Title

Staphylococcus aureus Induces Increased Serine Protease Activity in Keratinocytes

Journal

Journal of Investigative Dermatology, 137(2)

ISSN

0022-202X

Authors

Williams, Michael R
Nakatsuji, Teruaki
Sanford, James A
et al.

Publication Date

2017-02-01

DOI

10.1016/j.jid.2016.10.008

Peer reviewed

HHS Public Access

Author manuscript

J Invest Dermatol. Author manuscript; available in PMC 2018 February 01.

Published in final edited form as:

J Invest Dermatol. 2017 February ; 137(2): 377–384. doi:10.1016/j.jid.2016.10.008.

Staphylococcus aureus induces increased serine protease activity in keratinocytes

Michael R. Williams[1], **Teruaki Nakatsuji**[1], **James A. Sanford**[1], **Alison F. Vrbanac**[1], and **Richard L. Gallo**[1]

[1]Department of Dermatology, University of California, San Diego, CA, USA.

Abstract

Bacteria that reside on the skin can influence the behavior of the cutaneous immune system, but the mechanisms responsible for these effects are incompletely understood. Colonization of the skin by *Staphylococcus aureus* (*S. aureus*) is increased in atopic dermatitis (AD) and can result in increased severity of the disease. In this study we show that *S. aureus* stimulates human keratinocytes to increase their endogenous protease activity, including specific increases in trypsin activity. This increased protease activity coincided with increased expression of mRNA for kallikreins (KLKs), with KLK6, 13, and 14 showing the greatest induction after exposure to *S. aureus.* Suppression of mRNA for these KLKs in keratinocytes by targeted siRNA silencing prior to *S. aureus* exposure blocked the increase in protease activity. Keratinocytes exposed to *S. aureus* showed enhanced degradation of desmoglein-1 (DSG-1) and filaggrin (FLG) while siRNA for KLK6, KLK13, and KLK14 partially blocked this degradation. These data illustrate how *S. aureus* directly influences the skin barrier integrity by stimulating endogenous proteolytic activity and defines a previously unknown mechanism by which *S. aureus* may influence skin diseases.

Keywords

Staphylococcus aureus; atopic dermatitis; kallikreins; skin barrier

INTRODUCTION

The epidermis is the first line of immune defense and protects and regulates interactions between microbes and the host organism. Control of this interaction is important since bacteria not only reside on the surface where they influence superficial keratinocytes, but bacteria also penetrate below the stratum corneum and into the dermis (Nakatsuji *et al.*,

Corresponding Author: Richard L Gallo, University of California, San Diego, Department of Dermatology, 9500 Gillman Dr. #0869, La Jolla, California, 92093, USA., Tel: 858- 822-4608, Fax: 858-822-6985.

CONFLICT OF INTEREST

R.L.G. is a consultant for, receives compensation and has equity interest in, MatriSys Bioscience, Inc. and Sente Inc., and is a co-founder of MatriSys Bioscience, Inc. The terms of these arrangements have been reviewed and approved by the University of California, San Diego in accordance with its conflict of interest policies.

2013) where some bacterial species have been shown to influence immune function. For example, *Staphylococcus epidermidis* (*S. epidermidis*) interacts with epidermal keratinocytes to prevent TLR3 mediated inflammation, recruits mast cells and T cells, and increases tight junctions and antimicrobial peptide production (Belkaid and Segre, 2014; Lai *et al.*, 2010; Lai *et al.*, 2009; Wang *et al.*, 2012; Yuki *et al.*, 2011). In contrast to the common skin commensal bacteria, *S. epidermidis*, *Staphylococcus aureus* (*S. aureus*) is often pathogenic and can have a negative influence on skin function. This is especially evident in skin diseases such as atopic dermatitis (AD) where *S. aureus* promotes this disease (Kobayashi *et al.*, 2015).

The microbiome inhabiting the skin of subjects with AD has been clearly shown to have a decrease in overall microbial diversity and an increase in *S. aureus* abundance (Kong *et al.*, 2012). Increased *S. aureus* colonization has been linked to increased disease severity for AD patients (Zollner *et al.*, 2000). Mechanistically, it is unclear how *S. aureus* worsens disease. Several products from *S. aureus* have been shown to damage the barrier and/or trigger inflammation. These products include; α-toxin, superantigens, toxin shock syndrome toxin 1 (TSST-1), enterotoxins, phenol-soluble modulins (PSMs), Protein A, Panton-Valentine Leukocidin (PVL), exfoliative toxins, and V8 serine protease (Bantel *et al.*, 2001; Chi *et al.*, 2014; Ezepchuk *et al.*, 1996; Hanakawa *et al.*, 2003; Kim *et al.*, 2006; Proft and Fraser, 2003; Syed *et al.*, 2015). Because of the potential pathogenic effects of these molecules, understanding the response of the skin to *S. aureus* colonization in the absence of clear clinical signs of infection is critical to understanding the pathogenesis of AD and for developing future therapies.

Defects in skin barrier function are an important characteristic of AD. The skin barrier of AD patients may be compromised by increased proteolytic activity as they have been found to display increased kallikrein (KLK) expression (Komatsu *et al.*, 2007; Voegeli *et al.*, 2009). KLKs are a family of 15 serine proteases of which several are found predominately in the upper granular and stratum corneal layers of the epidermis. In Netherton syndrome (NS), increased serine protease activity is observed due to decreased activity of the serine protease inhibitor Kazal-type 5 (SPINK5) (Chavanas *et al.*, 2000). The resulting increase in enzymatic activity leads to increased desquamation, altered antimicrobial peptide (AMP) and filaggrin (FLG) processing, and PAR-2 activation and inflammation (Borgono *et al.*, 2007; Caubet *et al.*, 2004; Sakabe *et al.*, 2013; Stefansson *et al.*, 2008). Increased protease activity may also play an important role in the communication of the microbiome with the skin immune system, and has recently been shown to directly influence epidermal cytokine production and inflammation by enhancing penetration of bacteria through the epidermis (Nakatsuji *et al.*, 2016).

In this study, we sought to determine if the presence of bacteria could alter the production of endogenous proteases produced by the epidermis. Our findings demonstrate that *S. aureus* has the ability to induce expression of specific KLKs from keratinocytes and increase overall proteolytic activity in the skin. This illustrates a system by which bacteria on the skin communicate with the host and suggests a previously unknown but likely important mechanism for how *S. aureus* colonization can increase disease severity in patients with AD.

RESULTS

Staphylococci affect the protease activity of human keratinocytes

To evaluate if different strains of bacteria found on human skin can induce protease activity of keratinocytes, primary cultures of normal human epidermal keratinocytes (NHEK) were treated with sterile filtered culture supernatant from 4 different laboratory isolates of *S. aureus* including 2 methicillin resistant *S. aureus* (MRSA) strains (USA300 and SANGER252) and 2 methicillin sensitive *S. aureus* (MSSA) strains (Newman and 113). Two commensal *S. epidermidis* isolates (ATCC12228 and ATCC1457) were also tested. 24 hr after exposure to the sterile bacterial culture supernatants, the keratinocyte culture media was analyzed for protease activity with substrates selective for trypsin-like, elastase-like or matrix metalloproteinase (MMP) activity. NHEK conditioned medium contained significantly more trypsin activity after treatment with *S. aureus* strains Newman and USA300 (Figure 1a). Both MMP and elastase activity were increased by *S. epidermidis* strain ATCC12228 while the *S. aureus* strains USA300 and SANGER 252 and the *S. epidermidis* strain ATCC1457 increased elastase activity to a lesser extent in NHEK conditioned medium (Figure 1b,c). To confirm that the increased protease activity observed in NHEK conditioned medium was derived from NHEKs and not produced by the bacteria themselves, we analyzed trypsin activity after addition of *S. aureus* (Newman) supernatant to culture wells with and without the presence of NHEKs. No enzymatic activity was detected in the absence of NHEKs when the same concentration of diluted supernatant from *S. aureus* was added to the NHEK media alone (Figure 1d).

