

CXCL16 and CXCR6 Are Upregulated in Psoriasis and Mediate Cutaneous Recruitment of Human CD8⁺ T Cells

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Psoriatic skin lesions are characterized by an inflammatory infiltrate, consisting of dendritic cells, monocytes, and both CD4⁺ and CD8⁺ T lymphocytes. Although the chemokines involved in the migration of CD4⁺ T cells into psoriatic skin are well characterized, those regulating CD8⁺ T-cell recruitment are less understood. We found that the percentages of peripheral blood CD8⁺ T cells expressing CXCR6 were higher in psoriatic patients than in healthy or atopic individuals. In addition, CXCR6 expression in psoriatic patients was more abundant in the CD8⁺ than in the CD4⁺ T-cell compartment. CXCR6 mRNA expression was also stronger in skin CD8⁺ T cells than in the corresponding blood-derived counterparts. Immunofluorescence analysis revealed profound upregulation of the CXCR6 ligand CXCL16 by monocytes, keratinocytes, and dendritic cells in psoriatic skin compared with healthy or atopic dermatitis skin. In line with this, CXCR6⁺ CD8⁺ T cells also were most prevalent in psoriatic skin. Furthermore, CXCL16 induced Ca²⁺ influx and chemotactic migration of psoriatic skin-derived CD8⁺ T cells *in vitro*. Most importantly, CXCL16 potently recruited human CD8⁺ T cells to human skin grafts previously transplanted onto SCID mice *in vivo*. These investigations indicate that CXCL16–CXCR6 interactions mediate homing of CD8⁺ T cells into human skin, and thereby contribute to psoriasis pathogenesis.

Journal of Investigative Dermatology advance online publication, 24 November 2011; doi:10.1038/jid.2011.371

INTRODUCTION

Chemokines have an important role in immune cell trafficking in healthy and diseased skin and can be upregulated during inflammation for recruitment of leukocytes (Rot and von Andrian, 2004). The chemokine CXCL16, which has been described as type I transmembrane molecule and as a soluble molecule (Abel *et al.*, 2004; Gough *et al.*, 2004), is constitutively expressed on keratinocytes in healthy epidermis (Scholz *et al.*, 2007); CXCL16 secretion can be induced by tumor necrosis factor (TNF)- α , IFN- γ , and IL-1 β in keratinocytes (Tohyama *et al.*, 2007), synovial fibroblasts (Nanki *et al.*, 2005; Ruth *et al.*, 2006), and monocytes/macrophages (van der Voort *et al.*, 2005; van Lieshout *et al.*, 2009). In addition, CXCL16 can be expressed by dendritic

cells, B cells, T cells, smooth muscle cells, and umbilical endothelial cells (Matloubian *et al.*, 2000; Shimaoka *et al.*, 2000; Wilbanks *et al.*, 2001; Hofnagel *et al.*, 2002).

CXCL16 binds to the unique receptor CXCR6, which is expressed on a subset of type 1 polarized peripheral blood CD4⁺ and CD8⁺ T cells, $\gamma\delta$ T cells, natural killer T cells, natural killer cells, monocytes, and B cells (Unutmaz *et al.*, 2000; Kim *et al.*, 2001a; Thomas *et al.*, 2003; Ruth *et al.*, 2006; Huang *et al.*, 2008) and has been implicated in the pathogenesis of atherosclerosis (Minami *et al.*, 2001) and Graves' disease (Aust *et al.*, 2005). In psoriatic (Pso, Kim *et al.*, 2001a) and rheumatoid arthritis (van der Voort *et al.*, 2005), CXCR6⁺ CD4⁺ and CD8⁺ T cells accumulated in inflamed joints. Recently, the expression of CXCL16 has also been described in Pso epidermis (Oh *et al.*, 2009), but investigations on the role of CXCL16-mediated recruitment of leukocytes into Pso skin are still missing.

Psoriasis is a chronic inflammatory skin disease characterized by hyperproliferation of keratinocytes and a marked cellular infiltrate (Griffiths and Barker, 2007). T cells infiltrating Pso lesions predominately secrete a Th1/Tc1 cytokine profile, and more recent studies have identified an involvement of the Th17 pathway in psoriasis pathogenesis (Di Cesare *et al.*, 2009). In addition to skin lesions, Pso patients may develop Pso arthritis and often suffer from vascular comorbidities and diseases of the metabolic syndrome, indicating the 'systemic' nature of the disease (Griffiths and Barker, 2007).

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Abbreviations: AD, atopic dermatitis; Ca²⁺, calcium; Pso, psoriatic; TNF- α , tumor necrosis factor

Received 11 April 2011; revised 12 August 2011; accepted 4 September 2011

The importance of CD8⁺ T cells for the pathogenesis of psoriasis has long been anticipated because CD8⁺ T cells were found to be increased in the epidermis and papillary dermis of Pso skin (Paukkonen *et al.*, 1992), whereas CD4⁺ T cells were predominantly present in the dermis (Bos *et al.*, 1989). Moreover, there is a strong linkage of major histocompatibility complex class I haplotype (HLA)-Cw6 to family-linked psoriasis (Ikaheimo *et al.*, 1996), in which Pso CD8⁺ T cells have been found to clonally expand after major histocompatibility complex class I-mediated activation (Chang *et al.*, 1994). Furthermore, improvement of Pso lesions by various therapies including cyclosporine, PUVA, alefacept, and methotrexate was primarily preceded by a decrease in CD8⁺ CD45RO⁺ cutaneous T cells (Fife *et al.*, 2007). The observation that psoriasis frequently exacerbates in the immunocompromised state of HIV infection also contributed to the recognition of the importance of CD8⁺ T cells in psoriasis (Fife *et al.*, 2007). In addition, the percentage of skin-homing CLA⁺ CD8⁺ T cells in the peripheral blood of Pso patients correlated with disease severity (Sigmundsdottir *et al.*, 2001), and it is now believed that CD8⁺ T cells contribute to Pso inflammation mainly by secretion of inflammatory cytokines such as IFN- γ , TNF- α , IL-2, IL-17, IL-22, and IL-21 (Austin *et al.*, 1999; Ortega *et al.*, 2009). Therefore, it has also been elucidated that Pso CD8⁺ T cells expressed proinflammatory cytokines such as IFN- γ and TNF- α more frequently than did CD4⁺ T cells (Austin *et al.*, 1999; Friedrich *et al.*, 2000).

In contrast to CD4⁺ T cells, whose recruitment to the skin has been intensively investigated (Campbell *et al.*, 1999; Homey *et al.*, 2000; Andrew *et al.*, 2001), the migration of CD8⁺ T cells to the skin is less well characterized.

