UNIVERSITY OF CALIFORNIA SAN DIEGO

Staphylococcus aureus quorum sensing and the battle for control of epidermal proteases by the skin microbiome

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

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2018

The dissertation of Michael Rey Williams is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

To my beautiful daughter Itzel “Izzy” Rey Padilla-Williams:

You are my rainbow and my world. You inspire me every day to not only help all those I can through science but to be the best person I can be as well. You will always be remembered and live on through those who love you each and everyday. Until we meet again…. my sweet, sweet, girl. I hope to make you proud!

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LIST OF ABBREVIATIONS

AD—Atopic dermatitis

AGR—Accessory gene regulator

AMP—Antimicrobial peptide

AIP—Autoinducing peptide

CAMP—Cathelicidin antimicrobial peptide

CoNS—Coagulase-negative *Staphylococci*

DAMP—Danger-associated molecular pattern

DSG1—Desmoglein-1

FLG—Filaggrin

GPCR—G-protein-coupled receptor

hBD—Human beta-defensin

IL—Interleukin

KLK—Kallikrein

LB—Lamellar bodies

MAPK—Mitogen activated protein kinase

NS—Netherton syndrome

NHEK—Normal human epidermal keratinocyte

PAMP—Pathogen-associated molecular pattern

PAR—Protease-activated receptor

PSM—Phenol-soluble modulin

PRR—Pattern recognition receptor

SC—Stratum corneum

SA—*Staphylococcus aureus*

TLR—Toll-like receptor

TNFα—Tumor necrosis factor α

TSLP—Thymic stromal lymphopoietin

ACKNOWLEDGEMENTS

I would like to thank my great mentor throughout the entirety of this dissertation, Dr. Richard L. Gallo. Dr. Gallo always made time to provide me with scientific insight and guide me through my project. Overall he has helped me develop into a much stronger scientist. Even more than a mentor of science, Dr. Gallo was always there to mentor me in life and help me through various personal endeavors, and for this I am forever grateful. I would also like to thank all of the members of the Gallo Lab and the Dermatology Department as a whole as well for always having their doors open to help me with my project.

Finally, I would like to thank my personal support at home. My best friend and partner, Celeste Padilla, is one of the most inspiring people I know. Not only through her energy and passion for teaching her 4th grade students but also through her drive to help those around her each and everyday. She has always been there for me throughout my Ph.D. as well as put up with my long nights in the lab. She is also the strong mother of my two beautiful daughters Itzel and Zoey who are my greatest inspiration for finishing this dissertation and to continue to help others through science. Last but not least, my other family members have also been a driving force in my ability to finish this project, but mostly by feeding me all the time so I have plenty of energy to do science!

Chapter II is based on: Williams, M.R. and Gallo, R.L. The role of the skin microbiome in atopic dermatitis. *Curr Allergy Asthma Rep.* 2015, 15(11):65. I was the primary researcher and author for the manuscript. The co-authors listed above either

supervised or provided support for the research, and have given permission for the inclusion of the work in this dissertation.

Chapter III is based on: Williams, M.R., Nakatsuji, T., Sanford, J.A., Vrbanac, A.F., and Gallo, R.L. *Staphylococcus aureus* Induces Increased Serine Protease Activity in Keratinocytes. *J Invest Dermatol*. 2017, 137(2):377-384. I was the primary researcher and author for the manuscript. The co-authors listed above either supervised or provided support for the research, and have given permission for the inclusion of the work in this dissertation.

Chapter IV is based on unpublished data currently being prepared for submission for publication: Williams, M.R., Costa, S.K., Zaramela, L.S., Sanford, J.A., O'Neill, A.M., Liggins, M.C., Nakatsuji, T., Horswill, A.R., Cheung, A.L., Zengler, K., and Gallo, R.L. Quorum sensing between bacterial species on the skin protects against epidermal injury in atopic dermatitis. I was the primary researcher and author for the manuscript. The co-authors listed above either supervised or provided support for the research, and have given permission for the inclusion of the work in this dissertation.

My graduate studies were funded in part by the UCSD Biomedical Sciences Graduate Program, the UCSD Dermatology Investigator Training Program T32 pre-doctoral training grant (NIH5T32AR06249603), the NIH Diversity Supplement (NIH3R01AR064781-04W1), and overall support from the Gallo Laboratory.

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ABSTRACT OF THE DISSERTATION

Staphylococcus aureus quorum sensing and the battle for control of epidermal proteases by the skin microbiome

by

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Doctor of Philosophy in Biomedical Sciences

University of California San Diego, 2018

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The top layer of the skin provides our first line of innate defense against external stimuli and is regulated in part by serine protease activity. One stimulus is that of bacteria that cover the entirety of our skin surface and can directly interact with skin immune cells, including epidermal keratinocytes. During a disease state such as atopic dermatitis (AD), the composition of the skin bacteria, or skin microbiome, changes and there is increased colonization by the pathogenic *Staphylococcus aureus (S. aureus)*. In this dissertation, we hypothesized that *S. aureus* could alter the protease activity of the epidermal skin barrier. We found that *S. aureus* could increase serine protease activity in both human keratinocytes and the murine epidermis in a strain and species dependent manner. Specifically a group of serine proteases known as the kallikreins

(KLKs) showed increased expression and these are directly responsible for part of the increased serine protease activity and epidermal barrier damage caused by *S. aureus*.

Based upon the initial hypothesis, we further explored the mechanism of *S. aureus* induced serine protease activity in keratinocytes. We discovered that *S. aureus* phenol-soluble modulins (PSMs), specifically PSMα, are responsible for inducing serine protease activity in keratinocytes. Furthermore, PSMα as well as *S. aureus* secreted proteases are both necessary for inflammation and barrier damage on murine skin. Both of which are controlled via the *S. aureus* accessory gene regulator (agr) quorum sensing system.

This led us to further explore how to prevent *S. aureus* mediated epidermal barrier damage through targeting the agr system. We showed that coagulase-negative *Staphylococci* (CoNS) clinical isolates from AD patients could turn off the *S. aureus* agr system through use of their own agr systems and secretion of autoinducing peptides (AIPs). This prevented *S. aureus* induced barrier damage and inflammation in both human keratinocyte and mouse models. Overall, the data presented in this dissertation provides a novel mechanism for how *S. aureus* can drive AD–like skin inflammation and epidermal barrier damage through multiple mechanisms including the induction of endogenous serine protease activity. Secondly, targeting the agr quorum sensing system of *S. aureus* and virulence factor release by using our own healthy microbes can ultimately provide a novel therapeutic option to prevent skin inflammation and barrier damage.

Chapter I:

Introduction

The skin is a remarkable organ that covers the entirety of our body and provides our initial line of immune defense to the external environment. Generally our skin is constantly exposed to potentially harmful stimuli including heat, ultra-violet (UV) light, chemicals, physical interactions, and microbes. In order for the skin to protect the body from these environmental factors, it forms a barrier on the outermost layer of the skin, the epidermis. The epidermis, comprised primarily of keratinocytes that form a stratified epithelial layer, creates both a physical and an immunological barrier. The physical barrier is composed primarily of lipids (e.g. ceramides), proteins (e.g. keratin, filaggrin), and carbohydrates (e.g. hyaluronan, heparin sulfate) that gather on the top layer of the epidermis, known as the stratum corneum, to form a highly hydrophobic and tightly packed/structured layer of 'dead keratinocytes' or corneocytes (*1*). This physical barrier is important both for retaining water and preventing external stimuli from penetrating the skin. Meanwhile, the epidermal immunological barrier can produce antimicrobial peptides (AMP) including cathelicidin and β-defensins (*2*) that play an important role in preventing bacterial infection of the skin as well as controlling angiogenesis and wound healing (*3*-*5*). Together, both the epidermal physical and immune skin barriers ensure we are protected from the external stimuli that our skin is constantly bombarded with.

A complex network of proteases and protease inhibitors play a crucial role in regulating this epidermal skin barrier. For instance, matriptase and prostasin are membrane bound serine proteases that help control terminal differentiation of keratinocytes (*6*). The skin specific cysteine protease, caspase-14, regulates filaggrin processing (*7*), and the matrix-metallo proteases (MMPs) have been linked to serine protease activation as well as regulation of wound healing in the skin (*8*). Although the

network of skin protease and protease inhibitors is vast, perhaps the most well studied group includes that of the kallikreins (KLK), a 15 membered serine protease family that has been linked to skin disease.

KLKs play an essential role in the natural turnover/shedding of the stratum corneum in a process called desquamation. This process normally occurs every 28 days. In particular, KLK5, KLK7, and KLK8 have been shown to maintain the rate of desquamation by controlling cleavage of the corneodesmosome adhesion proteins desmoglein-1 (DSG-1), corneodesmosin (CDSN), and desmocollin 1 (DSC1) (*9, 10*). Other functions of KLKs include controlling skin inflammation and pro-inflammatory cytokine production through regulation of the protease-activated receptor 2 (PAR-2) (*11, 12*), KLK5 and KLK7 mediated processing of the AMP, cathelicidin (*13*), and regulation of the structural protein filaggrin (*14*). KLKs therefore control both the physical and immunological aspects of the epidermal skin barrier.

Control of unimpeded KLK activity by serine protease inhibitors is equally important to maintaining an intact skin barrier. The serine protease inhibitor Kazal-type 5 (SPINK5) has been shown to be the primary inhibitor of multiple members of the KLK family and thus plays a vital role in balancing KLK activity. Perhaps the importance of SPINK5 in controlling KLK activity is best displayed in the skin disease of Netherton syndrome (NS). NS patients display a genetic mutation in SPINK5 that leads to a hyperactive KLK response and an exposed skin barrier. These patients often are left very susceptible to dehydration, allergy, and infection as a result of this uncontrolled protease response (*15*). Mechanistically in NS patients KLKs make a dysfunctional skin barrier through enhanced levels of desquamation as well as increased protease

activated receptor 2 (PAR-2) activation/inflammation, AMP processing, and filaggrin cleavage. Thus the balance between KLKs and SPINK5 is key to maintaining an intact skin barrier and protecting us from dangerous environmental stimuli.

Based upon the importance of the epidermal barrier defense against external stimuli and the role of proteases in regulating this barrier, we initially wanted to explore how surface microbes might affect the skin barrier integrity. The entirety of our skin surface is covered by a culmination of bacteria, viruses, and fungi that together comprise the skin microbiome. In particular, the composition of bacteria on the skin has been well characterized through the use of next generation sequencing (NGS) techniques. Initially it was revealed through 16S ribosomal RNA (rRNA) DNA sequencing that four phyla of bacteria readily colonize the skin including Firmucetes, Proteobacteria, Corynebacterium, and Bacteroidetes. The regions of the skin these bacteria colonize depend both on the physical composition of the skin (e.g. sebaceous, moist, dry) and environmental factors including age, sex, and location (*16, 17*). More sensitive sequencing methods as well as culture techniques have further shown that specific commensal microbial species frequently inhabit our skin as well including *Staphylococcus epidermidis* (*S. epidermidis*) and *Propionibacterium acnes* (*P. acnes*). Interestingly, these commensal microbes have been shown to communicate in a symbiotic fashion with our skin barrier by presumably enhancing our skin barrier defense against external stimuli. It has been shown the *S. epidermidis* for instance can increase T cell recruitment into the epidermis as well as increase tight junctions, AMP production, mast cell recruitment, and prevent TLR3 mediated inflammation (*18-22*).

Aside from healthy skin, surface bacteria have also been shown to play a role in skin diseases such as atopic dermatitis (AD) (*23, 24*). The bacterial skin microbiome of AD patients has been shown to enter a state of dysbiosis, or a change from the normal bacterial composition. In particular, the pathogenic *Staphylococcus aureus* (*S. aureus*) displays increased skin colonization during AD while overall microbial diversity is decreased (*25, 26*). Increased *S. aureus* colonization is directly correlated with increased AD severity and recent clinical studies have even suggested *S. aureus* can have a causative effect on disease onset while more virulent strains of *S. aureus* can also cause worsened skin lesions in animal models (*27-29*). Due to these findings, multiple efforts have sought out new therapeutic strategies to combat changes to the skin microbiome during AD as well as to understand mechanistically how increased *S. aureus* colonization can cause or worsen the disease state.

S. aureus secretes an array of virulence factors including protein A, superantigens (e.g. enterotoxins, TSST-1), lysins (α-toxin, PVL, phenol-soluble modulins (PSMs)), and proteases (e.g. V8, aureolysin) (*30*). These factors have been shown both *in vitro* and *in vivo* to induce non-specific T cell responses, keratinocyte cytokine production and cytotoxicity, and increase the rate of desquamation in the skin amongst other functions. Most recent evidence has revealed that *S. aureus* can elicit an immune response on murine skin in a PSMα and secreted protease dependent manner (*31-33*). Although there is evidence clearly showing experimentally how *S. aureus* can elicit a direct inflammatory phenotype on the skin, further exploration into how it mechanistically alters the epidermal barrier and which toxins are responsible still remains unanswered.

The role of skin bacteria, both commensal and pathogenic, and proteases in regulating the epidermal skin barrier integrity led us to propose the initial question of do skin surface bacteria alter the epidermal skin barrier proteolytic balance? This dissertation goes on to define how *S. aureus*, in a species and strain dependent manner, plays a vital role in enhancing serine protease activity in epidermal keratinocytes, specifically that of the KLKs. Furthermore we define the role of both *S. aureus* secreted proteases and PSMs in stimulating this response in both keratinocyte and murine models.

Interestingly, the *S. aureus* virulence factors that have been shown to induce both epidermal barrier damage and skin inflammation, including increases to KLK activity, are both under control of the accessory gene regulator (agr) quorum sensing system. The agr system is activated when *S. aureus* reaches a high density by a short autoinducing peptide (AIP). Basically this AIP produced by the agrD gene leads to downstream activation of the P3 promoter leading to RNAIII transcription and in turn further transcription of many *S. aureus* virulence factors including secreted proteases. Furthermore the phosphorylated agrA protein can further directly activate the PSMα/β operons (*34, 35*). Since the *S. aureus* agr system controls production of important virulence factors that induce skin inflammation and barrier damage, we sought to further explore how to directly turn it off and thus present a novel therapeutic strategy to combat increased *S. aureus* colonization in disease states. In 2001, an essential finding to turn off the *S. aureus* agr induced virulence factors was made. It was determined that a particular strain of *S. epidermidis* can secrete an AIP using it's own agr system that can crosstalk with *S. aureus* and turn off the agr system (*36*). Based

upon these findings we further explored in this dissertation how commensal microbes, including *S. epidermidis* as well as other Coagulase-negative *Staphylococci* (CoNS) can turn off the *S. aureus* agr system in both human keratinocyte and murine models.

Overall this dissertation looks into a novel virulence mechanism by which *S. aureus* PSMs and proteases can target the epidermis to release KLKs leading to a disrupted skin barrier. Furthermore we explore how specific commensal microbes can prevent *S. aureus* from inducing skin barrier damage and increased KLK activity by turning off the agr quorum sensing system. Overall this work will allow us to therapeutically apply specific commensal microbes onto diseased skin in order to combat the effects of *S. aureus* agr regulated virulence factors on our epidermal barrier.

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Chapter II:

The role of the skin microbiome in atopic dermatitis

Abstract

Atopic dermatitis (AD) is a common skin disease that affects a large proportion of the population worldwide. The incidence of AD has increased over the last several decades along with AD's burden on the physical and psychological health of the patient and family. However, current advances in understanding the mechanisms behind the pathophysiology of AD are leading to a hopeful outlook for the future. *Staphylococcus aureus* (*S. aureus)* colonization on AD skin has been directly correlated to disease severity but the functions of other members of the skin bacterial community may be equally important. Applying knowledge gained from understanding the role of the skin microbiome in maintaining normal skin immune function, and addressing the detrimental consequences of microbial dysbiosis in driving inflammation, is a promising direction for development of new treatments. This review discusses current preclinical and clinical research focused on determining how the skin microbiome may influence the development of AD.

Introduction

Atopic dermatitis (AD) is a chronic allergic skin disease the includes frequent flares, and manifests with characteristic findings of dry, red, and pruritic skin in a typical distribution (*1*). AD is one of the most common skin disorders and affects 5-20% of infants worldwide with lesser prevalence in adulthood (*2*). Although AD is rarely lethal, several studies have shown that AD frequently leads to a severely compromised way of life for patients and their families, and thus is a major health care burden (*3, 4*). Furthermore, AD often associates with other allergic diseases including asthma and allergic rhinitis (*5*), thus further compounding the importance of this disease. Finally, the number of infants and adults affected by AD is higher in industrialized nations and has also grown over the past several decades. Many suspect a link to excessive hygiene in these industrialized regions leads to a lack of beneficial host immune education provided by microbes on or within the body (*6, 7*). Understanding the mechanisms behind AD and ways to treat it are therefore extremely important and highly relevant topics in the fields of allergy and dermatology.

Both genetic and environmental factors play a substantial role in the onset of AD. Genome-wide scans have revealed that there are several shared chromosome loci among AD patients where gene expression has been altered. These studies, however, clearly demonstrate that AD is a complex disease with many potential genetic factors at play (*1*). Specific genetic mutations that have been linked to AD Include genes involved in formation of the epidermal skin barrier such as filaggrin (FLG), genes involved in tight junctions (TJs), and the serine protease inhibitor Kazal-type 5 (SPINK5) (*8-12*). Mutations in T helper cell 2 (Th2) cytokines IL-4 and IL-13 have also been correlated in

AD pathogenesis (*13, 14*). In most cases, data support that environmental factors including food and aeroallergens, as well as physical stressors including hydration and scratching, can play a substantial role in disease severity as well (*15-17*).

A complete and in depth analysis of the hypothesized mechanisms that drive AD have been discussed previously (*1, 18, 19*). The purpose of this review is to focus on the rapidly advancing information that has shown a clear role for the skin microbiome in controlling the clinical manifestations of this disease (Figure 2.1). Determining how microorganisms that inhabit the skin regulate AD can help us to better understand both the mechanisms behind AD and provide new ways to apply therapeutic strategies to combat the disease.

The skin microbiome and changes in atopic dermatitis

Methods of determining the skin microbiome composition

The skin is colonized by numerous species of bacteria, fungi, and viruses that together are known as the skin microbiome. The composition of this community varies between individuals but varies even more depending on skin topography (*20*). The use of next-generation DNA sequencing methods have allowed us to better understand both the composition and potential roles that these microorganisms, in particular bacteria, play on our skin. Importantly, as the technology has improved, so has our understanding of what specific bacteria are associated with disease.

16S ribosomal RNA (16S rRNA) partial DNA sequencing was initially used to determine the microbial composition on the skin at the genus level, and has been followed by the development of full-length 16S rRNA DNA sequencing techniques to

allow for more specific identification of the organism at the species level (*20, 21*). These initial sequencing methods showed that our normal skin microbial flora consist of four common phyla of bacteria—Firmicutes, Proteobacteria, Bacteriodetes, and Actinobacteria (Table 2.1). Species of bacteria detected by 16S rRNA DNA sequencing show that hundreds of bacteria frequently colonize the skin, while culture techniques have found that the most commonly detected organisms include both *Propionibacterium acnes* and *Staphylococcus epidermidis* (*S. epidermidis*) among others (*22-25*). DNA sequencing has also provided a useful way to track temporal shifts in the skin microbial flora during normal versus disease states, and this will be discussed later in the review. Beyond 16S rRNA DNA sequencing, recent efforts have begun to apply metagenomics (*26*). Metagenomics is a method that has been used for studying the composition of microbial communities in both the gut and oral cavities. It uses DNA sequencing of full-length microbial genomes allowing for identification of microbes at the strain level. This degree of specificity provides a tool for determining which bacterial strains are present on the skin. Knowing the specific strains, and not just the species of bacteria, permits one to explore the molecular mechanisms by which individual bacteria influence skin immune function.

Normal skin microbial flora

The colonization of bacteria on specific regions on the skin depends upon the local skin environment including moisture levels, pH, and keratinocyte cell surface adhesion proteins. For instance, *Staphylococcus* and *Corynebacterium* species thrive in specific environments on the skin such as the sole of the foot and the popliteal fossa

(back of the knee). Dry environments such as the volar forearm (inside forearm) and hypothenar palm (palm of hand) are more prone to harbor a mixed population of bacteria including both β-Proteobacteria and Flavobacteriales (*20*). Environmental factors such as diet, age, and gender also play a role in the makeup of the skin microbial flora (*27*). Infants are essentially born microbe free until exposure to the external environment allows for immediate colonization of their skin (*28*). Throughout their lifespan, the microbial diversity can change. This environmental influence on our skin microbial flora means that each individual has a unique skin microbiome based in part upon life experiences and in part upon factors genetically predetermined by the host.

