-} mice. **A**, Flow cytometric (fluorescence-activated cell sorting [FACS]) analysis of skin lymphocyte preparations of CCR10^{-/-} and CCR10^{+/-} mice for EGFP⁺ total CD3⁺ T cells (top) and $\gamma\delta$ T cells (bottom). The number next to each gate is the percentage of gated cells of total events in the histogram. CD3^{high}CCR10^{low} cells are V γ 3⁺ $\gamma\delta$ T cells.²¹ **B**, FACS analysis of gated skin EGFP⁺CD3⁺ T cells of CCR10^{-/-} and CCR10^{+/-} mice for CD4⁺ and CD8⁺ subsets. **C**, Average percentages of EGFP⁺CD8⁺ and CD4⁺ cells of skin lymphocyte preparations in CCR10^{+/-} and CCR10^{-/-} mice calculated by multiplying percentages of total EGFP⁺ T cells (Fig 2, A) and percentages of CD4⁺ and CD8⁺ cells of the total EGFP⁺ T cells (Fig 2, B). **D**, FACS analysis of gated skin EGFP⁺CD4⁺ T cells of CCR10^{-/-} and CCR10^{+/-} mice for Foxp3⁺ Treg cells. **E**, Average percentages of Foxp3⁺CD25⁺ Treg cells of skin CD4⁺ T cells in CCR10^{+/-} and CCR10^{-/-} mice calculated from the FACS analysis in Fig 2, D (n $\geq$ 10). **F**, Average numbers of EGFP⁺CD4⁺Foxp3⁺, CD4⁺, and CD4⁻(CD8⁺) T cells isolated from skin of CCR10^{+/-} versus CCR10^{-/-} mice (n $\geq$ 10). **G**, FACS analysis of skin CD4⁺ T cells of CCR10^{-/-} and CCR10^{+/-} mice for the IL-10⁺ subset. Average percentages of IL-10⁺ cells of the EGFP⁺ or EGFP⁻ CD4⁺ cells is in the left panels (n = 4 each). **H**, FACS analysis of skin CD4⁺ T cells of CCR10^{+/-} and CCR10^{-/-} mice for the IL-17A⁺ subset. Bar graphs show average percentages of IL-17A⁺ cells of EGFP⁺ or EGFP⁻ CD4⁺ cells (middle) and their mean fluorescence intensity for IL-17A staining (right; n = 6 each). **I**, Levels of IL-17A production by purified CCR10^{+/-} or CCR10^{-/-} skin EGFP⁺CD4⁺CD25⁺ T cells stimulated with IL-2 and coated anti-CD28/anti-TCR β antibodies in culture (n = 4-5). **J**, Relative contribution of transferred CCR10^{+/-} versus CCR10^{-/-} BM cells to the indicated skin T-cell subsets in irradiated WT recipient mice (n = 5 each). **K**, FACS analysis of skin CD4⁺ T cells of CCR10^{-/-} versus CCR10^{+/-} BM donor origins in recipient mice for IL-17A⁺ cells, as presented in Fig 2, H (n = 5 each). **P* < .05, ***P* < .005, and ****P* < .001. NS, No significant difference.

compared with CCR10^{-/-} mice, CCR10^{+/-} mice had marginally enhanced innate responses to topical application of TPA, a strong mitogen that activates both keratinocytes and immune cells (Fig 1, F, and see Fig E1 in this article's Online Repository at www.jacionline.org). Considering the 1-time DNFB or FITC treatments are weaker stimulators than TPA or repeated DNFB treatments of the CHS assay, these results reveal that CCR10^{-/-} mice have a reduced activation threshold to the weak stimulation but still mount full-scale responses once the threshold is overcome by strong stimulators, suggesting a defect in the immune regulatory system in the skin.

Imbalanced maintenance of skin-resident Treg and Teff cells in CCR10^{-/-} mice

We next analyzed how CCR10 knockout affected skin-resident T cells, including Treg cells, which are critical in immune regulation. Based on the EGFP reporter for CCR10 in CCR10^{+/-} mice,^{21,22} the majority of skin-resident $\alpha\beta$ T cells highly expressed CCR10 (EGFP; Fig 2, A, and see Fig E2 in this article's Online Repository at www.jacionline.org) and memory T-cell markers (KLRG1⁻CD62L⁻CD44^{high}CD127⁺ for CD8⁺ and KLRG1⁺CD62L⁻CD44^{high}CD127⁺ for CD4⁺ cells, see Fig E3 in this article's Online Repository at www.jacionline.org).^{21,25}

Compared with CCR10^{+/-} mice, CCR10^{-/-} mice had fewer skin $\alpha\beta$ T (CD3⁺ $\gamma\delta$ TCR⁻) cells (Fig 2, A), of which the CD8⁺ subset was impaired more profoundly than the CD4⁺ subset (Fig 2, B and C). The CCR10^{low}CD3^{high} population of T cells were V γ 3⁺ $\gamma\delta$ T cells, numbers of which were increased in the dermis of CCR10^{-/-} mice because of dysregulated localization (Fig 2, A), as we previously reported.²¹ Notably, CCR10^{-/-} mice had significantly fewer forkhead box protein 3 (Foxp3)-positive Treg cells within the skin CD4⁺ population than CCR10^{+/-} mice (Fig 2, D and E). When total cell yields are considered, fewer Treg and CD4⁺ and CD8⁺(CD4⁻) Teff cells were isolated from the skin of CCR10^{-/-} mice than CCR10^{+/-} mice (Fig 2, F). In contrast to the skin, very few EGFP⁺ T cells were found in internal lymphoid organs, such as spleens, and their numbers were even higher in CCR10^{-/-} mice than in CCR10^{+/-} mice (see Fig E4 in this article's Online Repository at www.jacionline.org), suggesting that defective maintenance of T cells in the skin results in their abnormal accumulation at internal sites.

Impaired immune homeostasis in the skin of CCR10^{-/-} mice

The imbalanced presence of resident Treg versus Teff cells could impair skin immune homeostasis in CCR10^{-/-} mice. Indeed, the skin CD4⁺ T-cell subset of CCR10^{-/-} mice contained a significantly lower percentage of IL-10⁺ and higher percentage of IL-17A⁺ (T_H17) cells than the skin of CCR10^{+/-} control animals (Fig 2, G and H). EGFP⁺ T_H17 cells of CCR10^{-/-} mice also had markedly higher mean fluorescence intensity for IL-17A staining than EGFP⁺ T_H17 cells of CCR10^{+/-} mice (Fig 2, H), suggesting that CCR10 regulates the function of resident CCR10⁺ T_H17 cells, as well as their maintenance. Supporting this conclusion, purified EGFP⁺ skin CD4⁺ Teff cells of CCR10^{-/-} mice secreted much higher levels of IL-17A in culture than those of the CCR10^{+/-} control animals (Fig 2, I). Notably, EGFP(CCR10)⁻ skin T cells of CCR10^{+/-} mice contained a higher percentage of T_H17 cells with higher IL-17A production than EGFP(CCR10)⁺ skin T cells of the same mice (Fig 2, H), also supporting the role of CCR10 in suppression of T_H17 functions.

To determine whether CCR10 is directly involved in regulating skin Treg and Teff cell subsets, we performed competitive BM cotransfer experiments in which similar numbers of CCR10^{-/-} and CCR10^{+/-} BM cells were injected into irradiated WT mice. EGFP⁺ CD8⁺, CD4⁺, and Foxp3⁺ T-cell subsets of the CCR10^{-/-} donor were all greatly underrepresented in the skin compared with corresponding populations of the CCR10^{+/-} donor (Fig 2, J, and see Fig E5, A, in this article's Online Repository at www.jacionline.org). In addition, EGFP⁻ CD8⁺, CD4⁺, and Treg cell subsets of the CCR10^{-/-} donor were also underrepresented (to a lesser extent than the EGFP⁺ subsets) in the skin compared with corresponding populations of the CCR10^{+/-} donor (see Fig E5, B, in this article's Online Repository at www.jacionline.org). Because EGFP⁻ cells do not express CCR10, the effect of CCR10 knockout on these cells is likely secondary to the effect of CCR10 knockout on EGFP⁺ cells. Furthermore, EGFP⁺ skin CD4⁺ T cells of the CCR10^{-/-} donor still had higher percentages of T_H17 cells with higher IL-17 mean fluorescence intensity staining than those of the CCR10^{+/-} donor (Fig 2, K), demonstrating an intrinsic regulatory role of CCR10 in the maintenance and function of skin-resident T cells.

