EXPRESSON OF CHEMOKINE RECEPTOR CXCR3 BY LYMPHOCYTES AND PLASMACYTOID DENDRITIC CELLS IN HUMAN PSORIATIC LESIONS

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ABSTRACT

In psoriasis, leukocytes that infiltrate skin lesions have been shown to be involved in the pathogenesis of this disease. Previous investigations reporting the presence of CXCR3+ T lymphocytes in psoriatic lesional skin have suggested a role of this receptor in the recruitment of T cells into the lesion. The purpose of this study was to quantify the mRNA levels of CXCR3 and to perform a systematic analysis of the cell populations that express CXCR3 in human lesional and non-lesional psoriatic biopsies. We showed by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) that the mRNA levels of CXCR3 and its ligands, CXCL9-11, were significantly elevated in psoriatic lesions, as compared to non-lesional samples. Serial cryostat sections of psoriasis skin biopsies were evaluated by immunohistochemistry. The number of CXCR3+ cells was low in non-lesional tissues. Quantitative image analysis demonstrated significant increases in the number of both epidermal and dermal CXCR3+ cells in lesional compared to non-lesional biopsies. The majority of CXCR3+ cells were located in the dermis of the lesional skin and 74% were demonstrated to be CD3+ T lymphocytes. A small number of CXCR3+ cells were CD68+ myeloid cells. In addition, we found that nearly all BDCA-2+ plasmacytoid dendritic cells in the psoriatic biopsies were CXCR3+. These findings support and extend prior reports suggesting the potential role for CXCR3 in the pathophysiology of plaque psoriasis, by mediating the recruitment of plasmacytoid dendritic cells and T cells into the developing lesions.
INTRODUCTION

Psoriasis is an inflammatory skin disease with unique histopathological characteristics including the formation of rete peg morphology of the epidermis, thickening of epidermal layers, parakeratosis, increased vascularization and marked leukocyte infiltration into the dermis. The majority of the cellular infiltrates are mononuclear T lymphocytes. Other cell types, such as neutrophils, macrophages, dendritic cells and natural killer cells are among the infiltrating leukocytes. It was reported that the presence of infiltrating T lymphocytes and macrophages in the dermis preceded any significant epidermal changes\(^1\). The role of T lymphocytes in the maintenance of psoriasis was originally suggested by the efficacy of cyclosporine\(^2\) and a lymphocyte-specific toxin\(^3\). Moreover biological agents that either stimulate T lymphocyte apoptosis or block T lymphocyte activation, co-stimulation and migration have demonstrated efficacy and gained approval as therapeutics for psoriasis (for review, see \(^4\)). In xenograft models, injection of activated lymphocytes\(^5;6\) or expansion of local resident T lymphocytes\(^7\) was required to induce psoriasis-like phenotype in pre-symptomatic non-lesional human skin.

Both CD4\(^+\) and CD8\(^+\) T lymphocytes are present among the infiltrating T cells in psoriasis, although the role of each T lymphocyte subset in the pathogenesis of psoriasis is not well understood. It is thought that CD4\(^+\) T cells are essential for the induction of psoriasis based on the ability of purified CD4\(^+\), but not CD8\(^+\), T lymphocytes to induce psoriatic lesions in the SCID-hu xenograft model\(^8\). However, CD8\(^+\) T cells are the prevalent infiltrating T cells present in psoriatic epidermis\(^9\) and have been speculated to be responsible for the activation of psoriatic keratinocytes. Both CD4\(^+\) and CD8\(^+\) T cells in psoriasis express Th1 cytokines such as interferon-\(\gamma\) (IFN-\(\gamma\)), interleukin (IL)-2 and tumor necrosis factor (TNF)-\(\alpha\). Based on this cytokine profile, psoriasis was initially considered to be a T-helper type 1 (Th1)-mediated inflammatory skin disease. Moreover, recent evidence also suggests an important contribution by Th17 cells in the development of psoriatic disease\(^10;14\).

