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Immune Surveillance and Effector Functions of CCR10$^+$ Skin Homing T Cells

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Skin homing T cells carry memory for cutaneous Ags and play an important sentinel and effector role in host defense against pathogens that enter via the skin. CCR10 is a chemokine receptor that is preferentially expressed among blood leukocytes by a subset of memory CD4 and CD8 T cells that coexpress the skin-homing receptor cutaneous lymphocyte Ag (CLA), but not the gut-homing receptor $\alpha_4\beta_7$. Homing and chemokine receptor coexpression studies detailed in this study suggest that the CLA$^+$/CCR10$^+$ memory CD4 T cell population contains members that have access to both secondary lymphoid organ and skin compartments; and therefore, can act as both "central" and "effector" memory T cells. Consistent with this effector phenotype, CLA$^+$/CCR10$^+$ memory CD4 T cells from normal donors secrete TNF and IFN-γ but minimal IL-4 and IL-10 following in vitro stimulation. Interactions of CCR10 and its skin-associated ligand CC ligand 27 may play an important role in facilitating memory T cell entry into cutaneous sites during times of inflammation. *The Journal of Immunology,* **2002, 169: 1189–1196.**

Naive T cells exit the thymus and enter into secondary lymphoid organs such as lymph nodes, Peyer's patches, and spleen via the blood. They are endowed with a unique set of cell surface homing receptors that facilitate their entry into these specialized organs to search for foreign Ags presented by APCs. Once a naive T cell encounters a reactive Ag presented in the context of MHC plus costimulatory molecules, it will be activated and undergo a developmental switch to become an activated/memory T cell. There is a subsequent reprogramming of the cell to allow it to carry out its new duties. Memory T cells possess a reduced Ag activation threshold to allow nonprofessional APCs such as B cells, macrophages, and epithelial cells to present foreign Ags and initiate a secondary immune response. Memory T cells make a new panel of effector cytokines (e.g., IL-4, IL-10, IFN-γ) that direct how the immune system will combat the pathogen. Equally important is the reprogramming of surface homing receptors that enable memory T cells to enter into peripheral tissues where the foreign invader may be found.

A four-step model of leukocyte egress from the blood into lymphoid and extralymphoid organs has been proposed: step 1) blood-borne leukocytes roll along the vascular endothelium; step 2) a pertussis toxin-sensitive activation event occurs within the leukocyte; step 3) activation-dependent, integrin-dependent leukocyte adhesion to the vascular endothelium; and step 4) leukocyte diapedisis into the surrounding tissue that is mediated by a gradient of tissue-expressed chemoattractant (1, 2). Importantly, the molecules that participate in each of these steps during memory T cell homing into many organs are different and different types of memory T cells exhibit distinct homing patterns.

Skin-homing cutaneous lymphocyte-associated Ag (CLA$^+$)[3] memory T cells preferentially home to cutaneous sites and are found at high frequency in inflammatory cutaneous lesions. The CLA Ag is a posttranslational glycosylation of P-selectin glycoprotein ligand 1 (3) (and possibly other proteins) that allows skin-homing memory T cells to roll on superficial dermal endothelium-expressed E-selectin (CD62E) during times of cutaneous inflammation (4–8) and may fulfill the step 1 role in the multistep model of leukocyte extravasation. CLA$^+$ memory T cells express the integrins CD11a/CD18 ($\alpha_L\beta_2$, LFA-1) and CD49d/CD29 ($\alpha_4\beta_1$) that bind inflamed endothelium-expressed ICAM-1 and VCAM-1 and can mediate step 3 adhesion (8). Among leukocytes, the message for the G-protein-coupled receptor CCR10 is exclusively expressed by CLA$^+$ skin-homing memory T cells and the CCR10 ligand CC ligand (CCL)27 (CTACK/ALP/ILC/ESkine) is exclusively made by skin-associated keratinocytes (9–11). It has been proposed that CCL27 interacting with CCR10 on skin-homing memory T cells could play a role in either or both step 2 (activation) or step 4 (diapedisis) to facilitate CCR10$^+$ T cell entry into inflamed cutaneous sites.

To fully address the potential role that CCR10 plays in memory T cell entry into cutaneous (and other) sites and to address the likely outcome of CCR10$^+$ T cell entry into inflamed cutaneous lesions, a CCR10 Ab was developed to identify the panel of homing receptors that CCR10$^+$ T cells coexpress and to address the cytokine secretion profiles of these cells following activation. The results of this study will have broad implications into the mechanisms of T cell entry into skin during times of cutaneous infection and in T cell-mediated skin diseases such as contact hypersensitivity, psoriasis, and atopic dermatitis (AD).

Materials and Methods

Development of anti-human CCR10 Abs

Mice were immunized with Ba/F3 cells transfected with a human CCR10 construct. After a final boost, the spleen was harvested and fused with SP/0

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[3] Abbreviations used in this paper: CLA, cutaneous lymphocyte-associated Ag; AD, atopic dermatitis; CKR, chemokine receptor; CSC, cytokine secreting cell; Ion, ionomycin; CCL, CC ligand; MIP, macrophage inflammatory protein; IP-10, IFN-γ-inducible protein-10; h, human; EM, effector memory; CM, central memory.

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cells by conventional methods. Supernatants from wells containing viable cells were tested on rat Y3 myeloma cells transfected with CCR10. Confirmation of positive clones was performed by testing supernatants against CCR10 transfectants in human TF-1 and mouse Ba/F3 cells, a CCL27-responsive human T cell line (S. Hudak and L. McEvoy, manuscript in preparation), and the CCL27-responsive CLA$^+$ memory CD4 T cell population from peripheral blood. Hybridomas were subcloned by limiting dilutions two additional times, Ab purified (in-house and at Sierra Biosciences, Gilroy, CA) and biotinylated (EZ-Link Sulfo-NHS-LC-LC-Biotin; Pierce Rockford, IL). The selected clones 37, 1363, and 1908 were mouse IgG1, IgG2a, and IgG1 Abs, respectively (all have κ L chains). Unconjugated Abs were visualized with PE-conjugated goat anti-mouse IgG secondary reagents (Jackson ImmunoResearch Laboratories, West Grove, PA).