The current study was conducted to examine the importance of CXCL16 for recruitment of CD8⁺ proinflammatory T cells into the skin.

RESULTS

CXCR6 on CD8⁺ T cells in Pso donors

CD8⁺ T cells constitute an important effector cell population in Pso skin. To investigate the homing of these cells, we first studied chemokine receptor expression on T cells in the blood of patients with psoriasis. Investigating 10 different Pso donors, we found significantly more CD8⁺ CD3⁺ T cells expressing CXCR6 compared with CD4⁺ CD3⁺ T cells (Figure 1a and b; $P=0.002$, Figure 1c). This amount of CXCR6⁺ CD8⁺ T cells in Pso patients was significantly increased compared with healthy individuals ($n=8$, difference to psoriasis $P=0.02$) and patients with atopic dermatitis (AD, $n=9$, difference to psoriasis $P=0.003$). There was no significant difference detectable in the percentage of CXCR6⁺ CD4⁺ T cells among Pso, AD, and healthy individuals (Figure 1c). In contrast to the preferential expression of CXCR6 on CD8⁺ T cells, higher numbers of CD4⁺ T cells of Pso donors compared with CD8⁺ T cells expressed CCR4 (Figure 1a and b). CXCR6⁺ CD8⁺ T cells did not co-express CCR4, indicating that CCR4⁺ and CXCR6⁺ CD8⁺ T cells represent different T-cell subpopulations (Figure 1a and b). CXCR6⁺ CD8⁺ T cells also

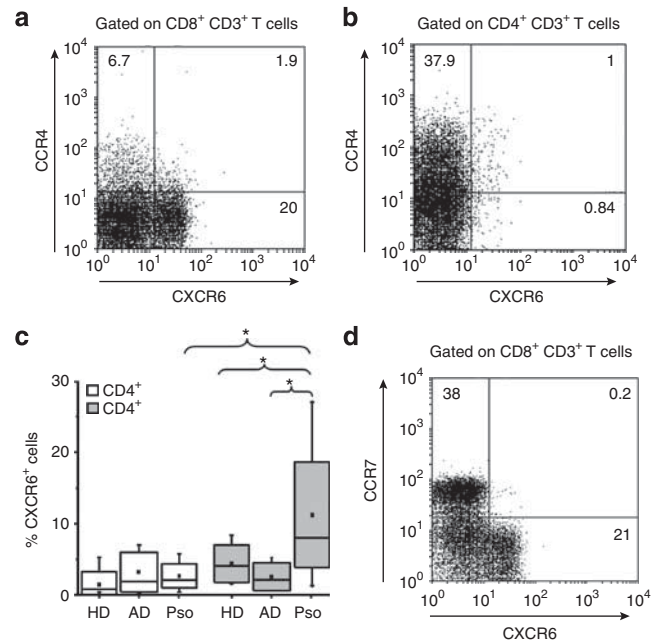


Figure 1. Detection of CXCR6 on CD8⁺ T cells in the blood of psoriatic (Pso) patients. Peripheral blood mononuclear cells of 10 Pso donors were stained for expression of CXCR6, CCR4, and CCR7 on CD8⁺ (a, d), or CD4⁺ T cells (b). The percentages of cells detected in the respective quadrants of representative stainings are given. (c) Boxplot graphic showing the mean (quadrant), median (line), SD (box), and 5th and 95th percentiles (whisker) of the percentage of CXCR6⁺ cells among CD3⁺ CD8⁺ T cells (filled gray boxes) and CD3⁺ CD4⁺ T cells (open white boxes) in the blood of eight healthy controls (HD), nine patients with atopic dermatitis (AD), and 10 Pso donors. *Indicates significance, with $P<0.05$ determined by Student's *t*-test.

lacked CCR7, marking these CXCR6⁺ CD8⁺ T cells as effector T cells (Figure 1d).

Enrichment of CXCR6⁺ CD8⁺ T cells in Pso skin

The preferential expression of CXCR6 on circulating CD8⁺ CD3⁺ T cells of Pso individuals prompted us to search for this chemokine receptor in inflamed Pso skin. First, CD8⁺ T cell lines from lesional skin and from the blood of the same patient with psoriasis were generated. Analysis of CXCR6 expression levels by real-time PCR demonstrated higher expression levels of CXCR6 on skin compared with blood-derived CD8⁺ T cells (Figure 2a). Analyzing other chemokine receptors relevant for skin-homing by real-time PCR, CD8⁺ T cells from skin and blood were found to express CXCR3 and CCR5, whereas CCR10 was not detectable. There was also expression of CCR6, but with much lower mRNA values compared with CXCR6. In summary, the highest mRNA amount was detected for CXCR6 in CD8⁺ skin-derived T cells (Figure 2a). These findings could be confirmed at the protein level by FACS staining. CXCR6 expression was found to a much higher extent on skin-derived CD8⁺ T cells (35%, MFI 117) compared with blood-derived CD8⁺ T cells (8%, MFI 59, Figure 2b and c). This indicates enrichment of CXCR6⁺ CD8⁺ T cells in the skin of Pso patients. Psoriatic T cells were cultured in parallel to AD skin-derived T cells

