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References

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Up-Regulation of Macrophage Inflammatory Protein-3α/CCL20 and CC Chemokine Receptor 6 in Psoriasis

Bernhard Homey,* 1 Marie-Caroline Dieu-Nosjean,2† Andrea Wiesenborn,‡ Catherine Massacrier,‡ Jean-Jacques Pin,‡ Elizabeth Oldham,* Daniel Catron,* Matthew E. Buchanan,* Anja Müller,* Rene deWaal Malefyt,* Glenn Deng,* Rocio Orozco,§ Thomas Ruzicka,‡ Percy Lehmann,‡ Serge Lebecque,‡ Christophe Caux,‡ and Albert Zlotnik3* 1

Autoimmunity plays a key role in the immunopathogenesis of psoriasis; however, little is known about the recruitment of pathogenic cells to skin lesions. We report here that the CC chemokine, macrophage inflammatory protein-3α, recently renamed CCL20, and its receptor CCR6 are markedly up-regulated in psoriasis. CCL20-expressing keratinocytes colocalize with skin-infiltrating T cells in lesional psoriatic skin. PBMCs derived from psoriatic patients show significantly increased CCR6 mRNA levels. Moreover, skin-homing CLA+ memory T cells express high levels of surface CCR6. Furthermore, the expression of CCR6 mRNA is 100- to 1000-fold higher on sorted CLA+ memory T cells than other chemokine receptors, including CXCR1, CXCR2, CXCR3, CCR2, CCR3, and CCR5. In vitro, CCL20 attracted skin-homing CLA+ T cells of both normal and psoriatic donors; however, psoriatic lymphocytes responded to lower concentrations of chemokine and showed higher chemotactic responses. Using ELISA as well as real-time quantitative PCR, we show that cultured primary keratinocytes, dermal fibroblasts, and dermal microvascular endothelial and dendritic cells are major sources of CCL20, and that the expression of this chemokine can be induced by proinflammatory mediators such as TNF-α/IL-1β, CD40 ligand, IFN-γ, or IL-17. Taken together, these findings strongly suggest that CCL20/CCR6 may play a role in the recruitment of T cells to lesional psoriatic skin. The Journal of Immunology, 2000, 164: 6621–6632.

Psoriasis is a common chronic inflammatory skin disease characterized by a marked inflammatory infiltrate and hyperproliferation of keratinocytes. The infiltrate is composed of skin-infiltrating T cells, predominantly of the memory phenotype, neutrophils, lining macrophages, and increased numbers of dendritic cells (1–3). There is evidence that T cells play a crucial role in the immunopathogenesis of this disease (4–14). An early cellular event in the development of psoriatic lesions is the infiltration of target sites by activated T cells, which, in turn, produce inflammatory mediators, such as IFN-γ, induce epidermal hyperplasia, and may act with keratinocytes and dermal macrophages to sustain a cycle of inflammation that finally leads to the psoriatic phenotype (15).

We have previously cloned and characterized a CC chemokine designated macrophage inflammatory protein-3α (MIP-3α;4 recently renamed CCL20 (16)) and identified CCR6 as its receptor (17, 18). CCL20 is known to attract both T and dendritic cells (19, 20). Among dendritic cells, CCL20 is a highly potent chemokine for the chemoattraction of epithelial Langerhans-type dendritic cells derived from CD34+ hematopoietic progenitor cells (19). Recently, CCL20 has been shown to preferentially attract the memory subset of T cells (21, 22).

We sought to identify chemokines and chemokine receptors involved in autoimmune diseases. To this end, we undertook a systematic analysis of the expression of mRNA of various chemokines and receptors in samples of inflammatory skin diseases using real-time quantitative PCR. Here, we report that the expression of the CC chemokine CCL20 and its receptor CCR6 is significantly up-regulated in psoriasis. Within psoriatic lesions, CCL20-expressing keratinocytes colocalize with skin-infiltrating T lymphocytes. Furthermore, CCR6 is expressed at high levels on the skin-homing CLA+ subset of memory T cells. Moreover, psoriatic skin-homing CLA+ T cells showed increased chemotactic responses to CCL20 gradients compared with those of normal donors. Finally, biologically active CCL20 is induced in cellular constituents of the skin by proinflammatory cytokines and T cell-derived inflammatory mediators. Taken together, these observations strongly suggest that this ligand/receptor pair is likely to play a unique role in the pathogenesis of psoriasis.

Materials and Methods

Patients

Six millimeter punch biopsies were taken, after obtaining informed consent, from either lesional (n = 10) and nonlesional (n = 5) skin of psoriatic patients or from normal (n = 5) healthy individuals. Skin samples were immediately frozen in liquid nitrogen and stored at −80°C. In addition, 50 ml of heparinized blood was drawn from either psoriatic patients (n = 15) in lesional phases of the disease or healthy donors (n = 3), and PBMC were prepared using standard protocols. The psoriatic patients used in this study had been untreated for at least 3 wk. This study was approved by the local ethics committees.
Quantitative real-time PCR (TagMan) analysis of MIP-3α/CCL20 and CCR6 mRNA expression

