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The Vast Majority of CLA⁺ T Cells Are Resident in Normal Skin¹

Rachael A. Clark,* Benjamin Chong,[†] Nina Mirchandani,[‡] Nooshin K. Brinster,[§] Kei-ichi Yamanaka,^{||} Rebecca K. Dowgiert,* and Thomas S. Kupper^{2,*}

There are T cells within normal, noninflamed skin that most likely conduct immunosurveillance and are implicated in the development of psoriasis. We isolated T cells from normal human skin using both established and novel methods. Skin resident T cells expressed high levels of CLA, CCR4, and CCR6, and a subset expressed CCR8 and CXCR6. Skin T cells had a remarkably diverse TCR repertoire and were mostly Th1 memory effector cells with smaller subsets of central memory, Th2, and functional T regulatory cells. We isolated a surprising number of nonexpanded T cells from normal skin. To validate this finding, we counted T cells in sections of normal skin and determined that there are $\sim 1 \times 10^6$ T cells/cm² normal skin and an estimated 2×10^{10} T cells in the entire skin surface, nearly twice the number of T cells in the circulation. Moreover, we estimate that 98% of CLA⁺ effector memory T cells are resident in normal skin under resting conditions. These findings demonstrate that there is a large pool of memory T cells in normal skin that can initiate and perpetuate immune reactions in the absence of T cell recruitment from the blood. *The Journal of Immunology*, 2006, 176: 4431–4439.

T cells are present in normal human skin, and it has been suggested that these cells provide cutaneous immunosurveillance (1). The importance of intact T cell responses in the skin is supported by the finding that transplant recipients receiving T cell immunosuppressive medications have a 65- to 250-fold increased risk of developing squamous cell carcinomas (2). Moreover, an increased percentage of these tumors metastasize, and metastasis is associated with a particularly poor prognosis. In addition to their role in immunosurveillance, skin resident T cells have been implicated recently in the development of psoriasis. T cells resident in nonlesional skin from psoriatic patients divided and induced spontaneous psoriasis lesions when this skin was transplanted to immunodeficient mice (3). T cells in normal skin can therefore contribute actively to inflammatory skin disease in addition to providing immunosurveillance.

Despite the importance of these cells, the study of T cells resident in normal skin has been hampered by the fact that conventional methods allow isolation of very few cells. As a result, previous studies performed only limited analyses or used T cell clones (4–6). We recently reported a novel method of isolating T cells from normal skin that uses the natural tendency of these cells to migrate toward areas of wound healing and tissue repair (37). This

method involves culture of skin explants on three-dimensional matrices that induce the ingrowth of dermal fibroblasts. Chemokines produced by these fibroblasts induce the migration of T cells out of explants, where they can be collected and studied. Although this method requires a culture period, it yields a significant number of T cells.

We report in this study the isolation and characterization of the T cells resident in normal human skin by both established techniques and explant cultures. We found that T cells isolated from explants are indistinguishable from those freshly isolated from skin, and that skin resident T cells are a remarkably diverse population with respect to their TCR repertoire, functional subtypes, and cytokine production. Last, we found that T cells are present in normal skin in substantial and previously unsuspected numbers.

Materials and Methods

Isolation of T cells from skin: EDTA and collagenase

Acquisition of skin samples and all scientific studies were approved by the Institutional Review Board of the Partners Human Research Committee. Samples of normal adult human skin were obtained as discarded human tissue from cutaneous surgeries. Subcutaneous fat was removed from samples of adult human skin, and the tissue was minced into fragments $\sim 3 \times 3$ mm. For EDTA isolation, T cells were isolated, as previously described (5). Briefly, skin fragments were incubated in cold 4 mM EDTA/HBSS for 120 min with vigorous stirring. The supernatant from this step was centrifuged to obtain released T cells, and the remaining tissue fragments were crushed through a 60- μ m pore-size strainer to obtain additional cells. For collagenase digestion, skin fragments were added to 10 ml of RPMI 1640 with 1 mg/ml collagenase D (Roche) and incubated at 37°C on a shaker for 30 min, as previously described (4). Digestion was halted by addition of 10 mM EDTA, and further processing was performed on ice. Floating cells were removed and pooled with cells obtained by washing the skin fragments three times with cold PBS/10 mM EDTA. Cells were isolated by centrifugation. When cell counts were required, T cells from both methods were incubated in 0.15 M NH₄Cl, 1.0 mM KHCO₃, and 0.1 mM EDTA for 5 min at room temperature to lyse RBC.

Isolation of skin T cells using three-dimensional skin explant cultures

Statamatrix-TM matrices $9 \times 9 \times 1.5$ mm (Cell Sciences) were autoclaved, then incubated in a solution of 100 μ g/ml rat tail collagen I (BD Biosciences) in PBS for 30 min at 37°C, and followed by two rinses in

*Department of Dermatology, Brigham and Women's Hospital and Harvard Skin Disease Research Center, Boston, MA 02115; [†]Division of Dermatology, Henry Ford Cancer Center, Detroit, MI 48202; [‡]Division of Dermatology, St. Luke's-Roosevelt Medical Center, New York, NY 10019; [§]Department of Dermatology, Lahey Clinic, Burlington, MA 01805; and ^{||}Department of Dermatology, Mie University, Graduate School of Medicine, Mie, Japan

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² Address correspondence and reprint requests to Dr. Thomas S. Kupper, Department of Dermatology, Harvard Skin Disease Research Center, Brigham and Women's Hospital, Harvard Institutes of Medicine Building 1, Room 660, 77 Avenue Louis Pasteur, Boston, MA 02115. E-mail address: tkupper@partners.org

PBS. In compliance with local institutional review board policies, samples of normal adult human skin were obtained as discarded human tissue from cutaneous surgeries. Subcutaneous fat was removed from samples of normal adult human skin, and the tissue was minced into explants $\sim 2 \times 2$ mm in size. Three skin explants were placed on the surface of each matrix; each matrix was placed into one well of a 24-well plate. The culture was maintained in 2 ml/well IDMEM (Mediatech) with 20% heat-inactivated FBS (Sigma-Aldrich), penicillin and streptomycin, and 3.5 μ L 2-ME. Cultures were fed three times per week by careful aspiration of 1 ml of culture medium and replacement with fresh medium. No exogenous cytokines were added. T cells were observed to spill from the matrices into the surrounding culture wells beginning between days 7 and 14. T cell production reproducibly peaked between 14 and 21 days. T cells were isolated by aspiration of the culture medium and a thorough flushing of the matrices. More than 95% of cells isolated were CD3⁺ T cells.