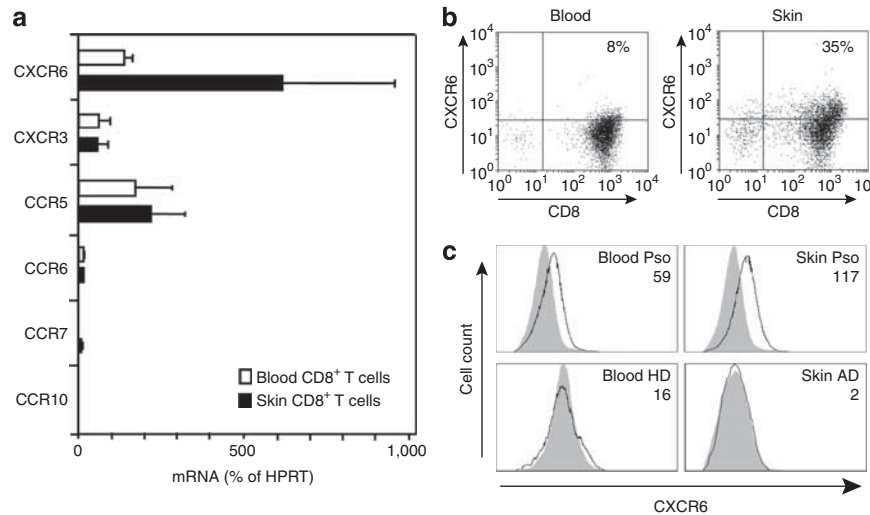


Figure 2. CXCR6 mRNA and protein in skin-derived psoriatic (Pso) CD8⁺ T cells. (a) T cell lines were generated from the blood and skin of Pso donors. Real-time RT-PCR analysis was carried out on skin- and blood-derived CD8⁺-sorted T cells for expression of chemokine receptors. The mRNA expression level of the respective receptor is given as a percentage of the mRNA of hypoxanthine phosphoribosyltransferase (HPRT). (b) Flow cytometry analysis revealed that a higher percentage of CD8⁺ T cells from skin were CXCR6⁺ compared with blood-derived T cells. The percentage of CXCR6⁺ CD8⁺ T cells is given in the right upper quadrant. The quadrant lines indicate isotype staining. (c) T cell lines from blood or skin of Pso patients, healthy donors (HD), or patients with atopic dermatitis (AD) were stained for CXCR6. Histograms show isotype (gray-filled area) and CXCR6 staining on CD8⁺ T cells (black line). The mean fluorescence intensities (MFI CXCR6 minus MFI isotype) are indicated in the upper right corner.

and to healthy peripheral blood mononuclear cell-derived T cells; however, analyses demonstrated CXCR6⁺ CD8⁺ T cells to be present almost exclusively on Pso CD8⁺ T cells (Figure 2c). Further characterization of the Pso skin-derived CD8⁺ T cells revealed expression of the integrins α E (CD103), α 4 (CD49d), CD11a, and the skin-homing selectin CLA, confirming the skin-homing phenotype of these CD8⁺ T cells (Supplementary Figure 1 online). Moreover, cytokine production upon activation marked skin- and blood-derived CD8⁺ T cells as polarized Tc1 cells (IL-2^{high}, TNF- α ^{high}, and IFN- γ ^{high}; data not shown).

The dominant expression of CXCR6 on skin-derived CD8⁺ T cells lead us to question whether CXCR6 can be found in Pso skin sections *in vivo*. By carrying out immunofluorescence stainings, we detected CD8⁺ T cells in the dermis and epidermis of Pso lesional skin. In both compartments, CD8⁺ T cells co-expressed CXCR6 (Figure 3a). Occasionally, CXCR6⁺ CD8⁺ T cells could be detected in healthy skin and AD lesions (Figure 3a). Compared with healthy skin, CXCR6⁺ CD8⁺ T cells were significantly increased in Pso dermis (29 ± 16 vs. 6 ± 2.7 cells per mm², $P=0.0001$) and Pso epidermis (9.1 ± 4 vs. 1.3 ± 1.9 cells per mm², $P=0.000005$; Figure 3b). The number of CXCR6⁺ CD8⁺ T cells in Pso lesions also exceeded those found in AD (dermis: 11.4 ± 3.3 cells per mm², $P=0.002$; epidermis: 1.5 ± 2.3 cells per mm², $P=0.00003$; Figure 3b). The percentage of CXCR6⁺ CD8⁺ T cells among all CD8⁺ T cells from Pso skin was 16% in the epidermis and dermis (Figure 3c), suggesting a similar efficiency of CXCL16-mediated recruitment of CXCR6⁺ CD8⁺ T cells to epidermis and dermis.

The CXCR6 ligand CXCL16 is expressed in Pso keratinocytes and dermal antigen-presenting cells and downregulated after successful treatment

After demonstrating enriched CXCR6⁺ CD8⁺ T cells in Pso skin, we aimed to detect the cutaneous source of its corresponding ligand CXCL16. In healthy skin, CXCL16 was found in the epidermis and in some dermal cells (Figure 4a), and, comparably, a faint staining for CXCL16 was also seen in lesional skin of patients with AD (Figure 4a). In contrast, CXCL16 was markedly upregulated in Pso skin with strong staining in the epidermis and in cells infiltrating the dermis (Figure 4a). In Pso lesions, CXCL16 was co-expressed by nearly all CD11c- and CD163-expressing cells, indicating that CD163⁺ monocytes/macrophages and CD11c⁺ dendritic cells are the main sources for CXCL16 in the dermis (Figure 4b). These strong differences in CXCL16 expression between healthy controls, AD, and Pso patients were skin specific, because serum concentrations of CXCL16 were not significantly different when comparing healthy individuals, patients with AD, and psoriasis patients (Figure 4c).

When Pso patients were treated with the soluble TNF- α -antagonist etanercept, a reduction in CXCL16 was already seen after 4 weeks of treatment and before complete resolution of the skin lesions (Figure 4d). The decrease of CXCL16 was accompanied by a significant reduction of infiltrating CXCR6⁺CD8⁺ T cells at this time point ($P=0.002$ for difference in dermal CXCR6⁺CD8⁺ T cells per mm² before treatment and at week 4; $P=0.01$ for epidermal CXCR6⁺CD8⁺ T cells per mm² during the same period). After clinical improvement of the skin lesions, when the psoriasis severity and activity index was reduced by

