

Expression of the chemokine receptor CCR5 in psoriasis and results of a randomized placebo controlled trial with a CCR5 inhibitor

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Abstract Several reports have indicated that the chemokine receptor CCR5 and its ligands, especially CCL5 (formerly known as RANTES), may play a role in the pathogenesis of psoriasis. The purpose of this investigation was to examine the expression of CCR5 and its ligands in chronic plaque psoriasis and to evaluate the clinical and immunohistochemical effect of a CCR5 receptor inhibitor. Immunohistochemical analysis showed low but significant increased total numbers of CCR5 positive cells in epidermis and dermis of lesional skin in comparison to non-lesional skin. However, relative expression of CCR5 proportional to the cells observed revealed that the difference between lesional and non-lesional skin was only statistically significant in the epidermis for CD3 positive cells and in the dermis for CD68 positive cells. Quantification of mRNA by reverse transcriptase-polymerase chain reaction

only showed an increased expression of CCL5 (RANTES) in lesional skin. A randomized placebo-controlled clinical trial in 32 psoriasis patients revealed no significant clinical effect and no changes at the immunohistochemical level comparing patients treated with placebo or a CCR5 inhibitor SCH351125. We conclude that although CCR5 expression is increased in psoriatic lesions, this receptor does not play a crucial role in the pathogenesis of psoriasis.

Keywords Psoriasis · CCR5 · Chemokine inhibitor

Introduction

Psoriasis is a chronic skin disease affecting approximately 2–3% of the population worldwide. Despite its common occurrence, the exact pathogenesis of psoriasis remains unclear and adaptations to the pathogenesis of this inflammatory disease are continuing [4, 9, 31, 35, 40, 52]. Although the specific effector cells responsible for the inflammatory process in psoriasis are not known, reported beneficial effects of specific T cell targeted therapies, such as cyclosporine A, DAB389IL-2 and alefacept support a central role for T cells in the pathogenesis of psoriasis [3, 5, 10, 13, 15, 24, 25]. Further investigation on the immunophenotype and cytokine secretion patterns of T cells have indicated that specifically Th1-cells are involved in psoriasis [2, 32, 46].

In the many aspects encompassing T-cell homeostasis, the trafficking of T cells from blood to tissues is thought to be relevant in chronic inflammatory diseases such as psoriasis. Key factors in this migration are chemo-attractant cytokine molecules known as chemokines and their receptors [30, 33, 37, 38, 57, 61, 62]. The predominant chemokine receptors expressed on Th1-cells are CCR5 and

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CXCR3 [7, 9, 29, 30, 33, 34, 47, 50]. Besides its preferential expression on Th1 cells, CCR5 is also expressed on monocytes, macrophages, natural killer and dendritic cells: all thought to be significant elements in the pathogenesis of psoriasis [8, 11, 12, 18, 26, 27, 36, 39, 41, 43, 54].

The ligands of CCR5 [CCL3, CCL4 and CCL5 (formerly known as MIP1 α , MIP1 β and RANTES, respectively)] are highly expressed by keratinocytes in psoriatic tissue [19, 22, 42, 48]. Furthermore, it has been demonstrated that the proinflammatory cytokines IFN- γ and TNF- α can induce the expression of these chemokines [19, 22] and that treatment of psoriasis resulted in a significant decrease of CCL5, as well as a reduction of CCR5⁺ T cells in the skin [19, 20, 58].

Several animal models resembling psoriasis have been developed, yet, none of these models imitates psoriasis completely, hence limiting their utility [51]. Investigations by Mack et al. [36] showed a different expression pattern of CCR5 in mice and humans. Additionally, different expression of a single amino acid in the CCR5 molecule between rhesus macaques and humans resulted in a different response to inhibitors of the receptor in the species [6]. Therefore, research on the expression of CCR5 in psoriasis, as well as clinical efficacy of a CCR5 inhibitor, is limited to humans. So far, the data available on the expression of CCR5 in psoriatic skin in humans are not univocal (varying from high to minimal) and were obtained with divergent methods in investigations in which CCR5 was never the main focus [20, 27, 49, 58, 59].

