

Intrinsic alterations of pro-inflammatory mediators in unstimulated and TLR-2 stimulated keratinocytes from atopic dermatitis patients

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Abstract: In many patients with atopic dermatitis (AD), the disease is complicated by their enhanced susceptibility to bacterial skin infections, especially with *Staphylococcus aureus* (*S. aureus*). Resistance to bacterial skin infections, e.g. *S. aureus*, is based on the function of intact innate immune mechanisms in the epidermis, mainly provided by keratinocytes. Toll-like receptor (TLR)-2 recognizes components of *S. aureus* and is known to be expressed on keratinocytes. The aim of this study was to investigate intrinsic TLR-2 expression and cytokine secretion upon TLR-2 stimulation with peptidoglycan (PGN), lipoteichoic acid (LTA) and N-palmitoyl-S-[2,3-bis(palmitoyl)-(2RS)-propyl]-(R)cysteiny-alanyl-glycine (Pam3Cys) in keratinocytes from patients with AD compared to healthy controls. Human primary keratinocytes (HPKs) were cultivated from hair follicles of patients with AD and non-atopic healthy controls and stimulated with Pam3Cys, LTA and PGN. TLR-2, TLR-1 and TLR-6 expression

were investigated at the mRNA level. IL-6, IL-8, chemokine C-C motif ligand (CCL)-20 and MMP-9 production were studied at the protein level. TLR-2, TLR-1 and TLR-6 were expressed on both HPKs from patients with AD as well as healthy controls without significant differences between these groups. HPKs from patients with AD had an intrinsically reduced capacity to produce IL-6, IL-8, CCL-20 and MMP-9 and responded less to TLR-2 stimulation compared to HPKs from healthy controls. Our findings show evidence for intrinsic alterations in HPKs from patients with AD compared to healthy controls and diminished responses upon TLR-2 stimulation that might contribute to the enhanced susceptibility to skin infections with *S. aureus*.

Key words: atopic dermatitis – chemokines – cytokines – keratinocytes – TLR-2

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Introduction

Atopic dermatitis (AD) is a chronic relapsing inflammatory skin disease characterized by eczematous skin lesions with 'lichenification', pruritic excoriations and an enhanced susceptibility to bacterial skin infections, particularly with *Staphylococcus aureus* (*S. aureus*). Keratinocytes represent the main cell population in the human epidermis and establish the local cytokine and chemokine milieu which is necessary for attracting other cells participating in an immune response against exogenous pathogens. They play a crucial role in innate immunity by expressing Toll-like receptors and producing antimicrobial peptides in response to invading microbes. Therefore, keratinocytes represent the first line of defence against pathogens in the skin (1–5).

Recognition of pathogens by innate immune cells is mediated by pattern recognition receptors (PRRs) that recognize conserved pathogen-associated molecular patterns. Toll-like receptors act as PRRs comprising a family of (currently) 10 receptors in humans with distinct recognition profiles (6–8). In this context, keratinocytes are known to express TLR-2 that has emerged as a principle receptor in combating Gram-positive bacteria, especially *S. aureus*

(6,8). TLR-2 forms homodimers and heterodimers with TLR-1 and TLR-6 to interact with a rather broad spectrum of ligands. Studies using knockout mice identified TLR-1 as the coreceptor required for the recognition of triacylated lipoproteins and lipopeptides such as N-palmitoyl-S-[2,3-bis(palmitoyl)-(2RS)-propyl]-(R)cysteiny-alanyl-glycine (Pam3Cys) (9). Diacylated components such as lipoteichoic acid (LTA), which is a component of the cell wall of *S. aureus*, interact with TLR-2/TLR-6 heterodimers (9,10). Peptidoglycan (PGN) is a major constituent of the cell wall of Gram-positive bacteria which induces signal transduction via TLR-2, NOD1 (Card4) and NOD2 (Card15) receptors, respectively. NOD molecules including NOD1 and NOD2 are a family of intracellular pattern recognition proteins involved in bacterial detection (11,12).

