

Differential suppression of epidermal antimicrobial protein expression in atopic dermatitis and in EFAD mice by pimecrolimus compared to corticosteroids

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Abstract: It has been suggested that the increased rate of bacterial infection in atopic dermatitis (AD) may be caused by reduced antimicrobial protein (AMP) expression. We were interested whether common treatments in AD affect antimicrobial defense. We investigated the effects of topically applied corticosteroids betamethasone valerate (BV) and triamcinolone acetonide (TA) and those of the calcineurin inhibitor pimecrolimus for 3 weeks on AMP expression in AD. BV and TA treatment in AD led to a significant reduction in AMP expression; protein expression of human beta-defensins (hBD)-2 and hBD-3, psoriasin, RNase 7 and cathelicidin LL-37 was below the level in skin of healthy controls. After pimecrolimus treatment, AMP expression was also reduced but less compared to BV and TA; the expression levels of hBD-2, psoriasin and RNase 7 still remained above the control levels. In essential fatty acid-deficient (EFAD) mice, a model of

chronic skin barrier disease with inflammation, expression of the mouse beta-defensins mBD-1, mBD-3 and mBD-14 (orthologues for hBD-1, hBD-2 and hBD-3, respectively), was reduced by both treatments, again more pronounced by BV compared to pimecrolimus. In summary, we found that treatment for AD with corticosteroids in human skin and EFAD mice caused a strong reduction in AMPs; reduction was less with pimecrolimus. This result may explain the clinical observation that prolonged treatment with topical corticosteroids sometimes leads to bacterial infection.

Key words: antimicrobial proteins – atopic dermatitis – corticosteroid – defensins – pimecrolimus

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Introduction

The epidermis is constantly exposed to a variety of microbial challenges. Antimicrobial defense is provided by the physical barrier of the stratum corneum, the physiological microflora, antimicrobial lipids and antimicrobial proteins (AMPs) (1–4). Several types of AMP including the human β -defensins (hBD)-1–3 (5–7), the cathelicidin LL-37 (8–10), sweat gland-derived dermcidin (11), RNase 7 (12) and psoriasin (13) have been identified in human skin. Some of the AMP (RNase 7, psoriasin) are constitutively expressed by keratinocytes, while others (hBD-2, -3, LL-37) are induced after bacterial contact, during epidermal differentiation, in wound healing or by 1,25(OH)₂ vitamin D3 (14–17).

In atopic dermatitis (AD) and – less well known – in psoriasis, diseases with a disrupted skin barrier, the epidermis is colonized at a high rate with potentially pathogenic microorganisms. The unexpectedly low incidence of infections in psoriatic skin (18) has been related to the abundant expression of keratinocyte-derived antimicrobial peptides (reviewed in 2). On the other hand, patients with AD often suffer from skin infections caused by bacteria and viruses (19). Among bacterial superinfections, *Staphylococcus aureus* is the predominant pathogen. It has been suggested that an impaired expression of AMPs may contribute to the increased susceptibility of AD skin to *S. aureus*. It has been demonstrated that hBD-2 and LL-37 are expressed to a lesser extent in the epidermis of patients with AD compared with the psoriatic

epidermis. Another study reported a disturbed upregulation of hBD-3 in AD skin (20). This may be of particular importance because hBD-3 has been shown to be an important AMP released by keratinocytes to control the growth of *S. aureus*. Recently, we found that the expression of psoriasin, which exhibits potent *Escherichia coli*-cidal activity, is increased in patients with AD (16). This unexpected finding may explain why patients with AD do not suffer from skin infections caused by the most common gut bacterium *E. coli*. Also, very recently, a number of authors described increased expression of several AMP in AD when compared to normal controls (21–24).

Overall, there is increasing evidence that the expression of several AMPs is upregulated in AD which may have a role in controlling bacterial colonization and disease manifestation associated with a disturbed skin barrier. However, the level of upregulation of AMPs in AD may not be sufficient to prevent bacterial colonization as compared to psoriasis.

