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A Skin-selective Homing Mechanism for Human Immune Surveillance T Cells

Patrick Schaerli,[1] Lisa Ebert,[1] Katharina Willimann,[1] Andrea Blaser,[1] Regula Stuber Roos,[1] Pius Loetscher,[2] and Bernhard Moser[1]

[1]*Theodor-Kocher Institute, University of Bern, CH-3000 Bern 9, Switzerland*
[2]*Novartis Institutes for Biomedical Research, Novartis Pharma AG, CH-4002 Basel, Switzerland*

Abstract

Effective immune surveillance is essential for maintaining protection and homeostasis of peripheral tissues. However, mechanisms controlling memory T cell migration to peripheral tissues such as the skin are poorly understood. Here, we show that the majority of human T cells in healthy skin express the chemokine receptor CCR8 and respond to its selective ligand I-309/CCL1. These $CCR8^+$ T cells are absent in small intestine and colon tissue, and are extremely rare in peripheral blood, suggesting healthy skin as their physiological target site. Cutaneous $CCR8^+$ T cells are preactivated and secrete proinflammatory cytokines such as tumor necrosis factor–α and interferon-γ, but lack markers of cytolytic T cells. Secretion of interleukin (IL)-4, IL-10, and transforming growth factor–β was low to undetectable, arguing against a strict association of CCR8 expression with either T helper cell 2 or regulatory T cell subsets. Potential precursors of skin surveillance T cells in peripheral blood may correspond to the minor subset of $CCR8^+CD25^-$ T cells. Importantly, CCL1 is constitutively expressed at strategic cutaneous locations, including dermal microvessels and epidermal antigen-presenting cells. For the first time, these findings define a chemokine system for homeostatic T cell traffic in normal human skin.

Key words: chemokine • CCR8 • peripheral tissue • memory T cells • migration

Introduction

Numerous chemokines contribute to the composition of inflammatory cell infiltrates (1–4). These "inflammatory" chemokines are produced locally at the site of pathogen entry or tissue damage in response to multiple proinflammatory and microbial stimuli and mediate extravasation and recruitment of effector leukocytes. Pathogen elimination ultimately results in the establishment of an immune memory for enhanced protection against repeated exposure to the same pathogen. Currently, two types of memory T cells in peripheral blood have been described that are characterized by mutually exclusive tissue-homing properties (5). Central memory T cells (T_{CM}) express L-selectin (CD62L) and the chemokine receptor CCR7 for efficient traffic through secondary lymphoid tissues. In contrast, effector memory T cells (T_{EM}) lack these migration receptors and, therefore, are excluded from LNs and splenic T cell areas, but can enter sites of inflammation in the periphery. In mice, long-lived T_{CM} may develop from T_{EM} (6). Of note, the T_{CM}–T_{EM} distinction is not strict because $CCR7^+$ T cells are also found at inflammatory sites, and $CCR7^-$ effector T cells may enter reactive LNs (4).

In contrast with LNs and sites of inflammation, the mechanisms controlling the homing of memory T cells to peripheral tissues under steady-state (noninflamed) conditions are poorly defined. Such "immune surveillance" T cells are likely to have tissue-selective address codes that differ from those of either T_{CM} (secondary lymphoid tissues) or T_{EM} (inflammation). Tissue selectivity is conferred in part by adhesion molecules, such as $\alpha_4\beta_7$ on gastrointestinal T cells and cutaneous lymphocyte-associated antigen (CLA) on cutaneous T cells, whose respective ligands (the mucosal vascular addressin MAdCAM-1 and E-selectin) are present on the microvasculature within gut and skin tissue (7). In addition, immune surveillance T cells need to respond to homeostatic chemokines that are constitutively expressed in healthy peripheral tissues, thereby clearly differing from effector cells that are recruited to inflamed sites in response

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Address correspondence to Bernhard Moser, Theodor-Kocher Institute, University of Bern, P.O. Box 99, CH-3000 Bern 9, Switzerland. Phone: 41-31-631-4157; Fax: 41-31-631-3799; email: bernhard.moser@tki.unibe.ch

Abbreviations used in this paper: CLA, cutaneous lymphocyte-associated antigen; LC, Langerhans cell; T_{CM}, central memory T cells; T_{EM}, effector memory T cells; Treg, T regulatory.

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to inducible chemokines (2–4). The chemokine receptor CCR9 is prominently expressed by small intestinal T cells, and its single ligand TECK/CCL25 is produced by crypt epithelial cells and proximal endothelial cells in the small intestine, which suggests that this chemokine may contribute to local T cell traffic (3, 8–10). It is unclear at present which chemokine systems contribute the steady-state traffic of immune surveillance T cells at other sites of the gastrointestinal tract.

The skin forms the primary barrier between the body and the environment and is subject to continuous insults that include microbes, chemicals, UV irradiation, and tissue disruption. Integrity of the skin critically depends on an adequate skin-selective immune system because malfunction can lead to a multitude of local diseases (e.g., tumors, psoriasis, and atopic dermatitis; references 11–13). The cutaneous immune system consists of sentinel cells, including macrophages, DCs, and mast cells as well as memory T cells that reflect previous vaccination responses. Understanding the mechanisms by which these cells are recruited and positioned within the skin is crucial to understand, and eventually manipulate, the cutaneous immune system for therapeutic benefits. Here, we report that I-309/CCL1 and its single receptor CCR8 fulfill the requirements for controlling the steady-state traffic of cutaneous memory T cells.

CCL1 was one of the first chemokines discovered, and after 16 yr of investigation, numerous target cells and cellular responses have been reported, including those related to tumor cell apoptosis, angiogenesis, and HIV-1 infection as well as blood and tissue cell chemotaxis. However, the cloning of CCR8 mRNA revealed the scarcity of this chemokine receptor, notably in peripheral blood leukocytes (14–17). Recently, CCR8 positivity was found in thymocytes (18–21), cultured Th2 (22–25), and $CD4^+CD25^+$ T regulatory (Treg) cells (20, 26, 27). The role of CCR8 in the physiology of these cell types is still unclear. Here, using a novel anti-CCR8 antibody reagent, we demonstrate that healthy skin is a major reservoir of $CCR8^+$ T cells, which consist of cytokine-producing but not cytotoxic $CD4^+$ and $CD8^+$ T cells. In peripheral blood, potential precursors of skin immune surveillance T cells were identified as $CCR8^+CD25^-$ T cells rather than Treg cells, which express low levels of CCR8. Importantly, CCL1 is constitutively expressed within the dermal microvasculature, as well as by epidermal Langerhans cells (LCs) and melanocytes. Together, these findings support a unique role for this chemokine system in the tissue localization of memory T cells during cutaneous immune surveillance.

