UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Role of *Staphylococcus epidermidis* in Cutaneous Defense

A dissertation submitted in partial satisfaction of the
requirements for the degree of Doctor of Philosophy

in

Bioengineering

by

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2009

The dissertation of Anna Lynn Cogen is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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2009

DEDICATION

I dedicate this dissertation to my family and friends

for their continued support and love

throughout my education.

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LIST OF COMMON ABBREVIATIONS

ANTS	8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt
AMP	antimicrobial peptide
cat	chloamphenicol acetyl transferase
cd	circular dichroism
CFU	colony forming units
CRAMP	cathelicidin related antimicrobial peptide
DNase	deoxyribonuclease
DPX	*p*-xylene-bis-pyridinium bromide
ELISA	enzyme-linked immunosorbant assay
GAS	Group A *Streptococcus*
hBD	human β-defensin
HPLC	high pressure liquid chromatography
Kpi	potassium phosphate buffer
L. lactis	*Lactococcus lactis*
LL	*Lactococcus lactis*
mg	milligram
ml	milliliter
NETs	neutrophil extracellular traps
NHEK	normal human epidermal keratinocyte
P. acnes	*Propionibacterium acnes*
PA	*Propionibacterium acnes*
PBS	phosphate buffered saline
POPC	palmitoyl-oleoyl-phosphatidyl-choline
POPG	palmitoyl-oleoyl-phosphatidyl-glycerol
PSM	Phenol Soluble Modulin
RDA	radial diffusion assay
RT-PCR	real time polymerase chain reaction
S. aureus	*Staphylococcus aureus*
SA	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
SE	*Staphylococcus epidermidis*
THA	Todd-Hewitt agar
THB	Todd-Hewitt broth
TLR	Toll-like Receptor
TSA	Tryptic Soy agar
TSB	Tryptic Soy broth
μg	microgram
μl	microliter
WT	wild-type

ACKNOWLEDGEMENTS

The chapters of this dissertation represent a collective body of work with contributions from multiple scientists. Chapter I of this dissertation, the introductory chapter, is a reprint of the publication: Cogen AL, Nizet V, Gallo RL. Skin microbiota: A source of disease or defence? *British Journal of Dermatology*. March 2008: 158(3): 442-55, with permission from all co-authors. Chapter III was modified from the publication: Cogen AL, Yamasaki K, Sanchez KM, Dorschner RA, Lai Y, MacLeod DT, Torpey JW, Otto M, Nizet V, Kim JE, Gallo RL. Selective antimicrobial action is provided by Phenol Soluble Modulins derived from *Staphylococcus epidermidis*, a normal resident of the skin. Accepted to the *Journal of Investigative Dermatology*, with permission from all co-authors. Chapter IV was modified from the manuscript: Cogen AL, Yamasaki K, Muto J, Sanchez KM, Crotty Alexander L, Tanios J, Lai Y, Kim JE, Nizet V, Gallo RL. *Staphylococcus epidermidis* antimicrobial Phenol Soluble Modulins cooperate with host antimicrobial peptides to kill Group A *Streptococcus,* with permission from all co-authors.

***

Throughout the last 5 years, I have leaned on many people for their support and guidance. First, I would like to thank my advisor, Dr. Richard Gallo. Under his guidance, I learned to think independently and creatively. Dr. Gallo allowed me to

direct my project and explore new avenues that I found rewarding. I would also like to thank Dr. Victor Nizet, who was effectively my co-advisor, for introducing me to the Gallo laboratory and for his continued support and encouragement throughout the years. Thank you also to Dr. Judy Kim for her scientific contributions in the tryptophan spectroscopy studies.

In the Gallo lab, my co-workers have been wonderful and I am so grateful for their kindness, patience, and advice. In particular, I'd like to extend a special thank you to Anna Di Nardo and Kenshi Yamasaki. I cannot thank them enough for their friendship, generosity, and compassion both scientifically and on a personal level. I always looked forward to coffee breaks with Anna and the times when Kenshi would listen to my crazy ideas (sometimes over a glass of wine!). I would also like to thank the other members of the lab (past and present) who helped me with experiments, made me laugh, and accompanied me on trips to the coffee cart.

