

Expression of chemokines and chemokine receptors in lesional and nonlesional upper skin of patients with atopic dermatitis

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Background: Trafficking of dendritic cell (DC) subtypes to and from the skin plays a pivotal role in atopic dermatitis (AD).

Objectives: We sought to determine the CCR pattern of epidermal DC subtypes and CCL expression in relation to the state of AD.

Methods: Shave biopsy specimens were taken from patients with AD before and after 24 and 72 hours of atopy patch testing and from the skin of patients with chronic AD, skin of patients with psoriasis, and healthy skin. CCR expression of epidermal DCs was studied by using flow cytometry, and chemokine mRNA levels in the skin were quantified by means of real-time PCR.

Results: The total number of CD1a⁺ epidermal DCs increased and the proportion of Langerin-positive CD1a⁺ DCs decreased whereas the percentage of Langerin-negative CD1a⁺ DCs increased after allergen application. Expression of CCR5 and CCR6 of Langerin-negative CD1a⁺ DCs was characteristic for acute AD. Expression of CCL1, CCL3, CCL4, and CCL11 mRNA was greater in patients with acute AD versus that seen in patients with chronic AD. Only a strong increase of CCLs, in particular CCL1, CCL17, and CCL18, went along with eczema development, and increased CCL1, CCL13, CCL17 and CCL18 expression was specific for patients with chronic AD compared with those with psoriasis.

Conclusion: Modified recruitment and differentiation of DCs from their dermal and blood precursors occurs in the acute phase of AD. A boost in the amplitude of CCLs after allergen

application goes along with eczema development. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Dendritic cells, chemokines, chemokine receptors, atopic dermatitis, atopy patch test

Trafficking of dendritic cells (DCs), including the recruitment from their blood precursors to the skin and emigration from the skin to the lymph nodes after activation and antigen uptake, plays a central role in both the maintenance of tissue homeostasis in healthy skin and the pathophysiology of chronic inflammatory skin diseases, such as atopic dermatitis (AD).¹⁻³ The cellular infiltrate, as well as the direction of trafficking cells, is regulated by the composition of chemokines released into the microenvironment on the one hand and the chemokine receptor repertoire with which the cells are equipped on the other hand.³⁻⁵

Because of the biphasic course of AD, disease-related and stage-specific differences of the chemokine expression pattern in the skin are more than likely.⁶ In this context a role of CCL18 in the amplification of allergic inflammation through increased homing of memory T cells has been demonstrated.^{7,8} Another chemokine shown to be selectively upregulated in patients with AD was CCL1, the ligand to CCR8, which promoted the recruitment of T cells and Langerhans cell (LC)-like DCs *in vitro*.⁹ In addition to the differences between acute and chronic AD, a previous study supported the concept of histologic, as well as immunologic, changes that were already detectable in subclinical eczema (ie, nonlesional skin of patients with AD).¹⁰ This implies that immunologic changes, including modifications in chemokine receptor and chemokine expression in the skin, have to exceed a specific threshold to encompass eczema development.

Two different myeloid CD1a⁺ DC subtypes predominate in the lesional skin of patients with AD. First are LCs, which are even detectable in nonlesional AD skin and which express the C-type lectin Langerin (CD207).^{1,11} In patients with AD, LCs are equipped with the high-affinity receptor for IgE (FcεRI) on their cellular surface, which enables specific allergen uptake.² Another subpopulation of epidermal DCs is represented by Langerin-negative CD1a⁺ inflammatory dendritic epidermal cells (IDECs).¹¹ IDECs are regarded as important amplifiers of the allergic-inflammatory reaction on the DC level.¹² The number of IDECs in the epidermis increases within 48 hours after allergen application.¹³ In contrast, the quantity of IDECs detectable in the epidermis after topical treatment with calcineurin inhibitors decreases.^{10,14-16} Mechanisms regulating the balance between rather protolerogenic LCs as opposed to proinflammatory DC subtypes are not clear. They comprise changes in the DC precursor types recruited

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Abbreviations used

AD:	Atopic dermatitis
APT:	Atopy patch test
DC:	Dendritic cell
FITC:	Fluorescein isothiocyanate
IDEC:	Inflammatory dendritic epidermal cell
LC:	Langerhans cell

from the dermal compartment and blood, as well as redifferentiation of DCs already present in the skin. However, a detailed knowledge about the complex network of CCR expression and chemokine-directed trafficking during different disease stages of AD would be indispensable to develop effective therapeutic strategies. These therapeutic measures should optimally provide tools to efficiently impede the key step in this complex cascade of events. In this study we performed a systematic analysis of CCRs expressed by DC subtypes in the epidermis of patients with nonlesional, acute, and chronic AD. In parallel, we assessed the kinetics of qualitative and quantitative changes in chemokine expression in the skin in response to allergen application that are involved in eczema development.

