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CXCR4 Negatively Regulates Keratinocyte Proliferation in IL-23-Mediated Psoriasiform Dermatitis

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CXCR4 is expressed by basal keratinocytes (KCs), but little is known about its function in inflamed skin. We crossed K14-Cre and CXCR4$^{flox/flox\ (f/f)}$ transgenic mice, resulting in mice with specific loss of the *CXCR4* gene in K14-expressing cells (K14-CXCR4KO), including basal KCs. K14-CXCR4KO pups had no obvious skin defects. We compared K14-CXCR4KO and CXCR4$^{f/f}$ control mice in an IL-23-mediated psoriasiform dermatitis model and measured skin edema, and histologic and immunohistological changes. IL-23-treated K14-CXCR4KO mice showed a 1.3-fold increase in mean ear swelling, a 2-fold increase in epidermal thickness, and greater parakeratosis. IL-23-treated wild-type (WT) mice showed weak CXCR4 expression in areas of severe epidermal hyperplasia, but strong CXCR4 expression in nonhyperplastic regions, suggesting that CXCR4 may regulate KC proliferation. To test this hypothesis, we overexpressed CXCR4 in HaCaT KC cells and treated them with IL-22 and/or CXCL12 (chemokine (C-X-C motif) ligand 12). CXCL12 blocked IL-22-mediated HaCaT cell proliferation *in vitro* and synergized with IL-22 in upregulating SOCS3 (suppressor of cytokine signaling 3), a key regulator of STAT3 (signal transducer and activator of transcription 3). SOCS3 was required for CXCR4-mediated growth inhibition. In human psoriatic skin, both CXCR4 and SOCS3 were upregulated in the junctional region at the border of psoriatic plaques. Thus, CXCR4 has an unexpected role in inhibiting KC proliferation and mitigating the effects of proliferative T helper type 17 cytokines.

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INTRODUCTION

Chemokine receptors comprise four homologous families of seven transmembrane-spanning, G protein–coupled receptors that activate key intracellular signaling pathways controlling cell shape, migration (chemotaxis), and proliferation (Nestle *et al.*, 2009). In inflammatory skin disease, chemokines and chemokine receptors also have important roles in immune cell migration into skin (Lonsdorf *et al.*, 2009). Keratinocytes (KCs) are considered to be a rich source of chemokines such as CXCL8 (chemokine (C-X-C motif) ligand 8) and CCL20, which are abundant in psoriatic plaques (Schon and Boehncke, 2005), but the role of chemokine receptors that are expressed by KCs has not been fully explored. CXCR4, a chemokine receptor that has multiple roles in cancer metastasis (Balkwill, 2004), vasculogenesis (Yamaguchi *et al.*, 2003; Urbich and Dimmeler, 2004), stem cell recruitment (Petit *et al.*, 2002), and HIV infection (Bleul *et al.*, 1996), has been detected in proliferating KCs after burn injury (Avniel *et al.*, 2006). Interestingly, inhibition of CXCR4 appeared to increase the rate of re-epithelialization following burn injury (Avniel *et al.*, 2006), suggesting a regulatory role for CXCR4 in skin repair or re-epithelialization.

In the psoriasis field, clinicians have long been aware of the Koebner phenomenon (Weiss *et al.*, 2002), and biochemical evidence has also suggested a link between wound healing and psoriatic plaques (Mansbridge and Knapp, 1987; Romanowska *et al.*, 2010). The above-mentioned link between CXCR4 and wound healing prompted us to explore the role of CXCR4 in the context of a well-known model of psoriasiform dermatitis (Chan *et al.*, 2006; Hedrick *et al.*, 2009; Mabuchi *et al.*, 2011) that is induced by repeated injections of the proinflammatory cytokine IL-23, a cytokine that is critical for the maintenance of T helper type 17 cells (Fitch *et al.*, 2007).

To determine the role of CXCR4 signaling in the epidermis in the setting of psoriasiform dermatitis, we specifically

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Abbreviations: CXCR, chemokine receptor; K14, keratin 14; KC, keratinocyte; PBS, phosphate-buffered saline; SOCS3, suppressor of cytokine signaling 3; siRNA, small interfering RNA; STAT3, signal transducer and activator of transcription 3; WT, wild type

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deleted CXCR4 in murine keratin 14 (K14)–positive basal KCs by crossing mice carrying floxed *CXCR4* alleles with those expressing *Cre* recombinase under the transcriptional control of the K14 promoter. We next induced skin inflammation via repeated injections of IL-23, resulting in psoriasiform dermatitis (Wilson *et al.*, 2007; Hedrick *et al.*, 2009). Surprisingly, targeted deletion of CXCR4 in basal KCs resulted in a markedly exaggerated response to IL-23 in the skin of K14-CXCR4KO mice. Furthermore, our *in vitro* studies indicated that CXCR4 signaling abrogates the proliferative response of KCs to IL-22 (Zheng *et al.*, 2007) through a mechanism that requires SOCS3 (suppressor of cytokine signaling 3). In aggregate, our data strongly suggest that CXCR4 may have an unsuspected role in regulating epidermal proliferation in some inflammatory skin conditions.

