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


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Atopic dermatitis represents a chronically relapsing skin disease with a steadily increasing prevalence ranging from 10–20% in children and from 1 to 3% in adults within developed countries. Clinically, atopic dermatitis is characterized by pruritic and erythematous papules and plaques with predilections at flexural parts of the body, intertrigines, 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. Histopathologically, atopic skin lesions show a marked inflammatory infiltrate composed of CD4+ or CD8+ cutaneous lymphocyte-associated Ag (CLA+3) and 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 sentinel cells 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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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.

Homoeopietic factors and reagents

Recombinant human GM-CSF (specific activity, 2 × 10^4 U/mg; Schering-Plough) was used at a saturating concentration of 100 ng/ml. Recombinant human IL-4 (specific activity, 2 × 10^3 U/mg; Schering-Plough) and recombinant human TGF-β1 (specific activity, 5 × 10^3 U/mg) were used at 10 ng/ml. Recombinant human TNF-α (specific activity, 2 × 10^4 U/mg) at either 3 ng/ml for DC culture or 10 ng/ml for nonhomoeopietic 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). 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-CD38 (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. pteronyssinus (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 punch biopsies.

Table I. Atopy patch tests in house dust mite-sensitized atopic dermatitis patients

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*House dust mite-sensitized atopic dermatitis patients were subjected to atopy patch tests with preparations containing a mixture of Dermatophagoides farinae (10%) and D. pteronyssinus (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, and vesicles, and spreading.
punches. Biopsy specimens were cut in half (quantitative PCR and immuno-
nohistochemistry) and stored at −70°C. All 10 patients had positive reac-
tions 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 Sys-
tems) was used according to the manufacturer’s instructions. Dilution se-
ries 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% en-
riched 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 recep-
tor (89% of the cells positive) expression. Murine bone marrow-derived
mast cells were incubated on 12-well microtiter plates (2 × 106 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 lysed 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 cytom-
try with the following Abs: Unlabelled anti-CCR8 (210-762-R100, goat
IgG; Alexis Biochemicals); FITC-conjugated anti-CD14 (MSE2, mouse
IgG2a), anti-CD19 (HIB19, mouse IgG1), anti-CD3 (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
IgG2a, anti-CD19 (HIB19, mouse IgG1), anti-CD8 (RPA-T8, mouse IgG1),
anti-CD117 (YB5.B8, mouse IgG1), swine anti-goat IgG (G50004; Caltag
Laboratories); PE-Cy5-conjugated anti-CD25 (M-A251, mouse IgG1), anti-
CD4 (RPA-T4, mouse IgG1), anti-CD123 (9F5, mouse IgG1), anti-mouse
IgG2a, anti-CD19 (HIB19, mouse IgG1), anti-CD8 (RPA-T8, mouse IgG1) from
BD Pharmingen. Briefly, 106 cells
were stained, fixed in 4% paraformaldehyde and then analyzed using FAC-
Scan or FACS Calibur 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% CO2, or left un-
treated. 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 I (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. Sub-
sequently cytospin slides of dermal single cell suspensions were prepared
using −5 × 106 cells on adhesive cover slides and subjected to immuno-
cytochemical analysis. Mast cells were identified by their typical morphol-
yre assay}

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 nonle-
sional 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 10^6 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% CO₂, 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% CO₂ 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. 1a) and CCL17 (TARC) (Fig. 1b) mRNAs were significantly (p < 0.05) up-regulated in lesional atopic skin compared with skin from healthy individuals, whereas CCL20 (MIP-3α) (Fig. 1c) 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. 2a) 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. 2b).

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), TH 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. 3a). 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 (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.
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 monocyctic precursors produced moderate to high amounts of CCL1 mRNA (Fig. 3a). Analyses of CCL1 protein expression (Fig. 3b) 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. 3b). 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. 4g). Furthermore, bone marrow-derived murine mast cells showed a dramatic (40,000-fold) increase in CCL1 mRNA expression after cross-linking of DNP-specific IgE with specific Ag/allergen on their surface for 8 h (Fig. 4h). Moreover, IgE binding alone also stimulated murine mast cells to produce high levels of CCL1 mRNA (Fig. 4b). Next to mRNA, CCL1 protein levels also markedly increased within the supernatant of IgE or IgE plus DNP-treated murine mast cells (Fig. 4i). 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 allergenspecific 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. 4d). 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. 4e). 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.

<table>
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<th>TABLE 3. CCL1 content in atopic dermatitis patients</th>
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**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, IFN-γ, IL-4 or left unstimulated for 6 and 18 h. b. Secretion of CCL1 by Langerhans-type DC and interstitial DC-type cells and their monocyctic 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 femtograms of target gene in 25 ng to total cDNA. Student t test was performed; *p* < 0.05. (representative of *n* = 5).
CD8 showed a 2-fold enhanced cell surface expression in activated CD14high monocyte subsets (Fig. 5). The expression of the skin-homing molecule, CLA (Fig. 5), and its expression was not associated with the Langerin lymphocytes store CCR8 in the cytoplasm (data not shown). Sub-permeabilized T cells indicated that the majority (>90%) of CD3

By flow cytometry, CCR8 was detected on the cell surface of a small subset of circulating CD4

In contrast to ConA, stimulation of lymphocytes with allergen caused CCR8 to rapidly recruit to the cell surface (Fig. 5). Moreover, staining of CCR8 in lymphocyte populations (Fig. 5) showed that CCR8 was also present on the surface of both CD14low and CD8 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

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). 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.

These observations indicate that CCR8 is expressed on leukocyte subsets known to play a role in atopic skin inflammation. Moreover, it is not associated with the expression of the skin-homing molecule, CLA (Fig. 5). Equal numbers of CCR8+ T cells could be detected in CLA+ or CLA- lymphocyte populations (Fig. 5). 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. 5e). 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. 5e).

CCR8 was also present on the surface of both CD14low and CD14high monocyte subsets (Fig. 5c). 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. 5a) and was also stored in the intracellular compartment as demonstrated with two different Abs directed against CCR8 (Fig. 5b).
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. 2g) and lesional atopic skin (Fig. 2g, 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 chemotactic responses, data not shown), CCL1+CLA+ (Fig. 6a), CCL1+ (data not shown), and CCL1+CLA+ (Fig. 6b) 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 CCL1+CLA+ T cell subset (Fig. 6b). 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 (c) 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 (c) was determined in comparison to the starting population from triplicate and duplicate measurements for T cells and monocytes, respectively. Langerhans-type DC (d) and interstitial DC (e) 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).

Thus 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 CCR5 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 T112-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 T112 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+/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 Schaeeri 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 CXL2 responsiveness on DC. At the beginning of the acute phase/T112 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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References


