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CCL1-CCR8 Interactions: An Axis Mediating the Recruitment of T Cells and Langerhans-Type Dendritic Cells to Sites of Atopic Skin Inflammation[1]

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Atopic dermatitis represents a chronically relapsing skin disease with a steadily increasing prevalence of 10–20% in children. Skin-infiltrating T cells, dendritic cells (DC), and mast cells are thought to play a crucial role in its pathogenesis. We report that the expression of the CC chemokine CCL1 (*I-309*) is significantly and selectively up-regulated in atopic dermatitis in comparison to psoriasis, cutaneous lupus erythematosus, or normal skin. CCL1 serum levels of atopic dermatitis patients are significantly higher than levels in healthy individuals. DC, mast cells, and dermal endothelial cells are abundant sources of CCL1 during atopic skin inflammation and allergen challenge, and *Staphylococcus aureus*-derived products induce its production. In vitro, binding and cross-linking of IgE on mast cells resulted in a significant up-regulation of this inflammatory chemokine. Its specific receptor, CCR8, is expressed on a small subset of circulating T cells and is abundantly expressed on interstitial DC, Langerhans cells generated in vitro, and their monocytic precursors. Although DC maintain their $CCR8^+$ status during maturation, brief activation of circulating T cells recruits CCR8 from intracytoplamic stores to the cell surface. Moreover, the inflammatory and atopy-associated chemokine CCL1 synergizes with the homeostatic chemokine CXCL12 (*SDF-1α*) resulting in the recruitment of T cell and Langerhans cell-like DC. Taken together, these findings suggest that the axis CCL1-CCR8 links adaptive and innate immune functions that play a role in the initiation and amplification of atopic skin inflammation. *The Journal of Immunology,* **2005, 174: 5082–5091.**

Atopic dermatitis is a chronically relapsing inflammatory skin disease with an increasing prevalence ranging from 10 to 20% in children and from 1 to 3% in adults within developed countries. Clinically, atopic dermatitis is characterized by pruritic and erythematosus papules and plaques with predilections at flexural parts of the body, intertrigenes, and the head and neck area. Elevated total serum IgE levels as well as Ag-specific IgE (e.g., house dust mite, pollen Ags) are frequently detected (1). Histopathologically, atopic skin lesions show a marked inflammatory infiltrate composed of $CD4^+$ or $CD8^+$ cutaneous lymphocyte-associated Ag (CLA^+)[4] memory T cells, eosinophils (2), as well as increased numbers of FcεRI-bearing dendritic cell (DC) subpopulations in epidermal and dermal compartments of the skin (3). Recent clinical and experimental evidence indicates that skin-infiltrating leukocytes play a crucial role in the initiation and maintenance of atopic dermatitis (1). Thus, unraveling of the mechanisms of leukocyte recruitment and activation in atopic dermatitis may identify novel targets for the development of innovative therapeutic strategies for patients with atopy. Although recent studies provided insights into the migration/trafficking pathways of skin-homing $CD4^+$ T lymphocytes, little is known about the recruitment of $CD8^+$ T cells and DC subsets in particular of Langerhans cells (LC) into the skin of atopic individuals. LC are believed to represent important sentinels of the immune system at the interface of the organism and the environment. In the pathogenesis of atopic dermatitis, this subset of DC is the first to capture "airborne" Ag via IgE on their cell surface, migrate to lymph nodes, and present relevant Ags to memory T cells.

With regard to leukocyte trafficking, the chemokine superfamily has gained increasing interest in recent years. To date, 45 human

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[4] Abbreviations used in this paper: CLA, cutaneous lymphocyte-associated antigen; DC, dendritic cell; LC, Langerhans cell; SEB, staphylococcal enterotoxin B; LTA, lipoteichoic acid; PGN, peptidoglycan.

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chemokine ligands binding to 18 G protein-coupled receptors are known (4). The chemokine superfamily is likely to be completely characterized at the molecular level, which offers for the first time the opportunity to identify all relevant members associated with physiologic or pathologic processes.

The present study aimed to systematically identify chemokines and receptors that are associated with an atopic dermatitis phenotype and elucidate their role in the pathogenesis of this chronically relapsing inflammatory skin disease. One such gene, CCL1 was found to be up-regulated in atopic dermatitis when compared with other chronic inflammatory skin diseases such as psoriasis and cutaneous lupus erythematosus or with normal skin. DC, mast cells, and endothelial cells were identified with the cellular origin of CCL1 production, which was regulated by FcεR engagement, allergen exposure as well as *Staphylococcus aureus*-derived products. Furthermore, this inflammatory chemokine synergizes with the homeostatic chemokine CXCL12 during the recruitment of activated T cells and $CCR8^+$ Langerhans-type DC.

Materials and Methods

Patients

Skin biopsies were taken, after obtaining informed consent, from either healthy individuals or patients suffering from atopic dermatitis, psoriasis, or lupus erythematosus. The clinical diagnosis of cutaneous lupus erythematosus was confirmed by histologic evaluation, serologic examinations, and UV-provocation testing. Atopic dermatitis patients were identified according to the criteria defined by Hanifin and Rajka (5). Furthermore, patients suffering from chronic plaque psoriasis in typical locations were enrolled into the study. Normal skin samples were obtained from patients undergoing plastic surgery. All studies were approved by the appropriate ethics committees.

Hemopoietic factors and reagents

Recombinant human GM-CSF (specific activity, 2×10^6 U/mg; Schering-Plough) was used at a saturating concentration of 100 ng/ml. Recombinant human IL-4 (specific activity, 2×10^7 U/mg; Schering-Plough) and recombinant human TGF-β1 (specific activity, 5×10^7 U/mg) were used at 10 ng/ml. Recombinant human TNF-α (specific activity, 2×10^7 U/mg) at either 3 ng/ml for DC culture or 10 ng/ml for nonhemopoietic cell culture, and recombinant human IL-1β at 5 ng/ml (R&D Systems) were used. Recombinant murine stem cell factor was used at 15 ng/ml (Sigma-Aldrich). Recombinant human chemokines CCL1 (specific activity, 4×10^5 U/mg), CXCL12 (specific activity, 2×10^5 U/mg), CCL21 (specific activity, 2×10^5 U/mg), and CCL7 (specific activity, 1×10^4 U/mg) were used at the indicated concentrations and were purchased through R&D Systems.

Quantitative real-time RT-PCR (TaqMan) analysis

Quantitative real-time RT-PCR analyses were performed, as previously described (6). Briefly, skin biopsy specimens were homogenized and RNA was extracted using TRIzol (Invitrogen Life Technologies). Total RNA of 4 μg was treated with DNase I (Boehringer Mannheim) and reverse transcribed. Primers and probes specific for chemokines and their receptors were obtained from Applied Biosystems. cDNA of 25 ng was assayed per reaction as earlier described (7). Briefly, gene-specific PCR products were measured by means of an ABI PRISM 7700 or 5700 Sequence Detection Systems (Applied Biosystems) continuously during 40 cycles. Ribosomal RNA (18 S) was used for normalization.

Immunohistochemistry

For immunohistochemistry of chemokine and chemokine receptor expression, frozen skin sections were fixed with acetone and preprocessed with H_2O_2 followed by an avidin and biotin blocking step (Blocking kit, Vector Laboratories). Sections were stained with Abs against human CCL1 (goat IgG; Santa Cruz Biotechnology), CD3 (A0452, mouse IgG1; DAKO), CXCL12 (K15C, mouse IgG2a; Unité d'Immunologie Virale, Institute Pasteur, Paris, France), and CCR8 (D6, mouse IgG; German Cancer Research Center, Heidelberg, Germany). Development of the staining was performed with Vectastain ABC kit (avidin-biotinylated enzyme complex; Vector Laboratories) and an AEC kit (3-amino-9-ethyl-carbazole solution; Vector Laboratories). Sections were counterstained with hematoxylin.