I have also been very lucky to be part of the Nizet lab, which has included fun times in Bay C, baseball games, happy hours, late nights in the fish lab, and a wonderful Nizet lab t-shirt for Christmas every year. The Nizet lab, like the Gallo lab, feels like an extended family. I'd like to thank all the lab members for teaching me so much about microbiology and molecular biology and I'd like to especially thank Jeff Locke for his friendship and help while making my knockout. I couldn't have done it without him.

Thank you also to the Kim lab members. Katie Sanchez taught me everything I know about tryptophan spectroscopy and was an incredible friend and collaborator throughout the years.

I'd like to thank my thesis committee: Amy Sung, Gabriel Silva, and Geert Schmid-Schönbein. I appreciate their willingness to meet with me on an individual basis and support my scientific endeavors.

On a more personal note, I'd like to acknowledge my friends in San Diego. The Brouha's, the chemistry crew, the happy hour girls (Morgan, Anjuli, and Laura), Farah and company, the fish lab (Jeff and Erica), my wonderful past roommates (Katie and Liz) and Dave: thank you all for your encouragement and friendship.

Finally, I'd like to thank my wonderful family for their love and support throughout my education. Thank you to my mother (Mary), father (Ken), stepmother (Caroline), and stepfather (Jerry) and sisters (Janie, Ellen, and Sylvia) for their love, understanding, confidence, and years of laugher. I have been blessed with an amazing family who will undoubtedly support my every endeavor.

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Patent

Anna L. Cogen, Victor Nizet, Richard L. Gallo
New antimicrobial to prevent skin infections. Patent application number: 60/890,683, Feb. 20, 2007

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Lai Y, Radek K, Park H, **Cogen A,** MacLeod DT, Ryan AF, Di Nardo A, Gallo RL. *Staphylococcus epidermidis* stimulates increased β-defensin expression in keratinocytes and enhanced antimicrobial function in the skin through activation of TLR2 and P38 MAPK. Society of Investigative Dermatology meeting, Montreal, Quebec, Canada. Poster.

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ABSTRACT OF THE DISSERTATION

The Role of *Staphylococcus epidermidis* in Cutaneous Defense

by

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Doctor of Philosophy in Bioengineering
University of California, San Diego, 2009

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Professor Richard L. Gallo, Co-Chair

The skin provides a protective niche and is home to a variety of microorganisms. Here, we hypothesize that the cutaneous resident microbe *Staphylococcus epidermidis* (*S. epidermidis*) plays a beneficial role by contributing to the skin's innate immune system.

We investigated the ability of *S. epidermidis* to provide an antimicrobial barrier. Our data demonstrate that *S. epidermidis* inhibits the pathogen Group A *Streptococcus* (GAS). Purification and sequencing identified 2 putative antimicrobial peptides (AMPs): Phenol Soluble Modulin (PSM)-γ and Phenol Soluble Modulin-δ. We found that both were α-helical, membrane-active, and formed complexes. Furthermore, the PSMs exerted selective antimicrobial activity on pathogens. Finally, the PSMs reduced the survival of GAS on explants. These studies illustrate that the

PSMs function as AMPs.

As *S. epidermidis* lives on the epidermis, the interaction of PSMγ and the host was evaluated. Immunostaining revealed deposition of PSMγ in human skin. PSMγ induced and bound neutrophils extracellular traps (NETs). In addition, PSMγ cooperated with host AMPs. Also, PSMγ rendered GAS bacteriostatic in blood and increased NET killing. Similarly, GAS survival in mouse wounds was reduced by pretreatment of wounds with PSMγ. To this end, PSMγ cooperates with the host AMPs to ward off bacterial infections.

To elucidate the components of PSMγ required for its antimicrobial properties, PSMγ analogs were investigated. Analogs included mutations in the c-terminus, n-terminus, helix disruption, polarity disruption, and charge neutralization. Although all analogs lost the ability to form complexes, only the c-terminus, polarity and charge neutralization analogs showed reduced membrane binding, vesicle leakage, and bacterial killing. Overall, these data demonstrate that the c-terminus, polarity and charge are critical for PSMγ's antimicrobial activity.

