Kinetics and differential expression of the skin-related chemokines CCL27 and CCL17 in psoriasis, atopic dermatitis and allergic contact dermatitis

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Abstract: CCL27 and CCL17 are chemokines believed to be involved in the process of establishing the inflammatory infiltrate, characteristic for the various inflammatory skin diseases. The skin-specific CCL27 binds the chemokine receptor-10 (CCR10), and CCL17 is a chemokine receptor-4 (CCR4) ligand. The purpose of our study was to characterize the expression of CCL27 and CCL17 in the inflammatory skin diseases: psoriasis, atopic dermatitis (AD) and allergic contact dermatitis (ACD) induced in nickel-sensitive individuals. Surprisingly, our studies revealed a markedly decreased CCL27 mRNA and protein expression in psoriatic lesions compared with non-lesional psoriatic skin. A minor CCL17 mRNA increase was measured in lesional psoriatic skin. No alterations were found in AD. In ACD, we found a pronounced (90-fold) raise in CCL17 mRNA and a 50-fold increase in CCL17 protein compared with normal skin. A kinetic ACD study of CCL17 expression showed the highest mean value 24 h after hapten application. Furthermore, we found the mRNA levels of CCR10 and CCR4 paralleling the results of their corresponding ligands. Overall, our principal findings were a distinct decrease in CCL27 in lesional psoriatic skin and a marked upregulation of CCL17 in ACD. These findings underscore the differential cutaneous T-cell recruitment in different inflammatory diseases.

Key words: CCL27 – CCL17 – CCR10 – CCR4 – inflammatory skin diseases

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Introduction

The common inflammatory skin diseases, such as psoriasis, atopic dermatitis (AD) and allergic contact dermatitis (ACD), are characterized by a distinct dermal inflammatory infiltrate primarily consisting of lymphocytes. It is well known that the genesis and the composition of this lymphocyte invasion are directed by the gradients of numerous chemoattractants produced by resident or immigrated cells (1). Chemoattractant cytokine molecules known as chemokines (2) regulate this lymphocyte traffic. The lymphocytes responsible for dermal immunity come from the blood to the skin through a multistep process involving vascular endothelial cell recognition and extravasation (3). Some chemokines including CCL27/CTACK (cutaneous T cell-attracting chemokine) and CCL17/TARC (thymus- and activation-regulated chemokine) contribute to a tissue-restricted leucocyte trafficking by exhibiting high receptor and tissue specificity (4).

CCL27 is a skin-specific CC chemokine constitutively expressed by keratinocytes (5). CCL27 specifically binds the chemokine receptor CCR10 (6) which is expressed almost exclusively on skin homing CLA+ (cutaneous lymphocyte-associated antigen) lymphocytes (5,7). Hereby, CCL27 induces inflammation by promoting Th1 and Th2 lymphocyte migration into the skin.

CCL17 is a Th2-type chemokine, which binds to the CC chemokine receptor 4 (CCR4) (8,9). CCL17 is constitutively expressed in the thymus and is produced by dendritic cells, endothelial cells, keratinocytes and fibroblasts. CCR4 is expressed by CLA+ skin homing cells, but not by, e.g., the intestinal $\gamma$/\(\delta\)$ T cells (10). Furthermore, CCL17 and CCR4 have been suggested to be important for the recognition of skin vasculature by circulating T cells and for the selective skin homing of CCR4+ T lymphocytes (10).

Thus, inflammatory skin diseases show distinct patterns of chemokine expression and lymphocyte profile. Psoriasis is considered a Th1/Th17-driven disease (11–13). While acute atopic dermatitis is a model of a Th2-dominated disorder, the chronic phase of AD show either coexistence of both Th2 and Th1 cells, or Th1 dominance (14–16). ACD is a T cell-mediated delayed-type hypersensitivity reaction (type IV) and is traditionally regarded as a mixed Th1/Th2 profile disease (17). However, evidence for the involvement of allergen-specific Th17-mediated inflammation in human ACD has recently been provided (18,19).

