

Original article

Malassezia sympodialis differently affects the expression of LL-37 in dendritic cells from atopic eczema patients and healthy individuals

Background: Atopic eczema (AE) is a multifactorial disease, which has increased in prevalence. The skin-colonizing yeast *Malassezia sympodialis* can induce IgE- and T-cell reactivity in patients with AE. LL-37 is an endogenous peptide antibiotic belonging to the cathelicidin family. The aim of this study was to examine whether exposure to *M. sympodialis* would affect the expression of LL-37 in dendritic cells.

Methods: The presence of LL-37 was analyzed in monocyte-derived dendritic cells (MDDCs) generated from healthy individuals and patients with AE by Western blotting and the corresponding cDNA by real-time quantitative RT-PCR. Antibacterial activity was measured with an inhibition zone assay in fractions after reverse phase chromatography.

Results: For the first time we here present data, showing that LL-37 is produced by MDDCs. Notably, the secretion of LL-37 was substantially enhanced in *M. sympodialis*-exposed MDDCs generated from patients with a high degree of eczema, as measured by SCORAD, compared to healthy controls and patients with a low SCORAD. The relative expression of LL-37 transcript in MDDCs generated from patients was up-regulated after 1 h of exposure to *M. sympodialis* and declined gradually at the time points analyzed, whereas the transcription was unaffected in the MDDCs of healthy controls.

Conclusions: Our results suggest that *M. sympodialis* can trigger the innate immune response differently in patients with AE and healthy individuals. The enhanced LL-37 secretion from the MDDCs in the patients with AE may reflect the severity of their inflammatory response to *M. sympodialis*.

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Key words: antimicrobial activity; cathelicidin; innate immunity; normal microflora; yeast cells.

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Accepted for publication 1 July 2005

Antimicrobial peptides are evolutionary ancient defense molecules and widespread in nature (1). These peptides can be considered as endogenous antibiotics that are included in the microbicidal armament of cells in innate immunity. Among the fundamentals in innate immunity are cells like neutrophils, macrophages and DCs that constantly screen for invaders with their pattern recognition receptors. Upon recognition these cells are armed for an immediate attack on the microbial intruders, where antimicrobial peptides are among the effector molecules. Besides their immediate defense function as microbicidal molecules, antimicrobial peptides exhibit additional activities such as chemotaxis and immunomodulation (2–4). This indicates an important role for these peptides in the interplay between innate and adaptive immunity during an immune response.

LL-37, a human antimicrobial peptide of the cathelicidin family (5) was originally characterized in granulocytes (6). Later LL-37 expression was found in epithelial

cells (7) and in different mononuclear cells (8). In keratinocytes LL-37 is induced during inflammatory skin disorders such as psoriasis and nickel allergy (9). A correlation between the degree of induction for both LL-37 and human β -defensin-2 (HBD-2) and infections in psoriatic lesions and atopic eczema (AE) has been suggested (10). It was found that patients with AE were lacking appropriate induction of these two antimicrobial peptides compared to psoriatic patients, suggesting an explanation for the frequent *Staphylococcus aureus* infections in the skin of AE patients (10).

The yeast *Malassezia*, formerly known as *Pityrosporum*, belongs to our normal cutaneous microflora but can cause skin infections and even systemic infections (11, 12). Monocyte-derived dendritic cells (MDDCs), when in an immature state, can internalize whole *Malassezia* yeast cells within 1 h (13), which leads to MDDC maturation, production of pro-inflammatory and immunoregulatory cytokines, favoring induction of a Th2-like immune

response (14). Several studies have shown the presence of specific serum IgE, positive skin prick test (SPT) and/or positive atopy patch test (APT) reactions to *Malassezia* in patients with AE (15). Therefore this yeast is considered to be one of the factors that can contribute to AE. In accordance, patients with AE show an altered gene expression profile of pro-inflammatory and co-stimulatory molecules in MDDCs exposed to *Malassezia* compared to healthy individuals (16). The possible interplay between the innate and adaptive immune system in triggering of MDDCs from AE by *Malassezia* has not been investigated.

In this study we have compared the expression of antimicrobial peptides, and in more detail LL-37, in MDDCs from healthy controls and AE patients. In addition, we have investigated if the expression is altered when MDDCs are exposed to the yeast *Malassezia sympodialis*.

Materials and methods

Subjects

Five patients with AE and seven healthy subjects participated in the study (Table 1). The patients were at the time of blood sampling diagnosed with moderate to severe AE according to SCORAD (16), had specific serum IgE to *M. sympodialis* (m70, Pharmacia Diagnostics, Uppsala, Sweden) and four of them had previously (17) proven to be positive for *M. sympodialis* in both SPT and APT. The healthy controls had no history of allergy, and except for HC4 they were analyzed for IgE antibodies and found to be ImmunoCap™ negative to a panel of common allergens (Phadiatop®, Pharmacia

Diagnostics) and to *M. sympodialis* (Table 1). The study was approved by the ethics committee of the Karolinska University Hospital, and all participating subjects gave their informed consent.

