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Staphylococcus aureus exploits epidermal barrier defects in atopic dermatitis to trigger cytokine expression

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Abstract

Patients with atopic dermatitis (AD) have an abnormal skin barrier and are frequently colonized by *S. aureus*. In this study we investigated if *S. aureus* penetrates the epidermal barrier of subjects with AD and sought to understand the mechanism and functional significance of this entry. *S. aureus* was observed to be more abundant in the dermis of lesional skin from AD patients. Bacterial entry past the epidermis was observed in cultured human skin equivalents and in mice, but found to be increased in the skin of cathelicidin knockout ($Camp^{-/-}$) and ovalbumin-sensitized filaggrin mutant ($FLG^{ft/ft}$) mice. *S. aureus* penetration through the epidermis was dependent on bacterial viability and protease activity as killed bacteria or a protease-null mutant strain of *S. aureus* was unable to penetrate. Entry of *S. aureus* directly correlated with increased expression of IL4, IL13, IL22, TSLP and other cytokines associated with AD, and with decreased expression of cathelicidin. These data illustrate how abnormalities of the epidermal barrier in AD can alter the balance of *S. aureus* entry into the dermis and provides an explanation for how such dermal dysbiosis results in increased inflammatory cytokines and exacerbation of disease.

Keywords

Atopic dermatitis; Cathelicidin; Microbiome; *Staphylococcus aureus*

INTRODUCTION

The microbial community "microbiome" can have both beneficial and detrimental functions (Gallo and Nakatsuji, 2011). For example, *Staphylococcus epidermidis*, a predominant resident on healthy human skin, can suppress inflammation after skin injury, maintain immune tolerance to commensals, modify cutaneous T-cell development, and enhance innate

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CONFLICT OF INTEREST

The authors state no conflict of interest.

immune defense by inducing expression of antimicrobial peptides (AMPs) (Cogen et al., 2010; Lai et al., 2010; Li et al., 2013; Naik et al., 2015; Naik et al., 2012; Scharschmidt et al., 2015; Wang et al., 2012; Wanke et al., 2011). Conversely, imbalance of the microbiome (dysbiosis), appears to contribute to the pathogenesis of some skin diseases. Strong associations have been shown between dysbiosis and the clinical phenotype of atopic dermatitis (AD) (Leung and Guttman-Yassky, 2014). For example, AD subjects are well known to have increased colonization by *Staphylococcus aureus* (*S. aureus*) (Leyden et al., 1974) and a loss in bacterial diversity on the skin (Kong et al., 2012). Furthermore, recent mechanistic studies have demonstrated that *S. aureus* can drive development of AD-like lesions in mice (Kobayashi et al., 2015). These findings suggest that a better understanding of how bacteria influence skin immunity may provide important clues to improve management of AD.

S. aureus can cause inflammation by inducing T cell-independent B cell expansion, initiating the production of proinflammatory cytokines such as thymic stromal lymphopoietin (TSLP) from keratinocytes, and stimulating mast cell degranulation, resulting in T_H2 skewing (Bekeredjian-Ding et al., 2007; Nakamura et al., 2013; Vu et al., 2010). *S. aureus* also disrupts proteolytic balance in the skin by inducing multiple metalloproteases in dermal fibroblasts (Kanangat et al., 2006). However, because of the complex structures and cell networks that comprise mammalian skin, the mechanism by which *S. aureus* disrupts cutaneous inflammatory homeostasis is incompletely understood. It appears that most beneficial and detrimental actions of skin bacteria are dependent on their capacity to interact with host cells that reside under the surface stratum corneum. Until recently it was unclear how skin surface microbes could influence immunological responses through a stratum corneum structure.

We recently observed that bacteria residing on the epidermis can be observed within the dermis of healthy normal human skin (Nakatsuji et al., 2013). This surprising observation that bacteria can penetrate the epidermis illustrated how bacteria position themselves to directly influence immune responses. The epidermis apparently acts as a regulator of microbiome entry rather than as an absolute barrier to microbes. This suggests that epidermal barrier defects such as the loss-of-function mutations found within the filaggrin gene (*FLG*) (Bisgaard et al., 2008; Palmer et al., 2006; Sandilands et al., 2007; Smith et al., 2006) could promote disease. In addition, the epidermis of AD subjects can have a decreased capacity to produce AMPs such as cathelicidin and β-defensins (Hata et al., 2010; Howell et al., 2006a; Howell et al., 2006b; Mallbris et al., 2010; Ong et al., 2002). Such antimicrobial or physical barrier defects may facilitate physical penetration of the epidermis by bacteria that otherwise would not trigger inflammation on normal skin.

In this study we sought to determine the mechanism by which *S. aureus* penetrates the epidermis. We hypothesized that the altered physical and antimicrobial barrier of the skin in AD will result in enhanced penetration of *S. aureus* across the epidermal surface, and that this can contribute to the loss of immune homeostasis. Such an interaction between bacteria on the skin surface and cells in the dermis provides a unifying hypothesis to explain why genetic or environmental defects in the skin barrier drive immunologic abnormalities of AD.