METHODS**Reagents**

For more information on reagents, see the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Skin samples and preparation of cell suspensions

After local ethics committee approval and written informed consent were obtained, shave biopsy specimens were taken from patients with AD ($n = 19$; age, 42.7 ± 11.2 years; total IgE level, 7877.9 ± 14096.3 kU/L; SCORAD score, 44.7 ± 18.1), healthy control volunteers ($n = 8$), and patients with psoriasis ($n = 8$) at the Department of Dermatology and Allergy of the University of Bonn. AD was diagnosed according to the criteria of Hanifin and Rajka.¹⁷ Skin lesions that had not been treated for 2 weeks were investigated. After administration of local anesthesia, skin-shave specimens were taken. Half of the skin sample was stored in RNeasy RNA Stabilization Reagent (Qiagen, Hilden, Germany) at 4°C until RNA isolation for real-time PCR analysis, and the other half was applied to FACS analysis. Therefore single-cell suspensions were prepared by means of trypsinization, as described previously.¹⁸ In brief, skin-shave specimens were incubated in 0.5% trypsin in PBS at 37°C for about 60 minutes. Epidermal sheets were vigorously shaken for 1 minute in RPMI 1640 medium supplemented with 10% FCS and filtrated through a 40- μ m nylon mesh. The cells were washed in PBS and immediately subjected to flow cytometric cell-surface staining.

Atopy patch tests

Atopy patch tests (APTs) were performed with a house dust mite preparation containing pure *Dermaphagoides pteronyssinus* allergen in petroleum (Stallergenes, Paris, France). Five patients with AD and increased IgE serum levels (>100 kU/L) against house dust mite allergens, as well as 3 patients with AD with low allergen-specific IgE levels (<25 kU/L) against house dust mite allergens, were selected (Table I). Test solutions were applied to large Finn Chambers (Epitest Ltd Oy, Tuusula, Finland) and patched on healthy-appearing dorsal skin. Shave biopsy specimens were taken before and 24 and 72 hours after allergen application from patients reacting with development of eczema in the tested region (responders), as well as from patients with AD without manifestation of eczema (nonresponders) to determine changes in subclinical eczema.

Flow cytometric analysis

Extracellular staining was performed as reported in detail elsewhere.¹⁹ The cells were acquired on a FACSCanto (BD Biosciences, Heidelberg, Germany) and analyzed with FACSDiva (BD Biosciences) or FlowJo (TreeStar, Inc, Ashland, Ore) software. For quantitative evaluation, the CD1a⁺ DC population was gated out manually and further separated into Langerin-positive CD1a⁺ and Langerin-negative CD1a⁺ cells according to their Langerin (CD207) cell-surface expression. CCR expression in both DC populations was determined as a percentage of positive cells.

Real-time PCR

For more information on real-time PCR, see the [Methods](#) section in this article's Online Repository.

Statistical analysis

Statistical analysis was performed with SPSS 17.0 for Windows (SPSS, Inc, Chicago, Ill). Quantitative values were compared between the different groups (unrelated samples) or DC populations (related samples) by using the Mann-Whitney *U* test or the Student *t* test if the results were normally distributed. Results are shown as means $\pm$ SEMs. Any *P* values are 2-sided and subject to a global significance level of less than .05.

RESULTS**Frequency of CCR1-, CCR5-, and CCR6-bearing DCs increases 24 and 72 hours after allergen application**

Information about the factors leading to the increase of inflammatory DC subtypes in the skin in response to epicutaneous allergen challenge is rare. Therefore we evaluated the expression pattern of CCRs on Langerin-positive CD1a⁺ and Langerin-negative CD1a⁺ DC subtypes in the skin of patients with AD before and after allergen application. Dot-plot analysis together with changes in the CCR pattern implied recruitment of inflammatory DC subtypes to the epidermis, which could be observed 24 hours after allergen application in patients reacting to APTs with an eczematous skin lesion (responders; Fig 1, A). Although the proportion of Langerin-positive CD1a⁺ DCs to Langerin-negative CD1a⁺ DCs was 87.7% to 12.3% (mean of $n = 5$) in nonlesional AD skin before the APT, it was modified to 64.6% to 35.4% 24 hours after allergen application (Table II). Finally, only 37.5% Langerin-positive CD1a⁺ DCs versus 62.5% Langerin-negative CD1a⁺ DCs were present in epidermal skin 72 hours after allergen challenge (Table II). Concomitantly with this shift of Langerin-positive CD1a⁺ to Langerin-negative CD1a⁺ DCs, the frequency of CD1a⁺ cells among total epidermal cells increased from 1.7% (mean of $n = 5$) before onset of the APT to 2.0% 24 hours and 4.2% 72 hours after allergen application (Table II). This implies recruitment of DCs from their blood and dermal precursor cells into the epidermis in the acute phase of AD.