S. aureus increases epidermal serine protease activity

Due to the large increase in trypsin activity induced by certain *S. aureus* strains (Newman and USA300), and the potential role this activity could have on diseases mediated by *S. aureus,* we next focused on this organism to better understand how the bacteria induces protease activity in NHEKs. To evaluate the kinetics of the protease response to *S. aureus,* keratinocytes were treated for 0, 8, 24, and 48h with sterile filtered culture supernatant from *S. aureus* (Newman) and then the NHEK conditioned medium was collected for protease analysis. Measurement of total protease activity in the conditioned medium of NHEKs showed a time dependent increase in total proteolytic activity after exposure to *S. aureus* supernatant (Figure 2a). Addition of the serine protease inhibitor aprotinin confirmed that this activity was due to serine proteases (Figure 2b), and this was consistent with the observation of an increase in trypsin-like activity shown in Figure 1a. Comparison of *S. aureus* USA300 LAC wild-type (WT) and a protease null strain demonstrated that both the WT and protease null strains increased trypsin activity in NHEK conditioned medium but the protease null strain had significantly decreased capacity to induce trypsin activity compared to that of the WT strain (Figure 2c). Together, these data confirm that *S. aureus* can increase endogenous NHEK serine protease activity and that *S. aureus* proteases and other *S. aureus* products contribute to the ability of this bacterium to activate keratinocytes.

To further validate the action of *S. aureus* on epidermal protease activity, we next applied live *S. aureus* (USA300) to the back skin of mice. Skin at the site of application was then biopsied and sectioned for analysis of total proteolytic activity by in situ zymography in the

presence or absence of the serine protease inhibitor AEBSF. Total epidermal protease activity was qualitatively increased in the epidermis after treatment with *S. aureus* compared to skin treated with agar disks alone, and the increased activity detected by increased fluorescence was largely eliminated by inhibition of serine protease activity with AEBSF (Figure 3a–e). Background auto-fluorescence at hair follicles is seen in all sections including the no substrate control (Figure 3f). These observations further demonstrate that the presence of *S. aureus* can increase protease activity in the epidermis.

S. aureus increases KLK expression in keratinocytes

KLKs are an abundant serine protease family in the epidermis that have trypsin-like or chymotrypsin-like activity (Emami and Diamandis, 2007; Prassas *et al.*, 2015). To determine if *S. aureus* could change the expression of KLK mRNA in keratinocytes, NHEKs were treated for 24h with *S. aureus* (Newman) supernatant and expression of KLK1–15 were measured by qPCR. KLK5 had the highest relative mRNA abundance while KLK6, 13, and 14 consistently displayed the largest fold increase after exposure to *S. aureus* (Figure 4a–e). All other KLKs analyzed showed subtle increases in mRNA expression after exposure to *S. aureus* except KLK1 that showed decreased expression. mRNA for KLK2, 3, and 15 were not detected (**data not shown**).

We next analyzed both cell lysates and NHEK conditioned medium for changes in KLK protein expression after *S. aureus* (Newman) supernatant treatment. Immunoblotting for KLK6 and 14 displayed increased expression of these KLK proteins after *S. aureus* supernatant treatment in both the cell lysate and the conditioned medium while KLK13 was only increased in the conditioned medium. KLK5 had no change in expression after *S. aureus* supernatant treatment (Figure 4f).

KLK6, 13, and 14 contribute to increased keratinocyte serine protease activity

Since KLK6, 13, and 14 showed the largest increase in expression in NHEKs after *S. aureus* exposure, we next examined if these KLKs were responsible for the observed increased serine protease activity. siRNA was used to selectively silence their expression. siRNA for KLK6 and KLK13 significantly decreased *S. aureus* induced trypsin activity while KLK14 decreased trypsin activity to a lesser extent. A triple knockdown of KLK6, 13, and 14 also showed a significant decrease in trypsin activity from the control siRNA although an additive effect was not observed (Figure 5a). Interestingly, triple knockdown of KLK6, 13, and 14 led to decreased knockdown efficiency of KLK13 and KLK14 which may account for the lack of an additive effect for trypsin activity (Figure 5b–d).

S. aureus promotes degradation of desmoglein-1 and filaggrin by induction of KLKs

Desmoglein-1 (DSG-1) and filaggrin (FLG) are both important for regulating the epidermal skin barrier integrity (Borgono *et al.*, 2007; Fortugno *et al.*, 2011; Sakabe *et al.*, 2013). We observed by immunoblotting that exposure of NHEKs to *S. aureus* (Newman) supernatant promoted the cleavage of full length DSG-1 (160kDa), and that DSG-1 cleavage was blocked by siRNA silencing of KLK6, 13, or 14 (Figure 6a). *S. aureus* mediated cleavage of Pro-filaggrin (Pro-FLG) in NHEKs, indicated by the >250kDa band on the immunoblot, was also partially blocked by siRNA silencing of KLK6 and KLK13 (Figure 6b). Densitometry

analysis further illustrates the ability of KLK6, 13, and 14 knockdowns to prevent either DSG-1 or Pro-FLG cleavage (Figure 6c). Overall, these observations demonstrate that the capacity of *S. aureus* to increase keratinocyte proteolytic activity by induction of KLK6, 13 and 14 can lead to digestion of molecules essential for maintaining a normal epidermal barrier.

DISCUSSION

Increasing evidence has shown that bacteria on the skin can influence epidermal biology. Here we show for the first time to our knowledge that some bacteria stimulate an increase in keratinocyte protease activity and that some strains of *S. aureus* can specifically activate keratinocytes to increase expression of endogenous serine proteases. Specifically, 3 members of the KLK family appear to play a substantial role in this increased enzymatic activity. Since *S. aureus* colonization has been associated with increased disease severity in AD, and an altered epidermal barrier is an integral part of the pathogenesis of this disorder, this discovery furthers our understanding of the molecular mechanisms through which *S. aureus* exacerbates disease.

S. aureus can secrete multiple proteases onto the skin that may directly alter skin barrier integrity. The serine protease V8 and serine-like protease exfoliative toxins have been shown to cleave corneodesmosome adhesion proteins including desmoglein-1 (DSG-1) leading to increased desquamation. Aureolysin, a MMP, is known to cleave and inactivate LL-37, an important antimicrobial peptide (AMP) on the skin (Hanakawa *et al.*, 2003; Hirasawa *et al.*, 2010; Ladhani, 2003; Sieprawska-Lupa *et al.*, 2004). However, these direct proteolytic actions of *S. aureus* products require high levels of the enzyme and bacteria, and are more consistent with events that occur during infection with this organism. We have shown here that a soluble factor(s) produced by *S. aureus* has a potent and previously unsuspected capacity to alter endogenous protease activity produced by the keratinocyte. This occurred at a dilution of *S. aureus* products from which the activity of the bacterial proteases was undetectable. Thus, *S. aureus* can promote the epidermis to increase expression of endogenous proteolytic activity, thus drastically altering the balance of total epidermal proteolytic activity.