Treg cell-regulated immune homeostasis is critical for maintenance of CCR10⁺ memory-like resident T cells in the skin

Although the imbalanced presence and dysregulated function of skin-resident Treg versus Teff cells are consistent with the overreactive innate response in CCR10^{-/-} mice, the normal CHS response (Fig 1, A) suggests that CCR10 is not critical for migration of infiltrating Teff cells during inflammation. We noted that DNFB-inflamed skin of the CHS assay had significantly lower percentages of CCR10⁺ T cells than untreated skin (Fig 3, A), which suggests that CCR10 is not expressed on many new infiltrating T cells and might explain the different effects of CCR10 knockout on skin-resident T cells under steady conditions versus infiltrating T cells during inflammation. Consistent with this, most Treg cells of TPA-treated inflamed ear skin did not express CCR10 (see Fig E6 in this article's Online Repository at www.jacionline.org). However, these assays could not distinguish infiltrating T cells from resident T cells that potentially downregulated CCR10. To dissect this further, we transferred EGFP⁻ splenic T cells of CCR10^{+/-} or CCR10^{-/-} mice into Rag1^{-/-} mice and analyzed the skin-infiltrating donor T cells at different times after induction of CHS. At the peak of inflammation (1 day after the DNFB challenge), only a few skin-infiltrating T cells were EGFP⁺ (Fig 3, B). In marked contrast, 1 month after the DNFB challenge, when inflammation was resolved, approximately half of the skin T cells of CCR10^{+/-} donors were EGFP⁺, and the percentage of EGFP⁺ skin T cells of the CCR10^{-/-} donor was significantly lower than that of the CCR10^{+/-} donor (Fig 3, C and D). Because the skin EGFP⁺ T cells of the donor in Rag1^{-/-} recipients had preferentially displayed memory phenotypes early after challenge, even during the active phase of inflammation (Fig 3, E), these results suggest that CCR10 is preferentially imprinted on memory T-cell progenitors for their maintenance in the skin after resolution of inflammation but not on Teff cells for their skin infiltration during inflammation.

We tested this notion further using 2 chronic inflammation models. First, we transferred Treg cell-depleted splenic naive CD4⁺ T cells into Rag1^{-/-} mice, which induces inflammation in the skin (and other tissues) because of the absence of Treg cells.^{26,27} Nearly no donor T cells expressed EGFP in the skin of recipients 2 months after transfer (Fig 3, F, right), whereas in Rag1^{-/-} mice receiving splenic naive CD4⁺ T and Treg cells, high percentages of skin T cells of both donor origins were EGFP⁺ (Fig 3, F, left), indicating that Treg cell-regulated immune homeostasis is critical for the establishment and maintenance of CCR10⁺ T cells in skin. Consistent with this idea, only a few skin T cells were EGFP⁺ in Foxp3-deficient Scurfy mice, which lack Treg cells and have skin inflammation soon after the birth (Fig 3, G). Together, these results reveal an important role of CCR10 in a positive feedback circuit of immune homeostatic regulation in the skin, where CCR10 maintains balanced presence and function of resident Treg versus Teff cells, which in turn helps to establish a homeostatic environment important for maintenance of the CCR10⁺ resident T cells.

CCR10 is critical for localization of CCR10⁺ T cells into homeostatic skin

A drawback with the DNFB/FITC model is the difficulty of following antigen-specific T cells. Therefore we crossed

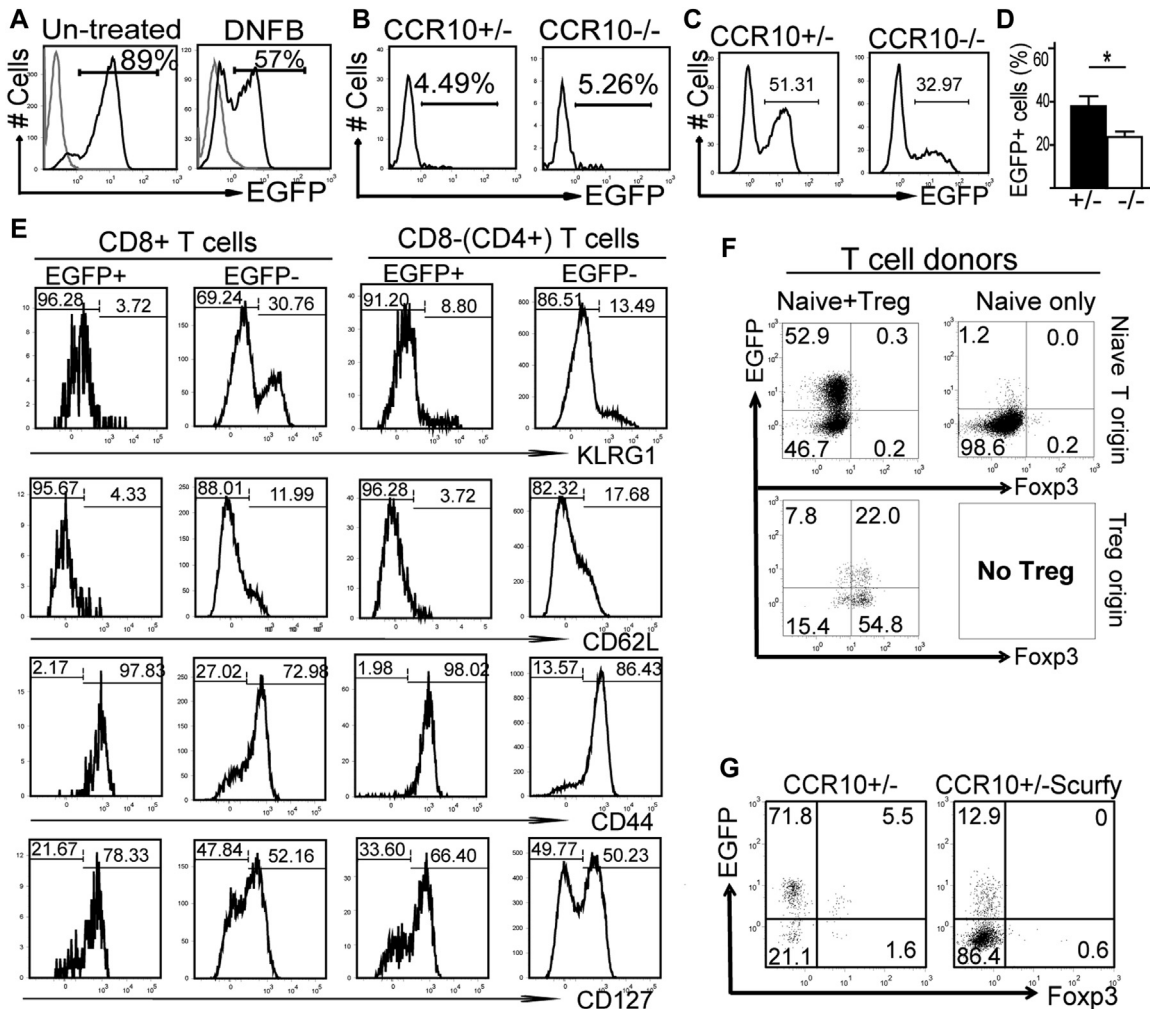


FIG 3. Treg cell-regulated immune homeostasis is critical for maintenance of CCR10⁺ memory-like resident T cells in the skin. **A**, FACS analysis of T cells of untreated or inflamed skin of CCR10^{+/+} mice for CCR10(EGFP) expression. The inflamed skin was of mice 1 day after DNFB-induced CHS response. The gray lines indicate WT cells as negative controls for EGFP (n = 2). **B-D**, FACS analysis for EGFP on skin T cells in Rag1^{-/-} mice transferred with CCR10^{+/+} or CCR10^{-/-} EGFP⁺ splenic T cells 1 day (Fig 3, B) or 1 month (Fig 3, C and D) after DNFB-induced CHS. Average percentages of EGFP⁺ skin T cells of CCR10^{+/+} versus CCR10^{-/-} donors 1 month after CHS induction are shown in Fig 3, D (n = 10 each). **E**, Representative FACS analysis for molecules associated with memory on skin EGFP⁺ versus EGFP⁻ T cells of CCR10^{+/+} donor T-cell origin in Rag1^{-/-} mice 1 day after DNFB-induced CHS (n = 4). **F**, FACS analysis of EGFP expression on Teff (top) and Treg (bottom) cells of the skin of Rag1^{-/-} mice 7 to 8 weeks after they were transferred with EGFP⁺ naive splenic CD4⁺ Teff cells only (right) or a mixture of EGFP⁺ naive splenic CD4⁺ Teff and Treg cells (left; n = 4). **G**, FACS analysis for EGFP on skin T cells of 1-month-old CCR10^{+/+} Scurfy and control mice (n = 5). *P < .05.