An increased CCL27 expression in the elicitation phase of ACD together with an increased CCR10 expression has been demonstrated (7). Furthermore, CCL27 and CCR10 have been reported upregulated in both atopic and psoriatic skin (7,20). A recent microarray study comparing inflammatory gene expression in AD and psoriasis has shown CCL27 transcripts to be increased in AD-affected skin compared with lesional psoriatic skin, and CCL27 mRNA significantly decreased in psoriatic skin compared with AD and normal skin (21).

CCL17 has been suggested to play an important role during ACD elicitation (22,23), and in patients suffering from AD, it may serve as an immunological marker in assessing clinical improvement following immunotherapy (24). However, the cellular source of this chemokine is a matter of debate. The mRNA of its corresponding receptor CCR4 has been located to dermal endothelial
cells and infiltrating cells in AD-affected skin, whereas CCL17 mRNA, in addition, was seen in epidermal keratinocytes (25). Furthermore, upregulation of CCL17 has been found in relation to acute barrier disruption (26). CCR4+ T cells has been reported well represented in psoriatic skin lesions (27), although this was not confirmed by others, who, on the other hand, found CCR4+ T cells in AD skin lesions (28).

It is noteworthy that although the role of these essential skin-related chemokines has been in focus for years, the studies in this field based on various methods such as immunohistochemistry, conventional PCR, in situ RT-PCR techniques, real-time RT-PCR along with microarray techniques have revealed contradicting results. Therefore, our objective was to conduct one study investigating the expression of CCL17, CCL27 and their receptors at the same time, using the same methods in the three prototypical inflammatory skin diseases: psoriasis, AD and acute ACD induced in nickel-sensitive individuals and to determine their contribution to the regulation of initiation and maintenance of cutaneous inflammation.

Materials and methods

Human biopsies

Punch biopsies were obtained from lesional and non-lesional skin from patients suffering from psoriasis and AD. Patients were untreated. Psoriasis biopsies were taken from the centre of a chronic stable plaque. In patients with acute guttate elements as well as chronic plaques, biopsies were obtained from both sites. Biopsies were also collected from NiSO4-challenged skin from patients suffering from nickel-induced ACD and from individuals non-sensitive to nickel. Patch test reactions were elicited by NiSO4 (0.16 mg/patch, 0.20 mg/cm²) (GE Healthcare, Hilleroed, Denmark). Biopsies were gathered as paired samples, and corresponding biopsies were taken from the same anatomical region. For PCR and ELISA procedures, punch biopsies were snap-frozen in N2(l). The Declaration of Helsinki protocols were followed, and the medical ethical committee of Aarhus approved the study. Informed, written consent was obtained from the patients included.

Mice

C57BL/6 mice were handled as earlier described (29). The Danish Committee for Animal Experiments approved all experiments.

Oxazolone-induced allergic contact dermatitis

The oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one) (Sigma-Aldrich, St. Louis, MO, USA)-induced ACD model has been described previously (29). Punch biopsies were obtained from the ears 2.5, 8, 24 h and 10 days postchallenge and snap-frozen in N2(l).

RNA isolation

Punch biopsies were transferred to RNAlater-ICE (Ambion Inc., Austin, TX, USA) prior to RNA purification. RNA from biopsies and keratinocytes was purified using SV Total RNA Isolation System (Promega, Madison, WI, USA) and completed according to the manufacturer’s instructions. Sample purity and RNA concentration were determined spectrophotometrically (Genequant; GE Healthcare).

Quantitative RT-PCR

For reverse transcription (RT), Taqman RT reagents (Applied Biosystems, Foster City, CA, USA) were used according to the manufacturer’s instructions with random hexamers as primers. PCR conditions were as follows: a 10-min 25°C incubation step, a 30-min 48°C RT step and finally a 5-min 95°C RT inactivation step. CCL17, CCL27, CCR4 and CCR10 mRNA expression was determined with TaqMan® Gene Expression Assays (Applied Biosystems) (Hs00171074_m1, Hs00171157_m1, Hs99999919_m1, and Hs00706455_s1, respectively). As housekeeping gene, RPLP0 (Hs00999902_m1) was used. We used AmpliTaq Gold® PCR Master Mix (Invitrogen, Carlsbad, CA, USA) when detecting CCL17, CCL27 and CCR4. For the detection of CCR10, TaqMan Universal PCR Master Mix (Applied Biosystems) was used. Murine CCL17 mRNA expression (Mm00516136_m1) was normalized to murine GAPDH (Mm99999915_g1). PCR conditions were as follows: 60°C (2 min), 95°C (10 min) followed by 50 cycles (15 s) at 95°C, and 60 s at 60°C. Gene expression was analyzed in triplicates using Rotorgene-3000 (Corbett, Sydney, Australia). Relative gene expression levels were determined using the relative standard curve method as outlined in User Bulletin 2 (ABI Prism 7700; Applied Biosystems).