Generation of interstitial DC and Langerhans-type DC from peripheral blood monocytes

PBMC were isolated by standard Ficoll-Paque method. Then, monocytes were enriched by 52% Percoll gradient and purified through negative selection (monocyte isolation kit) and Midimacs separation columns according to the manufacturer's instructions (Miltenyi Biotec). Freshly isolated monocytes were cultured for 6 days in the presence of either GM-CSF and IL-4 for the generation of interstitial DC-type cells (8) or GM-CSF, IL-4, and TGF-β1 plus TNF-α during the last 2 days of the culture for the generation of Langerhans-type DC (9). mAbs against Langerin (DC-GM4; Immunotech) and E-cadherin (HECD-1; Takara Shuzo) were used for the characterization of LC. Interstitial DC were characterized by their expression of CD1a (HI149, BD Biosciences), DC-SIGN (DCN46; BD Biosciences), and the absence of CD14 (M5E2; BD Pharmingen) and Langerin.

Activation of monocytes, interstitial DC, and Langerhans-type DC in the presence of microbial products

Cells were washed three times and cultivated in presence of either LPS (25 ng/ml), lipoteichoic acid (LTA) from *S. aureus* (10 μg/ml), peptidoglycan (PGN) from *S. aureus* (10 μg/ml), mannan from *Saccharomyces cerevisiae* (10 μg/ml), or the superantigen staphylococcal enterotoxin B (SEB) from *S. aureus* (10 ng/ml). All reagents were purchased from Sigma-Aldrich. The murine CD40L transfected fibroblast cell line (CD40L L cells) was kindly provided by Schering-Plough Laboratory, and was used as stimulator of DC maturation at the ratio 1:5 of CD40L L cell to DC (10). After 6 and 24 h, cells and supernatants were harvested for mRNA (quantitative PCR) and ELISA analysis, respectively. The activation stage of DC was controlled by using the following mAbs: anti-CD83 (HB15a; Immunotech), anti-CD40 (MAB89; Immunotech), and anti-HLA-DR (L243; BD Biosciences).

Cell culture of structural cells of the skin

Human primary epidermal keratinocytes, dermal fibroblasts, and dermal microvascular endothelial cells (Clonetics) were cultured in keratinocyte (KGM-2), fibroblast (FGM-2), or endothelial cell (EGM-2) growth medium (Clonetics), respectively, as previously described (6). Cells were treated with either TNF-α plus IL-1β or left untreated. RNA was extracted from cells as described.

Atopy patch test

Atopy patch tests were performed with house dust mite preparations containing a mixture of *Dermatophagoides farinae* (10%) and *D. pteronyssimus* (10%) Ags in petrolatum (Chemotechnique Diagnostics). A total of 17 patients with atopic dermatitis and positive house dust mite prick tests were selected for the study. Tests were performed with Finn Chambers (Epitest) on healthy appearing dorsal skin. Of those tested, nine patients reacted positively and were chosen for the study (Table I).

Patients were studied as follows: After 2, 6, and 48 h of application, the patches were removed and test results were read. Patch sites and an additional untreated site of healthy-looking back skin were biopsied with 6-mm

Table I. *Atopy patch tests in house dust mite-sensitized atopic dermatitis patients*[a]

Patient No.	Atopy Patch Test Response	
	First Test	Second Test
1	+++	+++
2	+++	++
3	+	+
4	++	+
5	++	++
6	++	++
7	+++	+
8	++	+
9	+	+

[a] House dust mite-sensitized atopic dermatitis patients were subjected to atopy patch tests with preparations containing a mixture of *Dermatophagoides farinae* (10%) and *D. pteronyssimus* (10%) Ags in petrolatum. After a first screening test, patients with positive skin reactions were exposed a second time, and clinical responses were determined as well as skin biopsies obtained. Skin reactions 48 hours after house dust mite exposure are shown. +, erythema and edema; ++, erythema, edema, and vesicles; +++, erythema, edema, vesicles, and spreading.

punches. Biopsy specimens were cut in half (quantitative PCR and immunohistochemistry) and stored at −70°C. All 10 patients had positive reactions to repeated atopy patch tests. Control testing done with petrolatum only was negative in all patients.

CCL1 protein analysis

For the detection of CCL1 protein in cell culture supernatants and human serum samples, a CCL1-specific ELISA (human CCL1 DuoSet; R&D Systems) was used according to the manufacturer's instructions. Dilution series of recombinant human CCL1 protein (R&D Systems) were used for standard curves.

Generation and stimulation of murine bone marrow-derived mast cells

Bone marrow cells of C57BL/6 mice were cultured for 4 wk in 70% enriched RPMI 1640 GlutaMAX I medium (10% heat inactivated FBS, 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.1 mM nonessential amino acids) (Invitrogen Life Technologies), 25 μM 2-ME (Sigma-Aldrich), and 30% WEHI-3B medium as a source of IL-3. After 2 wk of culture, murine stem cell factor was included in the culture medium. Nonadherent cells were transferred to fresh medium twice a week. The cells were identified as mast cells by May-Grünwald-Giemsa staining and by flow cytometric analysis of c-*kit* (>99% of c-*kit*$^+$ cells) and IgE receptor (89% of the cells positive) expression. Murine bone marrow-derived mast cells were incubated on 12-well microtiter plates (2×10^6 cells/well) with 10 μg of anti-DNP IgE (Sigma-Aldrich) in 2 ml of culture medium for 2 h on ice. The plates were centrifuged, supernatant discarded, and the cells resuspended in 2 ml of fresh medium with 200 ng of DNP-human serum albumin (Sigma-Aldrich). The cells were incubated for 6 and 24 h after which the cells were collected and lyzed with TRIzol (Invitrogen Life Technologies). RNA was extracted, reverse transcribed, and subjected to quantitative real-time RT-PCR analyses as described.

Flow cytometric analyses of CCR8 expression on leukocyte subsets

Cells from at least three different donors were analyzed using flow cytometry with the following Abs: Unlabelled anti-CCR8 (210-762-R100, goat IgG; Alexis Biochemicals); FITC-conjugated anti-CD14 (M5E2, mouse IgG2a), anti-CD19 (HIB19, mouse IgG1), anti-CD8 (RPA-T8, mouse IgG1), anti-CLA (HECA-542, rat IgM), and anti-CD4 (RPA-T4, mouse IgG1) from BD Pharmingen; PE-conjugated anti-CD8 (HIT8a, mouse IgG1), anti-CD117 (YB5.B8, mouse IgG1), swine anti-goat IgG (G50004; Caltag Laboratories); F(ab′)$_2$ donkey anti-goat IgG (Jackson ImmunoResearch Laboratories); PE-Cy5-conjugated anti-CD25 (M-A251, mouse IgG1), anti-CD4 (RPA-T4, mouse IgG1), anti-CD123 (9F5, mouse IgG1), anti-mouse IgG1 (MOPC-21) from BD Pharmingen; and allophycocyanin-conjugated anti-CD8 (RPA-T8, mouse IgG1) from BD Pharmingen. Briefly, 10^6 cells were stained, fixed in 4% paraformaldehyde and then analyzed using FACScan or FACSCalibur flow cytometers and CellQuest software (BD Biosciences).

Regulation of CCR8 expression on the surface of T cells

PBMC were isolated and stimulated with ConA (5 μg/ml; Sigma-Aldrich), PHA (5 μg/ml; Sigma-Aldrich), anti-CD3/CD28 (500 ng/ml, clone 37407.111/UCHT1; R&D Systems) for 2 h at 37°C, 5% CO_2, or left untreated. Subsequently, intracellular pools as well as cell surface expression of CCR8 was analyzed in CD3$^+$, CD4$^+$, or CD8$^+$ lymphocytes using flow cytometry.

