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CCL18 Is Expressed in Atopic Dermatitis and Mediates Skin Homing of Human Memory T Cells

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CCL18 is a human chemokine secreted by monocytes and dendritic cells. The receptor for CCL18 is not yet known and the functions of this chemokine on immune cells are not fully elucidated. In this study, we describe that CCL18 is present in skin biopsies of atopic dermatitis (AD) patients but not in normal or psoriatic skin. CCL18 was specifically expressed by APCs in the dermis and by Langerhans and inflammatory dendritic epidermal cells in the epidermis. In addition, the serum levels of CCL18 and the percentages of CCL18-producing monocyte/macrophages and dendritic cells were significantly increased in AD patients compared with healthy controls. Furthermore, we demonstrate that CCL18 binds to CLA+ T cells in peripheral blood of AD patients and healthy individuals and induces migration of AD-derived memory T cells in vitro and in human skin-transplanted SCID mice. These findings highlight a unique role of CCL18 in AD and reveal a novel function of this chemokine mediating skin homing of a subpopulation of human memory T cells. The Journal of Immunology, 2005, 174: 1723–1728.

The regulation of leukocyte trafficking during homeostatic and pathologic responses is achieved in great part by chemokines. Chemokines comprise a large group of homologous proteins that exert their biological effects by interactions with cell surface heptahedral G protein-coupled receptors (1). CCL18, also designated dendritic cell (DC)-derived CC chemokine 1, pulmonary and activation-regulated chemokine, alternative macrophage activation-associated CC chemokine 1, and MIP-4, is a human chemokine structurally related to CCL3 (2, 3). So far, CCL18 has no mouse equivalent and its receptor is not known. CCL18 was shown to be expressed in germinal centers of tonsils by dendritic cells and to attract mainly naive T cells, CD38+CD45RA+CD62L–CD27+CD8α+CD8β+CD69+ memory T cells (4). The production of CCL18 by APC is enhanced by Th2 cytokines like IL-4 and IL-13 and suppressed by IFN-γ (5, 6). Accordingly, CCL18 has been implicated in hypersensitivity pneumonitis (7), vernal keratoconjunctivitis (8), and contact hypersensitivity (9). Atopic dermatitis (AD) is a Th2-mediated, chronic inflammatory skin disease characterized by eczematous skin lesions (10). Histopathologically, skin lesions of AD reveal a mononuclear cell infiltrate consisting of macrophages and CD4+ Th cells. In acute lesions, these Th cells mainly produce Th2 cytokines like IL-4, IL-5, and IL-13, whereas in chronic AD, IFN-γ secreting Th cells are also present (11). The chemokines suggested to mediate the homing of Th cells into AD skin include the CCR4 ligands CCL17 (12) and CCL22 (13) and the CCR10 ligand CCL27 (14). However, additional chemotactic signals may be required for the specific recruitment of specialized T cell subpopulations (15). In this study, we demonstrate that CCL18 contributes to the leukocytic infiltration associated with AD. We found, that AD patients have elevated levels of CCL18 in serum and lesional skin and that CCL18 recruits human memory T cells derived from AD patients to human skin in vivo.

Materials and Methods

Patients

Blood samples were obtained with informed consent from Caucasian individuals, including 36 patients with AD and 28 nonatopic healthy controls. Clinical severity was documented independently and blindly by the clinicians using the eczema area and severity index (EASI) and the investigators’ global assessment (IGA) (16). The patient population was distributed according to IGA scores as follows: almost clear (n = 3), mild disease (n = 0), moderate disease (n = 15), severe disease (n = 10), and very severe disease (n = 4). The patients did not receive any systemic or topical treatment with immunosuppressive drugs for at least 4 wk before collection of blood samples and skin biopsies.

Purification and culture of cells

PBMC from human blood were prepared by density gradient separation on Ficoll-Paque Plus (Amersham Biosciences). The AD-derived T cell lines (NIT, SVT, DK2-JOT) and clones (98016T.02, 98016T.24), which expressed cutaneous lymphocyte Ag (CLA), CCR4, and displayed a Th2/Th0 cytokine secretion pattern following activation, were generated from lesional punch biopsies as previously described (17, 18). T cells were cultured in X-Vivo15 medium (BioWittaker) supplemented with recombinant human IL-2 (20 ng/ml; Novartis Institutes for Biomedical Research (NIBR) Vienna, Austria).