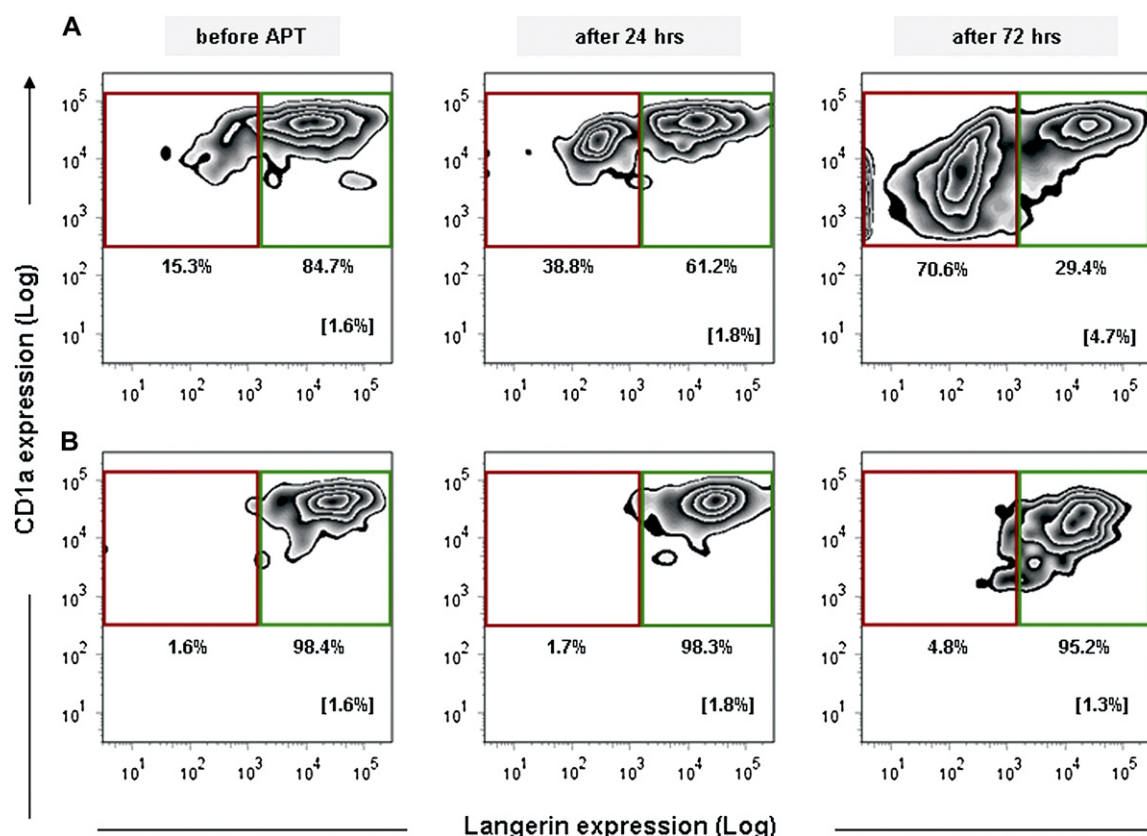
We observed increased number of CCR6-bearing, Langerin-positive CD1a⁺ DCs 24 hours after allergen application (Fig 2, A), as well as increased CCR1, CCR5, and CCR6 expression of Langerin-negative CD1a⁺ DCs 24 to 72 hours after allergen application in patients responding with an eczematous skin lesion (Fig 2, B).

In regard to the skin of patients without development of eczema after allergen application, increase of Langerin-negative CD1a⁺ DCs in the epidermis, as visible in responders, was missing (Fig 1, B). The proportion of Langerin-positive CD1a⁺ DCs to Langerin-negative CD1a⁺ DCs remained relatively stable: 94.5% to 5.5% (mean of $n = 3$) after 24 hours and 85.5% to 14.5% after

TABLE I. Clinical data of patients with AD in whom APTs were conducted

Patient no.	Age (y)	Sex	Total IgE (kU/L)	Allergen-specific IgE HDM (kU/L)	APT reaction after 24 h	APT reaction after 72 h
1	50	F	476	0.64	—	—
2	37	F	2263	24.1	—	—
3	39	M	2779	1.67	—	—
4	53	M	4072	>100	+	++
5	61	M	9386	>100	+	+++
6	37	M	10982	>100	—	+++
7	44	M	3492	>100	—	+++
8	39	M	58640	>100	++	+++

HDM, House dust mite; F, female; M, male.

**FIG 1.** Quantitative changes in the relation of epidermal DC subpopulations during APTs. Contour plot analysis of Langerin-positive CD1a⁺ DCs (green frame) and Langerin-negative CD1a⁺ DCs (red frame) of a representative responder (**A**) and a representative nonresponder (**B**) before (left column), 24 hours after (middle column), and 72 hours after (right column) allergen application is shown. Values represent percentages of Langerin-positive CD1a⁺ cells and Langerin-negative CD1a⁺ cells. Values in brackets indicate the proportion of CD1a⁺ cells of total epidermal cells.

72 hours (Table II). No remarkable changes in CCR expression were observed on the DC subpopulations of patients not responding (data not shown).

CCR5, CCR6, and CCR8 expression of Langerin-negative CD1a⁺ DCs is higher in acute than in chronic AD lesions

To determine whether different disease stages in patients with AD could be linked to a certain CCR expression profile of the inflammatory DC subtype, we examined CCR expression on

CD1a⁺ DCs in epidermal skin lesions of patients with chronic AD in relation to CCR expression in acute skin lesions 24 hours after allergen application during APTs. Concerning the subgroup of Langerin-positive CD1a⁺ DCs, a significantly higher expression of CCR6 in patients with acute AD compared with that seen in those with chronic AD was detectable, whereas expression of CCR5 was higher on Langerin-positive CD1a⁺ DCs in patients with chronic AD (data not shown).