The primary purpose of this study was to determine the expression of CCR5 and its ligands in chronic plaque psoriasis *in situ* compared to non-lesional skin, through analysis by immunohistochemistry and quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). In order to examine the possibility that CCR5 plays a functional role in the pathogenesis of psoriasis, we also analyzed clinical and immunohistochemical data obtained from lesional and non-lesional skin biopsies of psoriasis patients before and after treatment with a CCR5 inhibitor.

Materials and methods

Study design and patients

Lesional and non-lesional skin biopsies were obtained from nine patients with moderate to severe chronic plaque psoriasis, defined by the psoriasis area severity index (PASI) \geq 8. These skin biopsies were analyzed by manual quantification of immunohistochemical double-staining and quantitative RT-PCR. In order to get insight in the possibility of a functional role of CCR5 in the pathogenesis of psoriasis, 34 patients, including the previous 9 patients, participated in an

8 week, randomized, placebo-controlled, parallel group, multi-centre, double-blind clinical trial in which patients received either 50 mg twice daily of the CCR5 inhibitor SCH51125 (23 patients) or matched placebo (11 patients) orally for 28 days, followed by a follow-up period of 4 weeks. During the follow-up period patients were only allowed to use emollients as treatment and on day 56 vital signs, PASI and blood were assessed. Patients were included at the dermatology outpatient departments of four academic hospitals from April 2004 to December 2004. At baseline and the last day of treatment (day 28), lesional biopsies were taken to evaluate the immunohistochemical effect of the CCR5 inhibitor. For this immunohistochemical evaluation, single-stained sections were analyzed with digital image analysis, semi-quantitative analysis (SQA) and confocal scanning microscopy, and double-stained sections on baseline and day 28 were analyzed by manual quantification. To evaluate the clinical effect of the CCR5 inhibitor the PASI was assessed at baseline, day 28 and day 56.

In all patients, psoriasis was diagnosed at least 12 months prior to enrolment and patients were not allowed to use systemic psoriasis treatment or phototherapy within 4 weeks of study entry. Only emollients were allowed as topical treatment. All other topical anti-psoriasis therapy (e.g. corticosteroids, vitamin D derivatives, etc.) had to be stopped 2 weeks before study entry. The protocol was reviewed and approved by the medical ethical committees of all participating centres and all patients gave their written informed consent before enrolment. The study was conducted according to the Declaration of Helsinki principles and is registered at the ISRCT register (<http://www.controlled-trials.com/ISRCT14986467>).

Biopsies

Four-millimeter biopsies were taken from the inside border of a target psoriatic plaque, preferentially from a non-sun-exposed area. Lesional biopsies from each patient were obtained from the same target lesion, separated by at least 1 cm. The biopsy samples were randomly coded, snap-frozen in Tissue-Tek OCT compound (Sakura Finetek Europe, Zoeterwoude, The Netherlands) by immersion in liquid nitrogen and stored at -80°C until processing. Five-micrometer cryostat sections were cut and mounted on glass slides (Star Frost adhesive slides, Knittelgläser, Braunschweig, Germany), before being stored at -80°C until immunohistochemical staining. For each staining three sections of each biopsy were analyzed to minimize random variation.

Immunohistochemistry

After fixation of the slides, the endogenous peroxide activity was inhibited with 0.1% sodium azide and 0.3%