Flow cytometry

Cells were stained for 30 min on ice using FACS buffer (PBS containing 2% FCS and 0.1% sodium azide). The following mAbs and corresponding isotypes were used: CD3-PE, CD3-PerCP, CD4-PE, CD4-PerCP, CD8-PerCP, CD14-FITC, CD19-FITC, CD20-FITC, CD62-P, CD56-FITC, HLA-DR-CyChrome, CCR4-PE, CD54-PE, CLA-FITC, rat anti-CLA, mouse anti-CD20-FITC, rat anti-CD19-FITC, mouse anti-CD62-PE, mouse anti-CLA-FITC, mouse anti-CD14-FITC, mouse anti-CD19-FITC, mouse anti-CD20-FITC, mouse anti-CD56-FITC, and mouse isotype control (BD Pharmingen).
mouse anti-rat IgM-Fc (BD Pharmingen), CD3-allophycocyanin, CD3- FITC, CD4-allophycocyanin, CD11a-PE, CD11c-allophycocyanin, CD19- allophycocyanin (Caltag Laboratories), CCR3-PE, CCR5-PE, CCR7-PE, CXCR3-PE, CXCR4, and CXCR6-PE (R&D Systems). Intracellular cytokine production by T cells was determined after 5 h of stimulation with anti-CD3 mAb (1 μg/ml; BD Pharmingen) and 2 ng/ml PMA (Sigma-Aldrich). Brefeldin A (10 μg/ml; Epicentre Technologies) was added for the last 2 h. Thereafter, cells were fixed with 2% formaldehyde (Sigma-Aldrich), subsequently permeabilized with 0.5% saponin (Sigma-Aldrich), and intracytoplasmic cytokine production was detected using allophycocyanin- or PE-conjugated mAbs specific for IL-4 or IFN-γ, respectively (BD Pharmingen). CCL22-Alexa487 was purchased from Dictagene. CCL18 (PeproTech) was labeled in-house with Cy5 or biotin (Molecular Probes) according to the manufacturer’s instructions. A peptide of similar size (CHI domain of the intracellular protein P300), that has no known cell surface receptor, was also labeled with Cy5 and used as staining control. Chemokines were used for staining at a final concentration of 300 ng/ml. Analysis was performed on a FACS Calibur (BD Biosciences) using BD CellQuest software.

Detection of intracellular CCL18 in monocyte/macrophages and DC after stimulation with IL-4

PBMC (5 × 10^6 cells/ml) from AD patients (n = 8) and controls (n = 13) were cultured for 48 h in the presence or absence of 10 ng/ml recombinant human IL-4 (NIBR) and treated with brefeldin A (10 μg/ml; Sigma-Aldrich) for the last 3 h. Monocyte/macrophages were recognized by CD14 staining, and myeloid DC were identified as the CD11c^+ fraction of HLA-DR^+, Lin^− (CD3, CD14, CD19, CD20, CD56) PBMC. For intracellular staining of CCL18, surface-stained cells were fixed with 4% paraformaldehyde (Sigma-Aldrich), permeabilized with 0.5% saponin (Sigma-Aldrich), and stained with goat anti-human CCL18 mAb (R&D Systems) followed by donkey anti-goat-PE (DPC Bierrmann). Goat serum (Sigma-Aldrich) was used as isotype control.

ELISA

Serum CCL18 and CCL22 levels were determined using Duoset ELISA (R&D Systems) according to the manufacturer’s instructions.

Chemotaxis assay

Chemokines (R&D Systems) or control medium (RPMI 1640 without phenol red with 0.5% BSA) were added in 30 μl to the lower wells of a 96-well plate (Molecular Probes)-labeled cells were seeded at 3 × 10^5 cells/ml in the upper chamber and selected wells of the lower chamber (to obtain 100% migration value). The two chamber compartments were separated by a 3-μm pore size polycarbonate filter (NeuroProbe). After a 2.5-h incubation at 37°C, filters were removed, the plate was spun down, and supernatants were discarded. Then, 20 μl of 0.4% Triton X-100 (Sigma-Aldrich) was added to each well. The degree of migration was quantified by fluorescence reading using a Victor Ⅱ (Wallac, PerkinElmer Life Sciences) multwell plate reader.

