Psoriasis-Associated Late Cornified Envelope (LCE) Proteins Have Antibacterial Activity

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Terminally differentiating epidermal keratinocytes express a large number of structural and antimicrobial proteins that are involved in the physical barrier function of the stratum corneum and provide innate cutaneous host defense. Late cornified envelope (LCE) genes, located in the epidermal differentiation complex on chromosome 1, encode a family of 18 proteins of unknown function, whose expression is largely restricted to epidermis. Deletion of two members, LCE3B and LCE3C (LCE3B/C-del), is a widely-replicated psoriasis risk factor that interacts with the major psoriasis-psoriasis risk gene HLA-C*06. Here we performed quantitative trait locus analysis, utilizing RNA-seq data from human skin and found that LCE3B/C-del was associated with a markedly increased expression of LCE3A, a gene directly adjacent to LCE3B/C-del. We confirmed these findings in a 3-dimensional skin model using primary keratinocytes from LCE3B/C-del genotyped donors. Functional analysis revealed that LCE3 proteins, and LCE3A in particular, have defensin-like antimicrobial activity against a variety of bacterial taxa at low micromolar concentrations. No genotype-dependent effect was observed for the inside-out or outside-in physical skin barrier function. Our findings identify an unknown biological function for LCE3 proteins and suggest a role in epidermal host defense and LCE3B/C-del-mediated psoriasis risk.

INTRODUCTION
Psoriasis vulgaris is a common inflammatory skin disease determined by both genetic and environmental factors (Nestle et al., 2009). Based on a number of genome wide association studies and meta-analyses thereof, more than 60 susceptibility loci have been identified that account for 20–25% of psoriasis heritability (Tsoi et al., 2012; Tsoi et al., 2015b; Zuo et al., 2015). Psoriasis is characterized by dysregulated cutaneous immune responses involving innate immunity (tumor necrosis factor-α and NF-kB pathways) and exaggerated T helper type 1 (Th1) and T helper type 1 (Th17) lymphocyte activation (Johnston et al., 2013; Jordan et al., 2012; Lowes et al., 2014). In addition, psoriasis-associated genes expressed by keratinocytes and with a presumed role in epidermal homeostasis have also been genetically implicated in psoriasis, including the late cornified envelope (LCE) genes (de Cid et al., 2009; Huffmeier et al., 2010) and the beta-defensins (Hollox et al., 2008). Despite the identification of many candidate genes, functional studies that explain the contribution of genetic polymorphism to psoriasis risk are largely lacking. A number of studies, however, have shown a plausible link between genes from susceptibility loci and immunobiological features of psoriasis such as an association between variation at TNFAIP3 and response to tumor necrosis factor-α blockade (Tejasvi et al., 2012), and the association between the IL12B risk allele and increased Th1 cytokine levels (Johnston et al., 2013). In addition, only very few risk loci involve coding variants that are amenable to experimental verification in animal models or in vitro cellular models of skin biology or inflammation (Jordan et al., 2012; Tsoi et al., 2012). Among the psoriasis susceptibility regions, the major histocompatibility complex class I gene HLA-C*06:02 (PSORS1) and the LCE region harboring a deletion of the LCE3B and LCE3C genes (originally designated PSORS4) (Capon et al., 2001) provide...
plausible candidates to investigate at the functional level. 

HLA-C*06:02 is by far the strongest psoriasis risk factor, with an odds ratio estimated to be between 2.6 and 5 in Caucasians (Genetic Analysis of Psoriasis Consortium & the Wellcome Trust Case Control Consortium 2 et al., 2010; Nair et al., 2009), and the odds ratio for the LCE deletion (odds ratio ~ 1.3) (Huffmeier et al., 2010) is one of the highest among the remaining psoriasis-associated loci (Tsai et al., 2012). LCE genes are expressed only in epidermis and oral epithelia (Bergboer et al., 2011; Jackson et al., 2005) and are assumed to encode structural proteins with a role in epithelial barrier formation; however, no functional studies supporting this contention have been published so far. Remarkably, the LCE3 group, which encompasses the LCE3B and LCE3C genes, is under regulation of psoriasis-associated Th1 and Th17 cytokines (Bergboer et al., 2011; Niehues et al., 2016). LCE3B/C-del affects a 32 kb fragment in the epidermal differentiation complex (EDC) on chromosome 1, which is commonly deleted in the non-African population (allele frequency of LCE3B/C-del: 60–70%) (de Cid et al., 2009). In addition to the loss of the protein coding genes LCE3B and LCE3C, it also removes three intergenic fragments that harbor potential regulatory sequences (de Guzman Strong et al., 2010). In this study, we show that LCE3B/C-del causes an upregulation of the flanking LCE3A gene. Our hypothesis-driven functional studies have revealed that these three proteins are unlikely to be involved in skin barrier function and rather represent antimicrobial proteins.