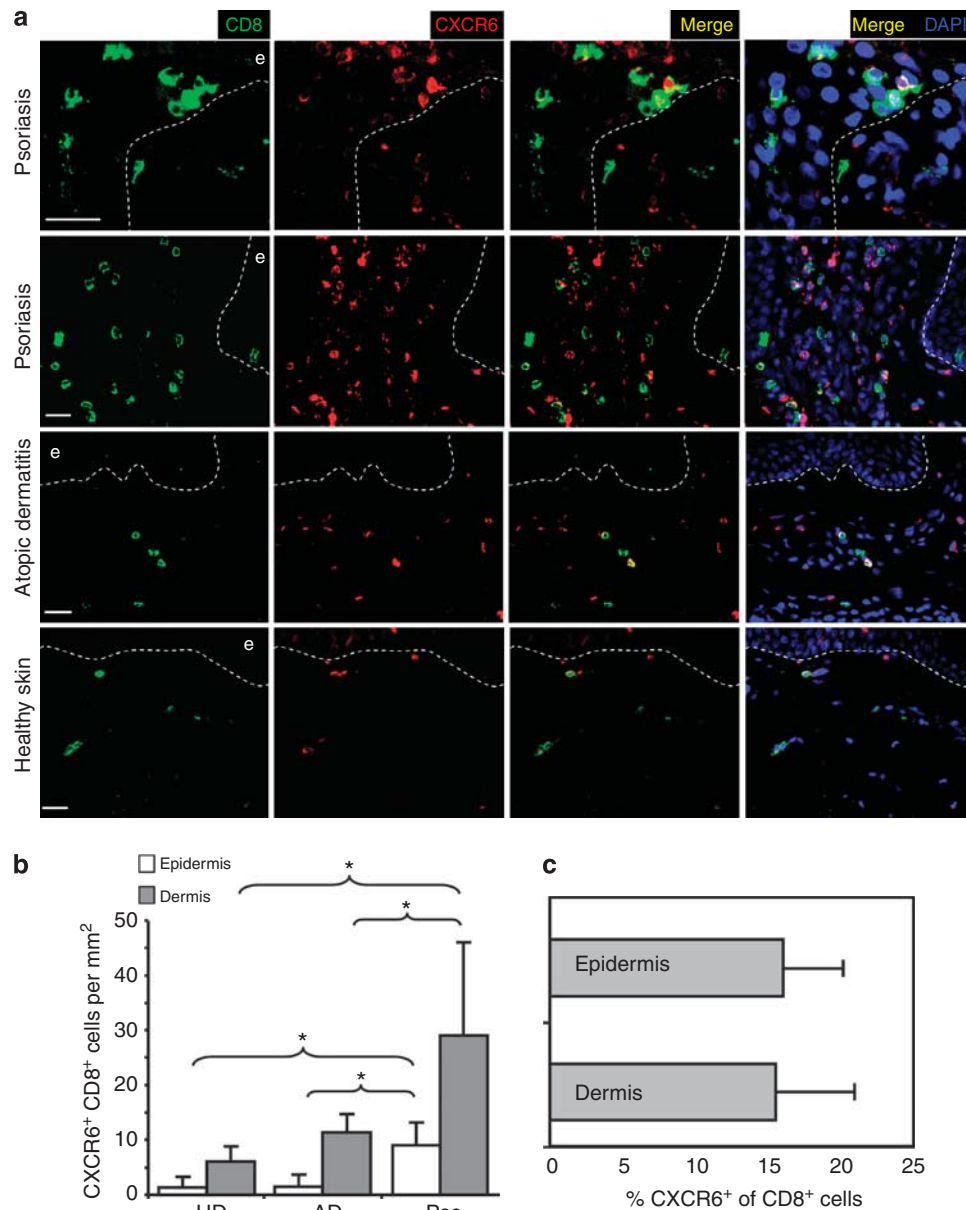


Figure 3. CXCR6 is expressed on CD8⁺ T cells in psoriatic (Pso) skin. (a) Double immunofluorescence stainings of Pso, atopic (AD), and healthy skin carried out for CXCR6 (red) and CD8 (green). Double-positive cells stand out by their yellow fluorescence in the merged figures. Nuclei are labeled by 4',6-diamidino-2-phenylindole in blue. CD8⁺ T cells infiltrated the epidermis and dermis of Pso lesions. The basement membrane zone is marked by the dotted line and the epidermis is indicated by "e". Scale bar = 25 μm. (b) Numbers of CXCR6⁺ CD8⁺ T cells per mm² were determined by manual counting in 16 Pso skin sections from eight patients, 12 sections from six patients with AD, and 11 healthy skin sections from six individuals. *Indicates significance with $P < 0.05$, Student's *t*-test. Bars indicate mean $\pm$ SD. (c) Double immunofluorescence stainings of CD8 and CXCR6 in skin sections from eight Pso patients were analyzed for the percentage of CD8⁺ T cells expressing CXCR6. Bars indicate mean $\pm$ SD.

80–90% at week 24, staining for CXCL16 was comparable to that of healthy skin. Moreover, at this time point, CXCR6⁺CD8⁺ T cells per mm² were reduced to the levels found in healthy skin, also demonstrating a significant difference compared with 4-week treatment ($P = 0.0001$ for difference in dermal CXCR6⁺CD8⁺ T cells per mm² between weeks 4 and 24; Figure 4d). These findings suggest a relevant contribution of CXCL16 in the pathogenesis of psoriasis and in particular for the recruitment of CXCR6⁺ CD8⁺ T cells to the skin (Figure 4d).

Migration of CXCR6⁺ CD8⁺ skin-derived Pso T cells in response to CXCL16

To investigate the functional relevance of CXCL16 and CXCR6 for Pso skin inflammation, we first evaluated chemokine-induced calcium (Ca^{2+}) influx in skin-derived CD8⁺ T cells. Expression of CXCR3 ligands was described in Pso lesions (Rottman *et al.*, 2001) and we could confirm these findings (data not shown). Moreover, further analyses indicated *in vivo* relevance of CXCR3 and CXCR3 ligands in psoriasis (Rottman *et al.*, 2001). Consequently, side-by-side