The skin microbiome has been investigated extensively for its role in communicating with the immune response. *S. epidermidis*, a highly abundant commensal bacteria, has been shown to modify innate inflammatory responses through a toll-like receptor (TLR) cross talk mechanism. In this study, it was revealed that *S. epidermidis* could suppress TLR3-mediated keratinocyte inflammation due to skin injury by stimulating TLR2 with lipoteichoic acid (LTA) (*29*). *S. epidermidis* can also increase T cell recruitment to the skin in germ-free mouse models and influence T cell maturation (*30*). Similarly, the host skin immune response can act on the microbiome. Keratinocytes in the epidermis detect microbial flora on the skin surface via pattern recognition receptors (PRRs). Such recognition by TLR2 leads to production of host antimicrobial peptides (AMPs) including the beta-defensins 2 and 3 (DEFB2/DEFB3) that defend the skin from microbial evasion. TLR2 activation in the skin by the microbial flora can also influence mast cell recruitment and increase tight barrier junctions leading

to a stronger skin barrier (*31-33*). Interestingly, commensal bacteria still survive on the skin surface despite TLR activation and AMP production by keratinocytes. These findings have led to the speculation that there must be a permissive relationship with common skin microbial flora. This prevents both a constitutively active immune response as well as allowing for commensal bacteria to help protect our skin from pathogens.

Recently, it was discovered that bacteria are normally present within the different layers of the skin and not just on the skin surface (*34*). This demonstrated that bacteria could penetrate the outer skin barrier and interact normally with many different cell types that exist in the deeper dermis. Based upon this finding, the capacity for the skin microbiome to influence immune homeostasis in normal and disease states becomes more apparent. In the future, it will be important to determine how microbes that have penetrated the skin barrier and that reside in either the epidermis, dermis, or subcutaneous layers communicate with the host and vice versa.

Commensal skin microbes seeking to retain their niche on specific skin sites have also developed defense mechanisms against other bacteria that may seek to colonize the skin surface. For example, some strains of *S. epidermidis* produce bacteriocins that that are toxic to other bacterial species such as *Staphylococcus aureus (35)*. *S. epidermidis* can also target *S. aureus* through production of both the serine protease Esp and phenol-soluble modulins (PSMs) that prevent *S. aureus* biofilm formation and growth, respectively (*36, 37*). Another common commensal bacteria on the skin, *P. acnes*, inhibits *S. aureus* growth as well (*38*). Thus, commensal skin

microbes can communicate with our skin in order to promote a stronger skin barrier and immune response, and have methods of their own to prevent invasion by pathogenic bacteria. Taken together, it is apparent that the normal skin microbiome has multiple essential actions to maintain immune homeostasis, and some commensal bacteria might even be considered as another immunocyte functioning in coordination with host derived immune cells.

Dysbiosis of the skin microbiome in AD

The skin microbial flora can enter a state of dysbiosis, defined as a change in relative composition of the different microbes compared to normal, during a disease state. This is most well described in AD. It was first observed in the 1970's that there was increased *S. aureus* colonization on AD lesions (*39*). Since then, multiple studies have observed this phenomenon, although the sheer complexity of how microbial composition changes in AD was not established until the use of 16S rRNA DNA sequencing (*40*). Sequencing revealed that AD patients have an overall increase of colonization by *Staphylococcae* species along with an overall decrease in the number of different types of bacteria (microbial diversity) on involved skin. It particular *Staphylococcus aureus* (*S. aureus*) colonization of the skin is increased, as previously shown, while *S. epidermidis* is increased to a lesser extent. These increases result in dysbiosis since the expansion of these populations is not accompanied by a proportional increase in the other bacteria found on normal skin.

The prevalence of *S. aureus* colonization on AD lesional skin varies, with some reporting the capacity to culture *S. aureus* from AD to be approximately 80-100% while

it is detectable with much lower frequency (5-20%) in healthy individuals (*41*). Increased colonization of *S. aureus* on AD skin is strongly linked to increased severity of the disease (*41, 42*). The role of *S. aureus* colonization in increasing AD severity will be discussed below, and this association highlights why it is important to understand the mechanism of *S. aureus* virulence on AD skin in order to come up with more appropriate therapeutic solutions. However, the association is not clearly one of cause and effect as elimination of *S. aureus* is not a solution for this disease.

Dysbiosis of the skin microbiome in other allergic skin diseases

Besides AD, other rare skin diseases associated with allergy also have been reported to experience shifts in the skin microbiome. Netherton syndrome (NS) is a rare skin disease caused by a loss-of-function mutation in SPINK5, a key serine protease inhibitor in the epidermis (*43*). Decreased SPINK5 expression leads to a hyperactive serine protease response and increased inflammation and desquamation, or stratum corneum shedding. This disease has been associated with both increased IgE levels in the blood as well as increased colonization by *S. aureus* although in depth 16S rRNA sequencing of NS patients has not yet been studied to reveal the total microbial composition (*44, 45*).

Hyper IgE Syndrome (HIES), a primary immunodeficiency disease, represents another allergic skin disease where STAT3, a crucial signaling kinase, is mutated. HIES patients experience eczema as well as increased blood IgE levels. 16S rRNA sequencing has revealed that the skin microbiome of HIES patients has increased colonization of *S. aureus* along with to a lesser extent, *Corynebacterium* (*46*). Thus

several allergic skin diseases that display dry and flaky skin and a compromised skin barrier, display dysbiosis of the skin microbiome and in particular increased *S. aureus* colonization.

Microbial mechanisms for increased AD severity

Physical changes to the skin barrier and the role of antimicrobial peptides in skin microbiome dysbiosis

AD skin harbors a very different environment for bacterial growth than that of normal skin and this may be the fundamental explanation for the dysbiosis observed in AD. A dysfunctional physical skin barrier leads to an increase in pH on the skin surface that favors *S. aureus* growth (*47, 48*). Differentiated keratinocytes in the epidermis that are directly exposed to microbes have altered cell surface marker expression as well. In particular it has been found that both fibronectin and fibrinogen expression is increased in keratinocytes from AD patients and these markers directly bind to *S. aureus in vitro* (*49, 50*).

Immunological forces at play in the skin also influence changes to the skin microbial composition during AD. Endogenous AMPs are important for preventing pathogenic microbes from infecting the skin. Two main classes of AMPs in the skin are cathelicidin and DEFβs (*51, 52*). Both of these have the ability to kill *S. aureus in vitro*. However, in AD skin cathelicidin as well as DEFβ-2 and DEFβ-3 expression is decreased in comparison to similarly inflamed psoriatic skin (*53*). Secondly, keratinocyte models show that the AD associated Th2 cytokines IL-4, IL-5, and IL-13 can decrease

expression of host AMPs *in vitro* (*54*). Besides Th2 specific cytokines, IL-10 has also been linked to decreasing AMP production (*55*). Overall, the lack of host AMPs represents another reason for decreased microbial diversity and enhanced *S.aureus* growth on AD skin.

S. aureus virulence factors in AD severity

It is proposed that the association of dysbiosis with AD disease severity is more complex than simply AD leading to changes in bacterial growth due to the factors described above. It is apparent that, in some cases, the bacterial community can further drive the disease. A well known example of this is the hypothesis that *S. aureus* can increase the severity of AD by secreting a variety of virulence factors (Table 2.2). The most well studied of these are the superantigens (SAgs). SAgs function mechanistically by binding to the non-peptide groove of major histocompatibility complex class II (MHC-II) on antigen presenting cells (APCs), including skin keratinocytes, as well as to the T cell receptor (TCR) β-chains. This leads to non-specific activation of approximately 5-20% of all naïve T cells and systemic inflammation through production of pro-inflammatory cytokines including TNF-α and IL-1β (*56-59*). SAgs produced by *S. aureus* include the staphylococcal enterotoxins (SE) and toxic shock syndrome toxin 1 (TSST-1) (*60-62*). Several studies have shown direct correlations between SAg producing *S. aureus* strains on AD patients and AD severity. It was observed in one study that 57% of AD patient *S. aureus* skin isolates produced SAgs as opposed to 33% of control patient isolates. The SAg positive *S. aureus* colonized AD patients also displayed increased AD severity based upon SCORAD (SCOring Atopic Dermatitis) with 58±17 as

opposed to 41±7 in control patients (*61*). A second study revealed that elevated levels of SEA and SEB specific IgE antibodies in the blood correlated with increased AD severity (*63*).

Another factor that is proposed to increase AD severity is that of hemolysin-α or α-toxin. α-toxin monomers form a heterodimer complex on the cell membrane that creates a porous channel leading to cell lysis. This has been shown in *in vitro* studies where α-toxin is severely toxic to keratinocytes (*64, 65*). It has been proposed that Th2 cytokines can also increase α-toxin induced keratinocyte toxicity (*66*). Murine models with subcutaneous injections of α-toxin displayed increased inflammation at the site of injection (*67*). Although high concentrations of α-toxin are toxic to host cells, low concentration can also stimulate keratinocyte cytokine production leading to increased inflammation as well (*68*).

Other *S. aureus* virulence factors have also can possibly influence AD severity. δ toxin can target skin mast cells leading to degranulation and increased inflammation (*69*). Protein A can induce TNF-α production in keratinocytes in a non-toxic manner (*68*). Finally, *S. aureus* secreted PSMs are also known to increase inflammation in keratinocytes (*70*). Although much is known of the virulence factors discussed above, more are still being discovered. Understanding all of these virulence factors and how they affect the skin barrier during AD is crucial to our ability to combat *S. aureus* mediated AD severity.

Role of proteases in microbial dysbiosis and AD severity

Maintaining the balance of activity between proteases and their inhibitors is essential to upholding a functional skin barrier. During AD, patients display an increase in serine protease activity. Specifically the serine protease family known as the kallikreins (KLKs) is observed to have increased activity (*71, 72*). Hyperactive KLK responses have been studied in NS patients and appear to be responsible for increased desquamation of the skin, altered cathelicidin and filaggrin processing, as well as increased PAR-2 activity and inflammation (*73-76*). Thus, the increased KLK activity in AD skin helps create a compromised skin barrier and possibly aids in increasing *S. aureus* colonization of the skin.

Exogenous serine proteases released from *S. aureus* have also been studied for their role in damaging the skin barrier. The V8 serine protease increases desquamation of the epidermis *in vitro* as well as being able to alter skin barrier integrity in murine models (*77, 78*). The serine-like proteases, exfoliative toxins A and B (ETA/B), have mostly been described for their role in inducing Staphyloccocal scalded skin syndrome (SSSS) (*79*). In SSSS, ETA/B cleave desmoglein-1, a corneodesmosomal adhesion protein that plays a crucial role in regulating desquamation. Excess shedding of the stratum corneum, or the uppermost epidermal layer, leads to a disrupted skin barrier and increased bacterial invasion. Although *S. aureus* serine proteases have not been directly linked to AD, their virulence can easily suggest another mechanism for enhanced AD severity.

Effect of other bacterial strains on AD pathogenesis

Besides increased *S. aureus* colonization of the skin in AD, 16S rRNA DNA sequencing has revealed increased *S. epidermidis* colonization. However, the function of increased *S. epidermidis* colonization on AD skin remains unclear. The presence of *S. epidermidis* on germ free mouse skin can lead to an increase in T cell effector function in an IL-1 dependent manner. This seems to have a protective role against infections (*30*). *S. epidermidis* has also been shown in both murine and keratinocyte models to have a protective role through the amplification of endogenous AMPs (*80, 81*). Finally there is evidence that *S. epidermidis* can prevent *S. aureus* biofilm formation in the nasal cavities as well as produce its own AMPs to prevent other pathogens from colonizing the skin (*35, 82*). Therefore increased *S. epidermidis* colonization in AD skin may represent a way for the skin to naturally prevent increased *S. aureus* colonization in AD. However, the increased abundance of *S. epidermidis* in AD may not correlate with the protective and beneficial effects discussed above since these effects are strain-dependent, and it is not clear if these beneficial strains are active in AD. The use of metagenomics and species specific bacterial sequencing should provide useful insight into the role of *S. epidermidis* on AD skin.

Recently it has been reported that *Corynebacterium bovis* colonization is increased in an ADAM17 knockout model of AD in mice (*83*). Increased *Corynebacterium bovis* colonization led to a robust Th2 response in the skin, a key characteristic of acute AD. 16S rRNA DNA sequencing has only revealed increased *Corynebacterium* in HIES though, and not in AD skin. Thus this might serve as a way

that dysbiosis of the skin microbiome can increase HIES severity by altering the immune response versus that seen in AD.

Therapeutic strategies to combat *S. aureus* on AD skin

Since *S. aureus* colonization has been linked to increased AD severity, multiple therapeutic strategies have attempted to target specifically *S. aureus* colonization. The most common used treatments for *S. aureus* on AD skin include both topical/oral antibiotics and mild bleach baths. Studies conducted so far however have produced conflicting results. Many topical/oral antibiotic treatments can kill *S. aureus* in the short term on AD patients including muripocin, flucloxacillin, retapamulin, and cephalexin (*84-86*). However, few of these patients see decreased AD severity. *S. aureus* colonization also typically relapses in AD patients after 4 to 8 weeks of antibiotic treatment (*87*). A study in 2002 showed that a combination therapy of multiple antibiotics including muripocin, chlohexidine, and cephalexin improved clinical AD scores in 9 out of 10 patients with *S. aureus* colonization (*88*), but this is not the typical clinical experience. Corticosteriods, anti-inflammatory reagents used as a staple for treatment of AD, have also been used in combination with antibiotics to improve AD severity (*41*). This suggests that perhaps removal of *S. aureus* alone is not sufficient for AD treatment but anti-inflammatory reagents are also necessary. Furthermore there also are other important issues that deter using antibiotics as a treatment method for AD. The recent rise of methicillin-resistant *S. aureus* (MRSA) strains and their ability to colonize AD skin is a clear demonstration of one major issue (*89*). Antibiotic therapy for AD patients is also relatively non-specific, targeting mostly all gram-positive bacteria. Thus treating *S.*

aureus colonization on AD skin with antibiotics could affect the beneficial microbes such as *S. epidermidis* on the skin as well.

Bleach baths in combination with antibiotics can also decrease the severity of AD (*90, 91*). In 2013, a study in Malaysia revealed that dilute bleach baths performed twice a week for 2 months both decreased AD severity and *S. aureus* colonization (*92*). Although bleach baths have potential to successfully treat AD through clearance of *S. aureus*, more studies need to be conducted to confirm the current results with or without antibiotic activity. In particular, it is unlikely that the highly dilute bleach bath solution is directly antimicrobial for the skin surface. Beneficial actions of the bleach bath solution may relate to other hydrating or immunological effects (*93*). In the few studies where *S. aureus* has decreased with bleach bath treatments it is important to understand if this effect occurred as a consequence of improved function of the skin rather that the action of the bath directly on the bacteria. It is also important to understand how bleach baths affect the total skin microbiome. Overall, it is clear that current treatment of *S. aureus* in AD patients represents at best a limited solution for some patients suffering from frank super-infection and other more targeted therapies are needed to correct the dysbiosis seen AD skin.

Conclusions

We have known for over 30 years that the community of bacteria changes on the skin during AD, and recent DNA sequencing approaches have further described in greater detail the magnitude of dysbiosis seen in this disease. *S. aureus* colonization has been studied in greatest depth, and appears to be a significant factor that

exacerbates the disorder. However, other species such as *S. epidermidis* also show increased colonization and may modulate AD pathogenesis. Today, it appears that understanding how the full community of different bacteria interact and communicate with each other is necessary to best treat this disease.

Since *S. aureus* colonization increases the most during AD flares and is correlated with AD severity, much work has focused on the mechanisms of *S. aureus* virulence on the skin. As discussed earlier, *S. aureus* produces a series of virulence factors that can directly disrupt the skin barrier and potentially act as the reason for *S. aureus* mediated increased AD severity. It is important however to consider mechanisms that still have yet to be studied. One possible future direction is to study how *S. aureus* colonization can lead to changes in gene expression by the host. If *S. aureus* virulence factors were to target the expression of skin barrier proteins (e.g. filaggrin, keratins, skin specific proteases and inhibitors) that are vital for maintaining an intact skin barrier against pathogens, than AD severity would likely be increased. Another interesting event to study is that of increased bacterial penetration into the skin. It is now known that the skin microbiome extends into the epidermis, dermis, and subcutaneous layers. Thus, an altered skin barrier in AD can result in increased *S. aureus* penetration into deeper layers of skin. Perhaps this could lead to increased disease severity as well by mechanisms yet to be investigated. Recently it has been published that skin adipocytes respond as immune cells to *S. aureus* infection (*94*). Perhaps *S. aureus* colonization and penetration can target adipocytes to mount an

immune response and increase AD severity. Thus, there remain various hypotheses to be studied in terms of how *S. aureus* or other microbes may modulate AD severity.

Current methods of treatment for AD do not address how resident microbes on the skin such as *S. epidermidis* are altered. Some novel small-scale studies have attempted to remove *S. aureus* colonization to improve the clinical symptoms of AD. Such approaches have even included adding antimicrobial factors to clothing (*95, 96*). These methods however face similar problems as previous studies in that they lack an approach to selectively protect the normal microbiome. Some of the most promising therapeutic strategies to combat *S. aureus* mediated AD severity may be linked to methods already being used in the gut. Fecal microbiota therapy (FMT) is a method for replacing a diseased gut microbiome in a state of dysbiosis with bacteria from normal patients. FMT has been shown to successfully revert gut microbial dysbiosis in patients with recurrent *Clostridium difficile* infection (CDI) as well as provided promising results for other gastrointestinal diseases (*97*). Similarly, the use of microbial skin transplant therapy could be useful in treating AD. It is worth exploring if the skin microbiome of a healthy donor can be transplanted onto a patient's skin with AD and determine if this could repair the skin microbiome. Overall, the most promising future therapeutic strategies will seek to prevent microbial dysbiosis.

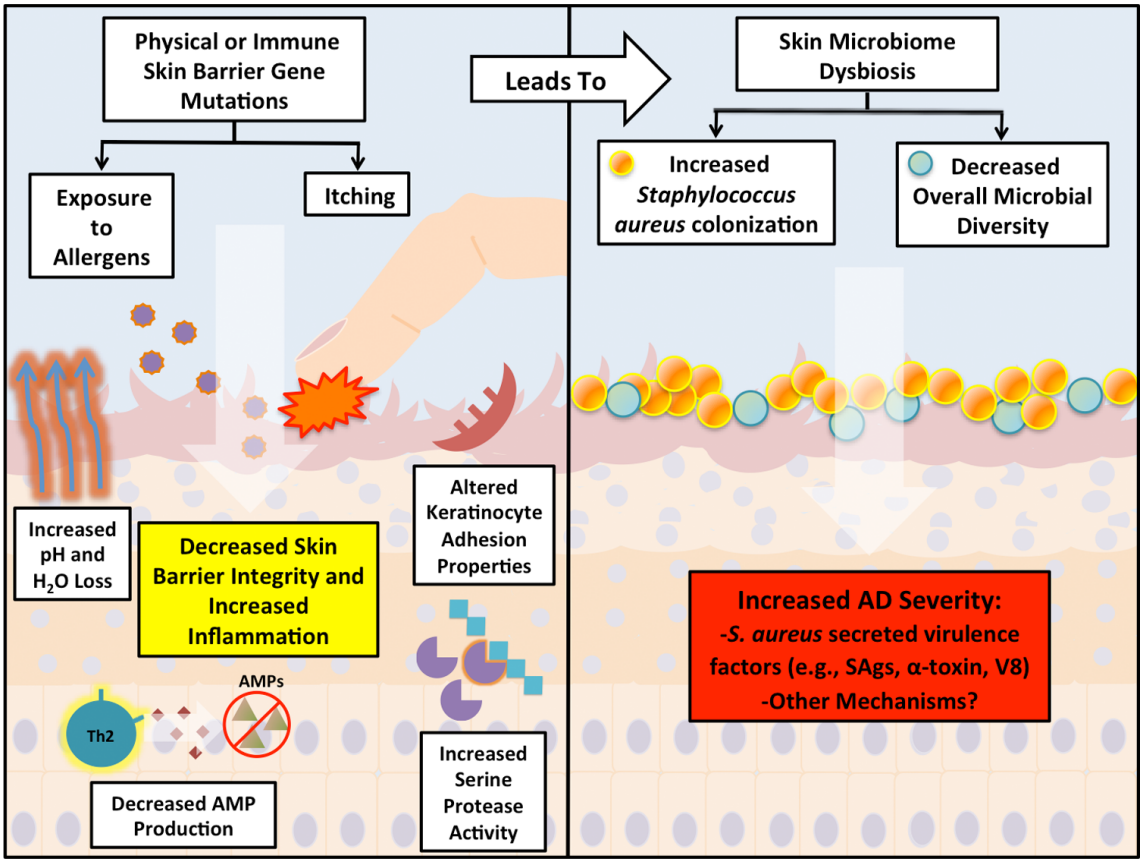

Figure 2.1. Dysbiosis of the Skin Microbiome in AD.
Genetic defects in both physical (e.g. FLG, SPINK5) and immune (e.g. IL-4, IL-13) skin barrier genes lead to increased skin barrier disruption. A decreased skin barrier response allows for increased susceptibility of the skin to allergens. Secondly, the skin becomes dry and itchy leading to more physical stress to the already damaged skin barrier. Overall the decreased skin barrier leads to an increase in skin pH, altered keratinocyte adhesion properties, and both increased serine protease activity and inflammation. An acute Th2 response is thought to dampen certain antimicrobial peptide (AMP) responses as well. These events all play a role in dysbiosis of the skin microbiome during AD leading to increased colonization by *S. aureus* along with decreased overall microbial diversity. *S. aureus* colonization is speculated to increase AD severity through secreting a series of virulence factors that damage the skin barrier and increase inflammation. Other mechanisms pertaining to the role of dysbiosis of the skin microbiome in altering AD pathogenesis have yet to be determined.