Materials and Methods

Antibodies and ELISA. Fluorochrome-conjugated antibodies to human proteins were purchased from the following sources: rat antibodies to CLA (HECA-452), IL-5 (JES1-39D10), IL-10 (JES3-9D7), and IL-13 (JES10-5A2); and mouse antibodies to CD3 (UCHT1), CD4 (RPA-T4), CD8 (HIT8a), CD11b (D12), CD25 (M-A251), CD27 (M-T271), CD45RA (HI100), CD45RO (UCHL1), CD57 (Leu-7), CD69 (FN50), HLA-DR (G46–6), TCR-α/β-1 (WT31), perforin (dG9), CCR4 (1G1), CCR5 (2D7), CCR6 (11A9), CXCR3 (1C6), IL-2 (5344.111), IL-4 (3010.211), IL-6 (AS12), IL-8 (AS14), TNF-α (6401.1111), and IFN-γ (25723.11) were obtained from BD Biosciences. Rat antibody to CCR7 (3D12; M. Lipp, Max-Delbruck-Center for Molecular Medicine, Berlin, Germany) was detected by a biotinylated donkey anti–rat IgG obtained from Jackson ImmunoResearch Laboratories, followed by PE-conjugated streptavidin (DakoCytomation). OptEIA ELISA kits for the detection of IL-4, IL-10, IFN-γ, TNF-α, and TGF-β in supernatants were obtained from BD Biosciences. The CCL1 ELISA assay was developed using monoclonal anti-CCL1 (clone 35305.11) as capture antibody and a biotinylated goat anti-CCL1 polyclonal antibody for detection (both obtained from R&D Systems) in combination with synthetic CCL1 for the generation of a standard curve.

Medium and Cell Preparations. Complete medium consisted of RPMI 1640, 2 mM L-glutamine, 1% nonessential amino acids, 1% sodium pyruvate, 50 μg/ml penicillin/streptomycin, 5×10^{-5} M 2-mercaptoethanol (Invitrogen), and 10% heat-inactivated FCS (Biological Ind.). Human PBMCs were isolated from fresh blood by centrifugation on Ficoll-Paque. Pure (>99%) $\alpha\beta TCR^+$ cells were obtained by positive selection using anti-αβTCR (BMA031) antibodies (Caltag Lab.) and goat anti–mouse MicroBead IgG (Miltenyi Biotec). Pure (>98%) $CD4^+CD25^+$ T cells from PBMCs were obtained by depletion of non-$CD4^+$ T cells followed by positive selection of $CD25^+$ cells (Miltenyi Biotec). Cell lines were generated by sorting $CCR8^+$ and $CCR8^-$ cells from $CD4^+$ (or $CD8^+$) $CD25^-CD45RA^+CD45RO^-$ T cells. 5,000 sorted cells were stimulated with 500,000 allogeneic PBMCs (irradiated 40 Gy) and twice 50,000 EBV-transformed B cells (from two donors, 70 Gy) in complete medium containing 1,000 U/ml IL-2. Skin T cells were isolated as follows: normal abdominal skin was excised using a dermatome (0.3–0.5 mm thick), and 100 cm^2 sections were digested in 10 ml RPMI 1640 containing 1 mg/ml collagenase D (Roche) for 30 min at 37°C on a shaker. Digestion was stopped by adding 10 mM EDTA and immediate cooling and processing on ice. Floating cells were collected and pooled with cells obtained by washing the remaining tissue several times with ice-cold PBS^-/10 mM EDTA (minimum 10× excess of digestion volume). Cells were passed through a 70-μm pore nylon mesh (BD Biosciences) and centrifuged (300 *g*) for 20 min at 4°C. After resuspension in cold complete medium, cells were passed through a 40-μm pore mesh and centrifuged on a Ficoll-Paque gradient (600 *g*; 20 min at 4°C). Interphase cells consisted of >90% viable T cells (as judged by CD45, αβTCR, and propidium iodide staining). Bulk T cells were sorted into $\alpha\beta TCR^+CCR8^+CD8^+$ or $\alpha\beta TCR^+CCR8^+CD8^-$ cells for single cell cloning or intracellular cytokine analysis. Skin T cells were allowed to recover for 3 h in complete medium at 37°C before chemotaxis assay was performed. Skin T cell clones were generated by single cell sorting and cultured under nonpolarizing conditions (10 ng/ml of recombinant TGF-β [R&D Systems], 1 μg/ml anti–IL-4 [MP4–25D2], and 2 μg/ml anti–IL-12 [C8.6; BD Biosciences]) as described previously (28). Clones were expanded in culture for 2 wk or longer before analysis. Gastrointestinal lamina propria and intraepithelial T cells were isolated from healthy small intestinal and colon tissue according to standard procedure (9).

Proliferation Assay. To assess inhibitory activities of $CD4^+$ $CD25^+$ T cell subsets, 50,000 allogeneic, irradiated (40 Gy) PBMCs (depleted from $CD3^+$ T cells) were mixed with 50,000 $CD4^+$ $CD25^-CD45RA^-$ (responder) T cells and 50,000 $CD4^+CD25^+$ T cells of the following phenotypes (obtained by cell sorting): $CD25^+$ CLA^-CCR8^-, $CD25^+CLA^+CCR8^+$, and $CD25^+CLA^+CCR8^-$.

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[^{3}H]Thymidine incorporation was measured in triplicates on day 5 after a 10-h pulse (0.5 μCi/round-bottom 96-well plates). Complete medium did not contain exogenous IL-2.

Intracellular Cytokine/Perforin Analysis. Sorted αβTCR$^+$ CCR8$^+$CD8$^+$ or αβTCR$^+$CCR8$^+$CD8$^-$ skin T cells, or skin T cell clones, were stimulated with 10 ng/ml PMA, 1 μg/ml ionomycin, and 10 μg/ml brefeldin A (Qbiogene) for 4.5–10 h. After fixing with 2% paraformaldehyde for 20 min at 4°C and permeabilization with 0.2% saponin/2% FCS in PBS$^-$, cells were stained with antibodies and analyzed by flow cytometry. Perforin was examined without prior cell activation.

Chemotaxis. Transwell chemotaxis assays were performed using a 5-μm pore polycarbonate filter (Corning Costar Corp.) loaded with 400,000 skin T cells. Migrated cells were collected from the lower well and stained and quantified by flow cytometry using a fixed number of polystyrene beads. The modified Boyden chamber assay (Neuroprobe) was conducted using collagen-coated 5-μm pore polycarbonate filters. Wells were loaded with 10^5 cells, and those that had migrated to the filter underside after 1 h at 37°C were stained and counted in five high-power fields.