hydrogen peroxide in Tris-buffered saline (TBS), before incubating for 15 min with 10% normal goat serum in TBS. Next, the sections were incubated for 1 h at room temperature (overnight at 4°C in the case of CCR5) with the primary antibody in 1% bovine serum albumin (BSA; Sigma-Chemical Co, St. Louis, MO, USA) in TBS. The following mouse anti-human monoclonal antibodies were used: FITC-conjugated anti-CD3 (BD Pharmingen, San Jose, CA, USA), FITC-conjugated-anti CD68 (clone EBM11; Dako), anti-human neutrophil elastase (Dako, Glostrup, Denmark), anti-cytokeratin 8.12 (keratin 16; Sigma, Saint Louis, MO, USA), anti-CD161 (NK-T cells; BD Pharmingen) and anti-CCR5 (CD195; BD Pharmingen). After rinsing with TBS, sections were further incubated with biotin-conjugated goat anti-mouse antibody (Dako) or, in case of CD3 and CD68 staining, with rabbit anti-FITC (Dako) in 10% normal human serum (NHS) in TBS for 30 min. Following a wash step with TBS, sections were subsequently incubated with horseradish peroxidase (HRP)-conjugated streptavidin (Dako) or, in case of CD3 staining, with HRP-conjugated goat anti-rabbit antibody (Dako), in 1% BSA in TBS for 30 min. In case of CCR5 staining an amplification step was performed with the TSA biotin system (Perkin Elmer, Boston, MA, USA). Sections were counterstained with Mayer's haematoxylin (Merck, Darmstadt, Germany) and mounted in Kaiser's glycerol gelatine (Merck). Skin sections were double stained with anti-CCR5 together with anti-CD3 or macrophage marker anti-CD68. The double stained sections were manually counted by two independent observers blinded for order, patient and clinical data. Using a 0.5×0.5 mm ocular grid and at $200\times$ magnification, single red (CCR5⁺), single blue (CD3⁺ or CD68⁺), and purple double-positive cells were counted in the entire section. The epidermal and dermal regions were separately counted. The results are expressed as the number of double-positive cells/mm².

RNA analysis

RNA was extracted from frozen skin biopsies using the RNeasy mini kit (Qiagen). RNA quantity was assessed by OD at 260 nm and RNA quality was analyzed by measuring the ratio of 28s and 18s rRNA using the Agilent 2100 bioanalyzer.

Quantitative PCR

Taqman primers and probes were designed with Primer Express software (ABI), and purchased from ABI. The sequences of the human primers and probes are available upon request. For the human skin tissue, quantitative PCR was carried out with an ABI Prism 7900HT

Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The PCR reactions were prepared using the components from the Invitrogen Platinum Quantitative RT-PCR One-Step kit and assembled according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The final concentrations of the primers and probe in the PCR reactions were 200 and 100 nM, respectively. The RT-PCR reactions for each gene were performed in a single 384-well plate. Separate plates of the same RNAs were used to quantitate 18S RNA as an internal control for RNA quality, and a primer/probe set for the CD4 promotor was used to check the RNAs for genomic contamination. The PCR data was quantitated based on a standard curve generated using fourfold serial dilutions of the target genes. The fourfold dilutions began at 0.25 ng, and eight dilutions were used to generate the standard curve. This procedure provides an absolute quantitation of the amount of CCR5 mRNA in a given tissue. Data were analyzed using Sequence Detection Systems software version 1.7 (Applied Biosystems, Foster City, CA, USA).

Digital image analysis

Single stained sections were randomly coded and analyzed by computer-assisted image analysis as described previously in detail [23]. In short, images were acquired and analyzed using Syndia algorithm on a Qwin based analysis system (Leica, Cambridge, UK). Twenty high power fields per section were analyzed. Positive staining of cellular markers was expressed as positive cells/mm².

Semi-quantitative analysis

For keratinocyte expression of K16 keratin, a semi-quantitative score was done by two independent observers, blinded for order, patient and clinical data, with a standard binocular light microscope (Olympus) at $200\times$ magnification. The semi-quantitative score ranged from 0 to 4+. A score of 0 represented no expression, while a score of 4 represented abundant expression in all layers of the epidermis.

Confocal scanning microscope

Cryosections of 5 μ m on silanized slides were fixed with acetone and dried at room temperature, and stored at -80°C . Sections were incubated in PBS-3% BSA for 30 min and washed with PBS before all steps. Next, monoclonal anti-human CCR-5 antibody (R&D Systems, MAB183, clone 45549.11), Texas red immunoglobulins diluted in PBS-3% BSA (1:100) was applied, followed by application of the primary monoclonal specific antibodies

CD3 (clone SK7; BD Biosciences), CD4 (clone SK3; BD Biosciences), CD8 (clone DK25, DakoCytomation) or CD68 (clone KP1, DakoCytomation). Next, FITC-conjugated affinity-purified rabbit anti-mouse IgG (Jackson, $A = 492$, $E = 520$) diluted in PSB-3% BSA (1:200) was applied and sections were mounted in fluorescent mounting medium (DakoCytomation). Of each double stained slide three pictures were taken with an image definition of 1024/1024 pixels at a magnification of $25\times$.