Table 2.1. Changes to skin microbial diversity in normal versus AD skin.

Normal Skin	Atopic Lesional Skin
Actinobacteria (Phylum) - *Corynecbacterium* (Genus) - *Propionibacterium* (Genus) - *Rothia* (Genus) - *Actinomyces* (Genus)	**Decreased relative abundance**
Bacteroidetes (Phylum) **-** *Prevotella* (Genus)	**No Change**
Proteobacteria (Phylum) - *Alphaproteobacteria* (Class) - *Betaproteobacteria* (Class) - *Gammaproteobacteria* (Class)	**Decreased relative abundance**
Firmicutes (Phylum) - *Streptococcus* (Genus) - *Staphylococcus* (Genus) - *Granulicatella* (Genus)	**Decreased relative abundance *Streptococcus/Granulicatella*** **Increased absolute and relative abundance *Staphylococcus***

(Data from [39, •40])

Table 2.2. Mechanisms of *S. aureus* mediated AD severity.

Virulence Factors	Mechanism for Increased AD Severity	Ref(s)
SAgs (SEA/B, TSST-1)	Non-specific APC mediated T cell activation, keratinocyte cytokine production	[60-63]
α-toxin	Keratinocyte lysis and TNF-α production	[64-68]
δ-toxin	Mast cell degranulation and inflammation	[69]
V8 and ETA/B	DSG-1 cleavage leading to both increased desquamation and skin inflammation	[77-79]
Protein A	Keratinocyte TNF-α production	[68]
PSM	Keratinocyte Inflammation	[70]

**Superantigen = SAg; enterotxoins A and B = SEA and SEB; toxin shock syndrome toxin 1 = TSST-1; exfoliative toxins A and B = ETA and ETB; Phenol-Soluble Modulins = PSM*

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Acknowledgements

Chapter II is based on: Williams, M.R. and Gallo, R.L. The Role of the Skin Microbiome in Atopic Dermatitis. *Curr Allergy Asthma Rep*. 2015, 15(11): 65-74.

I was the primary researcher and author for the manuscript. The co-authors listed above either supervised or provided support for the research, and have given permission for the inclusion of the work in this dissertation.

Chapter III:

Staphylococcus aureus induces keratinocytes to increase their serine protease activity

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.

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 (*1*) 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 (*2-6*). 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 (*7*).

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 (*8*). Increased *S. aureus* colonization has been linked to increased disease severity for AD patients (*9*). 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 (*10-16*). 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 (*17, 18*). 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) (*19*). The resulting increase in enzymatic activity leads to increased desquamation, altered antimicrobial peptide (AMP) and filaggrin (FLG) processing, and PAR-2 activation and inflammation (*20-23*). 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 (*24*).

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 3.1a). 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 3.1b,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 3.1d).

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 3.2a). Addition of the serine protease inhibitor aprotinin confirmed that this activity was due to serine proteases (Figure 3.2b), and this was consistent with the observation of an increase in trypsin-like activity shown in Figure 3.1a. 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 3.2c). 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 3.3a-e). Background auto-fluorescence at hair follicles is seen in all sections including the no substrate control (Figure 3.3f). 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 (*25, 26*). 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 3.4a-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 3.4f).

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 3.5a). 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 3.5b-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 (*20, 22, 27*). 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 3.6a). *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 3.6b). Densitometry analysis further illustrates the ability of KLK6, 13, and 14 knockdowns to prevent either DSG-1 or Pro-FLG cleavage (Figure 3.6c). 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 (*14, 28-30*). 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 (*31*). It has previously been shown that accelerated Pro-FLG cleavage could be linked to increased desquamation of the skin (*32*). 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 (*22*).

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 (*20*). 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 (*33*) or Netherton syndrome.

Different strains of *S. aureus* (Newman, USA300, SA113, 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 cutaneous 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 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 3.2c) 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 (*34*), have potent cell-specific proteases. Our whole tissue zymograms (Figure 3.3) 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 (*1, 24, 35*), 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 antibiotic-antimycotic (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. 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 3.7) 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 (*36*) 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, 3x 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.

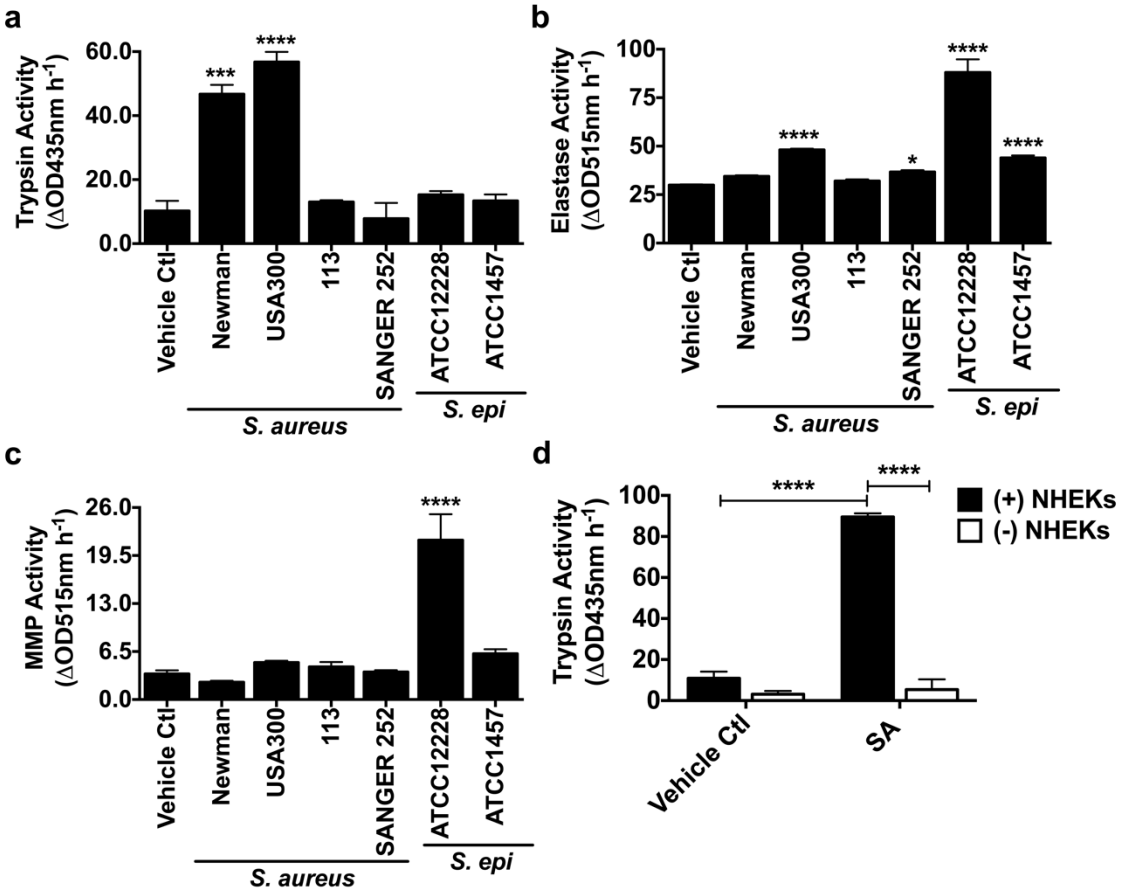


Figure 3.1. *Staphylococci* regulate human keratinocyte protease activity.
(a-c) NHEKs were treated for 24h with *S. aureus* (Newman, USA300, 113, SANGER252) and *S. epidermidis* (ATCC12228, ATCC1457) sterile filtered supernatants and NHEK conditioned medium was analyzed with specific trypsin-like, elastase-like, 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. One-way 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.

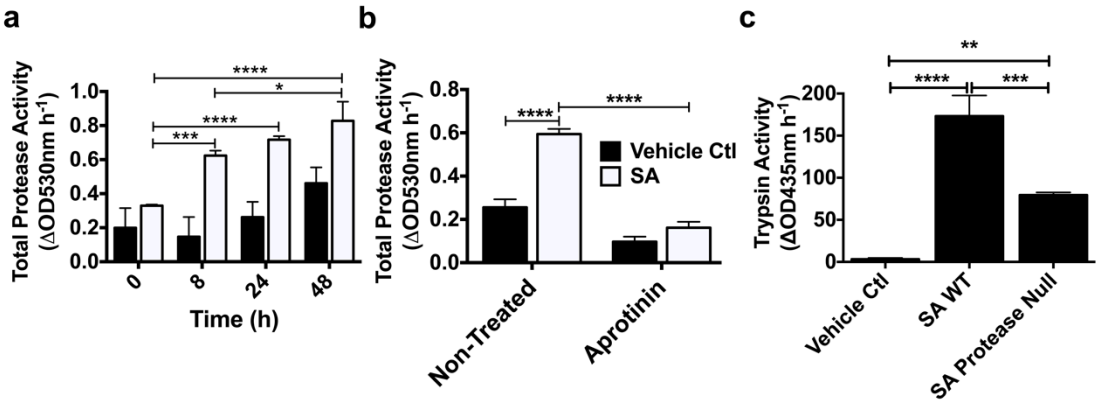


Figure 3.2. *S. aureus* increases human keratinocyte serine protease activity.
(a) Total protease activity (5µg-mL BODIPY FL casein) was measured in NHEK conditioned medium after *S. aureus* (SA, Newman) supernatant treatment for 0-48h, **(b)** while the serine protease inhibitor aprotinin (800µg-mL) 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.

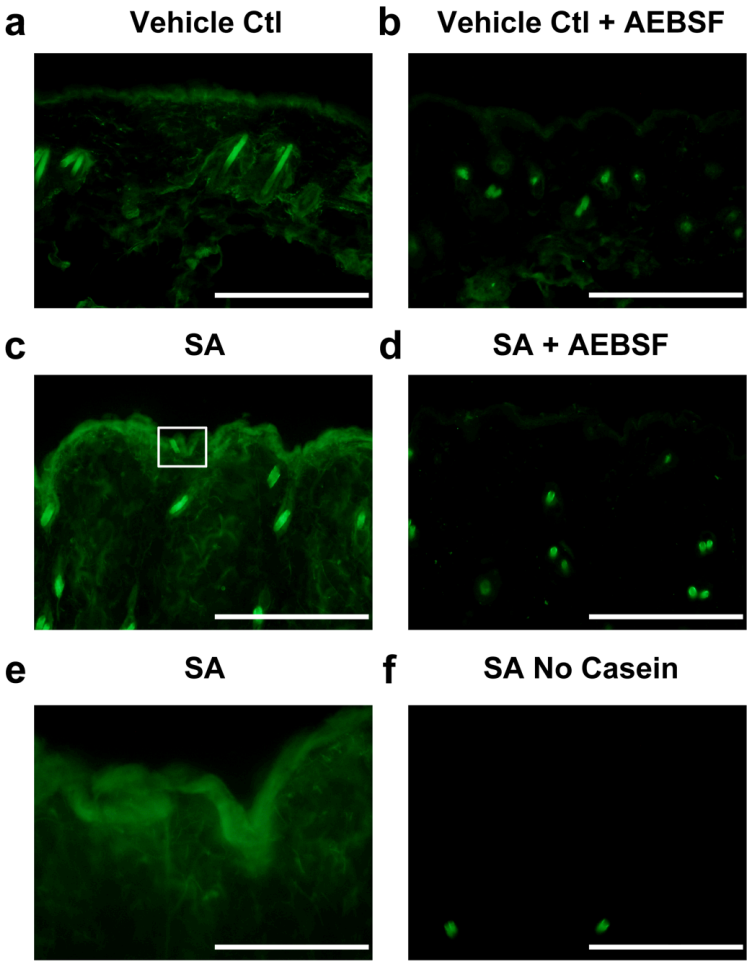


Figure 3.3. Murine epidermal serine protease activity is increased by *S. aureus.* Murine skin colonized with **(a,b)** TSB vehicle control or **(c,d)** $2e^6$ CFU *S. aureus* (SA, USA300) for 12h was assessed for changes to total protease activity (2µg-mL BODIPY FL casein) with or without the serine 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.

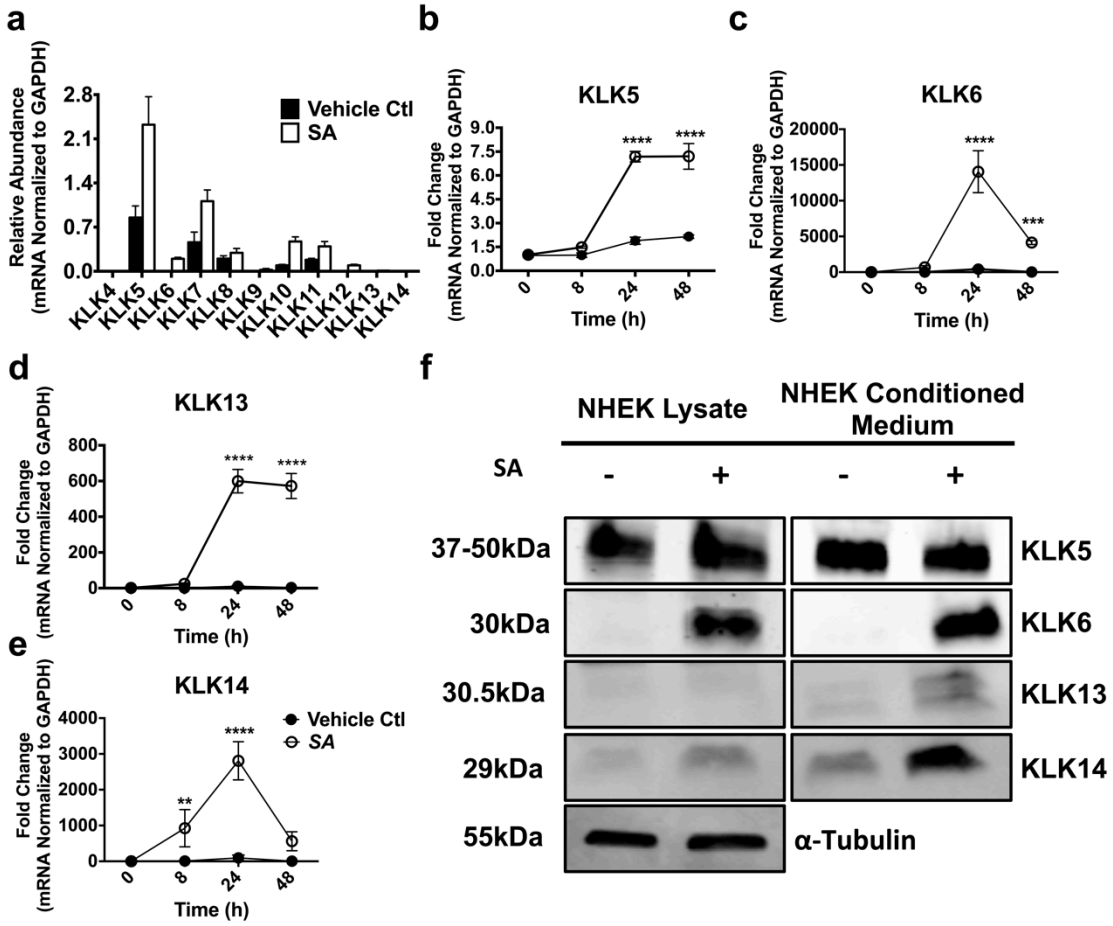


Figure 3.4. *S. aureus* increases KLK expression in human keratinocytes.
(a) Relative abundance of KLK mRNA expression in NHEKs after 24h *S. aureus* (SA, Newman) supernatant treatment was analyzed by qPCR. **(b-e)** KLK5, 6, 13, and 14 were analyzed for fold changes in mRNA expression in NHEKs treated 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. Two-way ANOVAs **(b-e)** were used and significance indicated by: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

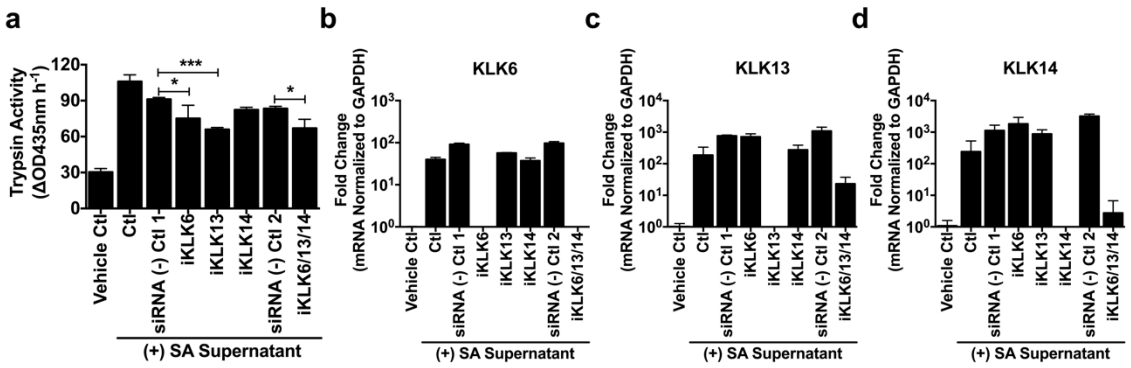


Figure 3.5. Multiple KLKs are responsible for *S. aureus* induced serine protease activity in human keratinocytes.

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.

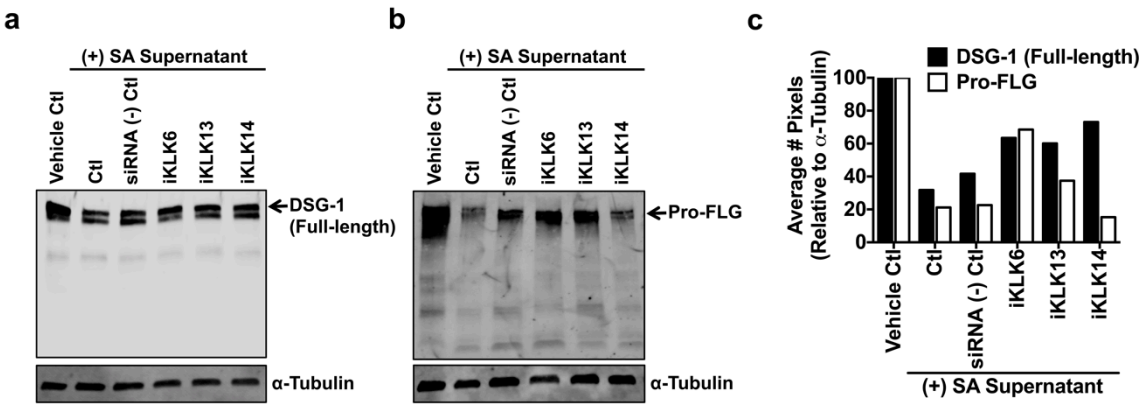
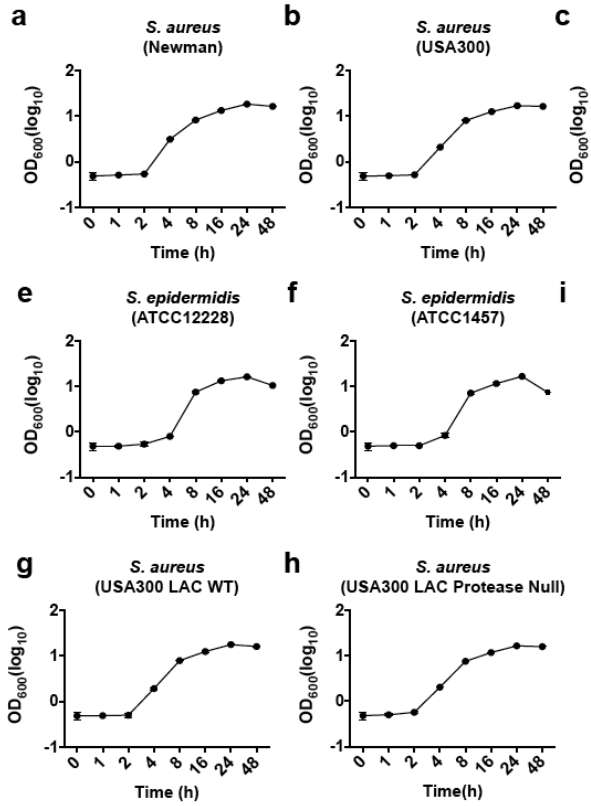


Figure 3.6. Multiple KLKs regulate *S. aureus* induced DSG-1 and FLG cleavage in human keratinocytes.

