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Identification of a Novel Proinflammatory Human Skin-Homing Vγ9Vδ2 T Cell Subset with a Potential Role in Psoriasis

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γδ T cells mediate rapid tissue responses in murine skin and participate in cutaneous immune regulation including protection against cancer. The role of human γδ cells in cutaneous homeostasis and pathology is characterized poorly. In this study, we show in vivo evidence that human blood contains a distinct subset of proinflammatory cutaneous lymphocyte Ag and CCR6-positive Vγ9Vδ2 T cells, which is rapidly recruited into perturbed human skin. Vγ9Vδ2 T cells produced an array of proinflammatory mediators including IL-17A and activated keratinocytes in a TNF-α- and IFN-γ-dependent manner. Examination of the common inflammatory skin disease psoriasis revealed a striking reduction of circulating Vγ9Vδ2 T cells in psoriasis patients compared with healthy controls and atopic dermatitis patients. Decreased numbers of circulating Vγ9Vδ2 T cells normalized after successful treatment with psoriasis-targeted therapy. Taken together with the increased presence of Vγ9Vδ2 T cells in psoriatic skin, these data indicate redistribution of Vγ9Vδ2 T cells from the blood to the skin compartment in psoriasis. In summary, we report a novel human proinflammatory γδ T cell involved in skin immune surveillance with immediate response characteristics and with potential clinical relevance in inflammatory skin disease.


In murine skin, the function of γδ T cells has been investigated extensively. Mouse epidermis contains large numbers of dendritic Vγ5Vδ1+ T cells, appropriately termed dendritic epidermal T cells (DETC) (1). DETC have been shown to participate in immunoregulation of the skin (2, 3) and to protect against epithelial malignancies (4, 5). Furthermore, γδ T cells in murine skin may produce growth factors that maintain epidermal integrity (6, 7). In contrast to mouse skin, γδ T cells are rare in healthy human skin, and an equivalent of mouse DETC does not appear to exist. Approximately 2–9% of all dermal and 1–10% of all epidermal T cells are γδ T cells (8–14). Vδ1 T cells are considered to be the primary γδ T cell subset in skin playing a role in skin cancer immune surveillance (14) and wound healing (13), whereas a direct association of Vγ9Vδ2 T cells with cutaneous immunology has to date been lacking. Because γδ+ T cells are less frequent in human skin compared with the frequency of DETC in murine skin, a key question is whether they perform a similar lymphoid stress–surveillance role as their murine counterpart (15).

Psoriasis is a common chronic inflammatory skin disease with a significant genetic disease susceptibility component (16, 17). Although the contribution of effector cells of the adaptive immune system including T cells to disease pathogenesis is well established, recent interest has focused on effector cells of the innate immune system and their putative roles in the initiation and maintenance of psoriatic inflammation (18).

In this article, we characterize a novel proinflammatory human skin homing Vγ9Vδ2 T cell subset, which is characterized by early migration to perturbed human skin in vivo, suggesting a role in tissue immunosurveillance. In plaque-type psoriasis, this subset is preferentially decreased in peripheral blood and increased in psoriatic skin, indicating a potential clinical relevance in the pathogenesis of this major inflammatory skin disease.

Materials and Methods

Study population

Sixty-six patients (age, 25–56 y) with plaque-type psoriasis (Psoriasis Area and Severity Index [PASI] 1–33) were enrolled in this study. The PASI measures extent and severity of skin inflammation. Venous blood and in some cases skin biopsies of patients were taken. Thirty-two age-matched healthy volunteers (age, 28–50 y) and eight age-matched atopic dermatitis patients (age, 26–65 y) were used as control. The patients were gender matched (male-to-female ratio healthy, 0.56; psoriasis, 0.52; atopic dermatitis, 0.33). Discarded healthy skin from female donors aged between 19 and 59 y was obtained after plastic surgery procedures. Human studies were conducted in accordance with the Helsinki Declaration and approved...
Patients included in the study received different treatment regimens at the time of sampling: 32 had not received any systemic treatment for at least 4 wk prior to the time the blood sample was taken (“no systemic treatment”). Thirty-four patients received systemic therapies: 16 patients were treated with different biologic therapies such as etanercept (n = 12), adalimumab (n = 3), and efalizumab (n = 1), 4 patients received biologic therapies in combination with methotrexate (methotrexate + infliximab (n = 3), methotrexate + etanercept, n = 2), 9 patients were treated with methotrexate only, and 5 patients received other therapies such as fumaric esters (n = 2) and acitretin (n = 3). Skin biopsies were taken exclusively from patients who had not received any systemic treatment for at least 4 wk prior. Patients who were evaluated before and 4 wk after treatment initiation received etanercept (patients C and E) and adalimumab (patient D), respectively. For clinical correlation, only patients not receiving systemic treatment were used.

**Cell isolation and culture**

PBMCs separated by density gradient centrifugation using lymphoprep (PAA), according to the manufacturer’s instruction. V-γ9Vδ2 T cells were expanded by culturing PBMCs with 1 μM 4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP, provided to A.C. Hayday by H. Jomaa, Justus Liebig University Giessen, Giessen, Germany), together with 60 IU/ml IL-2 (R&D Systems) and 12 ng/ml IL-15 (R&D Systems) in X-Vivo 15 medium (Lonza) supplemented with 10% human serum (PAA). For generation of skin T cell lines, skin explant cultures were performed as described by Clark et al. (19). For isolation of primary human keratinocytes, skin was incubated overnight in disase (Roche) on 4°C. Then, epidermis and dermis were separated, and epidermal sheets were immersed in trypsin for 10 min on 37°C. For keratinocyte suspension was seeded in 75 cm² flasks precoated with coating matrix (Life Technologies) in Epilife keratinocyte medium (Life Technologies) supplemented with human keratinocyte growth supplement (Life Technologies).