Culturing of *Malassezia sympodialis*

Malassezia sympodialis strain No. 42132 (American Type Culture Collection) was cultured on Dixon solid phase medium, at 37°C for 4 days (18). The culture was controlled for bacterial contamination and growth of other yeast using a blood agar plate and a Sabouraud dextrose agar plate. The yeast cells were harvested into sterile water and counted under a light microscope before incubation with MDDCs.

Generation of MDDCs from peripheral blood

Monocytes were isolated from 450 ml peripheral blood and MDDCs generated as previously described (14, 19). Briefly, the CD14⁺ cells separated by magnetic activated cell sorting (MACS, Miltenyi Biotec, Bergish Gladbach, Germany) were diluted to 4×10^5 cells/ml in complete culture medium, RPMIc [RPMI 1640 medium supplemented with 25 µg/ml gentamicin, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin (Gibco BRL, Life Technologies Ltd, Scotland), 10% (v/v) fetal calf serum (FCS, Hyclone, Logan, Utah), 50 µM 2-β-mercaptoethanol (Sigma Chemical, St Louis, MO, USA), 550 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF, Schering Plough, Kenilworth, NJ, USA) and 800 U/ml of recombinant human interleukin-4 (rIL-4, Nordic BioSite, Sweden)], and cultured in 25 or 75 cm² culture flasks (Costar Corporation, Cambridge, MA, USA). To generate immature MDDCs the CD14⁺ cells were cultured in a humidified incubator with 6% CO₂ in air for 6 days with refeeding on day 3. Immature MDDCs were harvested on day 6 by gentle flushing. Anti-CD14 staining was performed to evaluate the separation efficiency of monocytes, which was always above 92%.

Table 1. Characterization of patients with atopic eczema and healthy controls

Subject	Gender	Age (years)	SCORAD*	SPT†	APT‡ specific s-IgE (kU/l)	<i>M. sympodialis</i> § (kU/l)	Total s-IgE¶	Phadiatop®**	Asthma/Rhinitis
AE1	F	31	23	4.8	+	4.8	5300	Pos	+/-
AE2	M	25	28	5	+	14	2400	Pos	+/+
AE3	M	45	54	6.5	+++	2.4	4710	Pos	+/-
	One year later ⁹	46	42			21	2700	Pos	
AE4	M	33	46	8	++	5.4	1800	Pos	-/+
AE5	F	25	28	ND	ND	5.3	110	Pos	
HC1	M	45	NA	ND	ND	<0.35	45	Neg	-/-
HC2	M	48	NA	ND	ND	<0.35	15	Neg	-/-
HC3	F	30	NA	ND	ND	<0.35	2	Neg	-/-
HC4	NK	NK	NA	ND	ND	ND	ND	ND	-/-
HC5	NK	NK	NA	ND	ND	<0.35	17	Neg	-/-
HC6	NK	NK	NA	ND	ND	<0.35	13	Neg	-/-
HC7	NK	NK	NA	ND	ND	<0.35	3.5	Neg	-/-

F, female; M, male; NA, not applicable; ND, not done; NK, not known, healthy blood donor.

*Assessed by SCORAD (33) at the time of MDDC generation.

†The skin prick test (SPT) was performed with extract of *M. sympodialis*, 100 µg/ml, evaluated after 15 min and graded as mean diameter (mm) of the wheal (17). A reaction with a mean diameter of ≥3 mm was considered positive.

‡Atopy patch test (APT) was performed with extract of *M. sympodialis*, 5 mg/ml, on tape-stripped nonlesional skin (17). The test results were scored 0–3+, where 0 = negative reaction, 1+ = erythema, infiltration and possibly papules, 2+ = erythema, infiltration, papules and/or small vesicles and 3+ = erythema, infiltration, papules and large vesicles (34).

§ImmunoCap™ m70 (Pharmacia Diagnostics AB), reference range <0.35 kU/l.

¶ImmunoCap™ (Pharmacia Diagnostics AB), reference range 1.6–122 kU/l.

**Phadiatop® (Pharmacia Diagnostics AB), serum IgE to any of 11 common aeroallergens. Reference range <0.35 kU/l.

⁹Additional blood sample 1 year later.