In addition to T lymphocytes, dendritic cells (DCs) are also among the prominent cellular aggregates in the dermis of psoriatic lesions. There are two major subsets of dermal DCs in psoriatic skin, myeloid (mDCs) and the plasmacytoid DCs (pDCs). mDCs are CD11c\(^+\) while pDCs are CD11c\(^-\)/BDCA-2\(^+\)/CD123\(^+\). mDCs are the predominant DCs in psoriatic tissue, found in both the epidermis and dermis, and express high levels of proinflammatory molecules such as TNF-\(\alpha\), IL-23 and IL-20\(^12;15;16\), which may activate T lymphocytes and keratinocytes. In contrast, activated pDCs are present in lower numbers but express high levels of interferon-\(\alpha\) (IFN-\(\alpha\)). In contrast to normal human skin of healthy subjects, pDCs are present in normal-appearing skin of psoriasis patients, and considerable numbers of these cells can be present in lesional skin\(^17\). pDCs express intracellular toll-like receptor (TLR)7 and TLR9. These receptors recognize viral or microbial nucleic acids. The exacerbation of psoriasis in patients treated with TLR7 agonist imiquimod for other conditions was accompanied by a
large infiltrates of pDCs and a massive induction of type I IFN activity. Very recently, a report demonstrated a unique pathway of pDC activation and its induction to secrete high levels of IFN-α by the binding of self-DNA in complex with an antimicrobial peptide LL37 over expressed in psoriatic skin. These data suggest an upstream role of pDCs in the induction of the psoriatic lesion.

The role of chemokines and their receptors in the trafficking of various subtypes of leukocytes has been well documented. Chemokine receptor CXCR3 is known to be highly expressed by activated Th1 lymphocytes and has been suggested to be one of the major chemokine receptors responsible for their recruitment to inflamed sites in vivo. In addition, it can also be expressed by natural killer (NK) cell, B cells, pDCs and mDCs. The presence of infiltrating CXCR3+ T cells in psoriasis as well as other inflammatory skin disorders has been reported. The cognate ligands of CXCR3, CXCL9 (MIG), CXCL10 (IP-10) and CXCL11 (ITAC) are induced by IFN-γ and have been shown to be expressed by inflammatory cells and/or keratinocytes in psoriatic skin and lesional skin of other inflammatory skin diseases. Despite its documented role in the trafficking of activated T cells in vitro and the reported presence of CXCR3+ T lymphocytes in the lesional psoriatic skin, a systematic analysis of CXCR3 expression in lesional psoriatic versus non-lesional skin biopsies has not been performed previously. In order to fully appreciate its potential role in the pathogenesis of this inflammatory skin disease, we have extensively examined the level and pattern of CXCR3 expression in lesional psoriatic versus non-lesional skin biopsies.

METHODS

Skin biopsies

The human inflammatory skin disease panels were obtained from two independent clinical trials. One panel consists of lesional skin biopsies from 35 patients with moderate to severe chronic plaque psoriasis, defined by a Psoriasis Area Severity Index (PASI) ≥ 8. Skin samples from this collection were used in both quantitative PCR and immunohistochemistry. In 12 out of these 35 patients non-lesional skin biopsies were obtained. 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, either directly snap frozen in liquid nitrogen or 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. 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 derivates, etc) was stopped 2 weeks before study initiation. 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. Tissues from this panel were used in quantitative PCR and immunohistochemistry studies described in this report.

A second panel of skin biopsies was also analyzed by quantitative PCR for the expression of CXCR3 ligands. This panel includes 35 normal skin samples (15 from autopsy donors and 20 from normal donors in clinical trial setting; see below), 24 non-lesional psoriasis skin samples, 25 lesional psoriasis skin samples, 30 non-lesional atopic dermatitis skin samples, and 30 lesional atopic dermatitis skin samples. All non-lesional and lesional patient samples were ranked by severity using either the PASI score or EASI (eczema area and severity index) score. For psoriasis patients, the PASI scores were in the range of 9-20.75. For atopic dermatitis patients, the EASI scores were in the range of 1.85-35.95. Two 4 millimeter punch biopsies were taken from each patient. Samples were obtained in a clinical trial setting at Stanford University Dermatology Department37. Autopsy donor materials were obtained from Zoion Diagnostics (Hawthorne, NY, USA).

RNA isolation and amplification

Total RNA was isolated from skin by pulverization of tissue in a dry ice encased metal mortal and pestle, followed by extraction using the RNeasy fibrous kit (Qiagen, Valencia, CA, USA) according to manufacturer’s instructions. Total RNA (5 μg) was subjected to treatment with DNase (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to manufacturer’s instructions to eliminate possible genomic DNA contamination. 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 (Agilent Technologies, Germany). DNase-treated total RNA was reverse-transcribed using Superscript II (Invitrogen) according to manufacturer’s instructions.