**Flow cytometry**

An analysis of fresh PBMCs, expanded V-γ9Vδ2 T cell lines, and keratinocytes was performed with combinations of Abs directed against various surface markers. For intracellular staining, cells were activated with 50 ng/ml PMA and 1 μM ionomycin for 5 h in the presence of GolgiPlug (brefeldin A; BD Biosciences). After surface staining, cells were fixed (IC fixation buffer; Bioscience) and permeabilized (permeabilization buffer; Bioscience), according to the manufacturer’s instructions, before staining for intracellular proteins. Dead cells were excluded after staining with Aqua dye (LiveDead cell kit; Invitrogen). Acquisition of cells was performed using a BD LSRII (Becton Dickinson), together with 60 IU/ml IL-2 (R&D Systems) and 12 ng/ml IL-15 (R&D Systems) in X-Vivo 15 medium (Lonza) supplemented with 10% human serum (PAA). For generation of skin T cell lines, skin explant cultures were performed as described by Clark et al. (19). For isolation of primary human keratinocytes, skin was incubated overnight in disase (Roche) on 4°C. Then, epidermis and dermis were separated, and epidermal sheets were immersed in trypsin for 10 min on 37°C. For keratinocyte suspension was seeded in 75 cm² flasks precoated with coating matrix (Life Technologies) in Epilife keratinocyte medium (Life Technologies) supplemented with human keratinocyte growth supplement (Life Technologies).

**Immunohistochemistry and immunofluorescence**

Frozen 5-μm-thick sections of skin punch biopsies were fixed in ice-cold acetone for 10 min. For immunofluorescence staining, sections were incubated with 5% normal goat serum (DakoCytomation) for 20 min and then immunostained with Ab (DakoCytomation) and monoclonal mouse Ab (1:50; Beckman Coulter). The secondary Abs (goat anti-mouse 488 and goat anti-rabbit 555) at a concentration of 10 μg/ml were applied for 20 min. After nuclear staining, Topro-3 (1:500) was applied to the secondary Ab solution. Immunofluorescence pictures were taken using a Zeiss LSM Confocal microscope and analyzed with LSM image browser software. For immunohistochemical staining, we used the Vγ9 Ab (1:50; Beckman Coulter), LSAB kit (DakoCytomation) was used according to the manufacturer’s instructions.

**Determination of chemokine, cytokine, and growth factor levels**

HMB-PP-expanded CLA-Vγ9Vδ2 T cells were sorted, seeded at 1.5 x 10⁶ cells/well in 96-well plates, and cultured for 72 h with respective stimuli for analysis of supernatant. A total of 5–7.5 x 10⁶ cells/well in 48-well plates were cultured for 24 h for subsequent RNA isolation. The following stimuli were used: 10 nM HMB-PP + 60 IU/ml IL-2, 10 μM anti-CD3/CD28-coated beads + 60 IU/ml IL-2, 1000 IU/ml IFN-α, or 5 ng/ml PMA and 1 μM ionomycin. Medium only served as a negative control. Keratinocytes were activated as described in Keratinocyte activation assays. Supernatants were assayed using the Milliplex Map Human Cytokine/Chemokine kit (Millipore) and acquired on a Luminex 100 flow-based sorting and detection analyzer (Luminex). Cytokines in the panel were IFN-γ, TNF-α, IL-4, IL-6, IL-10, CCL20, CCL3, CCL4, CCL5, CXCL9, CXCL10, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)-2, epidermal growth factor (EGF), IL-8, and IL-17A. IL-22 and keratinocyte growth factor (KGF) levels were assayed using commercially available ELISA kits (R&D Systems), according to the manufacturer’s instructions.

**Keratinocyte activation assays**

Primary human keratinocytes at passages 2–4 were incubated with a 1:1 mixture of Epilife medium and supernatant of resting or activated CLA-Vγ9Vδ2 T cells. Epilife medium and RPMI 1640 medium at a ratio of 1:1 were used as a negative control, whereas addition of IFN-γ (200 U/ml) and TNF-α (50 ng/ml) to the culture served as a positive control. Activation was blocked with 5 μg/ml anti–IFN-γ and anti–TNF-α Abs (R&D Systems). For RNA extraction, keratinocytes were harvested after 24 h, and for flow cytometry and analysis of supernatant, keratinocytes were cultured for 48 h.

**Skin blisters**

Skin suction blisters were induced applying negative pressure of 25–40 KPa (200–300 mmHg) below atmospheric pressure via a suction chamber (Medical Engineering, Royal Free Hospital, London, U.K.) for 2–4 h using a clinical suction pump (VP25; Eschmann) until a uninodal blister measuring 10–15 mm in diameter was formed between dermis and epidermis at the level of the lamina lucida. Blisters were performed either on unperturbed skin or on skin perturbation (injection of 100 μl sterile saline 24 h prior to blister). Blister fluid was aspirated after 16–20 h using a sterile syringe. The amount of blister fluid was measured and then microcentrifuged at 650 x g for 4 min. Cells were stained for flow cytometry.

**CLA regulation**

Experiments were performed on fresh PBMCs and HMB-PP-expanded Vγ9Vδ2 T cell lines. A total of 1 x 10⁶ cells/96-well plate were cultured in 200 μl X-Vivo medium + 10% of human serum and stimulated with either 10 μM anti-CD3/CD28-coated beads (Invitrogen) or 10 nM HMB-PP + 60 IU/ml IL-2 or 100 ng/ml superantigen staphylococcal enterotoxin B as well as medium only as control. Every condition was performed with or without IL-12 (10 ng/ml) (R&D Systems). After 3 d, cells were harvested, stained with directly labeled Abs directed against CD3, Vγ9, and CLA, and analyzed by flow cytometry.