CCR10^{-/-} mice to OT-I or OT-II mice that carry CD8⁺ or CD4⁺ T cells expressing transgenic $\alpha\beta$ TCRs specific for OVA antigens, respectively. Purified naive splenic EGFP⁺ CCR10^{+/+} and CCR10^{-/-} OT-I (or OT-II) T cells were transferred into WT mice, followed by epicutaneous immunization with OVA on the ear (twice with a week interval).²³ One week after the second immunization, inflamed ear and uninflamed torso skin were analyzed for OVA-specific T cells.

In the inflamed ear skin, newly infiltrating, OVA-specific CD8⁺ OT-I T cells accounted for the majority of total T cells (Fig 4, A). However, only a small portion of this subset was EGFP⁺, and

CCR10 knockout did not affect the percentage of EGFP⁺ OT-I T cells in the ear (Fig 4, A and C). In striking contrast, much higher percentages of infiltrating OT-I T cells in the untreated (and therefore uninflamed) torso skin were EGFP⁺, and CCR10 knockout significantly reduced the EGFP⁺ OT-I cell numbers in the untreated skin (Fig 4, B and C), revealing that CCR10 is critical for localization of the CCR10⁺ CD8⁺ T cells into uninflamed but not inflamed skin, even in the same mouse. Similarly, CCR10 is also required for localization of CCR10⁺CD4⁺ OT-II T cells into untreated skin but dispensable for their localization into inflamed skin stimulated by OVA immunization (see Fig E7 in this article's Online Repository at www.jacionline.org).

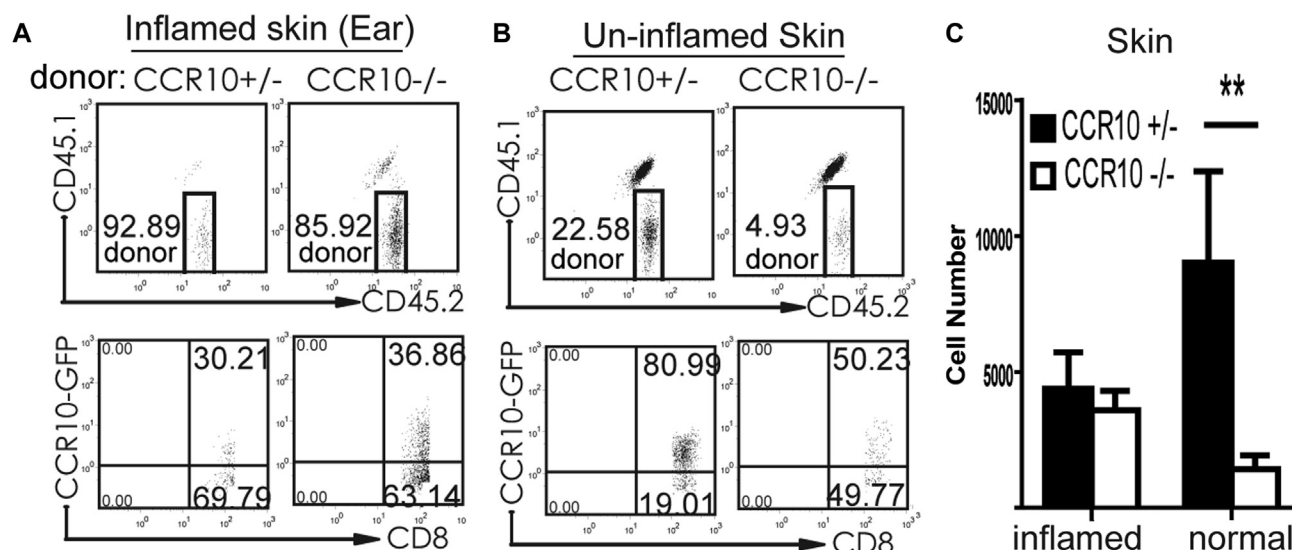


FIG 4. CCR10 is critical in localization of CCR10⁺ T cells into homeostatic skin. **A** and **B**, Flow cytometry of gated CD3⁺ CD8⁺ T cells isolated from OVA-treated inflamed ear skin (Fig 4, **A**) and untreated torso skin (Fig 4, **B**) for donor-derived OT-I T cells (CD45.1⁺ CD45.2⁺; top) and expression of EGFP of donor OT-I T cells (bottom). **C**, Average numbers of EGFP⁺ CCR10⁺/− and CCR10[−]/− OT-I T cells isolated from inflamed ear skin and unaffected torso skin based on analyses in Fig 4, **B** (n = 5-6). **P < .005.

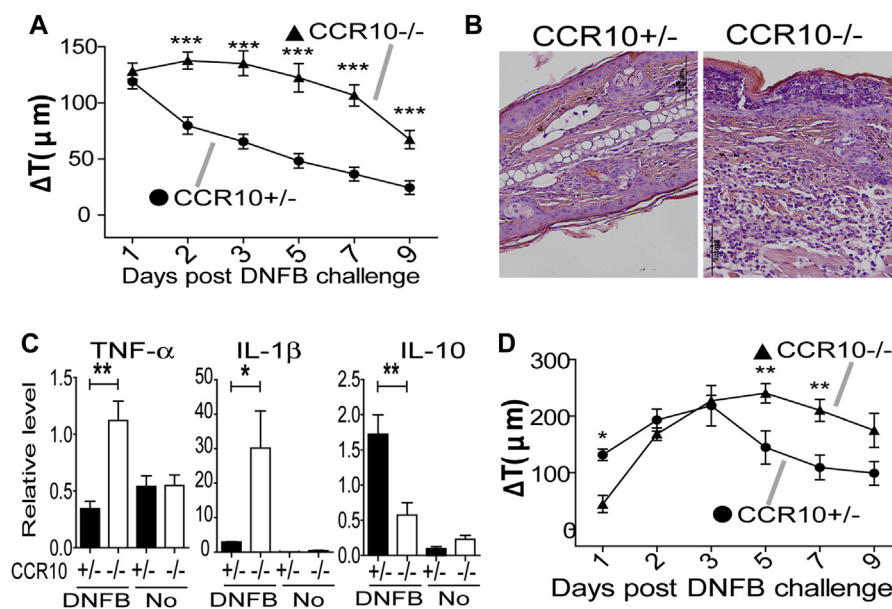


FIG 5. Defective resolution of immune memory responses in the skin of CCR10[−]/− mice. **A**, Ear thickness changes of CCR10[−]/− and CCR10⁺/− mice in a DNFB-induced memory CHS assay (n ≥ 6). **B**, Representative hematoxylin and eosin staining of ear sections 3 days after the DNFB-induced memory CHS response (magnification ×400; n = 4). **C**, Quantitative RT-PCR analysis of TNF-α, IL-1β, and IL-10 transcripts in treated skin 3 days after DNFB-induced memory CHS (n = 4). **D**, Ear thickness changes of Rag1[−]/− mice transferred with EGFP⁺ CCR10[−]/− and CCR10⁺/− splenic T cells in DNFB-induced memory CHS (n ≥ 6). *P < .05, **P < .005, and ***P < .001.

Defective resolution of immune memory responses in skin of CCR10[−]/− mice

Skin-resident memory T cells play important roles in memory responses.^{1,4-6} We tested CCR10[−]/− mice using a memory CHS assay, in which mice were challenged with DNFB 1 month (instead of 5 days in a classic CHS assay) after the initial DNFB sensitization. Compared with CCR10⁺/− mice, CCR10[−]/− mice had strikingly prolonged inflammation in the

skin associated with enhanced TNF-α and IL-1β and reduced IL-10 expression (Fig 5, A-C), suggesting that the imbalanced maintenance and function of the resident Treg versus Teff cells also causes defective resolution of memory responses. Further supporting this notion, memory CHS responses in Rag1[−]/− mice transferred with splenic CCR10[−]/− T cells, which had defective maintenance of the donor-derived EGFP⁺ memory-like T cells in the skin after resolution of inflammation in classic