Immunohistochemistry. Skin samples from healthy donors undergoing abdominal reduction were formaldehyde fixed and paraffin embedded. Freshly prepared 5-μm tissue sections were dewaxed and rehydrated. After pretreatment with 1 mg/ml protease from *Streptomyces griseus* (Sigma-Aldrich) in Tris-buffered saline for 6 min at 37°C or heat-induced epitope retrieval, sections were blocked with 3 mg/ml Redimune (ZLB Bioplasma AG), 10% donkey or rabbit serum (Jackson ImmunoResearch Laboratories and DakoCytomation), 0.01% Tween 20 and 0.01% sodium azide in Tris-buffered saline. Sections were incubated overnight at 4°C with primary antibodies to CCL1 (Santa Cruz Biotechnology, Inc.); podoplanin (AngioBio); CD31 (JC70A); S100, Vimentin (Vim3B4), CD1a (010), CD3, melan-A (A103), and isotype control antibodies mouse IgG2a (all obtained from DakoCytomation); rabbit IgG (Jackson ImmunoResearch Laboratories); and mouse IgG1 (MOPC21) and goat IgG (both obtained from Sigma-Aldrich). Subsequent detection of single bound antibodies was performed by the avidin–biotin enzyme complex method. Alternatively, multicolor staining was obtained by immunofluorescence using Alexa Fluor 488–conjugated chicken anti–mouse Ig (Molecular Probes) in combination with biotinylated donkey anti–sheep/goat Ig (The Binding Site) and Alexa Fluor 594–conjugated streptavidin (Molecular Probes). Primary rabbit antibodies were additionally bridged with mouse anti–rabbit antibodies (DakoCytomation). Cell nuclei were counterstained in blue by 4′,6′-diamino-2-phenylindole present in the mounting medium SlowFade Light (Molecular Probes). Two-dimensional images were recorded by wide-field microscopy. For statistical analysis of CCL1 coexpression with CD31 or S100, CD31 positive vessels of the superficial dermal plexus and S-100 positive cells (LCs and melanocytes) were analyzed in sections from eight different skin tissues samples obtained from three separate donors.

Online Supplemental Material. Anti–human CCR8 antibodies were generated by immunizing rabbits with a human CCR8 peptide conjugate, corresponding to positions 1–34 of the NH_2-terminal region of CCR8 coupled to either KLH or BSA in Specol (Institute for Animal Science and Health, The Netherlands), and purified by CCR8 peptide–agarose chromatography. Specificity was determined by flow cytometry using stably transfected 300-19 pre–B cells expressing CCR8 or other human chemokine receptors. Affinity-purified rabbit anti-CCR8 was detected by a biotinylated donkey anti–rabbit IgG (711-065-152) obtained from Jackson ImmunoResearch Laboratories, followed by PE-conjugated streptavidin (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20032177/DC1). The clones in Table S1 were generated from skin biopsies of patients with pustular psoriasis and Behcet's disease. Acute generalized exanthematous pustulosis (AGEP) clones were derived from positive epicutaneous test reactions. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20032177/DC1.

Results

CCR8$^+$ T Cells Predominate in Normal Human Skin. As part of our efforts to understand the control of peripheral

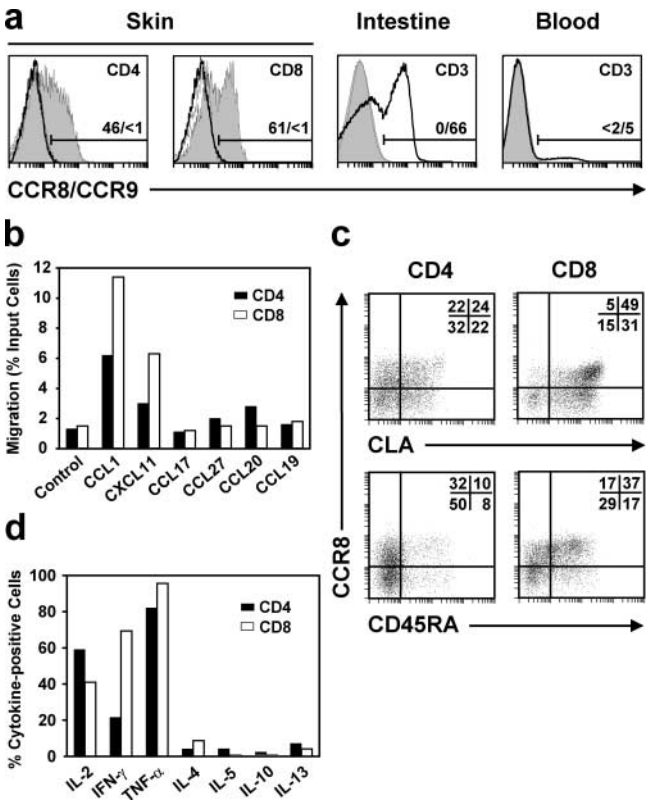

Figure 1. CCR8$^+$ T cells predominate in normal human skin. Analyses were performed with freshly isolated cells. (a) Flow cytometric detection of CCR8 (gray histograms) and CCR9 (bold line) in T cells isolated from human skin, lamina propria of small intestine, and peripheral blood. Peptide-blocked control for CCR8 staining is shown as a thin line. CD4, CD8, and CD3 denote the respective populations analyzed within the αβTCR$^+$ cell gate, and the numbers indicate percentages of CCR8- and CCR9-positive cells, respectively. Data for skin and intestinal cells are representative for nine and three donors, respectively. (b) Chemotactic migration of skin T cells, gated on CD4$^+$ cells (black bars) and CD8$^+$ (white bars), in response to chemokines as indicated. Values represent the percentage of migrated cells as a proportion of input cells, and control denotes the level of migration in the absence of chemokines. (c) Expression of CLA and CD45RA on CCR8$^+$ skin T cells. Gates were set for CD4$^+$αβTCR$^+$ cells (CD4) and CD8$^+$αβTCR$^+$ cells (CD8), and the numbers refer to the percentage of cells within each quadrant. (d) Production of various cytokines by CD4$^+$ (black bars) or CD8$^+$ (white bars) CCR8$^+$αβTCR$^+$ skin T cells. Intracellular accumulation of cytokines in response to PMA/ionomycin was measured by flow cytometry and is expressed as percent cytokine-positive cells. Chemotaxis and cytokine production data are representative of four independent experiments.

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leukocyte traffic under conditions of homeostasis, T cells were isolated from normal skin and assessed for unique patterns of chemokine receptor expression. Our focus on homeostatic T cell traffic required that we processed normal healthy skin samples with the least exposure to mechanical stress or UV light (skin specimen from abdominoplastic surgery). Furthermore, care was taken in the choice of the skin cell isolation procedure to prevent changes in cell migration properties and surface marker expression (i.e., skin tissue was processed without delay and recovered leukocytes were examined 2–4 h after isolation).

