Th17 Cytokines Stimulate CCL20 Expression in Keratinocytes *In Vitro* and *In Vivo*: Implications for Psoriasis Pathogenesis

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T helper (Th) 17 cells have recently been implicated in psoriasis pathogenesis, but mechanisms of how these cells traffic into inflamed skin are unknown. By immunostaining for interleukin (IL)-17A and IL-22, we show numerous cells present in psoriasis lesions that produce these cytokines. We next found that Th17 cytokines (IL-17A, IL-22, and tumor necrosis factor (TNF)-α) markedly increased the expression of CC chemokine ligand (CCL) 20, a CC chemokine receptor (CCR)6 ligand, in human keratinocyte monolayer and raft cultures in a dose- and time-dependent manner. Lastly, we showed in mice that subcutaneous injection with recombinant IL-17A, IL-22, or TNF-α led to the upregulation of both CCL20 and CCR6 expression in skin as well as cutaneous T-cell infiltration. Taken together, these data show that Th17 cytokines stimulate CCL20 production *in vitro* and *in vivo*, and thus provide a potential explanation of how CCR6-positive Th17 cells maintain their continual presence in psoriasis through a positive chemotactic feedback loop.

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INTRODUCTION

Interleukin (IL)-23 is a heterodimeric protein consisting of p19 and p40 subunits that is overexpressed in psoriasis lesional skin, as shown by increased *p19* and *p40* transcripts (Lee *et al*., 2004; Chan *et al*., 2006; Zaba *et al*., 2007) and by the presence of elevated numbers of p19-positive and p40-positive dendritic cells detected by immunohistochemistry (IHC) in the dermis of the affected skin (Lee *et al*., 2004; Piskin *et al*., 2006; Wilson *et al*., 2007). IL-23 is also expressed by keratinocytes (KC) in lesional psoriatic skin (Piskin *et al*., 2006), although transcripts and protein appear less abundant when compared with dendritic cells. By contrast, most recent reports show no increased expression of the IL-23-related protein, IL-12, in psoriasis; that is, transcripts for the IL-12-specific subunit *p35* are not increased in psoriatic plaques (Lee *et al*., 2004; Chan *et al*., 2006; Zaba *et al*., 2007). Furthermore, large-scale genomic studies have identified *IL-23R*, specific for IL-23 signaling, as a psoriasis susceptibility gene, whereas no psoriasis association was found for IL-12Rβ1, the signaling receptor for IL-12 (Capon *et al*., 2007; Cargill *et al*., 2007).

IL-23 promotes survival and proliferation of T helper (Th) 17 cells, whereas IL-12 promotes the development of Th1 cells (Blauvelt, 2007; Fitch *et al*., 2007). In psoriasis, a large body of literature supports the presence of interferon (IFN)-γ in psoriasis (Austin *et al*., 1999; Zhou *et al*., 2003; Lew *et al*., 2004), which has long been regarded as a prototypic Th1 cell-derived cytokine. Of recent relevance, a distinct subset of human Th17 cells have also been shown to produce IFN-γ (Annunziato *et al*., 2007; Acosta-Rodriguez *et al*., 2007a, b; Wilson *et al*., 2007), so that the presence of IFN-γ and downstream IFN-γ-regulated genes in psoriasis, as well as other human diseases, can no longer be equated with the presence of Th1 cells and with a ‘‘Th1 disease’’ state. Recently, transcripts for Th17 cell-specific cytokines, notably *IL-17A* and *IL-22*, have been documented at high levels in psoriasis (Chan *et al*., 2006; Wolk *et al*., 2006; Wilson *et al*., 2007; Zaba *et al*., 2007) and Th17 cells have been identified by flow cytometry in lesions of psoriasis (Lowes *et al*., 2008). Quantification of Th17 cytokine-producing cells within psoriatic tissue by IHC has not been published to date.

Little is known about how Th17 cells traffic into skin. Recently, several groups have characterized the chemokine receptor expression pattern on human Th17 cells, and found that Th17 cells, secreting IL-17A, but not IFN-γ, express CC chemokine receptors (CCR) 6 and CCR4, whereas CCR6 and CXC chemokine receptor 3 positivity identified both Th17 T

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Abbreviations: BKM, basal KC medium; CCL, CC chemokine ligand; CCR, CC chemokine receptor; HKGS, human keratinocyte growth supplement; IFN, interferon; IHC, immunohistochemistry; IL, interleukin; KC, human keratinocytes; RHE, reconstructed human epidermis; Th, T helper; TNF, tumor necrosis factor

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cells that secreted both IL-17A and IFN-γ and Th1 cells that secrete IFN-γ (Annunziato *et al.*, 2007; Acosta-Rodriguez *et al.*, 2007a, b). Consistently, differentiation of T cells into a Th17 phenotype resulted in a preferential induction of CCR6 expression and CCR6 was expressed on all IL-17-producing cells (Singh *et al.*, 2008). A recent report by Hirota *et al.* (2007) confirmed that Th17 T cells predominantly express CCR6, whereas Th1 cells do not. This group also reported that CCR6 was critical for leukocyte migration into inflamed joints in a mouse model rheumatoid arthritis (Hirota *et al.*, 2007). By contrast, Lim *et al.* (2008) reported that Th17, Th1, Th2, and regulatory T cells exhibit significant overlap in chemokine receptor expression patterns and this overlap allows for co-migration of different T-cell subsets to the site of tissue inflammation.