In vivo migration assay in SCID-hu Skin mice

Human skin pieces were obtained from the Western Hungarian Tissue Bank, Pécs Aladár County Hospital (Győr, Hungary) according to institutional and governmental ethical guidelines. SCID (C.B-17/GstTac-Pkdcre/tr Lystbg) mice (Taconic) were transplanted on their backs with two human skin pieces from the same donor as previously described (19). Chemokine-induced skin homing of human T cells was performed using a modification of the previously described method (18). AD skin-derived T cells were labeled with CFSE (1 μM in PBS for 5 min at room temperature; Molecular Probes) and 5 × 10^5 cells/mouse were injected i.v. into SCID mice transplanted with human skin (SCID-hu Skin mice). Immediately after, CCL18 or CCL22 (30 ng in 30 μl PBS; R&D Systems) or PBS alone were injected intradermally into human skin grafts. Each single mouse was injected with one chemokine in one human skin graft and with another chemokine or with PBS control in the other skin graft. After 24 h, skin grafts were explanted, processed into single-cell suspensions, stained, and analyzed in the FACS by quantifying the percentages of CD3^−, CFSE^− cells present in the living (propidium iodide negative) cell fraction. Alternatively, parts of the human skin grafts were frozen for further analysis by immunohistochemistry.

Immunohistochemistry

Acetone-fixed 5-μm cryostat sections of normal skin of healthy subjects (n = 3) and lesional skin from patients with AD (n = 10) or psoriasis (n = 3) were stained with goat anti-CCL18 mAb (R&D Systems) or goat serum and biotinylated horse anti-goat IgG (Vector Laboratories) followed by avidin-conjugated alkaline phosphatase (AP) and naphthol-AS phosphate/Fast Red TR substrate (Sigma-Aldrich). CCL18-binding cells in AD samples were detected using CCL18-biotin followed by avidin-AP and Fast Red. The samples were contained with anti-CD3 mAb (BD Biosciences) or anti-CCR4 mAb (Santa Cruz Biotechnology) followed by anti-mouse-IgG-biotin (Vector Laboratories), avidin-AP, and Fast Blue (Sigma-Aldrich) and counterstained with hematoxylin (Merck). For immunofluorescence analysis, cryostat sections were stained with goat anti-CCL18 (R&D Systems) and mouse anti-HLA-DR or anti-CD3 (BD Biosciences), anti-CD1a (Caltag Laboratories), anti-CD207 (Baxter) followed by rabbit anti-goat- Texas Red and horse anti-mouse- FITC (Vector Laboratories), respectively. Slides were mounted with 4',6-diamidino-2-phenylindole containing medium (Vector Laboratories), coverslipped, and analyzed with a confocal scanning microscope (Zeiss) using the Zeiss LSM5 Image Browser.

Statistical analysis

Data are presented as bars (indicating averages ± SE), curves (indicating averages ± SE), or box plots (indicating average, median, 25–75% percentiles and SD). Statistical analysis was performed using unpaired Student’s t test with the help of Origin 7.0 software (OriginLab). Clinical data were tested for simple correlations by determining Pearson’s correlation coefficient and uncorrected probability values with Systat 10 software (SST). Statistical evaluation of histologic samples was performed using Fisher’s exact test (Systat 10 software). In all cases, p < 0.05 was considered significant.