Finally, we evaluated the ability of the PSMs to induce AMPs in keratinocytes. Both PSMs induced hBD2 and hBD3 with synergy occurring with the toll-like receptor 2 agonists. Thus, PSMs increase the production of endogenous antimicrobial peptides.

As a whole, this work demonstrates the beneficial relationship that *S. epidermidis* has with the host. A deeper understanding of bacteria:host mutualism can lead to a greater understanding of the innate immune system as well as providing novel therapeutic strategies for the treatment of disease.

Chapter I.

Introduction:
Skin Microbiota: A source of disease or defense?

ABSTRACT

Microbes found on the skin are usually regarded as pathogens, potential pathogens, or unimportant symbiotic organisms. Advances in microbiology and immunology are revising our understanding of the molecular mechanisms of microbial virulence and the specific events involved in the host-microbe interaction. Current data contradict some historical classifications of cutaneous microbiota and suggest that these organisms may protect the host, defining them not as simple symbiotic microbes but rather as mutualistic. This review will summarize current information on bacterial skin flora including *Staphylococcus*, *Corynebacterium*, *Propionibacterium, Streptococcus,* and *Pseudomonas.* Specifically, the review will discuss our current understanding of the cutaneous microbiota as well as shifting paradigms in the interpretation of the role microbes play in skin health and disease.

INTRODUCTION: DOES THE HYGIENE HYPOTHESIS APPLY TO THE SKIN?

Most scholarly reviews of skin microbiota concentrate on understanding the population structure of the flora inhabiting the skin or how a subset of these microbes can become human pathogens. In the last decade, interdisciplinary collaborations at the interface of microbiology and immunology have greatly advanced our understanding of the host:symbiont and host:pathogen relationships. The goal of the present review is to summarize current information on bacterial skin flora with special emphasis on new concepts that go beyond the narrow perception of these organisms as potential agents of disease. Today, we are in a position to understand how the delicate balance of defenses provided by microbes, skin barrier formation, and innate immunity combine to maintain healthy skin, and explain the pathophysiologic mechanisms underlying a number of cutaneous infectious and inflammatory diseases.

Several studies on non-cutaneous epithelial surfaces have revealed the significance of microflora to the host innate immune system (Figure 1.1). Examples include of the importance of the indigenous microbiota on expansion and maintenance of the CD8 memory T cells in the lung.[1] Gut microflora maintain homeostasis and prevent ailments including inflammatory bowel disease.[2] *Lactobacilli* in the intestine are implicated in education of prenatal immune responses. Several studies have also shown that a disruption in microbial exposure early in development leads to allergic disease.[3,4]

These observations have lent support to the "hygiene hypothesis," which stipulates that exposure of T regulatory cells, T(regs), to intestinal microbes generates a mature immune response that decreases reactions to self-antigens, as well as harmless antigens from non-pathogenic microbes.[5] The beneficial effect of microbiota in the gut has substantiated the use of probiotics. Probiotics such as *Lactobacillus acidophilus* secrete antibacterial substances and can prevent adhesion and invasion of enteroinvasive pathogens in experimental models such as cultured intestinal Caco-2 cells.[6-8] Furthermore, oral administration of various probiotics has been associated with reduced colorectal cancer and active ulcerative colitis in some clinical studies.[9,10] Although these approaches remain controversial, the benefits of resident gut microbiota are being explored through a variety of trial therapeutics and disease prevention measures.

An incomplete understanding of the potential benefits of the cutaneous microflora stems from a poor understanding of their fundamental biology and the limited research efforts directed towards this goal to date. Existing clinical studies have provided invaluable information about the abundance and types of microbes on the skin, but fail to address their functions.[11-15] In light of the symbiotic relationships of microbial mutualism and commensalism that have been demonstrated as critical to human health in studies of gut microbiota, there is a need to expand this research in skin.