The secretion of cytokines and chemokines in keratinocytes is of great importance for the adequate immune response to different pathogens. We recently showed an enhanced secretion of chemokine C-C motif ligand (CCL)-2, CCL-20 and MMP-9 upon stimulation with the TLR-2/TLR-1 agonist Pam3Cys in human keratinocytes (13). We also confirmed the upregulation of IL-8 secretion upon Pam3Cys stimulation previously published by Köllisch et al. (7). However, the knowledge of cytokine and chemokine secretion in response to microbial stimuli such as TLR-2 ligands in keratinocytes from patients with AD compared to healthy controls still remains fragmented.

In this study, we have shown intrinsic alterations in keratinocytes from patients with AD compared to healthy controls, namely a reduced capacity to produce IL-6, IL-8, CCL-20 and MMP-9

Abbreviations: AD, atopic dermatitis; CCL, chemokine C-C motif ligand; IL, interleukin; LTA, lipoteichoic acid; MIP-3 α , macrophage inflammatory protein 3 α ; MMP, matrix metalloproteinase; Pam3Cys, N-palmitoyl-S-[2,3-bis(palmitoyl)-(2RS)-propyl]-(R)cysteiny-alanyl-glycine; PAMP, pathogen-associated molecular pattern; PGN, peptidoglycan; PRR, pattern recognition receptor; *S. aureus*, *Staphylococcus aureus*; TLR, Toll-like receptor.

and a diminished response upon TLR-2 stimulation. These alterations may contribute to the enhanced susceptibility to skin infections with *S. aureus*.

Materials and methods

Human primary keratinocyte (HPK) isolation from human hair follicles

Approximately five anagen hairs were plucked from the scalp of informed consenting healthy donors or patients with AD as previously described (14). AD was determined by the diagnostic criteria of Hanifin & Rajka. All patients had an exacerbated AD with a mean age $53 \text{ years} \pm 19$ and a mean total IgE $10620 \text{ kIU/l} \pm 6989 \text{ kIU/l}$ (15). The plucked hairs were cut down to the follicle, and the hair follicles were incubated for 2–24 h in Hanks' balanced salt solution buffered with 20 mM HEPES (Sigma-Aldrich, Deisenhofen, Germany) and supplemented with $50 \mu\text{g/ml}$ of gentamicin and $2.5 \mu\text{g/ml}$ of amphotericin B (PromoCell, Heidelberg, Germany). The hairs were then placed in a 6-well plate that had been covered with a feeder layer of 3T3 fibroblasts (catalogue no. ACC 173; DSMZ, Braunschweig, Germany) that had been treated with mitomycin C (Medac, Hamburg, Germany). Keratinocytes grew out within 1–2 weeks (mainly from the bulge region). The medium [high-glucose Dulbecco's modified Eagle's medium with Ham's F-12 supplemented with 10% fetal calf serum, $1.8 \times 10^{-4} \text{ M}$ adenine (Sigma-Aldrich), 10^{-10} M cholera toxin (Sigma-Aldrich), $2 \times 10^{-9} \text{ M}$ triiodothyronine (T3), 10 ng/ml epidermal growth factor, 4 $\mu\text{g/ml}$ of hydrocortisone, 5 $\mu\text{g/ml}$ of insulin, 5 $\mu\text{g/ml}$ of transferrin, 50 $\mu\text{g/ml}$ of gentamicin and 2.5 $\mu\text{g/ml}$ of amphotericin B (all components except adenine and cholera toxin were from PromoCell)] was changed every 2–3 days. When sufficient numbers of keratinocytes were outgrown, cells were detached using 0.05% trypsin/0.02% EDTA (PAN Biotech, Aidenbach, Germany). Trypsin activity was stopped by the addition of an equal volume of trypsin inhibitor (PAN Biotech). Keratinocytes were then cultured in normal medium (Keratinocyte Growth Medium 2 kit; PromoCell), which was changed every other day. When cells reached 70–80% confluence, they were used for further experiments or were passaged, and used between passage 3 and passage 7. When possible, some cells were cryopreserved at passage 3 and thawed at a later time (not exceeding a total of seven passages).