We were interested to examine how different treatment regimens in AD influence AMP expression. Here, we systematically evaluated the influence of common topical treatments, cream preparations of the corticosteroids betamethasone valerate (BV) and triamcinolone acetonide (TA) and those of the calcineurin inhibitor pimecrolimus on AMP expression in AD. Pimecrolimus cream is widely used for the treatment for AD to avoid corticosteroid side effects, particularly skin atrophy. The effect of pimecrolimus or corticosteroids on AMP

expression in AD *in vivo* has not yet been examined. In each of two randomized, double-blinded left–right comparison studies, 15 patients with AD were treated on one upper limb with pimecrolimus cream and on the other with BV or TA twice daily for 3 weeks. Expression of AMP was analysed by immunohistochemistry and DNA microarray. Clinical scoring, biophysical measurements, and analyses of epidermal proliferation, differentiation and skin barrier ultra structure after treatment with pimecrolimus and BV have already been reported (25).

The essential fatty acid deficient mouse (EFAD) is a model demonstrating chronic disturbed skin barrier function and skin inflammation. EFAD mice are characterized by a red and scaly skin with a disturbed skin permeability barrier, disturbed epidermal lipid composition as well epidermal hyperproliferation and inflammation similar as seen in AD (26,27). EFAD skin partly resembles AD, and the EFAD mouse model has been used to determine the influence of drugs on the skin (28,29). The mouse model was chosen for the present study to analyse the changes of AMP expression resulting from a disturbed skin barrier.

The present publication specifically examines AMP expression after treatment with BV or TA vs. pimecrolimus in skin of patients with AD compared to healthy volunteers and BV vs. pimecrolimus in the EFAD mouse model.

Material and methods

Patients and treatment regimens

In two separate studies, fifteen patients with symmetric AD lesions affecting the arms were treated twice daily for 3 weeks on one arm with 1% pimecrolimus cream and on the other with 0.1% BV or TA cream in a double-blinded study (clinical, biophysical and some analytical results from one of the two studies are already published in 25). Skin biopsies were taken on days 1 (before starting the topical treatment) and 22 (21 days after topical treatment) for immunohistological examination of AMPs. The study was approved by the local ethics commission and the German Federal Institute for Pharmaceuticals and Medical Products Bundesinstitut für Arzneimittel und Medizinprodukte and was conducted as an amendment to a previous study (EudraCT-No. 2004-004824-11), and all patients gave written informed consent.

Mice

Six- to eight-week-old male hairless mice [Crl:(hr/hr) BR; SKH1] were supplied by Charles River (Sulzfeld, Germany). EFAD mice were obtained by feeding 3-week-old hairless mice with an EFAD diet (30), modified by adding $\text{MgSO}_4 \times 7\text{H}_2\text{O}$. Mice were individually and conventionally maintained in plastic cages under standardized conditions (RT 25°C, relative humidity 45–55%, circadian rhythm 12 h, food (standard laboratory animal chow, V1534; SSNIFF Spezialdiäten GmbH, Soest, Germany) and water supplied *ad libitum*. SKH1 und EFAD-SKH1 mice were treated topically on one flank twice daily for 8 days with 30 μl 1% pimecrolimus or 0.1% betamethasone in solution or the vehicle solution (ethanol/propylene glycol 3/7), and skin samples were taken 16 h after the last application. The University of Kiel Committee for Animal Care approved the study (No. V 312-72241.121-5).

Immunohistochemistry

Skin samples of the skin of healthy volunteers and of patients with AD before and after treatment were fixed in formalin and embedded in paraffin. After deparaffinization and rehydration, 5- μm -thick sections were stained using Vectastain ABC System (Biologo,

Kronshagen, Germany) using specific antibodies: commercially available antibodies against hBD-2 and hBD-3 (Pepro Tech Cell Concepts GmbH, Umkirch, Germany), cathelicidin LL-37 (Phoenix Pharmaceuticals Inc., Burlingame, CA, USA) and self-generated antibodies against RNase 7 (31), and psoriasin (13) were used. The antibodies had their proof of value for clinical studies by Dressel et al. (17). Examination of immunochemical slides was blinded and independently performed by two trained researchers in seven microscopic fields/sections each and graded for the intensity of the staining: 0 = none, 1 = slight, 2 = moderate, 3 = strong and 4 = very strong.

