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

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Abstract

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 3D skin model using primary keratinocytes from *LCE3B/C-del*

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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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.

Keywords

Psoriasis; Late Cornified Envelope; LCE3B/C-del; defensins; skin barrier

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 (GWAS) and meta-analyses thereof, more than 60 susceptibility loci have been identified that account for 20–25% of psoriasis heritability (Tsoi *et al.*, 2015b; Tsoi *et al.*, 2012; Zuo *et al.*, 2015). Psoriasis is characterized by dysregulated cutaneous immune responses involving innate immunity (TNF-alpha and NFkB pathways) and exaggerated Th1 and 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 TNF-alpha 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 MHC class 1 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 to 5 in Caucasians (Genetic Analysis of Psoriasis *et al.*, 2010; Nair *et al.*, 2009), and the odds ratio for the *LCE* deletion (OR ~ 1.3) (Huffmeier *et al.*, 2010) is one of the highest among the remaining psoriasis-associated loci (Tsoi *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.*, 2015). *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

eQTL analysis of genes in the epidermal differentiation complex (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, while *LCE3B* could not be assessed as it was not expressed in more than 20% NN skin samples. In fact, out 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, out 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 to individuals with the AA genotype, in both NN and PP samples. *LCE3A* is strongly and significantly ($p=7.7\times 10^{-30}$) up-regulated in PP (mean expression level = 5.8×10^3) compared to 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 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 Table S4 for the normalized RNAseq expression data of all individuals.

Genotype dependent expression of LCE genes in 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 wt/wt (N=6) and del/del (N=6) keratinocytes. We have previously shown that in such a 3D reconstructed epidermis model, the spatio-temporal expression of LCE proteins is similar to *in vivo* epidermis (Niehues *et al.*, 2015). Histopathological examination of the 3D reconstructed epidermis did not reveal obvious morphological differences between the two genotypes (H&E staining), nor did immunohistochemical staining reveal abnormalities in expression of differentiation proteins (Figure S1). Confirming what was observed *in vivo* (Figure 1a–b), qPCR 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 to *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. (Figure S2a–b). Following Th1-, but not Th17-cytokine stimulation, we found a significant genotype effect (4.7-fold, $p < 0.05$, Figure 2b–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 to 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, i.e. 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 trans-epidermal water loss (TEWL) from 3D reconstructed epidermis of wt/wt and del/del genotypes to unveil a possible effect on inside-out barrier function. No difference in TEWL was observed between the genotypes (Figure 3a–c). We noticed that cytokine-stimulated 3D reconstructed epidermis had a lower TEWL compared to 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 analysis of the penetration of hydrophilic and hydrophobic low molecular weight tracers. Quantitative determination of hydrocortisone diffusion by UPLC analysis through the SC did not reveal a difference between the two genotypes (Figure 3e). Furthermore, we did not observe LY 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, SLPI, SKALP/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), cationic (isoelectric points > 8.6) having an extremely high cysteine content (18–23%) we tested the hypothesis that LCE proteins are antimicrobial. Based on 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 Table S3). 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 antibacterial activity of human beta-defensin 2 (hBD2), a prototypic cationic, epidermal cysteine-rich AMP (Harder *et al.*, 1997). High salt concentrations inhibited antimicrobial activity of LCE3 proteins (Figure S3a). LCE3A appeared to be the strongest antimicrobial protein of the tested LCE's, and its potency towards gram-negative bacteria is similar to the reported potency of hBD2 for *E. coli* and *P. aeruginosa* (Harder *et al.*, 1997) (Figure S3b).

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

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. qPCR 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). Upon 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). Based on this assumption, we performed detailed analysis of barrier properties of the *del/del* and *wt/wt* 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.*, 2015), we cannot exclude the possibility that the impact of LCE3B/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 site of entry of bacterial antigens. This potential mechanism clearly warrants further investigation.

An alternative explanation for increased penetration of external antigens in LCEB/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 defensin-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 about 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, SLPI 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 stratum corneum 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 stratum corneum, which is composed of lipids and crosslinked proteins, and devoid of free water. The (bound) water content of stratum corneum is extremely variable (Thakoersing *et al.*, 2013), and to our knowledge the ionic composition and concentration of the stratum corneum 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 hBD-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 like 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 which is reminiscent of the kinetics of killing by human neutrophil defensins where the outer and inner membrane of *Escherichia 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 the indel is only 37% in African populations but it is between 62–64% in East-Asian, South-Asian, American and European populations (Bassaganyas *et al.*, 2013; Genomes Project *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 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 to 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* genotype. 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*. Firstly, 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