The potential symbiosis between skin flora and the host falls into one of three categories: parasitism, commensalism, or mutualism (Figure 1.2). Commonly, a symbiotic relationship is understood as one in which both organisms benefit each other. This perception is not correct. Symbiotic relationships can exist in which only one organism benefits while the other is harmed (parasitism, predation, ammensalism, and competition), one organism benefits and no harm occurs to the other (commensalism), or both find benefit (mutualism and protocooperation). Microbes found on the surface of the skin that are only very infrequently associated with disease are typically referred to as commensal. This term implies that the microbe lives in peaceful coexistence with the host while benefiting from the sheltered ecological niche. An example of such a microbe is the Gram-positive bacterium *Staphylococcus epidermidis.* This species and other so-called skin commensals may play an active role in host defense, such that they may represent, in fact, mutuals. One must recognize, however, that distinct categorizations such as parasitic, commensalistic, or mutualistic may be oversimplified since the same microbe may function in different roles at different times. Understanding this, and the elements that dictate of the type of microbe-host symbiosis, could lead to effective treatment and prevention strategies against skin infection.

It is also important to recognize that the distinction between what we consider to be harmless flora vs. pathogenic agent often lies in the skin's capacity to resist infection, and not the inherent properties of the microbe. Host cutaneous defense occurs through the combined action of a large variety of complementary systems.

These include the physical barrier, a hostile surface pH, and the active synthesis of gene-encoded host defense molecules such as antimicrobial peptides, proteases, lysozymes, and cytokines and chemokines that serve as activators of the cellular and adaptive immune response. Virulence factors expressed by the microbe may enable it to avoid the host defense program, but it is ultimately the effectiveness of this host response that determines if a microbe is a commensal (or mutual) organism or a dangerous pathogen for the host.

In the following review we will focus on the microbe to illustrate for the reader what is currently understood about cutaneous microflora as pathogens and what can be hypothesized about these microbes as potential mutuals. The resident microflora on the skin includes bacteria, viruses and many types of fungi. To limit and focus the discussion, we will concentrate specifically on bacteria. The number of bacteria identified from human skin has expanded significantly, and will likely continue to increase in number as genotyping techniques advance.[11,12] Some of the best-studied long-term and transient bacterial residents isolated from the skin include those from the genus' *Staphylococcus*, *Corynebacterium*, *Propionibacterium, Streptococcus,* and *Pseudomonas* and therefore these will be described in greatest detail (Table 1.1). Unfortunately, little is known about many of the other bacterial species on skin due to their low abundance and apparent harmlessness.[11]

Staphylococcus epidermidis

Staphylococcus epidermidis (*S. epidermidis*), the most common clinical isolate of the cutaneous microbiota, is a Gram-positive coccus found in clusters. As a major inhabitant of the skin and mucosa it is thought that *S. epidermidis* comprises greater than 90% of the aerobic resident flora. Small white or beige colonies (1-2mm in diameter), desferrioxamine sensitivity, lack of trehalose production from acid, and coagulase-negative characteristics easily distinguish *S. epidermidis* from other bacteria in the same genus.

Despite its generally innocuous nature, over the past 20 years, *S. epidermidis* has emerged as a frequent cause of nosocomial infections. Several extrinsic factors contribute to the conversion of *S. epidermidis* from a resident microflora to an infectious agent. The bacteria primarily infect compromised patients including drug abusers, those on immunosuppressive therapy, AIDS patients, neonates (premature newborns), and patients with an indwelling device.[16] The major port of entry for these infections are foreign bodies such catheters and implants.[17] After entry, virulent strains of *S. epidermidis* form biofilms that partially shield the dividing bacteria from the host's immune system and exogenous antibiotics. Once systemic, *S. epidermidis* can cause sepsis, native valve endocarditis, or other conditions that are often subacute or chronic.[18,19] A major complicating factor in the management of *S. epidermidis* blood infections is the inadequacy of many common antibiotic treatments. Biofilm formation reduces the access of antibiotics to the bacteria and often necessitates the removal of indwelling devices.[20]

In addition to catheter infections, patients with necrotic tumor masses from ulcerated advanced squamous cell carcinomas, head and neck carcinomas, breast carcinomas, and sarcomas have a high propensity for infection by *S. epidermidis*.[21] Also, myelosuppressive chemotherapy renders patients neutropenic, thereby increasing the risk of septicemia. As abscesses infrequently form in neutropenic patients, *S. epidermidis* infections present as spreading cellulitis, associated with septicemia.[22] A recent case study also reported that methicillin-resistant *S. epidermidis* (MRSE) was responsible for acute febrile neutrophilic dermatosis, or Sweet's syndrome (SS), in an immuno-compromised patient with neutropenia from myelodyplastic syndrome (MDS).[23] These specific skin infections caused by *S. epidermidis* require a predisposed host and do not reflect the typical bacterial-host interaction. In fact, *S. epidermidis* resides benignly, if not as a mutual on the skin's surface, with infection arising only in conjunction with specific host predisposition.