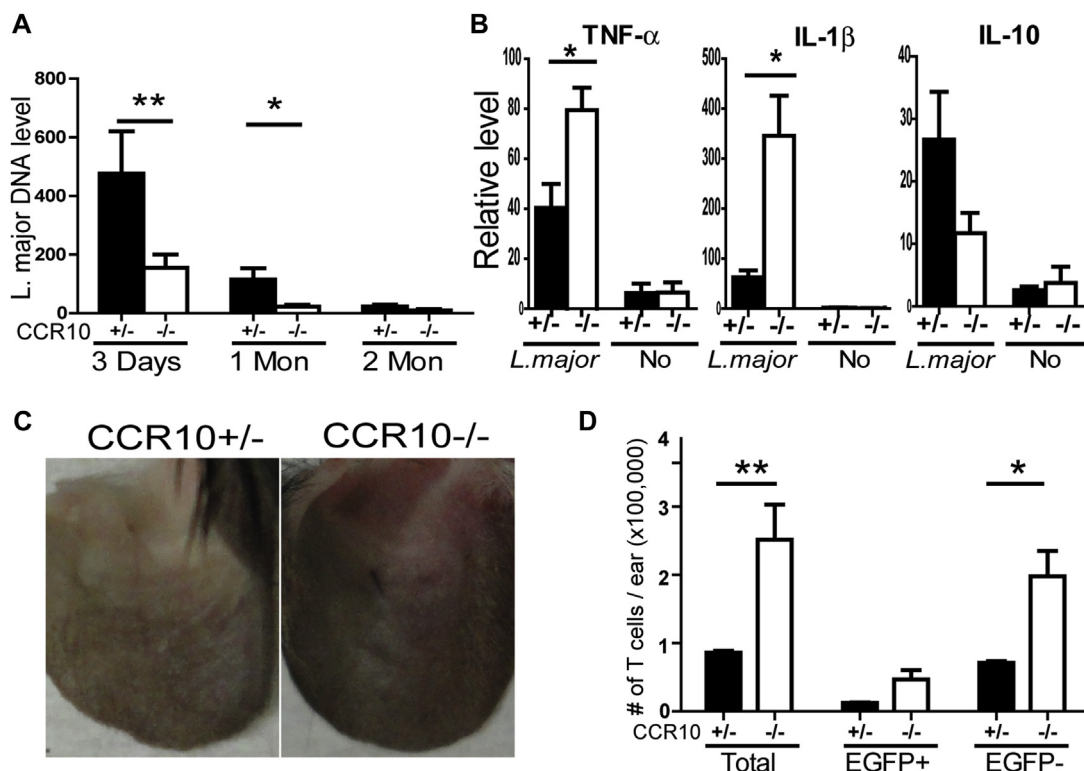


FIG 6. Accelerated clearance of *L. major* infection in skin of CCR10^{-/-} mice. **A**, Levels of *L. major*-specific 16S rDNA in skin of CCR10^{-/-} and CCR10^{+/-} mice at different time points after infection, as determined by using quantitative PCR and normalized on mouse β -actin ($n = 11$, 10 and 4 of each genotype for 3 days and 1 and 2 months after the infection, respectively). **B**, Quantitative RT-PCR analysis of TNF- α , IL-1 β , and IL-10 transcripts in infected ears of CCR10^{-/-} versus CCR10^{+/-} mice on day 3 after *L. major* infection ($n = 5$ each). **C**, Representative ear lesions at infection sites in CCR10^{-/-} and CCR10^{+/-} mice 1 month after *L. major* injection. **D**, Numbers of total, EGFP⁺, and EGFP⁻ T cells in infected ears of CCR10^{-/-} and CCR10^{+/-} mice 1 month after *L. major* infection ($n = 3$ each). * $P < .05$ and ** $P < .005$.

CHS (Fig 3, C and D), were also prolonged compared with those seen in recipients of CCR10^{+/-} T cells (Fig 5, D). Notably, although there was no difference in the early memory response between immunized CCR10^{+/-} and CCR10^{-/-} mice the first day after reapplication of DNFB (Fig 5, A), Rag1^{-/-} mice receiving CCR10^{-/-} T cells had slower memory responses than Rag1^{-/-} mice receiving CCR10^{+/-} T cells (Fig 5, D, day 1). The different effects of CCR10 knockout on the early response between the 2 models are likely because in direct comparison of CCR10^{-/-} and CCR10^{+/-} mice, the memory response could be affected by CCR10-regulated $\alpha\beta$ T cells, as well as other cells, whereas in Rag1^{-/-} mice transferred with splenic T cells, some of the CCR10-regulated cells, such as the epidermis-resident $\gamma\delta$ T cells, are not reconstituted.²¹ The epidermis-resident $\gamma\delta$ T cells are known to affect the skin immune response.²⁸

Enhanced immune response to and accelerated clearance of *L. major* infection in the skin of CCR10^{-/-} mice

Our finding of the important role of CCR10 in regulating skin-resident Treg and Teff cells to prevent overactive immune responses predicts that targeting CCR10 might enhance immune responses against skin-specific infections and other diseases. As a test, we infected CCR10^{-/-} mice with *L. major*, a parasitic

pathogen that could manipulate Treg cells to evade immune attack for its long-term survival in the skin.²⁹ Indeed, CCR10^{-/-} mice cleared the parasite much faster than CCR10^{+/-} mice (Fig 6, A). Consistent with an enhanced immune response, CCR10^{-/-} mice had higher TNF- α and IL-1 β production than CCR10^{+/-} mice early after infection (Fig 6, B), as in the case of chemical challenge (Fig 1). In addition, 1 month after infection, significantly higher percentages of CCR10^{-/-} mice had visible inflammation at infection sites than CCR10^{+/-} mice (76% [13/17] vs 38% [8/21], $P = .025$; Fig 6, C), and the infected ears of CCR10^{-/-} mice had many more T cells, most of which were EGFP⁻, than the CCR10^{+/-} control animals (Fig 6, D, and see Fig E8 in this article's Online Repository at www.jacionline.org), demonstrating that absence of CCR10 increased the Teff cell response for more efficient clearance of infections in the skin.

Significant percentages of T cells in healthy human skin express CCR10

In human subjects all blood CCR10⁺ T cells display memory cell markers.^{9,13} Therefore CCR10/CCL27 might promote preferential maintenance of the CCR10⁺ memory-like T cells in healthy skin. However, direct evidence of the enrichment of CCR10⁺ T cells in skin is lacking. Therefore we analyzed T cells isolated from human healthy skin for their expression of CCR10.

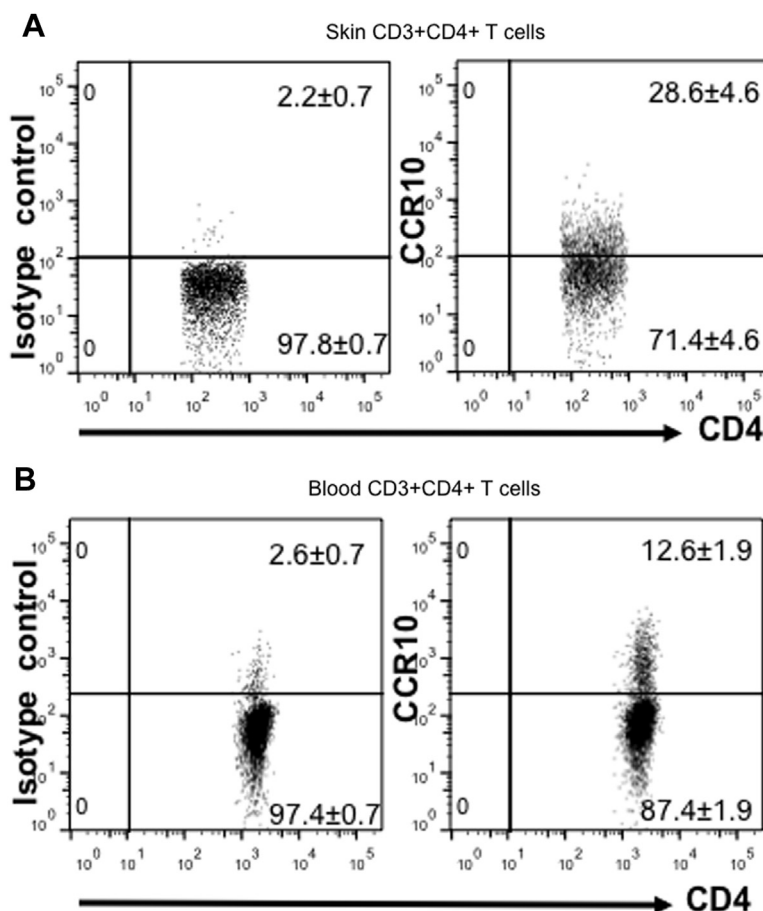


FIG 7. Preferential expression of CCR10 by T cells of healthy human skin. Flow cytometric analysis of gated CD3⁺CD4⁺ T cells isolated from the healthy skin (**A**) and blood (**B**) of human subjects for CCR10 expression. The number in each gate is the average percentage of cells in the gate of total events expressed as means $\pm$ SEs ($n = 3$ individual samples for each analysis). Isotype control staining for CCR10 staining is shown in the left panels.