RESULTS

Dysbiosis of the bacterial community in the dermis of patients with atopic dermatitis

To examine the microbial community in the dermis of skin from subjects with AD, skin biopsies from non-AD control subjects and lesional and nonlesional sites of AD patients were obtained. Biopsies of lesional AD skin did not include skin sites that were excoriated. Skin samples were separated into epidermal and dermal tissues by laser-capture microdissection (LCM) (Figure S1a) and bacterial DNA contained in each tissue was analyzed by qPCR and pyrosequencing. The absolute abundance of 16S rDNA in the LCM-dissected epidermis and dermis was higher from lesional skin than that of non-AD and nonlesional AD skin (Figure 1a). *S. aureus* was detected by qPCR in both epidermis and dermis of lesional skin, but not in non-AD and nonlesional AD skin (Figure 1b). *S. epidermidis* DNA was also detected in epidermis and dermis of non-AD and AD skin, and was higher in dermis of lesional skin than that of non-AD and nonlesional AD skin (Figure 1c). Pyrosequencing for 16S rDNA detected relatively higher abundance of Firmicutes (phylum of Staphylococcus species) in epidermal and dermal compartments of lesional AD skin than those of nonlesional AD skin (Figure 1d). To detect potential contamination, non-tissue controls (NTC) prepared from the same tissue blocks and assay reagents, and DNA was not detected in these samples (Figure 1a–c).

To further confirm these observations, lesional and nonlesional skin was stained for *S. aureus*, and this directly demonstrated that *S. aureus* was more abundant within the epidermis and dermis of lesional skin compared to nonlesional samples (Figure 1e–f). Notably, *S. aureus* was independently detected in the epidermis and dermis of lesional AD skin and not associated with $CD11c^+$ immune cells (Figure 1h), thus suggesting a penetration mechanism independent from classical phagocytic cells. No immunoreactivity was seen with control IgG (Figure 1g,i).

S. aureus penetrates cultured human skin equivalents

To begin to understand how *S. aureus* may enter the dermis, we next compared the capacity of live and dead *S. aureus* to penetrate the epidermis of an organotypic human skin equivalent. Immunohistochemical analysis was done on samples processed at 13, 24 and 48 hours after *S. aureus* application. Immunostaining of the skin equivalent demonstrated that *S. aureus* were present at progressively deeper layers in the epidermis in a time-dependent manner, and detectable below the basement membrane by 48hrs (Figure 2a–c). In contrast, despite application of a larger bacterial load, bacteria that were killed by UV radiation remained on the surface at 48 hrs (Figure 2d). These data demonstrated that *S. aureus* must be viable to penetrate the epidermis.

Cathelicidin inhibits bacterial entry into dermis

Since we observed that penetration of skin required viable bacteria, we hypothesized that AMP activity may act to limit bacterial entry into the dermis. To test this, *S. aureus* was applied to dorsal skin of cathelicidin (*Camp*) knock out or wild type (WT) mice. Mouse skin was then separated by LCM into control, epidermal, dermal and dermal adipose compartments (Figure S1b). After application to the skin surface, *S. aureus* abundance was

not statistically different on the epidermis of WT and *Camp*$^{-/-}$ mice (Figure 3a). However, *S. aureus* was significantly more abundant in the dermis and adipose tissue of *Camp*$^{-/-}$ mice than WT mice (Figure 3b–d). These data suggest that the antimicrobial barrier provided by cathelicidin limits entry of *S. aureus* into the skin and is one variable controlling bacterial penetration into the dermis.

S. aureus penetration is enhanced in a mouse loss-of-function of filaggrin model of atopic dermatitis

We next evaluated entry of *S. aureus* in loss-of-function of filaggrin (*FLG*$^{ft/ft}$) mice (Fallon et al., 2009). Similar to previous reports, *FLG*$^{ft/ft}$ mice developed AD-like skin inflammation after mechanical barrier disruption by tape-stripping and repeated application of ovalbumin (OVA), whereas WT mice or *FLG*$^{ft/ft}$ without OVA sensitization had much lesser inflammation (Figure S2). Consistent with this observation, the skin of *FLG*$^{ft/ft}$ mice after tape-stripping and OVA sensitization showed enhanced transepidermal water loss (TEWL) compared to WT mice (Figure 4a). Twenty hrs after application of *S. aureus* to the skin of these mice there was only a minimal difference in *S. aureus* detected in the surface epidermal compartment of the *FLG*$^{ft/ft}$ mice (Figure 4b), whereas a significantly increased amount of *S. aureus* was detected in the dermis and adipose tissue of *FLG*$^{ft/ft}$ than WT mice with similar tape-strip and OVA sensitization or PBS-treated *FLG*$^{ft/ft}$ mice (Figure 4c–d). *S. aureus* was undetectable in NTC (Figure 4e). These data demonstrate that in the setting of increased inflammation and enhanced TWEL, a loss-of-function mutation in *FLG* facilitates bacterial entry through the epidermal barrier.