Since KLKs are one of the most well recognized serine protease families in the epidermis, we sought to explore how KLK expression is altered by *S. aureus* in human keratinocytes. We observed that KLK6, 13, and 14 had the greatest relative increase in mRNA and protein expression. Specific siRNA knockdown suggested that the increased expression of these KLKs was responsible, at least in part, for the increased serine protease activity stimulated by *S. aureus*. Further analysis to better confirm the mammalian enzymes and enzyme inhibitors targeted by bacteria on the skin will undoubtedly reveal an important new mode of communication between the microbiome and the skin.

We observed increased digestion of barrier proteins after keratinocytes were activated by *S. aureus*. FLG is known to be cleaved from the larger Pro-FLG (400kDa) into a monomeric form (37kDa) that plays an important role in forming the physical barrier of the stratum corneum with keratin (Steinert *et al.*, 1981). It has previously been shown that accelerated

Pro-FLG cleavage could be linked to increased desquamation of the skin (Hewett *et al.*, 2005). Interestingly, we observed increased cleavage of Pro-FLG in human keratinocytes treated with *S. aureus* supernatant. Pro-FLG cleavage was partially blocked when KLK6 or KLK13 was silenced, indicating that *S. aureus* may decrease skin barrier integrity in a KLK dependent manner through cleavage of Pro-FLG. This finding both matches and expands upon previous finding that KLK5 can cleave FLG (Sakabe *et al.*, 2013).

DSG-1 is an important corneodesmosome adhesion protein that when cleaved leads to increased desquamation. We found that full-length DSG-1 (160kDa) in keratinocytes is readily cleaved by KLK activity stimulated by *S. aureus*. It has been reported that KLK5, 6, 7, and 14 can cleave DSG-1 while KLK13 could not (Borgono *et al.*, 2007). This study showed that up-regulated KLK6 and KLK14 can lead to enhanced cleavage of full-length DSG-1 while providing contrary evidence to the notion that KLK13 is not involved in DSG-1 cleavage. Thus we presume that not only can *S. aureus* use KLKs to alter FLG cleavage, but also increase DSG- 1 cleavage as another way to decrease the epidermal skin barrier integrity. These observations also inspire further investigation into how bacteria may influence other diseases mediated by KLK activity such as Rosacea (Yamasaki *et al.*, 2007) or Netherton syndrome.

Different strains of *S. aureus* (Newman, USA300, 113, and SANGER252) and *S. epidermidis* (ATCC12228 and ATCC1457) had different effects on human keratinocyte protease activity. Only certain *S. aureus* strains including Newman and USA300 increased trypsin activity while other strains of *S. aureus* and *S. epidermidis* increased elastase or MMP activity. Thus, we observed that bacteria could alter epidermal protease activity depending on both the species and strain of bacteria. It is possible that other bacterial species and strains of *S. aureus* not tested here could further uniquely influence the enzymatic balance of human skin. Interestingly, preliminary data has found that purified TLR ligands do not induce trypsin activity or KLK expression in keratinocytes. We are actively investigating the role of virulence factors secreted from *S. aureus* including α-toxin, TSST-1, PSMs, and *S. aureus* secreted proteases for their capacity to influence this response. Initial evidence (Figure 2c) has already shown that secreted proteases from *S. aureus* contribute to the induction of increased trypsin activity in keratinocytes. Overall, determining this factor(s) produced by specific bacterial strains that selectively regulate keratinocyte protease activity is a fascinating new target of research that may help explain strain-specific responses of the skin to the microbiome.

It will also be interesting to observe if other cell types in the skin in addition to keratinocytes play a role in changing skin protease activity in response to bacteria. Cells in the dermis and subcutaneous layers, such as mast cells (Pejler *et al.*, 2007), have potent cell-specific proteases. Our whole tissue zymograms (Figure 3) demonstrated that the casein substrate was degraded at sites other than the epidermis. Since bacteria including *S. aureus* can penetrate the skin surface and elicit strong dermal immune responses (Nakatsuji *et al.*, 2016; Nakatsuji *et al.*, 2013; Zhang *et al.*, 2015), it is possible these bacteria may also influence protease activity of dermal cells. It will be interesting to see if changes in skin protease activity are also dependent on skin barrier penetration by *S. aureus*. Ultimately, determining

if and how various strains of skin microbes influence the protease activity of mammalian host cells is of crucial importance to understanding this system.

The ability of cutaneous bacteria to communicate with the skin immune system is likely important to the pathogenesis of several skin diseases. Here we have described an unexpected response of keratinocytes to *S. aureus*. Due to the increased DSG-1 and FLG cleavage, we speculate that *S. aureus* will decrease the integrity of the skin barrier in a KLK dependent manner. This observation defines a link between increased *S. aureus* colonization and increased serine protease activity in AD skin and points to new directions for development of future therapeutic strategies.

MATERIALS & METHODS

Culture of primary human keratinocytes

Normal neonatal human epidermal keratinocytes (NHEKs; ThermoFisher Scientific, Waltham, MA) were cultured in Epilife medium (ThermoFisher Scientific) supplemented with 1x Epilife Defined Growth Supplement (EDGS; ThermoFisher Scientific), 60 μM $CaCl_2$, and 1x antibioticantimycotic (PSA; 100 U/mL penicillin, 100 U/mL streptomycin, 250ng/mL amphotericin B; ThermoFisher Scientific) at 37°C, 5% CO_2. For experiments, NHEKS were grown to 70% confluency followed by differentiation in high calcium EpiLife medium (2mM $CaCl_2$) for 48h prior to treatment with bacteria sterile filtered supernatant. Use of these human derived commercial cell products do not require informed consent. For bacterial supernatant treatments, differentiated NHEKs were treated with sterile filtered bacterial supernatant at 5% by volume to Epilife medium. NHEKs were only used for experiments between passages 3–5.

Bacterial culture

All bacteria were cultured in 3% tryptic soy broth (TSB; Sigma, St. Louis, MO) at 37°C with shaking at 300 RPM. *Staphylococcus aureus (S. aureus)* strains Newman, USA300, 113, SANGER252 and *Staphylococcus epidermidis* (*S. epidermidis*) strains ATCC12228 and ATCC1457 were grown for 24h to stationary phase (Figure S1) followed by centrifugation (4,000 RPM, room temperature (RT), 10 minutes) and sterile-filtration (0.22μm) of supernatants prior to addition to NHEKs. The *S. aureus* USA300 LAC wild-type (WT) and protease null strains (Kolar *et al.*, 2013) were a gift from Dr. Lindsey Shaw (University of South Florida, FL). Briefly the protease null strain was cultured for 24h in 3% TSB containing 25μg/mL Lincomycin and 5μg/mL Erythromycin followed by subculture in 3%TSB only for an additional 24h. For murine live *S. aureus* colonization assays, $2e^6$ colony-forming units (CFU) of bacteria were applied to 8mm TSB agar discs and allowed to dry for 30 minutes at RT prior to addition to murine dorsal skin.

Murine bacteria disc model

Female C57BJ/6L mice (8 weeks old) were housed at the University Research Center at the University of California, San Diego, and were used for a murine model of bacterial skin colonization. Briefly, to remove dorsal skin hair, mice were shaved and Nair was applied for 2–3 minutes followed by removal of hair with alcohol wipes. After 24h recovery, 3 × 8mm

TSB agar discs were applied to murine dorsal skin with TSB only (vehicle control) or $2e^6$ CFU *S. aureus* (USA300) per disc for 12h. Tegaderm was applied on top of agar discs to hold in place. Mice were euthanized followed by collection of 8mm whole skin punch biopsies for analysis. All animal experiments were approved by the UCSD (University of California, San Diego) Institutional Animal Care and Use Committee.