Furthermore, we observed significantly higher expression of CCR5, CCR6, and CCR8 on Langerin-negative CD1a⁺ DCs from acute skin lesions compared with Langerin-negative CD1a⁺ DCs

TABLE II. Relative amount of Langerin-positive CD1a⁺ and Langerin-negative CD1a⁺ epidermal DC subpopulations

	Percentage of CD1a ⁺ cells of all epidermal cells (%)	Proportion of Langerin- positive CD1a ⁺ cells (%)	Proportion of Langerin- negative CD1a ⁺ cells (%)
Healthy skin	1.6 ± 0.1	95.4 ± 0.8	4.6 ± 0.7
Chronic AD	2.7 ± 0.4	58.8 ± 5.5	41.2 ± 5.4
Psoriasis	2.7 ± 0.5	53.8 ± 9.4	45.2 ± 9.3
Responders			
Before APT	1.7 ± 0.2	87.7 ± 2.9	12.3 ± 2.9
APT 24 h	2.0 ± 0.3	64.6 ± 6.9	35.4 ± 6.8
APT 72 h	4.2 ± 0.7	37.5 ± 7.2	62.5 ± 6.7
Nonresponders			
Before APT	1.6 ± 0.1	94.5 ± 0.3	5.5 ± 0.7
APT 24 h	2.1 ± 0.1	93.5 ± 1.8	6.5 ± 1.7
APT 72 h	2.2 ± 0.3	85.5 ± 4.8	14.5 ± 4.4

Mean percentages of gated cells ± SEMs of healthy control subjects (n = 8), patients with psoriasis (n = 8), patients with chronic AD (n = 11), responders (n = 5), and nonresponders (n = 3) before and 24 and 72 hours after APTs are shown.

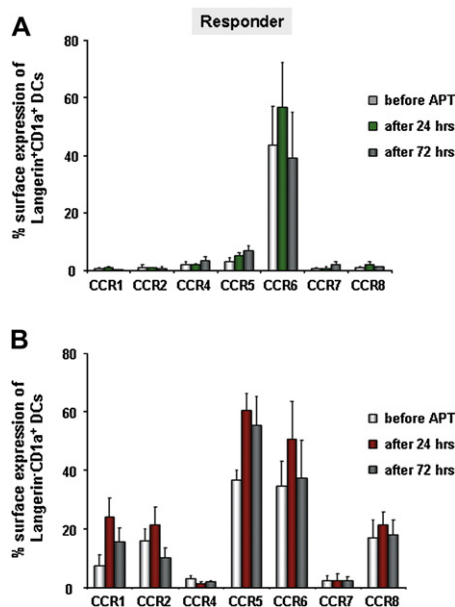


FIG 2. Different CCR expression on epidermal DC populations of responders during APTs. Expression of CCRs on Langerin-positive CD1a⁺ DCs (**A**) and Langerin-negative CD1a⁺ DCs (**B**) before APTs (white bars) and 24 hours (green and red bars) and 72 hours (gray bars) after allergen application (n = 5) is shown. Results are given as the mean percentage of positively gated cells ± SEMs.

in patients with chronic AD. Expression of CCR6 was almost 3-fold higher, and expression of CCR8 was 4-fold higher. In contrast, expression of CCR4 was significantly higher on Langerin-negative CD1a⁺ DC in patients with chronic AD (data not shown).

Different CCR expression on Langerin-positive CD1a⁺ and Langerin-negative CD1a⁺ epidermal DCs in patients with chronic AD versus those with psoriasis and healthy skin

We analyzed the chemokine receptor pattern of the CCR subfamily of Langerin-positive CD1a⁺ to delineate LCs and Langerin-negative CD1a⁺ epidermal subtypes to which inflammatory DC subtypes, such as IDECs, belong in the skin of healthy donors,

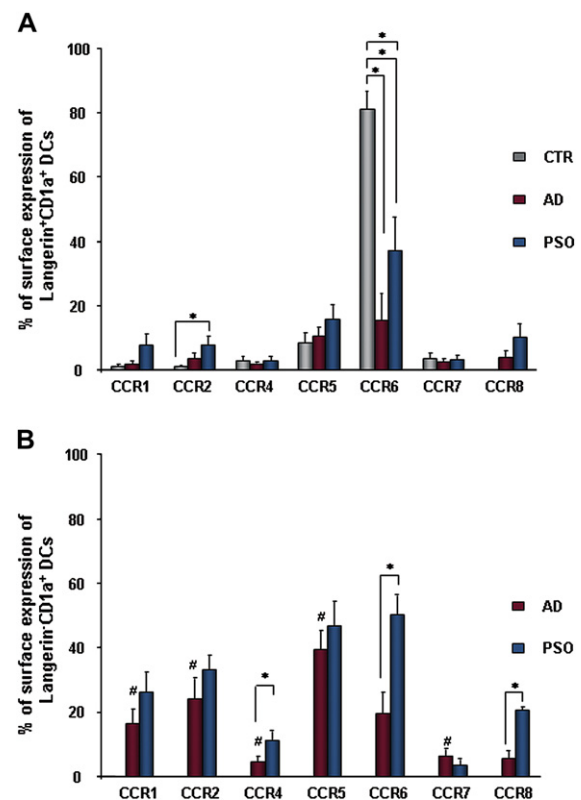
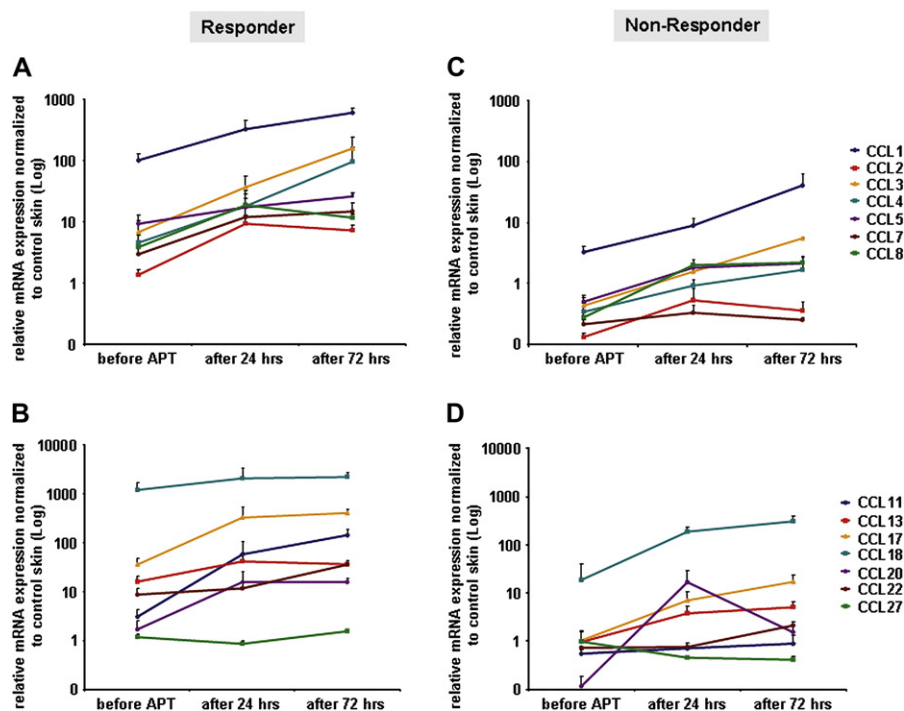