Generation of dermal single cell suspensions

Dermal single cell suspensions were dispersed from human infant foreskin as previously described (11). Briefly, human foreskin was disintegrated with scissors, washed two times, and incubated 1 h at 37°C with 1.5 mg/ml collagenase type I and 0.5 mg/ml hyaluronidase type 1 (Sigma-Aldrich). Cells were filtered through a 100-μm cell strainer (BD Falcon) and washed with HBSS two times. Digestion was repeated with undigested tissue. Subsequently cytospin slides of dermal single cell suspensions were prepared using $\sim 5 \times 10^4$ cells on adhesive cover slides and subjected to immunocytochemical analysis. Mast cells were identified by their typical morphology and tryptase staining (Santa Cruz Biotechnology).

Chemotaxis assays

DC migration was evaluated using a chemotaxis microchamber technique (48-well Boyden microchamber; NeuroProbe) as previously described (12). Briefly, chemokines were diluted and added to the lower wells of the

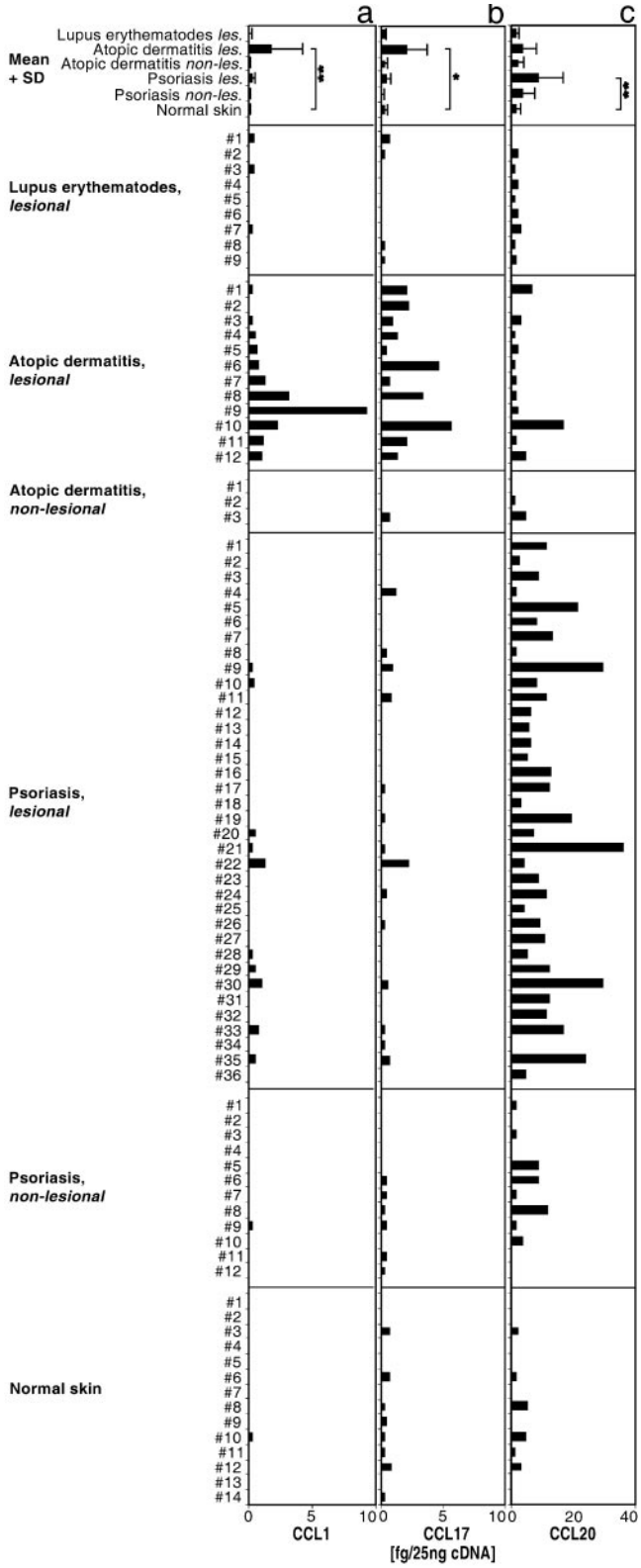

FIGURE 1. CCL1 and CCL17 are selectively up-regulated in lesional skin of atopic dermatitis patients. Quantitative real-time PCR analysis of CCL1 (*a*), CCL17 (*b*), and CCL20 (*c*) expression in lesional and nonlesional skin of atopic dermatitis patients ($n = 12$) compared with lesional and nonlesional skin of psoriatic patients ($n = 36$), to lesional skin of lupus erythematosus patients ($n = 9$) and to normal skin of healthy individuals ($n = 14$). Values are expressed in femtograms of target gene in 25 ng of total cDNA. Measurements of individual samples and the mean + SD are shown. Wilcoxon two sample test was performed: *, $p < 0.05$; **, $p < 0.01$.

chamber. A total of 10^5 cells/well in 50 μl of medium were applied to the upper wells of the chamber, with a standard 5-μm pore size polycarbonate filter (NeuroProbe) separating the lower wells. After 1 h at 37°C in humidified air with 5% CO_2, cells that migrated through the filter were stained and counted using a microscope (magnification: ×20). Each assay was performed in duplicate. Enriched monocytes and T cell migration was evaluated by Transwell chemotaxis assays as previously described (13). A total of 1×10^6 cells in 100 μl of RPMI 1640 supplemented with 1% BSA (Sigma-Aldrich) and 1% HEPES (Invitrogen Life Technologies) was placed in a 3-μm pore size polycarbonate Transwell culture insert (Costar). Chemokines were added in the bottom chamber. After 3 h in a 5% CO_2 environment at 37°C, migrated cells were recovered in the bottom compartments and their number was determined by flow cytometry using anti-CD14, anti-CD8, anti-CD4, and anti-CLA Abs. To determine the absolute number of migrated cells, a known number of 15-μm microsphere beads (Bangs Laboratories) was added to each sample before analysis. Assays were performed in triplicates.

Results

CCL1 is an inflammatory chemokine associated with atopic skin inflammation

We initially sought to identify members of the chemokine superfamily specifically associated with chronic inflammatory skin diseases such as atopic dermatitis, psoriasis, or cutaneous lupus erythematosus. A systematic analysis of chemokine superfamily members in nonlesional as well as lesional skin when compared with normal skin of healthy individuals identified several chemokines specifically overexpressed in inflammatory skin diseases (7, 14). We screened 33 human chemokine ligands in a panel of cDNAs derived from healthy ($n = 14$), nonlesional psoriatic ($n = 12$), lesional psoriatic ($n = 36$), nonlesional atopic ($n = 3$), lesional atopic ($n = 12$), and lesional lupus erythematosus ($n = 9$) skin. By quantitative real-time PCR analysis, we observed that both CCL1 (Fig. 1*a*) and CCL17 (TARC) (Fig. 1*b*) mRNAs were significantly ($p < 0.05$) up-regulated in lesional atopic skin compared with skin from healthy individuals, whereas CCL20 (MIP-3α) (Fig. 1*c*) showed a significant induction in psoriasis. To obtain insights into the anatomical location as well as the cellular origin of CCL1 and its specific receptor CCR8 in the skin, we performed immunohistochemical analyses using specific Abs directed against CCL1 (Fig. 2, *a–c*) and CCR8 (Fig. 2, *d–f*). CCL1 was not detected in normal skin (Fig. 2*a*) or in nonlesional atopic skin (data not shown). In contrast, CCL1 was abundantly expressed within the basal layer of the epidermis of lesional atopic skin (Fig. 2*b*). Furthermore, dermal endothelial cells markedly expressed CCL1 protein in atopic dermatitis patients (Fig. 2, *c* and *g*). Next to endothelial cells, cells with a dendritic morphology markedly expressed CCL1 protein in lesional skin of atopic dermatitis patients (Fig. 2, *b* and *c*).