The primary chemokine ligands for CCR6, CC chemokine ligand (CCL) 20 and β-defensin 2, are upregulated in psoriasis (Homey *et al.*, 2000; Ong *et al.*, 2002; Wilson *et al.*, 2007). In skin, these two chemotactic molecules are produced predominantly by KC and CCL20 is a key stimulus for chemoattracting both CCR6-positive immature dendritic cells (Le Borgne *et al.*, 2006) and T cells (Varona *et al.*, 2005; Paradis *et al.*, 2007) from blood into inflamed cutaneous tissue. Currently, little is known about whether Th17 cytokines affect CCL20 production in KC. In this report, we found that IL-17A, IL-22, and tumor necrosis factor (TNF)-α stimulated CCL20 expression by KC. These results suggest a mechanism by which Th17 cells can promote their continued presence in psoriatic tissue, by stimulating a positive chemotactic feedback loop that involves KC-derived CCL20 and CCR6 expressed on Th17 cell surfaces.

RESULTS AND DISCUSSION

IL-17A-positive and IL-22-positive cells are present in psoriasis lesional skin

Transcript levels for *IL-17A* and *IL-22* are increased in psoriasis plaques (Chan *et al.*, 2006; Wolk *et al.*, 2006; Wilson *et al.*, 2007; Zaba *et al.*, 2007), however, quantification of Th17 cells within lesions of psoriasis by IHC had not been previously published. We found abundant IL-17A-positive cells (Figure 1, Table 1) and many (although fewer) IL-22-positive cells (Figure 1, Table 2) in skin affected by psoriasis. We determined optimal IHC staining conditions for these cytokines using formalin-fixed (for IL-17A IHC) and fresh-frozen (for IL-22 IHC) tissue sections (data not shown). In these experiments, IL-17A-positive cells were more numerous than IL-22-positive cells, although this could simply be a feature of differing antibody affinities for each of these cytokines. By comparison, CD3 positive T cells were on average 2.4 times more abundant than IL-17A-positive cells and 5.7 times more abundant than IL-22-positive cells (Tables 1 and 2). Single IL-17A-positive or IL-22-positive immunoreactive cells were present throughout the dermal papillae and upper dermis. No positive cells were found in more superficial regions of the epidermis. The cells were small and mostly round, consistent with the morphology of T cells. Only rare IL-17A- or IL-22-positive cells were detected in tissue sections obtained from normal skin of healthy individuals (Figure 1).

The demonstration of numerous IL-17A-positive cells and less abundant IL-22-positive cells localizing to psoriasis lesions is consistent with the high levels of transcripts for these Th17 cytokines detected by previous RT-PCR studies (Chan *et al.*, 2006; Wolk *et al.*, 2006; Wilson *et al.*, 2007; Zaba *et al.*, 2007). These data are also consistent with recent flow cytometry studies on T-cell populations isolated from psoriatic skin explants, where IL-17A+ T cells were identified in less than 10% of T cells (Kryczek *et al.*, 2008; Lowes *et al.*, 2008). Because of the time tissue manipulation and *ex vivo* culture conditions required to quantify IL-17A+ cells in psoriatic lesions by flow cytometry, we believe these methods would be predicted to underestimate the actual number of IL-17A+ cells in psoriatic tissue when compared with IHC. We doubt that our IL-17A staining is due to extracellular-bound IL-17A, as keratinocytes express IL-17 receptors, and these cells did not stain positively in our experiments (Figure 1). A limitation of our study, however, is that we cannot definitively conclude that our IL-17A+ and IL-22+ cells represent Th17 cells. It will be important to perform IHC double-labeling experiments on psoriatic tissue to identify these cells as Th17 cells.

IL-17A, IL-22, and TNF-α increase CCL20 mRNA and protein expression by normal human KC *in vitro*

Using normal human KC cultured as monolayers on plastic dishes, we found that IL-17A, IL-22, and TNF-α markedly upregulated *CCL20* mRNA at 24 hours in a dose-dependent manner (Figure 2a–c). Optimal cytokine stimulation dosage was found to be 100 ng ml^{-1} for both IL-17A and TNF-α (19- and 45-fold increases, respectively, normalized to *GAPDH* expression and compared with no cytokine stimulation), whereas 10 ng ml^{-1} was optimal for IL-22 (17-fold increase) (Figure 2a–c). In contrast, neither TGF-β1 nor IFN-γ increased *CCL20* expression by KC (data not shown). Using these optimal doses, we also found Th17 cytokines upregulate *CCL20* mRNA expression in a time-dependent manner (Figure 2d–f). The various cytokines each had somewhat different time course patterns, with IL-17A and IL-22 showing maximal CCL20 mRNA expression at 24 hours post treatment, and TNF-α at 48 hours. Dose- and time-dependent increases in CCL20 protein were also demonstrated by ELISA using cell-free supernatants of KC cultures under the same cytokine-stimulated conditions described above (Figure 3a–f).