FIG 3. Different expression of CCRs on Langerin-positive CD1a⁺ DCs (**A**) and Langerin-negative CD1a⁺ DCs (**B**) in healthy control skin (n = 8; CTR; gray bars), skin of patients with chronic AD (n = 11; red bars), and skin of patients with psoriasis (n = 8; PSO; blue bars). Results are given as the mean percentage of positive cells ± SEMs. *Significantly different from patients with psoriasis; #significantly different compared with the Langerin-positive CD1a⁺ DC population in AD skin.

as well as in lesional skin from patients with chronic AD and psoriasis. LCs were the major DC subpopulation (95.4%, mean of n=8) of CD1a⁺ epidermal cells in healthy skin. In chronic AD and psoriatic lesions, the frequency of CD1a⁺ cells was slightly higher than in healthy skin, and the proportion of Langerin-positive CD1a⁺ DCs to Langerin-negative CD1a⁺ DCs was 58.8% to 41.2% in patients with AD (mean of n=11), whereas it was 53.8% to 45.2% (mean of n=8) in patients with psoriasis (Table II). The

TABLE III. Significance levels of differences of CC chemokine mRNA expression during APTs

CC chemokines (CCL)	Before APT to 24 h	Before APT to 72 h	Between APTs, 24-72 h
CCL1	.095	.016	.222
CCL2	.056	.008	1.000
CCL3	.032	.008	.095
CCL4	.222	.008	.095
CCL5	.286	.032	.151
CCL7	.151	.016	.310
CCL8	.222	.016	.222
CCL11	.087	.008	.087
CCL13	.238	.040	.690
CCL17	.095	.008	.151
CCL18	.548	.222	.421
CCL20	.032	.008	.222
CCL22	.690	.008	.008
CCL27	.151	.151	.008

**FIG 4.** Relative expression of CC chemokine mRNA in epidermal skin of patients with AD during APTs. CCL expression in the epidermis of responders (n=5; **A** and **B**) and CCL expression in the epidermis of nonresponders (n=3; **C** and **D**) before (left column) and 24 hours (middle column) and 72 hours (right column) after allergen application are shown. Top, Panel 1 of CCLs; bottom, panel 2 of CCLs. Results are given as the mean expression of CCL mRNA normalized to healthy control skin $\pm$ SEMs.

lowest expression of CCR6 could be observed on LCs from lesional skin of patients with AD (Fig 3, A). Furthermore, the surface expression of CCR1, CCR2, CCR4, CCR5, and CCR7 was significantly higher on Langerin-negative CD1a⁺ epidermal DCs than on Langerin-positive CD1a⁺ DCs (Fig 3). In addition, the Langerin-negative CD1a⁺ cells in patients with AD were in a more mature state than the Langerin-positive CD1a⁺ DCs, mirrored by increased CD83 surface expression ($4.7\% \pm 1.5\%$ vs $11.1\% \pm 2.1\%$, $P=.008$). Significantly higher expression of CCR1, CCR2, CCR4, and CCR5 could be observed also on Langerin-negative CD1a⁺ DCs in patients with psoriasis compared with Langerin-positive CD1a⁺ DCs (Fig 3). Expression of

CCR4, CCR6, and CCR8 on Langerin-negative CD1a⁺ DCs was significantly lower in AD than in psoriatic lesions (Fig 3, B).