RESULTS

Targeted deletion of CXCR4 in basal KCs results in exaggerated psoriasiform changes after IL-23 treatment

We first confirmed that K14-CXCR4KO mice were homozygous for the floxed *CXCR4* and *Cre* genes using PCR (Supplementary Figure S1 online). Because we were principally interested in the expression of CXCR4 in epidermal KCs after IL-23 treatment, we separated the epidermis from the dermis by standard techniques. Whereas epidermal CXCR4 mRNA was upregulated in CXCR4$^{f/f}$ control mice after IL-23 treatment, K14-CXCR4KO mouse skin showed minimal expression of epidermal CXCR4 (Figure 1a). On combining the results of three experiments, the *P*-value (after combining three experiments) did not reach significance ($P = 0.062$). It is known that Langerhans cells, which do not express K14, express high levels of CXCR4 upon activation, thus accounting for measurable (and variable) levels of CXCR4 in the epidermis of K14-CXCR4KO mice. Minimal levels of CXCR4 protein, possibly from Langerhans cells, were detected in the basal KCs of K14-CXCR4KO mice versus CXCR4$^{f/f}$ mice by antibody staining (Supplementary Figure S2 online). CXCR4 was not detected in phosphate-buffered saline (PBS)–injected CXCR4$^{f/f}$ (or wild-type (WT)) mice epidermis (data not shown). Thus, epidermal CXCR4 expression in CXCR4$^{f/f}$ control mice is upregulated following repeated injection of IL-23, but only minimally observed in CXCR4KO mouse skin (possibly because of incomplete deletion of the *CXCR4* gene by the *Cre* recombinase or unaffected expression of CXCR4 by Langerhans cells).

Histologically, IL-23-treated ears of K14-CXCR4KO mice exhibited exaggerated psoriasiform changes compared with control mice (Figure 1c and d). Specifically, they displayed a thicker epidermis (Figure 1b), increased parakeratosis with greater accumulation of neutrophils in the cornified layer, and a dense inflammatory infiltrate in the dermis (Figure 1c). The neutrophilic aggregates were subjectively observed more frequently in the K14-CXCR4KO mice. Although both control mice and K14-CXCR4KO mice showed epidermal thickening after IL-23 treatment, K14-CXCR4KO mice ears showed a 2-fold increase compared with CXCR4$^{f/f}$ control mice (Figure 2a). We also counted infiltrating cells in the dermis (Figure 1c) and found that the number of infiltrating cells

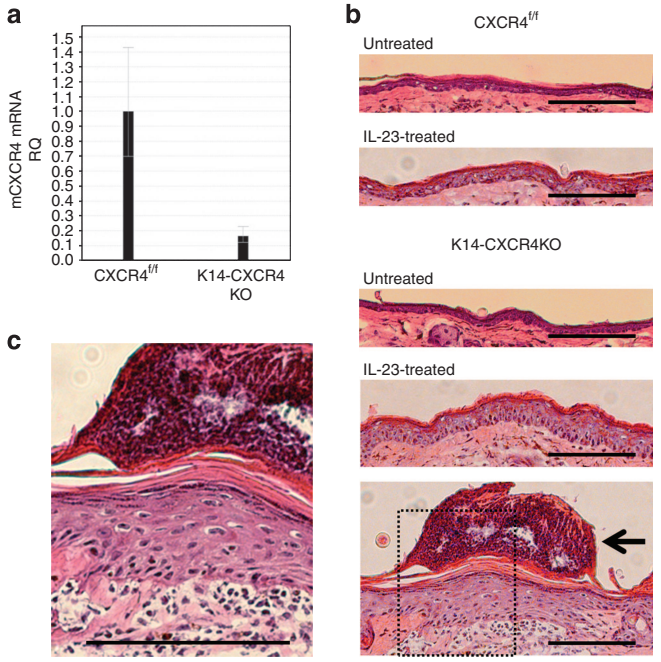

Figure 1. CXCR4 expression in CXCR4$^{f/f}$ control mice (CXCR4$^{f/f}$-Cre^{-} mice) and K14-CXCR4KO mice following IL-23 injection. CXCR4KO mice and CXCR4$^{f/f}$ control mice ears were injected with IL-23 every other day for 5 days and collected at day 5. Abrogation of CXCR4 expression was shown using real-time PCR (2–3 ears). (**a**) Representative results from one of three experiments with similar results are shown. Hematoxylin and eosin (H&E) staining was performed for ear sections from K14-CXCR4KO mice and control mice treated with or without IL-23. Neutrophils in the stratum corneum are indicated by a black arrow in **b** and shown at higher magnification in **c**. Scale bars = 100 μm. (**b**, **c**) Representative results from one of three experiments with similar results are shown.

increased 1.5-fold in K14-CXCR4KO mice compared with CXCR4$^{f/f}$ control mice (Figure 2b). Ear swelling, a measurement of dermal edema and inflammation, during IL-23 treatment was significantly increased (following analysis of variance) in K14-CXCR4KO mice on days 4 and 5 compared with CXCR4$^{f/f}$ control mice, which showed an increase above day 0 only on day 5 (Figure 2c). Thus, targeted deletion of CXCR4 in basal KCs results in exaggerated psoriasiform inflammatory changes after treatment with IL-23.

CXCR4 expression inversely correlates with epidermal hyperplasia in WT mice following IL-23 treatment

We observed that injection of IL-23 in WT C57BL/6 mice did not result in completely uniform epidermal hyperplasia throughout the entire epidermis above the injection site (Figure 3a). We hypothesized that the expression level of CXCR4 on basal KCs might regulate epidermal hyperplasia and thus examined CXCR4 expression by immunofluorescence microscopy in basal KCs of WT mice treated with IL-23. Indeed, CXCR4 expression was enhanced in nonhyperplastic regions, whereas hyperplastic regions showed little or no expression of CXCR4 (Figure 3b).