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 represent the average number of pixels normalized to α-Tubulin (n=1). Immunoblots are representative of at least 3 independent experiments.



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Species	Strain	OD (600nm)	CFU/mL
S. aureus	Newman	13.00	3.90×10^9
S. aureus	USA300	12.60	3.78×10^9
S. aureus	113	16.20	4.86×10^9
S. aureus	SANGER 252	14.00	4.20×10^9
S. epidermidis	ATCC12228	12.80	3.84×10^9
S. epidermidis	ATCC1457	10.60	3.18×10^9
S. aureus	USA300 LAC WT	14.00	4.20×10^9
S. aureus	USA300 LAC Protease Null	15.00	4.50×10^9

Figure 3.7. Growth curves for all *S. aureus* and *S. epidermidis* strains used.
(a-h) *S. aureus* (Newman, USA300, 113, SANGER252, USA300 LAC WT, and USA300 LAC Protease Null) and *S. epidermidis* (ATCC12228 and ATCC1457) strains were cultured in 3%TSB (37°C, 300RPM) for up to 48h with the optical density of the bacteria measured at an absorbance of 600nm (OD600). (i) Table indicates OD600 values for stationary phase bacteria at 24h as well as the approximate CFU/mL for each bacterial strain used before sterile filtration of bacteria supernatant and addition to human keratinocytes.

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Acknowledgements

Chapter III is based on: Williams, M.R., Nakatsuji, T., Sanford, J.A., Vrbanac, A.F., and Gallo, R.L. *Staphylococcus aureus* induces keratinocytes to increase their serine protease activity. *J Invest Dermatol*. 2017, 37(2):377-384. I was the primary researcher and author for the manuscript. The co-authors listed above either supervised or provided support for the research, and have given permission for the inclusion of the work in this dissertation.

Chapter IV:

Quorum sensing between bacterial species on the skin protects against epidermal injury in atopic dermatitis

Abstract

Dysbiosis of the microbiome and colonization of the skin by *Staphylococcus aureus* (*S. aureus)* is associated with exacerbations of atopic dermatitis (AD), but a direct mechanism through which the microbiome can influence the function of the epidermis and thus the development of AD was unknown. We show here that proteases and phenol-soluble modulin alpha (PSMα) secreted by *S. aureus* trigger keratinocyte protease activity and auto-digestion of the epidermis. Coagulase-negative *Staphylococci* (CoNS) species that normally reside on skin such as *S. epidermidis* and *S. hominis* protect against this by producing autoinducing peptides (AIP) that inhibit the accessory gene regulatory (agr) quorum sensing system of *S. aureus* and turn off PSMα secretion. Multiple clinical isolates of different CoNS species inhibited this process and prevented epithelial damage both *in vitro* and *in vivo* without changing the abundance of *S. aureus*. Patients with active AD showed a decrease in relative abundance of these beneficial microbes compared to *S. aureus*, thus overcoming inhibition of quorum sensing and enabling barrier disruption by *S. aureus*. Taken together, our findings show how members of the normal human skin microbiome maintain immune homeostasis by contributing as a community to the control of *S. aureus* toxin production.

Introduction

Atopic dermatitis (AD) is among the most common immune disorders, and causes a serious burden to patient quality of life and finances as well as posing a serious risk of comorbidities (*1, 2*). The risk of developing AD is increased in patients with genetic defects in skin barrier function and is also associated with early life environmental exposures (*3-5*). Furthermore, recent studies of the early life microbial community on the skin have suggested that the relative abundance of bacteria, such as *Staphylococcus aureus (S. aureus)* and coagulase negative Staphylococcal (CoNS) species, may predict the development of AD (*6, 7*). These observations follow several decades of reports that *S. aureus* often colonizes lesions on the skin of patients with AD (*8*), correlate with disease severity (*9-11*) and is a frequent cause of superinfections (*12, 13*). Despite the large body of work to identify genetic, environmental and microbial risk factors for AD, a cohesive hypothesis to link these observations together into a unifying pathophysiologic mechanism has not been validated.

Eczematous skin lesions of patients with AD have increased levels of Th2 cytokines such as IL4 and IL13 (*14, 15*). Th2 cytokines promote decreased function of the skin barrier by inhibiting expression of filaggrin (*16*). These cytokines also suppress expression of human antimicrobial peptides such as cathelicidin and b-defensin-2, a defect in AD that may lead to dysbiosis of the skin bacterial community and enhanced colonization by *S. aureus* (*17*). Therapy targeting IL4 receptor alpha result in significant improvement in disease (*18*). The strong association between Th2 cytokine activity, barrier function, antimicrobial activity, and disease outcome supports efforts to define a causal link between these essential epidermal functions.

Recent evidence in murine models has demonstrated that virulence factors produced by *S. aureus*, such as phenol-soluble modulin alpha (PSMα), can promote skin inflammation. These studies have shown that *S. aureus* PSMα can induce cytokine release from cultured keratinocytes, and that mice exposed to bacteria expressing this toxin develop an AD-like inflammatory signature (*19-22*). Other studies have shown that skin bacteria, in particular *S. aureus*, can penetrate the epidermis and that the potential to induce inflammation can be linked to genetic disorders in barrier assembly such as a defect in filaggrin expression (*23, 24*). Furthermore, penetration of the epidermis and induction of skin inflammation correlates with the capacity of *S. aureus* to induce expression of trypsin-like proteases by keratinocytes, suggesting that enzymatic digestion of the epidermal barrier promotes disease.(*23*).

This investigation sought to identify the molecular mechanism responsible for the deleterious effects of *S. aureus* on the epidermal barrier with an overall goal of understanding initial events at the skin surface that permit this microbe to promote inflammation. Our study uncovers a previously unappreciated interaction between microbial communities on the skin of patients with AD that reinforces the link between microbial dysbiosis and disease, and suggests that interspecies quorum sensing between bacteria and host may be an important and previously unrecognized defense mechanism for maintaining homeostasis and resisting development of AD.

Results

PSMα and proteases produced by S. aureus induce epidermal barrier damage

A primary function of human skin is to establish a physical barrier against the external environment. Specific toxins produced by *S. aureus* such as the phenol soluble modulins (PSM) can promote epithelial inflammation and have been proposed to be key to driving disease in AD(*19-22*). Therefore, to understand how *S. aureus* on the skin surface could influence inflammatory activity across the epidermal barrier we assessed normal human epidermal keratinocytes (NHEK) for their capacity to expresses proteolytic activity when exposed to a *S. aureus* USA300 LAC strain that has a targeted deletion in either the PSMα or PSMb operons. PSMα production was required for induction of trypsin-like serine protease activity and increased mRNA levels of kallikrein 6 (KLK6) (Figure 4.1a,b), The PSMα and PSMβ operons in *S. aureus* contain distinct peptides including PSMα1-4 and PSMβ1-2. Thus we tested synthetic PSMα1-4 and PSMβ2 peptides on NHEKs and found that (Figure 4.1c) all PSMα peptides could stimulate trypsin activity while PSMβ2 could not. We chose PSMα3, the strongest PSMα trypsin activity inducers in NHEKs, to further show it could stimulate trypsin activity and KLK6 mRNA expression in NHEKs in both a dose and time dependent manner (Figure 4.5). Furthermore, transcriptional profiling by RNA-Seq of NHEKs exposed to PSMα3 showed this toxin had a broad effect on expression of genes related to the skin barrier including multiple proteases (KLKs, MMPs), components of the physical barrier (filaggrin, desmoglein-1, loricrin, involucrin, keratins), antimicrobial peptides, and cytokines (Figure 4.1d,e; Figure 4.5).

To validate the role of the PSMα operon on the epidermal barrier *in vivo*, mice were colonized for 72h on the skin surface with equal numbers of the *S. aureus* USA300 LAC wild type or the PSMα mutant strain. Wild type *S. aureus* induced erythema, scaling, and epidermal thickening while no change in bacterial abundance was observed in the absence of PSMα (Figure 4.1f). Despite increased epidermal thickness, an increase in transepidermal water loss (TEWL), a well established method to assess skin barrier damage, was observed after exposure to wild-type *S. aureus* but not when PSMα was absent (Figure 4.1g). However, skin barrier disruption of a fully differentiated epidermis *in vivo* was also dependent on *S. aureus* protease expression. Using a *S. aureus* USA300 LAC mutant strain that lacks 10 major secreted proteases including aureolysin, V8, staphopain A/B, and SplA-F (*25*), we showed visible evidence of injury and increased TEWL was diminished in a *S. aureus* protease deficient manner despite fully intact PSMα expression (Figure 4.1f,h). Coincident with the gross and histologic changes observed to be associated with expression of either PSMα or bacterial proteases, an increase in keratinocyte trypsin activity, Klk6 transcript expression and cytokines Il6, Il17a, and Il17f was measured only in mice exposed to wild-type *S. aureus* but not in PSMα or protease deficient strains (Figure 4.6). Furthermore, despite changes in the skin barrier and inflammatory milieu of the skin, *S. aureus* abundance was unchanged on the skin surface under these conditions (Figure 4.1i,j). Taken together, these data suggest that production of PSMα and protease activity from *S. aureus* results in damage to the epidermal barrier and that this barrier damage is required for *S. aureus* to promote inflammation.

S. epidermidis autoinducing peptide inhibits S. aureus agr activity

Interestingly, both the *S. aureus* PSMα peptides and secreted proteases are under regulation of the agr quorum sensing system. *S. aureus* clinical isolates furthermore have been found to have four distinct agr types with agr type I being the most prominent in AD subjects (*26, 27*). Although *S. aureus* skin colonization increases in AD, other bacterial species such as coagulase-negative *Staphylococci* (CoNS) strains including the abundant human skin commensal organism *S. epidermidis* are also present making it essential to understand how these bacteria communicate. *S. epidermidis* agr type I lab isolates have been shown to produce an autoinducing peptide (AIP) that has was suggested to inhibit the *S. aureus* agr type I-III systems but not type IV through an agr crosstalk mechanism, while little is known of the other *S. epidermidis* agr types II and III on their influence on *S. aureus* agr activity (*28*). Conditioned culture supernatants from *S. epidermidis* strains with agr types I, II, or III were added to a *S. aureus* USA300 LAC agr type I reporter strain to explore if *S. epidermidis* agr activity could influence the *S. aureus* agr system. This experiment confirmed that *S. epidermidis* agr I was the only potent inhibitor of *S. aureus* agr activity while *S. epidermidis* agr type II and III had little effect (Figure 4.2a). Targeted deletion of the *S. epidermidis* agr type I AIP within the agrD gene region abolished the capacity of *S. epidermidis* to inhibit *S. aureus* agr activity (Figure 4.2b,c). Since *S. au*reus PSMα induced NHEK trypsin activity was previously established to be a key component to epidermal barrier damage, we tested if the *S. epidermidis* agr type I wild type or AIP knockout strain could effect this result. Thus we observed that *S. aureus* induced NHEK trypsin activity was inhibited when *S. aureus* was cultured in the presence of wild type *S. epidermidis* agr type I

supernatant but not by *S. epidermidis* lacking this AIP (Figure 4.2d). Overall, these experiments established that *S. aureus* capacity to induce NHEK barrier damage can be influenced by *S. epidermidis* agr type I AIP expression.

Deficiency in S. epidermidis agr type I relative abundance on AD skin

Having established the potential for a laboratory strain of *S. epidermidis* to influence the effects of *S. aureus* on the function of human keratinocytes, we next wanted to investigate the abundance of these bacteria in a clinical setting. Thus we interrogated metagenomic data available from the skin microbiome of 8 subjects with AD of different severity (based upon the objective SCORAD) collected from 7 body sites for *S. epidermidis* relative abundance based upon agr type (*29*). Sequence alignments identified *S. epidermidis* genomes based on agr types I-III on AD patients and determined the most frequent *S. epidermidis* agr type on AD skin is that of agr type I (Figure 4.2e). Comparison of *S. epidermidis* agr I to *S. aureus* showed that *S. epidermidis* agr type I became relatively less abundant in AD subjects with increased disease severity (Figure 4.2f,g). These observations confirmed the presence of *S. epidermidis* agr type I in the AD skin microbiome and suggest the potential for association with clinical disease.

Diverse Staphylococci species and strains inhibit S. aureus agr activity

To further establish the physiological significance of quorum sensing interactions between *S. aureus* and other members of the skin microbiome, we next tested different AD clinical isolates of CoNS for the capacity of their culture supernatant to inhibit *S.*

aureus USA300 LAC agr type I quorum sensing activity. Diverse species including *S. epidermidis, S. hominis, S warneri, and S capitis* showed potent inhibitory activity against agr activity of *S. aureus* (Figure 4.3a). Similar to the lab isolates of *S. epidermidis*, the CoNS strains inhibited *S. aureus* agr activity without inhibiting the growth rate (Figure 4.7). Furthermore, a genomic sequence analysis of the agrD AIP coding region of *S. hominis* strain C5 revealed a novel AIP sequence in the AIP coding region similar to the sequence of *S. epidermidis* agr type I coding region and thus we presented a predicted octomer AIP sequence for *S. hominis* C5 (Figure 4.3b). Biochemical techniques of the active *S. hominis* C5 supernatant showed that inhibition of *S. aureus* agr activity was dependent on a <3kDa (small size), pH 11 sensitive (thiolactone ring) factor that could be precipitated with 80% ammonium sulfate (peptide) (Figure 4.3c). These characteristics were consistent with previously purified AIPs (*30, 31*).

Next, *S. aureus* was cultured in the presence of *S. hominis* C5 sterile-filtered supernatant and the subsequent culture supernatant was applied to NHEKs as in Figure 4.1. Similar to *S. epidermidis* agr type I, *S. hominis* C5 inhibited *S. aureus* induced trypsin activity, KLK6 transcript production, and IL-6 protein expression in NHEKs (Figure 4.3d-f). Furthermore, *S. hominis* C5 could inhibit multiple *S. aureus* agr systems aside from most common clinical isolate of agr type I including agr types II and III but not IV (Figure 4.8). This finding coincides with what has been observed with the *S. epidermidis* agr type I system. Overall, these observations suggest clinical isolates of CoNS species in addition to *S. epidermidis* may use quorum sensing to suppress *S. aureus* damage to keratinocytes.

A clinical CoNS isolate inhibits S. aureus agr activity its ability to promote AD

To establish the physiological relevance of quorum sensing interactions between CoNS and *S. aureus in vivo*, *S. aureus* agr activity was assessed by IVIS using a *S. aureus* USA300 LAC agr type I P3-Lux promoter (luminescence) strain. *S. aureus* on back skin showed abundant agr activity but when in the presence of live *S. hominis* C5, *S. aureus* agr activity was inhibited (Fig 4.4a,b). Furthermore, *S. hominis* C5 also protected against *S. aureus* induced skin erythema and scaling (Figure 4.4c) without altering *S. aureus* abundance (Figure 4.4d). This phenotype was associated with improved evidence for inflammation, barrier disruption, and epidermal protease activity and Klk6 expression (Figure 4.4e-h). Furthermore, when *S. aureus* was applied to murine back skin in the presence of a <3kDa concentrated *S. hominis* C5 supernatant, similar reductions in barrier damage and inflammation were observed without changes to *S. aureus* abundance. (Figure 4.9). These data show the skin CoNS microbial community likely contains novel AIPs that promote epithelial barrier homeostasis by interspecies quorum sensing activity.

Discussion

Progress in understanding the pathophysiology of atopic dermatitis (AD) has identified strong associations with genetic defects in skin barrier formation (*32*), dysfunction of adaptive and innate immunity, and dysbiosis of the skin microbiome (*33*). Despite this progress however, a cohesive mechanism to link these observations into a causal model has not been established. Genetic defects in barrier function that have been identified in AD subjects are not causal as many individuals lack these mutations or have mutations without disease. This has suggested that additional events must occur to drive disease. Based on the increased risk for AD associated with excess hygiene or antibiotic use (*34-36*), the microbiome has been a prime suspect for providing the signal that promotes AD. However, a molecular mechanism to explain an interaction between barrier formation and microbiome composition was unknown.

In this study we provide two previously unreported and interrelated mechanisms that shed new light on the development and control of AD. Our findings show that *S. aureus* phenol-soluble modulin alpha (PSMα) peptides target human keratinocytes, the main cell type responsible for establishment of the physical barrier of the skin. Exposure to PSMα leads to increased proteolytic activity in the epidermis, breakdown of the epidermal barrier and initiation of local inflammation. This observation explains how *S. aureus* residing on the skin surface can promote and exacerbate disease prior to the uncontrolled tissue invasion that defines infection. Furthermore, based on earlier observations that PSMα is under control of the quorum sensing accessory gene regulator (*agr*) system (*37*), and that autoinducing peptide (AIP) pheromones from other bacterial species have been shown to repress the *S. aureus agr* system (*28, 30, 38*), we

asked if interspecies interactions within the human skin microbiome may influence this process. Our data demonstrate that several different Coagulase negative *Staphylococci* (CoNS) species, but not all strains within these species, encode AIPs and repress *S. aureus agr* activity. The stoichiometry of these interspecies interactions is unfavorable during disease flares in patients with AD of different severity, and a murine model of AD directly demonstrated that disease could be suppressed when cross-inhibitory activity is present from a beneficial CoNS strain.

S. aureus secreted proteases play a vital role in *S. aureus* penetration in to deeper layer of the epidermis and dermis (*23, 24*). It is also known that certain *S. aureus* proteases such as the serine protease V8 can directly cause skin barrier damage in murine models (*39*). In this study we found that *S. aureus* subverts the epidermal barrier by combining protease activity with the production of PSMα peptides. These *S. aureus* secreted proteases are essential to validate observations in the physiologically relevant system *in vivo* where an intact stratum corneum protects underlying live keratinocytes. *In vitro*, *S. aureus* proteases had little to no effect on human keratinocytes with the only observed changes being in that of minimal changes to SA induced keratinocyte trypsin activity. This suggests that *S. aureus* secreted proteases assist *S. aureus* to penetrate the upper layers of the skin in order for PSMα to further stimulate keratinocytes. This process of interkingdom signaling explains how minor colonization by *S. aureus* can be amplified to promote major defects in the skin barrier. Future work is needed to better understand how *S. aureus* can penetrate the epidermal barrier including analyzing exactly which of the 10 secreted *S. aureus*

proteases including aureolysin, V8, staphopainA/B, and SplA-F in the knockout strain are responsible for the effect on inducing skin barrier damage in mice.

We found that PSMα peptides could induce the pro-inflammatory cytokines IL-6, IL-1α, and TNFα in human keratinocytes. Keratinocyte secretion of IL-1α as well as IL-36α was recently shown to be important for *S. aureus* induced Th17 responses in murine skin (*19, 20*). Using human keratinocytes we established that IL-1α is indeed secreted in a PSMα dependent manner. Interestingly, IL-36α was secreted from human keratinocytes exposed to *S. aureus* but was not regulated by PSMα or products of the *agr* system. Thus, it remains unknown how IL-36α is influenced by *S. aureus*. This is a relevant question for future investigation since IL-36α production seems to be very important for Th17 induced skin inflammation (*19, 20*). It is also worth noting that our *S. aureus* epicutaneous mouse model led to a Th17 response similar to previous reports that showed a *S. aureus* induced Th17 response was essential for producing an AD-like phenotype on mouse skin (*19, 20*). Th17 responses have also been clinically found to be important in acute AD (*40-42*) and possibly explains an important component of how both *S. aureus* agr regulated toxins such as PSMα and secreted proteases stimulate AD-like skin phenotypes through a Th17 driven response. Interestingly, *S. aureus* epicutaneous colonization alone could not stimulate a Th2 responses in mice without the presence of a genetic barrier defect or allergic stimulus as we have previously shown in our lab using an ovalbumin stimulated filaggrin knockout strain (*23*). This further establishes the need for other factors in the environment to cause an AD phenotype aside from just *S. aureus* colonization on healthy skin.