The majority of freshly isolated skin T cells expressed CCR8, whereas other skin-derived leukocytes, including monocytes/macrophages ($CD1c^-/CD14^+$), DCs ($CD1c^+$), or B cells ($CD19^+$), were negative for this chemokine receptor (Fig. 1 a and not depicted). In clear contrast with skin, $CCR8^+$ T cells were rare in peripheral blood (see next paragraph), whereas gastrointestinal T cells completely lacked CCR8 expression (Fig. 1 a). Specifically, CCR8 expression was undetectable on intraepithelial and lamina propria $CD4^+$ and $CD8^+$ T cells from normal small intestine and colon, whereas the small intestinal homing receptor CCR9 was readily detected (Fig. 1 a and not depicted). Amongst skin T cells, CCR8 was present on 55 ± 19% SD of $CD4^+$ cells and 77 ± 18% SD of $CD8^+$ cells ($n = 9$). Skin $CCR8^+$ T cells did not express CCR4, a chemokine receptor that is commonly observed on effector T cells present at inflammatory skin lesions (Table I and references 29–32). These cells also lacked expression of CCR7, thereby highlighting the peripheral, rather than lymphoid homing propensity of these cells (2, 4). In contrast, numerous cells expressed CXCR3, a chemokine receptor broadly associated with activated T cells (2, 4). CCR5 and CCR6 positivity was <20%. In vitro chemotaxis responses to CCL1 and I-TAC/CXCL11, but not TARC/CCL17, LARC/CCL20, and ELC/CCL19, confirmed functional expression (CCR8 and CXCR3) or absence (CCR4, CCR6, and CCR7) of the corresponding receptors (Fig. 1 b).

Phenotypic and Functional Heterogeneity of Skin $CCR8^+$ T Cells. The majority of $CCR8^+$ T cells from normal skin carried the skin homing ligand CLA and showed clear signs of preactivation, including high levels of CD69, low but uniform levels of CD25, and reduced levels of αβTCRs (Fig. 1 c, Table I, and not depicted). All skin $CCR8^+$ T cells were antigen experienced ($CD45RO^+$), but, interestingly, substantial numbers of $CD4^+$ cells and even more $CD8^+$ cells coexpressed CD45RA, a marker typically associated with naive or cytolytic effector T cells (Fig. 1 c and Table I; references 5, 33). Strikingly, and in clear contrast with peripheral blood $CD8^+$ T cells, CCR8-expressing T cells in the skin lacked markers that are typical for cytolytic T cells (perforin and CD57; references 5, 33 and not depicted). Instead, the majority of skin $CD4^+$ and $CD8^+$ T cells produced IFN-γ and TNF-α (Fig. 1 d), indicating that antigen contact in the skin initiates preferentially type 1 T helper cell responses. Still, minor populations expressing the type 2 cytokines IL-4 and IL-13 or the antiinflammatory cytokine IL-10 were clearly distinguishable, demonstrating limited functional diversity.

Preferential production of type 1 cytokines was also observed in 32 T cell clones derived from single cell–sorted $CCR8^+$ T cells isolated from normal skin ($n = 3$). To preserve functional and phenotypic characteristics, skin T cells were expanded under nonpolarizing conditions (28). In agreement with the results for intracellular cytokine production by freshly isolated skin $CCR8^+$ T cells, only a few clones secreted substantial amounts of either IL-4 or IL-10, whereas most clones secreted large amounts of TNF-α and IFN-γ (Fig. 2 a). All clones tested produced IL-2, but not a single one secreted detectable levels (>50 pg/ml) of TGF-β (unpublished data), suggesting an absence of Treg cells. With the exception of two clones showing low-level staining, the T cell clones were predominantly negative for intracellular perforin (unpublished data), which fully matches our findings with freshly isolated skin $CCR8^+$ T cells. Of interest, the vast majority of $CCR8^+$ T cell clones secreted large amounts of CCL1 (50–1000 ng/ml) after stimulation with PMA plus ionomycin, implying a positive feedback loop in the control of $CCR8^+$ T cell recruitment (Fig. 2 b).

Of particular note, CCR8 expression was maintained, at varying levels of intensity, on skin T cell clones, even after four rounds of stimulation and extensive expansion (Fig. 2, c and d), suggesting that this chemokine receptor is a stable feature of skin surveillance T cells. This is in striking contrast to our findings with 37 T cell clones derived from var-

Table I. *Phenotypic Heterogeneity of Human $CCR8^+$ T Cells from Skin and Blood*

T cells	Subset	CLA	CD25	CD69	CD27	CD45RO	CD45RA	CCR4	CCR7	CXCR3
Skin	$CD4^+$	65 ± 13[a]	37 ± 7	83 ± 9	59 ± 9	93 ± 8	37 ± 10	0[b]	0	8 ± 2
	$CD8^+$	90 ± 1.5	63 ± 5	100	39 ± 14	100	65 ± 4	0	0	39 ± 4
Blood	$CD25^+$	100	100	0	100	100	0	100	40	0
	$CD25^-$	11 ± 4	0	0	100	45 ± 5	87 ± 3	0	64 ± 4	0

Freshly isolated $CCR8^+$ T cells from skin and blood were analyzed for coexpression of various markers by flow cytometry.
[a]Mean ± SEM of three to nine independent experiments.
[b]0 and 100 indicate <5 and >95% positive cells, respectively.

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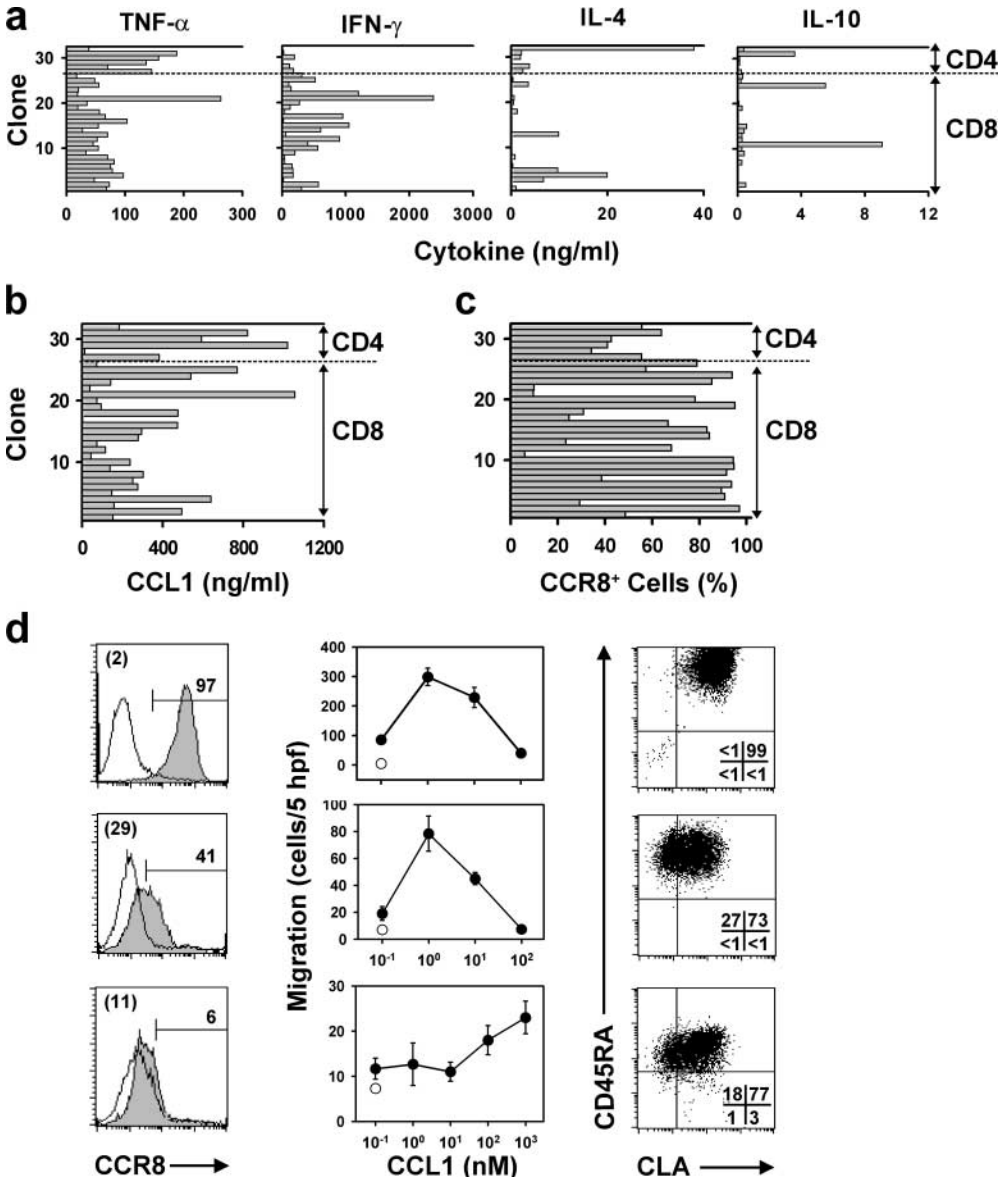