Human peripheral blood cell isolation

Human PBMCs were obtained from healthy donors by conventional Ficoll/Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) sedimentation of buffy coats obtained from the Stanford University Blood Bank (Stanford, CA). All donors were anonymous which precluded assessment of how CCR10 expression may differ based on age, sex, or race. Donors were negative for a panel of infectious agents (syphilis, hepatitis B and C, human T cell leukemia virus, and HIV). T cells were isolated using a Pan T cell isolation kit (Miltenyi Biotec, Auburn, CA) or T cell enrichment columns (R&D Systems, Minneapolis, MN) according to the manufacturer's protocols. Granulocytes were obtained by collecting venous blood and preparing according to Ref. 12. Platelets were obtained by collecting venous blood in yellow top ACDA tubes, low-speed centrifugation, and harvesting the top platelet-rich plasma layer (13).

Flow cytometry

Cells were stained on ice in HBSS (BioWhittaker, Walkersville, MD) containing 1–2% FBS (HyClone Laboratories, Logan, UT), 10 mM HEPES, and 0.1% sodium azide or PBS containing 1% FBS and 0.1% sodium azide. Analysis was performed on a FACSCalibur (BD Immunocytometry Systems, San Jose, CA). The biotinylated anti-CCR10 Ab was detected using PE- or allophycocyanin-conjugated streptavidin (BD PharMingen, San Diego, CA).

Lineage and naive/memory Ags were visualized using CyChrome- and allophycocyanin-conjugated mouse anti-hCD4 (clone RPA-T4; BD PharMingen), FITC- and PE-conjugated mouse anti-hCD8 (clone HIT8a; BD PharMingen), PE-conjugated mouse anti-hCD14 (clone M5E2; BD PharMingen), FITC-conjugated mouse anti-hCD16 (clone 3G8; BD PharMingen), FITC-conjugated mouse anti-hCD19 (clone HIB19; BD PharMingen), FITC-conjugated mouse anti-hCD41a (clone HIP8; BD PharMingen), CyChrome-conjugated mouse anti-hCD45RA (clone HI100; BD PharMingen), and PE- and CyChrome-conjugated mouse anti-hCD45RO (clone UCHL1; BD PharMingen).

Homing receptor expression was evaluated using PE-conjugated mouse anti-hCD11a (clone HI111; BD PharMingen), PE-conjugated mouse anti-hCD18 (clone L130; BD PharMingen), PE-conjugated mouse anti-hCD62L (clone DREG 56; BD PharMingen), PE-conjugated rat anti-human Integrin β_7 (clone FIB504; BD PharMingen), and FITC-conjugated rat anti-hCLA (clone HECA452; BD PharMingen).

Chemokine receptor (CKR) expression was evaluated using PE-conjugated mouse anti-hCCR6 (clone 11A9; BD PharMingen) and PE-conjugated mouse anti-CXCR3 (clone1C6; BD PharMingen), and PE-conjugated anti-hCXCR4 (R&D Systems). CCR7 was detected using an unconjugated mouse anti-hCCR7 mAb (clone 2H4; BD PharMingen) followed by PE-conjugated goat anti-mouse IgM (Jackson ImmunoResearch Laboratories). CCR4 was detected with an unconjugated mouse anti-hCCR4 mAb (clone 1G1; BD PharMingen) followed with PE-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories).

Migration assays

Migration assays were conducted as described in Ref. 14 with the following modifications. T cell populations were isolated as described above and incubated in Yssel's medium containing penicillin-streptomycin and 10 mM HEPES for >1 h. Chemokines were diluted in above medium. Following chemotaxis assay using 5-micron Transwell inserts, responding cells and added counting beads were stained as described in the figure legends and fixed in PBS/2% paraformaldehyde before analysis by flow cytometry. Calculation of percentage of migration was as described in Ref. 15.

ELISPOT analysis of sorted populations

Cells were stained with a mixture of labeled Abs described above and sorted. Sorted cells were pelleted and resuspended in 700 μl of Yssel's medium/penicillin-streptamycin/25 mM HEPES containing 50 U/ml of human IL-2 and an aliquot counted to determine cell concentration. Ninety-six-well MultiScreen-IP plates (Millipore, Bedford, MA) were precoated overnight with Abs against human IL-4, IL-10, IFN-γ, or TNF according to manufacturer's protocol (Diaclone, Cedex, France). For wells with anti-CD3 stimulation, 1 μg/ml anti-hCD3 was included with each anti-cytokine Ab. Plates were washed and blocked with PBS/2% skimmed dry milk. Plates were washed and wells filled with 150 μl of medium above (Basal), or medium containing 1 μg/ml anti-hCD28 (CD3/CD28) or 10 ng/ml PMA and 500 ng/ml ionomycin (Ion). A total of 50 μl of sorted cells were placed in the top well of each column and mixed with 150 μl of medium. A total of 50 μl of diluted cells was transferred to the next well in the column using a multichannel pipette and mixed. This was repeated down the column to dilute the number of cells plated by 4-fold in each row. Plates were placed in a nonvibrating incubator overnight and developed according to manufacturer's protocol. Briefly, plates were washed, plate-bound cytokine was detected with a biotinylated anti-cytokine Ab, plate washed, biotinylated Ab detected with HRP-conjugated streptavidin, and spots visualized with nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt after extensive washing. Number of spots was quantified using a dissecting microscope and the frequency of cytokine-secreting cells (CSCs) in a population presented as the percentage of plated cells that gave a detectable "spot" for each cytokine.

Quantification of cytokine production

Enriched T cell preparations were stained with Abs described in the figure legend and sorted. Sorted cells were pelleted and resuspended in Yssel's medium/penicillin-streptamycin/25 mM HEPES containing 50 U/ml human IL-2 and an aliquot counted to determine cell concentration. Wells of a 96-well tissue culture plate were filled with 150 μl of medium above (Basal) or 10 ng/ml PMA and 500 ng/ml Ion. A total of 50 μl of sorted cells were placed into a well of each stimulation condition. Plates were incubated for 20 h, and supernatants harvested, centrifuged; and cleared supernatant frozen till analyzed. The concentration of cytokines in the supernatants was quantified by a Cytometric Bead Array kit (BD Biosciences, Mountain View, CA) and concentrations normalized to nanograms per milliliter cytokine produced by 2E4 cells activated in 200 μL of medium for ~20 h.