Paraffin-embedded sections of untreated (normal) and EFAD mouse skins were prepared for mBD-1, mBD-3 and mBD-14 immunohistology. Paraffin sections were boiled in citrate acid buffer (10 mM, pH 6.0) at 80°C for 30 min. After cooling, sections were incubated with 3% aqueous H_2O_2 for 5 min at room temperature to block endogenous peroxidase activity, then rinsed with distilled water and washed three times with Tris-buffered saline buffer (TBS: 0.15 M NaCl, 0.05 M Tris, pH 7.6). After blocking non-specific antibody binding by incubation with normal goat serum (1:5 in TBS; DAKO Diagnostics, Hamburg, Germany), sections were incubated with antibodies for mBD-1, mBD-3 (both from Santa Cruz Technology, Santa Cruz, CA, USA) and mBD-14 (self-generated antibody) diluted in TBS over night at 4°C. After several washes in TBS, they were incubated for 30 min with a secondary biotinylated antibody followed by incubation for 30 min with StreptABComplex/HRP (Dako, Hamburg, Germany). Finally, sections were incubated with Liquid DAB (Biogenex, San Ramon, CA, USA), counterstained with Mayer's hemalaun (Merck, Darmstadt, Germany) and mounted in Glycergel® Mounting Medium (Dako).

mRNA isolation, reverse transcription and real-time PCR

Normal and EFAD mouse skin was harvested to investigate the mRNA expression of mBD-1, mBD-3 and mBD-14. Skin samples were minced in liquid nitrogen, and RNA was isolated using the TRIzol reagent (Invitrogen, Karlsruhe, Germany) according to the standard protocol. Reverse transcription reagents were obtained from Applied Biosystems (HighCap cDNA RT kit; Darmstadt, Germany) and reverse transcription followed a standard protocol. cDNA was analysed by real-time PCR using a fluorescence temperature cycler (LightCycler 2.0; Roche Diagnostics GmbH, Mannheim, Germany) and the SYBR Advantage qPCR Premix (TaKaRa Bio Europe, Saint-Germain-en-Laye, France) according to the manufacturer's instructions. For amplification, cDNA corresponding to 10 ng RNA was used as a template. The samples were incubated for an initial denaturation at 95°C for 10 min followed by 45 cycles, each cycle consisting of 95°C for 10 s, 60°C (touchdown of $-1^\circ\text{C}/\text{cycle}$ from 66 to 60°C) for 5 s and 72°C for 15 s. To confirm the amplification of specific transcripts, melting curve profiles were produced. The following primers were used: mBD-1, 5'-GGT GTT GGC ATT CTC ACA AG-3' (forward primer) and 5'-CCA TCG CTC GTC CTT TAT GT-3' (reverse primer); mBD-3, 5'-GTC AGA TTG GCA GTT GTG GA-3' (forward primer) and 5'-GCT AGG GAG CAC TTG TTT GC-3' (reverse primer); mBD-14, 5'-TCT TGT TCT TGG TGC CTG CT-3' (forward primer) and 5'-CGA CCG CTA TTA GAA CAT CGA C-3' (reverse primer); GAPDH, 5'-TGT TCC TAC CCC CAA TGT GT-3' (forward primer) and 5'-TGT GAG GGA GAT GCT CAG TG-3' (reverse

primer). Primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany). All quantifications were normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Relative expression is given as the ratio between target gene expression and GAPDH expression.

Gene chip experiments

RNA was amplified according to the manufacturer's instructions (Affymetrix, Palo Alto, CA, USA). Human HG-U133-plus 2 expression probe arrays [Affymetrix] were used, comprising more than 54 000 probe sets, analysing over 30 000 transcripts and variants from over 28 000 human genes. One GeneChip per sample per patient was used. Image files (dat files) were processed using the Microarray Analysis Suite 5 (MAS5) software [Affymetrix]. Tab-delimited files containing data regarding signal intensity (Signal) and categorical expression level measurement (Absolute Call) were obtained. Raw data were converted to expression levels using a 'target intensity' of 150. All DNA microarray experiments were conducted in the Genomics Factory EU, Basel, Switzerland, following the instructions of the manufacturer of the GeneChip system [Affymetrix, Inc., San Diego, CA, USA] and as previously described (32).