Figure 2. Analysis of T cell clones derived from skin CCR8$^+$ T cells. Sorted CCR8$^+$ T cells isolated from normal skin were cloned under nonpolarizing conditions. (a) For cytokine and (b) CCL1 secretion analysis, 6 CD4$^+$ and 26 CD8$^+$ T cell clones were stimulated with PMA/ionomycin for 24 h, and cell-free supernatants were tested in ELISA. (c) Expression of CCR8 by T cell clones, as determined by flow cytometry. Percent (%) CCR8$^+$ refers to the fraction of CCR8-positive cells present within individual clones. (d) Detailed analysis of three clones representing high, intermediate, and low level expression of CCR8. Cells stained with anti-CCR8 antibodies (left, shaded histogram) or peptide-blocked anti-CCR8 (unshaded histogram). Numbers in parentheses indicate the clone number, whereas numbers above the gate lines refer to the percentage of cells positive for CCR8. The corresponding center panels show chemotactic responses of the same clones toward CCL1, expressed as the number of migrated cells counted (mean of triplicate wells ± SEM) in five high-power fields. Background migration in the absence of chemokine is indicated by open circles. The expression of CLA versus CD45RA, with the percentage of cells positive within each quadrant indicated (right).

ious skin lesions (pustular psoriasis, AGEP, and Behcet's disease) that showed scarce CCR8 expression (4/37 positive) but elevated frequencies in CCR4 (13/37 positive) and most notably CCR6 expression (35/37 positive; Table S1, available at http://www.jem.org/cgi/content/full/jem.20032177/DC1). CCR8$^+$ cells are similarly underrepresented in T cell clones from atopic dermatitis (34). Of note, the distinct levels of CCR8 expression correlated well with the degree of responsiveness of these clones to CCL1 (Fig. 2 d). Interestingly, the expression of CLA and CD45RA was maintained by both CD4$^+$ and CD8$^+$ clones derived from CCR8$^+$ skin T cells (Fig. 2 d), supporting our observations in fresh skin T cells and setting these clones apart from those derived from either peripheral blood or sites of skin inflammation (35).

Collectively, the majority of both CD4$^+$ and CD8$^+$ T cells in normal skin are positive for CCR8, which is in clear contrast to the situation in peripheral blood (see next paragraph), intestine, or inflamed skin, and our analysis revealed a mixed cytokine production profile with a clear preference for proinflammatory cytokines. Lack of cytolytic capabilities suggests that CCR8$^+$ T cells may be more important in initiating recall responses via cytokine secretion as opposed to elimination of target cells. Of note, our findings with cutaneous CCR8$^+$ T cells do not support a close correlation between CCR8 expression and either Treg or Th2 cells (20, 22–27).

Distinct Populations of CCR8$^+$ T Cells in Peripheral Blood. In clear contrast with skin, CCR8$^+$ T cells in peripheral blood were extremely infrequent (<2% of CD4$^+$ or CD8$^+$ cells). These rare CCR8$^+$ T cells were contained within two distinct subpopulations that could be discriminated on the basis of CD25 expression. First, within the CD25-negative fraction, the CCR8$^+$ cells largely coex-

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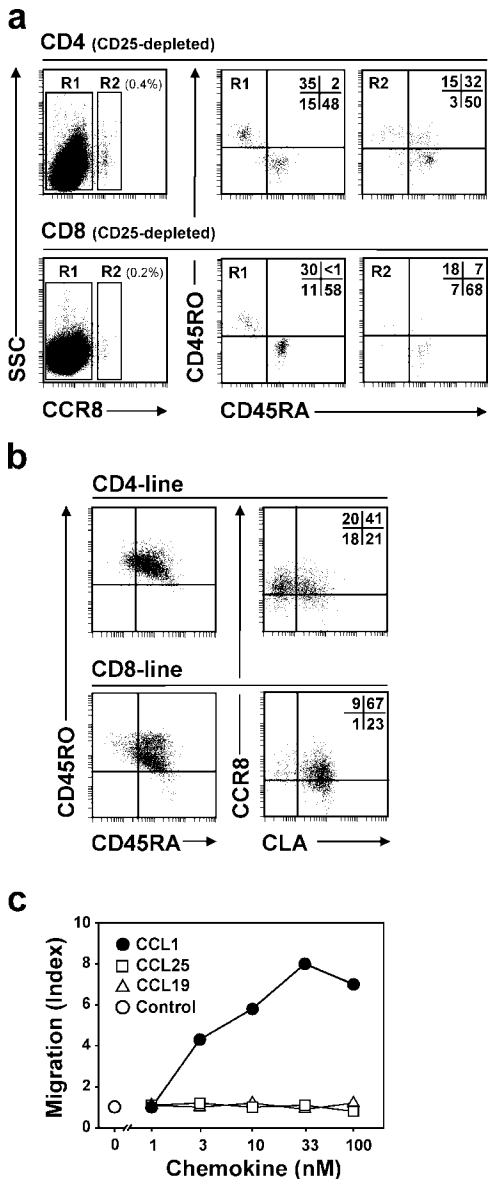

Figure 3. Expression of CCR8 on a minor subset of $CD25^-$ peripheral blood T cells. (a) Characterization of CCR8 expression on freshly isolated, CD25-depleted $CD4^+$ (top) or $CD8^+$ (bottom) T cells. (left) Some dot plots demonstrate staining for CCR8 versus side scatter, whereas (right) others demonstrate expression of CD45RO and CD45RA on cells gated for absence (R1) or presence (R2) of CCR8 expression. (b) Characterization of CCR8, CLA, CD45RA, and CD45RO expression on T cell lines derived from sorted $CCR8^+$ cells corresponding to gates R2 in a. Numbers in a and b represent the percentage of cells within each quadrant. (c) Chemotactic migration of cultured $CD4^+$ T cells as shown in b in response to chemokines as indicated. Migration is expressed as chemotactic index, which is the ratio of cells migrated in response to chemokines versus medium (no chemokine). $CD8^+$ T cell lines gave similar results (not depicted). Data are representative of three independent experiments.