**RNA extraction, cDNA generation, and real-time PCR analysis**

Total RNA was obtained using the RNeasy Plus Mini Kit (Qiagen). According to the manufacturer’s instructions, and reverse transcribed into cDNA (Superscript II; Invitrogen). TNF, IFN-γ, IL-17A, IL-6, CXCL9, CXCL10, CCL3, CCL4, CCL5, I-309, IL-12, VEGF, FGF, KGF, IL-22, IL-4, IL-6, IL-10, IL-17A, IFN-α, and IL-17A were measured by real-time quantitative RT-PCR using TaqMan assays (Applied Biosystems), according to the manufacturer’s instructions. For each sample, mRNA abundance was normalized to the amount of human GAPDH (Vγ9Vδ2 T cells) or cyclophilin (keratinocytes). Data analysis was performed using the ΔΔCt method; results are expressed as fold change.

**Quantification**

To calculate absolute numbers of CD3+ cells and Vγ9Vδ2 T cells in the blister, we used Trucount tubes (BD Biosciences) together with BD Tristell (CD3/CD16/CD56/CD45) (BD Biosciences), according to the manufacturer’s instructions. For absolute quantification of T cells in the skin, the
method published by Clark et al. (20) was used. Pictures of four randomly selected fields (×20 magnification) were taken with a Zeiss LSM. Pictures were then analyzed with the LSM image browser software. For each picture, V62 T cells were counted, the length of skin was recorded, and the number of V62 T cells in 1 cm skin was calculated.

For quantification of V62 percentage of total CD3 T cells in skin, T cell numbers in skin were counted for CD3- and V62/CD3-expressing cells using the ImageJ software.

Extrapolation of total absolute Vγ9Vδ2 T cell numbers in skin and blood

Absolute numbers of V62 expressing cells in 1 cm skin were acquired as described above and the resulting number was multiplied by 1000 to calculate V62 T cell numbers in 1 cm² of skin. Patient body surface area (BSA) was calculated using the following formula by Mosteller: BSA (m²) = [(height [cm] × weight [kg])/3600]¹/². Clinical examination revealed the approximate percentage of BSA affected by psoriasis from which we extrapolated absolute V62 T cell in psoriatic skin. For analysis of absolute numbers in blood, the absolute number of lymphocytes present in 1 μl blood was performed in the hematology laboratory at Guy’s and St. Thomas’ Hospital. Flow cytometry staining revealed the percentage of CD3 T cells and V62 T cells in the lymphocyte population. Patient blood volume was estimated using the following formula: 0.3669 × height (m²) + 0.32319 × weight (kg) + 0.6041, from which the absolute V62 T cells in peripheral circulation was calculated.

Statistical analysis

Data were assayed for normal distribution by using the D’Agostino–Pearson test and analyzed using unpaired two-tailed student t test for determination of significant differences between two unpaired groups normally distributed and one-way ANOVA for more than two unpaired normally distributed groups. For comparison of two paired groups with normal distribution, a paired t test was used, and repeated measure one-way ANOVA was used for more than two matched groups. To compare not normally distributed samples, we used Mann–Whitney U test to compare two groups and Kruskal–Wallis test for comparison of three groups. As a nonparametric test for two paired groups, we used Wilcoxon matched pair test. Pearson correlation was used to correlate clinical variables and percentage/absolute numbers of γδ T cells, whereas one-way exponential decay was applied for calculation of R². For all statistical tests, we considered p values <0.05 to be statistically significant. Results are expressed as mean ± SEM.

Results

Identification of a subset of circulating Vγ9Vδ2 T cells expressing CLA and skin homing chemokine receptors

We performed extensive phenotypic screening of innate and adaptive lymphocytes in peripheral blood from healthy volunteers and patients with psoriasis. The percentage of CD4 T cells, CD8 T cells, invariant NKT cells, CD3⁺CD161⁺ NK-like T cells, CD56⁺ CD16⁺ NK cells, and CD56 bright NK cells did not show any statistically significant difference between psoriasis patients and healthy individuals (Supplemental Fig. 1A–F). However, we found a significant decrease of circulating Vγ9Vδ2 T cells in psoriasis patients 2.16% (± 0.23%, n = 66) compared with healthy controls 4.21% (± 0.55%, n = 32; p = 0.01) (Fig. 1A). To investigate whether this observation was restricted to psoriasis, we assessed Vγ9Vδ2 T cells in atopic dermatitis, another common inflammatory skin disease with T cell involvement (21). Percentages of circulating Vγ9Vδ2 T cells in atopic dermatitis patients (n = 8) were significantly higher than in psoriasis patients (5.18% (± 1.75%); p < 0.01) (Fig. 1A), indicating that the reduction of Vγ9Vδ2 T cells in psoriasis was not due to unspecific skin inflammation. V61 T cells, the other main γδ T cell subset in humans, was unchanged in the peripheral blood of psoriasis compared with healthy controls and AD (Fig. 1B).

We then assessed the skin homing phenotype of circulating Vγ9Vδ2 T cells in psoriasis patients and healthy volunteers using flow cytometry analysis of fresh PBMCs. A proportion of Vγ9Vδ2 T cells in patients and controls both expressed CLA, a marker for skin homing T cells (Fig. 2A). Only the CLA⁺Vγ9Vδ2 T cell subset was significantly decreased in patients (n = 32) compared with controls (n = 19) (0.94% (± 0.11%) versus 2.22% (± 0.38%); p < 0.001), whereas the CLA⁻ subset was not significantly changed (1.74% (± 0.29%) versus 2.67% (± 0.58%)) (Fig. 2B), demonstrating a preferential reduction of peripheral CLA⁺Vγ9Vδ2 T cells in psoriasis. CLA⁻ total T cells were comparable in patients and controls (16.75% (± 1.39%) versus 18.76% (± 1.26%)) (data not shown).