Bacterial quorum sensing behaviors were first observed in the 1960s, but recent progress in defining the extracellular chemical signals and responses of many human pathogens has driven intense interest in use of quorum sensing inhibitors as a therapeutic alternative to antibiotics *(37, 43-45).* Progress in understanding the functions of the gut microbiome have suggested that interspecies quorum sensing interactions can promote colonization by Firmicutes (*46*), and protect against *Vibrio cholera* infections (*47*). Quorum sensing interactions that influence inflammatory skin diseases have not been previously described although prior observations that laboratory isolates of *S. epidermidis* and *S. caprea* can produce AIPs that inhibit the *S. aureus* agr system are consistent with the present findings (*28, 30*). Here we validated that *S. epidermidis* agr type I AIP was active against *S. aureus* USA300 LAC agr type I and protective for human keratinocytes, while *S. epidermidis* agr type II and III were not active. Analysis of clinical samples from AD subjects demonstrated the presence of these genes on human skin and suggested that disease flare severity can correlate with a decrease in the abundance of *S. epidermidis* agr type I relative to *S. aureus*, a condition that would promote PSMα expression and disease progression. However, the clinical situation is likely much more complex, as many unknown chemical signals that regulate *S. aureus* agr activity likely exist on the skin. This was illustrated by screening of AD clinical CoNS isolates and demonstration of a highly potent *S. hominis* strain that inhibited *S. aureus* agr type I activity as well as prevented *S. aureus* induced skin barrier damage and inflammation both *in vitro* and *in vivo*.

Taken together, we now provide evidence that interkingdom communication between *S. aureus* and human skin promotes AD, and interspecies communication

between CoNS species and *S. aureus* acts as a potential regulator to inhibit the negative consequences of *S. aureus* toxin production. These findings suggest that an understanding of the pathophysiology of AD requires better knowledge of the communication networks between bacteria living on the skin. This information will improve existing therapeutic strategies and may drive the development of novel new approaches for treatment.

Materials & Methods

Bacterial preparation

All bacteria used in this study are listed in Table 4.1. All *Staphylococci* strains (*S. aureus*, *S. epidermidis*, *S. hominis*, *S. warneri*, *S. capitis*, and *S. lugdunensis*) were grown to stationary phase in 3% tryptic soy broth (TSB) for 24h at 250RPM in a 37°C incubator at either 4mL or 400µL volumes depending on the assay. Specific strains were grown with antibiotic selection where indicated in Table 4.1 at the following concentrations: 5µg/mL Erm, 25µg/mL Lcm, and 10µg/mL Cm. For treatment of bacterial supernatant on human keratinocytes or murine skin, 24h cultured bacteria was pelleted (15 min, 4,000RPM, RT) followed by filtered-sterilization of the supernatant (0.22µm). For murine and human keratinocyte experiments with *S. hominis* C5 and *S. epidermidis* RP62A strains, bacteria sterile-filtered supernatant was filtered with a 3kDa size exclusion column (Amicon Ultra-15 centrifugual filter, Millipore) to collect the <3kDa fraction and further concentrated 10x using a lyophilizer and a re-suspended in molecular grade H_2O prior to treatment. *S. hominis* C5 supernatant was further biochemically tested with several techniques. Ammonium sulfate precipitation (80%) for 1h at room temperature followed by centrifugation (30 min, 4,000RPM, RT) and re-supsension of the precipitate (pellet) in H_2O was used for isolating small peptides. Furthermore, *S. hominis* C5 supernatant was raised to pH11 with 2M NaOH for 1h followed by using 2M HCl to return the supernatant pH to approximately the starting pH of 6.5 using pH 1-14 strips prior to addition to the *S. aureus* agr reporter strain.

Normal human epidermal keratinocyte culture

Normal neonatal human epidermal keratinocytes (NHEKs; Thermo Fisher Scientific) were cultured in Epilife medium containing 60 μM $CaCl_2$ (Thermo Fisher Scientific) supplemented with 1x Epilife Defined Growth Supplement (EDGS; Thermo Fisher Scientific) and 1x antibiotic-antimycotic (PSA; 100 U/mL penicillin, 100 U/mL streptomycin, 250ng/mL amphotericin B; Thermo Fisher Scientific) at 37°C, 5% CO_2. NHEKs were only used for experiments between passages 3-5. For experiments, NHEKS were grown to 70% confluency followed by differentiation in high calcium EpiLife medium (2mM $CaCl_2$) for 48h to simulate the upper layers of the epidermis. For bacterial supernatant treatments, differentiated NHEKs were treated with sterile-filtered bacterial supernatant at 5% by volume to Epilife medium for 24h. Similarly for synthetic PSM treatments, 5-50μg-mL of peptide were added to the NHEKs for 24h in DMSO.

S. aureus epicutaneous mouse model

Sex and age matched male or female C57BL/6 (Jackson) mice at 8 weeks age were used for all experiments (n=3-6) as specified in the figure legends. All animal experiments were approved by the UCSD (University of California, San Diego) Institutional Animal Care and Use Committee. Mouse hair was removed by shaving and application of Nair for 2-3 min followed by immediate removal with alcohol wipes. The skin barrier was allowed to recover from hair removal for 48h prior to application of bacteria. *S. aureus* ($1e^7$ CFU) in 3%TSB was applied to murine skin for 48-72h at a 100μL volume on a $1.5cm^2$ piece of sterile guaze. Tegaderm was applied on top of guaze to hold in place for duration of the treatment. For *S. aureus* agr inhibition experiments, live *S. hominis* C5 (10:1) or 10x concentrated <3kDa sterile-filtered

commensal bacterial supernatant (1:1) was combined with *S. aureus* in 3%TSB immediately before application on guaze.

Synthetic phenol-soluble modulin preparation

All synthetic phenol-soluble modluins (PSM) were produced by LifeTein (Hillsborough, NJ). Peptides were produced at 95% purity with N-terminal formylation (f). PSM sequences were as follows:

PSMα1:fMGIIAGIIKVIKSLIEQFTGK,

PSMα2: fMGIIAGIIKFIKGLIEKFTGK

PSMα3: fMEFVAKLFKFFKDLLGKFLGNN

PSMα4: fMAIVGTIIKIIKAIIDIFAK

PSMβ2: fMTGLAEAIANTVQAAQQHDSVKLGTSIVDIVANGVGLLGKLFGF

Peptides were re-suspended in DMSO and concentrated by speedvac into 500mg powdered stocks stored at -80°C prior to reconstitution in DMSO for experiments.

RNA isolation and quantitative real-time PCR

All RNA was isolated using the Purelink RNA isolation kit according to manufacturer's instructions (Thermo Fisher Scientific). For NHEKs, 350μL RNA lysis buffer (with 1% β-mercaptoethanol) was added directly to cells. For mouse tissue, 0.5cm^2 full thickness skin was bead beat (2x 30 sec, 2.0mm zirconia bead) in 750μL of RNA lysis buffer with 5 minutes on ice in between. Tissue was than centrifuged (10min, 13,000 RPM, 4°C), followed by adding 350μL of clear lysate to 70% EtOH and column based isolation of RNA. After RNA isolation, samples were quantified with a Nanodrop (ThermoFisher

Scientific), and 500ng of RNA was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad). qPCR reactions were ran on a CFX96 Real-Time Detection System (Bio-Rad). For mammalian cells, gene-specific primers and TaqMan probes (Thermo Fisher Scientific) were used with GAPDH as a housekeeping gene as listed in Table 4.2.

Generation of RP62A competent cells and transformation

Electro-competent RP62A cells were prepared as previously described (*48, 49*). Briefly, an overnight culture of *S. epidermidis* RP62A was diluted to an OD600nm of 0.5 in pre-warmed Brain Heart Infusion (BHI) broth, incubated for an additional 30 min at 37°C with shaking, transferred to centrifuge tubes and then chilled on ice for 10 min. Cells were harvested by centrifugation (10 min, 4000RPM, 4°C), washed serially with 1 volume, 1/10 volume and then 1/25 volume of cold autoclaved water followed by re-pelleting at 4°C after each wash. After the final wash, cells were re-suspended in 1/200 volume of cold 10% sterile glycerol and aliquoted at 50µL into tubes for storage at -80°C. Transformation of *S. epidermidis* RP62A was carried out as previously described (*48*). Briefly, frozen competent cells were thawed on ice for 5 min and then at RT for 5 min. Thawed cells were briefly centrifuged (1 min, 5000g, RT) and the pellet was re-suspended in 50µL of 10% glycerol supplemented with 500mM sucrose. After addition of DNA, cells were transferred to a 1 mm cuvette and pulsed on a Micropulser (Bio-Rad) at 2.1kV with a time constant of 1.1 msec. Immediately after electroporation, cells were re-suspended in 1mL of BHI broth supplemented with 500mM sucrose, shaken for 1hr at 30°C and then plated on BHI agar with 10 µg/mL chloramphenicol (Cm) at 30°C.

Allelic replacement of S. epidermidis RP62A AIP

The allelic replacement plasmid pMAD (*50*) was used to selectively generate an in-frame deletion of the AIP coding sequence of *agrD* in *S. epidermidis* RP62A. Briefly, approximately 1000bp fragments upstream and downstream of the AIP sequence of RP62A were amplified by PCR and joined by gene splicing by overlap extension or 'SOEing'. The sewn fragments and pMAD vector were digested with BamHI and SalI, ligated together by T4 DNA ligase (New England Biolabs) and subsequently used to chemically transform the *S. epidermidis* clonal complex 10 plasmid artificial modification *E. coli* strain, DC10B-CC10. Transformants were plated on LB with 100µg/mL Amp and 30µg/mL Cm at 37°C. Correct transformants were validated by restriction digest and sequencing. The verified construct was annotated as pMAD:: *ΔAIP*. Electro-competent RP62A was then transformed with ~5µg of pMAD:: *ΔAIP* derived from DC10B-CC10 and then plated on BHI agar with 10 µg/mL Cm and 50µL of 40 mg/mL 5-bromo-4-chloro-3-indolyl- β-D-galactopyranoside (X-Gal) at 30°C. A single blue colony was selected and grown in BHI with 10 µg/mL Cm overnight at 30°C. The overnight culture was then diluted 1:100 (for final volume of 100mL) into fresh, pre-warmed, BHI without antibiotics and incubated for 24 hrs at 43°C. The dilution and growth at 43°C was repeated an additional time to promote the single crossover event by selecting for light blue colonies grown on BHI agar supplemented with 10 µg/mL Cm and 50uL of 40 mg/mL X-Gal at 43°C. A light blue colony was selected and incubated in BHI without antibiotics overnight at 30°C to promote the double crossover event. Dilutions of this overnight were plated on BHI agar supplemented with 50µL of 40mg/mL X-Gal and incubated overnight at 37°C. White colonies were selected and patched on BHI agar

supplemented with either 10 μg/mL Cm or 50μL of 40 mg/mL X-Gal. Colonies that failed to grow in the presence of Cm and remained white in the presence of X-Gal were selected and screened for deletion of the AIP coding sequence by sequencing. The verified mutant strain was annotated as *S. epidermidis* RP62A *Δ*AIP.

RNA sequencing

RNA was submitted to the University of California, San Diego (UCSD) genomic core facility for library preparation and sequencing. TruSeq mRNA Library Prep Kit (Illumina) was used for library prep followed by high-throughput sequencing on a HiSeq 2500 sequencer (Illumina). Data was analyzed using Partek Flow and Partek Genomics Suite software and gene ontology analysis was performed using the PANTHER classification system (http://pantherdb.org).

Histology

Full-thickness murine skin (0.5cm^2) were collected, fixed in paraformaldehyde (4%), and washed in PBS prior to overnight incubations with 30% and 10% sucrose prior to freezing tissue in OCT mounting medium with dry ice. Cryostat cut sections (10 mm) were mounted onto Superfrost Plus glass slides (Fisher Scientific) and stained with hematoxylin and eosin (H&E). Sections were incubated for 5min intervals in EtOH gradient of 75%-100% prior to xylene incubation and mounting with paramount and glass slide. Pictures were taken on an Olympus BX51 (Tokyo, Japan) fluorescent microscope at a 200x magnification.

Cytokine Level Determination

Conditioned medium from NHEKs (25μL) were used to quantify protein concentration of various cytokines. Magnetic bead-based milliplex assay kits (Millipore) for 2 human cytokines (IL-6 and TNFα) was used according to manufacture's instructions on a Magpix 200 (Luminex) system. Human IL-1α and IL-36α were quantified by ELISA (R&D Systems).

Quantification of bacterial CFU

S. aureus colony-forming units (CFU) was quantified via plating out serial dilutions (10μL) of 10^{-1} to 10^{-5} on Baird-parker agar (BD) plates containing 3% egg yolk emulsion with tellurite for 24h in a 37°C incubator followed by counting the CFU. Bacterial CFU for all *Staphylococci* strains was also approximated using a spectrophotometer and measuring the OD600nm of cells diluted 1:20 in PBS as well.

Transepidermal water loss measurements

To determine damage to the epidermal skin barrier, transepidermal water loss (TEWL) of murine skin treated for 48-72h with *S. aureus* was measured using a TEWAMETER TM300 (C & K).

Trypsin activity analysis

NHEK conditioned medium was added at 50μL to black 96 well black bottom plates (Corning) followed by addition of 150μL of the peptide Boc-Val-Pro-Arg-AMC (trypsin-like substrate; BACHEM) at a final concentration of 200μM in 1x digestion buffer (10mM

Tris-HCl pH7.8) and incubated at 37°C for 24h. Relative fluorescent intensity (ex:354nm, em:435nm) was analyzed with a SpectraMAX Gemini EM fluorometer (Thermo Fisher Scientific). For murine skin trypsin activity analysis, 0.5cm^2 full-thickness skin was bead-beat (2.0mm zirconia beads, 2x 30sec with 5min after each) in 1mL of 1M acetic acid followed by an overnight rotation at 4°C. Samples were centrifuged (10min, 13,000RPM, 4°C), added to a new microcentrifuge tube followed by protein concentration using a speedvac to remove all remaining acetic acid. Proteins were re-suspended in molecular grade water (500µL) and rotated overnight at 4°C followed by another centrifugation. Clear protein lysates were added to a new tube, and BCA (Bio-rad) analysis used to determine protein concentration. Finally, 10µg of total protein was added to a 96 well plate followed by analysis with the trypsin substrate as above.

S. aureus agr activity

Either the *S. aureus* USA300 LAC agr type I P3-YFP (AH1677) or the *S. aureus* USA300 LAC agr type I pAmi P3-Lux (AH2759) reporter strains were used to detect *S. aureus* agr activity. For *in vitro* experiments, 1e^6 CFU of *S. aureus* USA300 LAC agr type I P3-YFP was added to 300µL of 3%TSB along with 100µL of sterile-filtered commensal supernatant (25% by volume), and shaken (250RPM) 24h at 37°C. Bacteria was than diluted 1:20 in PBS (200µL final) and YFP (ex:495nm, em:530nm) was detected using the fluorometer as above and bacterial density was determined using an OD600nm readout on a spectrophotometer. For murine experiments, *S. aureus* USA300 LAC agr type I pAmi P3-Lux activity was determined using an IVIS machine

and assessing luminescent intensity after a 2min exposure by measuring emitted photons (p/sec/cm^2/sr) using the LiveImaging software (PerkinElmer).

Genome sequencing and assembling

The *S. hominis* C5 genomic DNA was isolated using the DNeasy UltraClean Microbial Kit (Qiagen). The libraries were sequenced using the MiSeq platform (Illumina Inc., San Diego, CA) for two cycles, generating 2x250bp paired-end reads. Adapters were removed using cutadapt (version 1.9.1) (http://cutadapt .readthedocs.io/en/stable/). Low-quality sequences (quality score <30) were removed using the Trim Galore (version 1.9.1) (https://www.bioinformatics. babraham.ac.uk/projects/trim_galore/) with default parameters. Sequences mapping to the human genome were removed from the quality-trimmed dataset using the Bowtie2 (version 2.2.8) (*51*) with the following parameters (-D 20 -R 3 -N 1 -L 20 --very-sensitive-local) and the human reference genome hg19 (UCSC Genome Browser). The filtered reads were *de novo* assembled using SPAdes (version 3.8.0) (*52*) with k-mer length ranging from 33 to 127. The genome was annotated with rapid annotation of microbial genomes using subsystems technology (RAST) with default parameters (*53*). Amino acid sequences from annotated CDS (coding DNA sequence) were aligned to bacterial agr proteins obtained from Uniprot database (downloaded in October 2017). Agr genes from the assembled genome were identified following three criteria: i) sequence identity > 60%; ii) e-value < e100; and iii) the *agr* locus organization, an operon of four genes, *agrBDCA (54)*.

Microbiome data and genome comparative analysis

We analyzed publicly available shotgun metagenomic data for atopic dermatitis skin (*29*). Relative abundance of *S. aureus* and *S. epidermidis* strains were obtained directly from the published supplementary material (www.science translationalmedicine.org/cgi/ content/full/9/397/eaal4651/DC1). The agrD characterization analysis was restricted to eight patients (AD01, AD02, AD03, AD04, AD05, AD08, AD09, and AD11) with information at 7 distinct body sites on flared AD skin and differences in AD severity based upon objective SCORAD. The 61 *S. epidermidis* strains evaluated were classified as agr type I, II, or III through amino acid sequence comparison with known agr type I-III sequences within the agrD gene region (*55*).

Quantification and statistical analysis

The non-parametric Mann-Whitney test was used for statistical significance analysis of AD patient metagenomic data in Figure 4.2. All other figures utilized either One-way ANOVA or Two-way ANOVA for statistical analysis as indicated in the figure legends. All statistical analysis was performed using GraphPad Prism Version 6.0 (GraphPad, La Jolla, CA). All data is presented as mean ± standard error of the mean (SEM) and a *P-value* ≤ 0.05 considered significant.

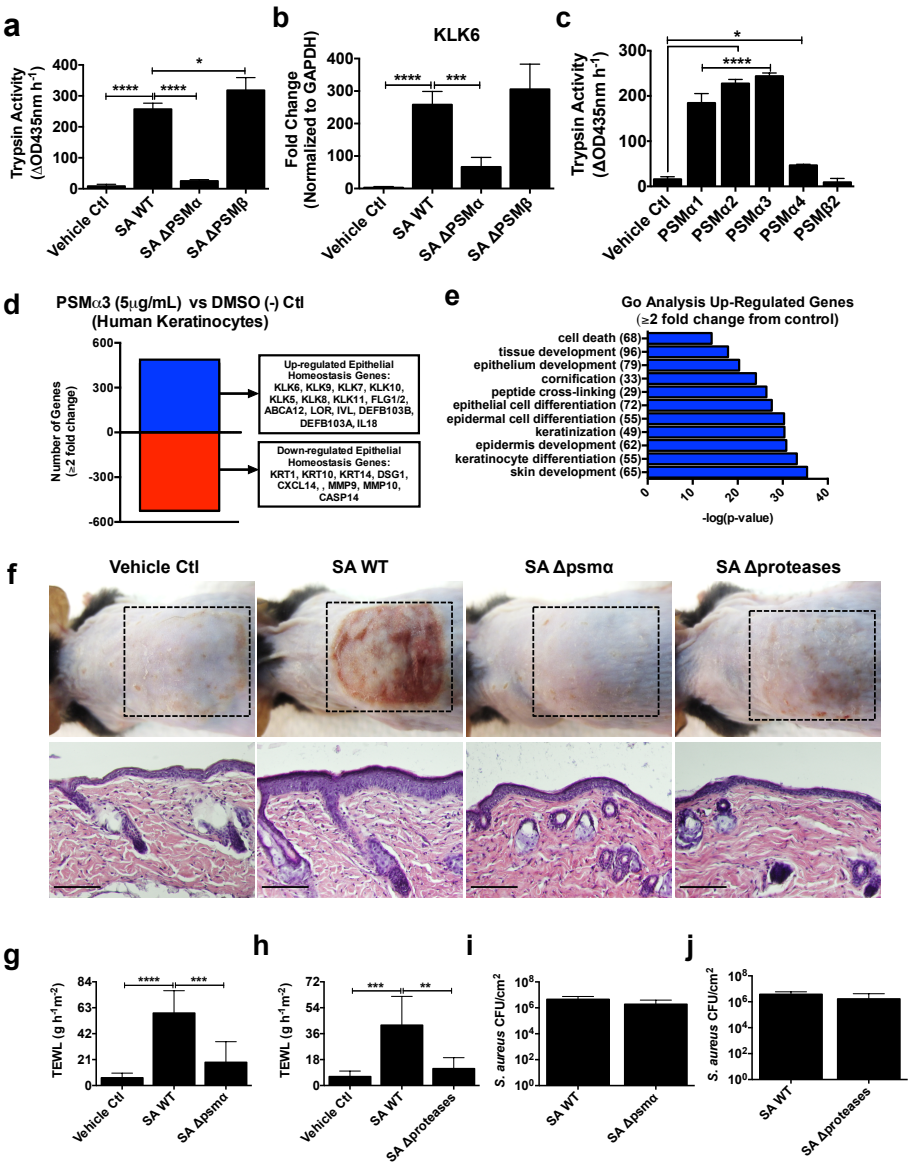

Figure 4.1. *S. aureus* PSMα leads to disruption of epithelial barrier homeostasis. Human keratinocytes (NHEKs) were stimulated with *S. aureus* (SA) sterile-filtered supernatant from wild type (WT), PSMα (ΔPSMα) or PSMβ (ΔPSMβ) knockout strains for 24h and **(a)** trypsin activity and **(b)** KLK6 mRNA compared to the housekeeping gene GAPDH were analyzed (n=4). **(c)** PSM synthetic peptides were added to NHEKs for up to 24h to analyze changes in trypsin activity. **(d,e)** Transcript analysis by RNA-Seq of genes that changed ≥2 fold after PSMα3 treatment was assessed followed by gene ontology (GO) analysis. 8 week male C57BL/6 mice (n=6) were treated for 72h with SA WT, SA ΔPSMα, or a SA 10 secreted protease knockout strain (Δproteases) ($1e^7$ CFU). **(f,g)** Murine skin representative pictures (dashed lines indicate treatment area) and changes to epidermal thickness after treatment (scale=200μm). **(h-k)** Changes in murine back skin with WT or mutant SA strains in transepidermal water loss (TEWL) and SA CFU/cm^2 were assessed as well. All error bars are represented at standard error of the mean (SEM) and One-way ANOVAs were used to determine statistical significance indicated by: $p<0.05$ *, $p<0.01$ **, $p<0.001$ ***, $p<0.0001$ ****.

