Human skin equivalents to study the prevention and treatment of wound infections

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CHAPTER 6

Reduced filaggrin expression is accompanied by increased *Staphylococcus aureus* colonization of epidermal skin models

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Abstract

Background Atopic dermatitis is an inflammatory skin disease that is characterized by a reduced skin barrier function, reduced filaggrin (FLG) expression as well as increased colonization by *Staphylococcus aureus.*

Objective The current study focused on the possible involvement of FLG in epidermal colonization by *S. aureus* and/or whether it affects the epidermal defence mechanisms, including the expression of antimicrobial peptides (AMPs) and enzymes involved in stratum corneum barrier lipid synthesis. Furthermore, IL-31 has been shown to reduce FLG expression, but its effects on bacterial colonization and on the expression of AMPs and enzymes involved in the barrier lipid synthesis are not known.

Material and Methods We established N/TERT-based epidermal models (NEMs), after FLG knockdown (FLG-KD) and/or cultured with IL-31, that were colonized with *S. aureus* for 24 hrs.

Results Both FLG-KD and IL-31 supplementation resulted in significantly increased epidermal *S. aureus* colonization, as well as in an upregulation of *S. aureus*-induced IL-8 expression. IL-31, but not FLG-KD, prevented *S. aureus*-induced upregulation of mRNA expression for the AMPs human-defensin 2 and -3 and RNAse7, whereas psoriasin expression remained unchanged. Furthermore, the *S. aureus* colonization induced changes in mRNA expression of ELOVL4 was not affected by FLG-KD, but was blocked by IL-31. Expression of SCD-1 and Gcase mRNA was reduced by IL-31, but not by FLG-KD.

Conclusion This study shows that NEMs, with FLG-KD and/or cultured in the presence of IL-31, mimics the skin of atopic dermatitis patients in several aspects, including enhanced bacterial colonization, increased inflammatory and reduced protective responses.

Introduction

Atopic dermatitis (AD) is a frequently occurring chronic inflammatory skin disease characterized by a reduced skin barrier function. Whereas over 90% of the patients with AD show skin colonization by *Staphylococcus aureus*, only 5% of the healthy individuals is colonized by this bacterium [1]. The mechanism underlying this increased host susceptibility in patients with AD is less understood. To survive on human skin, bacteria have to overcome various barriers, including acidic conditions, fatty acids and antimicrobial peptides (AMPs). *Staphylococcus aureus* colonization of healthy skin is usually transient. However, in case of a dysfunctional skin barrier as seen in patients with AD, *S. aureus* produces factors that promote adhesion, for example fibronectinbinding proteins, which result in an increased colonization and the subsequent inflammatory response [2]. In addition, S. aureus might play a role in the alterations in the stratum corneum (SC) lipid composition as seen in AD, through their suggested ability to alter the expression of enzymes involved in SC lipid synthesis [3].

To date, the association between AD and filaggrin (FLG) mutations is one of the strongest genotype linkage observed in complex human genetic disorders [4, 5]. FLG is initially transcribed as profilaggrin, which during epidermal differentiation, is cleaved into 10–12 FLG monomers that bind to keratin filaments to ensure corneocyte rigidity. Subsequently, FLG is broken down by various enzymes, including caspase-14, into small peptides and free amino acids, that is urocanic acid (UCA) and pyrrolidonic carboxylic acid (PCA), which are components of the natural moisturizing factor [6]. In addition to their contribution to skin hydration, UCA and PCA may also be involved in regulation of SC pH, and reduced UCA and PCA levels were found to be associated with FLG null mutations as well as with disease severity [7–9].

Lesional AD skin is characterized by increased levels of Th2 cytokines of which IL-31 has been shown to play an important role [10]. IL-31-overexpressing transgenic mice display skin inflammation, pruritus and severe dermatitis [11, 12]. Furthermore, IL-31 has been shown to reduce FLG expression in organotypic human skin models [13]. While the Th2 cytokines, IL-4 and IL-13, have been shown to reduce the expression of the AMPs human b-defensin 2 and 3 (hBD-2 and hBD-3) in vitro, the effect of IL-31 on AMP expression and the innate immune response is still unknown [14, 15]. Furthermore, IL-8 is known to play an important role in skin inflammation, both after injury and infection, through attracting various immune cells, for example neutrophils and dendritic cells, and

increased IL-8 expression was observed in AD which was associated with the presence of *S. aureus* [16].