Next, we investigated the expression of skin homing chemokine receptors. CCR6 expressing cells were the most represented within peripheral Vγ9Vδ2 T cells of patients and controls, whereas a variable Vγ9Vδ2 T cell subset expressed CCR4 and a small subset CCR10 (Fig. 2C). Comparing psoriasis patients and controls, we found that Vγ9Vδ2 T cells of patients showed decreased expression of CCR6 (16.5% [3.5–73%, n = 24] versus 30.61% [7.3–59%, n = 22]; p < 0.05) and CCR10 (16.6% [0–15%, n = 22] versus 5.0–10%, n = 17); p < 0.05) compared with healthy
controls (Fig. 2D), whereas there was no difference in CCR4 expression (2.75% [0–14.9%], n = 22) versus 2.8% [1–17%], n = 17]; ns (Fig. 2D). Vγ9Vδ2 T cells did not express CD103 or CCR9 associated with gut homing (Supplemental Fig. 2A, B).

Because Vγ9Vδ2 T cells are rare in peripheral blood, we generated Vγ9Vδ2 T cell lines by culturing PBMCs with the Vγ9Vδ2 T cell-specific Ag HMB-PP and IL-2 as described previously (24). Using these Vγ9Vδ2 T cell lines, we examined the regulation of CLA on Vγ9Vδ2 T cells. Activation with HMB-PP or the superantigen staphylococcal enterotoxin B did not upregulate CLA expression on peripheral Vγ9Vδ2 T cells. However, incubation with the cytokine IL-12, which has been shown to induce CLA on αβ T cells (25), induced CLA expression also on Vγ9Vδ2 T cells independent of activation (n = 3) (Supplemental Fig. 2C). IL-12–dependent CLA upregulation was confirmed on fresh peripheral Vγ9Vδ2 T cells (n = 4) (Supplemental Fig. 2D).

Expression of CLA on peripheral Vγ9Vδ2 T cells suggested the existence of a specialized skin homing Vγ9Vδ2 T cell subset possibly recruited to skin under conditions of skin perturbation. To assess in vivo dynamics of cutaneous Vγ9Vδ2 T cell homing, we performed skin suction blisters in healthy volunteers (Fig. 3A).

The skin suction blister model allows for the ex vivo analysis of cells present within skin under conditions of tissue homeostasis and pathology (26, 27). We compared Vγ9Vδ2 T cells present in skin blisters induced on normal skin ("nonperturbed" condition) with those induced on "perturbed" skin previously injected with physiological saline. Within the CD3+ T cell population, perturbed skin contained significantly higher percentages of Vγ9Vδ2 T cells than nonperturbed skin (1.2% [± 0.16%] versus 0.7% [± 0.08%]; p < 0.05) (Fig. 3B). Absolute numbers of Vγ9Vδ2 T cells (4.6 × 10^2 [± 1.06 × 10^2]) versus 1.02 × 10^3 [± 1.5 × 10^2]/ml blister...
Acute skin lesions. Therefore, we examined the presence of Vγ9Vδ2 T cells and non-Vγ9Vδ2 CD3+ cells. Perturbed skin contained significantly higher percentages of Vγ9Vδ2 T cells than nonperturbed skin (p < 0.05) (B). Absolute numbers of Vγ9Vδ2 T cells and non-Vγ9Vδ2 CD3+ cells were both significantly higher in perturbed compared with nonperturbed skin (both p < 0.05) (C, D), but Vγ9Vδ2 T cells increased to a significantly greater extent than non-Vγ9Vδ2 CD3+ cells (p < 0.05) (E). Vγ9Vδ2 T cells in perturbed skin were significantly enriched for CLA+Vγ9Vδ2 T cells as compared with circulating Vγ9Vδ2 T cells of the same individuals (p < 0.01) (F, G).

Vγ9Vδ2 T cells are increased in skin of psoriasis patients

Skin homing properties of peripheral Vγ9Vδ2 T cells suggested that the selective reduction of Vγ9Vδ2 T cells in the blood of psoriatic patients might be due to their redistribution into the skin. Therefore, we examined the presence of Vγ9Vδ2 T cells in psoriatic skin lesions. V62+ T cells coexpressing CD3 were preferentially located in psoriatic dermis (Fig. 4A). Few V62+ T cells were identified in clinically normal appearing psoriatic skin (Fig. 4B), whereas V62+ T cells were rarely found in healthy skin (Fig. 4C). Immunohistochemical staining for Vγ9 cells revealed positive cells around blood vessels in the dermis and scattered in the epidermis (Supplemental Fig. 2E). Vγ9 T cells were also detected in nonlesional psoriatic skin (Supplemental Fig. 2F) while being rare in normal skin (Supplemental Fig. 2G). V62 and CLA were commonly coexpressed in psoriatic skin confirming the infiltration of a skin homing Vγ9Vδ2 T cell subset (Fig. 4D).

Absolute numbers of Vγ9Vδ2 T cells were significantly higher in psoriatic lesions (23.62 ± 4.95/cm2) than in nonlesional psoriatic skin (8.00 ± 2.55/cm2; p < 0.01) and healthy skin (0.63 ± 0.63/cm2; p < 0.001) (Fig. 4E). Quantifying Vγ9Vδ2 T cells in skin sections as percentage of total CD3+ cells, we found a significantly higher percentage of Vγ9Vδ2 T cells in lesional (1.31% [± 0.19%]; n = 13) as well as nonlesional (1.23% [± 0.32%]; n = 8) psoriatic skin compared with healthy skin (0.12% [±0.12%]; n = 8; both p < 0.01) (Fig. 4F). Higher proportions of Vγ9Vδ2 T cells already strategically positioned in nonlesional psoriatic skin indicate a potential role in development of early lesions.