IL-17A, IL-22, and TNF-α increase CCL20 mRNA and protein expression by reconstructed human epidermis (RHE)

KC grown as monolayers on plastic dishes differentially respond to certain cytokine signals when compared with KC grown on dermal substrates at air–liquid interfaces, which allows for KC stratification and terminal differentiation (Poumay *et al.*, 2004; Farley *et al.*, 2006, 2008). These KC cultures are called as reconstructed human epidermis (RHE) because they resemble normal human epidermis, and may be a more physiologic model for studying KC biology when compared with KC grown in monolayers on plastic. To study

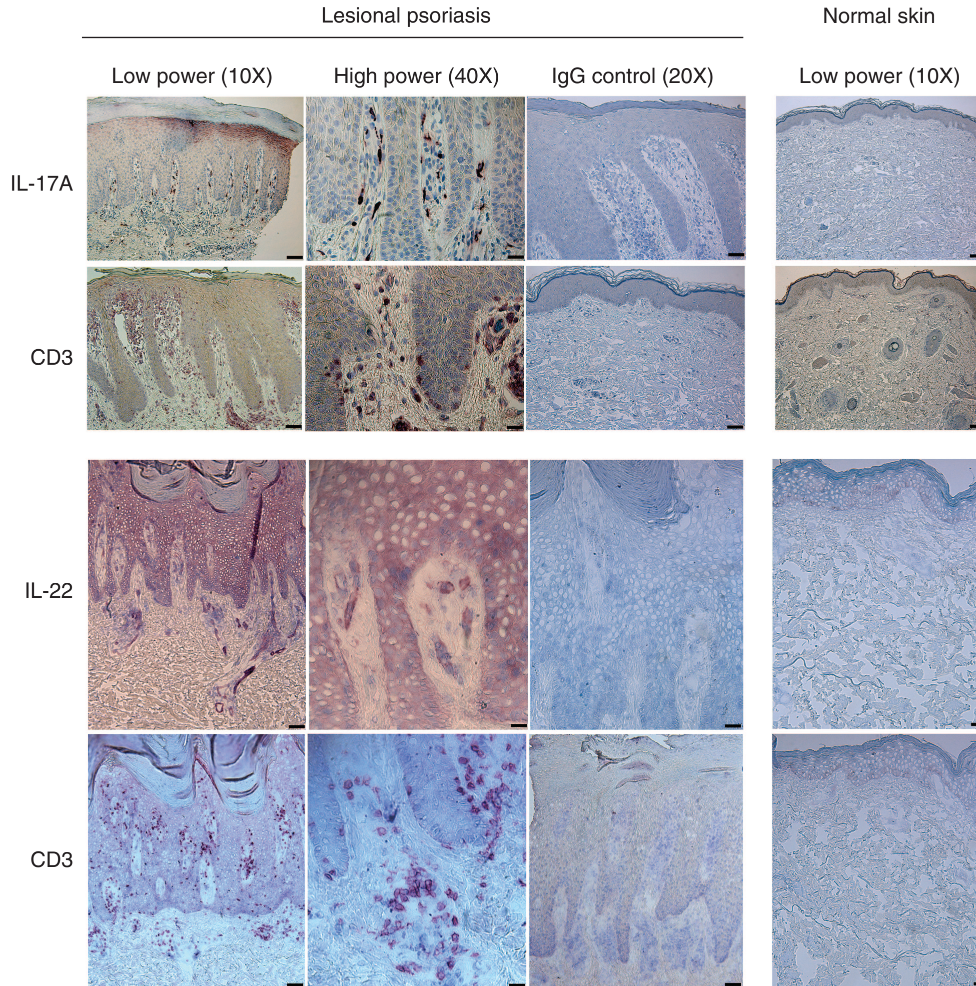

Figure 1. IL-17A-positive and IL-22-positive cells are abundant in psoriasis lesional skin. Formalin-fixed paraffin-embedded tissue sections obtained from seven psoriasis patients and three healthy individuals were examined by IHC. Tissue was stained with either anti-CD3 or anti-IL-17A antibodies. In six separate individuals with psoriasis, lesional skin was biopsied and snap frozen. Fresh-frozen sections were stained with either anti-CD3 or anti-IL-22 antibodies. Increased numbers of IL-17A- and IL-22-expressing cells (brown or purple in color) were present in lesional skin when compared with healthy skin. Representative photos are shown. Bar = 10 μm in figures labeled × 40, 20 μm in figures labeled × 20, and 40 μm in figures labeled × 10.

Table 1. IL-17A-positive cells are abundant in formalin-fixed psoriasis tissue

CASE No.	CD3 positive (cells per HPF in 10 h.p.f.)	IL-17A positive (cells per HPF in 10 h.p.f.)	CD3 positive/ IL-17A positive
1	39.6	15.5	2.6
2	43.8	24.9	1.8
3	79.3	29.4	2.7
4	89.9	43.6	2.1
5	99	49.8	2.0
6	86.1	27.2	3.2
7	103.6	43.3	2.4
Mean	77.3	33.4	2.4

HPF, high-power field.

Table 2. IL-22-positive cells are present in fresh-frozen lesional psoriasis tissue

CASE No.	CD3 positive (cells per HPF in 10 h.p.f.)	IL-22 positive (cells per HPF in 10 h.p.f.)	CD3 positive/ IL-22 positive
8	31.8	5.5	5.8
9	47.3	11.7	4.0
10	29.6	6.5	4.6
11	69.8	11.1	6.3
12	95.1	11.7	8.1
13	44.3	8.3	5.3
Mean	53.0	9.1	5.7

HPF, high-power field.