In the IL-23 injection model, IL-22 is produced by immune cells such as conventional and γδ T cells (Mabuchi *et al.*, 2011), and it induces KC proliferation via signal transducer

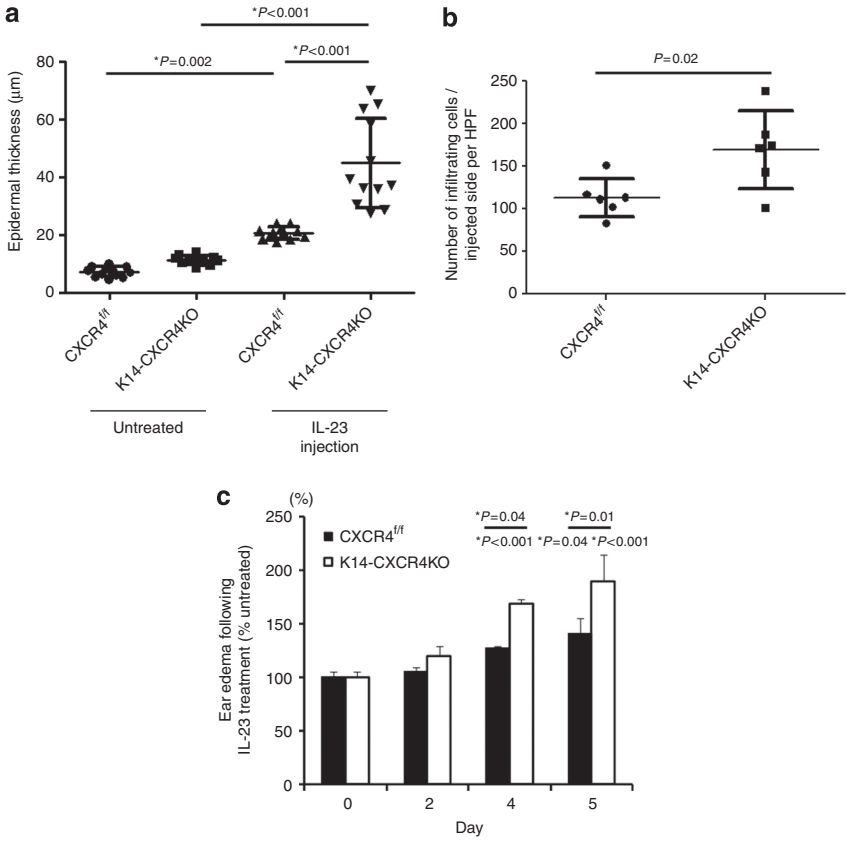

Figure 2. Epidermal thickening and psoriasiform changes in CXCR4$^{f/f}$ control and K14-CXCR4KO mice following IL-23 injection. Ear sections from K14-CXCR4KO mice ($n=3$) and CXCR4$^{f/f}$ control mice ($n=3$) were treated with IL-23 every other day for 5 days and were hematoxylin and eosin (H&E) stained. (**a**) The subjectively thickest epidermal regions were selected, and epidermal thicknesses were measured at four points in a blinded manner for each mouse sample. (**b**) Dermal infiltrating cells were counted on randomly selected sections of IL-23-treated mice ($n=3$). Ear swelling was measured before injection (days 0, 2, and 4) and before harvesting ears (day 5; $n=3$). (**a–c**) Representative data from one of two independent experiments with similar results are shown. *Significance using Scheffé's *post hoc* comparison test.

and activator of transcription 3 (STAT3) activation (Boniface *et al.*, 2005). As a negative feedback control system for STAT3 signaling, SOCS3 is known to block the STAT3 phosphorylation by JAK (Brantley and Benveniste, 2008). Thus, we asked whether SOCS3 expression differed between hyperplastic and nonhyperplastic regions. Similar to the expression pattern observed with CXCR4, SOCS3 expression in the basal epidermal layer was markedly reduced in hyperplastic regions compared with nonhyperplastic regions (Figure 3c). In summary, CXCR4 and SOCS3 expression on basal KCs correlates with regions of reduced epidermal hyperplasia following IL-23 injection, suggesting that CXCR4 may negatively regulate KC proliferation.

IL-22 and CXCL12 synergistically upregulate SOCS3 in CXCR4-overexpressing HaCaT cells

IL-22 is a key downstream effector molecule that is produced by both αβ and γδ T cells (Mabuchi *et al.*, 2012). It mediates epidermal hyperplasia and inflammation in response to IL-23. KCs are not believed to respond to IL-23 directly, but they do possess the IL-22 receptor, which acts through STAT3 to induce a variety of key changes found in psoriasis (Zheng *et al.*, 2007). HaCaT cells, a KC-derived cell line, express the IL-22 receptor (Kreis *et al.*, 2007) and are known to proliferate in response to IL-22 *in vitro* (Nakagawa *et al.*, 2011). To determine whether CXCR4 signaling regulated IL-22-mediated KC proliferation via SOCS3, we established CXCR4-overexpressing HaCaT (Boukamp *et al.*, 1988) immortalized human KC cells (CXCR4-HaCaT) and cultured them in the presence of IL-22 with or without CXCL12 (chemokine (C-X-C motif) ligand 12). As expected, IL-22 increased CXCR4-HaCaT proliferation, whereas CXCL12 alone had little effect on HaCaT cell proliferation (Figure 4a). Strikingly, co-addition of CXCL12 blocked the ability of IL-22 to stimulate HaCaT cell proliferation (Figure 4a), suggesting that CXCR4 activation functions as an inhibitor when IL-22 signaling is engaged.