Characterization of the MDDCs

At day 6 of culture, the MDDCs' surface phenotype was assessed by flow cytometry. The following fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugated mouse monoclonal antibodies (MoAbs) were used: anti-CD1a PE (T6-RD1, Coulter, Beckman-Coulter, Sweden), anti-CD14 FITC (Leu-M3), anti-CD40 FITC (5C3), anti-CD80 FITC (L307.4), anti-CD83 FITC (HB15e), anti-CD86 FITC (2331 FUN-1) and anti-HLA-DR FITC (L243) all from PharMingen/Becton Dickinson (San Jose, CA, USA). Isotype matched antibodies, mouse IgG₁ FITC- or PE-conjugated (X40) and mouse IgG_{2a} FITC-conjugated (X39) from Becton Dickinson were used as negative controls. Approximately 5×10^4 cells were incubated for 30 min on ice with MoAbs. Gates were set according to a characteristic forward-scatter pattern (20). The flow cytometer was calibrated according to the manufacturer's instructions before each acquisition, and a minimum of 10^4 cells was acquired on a FACSCalibur flow cytometer and analyzed using CellQuest software (Becton Dickinson). After 6 days of culture, the MDDCs displayed a typical immature DC phenotype (less than 5% CD83⁺ cells) (14) and purity with always less than 2% CD14⁺ cells and above 82% CD1a⁺ cells. No phenotypical differences were observed between MDDCs from healthy controls and AE patients.

Co-culture of MDDCs with *Malassezia sympodialis*

After 6 days of culture, the immature MDDCs were harvested and co-cultured with *M. sympodialis*, at a 1 : 5 ratio, in 25 cm² culture flasks (Costar Corporation) at 37°C, in RPMI supplemented with cytokines (550 U/ml GM-CSF and 800 U/ml rIL-4) or in complete culture medium alone, at a cell density of 4×10^5 MDDCs/ml, for different time points (1, 2, 4, 8, 18 and 24 h). In the 18 h cultures of AE1-4 and HC1-3 (Table 1), 5% FCS and 5% autologous serum replaced 10% FCS (16). Upon harvest, the cells were centrifuged and cell viability, as assessed by trypan blue exclusion, always exceeded 90%. After centrifugation the culture supernatants were collected and stored at -20°C until further analysis. The MDDCs were saved in RNeasy lysis buffer (Qiagen) and stored at -80°C for analysis of LL-37 mRNA by real time PCR.

Enrichment of peptides/proteins

Collected supernatants from MDDCs, cultured with or without *M. sympodialis*, were adjusted with concentrated trifluoroacetic acid (TFA) to a final concentration of 0.1% TFA. These solutions were applied onto OASISTM (Waters, Milford, MA, USA) cartridges activated in acetonitrile (AcN) and equilibrated in aqueous 0.1% TFA. The cartridges were then washed with 0.1% TFA, 10% AcN in 0.1% TFA, 20% AcN in 0.1% TFA and bound peptides/proteins were eluted with 80% AcN in 0.1% TFA and lyophilized. This concentrate of peptides/proteins was the starting material for further analyses.

Antibacterial assay

Thin plates (1 mm) were made of 1% agarose in Luria Bertani (LB) broth containing approximately 6×10^4 cells/ml of the test bacterium, *Bacillus megaterium*, strain Bm11 (6). For detection of lysozyme a zone clearing on plates with cell walls from *Micrococcus luteus* (1 mg/ml, Sigma Chemical) was used. Small wells (3 mm) were punched in the plates, and 3 µl of samples dissolved in 0.1% TFA were loaded in each well. As a positive control, synthetic LL-37 (9 µg) (Innovagen, Lund, Sweden) was used. After an overnight incubation at 30°C, the diameters of the inhibition zones were recorded.

Western blot analysis

The presence of LL-37 in lyophilized peptide/protein material (80 or 100 µg) was determined with Western blot analysis using a rabbit polyclonal antiserum specific for LL-37 (6). Polypeptides were separated by discontinuous sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) using 10–20% Tricine Ready Gels (Invitrogen, Carlsbad, CA, USA) and further blotted onto polyvinylidene difluoride (PVDF) membranes by electrophoretic transfer according to the instructions from the manufacturer. The membrane was blocked in PBS containing 5% fat free milk. The concentration of the polyclonal antibody for LL-37 was 2.5 µg/ml in PBS with 5% fat free milk. The second antibody was a donkey antirabbit IgG conjugated with horseradish peroxidase (Amersham Pharmacia Biotech, Uppsala, Sweden). An ECL Western blot detection system (Amersham Pharmacia Biotech) was used to visualize the results.

RT-PCR and TaqMan[®] real-time RT-PCR

Total RNA was prepared from MDDCs co-cultured with or without *M. sympodialis* for different time points, according to the manufacturer's (Qiagen Inc. Valencia, CA, USA) instructions. Reverse transcription of RNA into cDNA was performed essentially as previously described (21). For quantification the expression of LL-37 and the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were measured in triplicates from the different RNA extractions by real-time quantitative RT-PCR utilizing a PE Applied Biosystems PRISM model 7700 sequence detection instrument (ABI, Foster City, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase transcript numbers were measured by PCR reactions using the GAPDH-PDAR-Housekeeping Kit (ABI), which does not amplify GAPDH pseudogenes. The expression of GAPDH in MDDCs has been found to be unaffected after stimulation with *M. sympodialis* (16). The sequences of forward and reverse primers as designed by Primer Express (ABI) for quantification of LL-37 mRNA were 5'-ACCCAGCAGGGCAAATCTC-3' and 5'-GAAGGACGGGCTGGTGAAG-3', respectively. The TaqMan[®] (ABI) fluorogenic probe used for LL-37 was 5'-TGT-TATCCTTATCACAACCTGAT-3' FAM-label. A standard curve was made with serial dilutions of cDNA synthesized from total RNA from the monocytic cell-line U937 (ACC5, DSMZ Braunschweig, Germany). LL-37 and GAPDH starting transcript number values for the standard curve were set arbitrarily. The LL-37 and GAPDH values in unknown samples were quantified by measuring cycle threshold (Ct) and the corresponding value of the standard curve was read. LL-37 expression was then normalized to GAPDH expression and LL-37 expression levels in MDDCs in medium alone were considered to be '1'. Relative expression of LL-37 was calculated by relating LL-37 expression in stimulated cells to control cells. The samples were analyzed twice in triplicates and the results are expressed as mean ± SD.