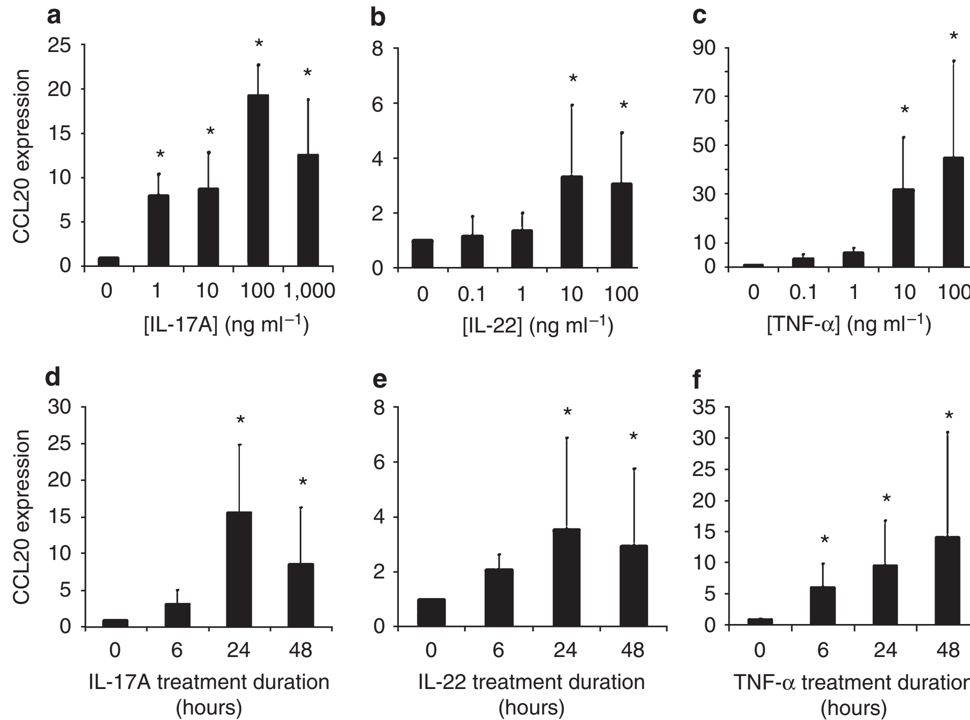

Figure 2. IL-17A, IL-22, and TNF-α increase *CCL20* mRNA expression by normal human KC in a dose- and time-dependent manner *in vitro*. Cells were treated with the indicated concentrations of (**a**) IL-17A, (**b**) IL-22, or (**c**) TNF-α for 24 hours. Cells were treated with the optimal cytokine concentrations of (**d**) IL-17A, (**e**) IL-22, or (**f**) TNF-α for 6, 24, or 48 hours. mRNA was harvested and *CCL20* transcripts were quantified by real-time RT-PCR analyses for all experiments. All experiments were performed in triplicate at least three separate times and the average mRNA increase and SD is reported. Significant differences were detected using the Mann–Whitney unpaired two-tailed *t*-test (*Indicates significance, $P<0.05$).

CCL20 mRNA and protein expression following stimulation with Th17 cytokines using this model, RHE were treated with 10 or 100 ng ml^{-1} of IL-17A, IL-22, or TNF-α for either 6 or 24 hours. IL-17A, IL-22, and TNF-α stimulated *CCL20* mRNA and protein production in a dose- and time-dependent manner (Figure 4a–f).

In both monolayers of KC and RHE, Th17 cytokines induced the expression of CCL20 mRNA and protein (Figures 2–4). In order of potency, all of the *in vitro* KC experiments suggest that TNF-α is the most potent Th17 cytokine that induces CCL20 expression, with IL-17A showing the next highest effects followed by IL-22. It is interesting to speculate that TNF-α-blocking agents for psoriasis may work at least in part by blocking TNF-α-induced upregulation of CCL20. These findings are consistent with previous reports indicating that IL-17A induces CCL20 expression by KC (Homey *et al.*, 2000), airway epithelial cells (Kao *et al.*, 2005), and synoviocytes (Hirota *et al.*, 2007). In addition, Kao *et al.* (2004) reported that IL-17A stimulated β-defensin 2, another CCR6 ligand, in airway epithelium. Importantly, we extend the KC results of (Homey *et al.*, (2000) by (1) showing that additional Th17 cytokines can stimulate CCL20 production (Figures 2–4), by (2) reporting that RHE, in addition to monolayers of KC, can be stimulated by Th17 cytokines to produce CCL20 (Figure 4), and by (3) showing that injection of these cytokines induces the upregulation of CCL20 and CCR6 as well as T-cell infiltration *in vivo* (see Figure 5 below).

CCL20 and CCR6 upregulation and T-cell infiltration in murine skin injected with Th17 cytokines

Ears of Balb/c mice were injected with 500 ng of recombinant murine IL-17A, IL-22, TNF-α, or PBS daily for 5 days. Total RNA or total protein were extracted from each ear and *CCL20* and *CCR6* mRNA expression was assessed by real-time RT-PCR, whereas CCL20 protein expression was assessed by ELISA. *CCL20* mRNA was markedly upregulated by all of the Th17 cytokines, with TNF-α causing the greatest increase (mean of 55-fold compared with PBS-injected ears), and IL-17A and IL-22 causing 3-fold and 2.5-fold elevations, respectively (Figure 5a). Both TNF-α and IL-17A injections led to concomitant increases in CCL20 protein levels in cytokine-injected ears (Figure 5b), whereas IL-22-injected ears showed no clear elevations in CCL20 protein expression in these experiments. Similarly, *CCR6* mRNA was elevated by TNF-α, IL-17A, and IL-22 by 4-, 2-, and 2.5-fold, respectively, when compared with PBS-injected ears (Figure 5c). Lastly, we showed that Th17 cytokine injections induced CD3-positive T-cell infiltration within murine skin (Figure 5d). Only rare T cells were observed in PBS-injected skin (Figure 5d).