Sample size calculation

The randomized placebo controlled clinical trial was targeted to randomize a total of 30 subjects (20 on active treatment and 10 on placebo). With this sample size, the trials would be able to detect a difference of 38% in the response rate from the placebo group assuming a 0% response rate in the placebo group with 80% power at an alpha level of 0.05 (two-sided test).

Randomization

Randomization was stratified by sites. Each site was assigned a fixed number of subjects numbers; e.g. site 1 would get numbers 1–9 and so on. Once the physician of the study site would enroll a subject, the subject would be assigned the next available subject number assigned to the site, starting with the bottom of the list; e.g. the first subject enrolled in site 1 would get number 1, the second subject would get number 2, and so on. Treatments would be assigned in an active to placebo ratio of 2:1 according to a computer generated randomization schedule. No stratification based on age, sex or other characteristics was performed. Throughout the study both patient and treating physician were blinded to the group assignment.

Statistical analysis

We used SPSS 12.0.1 for Windows (SPSS, Chicago, IL, USA) for the statistical analysis of clinical data. The Mann–Whitney test was used to compare lesional and non-lesional skin at baseline and to compare skin biopsies before and after treatment. All statistical tests were two-sided: P values less than 0.05 were considered significant. The results are expressed as median \pm standard error of the mean. To evaluate the clinical effect of treatment with a CCR5 inhibitor, an intention to treat analysis was performed. Quantitative PCR data analysis was performed by two-sided t test as implemented by Graphpad Prism (version 4.0 Graphpad Software, San Diego, CA, USA). A P value less than 0.05 was considered significant.

Results

Comparison of CCR5 expression in lesional versus non-lesional psoriatic skin

The expression of CCR5 in T cells (CD3) and macrophages (CD68) in lesional and non-lesional skin of nine psoriasis patients was compared at baseline. We observed a clear expression of CCR5 which was primarily present in the dermis. In absolute numbers approximately half of the T cells and half of the macrophages co-expressed CCR5 (Fig. 1). The $CD3^+CCR5^+$ and $CD68^+CCR5^+$ double positive cells showed a low but statistically significant increased expression of CCR5 in epidermis and dermis of lesional skin in comparison to non-lesional skin, as shown in Fig. 1. Focussing on the expression of CCR5 as the percentage of all T cells or macrophages present in the sections, the difference between lesional and non-lesional skin was only statistically significant in the epidermis for $CD3^+$ cells ($P < 0.05$) and in the dermis for $CD68^+$ cells ($P < 0.001$).

Quantitative RT-PCR analysis indicated no increased expression of mRNA for CCR5 and CCR5-ligand CCL4 (MIP-1 β) in lesional skin (Fig. 2), only the expression of CCR5-ligand CCL5 (RANTES) and IL-8 was significantly increased in lesional skin ($P < 0.0001$ and $P < 0.05$). The well-known enhanced expression of IL-8 in psoriatic skin was included as positive control.

Lack of clinical efficacy of SCH351125: a CCR5 inhibitor

In total 34 patients were randomized, as shown in Fig. 3. The demographical data of the patients are shown in Table 1. With regards to co-morbidities, one patient had hypercholesterolemia and one had obesity in the placebo group. In the SCH351125 group one patient also had obesity and two had a history of hypertension. After treatment with the CCR5 inhibitor there was no change in mean PASI in the SCH351125 group ($n = 23$) [15.5 ± 3.8 at baseline, 15.4 ± 7.4 at day 28 (Fig. 4a)]. Three of the patients treated with SCH351125 (13%) attained an improvement of 50% or more compared to baseline (PASI 50 responders), showing improvements of 67, 77 and 69%. In the placebo group ($n = 9$) the mean PASI slightly decreased (14.2 ± 4.7 at baseline, 12.9 ± 3.7 at day 28). None of the patients treated with placebo showed an improvement of more than 50%. All changes observed were not statistically significant. In the follow-up period no changes in mean PASI were seen in either treatment groups.