pressed CD45RA and CD45RO, whereas CD45RO single-positive memory cells were less frequent (Fig. 3 a and Table I). These $CCR8^+$ T cells lacked expression of CCR4 and did not bear markers of activation (CD69, HLA-DR, and CXCR3), but 10–20% were positive for CLA. They were too infrequent in peripheral blood for detailed analysis and, therefore, primary lines were generated by in vitro culture of freshly sorted $CCR8^+CD4^+$ or $CCR8^+CD8^+$ T cells. Virtually all cultured T cells coexpressed CD45RA and CD45RO, and numerous cells maintained CCR8 expression and expressed CLA (Fig. 3 b), thereby closely resembling T cells from normal skin. Culturing under nonpolarizing conditions did not affect their phenotype (unpublished data). Of interest, these cells responded well in a chemotaxis assay to CCL1, but failed to migrate to CCL19 and CCL25, which reflects the absence of CCR7 and CCR9, respectively, and their exclusion from LNs and intestinal tissue (Fig. 3 c). Most (>95%) cultured T cells produced TNF-α, 30–40% produced IFN-γ, and 10% were $IL\text{-}4^+$ ($IL\text{-}5^+$ and $IL\text{-}10^+$ cells were <5%; unpublished data). In control lines derived from sorted CCR8-negative blood T cells, CD45RO single-positive, CLA-negative cells predominated (unpublished data). Apparently, within the CD25-negative fraction of peripheral blood, CCR8 expression is associated with $CD45RA^+$ cells that secrete proinflammatory cytokines and up-regulate CLA during culture. These findings compare favorably with those obtained for skin $CCR8^+$ T cells (Table I).

Recently, a subset of $CD4^+CD25^+$ Treg cells in peripheral blood was shown to express CCR8 mRNA and to respond to CCL1 (20, 26). These $CCR8^+$ cells could represent "natural" Treg cells derived from $CCR8^+CD25^+$ thymocytes (20, 21). Coexpression of CLA and CCR4 suggests that circulating CCL1-responsive Treg cells preferentially home to inflamed skin tissue (20, 36). Using our anti-CCR8 antibody reagent, we confirm the presence of low level $CCR8^+$ T cells among $CD4^+CD25^+$ and $CD8^+CD25^+$ T cells (not depicted), which also coexpressed CLA, CCR4, and CD45RO, but not CD45RA (Fig. 4 a and not depicted). Of note, the $CCR8^+$ fraction of $CD4^+CD25^+$ Treg cells was able to suppress T cell proliferation during in vitro coculture, and was equally potent as the two CCR8-negative subsets distinguished by presence or absence of CLA expression (Fig. 4 b and reference 37). Finally, $CD4^+CD25^+$ T cells, and in particular those coexpressing CLA, strongly migrated in response to CCL17 and less so to CTACK/CCL27 and CCL1, in agreement with the distribution of the corresponding chemokine receptors (CCR4, CCR10, and CCR8) in these cells (Fig. 4 c and 29–32, 38, 39). Presently, it is unclear whether these $CCR8^+$ Treg cells can home to the skin, although the predominant phenotype of skin $CCR8^+$ T cells (production of TNF-α, IFN-γ, and IL-2, but not TGF-β, as well as lack of CCR4, but presence of CD45RA) implies that $CCR8^+$ Treg cells are not a major component at this site.

Localization of CCL1 in Normal Human Skin. Scattered cells in the superficial plexus and epidermis of normal healthy skin stained positive for CCL1 protein, as determined by immunohistochemistry (Fig. 5). Colocalization of CCL1 with $CD31^+$ endothelial cells was frequently observed in sequential skin sections (Fig. 5, a and b) and was confirmed in double immunofluorescence analysis (Fig. 5, f

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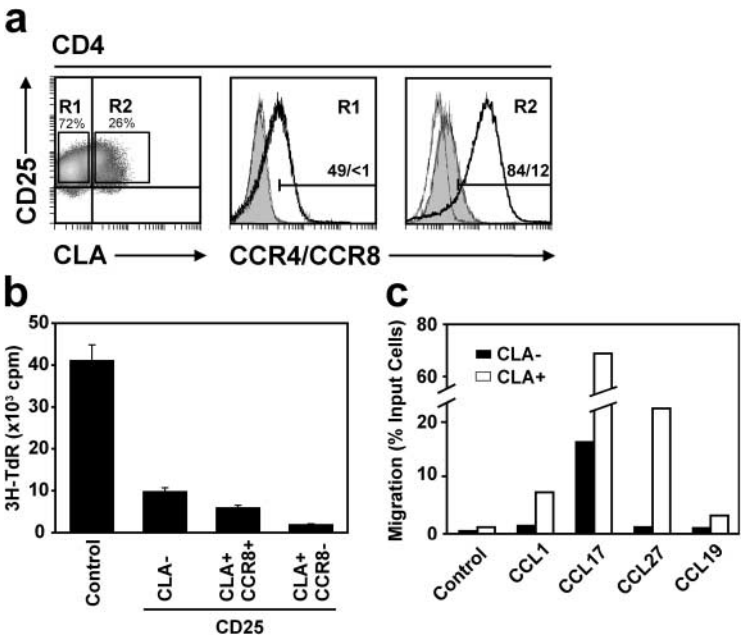

Figure 4. A small subset of peripheral blood $CD4^+CD25^+$ regulatory T cells expresses CCR8. (a) CCR8 expression (shaded histograms) was analyzed on positively selected $CD4^+CD25^+$ peripheral blood T cells after gating for CLA^- (R1) and CLA^+ (R2) cells. Expression of CCR4 is shown in bold lines, and control stainings are shown in thin lines. (b) CCR8-expressing $CD4^+CD25^+$ T cells suppress proliferation of autologous $CD4^+$ memory T cells as measured by [^{3}H]thymidine incorporation after 5 d coculture in the presence of irradiated heterologous PBMCs. Suppressive activity of sorted $CCR8^+$ Treg cells was compared with the two major subsets of CCR8-negative Treg cells distinguished by the presence ($CLA^+CCR8^-CD25^+$) or absence ($CLA^-CCR8^-CD25^+$) of CLA. Control denotes T cell proliferation in the absence of autologous Treg cells. (c) CLA^+ Treg cells show reduced migration responses to CCL1. Migration of CLA^- (black bars) and CLA^+ (white bars) Treg cells in response to indicated chemokines is expressed as migrated cells in percentage of input cells. CCR8 expression and function data are representative of three to seven independent experiments.