RESULTS

Expression quantitative trait loci analysis of genes in the EDC

The association results between the deletion surrogate rs4112788 and expression traits are shown in Figure 1a. Within the EDC region, only the expression levels of LCE3A, LCE3B, and LCE3C are significantly (false discovery rate < 0.1) associated with the marker in both normal (NN) and psoriatic (PP) skin (Table 1). As expected, expression of both LCE3B and LCE3C are decreased in PP skin in the presence of the G allele of rs4112788, which is in linkage disequilibrium with LCE3B/C-del. We observed the same result for LCE3C expression in NN, whereas LCE3B could not be assessed as it was not expressed in more than 20% NN skin samples. In fact, of the 80 NN samples, we detected expression of LCE3A and LCE3C in 43 and 40 samples, respectively, but LCE3B was expressed only in one sample. In contrast, of the 92 PP samples, we detected expression of LCE3A, LCE3B, and LCE3C in 92, 48, and 55 samples, respectively. Figure 1b shows that LCE3A expression levels were elevated in individuals with the GG genotype (surrogate for del/del) compared with individuals with the AA genotype, in both NN and PP samples. LCE3A is strongly and significantly (P = 7.7 x 10^{-30}) upregulated in PP (mean expression level = 5.8 x 10^{3}) compared with NN skin (mean expression level = 20). The fold change difference in expression between GG/AA genotypes is 2.1 in PP samples, and the contrast is even higher in NN samples (mean expression level is 35 for GG and 0 for AA). We did not observe a significant genotype difference for LCE3A expression in nonlesional psoriatic (PN) skin samples, likely due to the smaller sample size. RNA-seq data showed that expression of LCE3A behaves similarly between NN and PN skin (NN = 4.6 vs. 4.5 in PN skin). See Supplementary Table S1 online for the normalized RNAseq expression data of all individuals.

Genotype-dependent expression of LCE genes in 3-dimensional (3D) reconstructed epidermis

To substantiate these findings in vitro, and to determine LCE3B/C-del-dependent epidermal morphology and differentiation, we generated 3D reconstructed epidermis from wild type/wild type (wt/wt) (N = 6) and del/del (N = 6) keratinocytes. We have previously shown that in such a 3D reconstructed epidermis model, the spatiotemporal expression of LCE

Figure 1. In vivo eQTL analysis. (a) Quantile-quantile plot of rs4112788 versus expression traits in the epidermal differentiation complex region. The shaded area indicates 95% confidence interval of statistical significance. (b) Estimated effect size of LCE3B/C-del on in vivo LCE3A expression in the eQTL data set. Error bars = standard error of the mean. eQTL, expression quantitative trait loci; LCE, late cornified envelope; n.e., no expression; NN, normal skin; PN, nonlesional psoriatic skin; PP, lesional psoriatic skin.