Results

Three hybridomas (clones 37, 1363, and 1908) were isolated that specifically stained human CCR10 transfectants. In Fig. 1, an example of clone 37 staining a Ba/F3-hCCR10 transfectant (*left panel*) and CCL27-responsive human T cell line (*right panel*) is shown. Clone 37 did not stain a panel of human CKR transfectants (CCR1–9, CXCR1–5) confirming its specific binding to CCR10. None of the three Abs could block CCL27-stimulated calcium flux by human CCR10 transfectants (data not shown).

A recent report concluded that the only CCL27-responsive cells in human peripheral blood were CLA$^+$ memory CD4 and CD8 T cells (9). These CCR10 Abs were used in conjunction with lineage-specific Abs to investigate CCR10 expression by different

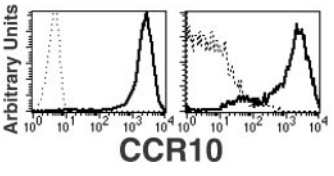

FIGURE 1. Characterization of anti-hCCR10 mAbs. BaF/3-hCCR10 transfectants (*left panel*) were stained with a mouse IgG1 isotype control mAb (dotted line) or mouse anti-hCCR10 mAb (clone 37, dark line) and visualized with PE-conjugated goat anti-mouse IgG polyclonal Ab. A CCL27-responsive human T cell line (*right panel*) was stained with biotinylated mouse IgG1 isotype control mAb (dashed line or mouse anti-hCCR10 mAb; clone 37, dark line) and visualized with PE-conjugated streptavidin.

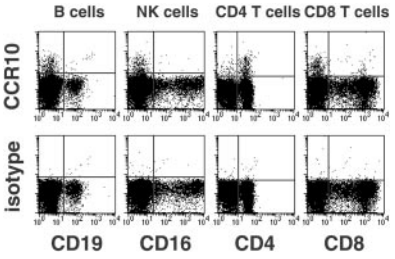

FIGURE 2. CCR10 is expressed by a subset of CD4 and CD8 T cells. Cell preparations were stained with biotinylated anti-CCR10 mAb (*upper row*) or isotype mAb (*lower row*). Mononuclear cell preparations were stained with the mAbs above in conjunction with lineage markers for B-cells (CD19), NK cells (CD16), and T cells (CD4 and CD8) and gated for lymphocyte scatter. Results are representative of >11 donors.

blood-derived cells to confirm and extend this observation. Fig. 2 shows that a minor subset of CD4 and $CD8^{high}$ T cells stained with an anti-CCR10 Ab, confirming our previous data. Isotype-staining controls are presented below the CCR10 staining panels for comparison. It should be noted that care was taken to exclude the apparently $CD4^+/CD8^+$ T cells that fall into the blast or large lymphocyte flow cytometric gate. These unusual cells or doublets can constitute a significant percentage of the minor number of $CCR10^+CD8^{high}$ T cell events analyzed. In contrast to the specific staining of a subset of CD4 and $CD8^{high}$ T cells, no specific staining was seen on $CD19^+$ B cells and $CD16^+$ NK cells (Fig. 2). $CD56^+/CD16^-$ NK cells, $CD56^+/CD3^+$ NK T cells, $CD14^+$ monocytes, $CD16^-$ eosinophils, and $CD16^+$ neutrophils were also negative for CCR10 expression (data not shown; $n = 5$ donors).

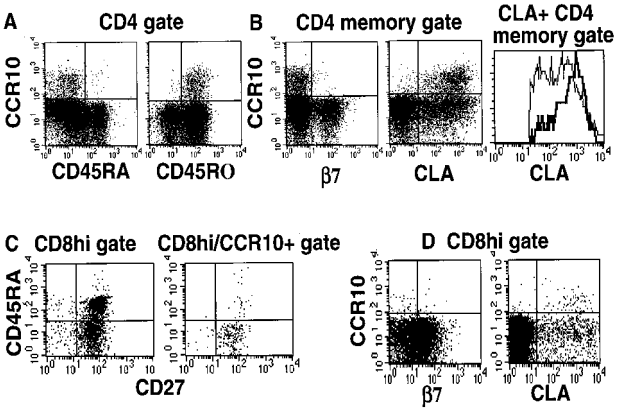

FIGURE 3. CCR10 is expressed on CLA^+ memory CD4 and CD8 T cells. A mononuclear cell preparation was stained with biotinylated anti-CCR10 and anti-CD4 or anti-CD8. *A*, The above staining was combined with anti-CD45RA (*left panel*) or anti-CD45RO (*right panel*) staining and gated on lymphocyte scatter and CD4 positivity. Results are representative of six donors. *B*, The above staining was combined with anti-β_7 (*left panel*) or anti-CLA (*middle panel*) staining and gated on lymphocyte scatter and CD4 positivity. The above staining was combined with anti-CD45RA and anti-CLA, and gated on $CD4^+/CD45RA^-/CLA^+$ lymphocytes that were either $CCR10^+$ (*right panel*, dark line) or $CCR10^-$ (*right panel*, light line). The expression of CLA by both of these populations is demonstrated as a histogram. Results are representative of 19 donors. *C*, The above staining was combined with anti-CD45RA and anti-CD27. Data are gated on lymphocyte scatter and $CD8^{high}$ positivity (*left panel*) or lymphocyte scatter and $CCR10^+/CD8^{high}$ positivity (*right panel*). Results are representative of six donors. *D*, The above staining was combined with anti-β_7 (*left panel*) or anti-CLA (*right panel*) and gated on lymphocyte scatter and $CD8^{high}$ positivity. Results are representative of 11 donors.

The two T cell populations were further subdivided to focus on the developmental stage that expressed CCR10. Costaining with the naive T cell marker CD45RA (Fig. 3*A*, *left panel*) and the memory marker CD45RO (Fig. 3*A*, *right panel*) demonstrated that the $CCR10^+CD4$ T cells were entirely contained in the $CD45RA^-/CD45RO^+$ memory population. Naive CD8 T cells are $CD45RA^+/CD27^+$, whereas memory CD8 T cells are split into $CD45RA^-/CD27^+$ and $CD45RA^+/CD27^-$ cells. Selective gating on $CCR10^+CD8^{high}$ T cells (Fig. 3*C*, *right panel*) compared with the bulk $CD8^{high}$ population (Fig. 3*C*, *left panel*) shows that the $CCR10^+$ population was primarily contained in the $CD45RA^-/CD27^+$ memory CD8 T cell population. Isotype staining indicated that most of the apparently $CCR10^+CD8^{high}$ T cells in the $CD45RA^+/CD27^+CD8^{high}$ T cell gate (Fig. 3*C*, *right panel*, *upper right quadrant*) was due to nonspecific staining (data not shown). Therefore, CCR10 expression is up-regulated during or after the developmental switch of naive CD4 and CD8 T cells to a memory phenotype and may play a prominent role in the homing of a subset of these memory T cells.