Flow cytometry studies

Flow cytometry analysis of T cells was performed using directly conjugated mAbs. CD3, CD45RO, CD45RA, CD25, and CD69 Abs were obtained from BD Biosciences; cutaneous lymphocyte-associated Ag (CLA),³ CD25, CCR4 (1G1), CCR5, CCR6, and CXCR3 Abs were purchased from BD Pharmingen; L-selectin Ab was purchased from Beckman Coulter; and CCR4 (150503), CXCR6, CCR7, CCR8, CCR9, and anti-human IFN- γ R α Abs were obtained from R&D Systems. Ab to human ST2L was purchased from MD Biosciences, and anti-human IFN- γ R β was obtained from Research Diagnostics. Analysis of flow cytometry samples was performed on a BD Biosciences FACScan instrument, and data were analyzed with CellQuest software.

Collagenase treatment of CLA⁺ T cells from skin and blood

PBMC were obtained as discarded material from RBC donors. T cells were purified by magnetic bead selection with the Miltenyi pan-T cell isolation kit (Miltenyi Biotec). CLA⁺ cells were isolated by subsequent incubation of T cells in biotin anti-human CLA (BD Pharmingen), followed by anti-biotin microbeads and magnetic separation (Miltenyi Biotec). Positively selected cells were collected as the CLA⁺-enriched fraction. T cells were isolated from normal human skin using explant cultures, as described above. Blood and skin T cells were resuspended in RPMI 1640 with 1 mg/ml collagenase D (Roche) and shaken at 37°C for 30 min. Digestion was neutralized by addition of 10 mM EDTA and cells were placed on ice. Cells were incubated with directly conjugated Abs and analyzed by flow cytometry, as above.

Analysis of T cell proliferation: [³H]thymidine incorporation

Normal skin explant cultures were prepared, as described above. On day 16, 2 μ Ci of [³H]thymidine was added to each culture well. Control PBMC wells contained 1×10^6 PBMC in RPMI 1640/10% FCS with or without 5 μ g/ml PHA (Sigma-Aldrich). Wells were incubated for 48 h at 37°C. Cells were isolated from all wells and counted with a disposable hemocytometer and then transferred to a 96-well plate and lysed. DNA was transferred to a glass fiber membrane with a Tomtec 96-well plate harvester. Membranes were counted in a Wallac TriLux liquid scintillation counter; results were expressed as cpm per 1×10^6 cells.

Analysis of T cell proliferation: BrdU incorporation

Normal skin explant cultures were prepared, as described above. BrdU (1 mM; BD Biosciences) was included in the culture medium for 1 wk, from days 14 to 21 of explant culture. On day 21, T cells were collected, stained for surface expression of CD3 using directly conjugated anti-CD3 (BD Biosciences), fixed in 0.5% paraformaldehyde, permeabilized, and stained for intracellular BrdU, as per protocol (BrdU Flow Kit; BD Biosciences). Cells were then analyzed with a BD Biosciences FACScan instrument and CellQuest software. The number of T cells was similar in explant cultures treated with BrdU and untreated cultures.

Cytokine Ab blockade

Normal skin explant cultures were prepared, as described above. Anticytokine Abs were included during the entire culture period and were refreshed with each feeding. Neutralizing IL-2, IL-7, and IL-15 Abs were purchased from R&D Systems and used at concentrations of 1, 5, and 2.5 μ g/ml, respectively. T cells were harvested from the matrices and counted on day 21.

TCR spectratype analysis

TCR-CDR3 length analysis was performed, as previously described (7). Briefly, total RNA was isolated from 2×10^6 cells (SV total RNA isolation system; Promega) and reverse transcribed into cDNA (PowerScript Reverse Transcriptase; BD Biosciences). PCR were performed with C β primers recognizing both the C β 1 and C β 2 regions and individual primers for the 26 TCR β -chains, as described previously (7). Additional runoff reactions were performed with fluorophore-labeled primers, and labeled products were analyzed with a DNA sequencer and Genescan software (7). A total of 2×10^6 T cells was analyzed in the skin T cell spectratype; the peripheral blood spectratype was generated using 10×10^6 cells.

E-selectin chimera binding

T cells isolated from normal skin via explant cultures were incubated with 1/200 dilution of murine E-selectin human IgG chimera (R&D Systems) in medium containing 2 mM Ca²⁺ for 30 min on ice. Cells were washed twice in calcium-containing medium, incubated in 1/200 dilution biotinylated anti-human IgG Fab for 30 min on ice, washed twice, and incubated with streptavidin-FITC at 1/200 in calcium-containing medium. Cells were then stained with CD3-PerCP, fixed, and analyzed by flow cytometry. Control experiments were performed using 5 mM EDTA to chelate calcium, and by omission of the chimera and staining with secondary Ab and streptavidin-FITC alone.

T regulatory assay

T cells were isolated from explant cultures of normal human skin cultured in the presence of IL-2 (100 U/ml) and IL-15 (20 ng/ml) to induce proliferation of skin resident T cells (37). Cells were harvested at 21 days, and CD4⁺ CD25^{high} and CD25^{low} populations were separated by staining with anti-CD4-PE-Cy5 and anti-CD25-PE and sorting on a FACSAria cell sorter. T cell regulatory assays were performed, as described previously (8). Irradiated (3000 rad) T cell-depleted PBMC prepared from an unrelated blood donor were used as accessory cells. Skin CD4⁺ CD25^{low} T cells (2,500 cells/well) were used as T responder cells. In combined CD25^{high+low} wells, 1,250 CD4⁺ CD25^{high} cells were added to 2,500 CD25^{low} T cells (1:2 T regulatory:T responder ratio). Cells were cultured for 5 days in a final volume of 200 μ l of RPMI 1640 medium with L-glutamine supplemented with 5 mM HEPES, 100 U/ml penicillin, 100 μ g/ml streptomycin (Invitrogen Life Technologies), 1 mM sodium pyruvate, nonessential amino acids (Mediatech), and 5% human AB serum (Fisher Scientific) in the presence of accessory cells (25,000 cells/well) in U-bottom 96-well plates (Costar). When indicated (Fig. 5F, +stim), the cells were stimulated with 1 ng/ml soluble anti-CD3 (HIT3a) plus 100 ng/ml soluble anti-CD28. All well cultures were performed in triplicate. After 5 days of culture, 1 μ Ci of [³H]thymidine was added to each well. The cells were harvested after 16 h, and radioactivity was measured using a liquid scintillation counter (Wallac). The results are expressed as the mean cpm \pm SEM of triplicate wells.

Counting T cells in histologic sections of normal skin

In compliance with local institutional review board policies, samples of normal adult human skin were obtained as discarded human tissue from cutaneous surgeries. Immunoperoxidase studies were performed on Formalin-fixed, paraffin-embedded sections following heat-induced epitope retrieval for the CD3 Ab (rabbit monoclonal, clone SP7; DakoCytomation). CD3-positive cells were counted manually using light microscopy on multiple samples from four separate donors.