The aim of this study was to evaluate whether reduced FLG expression contributes to epidermal *S. aureus* colonization. Therefore, we established a novel N/TERT-based epidermal model (NEM) after knockdown of filaggrin (FLG-KD) and/or IL-31 supplementation. These NEMs allowed us to study whether these two factors affect epidermal colonization with *S. aureus*. Moreover, we could study their effects on the expression of inflammatory mediators, AMPs and enzymes involved in SC barrier lipid synthesis.

Here we demonstrate for the first time that both FLGKD and IL-31 supplementation resulted in enhanced epidermal *S. aureus* colonization of NEMs. This *S. aureus* colonized in vitro epidermal model could therefore be a promising tool for testing novel antibacterial agents for the treatment of the infectious complications of AD.

Material and Methods

N/TERT keratinocyte cell line

The N/TERT keratinocyte cell line was purchased from Harvard Medical School (USA) and cultured under low confluency (<40%) at 37°C and 7.3% CO_2 in keratinocyte-serum free medium (K-SFM, Invitrogen, Breda, the Netherlands) supplemented with the following final concentrations; 25 µg/ml BPE, 0.4 mM $CaCl_2$, 0.2 ng/ml hEGF, 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Bleiswijk, the Netherlands). The culture medium was refreshed every 3 days.

Transfection

Knockdown of filaggrin in N/TERT cells was performed as described earlier [17]. In short, N/TERT cells were transfected with pLKO.1-puro plasmid containing shRNA against filaggrin (TRCN0000083680) or control (Mock, TRC1.5-SHC001) using the Amaxa human keratinocyte nucleofector kit (Lonza, Breda, the Netherlands). After transfection, the cells were cultured similar to the N/TERT cells, except for the addition of puromycin to the KSFM medium (1 µg/ml).

N/TERT-based epidermal model

The NEMs were constructed by seeding $2x10^5$ cells on insert filters (ThinCert 12 well, Greiner bio-one, Alphen aan den Rijn, the Netherlands) in Dermalife K medium including lifefactors (Lifeline Cell Technology, Walkersville, MD) till confluency. Dermalife medium was supplemented with 10 µM L-carnitine (Sigma, Zwijndrecht, the Netherlands), 10 mM L-serine (Sigma), 1 µM hydrocortisone, 1 µM isoproterenol, 0.1 µM insulin, 53 µM selenious acid (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen). Thereafter, they were cultured in CNT medium (basal medium plus supplement kit, CellnTec, Bern, Switserland), supplemented with 24 µM bovine serum albumin, 25 µM palmitic acid, 15 µM linoleic acid and 7 µM arachidonic acid (all from Sigma). Next, cultures were lifted to the air/liquid interface and after one day the linoleic acid concentration was increased to 30 µM. Medium was refreshed every 2 days. NEMs were cultured at the air/liquid interface for 10 days at 37 °C and 7.3% CO_2 prior to infection. For IL-31 supplementation, IL-31 (30 ng/ml, R&D Systems, Minneapolis, USA) was added to the medium once 5 days prior to inoculation and once on the day of inoculation.

Colonization of NEMs

Methicillin resistant *S. aureus* (MRSA) strain LUH14616, a clinical isolate that was kindly provided by S. Croes [18], was used in this study. Bacteria were preserved for prolonged periods in nutrient broth supplemented with 20% (vol/vol) glycerol at -80°C. Inocula from frozen cultures were grown overnight at 37°C on sheep blood agar plates (bioMérieux, Zaltbommel, the Netherlands). To create a log-phase growth culture, LUH14616 was cultured for 2.5 hrs at 37°C in Tryptic Soy Broth (TSB) (Oxoid, badhoevedorp, the Netherlands) at 200 rpm. This suspension was centrifuged for 10 min at 1200 rpm and resuspended in phosphate-buffered saline (PBS; pH 7.4) to a concentration of $3.3x10^5$ CFU/ml, calculated from the absorbance of the suspension at 600 nm. Next, NEMs were inoculated with 300 µl of the bacterial suspension at 37°C in 7.3% CO_2. After 1 hr, the bacterial suspension was aspirated to remove the non-adherent bacteria. After 24 hrs the non-adherent bacteria were removed by washing the NEMs with 1 ml of PBS. This bacterial suspension was serial diluted and plated onto diagnostic sensitivity test agar (DST) (Oxiod). These plates were incubated for 24 hrs at 37°C, after which the colony forming units (CFU) were counted. Lower limit of detection was 20 CFU/ml. To determine the number of adherent bacteria, the NEMs were mechanically homogenized using a glass Potter-Elvehjem tissue homogenizer and resuspended in 1 ml PBS, then serial diluted and plated onto

DST plates and the number of CFUs was determined as above and is described previously [19].