Data quality assessment and analysis were performed using Sili-con Genetics software package GeneSpring version 7.3.1 (Agilent Inc., Santa Clara, CA, USA), Partek Pro 6.4 (Partek Inc., St. Louis, MO, USA) and SIMCA-P+ (version 10.5) by Umetrics AB, Umeå, Sweden. ANCOVA (Analysis of covariance) was performed on floored data (minimum signal intensity set to a value of 25) for all genes. Each array was normalized by dividing the signal intensities by the 50th percentile of that array. The expression values for each gene were normalized by dividing them by the expression value for that gene in the individual baseline sample (i.e. Day 1 biopsy). As an additional filter, only probe sets with present or marginal calls in at least 70% of samples per analysis group were considered. Regression analysis was performed to explore genes whose expression changes correlate with degree of pEASI score for each sample. The analyses were performed for pimecrolimus and for betamethasone (both versus baseline) separately. Only probe sets with present or marginal calls in at least 70% of samples per analysis group were considered (about 18 000 probe sets for each pair of groups).

ANCOVA was performed on floored data (see above) for all genes. Change in pEASI score from day 0 to day 22 was used as covariate. This allowed the generation of mean expression values for each group and probe set and the calculation of fold changes. It is important to note that mean ratios do not reflect the high heterogeneity of the response of the patients to the treatments in this study, and the coefficient of variance is usually quite high. Therefore, the mean ratios as they are depicted in the graphs should be considered as indicators of trends of expression changes. The importance of a probe set in the context of this study is assessed by the regression analysis.

Statistical analysis

All calculations were performed using the unpaired Student's *t*-test.

Results

AMP expression in AD after treatment with pimecrolimus cream and corticosteroids

AMP protein expression was evaluated by immunostaining with specific antibodies, and mRNA expression was evaluated by gene

chip analysis. AMP protein expression in AD was increased compared to healthy skin confirming previous studies (16,21–24,33) and reduced by treatments containing pimecrolimus and corticosteroids. Representative histological slides are shown in Fig. 1a, and the scoring of the entire set of immunohistological slides is demonstrated in Fig. 1b. In healthy controls, skin protein expression was very low for hBD-2 and LL-37, low for hBD-3 and psoriasin, and moderate for RNase 7. In untreated AD, lesional skin expression of hBD-2, hBD-3, psoriasin, RNase 7 and LL-37 was significantly increased; however, the intensity of the staining was different. We found low expression of LL-37, moderate expression of hBD-2, hBD-3 and RNase 7, and strong expression of psoriasin. Pimecrolimus treatment led to a slight decrease in the protein expression of LL-37, hBD-2, hBD-3, and psoriasin. RNase 7 expression after pimecrolimus treatment was not significantly reduced compared to untreated AD. The expression levels for hBD-2, psoriasin and RNase 7 were still above normal. After 3 weeks of treatment with the corticosteroids, TA and BV, a significant decrease in all AMPs examined occurred. The reduction was less pronounced for hBD-3 and RNase 7 than for hBD-2 and psoriasin. There was no significant difference between TA and BV treatment.

AMP gene expression analysed by gene array is shown in Fig. 1c and is in line with the protein expression data. RNase 7 expression after pimecrolimus treatment was even slightly increased, whereas a slight decrease in the expression of CAMP (encoding LL-37), DEFB103 (encoding hBD-3), S100A7 (encoding psoriasin), and a moderate decrease in DEFB4 (encoding hBD-2) was noted. After BV treatment, a slight decrease occurred for RNase 7, CAMP and DEFB103. The expression of S100A7 and DEFB4A was strongly reduced after BV treatment.

AMP expression in EFAD mice after treatment with pimecrolimus and BV

AMP expression in EFAD mice was determined after treatment for 8 days with the vehicle (ethanol/propylene glycol 3/7), pimecrolimus and BV. In normal skin of mice, protein expression of mBDs was low. In EFAD mice, clear staining with the antibodies was seen for mBD-1, mBD-3 and mBD-14, as reported previously (34). The vehicle did not significantly affect protein expression. Protein expression was reduced after treatment with pimecrolimus and was more pronounced after treatment with BV (Fig. 2a). Further to this, a small reduction in epidermal thickness was observed following pimecrolimus and a pronounced reduction in after treatment with BV.