Quantitative PCR

TaqMan primers and probes were designed with PrimerExpress software (ABI), and purchased from ABI (Applied Biosystems, Foster City, CA, USA). The PCR reactions were prepared using the components from the iScript Custom One-Step RT-PCR Kit with ROX and assembled according to the manufacturer’s instructions (Bio-Rad, Hercules, CA, USA). The fluorogenic probes were labeled with 6-carboxyfluorescein (6FAM) as the reporter and 6-carboxy-4,7,2,7′-tetramethylrhodamine (TAMRA) as a quencher. Real-time quantitative PCR was performed using either of two methods. In the first method, each 10 μl PCR reaction contained 20 ng of total RNA in a TAQMAN™ real-time quantitative PCR reaction on an ABI 7900 sequence detection system. The final concentrations of the primers in the PCR reactions were at 200 nM and the probe at
100 nM respectively. The RT-PCR reactions were performed in triplicate in a 384-well plate. An eukaryotic 18S rRNA endogenous control probe/primer set (ABI) was used as an internal control for RNA quality, and a primer/probe set for the CD4 promoter was used to check the RNAs for genomic DNA contamination. The PCR data was quantified based on a 12-point standard curve generated using 4-fold serial dilutions of a cDNA containing the gene of interest. The fourfold dilutions began at 20,000 fg. This procedure provides an absolute quantification of the amounts of CXCR3, CXCL9, CXCL10 and CXCL11 mRNA in a given sample.

In the second method, 10 ng of cDNA from each sample was used. Two unlabelled primers at either 400 nM or 900 nM each were used with 250 nM of FAM-labelled probe (Applied Biosystems, Foster City, CA, USA) in a TAQMAN™ real-time quantitative PCR reaction on an ABI 7000, 7300 or 7700 sequence detection system. Ubiquitin levels were measured in a separate reaction and used to normalize the data by the Δ-Δ cycle threshold (Ct) method. (Using the mean cycle threshold value for ubiquitin and the gene of interests for each sample, the equation 1.8 e (Ct ubiquitin minus Ct gene of interest) x 10^4 was used to obtain the normalized values.) Measurement of Ct values for ubiquitin was also used as a secondary measurement of RNA/cDNA quality and samples were deemed acceptable if they were at a Ct of 23 or less. High quality RNA generally leads to ubiquitin Ct values between 17 and 23 for 10 ng of input cDNA (McClanahan, unpublished data). The absence of genomic DNA contamination was confirmed using primers that recognize a region of genomic DNA. Samples with Ct values for genomic DNA of 35-40 were considered acceptable for analysis. The Δ-Δ Ct method described above results in normalized expression values relative to the housekeeping gene ubiquitin. Normalized values less than 1.0 are considered to be at the limit of detection for this method and were considered to be negative for analysis. Kruskal-Wallis statistical analysis was performed on log transformed data (median method).

**Immunohistochemistry (IHC)**

Acetone-fixed fresh frozen sections of skin biopsies at 5 μm thickness were incubated with primary antibodies for 1 hour at room temperature. Following washes, the bound primary antibodies were detected by incubation with either biotinylated donkey anti-rabbit or horse anti-mouse for 30 min followed by incubation with an alkaline phosphatase kit, Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). Visualization of the IHC signal was accomplished by incubation with Liquid Permanent Red from Dako Cytomation (Carpinteria, CA, USA). After immunostaining tissue sections were counterstained with hematoxylin. Antibodies used were anti-CD8, anti-CD3, anti-CD68 (Dako Cytomation, Carpinteria, CA, USA), anti-CXCR3 (BD Biosciences, San Diego, CA, USA), Cytokeratin 16 (K16, Novacastra Lab., New Castle, UK), anti-BDCA-2 (Miltenyi Biotec, Auburn, CA, USA) and anti-CD123 (BioLegend, San Diego, CA, USA). Detection of anti-BDCA-2 was amplified using Tyramide Signal Amplification (TSA) kit from Perkin Elmer Life Sciences (Boston, MA, USA) according
to the manufacturer’s directions. A total of 45 fresh frozen skin biopsies with 11 paired (lesional and non-lesional) tissue blocks were included in the final IHC analysis. Adjacent sections were used to compare the distribution patterns of T cells and CXCR3+ cells.