In normal skin, dermal endothelial cells as well as epidermal and dermal cells with a dendritic morphology showed immunoreactivity for the CCL1 receptor CCR8 (Fig. 2*d*). In comparison to normal or nonlesional atopic skin, lesional atopic dermatitis showed a significant increase in $CCR8^+$ cells within epidermal and dermal compartments of the skin (Fig. 2). At higher magnification, a subset of $CCR8^+$ cells in the dermis as well as in the epidermis exhibited a dendritic morphology (Fig. 2, *e* and *f*). Counting two medium power fields, we detected on average 13.8 ± 7.2 $CCR8^+$ leukocytes in healthy skin vs 90.3 ± 19.2 $CCR8^+$ leukocytes in lesional atopic skin, resulting in a 6.5-fold increase of $CCR8^+$ skin-infiltrating leukocytes in atopic dermatitis.

Taken together, these observations identify CCL1 as an inflammatory and atopy-associated chemokine produced within epidermal and dermal compartments of the skin. Furthermore, its specific receptor CCR8 was detected in skin-infiltrating cells among which a subset exhibits a dendritic morphology.

Inflammatory cytokines and microbial products regulate CCL1 expression

Next, we investigated the regulation of CCL1 in cellular constituents of the skin. Previous immunohistochemical data suggested that endothelial cells and DC could be major sources of CCL1 in lesional atopic skin. Because primary proinflammatory cytokines (i.e., TNF-α and IL-1β), T_H cell-derived effector cytokines (i.e., IL-4 and IFN-γ), and microbial products are known to play a crucial role in the initiation and amplification of atopic skin inflammation (1), we investigated their role in the regulation of CCL1. Quantitative real-time PCR analyses showed that CCL1 mRNA was constitutively expressed in resting dermal endothelial cells and showed a marked induction upon TNF-α plus IL-1β activation (Fig. 3*a*). Cultured epidermal keratinocytes and dermal fibroblasts did not express significant amounts of CCL1 transcripts under resting or activated conditions (Fig. 3). In contrast to interstitial DC

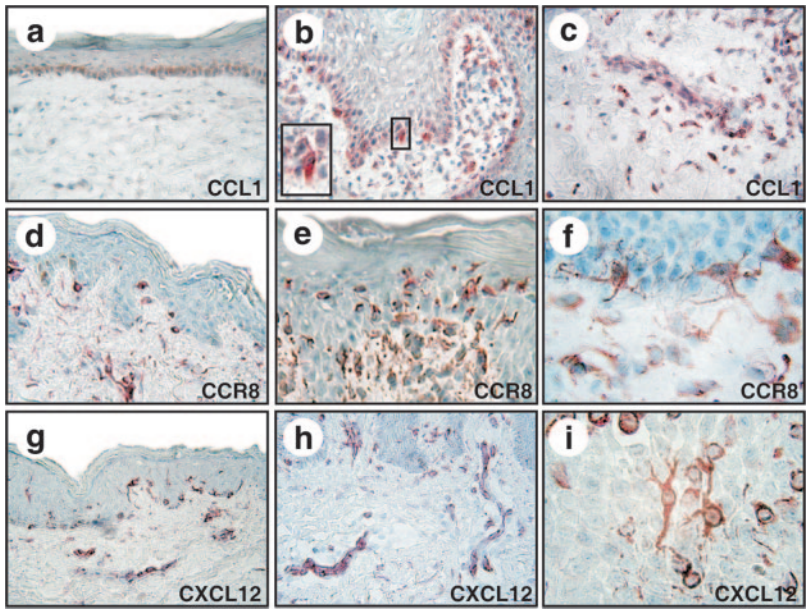

FIGURE 2. Induction of CCL1 within epidermal and dermal compartments of lesional skin in atopic dermatitis patients. Immunohistochemical analysis demonstrates that CCL1 protein is absent in normal skin (*a*) but abundantly expressed in lesional skin of atopic dermatitis patients (*a* and *b*), where a marked staining is observed within the basal layer of the epidermis (*b*), in cells with a dendritic morphology (*inset*), and in endothelial cells of the superficial plexus (*c*). Compared with normal skin (*d*) lesional atopic dermatitis (*e*) exhibits a marked increase of skin-infiltrating $CCR8^+$ cells. Within the population of $CCR8^+$ mononuclear cells infiltrating the skin a large subsets shows a dendritic morphology (*e*). *g–i*, In contrast to CCL1, the homeostatic chemokine CXCL12 is expressed by epidermal and dermal DC populations and endothelial cells in normal skin as well as lesional atopic dermatitis skin. *i*, CXCL12-expressing DC within the epidermis of lesional skin of an atopic dermatitis patient. Original magnification: *a–e*, *g*, and *h*, ×400; *f* and *i*, ×1000.

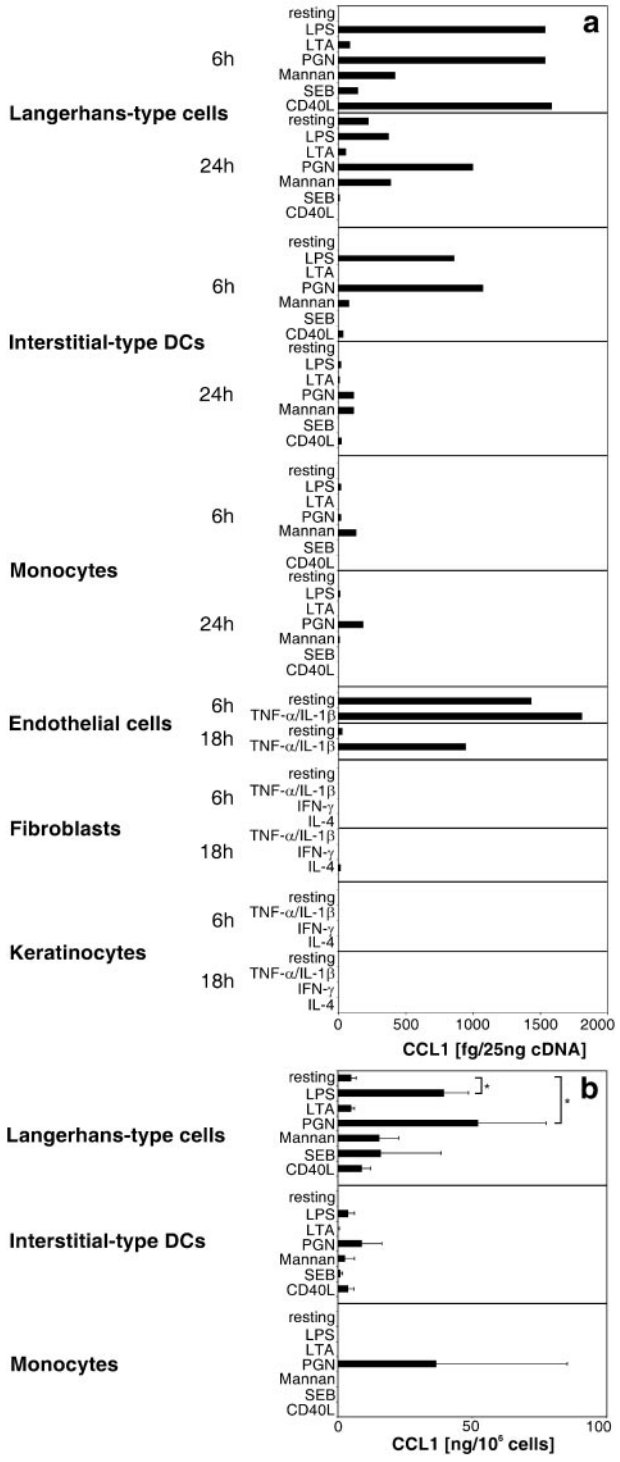

FIGURE 3. Langerhans-type DC and endothelial cells are the major sources of CCL1 among the cellular constituents of the skin. *a*, Quantitative real-time PCR analysis of CCL1 expression in cDNA obtained from cultured structural cells of the skin. Monocytes, Langerhans-type DC and interstitial DC-type cells were cultured with or without LPS, LTA, PGN, mannan, SEB, or CD40L for 6 and 24 h. Human primary dermal endothelial cells, dermal fibroblasts and keratinocytes, were activated by TNF-α/IL-1β, IFN-γ, IL-4 or left unstimulated for 6 and 18 h. Values are expressed in femtograms of target gene in 25 ng to total cDNA. *b*, Secretion of CCL1 by Langerhans-type DC and interstitial DC-type cells and their monocytic precursors. Cells were treated with medium alone or in presence of LPS, LTA, PGN, Mannan, SEB, and CD40L for 24 h. Supernatants were collected and measured for CCL1 content using a specific ELISA. Values are expressed in nanograms of target protein per 10^6 cells. Student *t* test was performed; $*$, $p < 0.05$. (representative of $n = 5$).