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proteins is similar to in vivo epidermis (Niehues et al., 2016). Histopathological examination of the 3D reconstructed epidermis did not reveal obvious morphological differences between the two genotypes (hematoxylin and eosin staining), nor did immunohistochemical staining reveal abnormalities in expression of differentiation proteins (Supplementary Figure S1 online). Confirming what was observed in vivo (Figure 1a and b), quantitative PCR analysis showed a significant effect of the LCE3B/C-del on LCE3A expression (Figure 2a–c): LCE3A mRNA expression was 6.6-fold higher in del/del keratinocytes compared with wt/wt keratinocytes ($P < 0.003$). To mimic psoriasis in vitro and confirm the in vivo findings, we stimulated 3D reconstructed epidermis with psoriasis-associated cytokines. To verify the effect of cytokine stimulation, expression levels of known Th1 and Th1/Th17 responsive genes CXCL10 and DEFB4 were analyzed and found to be strongly induced irrespective of the LCE3B/C-del genotype (Supplementary Figure S2a and b online). After Th1- but not Th17-cytokine stimulation, we found a significant genotype effect (4.7-fold, $P < 0.05$, Figure 2b and c). Generally, Th1 cytokines induced expression of all tested LCE family genes (Figure 2d), whereas Th17 cytokines specifically increased LCE2 and LCE3 genes (Figure 2e). These in vitro findings corroborate the in vivo findings of the effect of LCE3B/C-del on LCE3A expression. The fold increase in 3D reconstructed epidermis of del/del keratinocytes is modest compared with the huge relative increase in vivo, which is caused by the relatively high basal expression of LCE3A in wt/wt cells in the skin equivalents, whereas LCE3A expression in vivo for the AA (wt/wt) genotype is zero.

### LCE3B/C del is not associated with skin barrier alterations

We asked whether genotype-dependent alterations of LCE3A expression, that is, the absence of LCE3B and LCE3C and the upregulation of LCE3A, had functional consequences that would provide a clue to its genetic association with psoriasis. Many epidermis-specific genes encode either structural proteins involved in physical barrier function (Candi et al., 2005) or small proteins that provide an antimicrobial barrier (Gallo and Hooper, 2012; Schroder and Harder, 2006). Limited proteomic data suggest that LCE proteins are part of the stratum corneum (SC), hence participate in physical skin barrier function, although direct proof is lacking. We therefore assessed transepidermal water loss from 3D reconstructed epidermis of wt/wt and del/del genotypes to unveil a possible effect on inside-out barrier function. No difference in transepidermal water loss was observed between the genotypes (Figure 3a–c). We noticed that cytokine-stimulated 3D reconstructed epidermis had a lower transepidermal water loss compared with control 3D constructs (control vs. Th17, $P < 0.001$), but again no differences between the two genotypes were observed (Figure 3d). The outside-in barrier function was investigated by the analysis of the penetration of hydrophilic and hydrophobic low molecular weight tracers. Quantitative determination of hydrocortisone diffusion by ultra performance liquid chromatography analysis through the SC did not reveal a difference between the two genotypes (Figure 3e). Furthermore, we did not observe Lucifer yellow penetration in constructs of any of the genotypes, under unstimulated and Th1- or Th17-cytokine conditions (Figure 3f). Collectively, these data indicate that LCE3B/C-del is not associated with altered skin barrier function.

### LCE3 proteins have antibacterial activity

Keratinocytes are a known source of antimicrobial peptides (AMPs) such as defensins, secretory leukocyte protease inhibitor, skin-derived antileukoprotease/Elafin, psoriasin, and LL37, many of which are small, cationic, and/or cysteine-rich proteins (Harder et al., 2013). As LCE3 proteins are also small (9–11 kD) and cationic (isoelectric points $> 8.6$) having an extremely high cysteine content (18–23%), we tested the hypothesis that LCE proteins are antimicrobial. On the basis of the finding that LCE3A expression is firmly increased in LCE3B/C-del carriers, we decided to study the antimicrobial activity for all three LCE3 proteins (LCE3A/B/C). We used chemically synthesized LCE3A, LCE3B, and LCE3C, which demonstrated clear antimicrobial activity against 10 commensal and pathogenic bacteria (Figure 4 and Supplementary Table S2 online). Remarkably, even at submicromolar concentrations, activity was observed against a...
variety of taxa, including Gram-negative (Figure 4a–d) and Gram-positive (Figure 4e–j) microorganisms, including both aerobic and aerotolerant species. Notably, antimicrobial activity was present under the same experimental (low salt) conditions that reveal the antibacterial activity of human beta-defensin 2, a prototypic cationic, epidermal cysteine-rich AMP (Harder et al., 1997). High salt concentrations inhibited the antimicrobial activity of LCE3 proteins (Supplementary Figure S3a online). LCE3A appeared to be the strongest antimicrobial protein of the tested LCEs, and its potency toward Gram-negative bacteria is similar to the reported potency of human beta-defensin 2 for Escherichia coli and Pseudomonas aeruginosa (Harder et al., 1997) (Supplementary Figure S3b).