The co-localization of cell type markers and CXCR3 was evaluated by double immunofluorescent (IFC) staining. Alexa Fluor 488 linked secondary antibodies, Alexa Fluor 488 linked streptavidin, Cy3 linked streptavidin and fluorescein (FITC)- or Cy3-linked TSA kits were used to detect the binding of primary antibodies. Sometimes both primary antibodies in the double staining were raised in the same species (such as CD8 vs. CXCR3 or CXCR3 vs. BDCA-2). In this situation, the first primary antibody was carefully titered to a concentration that would not be detected by the conditions used to detect the second primary antibody under the experimental condition. Detection of the binding of the first antibody was then amplified with a TSA kit while no TSA amplification was used to detect the binding of the second antibody. The reliability of this approach to avoid artificial co-localization generated from cross reactivity was evident by the co-existence of single and double positive signal of each marker, and was further confirmed by the lack of double positive cells when eliminating the second primary antibody in the staining protocol.

Quantification of IHC data

Single stained sections were randomly coded and analyzed by computer-assisted image analysis as described previously in detail. Briefly, images were acquired and analyzed using Syndia algorithm on a Qwin based analysis system (Leica, Cambridge, U.K.). Five random, non-overlapping, high power fields (100X) per section were analyzed.

The percentage of CXCR3+ T cells in the psoriatic lesions were analysed by double IFC staining and counted manually. Six random, non-overlapping, high power fields (200X) per section were counted twice. The sum of the total cell number was calculated for the epidermis and dermis compartments separately of the six fields and used to calculate the percentage of CXCR3+CD3+ in either the CXCR3+ or CD3+ cell populations.

RESULTS

Up-regulated expression of CXCR3 and its ligands in psoriatic lesional skin

To evaluate the expression of CXCR3 in the psoriatic lesions of moderate to severe psoriasis patients in comparison to non-lesional skin, the steady-state mRNA levels of CXCR3 and its ligands were analyzed by real-time RT-PCR. Thirty lesional and twelve non-lesional skin biopsies were included in the analysis. As shown in Figure 1, CXCR3 expression was significantly up-regulated in the lesions as compared to non-lesional biopsies. The same samples were also analyzed for the expression of its cognate
ligands, CXCL9, CXCL10 and CXCL11. A significant increase of the mRNA levels of all three ligands in psoriatic lesions was observed as compared to non-lesional samples (Figure 1).

In contrast to our findings, it was previously reported that the message levels of CXCR3 ligands were not significant upregulated in psoriatic lesional skin as compared to normal human skin or non-lesional skin. To confirm our initial observation, a second panel of inflammatory skin biopsies which was obtained independently was analyzed for the expression of CXCL9 and CXCL10. This panel of samples includes skin biopsies from healthy donors and donors from patients with psoriasis and atopic dermatitis. As shown in Figure 2, significant increases in the expression of both ligands were observed in the lesional tissues from each patient population compared with normal skin or the corresponding non-lesional skin samples of each disease. In addition, the message levels of both ligands were also significantly elevated in non-lesional skin biopsies from both disease populations compared with normal skin.

Similar distribution patterns of T lymphocytes and CXCR3+ cells in psoriatic skin
To qualify the lesional versus non-lesional biopsies for further analysis, the histopathology of the biopsies was first examined for epidermis morphology and

Figure 1 Real-time RT-PCR analysis of mRNA of CXCR3 and its ligands, CXCL9, CXCL10 and CXCL11, in psoriatic skin biopsies. The message levels of CXCR3 and its ligands from 12 non-lesional and 30 psoriatic lesional skin biopsies were evaluated by RT-PCR. **p< 0.01; *p< 0.05 versus non-lesional biopsies.
Figure 2 Real-time RT-PCR analysis of mRNA of CXCR3 ligands, CXCL9 and CXCL10, in psoriatic and atopic dermatitis skin biopsies. The message levels of CXCL9 and CXCL10 were evaluated by RT-PCR in 35 normal skin samples, 24 non-lesional psoriasis skin samples, 25 lesional psoriasis skin samples, 30 non-lesional atopic dermatitis skin samples and 30 lesional atopic dermatitis skin samples. CXCL9 and CXCL10 mRNA levels were normalized against the ubiquitin expression level and log transformed as described in Methods. *p< 0.01 versus normal skin; @p< 0.05 versus respective non-lesional biopsies.