In situ zymography

Murine skin sections (10μm thickness) were rinsed 1x with 1% Tween-20 in water for 5 minutes. Sections were treated with 2μg-mL of BODIPY FL casein total protease activity substrate (ThermoFisher Scientific) for 4h at 37°C in a humidified chamber for measurement of total protease activity. The serine protease inhibitor AEBSF (50mM; Sigma) was applied to sections 30 minutes prior to addition of the BODIPY FL casein as well. Slides were rinsed 1x in PBS followed by application of ProLong Gold Antifade mounting medium without DAPI (ThermoFisher Scientific) and a cover slide. Fluorescent signal was measured using an Olympus BX51 (Tokyo, Japan) fluorescent microscope.

Protease activity assays

NHEK conditioned medium was added at 50μL to black 96 well black bottom plates (Corning, Corning, NY) followed by addition of 150μL of 5μg-mL BODIPY FL casein substrate, 2μg-mL of elastin (elastase-like substrate; ThermoFisher Scientific), or 4μg-mL gelatin (MMP substrate; ThermoFisher Scientific) according to manufacture's instructions. Additionally, 200μM of the peptide Boc-Val-Pro-Arg-AMC (trypsin-like substrate; BACHEM, Bubendorf, Switzerland) was added to NHEK conditioned medium at 150μL in 1x digestion buffer (ThermoFisher Scientific). Relative fluorescent intensity was analyzed with a SpectraMAX Gemini EM fluorometer (ThermoFisher Scientific) at RT with readings every 2h for 24h. BODIPY FL casein plates were read at ex: 485nm and em: 530nm. Elastin-like and MMP substrate plates were read at ex: 485nm and em: 515nm. Trypsin-like substrate plates were read at ex: 354nm and em: 435nm.

Quantitative real-time PCR (qPCR)

RNA was isolated from NHEKs using Purelink RNA isolation columns (ThermoFisher Scientific) according to manufacturer's instructions. RNA was quantified using a Nanodrop spectrophotometer (ThermoFisher Scientific), and 500ng of RNA was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad, Irvine, CA). qPCR reactions were ran on a CFX96 Real-Time Detection System (Bio-Rad) using gene-specific primers and TaqMan probes (ThermoFisher Scientific).

Immunoblotting

For cell lysis, cold 1x RIPA buffer (Sigma) containing 1x protease inhibitor cocktail (Cell Signaling Technology, Danvers, MA) was applied to NHEKs followed by scraping. Cell lysates were incubated for 30 minutes on ice and centrifuged (13,000 RPM, 15 minutes, 4°C) to remove cell debris. Samples were prepared by determining protein concentration with BCA assays (Pierce, Rockford, IL) followed by the addition of 40μg of sample to 4x Laemmli sample buffer (Bio-Rad) containing 1% β-mercaptoethanol and heating for 7

minutes at 95°C. Samples were ran on 4–20% Tris-Glycine precast TGX gels (Bio-Rad), transferred to 0.22μm PVDF membranes (Bio-Rad) using a Trans-blot Turbo Transfer System (Bio-Rad), blocked for 1h at RT in 1x Odyssey blocking solution containing 0.1% Tween-20 (LI-COR, Lincoln, NE), and stained overnight at 4°C with primary antibodies. Odyssey (LI-COR) fluorescent secondary antibodies were applied to membranes for 1h at RT on an orbital shaker after 3x PBST (PBS with 0.1%Tween-20) washes. 3x additional PBST washes were applied before analysis on an infrared imager (LI-COR). The primary antibodies KLK5 (H-55), KLK6 (H-60), DSG-1 (H- 290), FLG (H-300), and α-Tubulin (TU-02) from Santa Cruz Biotechnologies (Santa Cruz, CA) were used at 1:100 dilutions. KLK13 (ab28569) and KLK14 (ab128957) antibodies from Abcam (Cambridge, UK) were used at 1:1000 dilutions.

KLK gene silencing

NHEKs were treated for 24h with 15nM or 45nM of specific KLK silencer select siRNA or a siRNA scrambled (-) control (ThermoFisher Scientific) using RNAiMAX (ThermoFisher Scientific) and OptiMEM medium (ThermoFisher Scientific). NHEKs were differentiated in high calcium medium (2mM $CaCl_2$) for 48h followed by a 24h treatment with sterile filtered *S. aureus* (Newman) supernatant prior to analysis of NHEK lysates and conditioned medium.

Statistical analysis

Both One-way ANOVAs and Two-way ANOVAs were used for statistical analysis with a *P-value* < 0.05 being significant. GraphPad Prism Version 6.0 (GraphPad, La Jolla, CA) was used for statistical analysis of results.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was funded by National Institute of Health R01 grants, R01AR064781, R01AI116576, R01AI052453 (RLG), and 0T32AR062496 (MW).

Abbreviations used:

AD	Atopic dermatitis
AMP	antimicrobial peptide
CFU	colony-forming units
DSG-1	desmoglein-1
FLG	filaggrin
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
KLK	kallikrein

NHEK	normal human epidermal keratinocytes
MMP	matrix metalloproteinase
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-sensitive Staphylococcus aureus
SPINK5	serine protease inhibitor Kazal-type 5
WT	Wild type: WT

REFERENCES

Bantel H, Sinha B, Domschke W, Peters G, Schulze-Osthoff K, Janicke RU. alpha-Toxin is a mediator of Staphylococcus aureus-induced cell death and activates caspases via the intrinsic death pathway independently of death receptor signaling. J Cell Biol. 2001; 155(4):637–648. [PubMed: 11696559]

Belkaid Y, Segre JA. Dialogue between skin microbiota and immunity. Science. 2014; 346(6212):7.

Borgono CA, Michael IP, Komatsu N, Jayakumar A, Kapadia R, Clayman GL, et al. A potential role for multiple tissue kallikrein serine proteases in epidermal desquamation. J Biol Chem. 2007; 282(6):3640–3652. [PubMed: 17158887]

Caubet C, Jonca N, Brattsand M, Guerrin M, Bernard D, Schmidt R, et al. Degradation of corneodesmosome proteins by two serine proteases of the kallikrein family, SCTE/KLK5/hK5 and SCCE/KLK7/hK7. J Invest Dermatol. 2004; 122(5):1235–1244. [PubMed: 15140227]

Chavanas S, Bodemer C, Rochat A, Hamel-Teillac D, Ali M, Irvine AD, et al. Mutations in SPINK5, encoding a serine protease inhibitor, cause Netherton syndrome. Nature Genetics. 2000; 25(2):2.