The level of mRNA expression after vehicle treatment was unchanged for mBD-1, reduced for mBD-3 and increased for mBD-14. A significant reduction in the expression of the mBDs was noted after treatment with pimecrolimus compared to untreated and to vehicle treated skin. After BV treatment, mRNA expression of the mBDs was dramatically reduced to almost undetectable levels (Fig. 2b).

Discussion

This study is divided into two parts: topical treatment with two corticosteroids (BV and TA) and pimecrolimus in AD in the human model, and topical treatment of EFAD-treated mouse skin with BV and pimecrolimus. The EFAD mouse model is a model for a skin disease displaying chronic barrier dysfunction similar to that seen in AD. In both parts of the study, we found reduced expression of AMP, although to different degrees in the various models.

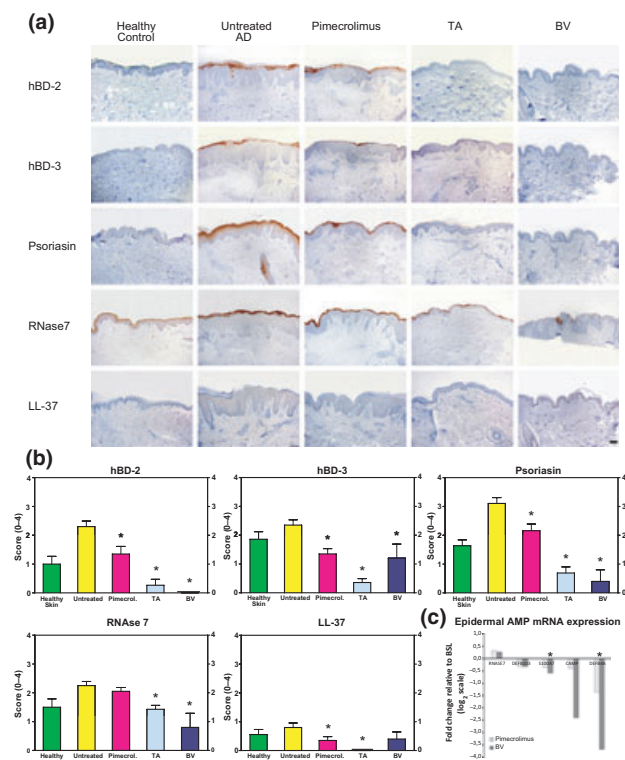


Figure 1. Epidermal antimicrobial protein (AMP) expression in atopic dermatitis (AD) skin before and after treatment. (a) Epidermal AMP expression in healthy skin, in untreated AD and after treatments with pimecrolimus, triamcinolone acetonide (TA) and betamethasone valerate (BV) cream ($n = 15$) was determined by immunostaining in skin biopsy samples using specific antibodies. (b) Grading of the staining intensity was performed (0 = no, 1 = slight, 2 = moderate, 3 = strong, and 4 = very strong staining) ($n = 5-15$; $P < 0.05$). (c) Epidermal AMP mRNA expression was determined after RNA amplification and gene chip analyses (Affymetrix, Palo Alto, CA, USA) ($n = 10$; $P < 0.05$ BV vs. untreated). The bar represents 100 μm .