CCR6–CCL20 interactions have been studied in the context of dendritic cell and T-cell trafficking in other murine diseases. Specifically, immature CCR6-positive dendritic cells were recruited to sites of inflamed epithelial tissue in mice following trauma (Le Borgne *et al.*, 2006). CCR6 was also shown to be critical in T-cell trafficking into skin in murine models of graft-versus-host disease (Varona *et al.*,

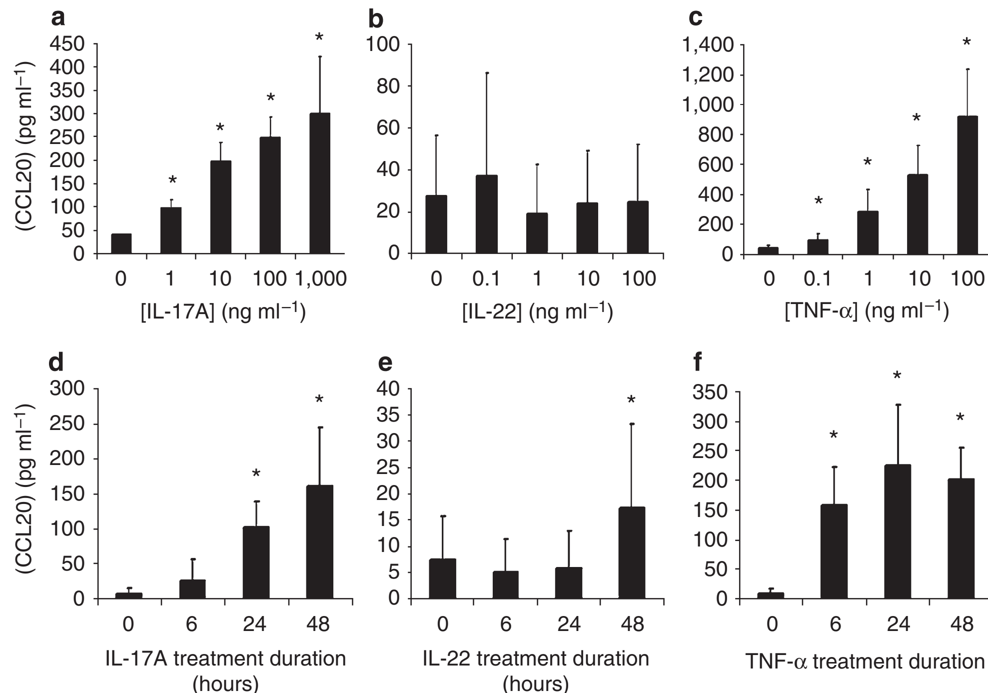

Figure 3. IL-17A, IL-22, and TNF-α increase CCL20 protein expression by normal human KC in a dose- and time-dependent manner *in vitro*. Cells were treated with the indicated concentrations of (**a**) IL-17A, (**b**) IL-22, or (**c**) TNF-α for 24 hours. Cells were treated with the optimal cytokine concentrations of (**d**) IL-17A, (**e**) IL-22, or (**f**) TNF-α for 6, 24, or 48 hours. Cell-free supernatants were harvested and CCL20 protein levels were quantified by ELISA for all experiments. All experiments were performed in triplicate at least three separate times and the average protein levels and SD are reported. Significant differences were detected using the Mann–Whitney unpaired two-tailed *t*-test (*Indicates significance, $P<0.05$).

2005) and contact hypersensitivity (Paradis *et al.*, 2007). Recently, CCR6–CCL20 interactions were shown to be critical in mediating leukocyte migration into inflamed joints in a mouse model of rheumatoid arthritis; anti-CCR6 monoclonal antibodies inhibited Th17 T-cell trafficking and early disease development in this model (Hirota *et al.*, 2007). In short-term 5-day experiments, we show for the first time that Th17 cytokines induce CCL20 and CCR6 *in vivo*, with influx of T cells into skin. It is noteworthy that we did not observe keratinocyte hyperproliferation following 5 days of IL-22 injections (Figure 5d). This is not surprising, given that IL-22-mediated keratinocyte hyperproliferation *in vivo* takes longer to occur (approximately 2 weeks) (Ma *et al.*, 2008). Our results do not exclude other chemokine–chemokine receptor interactions in mediating Th17 cell chemotaxis (for example, involvement of CCR4). In fact, prominent redundancy in leukocyte-trafficking pathways has been well documented. Thus, elucidation of additional mechanisms by which Th17 cells may enter the skin requires further study.

Our findings suggest a novel paradigm for human Th17 disease. Human Th17 cells are able to maintain their own presence in peripheral inflamed tissue by secreting cytokines that stimulate epithelial cells to produce the chemokine CCL20, which in turn induces chemotaxis of additional Th17 cells. This biologic paradigm fits with clinical disease features of psoriasis. Clinical features of skin affected by psoriasis (for example, erythema, induration, and scaling) are remarkably stable when untreated. Persistent presence of Th17 cells within the dermis driven by a continual chemotactic gradient coming from epidermal KC would manifest as phenotypically stable skin. Breaking this cycle would require the death of Th17 cells. The experimental biologic agents ustekinumab and ABT 874, which are monoclonal antibodies directed against the p40 subunit of IL-23, lead to dramatic clinical improvement in psoriasis (Krueger *et al.*, 2007; Kimball *et al.*, 2008; Leonardi *et al.*, 2008; Papp *et al.*, 2008). We postulate that these agents are clinically effective in psoriasis because they block IL-23, the cytokine signal needed by Th17 cells to survive. Death of Th17 cells would stop Th17 cytokine production, and thus break the chemotactic gradient present in psoriasis. Future mechanism of action studies using psoriatic skin obtained from individuals receiving anti-p40 monoclonal antibody therapy are necessary to test this hypothesis. Our findings also suggest the possible therapeutic use of agents designed to block chemokine–chemokine receptor interactions in patients with psoriasis.