Naive CD4 and CD8 T cells are identical in their respective preferential homing to secondary lymphoid organs, due in part to their uniform surface expression of a set of secondary lymphoid organ-targeting homing receptors. In contrast, memory T cells express additional combinations of other homing receptors that facilitate their survey of peripheral tissues, but importantly not all memory T cells express the same combinations. Thus, the memory T cell population can be subdivided functionally into subsets that exhibit preferential homing to particular peripheral sites. The integrin $\alpha_4\beta_7$ is expressed at low levels on naive T cells, but a subset of memory T cells express high levels and preferentially home to gut peripheral tissues (i.e., the lamina propria and the intraepithelial lymphocyte compartment). In contrast, CLA is not expressed by naive T cells, but is expressed on a memory T cell subset that preferentially homes to the peripheral skin compartment. A third memory population is the $\alpha_4\beta_7^-/CLA^-$ memory T cell population that contains cells that may home to other extralymphoid sites (1).

Fig. 3*B* shows that practically no $CCR10^+$ memory CD4 T cells express detectable levels of β_7 integrin (Fig. 3*B*, *left panel*). In contrast, most $CCR10^+$ cells are found within the CLA^+ memory CD4 T cell subset (Fig. 3*B*, *middle panel*). The $CLA^+/CCR10^+$ subset is $9 \pm 2\%$ (mean $\pm$ SD, $n = 5$ donors) of the memory CD4 T cell population, and 28% of CLA^+ memory CD4 T cells express CCR10. All donors tested have a minor population of $CCR10^+$ memory CD4 T cells that express undetectable levels of CLA (Fig. 3*B*, *middle panel*, *upper left quadrant*). The percentage of these cells in the memory CD4 T cell population is $1.2 \pm 0.7\%$ (mean $\pm$ SD, $n = 5$ donors). Importantly, $CLA^+/CCR10^+$ memory CD4 T cells (Fig. 3*B*, *right panel*, dark line) have higher levels of CLA than the $CLA^+/CCR10^-$ memory CD4 T cell population (Fig. 3*B*, *right panel*, light line). Additionally, $CLA^+/CCR10^+CD4$ T cells express high levels of the integrin CD11a/CD18 (LFA-1, $\alpha_L\beta_2$) and have a range of CD62L expression from high to undetectable (data not shown).

The bulk of the $CCR10^+CD8^{high}$ T cells are also found in the CLA^+ (Fig. 3*D*, *right panel*) and β_7^- (Fig. 3*D*, *left panel*) subpopulation. The $CLA^+/CCR10^+$ subset is $0.6 \pm 0.5\%$ (mean $\pm$ SD, $n = 8$ donors) of the $CD8^{high}$ T cell population, and ~10% of CLA^+ $CD8^{high}$ T cells express CCR10. There is little evidence for a $CLA^-/CCR10^+CD8^{high}$ T cell population in the normal donors tested. Further characterization of the $CCR10^+CD8^{high}$ T cell population is hampered by its very low frequency in blood and problematic background staining with isotype control Ab; therefore, this aspect of CCR10 biology will not be further characterized in this report.

Chemokines interacting with pertussis toxin-sensitive CKRs are postulated to facilitate leukocyte entry into tissues by acting as either or both step 2 activation ligands or step 4 chemoattractants. In addition to CCR10, there are 18 G-protein-coupled receptors that have chemokine ligands. We have analyzed a subset of CKRs that are important in memory T cell biology, and addressed which of these CKRs are coexpressed on CCR10$^+$ memory CD4 T cells. The ligands for CCR4 (16–18), CCR6 (19, 20), and CXCR3 (18, 21) are found in inflamed skin, and ligands for CCR7 are found in both lymphoid and peripheral tissues (1). Coexpression of different CKRs investigates the potential for cooperative or redundant mechanisms to facilitate T cell entry into peripheral tissues during immune surveillance or during T cell-mediated disease episodes. The different respective isotype Ab staining controls on CLA$^+$ memory CD4 T cells are shown in the second row (Fig. 4, *F–J*).

Most or all CLA$^+$/CCR10$^+$ memory CD4 T cells coexpress CCR4 in contrast to CLA$^+$/CCR10$^-$ memory CD4 T cells, which only a portion express CCR4 (Fig. 4*A*). Conversely, a much greater percentage of CLA$^+$/CCR10$^+$ memory CD4 T cells do not express detectable levels of CXCR3 compared with CLA$^+$/CCR10$^-$ memory CD4 T cells (Fig. 4*D*). A significant subset of both CCR10$^+$ and CCR10$^-$ CLA$^+$ memory CD4 T cells coexpressed CCR6 (Fig. 4*B*) and CCR7 (Fig. 4*C*), and practically all CLA$^+$ memory CD4 T cells express CXCR4 regardless of CCR10 expression (Fig. 4*E*). Similar conclusions were reached when analysis was focused on the minor CLA$^-$/CCR10$^+$ memory CD4 T cell population (data not shown). The analysis of CCR10 coexpression with these CKRs in both the CLA$^+$ and CLA$^-$ memory CD4 T cell population is presented in Table I. These results underscore the fact that CCR10 is the most preferentially restricted receptor of the skin-homing CLA$^+$ memory T cell subset. Preliminary experiments on normal donors showed that CCR10 is not coexpressed on CD4 T cells with CCR1, CCR2, CCR3, CCR5, CCR9, CXCR1, CXCR2, CXCR5, or CXCR6 (data not shown).