The IL-22 receptor was only modestly upregulated in the presence of IL-22 and CXCL12 compared with IL-22 or CXCL12 alone (data not shown), indicating that CXCR4 did not directly regulate the expression of this receptor. We next asked whether the inhibitors of JAK/STAT3 pathway were upregulated by coculture of CXCL12 and IL-22. IL-22 induces the phosphorylation of STAT3, thus promoting the expression of JAK/STAT3 inhibitors such as SOCS3 and PIAS3 (protein inhibitor of activated STAT3) as potential negative feedback control mechanisms (Brantley and Benveniste, 2008).

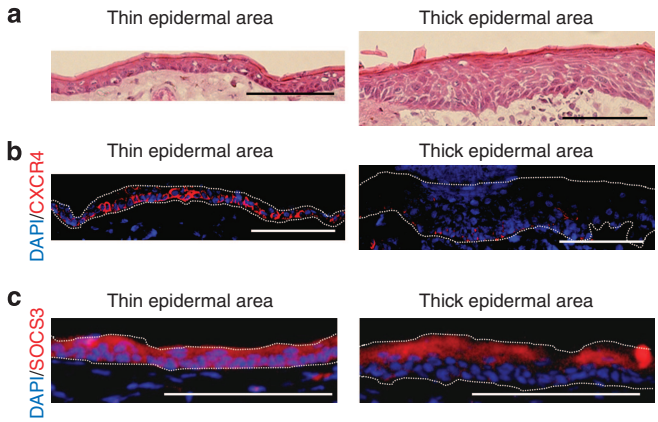

Figure 3. Epidermal expression of CXCR4 and SOCS3 (suppressor of cytokine signaling 3) in wild type (WT) mice following IL-23 treatment. Ear sections from WT mice ($n = 3$) treated with IL-23 were hematoxylin and eosin (H&E) stained and digitally photographed. (**a**) Representative H&E-stained sections from thick and thin epidermal regions of WT mice treated with IL-23 are shown. Skin from WT mice treated with IL-23 were stained with (**b**) anti-CXCR4 and (**c**) anti-SOCS3 antibodies, and representative thick and thin epidermal regions were selected for illustration of differential antibody staining. Scale bars = 100 μm. The superior dotted lines in **b** and **c** represent the approximate level of the cornified layer, whereas the inferior (or single) dotted lines in **b** and **c** represent the approximate level of the epidermal basement membrane. DAPI, 4′,6-diamidino-2-phenylindole.

PIAS3 mRNA expression, however, was not affected by CXCL12, IL-22, or the combination of the two (data not shown). As expected based on previous reports, SOCS3 mRNA was upregulated in CXCR4-HaCaT cells cultured with IL-22 for 1 hour (Figure 4b). CXCL12 had little impact on SOCS3 expression by itself, but the addition of CXCL12 in the presence of IL-22 synergistically enhanced SOCS3 mRNA level (Figure 4b). To confirm this result at the protein level, we observed enhanced SOCS3 upregulation in CXCR4-HaCaT cells cultured with IL-22 and CXCL12 using quantitative immunofluorescence microscopy (Supplementary Figure S3 online).

We next asked whether the observed SOCS3 upregulation inhibited the JAK/STAT3 pathway with the knowledge that SOCS3 attenuates STAT3 phosphorylation at Tyr705 (Linke *et al.*, 2010), a key activation site, following exposure to IL-22 (Sestito *et al.*, 2011). As shown in Figure 4c (full-length blot is shown in Supplementary Figure S4 online), IL-22, but not CXCL12, induced marked phosphorylation of STAT3. The addition of CXCL12 with IL-22, however, reduced the levels of phosphorylated STAT3 by 33.4 ± 2.5% (mean ± SD from three independent experiments) without affecting total STAT3. Thus, CXCR4-HaCAt cells cultured with IL-22 and CXCL12 show reduction in activated pSTAT3 at a key phosphorylation site.

We next asked whether SOCS3 was required for CXCR4-mediated inhibition of CXCR4-HaCaT cell proliferation. CXCR4-HaCaT cells were transiently transfected with small interfering RNA (siRNA) that specifically targeted SOCS3 and were then cultured with IL-22 and CXCL12. We confirmed that SOCS3 siRNA inhibited SOCS3 expression in CXCR4-HaCaT cells cultured with IL-22 and CXCL12 (Supplementary Figure S5 online). It is noteworthy that CXCR4-HaCaT cell proliferation was restored when cells were treated with SOCS3-specific siRNA in the presence of IL-22 and CXCL12, but not with nonspecific control siRNA (Figure 4d). Thus, CXCL12 in combination with IL-22 synergistically enhances SOCS3 expression in CXCR4-HaCaT cells, reduces phosphorylation of STAT3 at a critical tyrosine site, and blocks HaCaT cell proliferation via a SOCS3-dependent mechanism.