MATERIALS AND METHODS

IHC

The OHSU Institutional Review Board approved all of the described studies, and the study was conducted according to the Declaration of Helsinki Principles. For archival tissue, written informed consent was waived as the tissue specimens were anonymized. Formalin-fixed, paraffin-embedded lesional skin obtained from seven individuals with psoriasis and three healthy individuals were retrieved from the OHSU dermatopathology archives maintained by Dr Clifton White. Sections were de-paraffinized and hydrated through heating and washing sections in a graded alcohol series. The sections

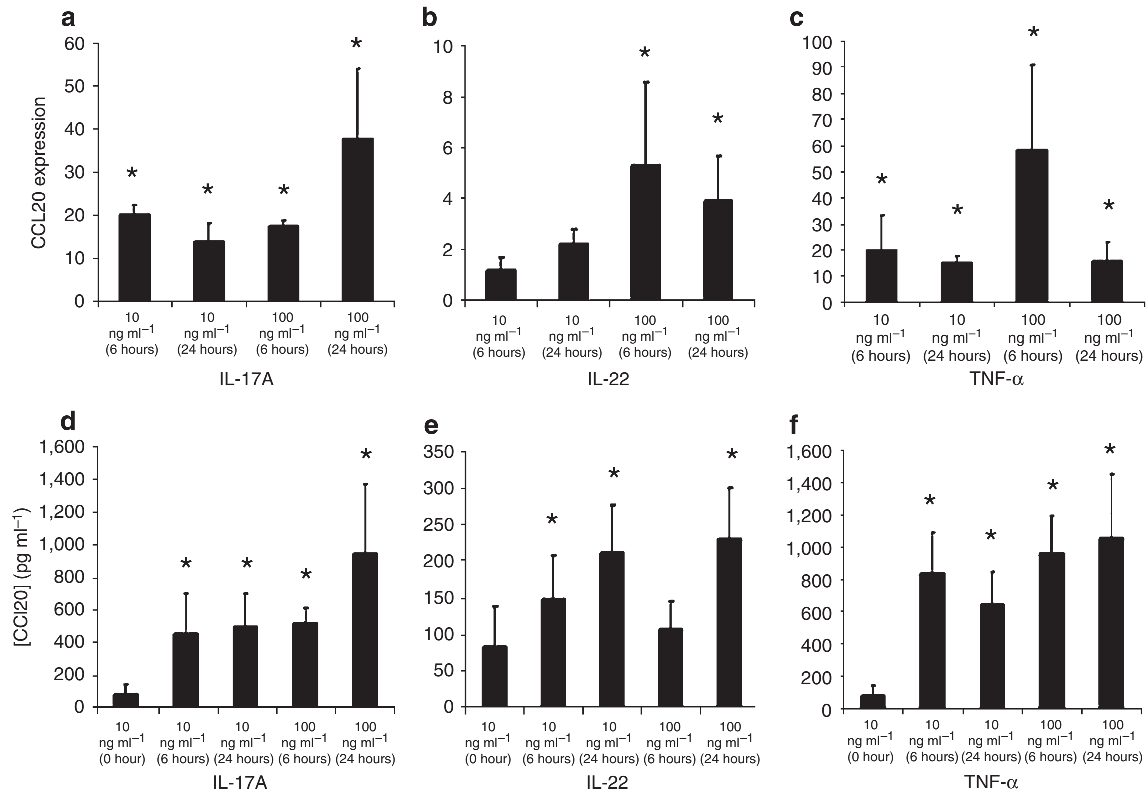

Figure 4. IL-17A, IL-22, and TNF-α increase CCL20 mRNA and protein expression by RHE in a dose- and time-dependent manner. Stratified KC in RHE were treated with the indicated concentrations of (**a**) IL-17A, (**b**) IL-22, or (**c**) TNF-α for 6 or 24 hours. Total RNA was harvested and *CCL20* transcripts were quantified by real-time RT-PCR analyses. RHE were treated with the optimal cytokine concentrations of (**d**) IL-17A, (**e**) IL-22, or (**f**) TNF-α for 6 or 24 hours. Cell-free supernatants were harvested and CCL20 protein levels were quantified by ELISA. All experiments were performed in triplicate at least three separate times and the average protein levels and SD are reported. Significant differences were detected using the Mann–Whitney unpaired two-tailed *t*-test (*Indicates significance, $P<0.05$).

were incubated in antigen-unmasking solution (Vector Laboratories, Burlingame, CA) at 95 °C for 20 minutes, and then exposed to either goat anti-human IL-17A polyclonal Abs (R&D Systems, Minneapolis, MN) or rabbit anti-human CD3 (Thermo Fisher Scientific, Waltham, MA) monoclonal Abs overnight at 4 °C. Samples were washed and incubated for 1 hour with biotinylated anti-goat or anti-rabbit secondary Abs (Vector Laboratories), followed by 1 hour incubation with streptavidin-conjugated horseradish peroxidase (Vectastain Elite ABC kit, Vector Laboratories), developed using Vector VIP substrate kit for peroxidases (Vector Laboratories), and counter-stained with hematoxylin (Vector Laboratories).