Skin biopsies were homogenized in liquid nitrogen using a Mikro-Dismembrator U (Braun Biotech, San Diego, CA), and RNA was extracted with RNeasyol according to the manufacturer’s protocol (Tel-Test, Friendsville, TX). Four micrograms of RNA was treated with DNase I (Roche, Mannheim, Germany) and reverse transcribed with oligo(dT)12-18 (Life Technologies, Gaithersburg, MD) and random hexamer primers (Promega, Madison, WI) using standard protocols. cDNA was diluted to a final concentration of 5 ng/μl. Ten microliters of cDNA was amplified in the presence of 12.5 μl of TaqMan universal master mix (Perkin-Elmer, Foster City, CA), 0.625 μl of gene-specific TaqMan probe, 0.5 μl of gene-specific forward and reverse primers, and 0.5 μl of water. As an internal positive control, 0.125 μl of 18S RNA-specific TaqMan probe and 0.125 μl of 18S RNA-specific forward and reverse primers were added to each reaction. Specific primers and probes for CCL20, CCR6, and the other chemokine receptors measured were obtained from Perkin-Elmer. Gene-specific probes used FAM as reporter, whereas probes for the internal positive control (18S RNA) were associated with either the JOE or VIC reporter. Samples underwent the following stages: stage 1, 50°C for 2 min; stage 2, 95°C for 10 min; and stage 3, 95°C for 15 s followed by 60°C for 1 min. Stage 3 was repeated 40 times. Gene-specific PCR products were measured by means of an ABI PRISM 7700 Sequence Detection System (Perkin-Elmer) continuously during 40 cycles. The specificity of primer probe combination was confirmed in cross-reactivity studies performed against plasmids of all known chemokine receptors (CCR1-CCR9, CXCR1-CXCR5, CCR10, CX3CR1) and the following panel of chemokines: MIP-1α/CCL3, MIP-1β/CCL4, MIP-1α/CCL15, MIP-8/CCL19, 6-cloneCCL21, IL-10/CXCL10, MIG/CXCL9, I-309/CCL1, I-TAC/CXCL11, HCC-1/CCL14, HCC-4/CCL16, GRO-α/GCP1/CCL2, ENA/78/CCL5, eotaxin/CCL11, eotaxin-2/CCL24, DC-CK1/CCL18, BCA-1/CXCL13, fractalkine/CX3CL1, stromal-derived factor (SDF)-1α/CXCL12, RANTES/CCL5, PF4/CXCL4, macrophage-derived chemokine/CCL22, lymphotactin/CXCL1, IL-8/CXCL8, thymus and activation-regulated chemokine/CCL17, thymus-expressed chemokine/CCL25, MCP-1/CCL2, MCP-2/CCL4, MCP-3/CCL7, and MCP-4/CCL13. Target gene expression was normalized between different samples based on the values of the expression of the internal positive control. Human cDNA libraries used in this study were generated as described previously (17, 23, 24).

Cell culture

Human primary epidermal keratinocytes, dermal fibroblasts, melanocytes and dermal microvascular endothelial cells were purchased from Clonetics (San Diego, CA) and cultured in keratinocyte, fibroblast, melanocyte, or dermal microvascular endothelial cell growth medium (Clonetics) as previously described (25). Cells were treated with TNF-α (10 ng/ml)/IL-1β (5 ng/ml), IFN-γ (20 ng/ml), IL-4 (50 ng/ml), and IL-17 (100 ng/ml; R&D Systems, Minneapolis, MN) or were left untreated. The epithelial y6 T cell line, 7-17, was provided by Richard Boismenu (The Scripps Institute, La Jolla, CA) and cultured as described previously (26). Epidermal y6 T cells were cultured with 1 μg/ml IL-2 (rIL-2, Genzyme) for medium alone and 10 ng/ml IL-1β, or 5% FBS, as indicated. Generation of dendritic cells either from cord blood CD34+ hematopoietic progenitor cells or from peripheral blood monocytes was performed as described previously (19). Immature dendritic cells from CD34+ hematopoietic progenitor cells or monocyte-derived dendritic cells were activated for 3–24 h in the presence of CD40 ligand (CD40L)-transfected L cells (one per five dendritic cells) as described previously (27). Cells and supernatants were harvested, 3, 12, 24, and 48 h after CD40L stimulation. RNA was extracted from cells as described above.

Flow cytometry and cell sorting

To analyze chemokine receptor expression of skin-homing T cell subsets, CLA+/CD45RO−/CD4+ cells were sorted from PBMCs isolated from two different donor pools comprising buffy coats (70 ml) from three individual donors, each using a FACS Vantage (Becton Dickinson, San Jose, CA) and the following mAbs: PE-conjugated anti-CLA, FITC-conjugated anti-CD45RO, APC-conjugated anti-CD4, FITC-conjugated anti-CD45RO (UCHL1) mAb (PharMingen), APC-conjugated anti-CD8 (RPA-T8) mAb (PharMingen), Cy-Chrome-conjugated anti-CD4 (RPA-T4) mAb (PharMingen), anti-CCR6-PE-conjugated (53103.111) mAb (R&D Systems), and mouse IgG2b-PE-conjugated (R&D Systems). Briefly, 105 PBMCs were stained with anti-CD4, anti-CD8, anti-CLA, or anti-CCR6 mAb or isotype and analyzed using a FACS Calibur and CellQuest software (Becton Dickinson).