Immunohistochemistry

Morphological and immunofluorescent analysis was performed on 5 µm paraffin-embedded NEM sections. For analysis of morphology sections were cut, deparaffinized, rehydrated, and stained with haematoxylin and eosin (HE). For analysis of filaggrin expression, sections were cut, deparaffinised and rehydrated, followed by heat-mediated antigen retrieval. After blocking non-specific binding using PBS containing 1% bovine serum albumin (BSA, Sigma) and 2% normal human serum (NHS, Sanquin, Leiden, the Netherlands), the sections were incubated overnight at 4°C with primary antibody for filaggrin (1:1000; Covance, Rotterdam, the Netherlands). After washing with PBS sections were incubated with secondary antibody Goat anti-Rabbit (Rhodamine Red, 1:300, Jackson ImmunoResearch, Amsterdam, the Netherlands). The sections were mounted with Vectashield containing DAPI for visualization of the nuclei (Vector Laboratories, Amsterdam, the Netherlands).

RNA isolation, cDNA synthesis and qPCR analysis

RNA isolation was performed using the Qiagen RNeasy mini kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's instructions. Prior to RNA isolation, NEMs were incubated in 500 µl of RLT buffer (Qiagen) and homogenized using a fine syringe. To remove bacterial residues, the samples were spun down at 10.000 rpm for 5 min after which 350 µl of RLT buffer was aspirated.

cDNA was synthesized with 1 µg RNA using the iScript cDNA synthesis kit (BioRad, Veenendaal, The Netherlands) according to the manufacturer's instructions. PCR reactions were based on the SYBR Green method (BioRad) using the CFX384 system (BioRad) and the primers listed in table 1. Expression analysis was performed using the CFX software with the ΔΔCt method and the reference genes β-2-microglobulin (B2M) and β glucuronidase (GUSB).

Table 1: primer sequences

target	Forward	Reverse
Filaggrin	GGGAAGTTATCTTTTCCTGTC	GATGTGCTAGCCCTGATGTTG
hBD-2	TGATGCCTCTTCCAGGTGTTT	GGATGACATATGGCTCCACTCTTA
hBD-3	TTATTGCAGAGTCAGAGGCGG	CGAGCACTTGCCGATCTGTT
Rnase 7	GGAGTCACAGCACGAAGACCA	CATGGCTGAGTTGCATGCTTGA
Psoriasin	AGACGTGATGACAAGATTGAC	TGTCCTTTTTCTCAAAGACGTC
Elovl 1	GGAGCTCCAGGTATTGCCAAGG	AGCCGTGGTCCCTGTAGAGCA
Elovl 4	GGGTTGCAGGAGGACAAGCATT	GAGACAGTGCCGTGTGCCCAA
Elovl 6	TCGGTGCTCTTCGAACTGGTGC	GTATCTCCTAGTTCGGGTGCTTTGC
SCD-1	ACAGTGCTGCCCACCTCTTCG	CCCTCACCCACAGCTCCAAGTG
aSmase	CTCGGGCTGAAGAAGGAACCCAA	ATTGGCACACGGCAGGTGGT
Gcase	ACCACCTTGGCCACTTCAGCAAG	TCCAGGAAGCCCACAGCAGGA
B2M	GATGAGTATGCCTGCCGTGTG	CAAACCTCGGGTAGCATCAT
GUSB	CTCATTTGGAATTTTGCCGATT	CCGAGTGAAGATCCCCTTTTTA

Enzyme-linked immunosorbent assays

Protein content of culture media was determined by enzyme-linked immunosorbent assays (ELISA). Measured were the levels of interleukin-8 (IL-8) (Biosource, Invitrogen), and human beta defensin-2 (hBD-2) (Phoenix Pharmaceuticals, Karlsruhe, Germany). All measurements were performed according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was conducted using Graph Pad Prism, version 5.04. The effect of IL-31 on FLG mRNA expression was analysed using a paired t-test. CFU data and IL-8 ELISA data was analysed using one-way ANOVA. qPCR data was analyzed by two-way ANOVA. P values of <0.05 were considered significant.