Results

Comparison of skin resident T cells isolated by three different methods

We isolated T cells from samples of normal, noninflamed human skin by two established methods, mechanical mincing followed by stirring in EDTA (5) or collagenase digestion (4), as well as by the novel explant method described above. All three methods yielded T cells of a similar phenotype (Fig. 1A and Table I): CLA and CCR4 were expressed at high levels, and CCR8 and CXCR6 were expressed on $\sim 50\%$ of T cells. Expression of CCR6 was more variable; the majority of donors showed high expression of CCR6 on skin resident T cells, but rare donors had lower levels. CCR8⁺ T cells isolated from skin by either the EDTA or skin explant method were uniformly positive for both CCR4 and CCR6, in

³ Abbreviation used in this paper: CLA, cutaneous lymphocyte-associated Ag.

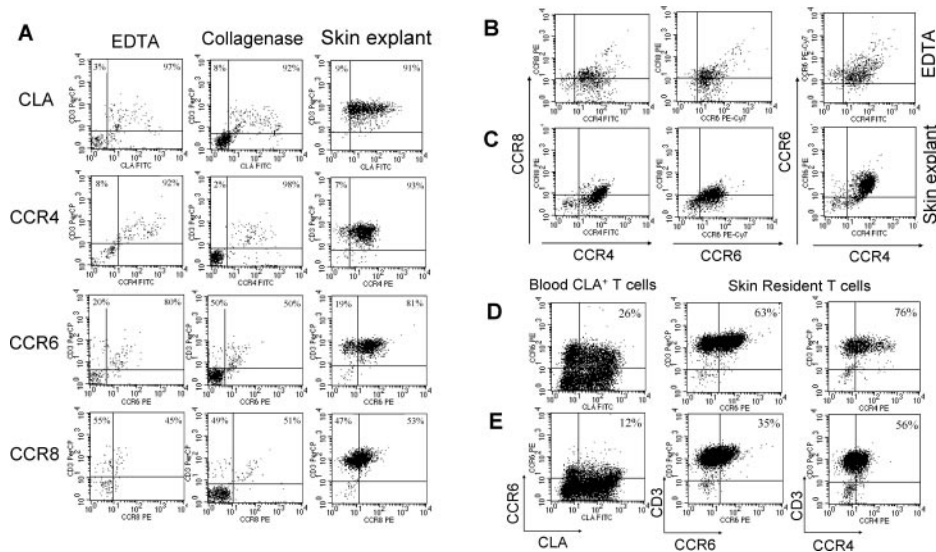


FIGURE 1. Characterization of skin resident T cells isolated by different methods. *A*, Expression of skin-homing markers on skin resident T cells isolated by EDTA treatment, collagenase treatment, or explant culture. A representative experiment is shown; mean levels of expression for three experiments are included in Table I. *B* and *C*, CCR8⁺ T cells are a subset of CCR4⁺CCR6⁺ skin-homing T cells. Identical results are obtained with skin resident T cells isolated by EDTA treatment (*B*) or skin explant culture (*C*). *D* and *E*, Collagenase treatment destroys CCR6 and CCR4 epitopes. CCR6 expression on peripheral blood CLA⁺ T cells and on skin resident T cells isolated from skin explants before (*D*) and after (*E*) treatment with collagenase. Percentage of positive cells is shown in upper right corners. A representative experiment is shown; two additional experiments from different donors gave comparable results.

contrast to the CCR8⁺ T cells described in an earlier report that used collagenase to isolate skin T cells (Fig. 1, *B* and *C*) (4). CCR8⁺ T cells were therefore a subset of CCR4⁺CCR6⁺ T cells. This was also the case for CXCR6⁺ T cells (data not shown).

Levels of CCR6 on skin T cells were lower on skin T cells from all donors when T cells were isolated using collagenase (Table I). Moreover, a previous report that used collagenase to isolate T cells from skin reported that CCR8⁺ T cells were negative for both CCR4 and CCR6 (4). Collagenase preparations have varying amounts of neutral protease activity that can degrade cell surface proteins. We found that treatment of blood-derived CLA⁺ T cells with collagenase destroyed CCR6 epitopes (Fig. 1, *D* and *E*). Additionally, we isolated T cells by the skin explant method and subsequently treated them with collagenase. We observed a mean reduction of 50% in the expression of detectable CCR6 (Fig. 1, *D* and *E*; SD = 4, *n* = 3) and a mean reduction of 22% in CCR4 expression when the Ab clone 1G1 was used (SD = 13, *n* = 3). There was no loss of CCR4 expression when the CCR4 Ab clone 205410 was used, suggesting variable sensitivity of CCR4 epitopes to collagenase.

Isolation of large numbers of nonexpanded skin resident T cells by skin explant culture

EDTA treatment and skin explant culture yielded T cells that expressed similar levels of several skin-homing surface markers (Fig.

1A; Table I). However, there was a dramatic difference in the number of cells isolated by these methods. EDTA treatment had the advantage of isolating T cells without enzymatic degradation, but the disadvantage of isolating only a few T cells (Fig. 2A; 374 cells/cm² skin). Collagenase treatment yielded more cells than did EDTA treatment (Fig. 2A; 2.1 × 10³ cells/cm² skin), but these cells had altered cell surface phenotypes, as described above. However, the skin explant method yielded a large number of T cells with a phenotype indistinguishable from that of T cells isolated by the EDTA method (Figs. 1A and 2A; 2.5 × 10⁵ cells/cm² skin).