For fresh tissue, patients first gave their written informed consent. Four-mm punch biopsies of lesional skin from six individuals with psoriasis were obtained and frozen immediately in OCT compound. Frozen sections were cut, fixed with methanol for 10 minutes, and then incubated with either rabbit anti-human IL-22 polyclonal Abs (Capralogics, Hardwick, MA) or CD3 mAbs overnight at 4 °C. Samples were washed and incubated for 1 hour with biotinylated anti-rabbit secondary Abs (Vector Laboratories) and developed and counter-stained as above.

Frozen mouse skin sections were cut and fixed in methanol for 10 minutes and incubated overnight with rat anti-mouse CD3 monoclonal Abs (Biolegend, San Diego, CA). Sections were then incubated for 1 hour with biotinylated goat anti-rat secondary Abs (Jackson ImmunoResearch Laboratories, West Grove, PA), for 45 minutes with streptavidin-conjugated horseradish peroxidase (Vectastain Elite ABC kit, Vector Laboratories), and developed and counter-stained as above.

All IHC images were captured using a microscope equipped with Imagepro software. For each antibody, the number of positive cells was counted in 10 separate high-powered ($\times 40$) fields and then divided by 10 to obtain the average number of positive cells per high-powered field.

Normal human KC cultures

Primary normal human KC (Cascade Biologics, Portland, OR) were cultured in Keratinocyte Growth Media containing bovine pituitary extract, human epidermal growth factor, insulin, hydrocortisone, gentamicin, and amphotericin B. Cytokine stimulation experiments were initiated on 60% confluent KC cultured between passages 2–5 in 6 cm plastic dishes at 37 °C in the presence of 0.05% heat-inactivated bovine serum albumin and in the absence of hydrocortisone. KC were cultured either alone or with one of the following cytokines: 1, 10, 100, or 1,000 ng ml^{-1} of IL-17A; 0.1, 1, 10, or 100 ng ml^{-1} of IL-22, TNF-α, or IFN-γ; or 0.002, 0.02, 0.2, or 2 ng ml^{-1} of TGF-β1 (R&D Systems). At indicated time points following cytokine exposure, conditioned culture media and cells were collected for CCL20 protein and mRNA quantification, respectively.