Compared with blood T cells, significantly higher percentages of T cells isolated from normal human skin expressed CCR10 (Fig 7), correlating with the different expression of CCR10 on circulating and skin-resident T cells in mice (Fig 2 and see Fig E4). The similar expression pattern of CCR10 in the skin T cells of mice and human subjects suggests that CCR10 might function in a similar fashion in both human and mouse skin.

DISCUSSION

CCR10/CCL27 are implicated in various skin allergy and inflammatory diseases, but the *in vivo* function of the pair has been elusive, hindering our effort in understanding their involvement in disease development.¹² In this report we provide the first definite evidence that CCR10 is critical in the regulation of immune homeostasis and resolution of inflammation, an advance from the current focus on the role of CCR10 as a homing molecule for infiltrating T cells to promote the immune response in the skin. Mechanistically, CCR10 could potentially function in several aspects to regulate skin T cells (see Fig E9 in this article's Online Repository at www.jacionline.org). First, CCR10 is critical in the migration of CCR10⁺ memory (or memory-like) T-cell precursors into the skin under homeostatic conditions, whereas it is

dispensable for infiltration of T cells into the inflamed skin. In addition, CCR10 expressed on skin-resident Treg and Teff cells is important for their balanced maintenance in the skin under homeostatic conditions, potentially by regulating their retention, proliferation, and survival in the skin. Furthermore, our studies also suggest that CCR10 might signal to regulate functions of the CCR10⁺ skin-resident T cells, such as their production of inflammatory and regulatory cytokines. In the absence of CCR10, the dysregulated maintenance and functions of resident Teff and Treg cells in the skin could result in the overactive immune response and impaired resolution of skin inflammation. In light of our finding of CCR10 as a critical regulator of skin immune homeostasis in mice, the roles of the CCR10/CCL27 axis in skin allergy and inflammatory diseases in patients need to be carefully investigated before targeting the axis for treatment. It is possible that the upregulated expression of CCL27 in inflamed skin is secondary to the inflammation for the purpose of regulation. Therefore developing a strategy to increase the number and activity of CCR10⁺ resident Treg cells might help to restore immune homeostasis and treat inflammatory diseases of the skin.

Considering both resident Treg and Teff cells are reduced in the skin of CCR10^{-/-} mice, the overactive immune response to skin stimulation is likely due to the impaired presence and

dysregulated function of both populations. Consistent with this, our study suggests that CCR10 has an intrinsic regulatory role in controlling the function of Teff cells (T_H17), which is in addition to potential regulatory effect of Treg cells on these Teff cells. Although mechanisms underlying the intrinsic regulatory functions of CCR10 in the Teff and Treg cells are not clear, our finding is reminiscent of functions of some other T-cell regulatory receptors, such as programmed cell death 1 and cytotoxic T lymphocyte-associated antigen, that inhibit Teff cell functions and enhance Treg cell functions.^{30,31} Considering the importance of balanced production of inflammatory and regulatory cytokines in immune homeostasis and inflammation, it would be important to elucidate the mechanism of CCR10 in regulating T-cell activation and inflammatory cytokine production. In addition, addressing the origin of CCR10⁺ memory (or memory-like) Teff and Treg cells is another important question to assess.

CCR10 knockout affects the presence of CD8⁺ Teff and Treg cell subsets in the skin to a larger extent than CD4⁺ Teff cells, suggesting that the different resident T-cell subsets are intrinsically different in their requirements of CCR10 for maintenance. Although the underlying mechanism is not clear, it seems to be associated with the different mobility and expression of skin retention molecules of the different T-cell subsets.^{2,32,33} Of note, the skin-resident CD8⁺ memory T cells do not move, whereas the CD4⁺ cells are highly mobile.^{3,32,33} Although the mobility of skin-resident Treg cells is well studied, they express the skin adhesion molecules CD103, as do the resident CD8⁺ cells, whereas the resident CD4⁺ Teff cells do not.^{2,32,33}

Although a predominantly net effect of CCR10 knockout on skin immune responses is overreactive and prolonged inflammation, CCR10 might be important for an efficient effector memory T-cell response. Rag1^{-/-} mice reconstituted with CCR10^{-/-} T cells had a slower and prolonged memory response compared with those reconstituted with CCR10^{+/+} T cells (Fig 5, D), suggesting that both effector and resolution phases of the memory response are impaired. It is likely that the CCR10-dependent efficient and balanced maintenance of resident Teff and Treg memory T cells in the skin allows a rapid Teff response to a challenge, as well as proper resolution of the effector response after clearance of the challenge. Such efficient effector and regulatory responses are important for preservation of skin function while dealing with harmful environmental challenges. Dysregulation of either phase of the response could result in skin disease.

Key messages

- CCR10 is critical for skin immune homeostasis to control allergic responses.
- CCR10 regulates balanced maintenance and function of skin-resident Treg and Teff cells.
- CCR10 is not expressed on infiltrating Teff cells for their migration during skin inflammation.

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METHODS

Antibodies used in flow cytometry and analyzing strategies

Phycoerythrin (PE)- or PeCy7-anti-mouse CD62L (MEL-14, 1:200 dilution); PE-, PeCy5-, or PeCy7-antimouse CD44 (IM7, 1:400); PE-, biotin-, or PeCy7-anti-mouse CD4 (GK1.5, 1:200); PE-, PeCy5-, or biotin-anti-mouse CD8 α (53-6.7, 1:100); PE- or biotin-anti-mouse TCR $\gamma\delta$ (GL3, 1:100); Alexa Fluor 647-anti-mouse/rat Foxp3 (FJK-16s, 1:50); and PE-anti-mouse TCR β (H57-597, 1:100) were purchased from eBioscience (San Diego, Calif). Alexa Fluor 647-anti-mouse IL-17A (TC11-18H10.1, 1:250), PeCy7- or Pacific Blue anti-mouse CD45.1 (A20, 1:200), Alexa Fluor 647- or Alexa Fluor 700-anti-mouse CD45.2 (104, 1:100), Alexa Fluor 647-anti-mouse CD127 (A7R34, 1:100), PE- or PeCy7- or Biotin-anti-mouse CD3e (145-2C11, 1:100), PE-CD25 (PC61, 1:100), and PE-KLRG1 (2F1/KLRG1,

1:100) were from BioLegend (San Diego, Calif). Allophycocyanin-anti-mouse IL-10 (JES5-16E3, 1:100), FITC-anti-human CD3 (UCHT1, 1:50), PeCy7-anti-human CD4 (SK3, 1:50) and streptavidin-PE-Texas Red (1:200) were from BD Biosciences (San Jose, Calif). PE-anti-human CCR10 (314305, 1:10) was from R&D Systems (Minneapolis, Minn). PE-Alexa Fluor 610-anti-mouse CD4 (RM4-5, 1:100) and PE-Alexa Fluor 610-anti-mouse CD8 α (5H10, 1:100) were from Invitrogen (Camarillo, Calif). The Foxp3/Transcription Factor Staining Buffer Set was from eBioscience. Cells stained with a proper combination of antibodies and analyzed on a flow cytometer FC500 (Beckman Coulter) or BD Fortessa LSRII (San Jose, Calif). Cells stained with a single antibody were used to calibrate signal output and compensation. Staining with isotype control antibodies was included to confirm the specific signal for their corresponding antigen-specific antibody staining. Flow data were analyzed with FlowJo software (Ashland, Ore).