S. aureus proteolytic activity enhances penetration of the epidermal barrier

As *Staphylococcus* extracellular proteases have been known to interact with the epidermal barrier components, we hypothesized that bacteria enter the dermis through the action of their proteases. To address this, we compared entry of WT methicillin-resistant *S. aureus* (MRSA) USA300 to an extracellular protease-mutant of this strain which lacks 10 major proteolytic enzymes, including aureolysin metalloprotease, V8 and SspA serine proteases, ScpA and SspB cysteine proteases, and 6 other serine-like protease homologs (Kolar et al., 2013). We confirmed that the culture supernatant of the mutant strain contained less proteolytic activity than that of WT of the same background strain (Figure S3). First, as in Figure 2, an equal number of bacteria were applied on the epidermis of cultured human skin equivalents to compare the capacity of each strain to penetrate the epidermis. Both strains grew at the same rate in this model since after 48 hrs, comparable colony-forming units (CFUs) of the live WT and mutant strains were detected in full-thickness biopsies of the skin construct (Figure 5a). More WT *S. aureus* was detected in the dermis than the protease deficient mutant strain. Next, when these bacteria were applied to mice in the AD skin model of OVA-sensitized and tape-stripped *FLG*$^{ft/ft}$ mice shown in Figure 4, a similar behavior of *S. aureus* was observed. Both bacterial strains were detected in similar quantities in the mouse epidermis (Figure 5b), whereas the only the WT strain showed a significant capacity to enter the dermis and adipose tissue (Figure 5c–e). These data suggest that skin entry by bacteria is, at least in part, facilitated by microbial proteases.

Entry of *S. aureus* into the dermis triggers immune abnormalities seen in AD skin

With the observation that WT and protease deficient strains of *S. aureus* penetrated differently below the epidermis, it became possible to examine if a correlation exists between entry of bacteria into the dermis and an immunological response. The expression of mRNA for inflammatory cytokines (IL-4, IL-13, CXCL2, TSLP, IL-17a, IL-22 and IFNs), and cathelicidin (*Camp*) was therefore measured in each condition. In accordance with the capacity to enter the skin as seen in Figure 5b–c, live WT *S. aureus* induced more expression of IL-4, IL-13, CXCL2, TSLP, IL-17 and IL-22, but not IFNs (Figure 5f–n). Furthermore, similar to observations in human subjects with AD, *S. aureus* entry resulted in suppression of *Camp* expression (Figure 5o). In contrast, *S. aureus* entry was correlated with a slight increase in β-defensins-14 and -4 expression, but the relative expression level of these β-defensins was much lower than that of *Camp*.

Application of a barrier repair cream decreases bacterial penetration of skin

An essential element in the therapy of AD is the topical application of moisturizers or barrier repair products. This can result in significant decrease in inflammation, but the immunological mechanism responsible for this improvement is unclear. To examine if restoration of skin barrier function could benefit the immune response by limiting bacterial entry into the dermis, AD-like skin lesions in OVA-sensitized $FLG^{ft/ft}$ mice were treated with a barrier repair formulation consisting of optimized ceramide-triple lipid mixture (Man et al., 1996; Mao-Qiang et al., 1995) or vehicle. Subsequently, *S. aureus* was applied to the surface and entry was tracked with LCM and qPCR as described previously. Treatment with the ceramide-triple lipid mixture restored barrier function as measured by TEWL (Figure 6a). Following barrier repair, *S. aureus* penetration decreased into the subepidermal tissues in comparison to the control skin treated with vehicle (Figure 6b–e). Application of the ceramide-triple lipid mixture did not exert antimicrobial effect on the *S. aureus* colonized on the epidermis (Figure 6b). In addition, barrier repair partially limited cytokine induction and failure of *Camp* induction caused by *S. aureus* application (Figure 6f–i).

DISCUSSION

Defects in the skin barrier have been associated with the pathogenesis of AD and are frequently associated with colonization by *S. aureus*, a factor that exacerbates disease (De Benedetto et al., 2011; Leung and Guttman-Yassky, 2014). It was unclear how dysbiosis at the skin surface could trigger inflammation that arises below the stratum corneum. We report herein that *S. aureus* penetrates the epidermis by a proteolytic mechanism and that failure of the antimicrobial or physical skin barrier of the epidermis enhances entry of *S. aureus* into the dermis. Entry of bacteria enables them to come into direct contact with viable immunocytes and stimulates production of pro-inflammatory cytokines. These data directly demonstrate how the skin barrier controls the interaction between the microbiome and the cutaneous immune system, and illustrate how abnormal penetration of surface microbes can mediate immune dysregulation associated with AD. Importantly, since this immune dysregulation further disrupts the barrier function of the skin, this relationship provides an explanation for the chronic nature of inflammation observed in this disorder.