Figure 3 The distribution patterns of CD8⁺, CD3⁺, and CXCR3⁺ positive cells in psoriatic skin biopsies. Representative images of CD3 (A, D, G), CXCR3 (B, E, H) and CD8 (C, F, I) stained sections, showing the presence and distribution patterns of these cells in non-lesional skin biopsies (A-C) and psoriatic plaque lesions (D-I). All images are at the same magnification, 100x. Positively stained cells are in red. Yellow arrowheads in (E) highlight the CXCR3⁺ cells located at the basal keratinocyte layer.
thickness followed by staining with anti-cytokeratin 16 (K16) for the proliferation/differentiation of keratinocytes. All non-lesional skin biopsies resembled normal skin histologically and were completely devoid of K16 staining, whereas all lesional tissue samples showed intense K16 staining (data not shown) and demonstrated histopathological characteristics of psoriasis, including rete elongation and thickening of epidermis (Figure 3). These observations are consistent with previous reports regarding the pattern of K16 expression.\textsuperscript{40}

Adjacent skin sections were used to compare the distribution pattern of CXCR3⁺ cells to those of CD3⁺ and CD8⁺ T lymphocytes. Owing to the expression of CD4 by cell populations other than T lymphocytes,\textsuperscript{41} we excluded this cell marker in our analysis of T lymphocyte expression. In the non-lesional skin biopsies, small numbers of CD3⁺ and CD8⁺ T lymphocytes were detected and were mainly present in the dermis and occasionally in the epidermis (Figure 3). In psoriatic lesions, the majority of CD3⁺ lymphocytes were found in the dermis, while CD8⁺ cells appeared to be evenly distributed in the dermis and epidermis or slightly more prevalent in the epidermis. In most samples, the T lymphocytes had a scattered distribution, but in a small number of lesional biopsies, we observed large massive infiltrates of CD3⁺ as well as CD8⁺ T lymphocytes in the dermis (Figure 3G-I). The distribution pattern of dermal CXCR3⁺ cells was similar to that of CD3⁺ T cells (compare Figure 3E versus 3D; and 3H versus 3G) in the psoriatic lesions. There appeared to be fewer CXCR3⁺ cells in the lesional epidermis as compared to those of CD8⁺ and CD3⁺ T lymphocytes (Figure 3D-F), indicating that the frequency of CXCR3⁺ T cells in the epidermis is lower than in the dermis. Interestingly, the majority of epidermal CXCR3⁺ cells in psoriatic lesions were frequently located at the basal keratinocyte layer as shown in Figure 3E.

**Quantification of T lymphocytes and CXCR3⁺ cells by digital image analysis**

To quantify the numbers of CD3⁺, CD8⁺, and CXCR3⁺ cells in the skin biopsies, single positive cells were counted using a computer-aided image analysis program. Consistent with our visual observation, all three cell populations were significantly increased in both epidermis and dermis of lesional biopsies compared to the non-lesional samples (Table 1). Overall, there was a mean 6.9-fold increase of CD3⁺ T lymphocytes in the lesional biopsies as compared to those in non-lesional tissue. The increase in CD3⁺ positive cells in lesional compared to non-lesional tissue was proportionally greater in the dermis than the epidermis (Table 2). The numbers of CD8⁺ T lymphocytes also were increased in lesional compared to non-lesional tissue and they were more evenly distributed between epidermis and dermis in both lesional and non-lesional biopsies (Table 1 and 2). There was a very large fold increase in the numbers of epidermal CXCR3⁺ cells in lesional compared to non-lesional tissue (Table 1), and this large increase was heavily skewed towards the dermis over the epidermis (Table 2). These data indicate the selective accumulation of CXCR3⁺ leukocytes associated with lesional tissues in psoriasis patients, especially in the dermis.
Table 1: Fold increase in cell numbers in the lesional skin biopsies