As defensins can exert a toxic effect on mammalian cells as well (Lichtenstein et al., 1986), we tested LCE proteins effects up to 10 μM on submerged keratinocytes cultures for 4 or 20 hours and did not encounter any effect on cell viability or morphology (Supplementary Figure S4a and b online).

**DISCUSSION**

LCE expression is largely restricted to skin and oral epithelial tissue, and is absent in cells of the immune system. This indicates that the biological mechanism conferring an increased psoriasis risk is linked to specific epithelial functions of these tissues. Quantitative PCR studies have shown that LCE3 gene expression is low to undetectable in normal skin, but is readily detectable in oral epithelia (Jackson et al., 2005). On skin injury and inflammation, however, LCE3 genes are induced (Bergboer et al., 2011; Jackson et al., 2005) suggesting that LCE3 proteins might be involved in skin barrier function or repair. The reported epistasis between the major risk factor HLA-C*06:02 and LCE3B/C-del (Chandra et al., 2016; de Cid et al., 2009; Huffmeier et al., 2010) supports the idea that an impaired barrier in LCE3B/C-deficient skin would facilitate the penetration of environmental antigens, which in turn could trigger an HLA-C*06:02-restricted immune response (Bergboer et al., 2012). On the basis of this assumption, we performed a detailed analysis of the barrier properties of the del/del and wt/wt...
Repeated measures ANOVA plus Bonferroni post hoc with LCE3B/C-del and inside-out barrier function in in vitro models did not reveal an impairment of outside-in genotypes. However, for the molecules studied here, our in vitro models did not reveal an impairment of outside-in and inside-out barrier function in LCE3B/C-del epidermis. As the LCE3 baseline expression levels are higher in stratified oral epithelia (tonsil, gingiva, pharynx) than in normal skin (Niehues et al., 2016), we cannot exclude the possibility that the impact of LCE3/C-loss could lead to leaky oral epithelia that are prone to penetration of external antigens. The observation that many psoriasis patients have their first flare after streptococcal tonsillitis (McFadden et al., 2009; Valdimarsson et al., 2009) would be compatible with the oral cavity as the site of entry of bacterial antigens. This potential mechanism clearly warrants further investigation.

An alternative explanation for increased penetration of external antigens in LCE3B/C-deficient epidermis would point to a more direct antimicrobial role of these LCE proteins. Mammalian epidermis is known for its antimicrobial shield of keratinocyte-expressed AMPs (Harder et al., 2013). We found a rather broad defense-like spectrum of antibacterial activity that includes Gram-positive and Gram-negative species, and anaerobic as well as aerotolerant organisms. In view of their high homology, other LCE members may have antimicrobial properties as well. It will be interesting to determine whether this activity extends to other organisms such as yeasts and fungi.

LCE3 proteins are small (9–10 kDa), cationic proteins (isoelectric points approximately 9.0), and their antimicrobial activity is inhibited at high ionic strength. LCE3 proteins share these properties with many previously described AMPs such as human beta defensin-1, human beta defensin-2, human alpha-defensin-1, secretory leukocyte protease inhibitor, and LL37. All these proteins lose antimicrobial activity in aqueous solutions of physiological ionic strength, which raises the question whether the observed in vitro activity has any relevance under physiological conditions. It has been argued that in vitro assays poorly capture the physiological conditions in which these proteins work in vivo, such as SC of the skin, or mucous surfaces of the oral cavity or respiratory system. Epidermis-expressed LCE proteins or peptides derived thereof will end up in the SC, which is composed of lipids and crosslinked proteins, and devoid of free water. The (bound) water content of SC is extremely variable (Thakoersing et al., 2013), and to our knowledge, the ionic composition and concentration of the SC is unknown. The antimicrobial activity of AMPs is obviously concentration dependent, and so is their salt sensitivity. Although AMPs are typically tested in the low micromolar range, their actual concentration in vivo may be orders of magnitude higher. We have modeled the epidermal concentration of human beta-defensin 2 using an in vitro reconstructed skin model, which suggested that a concentration of 300 μM is reached (Jansen et al., 2009), which is far higher than required for antimicrobial activity, and which may offset the effect of high ionic strength. For all these reasons, the in vitro antimicrobial assay conditions are quite distinct from the environment in which AMPs such as LCE and defensins operate, and in vitro models to test AMPs under these tissue-specific conditions are currently lacking. Indirect evidence that these molecules are genuine antimicrobial agents in vivo is provided by the observation that mice in which these genes have been knocked out have a compromised innate immune system (Moser et al., 2002).