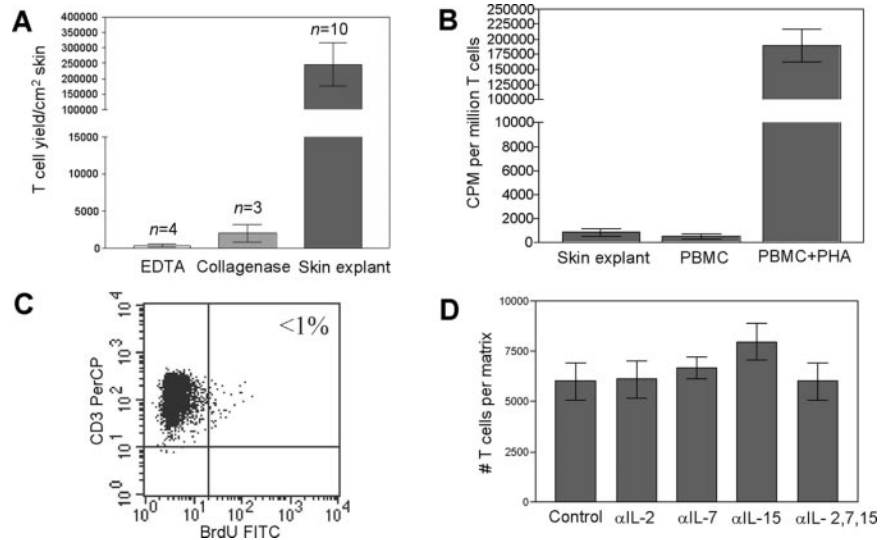
The large number of T cells that we isolated from normal human skin led us to investigate whether T cells were in fact dividing in skin explant cultures. Analysis of [³H]thymidine incorporation during periods of peak T cell production failed to demonstrate any evidence of T cell proliferation (Fig. 2B). Incorporation of [³H]thymidine by T cells in explant cultures was not significantly different from that of resting PBMC kept in culture medium without cytokines. We next assayed for cell proliferation over an extended 7-day period during peak T cell production using incorporation of BrdU to detect cells that were the products of cell division, and again did not detect any significant proliferation (Fig. 2C). We measured explant culture supernatants for the pro-proliferative cytokines IL-2, IL-7, and IL-15 and found low to undetectable levels by both ELISA and protein microarray analyses

Table I. Expression of homing molecules on T cells isolated by various methods from normal skin^a

	EDTA		Collagenase		Skin Explant	
	% Positive	SD	% Positive	SD	% Positive	SD
CLA	93	3	90	8	92	2
CCR4	87	5	97	1	95	3
CCR6	74	7	54	10	82	9
CCR8	50	4	51	2	52	2

^a Values represent percentage of CD3⁺ T cells positive for the indicated marker. Percentages reflect mean values from three different donors.

FIGURE 2. Skin resident T cells isolated from skin explants do not proliferate while in culture. *A*, Comparison of number of T cells isolated from skin using EDTA, collagenase, and skin explant culture. Brackets indicate SD. *B*, T cells from skin explant cultures do not incorporate [³H]thymidine during a 48-h incubation. Comparison is made to resting PBMCs and PBMCs treated with PHA. Brackets indicate SD; skin explant, *n* = 4; PBMC experiments, *n* = 8. *C*, T cells from skin explant cultures do not incorporate BrdU. BrdU was included in the culture system for 1 wk; cells were then fixed, permeabilized, stained for BrdU, and analyzed by flow cytometry. A representative experiment is shown. Four additional experiments produced identical results. *D*, Neutralizing Abs to IL-2, IL-7, and IL-15 did not reduce the numbers of T cells isolated. Abs were included for the entire culture period. Brackets indicate the SD of three experiments.



(data not shown). Moreover, inclusion of neutralizing Abs to these cytokines during the entire period of explant culture did not significantly reduce cell numbers (Fig. 2*D*). Multiple repetitions of these experiments with skin from numerous donors failed to provide any evidence of T cell proliferation.

Comparison of skin resident T cells with CLA⁺ cells from peripheral blood

We next isolated CLA⁺ T cells from peripheral blood and compared the characteristics of these cells to T cells isolated from normal skin in explant culture (Fig. 3). Both populations were universally CD45RO⁺ memory T cells, and the vast majority were CD4⁺. Skin resident T cells expressed high levels of CLA, CCR4, and CCR6, as described above. In contrast, only 60% of CLA⁺ cells from the circulation expressed CCR4 or CCR6. CCR8 and CXCR6, both expressed on ~50% of skin resident T cells, were detectable on <2% of circulating CLA⁺ T cells. CCR5 and CCR9 were undetectable on blood CLA⁺ and skin resident T cells (data not shown). CCR7 and L-selectin were expressed by only 50 and 40% of skin resident T cells, respectively. Expression of the activation Ags CD69 and CD25 on skin resident T cells was increased, consistent with earlier findings that tissue-infiltrating lymphocytes from a variety of sites display an activated phenotype (9, 10). We isolated T cells from both sun-exposed (face and neck) and sun-protected skin (abdomen, breast) from multiple donors and found no significant differences in surface marker expression between T cells from sun-exposed and sun-protected sites (data not shown).

Skin resident T cells have a highly diverse TCR repertoire

We investigated the complexity of the T cell population resident in normal skin by CDR3 length analysis (TCR spectratyping). TCR gene rearrangement produces a unique Ag receptor for each T cell clone. Individual TCR gene rearrangements give rise to TCR with varying CDR3 lengths, and spectratyping of a diverse population of T cells produces a roughly Gaussian distribution of CDR3 lengths within all Vβ families. By contrast, a clonal population of T cells will appear on a spectratype as a single peak in a single Vβ family. We found a remarkable diversity among the T cells resident in normal human skin (Fig. 4). All 26 Vβ families studied were represented, and there was significant diversity within each family. The complexity of the TCR spectratype of cells from skin was only slightly less than that of peripheral blood T cells (Fig. 4). In contrast to the previously described limited expression of Vβ by

T cells from mycosis fungoides and psoriasis skin lesions, we found no discernable Vβ bias among T cells infiltrating normal skin (11–16).

E-selectin-binding activity of skin resident T cells

Skin resident T cells express high levels of the skin-homing marker CLA. To determine whether this CLA expression represents functional E-selectin-binding activity, we stained skin resident T cells with E-selectin chimera. Skin resident T cells bound to E-selectin chimera, and their binding increased linearly with increasing expression of CLA (Fig. 5*A*). Binding was calcium dependent and abrogated by chelation of calcium with EDTA. The high CLA expression observed on skin resident T cells correlated with high E-selectin-binding activity.

Skin resident T cells are predominantly Th1 effector memory cells

Memory T cells can be functionally divided into CCR7⁺L-selectin⁺ central memory T cells and effector memory T cells (17). Central memory T cells are thought to recirculate primarily between blood and lymph nodes, but a subset of these cells expresses CLA, suggesting that they may also be able to enter peripheral tissues (5). We analyzed skin resident T cells and found that only 20% expressed both L-selectin and CCR7 (Fig. 5*B*, mean = 21%, *n* = 3). We thus concluded that the majority of skin resident T cells are effector memory T cells. Among the effector memory subset, ~25% expressed L-selectin alone, a variable number (10–35%) expressed CCR7 alone, and the majority expressed neither CCR7 nor L-selectin.