Flow cytometry analysis of T cell lines from lesional (n = 12), nonlesional (n = 11) and healthy (n = 8) dermis revealed significantly higher percentages of Vγ9Vδ2 T cells in lesional psoriatic skin compared with healthy skin (1.03% [± 0.31%] versus 0.21% [± 0.18%]; p < 0.05), whereas the percentage of Vγ9Vδ2 T cells in nonlesional psoriatic skin was also slightly increased (0.39% [± 0.16%]; ns) (Fig. 4G).

Correlation between severity of psoriatic disease and percentage of circulating Vγ9Vδ2 T cells

To evaluate the clinical relevance of Vγ9Vδ2 T cells, we analyzed circulating Vγ9Vδ2 T cells in various disease severity states as reflected by the PASI. We found a significant correlation between severe clinical disease (higher PASI) and lower percentages of circulating Vγ9Vδ2 T cells (R2 = 0.53, p < 0.001, n = 30) (Fig. 5A). Absolute Vγ9Vδ2 T cell numbers were obtained in a subpopulation of patients (n = 16) and also negatively correlated with disease severity (R2 = 0.92, n = 16; p = 0.05) (Fig. 5B). In addition, there was no correlation between age or gender with levels of peripheral Vγ9Vδ2 T cells (data not shown). None of the other immune cell subsets investigated (total CD3+, CD4+, CD8+, V61+, CD56+, and CD161+ NK-like T cells, CD56CD16+ NK cells, and CD56bright NK cells) showed a significant positive or negative correlation with disease severity as indicated by the PASI index (data not shown).

We selected two patients with a PASI >20 for detailed calculation of Vγ9Vδ2 T cell numbers. We took advantage of a method developed for quantification of T cell numbers in skin by the group of Kupper and Clark (20). On the basis of this method,
analysed for gd skin pieces in the presence of IL-2 (60 IU/ml) and IL-15 (12 ng/ml) for 2–3 wk and harvesting cells that migrated out of the tissue. Skin T cell lines were quantification of Vg psoriatic skin express Vd higher absolute numbers of Vg (green) and anti-Vd T cells were found in nonlesional psoriatic skin (BT cells were present in lesional psoriatic dermis as well as scattered in the epidermis. Scale bars, 20 m

...g for an additional patient B with a PASI score.

A). Similar numbers were obtained T cells) (Supplemental Fig. 3). We established T cell lines from lesional (Fig. 5). Additionally, we found that alleviation of psoriasis was

...tions in patient A (with 50% of the body surface involved) showed a total of 7.7 × 10⁸ VγVδ2 T cells present in inflamed skin. This patient had ~14 times more VγVδ2 T cells in inflamed psoriatic skin than in peripheral blood (0.53 × 10⁸ VγVδ2 T cells) (Supplemental Fig. 3A). Similar numbers were obtained for an additional patient B with a PASI score > 20 (Supplemental Fig. 3B). These data are in line with our hypothesis that VγVδ2 T cells redistribute from blood to skin during skin inflammation.

Next, we sought to investigate whether treatment would affect peripheral VγVδ2 T cells. We found that VγVδ2 T cells percentage (2.44% [± 0.35%] versus 1.93 [± 0.31%]; ns) (Fig. 5C) and absolute number (data not shown) were similar in peripheral blood of untreated patients and patients on systemic therapy (Fig. 5C) having the same median PASI score (5.95 [1.2–32.2] versus 5.35 [0.6–22.4]). However, when we followed three patients before and 4 wk after successful therapy (PASD score reduced by >40% from baseline), we found that alleviation of psoriasis was accompanied by an increase of absolute T cell numbers (Fig. 5D) and percentage (data not shown) in peripheral blood. Possibly as a result of residual disease, VγVδ2 T cell levels did not consistently reach levels seen in healthy controls (data not shown).

Taken together, psoriasis severity correlated with decreased numbers of circulating VγVδ2 T cells and was reversed after successful treatment with psoriasis-targeted therapy, suggesting a clinically significant role of VγVδ2 T cells in psoriasis.

CLA⁺VγVδ2 T cells produce psoriasis-relevant proinflammatory cytokines, chemokines and growth factors

To further define the role of VγVδ2 T cells in psoriasis, we investigated a range of proinflammatory mediators with potential relevance to psoriasis pathogenesis. CLA⁺VγVδ2 T cells produced high levels of TNF-α and IFN-γ upon TCR-specific (HMB-PP) and unspecific stimulation, whereas constitutive production in resting cells was low to undetectable (representative experiment, n = 8) (Fig. 6A). VγVδ2 T cells produced IL-17A and IL-17A–producing VγVδ2 T cells were enriched in the CLA⁺ population...
Clinical correlation between psoriasis severity and circulating Vy9V82 T cells. Lower percentages of circulating Vy9V82 T cells significantly correlated with severe clinical disease as reflected in a higher PASI score ($R^2 = 0.53$, $n = 30$; $p < 0.001$) (A). Absolute Vy9V82 T cell numbers were obtained in a subpopulation of patients ($n = 16$) and also correlated with disease severity ($R^2 = 0.92$; $p < 0.05$) (B). Comparing percentage of peripheral Vy9V82 T cells of patients without systemic treatment to treated patients with the same PASI score, we did not observe any significant difference (2.44% [+ 0.35%] versus 1.93% [+ 0.31%]; ns) (C). We analyzed three patients before and during the course of their treatment at weeks 0 and 4. In all of these patients, PASI had dropped by at least 40% after 4 wk of systemic treatment (patient C, 4 wk, 40%; patient D, 4 wk, 48%; patient E, 45%). In all three patients, the absolute numbers of peripheral Vy9V82 T cell increased with successful treatment (patient C, 4 wk, 10%; patient D, 4 wk, 360%; patient E, 4 wk, 67%) (D).