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<tr>
<th></th>
<th>CD3&lt;sup&gt;e&lt;/sup&gt;</th>
<th>CD3&lt;sup&gt;d&lt;/sup&gt;</th>
<th>CD8&lt;sup&gt;e&lt;/sup&gt;</th>
<th>CD8&lt;sup&gt;d&lt;/sup&gt;</th>
<th>CXCR3&lt;sup&gt;e&lt;/sup&gt;</th>
<th>CXCR3&lt;sup&gt;d&lt;/sup&gt;</th>
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<tr>
<td>Folds (L / NL)</td>
<td>4.4</td>
<td>8.6</td>
<td>3.1</td>
<td>3.4</td>
<td>15.4</td>
<td>6.8</td>
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The difference in cell numbers between NL and L is statistically significant for each cell marker. L, lesional skin biopsies; NL, non-lesional skin biopsies; <sup>e</sup>, e stands for epidermis; <sup>d</sup>, d stands for dermis.

Table 2: Fold increase of each cell population in dermis as compared to epidermis

<table>
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<tr>
<th></th>
<th>CD3 (NL)</th>
<th>CD3 (L)</th>
<th>CD8 (NL)</th>
<th>CD8 (L)</th>
<th>CXCR3 (NL)</th>
<th>CXCR3 (L)</th>
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<tr>
<td>Folds (dermis / epidermis)</td>
<td>1.5</td>
<td>2.8</td>
<td>1.4</td>
<td>1.6</td>
<td>21.4</td>
<td>9.5</td>
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The difference in cell numbers between dermis and epidermis is statistically significant for each cell marker in lesional skin samples and CXCR3 in non-lesional skin samples. L, lesional skin biopsy; NL, non-lesional skin biopsy.

Double immunofluorescent staining of cellular infiltrates in the lesional plaques

To identify the subtypes of CXCR3<sup>+</sup> cells in the psoriatic lesions, we performed double immunofluorescent staining with cell surface markers in combination with anti-CXCR3 antibody. As shown in Figure 4A, the majority of CXCR3<sup>+</sup> cells are clearly CD3<sup>+</sup> T lymphocytes. The proportion of CXCR3<sup>+</sup> T cells in the lesional biopsies was further quantified by manual counting of five subjects. Our analysis indicated that 71.3 ± 2.2% of CXCR3<sup>+</sup> cells co-stain as CD3<sup>+</sup> T lymphocytes, while 54.3 ± 3.7% of CD3<sup>+</sup> T cells co-express CXCR3. Interestingly, while the percentage of CXCR3<sup>+</sup> T cells in the CXCR3<sup>+</sup> cell population remains constant in both dermis (71.4% ± 2.1%) and epidermis (71.1% ± 3.6%) of the psoriatic lesions, the frequency of epidermal CXCR3<sup>+</sup> T lymphocytes (30.7 ± 5.2%) was significantly lower that of the dermis (59.5 ± 4.5%, p<0.003). Among the CXCR3<sup>+</sup> T lymphocytes in the lesional skin, a subset of these also expresses the CD8 marker (Figure 4B). CD8<sup>+</sup>CXCR3<sup>+</sup> T cells were also found in the epidermis of psoriatic lesions albeit at a much lower frequency than CD3<sup>+</sup>CXCR3<sup>+</sup>.

To further identify additional subsets of CXCR3<sup>+</sup> cells, we investigated the co-expression of the myeloid marker CD68 and the pDC marker BDCA-2. Although high numbers of CD68<sup>+</sup> cells were present in lesional skin, only a small percentage of these were CXCR3<sup>+</sup> (Figure 4C). We next examined the expression of CXCR3 by pDCs and found that nearly all BDCA-2<sup>+</sup> cells were CXCR3<sup>+</sup>. In addition, the pDCs appeared to express the highest levels of this receptor (Figure 4D). To ensure that this observation did not result from a technical artifact, we validated the identity of the pDCs and the expression patterns of BDCA-2 and CXCR3 by analyzing the adjacent sections for the expression of CD3 versus BDCA-2 and CXCR3 versus CD3. As shown on
Figure 4 Expression of CXCR3 by CD3⁺, CD8⁺ T lymphocytes, CD68⁺ myeloid cells and BDCA-2⁺ pDCs in psoriatic lesional skin. Representative double-stained sections showing expression of CXCR3 by CD3⁺ T lymphocytes (A, F), CD8⁺ T lymphocytes (B), CD68⁺ myeloid cells (C) and BDCA-2⁺ pDCs (D). (A), CD3, red, CXCR3, green; (B), CXCR3, red, CD8, green; (C), CXCR3, red, CD68, green; (D), BDCA-2, red, CXCR3, green; (E), CD3, red, BDCA-2, green; (F), CD3, red, CXCR3, green. Double-positive cells are revealed as yellow. All images are at the same magnification, 100x. White arrowheads in (C) point to CD68/CXCR3 double-positive cells. (D-F), white circles illustrate that the BDCA-2/CXCR3 double-positive cells in (D) are not CD3⁺ T lymphocytes (E) and neither are they CD3/CXCR3 double-positive (F).