Chemotaxis

PBMCs from either normal or psoriatic donors were incubated for 1 h at 37°C. Nonadherent cells were harvested and used for Transwell chemotaxis assays. Enriched lymphocytes in DMEM (pH 6.9)1%/BSA were added to the top chamber of 3-μm pore size polycarbonate Transwell culture insert (Costar, Cambridge, MA) and incubated for 4 h. Supernatants as well as cells were harvested after 6 or 18 h. The number of migrating cells was determined by flow cytometry with anti-CLA (HECA-452) and anti-CD3 mAbs (PharMingen). A known number of 15-mm microsphere beads (Bangs Laboratories, Fishers, IN) was added to each sample before analysis to determine the absolute number of migrating cells. Chemotaxis indices were calculated as the ratio of the number of cells migrating toward CCL20 divided by the number of migrating cells in the negative control.

In situ hybridization

In situ hybridization was performed as previously described (19). Coupled primers were used for amplifying by RT-PCR the majority of the open reading frame of the CCL20 gene. +77/MIP-3α/CCL20 (5′-TTGCTTCTGGCTGTCTTTG-3′, forward primer) and −425/MIP-3α/CCL20 (5′-AC CCTCCATGATGATGTGCAAG-3′) were used with an annealing temperature of 62°C. Then, PCR products were cloned into pCRII TA cloning vector (Invitrogen, Leek, The Netherlands). RNA probes were synthesized using Sp6 and T7 RNA polymerases (Roche) and radio labeled with [35S]UTP (Amersham, Aylesbury, U.K.). Sense and antisense 35S-labeled probes of CCL20 were obtained by run-off transcription of the 367-bp fragment and then partially degraded by alkaline hydrolisis for 20 min at 60°C. Six-micron cryostat sections were prepared on charged electrotastic slides (SuperFrost/Plus, Polylabo, Strasbourg, France) and fixed with cold acetone for 20 min and with 4% paraformaldehyde for 20 min, followed by 0.1 M triethanolamine/0.25% acetic anhydride. The sections were hybridized overnight at 50°C (2.5 × 106 cpm/slide), RNase A treated, and exposed for 40 days. After development, the sections were stained with hematoxylin.

Immunohistochemistry

Frozen 6-μm tissue sections were fixed in acetone and paraformaldehyde before the immunostaining. To block the nonspecific binding of avidin, biotin system components, or endogenous peroxidase activity, sections were pretreated with avidin D and biotin solutions (Blocking kit, Vector, Biosys, Compiegne, France) for 10 min each step and with PBS containing 0.3% hydrogen peroxide (Sigma, St. Louis, MO) for 15 min at room temperature. After brief washing in PBS, the sections were incubated with 1% goat serum (SuperFrost/Plus, Polylabo, Strasbourg, France) for at least 30 min. Negative controls were established by adding goat IgG to the secondary Ab. Sections were double stained simultaneously with anti-human MIP-3α/CCL20 and goat polyclonal Ab (IgG isotype, R&D Systems) and anti-human CD3 mouse Ab (Leu 4, IgG1 isotype, Becton Dickinson, Mountain View, CA) for 1 h at room temperature in a humid atmosphere. The binding of goat IgG was detected using biotinylated rabbit anti-goat IgG followed by streptavidin-peroxidase (both included in the Vectastain ABC kit: Vector, Burlingame, CA) for1 h at room temperature in a humid atmosphere. The peroxidase and alkaline phosphatase activities were revealed using 3-amin-9-ethylcarbazole substrate (SK-4200, Vector) and alkaline phosphatase substrate III (SK-5300, Vector) for 5–10 min at room temperature, respectively. Negative controls were established by adding nonspecific isotype controls as primary Abs.

Generation of mouse Abs against hMIP-3α/CCL20 and development of an hMIP-3α/CCL20 ELISA

Inbred BALB/c mice were immunized with three successive i.p. injections of CFA (Sigma, St. Louis, MO) or IFA or without Freund’s adjuvant, respectively, with 50 ng of purified hCCL20 obtained from supernatants of hCCL20 transient-transfected COPI cells. Splenes were removed for fusion 3 days after a final i.v. injection of hCCL20. Hybridization was conducted using the nonsecreting myeloma cell line SP2/0-Ag14 with polyethylene glycol 1000 (Sigma). Human CCL20 transient-transfected COPI cells were cultured for 2 days in 96-well plates and fixed in acetone. Then, hybridoma supernatants were harvested after 6 days and incubated for 30
min on fixed hCCL20 transient-transfected COP5 cells. Ab binding was then revealed with peroxidase-conjugated sheep anti-mouse IgG (Biosys) at a 1/200 dilution in PBS for 30 min at 37°C. Positive hybridomas were cloned by limiting dilution and expanded using a high density culture system (Integra cell line CL1000, Integra Biosciences, Paris, France). After sodium sulfate precipitation, the mAbs were purified by anion exchange chromatography on a HyperD column and peroxidase-labeled (Sepracor, Villeneuve, France). An ELISA was set up using one of the hCCL20 mAbs, 319F6, as a capture mAb and a peroxidase-coupled mouse anti-hCCL20 mAb to reveal the captured hCCL20. The assay proved to be specific for hCCL20 with a sensitivity of 0.2 ng/ml.