Chi CY, Lin CC, Liao IC, Yao YC, Shen FC, Liu CC, et al. Panton-Valentine leukocidin facilitates the escape of Staphylococcus aureus from human keratinocyte endosomes and induces apoptosis. J Infect Dis. 2014; 209(2):224–235. [PubMed: 23956440]

Emami N, Diamandis EP. New insights into the functional mechanisms and clinical applications of the kallikrein-related peptidase family. Molecular oncology. 2007; 1(3):269–287. [PubMed: 19383303]

Ezepchuk YV, Leung DY, Middleton MH, Bina P, Reiser R, Norris DA. Staphylococcal toxins and protein A differentially induce cytotoxicity and release of tumor necrosis factor-alpha from human keratinocytes. J Invest Dermatol. 1996; 107(4):603–609. [PubMed: 8823368]

Fortugno P, Bresciani A, Paolini C, Pazzagli C, El Hachem M, D'Alessio M, et al. Proteolytic activation cascade of the Netherton syndrome-defective protein, LEKTI, in the epidermis: implications for skin homeostasis. J Invest Dermatol. 2011; 131(11):2223–2232. [PubMed: 21697885]

Hanakawa Y, Selwood T, Woo D, Lin C, Schechter NM, Stanley JR. Calcium-dependent conformation of desmoglein 1 is required for its cleavage by exfoliative toxin. J Invest Dermatol. 2003; 121(2): 383–389. [PubMed: 12880431]

Hewett DR, Simons AL, Mangan NE, Jolin HE, Green SM, Fallon PG, et al. Lethal, neonatal ichthyosis with increased proteolytic processing of filaggrin in a mouse model of Netherton syndrome. Human molecular genetics. 2005; 14(2):335–346. [PubMed: 15590704]

Hirasawa Y, Takai T, Nakamura T, Mitsuishi K, Gunawan H, Suto H, et al. Staphylococcus aureus extracellular protease causes epidermal barrier dysfunction. J Invest Dermatol. 2010; 130(2):614–617. [PubMed: 19812593]

Kim KH, Han JH, CHung JH, Cho KH, Eun HC. Role of Staphylococcal Superantigen in Atopic Dermatitis: Influence on Keratinocytes. J Korean Med Sci. 2006; 21:9.

Kobayashi T, Glatz M, Horiuchi K, Kawasaki H, Akiyama H, Kaplan DH, et al. Dysbiosis and Staphyloccus aureus Colonization Drives Inflammation in Atopic Dermatitis. Immunity. 2015; 42(4):756–766. [PubMed: 25902485]

Kolar SL, Ibarra JA, Rivera FE, Mootz JM, Davenport JE, Stevens SM, et al. Extracellular proteases are key mediators of Staphylococcus aureus virulence via the global modulation of virulence-determinant stability. Microbiologyopen. 2013; 2(1):18–34. [PubMed: 23233325]

Komatsu N, Saijoh K, Kuk C, Liu AC, Khan S, Shirasaki F, et al. Human tissue kallikrein expression in the stratum corneum and serum of atopic dermatitis patients. Exp Dermatol. 2007; 16(6):513–519. [PubMed: 17518992]

Kong HH, Oh J, Deming C, Conlan S, Grice EA, Beatson MA, et al. Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis. Genome Res. 2012; 22(5):850–859. [PubMed: 22310478]

Ladhani S. Understanding the mechanism of action of the exfoliative toxins of Staphylococcus aureus. FEMS Immunol Med Microbiol. 2003; 39(2):181–189. [PubMed: 14625102]

Lai Y, Cogen AL, Radek KA, Park HJ, Macleod DT, Leichtle A, et al. Activation of TLR2 by a small molecule produced by Staphylococcus epidermidis increases antimicrobial defense against bacterial skin infections. J Invest Dermatol. 2010; 130(9):2211–2221. [PubMed: 20463690]

Lai Y, Di Nardo A, Nakatsuji T, Leichtle A, Yang Y, Cogen AL, et al. Commensal bacteria regulate Toll-like receptor 3-dependent inflammation after skin injury. Nat Med. 2009; 15(12):1377–1382. [PubMed: 19966777]

Nakatsuji T, Chen TH, Two AM, Chun KA, Narala S, Geha RS, et al. Staphylococcus aureus Exploits Epidermal Barrier Defects in Atopic Dermatitis to Trigger Cytokine Expression. J Invest Dermatol. 2016

Nakatsuji T, Chiang HI, Jiang SB, Nagarajan H, Zengler K, Gallo RL. The microbiome extends to subepidermal compartments of normal skin. Nat Commun. 2013; 4:1431. [PubMed: 23385576]

Pejler G, Abrink M, Ringvall M, Wernersson S. Mast cell proteases. Adv Immunol. 2007; 95:167–255. [PubMed: 17869614]

Prassas I, Eissa A, Poda G, Diamandis EP. Unleashing the therapeutic potential of human kallikrein-related serine proteases. Nat Rev Drug Discov. 2015; 14(3):183–202. [PubMed: 25698643]

Proft T, Fraser JD. Bacterial superantigens. Clinical and experimental immunology. 2003; 133(3):299–306. [PubMed: 12930353]

Sakabe J, Yamamoto M, Hirakawa S, Motoyama A, Ohta I, Tatsuno K, et al. Kallikrein-related peptidase 5 functions in proteolytic processing of profilaggrin in cultured human keratinocytes. J Biol Chem. 2013; 288(24):17179–17189. [PubMed: 23629652]

Sieprawska-Lupa M, Mydel P, Krawczyk K, Wojcik K, Puklo M, Lupa B, et al. Degradation of human antimicrobial peptide LL-37 by Staphylococcus aureus-derived proteinases. Antimicrobial agents and chemotherapy. 2004; 48(12):4673–4679. [PubMed: 15561843]

Stefansson K, Brattsand M, Roosterman D, Kempkes C, Bocheva G, Steinhoff M, et al. Activation of proteinase-activated receptor-2 by human kallikrein-related peptidases. J Invest Dermatol. 2008; 128(1):18–25. [PubMed: 17625593]

Steinert PM, Cantieri JS, Teller DC, Lonsdale-Eccles JD, Dale BA. Characterization of a class of cationic proteins that specifically interact with intermediate filaments. Proc Natl Acad Sci USA. 1981; 78(7):5.

Syed AK, Reed TJ, Clark KL, Boles BR, Kahlenberg JM. Staphlyococcus aureus phenol-soluble modulins stimulate the release of proinflammatory cytokines from keratinocytes and are required for induction of skin inflammation. Infect Immun. 2015; 83(9):3428–3437. [PubMed: 26077761]

Voegeli R, Rawlings AV, Breternitz M, Doppler S, Schreier T, Fluhr JW. Increased stratum corneum serine protease activity in acute eczematous atopic skin. Br J Dermatol. 2009; 161(1):70–77. [PubMed: 19416247]

Wang Z, MacLeod DT, Di Nardo A. Commensal bacteria lipoteichoic acid increases skin mast cell antimicrobial activity against vaccinia viruses. J Immunol. 2012; 189(4):1551–1558. [PubMed: 22772452]

Yamasaki K, Di Nardo A, Bardan A, Murakami M, Ohtake T, Coda A, et al. Increased serine protease activity and cathelicidin promotes skin inflammation in rosacea. Nat Med. 2007; 13(8):975–980. [PubMed: 17676051]

Yuki T, Yoshida H, Akazawa Y, Komiya A, Sugiyama Y, Inoue S. Activation of TLR2 enhances tight junction barrier in epidermal keratinocytes. J Immunol. 2011; 187(6):3230–3237. [PubMed: 21841130]

Zhang LJ, Guerrero-Juarez CF, Hata T, Bapat SP, Ramos R, Plikus MV, et al. Innate immunity. Dermal adipocytes protect against invasive Staphylococcus aureus skin infection. Science. 2015; 347(6217):67–71. [PubMed: 25554785]

Zollner TM, Wichelhaus TA, Hartung A, Von Mallinckrodt C, Wagner TO, Brade V, et al. Colonization with superantigen-producing Staphylococcus aureus is associated with increased severity of atopic dermatitis. Clinical and Experimental Allergy. 2000; 30:7. [PubMed: 10606925]