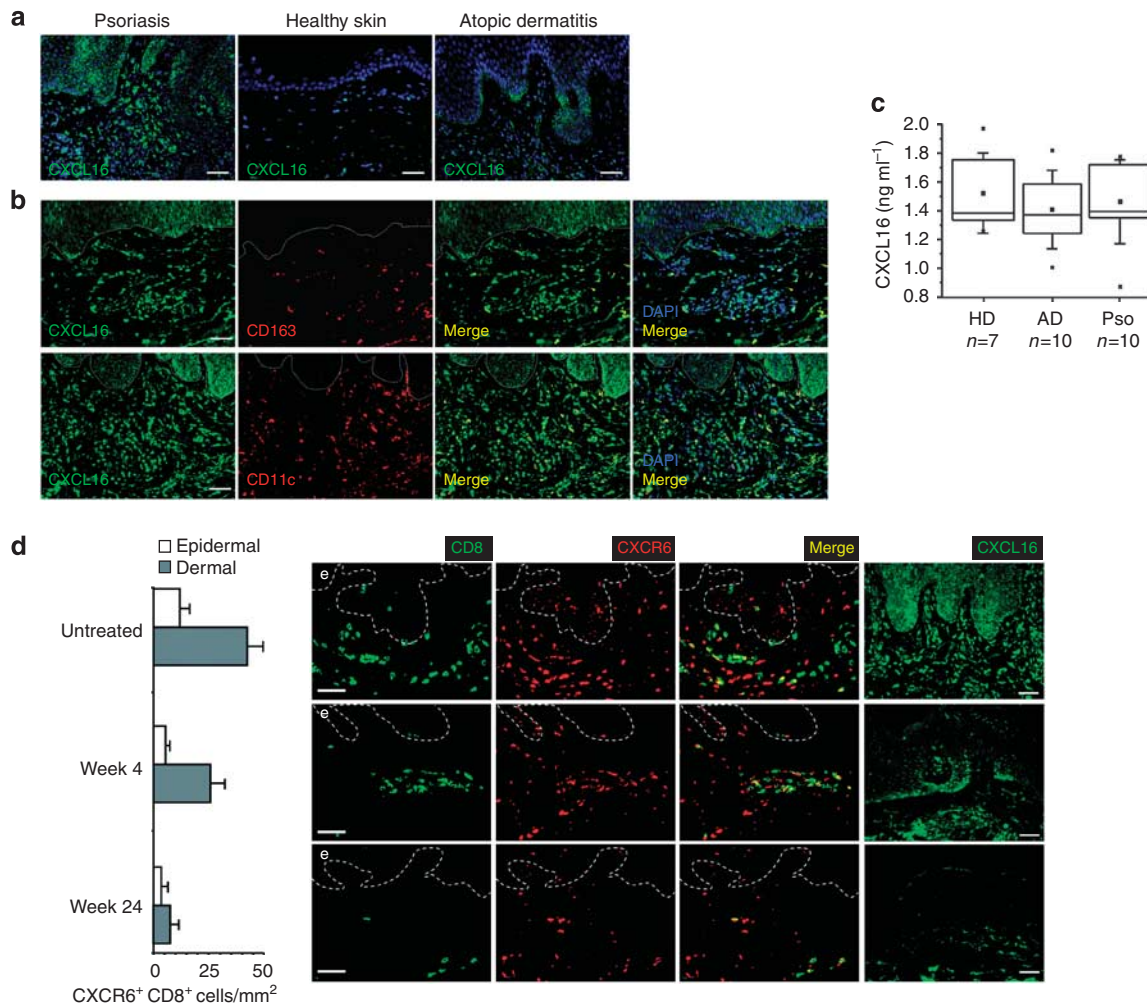


Figure 4. CXCL16 is expressed by keratinocytes, dendritic cells, and monocytes in psoriatic (Pso) skin. (a) Immunofluorescence staining for CXCL16 (green) was carried out on paraffin-embedded skin of Pso, healthy, and atopic dermatitis (AD) skin. Positive staining was stronger in the epidermis and dermis of Pso skin ($n=8$) compared with healthy skin ($n=3$) and skin from patients with AD ($n=5$). Representative stainings are shown. Scale bar = 50 μ m. (b) Double immunofluorescence staining was carried out investigating co-expression (yellow) of CXCL16 (green) and CD11c (red, upper panel), or CD163 (red, lower panel). Scale bar = 50 μ m. (c) The concentration of CXCL16 was determined by ELISA in the sera of healthy donors (HD; $n=6$), patients with AD ($n=7$), and patients with Pso ($n=10$). Boxplots indicate mean, median, 25th and 75th percentiles (box), and SD (whisker). CXCL16 levels of the three groups were not significantly different. (d) Three patients with chronic plaque-type psoriasis were treated for 24 weeks with etanercept. Lesional biopsies before treatment and at weeks 4 and 24 were stained for the expression of CXCL16 (green) or CXCR6 (red), and CD8 (green). Improvement of psoriasis severity and activity index by 80–90% was achieved in all patients after 24 weeks. Representative stainings of CXCL16 of one patient are shown. The basement membrane zone is marked by the dotted line and the epidermis is indicated by "e". The means $\pm$ SD of CXCR6⁺ CD8⁺ T cells per mm² skin of three patients are indicated ($P=0.002$ for difference of dermal CXCR6⁺ CD8⁺ T cells per mm² before treatment and at week 4; $P=0.01$ for epidermal CXCR6⁺ CD8⁺ T cells per mm² in the same period; $P=0.0001$ for difference of dermal CXCR6⁺ CD8⁺ T cells per mm² between week 4 and week 24; Student's *t*-test).

analyses were carried out, which showed that CXCL16 induced profound and fast Ca²⁺ influx comparable to the responses to the CXCR3 ligand CXCL10, indicating functional CXCR6 activation (Figure 5a).

Compared with CXCL16, CCL4, a ligand of the receptor CCR5, induced a less pronounced Ca²⁺ influx, and, interestingly, CCL20 and CCL27 induced no Ca²⁺ influx in skin-derived CD8⁺ T cells (Figure 5a). This was consistent with the low detection of CCR6 mRNA and the failure to detect CCR10 mRNA on CD8⁺ T cells derived from Pso skin (Figure 2a).

The response pattern of the Ca²⁺ flux assays was confirmed by analyzing the chemokine-induced *in vitro*

migration of skin-derived Pso CD8⁺ T cells (Figure 5b). CXCL16 induced a strong and dose-dependent migration of these CD8⁺ T cells. Less potently, migration was also induced by the CXCR3 ligand CXCL9. In line with the Ca²⁺ flux data, ligation of CCR5 by CCL5 induced a significant migratory response, whereas the migration induced by CCL20 was very weak, and CCL27 did not induce chemotaxis in CD8⁺ T cells.

Cutaneous recruitment of blood-derived CD8⁺ T cells by CXCL16 *in vivo*

After demonstrating that Pso skin-derived CD8⁺ T cells are responsive to CXCL16, the potency of CXCL16 to induce