Medical treatments for *S. epidermidis* infection range from systemic antibiotics to device modification and removal. Current research suggests that bacterial attachment to materials is dependent on the physico-chemical properties of the bacterial surface and the plastic.[24-26] In particular, *S. epidermidis* has been shown to adhere to highly hydrophobic surfaces, while detergent-like substances and electric currents reduce attachment to the surfaces of the prosthetics or catheters. [26,27] The autolysin protein AtlE, which possess a vitronectin-binding domain, has been identified as likely attachment factor. When the *altE* gene is disrupted, the resulting *S. epidermidis* mutant exhibits reduced surface hydrophobicity and impaired attachment

to a polystyrene surface. [28] Other adhesion factors include MSCRAMM (Microbial Surface Components Recognizing Adhesive Matrix Molecules) and fibrinogen-binding protein, Fbe, which may be involved in attachment of the peptidoglycans in the bacterial cell wall to a plastic surface.[29,30] Several other proposed proteins that may be involved in attachment to plastic-coated materials include Aas1, Aas2, SdrF, and AAP (accumulation-associated protein). [31,32]

Increased virulence of *S. epidermidis* has also been attributed to a process known as intercellular adhesion (Figure 1.3). Once the bacteria have gained entry, through a catheter for example, *S. epidermidis* produces factors responsible for growth, immune evasion, and adhesion. In particular, PIA (polysaccharide intercellular adhesion) and PNSG (poly-*N*-succinyl-glucosamine), both encoded by the *ica* locus, mediate intercellular adhesion and have been implicated in virulence.[33,34] Only a fraction of the *S. epidermidis* strains contain these genes, with the majority of the positive strains isolated from catheter infections and not from healthy skin.[35] Other virulence factors are regulated by the *agr* (accessory gene regulator), *sar*, and *sigB* loci. In a complex regulatory system, these three loci are involved in quorum-sensing and potentially biofilm (slime capsule) formation.[24,36] The understanding and inhibition of biofilms is of great interest and may increase the effectiveness of antibiotics against *S. epidermidis* catheter infections or sepsis. In addition, anti-PIA antibodies are being investigated in biofilm formation prevention.[37] Interferon-γ therapy in addition to antibodies against specific *S. epidermidis* surface binding proteins have also been proven effective in preventing catheter adhesion.[38]

Despite focused investigation of *S. epidermidis* as a pathogen, the bacterium normally exists as an inoffensive member of the cutaneous microflora. Recent studies can be interpreted to suggest that *S. epidermidis* is a mutualistic organism, much like the bacteria of the gut. Many strains of *S. epidermidis* produce lantibiotics, which are lanthionine-containing antibacterial peptides, also known as bacteriocins (Figure 1.3). Among the several identified bacteriocins are epidermin, epilancin K7, epilancin 15X, Pep5, and staphylococcin 1580.[39-41] Additional antimicrobial peptides on the surface of the skin have recently been identified as originating from *S epidermidis*.[42] These bacteriocins appear to play a role in intra-species competition on the skin's surface. Although *S. epidermidis* rarely damages the keratinocytes in the epidermis, the bacteria produce peptides toxic to other organisms, such as *Staphylococcus aureus (S. aureus)* and Group A *Streptococcus* (GAS, *Streptococcus pyogenes*). The host epidermis permits *S. epidermidis* growth since the bacterium may provide and added level of protection against certain common pathogens, making the host:bacterium relationship one of mutualism. Protection afforded by *S. epidermidis* is further demonstrated in recent studies on pheromone cross inhibition. The *agr* locus produces modified peptide pheromones, which subsequently affect the *agr* systems of various species by activating self and inhibiting non-self *agr* loci.[43,44] The activation of *agr* signals to the bacterium that an appropriate density is reached and leads to a down-regulation of virulence factors.[45] Quorum-sensing decreases colonization-promoting factors and increases pheromones such as the phenol soluble modulin γ (δ-haemolysin, δ-toxin, δ-lysin).[43] These pheromones affect the *agr* signaling of competing bacteria