Before stimulation, hydrocortisone and epidermal growth factor were omitted from the medium. The purity of HPKs was verified by the expression of the epithelial marker cytokeratin (anti-human cytokeratin antibody, clone: MNF-116; DakoCytomation, Hamburg, Germany). All cells were found to be uniformly positive for cytokeratin.

Stimulation of HPKs

Cells were either unstimulated or stimulated for various periods of time with PGN from *S. aureus* (10 $\mu\text{g/ml}$; Invivogen, Toulouse, France) which acts via TLR-2 and NOD. Cells were stimulated with LTA from *S. aureus*, isolated as previously described (16) (10 $\mu\text{g/ml}$; University of Konstanz, Germany) which acts via TLR-2/TLR-6 and Pam3Cys (10 $\mu\text{g/ml}$; EMC microcollections, Tübingen, Germany) which acts via TLR-2/TLR-1. For TLR-2-independent stimulation, lipopolysaccharide from *E. coli* (LPS, 50 ng/ml; Sigma-Aldrich) and phorbol 12-myristate 13-acetate (PMA, 10 ng/ml; Sigma-Aldrich) were used.

Cytokine assessment

Human primary keratinocytes were grown in a 24-well plate (Nunc Inc., Wiesbaden, Germany) to a 60–90% cell density and were stimulated as indicated. Cell-free supernatants were harvested and analysed for IL-6, IL-8, CCL-20 and MMP-9 (Duo Set; R&D-Systems, Minneapolis, MN, USA) using a commercially available enzyme-linked immunosorbent assay (ELISA) system following the manufacturer's instructions.

mRNA isolation, RT and Light Cycler PCR

mRNA was isolated from HPKs by using the Mini RNA Isolation II kit (HiSS Diagnostics GmbH, Freiburg, Germany) according to the supplier's instructions.

The cDNA was synthesized in the samples by using the Revert Aid First Strand cDNA Synthesis kit (Fermentas, St. Leon-Rot, Germany). The quantitative real-time RT-PCR (qRT-PCR) was performed on a LightCycler PCR (Roche, Molecular Biochemicals, Mannheim, Germany) by using the Quantitect SYBR Green PCR kit (Qiagen, Hilden, Germany) with Quantitect Primer assays (Qiagen) for TLR-1, TLR-2, TLR-6 and GAPDH (housekeeping gene) according to the manufacturer's instructions.

Specific targets were amplified using the following program: (PCR initial activation step) 15 min, 95°C and ramp 20°C per min; (denaturation) 15 s, 94°C, ramp 2°C per min; (annealing) 20 s, 55°C, ramp 2°C per min; (extension) 20 s, 72°C, ramp 2°C per min. Melting curve analysis was performed from 60 to 90°C with ramp 20°Cs^{-1} . The amount of TLR-1, TLR-2 and TLR-6 relative to the reference GAPDH was quantified using the Relative Quantification software (Roche, Molecular Biochemicals).

Statistical analysis

Statistical analyses were performed using the Student's *t* test. The software used to perform the statistical analysis was Sigma stat for Windows. Data are shown as the mean + SEM (standard error of the mean). In the figures, **P*-value < 0.05, ***P*-value < 0.01, ****P*-value < 0.001.

Results

Expression of TLR-1, TLR-2 and TLR-6 in HPKs from patients with AD and healthy controls

Human primary keratinocytes expressed constitutively TLR-1, TLR-2 and TLR-6 at the mRNA level. We found no significant differences in TLR-1, TLR-2 or TLR-6 expression between HPKs from patients with AD and healthy controls (Fig. 1).