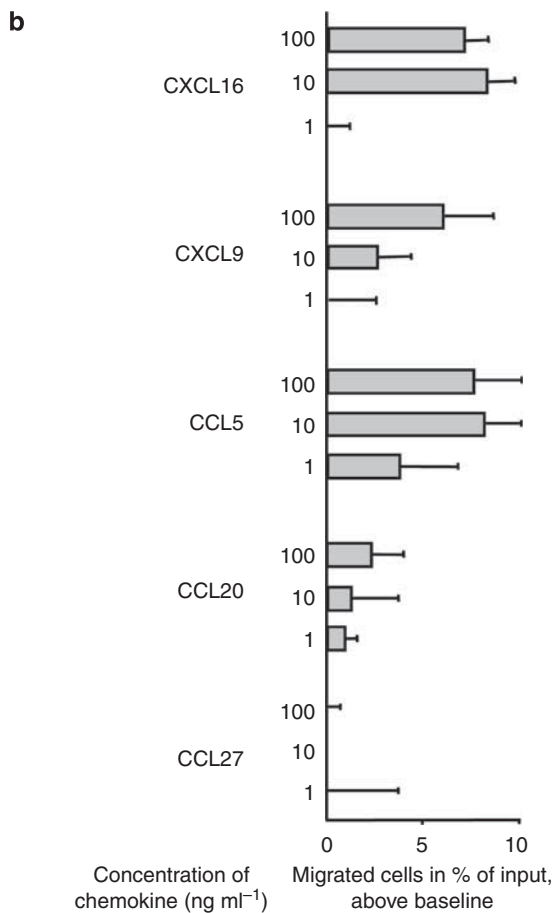
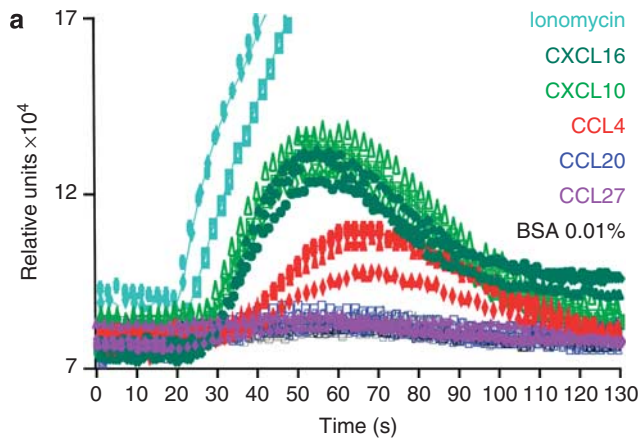


Figure 5. CXCL16 induced calcium (Ca²⁺) influx and migration in CXCR6⁺ CD8⁺ T cells derived from psoriatic (Pso) skin. (a) Ca²⁺ flux was detected over time after challenging T cells with the respective chemokine receptor ligands using a Flex station. Ionomycin was used as a positive control. Colors indicate triplicates. Fluorescence induced by Ca²⁺ mobilization is indicated by an increase of the units on the y-axis. (b) *In vitro* migration of skin-derived Pso CD8⁺ T cells is indicated by demonstrating the percentage of migrated cells of input cells for each concentration of the respective chemokine. The migration to medium (= baseline) was subtracted. Analysis was performed in quadruplicates and each bar represents the mean $\pm$ SD. One of three similar experiments is shown.

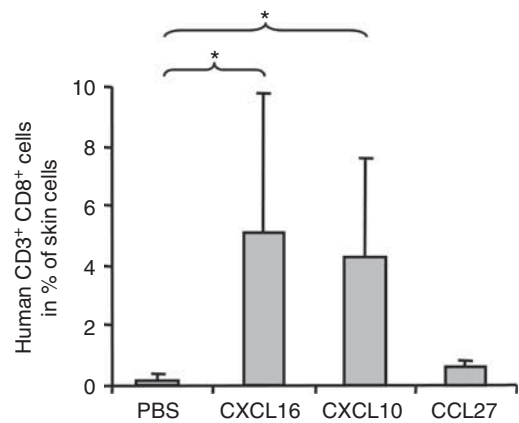


Figure 6. CXCL16 induced T cell recruitment to the skin *in vivo*.

SCID mice, transplanted with two pieces of human skin each, were reconstituted (i.p.) with peripheral blood mononuclear cells on experimental day 0. Phosphate-buffered saline (PBS) or the respective chemokine was injected intracutaneously into human skin grafts on days 1, 2, 4, and 6. For each chemokine, four, or (for PBS) eight, skin pieces were injected and analyzed. Human skin grafts were harvested on day 8 and single-cell suspensions were stained for human CD3 and CD8 T-cell antigens. The amount of CD3⁺ CD8⁺ T cells recruited by the respective chemokine or PBS is given with mean $\pm$ SD (* P =0.05 for CXCL10 vs. PBS, P =0.009 for CXCL16 versus PBS, determined by Student's *t*-test). One experiment out of two with similar results is shown.

migration of CD8⁺ T cells from the circulation into the skin was investigated *in vivo*, using the previously described SCID-hu skin mouse model (Biedermann *et al.*, 2002). SCID-hu skin mice were reconstituted with peripheral blood mononuclear cells intraperitoneally. T-cell recruitment into skin grafts was induced by injection of chemokines or phosphate-buffered saline (PBS) to the grafts. Analyzing explanted skin grafts at day 8 for recruited T cells by flow cytometry demonstrated a marked recruitment of blood-derived CD8⁺ T cells in response to CXCL16, which was significantly higher compared with PBS-injected grafts (P =0.009; Figure 6). In addition to CXCL16, CXCL10 also recruited circulating CD8⁺ T cells into the skin (P =0.05; Figure 6). This is in line with previous studies demonstrating that CD8⁺ T cells in the blood of healthy and Pso individuals express the receptor of CXCL10, namely, CXCR3 (Teraki *et al.*, 2004). In contrast to this recruitment, CCL27 failed to generate a significant migration of CD8⁺ T cells to the skin, confirming our *in vitro* analyses on mRNA expression, negative Ca²⁺ flux, and *in vitro* lack of migratory response of Pso CD8⁺ T cells to CCL27 (Figure 6).

DISCUSSION

The data of this study show that CXCL16 is upregulated in Pso lesional skin and attracts CD8⁺ T cells *in vitro* and *in vivo* into human skin. Consequently, increased numbers of CXCR6⁺ CD8⁺ T cells were found in the epidermis and dermis of lesional Pso skin. Notably, as we could not detect CXCR6⁺ CD8⁺ T cells in this range in healthy individuals and in patients with AD, these data indicate specificity for

psoriasis. These findings broaden our understanding on how recruitment of CD8⁺ T cells to the skin could be achieved.

The importance of CD8⁺ T cells for Pso inflammation has long been recognized and is based on their cutaneous infiltration associated with Pso exacerbation (Baker *et al.*, 1988; Gudjonsson *et al.*, 2004; Vissers *et al.*, 2004). Recent functional characterization of the CD8⁺ T-cell population in psoriasis highlighted their relevant Th1/17/Tc1 cytokine production for the development of the disease (Kryczek *et al.*, 2008; Ortega *et al.*, 2009). Thus, understanding the recruitment of CD8⁺ T cells to the skin might elucidate new targets for intervention in Pso inflammation.

Compared with the strong CXCL16 staining in psoriasis, staining of CXCL16 was weaker in healthy skin and in AD (Figure 4) (Scholz *et al.*, 2007). Constitutive low-level expression of CXCL16 may function as a very early signal for recruitment of CD8⁺ T cells in the initiation phase of psoriasis. This hypothesis is supported by our data demonstrating a sparse infiltrate of CXCR6⁺ CD8⁺ T cells already in healthy skin and a previous study demonstrating accumulation of CD8⁺ effector T cells already in the early phase of the Pso inflammation in the skin (Vissers *et al.*, 2004).