and g). Antibodies to CD31 stain blood endothelia more brightly than lymphatic endothelia, suggesting an involvement of CCL1 in the recruitment of blood $CCR8^+$ cells. In contrast, this chemokine probably does not play a role in the exit of tissue-resident $CCR8^+$ cells because lymphatic vessels, identified by podoplanin staining (40, 41), were negative for CCL1 (Fig. 5 b). 57% of $CD31^+$ vessels (717/1,258 positive; $n = 3$) stained positive for CCL1, although the actual degree of CCL1 positivity might be even higher due to a patchy expression profile of this chemokine (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20032177/DC1). CCL1 and CD31 also differed in their subcellular localization, with staining for CCL1 being more diffuse, whereas CD31 staining was highest at endothelial cell junctions (Fig. 5 g). Mononuclear cells positive for CCL1 were also occasionally detected within the perivascular infiltrates; however, CCL1 expression by T cells at this location was extremely rare (Fig. 5, c and g). In the epidermis of normal healthy skin, melanocytes and LCs, but not keratinocytes, produced CCL1, characterizing an expression profile not previously seen with chemokines. In sequential sections, CCL1 immunostaining overlapped with loosely scattered melanocytes interspersed in the basal layer of keratinocytes that stained positive for melan-A and LCs in the basal and suprabasal layers of the epidermis showing strong staining for S-100 and CD1a (Fig. 5, d and e). In confirmation, colocalization of CCL1 with LCs and melanocytes were readily observed by double immunofluorescence analysis (Fig. 5, h and i). Numerous LCs, identified by staining for either S-100 or vimentin (42), were found to express CCL1. Melanocytes in the basal layer of keratinocytes, identified by differential staining for S-100 (dim) and vimentin (bright), also appeared to secrete this chemokine. Staining for CCL1 was more intense in dendritic processes as opposed to the central body of LCs and melanocytes (Fig. 5, h and i, and Fig. S2). Of note, no CCL1 staining was observed in epidermal keratinocytes or dermal fibroblasts, macrophages or B cells. In agreement with the scattered distribution of CCL1-producing cells, CCL1 mRNA was detected in unfractionated skin tissue by RT-PCR but not by Northern blot analysis (unpublished data).

Discussion

The constitutive recruitment of immune surveillance T cells critically depends on chemokines that are expressed at strategic locations in healthy peripheral tissue. In addition, immune surveillance T cells need to express inflammation-independent address cues, including adhesion molecules and receptors for corresponding tissue-selective chemokines. Those cues, which determine inflammatory- or LN-homing in T cells, are obsolete. Here, we provide evidence that CCL1 and its only receptor CCR8 meet both requirements that are essential for controlling cutaneous homeostatic T cell traffic: selective expression of CCR8 by skin-homing memory T cells and constitutive production of CCL1 in healthy (noninflamed) skin. Possibly, CCL1 may also contribute to the survival of local ($CCR8^+$) immune surveillance T cells. This notion is supported by previous studies demonstrating an antiapoptotic effect of CCL1 on cells in vitro (43–46).

CCR8 is present on the majority of T cells isolated from normal human skin, but is absent on T cells from small intestine, which are characterized by CCR9 expression and colon. In agreement with previous studies (14–17, 26), $CCR8^+$ T cells are scarce in peripheral blood, indicating that healthy skin is their primary site of residence. Of note, CCR8 expression is not induced on unfractionated peripheral blood T cells during in vitro stimulation (PHA, Th1/Th2-polarizing conditions, and superantigen-present-

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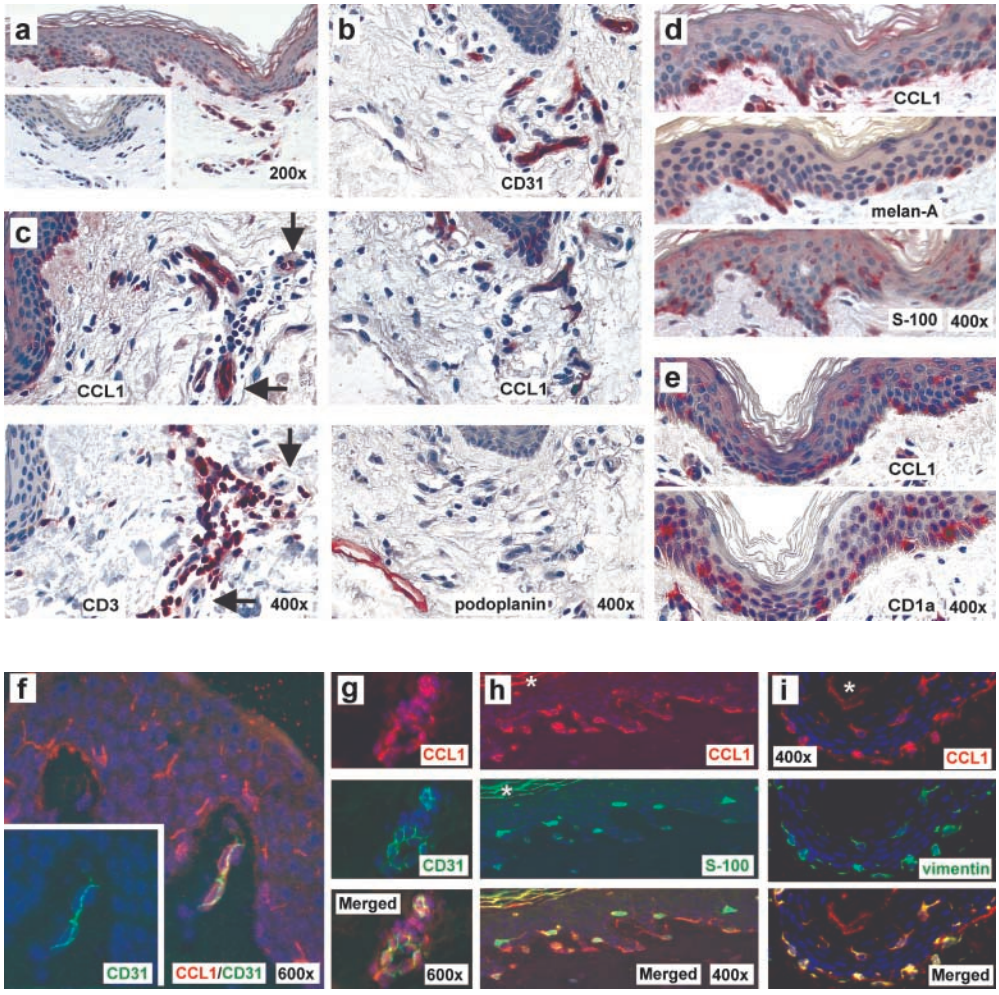