Protein extraction

Biopsies were homogenized (TissueLyser; Qiagen, Haan, Germany), and lysis buffer was added. Samples were homogenized, sonicated and centrifuged (10 000 g). The supernatant constitutes the total whole-cell protein extract. Protein concentration was determined by Bio-Rad Protein Assay (Bio-Rad, Copenhagen, Denmark).

Enzyme-linked immunosorbent assay

CCL17 and CCL27 protein were measured by Duo-Set® ELISA Development System (DY364 and DY376, R&D Systems, Oxon, UK) according to the manufacturer’s instructions. Wells were incubated with capture antibody, blocked with PBS with bovine serum albumin, and incubated overnight with 1:1 diluted samples. Biotinylated detection antibody was added followed by 2 h of incubation. HRP-conjugated streptavidin (1:200) was added prior to visualization with substrate solution (R&D Systems). The reactions were stopped using H2SO4. Results were double-determined at 450 nm (iEMS Reader, Lab Systems, Copenhagen, Denmark), and 540 nm readings were substracted correcting for optical imperfections.

Immunohistochemistry

Biopsies were immediately formalin-fixed and paraffin-embedded. Serial sections were mounted on poly-L-lysine-coated slides and dried. Slides were xylene-deparaffinized, rehydrated through a descending ethanol series, and for antigen unmasking heated in citrate buffer, cooled and placed in humidity chamber. After endogenous biotin blocking, sections were serum-incubated (10%) and immunostained with mouse anti-human CCR4 overnight (kindly provided by Matsushima) (2 µg/ml). Next day, sections were incubated with secondary antibody (Donkey anti-mouse IgG, 715-025-150; Jackson, West Grove, PA, USA). After streptavidin-incubation, New-Fuchsin and Levamisol-staining (K0624, X3021; Dako, Glostrup, Denmark) was performed. Finally, sections were haematoxylin-stained (Bie&Bernten). For negative controls, either no primary antibody or normal mouse IgG (Santa Cruz, Santa Cruz, CA, USA) was used.

Statistics

Results were expressed as mean ± standard deviation (SD). Statistical significance (P < 0.05) was assessed by Student’s t-test (two tailed). To test for normal distribution, a probability test was conducted.
Results

CCL27 and CCR10 expression is low in psoriasis

CCL27 mRNA levels relative to the housekeeping gene RPLP0 mRNA were determined by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) in punch biopsies from patients suffering from psoriasis, AD and nickel-induced ACD. We found the expression of CCL27 mRNA in psoriatic skin lesions (acute guttate elements as well as chronic plaques from the same patient) markedly decreased compared with non-lesional psoriatic skin (Fig. 1a). This decrease in CCL27 expression was consistent throughout all 29 psoriatic biopsy pairs. The mean CCL27 mRNA expression level in lesional chronic psoriatic skin was decreased eightfold compared with non-lesional psoriatic skin, and a fourfold decrease was measured in acute guttate psoriatic lesions from the same patients. As determined by ELISA, the protein level of CCL27 was decreased almost fivefold in chronic psoriatic skin compared with non-lesional psoriatic skin (Fig. 1b). In skin from lesional nickel-induced ACD, a significant decrease in the CCL27 protein level was seen compared with normal skin from nickel-sensitive individuals (Fig. 1b). This finding was not paralleled by a similar decrease at the mRNA expression level (Fig. 1a). In AD lesions, we observed a slight increase in CCL27 transcripts and protein; however, this was not statistically significant (Fig. 1a,b).