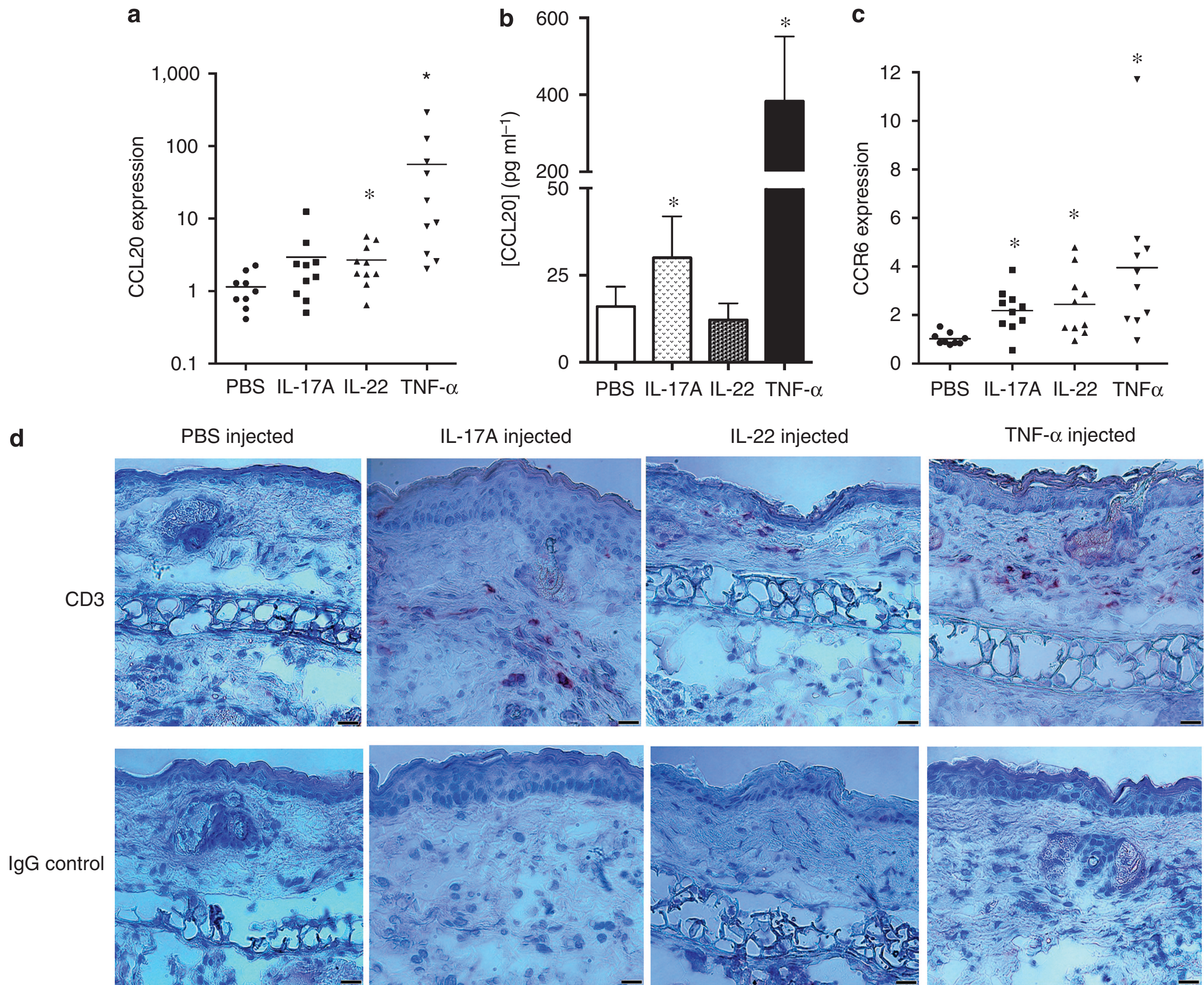

Figure 5. CCL20 and CCR6 upregulation and T-cell infiltration in murine skin injected with Th17 cytokines. (**a–c**) Balb/c mouse ears were injected with 500 ng of IL-17A, IL-22, TNF-α, or PBS daily for 5 days. (**a** and **c**) *CCL20* and *CCR6* mRNA expression levels in injected ears were determined by real-time RT-PCR. Significant differences were detected using the Mann–Whitney unpaired two-tailed *t*-test (*Indicates significance, $P<0.05$). (**b**) CCL20 protein expression was measured by ELISA. Significant differences were detected using the unpaired *t*-test with Welch's correction (*Indicates significance, $P<0.05$). (**a–c**) Data from 10 mice in each treatment group are shown. (**d**) Representative histologic photos showing CD3 positive T cells in murine skin injected with Th17 cytokines. Bar = 40 μm.

RHE cultures

HEKn-E6/E7 cells were established and propagated as described (Iordanov *et al.*, 2002, 2005). HEKn-E6/E7 were propagated in EpiLife basal KC medium (BKM) (Cascade Biologics) supplemented with a semi-defined human keratinocyte growth supplement (HKGS) (Cascade Biologics), which included bovine pituitary extract, bovine insulin, hydrocortisone, bovine transferrin, and human epidermal growth factor. RHE were established as described (Poumay and Coquette, 2007). Briefly, HEKn-E6/E7 cells were plated at 5×10^5cells per ml on 0.4 μm, 12 mm-diameter polycarbonate membranes (Millipore, Billerica, MA) and allowed to reach confluence in BKM + HKGS. Cells were then exposed to the air–liquid interface by removing the medium above the confluent monolayer. Simultaneously, the cells were exposed from underneath the monolayer to a differentiation medium (BKM + HKGSF supplemented with 1.5 mM $CaCl_2$, 50 μg ml^{-1} ascorbic acid, and 10 ng ml^{-1} rhKGF (Peprotech, Rocky Hill, NJ)). The differentiation medium was changed every 48 hours for 14 days. RHE cultures were treated with 10 or 100 ng ml^{-1} of IL-17A, IL-22, or TNF-α, and incubated at 37 °C. At indicated time points following cytokine exposure, conditioned culture media and cells were collected for CCL20 protein and mRNA quantification, respectively.

Real-time RT-PCR

Cells from normal human KC monolayer and RHE cultures were lysed directly in TRIzol, whereas mouse ear tissue was minced first and then placed in TRIzol and total RNA was extracted according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Integrity of RNA was determined by the appearance of distinct 28S and 18S rRNA bands when analyzed by electrophoresis on 1% agarose gels. The iScript kit (Bio-Rad, Hercules, CA) was employed for cDNA synthesis and resulting cDNA was analyzed to determine CCL20

mRNA levels relative to GAPDH mRNA levels. cDNA was amplified using SYBR green (Bio-Rad) and the following primers: human CCL20, forward TACTCCACCTCTGCGGCGAATCAGAA, and reverse GTGAAACCTCCAACCCCAGCAAGGTT; human GAPDH, forward GAGTCAACGGATTTGGTCGT, and reverse TTGATTTTG GAGGGATCTCG. Primers were annealed at 60 °C and amplified using My IQ single color RT-PCR detection system (Bio-Rad) for a total of 40 cycles.

ELISA

Secreted CCL20 protein levels in conditioned human KC culture media were quantified using the Quantikine ELISA kit according to the manufacturer's protocol (R&D Systems). For the murine studies, total protein was extracted from ears by snap-freezing tissue in liquid nitrogen, pulverizing tissue while immersed in liquid nitrogen, and subsequently re-suspending in cell extraction buffer (Invitrogen). Samples underwent three freeze-thaw cycles and were centrifuged at full speed for 15 minutes to fractionate samples and remove protein. Mouse CCL20 ELISA (R&D Systems) was performed according to the manufacturer's instructions using 150 μg total protein.

Animal studies

The Portland VA Medical Center Institutional Animal Care and Use Committee approved all of the described studies using mice. Wild type Balb/c mice were injected in each ear with either 500 ng of IL-17A, IL-22, or TNF-α in 25 μl of PBS/0.1% of BSA, or with 25 μl of PBS/0.1% BSA alone. Ten animals per cytokine treatment group were injected daily for 5 days. All animals were killed at day 5 and mRNA was extracted from ears using a standard Trizol protocol, followed by further purification using an RNeasy kit (Qiagen, Valencia, CA). cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad) and amplified using Taqman primers and fluorescent probes for *GAPDH*, *CCL20*, and *CCR6* using My IQ single color RT-PCR detection system for a total of 40 cycles.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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