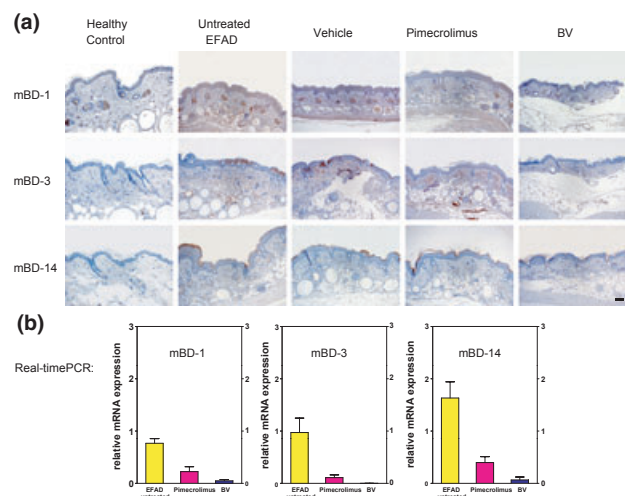


Figure 2. Epidermal defensin expression in essential fatty acid-deficient (EFAD) mouse skin. (a) Epidermal antimicrobial protein expression was determined by immunostaining in healthy control mice, in EFAD mice and after treatments with the vehicle and solutions with pimecrolimus and betamethasone valerate. (b) Epidermal mRNA expression was determined using real-time PCR ($n = 3-5$; $P < 0.05$ vs. EFAD untreated). The bar represents 100 μm .

First, we confirmed our recent results of increased expression of hBD-2, hBD-3, psoriasin and RNase 7 in untreated AD compared to normal skin (16,21-24,33) (Fig. 1a). Also, a slight induction was found for LL-37 (23). A moderate to strong induction was noted for hBD-2, hBD-3 and RNase 7, and a strong induction for psoriasin (Fig. 1b). These results suggest an important role for hBD-2, hBD-3, RNase 7 and psoriasin in antimicrobial defense in AD.

We then found that the corticosteroids BV and TA, as well as pimecrolimus treatment, resulted in a reduction in antimicrobial peptide expression in AD during improvement of the skin conditions. There were only small differences between the two corticosteroids, with BV suppressing AMP expression somewhat more than TA. BV (corticosteroid class 3 using the US system utilizing seven classes) is regarded as more potent than TA (class 4), and usually, the potency correlates with the side effects of the corticosteroids (35). Pimecrolimus' suppressive effect on antimicrobial peptide expression in AD was clearly less pronounced than that of the two corticosteroids (Fig. 1a-c).

A detailed examination of the data revealed that pimecrolimus and the corticosteroids showed a certain kind of selectivity in the suppression of AMPs. The corticosteroids suppressed the expression of all AMPs below the level of normal skin. Strong suppression in protein and mRNA expression resulted in particular for hBD-2 and psoriasin. Suppression of hBD-3 and RNase 7 expression was less pronounced and was also confirmed by gene chip analysis (Fig. 1c). Pimecrolimus treatment resulted in a moderate reduction in the expression of AMPs compared to untreated skin. Values below the level of normal skin were only obtained for hBD-3 and LL-37. Protein expression for hBD-2 was still above the normal levels. Strong expression and above normal levels were noted for psoriasin and RNase 7 after pimecrolimus treatment. The preservation of high levels of AMP expression is most likely very important for the antimicrobial defense in AD after treatment. Reduction in hBD-2, hBD-3, psoriasin and RNase 7 after corticosteroid treatment may be a risk factor for bacterial infection.

The mRNA and protein expression of LL-37 was low in untreated AD and a slight reduction occurred after betamethasone treatment. LL-37 is produced preferentially by neutrophils and under specific conditions also in epithelial cells and was shown to be upregulated during inflammation (9,10,36). Our results suggest that LL-37 does not play a major role in first-line antimicrobial defense in AD. However, in the case of a manifest bacterial infection, neutrophils invade the skin which may be followed by increased LL-37 expression. In this study, we did not examine AD lesions for bacterial infection.

In the EFAD mouse, a model for a metabolically induced skin barrier disruption, we found a significant reduction in the expression of the defensins mBD-1, mBD-3 and mBD-14, which are the mouse orthologues of the human defensins hBD-1, hBD-2 and hBD-3, respectively, by pimecrolimus. An even more pronounced reduction occurred after BV treatment such that mBDs were nearly undetectable. This is in line with the studies in human AD and show that beta-defensins are particularly sensitive to suppression by drug treatment. The influence of the calcineurin inhibitors on some AMP has previously been studied in mouse skin and in cell culture only. A reduced expression of mBD-3 and CRAMP (mouse

orthologue of cathelicidin LL-37) in mouse skin after treatment with calcineurin inhibitors has been described (37). This report is in line with our results. In contrast, in keratinocyte cell culture, increased expression of LL-37, hBD-2 and hBD-3 after pimecrolimus treatment has been found; however, such effects could only be shown at 10–20 nM concentrations (38). Also, a small study with three patients with AD showed increased expression of hBD-2 and LL-37 after treatment with the calcineurin inhibitor tacrolimus (39). The different findings may be related to the different conditions *in vitro* versus *in vivo* or different stages of the disease.