We next examined CD4⁺ skin resident T cells for Th1/Th2 polarization by analyzing secreted cytokines and surface markers. Mitogen-stimulated T cells demonstrated a predominance of cells secreting the Th1 cytokines IFN-γ and IL-2, although a small population produced the Th2 cytokine IL-4 (Fig. 5*C*). Several surface markers are differentially expressed on Th1 and Th2 T cells. Th2 cells reportedly express both α and β IFN-γR chains, but Th1 cells express only IFN-γRα (18, 19). Expression of the ST2L molecule is also limited to Th2 cells (20). We found that ~7% of skin resident T cells expressed both IFN-γRα and IFN-γRβ, consistent with a Th2 phenotype, and that a similar percentage expressed the additional Th2 marker ST2L (mean = 7%, range 5–11%, *n* = 3 donors; Fig. 5*D*). Therefore, the large majority T cells resident in normal skin appear to be Th1 biased.

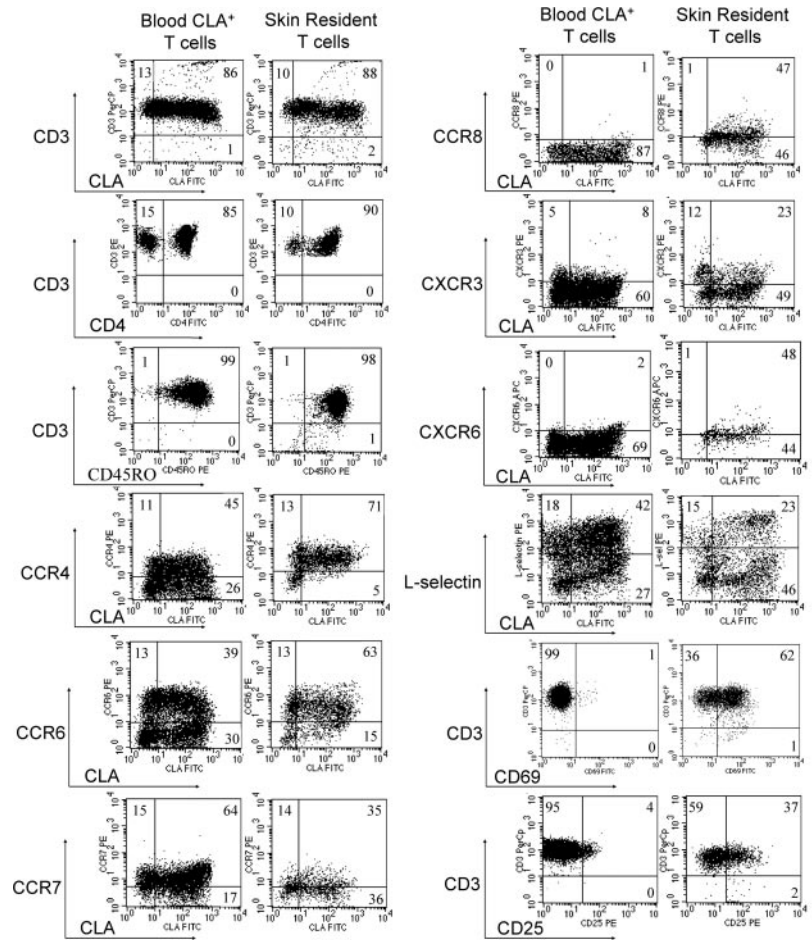


FIGURE 3. Comparison of surface phenotypes of blood CLA⁺ T cells and skin resident T cells. A representative experiment is shown. Five additional experiments with different donors produced similar results.

Normal skin contains a functional population of T regulatory cells

A subpopulation of CD4⁺CD25^{high}CD69⁻ T cells has T regulatory activity (21). We found that a small, but reproducible population of skin resident CD4⁺ T cells expressed CD25, but were negative for CD69, a surface phenotype consistent with that of T regulatory cells (Fig. 5E). To determine whether these cells have regulatory activity, we investigated whether CD25^{high} T cells inhibited the proliferation of CD25^{low} cells. We found that CD25^{high} skin resident T cells proliferated poorly when stimulated with soluble anti-CD3 and anti-CD28, in contrast to the robust proliferation seen in the CD25^{low} subset (Fig. 5F). Moreover, CD25^{high} T cells isolated from normal skin suppressed the proliferation of CD25^{low} skin resident T cells isolated from the same skin sample (Fig. 5F).

Enumeration of T cells in histologic sections of normal skin

We were able to reproducibly isolate significant numbers of T cells from normal skin. Moreover, these cells did not proliferate and had a highly diverse TCR repertoire. To validate this finding, we counted the T cells directly in fixed and immunostained sections of normal skin from various sites (Fig. 6; Table II). The majority of T cells were present in the dermis, and most were near either blood vessels or skin appendages. We counted all T cells in the epidermis and dermis in all sections. Because the depth of the dermis varied by anatomic site, we based T cell counts on the anatomic compartments (epidermis and dermis) rather than on the depth of the section in millimeters. Thus, we counted all of the T cells present within the epidermis and the entire thickness of the dermis, in histologic sections of 1 cm width and 5 μm thickness (Fig. 7).