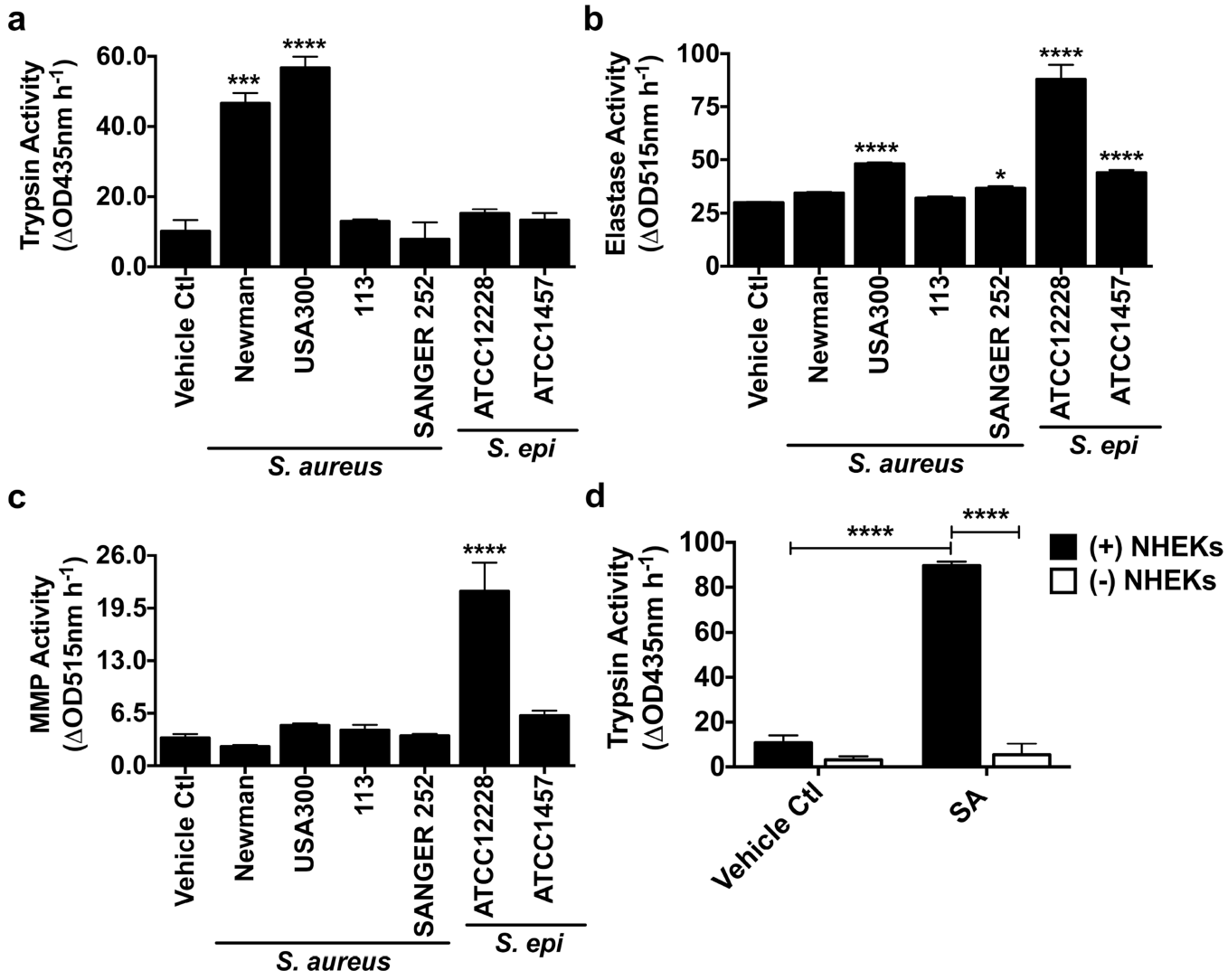

Figure 1. ***Staphylococci*** **regulate human keratinocyte protease activity**

(a–c) NHEKs were treated for 24h with *S. aureus* (Newman, USA300, 113, SANGER252) and *S. epidermidis* (ATCC12228, ATCC1457) sterile filtered supernatants and NHEK conditioned medium was analyzed with specific trypsin-like, elastase-like, and MMP protease substrates. **(d)** *S. aureus* (Newman) secreted proteases were analyzed for their influence on trypsin activity. Data represent mean ± SEM (n=4) and are representative of at least 3 independent experiments. Oneway ANOVAs **(a–c)** and two-way ANOVAs **(d)** were used and significance indicated by: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

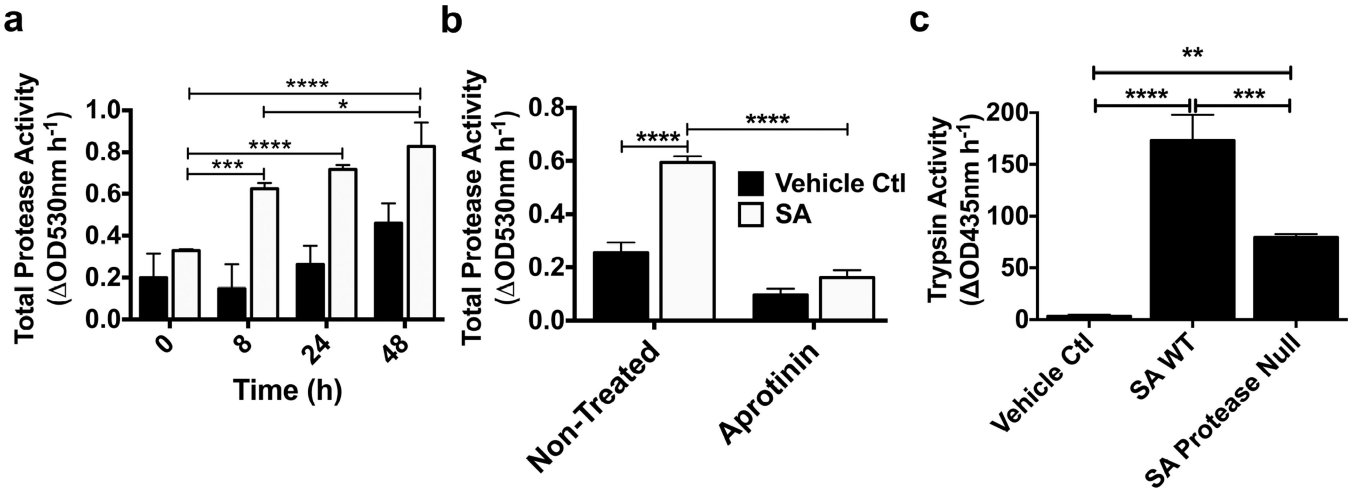

Figure 2. *S. aureus* increases human keratinocyte serine protease activity

(a) Total protease activity (5μg-mL BODIPY FL casein) was measured in NHEK conditioned medium after *S. aureus* (SA, Newman) supernatant treatment for 0–48h, **(b)** while the serine protease inhibitor aprotinin (800μg-mL) was applied to 24h post treatment conditioned medium. **(c)** *S. aureus* (USA300 LAC) WT and protease null strains were compared for effects on NHEK conditioned medium trypsin activity (Boc-Val-Pro-Arg-AMC, 200μM). Both two-way ANOVAs **(a,b)** and one-way ANOVAs (c) were used and significance indicated by: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