CKR coexpression analysis was confirmed by studying the functional chemotactic response profiles of CLA$^+$/CCR10$^+$, CLA$^+$/CCR10$^-$, and CLA$^-$/CCR10$^+$ memory CD4 T cells (Fig. 5). CLA$^+$/CCR10$^+$ (■) but not CLA$^+$/CCR10$^-$ (□) memory CD4 T cells migrate to the CCR10 ligand CCL27 confirming that anti-CCR10 Ab staining identifies the CCL27-responsive cells within the CLA$^+$ memory CD4 T cell population. The CLA$^+$/CCR10$^+$ memory CD4 T cell population also had a more prominent response to the CCR4 ligand CCL17/thymus and activation-regulated chemokine than CLA$^+$/CCR10$^-$ memory CD4 T cells confirming the positive association between CCR10 and CCR4 expression (Fig. 5*B*). Similar chemotactic responses by both populations were seen to CCR6 (CCL20/macrophage inflammatory protein (MIP)-3α, Fig. 5*C*) and CCR7 (CCL21/secondary lymphoid tissue chemokine, Fig. 5*D*) ligands confirming the neutral association of CCR10 with CCR6 and CCR7 in CLA$^+$ memory CD4 T cells seen by flow cytometry. Unexpectedly, similar chemotactic responses were seen by both CLA$^+$/CCR10$^+$ and CLA$^+$/CCR10$^-$ memory CD4 T cells to the CXCR3 ligands CXCL10/IFN-γ-inducible protein (IP)-10 (Fig. 5*E*) and CXCL9/monokine induced by IFN-γ (data not shown). In sharp contrast to the flow cytometric analysis, CLA$^-$/CCR10$^+$ memory CD4 T cells (◆) did not respond significantly to CCL27. They also did not respond appreciably to CCL17/TARC, CCL20/MIP-3α, and CXCL10/IP-10. However, these cells were fully capable of migration based on their high chemotactic response to the CCR7 ligand CCL21/SLC and CXCR4 ligand CXCL12/stromal cell-derived factor-1α (data

Table I. *Coexpression of CCR10 and other CKRs by CLA$^+$ and CLA$^-$ memory CD4 T-cells.*[a]

	CCR4	CCR6	CCR7	CXCR3
CLA$^+$gated				
CCR10$^+$CKR$^-$	**7 ± 6**	**17 ± 9**	**9 ± 4**	**23 ± 7**
CCR10$^+$CKR$^+$	**15 ± 7**	**11 ± 3**	**17 ± 6**	**5 ± 1**
CCR10$^-$CKR$^+$	40 ± 20	34 ± 9	50 ± 10	28 ± 8
CCR10$^-$CKR$^-$	40 ± 10	38 ± 6	20 ± 6	44 ± 3
CLA$^-$ gated				
CCR10$^+$CKR$^-$	**2 ± 4**	**0.8 ± 0.5**	**0.2 ± 0.2**	**1.1 ± 0.9**
CCR10$^+$CKR$^+$	**0.8 ± 0.8**	**0.4 ± 0.3**	**1 ± 1**	**0.3 ± 0.1**
CCR10$^-$CKR$^+$	22 ± 9	30 ± 10	79 ± 8	39 ± 7
CCR10$^-$CKR$^-$	76 ± 8	67 ± 9	19 ± 8	50 ± 10

[a] The percentages of CLA$^+$ (top group) or CLA$^-$ (bottom group) memory CD4 T cells coexpressing the indicated CKR with (bold text) or without (normal text) CCR10 are shown. Data are from three to five donors and are the means ± SD.

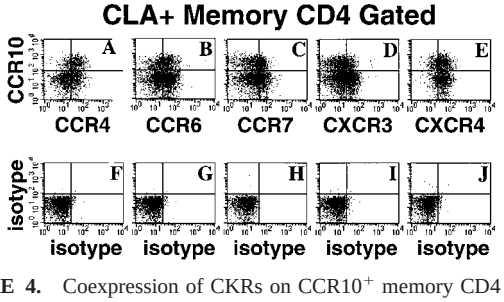

FIGURE 4. Coexpression of CKRs on CCR10$^+$ memory CD4 T cells. T cell preparations were stained with biotinylated anti-CCR10 mAb (*upper row*) or isotype control mAb (*lower row*) in conjunction with CyChrome-conjugated anti-CD45RA, CyChrome-conjugated anti-CD8, FITC-conjugated anti-CLA, and either PE-conjugated anti-CCR6 (*B*), anti-CXCR3 (*D*), anti-CXCR4 (*E*), or their respective PE-conjugated isotype mAbs (*G*, *I*, and *J*). T cell preparations were stained with unconjugated CCR4 (*A*), CCR7 (*C*), or their respective unconjugated isotype mAbs (*F* and *H*), and then PE-conjugated goat anti-mouse IgG or anti-mouse IgM, respectively, before addition of the conjugated mAbs listed above. All presented data are gated on lymphocyte scatter and CLA$^+$/CD8$^-$/CD45RA$^-$ cells. The data are representative of staining from three to five donors.

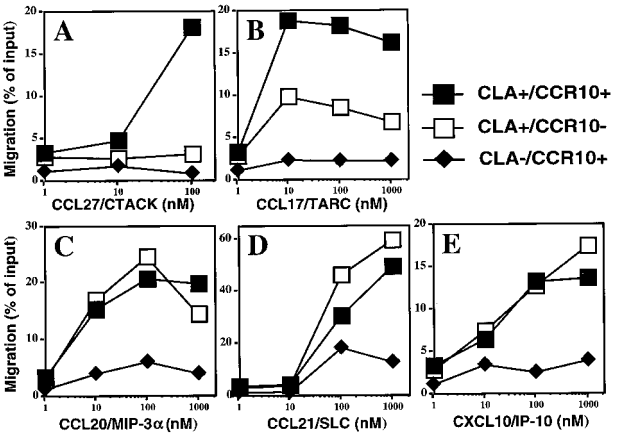

FIGURE 5. CLA$^+$/CCR10$^+$ but not CLA$^-$/CCR10$^+$ memory CD4 T cells respond to a variety of chemokines. T cell preparations were allowed to migrate to medium or medium containing the indicated concentrations of a CCR10 ligand (*A*, CCL27/CTACK), a CCR4 ligand (*B*, CCL17/TARC), a CCR6 ligand (*C*, CCL20/MIP-3α), a CCR7 ligand (*D*, CCL21/SLC), and a CXCR3 ligand (*E*, CXCL10/IP-10). Responding cells were harvested, stained with biotinylated anti-CCR10 in combination with FITC-conjugated anti-CLA and allophycocyanin-conjugated anti-CD4, and migration quantified. Migration by CLA$^+$/CCR10$^+$ (■), CLA$^+$/CCR10$^-$ (□), and CLA$^-$/CCR10$^+$ (◆) memory CD4 T cells is presented for a donor. Data are representative of four donors tested.

not shown). The specificity and selectivity of the migratory response by the two CLA^+ populations to skin-associated chemokines were demonstrated by their nonresponsiveness to the B cell follicle-associated chemokine CXCL13/B cell-attracting chemokine-1 (data not shown).