Cytokine and chemokine (IL-6, IL-8 and CCL-20) as well as MMP-9 production is intrinsically altered in HPKs from patients with AD compared to healthy controls and diminished upon TLR-2 stimulation

To study intrinsic as well as TLR-2-mediated differences in keratinocytes from patients with AD and healthy controls, HPKs were either left unstimulated or were stimulated with Pam3Cys, LTA and PGN. This was carried out to elucidate whether signalling of ligands requiring the TLR-2/TLR-1 heterodimer (Pam3Cys), the TLR-2/TLR-6 heterodimer (LTA) and the TLR-2/NOD pathway (PGN) is affected. In a previous study, we could show induction of IL-8, CCL-20 and MMP-9 in foreskin-derived HPKs upon Pam3Cys stimulation. Therefore, Pam3Cys served as a positive control (13). Cell culture supernatants were analysed for IL-6, IL-8, CCL-20 and MMP-9 secretion by ELISA.

Human primary keratinocytes from patients with AD secreted significantly less IL-6 compared to HPKs from healthy controls

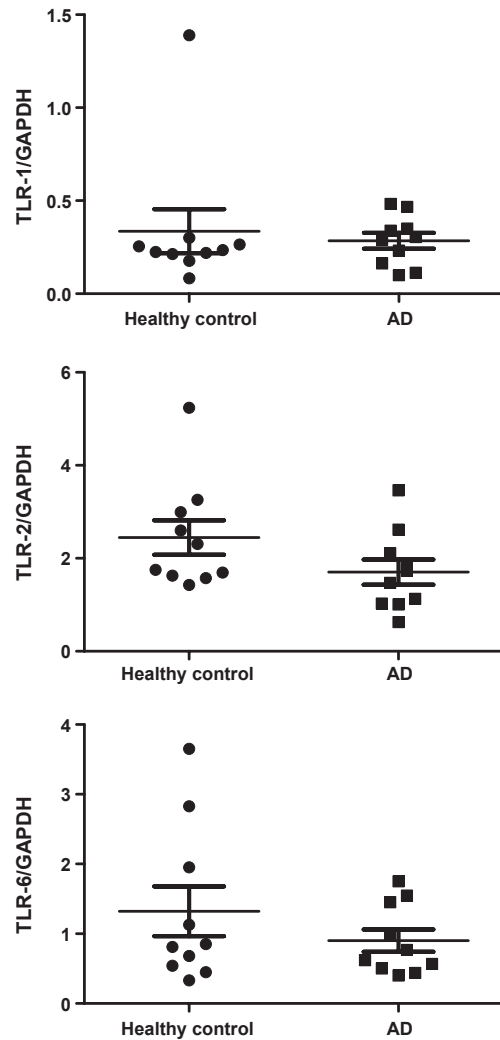


Figure 1. Expression of TLR-1, TLR-2 and TLR-6 in human primary keratinocytes from patients with atopic dermatitis and healthy controls. qRT-PCR was employed to determine the TLR-1 or TLR-2 or TLR-6 expression at the mRNA level. Data are shown as individual values as well as mean TLR-1 or TLR-2 or TLR-6/GAPDH ratio + SEM of $n = 10$ independent experiments per group.

after 24- and 48-h incubation in cell culture medium or stimulation with Pam3Cys, LTA or PGN. TLR-2 stimulation with Pam3Cys yielded a significantly enhanced IL-6 secretion in HPKs from healthy controls and patients with AD. Stimulation with LTA or PGN did not enhance IL-6 in HPKs from patients with AD or healthy controls. TLR-2-independent stimulation with PMA or LPS, respectively, induced a significant upregulation of IL-6 in HPKs from healthy controls compared to patients with AD (Fig. 2a).