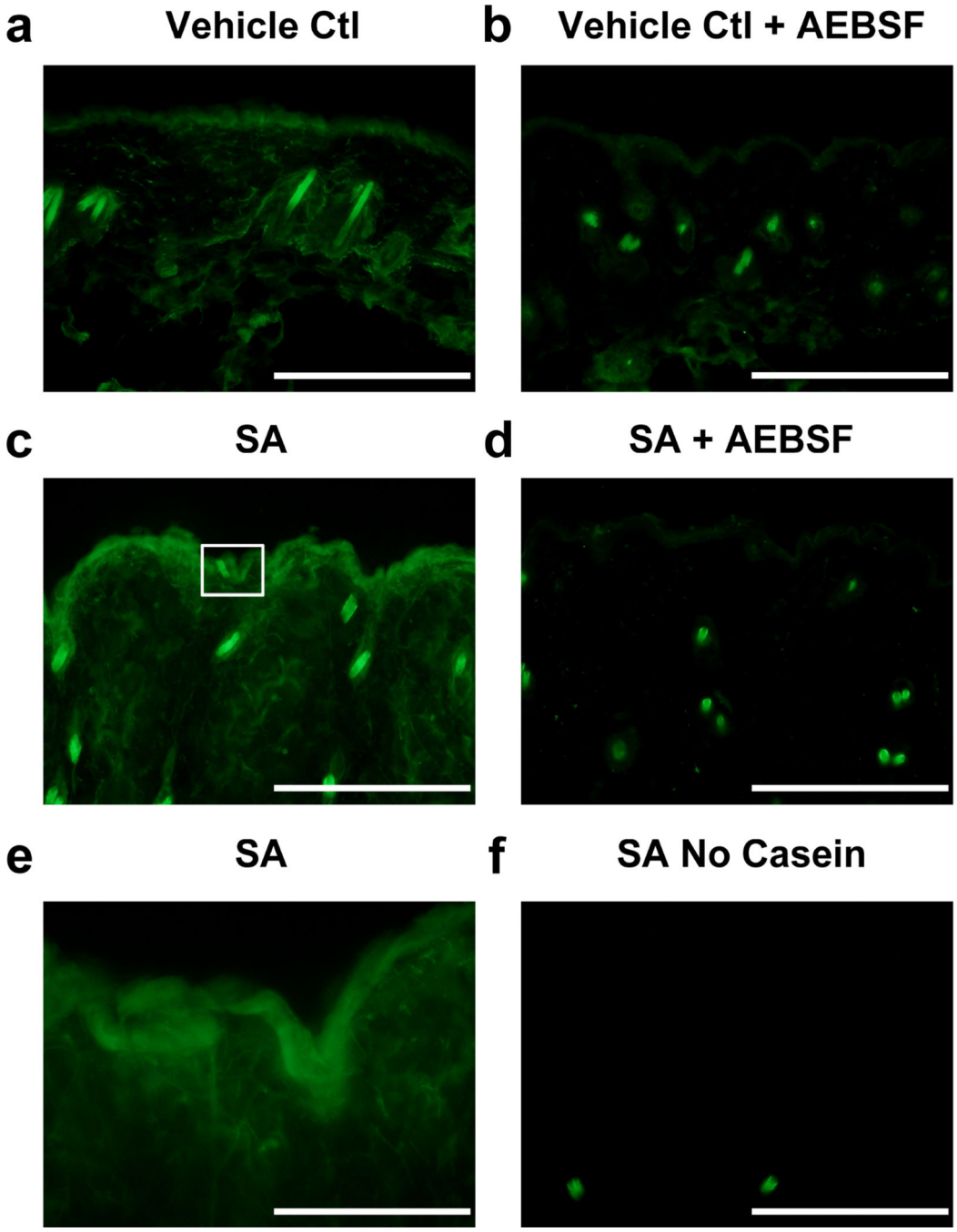

Figure 3. Murine epidermal serine protease activity is increased by *S. aureus*

Murine skin colonized with **(a,b)** TSB vehicle control or **(c,d)** $2e^6$ CFU *S. aureus* (SA, USA300) for 12h was assessed for changes to total protease activity (2μg-mL BODIPY FL casein) with or without the serine protease inhibitor AEBSF (50mM) by in situ zymography; scale bars=200μm. **(e)** Increased magnification of epidermal total protease activity from panel c as indicated by white box; scale bar=100μm. **(f)** A 12h *S. aureus* treated no BODIPY FL casein added control was included to show background staining; scale bar=200μm.