Recent reports have proposed subdividing the bulk memory T cell population into cells that constitute "central" memory (CM) and others that are "effector" memory (EM) cells (22). Conceptually, CM cells may enter secondary lymphoid organs based on their homing receptor repertoire, scan for the presence of previously seen foreign Ags, and proliferate in the presence of these Ag to provide an army of further differentiated effector cells to fight the pathogens. But CM T cells themselves may be inefficient in the effector mechanisms (e.g., cytokine secretion) needed to clear infection. In contrast, EM cells may exclusively home to extralymphoid sites to efficiently produce effector cytokines and/or cytotoxic molecules that directly clear or can direct other aspects of the immune system to clear the pathogen.

The homing receptors and CKRs expressed by $CCR10^+$ memory T cells suggested that they would access both inflamed cutaneous sites (CLA, CD11a/CD18, CCR4/6/10, CXCR3) and secondary lymphoid organs (CD62L, CD11a/CD18, CCR7) to search for foreign Ags and establish an appropriate immune response to combat the pathogen. A primary mechanism to establish the type of immune reaction is by Ag-stimulated secretion of proinflammatory cytokines (e.g., TNF) in concert with Th1 (e.g., IFN-γ), Th2 (e.g., IL-4), and/or other (e.g., IL-10) cytokines. The cytokine secretion profiles of $CLA^+/CCR10^+$ memory CD4 T cells was compared with $CLA^+/CCR10^-$ and $CLA^-/CCR10^+$ memory CD4 T cells by direct measurement of the frequency of CSCs within each population and by the bulk cytokine secretion capacity of each population following anti-CD3/CD28 or PMA/Ion stimulation.

The sorting regions used to isolate the three populations are shown in Fig. 6*A*. No or very few cells secreting IFN-γ, IL-4, IL-10, or TNF were detected in any population in the absence of in vitro stimulation (Fig. 6*C*, *open bars*), demonstrating that these sorted memory CD4 T cells are not in vivo activated in the blood from the nondiseased healthy donors tested. PMA/Ion activation (Fig. 6*C*, *right panel*, hatched bars) gave the maximal number of detectable CSCs in each sorted population compared with anti-CD3/CD28 stimulation (Fig. 6*C*, *left panel*, filled bars), but the cytokine secretion profile differences among the three populations was similar regardless of the type of stimulation. IL-4 and IL-10 CSCs were either undetectable or marginally observable in $CLA^-/CCR10^+$ (R1), $CLA^+/CCR10^+$ (R2), and $CLA^+/CCR10^-$ (R3) memory CD4 T cell populations regardless of the activation protocol. In contrast, a great percentage of cells in all three populations secreted detectable levels of TNF following PMA/Ion stimulation (Fig. 6*C*, *right panel*, hatched bars), and TNF-CSCs were the most numerous CSCs following anti-CD3/CD28 stimulation (Fig. 6*C*, *left panel*, filled bars). The IFN-γ CSCs frequency was intermediate between TNF and IL-4/10 CSC frequencies for both activation protocols.

Similar conclusions were obtained when the levels of cytokines secreted by these sorted populations following PMA/Ion stimulation were measured (Fig. 6*D*). TNF and IFN-γ were the most abundant cytokines secreted by all three populations. All three sorted populations also expressed very low levels of IL-4 (~0.2 ng/ml) and IL-10 (~0.1 ng/ml) following PMA/Ion stimulation; levels that are barely detectable on the scale shown in Fig. 6*D*. The CSC frequency (Fig. 6*E*) and cytokine production capabilities (Fig. 6*F*) of naive CD4 T cells and the previously described EM and CM populations (sort gates shown in Fig. 6*B*; Ref. 22) are shown for comparison purposes.