The effect of corticosteroids on AMP expression has also been studied in the lung. In cultured bronchial epithelial cells, dexamethasone inhibited the stimulated expression of mRNA for hBD-3, but not hBD-1 and -2 (40). Also, dexamethasone inhibited IL-1 β -induced hBD-2 mRNA expression (41). *In vivo* studies showed that bronchial biopsy specimens from dexamethasone-treated calves had significantly lower expression of tracheal antimicrobial peptide (TAP) and lingual antimicrobial peptide (LAP) mRNA than saline-treated controls (42). Our results showing a pronounced reduction in several AMPs after corticosteroid treatment for AD are in line with the results in the lung.

The different effects of calcineurin inhibitors and corticosteroids on AMPs may be caused by differences in their effects on the regulation mechanisms of AMPs. AMP expression is regulated by cytokines (hBD-2) and growth factors (hBD-3, LL-37) (5,43,44). Corticosteroids reduce the release of growth factors and cytokines (45). Therefore, the reduction in the AMPs by corticosteroids may be related to the reduction in cytokines and growth factors. In addition, the well-known immunosuppressive and cytostatic effect of corticosteroids, which includes suppression of protein synthesis (46), may be responsible for the reduction in AMP expression after corticosteroid treatment. In contrast, a general immunosuppressive effect and skin atrophy does not occur after pimecrolimus treatment (25,47); this may be the reason why the effect of pimecrolimus on AMPs is less compared to corticosteroids.

Recently, we showed that BV treatment for AD, which results in very good clinical improvement, does not lead to a repair of the skin's physical (permeability) barrier (25). The physical barrier is very important in the defense against infection. Impairment of the physical barrier is a prerequisite for experimental skin infection in humans by application of a bacterial suspension (48). Reduced AMP expression following corticosteroid treatment together with an ongoing defect in the skin permeability may lead to a pronounced reduction in antimicrobial defense. The skin of

patients with AD is often colonized with *S. aureus*, and this is associated with a worsening of clinical symptoms. *Staphylococcus aureus* produces toxins and superantigens that may penetrate into the skin and contribute to cutaneous inflammation and corticosteroid (CS) resistance (49,50). In clinical observations, prolonged treatment with topical corticosteroids sometimes leads to bacterial folliculitis. Therefore, treatment for AD with corticosteroids is often combined with local antiseptics or antibiotics or with systemic antibiotics; though, the efficacy of this combination is a subject of discussion (51–54). Pimecrolimus treatment in AD preserves a certain level of AMP expression and simultaneously repairs skin barrier function. Our results suggest that after treatment for AD with pimecrolimus, the natural protection against bacterial infection may be preserved. In contrast, treatment with corticosteroids leads to a significant reduction in AMP expression. This may be the reason that in cases of clinical insensitivity to corticosteroids, treatment with pimecrolimus was successful (50). In summary, we found that the corticosteroids BV and TA lead to a significant reduction in AMP expression in AD, whereas the reduction was less pronounced after pimecrolimus treatment.

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Author's contributions

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(A) Substantial contributions to research design, or the acquisition, analysis or interpretation of data, (B) drafting the paper or revising it critically, (C) approval of the submitted and final versions, (D) performed the research, (E) designed the research study, (F) contributed essential reagents or tools, (G) analysed the data and (H) wrote the paper.

Conflict of interest

This work was supported by a grant of Novartis Pharma, Nürnberg (Germany) given to Ehrhardt Proksch and Jens-Michael Jensen. Ehrhardt Proksch and Thomas Schwarz have acted as consultants to Novartis. Josef Meingassner, Anton Stütz and Matthias Bräutigam are employed by Novartis. Andreas Scherer was previously employed by Novartis.

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