and monocytes, resting Langerhans-type DC expressed constitutive levels of CCL1 in vitro. However, upon activation with *Escherichia coli*-derived LPS, *S. aureus*-derived PGN, or *S. cerevisiae*-derived mannan, both DC subsets and their monocytic precursors produced moderate to high amounts of CCL1 mRNA (Fig. 3*a*). Analyses of CCL1 protein expression (Fig. 3*b*) confirmed that Langerhans-type DC constitutively produced CCL1 at a moderate level under resting conditions (ranging from 4.3 to 7.4 ng/10^6 cells). With an exception for LTA, an up-regulation was seen after stimulation with all activators. The better inducers were PGN (level range from 27.6 to 85.4 ng/10^6 cells) and LPS (from 26.8 to 47.2 ng/10^6 cells) showing significant differences in CCL1 protein production compared with resting Langerhans-type DC ($p < 0.05$; Fig. 3*b*). CCL1 protein production could also be induced in PGN-activated monocytes and in PGN-, mannan- or LPS-stimulated interstitial DC. In general for the 24 h time point, mRNA and protein expression are in accordance with each other; however, small differences between mRNA and protein expression exist. These differences may reflect different kinetics of mRNA and protein expression as well as chemokine secretion.

These observations support an active role of inflammatory cytokines and relevant microbial products (i.e., LPS, PGN, and mannan) in the production of CCL1 by endothelial cells, DC, and their precursors.

Binding and cross-linking of IgE induces CCL1 in mast cells

IgE-mediated activation of mast cells and DC is a hallmark of atopic skin inflammation. Immunohistochemical analysis of dermal single cell suspensions identified human dermal mast cells as an abundant source of CCL1 protein. Dermal tissue mast cells stored considerable amounts of CCL1 protein in their cytoplasm (Fig. 4*g*). Furthermore, bone marrow-derived murine mast cells showed a dramatic (40,000-fold) increase of CCL1 mRNA expression after cross-linking of DNP-specific IgE with specific Ag/allergen on their surface for 8 h (Fig. 4*h*). Moreover, IgE binding alone also stimulated murine mast cells to produce high levels of CCL1 mRNA (Fig. 4*h*). Next to mRNA, CCL1 protein levels also markedly increased within the supernatant of IgE or IgE plus DNP-treated murine mast cells (Fig. 4*i*). In contrast to IgE plus DNP, IgE alone did not induce the degranulation of mast cells using a β-hexosaminidase release assay (data not shown). Hence, we conclude that CCL1 production and release following allergen-specific cross-linking represents a downstream event of IgE-mediated allergic/atopic inflammation, suggesting CCL1-CCR8 interactions might play a role during the immunologic cascade of allergen-induced skin inflammation in vivo.

Allergen challenge induces CCL1 production in atopic dermatitis patients

To investigate the relevance of CCL1 in the pathogenesis of atopic dermatitis, patients with a history of house dust mite allergy were subjected to atopy patch testing. Skin specimens were obtained before as well as 2, 6, and 48 h after allergen exposure and analyzed by immunohistochemistry (Fig. 4, *a–c*) or quantitative real-time PCR (Fig. 4*d*). Eight of nine patients showed the induction of CCL1 mRNA and protein 6 to 48 h after relevant Ag exposure (Fig. 4, *b–d*). Subsequently, we compared serum levels of CCL1 in healthy individuals ($n = 11$) with those detected in atopic dermatitis patients during nonlesional ($n = 2$) or lesional ($n = 32$) phases of the disease (Fig. 4*e*). We observed that patients suffering from atopic dermatitis showed 3-fold higher ($p < 0.05$) CCL1 levels in their serum when compared with serum samples from nonlesional atopic dermatitis patients or healthy donors.

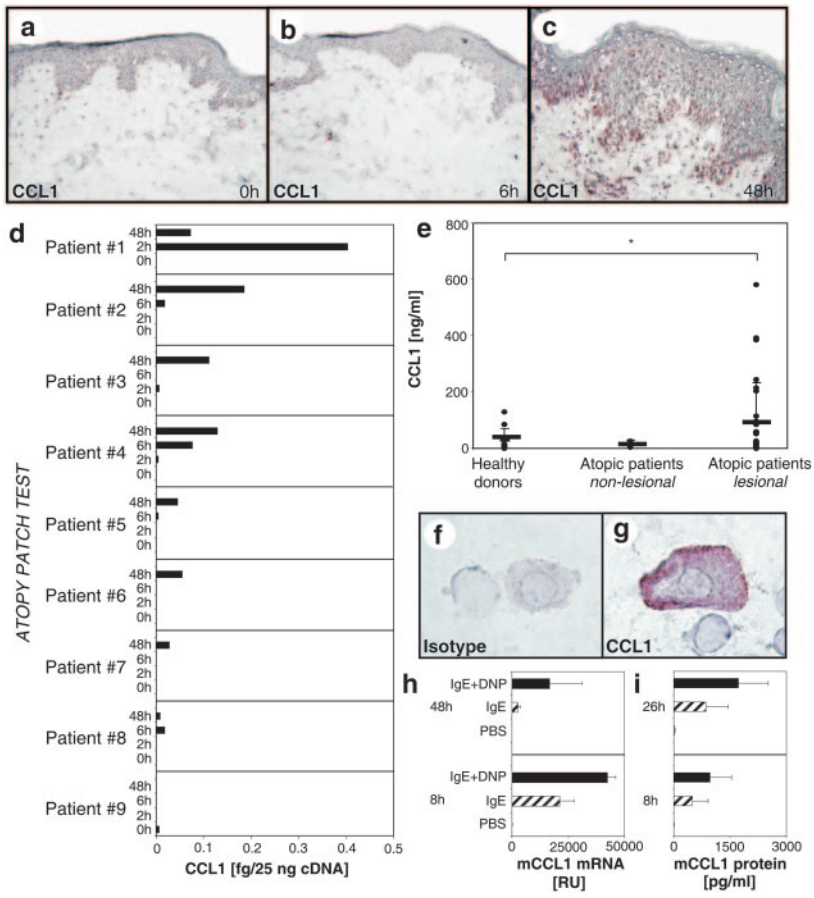

FIGURE 4. Allergen exposure induces CCL1 expression. *a–c*, The detection of CCL1 expression was performed by immunohistochemistry in the skin of atopic dermatitis patients after topical exposure with house dust mite allergen (atopy patch test). Negligible CCL1 expression before (*a*) as well as 6 h after (*b*) allergen exposure. However, a marked induction of CCL1 is observed 48 h (*c*) after house dust mite exposure. Original magnification: *a–c*, ×250. *d*, Quantitative real-time PCR analysis of CCL1 was performed in atopic dermatitis patients ($n = 9$) subjected to atopy patch tests with house dust mite allergen. Skin samples were obtained before as well as 2, 6, and 24 h after allergen exposure. Values are expressed as femtograms of target gene in 25 ng of total cDNA. *e*, Atopic dermatitis patients show increased CCL1 serum levels. CCL1-specific ELISA was performed with serum samples of healthy individuals ($n = 11$) and atopic dermatitis patients in lesional ($n = 32$) or nonlesional ($n = 2$) phases of the disease. Values are expressed in nanograms of target protein per milliliter of serum. Measurements of individual samples and the mean + SD are shown. Student *t* test was performed; *, $p < 0.05$. *f* and *g*, Allergen-specific cross-linking of IgE induces CCL1 in mast cells. Immunocytochemistry reveals abundant CCL1 protein expression in human dermal mast cells (*g*) compared with the isotype control (*f*). Original magnification, ×1000. *h*, Quantitative real-time PCR analysis of CCL1 expression was performed in murine bone marrow-derived mast cells treated with either PBS alone, DNP-specific IgE or DNP-specific IgE plus DNP for 8 and 48 h. Values are expressed as relative units of target gene expression compared with the level of 18 S RNA. *i*, CCL1 protein expression in murine bone marrow-derived mast cells treated either with PBS alone, DNP-specific IgE or DNP-specific IgE plus DNP for 8 or 48 h. Mean + SD are shown.