**Analysis of hMIP-3α/CCL20 bioactivity by calcium mobilization assay**

A cell line expressing the human CCR6 chemokine receptor was provided by Chuan Chu Chou (SPRI, Kennilworth, NJ). Briefly, the CCR6 open reading frame was cloned into the pME18sneo eukaryotic expression vector and transfected into the murine B cell line, BAF/3, by electroporation. Stable transfectants were isolated by selection in medium containing 1 mg/ml G418. CCR6 expression was confirmed using calcium signaling, ligand binding analysis with recombinant human CCL20 (R&D Systems), and immunohistochemistry with anti-CCR6 (53103.111) mAb (R&D Systems). The average number of binding sites per cell was 220,000. To measure the biological activity of the CCL20 produced by keratinocytes, fibroblasts, or endothelial cells, supernatants from these cell cultures were concentrated 20-fold using Centriplus concentrators with a cutoff of 3 kDa (Amicon, Beverly, MA). The calcium response to supernatants from these resting or activated cells was measured using standard protocols (18). Briefly, the BAF/3 parental and CCR6 transfectant were loaded for 60 min at 37°C with 3 μM Indo-1A (Molecular Probes, Eugene, OR). Cells were washed and resuspended in HBSS (Life Technologies/BRL, Grand Island, NY) to a final concentration of 10⁷ cells/ml. Calcium mobilization was quantified using a fluorometer (Molecular Probes) and corrected for cell numbers. The calcium responses were normalized to total cell numbers and expressed as percent change from basal levels.

**FIGURE 1.** Quantitative TaqMan PCR analysis of MIP-3α/CCL20 and CCR6 expression in psoriasis. (A and B) Pattern of CCR6 and CCL20 expression in normal (n = 5) and nonlesional (n = 5) psoriatic skin vs lesional (n = 10) psoriatic skin. Values are expressed as femtograms of target gene in 50 ng of total cDNA. Measurements of individual samples and the mean ± SD are shown. A Student t test was performed. *p < 0.005. C. Level of CCR6 expression in PBMCs derived from either psoriatic patients in lesional phases of the disease (n = 10) or healthy donors (n = 5). Values are expressed as femtograms of target gene in 50 ng of total cDNA (mean ± SD). Student t test was performed. **p < 0.001.
measured using a spectrophotometer (Photon Technology International, South Brunswick, NJ) with excitation at 350 nm and dual simultaneous recording of fluorescence emission at 400 and 490 nm. Relative intracellular calcium levels are expressed as the 400 nm/490 nm emission ratio. Experiments were performed at 37°C with constant mixing in cuvettes containing 10^6 cells in 2 ml of HBSS with 1 mM CaCl_2. To demonstrate the specificity of CCL20-induced calcium mobilization, neutralization studies were performed using a blocking mouse anti-human CCL20 (IgG1; 67310.111) mAb (R&D Systems) or isotype control (IgG1; Sigma).

**Results**

CCL20 and its receptor CCR6 are significantly up-regulated in lesional psoriatic vs nonlesional or normal skin

After cloning and initial characterization of mouse and human CCL20 and identification of its receptor, CCR6, we were interested in investigating the potential role of this ligand-receptor pair in human diseases (17, 18). To this end, we undertook a systematic screening of human tissue cDNA libraries with CCL20- and CCR6-specific TaqMan probe and primers. Our cDNA library panel included various libraries derived from human autoimmune disease samples. This initial screening showed that CCL20 was expressed >100 times higher in a cDNA library derived from lesional psoriatic skin (568,000 fg/50 ng of cDNA) compared with normal skin (5,530 fg/50 ng of cDNA). Moreover, TaqMan analyses showed abundant CCR6 message (864 fg/50 ng of cDNA) in the cDNA library generated from psoriatic skin. In contrast, CCR6 was undetectable in a cDNA library derived from normal skin. We then sought to analyze other chemokine receptors that have been reported to be up-regulated in psoriasis in our cDNA libraries from normal and psoriatic skin. To this end, we studied the expression of the IL-8R and confirmed previous reports (28, 29). CXCR1 and CXCR2 were constitutively expressed in the cDNA library derived from normal skin and markedly up-regulated in the psoriatic skin cDNA library (data not shown). Thus, our cDNA libraries derived from normal or psoriatic skin provided representative tools to study gene expression. These initial observations prompted us to investigate a possible role for CCL20 and CCR6 in the pathogenesis of psoriasis in more detail. We initially sought to validate these observations in more patient samples.

Consecutive quantitative real-time PCR analyses of individual cDNAs derived from lesional (n = 10), nonlesional psoriatic (n = 5), or normal (n = 5) skin confirmed that both CCL20 and CCR6 were significantly up-regulated in lesional psoriatic vs nonlesional or normal skin (p < 0.005; Fig. 1, A and B). An average 7- and 4-fold induction of CCL20 and CCR6 could be detected, respectively. The differences in CCL20 expression levels probably reflect interindividual differences of patients or various clinical stages of the disease. We did observe, however, that differences in CCL20 expression generally correlated with disease severity. Notably, although CCL20 expression was variable within lesional psoriatic skin, direct comparison with nonlesional psoriatic skin consistently showed that CCL20 mRNA was up-regulated in inflamed skin (Fig. 1A).

To look more precisely at the distribution of CCL20, we performed in situ hybridization (Fig. 2) and immunohistochemistry (Fig. 3) using specific probes and polyclonal Abs directed against CCL20. In situ hybridization with a CCL20-specific probe showed marked expression of this chemokine in the basal as well as
suprabasal layers of the epidermis of lesional psoriatic skin (Fig. 2, A–C). Two different expression pattern of CCL20 mRNA could be observed. First, in more chronic lesions CCL20 mRNA expression was predominantly located within the stratum granulosum of the epidermis (Fig. 2, A and C). In contrast, more active and severe psoriatic lesions showed CCL20-specific hybridization signals within the stratum spinosum and partly in the stratum basale of the epidermis (Fig. 2B). Sense (vs antisense) controls (Fig. 2D) as well as in situ hybridization with normal (Fig. 2E) and nonlesional psoriatic (Fig. 2F) skin confirmed the specific detection of CCL20 hybridization in lesional psoriatic skin.