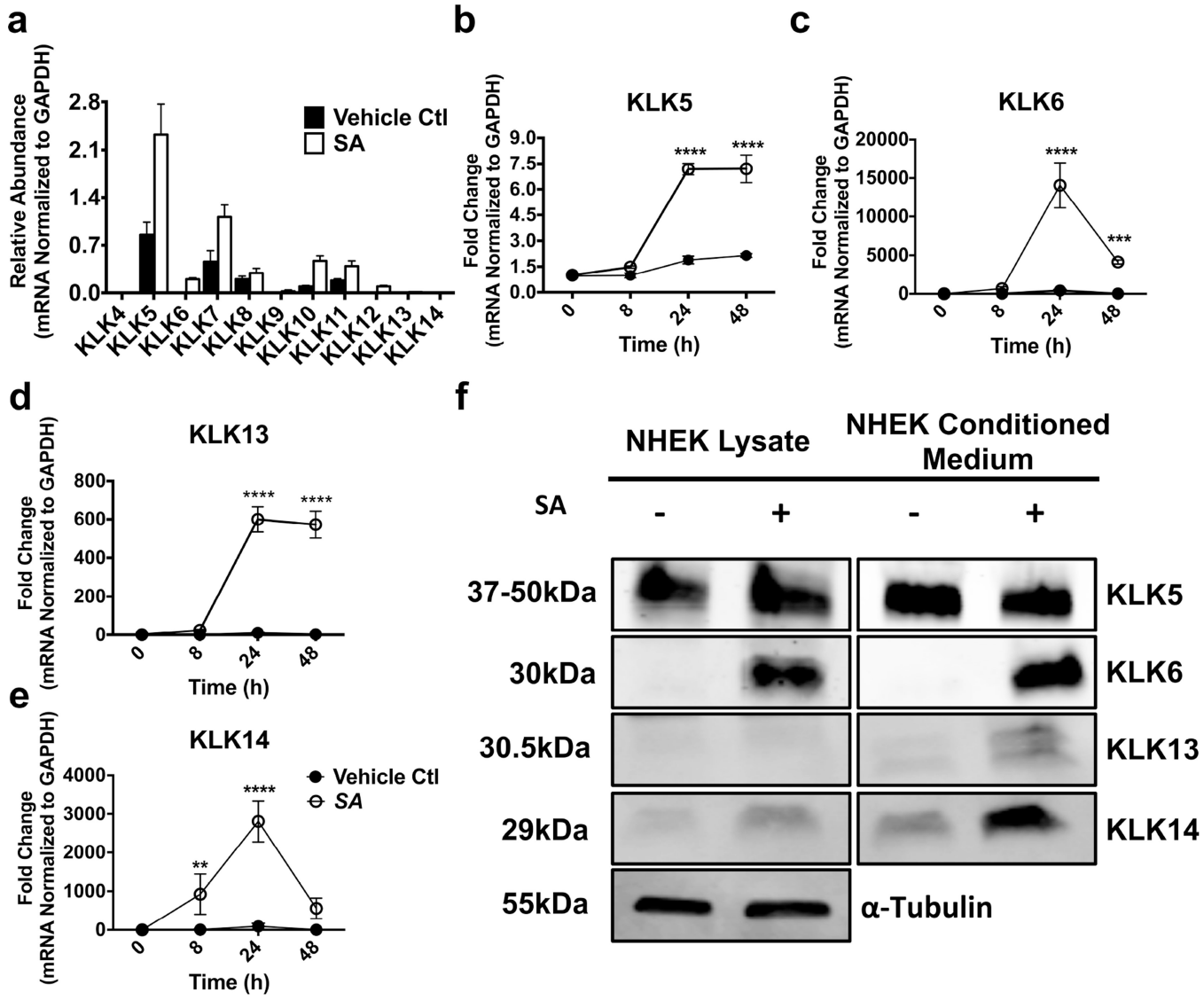

Figure 4. *S. aureus* increases KLK expression in human keratinocytes

(a) Relative abundance of KLK mRNA expression in NHEKs after 24h *S. aureus* (SA, Newman) supernatant treatment was analyzed by qPCR. **(b–e)** KLK5, 6, 13, and 14 were analyzed for fold changes in mRNA expression in NHEKs treated with *S. aureus* supernatant for 0–48h. All mRNA expression levels were normalized with the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). **(f)** NHEK conditioned medium and cell lysates were analyzed for changes in protein expression of KLK5, 6, 13, and 14 by immunoblotting after a 24h treatment with *S. aureus* (SA, Newman) supernatant using both published and predicted molecular weights. The housekeeping gene, α-Tubulin, was used as a loading control for cell lysates. Data represent mean ± SEM (n=3) and are representative of at least 3 independent experiments. Twoway ANOVAs **(b–e)** were used and significance indicated by: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

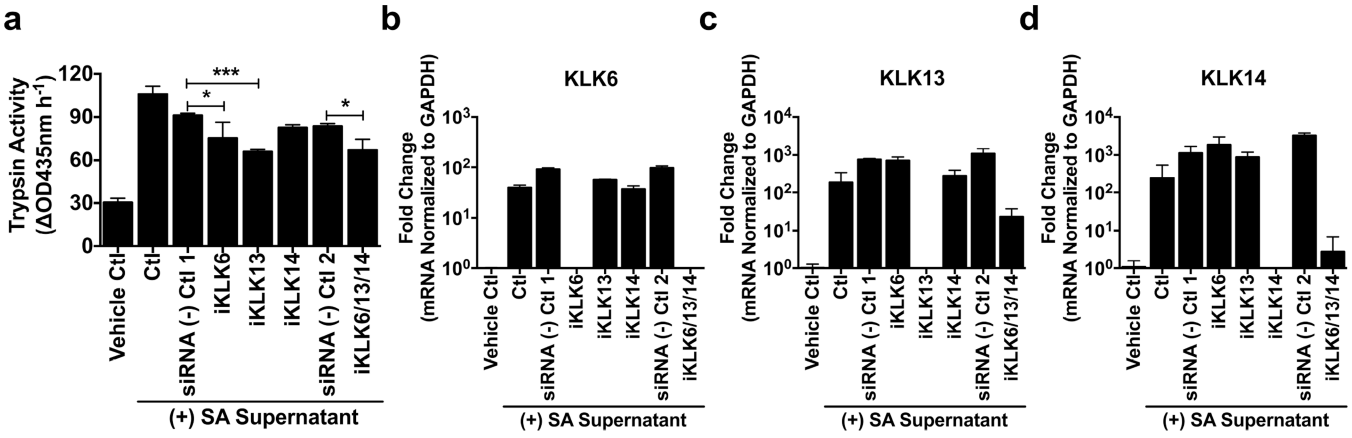

Figure 5. Multiple KLKs are responsible for *S. aureus* induced serine protease activity in human keratinocytes

NHEKs were treated with KLK6, 13, or 14 siRNA (15nM) prior to $CaCl_2$ differentiation and the addition of *S. aureus* (Newman) supernatant. siRNA scrambled (−) controls 1 and 2 were used at 15nM and 45nM respectively. **(a)** Conditioned medium was analyzed for changes in trypsin activity (Boc-Val-Pro-Arg-AMC, 200μM). **(b–d)** Transcript levels of KLK6, KLK13, and KLK14 were assessed by qPCR and normalized to the housekeeping gene, GAPDH, to confirm siRNA knockdown efficiency. Data represents mean ± SEM (n=4) and is representative of at least 3 independent experiments. One-way ANOVA **(a)** was used and significance indicated by: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

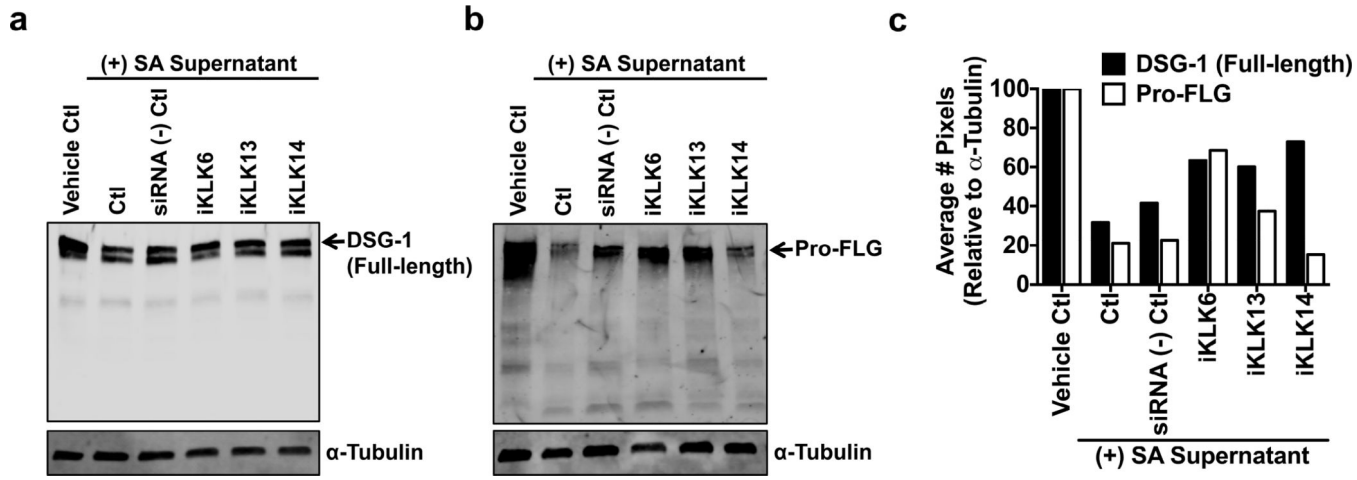

Figure 6. Multiple KLKs regulate *S. aureus* induced DSG-1 and FLG cleavage in human keratinocytes

NHEKs treated with *S. aureus* (Newman) supernatant for 24h were assessed for changes to **(a)** desmoglein-1 (DSG-1) and **(b)** Pro-filaggrin (Pro-FLG) cleavage after siRNA knockdown of KLK6, 13, and 14 (15nM) by immunoblotting. The housekeeping gene, α-Tubulin, was used as a loading control. DSG-1 (full-length) and Pro-FLG are indicated by black arrows. **(c)** Densitometry analysis of both DSG-1 (full-length) and Pro-FLG represented by the average number of pixels normalized to α-Tubulin (n=1). Immunoblots are representative of at least 3 independent experiments.