T cell subsets, monocytes, and DC subpopulations express CCR8

To assess the biologic relevance of CCL1, we investigated CCR8 expression on peripheral blood T cells, monocytes, and in vitro-derived DC subsets.

By flow cytometry, CCR8 was detected on the cell surface of a small subset of circulating $CD4^+$ and $CD8^+$ T lymphocytes (1.2–2.7%) (Fig. 5*d*), and its expression was not associated with the expression of the skin-homing molecule, CLA (Fig. 5*d*). Equal numbers of $CCR8^+$ T cells could be detected in CLA^+ or CLA^- lymphocyte populations (Fig. 5*d*). Moreover, staining of CCR8 in permeabilized T cells indicated that the majority (>90%) of $CD3^+$ lymphocytes store CCR8 in the cytoplasm (data not shown). Subsequent analyses demonstrated that a 2 h stimulation of lymphocytes with ConA rapidly recruits CCR8 to the cell surface (Fig. 5*e*). Comparing $CD4^+$ and $CD8^+$ lymphocyte subsets, CCR8 showed a 2-fold enhanced cell surface expression in activated $CD8^+$ cells. In contrast to ConA, stimulation of lymphocytes with anti-CD3/CD28 or PHA did not induce CCR8 on the cell surface (Fig. 5*e*).

CCR8 was also present on the surface of both $CD14^{low}$ and $CD14^{high}$ monocyte subsets (Fig. 5*c*). As previously described (8, 9), E-cadherin$^-$ Langerin$^-$ interstitial DC were differentiated from monocytes in presence of GM-CSF plus IL-4, and E-cadherin$^+$ Langerin$^+$ LC were differentiated in the presence of GM-CSF, IL-4, TGF-β plus TNF-α during the last 2 days of culture. In both DC subsets, CCR8 was expressed at the cell surface (Fig. 5*a*) and was also stored in the intracellular compartment as demonstrated with two different Abs directed against CCR8 (Fig. 5*b*).

These observations indicate that CCR8 is expressed on leukocyte subsets known to play a role in atopic skin inflammation. Furthermore, they demonstrate a novel regulation pathway for a chemokine receptor arguing for a rapid and local modulation of the responsiveness of T cell subsets.

CCR8 expression is maintained during early DC differentiation and activation

To obtain insights into the recruitment pathways of DC during the initiation and amplification of atopic dermatitis, we studied the modulation of CCR8 expression during DC differentiation from peripheral blood monocytes and DC maturation in presence of atopic dermatitis-associated bacterial products or T cell signals (Fig. 5). Monocytes were cultured in presence of GM-CSF plus IL-4 until day 6 for DC differentiation and then seeded with GM-CSF with or without IL-4 until day 10 to preserve their immature phenotype. In both conditions, cells were stimulated in the presence of the T cell signal, CD40L or left alone during the last 2 days of culture. CCR8 was detected on freshly isolated monocytes (day 0). During DC differentiation in the presence of IL-4, its expression decreased progressively until the end of culture (day 10). Upon withdrawal of IL-4 for 2 days (days 6 to 8), the expression of CCR8 was recovered (from 40 to 70%) compared with cells that have constantly been exposed to IL-4 (Fig. 5*c*). Subsequently, CCR8 expression was progressively lost during the terminal differentiation of DC in the presence of GM-CSF alone (days 8 to 10). Contrary to other chemokine receptors known to be expressed on DC (e.g., CCR1, CCR2, CCR5, CCR6, CCR7), the addition of the CD40L activation did not change the level of CCR8 expression

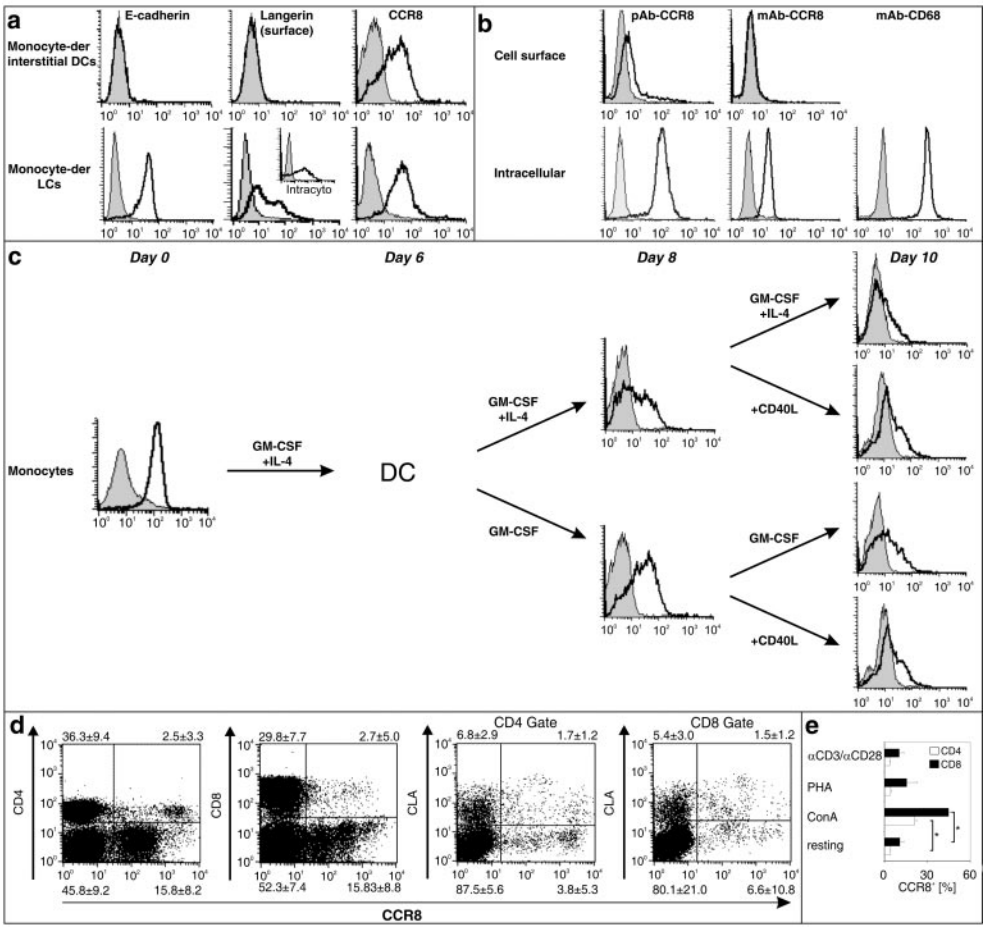

FIGURE 5. CCR8 is expressed on DC, their precursors and a subset of T cells. CCR8 expression was determined by flow cytometry on monocytes, interstitial DC, Langerhans-type DC, and peripheral blood T lymphocytes. *a*, Cell surface expression of CCR8 on E-cadherin$^-$, Langerin/CD207$^-$, interstitial DC, and E-cadherin$^+$ Langerin/CD207$^+$ Langerhans-type cells. *b*, Cell surface and intracellular stainings of CCR8 on Langerhans-type DC with two different anti-human CCR8 (goat polyclonal and mouse monoclonal). Cell permeabilization was determined by staining with an anti-CD68. *c*, Regulation of CCR8 expression during DC differentiation. Monocytes were differentiated into DC in presence of GM-CSF plus IL-4 for 6 days. Subsequently, DC were seeded in presence of GM-CSF alone or GM-CSF+IL-4 until day 10 and either activated by CD40L or left unstimulated during the last 2 days of the culture (*c*). *d* and *e*, CCR8 expression on resting and activated peripheral blood T cells. PBMC were stained with mAbs directed against CD4, CD8, CLA, and CCR8. Percentages indicate the relative number of double-positive cells analyzed in total PBMC. The expression of CCR8 on skin-homing CLA$^+$ T cells was determined on gated CD4$^+$ and CD8$^+$ T cells. The results are representative of 5–10 experiments. *e*, CCR8 expression on CD4$^+$ (□) and CD8$^+$ (■) T cells after stimulation with either anti-CD3/CD28, PHA, or ConA for 2 h compared with unstimulated cells. Values indicate the percentage of CCR8$^+$ T cells (representative of $n \geq 3$ different donors). Mean + SD are shown. Student's t test was performed.