CXCR4 and SOCS3 are upregulated in the junctional zone between lesional and perilesional regions in human psoriatic skin

Finally, we asked whether CXCR4 was upregulated in human psoriatic skin lesions. The biopsied samples that we examined were taken from the border of psoriatic plaques and contained lesional hyperplastic areas as well as the transitional area (junctional zone) between clinically psoriatic and nonpsoriatic skin (Figure 5a). The junctional zone showed moderate epidermal hyperplasia compared with perilesional regions (Figure 5a). Strong CXCR4 expression was observed in basal KCs in the junctional zone (Figure 5b; upper-left panel) between lesional and perilesional regions. CXCR4, however, was only focally present in lesional regions (Figure 5b; middle panel) and was nearly completely absent in perilesional regions (data not shown). We calculated the ratio of CXCR4-positive area to either the junctional or lesional zone areas of psoriatic epidermis and found a significant difference (Figure 5b; right panel). Control human skin from nonpsoriatic, healthy patients showed little or no CXCR4 staining (Figure 5b; lower-middle panel). We next stained psoriatic skin sections with anti-SOCS3 antibody and noted increased SOCS3 expression in the junctional zone (Figure 5c; left panel). In samples taken from the same patient, lesional psoriasis regions that showed weak CXCR4 staining at the basal layer of the epidermis (Figure 5b; middle panel) also showed weak SOCS3 staining (Figure 5c; middle panel). The ratio of SOCS3-positive area to the entire junctional or lesional zones was also significantly higher in junctional zones (Figure 5c; right panel). Weak SOCS3 expression in normal human skin was shown to be almost the same as that in psoriasis lesional skin (Horiuchi *et al.*, 2006). Similar results were noted in two additional psoriatic patients (data not shown). Thus, CXCR4 and SOCS3 were both upregulated at the junctional zone of psoriatic lesions, suggesting that the expression of these two proteins may be a biomarker for the transition between hyperplastic psoriatic plaques and clinically nonaffected skin.

DISCUSSION

CXCR4 has been shown in several cancer systems to positively regulate proliferation, survival, and metastasis (Murakami *et al.*, 2002, 2003; Dell'Agnola and Biragyn, 2007). Thus, we were surprised to see strikingly enhanced epidermal hyperplasia and parakeratosis in K14-CXCR4KO mice following IL-23 treatment. Previous reports showed that CXCR4 signaling can upregulate SOCS expression and inactive growth hormone function (Garzon *et al.*, 2004). In our *in vitro* model, combined treatment with CXCL12 and IL-22 synergistically induced SOCS3 expression and reduced phosphorylation of STAT3. In contrast to the work of Garzon

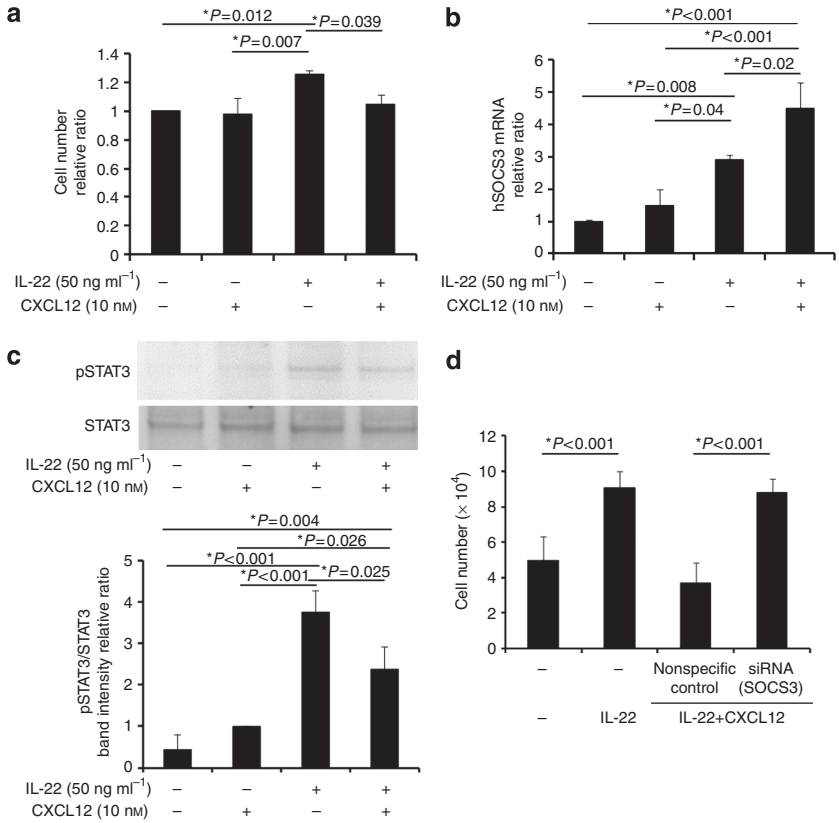

Figure 4. Effects of CXCL12 (chemokine (C-X-C motif) ligand 12) on HaCaT cell proliferation, SOCS3 expression, and phosphorylation of STAT3 (signal transducer and activator of transcription 3). CXCR4-HaCaT cells were cultured for 1 day, and then with human CXCL12 and/or human IL-22. After 2 more days ($n = 5$ wells/condition), cells were counted. (**a**) Summary of three independent experiments is shown. CXCR4-HaCaT cells were stimulated with CXCL12 and/or IL-22 for 1 hour. (**b**) SOCS3 mRNA expression was measured using real-time reverse transcriptase–PCR (RT–PCR; $n = 3$ wells/condition; one of three experiments shown). (**c**) Phospho-STAT3 (pSTAT3) and total STAT3 were examined by western blotting (representative experiment shown). (**c**) Analysis by densitometry (normalized using vehicle-treated cells) of three independent experiments is shown in the lower panel. (**d**) CXCR4-HaCaT cells were transfected with SOCS3 small interfering RNA (siRNA) and nonspecific control siRNA, cultured with CXCL12 and IL-22 for 2 days, and counted ($n = 5$). *Significance using Scheffé's *post hoc* comparison test.

et al. (2004), CXCL12 alone induced little, or no, SOCS3 expression, which may be a reflection of the different cell type used in our system.