CLA$^+$, 0.4% [± 0.07] versus CLA$^-$, 0.15% [± 0.05%]; $p = 0.0313$) (Fig. 6B). CLA$^+$Vy9V82 T cells did not produce IL-22 (data not shown).

Activated CLA$^+$Vy9V82 T cells produced the psoriasis-relevant chemokine IL-8 (Fig. 6C). In addition, they produced the proinflammatory chemokines CCL3 (MIP-1$\alpha$) and CCL4 (MIP-1$\beta$), which were markedly upregulated upon activation (Fig. 6C). CCL5 (RANTES) was constitutively produced at high levels (Fig. 6C).

Psoriasis is an immune-mediated disease associated with hyperproliferation of keratinocytes and angiogenesis; we therefore assessed whether CLA$^+$Vy9V82 T cells produced growth factors such as VEGF, KGF, FGF-2, EGF, and IFN-$\gamma$. CLA$^+$Vy9V82 T cells upregulated IGF-1 production when stimulated with HMB-PP or PMA/ß-omnomycin (Fig. 6D). This was confirmed at the RNA level where the psoriasis-relevant cytokine IFN-$\gamma$ was identified as a strong inducer of IGF-1 mRNA ($n = 3$) (Fig. 6D). CLA$^+$Vy9V82 T cells produced low amounts of VEGF (<100 pg/ml) and FGF-2 (<15 pg/ml) upon activation, whereas KGF and EGF production was absent (data not shown). These data establish CLA$^+$Vy9V82 T cells as potent proinflammatory cells and potential contributors to psoriasis immunopathogenesis.

CLA$^+$Vy9V82 T cells activate keratinocytes

We next investigated the effects of CLA$^+$Vy9V82 T cells on skin epithelial cells. Incubation of primary human keratinocytes with CLA$^+$Vy9V82 T cell supernatant upregulated the activation markers ICAM-1, HLA-DR, and HLA-ABC on keratinocytes (Fig. 7A). Keratinocyte activation with CLA$^+$Vy9V82 T cell supernatant was partly blocked by adding anti–IFN-$\gamma$ and TNF-$\alpha$ Abs to the culture (Fig. 7B). CLA$^+$Vy9V82 T cell supernatant induced keratinocytes to produce the psoriasis-relevant mediators TNF-$\alpha$, IL-6, CXCL9, and CXCL10 in an IFN-$\gamma$– and TNF-$\alpha$–dependent manner (Fig. 7C). A major defense mechanism of keratinocytes against invading pathogens is their production of antimicrobial peptides (28), which are also upregulated in psoriasis (29). CLA$^+$Vy9V82 T cell supernatant induced production of $\beta$-defensin 2, S100A7, and S100A8 but not LL37 in keratinocytes ($n = 6$) (Fig. 7D and data not shown). These data demonstrate a proinflammatory cross-talk between CLA$^+$Vy9V82 T cells and keratinocytes.

Discussion

Murine skin has an abundant population of epidermal $\gamma$6 T cells, which serve an important role as early immune sentinels. $\gamma$6 T cells are exceedingly rare in human skin, and it has remained controversial whether and to what extent $\gamma$6 T cells play a role in human skin homeostasis or pathology. This study revisits the role of $\gamma$6 T cells in skin of healthy individuals and in the chronic inflammatory skin disease psoriasis.

In a first instance, we had performed an extensive flow cytometry-based screen of unconventional T cell populations including NKT cells and $\gamma$6 T cells in healthy subjects and psoriasis patients. Although we were unable to find significant differences in most of the cell populations investigated, there was a reproducible and significant reduction of V$\gamma$9V82 cells in the blood of psoriasis patients compared with healthy controls.

In this study, we identify and characterize a novel subset of skin-homing, proinflammatory Vy9V82 T cells that migrates to perturbed human skin in vivo. We suggest that Vy9V82 T cells are clinically relevant in psoriasis because reduction of circulating Vy9V82 T cell numbers correlated with increased psoriasis severity and was restored by successful psoriasis-targeted therapy.

Few studies have investigated Vy9V82 T cell in skin homeostasis and pathology, and even though several reports show that Vy9V82 T cells are present in skin, their role and function has remained ill explored. Using spectrotyping techniques, Holtmeier et al. (12) described the presence of Vy9V82 T cells in healthy...
human skin. Other studies using immunohistology techniques detected Vγ9Vδ2 T cells in a variety of infectious, inflammatory, and malignant cutaneous diseases (8, 9, 30–32). Interestingly, the majority of primary cutaneous γδ T cell lymphomas display a Vδ2 gene usage (33) supporting the concept of a specialized subset of skin homing Vγ9Vδ2 T cells.

We show that a reduction of circulating Vγ9Vδ2 T cells in psoriasis patients compared with healthy controls is specific for Vγ9Vδ2 T cells as other circulating lymphocyte subsets, such as conventional T cells, V61 T cells, NKT cells, or NK cells, did not show significant differences. More detailed analysis revealed the existence of a Vγ9Vδ2 T cell subset expressing the E-selectin ligand and skin homing marker CLA. This subset was selectively decreased in psoriasis compared with the CLA− subset, indicating the existence of a skin homing Vγ9Vδ2 T cell subset with the potential to be recruited into psoriatic skin. This was further supported by the reduction of peripheral Vγ9Vδ2 T cells expressing skin homing chemokine receptors in psoriasis.

Although the majority of CLA+ conventional αβ T cells coexpresses the homeostatic skin homing chemokine receptors CCR4 and CCR10 (20), only a minority of Vγ9Vδ2 T cells expressed these receptors indicating differences in skin homing behavior between conventional αβ T cells and Vγ9Vδ2 T cells. Predominant expression of the inflammatory skin chemokine receptor CCR6 on Vγ9Vδ2 T cells supported the possibility of a coordinated and selective recruitment of circulating Vγ9Vδ2 T cells into skin upon skin perturbation as opposed to homeostatic migration into the skin.