(Fig. 5*c*). Hence, we investigated the effects of microbial products on the expression of CCR8 on DC. Cells were stimulated for 2 days with LPS, LTA, PGN, mannan, SEB, or CD40L or left unstimulated. CCR8 expression was not modulated after stimulation of DC by those compounds. Depending on the experiments, CCR8 can be slightly down-regulated in presence of LPS (data not shown).

These observations indicate that CCR8 expression is down-regulated by the Th2 cytokine IL-4 during DC differentiation and that this effect is reversible. Furthermore activation of LC by atopy-associated bacterial products or the T cell signal CD40L has no effect on its expression.

The inflammatory chemokine CCL1 synergizes with the homeostatic chemokine CXCL12 during the recruitment of CCR8$^+$ T and DC subsets

During the next step we investigated the biologic activity of CCR8 on leukocyte subsets. Surprisingly, CCL1 alone failed to induce significant migration of CCR8-expressing leukocytes subsets (Fig. 6) at any concentration. Because Vanbervliet et al. (15) obtained similar results with CXCR3 expression on plasmacytoid DC and demonstrated the synergistic cooperation of CXCR3 ligands with the homeostatic chemokine CXCL12, we first investigated the anatomical distribution of CXCL12 expression in atopic dermatitis. By immunohistochemistry, CXCL12 was detected in endothelial cells as well as in cells with dendritic morphology in both normal skin (Fig. 2*g*) and lesional atopic skin (Fig. 2, *h* and *i*). In contrast to normal skin in which CCL1 was not observed, expression of CXCL12 and CCL1 overlapped in atopic dermatitis. Indeed, CCL1 in combination with suboptimal doses of CXCL12 (10 ng/ml; equals 20–50% of maximal CXCL12-mediated chemotatic responses, data not shown) synergistically attracted CD4$^+$ (data not shown), CD4$^+$CLA$^+$ (Fig. 6*a*), CD8$^+$ (data not shown), and CD8$^+$CLA$^+$ (Fig. 6*b*) T cells in a dose-dependent manner. At a concentration of 1000 ng/ml CCL1 and 10 ng/ml CXCL12, a significant increase in migration was shown in all T cell subsets, with the highest migration percentage in the CD8$^+$CLA$^+$ T cell subset (Fig. 6*b*). Overall, between 8 and 16% of the starting population of T cells migrated in response to CCL1/CXCL12 gradients. In accordance to this, the CCL1/CXCL12 mix synergistically induced

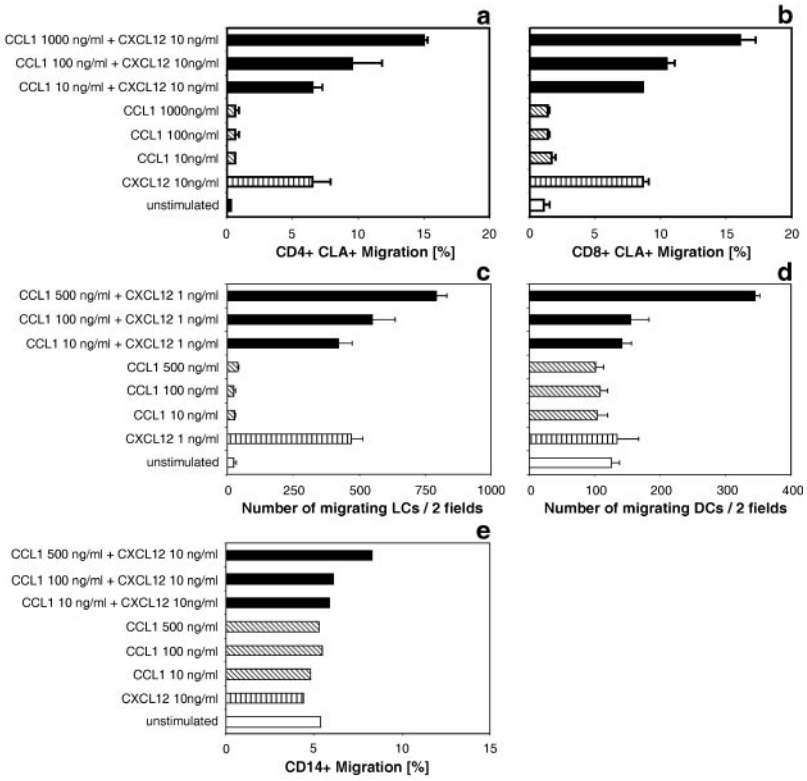

FIGURE 6. CCL1 synergizes with the homeostatic chemokine CXCL12 in the recruitment of distinct leukocyte subsets. Peripheral blood T cells (*a* and *b*) and monocytes (*e*) from healthy donors were analyzed in Transwell chemotaxis assays for their capacity to migrate in response to either CCL1 (10, 100, 1000 ng/ml) or CXCL12 (10 ng/ml; suboptimal concentration), or the combination of CCL1 plus CXCL12. The percentage of migrated $CD4^+CLA^+$ (*a*), $CD8^+CLA^+$ (*b*) T lymphocytes and $CD14^+$ monocytes (*e*) was determined in comparison to the starting population from triplicate and duplicate measurements for T cells and monocytes, respectively. Langerhans-type DC (*c*) and interstitial DC (*d*) were analyzed in Boyden chamber assays for their capacity to respond to either CCL1 (10, 100, 1000 ng/ml) or CXCL12 (1 or 10 ng/ml, suboptimal concentration) or the mixture of CCL1 and CXCL12. Results are expressed as number of migrated DC per two low power fields (magnification, ×20). (Results of one donor is shown representative for $n \geq 4$ different donors).

the migration of three and two times more interstitial DC (Fig. 6*d*) and LC (Fig. 6*c*), respectively, compared with CXCL12 alone. The best synergism for DC was observed at a concentration of 500 ng/ml CCL1 and 1 ng/ml CXCL12. However, the synergism between those chemokines was not observed in monocytes (Fig. 6*e*).

Therefore, although T cell subsets, interstitial DC, and LC express CCR8, they do not migrate in response to CCL1 alone. As CCL1 and CXCL12 have a common cellular origin in endothelial cells and cells with dendritic morphology, we have demonstrated that CCL1 potentiates the chemotactic responsiveness of relevant leukocyte subsets to CXCL12.

Discussion

Atopic disorders are a group of increasingly common multifactorial chronic diseases, which cause inflammatory and degenerative changes in the skin and mucosal surfaces. In atopic dermatitis patients, skin-infiltrating leukocytes play a crucial role in the initiation and maintenance of atopic skin inflammation. Predominantly CLA^+ memory T cells, FcεR-bearing DC, and eosinophils constitute the inflammatory infiltrate (2, 3). A better understanding of their recruitment pathways may help develop new approaches for the prevention, diagnosis, and treatment of atopic disorders.