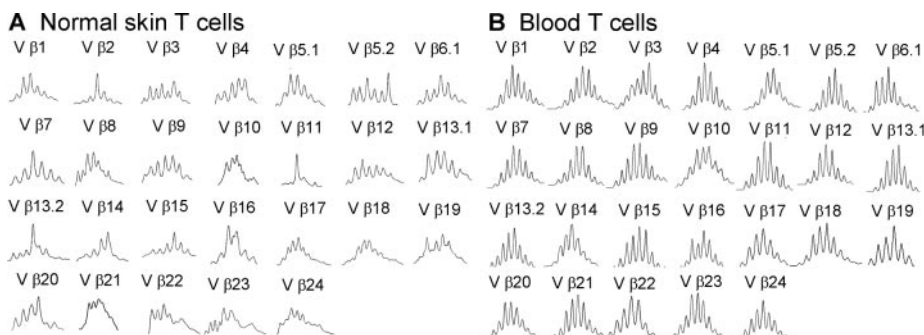
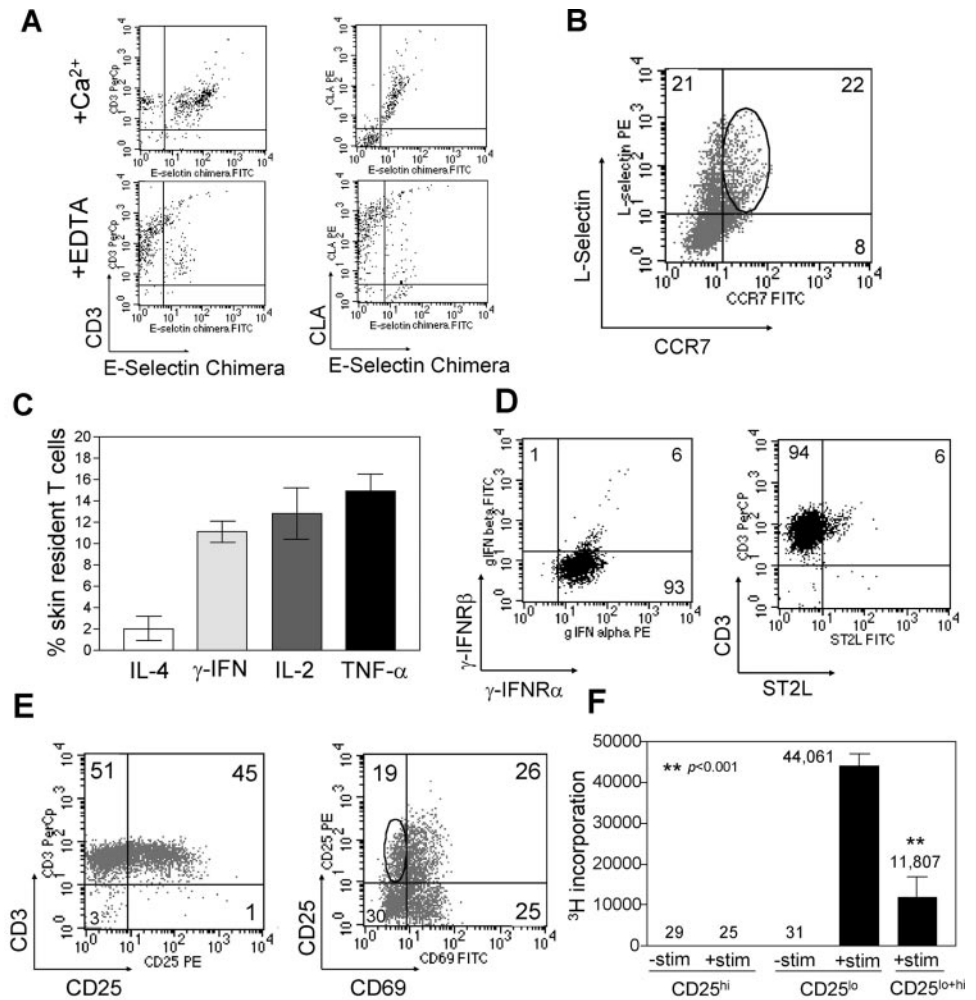


FIGURE 4. Spectratype analysis of skin resident (A) and peripheral blood (B) T cells. A total of 2 × 10⁶ skin resident T cells and 5 × 10⁶ peripheral blood T cells was analyzed. Diversity within each Vβ family is denoted by multiple peaks.

FIGURE 5. E-selectin-binding activity and subset characterization of skin resident T cells. *A*, CLA⁺ skin resident T cells bound to E-selectin chimera; binding was abrogated by addition of EDTA. *B*, Approximately 20% of skin resident T cells are CCR7⁺ L-selectin⁺, consistent with a central memory phenotype. *C* and *D*, Skin resident T cells are primarily Th1 T cells. *C*, Cytokine production by skin resident T cells after stimulation with Con A. Error bars represent the SDs of three measurements. *D*, Th1 and Th2 subsets based on IFN- γ R α β and ST2L expression. *E*, A small subset of skin resident T cells is CD4⁺CD25⁺CD69⁻, consistent with a T regulatory phenotype. Histogram shown is gated on CD4⁺ T cells. All histograms in panels (*A*–*E*) are representative of at least three separate experiments. *F*, CD25^{high} skin resident T cells are functional T regulatory cells. CD4⁺CD25^{high} skin resident T cells suppressed the proliferation of CD4⁺CD25^{low} skin resident T cells isolated from the same skin sample in response to soluble anti-CD3 and anti-CD28 (+stim). The mean and SD of triplicate wells are shown. Duplicate experiments with skin from different donors produced comparable results.



The abundance of T cells in normal skin was striking: there was a mean of 590 T cells in each 1 cm (width) \times 5- μ m (thickness) section of normal sun-exposed skin (SD = 105, n = 25) and a mean of 520 T cells in each 1 cm \times 5- μ m section of normal sun-protected skin (SD = 245, n = 11). None of the differences between the number of T cells from sun-exposed and sun-protected sites was significant. We next used these counts to estimate the number of T cells/cm² of skin surface area. A 1-cm² area of skin is equivalent to 2×10^3 sections that are 1 cm wide \times 5 μ m thick. We used the actual T cell counts noted above to estimate the number of T cells present per cm² of surface area (Table II; Fig. 7). We estimated that there are \sim 1 million T cells resident in each cm² of normal human skin. An average 70-kg male has \sim 1.8 meter (2) of skin surface area (22). We therefore estimate that an average individual of that size has 1.96×10^{10} to 2.12×10^{10} total skin resident T cells.

We next wished to compare the number of skin resident T cells with the number of T cells of various types in the blood. Given that the average 70-kg male has \sim 5 L of blood (23), an average of 7.55 white blood cells/mm³ blood, and an average percentage of lymphocytes of 29.5%, we estimate that he would have 1.11×10^{10} total T cells in the circulation. Remarkably, these estimates suggest that there are twice as many T cells resident in normal human skin than are present in the entire circulation.

Given the large number of T cells resident in normal skin, we were interested in the distribution of CLA⁺ skin-homing T cells within the body. There are two distinct populations of CLA⁺ T cells that differ in their homing behaviors. The most well-described

skin-homing T cells are the cutaneous effector memory T cells. These cells express CLA, but not CCR7 and L-selectin. CLA⁺ effector memory T cells recirculate between the blood and skin, but do not enter the lymph nodes. When the concept of central and effector memory T cells was first described, all T cells that expressed homing receptors for peripheral tissues such as skin were considered to be effector memory T cells (17). However, in the case of skin-homing T cells, the division between central and effector memory T cells is less clear. A subset of CLA⁺ T cells also

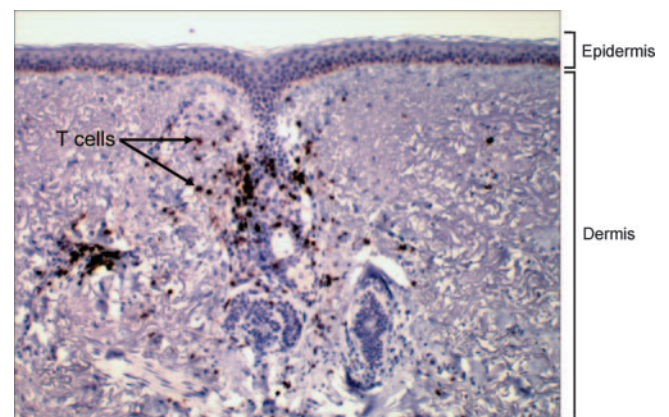


FIGURE 6. Photomicrograph of CD3 immunoperoxidase staining of Formalin-fixed sections of normal human skin. T cells are visualized most frequently near vessels and skin appendages. Magnification: \times 25.