Chromatography of the culture supernatants from MDDCs

A ÄKTA purifier system (Amersham Pharmacia Biotech) was utilized in high performance liquid chromatography (HPLC) steps and the column effluent was monitored at 214 and 280 nm. Reverse phase HPLC was performed on a Vydac C₁₈ column, 4.6 × 250 mm (Separations Group, Hesperia, CA, USA) equilibrated in 0.1% TFA, at a flow rate of 1 ml/min. A gradient of 0–15%, 15–60% and 60–100% AcN for 4, 45 and 12 min, respectively, was employed.

Results

LL-37 in the culture supernatants of MDDCs

The presence of the human antimicrobial peptide LL-37 was investigated by Western blot analysis in the supernatants of MDDCs, which had been cultured with or without *M. sympodialis*. The amount of LL-37 was highly increased in the supernatants of the MDDCs exposed to *M. sympodialis* for 18 h generated from the patients AE3 and AE4 (Fig. 1). These two patients had the highest SCORAD values (Table 1). However, in the supernatants derived from patients with low SCORAD values the levels of LL-37 were lower or the peptide was hardly detectable, both for MDDCs cultured alone and exposed to *M. sympodialis* (Fig. 1). Similar results were obtained for HC1 and HC2 (Fig. 1). In contrast, the band corresponding to LL-37 was strong, indicating high LL-37 secretion, in the supernatant of unstimulated MDDCs from HC3. After *M. sympodialis* exposure the band strength for LL-37 decreased, revealing lower levels of secreted LL-37 (Fig. 1). Interestingly, this individual had an altered response pattern to *M. sympodialis* in terms of inflammatory related genes compared to other healthy individuals in a microarray study (16), which could reflect ongoing disease or an immune dysregulation.

Effect of *Malassezia* exposure on the expression of LL-37 in MDDCs over time

Transcriptional level. To evaluate the transcriptional expression of LL-37 in MDDCs and to investigate if *M. sympodialis* affects this expression, a kinetic study was conducted. Immature MDDCs originated from four

healthy blood donors (HC4-7) and two AE patients (AE3^g and AE5 see Table 1) were cultured in medium alone or co-cultured with *M. sympodialis* for different periods of time. In the healthy individuals the relative expression of LL-37 in MDDCs was unaffected at the different time-points analyzed (Fig. 2), since the average level of LL-37 transcript in *M. sympodialis* treated cells was the same as in control cells (fold-induction = 1). In MDDCs derived from the two AE patients the relative expression of LL-37 was up-regulated after 1 h of exposure to *M. sympodialis*. This induction was more prominent in patient AE3^g, who had a SCORAD value of 42 at the time of blood sampling (Table 1) compared to the lower SCORAD value of 28 in AE5 (data not shown). At the additional time-points analyzed (Fig. 2) the relative expression of LL-37 declined gradually and at 8 h of exposure with *M. sympodialis* the fold-induction was around 0.5, a value that represents 50% down-regulation.

Peptide level. In the kinetic study the presence of the peptide LL-37 was investigated in the respective supernatants of cultured MDDCs. At each time point peptides/proteins derived from HC7 and patients AE3^g and AE5 were analyzed by Western blot with the specific polyclonal LL-37 antiserum. In the HC sample an overall decrease in the amount of LL-37 was seen in the supernatants of MDDCs after exposure to *M. sympodialis* compared to the unstimulated MDDCs (Fig. 3A). This reduction was most prominent after 4 and 48 h, where LL-37 was hardly detected. In AE5, a patient with a SCORAD value of 28, the level of LL-37 increased after 1 and 2 h of co-culture of the MDDCs with *M. sympodialis*. At the other time-points analyzed LL-37 was either not detected, or very faint bands were present (Fig. 3B). For patient AE3^g, exhibiting the higher SCORAD value