It is known that a family of small cytokine-like chemoattractive proteins, called chemokines, regulate leukocyte trafficking under homeostatic and inflammatory conditions (16, 17). Members of the chemokine superfamily can be divided into so called cluster and noncluster chemokines (4). A chemokine gene cluster including 15 different ligands is located on chromosome 17q11.2. Many of the CC chemokines in this cluster bind the same receptors, namely CCR1, CCR2, CCR3, and CCR5. Interestingly, although the gene encoding CCL1 is located in this chemokine gene cluster (18) it is the only CC chemokine from this cluster that specifically binds CCR8, or in other words, has a single ligand/receptor relationship. This suggests that CCL1-CCR8 interactions may mediate nonredundant functions.

Recent insights into the relationship between DNA viruses and hosts showed that during the evolution of the complex virus-host interactions viruses adapted to the immune system of their hosts and developed viral chemokine receptor antagonists to escape the host immune defense. A critical feature of this strategy was to prevent recognition by the immune system without severely interfering with the viability of the host. One such example is the epidermotropic DNA virus *Molluscum contagiosum* expressing the viral CCR8 antagonist MC148 (19). Interestingly, Molluscum contagiosum infections show a markedly increased frequency in individuals with atopic dermatitis (20) suggesting that this DNA virus adapted to preferentially CCL1-CCR8-driven skin immune responses in atopic individuals.

Recently, CCL17 has been shown to be selectively expressed in lesional atopic dermatitis skin and serum levels of CCL17 (TARC) correlate with disease activity of atopic dermatitis patients (21). CCL17 binds CCR4, which is abundantly and preferentially expressed on CLA^+CD4^+ skin-homing memory T cells. Current concepts suggest that CCR4 and CCR10 ligands cooperate in the recruitment of skin-homing $CD4^+$ memory T cells, however, very little is known about the recruitment pathways of $CD8^+$ T cells into the skin.

In the present study, we show that activation rapidly up-regulates the expression of CCR8 on the surface of $CD8^+$ T cells. Furthermore, the synergism between CXCL12 and CCL1 attracts $CD8^+$ memory T cells providing a novel mechanism for the recruitment of CD8 cells to sites of atopic inflammation. Moreover, DC and their precursors do not express CCR4 or CCR10. Hence, these receptors cannot explain the DC accumulation observed during atopic skin inflammation (22–24). In this study we show that CCL1 represents an inflammatory and atopy-associated chemokine induced during atopic skin inflammation. Serum levels of CCL1

were significantly increased during lesional phases of atopic dermatitis linking this chemokine with at least a partly T_H2-driven skin inflammation. Among the cellular constituents of the skin, major sources of CCL1 are endothelial cells, mast cells, and LC. These observations support recent findings associating the CCL1-CCR8 with a T_H2 phenotype (25) and demonstrating endothelial cells and LC (26) as a cellular origin of CCL1 production in vitro.

There is increasing evidence but as yet no demonstration that macrophages and DC-like cells represent a target for CCL1. Recently, Mahad et al. (27) showed that macrophages of patients suffering from multiple sclerosis abundantly express CCR8 on their cell surface. We show that CCR8 is expressed on the surface of monocytes, both interstitial as well as Langerhans-type DCs and on a minor subset of circulating $CD4^+$ and $CD8^+$ T cells. Surprisingly, they do not migrate in response to CCL1 alone. We observed that CXCL12 synergizes with CCL1 in recruiting a subset of $CD4^+$ and $CD8^+$ skin-homing CLA^+ memory T cells and both DC subsets in vitro. In particular, LC migrate in response to a restricted set of chemokines (CCL20, CXCL12 and to a lesser extend CCL5 (RANTES) and CCL7 (MCP-3)), in accordance with the expression of a very restricted repertoire of chemokine receptors (13). As DC are crucial in the initiation of immune responses, CCL1 may play a key role in the recruitment of LC in atopic dermatitis. In comparison, monocytes displayed minor chemotactic responses toward CXCL12 and CCL1. As they express a broad range of chemokine receptors, we can speculate that monocytes might extravasate from the blood in response to other inflammatory chemokines. Notably we show that CCL1 and CXCL12 are produced at similar anatomical locations in vivo. Both chemokines are expressed by endothelial cells of the superficial dermal plexus, DC and show immunoreactivity within the epidermal compartment of atopic dermatitis patients. Thus, both chemokines are well suited to cooperate in the recruitment of T cell and DC subsets to sites of atopic skin inflammation. Results of the present study extend previous findings showing that the inducible inflammatory ligands of CXCR3 (CXCL9, CXCL10, and CXCL11) control the migratory responsiveness of plasmacytoid DC in cooperation with the homeostatic chemokine CXCL12 (15). Together with the observations of Vanbervliet et al. (15), our study supports the concept that the cooperation of inflammatory and homeostatic chemokines plays an important role in the recruitment of distinct leukocyte subsets to sites of inflammation in vivo.

Our study also supports the findings of Schaerli et al. (26) showing the presence of a rare subset of $CCR8^+$ T cells in peripheral blood. However in normal skin, they detected CCL1 protein in the epidermis and endothelial blood vessels, whereas we did not observe significant expression by quantitative real-time PCR or immunohistochemistry. The authors argued for a role of CCL1 in the homeostatic trafficking of T cells into the skin. Because we have detected few $CCR8^+$ cells in normal skin, we cannot exclude a putative role for CCL1 in T cell trafficking under homeostatic conditions. However in the present study, we extend previous observations and demonstrate a significant and selective up-regulation of CCL1 mRNA and protein in lesional skin and serum of atopic patients. We also demonstrate CCL1 mRNA induction after allergen challenge, stimulation with microbial products, and cross-linking of allergen-specific IgE on the surface of mast cells, suggesting a major role of CCL1 during atopic skin inflammation.

Moreover, we have studied the effect of microbial or T cell-derived factors on the regulation of CCR8 during DC differentiation and maturation. CCR8 expression is maintained during the early differentiation of monocytes into interstitial-type or Langerhans-type DC and in turn, is progressively lost during their terminal stage of differentiation. We have also observed that IL-4 down-regulates CCR8. Thus, IL-4 might switch off CCL1 and CXL12 responsiveness on DC. At the beginning of the acute phase/T_H2 response, the local production of IL-4 by T cells and mast cells might allow DC still recruited to respond to other chemokines like CCR7 ligands known to be expressed by lymphatic vessels. Surprisingly unlike most of chemokine receptors (CCR1, CCR2, CCR5, CCR6), DC maintain cell surface expression of CCR8 during the presence of microbial products or T cell signals. The secretion of high amounts of CCL1 by activated DC might have two direct effects. First, CCL1 autodesensitizes its receptor on DC allowing them to become responsive to other chemokines like CCR7 ligands (CCL19 (MIP-3β) and CCL21 (6Ckine/SLC)). At the same time, CCL1 amplifies the immune response by recruiting more $CCR8^+$ cells to sites of atopic skin inflammation. The maintenance of CCR8 expression during DC activation indicates that CCR8 might also be involved in functions other than chemotaxis. CCL1 has been described as a potent antiapoptotic factor for thymocytes (28) suggesting that CCL1-CCR8 interactions may provide survival signals for T cells and DC at sites of atopic skin inflammation. This interesting aspect is currently under investigation.

Taken together, we suggest a model in which allergen or microbial products (*S. aureus*) may trigger CCL1 production in atopic individuals which in turn recruits memory T cells and in particular LC into the skin and leads to their accumulation in subepidermal and intraepidermal locations. Because activated DC secrete large amounts of CCL1, CCL1-CCR8-driven recruitment pathways may facilitate DC-T cell interactions at sites of atopic skin inflammation. Moreover, CCR8 signaling may enhance cell survival providing a potential mechanism that sustains atopic inflammation and prevents activation-induced apoptosis of skin-infiltrating leukocytes. Thus, CCL1 and CCR8 may provide an axis linking adaptive and innate immune functions, leading to the accumulation of relevant leukocyte subsets at sites of atopic skin inflammation and supporting the initiation and amplification of atopic dermatitis.

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Disclosures

The authors have no financial conflict of interest.

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