An important variable that influenced microbial entry into the dermis was AMP expression. Cathelicidin exhibits direct antimicrobial action against a wide range of pathogens (Dorschner et al., 2001; Gallo et al., 1994; Nizet et al., 2001). In healthy skin, cathelicidin expression is increased upon infection, inflammation and injury (Gallo and Hooper, 2012; Lai and Gallo, 2009). However, the skin of patients with AD has been shown in some studies to have a decreased capacity to produce an adequate amount of this AMP and β-defensins-2 and -3 (Hata et al., 2010; Howell et al., 2006a; Howell et al., 2006b; Mallbris et al., 2010; Ong et al., 2002). This AMP deficiency may result in an inability of the skin to resist infection by several pathogens including *S. aureus*. However, it was not known if the constitutive presence of cathelicidin expressed in healthy skin contributed to antibacterial defense or how this influenced colonization of skin microbes. In this study, cathelicidin was shown to regulate entry of *S. aureus*. This finding may be clinically relevant when considered with observations that cathelicidin expression is enhanced by vitamin D and improves clinical outcome in AD (Liu et al., 2006; Schauber et al., 2007; Schauber et al., 2006; Camargo et al., 2014; Hata et al., 2008). Therefore, although many variables may confound this response, the capacity of vitamin D to enhance the antimicrobial barrier may partially explain reported benefits of vitamin D3 in AD and other allergic disorders (Goetz, 2011; Malley et al., 2009).

Another variable found here to influence penetration of *S. aureus* was the expression of filaggrin. Filaggrin is a structural protein that is fundamental in the development and maintenance of the physical skin barrier (Sandilands et al., 2009). Loss-of-function mutations in *FLG* represent a significant genetic factor predisposing the development of AD in some populations (Bisgaard et al., 2008; Palmer et al., 2006; Sandilands et al., 2007; Smith et al., 2006). Two of the most studied mutations (R501X and 2282del4) are common in European populations and result in loss-of-function (Weidinger et al., 2006). The flaky-tail mouse used here has a naturally-occurring single-base-pair deletion ($FLG^{ft/ft}$) that induces a premature stop codon and also results in loss-of-function (Fallon et al., 2009; Moniaga and Kabashima, 2011). This mouse differs from the original flaky-tail mouse that has an additional mutation in *Tmem79/Matt*, a mutation that is also associated with AD in humans (Saunders et al., 2013). In contrast to the double mutation original flaky-tail mouse, this $FLG^{ft/ft}$ mouse line does not develop spontaneous inflammation (Hoff et al., 2015). However, out data illustrate how mutations in only *FLG* increase the risk of inflammation and enhance *S. aureus* entry. Other epidermal barrier proteins, such as envoplakin, periplakin, and involucrin, may also control microbiome penetration into the skin (Natsuga, et al., 2016). Barrier defects permitted increased *S. aureus* entry and subsequent enhanced expression of T_H2 cytokines, IL-17 and TSLP, and decreased expression of cathelicidin. These results are consistent with previous reports demonstrating that T_H2 cytokines directly downregulate the induction of cathelicidin in the skin (Howell et al., 2006b). Such changes are characteristic of AD and may illustrate how mutations of *FLG* in the human population may confer risk of AD by enabling the abnormal entry of microbes into the dermis.

We previously reported that most of the microbes in the dermis of human skin are not present within classical $CD11c^+$ phagocytic immune cells (Nakatsuji et al., 2013). Similarly *S. aureus* was detected outside of $CD11c^+$ cells in lesional AD skin. These data suggest that these microbes entered across the epidermal barrier rather than carried in by phagocytosis.

Here, we directly demonstrated in cultured skin equivalents that antigen presenting cells are not required for *S. aureus* to enter the dermis. In contrast penetration of the stratum corneum by *S. aureus* was dependent on protease activity. Future work will determine if a specific protease mediates entry although there are several proteases produced by *Staphylococcal* species that are of interest. For example, *S. aureus* produces an extracellular zinc-calcium-dependent metalloproteinase called aureolysin which can proteolytically degrade cathelicidin and neutralize its antimicrobial activity (Sieprawska-Lupa et al., 2004). *S. aureus* also produces a serine protease, commonly referred to as V8 protease, that is known to impair epidermal barrier function in mice (Hirasawa et al., 2010) and has been known as the “epidermolytic toxin” (Dancer et al., 1990; Redpath et al., 1991). *S. aureus* strains isolated from AD patients have found to produce high extracellular proteolytic activity, and aureolysin and V8 protease are predominantly contribute to their total proteolytic activity (Miedzobrodzki et al., 2002). In the current study we showed that the extracellular protease-null mutant of MRSA USA300, which lacks both proteases, had less capacity to break through the epidermal barrier of the murine skin and organotypic human skin constructs. Thus, aureolysin and the V8 serine protease are two prime specific candidates to examine for their role to permit bacteria to penetrate the skin, and may explain the higher amount of *S. aureus* seen in the dermis of AD subjects. Blocking specific bacterial proteases could therefore be a useful therapeutic approach for AD though this therapeutic use of protease inhibitors for AD has been controversial (Foelster Holst et al., 2010; Wachter and Lezdey, 1992).

This study demonstrates that *S. aureus* can directly penetrate the stratum corneum and epidermis, a behavior that explains how this microbe can disrupt skin immune homeostasis. Defects in the skin barrier enable enhanced entry from the surface, and although this entry does not show characteristics of infection, this penetration alters cytokine and AMP responses. Such inflammatory triggers may then further alter the surface microbiome and perpetuate disease by adding to the process of barrier disruption. Notably, we demonstrated that barrier repair reduces *S. aureus* penetration into skin and normalizes immune abnormality triggered by the *S. aureus* penetration. Thus, these observations provide clinical insight into how physical and innate immune barrier defects influence the pathogenesis of AD and provide guidance for optimizing therapeutic approaches to this disorder.