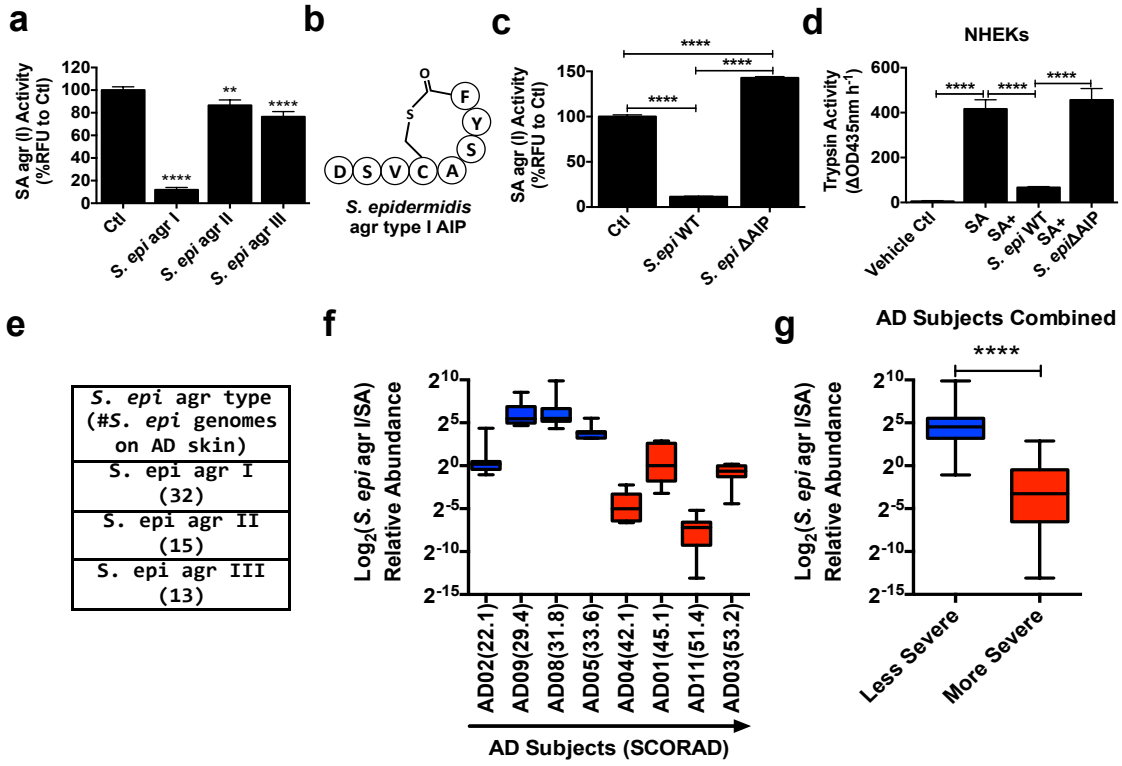

Figure 4.2. *Staphylococcus epidermidis* agr type I autoinducing peptide characterization and deficiency in AD skin.

(a, b) *S. epidermidis* agr types I-III supernatant inhibition of *S. aureus* (SA) USA300 LAC agr type I activity after 24h (n=4) and representation of known structure of S. epidermidis agr type I autoinducing peptide (AIP). **(c)** *Staphylococcus epidermidis* (*S. epi*) agr type I strain RP62A wild-type (WT) or autoinducing peptide knockout (ΔAIP) effect on SA agr activity after 24h. **(d)** SA sterile-filtered supernatant growth with or without *S. epi* WT or ΔAIP supernatant was applied to NHEKs for an additionally 24h followed by measurement of NHEK trypsin activity (n=4). **(e)** Consensus of *S. epidermidis* agr types I-III genomes found on AD skin. **(f,g)** Ratio of *S. epidermidis* agr type I to SA relative abundance on flare regions of 8 individual AD subjects from 'least severe' to 'most severe' AD score based upon objective SCORAD and overall combined data of all subjects based upon AD severity. All error bars are represented at standard error of the mean (SEM) and One-way ANOVAs **(a,c,d)** and a (nonparametric) unpaired Mann-Whitney test **(f)** were used to determine statistical significance indicated by: p<0.05 *, p<0.01 **, p<0.001 ***, p<0.0001 ****.

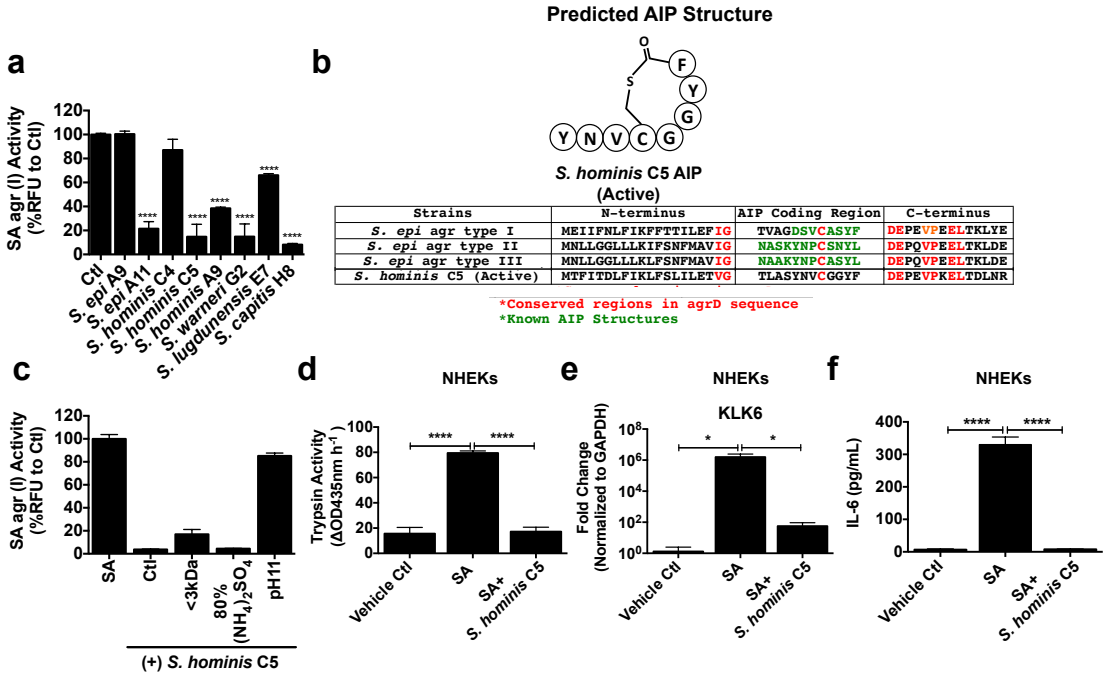

Figure 4.3. Multiple clinically isolated Coagulase-negative *Staphylococci* inhibit *S. aureus* agr activity.

(a) Sterile-filtered supernatants of clinically isolated Coagulase-negative Staphylococci (CoNS) were added to *S. aureus* (SA) USA300 LAC agr type I P3-YFP reporter strain for 24h followed by analysis for SA agr activity (n=3). **(b,c)** *S. hominis* C5 strain genome was further sequenced and analyzed at the agrD gene for the autoinducing peptide (AIP) sequence. Biochemical analysis of *S. hominis* C5 supernatant tested the ability of a <3kDa size exclusion centrifugation filtration, 80% ammonium sulfate precipitate, and pH11 1h treated supernatant to effect SA agr activity as well. **(d-f)** SA grown in presence of *S. hominis* C5 supernatant for 24h was sterile-filtered and added to human keratinocytes (NHEKs) for 24h followed by analysis of trypsin activity, KLK6 mRNA expression compared to the housekeeping gene GAPDH, and IL-6 protein levels. All error bars are represented at standard error of the mean (SEM) and One-way ANOVAs were used to determine statistical significance indicated by: $p<0.05$ *, $p<0.01$ **, $p<0.001$ ***, $p<0.0001$ ****.

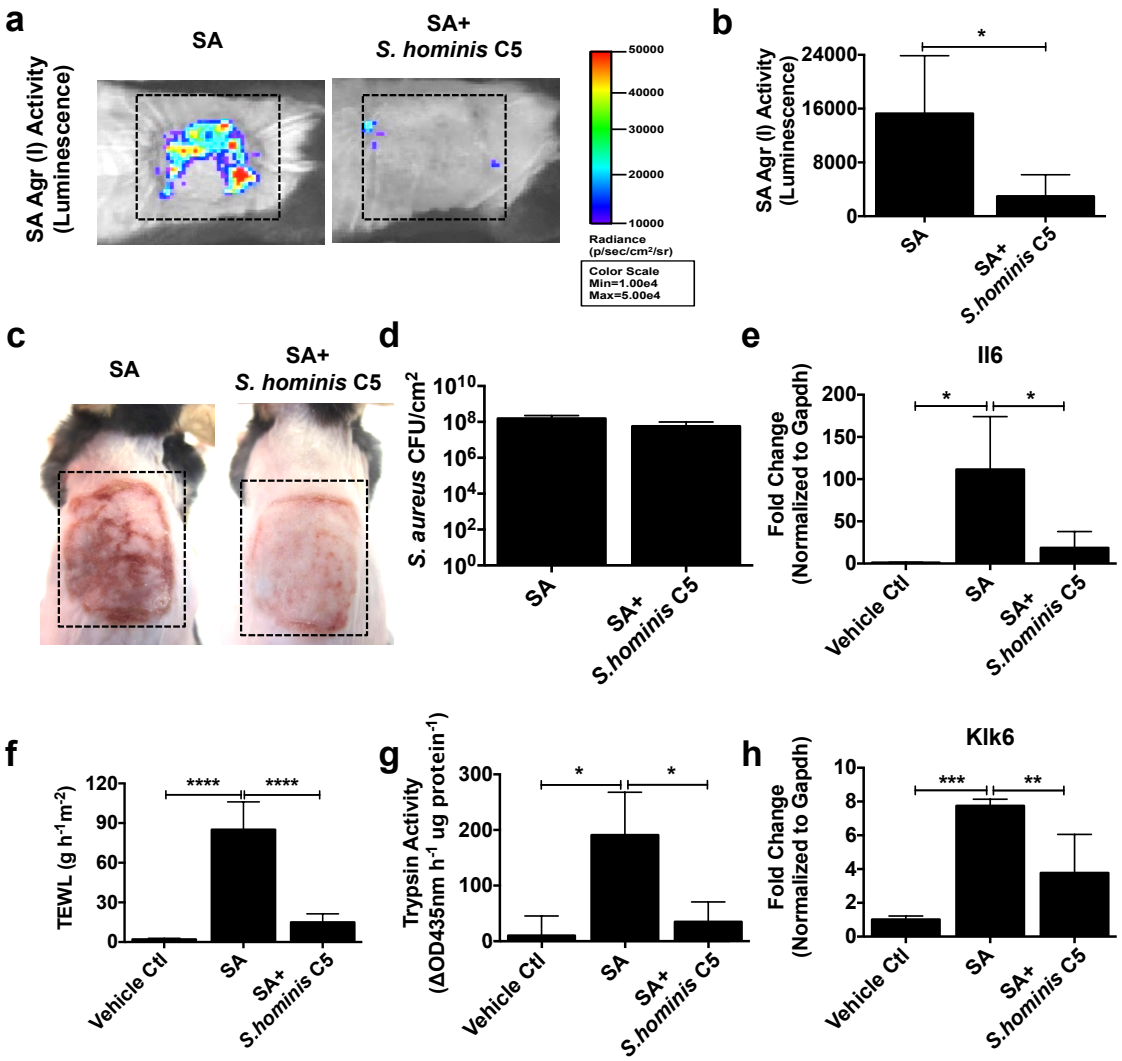

Figure 4.4. AD clinical CoNS isolate inhibits SA induced murine skin barrier damage.

S. aureus (SA) USA300 LAC agr type I pAmi P3-Lux reporter strain ($1e^7$ CFU) with or without live *S. hominis* C5 ($1e^8$ CFU) was applied to 8 week female C57BL/6 mice for 48h (n=5). **(a,b)** SA agr activity was assessed on murine back skin by changes in luminescence. **(c)** Representative images of murine skin after 48h SA treatment (dashed boxes indicate treatment area). **(d-h)** SA CFU/cm^2 was determined and murine skin barrier damage and inflammation was assessed by analyzing changes in Il6 mRNA expression, transepidermal water loss (TEWL), trypsin activity, and Klk6 mRNA expression normalized to the housekeeping gene Gapdh. All error bars are represented at standard error of the mean (SEM) and One-way ANOVAs were used to determine statistical significance indicated by: $p<0.05$ *, $p<0.01$ **, $p<0.001$ ***, $p<0.0001$ ****.

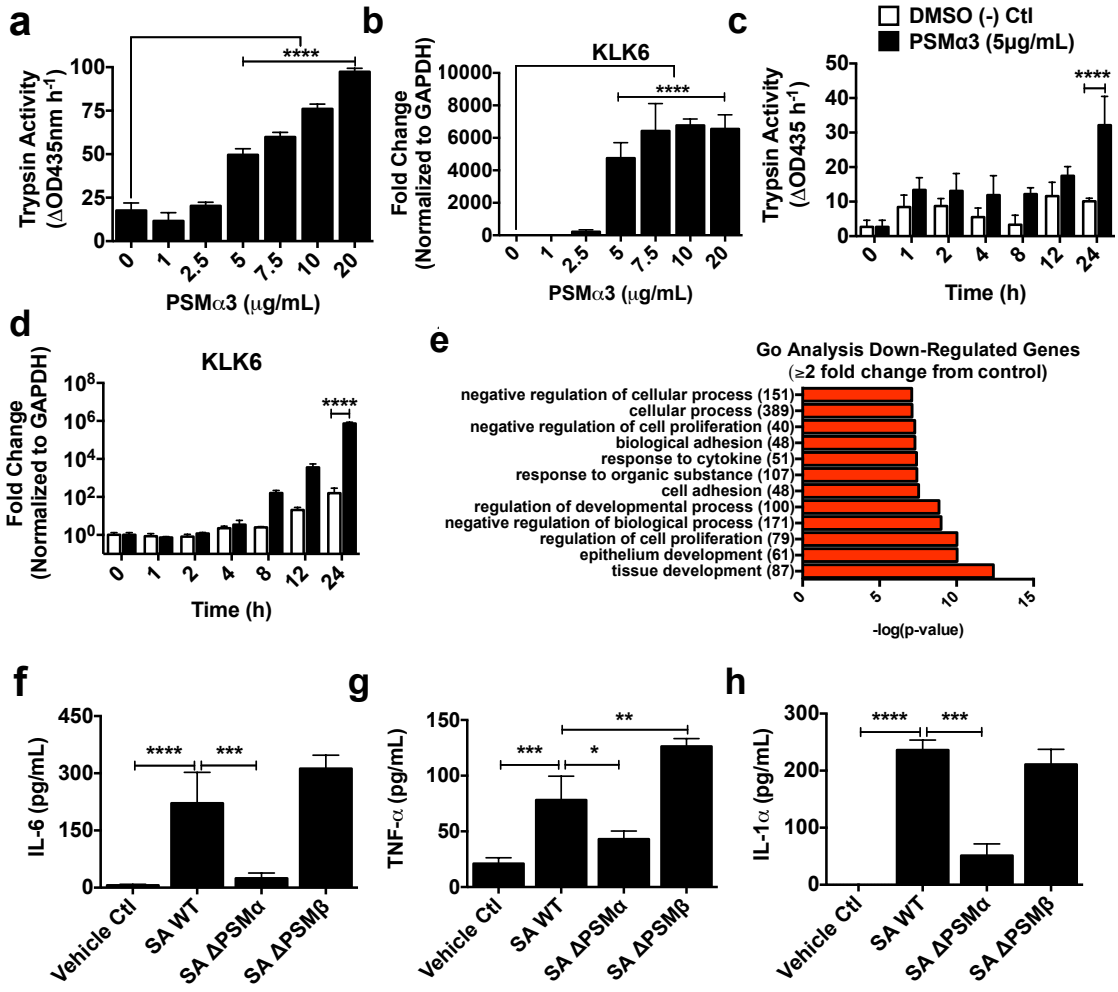

Figure 4.5. *S. aureus* PSMα changes essential barrier genes and cytokine expression in human keratinocytes.
(a-d) Human keratinocytes treated with synthetic PSMα3 were assessed for changes in trypsin activity and KLK6 transcript expression normalized to the housekeeping gene GAPDH in both a dose and time dependent manner. **(e)** GO-term analysis of genes down-regulated ≥2 fold from the control in human keratinocytes treated with PSMα3 for 24h. **(f-h)** Changes in human keratinocyte cytokine protein expression of IL-6, TNF-α, or IL-1α treated with SA WT, SA Δpsmα, or SA Δpsmβ supernatant for 24h. All error bars are represented at standard error of the mean (SEM) and One-way ANOVAs were used to determine statistical significance indicated by: $p<0.05$ *, $p<0.01$ **, $p<0.001$ ***, $p<0.0001$ ****.

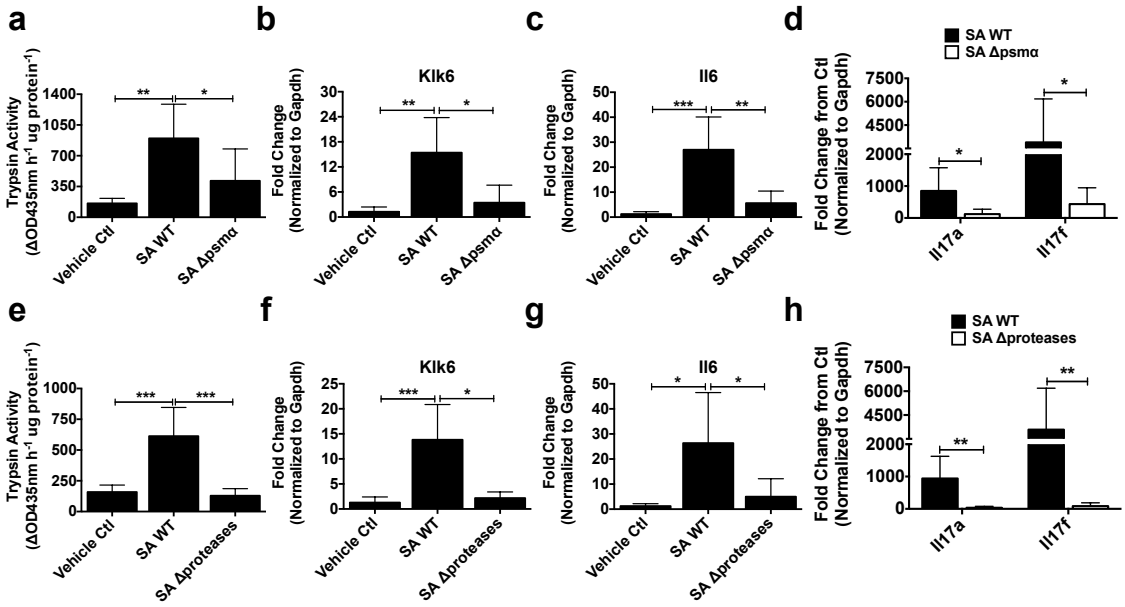

Figure 4.6. ***S. aureus PSMα and proteases are responsible for barrier damage and induction of inflammation on murine skin.***

S. aureus (SA) ($1e^7$ CFU) wild type (WT), PSMα knockout (Δpsmα), and protease null (Δproteases) strains were applied to male murine back skin for 72h (n=6) and changes in **(a,e)** trypsin activity, (b,f) Klk6, (c,g) Il6, and (d,h) IL17a/f mRNA expression normalized to the housekeeping gene Gapdh were measured. All error bars are represented at standard error of the mean (SEM) and One-way ANOVAs were used to determine statistical significance indicated by: $p<0.05$ *, $p<0.01$ **, $p<0.001$ ***, $p<0.0001$ ****.