We studied the skin homing capability of Vγ9Vδ2 T cells in vivo using a skin suction blister model. This model has been widely used to study skin immune cells under homeostasis and in the context of skin perturbation (26, 27, 34). Immune cells present in blister fluid have been shown to represent cells, phenotypically and numerically, found in situ by histology (35). Vγ9Vδ2 T cells were significantly increased following skin perturbation against a background of Vγ9Vδ2 T cells present in unperturbed skin. Vγ9Vδ2 T cells in unperturbed skin could represent a rare skin resident population (14). Vγ9Vδ2 T cells in perturbed skin increased to a significantly higher extent than total T cells and expressed significantly more CLA than circulating Vγ9Vδ2 T cells. Taken together, these results support the existence of a specialized circulating skin homing Vγ9Vδ2 T cell subset in vivo.

To place Vγ9Vδ2 T cells in the context of pathology we investigated psoriasis, one of the most common inflammatory skin conditions. Psoriasis has served a model of inflammatory disease in which to study the involvement of immune cell subsets.

Although the presence of cells expressing the pan-γδ TCR in psoriasis has been previously described (30), our results show for the first time, to our knowledge, the infiltration of Vγ9Vδ2 T cells...
in psoriatic skin. Calculating absolute V_{g9}V_{d2} T cell numbers in psoriatic skin, we found substantial amounts of V_{g9}V_{d2} T cells in psoriatic lesions. The number of V_{g9}V_{d2} T cells in the skin of patients with severe psoriasis was an order of magnitude higher than the number of circulating V_{g9}V_{d2} T cells (e.g., patient A had 14-fold higher numbers of V_{g9}V_{d2} T cells in inflamed skin compared with that in the peripheral circulation [770 million V_{g9}V_{d2} T cells in skin versus 53 million circulating V_{g9}V_{d2} T cells]).

Perhaps our most striking finding was the fact that psoriasis disease severity significantly correlated with lower relative and absolute numbers of V_{g9}V_{d2} T cells in the circulation and that successful antipsoriatic therapy leads to increase of peripheral V_{g9}V_{d2} T cells. This establishes V_{g9}V_{d2} T cells as potential biomarkers for psoriasis and supports their clinical significance.

We suggest that circulating CLA^+V_{g9}V_{d2} T cells are early immune sentinels recruited to perturbed skin in the context of trauma or infection. In this scenario, V_{g9}V_{d2} T cells might be protective/beneficial in conditions of skin homeostasis and help in maintenance of skin integrity and in antimicrobial responses (31, 32). In contrast, V_{g9}V_{d2} T cells might be pathogenic in an inflammatory environment contributing to psoriasis pathogenesis as potentially early players in the development of a psoriatic plaque (Supplemental Fig. 4).

Plasmacytoid DCs triggering the development of psoriatic lesions through their IFN-α production (36). Thus, it is conceivable that V_{g9}V_{d2} T cells are activated by IFN-α in psoriatic skin as IFN-α potently induces IFN-γ production in V_{g9}V_{d2} T cells (37). In addition we show that IFN-α induces production of the psoriasis relevant growth factor IGF-1 (38, 39) in V_{g9}V_{d2} T cells. Furthermore, V_{g9}V_{d2} T cells produce the psoriasis-relevant cytokines IFN-γ, TNF-α, and IL-17A, in line with previously reported IL-17A production of blood-derived V_{g9}V_{d2} T cells (40). V_{g9}V_{d2} T cells also release large amounts of proinflammatory chemokines such as IL-8, CCL3, CCL4, and CCL5 which have been shown to be of importance for recruitment of...
key immune effector cells to skin (41, 42) and efficiently activate keratinocytes. Thus, Vγ9Vδ2 T cells might play an important role in psoriasis disease pathogenesis.

There are questions remaining. Although it is the nature of translational human immunology studies that most of the data are observational, it will be interesting to study the role of Vγ9Vδ2 T cells in the context of targeted interventions using biologics. Also, more insights into the functional nature of these cells might be obtained in clinically relevant model systems such as animal xenotransplantation models.

Taken together, we propose that CLA+Vγ9Vδ2 T cells represent an innate immediate response tissue surveillance cell, constituting a first wave of T cells that enter skin upon tissue perturbation, such as trauma, adding to the intricate immune surveillance network that operates in the skin (43). Our data point toward three major roles for skin homing Vγ9Vδ2 T cells: 1) the rapid release of proinflammatory cytokines influencing resident immune and epithelial cells; 2) the recruitment of immune cells from the circulation; and 3) the release of growth factors resulting in tissue remodeling.

Our data put the emphasis on a previously overlooked T cell subset in skin immunology. The discovery of a potential role of Vγ9Vδ2 T cells in psoriasis disease pathogenesis provides the basis for the discovery of novel biomarkers and potential therapeutic targets for the discovery of chronic inflammatory skin diseases.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure 1. Adaptive and innate lymphocytes in psoriasis and healthy controls. Percentage of circulating blood CD4+ T cells (CD3+CD4+) (A), CD8+ T cells (CD3+CD8+) (B), NK-like T cells (CD3+CD161+) (C) NKT cells (CD3+6B11+) (D), and NK cells (CD56brightCD3- (E) and CD56+CD16+CD3- (F)) was measured by flow cytometry, gated on CD3 (CD4, CD8, 6B11) and lymphocytes (NK cells, CD161+ T cells), respectively. Psoriasis patients and healthy controls did not show significant differences in any of the cell types analyzed. Psoriasis patients had a mean of 29 (+ 4.7) Vγ9Vδ2 T cells / µl peripheral blood (G).
Supplementary Figure 2