In the treatment group four patients discontinued. One patient developed an erythrodermic eruption after 4 days of treatment, which was considered by the site physician as a

Fig. 1 Immunohistochemical analysis of CCR5 in lesional versus non-lesional skin. Data are shown as mean \pm SD; *ns* non-significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

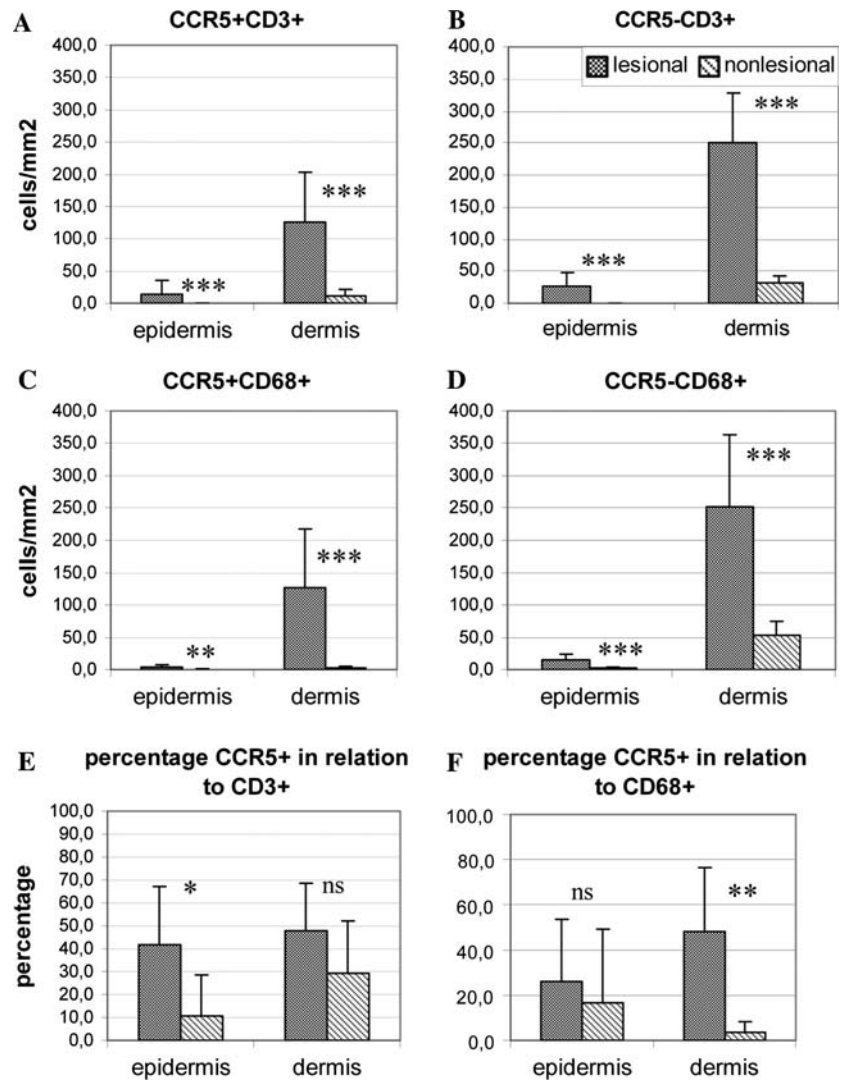
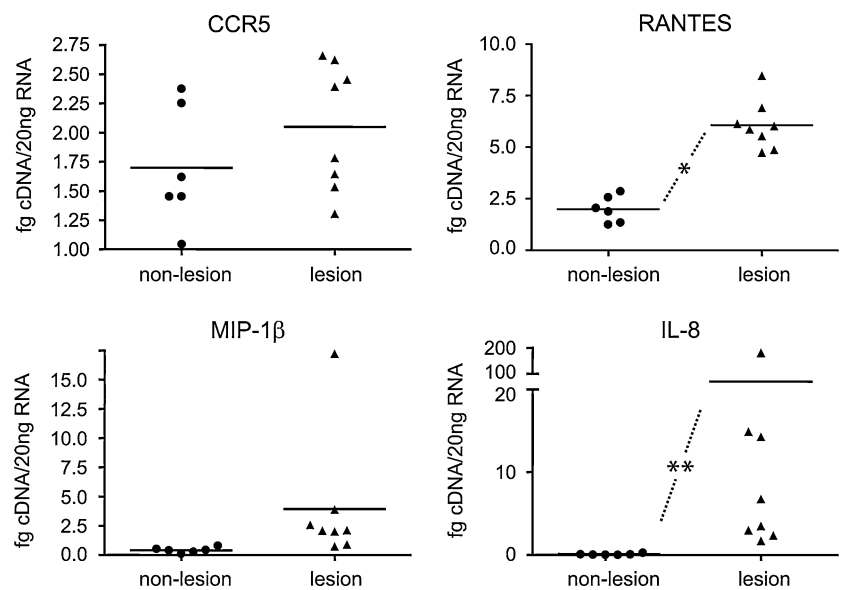