MATERIALS AND METHODS

Analysis of skin from human subjects with AD

All sample acquisition, including biopsies of lesional and nonlesional skin from patients with AD, and normal skin from non-AD subjects, was approved and performed in accordance with the Human Research Protections Program at the University of California, San Diego (reference number: 071032). Written informed consent was obtained from all subjects prior to performing the skin biopsies. Demographic data of AD patients recruited are shown in Table S1. Subepidermal compartments of lesional and nonlesional skin from patients with AD were excised from the skin biopsy by using LCM without inclusion of appendageal structures. Total genomic DNA was extracted from LCM sections and

subjected to real-time qPCR or pyrosequencing for 16S rRNA using universal 16S rRNA or species-specific primers/probe (Table S2). For details, see Supplementary Material.

Tracking bacteria entry into mouse skin

All animal protocols were reviewed and approved by the UC San Diego (approval number: S09074). After shaving and disinfecting the dorsal skin of OVA-sensitized *FLG*$^{ft/ft}$ or *Camp*$^{-/-}$ mice, an agar disk (6 mm) containing *S. aureus* ATCC35556 or MRSA USA300 LAC strains (1×10^6 CFU) was applied on the skin, and the entire dorsal skin was then covered with wound dressing film for 20 hrs. An agar disc without bacteria or with UV-killed *S. aureus* (1×10^7 CFU equivalent) was used as controls. Following disk application, skin was carefully cleaned with alcohol swabs and frozen in tissue embedding compound. Epidermis, dermis and adipose tissue sections were excised by LCM without inclusion of appendageal structures. Total genomic DNA was extracted from each section for qPCR. For details, see Supplementary Material.

Skin barrier disruption and restoration

A sterile patch with OVA solution or PBS was placed on tape-stripped dorsal skin of *FLG*$^{ft/ft}$ Balb/c mice for 8 days (the patch was replaced every 2 days) as described previously (Jin et al., 2009). Each mouse received three 8-day exposures in 2 week intervals. Twenty four hrs after the third sensitization, dorsal skin was tape-stripped. For skin barrier repair experiments an optimized formula of cholesterol, ceramide, linoleate and palmitate (3:1:1:1), or propylene glycol-ethanol (7:3) (vehicle) was applied twice every 4 hrs as previously described (Man et al., 1996; Mao-Qiang et al., 1995). *S. aureus* was applied 4 hrs after the second application of barrier repair mixture or vehicle. For details, see Supplementary Material.

Data access

The 16S pyrosequencing data for this study has been submitted to DDBJ Sequence Read Achieve (http://trace.ddbj.nig.ac.jp/dra/index_e.html) through the BioProject ID PRJDB4882 and published under the Accession Code DRA004759.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 software (GraphPad, La Jolla, CA). Independent *t*-test was used for significance of differences.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

AD	Atopic dermatitis
AMP	Antimicrobial peptide
CFU	Colony-forming unit
DAPI	4′,6-diamidino-2-phenylindole
IFN	Interferon
IL	Interleukin
LCM	Laser-capture microdissection
MRSA	Methicillin-resistant *Staphylococcus aureus*
NTC	Non-tissue control
OVA	Ovalbumin
TEWL	Transepidermal water loss
TSLP	Thymic stromal lymphopoietin
WT	Wild type: WT

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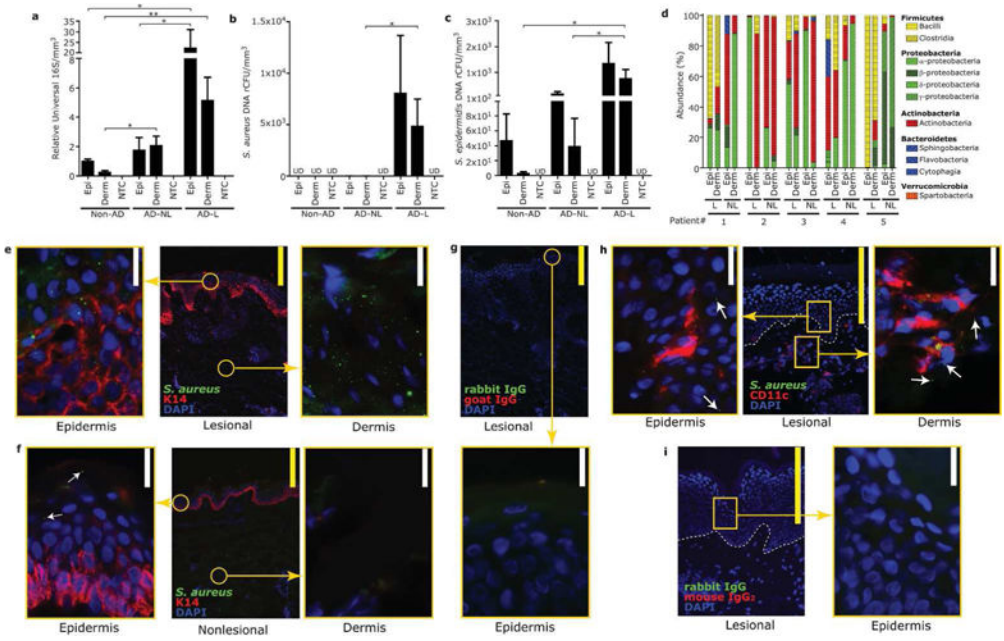