Table II. T cell counts in histologic sections of normal human skin

Anatomic Location	Mean T Cells/cm ²	SD	n	Projected No. T Cells Entire Cutaneous Surface
Sun exposed (face, neck)	1.18×10^6	2.1×10^5	25	2.12×10^{10}
Non-sun exposed (breast, abdomen)	1.04×10^6	4.9×10^5	11	1.96×10^{10}

expresses L-selectin and CCR7; these cells actually comprise 80% of CLA⁺ T cells in the blood (5). These cells, referred to in this study as central memory skin-homing T cells, can enter the skin, but most likely also have access to the lymph nodes.

Estimating the distribution of effector memory CLA⁺ T cells is fairly straightforward because these cells recirculate primarily between the skin and the blood. The vast majority of skin T cells express CLA, but <5% of T cells at extracutaneous sites are CLA⁺ (24). To estimate the number of CLA⁺ effector memory T cells in the skin, we conservatively estimated that only 90% of skin resident T cells express CLA, and, as we have shown above, 80% of these cells are effector memory T cells. Thus, 72% of 2.04×10^{10} total skin resident T cells (a mean value of sun-exposed and sun-protected sites), or 1.47×10^{10} cells, are CLA⁺ effector memory T cells. In the blood, between 10 and 20% of total T cells express CLA (mean 15%), and ~20% of these CLA⁺ T cells do not express L-selectin and CCR7 (5). Therefore, 3% of T cells in the blood, or 3.33×10^8 T cells, are CLA⁺ effector memory T cells. There are thus 1.47×10^{10} of these cells in the skin, 3.33×10^8 in the blood, and a total of 1.50×10^{10} in the body. From these estimates, 98% (1.47/1.5) of CLA⁺ effector memory T cells reside with normal skin under resting, noninflamed conditions.

Estimating the distribution of central memory skin-homing T cells is more complicated. These T cells should be present within three compartments: skin, blood, and lymph nodes. We can estimate the number of these cells within the skin and blood, but not in the lymph nodes. The number of CLA⁺L-selectin⁺CCR7⁺ T cells within lymph nodes has not been described and may vary among cutaneous lymph nodes vs lymph nodes draining other tissues. However, we can compare the number of central memory skin-homing T cells in the skin with that in the blood. We estimate that 90% of T cells in the skin express CLA and have shown that 20% of these cells are central memory T cells, leading to an estimate of 3.67×10^9 central memory skin-homing T cells within the skin. Within the blood, ~15% of T cells express CLA and 80% are central memory T cells, leading to an estimate of 1.33×10^9 cells. Thus, there are 2.8-fold more central memory skin-homing T cells in the skin than in the blood.

In summary, more T cells are present in normal skin than in the entire circulation (2.0×10^{10} vs 1.1×10^{10}), 98% of skin homing effector memory cells are in the skin under resting conditions, and

2.8 times more central memory CLA⁺ T cells are in the skin than in the blood.

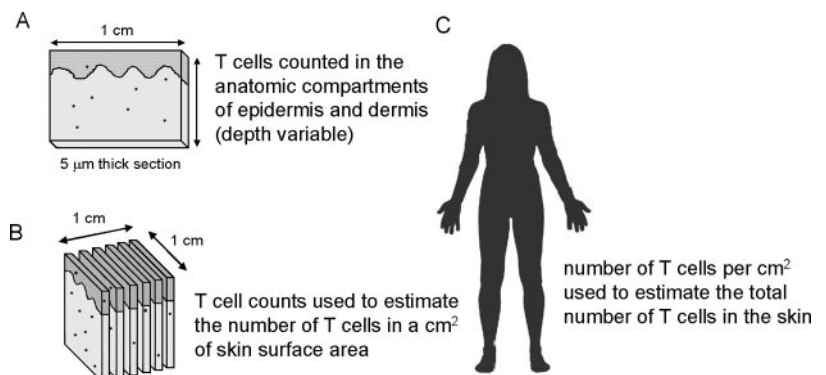
Lastly, we determined the percentage of the total skin resident T cells that we were able to isolate using explant cultures. From three different skin donors, we counted the number of T cells present in sections of skin and used this information to estimate the total number of T cells in the volume of skin cultured on each matrix. We then compared this estimate with the actual number of T cells isolated using explant cultures. We estimated that the skin explants applied to a single matrix contained a mean 4.72×10^5 T cells. However, we isolated from each matrix a mean 1.02×10^5 T cells. Thus, explant cultures allow isolation of ~20% of the T cells actually present in normal human skin.

Discussion

We report in this study isolation, characterization, and enumeration of T cells resident in normal human skin by both established and novel techniques. T cells from normal skin expressed high levels of CLA, CCR4, and CCR6. Skin resident T cells had a remarkably diverse TCR repertoire, and most had the phenotype of Th1 effector memory cells, although Th2 cells, central memory cells, and functional T regulatory cells were also present. Using explant cultures, we recovered significant numbers of T cells from normal human skin that did not appear to be proliferating in culture, leading us to directly count the number of T cells in histologic sections of normal human skin. We found that T cells are present in normal skin in substantial and previously unsuspected numbers.

T cells specialized for entry into cutaneous sites are characterized by expression of CLA, a ligand for E-selectin (1). Additional vascular addressins thought to contribute to the selective traffic of T cells to skin include CCR4, CCR6, and CCR10 (1). T cells must enter noninflamed skin by interacting with the low constitutive levels of homing molecules expressed on resting endothelium. E-selectin, CCL17/thymus and activation-regulated chemokine, CCL20/liver and activation-regulated chemokine, and ICAM-1 are expressed on normal cutaneous endothelium, providing potential interactions with their respective lymphocyte ligands CLA, CCR4, CCR6, and LFA-1 (25–27). Unlike CLA, CCR4 and CCR6 are expressed on both cutaneous and noncutaneous subsets of T cells, and it has been proposed that these molecules must work together with CLA to support selective trafficking to skin (28). Indeed, we

FIGURE 7. Approach used for estimating the total number of T cells in the skin. *A*, The total number of T cells present in the anatomic compartments of the epidermis and the dermis (the depth of which varies depending on site) were counted in sections 5 μ m thick and 1 cm wide. *B*, T cell counts were used to calculate the number of T cells present in a 1 \times 1-cm area of skin. *C*, The number of T cells/cm² surface area and the total surface area of the skin (1.8 meter²) were used to estimate the total number of T cells resident in the skin.



found that >80% of skin resident T cells coexpress CLA, CCR4, and CCR6. Our results are consistent with previous reports showing that CCR4 and CLA are expressed on normal skin T cells (6, 10, 28). CCR8 and CXCR6 have also been proposed to mediate trafficking of T cells to normal skin (K. Ferenczi, submitted for publication) (4). Both CCR8 and CXCR6 are expressed on <1% of circulating CLA⁺ T cells and 50% of skin resident T cells. The enrichment of these cells in skin is consistent with a role in cutaneous homing, although 50% of T cells must have accessed the skin by different mechanisms. Last, we found that CCR8⁺ T cells are a subset of CCR4⁺CCR6⁺ T cells. A previous report describing these cells as CCR4⁻CCR6⁻ used collagenase to isolate cells, a method we have shown to destroy at least some CCR4 and CCR8 epitopes (4).