Results

CCL18 is expressed in human AD skin

AD is an inflammatory skin disease associated with cutaneous hyperreactivity to allergens and high IL-4 production (10). Consequently, AD can provide a suitable environment for CCL18 up-regulation. To test this hypothesis, we performed an immunohistochemistry evaluation of CCL18 expression in skin samples of AD patients (n = 10) in comparison to normal (n = 3) and psoriatic (n = 3) skin. CCL18 expression was detected in all AD skin samples but was absent in the skin from healthy or psoriatic individuals (Fig. 1A). The differences in the expression of CCL18 in those skin samples were significant as assessed by Fisher’s exact test (p < 0.003). In AD skin, CCL18 was strongly expressed in the upper dermal vascular plexus, in close association with the mononuclear cell infiltrates. Immunofluorescence staining demonstrated that CCL18 expression was restricted to HLA-DR^+ APC (Fig. 1B). Particularly, all CCL18-expressing cells in the epidermis were CD1a^+. Subsequent staining with Abs against CD207 (langerin) demonstrated that most of the CCL18-expressing cells were Langherhans cells whereas the remaining population consisted of inflammatory dendritic epidermal cells. Keratinocytes, fibroblasts, and CD3-positive T cells did not express CCL18 (Fig. 1B).

CCL18-producing APC are increased in blood of AD patients

Our findings demonstrating a selective expression of CCL18 by APC in the skin of atopic individuals prompted us to investigate whether CCL18-producing APC are also enriched in the circulation of these patients. CCL18 expression by APC of AD patients and healthy controls was assessed by intracellular immunofluorescence staining following stimulation with IL-4. As shown in Fig. 2A, the percentages of CCL18-producing monocyte/macrophages (CD14^+) in blood of AD patients were significantly higher (5-fold, p < 0.003) than those observed in healthy individuals. Similarly, the percentages of CD11c^+, HLA-DR^−, and in CCL18-producing DC from AD patients were also higher (3-fold, p < 0.001) than those of healthy donors (Fig. 2A). In both groups, CCL18-expressing cells were detected following stimulation with IL-4, suggesting that CCL18 was produced de novo and that the reactivity was not due to staining of prestored CCL18 in cellular compartments (data not shown). CCL18...
production was undetectable in the CD3+ T cell and CD19+ B cell fractions of PBMC cultured for 48 h in the presence or absence of IL-4 (data not shown).

**AD patients have higher CCL18 serum levels than normal individuals**

Serum values of CCL18 were 3-fold higher ($p < 0.0003$) in AD patients (34.9 ± 5.3 ng/ml, mean ± SE) than in healthy, nonallergic, age- and sex-matched controls (10.7 ± 0.9 ng/ml, mean ± SE; Fig. 2B). When the atopic patients were grouped according to their disease stage (determined by the clinical scores IGA and EASI), we observed that the expression levels of CCL18 in moderate and severe AD were higher than in mild disease manifestations (Fig. 2C). However, the serum levels of CCL18 decreased in patients with very severe disease scores (Fig. 2C).

Other chemokines associated with AD, such as CCL17, CCL22, CCL26, and CCL27, have been described to correlate with disease severity scores (12–14, 20). In our patient population, we also found a correlation between EASI and serum CCL22 levels ($r = 0.4, p < 0.05$; data not shown). Furthermore, we found a strong correlation ($r = 0.6, p < 0.003$, Fig. 2D) between CCL18 and CCL22 expression in patients with mild to severe disease. However, patients with the highest CCL22 serum levels, who also had the highest disease scores, showed only moderate CCL18 expression (Fig. 2D). Thus, the selective increase of CCL18 expression during the development of AD and its subsequent decline in patients with the highest clinical disease scores suggests a unique pattern of CCL18 regulation.