Figure 5. Localization of CCL1 in normal human skin. Immunohistochemistry was performed on paraffin-embedded sections from healthy abdominal skin tissue. (a) CCL1 (red staining) is shown in scattered cells throughout the superficial dermal plexus and epidermis; inset depicts isotype control antibody staining. (b) Staining of serial skin sections demonstrates overlapping expression of CCL1 and CD31 (blood vessels), but not podoplanin (lymphatic vessels) within the superficial dermal plexus. (c) Sequential for CCL1 and CD3 excludes T cells as major CCL1 producers. (d) Serial section analysis of healthy epidermis indicates CCL1 expression in melan-A–positive melanocytes in the basal layer of keratinocytes and S-100–positive LCs in the upper epidermis. (e) Serial staining for CCL1 and CD1a in normal epidermis. (f and g) Double immunofluorescence analysis demonstrates the presence of CCL1 (red) in CD31-positive dermal microvessels (green). (h and i) In agreement with serial section analysis, LCs (S-100^{bright}, vimentin^{bright}, in green) in the upper epidermis and melanocytes (S-100^{dim}, vimentin^{bright}, in green) in the basal keratinocyte layer co-express CCL1 (red). Asterisks mark nonspecific staining of stratum corneum.

ing DCs), supporting the notion that induction of cell surface CCR8 expression requires "skin-specific" costimulation (47–49). However, this chemokine receptor is not induced under in vitro conditions favoring CLA expression (reference 50 and unpublished data). Of note, sorted CCR8$^+$ skin T cells maintained their characteristic level of CCR8 during repeated rounds of in vitro expansion. The identification of CCR8 as a stable marker for skin immune surveillance T cells will enable us to examine the formation of such cells during an immune response and, eventually, clarify their relationship to the fraction of CCR8-negative T cells present in healthy skin. Lack of cytolytic function in CD8$^+$ T cells suggests that the process of in vivo CCR8 induction occurs separately from effector cell generation.

CCL1, the selective and only ligand for CCR8, is present at critical sites for recruitment and tissue localization of blood CCR8$^+$ cells, including blood portals (vasculature in the superficial dermal plexus) and epidermis (melanocytes and LCs). CCL1 production by LCs and melanocytes suggests that this chemokine controls the colocalization of memory T cells with epidermal sentinel cells for steady-state surveillance and optimal response to local infection and tissue damage. Preliminary data indicate that the vascular and epidermal expression of CCL1 is not greatly enhanced during skin inflammation (psoriasis and atopic dermatitis; unpublished data), further arguing for a principal role of this chemokine system in the control of homeostatic as opposed to inflammatory T cell traffic. This fully agrees with the general absence of CCR8 expression in cultured T cells derived from various inflammatory skin lesions. In addition, RNA dot-blot and Northern blot hybridization analysis excludes alternative tissues with prominent CCL1 expression (unpublished data), supporting our view that this chemokine is selective for skin-homing T cells. As an exception, CCL1 is expressed in the thymus and, thus, may contribute to thymocyte development and/or Treg cell generation (20). We did not detect CCL1 secretion in primary cultures of human keratinocytes and dermal fibroblasts, irrespective of the culture conditions (treatment with IL-1, TNF-α, IFN-γ, LPS, etc.), putting it apart from most other chemokines (reference 4 and unpublished data). However, in vitro–stimulated CCR8$^+$ skin T cells produced substantial amounts of this chemokine, suggesting a positive feedback mechanism in the recruitment of immune surveillance T cells to the site of initial pathogen exposure.

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Other chemokines with known expression in normal skin include SDF-1/CXCL12 (51), BRAK/CXCL14 (52, 53), CCL27 (38, 54), and CCL20 (55). However, these chemokines are also found in other epithelial tissues and neither shows selectivity for cutaneous memory T cells. CCL27, the ligand for CCR10, is highly up-regulated in inflamed skin and is known to orchestrate, together with the CCR4 ligands CCL17 and MDC/CCL22, the composition of effector T cells at murine skin lesions (38, 54, 56). CCR10 is enriched among CLA^+CD4^+ effector T cells as well as gut-homing plasma B cells (32, 38, 39, 57–59), whereas CCR4 shows preference for effector $CD4^+$ T cells with (CLA^+) and without (CLA^-) skin-homing phenotype (29–32, 60). Both receptors are less frequent on $CD8^+$ as opposed to $CD4^+$ blood T cells. In summary, these chemokines and their receptors represent unlikely candidates for controlling memory T cell localization within healthy human skin.

Skin $CCR8^+$ T cells secrete cytokines that may be involved in the initiation of type 1 recall responses (TNF-α and IFN-γ), whereas cytokines typical of Th2 cells (IL-4 and IL-13) or Treg cells (IL-10 and TGF-β) were low to undetectable. Therefore, our results support the view that, at least in normal skin, CCR8 expression is not confined to Th2 and/or Treg cells (20, 22–27). We could confirm the expression of low levels of CCR8 by peripheral blood $CD25^+$ Treg cells, suggesting that some cutaneous $CCR8^+$ T cells may have Treg function. However, several features in cutaneous $CCR8^+$ T cells, including cytokine secretion, chemokine receptor, and CD45RA expression profiles as well as unproblematic in vitro culture, argue against a prevalence of Treg cells in normal skin. We will examine the distribution of the Treg cell marker FoxP3 among skin T cells as soon as reagents for immunohistochemistry are available (61). The alternative and equally minor subset of $CCR8^+$ T cells in peripheral blood, characterized by lack of CD25 but presence of CD45RA and CLA, resembles better $CCR8^+$ immune surveillance T cells within skin and, thus, may represent their circulating precursors. Importantly, our studies demonstrate that normal healthy skin but not peripheral blood is the major reservoir of $CCR8^+$ T cells.

We propose that CCL1 and CCR8 contribute in multiple ways to immune protection of the skin (11–13). First, the constitutive production of CCL1 by noninflamed skin endothelium is likely to mediate the steady-state extravasation of $CCR8^+$ blood precursors of skin-tropic immune surveillance T cells. Second, CCL1 produced by LCs and melanocytes may allow colocalization of the recruited T cells with epidermal APCs, allowing the detection of MHC–peptide complexes. Successful TCR engagement together with appropriate costimulation will trigger an immune defense program that may involve monocytes, granulocytes, inflammatory T cells, and antibodies. In this scheme, activated immune surveillance T cells are proposed to play a dual role. Secretion of TNF-α and IFN-γ initiates inflammatory sequelae in local sentinel and tissue cells, leading to acute phase responses via induction of inflammatory chemokines, up-regulation of vascular adhesion receptors, and consequent recruitment of leukocytes of the innate immune system. Antigen specificity in memory T cells provides enhanced tissue protection while allowing the induction of selective proinflammatory responses in case of local antigenic contact. In addition, immune surveillance T cells may function as ambassadors of cutaneous LNs, and their relocation to draining LNs may accelerate appropriate T and B cell responses. Understanding the mechanisms by which immune surveillance T cells are recruited and positioned within the skin provides an important insight into the biology of these cells, and potentially a means by which to manipulate the cutaneous immune system for vaccination purposes. Here, we provide evidence that $CCR8^+$, together with its single ligand CCL1, represent the first chemokine system that meets the requirements for controlling T cell–mediated immune protection in normal, healthy skin.

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