Skin resident T cells had a remarkably diverse T cell repertoire. Spectratyping analysis demonstrated that skin resident T cells were only slightly less diverse than PBLs. T cells from all 26 V β subgroups tested were present within normal skin and demonstrated significant diversity within each subgroup. We observed no evidence for preferential use of specific V β subgroups among skin resident T cells, as had been suggested previously (15, 16). Whereas T cells responding to bacterial superantigens in the skin would be expected to express particular V β receptors, we would argue that T cells in normal, noninflamed skin are unlikely to be preferentially recruited or expanded on the basis of their V β usage. It appears instead that the diversity of skin resident T cells accurately reflects the diversity of circulating CLA⁺ T cells, as would be the case if one large pool of cells cycles between the skin and blood.

We found that ~80% of T cells residing in normal skin were effector memory T cells. These make up only a minority (20%) of circulating CLA⁺ T cells (5), highlighting the tendency of these cells to home to and reside within normal skin. The preferential accumulation of effector memory T cells within the skin is consistent with a primary role of these cells in cutaneous immunosurveillance.

Approximately 95% of CD4⁺ T cells resident in normal skin were Th1 polarized. In the blood, Th1 and Th2 T cells are normally present in a 7:1 ratio, for both CLA⁻ and CLA⁺ subsets (29). Keratinocyte production of cytokines plays a role in the selective recruitment of T cells to inflamed skin (30) and may also participate in recruitment of T cells into normal skin. Keratinocytes respond to Th1 cytokines by inducing the migration of Th1 T cells and to Th2 cytokines by recruiting both Th1 and Th2 cells (31). Keratinocytes appear to be more responsive to Th1 cytokines, and this characteristic has been postulated to underlie the preferential accumulation of Th1 cells in chronic inflammatory skin diseases. If similar mechanisms of keratinocyte recruitment are operating at lower levels in noninflamed skin, this could explain the preponderance of Th1 T cells in normal skin. Patients with atopic dermatitis have increased numbers of Th2-polarized cutaneous T cells in the blood, and lesions of atopic dermatitis are characterized by infiltration of these cells into the skin (32, 33). It is possible that T cells resident in the normal skin of patients with atopic dermatitis may have a more Th2-skewed phenotype, reflecting a preferential recruitment and expansion of these cells even within normal skin.

We have shown that a population of functional T regulatory cells is resident in normal human skin. These results are consistent with recent findings from our laboratory that a significant proportion of T regulatory cells in the circulation expresses CLA and is most likely specialized for entry into the skin (K. Hirahara, manuscript in preparation). Cutaneous T regulatory cells have a potential role

both in serving as a brake for cutaneous inflammation and in mediating tolerance to normal skin flora.

Our most striking observation is the sheer number of T cells present in normal, noninflamed human skin. Using explant cultures, we isolated an average of 2.5×10^5 T cells from 1 cm² of skin. By histologic counts, an average of 1.1×10^6 T cells are present in each square centimeter of normal skin, suggesting that we were able to isolate only ~20% of skin resident T cells using explant cultures. By extrapolating our histologic T cell counts to the entire cutaneous area, we estimated that 2×10^{10} T cells are present in skin, almost twice the number of T cells in the entire circulation. Moreover, our estimates suggested that the vast majority (98%) of effector memory CLA⁺ T cells are resident within normal skin under resting conditions.

Our findings suggest that there is a large pool of memory T cells within normal skin that is poised to respond immediately to antigenic challenges. Previously, it was assumed that skin-homing T cells are primarily present in the circulation and only extravasate when danger signals are encountered on the vascular endothelium at sites of inflammation (1). Instead, we find that large numbers of T cells enter and populate normal skin under normal, resting conditions. This finding has several implications: first, that skin-homing T cells are remarkably efficient at accessing the skin using only the low, constitutive levels of homing receptors present on resting cutaneous endothelium (25), and, second, that all the components necessary for an adaptive immunologic response, namely, dermal APC and memory T cells, are present in normal skin. Thus, whereas recruitment of additional T cells from the circulation may be a feature of some cutaneous immune responses and inflammatory conditions, it may not be required.

For example, T cells resident in normal skin have been shown recently to be both necessary and sufficient for the development of psoriatic skin lesions. Samples of normal, nonlesional skin from psoriatic patients developed spontaneous psoriatic lesions when transplanted onto immunodeficient mice (3). T cells resident in these samples of nonlesional human skin underwent proliferation within the dermis in response to TNF- α produced by APC, most likely in response to transplantation-induced skin injury. These proliferating T cells were derived entirely from the samples of normal skin and were uniquely responsible for the development of the psoriatic lesions. This study and our results may explain the previously puzzling result that E-selectin blockade, which should efficiently block E-selectin-mediated entry of cells into inflammatory cutaneous sites, does not improve psoriasis, but that TNF antagonists (etanercept, infliximab) and integrin Abs that disrupt the immunologic synapse (efalizumab, alefacept) are effective (34, 35).

Other epithelial surfaces, most notably the gut and pulmonary epithelia, also have large surface areas that interact with the external environment. Gut-homing T cells are a distinct population identified by their expression of $\alpha_{4\beta7}$ integrin and CCR9 (36). By analogy with our findings, it is tempting to speculate that the vast majority of mucosal T cells may be localized to gastrointestinal tissues under noninflamed conditions. If this proves to be the case, T cells resident in these tissues may also play key roles in defense against infections and in the development of inflammatory diseases such as Crohn's disease.

In conclusion, we find that memory T cells are present in normal human skin in substantial and previously unsuspected numbers. T cells from normal skin had a diverse T cell repertoire, were largely Th1 biased, and comprised primarily of effector memory cells, although subpopulations of central memory and functional T regulatory cells were also present. These skin resident T cells form a large pool of cells poised to respond immediately to stimulation by

dermal dendritic cells and other skin-associated APC. This situation is advantageous for prompt defense against microbial invasion, but may also contribute to the development and perpetuation of inflammatory skin diseases.

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Disclosures

The authors have no financial conflict of interest.

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