Results

Reduced filaggrin expression after knockdown and/or IL-31 supplementation in NEMs

To study the role of FLG in epidermal *S. aureus* colonization, we established a NEM after FLG-KD. In addition, since the Th2 cytokine IL-31, was shown to reduce FLG expression in human organotypic skin models and is associated with AD, IL-31 (30 ng/ml) was supplemented to the culture medium of both Mock

and FLG-KD NEMs [13]. After 10 days of air-exposed culturing, the NEMs were evaluated for their morphology. NEMs that were generated after FLG-KD displayed an epidermal morphology similar to native human skin, i.e. all epidermal layers, including the SC, were present (figure 1a). FLG-KD resulted in 70% reduction in FLG mRNA expression compared to the control, Mock ($P<0.01$, Fig. 1b). Reduced FLG expression was confirmed at protein level by immunofluorescent staining for FLG (figure 1c).

Supplementation of the NEMs with IL-31 for five days prior to termination of the cultures did not affect epidermal morphology (figure 1a). IL-31 supplementation resulted in a reduction of FLG mRNA expression of approximately 50% compared to control NEMs (figure 1b). The IL-31 induced reduction of FLG expression was confirmed on protein levels by immunofluorescent staining (figure. 1c).

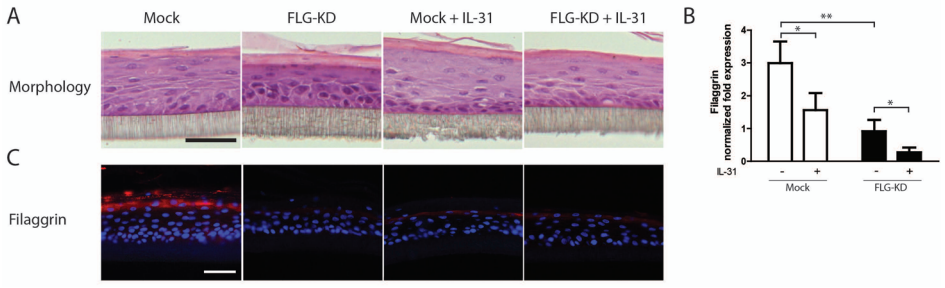

Figure 1. FLG-KD and IL-31 supplementation reduce filaggrin mRNA and protein expression in NEMs. (a) HE staining of the NEMs. Both the Mock and the FLG-KD NEMs show a normal differentiated epidermis with the presence of all epidermal layers. Supplementation of IL-31 (30 ng/mL) for 5 days did not affect epidermal morphology; scale bar indicates 50 µm (b) Reduced FLG mRNA expression after FLG-KD and/or IL-31 supplementation. mRNA data represent mean + SEM of three independent experiments. White bars indicate Mock NEMs, black bars indicate FLG-KD NEMs. *indicates $P < 0.05$; **indicates $P < 0.01$. (c) Reduced immunofluorescent staining for FLG after FLG-KD and IL-31 supplementation. Scale bar indicates 50 µm.

FLG-KD and IL-31 increase *S. aureus* colonization of NEMs

Next, we evaluated the effect of FLG-KD and/or presence of IL-31 on epidermal *S. aureus* colonization. For this purpose, we used the clinical relevant MRSA strain LUH14616. After inoculating 1 x 10^5 bacteria onto the skin model for 1 h, the suspensions were aspirated from the skin models to remove the non-adherent bacteria. After 24 h, the number of detachable and adherent bacteria were determined by CFU counting. As shown in figure 2a, 24 h post inoculation *S. aureus* colonies were present on the SC of the NEMs (figure 2a, right panel). No effects of bacterial colonization on the epidermal morphology were observed.

We did not observe significant differences in the number of detachable bacteria between the different models (figure 2b). However, compared to the control (inoculated Mock NEMs) the number of adherent bacteria after IL-31 supplementation or FLG-KD was increased by 2.3-fold ($P<0.05$) and 2.4-fold ($P<0.05$), respectively (figure 2c). Supplementation of IL-31 to FLG-KD NEMs resulted in a 3.3-fold increase ($P<0.01$) in adherent bacteria compared to control NEMs. After IL-31 supplementation, the number of adherent bacteria on FLG-KD NEMs was 1.4-fold higher ($P<0.05$) compared to IL-31 supplemented Mock NEMs, indicating an additional effect of FLG-KD in presence of IL-31. Supplementation of IL-31 to FLG-KD NEMs did not result in a significant increase in the number of adherent bacteria compared to FLG-KD alone (figure 2c).