Figure 4D – 4F, the CXCR3⁺BDCA-2⁺ cells were in fact CD3⁻. The identity of CXCR3⁺ pDCs was also confirmed by their expression of CD123 (data not shown). Thus, both T lymphocytes and pDCs appear to be the predominant infiltrating leukocyte populations expressing CXCR3 in human psoriatic lesions.

DISCUSSION

CXCR3 has been suggested to be one of the major chemokine receptors responsible for the trafficking of T lymphocytes to the psoriatic dermis. Intraepidermal T lymphocytes that expressed cutaneous lymphocyte antigen (CLA) and αEβ7 integrin have been demonstrated to selectively express CXCR3 and these data suggest that CXCR3 may also be responsible for T cell homing to the psoriatic epidermis. However, a thorough analysis of CXCR3 expression in human psoriatic lesions has been lacking, which has limited the understanding of the role of this receptor in the etiology of this disease. In this study, we have performed a systematic analysis of the expression and pattern of CXCR3 in these tissues, and the identification of the cell types that express this receptor. We have demonstrated a significant increase of mRNA levels of CXCR3 and its cognate ligands in the psoriatic lesions as compared to non-lesional.
skin from the same patients. In addition, using a distinct panel of biopsy samples, we have confirmed our observation for CXCL9 and CXCL10 expression and extended the study to include another inflammatory skin disease, atopic dermatitis. Our data contradicts a previous report by Meller et al., which showed a lack of significant difference in the levels of these chemokines by RT-PCR between normal skin and the lesional skin biopsies from psoriatic or atopic dermatitis patients. Transcript levels of CXCR3 ligands in psoriatic lesions were relatively low and within similar ranges in both studies (see Figure 1 in this report and Figure 2 in). Only patients with moderate to severe psoriasis were included in our study. It is likely that the difference between lesional skin and normal skin could be obscured if skin samples from milder disease or a wider range of disease scores were included in a study. Furthermore, differences in current or prior use of medications by the patient populations included in a study could also contribute to the variations in experimental results. The low expression levels of CXCR3 ligands detected in our study is consistent with previous observations made with in situ hybridization, microarray analysis or immunohistochemistry. In spite of the low message levels observed a significant increase of CXCL9 protein in lesional skin from psoriasis and atopic dermatitis patients compared to normal skin has been reported. Given the high affinity of these ligands to CXCR3, a relatively small but significant increase in their levels can still be envisioned to play a role in the etiology of the disease.

The elevation of CXCR3 messages in psoriatic lesions correlates with the marked increase in the number of CXCR3+ leukocytes observed in situ. Most of the CXCR3+ cells are located in the dermis of both non-lesional skin and psoriatic lesions. More than 50% of CD3+ T lymphocytes present in lesional biopsies were CXCR3+ and a subset of these CXCR3+ T lymphocytes belonged to the CD8 subpopulation. In the epidermis, we also observed CXCR3+CD8+ T cells but their absolute numbers were relatively small. The numbers of CXCR3+ cells were dramatically increased in both the epidermis (15-fold) and dermis (7-fold) of lesional tissues as compared to paired non-lesional samples. Interestingly, we also confirmed the prior observation that epidermal CXCR3+ cells are located in the basal keratinocyte layer. Based on these findings, our data support and extend the hypothesis that CXCR3 likely plays an important role in the recruitment of T lymphocytes into the dermis of psoriatic lesions. It is intriguing that despite high levels of CXCL9 and CXCL10 shown to be expressed by psoriatic keratinocytes and the large numbers of CXCR3+ T lymphocytes present in the lesional dermis, there was only a low frequency of T cells in the epidermis which expressed CXCR3 by our analysis. This observation suggests that this chemokine receptor may not be essential for the migration of T cells into the epidermis in psoriatic tissues and that the T lymphocytes that migrated into the epidermis were predominantly CXCR3- cells. Thus, other mechanisms, such as those involving α1β1 integrin, may be more integral for the accumulation of epidermal T cells and the development of psoriasis. Alternatively, we cannot rule out the possibility that the apparent low numbers of CXCR3+ cells in the epidermis may have resulted from the internalization of this receptor following exposure to high levels of its cognate
CXCR3 in Psoriasis