Figure 1. Dysbiosis of the subepidermal compartments from skin of AD patients

(a) qPCR results for relative abundance of DNA for 16S rRNA, **(b)** *S. aureus* (**c**) and *S. epidermidis* detected in epidermal or dermal compartments, isolated by laser-capture microdissection (LCM), of skin from normal skin of non-AD subjects, nonlesional and lesional skin of patients with AD. Non-tissue controls (NTC) were simultaneously processed. Data represent mean ± SEM of 11 subjects. **P*<0.05, ***P*<0.01. Epi=epidermis, Derm=dermis, UD=undetectable. **(d)** 16S rRNA pyrosequencing results from samples isolated by LCM of the epidermis and dermis of nonlesional (NL) and lesional (L) skin of AD subjects. Each bacterial phylum is shown in different color. **(e–g)** Immunofluorescence for *S. aureus* and keratin-14 in lesional (e) or nonlesional (f) skin of AD subject. Staining with isotype control (g). **(h,i)** Immunofluorescense for *S. aureus* and CD11c in lesional skin of AD subject. Staining with isotype control (i). Arrows indicate *S. aureus* staining detected outside of $CD11c^+$ immune cells. Immunostaining shown is a representative of 3 biopsies from different donors. Scale bar= 20μm (white) or 200μm (yellow).

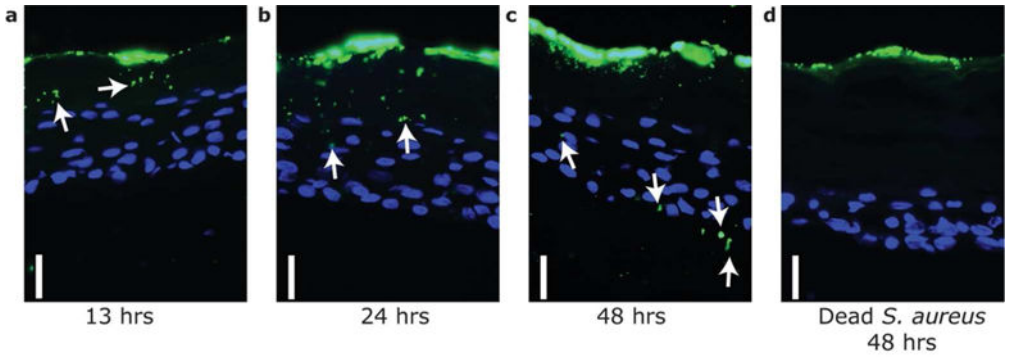

Figure 2. *S. aureus* actively penetrates human skin organotypic equivalents

Time-dependent entry of *S. aureus* across the epidermis of a human skin organotypic construct. Viable *S. aureus* (1×10^6 CFU) (a–c) or UV-killed *S. aureus* (1×10^7 CFU) (d) were applied on the stratum corneum surface and individual constructs fixed at the indicated time after bacterial application. Skin constructs were then sectioned and stained with anti-*S. aureus* (green) to visualize bacteria. Keratinocyte nuclei were counter stained with DAPI (4′, 6-diamidino-2-phenylindole) (blue). Scale bar=20 μm. Arrows indicate immunoreactivity for *S. aureus* under the epidermal surface when live bacteria were applied to the surface.