A variety of AMP mechanisms that mediate bacterial killing or toxin inactivation have been described, including membrane permeabilization and depolarization (Selsted and Ouellette, 2005), peptide nanonets (Chu et al., 2012), and local protein unfolding (Kudryashova et al., 2014). LCE3 proteins cause a rapid killing that is reminiscent of the kinetics of killing by human neutrophil defensins where the
outer and inner membrane of *E. coli* are sequentially permeabilized within 30–60 minutes (Lehrer et al., 1989). Further analysis is needed to better understand the mechanism of bacterial killing and its relevance in the antimicrobial shield of the epidermis.

How can we integrate these findings into a comprehensive picture of the pathogenesis of psoriasis? From an evolutionary perspective, the LCE3B/C indel is likely a derived rather than an ancestral allele, as the deletion has not been reported in non-human primate genomes. In addition, the frequency of

Figure 4. Antimicrobial activity of LCE3 proteins. LCE3A, B, C were incubated with Gram-negative (a–d) and Gram-positive commensal and pathogenic bacteria (e–j) between 0.1 to 10 μM protein. For specifications see Supplementary Table S4.2. 2 × 10^4 CFU/ml is the detection limit; therefore values <2 × 10^4 CFU/ml are not depicted. Error bars = standard error of the mean. CFU, colony forming units; LCE, late cornified envelope.
the indel is only 37% in African populations, but it is between 62 and 64% in East-Asian, South-Asian, American, and European populations (Bassaganyas et al., 2013; 1000 Genomes Project Consortium et al., 2010). This may possibly indicate a selective advantage in out-of-Africa populations. In view of the antimicrobial activity of LCE3 proteins, the deletion could cause a different antimicrobial shield in the epidermis. In wt/wt individuals (AA genotype), LCE3A is not expressed in normal skin, and also the expression levels of LCE3B/C are also very low or absent. LCE3B/C-del thus causes the LCE3A gene to be expressed at significant levels in the normal skin of these individuals (Figure 1b). The loss of LCE3B/C would not have a noticeable effect in normal skin, as they were not expressed anyway. The net result of the indel would be a quantitatively (and possibly qualitatively) different epidermal host defense repertoire. Speculatively, this could provide an evolutionary benefit and hence favor the spread of LCE3B/C-del. An increased risk to develop psoriasis could be regarded as the evolutionary cost of having this stronger antimicrobial shield in the epidermis and oral cavity.

As there is an epistatic interaction with HLA*C06:02 (Tsoi et al., 2012), LCE3B/C-del could alter the self-peptide profile of the skin by altering the balance of LCE3A self-peptides, compared with LCE3B and LCE3C. It could also alter the cutaneous microbiome in such a way as to promote the survival and colonization of taxa that elicit an HLA*C06:02-restricted response. A precedent comes from a recent microbiome study showing that filaggrin deficiency in ichthyosis vulgaris patients causes marked changes in the bacterial species composition (Zeeuwen et al., 2016). Microbiome studies on psoriasis have been performed, and shifts in microbiome composition have been reported, but unfortunately none of these studies have included the host HLA*C06:02 and LCE3B/C-del genotypes. In addition to psoriasis, the LCE3B/C-del has been associated with different other diseases, including lupus (Lu et al., 2011) and rheumatoid arthritis (Docampo et al., 2010); our findings will provide further insights for the causal mechanism of this locus to the associated autoimmune disorders. Clearly, future studies should aim to establish the effects of LCE3B/C-del on the tissue microbiome, and their potential effects on HLA*C06:02-dependent immune processes that lead to disease.