Fig. 2 mRNA analysis of CCR5 in lesional psoriatic skin in comparison with non-lesional skin. IL-8 is used as a control marker. * $P < 0.0001$, ** $P < 0.05$



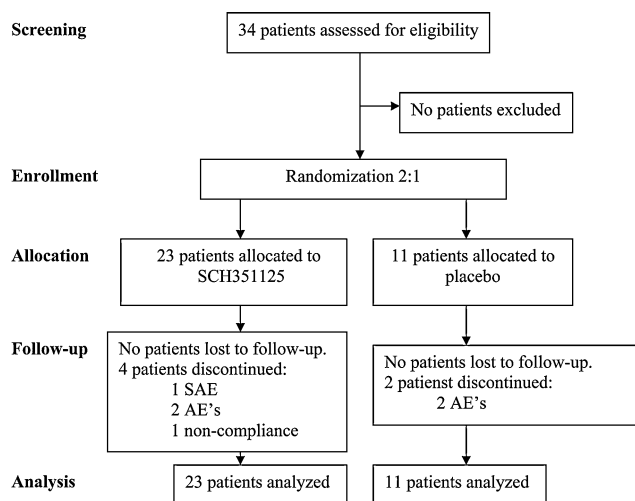


Fig. 3 Flow chart of randomized, placebo controlled clinical trial. SCH351125 CCR5 ligand inhibitor, SAE serious adverse event, AE adverse event

Table 1 Demographical data patients

	Randomized clinical trial	
	Placebo	SCH351125
Number	11	23
Male:female	7:4	18:5
Age (years) ^a	41.8 (10.2)	49.4 (14.3)
Duration of skin disease (years) ^a	20.6 (9.8)	19.8 (11.6)
Baseline PASI ^a	14.9 (4.7)	15.7 (4.3)

^a Mean (\pm SD)

SCH351125 CCR5 ligand inhibitor, PASI psoriasis area and severity index, BSA body surface area

serious adverse event (SAE). Two patients discontinued due to adverse events (AEs): one developed shingles in the n.trigeminus area of the right side of his face after 8 days of treatment and one patient discontinued due to hair loss after 21 days of treatment. One patient discontinued due to non-compliance. In the placebo group two patients discontinued due to AEs: both exacerbation of their psoriasis after 2 weeks of treatment.

CCR5 expression before and after treatment with SCH351125

Immunohistochemical analysis of lesional tissue samples from the SCH351125 group and the placebo group revealed no statistically different expression of CCR5 between baseline and day 28 in both treatment groups, as illustrated by Fig. 4b, c. When focusing on the markers CD3, CD68, CD161, elastase and K16 in relation to the clinical response, no statistically significant difference after 28 days

of treatment with either SCH351125 or placebo was found (Fig. 4d), except for elastase and dermal CCR5⁺CD3⁺ cells, which were statistically significantly lowered in the three PASI 50 responders treated with SCH351125. Additional data obtained by confocal scanning microscopy corresponded with the digital image and SQA (data not shown).