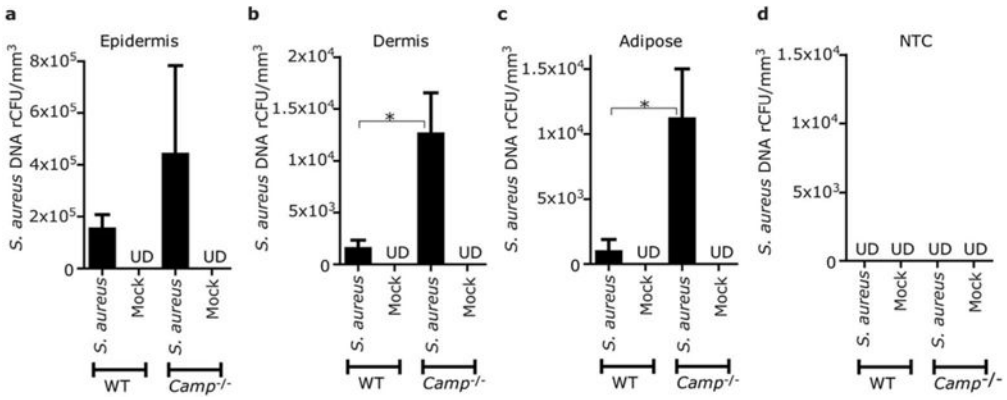

Figure 3. Cathelicidin inhibits *S. aureus* entry into the mouse dermis

(a–c) *S. aureus* (ATCC35556) or vehicle (mock) were loaded in agar discs and applied on dorsal skin of *Camp*$^{-/-}$ or WT mice for 20 hrs. Skin was then excised and DNA extracted from epidermis (a), dermis (b) or adipose tissue (c) isolated by LCM. Relative colony forming units (rCFU) of *S. aureus* DNA was determined by real-time qPCR by comparison to a standard of known CFUs of *S. aureus* (ATCC35556). **(d)** As negative control a non-tissue control (NTC) was simultaneously processed with the same reagents from embedding material adjacent to each tissue section. The data were normalized against tissue volume excised by LCM. Data represent mean±SEM of results from 4 independent experiments.* $P<0.05$. UD=undetectable

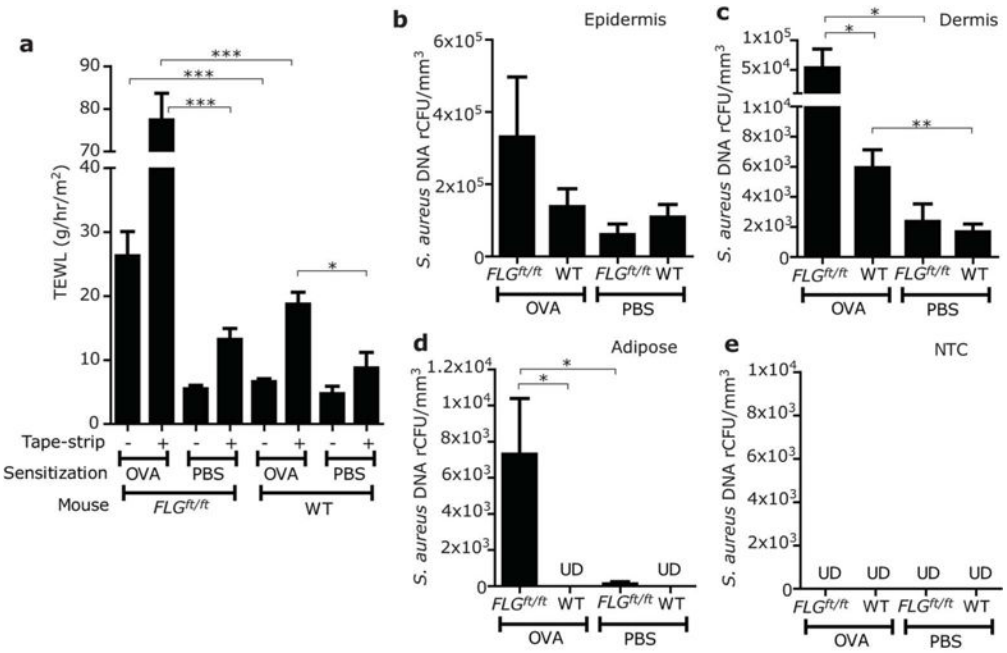

Figure 4. A loss-of-function mutation in filaggrin increases *S. aureus* entry into the mouse dermis after ovalbumin sensitization

(a) Transepidermal water loss (TEWL) determined before (−) or after tape-stripping (+) on the dorsal skin of *FLG*$^{ft/ft}$ Balb/c or WT mice which were treated by repeated applications of OVA or PBS. **(b–e)** The backs of *FLG*$^{ft/ft}$ Balb/c mice, or wild type (WT) Balb/c mice were treated with tape-stripping and OVA as described in panel (a). Abundance of *S. aureus* (ATCC3555) in epidermis (b), dermis (c) and adipose tissue (d) was measured by qPCR and LCM as described in Figure 3. (e) NTC was simultaneously processed as negative control. Data represent mean ± SEM of results from 6 independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001. UD=undetectable