We then studied the expression of the CCL27 receptor, CCR10, in the various inflammatory skin diseases. A significant downregulation of CCR10 mRNA expression was seen in biopsies from chronic psoriatic plaques when compared with paired tissue samples from non-lesional psoriatic skin. No changes were seen in either AD or nickel-induced ACD (Fig. 1c).

To investigate whether the CCL27 and CCR10 mRNA expression level varies between different stages of psoriasis, skin samples were obtained from eight patients having both acute guttate and chronic plaque lesions. We found no significant differences in the mRNA expression level of CCL27 (Fig. 1a) or CCR10 (Fig. 1c) when comparing guttate psoriatic elements with plaque-type psoriasis from the same patient.

CCL17 and CCR4 expression is increased in ACD

Interestingly, when the mRNA expression of CCL17 was measured, a highly pronounced (90-fold) and statistically significant increase in CCL17 mRNA was found when comparing nickel-induced ACD with nickel-sensitive normal skin (Fig. 2a). A marked (30-fold) raise in CCL17 protein level (Fig. 2b) accompanied this result. In both lesional chronic plaque-type and acute guttate psoriatic skin, only a minor, but consistent and statistically significant increase in CCL17 mRNA was found when compared with non-lesional skin (Fig. 2a). This increase was not seen at the protein level (Fig. 2b). Furthermore, a slight increase ($P = 0.075$) in CCL17 mRNA expression was found in lesional atopic skin, and a non-significant threefold increase in CCL17 protein was observed (Fig. 2a,b).

The elevated CCL17 chemokine expression in nickel-induced ACD was accompanied by a statistically significant sixfold increase in the expression of its corresponding receptor, CCR4 (Fig. 2c). In psoriatic and atopic skin, we found no significant changes in CCR4 mRNA expression between lesional and non-lesional skin. Guttate and plaque-type psoriasis lesions did not differ significantly in either CCL17 or CCR4 mRNA expression.

CCR4 expression increases visibly in nickel-induced ACD involved skin

To examine the localization of CCR4 in ACD-affected skin, immunohistochemistry was performed on paraffin-embedded tissue obtained after 72 h of NiSO$_4$ application. In nickel-induced ACD, thickened epidermis with spongiosis and acanthosis and a solid mononuclear dermal infiltration were conspicuous. CCR4$^+$ cells were primarily apparent in the dermal infiltrate, and the CCR4 staining was substantially augmented in nickel-induced ACD (Figure S1d: III,IV). In the corresponding normal skin sections from the nickel-sensitive individuals, a few CCR4$^+$ cells were found at perivascular locations (Figure S1d: I,II).

Kinetics of CCL17 and CCR4 expression in nickel-induced ACD

To further characterize the mRNA expression of CCL17 and CCR4 in nickel-induced ACD, we conducted a kinetic patch test study over a 72-h time span. Biopsies were obtained 0, 6, 24 and 72 h after NiSO$_4$ application. After 6 h, no significant alterations were seen in CCL17 mRNA. The highest mean of the four
patient-specific CCL17 mRNA values was found at 24 h (Fig. 3a). The mean CCR4 value at 24 h was increased sixfold compared with the mean value at 0 h, whereas after 72 h the mean CCR4 value was increased 11-fold (Fig. 3b). At the 72-h time point, the mean CCL17 value as well as the mean CCR4 value were found significantly increased.

To ensure that the strong upregulation in CCL17 expression was not just because of NiSO₄ application, we also obtained biopsies from NiSO₄-challenged skin and normal skin from donors not suffering from nickel ACD. The biopsies were obtained at 0 and 72 h after application of NiSO₄. No differences in the mRNA expression of either CCL17 or CCR4 were found in these individuals (Fig. 3a,b).