Effect of FLG-KD and/or IL-31 on IL-8 release by NEMs after *S. aureus* colonization

Next, we wanted to know whether the epidermal inflammatory response of NEMs was affected by *S. aureus*, by FLG-KD and/or by IL-31. Therefore, we assessed the levels of secreted IL-8 in the culture medium after 24 h of *S. aureus* colonization. Our results show that *S. aureus* colonization on both the Mock and the FLG-KD NEMs resulted in a significant two fold increase of IL-8 ($P<0.001$) (Fig. 2d). Although we observed higher levels of IL-8 in the FLG-KD NEM medium before and after *S. aureus* colonization compared to Mock NEMs, these differences were not significantly different (figure 2d). Supplementation of IL-31 did not affect IL-8 secretion by NEMs. However, subsequent *S. aureus* colonization resulted in 4.5-6.5-fold increase ($P<0.001$) in IL-8 release from the NEMs, irrespective of FLG-KD when compared to non-supplemented or to IL-31 supplementation alone (figure 2d). No significant additional effect of FLG-KD on the IL-8 release after IL-31 supplementation was observed (figure 2d).

IL-31 prevents upregulation of AMPs after *S. aureus* colonization

AMPs are associated with local antimicrobial activity, immunomodulation and wound healing. Furthermore, it is known that AMP expression is altered in AD [20-22]. Therefore, we evaluated the expression of several AMPs, hBD-2 and hBD-3, RNAse7 and psoriasin after *S. aureus* colonization on the FLG-KD and IL-31 supplemented NEMs. The mRNA expression for hBD-2, hBD-3 and RNase7 was not affected by FLG-KD or IL-31 (figure 3). *S. aureus* colonization resulted in a significant increase of hBD-2, hBD-3 and RNase7 mRNA expression, irrespective of FLG-KD (figure 3a, c,d). IL-31 supplementation to the medium followed by *S. aureus* colonization of the NEMs completely prevented upregulation of hBD-2, hBD-3 and RNase7 mRNA expression (figure 3a-c). For hBD-2, protein analysis revealed similar results, IL-31 blocked the *S. aureus*-induced increase in hBD-2

expression (figure 3b) The mRNA expression of psoriasin was not affected by either FLG-KD, IL-31 supplementation or *S. aureus* colonization (figure 3e).