Human primary keratinocytes from patients with AD secreted significantly less IL-8 compared to HPKs from healthy controls after 48-h incubation in cell culture medium or stimulation with LTA or PGN. Twenty-four-hour stimulation with Pam3Cys as well as LTA led to a significant induction of IL-8 both in HPKs from healthy controls and patients with AD. Forty-eight-hour stimulation with Pam3Cys, LTA or PGN significantly induced IL-8 in HPKs from healthy controls, whereas solely Pam3Cys enhanced

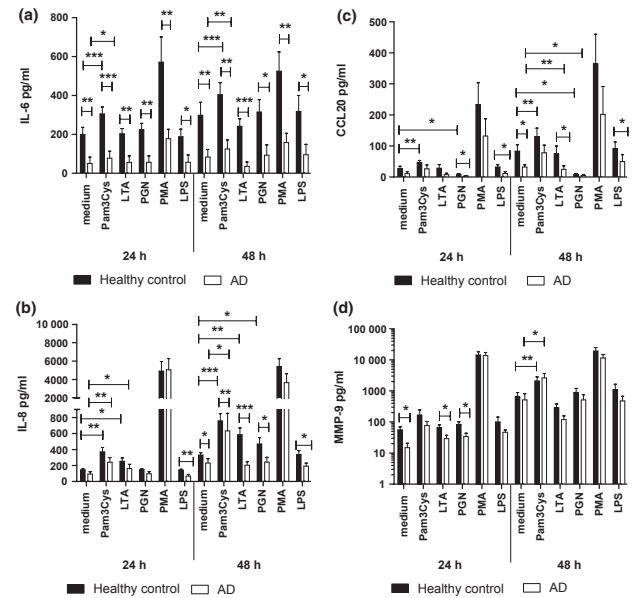


Figure 2. Cytokine and chemokine [IL-6, IL-8, chemokine C-C motif ligand (CCL)-20] and MMP-9 production in human primary keratinocytes from patients with atopic dermatitis and healthy controls upon TLR-2 stimulation. Cells were either not stimulated (medium) or stimulated with N-palmitoyl-S-[2,3-bis(palmitoyl)-(2RS)-propyl]-L-cysteiny-alanyl-glycine ($10 \mu\text{g/ml}$), lipoteichoic acid ($10 \mu\text{g/ml}$), peptidoglycan ($10 \mu\text{g/ml}$), PMA (10 ng/ml) or LPS (50 ng/ml), respectively, for 24 or 48 h. Cell culture supernatants were analysed for concentrations of IL-6 (a), IL-8 (b), CCL-20 (c) and MMP-9 (d) by ELISA. Data are mean values + SEM of $n = 9-10$ independent experiments per group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ as indicated with brackets. Figure 'a-c' linear scale, 'd' logarithmic scale.

IL-8 secretion in HPKs from patients with AD. TLR-2-independent intrinsic differences in HPKs from patients with AD compared to healthy controls were reflected upon TLR-2-independent stimulation with LPS as well (Fig. 2b).

Human primary keratinocytes from patients with AD secreted significantly less CCL-20 compared to HPKs from healthy controls after 48-h incubation in cell culture medium or stimulation with PGN for 24 h and LTA for 48 h, respectively. This observation was observed upon TLR-2-independent stimulation with PMA or LPS as well. Stimulation with Pam3Cys led to a significant upregulation of CCL-20 in HPKs from healthy controls and patients with AD. HPKs from patients with AD as well as healthy controls secreted significantly lower amounts of CCL-20 upon stimulation with PGN (Fig. 2c).

MMP-9 secretion was significantly impaired in HPKs from patients with AD compared to HPKs from healthy controls after 24-h incubation in cell culture medium or stimulation with PGN and LTA, respectively. Stimulation with Pam3Cys led to an upregulation of MMP-9 in HPKs from healthy controls and patients with AD (Fig. 2d).