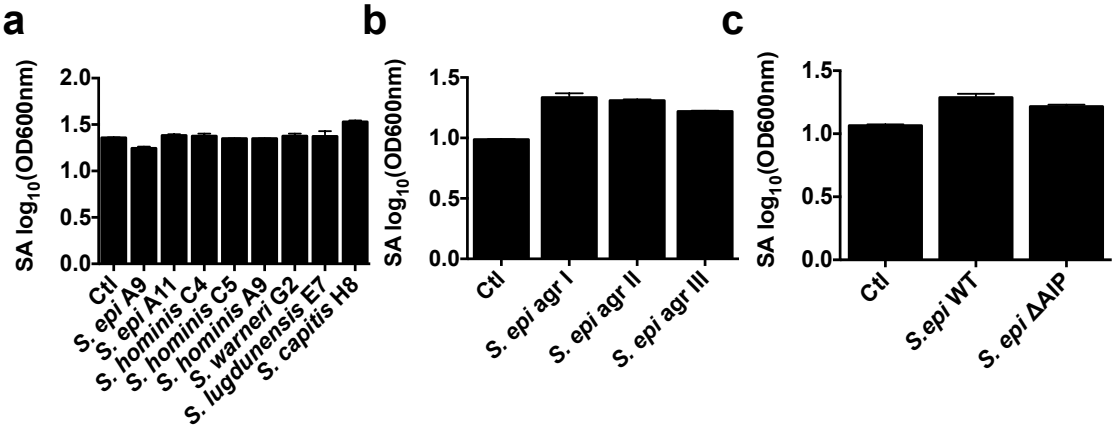

Figure 4.7. CoNS strains do not affect SA growth.
Coagulase-negative *Staphylococci* (CoNS) supernatant affect on SA agr type I P3-YFP reporter strain growth as assessed by OD600nm (n=3-4) including **(a)** CoNS clinical isolates, **(b)** *S. epidermidis (S. epi)* agr type I-III, and **(c)** *S. epidermidis* (*S. epi*) wild type (WT) or autoinducing peptide knockout (ΔAIP) supernatant added to SA agr type I reporter strain for 24h. All error bars are represented at standard error of the mean (SEM).

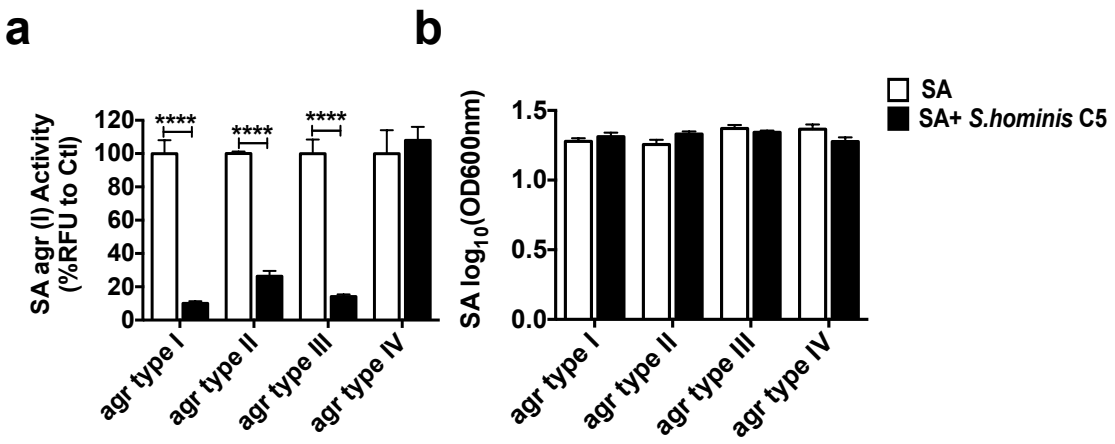

Figure 4.8. *S. hominis* C5 inhibits SA agr type I-III but not type IV.
S. hominis C5 supernatant added to SA agr types I-IV P3-YFP reporter strains for 24h (n=3). **(a)** SA reporter strain agr type I-IV activity and **(b)** measurement of growth by OD600nm when cultured in presence of *S. hominis* C5 supernatant. All error bars are represented at standard error of the mean (SEM) and One-way ANOVAs were used to determine statistical significance indicated by: $p<0.05$ *, $p<0.01$ **, $p<0.001$ ***, $p<0.0001$ ****.

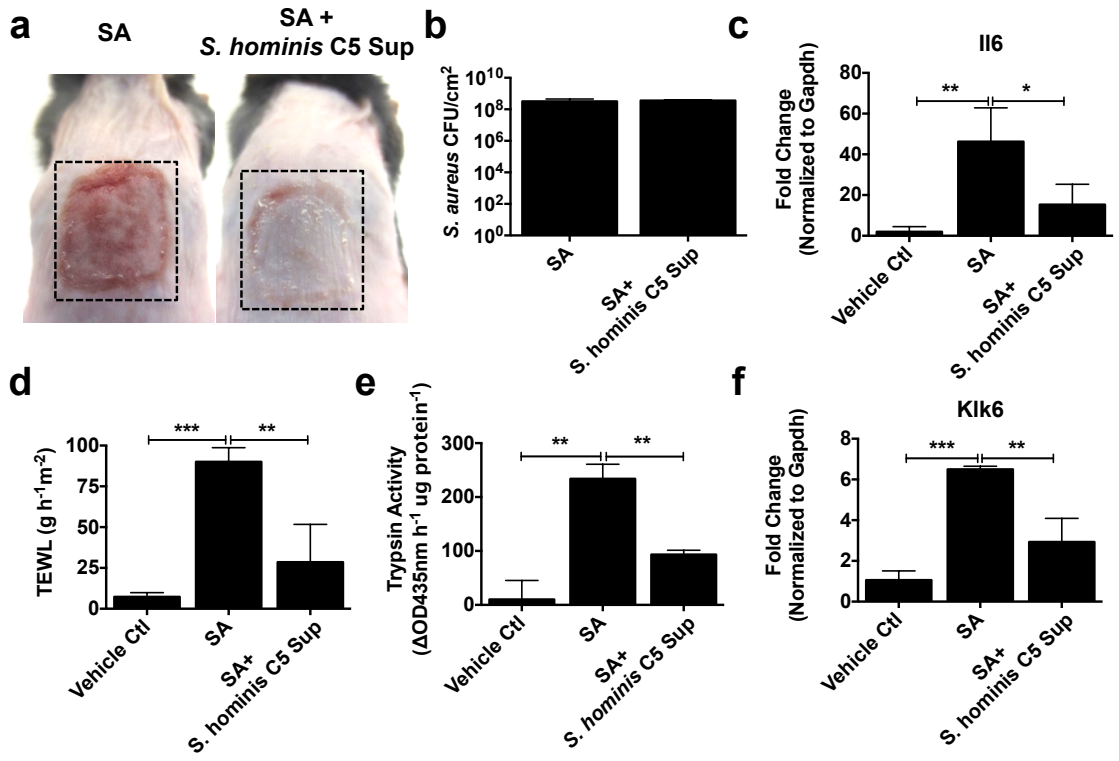

Figure 4.9. *S. hominis* C5 supernatant inhibits SA induced skin barrier damage. *S. aureus* (SA) (1e^7 CFU) with or without 10x concentrated <3kDa *S. hominis* C5 supernatant was applied to female murine back skin for 48h (n=3). **(a)** Representative images of murine back (dashed lines indicate treatment area) and SA CFU/cm^2 recovered from murine skin after SA treatment. (c-f) SA induced skin barrier damage markers including Il6, transepidermal water loss (TEWL), trypsin activity, and Klk6 mRNA expression compared to the housekeeping gene Gapdh. All error bars are represented at standard error of the mean (SEM) and One-way ANOVAs were used to determine statistical significance indicated by: p<0.05 *, p<0.01 **, p<0.001 ***, p<0.0001 ****.

Table 4.1. Bacteria strains and plasmids.

Bacterial and Plasmids		
Bacteria		
Strain Name	Source	Reference
S. epidermidis RP62A WT (agr type I)	Gallo (UCSD) Cheung (Darmouth)	This Study
S. epidermidis RP62A ΔAIP (#47)	Gallo (UCSD) Cheung (Darmouth)	This Study
S. epidermidis ATCC1457 (agr type II)	Gallo (UCSD)	This Study
S. epidermidis 8247 (agr type III)	Horswill (UC Denver)	(*53*)
S. epidermidis A9	Gallo (UCSD)	(*56*)
S. epidermidis A11	This Study	This Study
S. aureus USA300 LAC WT	Otto (NIH/NIAID)	(*57*)
S. aureus USA300 Δpsmα	Otto (NIH/NIAID)	(*57*)
S. aureus USA300 Δpsmβ	Otto (NIH/NIAID)	(*57*)
S. aureus USA300 LAC WT (AH1263)	Horswill (UC Denver)	*(25)*
S. aureus USA300 LAC Δprotease (AH1919), Erm^R, Lcm^R	Horswill (UC Denver)	*(25)*
S. aureus USA300 LAC agr type I pAmi P3-Lux (AH2759), Cm^R	Horswill (UC Denver)	(*58,59*)
S. aureus USA300 LAC agr type I P3-YFP (AH1677), Cm^R	Horswill (UC Denver)	(*60*)
S. aureus 502a agr type II P3- YFP (AH430), Cm^R	Horswill (UC Denver)	(*60*)
S. aureus MW2 agr type III P3- YFP (AH1747), Cm^R	Horswill (UC Denver)	(*60*)
S. aureus MN TG agr type IV P3-YFP (AH1872), Cm^R	Horswill (UC Denver)	(*60*)
S. hominis C4	Gallo (UCSD)	(*56*)
S. hominis C5	Gallo (UCSD)	(*56*)
S. hominis A9	Gallo (UCSD)	(*56*)
S. warneri G25	Gallo (UCSD)	This Study
S. capitis H8	Gallo (UCSD)	This Study
S. lugdunensis E7	Gallo (UCSD)	This Study
DC10B-CC10	Cheung (Dartmouth)	(*48)*
Plasmids		
Strain Name	Source	Reference
pMAD (Amp^R in *E. coli*, Cm^R in *Staphylococci*)	Cheung (Dartmouth)	(*48-50*)
pMAD:: *ΔAIP*	Cheung (Dartmouth)	This Study

Table 4.2. Primers used for qPCR and *S. epidermidis* allelic replacement.

Primers	Description
TaqMan Murine Gapdh primers	Mm99999915_g1 (Applied Biosystems)
TaqMan Murine Klk6 primers	Mm00478322_m1 (Applied Biosystems)
TaqMan Murine Il6 primers	Mm00446190_m1 (Applied Biosystems)
TaqMan Murine Ifng primers	Mm01168134_m1 (Applied Biosystems)
TaqMan Murine Il4 primers	Mm00445259_m1 (Applied Biosystems)
TaqMan Murine Il17a primers	Mm00439618_m1 (Applied Biosystems)
TaqMan Murine Il17f primers	Mm00521423_m1 (Applied Biosystems)
TaqMan Human KLK6 primers	Hs00160519_m1 (Applied Biosystems)
TaqMan Human GAPDH primers	Hs02786624_g1 (Applied Biosystems)
agrD-Up (This Study; *S. epidermidis* RP62A ΔAIP Construction)	Forward-Bam: AGCTA**GGATCC**GTTGGGATGGCTCAACAAC TCACTAATAATAT Reverse: AGTTCTTCTGGTACTTCTGGTTCGTCTCCTG CTACAGTACCAATAAATTCCAAGATTG
agrD-Down (This Study; *S. epidermidis* RP62A ΔAIP Construction)	*Forward:* CAATCTTGGAATTTATTGGTACTGTAGCAG GAGACGAACCAGAAGTACCAGAAGAACT *Reverse-Sal:* AGCTA**GTCGAC**TTCCTCATTATCGATGAAT GCGATATTGATGAG
agrD Seq (This Study; *S. epidermidis* RP62A ΔAIP Construction)	*Forward:* GTATAATGACACTTGAGGAGAGTAGTAAA *Reverse:* CCATATCTTACACACTTTCTAGGGTTATA

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Acknowledgements

Chapter IV is based on unpublished data currently being prepared for submission for publication: Williams, M.R., Costa, S.K., Zaramela, L.S., Sanford, J.A., O'Neill, A.M., Liggins, M.C., Nakatsuji, T., Horswill, A.R., Cheung, A.L., Zengler, K., and Gallo, R.L. Quorum sensing between bacterial species on the skin protects against epidermal injury in atopic dermatitis. I was the primary researcher and author for the manuscript. The co-authors listed above either supervised or provided support for the research, and have given permission for the inclusion of the work in this dissertation.

Chapter V:

Conclusions and perspectives

Summary

The epidermal skin barrier plays a very unique role in providing the first line of defense against multiple environmental stimuli and regulation of protease activity is vital to maintaining an intact skin barrier. Bacteria cover the entirety of our skin surface and often can modulate our skin barrier response. Thus we first posed the simple question in this dissertation of do bacteria alter the proteolytic balance of the skin barrier? After screening multiple strains of bacteria on human keratinocytes, it became clear that the ability of *S. aureus* to induce endogenous serine proteases would be an interesting topic to focus on. Especially since increased serine protease responses have a negative effect on the skin barrier integrity as seen in the skin disease of Netherton syndrome. We determined that *S. aureus* could induce expression of a specific family of serine proteases known as the kallikreins (KLKs) and knockdown of specific KLKs in keratinocytes prevented cleavage of essential skin barrier proteins. Furthermore, we determined mechanistically that *S. aureus* phenol-soluble modulin α (PSMα) was responsible for induction of serine protease activity in human keratinocytes and that both *S. aureus* secreted proteases and PSMα were required for induction of skin barrier damage and inflammation in murine models.

Based upon these initial observations, we shifted our focus to determine how to prevent *S. aureus* induced serine protease activity and overall epidermal barrier damage and inflammation. Interestingly, both *S. aureus* PSMα and secreted proteases are regulated by the accessory gene regulatory (agr) quorum sensing system (*1*). Furthermore *S. aureus* agr activity could be inhibited by the agr systems of certain commensal microbes by secretion of autoinducing peptides (AIPs), the main component

that turns on the agr system in bacteria (*2-4*). We determined using a *S. epidermidis* agr type I AIP knockout strain that this commensal AIP was directly responsible for inhibiting the *S. aureus* agr system. In order to make this clinically relevant, we also found that the majority of *S. epidermidis* strains on atopic dermatitis (AD) skin were agr type I and produce this AIP. Furthermore, in comparison to *S. aureus*, which frequently colonized AD skin and is related to increased diseased severity, the relative abundance of these *S. epidermidis* agr type I strains were deficient in AD patients with higher disease severity. We further explored if clinical isolates of commensal coagulase negative *Staphylococci* (CoNS) strains could also inhibit *S. aureus* agr activity. We determined that the majority of commensal CoNS strains from AD skin swabs could strongly inhibit *S. aureus* agr activity including the species of *S. epidermidis*, *S. hominis*, *S. warneri*, and *S. capitis*. Finally a novel AIP was discovered from a *S. hominis* strain that strongly inhibited *S. aureus* agr activity and further blocked inflammation and skin barrier damage in both human keratinocytes and murine models.

Overall this work clearly outlined how S. aureus can secrete virulence factors that cause AD-like skin phenotypes by initially disrupting the epithelial skin barrier by induction of serine protease activity. Furthermore, we determined that clinical isolates of commensal microbes could be used to block the virulence of *S. aureus* through inhibition of the agr quorum sensing system. Thus providing a possible therapeutic strategy to combat the *S. aureus* driven skin damage on AD patients.

Further exploration of mechanisms for serine protease induction in human keratinocytes

Although we characterized *S. aureus* secreted PSMα peptides as the primary culprit for both increased KLK expression and serine protease activity in keratinocytes, it was important to further explore how PSMα mechanistically induces these changes in the cell. It is known that PSMα peptides can influence cells in two distinct ways. PSMα can readily form pores in cell membranes that leads to a lytic pathway (*5, 6*). Secondly, it was shown several times that these amphipathic peptides would also stimulate formyl peptide receptors 1/2 (FPR1/2), a family of cell surface G protein- coupled receptors (GPCRs), with different concentrations of PSMα affecting the stimulation of these receptors (*7, 8*). Thus, we started to explore the mechanism by which *S. aureus* PSMα3 could stimulate serine protease activity and KLK expression in human keratinocytes. We showed that PSMα3 could immediately increase serine protease activity and release of KLKs including KLK5 and KLK6 from human keratinocytes into the surrounding supernatant within a 1h treatment time and in a dose dependent manner. This correlated with increased LDH release and propidium iodide staining, two indicators of keratinocyte lysis. Furthermore transcript expression levels did not change for KLK6 during this period indicating that this initial spike in serine protease activity was due to release of preformed proteases from the keratinocytes matching previous reports that keratinocytes store proteases in lamellar bodies (*9*). The detergent triton X-100 showed that direct lysis of keratinocytes could induce release of active serine proteases as well independently of any receptor binding (Figure 5.1). Overall, this indicated that PSMα could indeed induce a rapid release of preformed serine proteases from human keratinocytes in most likely a lytic fashion. Interestingly, as shown with previous papers where an immediate treatment with PSMs could stimulate cytokine release, the same

seems to be true for KLKs and serine protease activity as well. Thus this has to be taken into account aside from just changes in transcript expression of serine proteases at later time points when looking at how *S. aureus* overall influences human keratinocytes protease activity.

Aside from PSMα3 induced lysis of human keratinocytes, we also started to explore how transcription of the KLKs is altered at later time points. We used a series of cell pathway inhibitors to determine that KLK6 expression is dependent on GPCRs and the MAPK signaling pathway (ERK1/2, p38, src). Furthermore, phosphorylation of key MAPK pathway proteins ERK1/2 and p38 was increased by PSMα3 treatment of human keratinocytes (Figure 5.2). This initial evidence led us to confirm that KLK6 expression could be dependent on the same pathways that have been explored with PSMα peptides and FPR1/2 receptors. However, we need to further identify if specific inhibition of the FPR1/2 receptors on human keratinocytes effects KLK expression versus that of GPCRs as a whole. Secondly, it is important to identify the transcription factors associated with MAPK signaling that ultimately stimulate KLK expression to further understand this pathway. Unfortunately little is known of how KLKs are expressed mechanistically in human keratinocytes so much is still left to explore. Interestingly, we now know that there also is a difference between KLK expression and trypsin activity measurements. Depending on the PSMα3 concentration trypsin activity can be released within 1h from keratinocytes without changes in KLK expression, but if we assess cells treated up to 24h with PSMα3 we observe a robust increase in KLK expression. Overall, it seems serine protease activity is increased by *S. aureus* PSMα

peptides through multiple pathways including both cell lysis and possible FPR stimulation, while more in depth analysis is necessary to fully understand this mechanism.

Defense mechanism of endogenous serine proteases against *S. aureus*?

The focus of this study was on how *S. aureus* could induce damage to the epidermal skin barrier by increasing serine protease activity in keratinocytes. However it is equally important to recognize how keratinocytes might react defensively by increasing expression of serine proteases to prevent *S. aureus* induced damage as well. It is known that host cells always mount an immune defense to a foreign threat. For instance, keratinocytes have been found to induce antimicrobial peptides (AMPs) such as the β-defensins or cathlecidin when exposed to bacteria (*10, 11*). Interestingly, serine proteases such as the KLKs control a plethora of mechanisms within the skin barrier including cleavage of corneodesmosome adhesion proteins and desquamation, or shedding, of the top layer of the skin. One interesting idea is perhaps that serine protease activity is increased in order to remove colonization of pathogenic bacteria such as *S. aureus* from our skin by allowing the skin to peel away faster. We minimally explored this concept by treating mice for 1, 3, 5, and 7 days with *S. aureus* and removed the adhesive that keeps *S. aureus* protected on the mouse skin at day 3 to allow *S. aureus* to be removed. We observed *S. aureus* reaches a peak colonization at day 1 on the skin and stays constant at day 3 prior to the removal of the protective adhesive. It is also at day 3 that there is a peak in serine protease activity, KLK expression, and barrier damage as assessed by transepidermal water loss (TEWL). Once the adhesive is removed, the *S. aureus* colonization rapidly declines at days 5

and 7 while all of the barrier damage markers including trypsin activity and KLK expression stay elevated throughout day 7 (Figure 5.3). Overall these data suggest that increased serine protease activity, although bad for our epidermal barrier integrity, could also be a defense mechanism for our skin against increased colonization with *S. aureus*. Obviously this concept would have to be explored further to completely understand how keratinocytes react defensively against the presence of *S. aureus*. However it is clear that the skin of mice with significantly less *S. aureus* colonization shows an improved phenotype while retaining elevated protease activity.