In human psoriasis skin samples, we observed strong CXCR4 expression in the junctional zone between lesional and perilesional regions. Increased SOCS3 expression was also detected in this area, further strengthening a possible relationship between CXCR4 and SOCS3 in controlling epidermal proliferation. We hypothesize that the coordinated upregulation of CXCR4 and SOCS3 at the border between affected and unaffected skin may provide a molecular explanation for the abrupt transition from affected to unaffected skin that is so highly characteristic of psoriasis lesions.

In this work, we do not directly address which signaling mechanisms govern CXCR4 upregulation in the junctional zones of lesional skin. CXCR4, however, is known to be positively regulated by hypoxia-inducible factor (Staller *et al.*, 2003), which is strongly upregulated in psoriatic skin (Rosenberger *et al.*, 2007). Our results suggest that CXCR4 (and SOCS3) upregulation may be a counter mechanism to balance the highly proliferative cytokine signals that are present in psoriatic lesions and to limit the circumferential expansion of psoriatic plaques. Given the limited amount of human tissue available to us that contained lesional, junction, and nonlesional areas, our results will need to be confirmed with larger numbers of psoriatic tissue samples. In aggregate, our data suggest that drugs that upregulate CXCR4 function or expression might be helpful in the setting of psoriasis or other hyperproliferative diseases.

As illustrated in Figure 6a, upregulation of SOCS3 is a well-known negative feedback mechanism that balances the effect of STAT3 activation. In psoriatic lesions, however, SOCS3 is apparently not expressed at sufficient levels to block KC proliferation in the presence of STAT3-dependent inflammatory cytokines such as IL-22. CXCR4-mediated signaling, however, synergistically upregulates SOCS3, leading to reduced phosphorylation of STAT3, and, subsequently, to the reduced ability of HaCaT cells to proliferate *in vitro* in response to IL-22 (Figure 6b). Our *in vitro* data are complemented by our *in vivo* data showing profound loss of CXCR4 and SOCS3 expression in thick epidermal regions compared with the thin epidermal regions of IL-23-treated WT mice. CXCR4 may be essential for downregulating KC proliferation and thus mitigating the effects of proliferative cytokines such as IL-23 and IL-22 under inflammatory conditions. It remains to be determined whether or not CXCR4 affects the degree of