**Kinetics of CCL17 mRNA expression in a murine allergic contact dermatitis model**

Finally, we wanted to investigate whether the prominent increase in CCL17 mRNA expression found in nickel-induced ACD was an ACD-specific phenomenon or rather evidence of an acute inflammatory reaction. Because of difficulties obtaining standardized biopsies from patients suffering from chronic ACD induced by only one allergen and with no irritant component, we decided to make use of the murine oxazolone-induced ACD model (30,31). CCL17 mRNA expression was determined 0, 2.5, 8, 24 h, and 10 days postchallenge (Fig. 4). The CCL17 mRNA expression 2.5 h after oxazolone treatment increased 2.3-fold compared with controls, and a 17-fold increase was found at the 8-h time point. The CCL17 mRNA expression peaked 24 h postchallenge (29-fold compared with controls), and 10 days postchallenge, the oxazolone-induced CCL17 mRNA expression was abolished and the mean CCL17 mRNA value was below that of controls.

**Discussion**

Chemokines, major promoters of recruiting lymphocytes into sites of inflammation, have distinct modes of action. CCL27 mediates its function by attracting Th1 and Th2 T cells expressing the CCR10 chemokine receptor. CCL17 binds to CCR4, and because

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**Figure 2.** Intense CCL17 upregulation and increased CCR4 expression in allergic contact dermatitis (ACD). (a) CCL17 mRNA expression. (b) CCL17 protein expression. The CCL17 protein data mimics the CCL17 mRNA expression in atopic dermatitis (AD) and ACD. (c) CCR4 mRNA expression is statistically significantly upregulated in nickel-induced ACD. No alterations in CCR4 expression were found in either psoriasis or AD. Bars represent mean ± SD. n = number of biopsies. *P < 0.05 compared with the corresponding non-lesional skin.

**Figure 3.** Kinetic study of CCL17 and CCR4 mRNA expression in allergic contact dermatitis (ACD). CCL17 (a) and CCR4 (b) mRNA was determined in biopsies collected 0, 6, 24, and 72 h after NiSO₄-application to nickel-sensitized skin (pt.) and in biopsies obtained 0 and 72 h after NiSO₄-challenged skin of individuals not suffering from nickel ACD (ct.). The NiSO₄ patch was removed after 48 h at the latest. *P < 0.05 compared with controls (0 h after NiSO₄-application).

**Figure 4.** CCL17 mRNA expression peaks at early time point in murine allergic contact dermatitis. C57BL/6 mice were sensitized by topical oxazolone-application. Biopsies were obtained from the ears of the mice 2.5, 8, 24 h, and 10 days postchallenge and from acetone-treated control mice. Bars represent mean ± SD. n = number of biopsies. *P < 0.05 compared with controls.
CCR4 has been described a marker for Th2 lymphocytes, CCL17 has been designated a Th2 chemokine. However, later studies have also shown CCR4 to be expressed on Th17 cells (32).

In this study focusing on the differential expression of the chemokines involved in the establishment of the lymphocyte profile characteristic for various inflammatory skin diseases, we have found several differences and variations in the expression patterns of the chemokines CCL27 and CCL17, along with their receptors, in the inflammatory skin diseases psoriasis, AD and ACD. Our main findings were a downregulation of the CCL27 expression in psoriasis and a pronounced increase in the CCL17 expression in ACD.

Our finding of a markedly decreased CCL27 expression in acute and chronic psoriatic skin lesions when compared with non-lesional psoriatic skin is clearly not in line with the reported upregulation of CCL27 in psoriasis, AD and ACD by immunohistochemistry (7). However, our result confirmed the findings of Nomura et al., who found lower CCL27 expression in psoriatic skin compared with atopic and normal skin samples by the use of both gene microarray analysis and real-time PCR (21). Furthermore, our results are in line with the recently published results by Guijonson et al. (33), a study reporting the CCL27 gene strongly downregulated in involved psoriasis skin compared with normal skin. In terms of CCR10 expression in AD and psoriasis, the chemokine receptor protein is reported immunohistochemically increased (7), a finding we were not able to confirm at mRNA level using the real-time RT-PCR technique.

During the elicitation phase of nickel-induced ACD, increased epidermal CCL27 expression has been shown immunohistochemically as early as 6 h after hapten application and until 48 h after application (7). This increase in CCL27 was followed by a dramatic raise in CCR10+ T cells at 24–48 h (7). In our kinetic study of provoked ACD with a time span of 72 h, we found no expression changes in either CCL27 or in its corresponding receptor CCR10 measured by PCR (not shown). However, a significant decrease in CCL27 protein expression was seen 72 h after NiSO4 application.