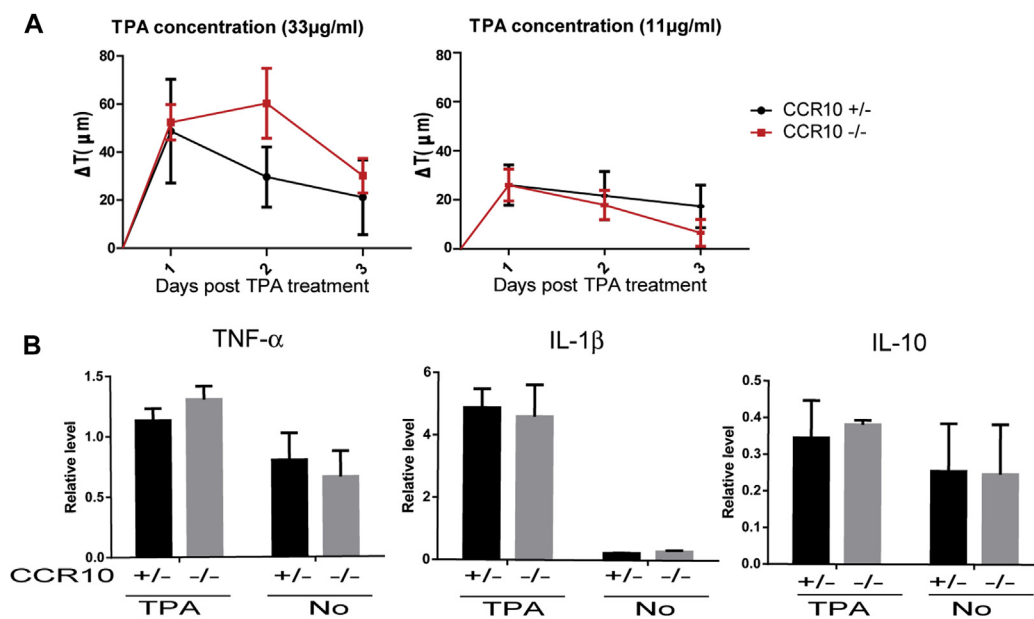


FIG E1. A, Ear thickness changes of CCR10^{-/-} and CCR10^{+/+} mice after 1-time topical application of indicated concentrations of TPA on the ear ($n \geq 6$). **B,** Quantitative real-time RT-PCR analysis of RNA isolated from treated ears 3 days after 1-time TPA application for TNF- α , IL-1 β , and IL-10. No indicates untreated skin samples. Values are relative levels normalized on β -actin ($n = 4$ CCR10^{+/+} and 5 CCR10^{-/-} mice).

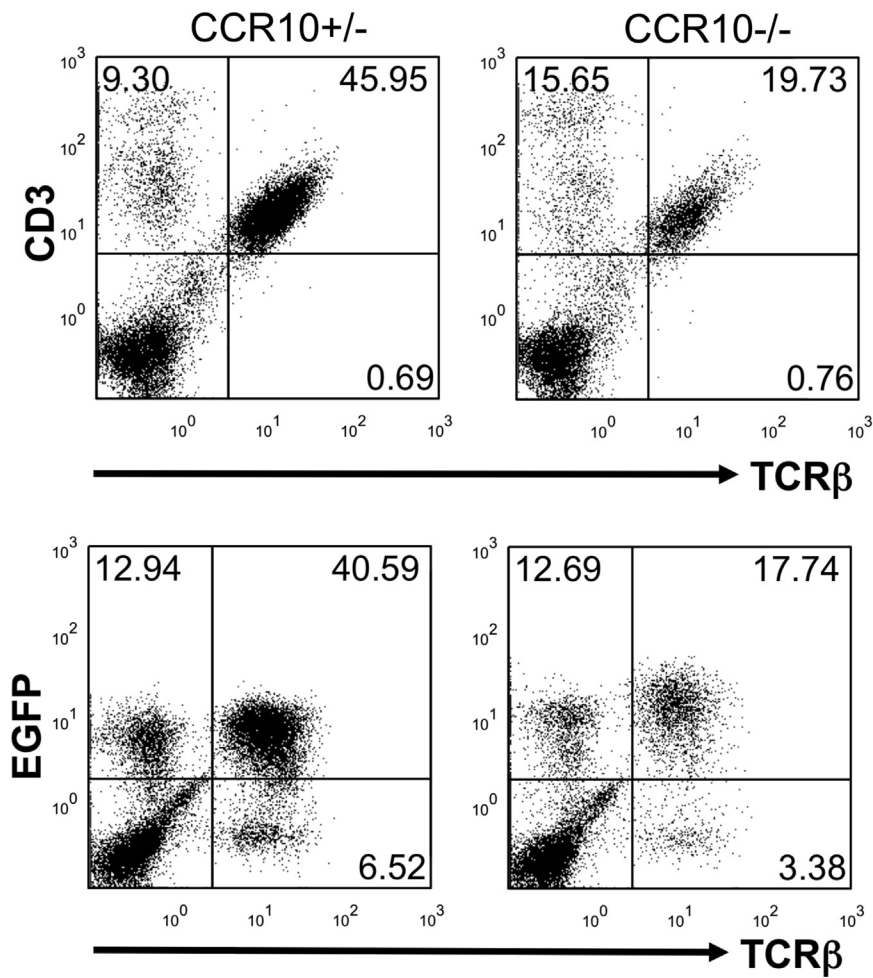


FIG E2. Representative flow cytometric analysis of gated lymphocytes isolated from skin of CCR10^{+/-} and CCR10^{-/-} mice for CD3⁺TCRβ⁺ T cells and their expression of EGFP (CCR10).

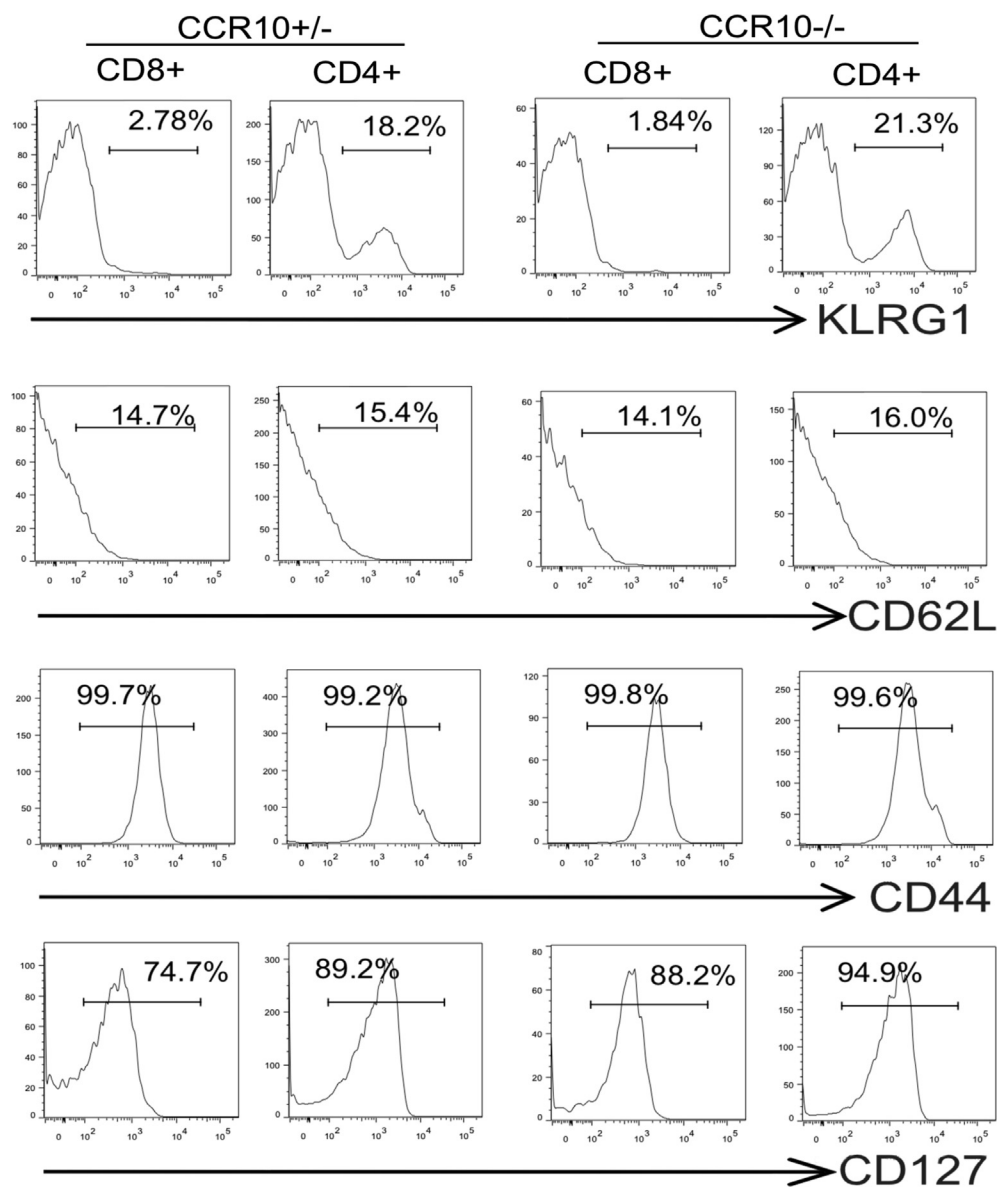


FIG E3. Representative flow cytometric analysis of gated EGFP⁺ CD4⁺ and CD8⁺ T cells isolated from skin of CCR10^{+/-} and CCR10^{-/-} mice for molecular markers associated with T-cell memory.