Membrane-bound CXCL16 has been proposed to mediate not only migration but also adhesion of CXCR6⁺ cells to CXCL16⁺ cells (Shimaoka *et al.*, 2004). CXCL16-mediated adhesion may support a prolonged interaction between antigen-presenting cells and CD8⁺ T cells, ensuring T-cell activation and further amplification of cutaneous inflammation.

In addition, CXCL16 has been reported to mediate adhesion and phagocytosis of both Gram-negative and Gram-positive bacteria (Shimaoka *et al.*, 2003). Upregulation of CXCL16 may therefore contribute to the upregulated innate bacterial host defense mechanisms in Pso skin, which has been proposed in numerous studies (Schroder and Harder, 2006; Buchau and Gallo, 2007).

In addition to its importance for the pathogenesis of cutaneous Pso inflammation, CXCL16 might also contribute to systemic manifestations of psoriasis. Patients often suffer not only from inflammatory skin lesions but also from associated arthritis, atherosclerosis, and cardiovascular disease (Griffiths and Barker, 2007). With regard to CXCL16, it is noteworthy that it has been shown to mediate attraction of T cells to inflamed joints of arthritis patients as well (Nanki *et al.*, 2005; van der Voort *et al.*, 2005; Ruth *et al.*, 2006). Inhibition of CXCL16 with a monoclonal antibody reduced the clinical arthritis score in the collagen-induced arthritis mouse model (Nanki *et al.*, 2005). Furthermore, membrane-bound CXCL16 has initially been described as the scavenger receptor SR-PSOX for oxidized low-density lipoprotein (Kim *et al.*, 2001b). CXCL16 might therefore have a role in the transformation of CXCL16⁺ macrophages into cholesterol-loaded foam cells and the subsequent development of atherosclerosis associated with psoriasis.

In conclusion, our data suggest that CXCL16–CXCR6 interactions are an effective mechanism for CD8⁺ T effector cell recruitment into Pso skin, and extend our understanding of the inflammatory processes in psoriasis.

MATERIALS AND METHODS

Patients

Diagnosis of psoriasis and AD was confirmed by clinical and histological criteria. Patients and healthy controls did not receive systemic or topical treatment at the time point they were included in the study, and the lesions did not show signs of superinfection. The investigational protocol including written, informed patient consent was approved by the Ethics Committee of the University hospital of the Technical University Dresden and of the University of Tübingen in adherence to the Declaration of Helsinki Principles.

Purification and culture of cells and generation of T cell lines from Pso skin

Peripheral blood mononuclear cells from human blood were prepared by density gradient separation using Ficoll-Paque Plus (Amersham Biosciences, GE Healthcare Europe GmbH, Freiburg, Germany). The skin-derived T cell lines from Pso patients were generated from lesional punch biopsies, as previously described (Carballido *et al.*, 1997). T cell lines were maintained by stimulation with phytohaemagglutinin and irradiated allogenic peripheral blood mononuclear cells every 2 weeks. After staining with CD8- and CD3-specific antibodies conjugated to Dynabeads (Invitrogen Life Technologies, Darmstadt, Germany), CD8⁺ T cell lines were isolated by magnetic cell sorting and were cultured as pure CD8⁺ T cell lines.

Flow cytometry (FACS)

For FACS staining the following monoclonal antibodies were used: CD3-Pe, CD3-Percp, CCR4-Pe, CLA-Fitc (BD Pharmingen, Heidelberg, Germany), CCR4-Fitc, CD8-Pe, CD3-APC (Caltag Laboratories, Hamburg, Germany), CCR7-Fitc (R&D Systems, Minneapolis, MN), and CXCR6-AF647 (BioLegend, Uithoorn, the Netherlands). Analysis was carried out on a FACSCalibur (Becton Dickinson, Heidelberg, Germany), using BD CELLQuest- (Becton Dickinson), WinMDI- (Joseph Trotter, Scripps Research Institute, La Jolla, CA), or WEASEL-software (Walter and Eliza Hall Institute, Parkville, Australia).

Immunofluorescence analysis

Antigen retrieval of paraffin-embedded sections was carried out at pH 7.0. Antibodies and reagents applied for immunofluorescence staining are listed in Supplementary Table S1 online. Slides were mounted with a 4',6-diamidino-2-phenylindole-containing medium (Vector Laboratories, Peterborough, UK) staining DNA, coverslipped, and analyzed with a Zeiss Axioimager A1 microscope (Zeiss, Jena, Germany).

For calculation of the percentage of CXCR6⁺ cells among CD8⁺ T cells in the epidermis and dermis, double-stained skin sections from five different Pso patients and six healthy controls (non-UV-irradiated skin left over from plastic surgery) were analyzed by manual counting of each complete slide on a Zeiss Axioimager A1 microscope (Zeiss). The area (mm²) of epidermis and dermis was determined in photographed sections using Image J software.

ELISA

Serum concentrations of CXCL16 were determined using DuoSet ELISA assays (R&D Systems), according to the manufacturer's instructions.

RT-PCR and quantitative real-time RT-PCR analysis

Extraction of mRNA from CD8⁺ T cells was carried out using a SNAP KIT (Invitrogen Life Technologies), according to the manufacturer's instructions. Primers for PCR analyses were custom ordered from Invitrogen Life Technologies (Supplementary Table S2 online). The primers were designed to match the requirements for the TaqMan Real-Time PCR system on an ABI7700 sequence detection system (Applied Biosystems, Carlsbad, CA) using Primer-Express Software. PCR was carried out as described previously (Biedermann et al., 2002). Concentrations of chemokines were expressed as a percentage of hypoxanthine phosphoribosyltransferase using the ΔC_T method (sequence detector 3.1; Applied Biosystems) on the basis of threshold cycle differences.

Analysis of intracellular Ca²⁺ mobilization

Cells (5×10^6 per ml) were loaded with Fluo-4/acetoxymethyl ester (60 minutes at 37 °C using 0.02% Pluronic in DMSO, 2 μ M Fluo-4 in HBSS, Molecular probes, Eugene, OR). Thereafter, cells were adjusted to 8×10^6 cells per ml, plated (50 μ l per well) in 96-well Costar Viewplates (Corning Costar, Kaiserslautern, Germany), and incubated for 20 minutes at room temperature for de-esterification of intracellular AM esters. All chemokines (R&D systems) and the positive control ionomycin were reconstituted in PBS and used at 300 ng ml⁻¹. Chemokine-induced Ca²⁺ mobilization corresponded to the fluorescence intensity and was quantified after injection of chemokines by fluorescence reading on a FLEXstation reader (Molecular Devices, Munich, Germany) and analyzed using SOFT-Max Pro software (Molecular Devices).