QTL 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 151.5–154 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 normal (NN), uninvolved (PN), and psoriatic (PP) skin samples, respectively. Most of the samples in the RNA-seq cohort were genotyped in our Exomechip cohort. Altogether 80 non-psoriatic 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 eQTL analysis only genes in the EDC region (chromosome 1: 151.5–154Mb) 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 SNP as a surrogate for the deletion and correlated its genotypes with expression traits using a linear model. The eQTL 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 non-deletion allele (i.e. containing *LCE3B* and *3C*) is designated as wild type (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.*, 2016). To model the response of Th1- or Th 17 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-1 α , 0.5 ng/ml TNF-1 α , 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, qPCR analysis and primer design was performed as described previously (Bergboer *et al.*, 2011; Zeeuwen *et al.*, 2008) (Table S1). Target gene expression was normalized to the expression of the housekeeping gene human acidic ribosomal phosphoprotein P0 (*RPLP0*). The $\Delta\Delta$ Ct method was used to calculate relative mRNA expression levels (Livak and Schmittgen, 2001).

Statistics

Statistical analysis of qPCR data was performed on $\Delta\Delta$ Ct values using commercially available software (IBM, SPSS Statistics 22). Repeated measures ANOVA, 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. 6 μ m paraffin sections were stained with antibodies using an indirect immunoperoxidase (Vectastain, Vector Laboratories) or immunofluorescence technique. Details on antibodies are presented in Table S2.

Transepidermal water loss measurement

Transepidermal water loss (TEWL) was determined according to condenser-chamber method (Aquaflux AF200, Biox Systems, London, UK), as described before (Niehues *et al.*, 2016).

In vitro outside-in dye penetration

1 mM Lucifer Yellow (LY, Sigma Aldrich) was added to the upper chambers of 3D reconstructed epidermis in 12-well dishes for one hour. Immunohistological sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Boehringer Mannheim) and assessed with fluorescence microscopy for LY penetration. Validation of this model is described in Niehues *et al.* (Niehues *et al.*, 2016).

Hydrocortisone diffusion analysis

Stratum corneum (SC) isolation was performed according to the procedure as earlier described^{20,21}. In short, SC was isolated from *ex vivo* ski or 3D epidermal equivalents and used in the diffusion set-up was 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/hour flow speed. Samples were collected for a period of 15 hours, with a sampling time of 60 minutes. Hydrocortisone content was analyzed by UPLC (Acquity UPLC-UV system, Milford, USA) 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 $\mu\text{g}/\text{cm}^2/\text{hour}$.

Expression and synthesis of LCE proteins

The cloning of LCE genes has been described previously¹⁶. LCE fusion-proteins of glutathione S-transferase (GST) were produced according to standard procedures (Frangioni and Neel, 1993). Fusion-proteins were thrombin-cleaved to liberate the LCE protein from GST. LCE3B-GST fusion-protein did not show any antibacterial activity, but after GST-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 (Pepmic, Suzhou, China). LCE proteins were delivered in reduced form. Reducing agents and other low molecular weight components were removed by spin column dialysis (Amicon Ultra Centrifugal Filters 3K, Merck Millipore) and LCE proteins were extensively dialyzed against 10mM sodium phosphate buffer (PB, pH 7.4).

Bacteria culture

Diverse bacterial strains were grown on Columbia agar with 5% sheep blood (Becton, Dickinson and Co.) 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 10^2 times in BHI medium and allowed to grow for another 2.5 hours to reach exponential growth (except for *P. acnes*). For strains and specifications of all bacterial strains see Table S3.

Antimicrobial assay

Bacteria were harvested from exponential growth cultures by centrifugation (2100 g, 5 min), washed with PB, and resuspended in PB at a concentration of $10^4 \sim 10^5$ CFU/ml. Bacterial suspensions were exposed to LCE peptides in an assay volume of 100 μ l, for 2 hours at 37°C in a 96 wells microplate (Greiner Bio-one). The suspensions were serially diluted in steps of ten and 10 μ l of each dilution was plated on 5% sheep blood containing Columbia agar plates overnight at 37°C. Antimicrobial effects were determined by counting CFU.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AMP	antimicrobial peptide
CE	cornified envelope
CFU	colony forming units
EDC	epidermal differentiation complex
EM	electron microscopy
eQTL	expression quantitative trait loci
GST	glutathione S-transferase
GWAS	genome wide association studies
hBD2	human beta-defensin 2
IL	interleukin
LCE	late cornified envelope
<i>LCE3B/C-del</i>	the 32 kb deleted fragment comprising <i>LCE3B</i> and <i>3C</i>
LY	Lucifer Yellow
NN	normal skin
PB	sodium phosphate buffer