Discussion

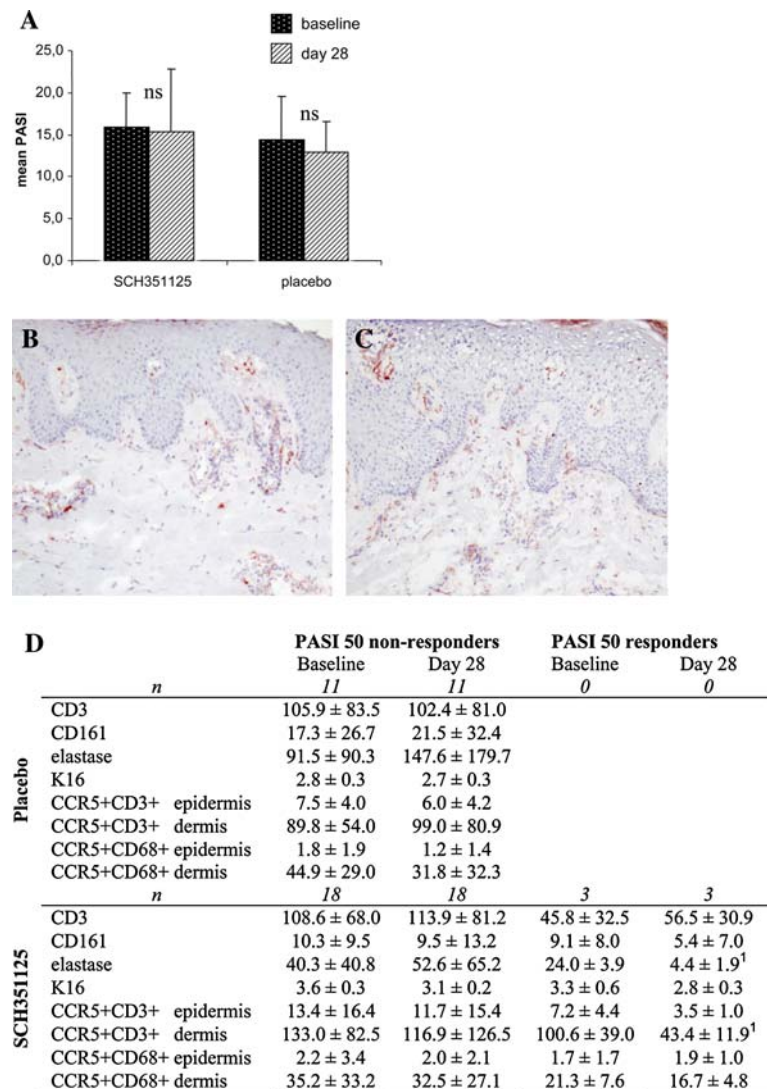
The primary objective of this study was to explore the possibility of involvement of CCR5 in the pathogenesis of chronic plaque psoriasis. Therefore we determined the expression of CCR5 in situ at the protein and mRNA level by immunohistochemical analysis and, quantitative RT-PCR, respectively. The total number of single positive (CCR5⁺) and double positive (CCR5⁺CD3⁺ and CCR5⁺CD68⁺) cells in lesional psoriatic skin significantly outnumbered those in non-lesional skin. However, when expressed as percentage of CD3 or CD68 cells, the difference between lesional versus non-lesional expression was less clear. With the latter approach we found that the proportion of CCR5 expression was significantly higher in the epidermal CD3⁺ cells and dermal CD68⁺ cells only, when comparing lesional skin to non-lesional skin.

Analysis of CCR5 mRNA expression demonstrated a slight, though not significant, increased expression of CCR5 in lesional psoriatic skin, perhaps due to the small numbers of patients. In line with earlier observations we found that the mRNA expression for RANTES [19, 48] and IL-8 [1, 17, 21, 45, 53] was significantly higher expressed in the lesional samples. However, in contrast to previous research [42] the expression of MIP1 β was not increased in lesional psoriatic skin.

In summary, our results do not provide a clear answer to our objective of determining whether the percentage of CCR5 expressing cells is similar in lesional and non-lesional skin, or if this percentage is increased in lesional skin.