In conclusion, our study establishes two findings regarding functional genomics of the psoriasis risk factor LCE3B/C-del. First, we show that LCE3B/C-del, a risk allele for psoriasis, is significantly associated with the elevated expression of the upstream LCE3A gene in human skin. Secondly, we have uncovered a hitherto unknown antibacterial function of the proteins encoded by these three genes of the LCE3 family. Overall, these findings suggest a central role for LCE3A in epidermal host defense and LCE3B/C-del-mediated psoriasis risk.

**MATERIALS AND METHODS**

**Quantitative trait loci analysis of skin biopsies**

All subjects involved in this study provided written informed consent according to the Helsinki Guidelines and approved by the Institutional Review Board of the University of Michigan Medical School. To evaluate the associations between the LCE3B/C-del and expression traits in the EDC region (between 151.5 and 154 Mb of chromosome 1), we utilized our RNA-seq cohort for psoriasis (Tsoi, 2014; Tsoi et al., 2015a) to measure the gene expression levels in NN, PN, and PP skin samples, respectively. Most of the samples in the RNA-seq cohort were genotyped in our Exomechip cohort. Altogether 80 nonpsoriatic controls and 92 psoriatic patients with both genetic and expression data were included, with 27 of the patients also providing expression data for PN skin. The samples and data processing procedures for the RNA-seq data were described previously (Li et al., 2014; Tsoi et al., 2015a). Briefly, we employed Tophat and Cufflink to align reads and estimate expression levels, respectively. We included in the expression quantitative trait loci analysis only genes in the EDC region (chromosome 1: 151.5–154 Mb) that were expressed in at least 20% of the samples in the condition being studied (i.e., NN, PN, or PP skin). For each expression trait, we removed expression outliers (i.e., three times the interquartile range smaller than the lower quartile or three times the interquartile range larger than the upper quartile), and we further performed inverse normal transformation on each gene’s expression values. Genetic marker rs4112788 has been reported to be a close proxy of LCE3B/C-del (de Cid et al., 2009), and it was genotyped in the Exomechip cohort; therefore, we used this single nucleotide polymorphism as a surrogate for the deletion and correlated its genotypes with expression traits using a linear model. The expression quantitative trait loci analysis was performed for NN, PN, and PP skin separately.

**LCE3B/C-del genotyping of keratinocytes used for in vitro analyses**

All subjects involved in this study were healthy (no skin diseases) and provided written informed consent according to the Helsinki Guidelines and approved by the local Medical Ethics Committee in The Netherlands (CMO Regio Arnhem-Nijmegen, The Netherlands). Keratinocytes were isolated and genotyped for their LCE3B/C-del status as previously described (de Cid et al., 2009). The nondeletion allele (i.e., containing LCE3B and 3C) is designated as wt. The deletion allele is designated del. Genotypes are referred to as wt/wt, del/wt, and del/del.

**3D reconstructed epidermis**

3D reconstructed epidermis was generated as described previously (Niehues et al., 2017). To model the response of Th1 or Th17 T cells, combinations of cytokines were added into the medium during days 5–8 of the air-liquid interface culture (Th1 mix: 10 ng/ml IL-12, 0.5 ng/ml tumor necrosis factor-1α, 250 U/ml IFN-γ; Th17 mix: 50 ng/ml IL-17, 50 ng/ml IL-22; Preprotech, Rocky Hill, NJ).

**Quantitative real-time PCR**

RNA isolation, cDNA synthesis, quantitative PCR analysis, and primer design were performed as described previously (Berghofer et al., 2011; Zeeuwen et al., 2008) (Supplementary Table S3 online). Target gene expression was normalized to the expression of the housekeeping gene human acidic ribosomal phosphoprotein P0 (RPLP0). The ΔΔCt method was used to calculate relative mRNA expression levels (Livak and Schmittgen, 2001).

**Statistics**

Statistical analysis of quantitative PCR data was performed on ΔΔCt values using commercially available software (IBM, SPSS Statistics 22). Repeated measures analysis of variance, followed by Bonferroni
post hoc testing, was performed. \( P < 0.05 \) was considered statistically significant.