PN	psoriasis uninvolved skin
PP	psoriasis lesional skin
SC	stratum corneum
SNP	single nucleotide polymorphism
TCEP	tris(2-carboxyethyl) phosphine
TEM	transmission electron microscopy
TEWL	transepidermal water loss
Th1/17	T helper 1/17

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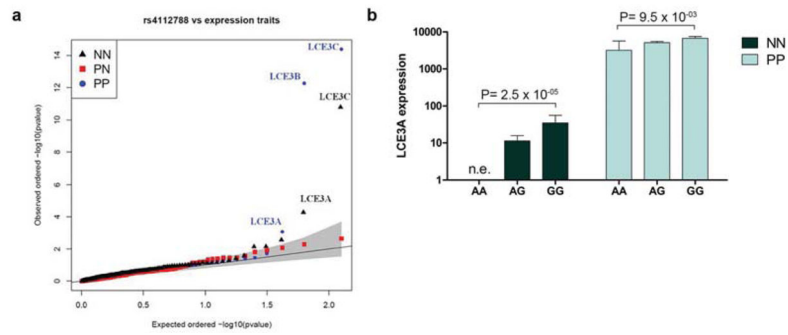


Figure 1. *In vivo* eQTL analysis

(a) Quantile-quantile plot of rs4112788 versus expression traits in the EDC region. Shaded area indicates 95% confidence interval of statistical significance. **(b)** Estimated effect size of LCE3B/C-del on *in vivo* LCE3A expression in the e-QTL data set. NN, normal skin; PN; non-lesional psoriatic skin; PP, lesional psoriatic skin. Error bars = SEM. n.e.=no expression.

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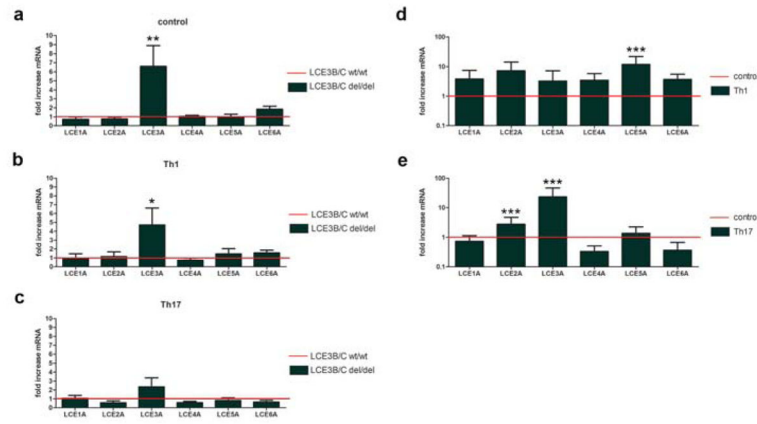


Figure 2. *In vitro* mRNA analysis mRNA expression of (a) control, (b) Th1- and (c) Th17-cytokine stimulated cells (N=5 per group) in wt/wt and del/del 3D reconstructed epidermis. (d–e) mRNA expression upon Th1- and Th17-cytokine stimulation (N=10). Error bars = SEM. Red lines = control (wt/wt or unstimulated, set to 1), * P<0.05, ** P<0.005, ***P<0.0005.

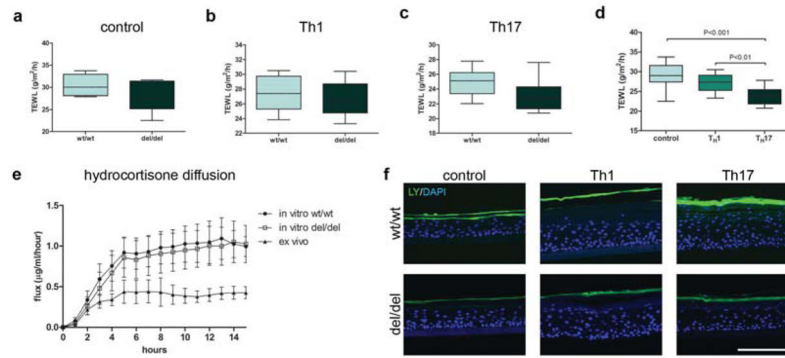


Figure 3. Barrier function of *LCE3B/C* wt/wt and del/del 3D reconstructed epidermis (a–d) TEWL of unstimulated, Th1- or Th17-stimulated epidermis. Repeated measures ANOVA plus Bonferroni post hoc with $P < 0.05$ significance. (e) SC hydrocortisone-diffusion assay. (f) Lucifer yellow (LY) penetration in unstimulated, Th1 or Th17-cytokine stimulated epidermis. Scale bar = 100 µm. N=5 per genotype group. Error bars = SEM.