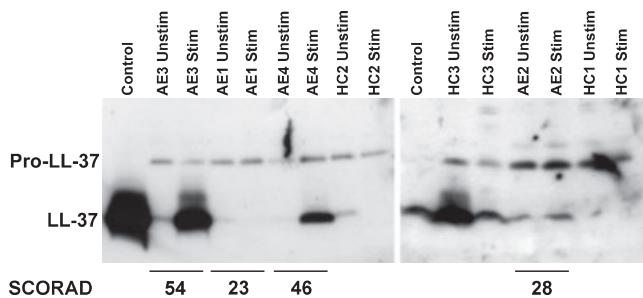


Figure 1. Secreted LL-37 in supernatants of MDDCs cultured with or without *M. sympodialis*. Western blot analysis of LL-37 in supernatants derived from MDDCs of three healthy individuals and four AE patients with SCORAD values indicated (80 µg of the lyophilized peptide/protein concentrate was loaded of each sample). The MDDCs are cultured in medium alone (Unstim) or co-cultured for 18 h with *M. sympodialis* (Stim). Lane 1 and 10: synthetic LL-37 (40 and 1 ng, respectively), indicated as control, other lanes: as indicated. The high molecular weight bands correspond to pro-LL-37. In samples with high levels of LL-37 oligomeric forms of LL-37 are detected.

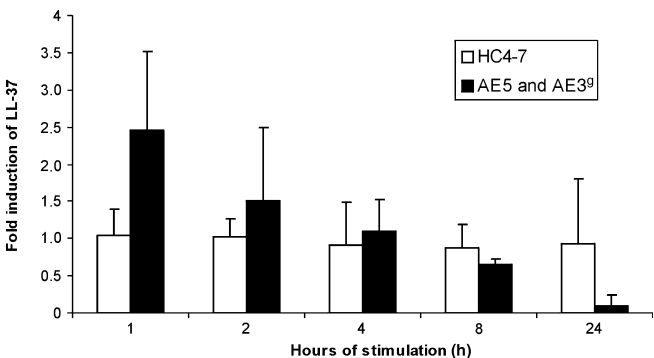


Figure 2. Kinetic of LL-37 transcription in MDDCs before and after stimulation with *M. sympodialis*. Quantitative real-time RT-PCR of LL-37 transcript in MDDCs after different time-points of exposure with *M. sympodialis* compared to MDDCs cultured only in medium. The relative expression of LL-37 in *M. sympodialis* treated MDDCs over-time was determined from HC4-7 and AE3^g and AE5 (see Table 1). Results are expressed as mean ± SD of triplicates analyzed twice.

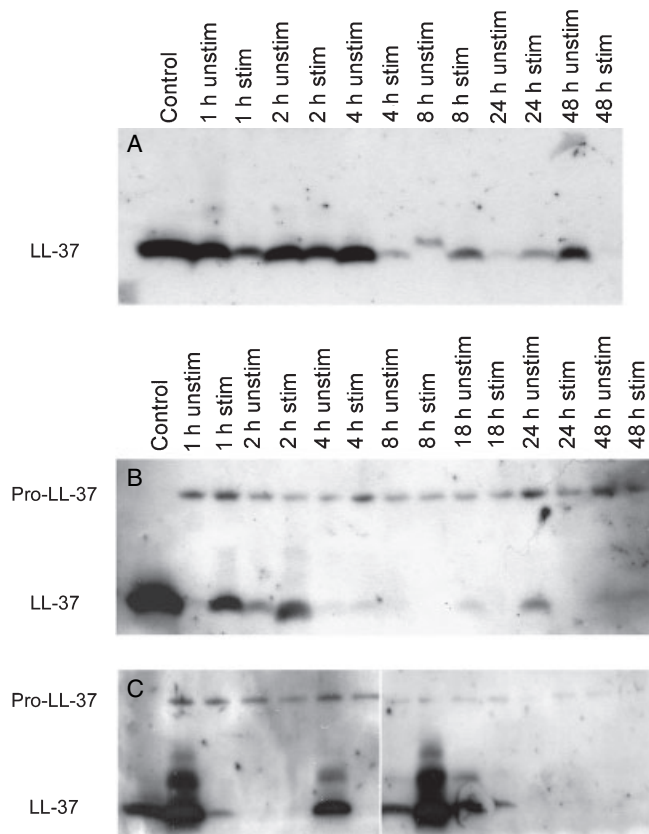


Figure 3. Kinetic of secreted LL-37 from MDDCs cultured with or without *M. sympodialis*. Western blot analyses of LL-37 on supernatants of MDDCs after different time-points (100 µg of the lyophilized peptide/protein concentrate was loaded of each sample). The MDDCs were cultured in medium (Unstim) or co-cultured with *M. sympodialis* (Stim). The supernatants were derived from (A) HC7, (B) AE patient No. 5 with a SCORAD value of 28 and (C) AE patient No. 3^g with a SCORAD of 42 at the time of blood sampling. The control was the synthetic LL-37 peptide (5 ng).

42, the amount of LL-37 in the supernatants of MDDCs was reduced after 1 and 4 h of stimulation with *M. sympodialis* (Fig. 3C). In contrast the amount of LL-37 was highly enhanced after 8 h of stimulation. After 18 h of stimulation the level of LL-37 declined and at the other time-points analyzed LL-37 was not detected (Fig. 3C).