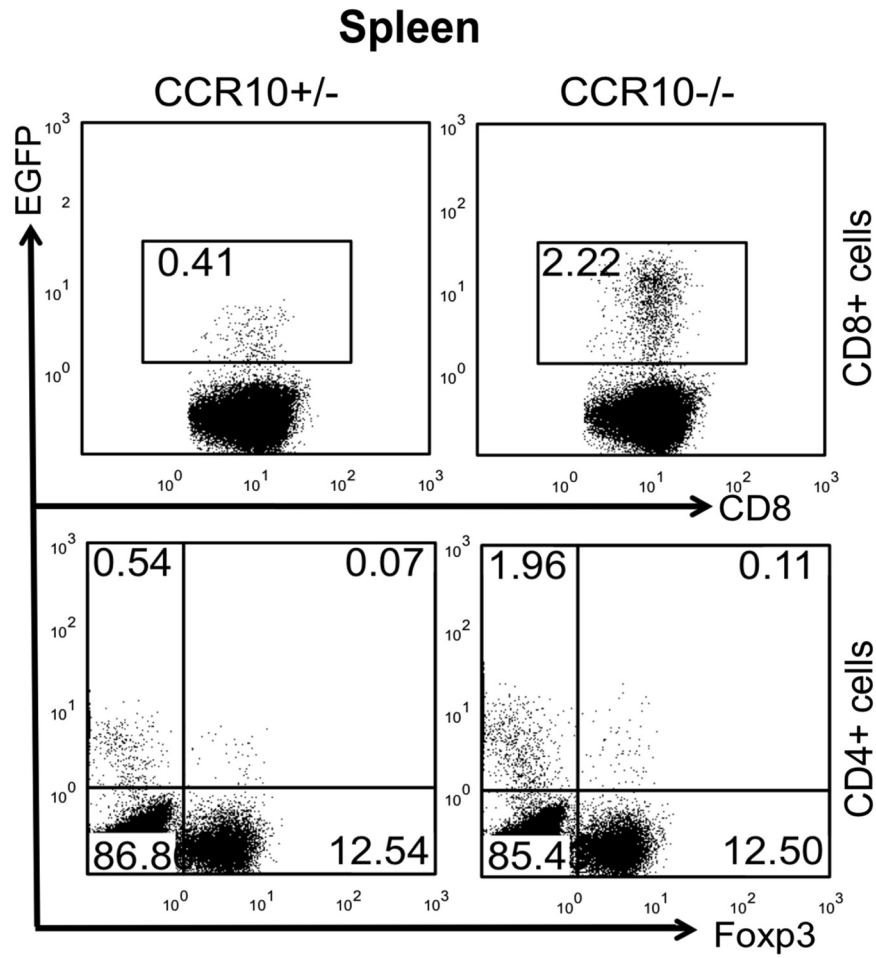


FIG E4. Representative FACS analysis of gated splenic CD3⁺CD8⁺ (*top*) and CD3⁺CD4⁺ (*bottom*) T cells for EGFP⁺ subsets in CCR10^{-/-} and CCR10^{+/-} mice.

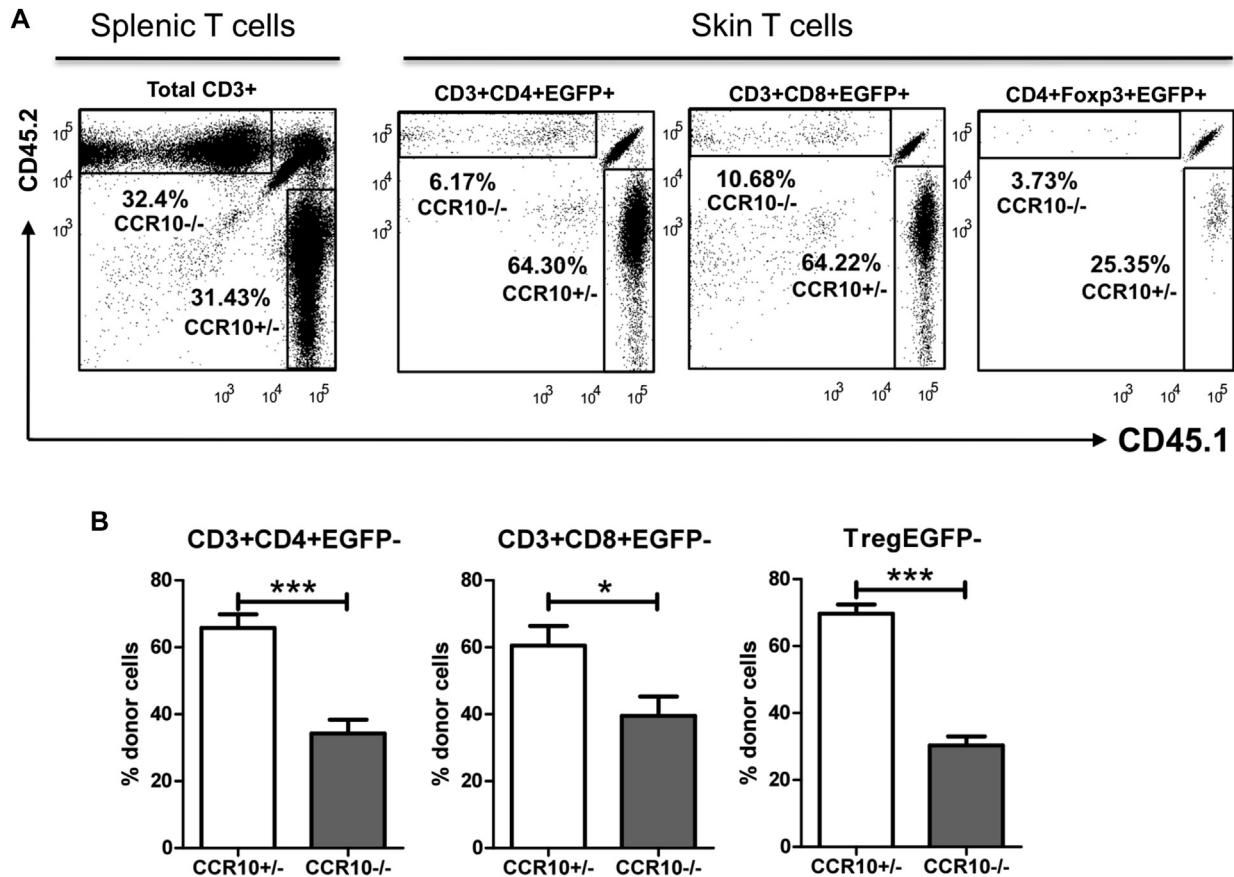


FIG E5. A, Representative flow cytometric analysis of gated skin T-cell populations (indicated in the figure) for their CCR10^{-/-} versus CCR10^{+/-} BM origins in irradiated WT recipients 2 months after BM transfer. CD45.1 and CD45.2 polymorphisms were used to distinguish T cells of different donor and host origins. Total splenic T cells were analyzed for their CCR10^{-/-} versus CCR10^{+/-} BM origins and used as the normalized reference to corresponding skin T-cell populations. **B**, Relative contribution of transferred CCR10^{-/-} versus CCR10^{+/-} BM cells to the indicated EGFP⁻ skin T-cell subsets in irradiated WT recipient mice (n = 5 each). **P* < .05 and ****P* < .001