Role of CoNS in protecting against *S. aureus* driven inflammation and skin barrier damage

Coagulase-negative *Staphyoccoci* (CoNS) have been shown to inhibit *S. aureus* agr activity and thus prevent that ability of *S. aureus* virulence factors such as PSMs and secreted proteases to damage the host skin. This was first evidenced in 2001 that the *S. epidermidis* strain ATCC14990 produced a novel autoinducing peptide (AIP) that could inhibit *S. aureus agr* types I-III, and more recently it has been determined that multiple types of CoNS might play a role in this inhibition as well (*2-4*). We built upon this idea by first showing that the *S. epidermidis* agr type I AIP was directly responsible for inhibiting *S. aureus* agr activity and showing that deficiency of these bacteria is clinically relevant to atopic dermatitis (AD) patients who often have elevated *S. aureus* colonization. We further showed that different types of CoNS species from AD patients including *S. hominis*, *S. warneri*, and *S. capitis* could also produce unique AIPs that inhibit *S. aureus* agr activity.

We have started to understand how commensal microbes can combat *S. aureus* virulence and improve skin barrier integrity. However, these studies were done in an isolated environment without the full complexity of skin microbes on patients. Ultimately, understanding how multiple bacteria in a given environment interact is a topic that needs to be explored further. For instance, it was recently shown that while *S. epidermidis* agr type I produces an AIP that can inhibit *S. aureus* agr activity, *S. epidermids* agr types II and III can inhibit the *S. epidermidis* agr type I system (*12*). It is reasonable to conclude from these experiments that perhaps in isolation CoNS are quite good at inhibiting *S. aureus* agr activity, but in a real skin microenvironment where multiple CoNS strains as well are other species of bacteria are present, there could be competition for which agr systems prevail. This definitely complicates how we interpret the composition of agr systems present on the skin, but also further illustrates why putting an overabundance of CoNS that inhibit the *S. aureus* agr system to outcompete other microbes on the skin, or even applying the synthetic AIP itself would be useful therapeutic strategies to turn off the *S. aureus* agr system in the future.

In this dissertation we further characterized *S. hominis* C5 as an AD clinical isolate that produces a novel AIP that strongly inhibits *S. aureus* agr activity. We also showed that application of this bacteria to mouse skin at the same time as *S. aureus* could block skin inflammation and barrier damage. Thus, this proves the therapeutic potential of these AIP producing strains to combat skin disease where *S. aureus* colonization is elevated.

Concluding Remarks

We show that *S. aureus* can induce serine protease activity in keratinocytes that leads to skin barrier damage. Furthermore *S. aureus* agr regulated virulence factors including PSMα and secreted proteases are necessary for increased inflammation and epidermal skin barrier damage, including changes in serine protease activity. Than we defined how certain clinical isolates of CoNS strains, including *S. hominis* C5, can inhibit *S. aureus* agr activity leading to a blockade of *S. aureus* virulence factor driven skin barrier damage and inflammation (Figure 5.4). Ultimately we hope that this work can lead the way in using certain CoNS strains therapeutically to help prevent the damage to our skin barrier caused by an overabundance of *S. aureus* on the skin in various skin diseases including atopic dermatitis.

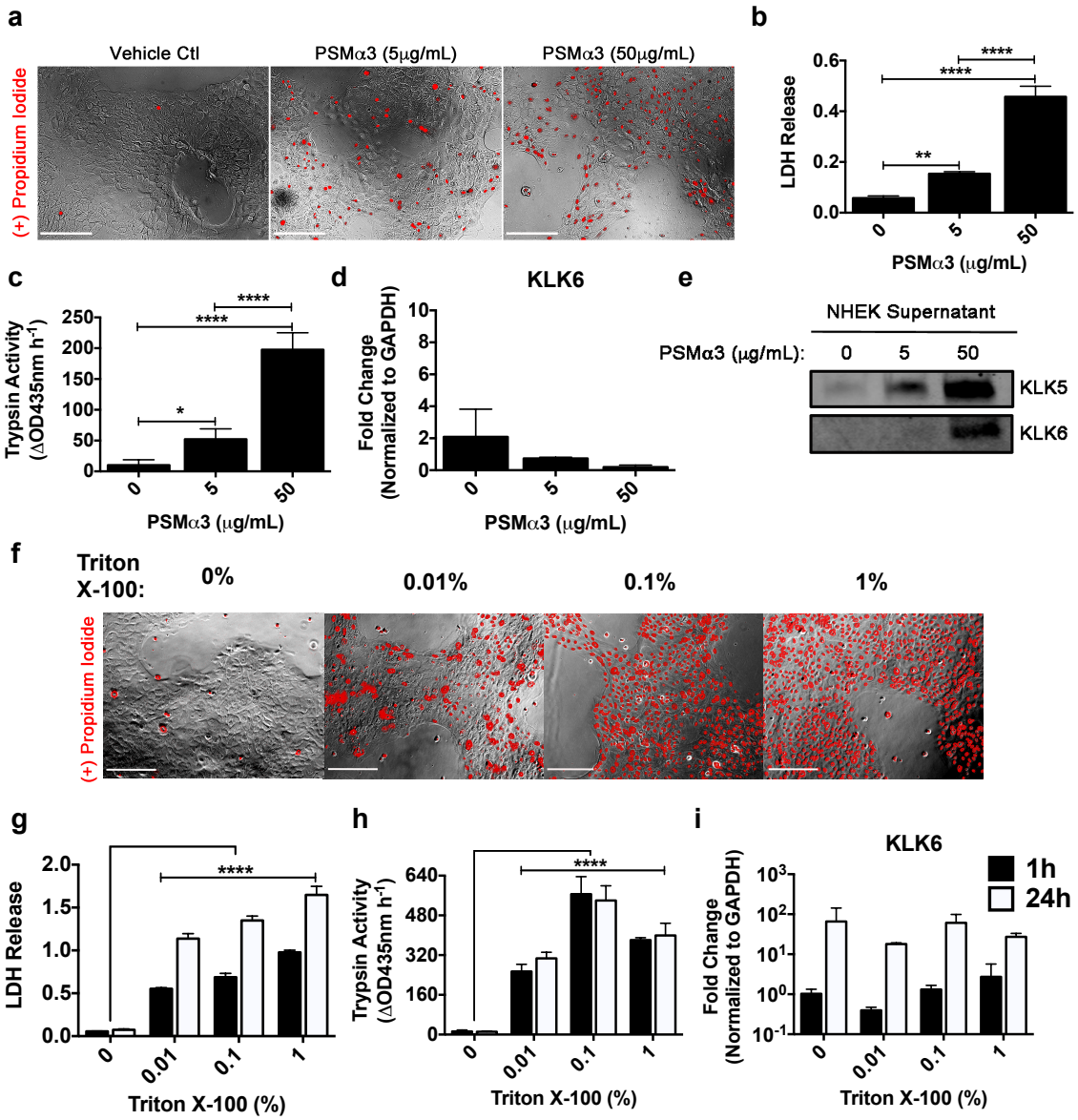

Figure 5.1. Immediate exposure of human keratinocytes to PSMα induces cell toxicity and release of proteases.

PSMα3 stimulation of human keratinocytes for 1h leads to increased **(a)** propidium iodide staining and **(b)** LDH release. Similarly **(c)** trypsin activity is increased along with **(e)** KLK5 and KLK6 protein expression in the human keratinocyte conditioned medium after 1h while **(d)** KLK6 transcript expression does not change. **(f)** Triton X-100 treatment for 1h also leads to increased propidium iodide staining and LDH release. **(h,i)** Trypsin activity is similarly increased while KLK6 transcript expression does not change. Data represents mean ± SEM (n=3) and one-way ANOVA **(a)** was used with significance indicated by: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

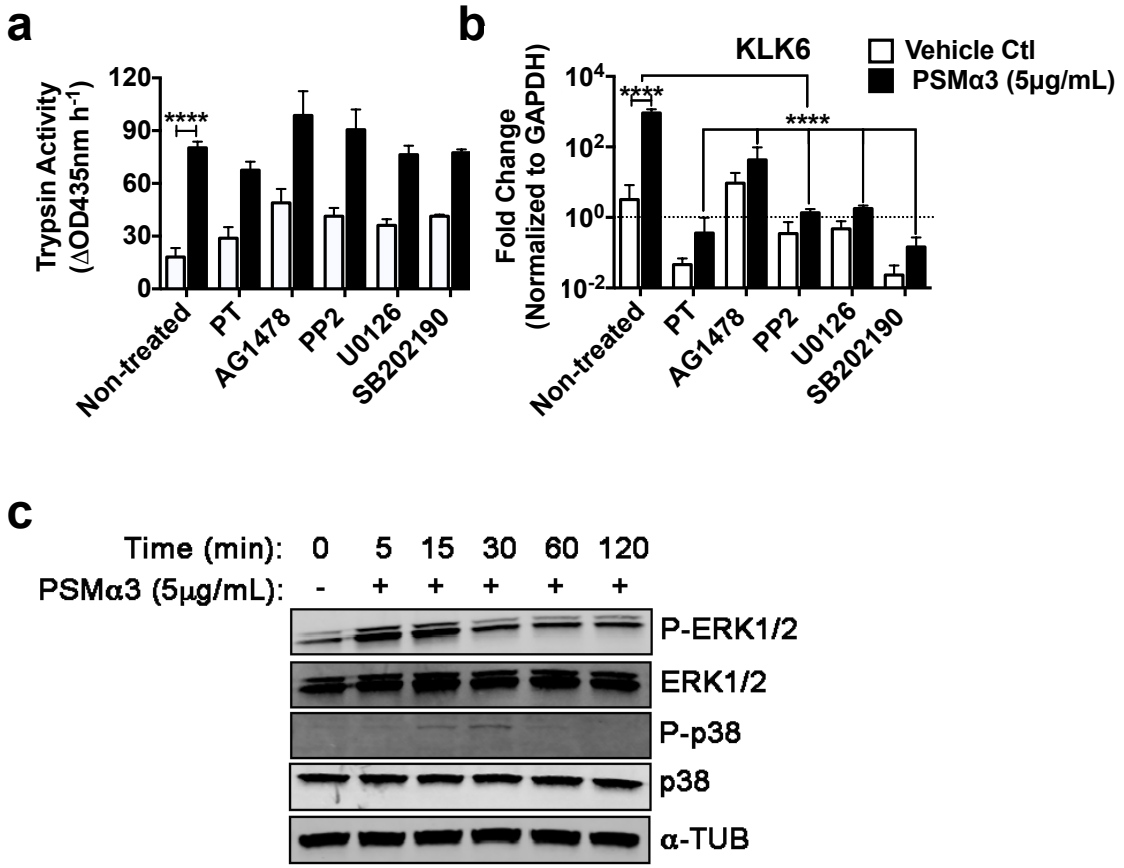

Figure 5.2. Human keratinocytes exposed to PSMα stimulates KLK expression in a GPCR and MAPK mediated pathway.

Human keratinocytes were treated with PSMα3 for 24h with a 1h pretreatment with or without the inhibitors for GPCR (pertussis toxin; PT), EGFR (AG1478), src (PP2), ERK1/2 (U0126), and p38 (SB202190). **(a)** Trypsin activity and **(b)** KLK6 transcript expression were measured after 24h. **(c)** Analysis of ERK1/2 and p38 phosphorylation by PSMα3 in human keratinocytes was assessed by phosphoblot as well. Data represents mean ± SEM (n=3) and one-way ANOVA **(a)** was used with significance indicated by: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

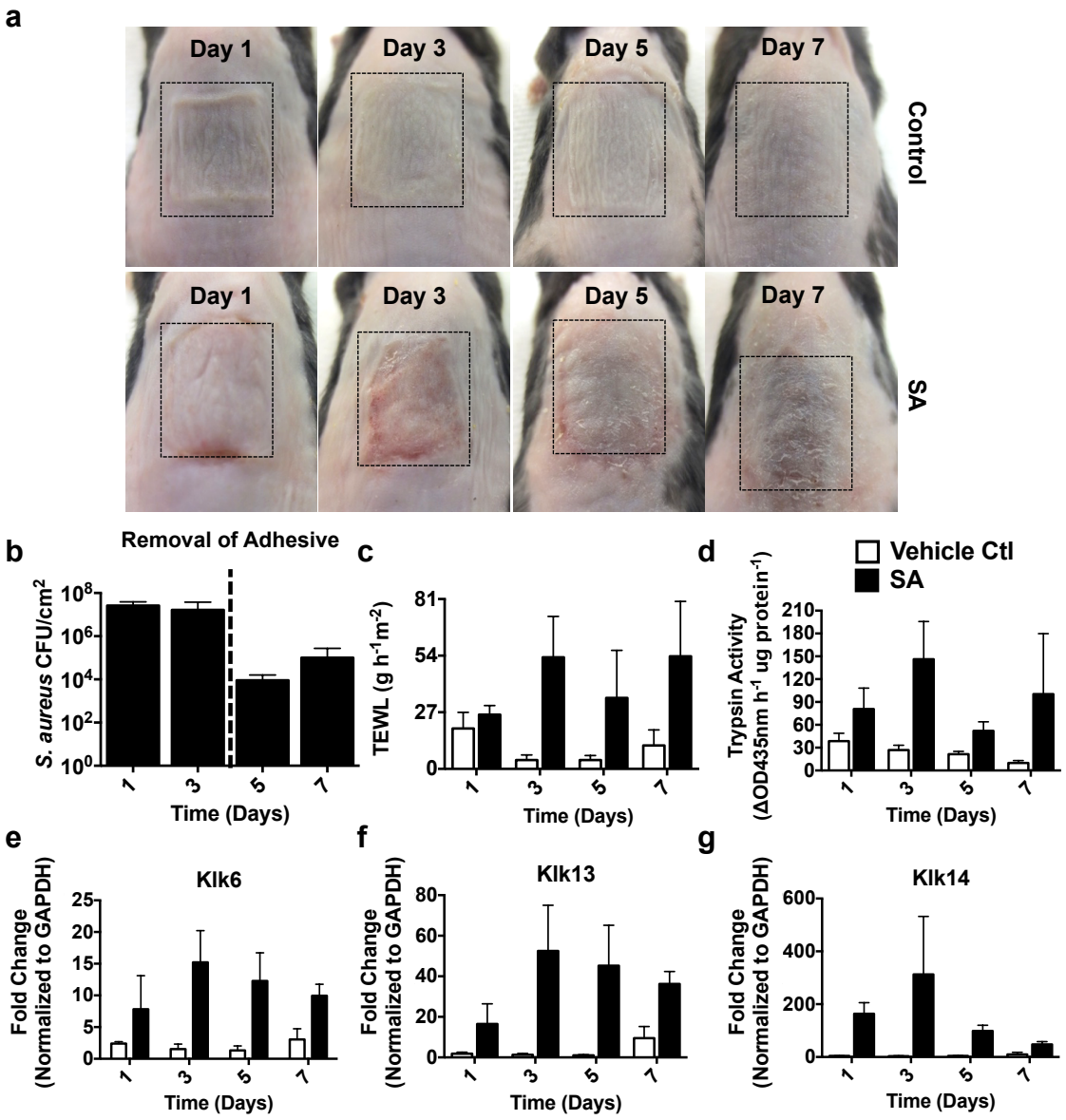

Figure 5.3. Epicutaneous *S. aureus* colonization of murine skin elevates trypsin activity and barrier damage for up to 7 days.

(a) Representative images of murine skin colonized with 1e7 CFU *S. aureus* USA300 (SA) for up to 7 days; black dashed lines represent treatment area. **(b)** SA CFU per cm^2 of skin recovered after days 1, 3, 5, and 7 (Dashed lines represents when protective adhesive was removed from skin). **(c-g)** Transepidermal water loss (TEWL), trypsin activity, and transcript levels of Klk6, Klk13, and Klk14 compared to the housekeeping gene Gapdh were assessed up to day 7 of treatment. Data represents mean ± SEM (n=3) and one-way ANOVA **(a)** was used with significance indicated by: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

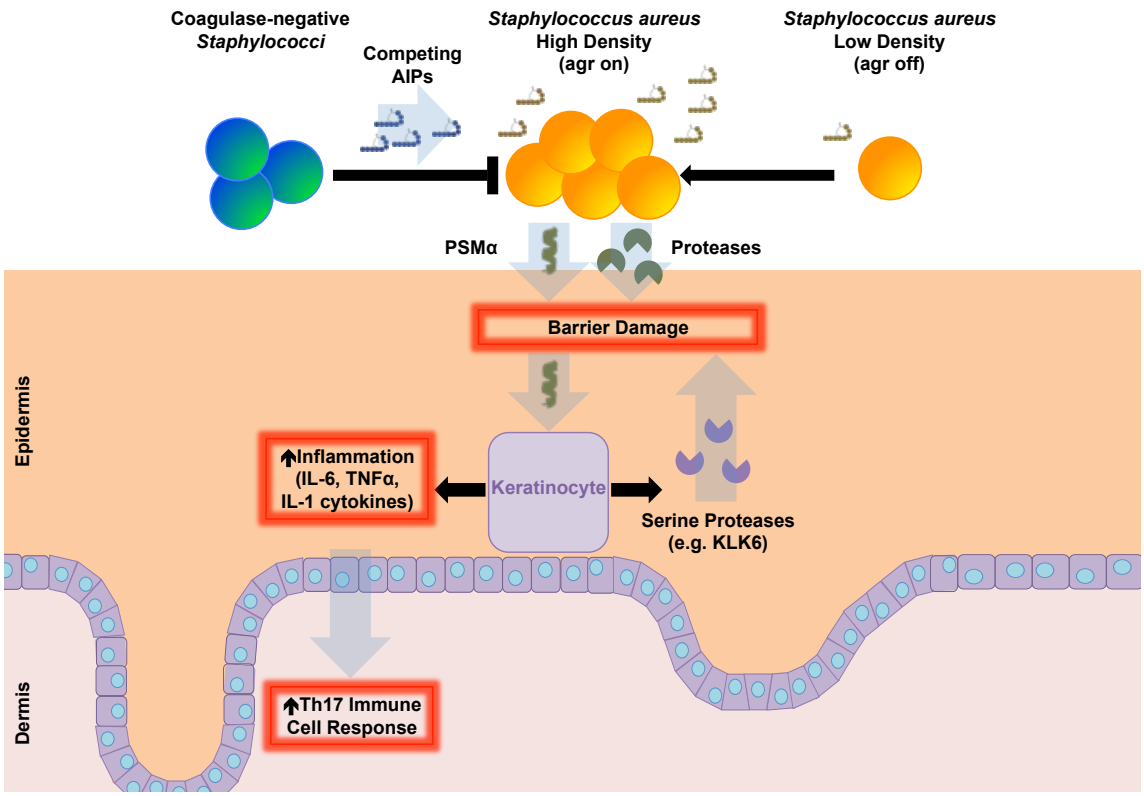

Figure 5.4. Overview of *S. aureus* effect on epidermal barrier homeostasis.
High abundance of *Staphylococcus aureus* (*S. aureus*) on the skin surface leads to activation of the agr quorum sensing system and secretion of virulence factors including phenol-soluble modulin α (PSMα) and secreted proteases. These virulence factors combine to damage the epidermal barrier and PSMα than can stimulate keratinocytes to secrete/express endogenous serine protease activity (e.g. Kallikrein 6) that can further damage the epidermal barrier. Both *S. aureus* PSMα and secreted proteases can induce pro-inflammatory cytokines and a Th17 immune response as well. Furthermore, clinical isolates of coagulase-negative *Staphylococci* species found on the skin can prevent *S. aureus* agr system activation via a cross talk mechanism with their own agr systems and secretion of small autoinducing peptides. Thus blocking *S. aureus* agr activation in diseases where *S. aureus* colonization is increased such as in atopic dermatitis, provides a novel therapeutic approach to combat *S. aureus* driven skin barrier damage.

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