Furthermore, immunohistochemical staining of lesional psoriatic skin confirmed the focal up-regulation of CCL20 protein within basal and suprabasal layers of the epidermis (Fig. 3, A, B, D, F, and H). In contrast, nonlesional psoriatic (Fig. 3C) and normal skin (Fig. 3E) showed no specific staining for CCL20. Staining with isotype controls confirmed the specificity of CCL20 detection (Fig. 3G). Importantly, there was a very good correlation between the detection of CCL20 mRNA by in situ hybridization and CCL20 protein by immunohistochemistry (see, for example, Fig. 2, A and B, and Fig. 3, A and B), confirming the two different expression patterns and the specificity of CCL20 detection in lesional psoriatic skin. Furthermore, CCL20 staining could be completely blocked by preincubation of anti-CCL20 Abs with recombinant CCL20 (data not shown).

Attempts to detect CCR6 by immunohistochemistry for CCR6 were inconclusive due to the low sensitivity of the Ab. Therefore, we performed double stainings for CCL20 and CD3 to study localization of CCL20-expressing cells and T cells (Fig. 3, B, D, and F). Immunohistochemistry of lesional psoriatic skin showed that the focal accumulation of T cells in the papillary dermis of lesional psoriatic skin was directly adjacent to foci of CCL20-expressing epidermal cells (Fig. 3, B and D). Furthermore, CCL20-expressing keratinocytes within the epidermis colocalized with intraepidermal
CD3\(^+\) T cells (Fig. 3, D and F). Moreover, Fig. 3H confirms that CCL20 is strongly expressed within the cytoplasm of keratinocytes of lesional psoriatic skin.

**Skin homing CLA\(^+\) T cells of psoriatic patients respond to lower concentrations of CCL20 and show higher chemotactic responses compared with those of normal donors**

Clinically, it is well known that infections may trigger psoriatic episodes, and recently, it has been suggested that superantigens may play a role in T cell activation during the pathogenesis of psoriasis (30–32). However, very little is known about chemokine receptor expression on PBMCs of normal healthy donors vs psoriatic patients. Interestingly, CCR6 was also significantly up-regulated in PBMCs derived from psoriasis patients (n = 10) vs PBMC from healthy donors (n = 5; p < 0.001). PBMCs from psoriatic patients expressed, on the average, 4-fold higher levels of CCR6 mRNA compared with PBMCs from healthy donors (Fig. 1C). The CLA\(^+\) T cell subset represents a skin-associated population of memory T cells that migrates preferentially to normal and chronically inflamed cutaneous sites (33).

In subsequent experiments we focused on the chemokine receptor profile of pathologically relevant skin-homing CLA\(^+\) memory T cells. Flow cytometric analyses revealed that CCR6 was expressed at high levels on the surface of skin-homing CLA\(^+\) T cells of normal donors (Fig. 4). Moreover, CCR6 was predominantly expressed on the CD4\(^+\) subset of CLA\(^+\) T cells (Fig. 4, C and D). The latter observation may account for the therapeutic effect of anti-hCD4 Abs in the treatment of psoriasis (34–36).

In agreement with flow cytometric analyses, TaqMan analyses on sorted CLA\(^+\)/CD4\(^+\)/CD45RO\(^+\) memory T cells from two different donor pools comprising cells from three different donors in each pool were analyzed for chemokine receptors (CCR2, CCR3, CCR5, CXCR1, CXCR2, CXCR3) that are either known or are likely to be involved in skin inflammation and were compared with the expression of CCR6. Values are expressed as femtograms of target gene in 50 ng of total cDNA.
higher than that of CXCR1, CXCR2, CXCR3, CCR2, CCR3, and CCR5 on this skin-homing subset of memory T cells (Fig. 4E).

The next question we addressed was whether differences of CCR6 mRNA expression in psoriatic vs normal PBMCs could be confirmed at the protein level and further characterized using flow cytometric analyses of T lymphocyte subpopulations (Table I). Within the T cell compartment, CCR6 is preferentially expressed on CD4+ T cells in both normal donors (n = 5) and psoriatic patients (n = 3). In total CD4+ T cells CCR6 expression was lower on psoriatic T cells compared with those from normal individuals. However, within the subpopulation of skin-homing CLA+ T cells, CCR6 expression was higher on psoriatic than on normal lymphocytes. Furthermore, we noticed that more CLA+ T cells could be detected within PBMCs of psoriatic vs normal donors (Table I). This observation parallels recent findings of Davison et al. (42).

The latter observations had functional implications, as psoriatic CLA+ T cells responded in Transwell chemotaxis assays to lower concentrations of CCL20 and showed higher chemotactic responses than CLA+ T cells from normal donors (Fig. 5). CCL20 significantly attracted CLA+ skin-homing T cells from both normal and psoriatic donors. Overall, 2–21% of the starting population of CD3+/CLA+ lymphocytes migrated toward CCL20.