Development of skin lesions in patients with AD goes along with an increase in epidermal CCL1, CCL17, and CCL18 mRNA expression

To collect evidence for an altered expression profile of chemokines during specific disease stages of AD, in particular during acute eczema development, we studied chemokine expression in the epidermis of patients with AD during APTs. mRNA was isolated from sequential shave biopsy specimens

taken before and after 24 and 72 hours of allergen application. Patients responded with an eczematous skin lesion to allergen application or exhibited no development of eczema in the particular skin region. The earliest significant increase (already after 24 hours) could be observed for CCL3 and CCL20 mRNA, whereas nearly all CCL mRNA levels increased significantly 72 hours after allergen application (Table III) in the subgroup of patients responding with the development of eczematous skin lesions (responders).

Concerning the comparison of patients responding and not responding with eczema, an increase of mRNA levels of up to 800-fold for CCL1, 400-fold for CCL17, and 3000-fold for CCL18 mRNA was detectable in patients with a positive APT reaction (Fig 4, A and B). In contrast, in the epidermal skin of non-responders, the mRNA level of these chemokines increased as well, although to a much lower extent (only about 20- to 300-fold; Fig 4, C and D). From these data we conclude that a strong increase of CCLs, in particular CCL1, CCL17, and CCL18, after allergen application goes along with eczema development.

Enhanced CCL1, CCL3, CCL4, CCL11, and CCL17 mRNA expression is characteristic for acute AD

Concerning the clinical picture of AD, acute lesions, which show up during the flare-ups, and chronic eczematous lesions, which are present to some degree even during remission phases of AD, have to be distinguished. Therefore as a next step we wanted to delineate changes in the CCL skin microenvironment characteristic for acute versus chronic AD lesions. For this purpose, we compared the CCL mRNA expression pattern of patients 72 hours after allergen application with the CCL mRNA expression patterns in chronic AD skin. As a result, we observed a higher expression of CCL1, CCL3, CCL4, CCL11, and CCL17 mRNA in acute skin lesions (72 hours after allergen application; Fig 5). From these data, we conclude that higher expression of CCL1, CCL3, CCL4, CCL11 and CCL17 mRNA is characteristic for acute compared with chronic eczema.

CCL1, CCL13, CCL17, and CCL18 mRNA levels are higher in patients with chronic AD than in patients with psoriasis

It is important to distinguish AD-specific changes from modifications related to chronic inflammation. To investigate this, we analyzed the expression of CCLs in the epidermal skin of healthy donors, as well as in the lesional skin of patients with chronic AD and psoriasis. mRNA was isolated from skin-shave specimens and subjected to quantitative real-time PCR analysis. Increased expression of all CCLs studied except for CCL27 was observed in both AD and psoriatic lesions compared with healthy skin. However, much higher expression of CCL1, CCL13, CCL17, and CCL18 mRNA was observed in the epidermis of patients with chronic AD compared with that of patients with psoriasis, implying that these CCLs are relatively specific for AD (Fig 6). In relation to healthy control skin, mRNA expression of these chemokines was enhanced up to 50- to 10,000-fold. In contrast, the epidermis of patients with psoriasis was characterized by higher expression of CCL3, CCL4, CCL5, CCL7, CCL11, and CCL20 mRNA.

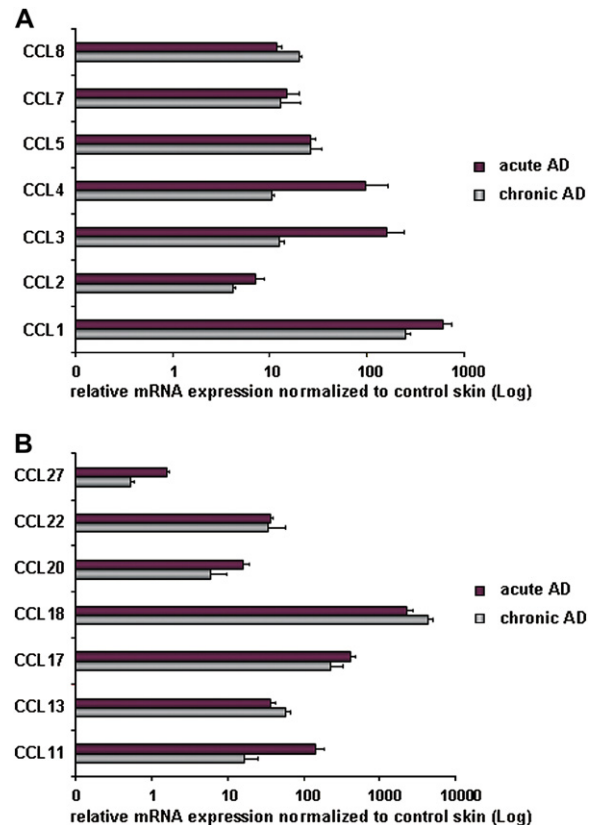


FIG 5. Different CCL mRNA expression levels in acute versus chronic AD skin lesions. Relative expression of mRNA of patients with acute AD 72 hours after APT (red bars) and patients with chronic AD (gray bars) was normalized to the mRNA level in healthy control skin. **A**, Panel 1 of CCLs. **B**, Panel 2 of CCLs. Results are given as the mean expression of CCL mRNA ($n=5$) $\pm$ SEMs.