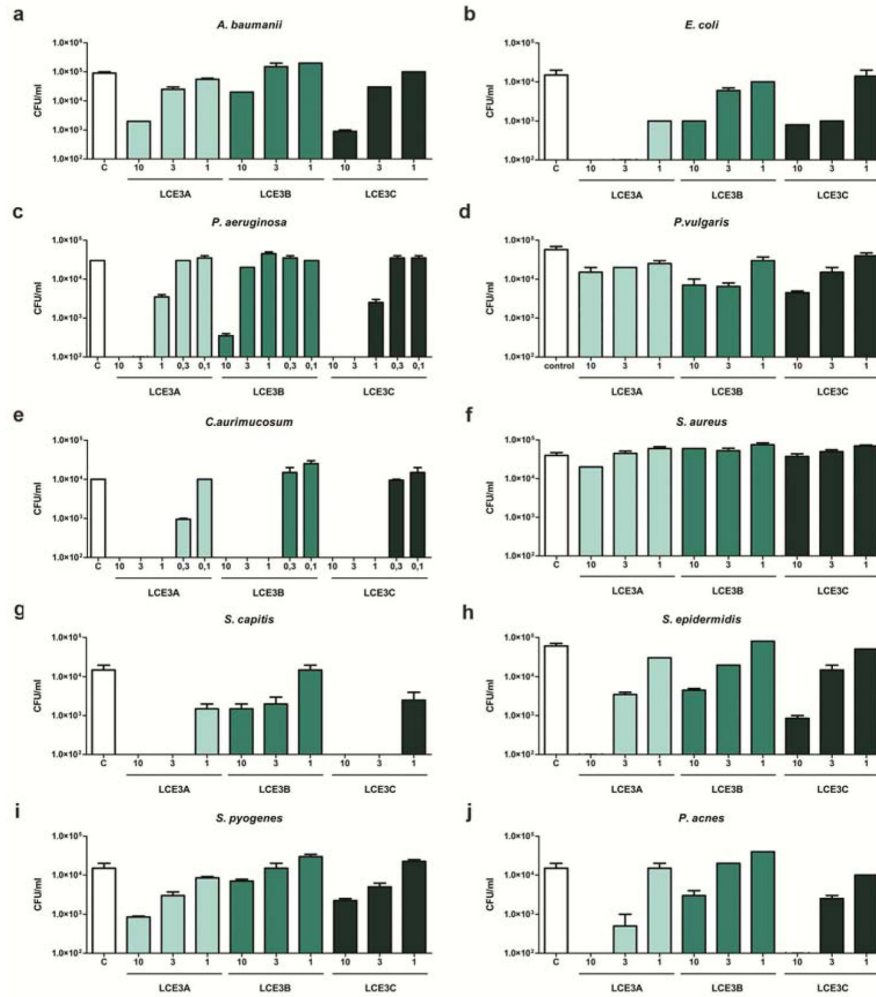


Figure 4. Antimicrobial activity of LCE3 proteins
 LCE3A, B, C were incubated with gram negative (a–d) and gram positive commensal and pathogenic bacteria (e–j) between 0.1 to 10 μ M protein. For specifications see Supplemental Table 4. 2×10^2 CFU/ml is the detection limit, therefore values $< 2 \times 10^2$ CFU/ml are not depicted. Error bars = SEM. CFU = colony forming units.

Table 1
Top significant e-QTL results in NN and PP skin between rs4112788 versus EDC genes

Note LCE3B is not expressed in more than 20% of the NN skin samples, therefore the eQTL analysis was not conducted.

Skin type	Gene	Relative location to deletion	Effect	p	FDR
Normal	<i>LCE3C</i> *	within	-	1.60×10^{-11}	1.96×10^{-9}
	<i>LCE3A</i> *	downstream	+	5.36×10^{-5}	3.30×10^{-3}
	<i>RPI1-216N14,9</i>	downstream	+	2.75×10^{-3}	1.13×10^{-1}
	<i>LCE1D</i>	downstream	-	7.1×10^{-3}	0.18
	<i>LINGO4</i>	upstream	-	7.29×10^{-3}	0.18
Psoriasis	<i>LCE3C</i> *	within	-	4.09×10^{-15}	5.11×10^{-13}
	<i>LCE3B</i> *	within	-	5.34×10^{-13}	3.34×10^{-11}
	<i>LCE3A</i> *	downstream	+	8.73×10^{-4}	3.64×10^{-2}
	<i>LCE1D</i>	downstream	-	1.92×10^{-2}	0.6
	<i>ILF2</i>	downstream	-	3.49×10^{-2}	0.67

* indicates significant at the false discovery rate (FDR \leq 0.1) level. "Effect" refers to whether the gene is up (+) or down (-) regulated in the presence of the G allele for rs4112788 (surrogate for del/del).