Transwell migration assay

T cells (5×10^6 per ml; 0.1 ml per well) were plated into the top chamber of Transwell plates (Costar; 24-well plates; 6.5 mm diameter, 5.0 μ m pore size) and medium (RPMI, 0.5% BSA, Novartis Institutes for Biomedical Research, Vienna, Austria), with or without indicated concentrations of chemokines (in ng ml⁻¹; R&D Systems), was added to the lower chamber of the transwells. Plates were left at 37 °C for 3 hours, and transwell migration was assessed by FACS acquisition thereafter. Transwell migration is expressed as the percentage of input cells minus the percentage of cells migrating to the medium alone (baseline).

Transplantation of human skin on SCID mice and recruitment of human T cells to skin grafts of SCID-hu skin mice

Transplantation of skin onto SCID mice (SCID-hu skin mice) and recruitment experiments were conducted, as described (Biedermann et al., 2002). Briefly, SCID mice were transplanted with two pieces of human adult skin on their back, replacing ~ 0.8 mm² of mouse skin each. Human skin pieces were obtained from the West Hungarian Tissue Bank, Petz Aladár County Hospital (Győr, Hungary), according to the institutional and governmental ethical guidelines. After 6–8 weeks, when human skin pieces resembled normal human skin, SCID-hu skin mice were reconstituted (i.p.) with 1.5×10^8 peripheral blood mononuclear cells (experimental day 0). PBS or the respective chemokine (R&D Systems) was injected intracutaneously (300 ng in 30 μ l) into human skin grafts at days 1, 2, 4, and 6. For each chemokine, four, or (for PBS) eight, skin pieces were injected. Human skin grafts were harvested on day 8, processed into single-cell suspensions using a mechanical tissue disaggregator (Medimachine;

DakoCytomation, Hamburg, Germany), and analyzed by flow cytometry. Skin-homing was expressed as the percentage of double-positive CD8⁺ CD3⁺ cells present in the mononuclear living cell fraction, which was determined using forward and side scatter and propidium iodide-negative gates. The *in vivo* experiments were conducted according to "Tierversuchsbeschreibung Nr. 167/C-3", approved by Wiener Landesregierung MA58.

CONFLICT OF INTEREST

NC-P and JMC are employees of Novartis Institutes for Biomedical Research and engaged in drug development activities. CG and TB are former employees of Novartis Institutes for Biomedical Research, Vienna, engaged in drug discovery activities, and have currently no conflict of interest. SK states no conflict of interest.

ACKNOWLEDGMENTS

We acknowledge the excellent technical assistance of Sonja Hinteregger, Julia Kund, Sandra Fassl (Novartis Institutes for Biomedical Research, Vienna, Austria), and Nick Zimmermann (Department of Dermatology, University Hospital Carl Gustav Carus, Technical University Dresden, Germany). This work was supported by SFB 685 grant (SK and TB), DFG grant BI 696/5-1 (TB) and MEDDRIVE, and a habilitation grant from the medical faculty of the Technical University of Dresden (CG).

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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Supplementary material JID-2011-0283.R1

Table S1 Antibodies used for immunofluorescence staining

antigen	manufacturer	clone	isotype	dilution	amplification/detection	
CD1a	DakoCytomation	O10	m IgG1, κ	1:50	goat a-mouse IgG1	Alexa Fluor 488 Molecular Probes
CD3	abcam	SP7	rabbit IgG	1:300	goat F(ab') ₂ a-rabbit IgG	Alexa Fluor 488 Molecular Probes
CD11c	abcam	EP1347Y	rabbit IgG	1:50	goat F(ab') ₂ a-rabbit IgG	Alexa Fluor 488 Molecular Probes
CD163	Novocastra	10D6	m IgG1	1:50	goat a-mouse IgG1	Alexa Fluor 488 Molecular Probes
CXCL16	R&D Systems	polyclonal	goat IgG	1:5	donkey a-goat IgG (H+L)	Alexa Fluor 488 Molecular Probes
CD8	DakoCytomation	C8/144B	m IgG1	1:50	goat a-mouse IgG1	Alexa Fluor 488 Molecular Probes
CXCR6	abcam	polyclonal	rabbit IgG	1:25	goat F(ab') ₂ a-rabbit IgG	Alexa Fluor 546 Molecular Probes
rabbit IgG	abcam			1:100		
normal goat serum	Santa Cruz			1:100		
normal mouse serum	DakoCytomation			1:500		

Table S2 Primer and probes for quantitative real-time RT-PCR analysis

primer	sense	antisense	Probe Tamra/FAM (Eurogentech, Seraing, Belgium)
CXCR3 (151bp)	GGT GGA CAT CCT CAT GGA CC	CCG GAA CTT GAC CCC TAC AA	TTG GCC CGC AAC TGT GGC C
CCR6 (129 bp)	AGT CAT GCC ACT GGT GCG T	AAT GGC GAT GTA CCG GTC C	CAG CAA TGC CAC GTG CAA CTT GC
CXCR6 (150 bp)	CCA GAT GCC CTT CAA CCT CA	CAG GCT GAC AAA GGC ATA GAG C	TCG CAT ACC TGA GGG CCT GCC TT
CCR10 (68 bp)	GGG ACG GAG GCC ACA GA	TCA GGG AGA CAC TGG GTT GG	TGA GCC ACT GCC GGA GCT TTG C
CCR5 (151bp)	ATG TGA AGC AAA TCG CAG CC	ATG GCC AGG TTG AGC AGG TA	TGG GCA ACA TGC TGG TCA TCC TGA T
CCR7 (15 bp)	CTT CCT CCT GAG CCT TCC CT	CAC GTA GCG GTC AAT GCT GA	CGC GGC CAA GTG CTG GGT CTT
			Probe Tamra/Vic (Applied Biosystems)
Hypoxanthine-phosphoribosyl-transferase (HPRT, 151 bp)	AGA CTT TGC TTT CCT TGG TCA GG	TCA AGG GCA TAT CCT ACA ACA AAC	CAA GCT TGC TGG TGA AAA GGA CCC C

Fig. S1 Skin derived CD8⁺ T cells were analyzed for surface expression of adhesion molecules by flow cytometry. Histograms show staining of adhesion markers in green. The respective isotypes are shown by dashed lines.