DISCUSSION

In this study we analyzed systematically the expression pattern of CCRs on epidermal DC subtypes in parallel to CCL mRNA levels in nonlesional acute and chronic AD skin compared with healthy epidermal skin and lesional skin of patients with psoriasis. We could show that development of eczematous skin lesions after allergen challenge was accompanied by an increase of CCR1-, CCR5-, and CCR6-expressing Langerin-negative CD1a⁺ DCs and a decrease of Langerin-positive CD1a⁺ DCs. In view of the increasing total number of CD1a⁺ cells detectable in the epidermis after allergen application together with the profound shift of Langerin-positive CD1a⁺ DCs into Langerin-negative CD1a⁺ DCs, increased recruitment and generation of inflammatory DCs, in particular CCR5- and CCR6-expressing DC subtypes from blood but, in view of the short time interval, foremost from dermal precursor cells, is very likely to occur. In addition, diverse inflammatory DC subtypes or differentiation stages of inflammatory subtypes in the acute versus chronic phases of AD would explain variations in the CCR expression pattern, such as upregulation of CCR1, CCR5, and CCR6 after allergen challenge and higher CCR5, CCR6, and CCR8 expression of Langerin-negative CD1a⁺ DCs in patients with acute versus chronic AD, as observed here. In particular, upregulated CCL1, CCL3, CCL4, and CCL20 mRNA expression and CCR5, CCR6, and CCR8 might be responsible for recruitment of these cells.

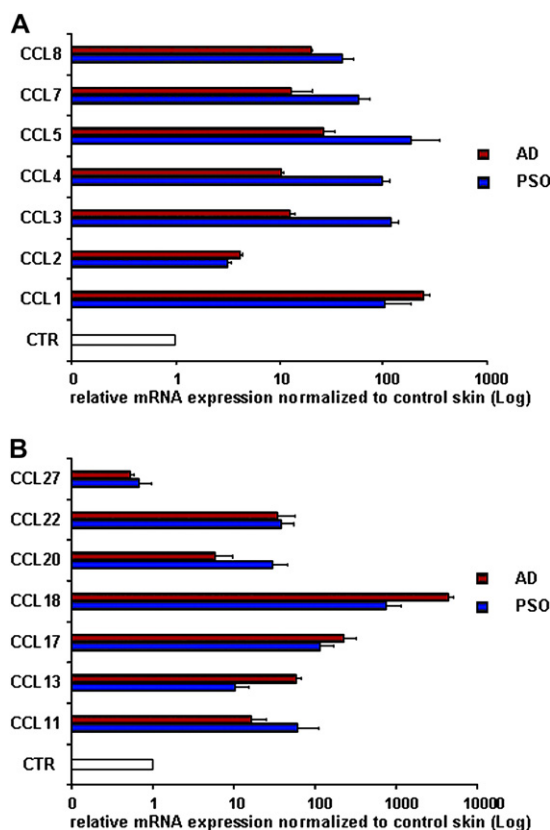


FIG 6. Relative expression of CC chemokine mRNA in the epidermis of healthy skin and skin from patients with chronic AD and psoriasis. Relative expression of mRNA in patients with chronic AD (red bars) and psoriasis (PSO; blue bars) was normalized to the mRNA level in healthy control skin (white bars; $n=2$). **A**, Panel 1 of CCLs. **B**, Panel 2 of CCLs $\pm$ SEMs.

Changes in the subtype of Langerin-positive CD1a⁺ DCs according to the stage of AD observed here are in line with data provided in previous studies that have shown changes of CD36 expression of Langerin-positive CD1a⁺ DCs in early versus chronic AD lesions.¹³

We observed a significantly higher expression of CCR1, CCR2, CCR5, and CCR7 on Langerin-negative CD1a⁺ DCs compared with that seen on Langerin-positive CD1a⁺ DCs in patients with chronic AD. Because increased expression of these CCRs was also detectable on Langerin-negative CD1a⁺ DCs in patients with psoriasis, high CCR expression seems to be in part a general feature of inflammatory DCs, underlining the crucial role of this DC type in recruitment and migration during ongoing inflammation. Relatively low CCR6 expression of epidermal DCs in patients with AD compared with that seen in patients with psoriasis observed in this study is in line with previous studies, which revealed lower expression of CCR6 on CD4⁺, CD8⁺, or CD3⁺ cells in patients with AD.²⁰ Differences of the CCR6 expression of epidermal DCs in nonlesional acute and chronic AD lesions might result also from interindividual variations or different expression patterns of CCR6 on epidermal DCs at distinct body sites.

Several chemokines have been linked to recruitment of inflammatory cell subtypes to the skin in patients with AD, including CCL1, CCL2, CCL3, CCL4, CCL5, CCL11, CCL13, CCL18, CCL20, CCL22, CCL26, and CCL27.³ Moreover, serum

levels of some of these chemokines correlate directly with disease activity.³ In line with this observation, both serum levels and tissue expression of selected chemokines decrease in response to successful topical treatment, such as the reduction of CCL5 and CCL11 levels after tacrolimus treatment.²¹

Chemokines, such as CCL3, CCL5, CCL11, CCL24, and CCL26, have also been demonstrated to be of relevance for the recruitment of eosinophils, T cells, and inflammatory cells to the lung in asthmatic patients.^{22,23}