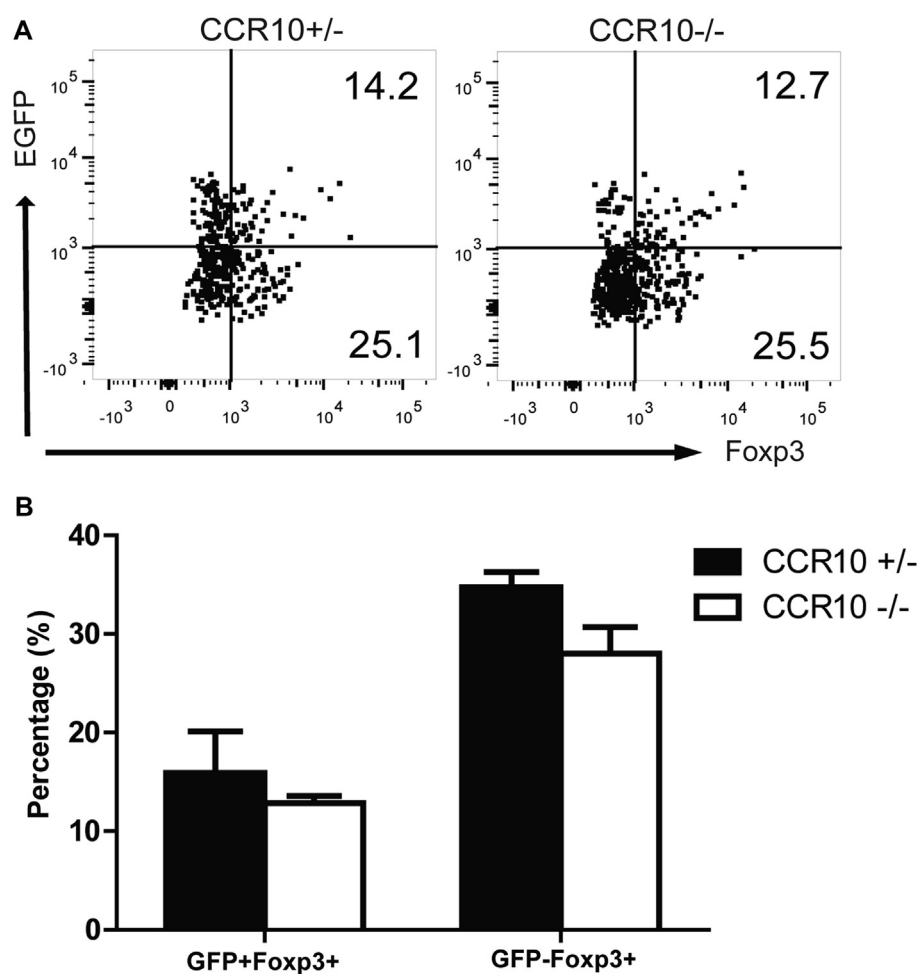


FIG E6. A, Representative FACS analysis of gated CD3⁺CD4⁺ T cells of TPA-treated ear skin of CCR10^{+/-} and CCR10^{-/-} mice for EGFP⁺ and EGFP⁻ Treg cells. TPA (33 μg/mL) was topically applied to ears. Two days after TPA application, cells isolated from treated ears were analyzed. **B**, Average percentages of EGFP⁺ and EGFP⁻ Treg cells of total CD4⁺ T cells isolated from TPA-treated ear skin of CCR10^{+/-} and CCR10^{-/-} mice based on the analysis in Fig E6, A (n = 3 each).

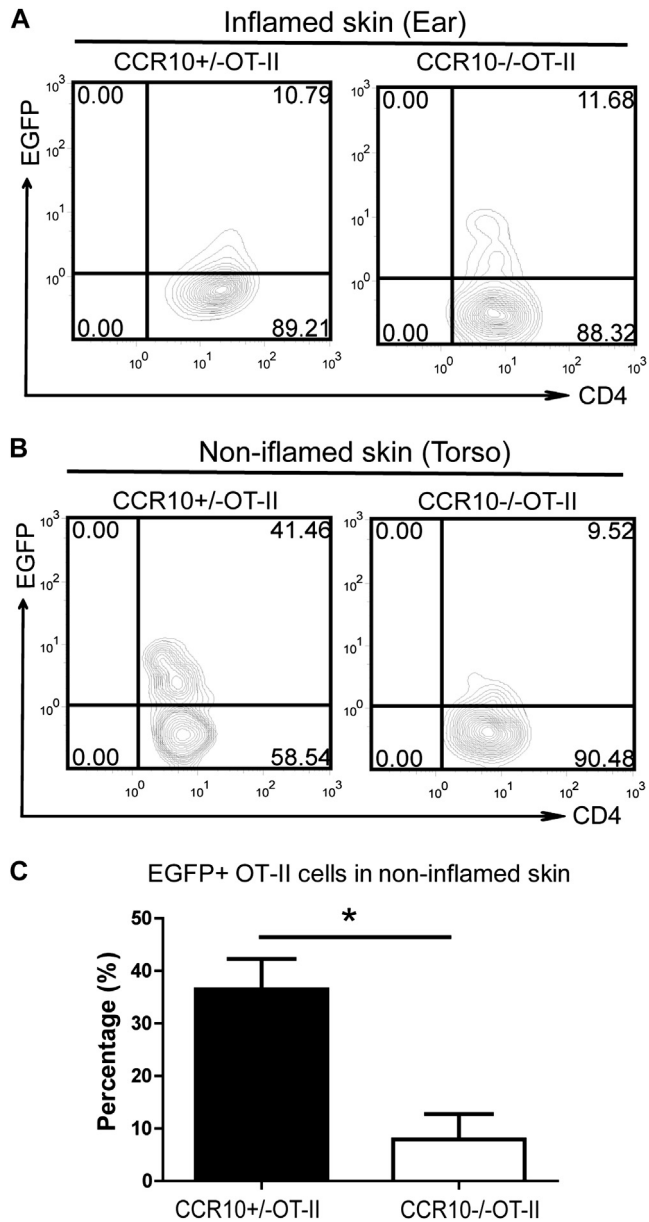


FIG E7. CCR10 is critical in localization of CCR10⁺CD4⁺ OT-II T cells into homeostatic skin. **A** and **B**, Flow cytometry of gated CD3⁺CD4⁺ OT-II cells isolated from OVA-treated inflamed ear skin (Fig E7, **A**) and untreated torso skin (Fig E7, **B**) for their expression of EGFP. **C**, Average percentages of CCR10^{+/-} and CCR10^{-/-} OT-II T cells of the inflamed ear skin and uninflamed torso skin that express EGFP (CCR10; n = 3 each). **P* < .05.

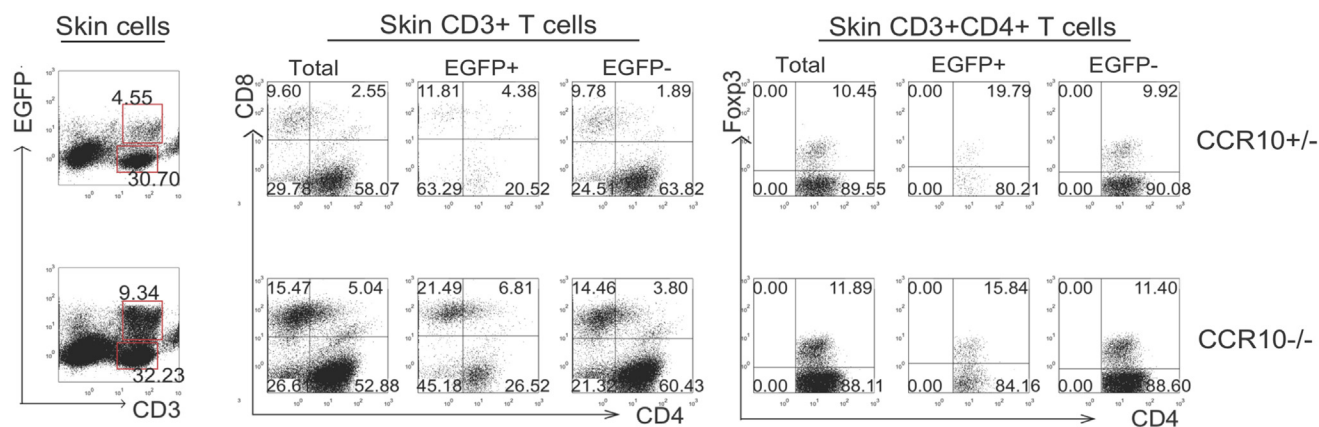


FIG E8. Representative flow cytometric analysis of T cells isolated from *L. major*-infected ears of CCR10^{-/-} versus CCR10^{+/-} mice for different T-cell subsets and their expression of EGFP. Mice were analyzed 1 month after infection.

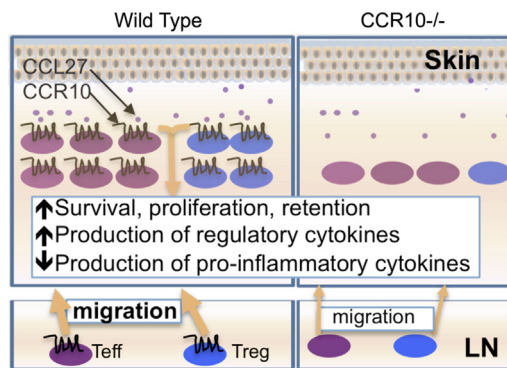


FIG E9. A schematic illustration of the roles of CCR10 in regulation of skin Treg and Teff cells under homeostatic conditions. *LN*, Lymph node.