**CCL18 binds to memory Th cells in AD**

The detection of the cell population targeted by CCL18 is hampered by the fact that the CCL18 receptor is not known. To overcome this problem, we generated Cy5- and biotin-labeled CCL18 without altering their biological properties (e.g., Cy5- and biotin-labeled CCL18 were as potent as unlabeled CCL18 to induce concentration-dependent migration of PBMC in vitro; data not shown). Using these tools, we found CCL18 binding to a fraction of CD3+ T cells infiltrating the skin of AD patients (Fig. 3A). CCL18-binding cells represented approximately one-half of the CCL18-producing APC and serum levels of CCL18 are increased in AD patients. A, PBMC from AD patients ($n = 8$) and controls ($n = 13$) were stimulated with IL-4. The percentages of CCL18-producing monocyte/macrophages in PBMC and CCL18-producing CD11c+ blood DC are represented as box plots. The analysis shows significant differences between monocyte/macrophages of AD and control individuals (averages, 7.1 and 1.5%, respectively, $p < 0.003$) and between DC of AD and control individuals (averages, 5.6 and 1.9%, respectively, $p < 0.001$). B, CCL18 levels in serum from AD patients ($n = 36$) are significantly higher than those of healthy control ($n = 28$) subjects (averages, 34.9 and 10.7 ng/ml, respectively, $p < 0.0003$). C, Graphical distribution of serum levels of CCL18 among AD patients with different severity disease scores (IGA). D, Correlation between CCL18 and CCL22 serum levels in AD patients. Patients with almost clear to severe manifestations ($r = 0.6, p < 0.003$). Patients with highest severity scores, represented by C, are excluded from the correlation analysis.
healthy controls (Fig. 3B). The percentages of cells reacting to an irrelevant fluorescently labeled peptide (P300) of similar size as CCL18 were indistinguishable from the percentages obtained in the absence of labeled chemokine and consistently below 1%.

In addition, CCL18 also bound to CLA+/Th1/Th0 memory T cell lines and clones isolated from the skin of AD patients, independently of whether they displayed a Th2 or Th1/0 cytokine production pattern following stimulation (Fig. 3C). Similarly, fluorescently labeled CCL22 reacted positively with all skin-derived T cells tested, whereas the control peptide P300 did not elicit any significant reactivity (Fig. 3C). Particularly, CCL11 did not compete with CCL18. This is important since CCL11 is a ligand for CCR3 and this receptor. Using specific mAbs, we demonstrated that CCL18-binding cells were CCR4+ but CCR3−, further excluding a putative binding of CCL18 to CCR3 (Fig. 3E).

CCL18 recruits memory Th cells to the skin

The functional relevance of CCL18 binding to memory Th cells was assessed in in vitro and in vivo migration assays. In vitro, all AD-derived T cell lines and clones tested, migrated significantly, and in a dose-dependent manner in response to CCL18 and to CCL22. The extent of CCL18-induced migration was comparable to that induced by CCL22 (Fig. 4A). Both CCL18- and CCL22-induced migration could be blocked using specific neutralizing mAbs, demonstrating the specificity of the responses (Fig. 4A).

The role of CCL18 in vivo was investigated using a modification of the previously described SCID-hu Skin mouse model (18). AD-derived memory T cell lines and clones migrated significantly to human skin grafts transplanted onto SCID mice in response to CCL18 alone (Fig. 4, B and C) with magnitudes that were comparable to that mediated by CCL22. Under these conditions, the migratory response induced by CCL11 was insignificant and similar to that mediated by the control PBS (Fig. 4C), which is in agreement with the lack of CCR3 expression by these AD-derived T cells (Fig. 3E). The extent of CCL18-mediated T cell infiltration observed in the mechanistic SCID-hu Skin mouse model was comparable to that generally observed in inflamed skin of AD patients (Fig. 4C).

Discussion

In this report, we demonstrate that CCL18 is associated with AD. We found that CCL18 is produced by APC in the dermis