**TNF-α, IL-1β, IFN-γ, IL-17, and CD40L regulate the expression of CCL20 in cellular constituents of the skin**

The pattern of CCL20 expression within the epidermis suggested that keratinocytes may be a major source of CCL20 in the skin. To further investigate the cellular origin of CCL20 within the skin and to gain insights into its regulation, we cultured human primary keratinocytes, melanocytes, and dermal fibroblasts with TNF-α/IL-1β, IFN-γ, IL-4, IL-17, or medium alone as a control. Furthermore, we determined whether cultured epidermal γδ T cells, resting or stimulated with either TNF-α/IL-1β or Con A, may express CCL20. Human primary dermal microvascular endothelial cells were also cultured in the presence or the absence of TNF-α/IL-1β. We used TNF-α and IL-1β stimulation, since these proinflammatory cytokines are known to be up-regulated during inflammatory conditions and in lesional psoriatic skin (43–47). Furthermore, we were interested in the effects of Th cell-derived cytokines such as IL-4, IFN-γ, and IL-17 on cellular constituents of the skin. TaqMan analyses showed that only keratinocytes and dermal microvascular endothelial cells constitutively express low levels of CCL20, and that TNF-α/IL-1β can induce CCL20 mRNA expression in both keratinocytes and dermal microvascular endothelial cells (Fig. 6A). In addition, TNF-α/IL-1β stimulation of dermal microvascular endothelial cells induced strong up-regulation of CCL20 expression (Fig. 6A). Activation of both CD34+ hemopoietic progenitor cell- or monocyte-derived dendritic cells with CD40L also induced CCL20-specific transcripts (Fig. 6A). Interestingly, primary melanocytes also showed significant expression of CCL20 following TNF-α/IL-1β stimulation. Moreover, CCL20 expression could be markedly induced in these cells by IFN-γ or IL-4 stimulation (Fig. 4A). Keratinocytes showed a weak (2- to 4-fold) up-regulation of CCL20 mRNA after activation with either IFN-γ or IL-4 (Fig. 4A). In contrast, resting or stimulated epidermal γδ T cells failed to express CCL20 mRNA (Fig. 6) under any conditions. Similar results were observed in keratinocytes (n = 4), melanocytes (n = 2), CD34+ hemopoietic progenitor cell-derived dendritic cells

<table>
<thead>
<tr>
<th>Donors</th>
<th>CD4/CCR6 (%)</th>
<th>CD8/CCR6 (%)</th>
<th>CLA/CCR6 (%)</th>
<th>CLA (%)</th>
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</thead>
<tbody>
<tr>
<td>Normal (n = 5)</td>
<td>12.09 ± 0.75</td>
<td>3.45 ± 0.76</td>
<td>4.2 ± 0.33</td>
<td>9.23 ± 0.29</td>
</tr>
<tr>
<td>Psoriasis (n = 3)</td>
<td>9.73 ± 1.59</td>
<td>1.95 ± 0.22</td>
<td>6.02 ± 0.29</td>
<td>12.16 ± 0.73</td>
</tr>
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* PBMCs of either normal or psoriatic donors were stained with mAb directed against CD4, CD8, CLA, and CCR6. Analyses were performed using a lymphocyte-sized gate. Percentages indicate the mean ± SD of positive lymphocytes.

![FIGURE 5](https://www.jimmunol.org/) MIP-3α/CCL20-induced chemotaxis of skin-homing CLA+ T cells in normal and psoriatic donors. Enriched lymphocytes from either normal (A) or psoriatic donors (B) were analyzed in Transwell chemotaxis assays for their response to hCCL20. The number of CD3+/CLA+ migrating cells was determined, and chemotaxis indices were calculated from triplicate measurements. Representative data for one of three normal or psoriatic donors are shown.
(n = 2), monocyte-derived dendritic cells (n = 2), dermal microvascular endothelial cells (n = 2), and dermal fibroblasts (n = 2) of different donors. Interestingly, keratinocytes, fibroblasts, melanocytes, or epidermal γδ T cells were never observed to express significant levels of CCR6 mRNA (data not shown).

Analyses of CCL20 protein expression by ELISA confirmed that activated keratinocytes, dermal microvascular endothelial cells, dermal fibroblasts, and monocyte-derived dendritic cells are potent producers of this chemokine. Supernatants from cultured primary human keratinocytes, dermal microvascular endothelial cells, and dermal fibroblasts activated with TNF-α and IL-1β showed a marked induction of CCL20 protein, while resting cells showed little or no production of CCL20 (Table II). These levels of hCCL20 production are in the range of biological activity reported for prokaryote-derived recombinant hCCL20. Low levels of CCL20 protein were also detected after stimulation of keratinocytes with IFN-γ or IL-17; however, additional TNF-α stimulation showed synergistic effects and markedly enhanced CCL20 protein production (Table II).

Supernatants of monocyte-derived dendritic cells showed significant production of CCL20 protein (1.14–8.76 ng/ml) 12–48 h following CD40L stimulation (Table II). CCL20 protein expression was confirmed using time-gated quantitative PCR (TaqMan). However, supernatants from CD34+ hematopoietic progenitor cells stimulated with CD40L did not contain detectable CCL20 protein despite a marked induction of CCL20 mRNA expression. This may be due to the high level of CCR6 expression by these cells, suggesting that they may be binding and internalizing CCL20.