Peripheral Vγ9Vδ2 T cells do not express gut homing markers, up-regulate CLA upon exposure to IL-12 and are present in psoriatic skin. Expression of the gut homing markers CCR9 and CD103 (αEβ7 integrin) were analyzed on peripheral T cells of 5 healthy individuals by flow cytometry. While total CD3+ T cells expressed low levels of CCR9 and CD103 (A), no circulating Vγ9Vδ2 T cells with gut homing phenotype could be detected (B). To investigate CLA regulation peripheral Vγ9Vδ2 T cell lines were either left unstimulated or stimulated with HMB-PP (1nM) or SEB (100ng/ml) for 3 days (all with and without IL-12). IL-12 induced a more than two fold up-regulation of CLA in all conditions independent of activation while activation by itself did not induce CLA (one representative experiment (n=3), done in triplicates) (C). To verify the exposure to IL-12 on CLA expression we analysed Vγ9Vδ2 T cells by flow cytometry using fresh PBMCs. Fresh PBMCs were cultured for 3 days with 10 ng/ml IL-12 or with medium alone before staining for flow cytometry. IL-12 induced a distinct up-regulation of CLA on fresh Vγ9Vδ2 T cells (one representative experiment, n=4) (D). 5 µm sections of frozen healthy and psoriatic lesional and non-lesional skin were stained for the Vγ9 antigen by immunohistochemistry. The arrows indicate Vγ9+ expressing cells. Vγ9+ cells were present in dermis and epidermis of psoriasis skin (E). In addition they were detected in non-lesional skin of psoriasis patients (F). Vγ9+ cells were rarely seen in healthy skin (G).
Supplementary Figure 3

A  Vγ9Vδ2 cells counts in psoriatic skin (A)

37.5 Vγ9Vδ2 T cells in 1 cm skin section

2000 5 μm-thick sections correspond to 1 cm² of skin

37.5 x 2000 = 75 000 Vγ9Vδ2 T cells estimated in 1 cm² of skin

A patient with 50% of body surface area affected (= 10300 cm² of skin) has 75,000 cells/cm² skin x 10300 cm² = 7.7 X10⁸ Vγ9Vδ2 T cells in affected psoriatic skin

Vγ9Vδ2 cells counts in psoriatic blood (A)

9.8 Vγ9Vδ2 T cells in 1 µl peripheral blood

9.8 Vγ9Vδ2 T cells x 5.4 L blood = 0.53x10⁸ Vγ9Vδ2 T cells in peripheral blood

14 times more Vγ9Vδ2 cells in psoriatic skin than in peripheral blood

B

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Supplementary Figure 3. Calculation of absolute numbers of Vγ9Vδ2 T cells in total psoriatic skin. Vγ9Vδ2 T cells in blood and psoriatic skin of one patient (Patient A) were estimated. In his lesional psoriatic skin, 6 Vδ2 expressing T cells were detected in 1.6 mm of a 5 µm section corresponding to 37.5 cells in 1 cm of 5 µm thick skin. A 1 cm² area of skin is equivalent to 2x10³ 0.5 µm-thick sections, therefore we multiplied 37.5 by 2x10³. This resulted in 7.5x10⁴ Vγ9Vδ2 T cells / 1 cm² of psoriatic skin. Clinical examination revealed that 50% of the body surface area were covered by psoriasis plaques. Patient A had – as calculated from his body size (178 cm) and weight (86 kilos) - a total body surface area of 2.06 cm², 50% of which corresponds to 10300 cm² of psoriatic skin. We calculated Vγ9Vδ2 T cell numbers in this patient’s affected skin by multiplying Vγ9Vδ2 T cell number in 1 cm² by the affected skin surface area resulting in an approximate number of 7.7x10⁸ Vγ9Vδ2 T cells (STD: +1.3*10⁸). To put this number into context we calculated the approximate number of Vγ9Vδ2 T cells in his peripheral blood. A differential blood count and flow cytometry for Vγ9Vδ2 T cells in peripheral blood taken at the same time as the biopsy was used to deduct absolute T cell numbers in peripheral blood taken at the same time as the biopsy was used to deduct absolute T cell numbers in peripheral blood. The total blood volume of this patient was calculated to be 5.44 l, resulting in an approximate total of 0.53x10⁸ Vγ9Vδ2 T cells in his circulation. Remarkably at the time of biopsy, Patient A had more than 14 times higher numbers of Vγ9Vδ2 T cells in his psoriatic skin than in his peripheral blood (A). The calculation for Patient A is summarized in (B). We could confirm these data in a further patient (Patient B) with a PASI of 24.1 with a 25 fold increase of Vγ9Vδ2 T cells in his psoriatic skin compared to peripheral blood (8.66 *10⁸ (± STD 1.5*10⁸) in skin, 3.4*10⁷ in peripheral blood) (B).
Supplementary Figure 4. The potential role of Vγ9Vδ2 T cells in skin immunology. We propose that Vγ9Vδ2 T cells are immediate response tissue surveillance cells that can have both, protective and pathogenic roles. Vγ9Vδ2 T cells are attracted to perturbed skin via CCL20 released by keratinocytes and produce growth factors such as IGF-1, FGF-2 and VEGF important for wound healing and angiogenesis. In addition Vγ9Vδ2 T cells are possibly involved in tumour immunosurveillance through their recognition of stress-upregulated self antigens such as IPP and MICA/B.

However, Vγ9Vδ2 T cells could be pathogenic in psoriasis where they might initiate and amplify the inflammatory loop by producing psoriasis-relevant cytokines (IL-17, IFN-γ and TNF-α) and chemokines (CCL3, CCL4, CCL5 and IL-8), thus attracting a plethora of immune cells to the evolving psoriatic lesions. Finally, they produce growth factors (IGF-1, FGF-2, VEGF) and antimicrobial peptides (S100A7, S100A8, β-defensin-2) also playing a role in psoriasis pathogenesis.