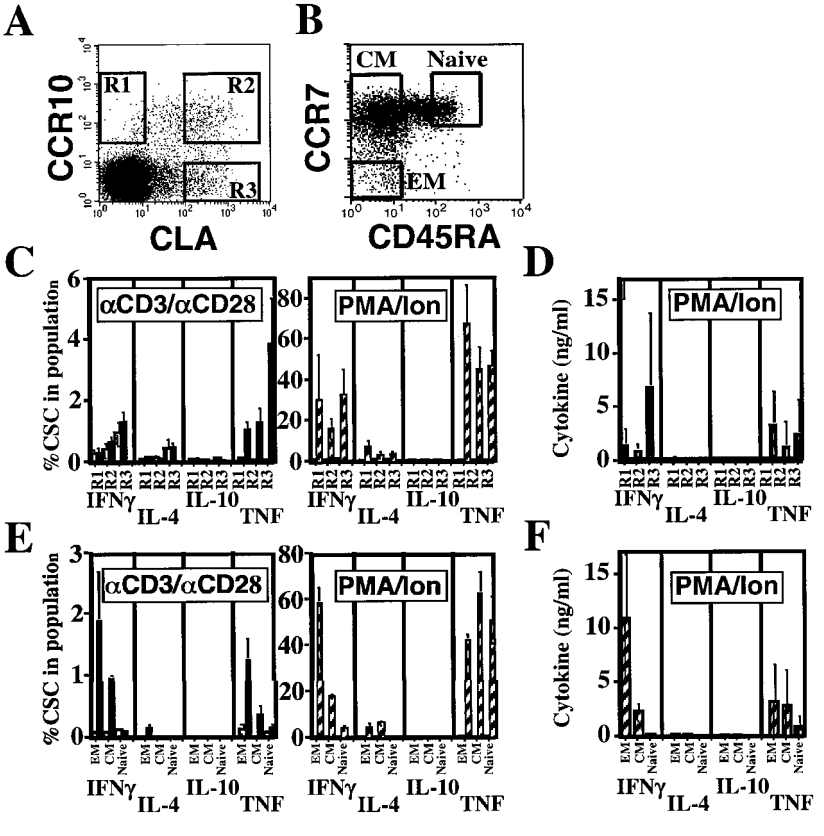

FIGURE 6. $CCR10^+$ memory CD4 T cells from normal donors secrete proinflammatory cytokines. *A*, T cell preparations were stained with biotinylated anti-CCR10, FITC-conjugated CLA, and allophycocyanin-conjugated CD4 and sorted for $CLA^-/CCR10^+$ (R1), $CLA^+/CCR10^+$ (R2), and $CLA^+/CCR10^-$ (R3) memory CD4 T cells. *B*, T cell preparations were stained for CCR7 (visualized with PE-conjugated goat anti-mouse IgM), FITC-conjugated CD45RA, and allophycocyanin-conjugated CD4 and sorted for $CD45RA^-/CCR7^-$ EM, $CD45RA^-/CCR7^+$ CM, and $CD45RA^+/CCR7^+$ naive CD4 T cells. *C*, Sorted cells were plated in an ELISPOT assay and left unstimulated (open bars) or stimulated by anti-CD3/CD28 (*left panel*, filled bars) or PMA/Ion (*right panel*, hatched bars) overnight before developing the plates. Presented data are the means ± SEM from six donors. *D*, Sorted cells were activated with PMA/Ion and secreted cytokines quantitated. Data are means ± SEM of four donors. *E*, Sorted cells were plated in an ELISPOT assay and left unstimulated (open bars) or stimulated by anti-CD3/CD28 (*left panel*, filled bars) or PMA/Ion (*right panel*, hatched bars) overnight before developing the plates. Presented data are the means ± range from two donors. *F*, Sorted cells were activated with PMA/Ion and secreted cytokines quantitated. Data are means ± SEM of four donors.

Discussion

Skin is under constant attack by UV light, environmental chemicals, and pathogens. Because it is our primary protection, the immune system devotes a significant portion of its arsenal toward defense in and near the skin. Immune cells acting as sentinels and effector cells reside among the keratinocytes, fibroblasts, melanocytes, and endothelial cells that constitute skin. Langerhans cells internalize Ags and migrate to draining cutaneous lymph nodes to present these Ags to circulating naive and CM T cells. Blood-borne skin-homing memory T cells enter inflamed cutaneous sites, recognize Ag presentation by Langerhans cells and/or nonprofessional APCs, and then direct the immune system's response to foreign pathogens. Eosinophils, mast cells, basophils, and macrophages also contribute to the immune system's vast complexity within skin under both normal and diseased situations (23).

CLA$^+$ memory T cells home to inflamed cutaneous lesions (5, 6, 24–32) by binding E-selectin expressed by superficial dermal postcapillary venules (4–8, 33). They can constitute the major hematopoietic cell type in a skin lesion and in afferent lymph draining the skin (34), even though these cells are a minor population in blood (8, 24, 25). Immunological memory to cutaneous Ags (e.g., nickel, house dust mite) is mainly carried within this population compared with memory for systemic Ags (e.g., tetanus toxid) that is found in the much larger CLA$^-$ memory T cell population (5, 32, 35–37). CLA$^+$ T cells make Th1 (IFN-γ), Th2 (IL-4, IL-5), and other (IL-10) cytokines that may direct how the immune system will mount a response (25, 27, 32, 35, 37–40). Based on these attributes, CLA$^+$ T cells are thought to contribute significantly to the host response to cutaneous encountered Ags.

E-selectin is absent or expressed at very low levels under normal conditions, but is up-regulated on endothelial cells in response to inflammation (41–43). Leukocyte-expressed CLA binding endothelium-expressed E-selectin facilitates leukocyte slow rolling on the endothelium. Importantly, E-selectin is up-regulated in other inflamed organs, not just skin (42, 44), but CLA$^+$ T cells are mostly found in inflamed cutaneous sites (24, 45–49). What can explain the dichotomy between E-selectin expression in many sites, yet CLA$^+$ T cells only being found in cutaneous lesions? Leukocyte integrins binding endothelium-expressed ICAM-1 and VCAM-1 can mediate step 3 activation-dependent adhesion (8), but these integrin ligands are present in many organs and would not provide a skin selectivity step (1). Therefore, it was postulated that a skin-specific activation cue (step 2) or chemoattractant (step 4) might confer skin-specific homing to the CLA$^+$ T cell population or to a subset of cells within this population. CCL27 is the only skin-specific chemokine known (9) and by interacting with CLA$^+$/CCR10$^+$ memory T cells, it may provide the postulated skin-specific step that facilitates these cells' entry into cutaneous sites.

CCR10 is not found on any peripheral blood leukocyte population with the exception of a small subset of memory CD4 and CD8^{high} T cells that predominately express high levels of CLA in the donors tested in this study. The restricted CCR10 expression to these cells correlated with the restricted migratory response by only these cells to the CCR10 ligand CCL27, suggesting that CCL27 does not have another receptor (9). However, other leukocytes may express CCR10 at developmental stages that are not found in peripheral blood, or at developmental stages in blood that are below our level of detection. Importantly, CCR10 is expressed by other nonleukocyte populations in skin, as assessed by both message (10) and by Ab staining (50).

CCR10 coexpression with some but not other CKRs on a population level suggests a wide array of combinatorial interactions with different sets of chemokines that may target the CCR10$^+$ memory CD4 T cell population into a variety of microenvironmental niches. Additionally, this complexity may provide redundant mechanisms to allow cell entry and dispersion within a tissue. CLA$^+$/CCR10$^+$ memory CD4 T cells tended to have undetectable or low levels of CXCR3 in comparison to the CLA$^+$/CCR10$^-$ memory CD4 T cell population. However, the functional chemotactic responses by both populations to CXCR3 ligands suggest the lower CXCR3 levels are sufficient to mediate step 4 diapedisis. The in vivo significance of lower CXCR3 expression by CLA$^+$/CCR10$^+$ vs CLA$^+$/CCR10$^-$ T cells is unclear at present, but it will be interesting to determine whether this observation is applicable to Th1-associated skin diseases where CLA$^+$/CXCR3$^+$ memory T cells are prevalent, such as psoriasis (18).

Practically all CCR10$^+$ memory CD4 T cells coexpressed CCR4, regardless of CLA expression. Both CCR4 and CCR10 ligands stimulated chemotaxis and triggered rapid integrin-dependent adhesion of CLA$^+$ memory CD4 T cells to ICAM-1 (Ref. 16 and S. Hudak, unpublished observations). However, CCR4 is expressed on most (>75%) CLA$^+$ memory CD4 T cells, and is also expressed on a significant subset of CLA$^-$ memory CD4 T cells (Fig. 4 and Refs. 16 and 40) unlike CCR10, which is on a smaller subset of CLA$^+$ memory CD4 T cells (28%) and very few (1.2%) CLA$^-$ memory CD4 T cells.