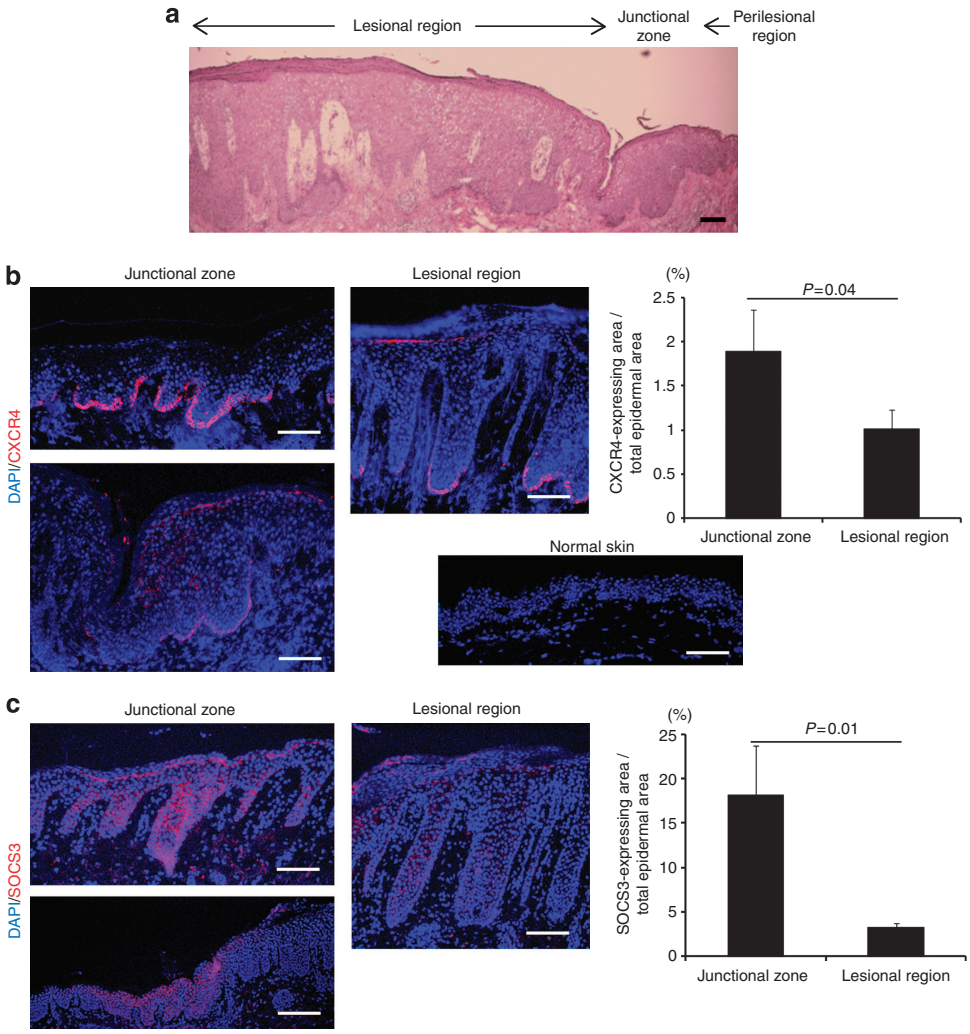

Figure 5. CXCR4 and SOCS3 expression in human psoriatic skin. Three human psoriasis skin samples containing the junctional zone between lesional and perilesional regions were biopsied (see Supplementary Table S1 online). (**a**) An example of hematoxylin and eosin (H&E) stainings showing these regions is shown (patient 1). (**b**) CXCR4 and (**c**) SOCS3 stainings of the junctional and lesional area were performed, and representative images of similar findings are depicted (patient 2; see Supplementary Figure S6online). A healthy human skin sample was stained using anti-CXCR4 Ab (**b**; lower-left panel). CXCR4- and SOCS3-positive areas of three psoriasis patients were quantified using ImageJ software and used for the calculation of the ratio (%) of marker-positive area to epidermal junctional or lesional zone areas (right panels of **b** and **c**). Scale bars = 100 μm.

inflammation in other inflammatory skin diseases that do not show enhanced epidermal hyperplasia.

MATERIALS AND METHODS

Mice

C57BL/6 WT mice, 8 and 12 weeks old, were purchased from Charles River Laboratories. Tissue-specific knockout mice were generated using a Cre-loxP (Wilminton, MA) approach (Nagy, 2000; Zimmerman *et al.*, 2011). Heterozygous C57BL/6J mice carrying a floxed *CXCR4* allele ($CXCR4^{f/wt}$) were originally obtained from Dr Daniel Littman (Howard Hughes Medical Institute, New York University, New York, NY). To selectively inactivate CXCR4 in basal KCs, $CXCR4^{f/f}$ mice were crossed with transgenic mice expressing the *Cre* recombinase under the transcriptional control of the K14 promoter (STOCK Tg (KRT14-cre) 1Amc/J; Jackson Laboratories, Bar Harbor, ME). Animal protocols were approved by the institutional animal care and use committee at the Medical College of Wisconsin.

Human psoriasis skin samples

Clinical protocols in accordance with the Declaration of Helsinki Principles that approved collection of human psoriasis skin sample were obtained from the institutional ethical committee of the Faculty of Medicine, University of Tokyo. All subjects signed written informed consent. Skin biopsies from the border of psoriatic lesions were taken from three patients (Supplementary Table S1 online) with typical psoriasis lesion by excisional biopsy. Skin samples were embedded in optimal cutting temperature compound, frozen, and then sectioned.

Genotyping of tissue-specific knockout mice

Mouse DNA was extracted from tail tissue using DNeasy Blood & Tissue Kit (Qiagen). PCR using Taq DNA polymerase (Qiagen, Germantown, MD) was performed. The forward and reverse primers used for PCR are shown in Supplementary Table S2 online.

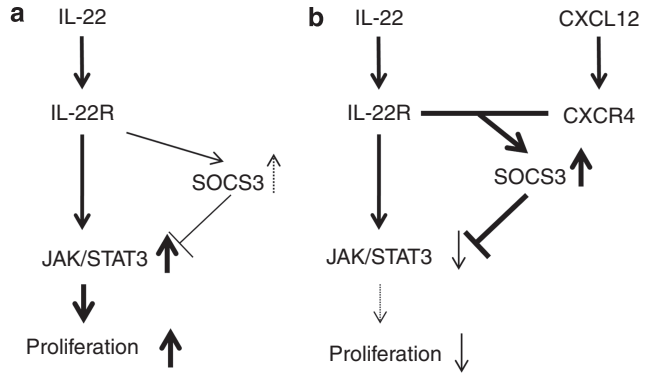

Figure 6. Schematic model of the role of CXCR4 and CXCL12 (chemokine (C-X-C motif) ligand 12) in IL-22-mediated keratinocyte (KC) proliferation. A schematic model for IL-22, CXCL12, and SOCS3 (suppressor of cytokine signaling 3) regulation of KC proliferation is shown. (**a**) JAK/signal transducer and activator of transcription 3 (STAT3) pathway is activated by IL-22 and results in modest upregulation of SOCS3, which is insufficient to downregulate keratinocyte proliferation. (**b**) However, SOCS3 expression is synergistically upregulated in the presence of IL-22, CXCR4, and CXCL12, resulting in the abrogation of STAT3 signaling and marked inhibition of KC proliferation.

Retroviral transduction of HaCaT human KC cell line

HaCaT cells were transduced with hCXCR4 complementary DNA using the pLNCX2 retroviral vector (Clontech, Mountain View, CA) as previously described (Fang *et al.*, 2008). CXCR4-overexpressing HaCaT cells (CXCR4-HaCaT) were grown in MEM (Invitrogen, Grand Island, NY) with 10% heat-inactivated fetal bovine serum (Biowest, Kansos City, MO).