FIGURE 3. CCL18 binds to memory T cells of AD patients. A, Immunohistochemistry analysis of CCL18-binding cells in AD skin, faintly counterstained by hematoxylin. Original magnification, ×200. B, CCL18 binding on PBMC from AD patients (n = 10) and healthy donors (n = 5). Bars represent average ± SE of CCL18 or control P300 peptide-reacting cells in the CD3+CLA− and CD3+CLA+CCR4+ T cell fractions. C, Phenotype analysis of human AD skin-derived T cell lines and clones. IL-4 and IFN-γ production was analyzed by intracellular cytokine staining after stimulation with anti-CD3 and PMA. All examined T cell lines and clones bound fluorescently labeled CCL18 (red line) and CCL22 (blue line) but not control P300 peptide (black line). The percentages of CCL18bright cells are indicated in red. D, Competition binding for CCL18 on T cells. CCL18 binding (black line) could be competed by simultaneous incubation with unlabeled CCL18 (blue line), but not by CCL11 (green line) or CCL22 (orange line). Unspecific binding with control P300 peptide is shown as a gray line. E, Costaining of CCL18 and CCR3 or CCR4 on AD-derived T cells. CCR4 but not CCR3 expression in the whole cell population is demonstrated in the dot plots by a shift in mean fluorescence intensity (MFI). The histogram shows mean fluorescence intensity of CCL18-binding T cells (blue gate in dot plots) stained with control mlgG1 (black), CCR3 (blue), and CCR4 (red) demonstrating that the CCL18-binding cells are an homogeneous CCR4+ population.
and epidermis of AD patients but not in skin samples from normal individuals or psoriatic patients. Furthermore, following stimulation with IL-4, there were higher percentages of CCL18-producing cells present in PBMC from AD patients than in healthy individuals. The mechanisms causing those differences are not known. It could be that APC from AD patients are already primed in vivo by Th2 cytokines. However, other explanations, including genetic factors predisposing for atopy, cannot be excluded. Our observations are novel and in line with other studies reporting notable differences between APC from atopic patients and healthy donors (23, 24).

CCL18 levels in the sera of AD patients were 3-fold higher than in normal individuals. Serum levels of CCL18 increased with the transition from mild to severe atopic dermatitis disease stages correlating with the levels of CCL22, a chemokine that has been previously associated with AD (13). However, CCL18 levels were down-regulated in patients with very severe disease scores, whereas CCL22 serum levels were further increased, suggesting a differential role of each of these chemokines in AD. IFN-γ-producing Th cells are also detected in the skin lesions of severe/chronic AD (11). Therefore, it is tempting to speculate that in these situations, when the microenvironment is less rich in IL-4 and contains IFN-γ, lower amounts of CCL18 are produced. This could account for the reduction in CCL18 expression observed in the serum of patients with the highest clinical disease scores, suggesting that CCL18 plays a role in the early pathogenesis of AD.

A report by Hieshima et al. (3) described that CCL18 is chemotactic for activated T cells. However, these authors did not differentiate between naive or memory T cell subpopulations and, therefore, the original report by Adema et al. (2) using naive T cells has led to the current belief that CCL18 is mostly a chemottractant for naive T cells. In agreement with this report, we found that CCL18 binds to a distinct population of human naive CD45RA⁺, CCR7hi, CD62Lhi, CD25⁻, CD4⁺, or CD8⁺ T cells in PBMC of normal individuals (data not shown). In contrast, in this study we demonstrate that CCL18 also binds to memory T cell lines and T cell clones isolated from the skin of AD patients. The binding was specific, since it could be competed with unlabeled CCL18 but not with other chemokines. CCL18 binding was also functionally relevant since CLA⁺ skin-homing memory T cells reacting with CCL18 could be demonstrated in the circulation of both AD patients and normal individuals, and AD-derived memory T cells migrated in vitro and in vivo in response to CCL18 alone.

The recruitment of Th cells into atopic skin lesions has been attributed to the concerted action of the CCR4 ligands CCL17 and CCL22 and the CCR10 ligand CCL27 (14, 25–28). In this study, we demonstrate that CCL18 also induces homing of memory T cells to human skin in vivo. The expression pattern of CCL18 is partially overlapping with that of CCL17 and CCL22 and complementary to that of CCL27 (25, 29), which is in line with the idea that individual chemokines play redundant roles and unique functions during the homing processes of various T cell populations (15). In addition, the finding that CCL18 is expressed in a Th2 cytokine-mediated inflammatory process such as AD, but is not present in a Th1-mediated disease like psoriasis, implies that the CCL18-responding T cells represent a specialized population. This is in contrast to CCR4 ligands, which are general T cell chemoattractants involved in Th1- and Th2-mediated skin diseases (15). This concept is supported by recent microarray studies demonstrating that CCL18 mRNA is up-regulated in skin lesions of AD patients compared with samples from psoriatic and normal individuals (30).

In summary, our results provide conclusive evidence linking CCL18 protein expression with various stages of AD and demonstrating the capacity of CCL18 to recruit memory T cells into human skin in vivo.
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