Discussion

Resistance to skin infections is based on the function of intact innate immune mechanisms in the epidermis. In this context, TLR-2 has emerged as a principle receptor in combating Gram-positive bacteria, especially *S. aureus* (8). Skin colonization as well as skin infection with this germ plays a pivotal role as complicating and exacerbating factors in AD and is therefore of extraordinary clinical interest (4). Keratinocytes represent the major cell

fraction in the epidermis and are thus the first line of defence against pathogens in the skin (1). However, the knowledge of cytokine and chemokine secretion in response to microbial stimuli such as TLR-2 ligands in keratinocytes from patients with AD compared to healthy controls still remains fragmented. Because there is no selective TLR-2 ligand known so far, we stimulated HPKs from patients with AD and healthy controls with two natural staphylococcal components, namely the TLR-2/TLR-6 ligand LTA and the TLR-2/NOD ligand PGN in parallel with the synthetic TLR-2/TLR-1 ligand Pam3Cys. This was carried out to elucidate whether signalling of ligands requiring the TLR-2/TLR-1 heterodimer (Pam3Cys), the TLR-2/TLR-6 heterodimer (LTA) and the TLR-2/NOD pathway (PGN) is affected. TLR-1, TLR-2 and TLR-6 expression were investigated at the mRNA level in parallel. Recently, we have shown an enhanced secretion of CCL-2, CCL-20 and MMP-9 upon stimulation with the TLR-2/TLR-1 agonist Pam3Cys in human foreskin-derived keratinocytes (13). We also confirmed the upregulation of IL-8 secretion upon Pam3Cys stimulation previously published by Köllisch et al. (7). Therefore, Pam3Cys served as a positive control in this study and upregulated IL-6, IL-8, CCL-20 as well as MMP-9 both in HPKs from patients with AD and healthy controls. Of note, our preparations of LTA as well as PGN had been shown to be biologically active on human monocytes and macrophages (17–19).

We have confirmed that keratinocytes both from patients with AD as well as healthy controls expressed TLR-1, TLR-2 and TLR-6 constitutively and have shown no significant intrinsic differences between HPKs from patients with AD and healthy controls. By trend, HPKs from patients with AD expressed less TLR-2 compared to healthy controls ($P = 0.1$), whereas this trend was not observed for TLR-1 ($P = 0.7$) and TLR-6 ($P = 0.3$) expression. In a different study, we have shown an impaired TLR-2 expression in keratinocytes from inflamed skin of patients with AD compared to healthy controls using immunohistochemistry (20). Therefore, the lack of significant differences in TLR-1, TLR-2 and TLR-6 expression in hair-derived HPKs from patients with AD compared to healthy controls may solely reflect intrinsic conditions and not the situation in inflamed skin. Moreover, differences in TLR-2 regulation in keratinocytes from patients with AD compared to healthy controls upon stimulation with well-recognized TLR-2 upregulating stimuli such as 1,25 vitamin D3 (21) should be addressed in further studies.

An interesting observation of this study was that HPKs from patients with AD produced reduced levels of IL-6, IL-8, CCL-20 and MMP-9 when compared to keratinocytes from healthy controls. These results may indicate the existence of genetically determined intrinsic defects in the constitutive cytokine and chemokine production by keratinocytes from patients with AD compared to healthy controls. Stimulation with TLR-2 ligands led to an upregulation of these cytokines both in keratinocytes from patients with AD as well as healthy controls. However, keratinocytes from patients with AD showed a diminished TLR-2 response compared to keratinocytes from healthy controls that reflected these intrinsic differences.

Keratinocytes can participate in skin inflammation through the regulated expression of chemokines that are divided into two sub-families, namely CCL and CXCL chemokines. Several studies have suggested a crucial involvement of CCL chemokines in allergic inflammation because they potently attract eosinophils, basophils,

monocytes and Th2 cells. CXCL chemokines are known to attract primarily neutrophils and Th1 lymphocytes (22,23).