Cell proliferation assay

CXCR4-HaCaT cells (2×10^4) were cultured in a six-well plate for 1 day, followed by incubation with recombinant human CXCL12 (Dr Brian Volkman, Biochemistry, Medical College of Wisconsin; Veldkamp *et al.*, 2009) and/or recombinant human IL-22 for 2 days. Cells were trypsinized, washed with PBS, and resuspended in CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) for measurement of luciferase activity.

siRNA transfection

siRNAs for SOCS3 (Silencer Select Pre-designed siRNA, s17189) and nonspecific control (Silencer Select Negative Control #1 siRNA) were purchased from Ambion (Grand Island, NY). They were used for transfection of CXCR4-HaCaT cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

IL-23 injection model

Recombinant mouse IL-23 (500 ng per site in 20 μl PBS; eBioscience, San Diego, CA) was injected into the ears of anesthetized mice every other day for 6 days, as described previously (Hedrick *et al.*, 2009). After harvesting mice ears, ventral skin sheets were incubated in PBS containing 0.5% trypsin (US Biochemical, Clevelond, OH) for 40 minutes at 37 °C to separate the epidermis from the dermis. To obtain cell suspensions, epidermal sheets were repeatedly minced with scissors (Salgado *et al.*, 1999). Cells were then filtered and washed with PBS before use.

Quantitative real-time reverse transcriptase–PCR

The extraction of RNA and complementary DNA synthesis from epidermal or dermal cell suspensions were performed using an RNeasy Kit (Qiagen) and *High Capacity complementary DNA Reverse Transcription Kits* (Applied Biosystems, Carlsbad, CA). Reverse transcriptase–PCR was performed via StepOnePlus Real-Time PCR System (Applied Biosystems) using the SYBR Green PCR master mix (Applied Biosystems). We confirmed PCR quality by performing melting curve analysis each time. The forward and reverse primers used for real-time reverse transcriptase–PCR are shown in Supplementary Table S2 online (Integrated DNA Technologies, Coralville, IA).

Western blotting

Total proteins were extracted using RIPA buffer (Sigma-Aldrich, St Louis, MO) containing protease inhibitor cocktail (Roche, Branford, CT). After centrifugation, supernatants were collected and cell lysates were reduced, boiled, loaded onto 4–12% Bis-Tris gel (Invitrogen), and transferred to nitrocellulose membranes (Invitrogen). Membranes were blocked with 2% BSA (Sigma-Aldrich) for 1 hour and incubated with anti-phosphorylated STAT3 mAb (Santa Cruz, Dallas, TX) at 1:200 dilutions for 1 hour or with anti-STAT3 p92 polyclonal antibody (eBioscience) at 1:500 overnight. Membranes were then washed three times and incubated with horseradish peroxidase–conjugated goat anti-mouse IgG (Santa Cruz) or with horseradish peroxidase–conjugated donkey anti-rabbit IgG (Santa Cruz) at 1:500 dilutions for 1 hour. After washing, membranes were incubated with chemiluminescent solution (ECL Western Blotting Detection Reagents; GE Healthcare (Pittsburgh, PA)) for 5 minutes at room temperature. Images were acquired using the ChemiDoc XRS + system (Bio-Rad, Hercules, CA). Densitometry analysis was performed using the ImageJ 1.45s software (NIH, Bethesda, MD). When required, membranes were incubated in stripping buffer (Thermo Fisher Scientific, Waltham, MA) for 30 minutes at 37 °C to remove bound antibodies.

Immunohistochemistry

Anti-CXCR4 (C-20) and anti-SOCS3 antibody (M-20; Santa Cruz) in combination with Alexa Fluor 568 donkey anti-goat IgG (H + L) secondary antibodies were used alone or with corresponding target peptides as specificity controls.

Mouse ears were excised, embedded in optimal cutting temperature compound without fixation, frozen, and then sectioned. CXCR4-HaCaT cells were cultured in Lab-TekII Chamber Slides (Thermo Scientific) for 1 day, followed by serum deprivation for 1 day. Cells were then incubated with cytokines for 1 hour and were air-dried. For CXCR4 staining, tissue sections were fixed with ice-cold acetone for 10 minutes (CXCR4 staining) or with 4% paraformaldehyde PBS solution for 10 minutes, followed by incubation with 95% ethanol for 20 minutes (SOCS3 staining); they were then blocked for 1 hour at room temperature with 5% donkey serum, Fc-blocker (2.4G2; Bio X Cell, W. Lebanon, NH), and donkey anti-goat IgG horseradish peroxidase (Santa Cruz) in PBS containing 3% skim milk. Sections were incubated with primary antibody overnight at 4 °C, followed by incubation with secondary antibody for 30 minutes at room temperature and with SlowFade Gold Antifade Reagent with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen). After image acquisition, the ImageJ 1.45s software (NIH) was used for quantification. For measuring epidermal hyperplasia, we selected the thickest epidermal regions from each mouse ear sample and measured the epidermal

thickness at four random points in a blinded manner. We counted infiltrating cells to the dermis in a blinded manner.

Statistical analysis

All quantitative data were shown as the mean ± SD unless otherwise indicated. Simple comparisons of means and SD of data were made by using Student's *t*-test, and *post hoc* multiple comparisons (indicated by * next to *P*-values) were made with one-way analysis of variance and Scheffé's test, using the Statcel statistical analysis software package (OMS Publishing, Saitama, Japan).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

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

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