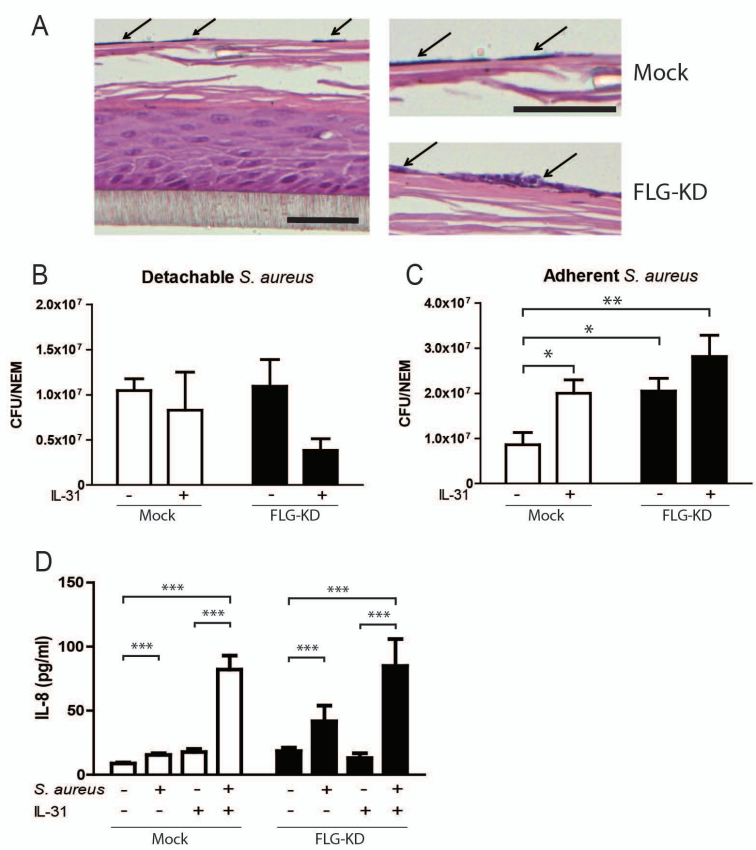

Figure 2. FLG-KD and/or IL-31 supplementation results in increased S. *aureus* colonization of NEMs. (a) *S. aureus* on the SC of NEMs is indicated with an arrow. The right panel shows a close-up of adherent *S. aureus* on the SC of a Mock and FLG-KD NEM. Scale bars indicate 50 µm. (b) The number of detachable bacteria 24 h after inoculation of the NEMs was not affected by FLG-KD and/or IL-31 supplementation. (c) The number of adherent S. aureus 24 h after inoculation of NEMs with S. aureus was increased on FLG-KD NEMs and NEMs cultured with IL-31, respectively, compared to the inoculated Mock NEMs. FLG-KD with IL-31 supplementation resulted increased number of adherent S. aureus compared to the inoculated Mock NEMs. Data represent mean + SEM of four independent experiments (d) IL-8 levels in culture media of FLG-KD and/or IL-31 cultured NEMs colonized for 24 h by S. aureus or without S. aureus colonization were measured by ELISA. Data represent mean + SEM of 3 independent experiments. White bars indicate Mock NEMs; black bars indicate FLG-KD NEMs. *indicates $P < 0.05$; **indicates $P < 0.01$ and ***indicates $P < 0.001$.

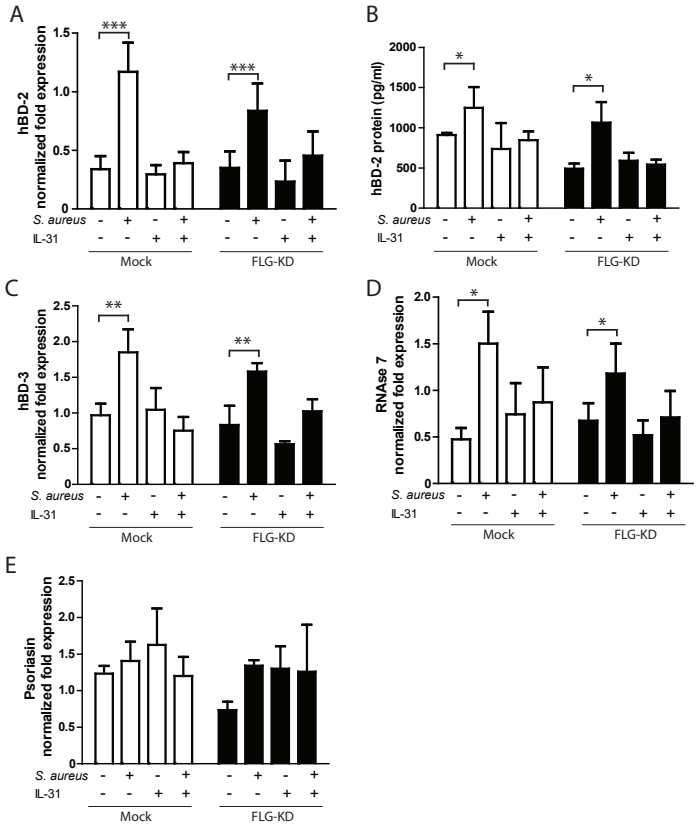

Figure 3. Effects of FLG-KD and IL-31 supplementation on AMPs mRNA expression by NEMs after *S. aureus* colonization. (a) hBD-2 mRNA expression, (b) hBD-2 protein expression, (c) hBD-3 mRNA expression, (d) RNAse 7 mRNA expression and (e) psoriasin mRNA expression. White bars represent the Mock NEMs, and black bars FLG-KD NEMs. Data represent mean + SEM of three independent experiments. *indicates $P < 0.05$; **indicates $P < 0.01$ and ***indicates $P < 0.001$.

Effect of FLG-KD and/or IL-31 on expression of enzymes involved in SC lipid synthesis after *Staphylococcus aureus* colonization

In addition to their effects on the expression of AMPs, we wondered whether FLG-KD and/or IL-31 supplementation to NEMs affects the expression of enzymes that are involved in SC lipid synthesis following *S. aureus* colonization. Free fatty acids (FFA) and ceramides (CERs) are two SC lipid classes that are crucial components of the SC, which protects against external factors, including bacteria. We evaluated whether *S. aureus*, FLG-KD and/or IL-31 supplementation affected mRNA expression of the elongases ELOVL1, -4 -6, and of Stearly-CoA desaturase 1 (SCD-1), which are involved in FFA synthesis, and of glucocerebrosidase (Gcase) and acid Sphingomyelinase (aSmase), which are involved in

CER synthesis were altered by *S. aureus* colonization. The expression of ELOVL1 and ELOVL6 was not significantly altered by *S. aureus* colonization or IL-31 supplementation (figure 4a and c). However, colonization of NEMs by *S. aureus* resulted in increased ELOVL4 mRNA expression, irrespective of FLG-KD (figure 4b). However, IL-31 supplementation prevented the *S. aureus*-induced increase in expression irrespective of FLG-KD (figure 4b). SCD-1 and Gcase mRNA expression by NEMs was not affected by *S.aureus* colonization alone, however supplementation of IL-31 prior to *S.aureus* colonization resulted in significant down-regulation of these enzymes in both the Mock and FLG-KD NEMs (Fig. 4d). In addition, the mRNA expression of aSmase was not affected by bacterial colonization, FLG-KD or IL-31 (figure 4f).