Concerning changes in the chemokine pattern in the upper skin, which occur in response to local allergen challenge and go along with the development of eczematous skin lesions, we detected a significant increase in the mRNA level of nearly all CCLs studied in patients with positive responses during APTs. An increase in the mRNA level of these chemokines after 24 to 72 hours was observed also in patients with AD with subclinical eczema who did not respond with the manifestation of an eczematous skin lesion to allergen application. However, upregulation of these chemokines was much lower in this group compared with that seen in responders, implying that the increase in CCLs, in particular CCL1, CCL17, and CCL18, has to overstep a specific threshold. Keratinocytes and epidermal DCs might represent a source of these chemokines in addition to several other cells in the skin.³ Subtypes of cells known to be recruited by these CCLs include T cells, LC and DC precursors, and eosinophils.³ The sequence and amplitude of the increase of chemokine mRNA expression is further in line with the CCR expression pattern of the invading cells. Similar to the data obtained in previous studies,^{8,9} we observed higher CCL1, CCL17, CCL18, and CCL20 expression in lesional versus nonlesional skin and higher CCL20 expression in patients with psoriasis.

In addition, we provide evidence for an upregulated mRNA expression of CCL1, CCL3, CCL4, CCL5, CCL7, CCL8, CCL11, CCL13, CCL17, CCL18, CCL20, and CCL22 in the upper compartment of chronic AD lesions, which is also detectable in patients with other inflammatory skin diseases, such as psoriasis. Therefore this upregulation represents rather a general feature of chronic inflammation in the skin. In contrast, a 10- to 100-fold higher expression of CCL1, CCL17, and CCL18 was observed in patients with chronic AD versus that seen in those with psoriasis and could be regarded as AD specific.

Taken together, these insights into the mechanisms that regulate DC trafficking to and from the skin under inflammatory conditions open new opportunities for therapeutic intervention, which might attenuate or impede early steps of eczema development during the flare-ups, as well as mechanisms aggravating inflammation during chronic AD.

Clinical implications: CCR- and chemokine-directed trafficking of DC subtypes might be targeted for therapeutic purposes.

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METHODS

Reagents

Unconjugated mAbs against CCR1 (53504; mIgG2b), CCR2 (48607; mIgG2b), CCR4 (205410; mIgG2b), CCR5 (CTC5; mIgG1), CCR6 (53103.111; mIgG2b), CCR7 (150503; mIgG2a), and CCR8 (191704; ratIgG2a) and mAb against mouse IgG2b as an isotype control were from R&D Systems (Minneapolis, Min). Phycoerythrin-Cy5-labeled monoclonal anti-CD1a (HI149; mIgG1) and the respective isotype control, unconjugated mAb against CD83 (HB15e; mIgG1) and rat IgG2a, and phycoerythrin-labeled IgG1 were purchased from BD Systems (San Jose, Calif). Phycoerythrin-conjugated monoclonal anti-CD207/Langerin (DCGM4; mIgG1) was from Beckman Coulter, Inc (Fullerton, Calif). Fluorescein isothiocyanate (FITC)-conjugated F(ab')₂ of goat anti-mouse IgG (GaM/FITC) and FITC-conjugated F(ab')₂ of goat anti-rat IgG (GaR/FITC) antibodies and normal mouse serum for blocking purposes were obtained from Dianova (Hamburg, Germany). Trizol Reagent for total RNA isolation and RPMI 1640 medium were from Invitrogen Ltd (Paisley, United Kingdom). All other reagents were obtained from Sigma Chemical Co (St Louis, Mo) unless otherwise indicated. Trypsin sensitivity of the different primary antibodies used for flow cytometric staining of epidermal skin cells was tested.

Real-time PCR

For mRNA analysis of CCL expression, skin-shave specimens were homogenized with the Precellys 24 homogenizer (Peqlab, Erlangen, Germany) together with an appropriate homogenization kit (Peqlab), and total

RNA was extracted according to the Trizol method. After phase separation through addition of chloroform and centrifugation, the aqueous phase containing RNA was selected and washed in 70% ethanol. RNA was cleaned up with the help of the RNeasy Mini Kit (Qiagen), including digestion of genomic DNA, and was subjected to cDNA synthesis with TaqMan reverse transcription reagents with random hexamers according to the manufacturer's instructions (Applied Biosystems, Darmstadt, Germany). The prepared cDNA was amplified with TaqMan assay Master Mix (Applied Biosystems), according to the recommendations of the manufacturer, in an ABI Prism 7300 Sequence Detection System (Applied Biosystems). Primers CCL1: Hs00171072_m1, CCL2: Hs00234140_m1, CCL3: Hs00234142_m1, CCL4: Hs99999148_m1, CCL5: Hs99999048_m1, CCL7: Hs00171147_m1, CCL8: Hs00271615_m1, CCL11: Hs00237013_m1, CCL13: Hs00234646_m1, CCL17: Hs00171074_m1, CCL18: Hs00268113_m1, CCL20: Hs00171125_m1, CCL22: Hs00171080_m1, and CCL27: Hs00171157_m1, as well as endogenous controls (18s), were from Applied Biosystems. All assays were performed according to the manufacturer's instructions. Relative quantification and calculation of the range of confidence was performed with comparative $\Delta\Delta CT$ methods.^{E1} All analyses were conducted in duplicates, and experimental samples were quantitated by using sample dilutions, ensuring that the cycle threshold values were in the linear proportion of the standard curve.

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