Antibacterial activity in fractions derived from MDDCs supernatants separated by HPLC

Since the MDDC supernatants contained antibacterial activity, as detected by the inhibition zone assay (data not shown), we wanted to evaluate the complexity of the material giving rise to this activity. Therefore, the enriched peptide/protein material (1 mg) originated from patients AE1, AE3, and AE4 and control HC2 were fractionated by reverse phase HPLC into 62 fractions.

After lyophilization, all fractions were dissolved in 12 µl of aqueous 0.1% TFA and 3 µl thereof was analyzed for antibacterial activity against *B. megaterium*. The chromatographic profiles appear similar between the *M. sympodialis* exposed and nonexposed material derived from both the healthy control (Fig. 4A, B) and the AE3 patient (Fig. 4C, D). Interestingly, the profile varies, however, between the patient and the healthy control by means of many more peaks in the chromatogram originated from the patient, representing more peptides and proteins. The combined antibacterial activity, i.e. the sum of the inhibition zones from material in all 62 chromatographic fractions is illustrated in Fig. 4E. This shows that the combined antibacterial activity in material originated from patient AE3 and patient AE4 with high SCORAD is decreased compared to the healthy control and the patient with a low SCORAD value. However, after exposure of the MDDCs to *M. sympodialis* the combined activity is enhanced in the high SCORAD patients (AE3 and AE4), while this activity is decreased, when the material is originated from patient AE1 with a lower SCORAD value. The combined activity is hardly affected in material from the healthy control (Fig. 4E). Furthermore, lysozyme was detected in fraction No. 31 by the *Micrococcus* clearance zone assay. By this method a reduction of the clearance zones was detected, which represents a decrease of lysozyme, after exposure of the MDDCs with *M. sympodialis* in all samples derived from AE1, AE3, AE4 and HC2 (not shown).

Discussion

In this study we present novel data, demonstrating that the human antimicrobial peptide LL-37 is secreted from human MDDCs. Interestingly, the secretion of LL-37 is enhanced in *M. sympodialis* exposed MDDCs generated from patients with AE, sensitized to *M. sympodialis*, and with a high SCORAD value compared to healthy controls.

Atopic eczema is a complex multifactorial skin disorder manifested with chronic inflammation. The skin barrier is dysfunctional with cutaneous hyper-reactivity to environmental allergens and susceptibility to infections (22). Today the disease is under extensive revision and new insights are rapidly developing (22, 23) not the least regarding antigen presenting cells (24). Components of innate immunity have entered the spotlight of allergy research and limited stimulation of dendritic cells (DCs) by microbes and parasites, leading to impaired development of T-regulatory cells have been suggested as critical for development of certain immunological disorders such as AE (25). Among the natural microbes, which have been suspected to contribute to the pathogenesis of AE, is the opportunistic yeast *Malassezia* spp. and its involvement has gradually been proven to be one contributing factor (15).