These findings are in line with a murine study where exposure to topical application of a contact sensitizer (DNFB) resulted in no change of CCL27 mRNA expression in the skin, whereas CCL27 protein expression was highly reduced (34). Furthermore, CCL27 protein was detected in skin-draining lymph nodes while CCL27 mRNA expression was low (34). This release of CCL27 protein from keratinocytes and accumulation in skin-draining lymph nodes was temporally paralleled by an increase in CCR10+ T cells in the lymph nodes.

Furthermore, in accordance to Goebeler et al., our work detected a dramatic increase in CCL17 expression at the 72-h time point in provoked nickel-induced ACD and a simultaneous upregulation of CCR4 mRNA. These data, together with our immunohistochemical data showing increased number of CCR4+ T cells in nickel-induced ACD skin compared with normal skin from nickel-sensitive individuals, provide further evidence to the findings of Goebeler et al. (22) that migrating effector cells encounter multiple chemotactic signals in a complex spatial and temporal pattern. In the early elicitation phase of ACD, Th1 cells and CD8+ cytotoxic T cells are the main effector cells (especially CD8+), whereas CD4+ T cells containing Th2 and regulatory T cells responsible for the resolution of the inflammation arrive in the later phase (35). Sebastiani et al. (36) have reported that CCL17 is crucial to CD4+ T-cell migration, but only plays a minor role in CD8+ T-cell attraction. Hence, our data suggest that CCL17 plays an important role during the late rather than in the early elicitation phase of ACD. Based on the recently verified presence of both Th1- (CCR2, CXCR3, CCR5, and CXCR6) and Th2-associated (CCR4) trafficking receptors on human Th17 lymphocytes (32), one may consider the potential role of CCL17 in Th17-mediated immunopathology in ACD.

The atopic cytokine milieu has been intensely investigated, and many have reported the involvement of CCL17 and CCL27 in AD. In this study, we only found small changes in chemokine expression in atopic skin, but neither CCL17 nor CCL27 expression was statistically significantly altered between non-lesional and lesional atopic skin. CCL27 and its receptor CCR10 has formerly been reported not significantly altered between normal skin and psoriasis, AD and lupus erythematosus (6). Our AD biopsies were obtained in the chronic phase and because AD is regarded as a Th2-dominated disease primarily in the acute phase; this may explain the absence of enhanced CCL17 expression in lesional AD skin.

In this study, we have investigated both acute and chronic inflammatory lesions. Biopsies from the nickel-induced ACD lesions were taken 72 h after hapten application at the latest, while the psoriatic and atopic lesions were chronic. The acute guttate elements from patients suffering from plaque-type psoriasis have been developing for weeks. Therefore, one may hypothesize that the pronounced CCL17 expression increase found in ACD is evidence of an acute inflammation rather than an ACD-specific phenomenon. To address this question, we investigated the kinetics of CCL17 mRNA expression in the murine model of oxazolone-induced ACD. In this study, the murine CCL17 mRNA expression has levelled out after 10 days of ACD after peaking at the 24-h time point, suggesting that the increase in CCL17 mRNA expression reflects the acute phase of inflammatory T-cell recruitment.

This differential expression of CCL17 and CCL27 in psoriasis, AD and ACD is just one part of the explanation of the various lymphocyte profiles seen in inflammatory skin diseases. Definitely, more knowledge on this chemokine–chemokine receptor network would improve our understanding of the differentiated recruitment of T cells into sites of inflammation and may therefore contribute to the search for potential targets in future therapy of inflammatory skin diseases.

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Author’s contributions
Jette L. Riis, Claus Johansen and Lars Iversen designed the research study. Jette L. Riis performed the research, analysed the data and wrote the article. Christian Vestergaard and Knud Kragballe helped with interpretation of data. Rikke Bech contributed with essential reagents and tools.

Conflict of interest
The authors state no conflict of interest.
References


Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. CCR4 expression visually increased in ACD.

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