We then sought to determine whether the CCL20 protein detected in these supernatants was biologically active. To this end, we tested supernatants of keratinocytes, fibroblasts, or endothelial cells, either resting or following stimulation with TNF-α/IL-1β, IFN-γ, or IL-4, in a calcium signaling assay using CCR6-transfected BAF3 cells. These cells are known to express endogenous CXCRI4. Therefore, to obtain a CCR6-specific assay we blocked the endogenous CXCRI4 with human SDF-1α before testing the supernatants for CCL20 activity (Fig. 4B). In agreement with the ELISA data, supernatants from keratinocytes (Fig. 4B), dermal fibroblasts (data not shown), and dermal microvascular endothelial cells (data not shown) stimulated with TNF-α/IL-1β induced significant calcium mobilization responses in CCR6-transfected BAF3 cells, but not in the parental untransfected BAF3 cells (Fig. 6B). However, the parental BAF3 cell line showed the expected calcium mobilization response due to the triggering of endogenous CXCRI4 by SDF-1α/CXCL12 (Fig. 6B). Furthermore, treatment with anti-CCL20 mAb completely neutralized supernatant-induced calcium mobilization in CCR6-transfected BAF3 cells; however, supernatants from CD34+ hematopoietic progenitor cells stimulated with CD40L did not contain detectable CCL20 protein despite a marked induction of CCL20 mRNA expression. This may be due to the high level of CCR6 expression by these cells, suggesting that they may be binding and internalizing CCL20.

![FIGURE 6](A) A, MIP-3α/CCL20 mRNA expression in cellular constituents of the skin. Quantitative TaqMan PCR analysis of CCL20 expression in cDNA obtained from cultured human primary keratinocytes, dermal fibroblasts, melanocytes, dermal microvascular endothelial cells, and epidermal γδ T cells (7–17) treated with medium alone or with TNF-α/IL-1β, IFN-γ, or IL-4 for 6 or 18 h. Analysis of MIP-3α/CCL20 expression in dendritic cells derived from CD34+ hematopoietic progenitor cells or monocytes that were cocultured with or without CD40L. Values are expressed as femtograms of target gene in 50 ng of total cDNA. Representative data from single donors are shown. B, Human primary keratinocytes produce biologically active CCL20. Concentrated supernatant harvested from resting and stimulated keratinocytes (KC) as well as the medium controls were assayed for intracellular calcium mobilization in CCR6-transfected BAF3 cells. The parental BAF3 cells showed no responses to any of the supernatants; however, calcium mobilization could be induced by hSDF-1α because BAF3 cells express endogenous CXCRI4. Despite the desensitization of endogenous CXCRI4, supernatants from keratinocytes activated with TNF-α/IL-1β could induce marked Ca2+ mobilization responses in CCR6 transfecants. Treatment with anti-MIP-3α/CCL20 mAb completely neutralized supernatant-induced calcium mobilization in CCR6-transfected BAF3 cells; however, the isotype control showed no effect. Similar results were obtained for dermal fibroblasts and dermal microvascular endothelial cells (data not shown). The addition of concentrated culture medium (with or without cytokines: TNF-α/IL-1β, IL-4, IFN-γ) did not produce any intracellular Ca2+ mobilization in parental or CCR6-transfected cells.}

<table>
<thead>
<tr>
<th>Supernatants*</th>
<th>MIP-3α/CCL20 (ng/ml) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keratinocytes untreated</td>
<td>0.18 ± 0.35</td>
</tr>
<tr>
<td>Keratinocytes TNF-α/IL-1β</td>
<td>57.25 ± 37.46</td>
</tr>
<tr>
<td>Keratinocytes untreated</td>
<td>0.03 ± 0.06</td>
</tr>
<tr>
<td>Keratinocytes TNF-α</td>
<td>0.93 ± 0.12</td>
</tr>
<tr>
<td>Keratinocytes IFN-γ</td>
<td>0.15 ± 0.07</td>
</tr>
<tr>
<td>Keratinocytes TNF-α/IFN-γ</td>
<td>2.6 ± 0.42</td>
</tr>
<tr>
<td>Keratinocytes IL-17</td>
<td>0.63 ± 0.71</td>
</tr>
<tr>
<td>Keratinocytes TNF-α/IL-17</td>
<td>8.93 ± 6.6</td>
</tr>
<tr>
<td>Microvascular endothelial cells untreated</td>
<td>0.2 ± 0.28</td>
</tr>
<tr>
<td>Microvascular endothelial cells TNF-α/IL-1β</td>
<td>40.5 ± 4.95</td>
</tr>
<tr>
<td>Monocyte-derived DC GM-CSF + IL-4 (d6)</td>
<td>0</td>
</tr>
<tr>
<td>Monocyte-derived DC GM-CSF + IL-4 (d6) CD40L 24 h</td>
<td>1.37 ± 0.16</td>
</tr>
<tr>
<td>Monocyte-derived DC GM-CSF + IL-4 (d6) CD40L 48 h</td>
<td>5.36 ± 3.4</td>
</tr>
<tr>
<td>Fibroblasts untreated</td>
<td>0</td>
</tr>
<tr>
<td>Fibroblasts TNF-α/IL-1β</td>
<td>52</td>
</tr>
</tbody>
</table>