Keratinocytes produce CCL-20 (MIP-3 α) which activates Langerhans cells and memory T cells via its receptor CCR-6 and has been shown to be enhanced in AD (24). A recent study by Nograles et al. (25) showed no intrinsic differences in CXCL-8 (IL-8) and CCL-20 mRNA expression in keratinocytes from patients with AD and healthy controls. Both chemokines were likewise strongly induced in both AD and healthy control keratinocytes upon IL-17 stimulation. Giustuzieri et al. investigated IL-8 secretion in supernatants from keratinocytes of patients with AD and psoriasis as well as healthy controls by ELISA and stained lesional skin sections for IL-8 by immunohistochemistry. They found no intrinsic differences in IL-8 production between patients with AD and healthy controls by neither ELISA nor immunohistochemistry which is in contrast to our findings but might be explained by the fact that keratinocytes from inflamed skin were used (26). The discrepancies between Nograles et al. (25) and our study could be explained by the different methods used: Nograles et al. investigated gene expression at the mRNA level, whereas we studied cytokine secretion at the protein level. Moreover, Nograles et al. investigated keratinocytes from inflamed AD skin, whereas we generated keratinocytes from hair follicles. A striking finding in lesional skin from patients with AD is the absence of neutrophil granulocytes polymorphonuclear neutrophils (PMNs), even in the setting of intense scratching or *S. aureus* colonization and/or infection. A number of studies pointed to a chemotactic defect in AD PMNs, e.g. with reduced production of the chemoattractants CXCL-8 (IL-8) or LL-37 (2,27). As PMNs are critical cells in the initial response to all pathogens, it is not surprising that a defect in PMN recruitment to the skin, e.g. by an impaired IL-8 secretion from keratinocytes in our study, would make patients with AD susceptible to a wide range of cutaneous microbes.

Here, we also investigated IL-6 that has previously been shown to be upregulated in human monocytes upon stimulation with PGN and LTA (28) and has been shown to play an important role as a pro-inflammatory cytokine in AD. IL-6 is upregulated in the skin of humans and in animal models of AD (29). We revealed an intrinsically impaired IL-6 secretion in keratinocytes from patients with AD compared to healthy controls. Keratinocytes from patients with AD produced lower amounts of IL-6 upon TLR-2 stimulation compared to keratinocytes from healthy controls. An impaired IL-6 secretion in keratinocytes from patients with AD upon stimulation with TLR-2 ligands in our study might point to an impaired *S. aureus* processing and consecutively explain the enhanced susceptibility of bacterial skin infections with this germ. This should be studied further in inflamed skin from patients with AD compared to healthy controls.

MMP-9 can cleave gelatins, type IV collagen, fibronectin, laminin and elastin (30). As most of these substances are found predominantly at the basement membrane, MMP-9 is of particular interest in inflammatory skin diseases, where migration of cells across the basal layer to enter and leave the site of inflammation is required (31). For various cell types relevant in inflammatory skin diseases, the ability to produce MMP-9 has been shown: epidermal keratinocytes (32), Langerhans cells (33), mast cells (34), T cells (35) and eosinophils (36). Here, we show that keratinocytes from patients with AD produce less MMP-9 compared to

keratinocytes from healthy controls. This might lead to reduced migration of inflammatory cells in AD after skin contact to *S. aureus* and contribute to the impaired immune response against this germ.

In this study, we could show intrinsic alterations in keratinocytes from patients with AD compared to healthy controls, namely a reduced capacity to produce IL-6, IL-8, CCL-20 and MMP-9 and consecutively less response to TLR-2 stimulation that might contribute to the enhanced susceptibility to skin infections with *S. aureus*.

Whether our findings can be translated to the situation in inflamed AD skin should be addressed in further studies, e.g. in

keratinocytes from lesional skin of patients with AD with *S. aureus* infection in comparison with keratinocytes from lesional AD skin without *S. aureus* infection.

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Conflict of interest

The authors declare no conflict of interest.

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