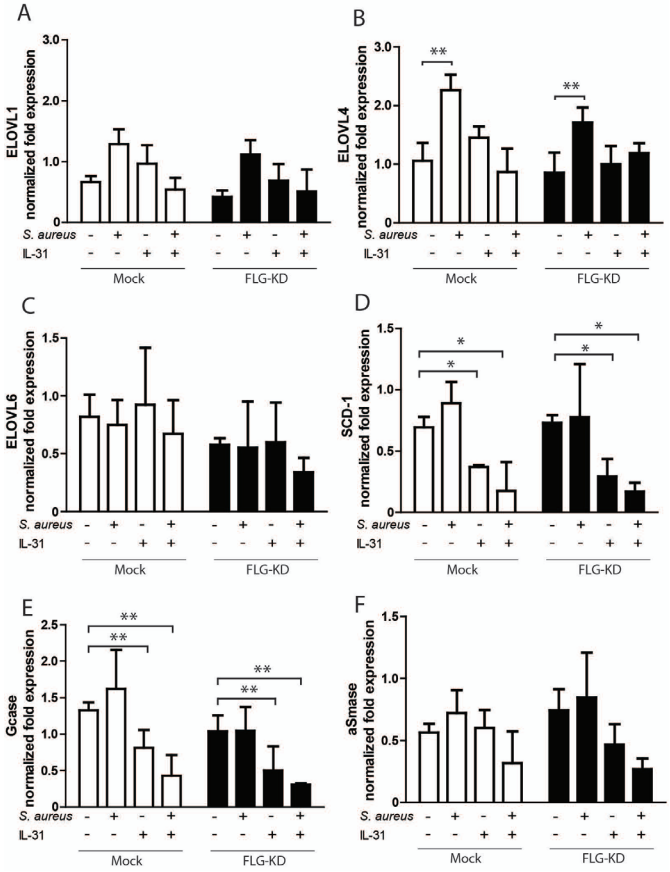

Figure 4. Effects of FLG-KD and IL-31 supplementation on the mRNA expression of enzymes involved in SC lipid synthesis, following Staphylococcus aureus colonization. (a) ELOVL1, (b) ELOVL4, (c) ELOVL6, (d) SCD-1, (e) Gcase and (f) aSmase mRNA expression. White bars represent the Mock NEMs, and black bars FLG-KD NEMs. Data represent mean + SEM of three independent experiments. *indicates $P < 0.05$; **indicates $P < 0.01$.

Discussion

Skin of patients with AD is characterized by a high frequency of *S. aureus* colonization [1]. Impaired skin barrier function is a hallmark of AD, but the underlying cause of this barrier defect is still unknown [23, 24]. Despite the highly reproducible finding that FLG mutations are a predisposing factor for AD development, its exact role in AD pathogenesis is not fully understood. Previously, we have shown that FLG-KD did not affect the SC lipid properties of reconstructed full thickness skin models [17]. Reduced FLG expression, due to FLG mutations or due to the presence of Th2 cytokines, for example IL-31, has been proposed to be an important contributor to skin barrier defects in AD [13, 25]. However, whether increased colonization is a cause or a con- sequence of a defective skin barrier or whether the presence of cytokines is the initiating cause of AD remains subject to extensive research.

In the current study, we utilized a NEM with reduced FLG expression, through FLG-KD or IL-31 supplementation, to evaluate its role in epidermal colonization by *S. aureus*. These NEMs were evaluated for their epidermal responses to this bacterium, such as the production of the pro-inflammatory chemokine IL-8, AMPs and expression of enzymes involved in the SC lipid synthesis. Due to their similarities with native human skin, for example presence of all epidermal layers including the SC, and the expression epidermal markers, for example proliferation marker Ki67, early differentiation marker K10 and late differentiation marker FLG, NEMs are suitable for studying many aspects of epidermal barrier function [26]. However, such epidermal models lack immune cells such as Langerhans cells.