* Supernatants of primary keratinocytes (n = 4 donors), dermal microvascular endothelial cells (n = 2 donors), and dermal fibroblasts (n = 1 donor) were harvested after treatment with TNF-α/IL-1β, IFN-γ, IL-17, TNF-α, IFN-γ/TNF-α, IL-17/TNF-α, or medium alone for 18 h. Supernatants of monocyte-derived dendritic cells (DC) cultured with GM-CSF and IL-4 for 6 days were harvested 24 and 48 h (n = 2 donors) after stimulation with CD40L or medium alone. Supernatants were analyzed by means of a MIP-3α/CCL20-specific ELISA. Values are expressed as mean ± SD.
The epidermis colocalized with intraepidermal CD3+ dermal cells. Moreover, CCL20-expressing keratinocytes within treating T cells in the papillary dermis of lesional psoriatic skin did observe a correlation between the level of CCL20 detected and heterogeneity in the patient population from either the genetic among the psoriatic patients tested. These probably represent either some variation in the levels of CCL20 and CCR6 detected significantly up-regulated in lesional psoriatic skin. There was, however, some level in lesional psoriatic skin. In lesional psoriatic skin, large numbers of dermal dendritic cells are present and show potent stimulatory functions (51). Activation of dendritic cells via C40 triggering resulted in a marked up-regulation of CCL20, suggesting that dendritic cell-T lymphocyte interactions may amplify inflammatory processes in psoriasis.

A recent report has implicated CCL20 in the constitutive trafficking of epidermal Langerhans cells and shows CCL20 expression in clinically normal appearing skin (52). However, some differences in results with the present study are probably related to the use of different Abs for immunohistochemistry or differences in the populations studied. Charbonnier et al. provided only limited data on immunohistochemical stainings; however, the present study shows quantitative real-time PCR on a significant number of patients’ samples as well as in situ hybridization and immunohistochemistry results that consistently showed that CCL20 expression was associated with inflamed, rather than normal, skin.

Along with its expression in intestinal epithelial cells, cutaneous CCL20 expression supports the hypothesis that this inflammatory chemokine plays an important role in the interface between the organism and the environment (53). Other chemokines have been shown to be associated with psoriasis, including RANTES/CCL5, which has been reported to be expressed in psoriatic lesions by activated keratinocytes (40, 54). However, peak levels of CCL5 expression (2.072 ng/ml) in activated keratinocytes were 10–50 times lower than those detected for CCL20 in our present study (40). Goebeler et al. detected selective expression of MIG/CXCL9 in the upper dermal lesions, with pronounced clustering in the tips of the papillae, whereas expression in normal or nonlesional psoriatic or normal skin was quiescent. Colocalization studies have suggested that highly activated dermal macrophages and dermal microvascular endothelial cells are major sources of CXCL9 in lesional psoriatic skin (41). The proinflammatory CXC chemokines, IP-10/CXCL10 and CXCL9, are able to attract activated T cells and are mainly regulated by T cell-produced cytokines, such as IFN-γ or TNF-α. Thus, skin-infiltrating T cells release inflammatory mediators that, in turn, induce CXCL9, CXCL10,
and CCL20, contributing to the amplification of inflammatory responses and the chronicity of psoriatic lesions. Moreover, MCP-1/CCL2 expression of keratinocytes in the stratum basale of lesional psoriatic skin is associated with chemotrafficking of dermal macrophages to lesional sites (39, 41, 55).

Given the cumulative evidence that psoriasis is a T cell-mediated disease (4–14), CCL20/CRCR6 is the first ligand/receptor pair identified in this disease that is directly associated with memory T cell recruitment to lesional psoriatic skin. The only other chemo-/receptor partners reported in psoriasis, such as IL-8/CXCL8 and GROα/CXCL1, with their receptors CXCR1 and CXCR2, are mainly involved in the recruitment of neutrophils to lesional psoriatic skin (56–58). In addition, the expression pattern of those CXC chemokines did not fully coincide with the pattern of T cell accumulation (56–58).

More recently, a nonchemokine ligand for CCR6 has been identified. Yang et al. showed that human β-defensin-2 is able to bind CCR6-transfected cells and to induce chemotaxis; however, its chemotactic activity was considerably lower than that of CCL20 (59). However, we found no evidence for a role for human β-defensin-2 in our study, since the intracellular Ca2+ mobilization induced by supernatants of TNF-α/IL-1β-stimulated primary keratinocytes in CRCR6 transfecants was completely blocked by an anti-CCL20 Ab.

Our findings suggest the following model for the involvement of CCL20 and CRCR6 in the pathogenesis of psoriasis. CCL20 may be induced in keratinocytes and/or dermal microvascular endothelial cells at sites of physical injury or infection due to the release of proinflammatory cytokines, such as TNF-α and IL-1. In turn, thymus and activation-regulated chemokine/CCL17, CTACK/CCL27, and CCL20 may induce adhesion (22, 60) and chemotaxis of skin-homing memory T cells (21) through the endothelium into the skin. Subsequently, the skin-homing CLA+ T cells may encounter their specific Ag presented by dendritic cells, get activated, and produce inflammatory mediators, such as IFN-γ, IL-17, or CD40L, which, in turn, induce additional CCL20, CXCL9, and CXCL10 production by activated keratinocytes, dendritic cells, and dermal macrophages (37, 41, 61). This “second wave” of chemokine production may complete a self-sustaining cycle of inflammation that may finally lead to the development of a psoriatic phenotype.

In conclusion, our study shows the potential of a highly specific and sensitive quantitative real-time PCR technique (TaqMan) to identify novel disease associations with the expression of specific genes. This technology allowed us to identify CCL20/CRCR6 as a new ligand/receptor pair potentially involved in the pathogenesis of psoriasis.

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