ligands present in the tissues as the cells migrated into this anatomical site, as has been previously reported in the case of the asthmatic lung\textsuperscript{44,45}. This possibility may also account for the predominant localization of epidermal CXCR3\(^+\) T cells detected at the basal layer of the lesional epidermis.

In addition to T cells, we found that nearly all BDCA-2\(^+\) pDCs express high levels of CXCR3. Consistent with previous reports we also observed higher numbers of pDCs in biopsies taken from lesional skin compared to non-lesional skin. It is relevant that pDCs are believed to be the major type I IFN-\(\alpha\) producing cells in vivo in response to viral and certain microbial infections, and that accumulating data suggest that pDCs and type I IFN pathways play an integral role in the early events of the immunopathogenesis of psoriasis. For example, locally activated IFN-\(\alpha\) signaling pathway has been reported in psoriatic skins with no alteration in IFN-\(\alpha\) sensitivity\textsuperscript{46,47}. Psoriatic T cells have increased sensitivity to IFN-\(\alpha\) which resulted in prolonged activation of several STAT transcription factors and increased IFN-\(\gamma\) production\textsuperscript{48}, a cytokine known to induce the expression of CXCR3 ligands. In a xenograft model in AKG-129 mice, blocking IFN-\(\alpha\) signaling or inhibiting the ability of pDCs to produce IFN-\(\alpha\) prevented the T cell-dependent development of psoriasis\textsuperscript{17}. Enhanced type I interferon signaling and recruitment of CXCR3-expressing cells into the skin has been demonstrated following treatment with the TLR7-agonist imiquimod\textsuperscript{32}. CXCR3 ligands have been shown to cooperate with CXCL12 to induce migration of pDC\textsuperscript{28}. CXCR3-dependent pDC migration had also been shown in response to immobilized CXCR3 ligands\textsuperscript{24}. Very recently another chemokine, chemerin, has been described to play a critical role for the recruitment of pDC into psoriatic lesion\textsuperscript{49} and its combination with CXCL10 and CXCL12 leads to a significantly higher migratory response of this cell type than the response to each individual chemokine\textsuperscript{50}. Based on the data described here, along with the putative collaborative role of these three distinct molecules in mediating pDC migration, it is conceivable that interfering with one arm of this process, such as selective CXCR3 antagonism, could directly impact pDC migration into the skin of psoriatic patients. Together with its role in the trafficking of activated T lymphocytes into the dermis of psoriatic skin, CXCR3 represents a highly attractive target for the treatment of this disease.

Interestingly, a selective CXCR3 antagonist, T0906487, was found to be ineffective in a 28 day clinical psoriasis trial\textsuperscript{51}. Pharmacodynamics responses were highly variable in all treatment groups in this study, and this was suggested to be due to the highly variable pharmacokinetics. The possibility also exists that the timeframe of this study was insufficient to optimally inhibit T cell-dependent activity in this disease, and that a longer (eg. 12 weeks study) may be preferable. Based on our observation that more than 40% of the T cells in the psoriatic lesions are CXCR3\(^+\), the potential for redundancies among chemokine-dependent pathways in this disease may also be considered. With other CXCR3 antagonists currently in development\textsuperscript{52,53} the therapeutic value of targeting CXCR3 in psoriasis will likely be conclusively defined with these emerging reagents.
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REFERENCE LIST


47. van der Fits L, van der Wel LI, Laman JD et al. In psoriasis lesional skin the type I interferon signaling pathway is activated, whereas interferon-alpha sensitivity is unaltered. J Invest Dermatol 2004; 122: 51-60.