The ligands for CCR4 and CCR10 are found in cutaneous sites, suggesting their involvement in T cell entry and dispersion within the skin. CCL27 message is constitutively expressed and is unchanged in various skin diseases (10), although CCL27 protein is increased in psoriasis, AD, and allergic contact dermatitis by immunohistochemistry (50). In vitro results indicate that CCL27 is expressed by keratinocytes and not by human dermal microvascular endothelial cells where the initial steps in skin homing occur (10), although CCL27 protein is associated with the inflamed endothelium by immunohistochemistry (Ref. 50 and S. Hudak, unpublished observations). This may be due to CCL27 secretion by keratinocytes and subsequent binding of CCL27 to endothelial cell-associated glucosaminoglycans as has been described for CXCL8/IL-8 and CCL5/RANTES (51).

The CCR4 ligand CCL17/TARC protein is found on many venules in normal skin, but was drastically up-regulated on most (but not limited to) E-selectin$^+$ venules in human psoriatic skin (16). These results contrast somewhat with Vestergaard et al. (17) who concluded that CCL17/TARC was not in normal skin, but was expressed by keratinocytes but not endothelial cells in AD patients. Because both CCL27 and CCL17/TARC protein are present on inflamed cutaneous endothelium, trigger rapid adhesion of CLA$^+$ memory T cells, and stimulate CLA$^+$ memory T cell migration, CCR4 and CCR10 may provide redundant mechanisms to allow CLA$^+$/CCR4$^+$/CCR10$^+$ memory T cell entry into inflamed cutaneous sites. Importantly, CCR10 could not provide a redundant mechanism for all cells because it is only expressed by a subset of CLA$^+$/CCR4$^+$ T cells. Alternatively, CCR10 and CCR4 may act in a sequential manner to facilitate the specific microenvironmental homing of CLA$^+$/CCR4$^+$/CCR10$^+$ memory T cells compared with the CLA$^+$/CCR4$^+$/CCR10$^-$ T cell population. The in vivo implications of specific spatial homing of these two populations are unclear at present.

CCR6$^+$ memory T cells have also been implicated in a variety of inflammatory skin conditions. CCR6 is expressed on some CLA$^+$ memory CD4 T cells, but is also found on the $\alpha_4\beta_7^+$ and a subset of the CLA$^-$/$\alpha_4\beta_7^-$ memory CD4 populations (52). CCR6 and its ligand CCL20/MIP-3α are weakly expressed in normal skin, but are up-regulated in psoriatic and AD lesions (19, 20), and CCR6$^+$ but not CCR6$^-$ memory T cells roll and adhere to

TNF-activated human dermal microvascular endothelial cells (43). Figs. 4*B* and 5, and Table I demonstrate that CCR6 is expressed on a subset of $CLA^+/CCR10^+$ memory CD4 T cells in normal peripheral blood. This is similar to the distribution of CCR6 on the $CLA^+/CCR10^-$ and CLA^- populations, suggesting a neutral correlation between CCR10 and CCR6 expression in contrast to CCR10 and CCR4 (positive association) or with CCR10 and CXCR3 (negative association). Coexpression of CCR6 and CCR10 on some cells may provide redundant mechanisms to facilitate their entry into inflamed cutaneous sites or these chemokines may act sequentially to transport $CCR6^+/CCR10^+$ memory CD4 T cells into different microenvironments than would be accessible to either $CCR6^+/CCR10^-$ or $CCR6^-/CCR10^+CLA^+$ memory CD4 T cells.

$CLA^+/CCR10^+$ memory T cell production of inflammatory cytokines following stimulation is consistent with a significant number of cells being part of the EM population. Importantly, basal mRNA levels (data not shown) and basal CSC frequency measurement (i.e., in the absence of in vitro stimulation) for inflammatory cytokines is consistent with $CCR10^+$ T cells in blood being memory, but not acutely activated cells in normal donors. It will be interesting to determine in various T cell-mediated skin disease patients whether blood-derived $CCR10^+$ cells have been acutely activated and are actively secreting cytokines. However, $CCR10^+$ CD4 T cells can be induced to secrete inflammatory cytokines such as the proinflammatory TNF and the Th1-associated IFN-γ. Production of TNF by activated skin-homing cells within skin may provide a positive feedback loop to up-regulate endothelial E-selectin expression, and thereby facilitate increased leukocyte entry into the inflamed tissue. IFN-γ production may up-regulate MHC class II expression on keratinocytes, and with TNF induce the expression of a wide variety of inflammatory chemokines such as CXCL9/monokine induced by IFN-γ, CXCL10/IP-10, CXCL11/IFN-inducible T cell α-chemoattractant, CCL17/TARC, and CCL20/MIP-3α. The combined actions of increased E-selectin, inflammatory cytokines and chemokines, and increased class II expression may establish a rich microenvironment to promote the migration and activation of other leukocyte subsets in the lesion. These cells under in vitro activation conditions produced little to no IL-10 or Th2-associated IL-4 message (data not shown) or protein (Fig. 6, *C* and *D*). It should be cautioned that in disease situations such as the Th2-associated AD, there may be an up-regulation of IL-4 production by $CCR10^+CD4$ T cells or a selective expansion of these cells that would change the overall character of the cytokine production capabilities of the $CCR10^+$ memory CD4 T cell population.

In summary, a subset of blood-derived skin-homing memory CD4 and CD8 T cells express CCR10. $CLA^+/CCR10^+$ memory CD4 T cells express a variety of homing and CKRs that may enable them to scan both lymphoid and extralymphoid sites for the presence of non-self Ags. Once activated, these cells express a variety of inflammatory cytokines that would establish and/or maintain a rich inflammatory environment in the skin. The homing of these cells to skin and the subsequent cytokine production by them may have both beneficial and detrimental outcomes. Beneficial responses by these cells may protect the host from pathogenic microbes and skin-derived tumors. However, disregulated responses initiated by environmental factors or host genetic background may lead to inappropriate inflammation as seen in T cell-mediated skin diseases such as psoriasis and AD. Future studies devoted toward understanding CCR10's role in these diseases should be enlightening.

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