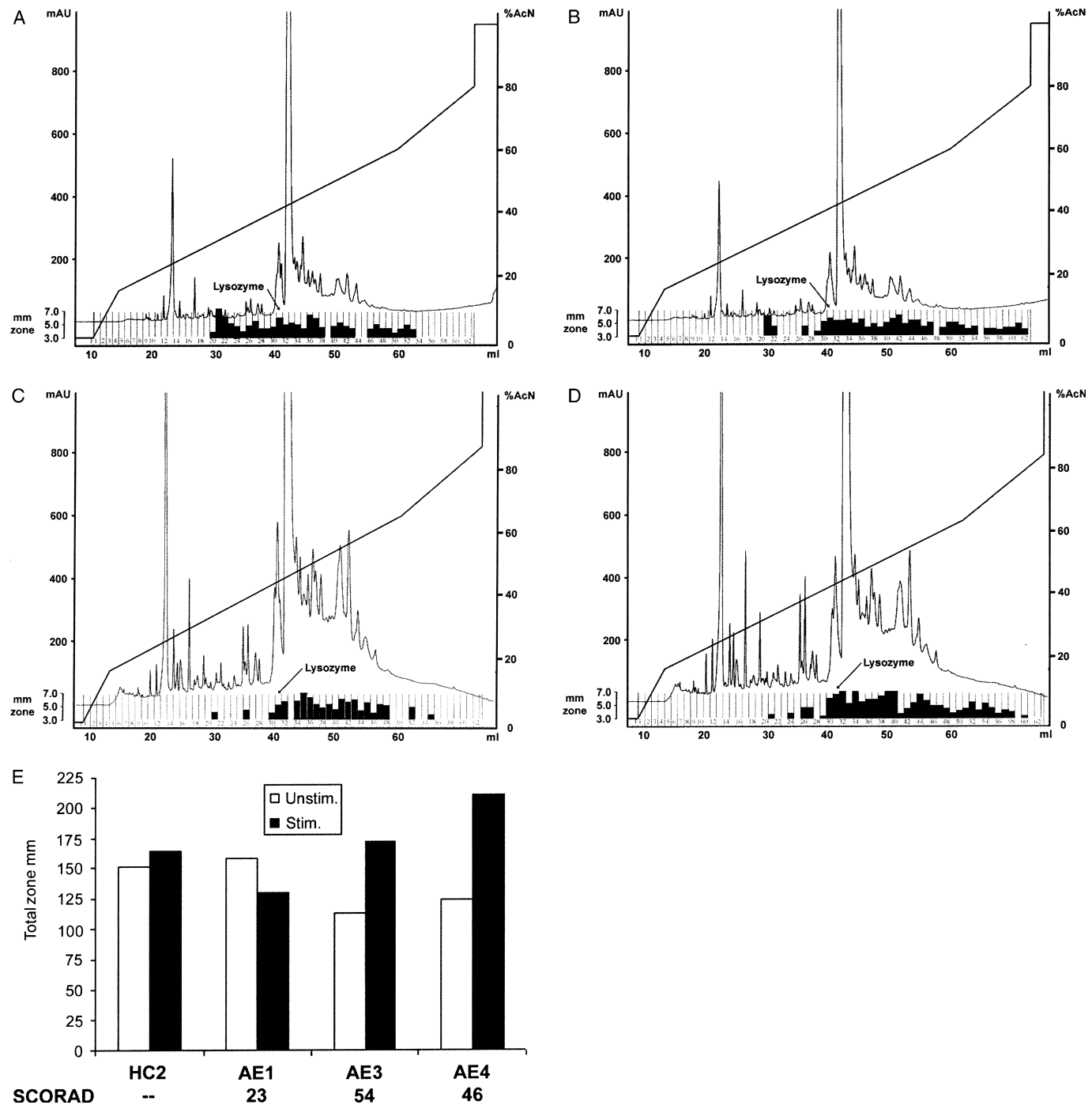


Figure 4. Separation of antimicrobial components. Reverse phase chromatography of lyophilized culture supernatants from MDDCs (1 mg) of HC2 (A and B) and AE3, SCORAD 54 (C and D). The MDDCs were cultured in medium alone (A and C) or co-cultured with *M. sympodialis* for 18 h (B and D). The distribution of the antibacterial activity against *B. megaterium* is depicted in the chromatogram as filled bars, where the amount of activity is given in mm of the diameter in the inhibition zone assay. The fraction containing lysozyme is indicated. Absorbance at 214 nm and the zone diameters are indicated on the left Y-axis and % acetonitrile (AcN) on the right. On the X-axis the fraction (F) numbers and eluting volume (ml) is shown. The combined antimicrobial activity in the 62 fractions from the different HPLC-runs was calculated by adding all recorded diameters in mm (E). These results are depicted for one healthy control (HC2) and three patients (AE1, AE3 and AE4) with the corresponding SCORAD-values indicated.

The antimicrobial peptides LL-37 and HBD-2 have a reduced expression in AE compared to psoriasis, where a prominent induction occurs (9, 10). This diminished induction may explain the frequent infections in lesions of AE. However, not only the barrier dysfunction is critical, also the normal up-regulation of proteins connected to antigen presentation and T-cell recruitment may be impaired. Interestingly, antimicrobial peptides exhibit both chemotactic and immunomodulatory properties (2–4, 8).

Dendritic cells are key players in the innate immune response, capable of inducing early defense mechanisms against intruders (26). Maybe it can be argued that DCs are in balance with the natural flora, while invasive pathogens trigger a response. Notably, LL-37 was shown to be localized in DCs in the skin of neonates (27), suggesting a role of LL-37 in DC function. This background was the motive to investigate antimicrobial peptide expression in MDDCs originated from AE patients and to examine the effects in these cells after exposure to the yeast *M. sympodialis*.

Since the culture supernatants of MDDCs from both healthy individuals and patients with AE contained antibacterial activity, peptides/proteins of the secreted material were separated on HPLC and fractions were screened for antibacterial activity. No dominating antibacterial component was obvious, instead many antibacterial components were present, since we detected activity in several fractions (see Fig. 4). Notably, the sum of the antibacterial activity in the separated fractions was decreased in the patients with a high SCORAD compared to the healthy individual and the patient with a low SCORAD value. This finding is in line with a study by Ong et al., where lack of antimicrobial peptides have been proposed to account for the frequent infections in eczemas of AD patients (10). Interestingly, *M. sympodialis* stimulation induced this total antibacterial activity in patients AE3 and AE4 (see Fig. 4E). This can be interpreted that the levels of antimicrobial components are restored to normal levels, suggesting that *Malassezia* infection would be beneficial for the antimicrobial defense. On the other hand, high levels of LL-37 and other antimicrobial peptides may lead to influx of neutrophils and sustained skin inflammation.