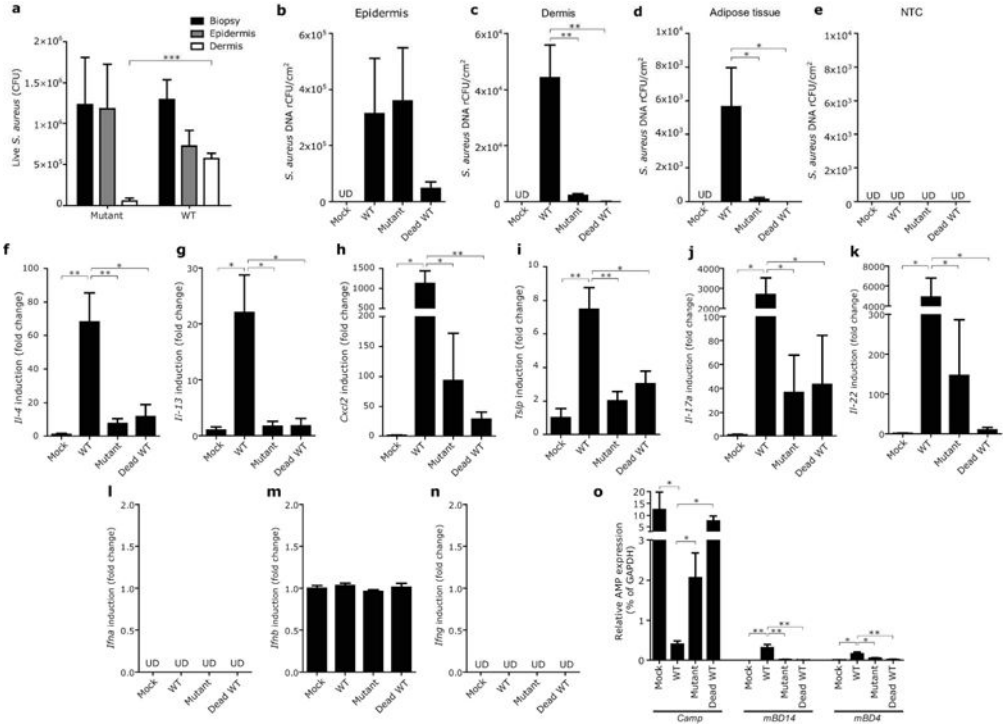

Figure 5. *S. aureus* protease activity is required for penetration of the epidermis and induction of inflammatory cytokines

(a) Entry of WT or an extracellular protease-null mutant strain of MRSA into organotypic human skin constructs **. (b–e)** Entry of WT, extracellular protease-null mutant strain and UV-killed WT strain of MRSA into epidermis (b), dermis (c) and adipose tissue (d) of *FLG*$^{ft/ft}$ Balb/c mice sensitized by OVA was tracked as described in Figure 3. NTC was processed as negative control (e). **(f–o)** To correlate entry of MRSA strains with cutaneous immune response, gene expression of indicated cytokines (f–n) and indicated AMPs (o) was measured in the same whole skin biopsies from panel (b–d). To compare relative expression level of each AMP, data was shown as relative to GAPDH expression. Data represent mean ± SEM of results from 5–6 independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001.

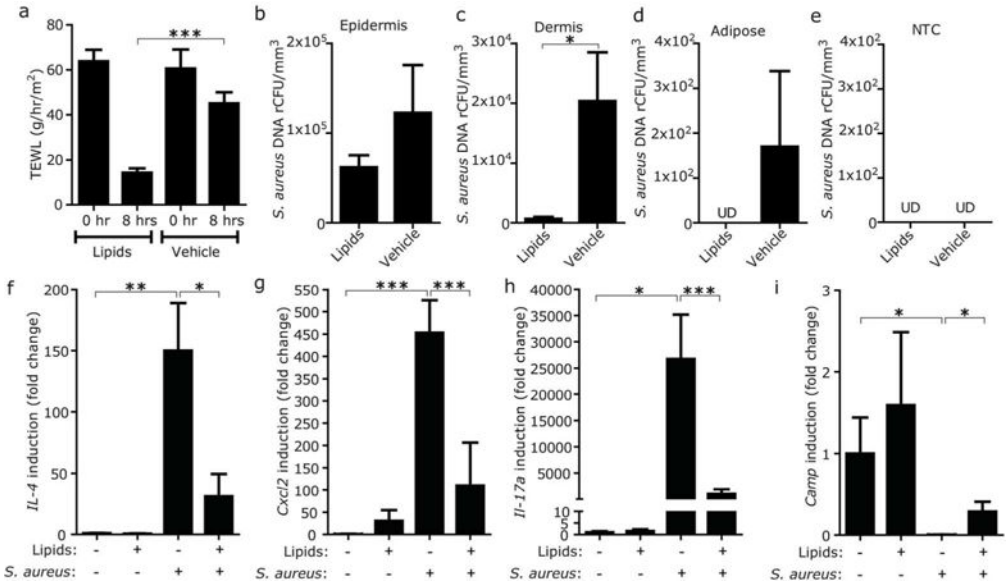

Figure 6. Skin barrier repair decreases *S. aureus* entry into the dermis and suppresses subsequent immune response in skin with *FLG* mutation

(a) Effect of the application of barrier repair formula of ceramide-triple lipid mixture on TEWL from $FLG^{ft/ft}$ Balb/c mice sensitized with OVA. TEWL was measured 4 hrs after application of barrier repair lipids or vehicle. **(b–e)** Effect of the application of barrier repair lipid mixture on entry of *S. aureus* into the skin of $FLG^{ft/ft}$ Balb/c mice with AD-like inflammation. Entry of *S. aureus* (ATCC3555) into epidermis (b), dermis (c) and adipose tissue (d) was tracked 4 hrs after application of barrier repair lipids or vehicle. NTC was simultaneously processed as negative control (e). Data represent mean ± SEM of results from 5–6 independent experiments. **(f–i)** Effect of the application of barrier repair lipid mixture on cytokine and *Camp* inductions after epicutaneous application of *S. aureus* on the OVA-sensitized skin of $FLG^{ft/ft}$ Balb/c mice. Data represent mean ± SEM of results from 6–7 independent experiments. *P<0.05, **<0.01, ***P<0.001. UD=undetectable