To assess any possible functional participation of CCR5 in the development or maintenance of psoriatic plaques we investigated the clinical response to treatment with a CCR5 inhibitor as well as the effect of this drug on the inflamed skin in situ. The randomized placebo-controlled clinical trial revealed no significant clinical effect and changes at the immunohistochemical level between patients treated with placebo or the CCR5 inhibitor. The specific type and dose of the CCR5 inhibitor used in this clinical trial has proven its efficacy previously in vitro, in vivo and clinical studies with other diseases in which CCR5 is known to play a pivotal or significant role [14, 44, 55, 56, 60]. However, only three patients treated with the CCR5 inhibitor demonstrated a clinical improvement of 50% or more. It cannot be

Fig. 4 Clinical and immunohistochemical responses after treatment with a CCR5 receptor inhibitor. In a randomized placebo controlled clinical trial 34 psoriasis patients were randomized for treatment with a CCR5 receptor inhibitor (*SCH351125*) or placebo for 28 days. Clinical efficacy was measured by psoriasis area and severity index (PASI) (a). Lesional skin biopsies were taken from all patients at baseline and day 28 to evaluate immunohistochemical effect. Immunohistochemical single staining of CCR5 of a patient treated with *SCH351125* at baseline (b) and day 28 (c). Immunohistochemical markers in relation to clinical response (d). PASI 50 non-responder, improvement of PASI of less than 50%; PASI 50 responder, improvement of PASI of 50 or more percent; data are shown as mean \pm SD; *ns* non-significant; ¹*P* = 0.05



excluded that this low and not statistically significant number of patients is due to a spontaneous improvement, reflecting the unpredictability of psoriasis. Surprisingly, the immunohistochemical markers analyzed in the skin biopsies of the so-called PASI 50 responders, did only partially correspond with the clinical response. Of all immunohistochemical markers only elastase and dermal CCR5+CD3+ showed a significant decline after 28 days of treatment. Notably, the expression of CD3, a marker known to correspond well with the clinical severity as measured by PASI, was increased after treatment with *SCH351125* in two out of the three responding patients (Fig. 4d). In addition, their baseline expression of CD3 was lower in comparison with the other patients while their PASI was similar. This inconsistency could be due to the low number of patients or suggests an individual difference in CD3 kinetics.

The increased expression of CCR5 observed by immunohistochemistry and the increased mRNA expression of CCR5-ligand RANTES in lesional psoriatic skin may

suggest an involvement of this receptor and ligand in psoriasis. However, our clinical trial with an effective CCR5 inhibitor unequivocally demonstrated that this is not the case. Previous research has shown increased expression of several chemokines and chemokine receptors in psoriasis, [16, 21, 27, 28, 49] indicating that multiple receptors may participate in regulating T cell recruitment to the inflamed skin. Furthermore, RANTES is known to also bind with CCR1 and CCR3, whereas MIP1 β is solely connected to CCR5. Given this complexity of interactions between chemokines and chemokine receptors, it is not unlikely that blocking a single chemokine receptor (i.e. CCR5) would have been insufficient in diminishing the inflammatory process.

According to Homey [26], chemokine antagonistic approaches to impede with the inflammatory process may perhaps be preventive rather than therapeutic. Chemokines and their receptors play an essential role in the trafficking of T cells to all kinds of tissue, including the skin. Yet, once

leucocytes have entered the target organ and underwent activation processes, impairment of recruitment of pathogenic T cells is likely to be less effective in reducing the clinical symptoms. When combined with a successful eradication treatment of the T cells, chemokine antagonists could perhaps be promising candidates for prevention of acute flares, prolongation of lesion-free interval and therefore provide optimized long-term management of patients suffering from chronically relapsing inflammatory skin disease such as psoriasis. So, although CCR5 does not seem to be a key chemokine receptor in the pathogenesis of psoriasis, further efforts are needed to unravel the complete set of chemokines and chemokine receptors significant in the recruitment of inflammatory cells in psoriasis, and may help to identify crucial molecules, as demonstrated previously by TNF in various immune mediated inflammatory diseases.

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