**Immunohistochemistry**

3D reconstructed epidermis was formalin-fixed and processed for routine histology. Paraffin sections (6 \( \mu \)m) were stained with antibodies using an indirect immunoperoxidase (Vectorstain, Vector Laboratories, Burlingame, CA) or immunofluorescence technique. Details of antibodies are presented in Supplementary Table S4 online.

**Transepidermal water loss measurement**

Transepidermal water loss was determined according to the condenser-chamber method (Aquaflex AF200, Biox Systems, London, UK), as described before (Niehues et al., 2017).

**In vitro outside-in dye penetration**

Lucifer yellow (1 \( \mu \)M Sigma Aldrich) was added to the upper chambers of 3D reconstructed epidermis in 12-well dishes for 1 hour. Immunohistological sections were counterstained with DAPI (Boehringer Mannheim) and assessed with fluorescence microscopy for Lucifer yellow penetration. Validation of this model is described in (Niehues et al. 2017).

**Hydrocortisone diffusion analysis**

SC isolation was performed according to the procedure as described earlier (van Smeden et al., 2011; Nugroho et al., 2006). In short, SC was isolated from ex vivo skin or 3D epidermal equivalents and used in the diffusion setup as described previously (Mojumdar et al., 2014) using 0.34 mg/ml hydrocortisone in acetate buffer (pH 5.0) as diffusion fluid with 2–2.5 ml/h flow speed. Samples were collected for a period of 15 hours, with a sampling time of 60 minutes. Hydrocortisone content was analyzed by ultra performance liquid chromatography (Acquity UPLC-UV system, Milford, MA) at 243 nm and analysis was performed using Masslynx v4.1 for peak integration, calculation of the concentration, and flux determination, which is represented as flux in \( \mu \)g/cm²/h.

**Expression and synthesis of LCE proteins**

The cloning of LCE genes has been described previously (Niehues et al., 2016). LCE fusion proteins of glutathione S-transferase were produced according to standard procedures (Frangioni and Neel, 1993). Fusion-proteins were thrombin-cleaved to liberate the LCE protein from glutathione S-transferase. LCE3B-glutathione S-transferase fusion protein did not show any antibacterial activity, but after protein from glutathione S-transferase removal, the LCE protein clearly displayed antimicrobial activity. As pure LCE proteins were difficult to obtain, most likely due to their extremely high cysteine content, LCE3A,B,C proteins were synthesized by solid phase peptide synthesis, purified to 85–90% purity by reverse-phase HPLC and characterized by electrospray mass spectroscopy (Pepeomic, Suzhou, China). LCE proteins were delivered in a reduced form. Reducing agents and other low molecular weight components were removed by spin column dialysis (Amicon Ultra Centrifugal Filters 3K, Merck Millipore, Billerica, MA), and LCE proteins were extensively dialyzed against 10 mM sodium phosphate buffer (pH 7.4).

**Bacteria culture**

Diverse bacterial strains were grown on Columbia agar with 5% sheep blood (Becton, Dickinson), and single colonies were used to inoculate cultures in brain heart infusion medium (Mediaproducts BV, Groningen, The Netherlands) overnight at 37 °C. Bacterial cultures were diluted 10² times in brain-heart infusion medium and allowed to grow for another 2.5 hours to reach exponential growth (except for *Propionibacterium acnes*). For strains and specifications of all bacterial strains, see Supplementary Table S2.

**Antimicrobial assay**

Bacteria were harvested from exponential growth cultures by centrifugation (2,100g, 5 minutes), washed with sodium phosphate buffer, and resuspended in sodium phosphate buffer at a concentration of 10⁴–10⁵ colony forming units/ml. Bacterial suspensions were exposed to LCE peptides in an assay volume of 100 \( \mu \)l, for 2 hours at 37 °C in a 96-well microplate (Greiner Bio-one, Kremsmünster, Austria). The suspensions were serially diluted in steps of 10, and 10 \( \mu \)l of each dilution was plated on 5% sheep blood containing Columbia agar plates overnight at 37 °C. Antimicrobial effects were determined by counting colony forming units.

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**CONFLICT OF INTEREST**

The authors state no conflict of interest.

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**SUPPLEMENTARY MATERIAL**

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2017.06.003.

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