Most data obtained for studying the effects of staphylococci, including *S. aureus*, and their secreted products on keratinocytes are performed using keratinocytes cultured in monolayer. Although keratinocytes cultured in monolayer are able to differentiate under submerged culture conditions, these keratinocytes do not form a SC. The SC is the epidermal layer mainly responsible for epidermal barrier function and is therefore necessary for studying epidermal S. aureus colonization and its consequences. Miajlovic and colleagues have shown that PCA and UCA, both FLG breakdown products, can reduce *S. aureus* growth rate as observed by measuring cell densities in vitro, which suggests that the epidermal 'acid mantle' functions as an additional antimicrobial barrier [27]. Using NEMs, we found that both FLG-KD and IL-31 supplementation resulted in increased epidermal colonization by *S. aureus*, similar to what is seen in AD [1, 28]. These findings implicate that FLG is important for protection against epidermal *S. aureus*

colonization. Using full thickness skin models, *S. aureus* strain LUH14616 was shown to induce the release of the inflammatory chemokine IL-8 by epidermal keratinocytes [29]. Although no much is known about the role of IL-8 in AD, its up-regulation in AD was shown to be associated with the presence of *S. aureus* [16]. We have found that the S. aureus-induced IL-8 response by epidermal models was not affected by FLG-KD, but significantly enhanced following exposure to IL-31, suggesting that enhanced S. aureus colonization alone due to FLG-KD is not sufficient to evoke an inflammatory response and requires the presence of (AD-related) cytokines such as IL-31.

While the role of AMPs in psoriasis is well established, their role in AD is still not completely known. Recent studies have shown that both lesional and nonlesional AD skin showed alterations in the expression of various AMPs, including hBD-2, hBD-3, RNAse7 and psoriasin [20, 21]. In line with a previous study, using full thickness skin models [26], we found that following *S. aureus* colonization of NEMs, the mRNA expression of hBD-2, hBD-3 and RNAse7 was increased, whereas psoriasin mRNA expression was not affected. FLG-KD did not affect the increase in AMP expression, whereas IL-31 supplementation prior to inoculation prevented the increase in AMP expression. The bacterial superantigen, staphylococcal enterotoxin B, has been shown to rapidly induce IL-31 mRNA expression in skin from patients with AD, whereas other studies using mouse models revealed that IL-31 is important into the induction of scratching behaviour [20, 30–32]. Furthermore, AMPs have been shown to induce IL-31 secretion by mast cells [33]. These studies clearly show the importance of *S. aureus* and IL-31 in the development of AD. Our results suggest that IL-31 might act as a repressor of up-regulation of AMP expression by keratinocytes in response to *S. aureus*.

The SC consists of corneocytes surrounded by an extracellular lipid matrix mainly composed of cholesterol, CERs and FFAs. Besides their function in maintaining proper SC barrier function, some lipids such as certain FFAs and CERs have roles in host defences against potential pathogenic or opportunistic microorganisms such as *S. aureus* [34–36]. Furthermore, the reduced CER and FFA chain lengths that were found in the SC of patients with AD were suggested to be caused by *S. aureus*-induced changes in expression of enzymes involved in SC lipid synthesis [3, 37]. Two enzymes involved in FFA synthesis, ELOVL1 and ELOVL4, have been shown to be important for the skin barrier function, and absence of one of these enzymes resulted in impaired barrier function in mice [38, 39]. Using a NEM, we found that ELOVL4 expression was up-regulated in response to *S. aureus* colonization, irrespective of FLG-KD, but was prevented by IL-31. In

addition, we have found down-regulation of SCD-1 and Gcase after S. aureus colonization in the presence of IL-31. These alterations in enzyme expression suggest bacterial colonization as an additional factor for alterations in the skin barrier function, besides the presence of inflammatory cytokines. However, future research is needed to evaluate whether *S. aureus* indeed contributes to the changes in lipid composition and reduced barrier function as seen in AD.

In conclusion, we have demonstrated that reduced FLG expression resulted in increased epidermal *S. aureus* colonization. However, increased colonization due to FLG-KD alone was not sufficient to alter the epidermal responses to these bacteria. On the contrary, IL-31 shifted a defensive response towards a pro-inflammatory response, by inducing more IL-8 production, as well as repressing AMP up-regulation. In addition, we have shown that *S. aureus* colonization of NEMs after IL-31 supplementation resulted in the down-regulation of Gcase and SCD-1 which are involved in CER and FFA synthesis. The FLG-KD epidermal skin model used in this study resembles non-lesional AD skin in terms of increased *S. aureus* colonization and altered AMP expression, whereas supplementation of this model with Th2 cytokines can be used to mimic features that are seen in lesional AD skin. These human skin models might therefore be a useful tool for screening of novel therapeutic antimicrobial compounds that targets the infectious complications of *S. aureus* in AD.

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Conflict of interest

The authors declare no conflict of interest .

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