To further investigate effects of *M. sympodialis* exposure on MDDCs connected to antimicrobial peptides, we have analyzed the response with respect to the human cathelicidin LL-37. Interestingly, in the healthy individuals the exposure of their MDDCs to *M. sympodialis* had no or a negative effect on the amount of secreted LL-37. In contrast 18 h exposure to *M. sympodialis* stimulated secretion of LL-37 from MDDCs derived from the AE patients with a high SCORAD value (Fig. 1). Keeping in mind that *Malassezia* spp. is part of the normal skin-flora (15), it might be expected that DCs from healthy individuals would not respond vigorously when encountering the yeast. The strong induction of LL-37 in patients

with a high SCORAD, and the increase in the combined antimicrobial activity in the same patients (Fig. 4E), might reflect a general hyper-responsive state of their MDDCs. The stimulation of antimicrobial activity could also be a way for the yeast to get an advantage over other microbes of the natural flora on the skin.

After detecting the levels of LL-37 in supernatants from 18 h co-cultures we studied the kinetics of the production and secretion of LL-37 to get a more complete understanding of the regulation of LL-37 in MDDCs after *M. sympodialis* stimulation. A kinetic study could also possibly indicate if there had been consumption of the peptide during the 18 h culture period. The LL-37 transcription in MDDCs from healthy individuals was not influenced by the presence of *M. sympodialis*. In patients with AE, however, we observed an initial transcriptional up-regulation followed by down-regulation of LL-37 transcription after *M. sympodialis* exposure. These results showed that also on the transcriptional level cells from healthy individuals did not respond to *M. sympodialis* by LL-37 induction, whereas LL-37 production was initially induced in the patients and with a higher fold induction in the patient with the higher SCORAD, than in the patient with less severe eczema. After the first induction the transcription was suppressed. This suppression can be caused directly by *M. sympodialis* or by the LL-37 peptide that has been secreted after the initial induction. Indeed we have observed that the peptide LL-37 can down-regulate its own transcription (Peter Bergman, unpublished). Down-regulation of LL-37 at transcriptional level has only been observed at invasive barriers, i.e. epithelial cells, in relation to pathogens, such as *Shigella* spp. (28) and *Neisseria gonorrhoeae* (29). If the suppression of LL-37 we have observed here in the patients is an effect of *M. sympodialis*, it supports that a microbe of our normal flora can behave as a pathogen.

The picture of LL-37 expression over time is more complex. A possible scenario could be that the secreted LL-37, when reached a certain concentration, is internalized by the MDDCs and may affect its own transcription and secretion in an autocrine manner. Recently we have obtained results, demonstrating that the peptide LL-37 is taken up by MDDCs, leading to up-regulation of co-stimulatory molecules and HLA-DR (L. Bandholtz, G. Jacobsson Ekman, M. Vilhelmsson, E. Buentke, B. Agerberth, A. Scheynius, G.H. Gudmundsson, unpublished results). An uptake of LL-37 has also been shown in lung epithelial cells (30). In addition, LL-37 is harmful to our own cells since in higher concentration than that of the microbicidal the peptide is cytotoxic (31). Thus, it is critical to regulate the synthesis and secretion of LL-37. To turn off the transcription after a period of induced production as we have observed is one regulatory motive. Furthermore, to affect the secretory machinery of the cell is an additional level of regulation.

We have only observed changes of LL-37 expression and secretion in the more severe state of the AE, referring

to the SCORAD of the patients. It is possible that the MDDCs derived from these patients are in a different activation state compared to MDDCs derived from patients with a low SCORAD value. Indeed, previous studies have indicated that DCs from AE patients are in a more activated stage with up-regulation of molecules involved in co-stimulation, cell-cell adhesion and chemoattraction as compared to healthy individuals (16, 32). To clarify these issues further studies are needed on different subgroups of patients with AE using refined techniques to obtain enough yields of Langerhans cells and inflammatory dendritic epidermal cells (IDEC).

In conclusion, MDDCs secrete various active bactericidal components. To our knowledge, this is the first time that LL-37 has been shown to be produced and secreted by MDDCs. Furthermore, *M. sympodialis* differently affects the amounts of antimicrobial components secreted by MDDCs with a prominent secretion of LL-37 by

MDDCs from patients with severe AE. Molecular details of the alterations may contribute to the understanding of the complex pathogenesis of AE.

Acknowledgments

This study was supported by grants from the Swedish Research Council (project Nos 16x-11217, 74x-7924 and 74EF-15193), the Swedish Foundation for International Cooperation in Research and Higher Education (STINT), Petrus and Augusta Hedlund's Foundation, Magnus Bergvall's Foundation, the Swedish Asthma and Allergy Association's Research Foundation, the Swedish Foundation for Health Care Sciences and Allergy Research, the Queen Silvia Jubilee Foundation, the Hesselman Foundation, the Swedish Society for Medical Research and Karolinska Institutet. We wish to thank Monica Lindh for technical assistance with real-time PCR. E. Buentke and P. Bergman contributed equally to this work.

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