(such as *S. aureus*) and ultimately lead to colonization inhibition.[44] Pheromones are being investigated for their therapeutic potential, such as δ-toxin, which reduces *S. aureus* attachment to polymer surfaces.[46]

Finally, *S. epidermidis* promotes the integrity of cutaneous defense through elicitation of host immune responses. Our own preliminary data suggests that *S. epidermidis* plays an additional protective role by influencing the innate-immune response of keratinocytes through Toll-like receptor (TLR) signaling (Figure 1.3). TLRs are pattern recognition receptors (PRRs) that specifically recognize molecules produced from pathogens collectively known as pathogen-associated molecular patterns (PAMPs).

In this light, *S. epidermidis* may be thought of as a mutual, thus, adding to the human innate immune system. Therefore, maintaining this type of mutualistic cutaneous microflora may prevent infections while understanding this interaction may advance our understanding of cutaneous diseases.

Staphylococcus aureus

Characterized by circular, golden-yellow colonies, and β-hemolysis of blood agar, the coagulase-positive *Staphylococcus aureus* (*S. aureus*) is a leading human pathogen.

S. aureus clinical disease ranges from minor and self-limited skin infections to invasive and life threatening diseases. *S. aureus* skin infections include impetigo, folliculitis, furuncles, and subcutaneous abscesses, and through the production of exfoliative toxins, staphylococcal scalded skin syndrome (SSSS).[47] The bacterium can also cause serious invasive infections such septic arthritis, osteomyelitis, pneumonia, meningitis, septicemia and endocarditis.[47-49] Elaboration of superantigen toxins can trigger staphylococcal toxic shock syndrome.

Particular conditions predispose the skin to *S. aureus* infections, such as atopic dermatitis (AD).[50,51] While viruses (ex. herpes simplex type 1 virus and human papillomavirus) and fungi (ex. *Trichophyton rubrum*) also opportunistically infect lesional and nonlesional AD skin, *S. aureus* is the by far the most common superinfecting agent.[50] Like *S. epidermidis, S. aureus* is a frequent cause of infection in catheterized patients.[52]

At present, *S. aureus* infections are treated with antibiotics and with the removal of infected implants as necessary.[53] Unfortunately, there has been a dramatic rise in antibiotic resistant strains, including methicillin-resistant *S. aureus* (MRSA) in both hospital and community settings, and even documented reports of vancomycin-intermediate and vancomycin-resistant S. aureus strains (VISA and VRSA).[48,54]

The emergence of methicillin resistance is due to the acquisition of a transferable DNA element called Staphylococcal Cassette Chromosome *mec*

(SCC*mec*), a cassette (types I-V) carrying the *mec*A gene, encoding the protein PBP2a, penicillin binding protein 2a.[55-57] Through site-specific recombination, the DNA element integrates into the genome. Normally, β-lactam antibiotics bind to the PBPs in the cell wall, disrupt peptidoglycan layer synthesis and kill the bacterium. However, β-lactam antibiotics cannot bind to PBP2a, allowing bacterium containing the *mec*A gene survive β-lactam killing.[57] Plasmids have also been found to confer *Staphylococcus* resistance to kanamycin, tobramycin, bleomycin, tetracycline, and vancomycin.[58,59]

S. aureus expresses many virulence factors, both secreted and cell-surface associated, that contribute to evasion (Figure 1.3). *S. aureus* secretes the chemotaxis inhibitory protein of staphylococci (CHIPS) which binds to the formyl peptide receptor and C5a receptor on neutrophils, thereby interfering with neutrophil chemotaxis.[60] Eap (also called major histocompatibility class II analog protein Map) adheres to ICAM-1 on neutrophils, and prevents leukocytes adhesion and extravasion.[61] *S. aureus* also secretes an arsenal of toxins that damage host cells. Such toxins include superantigens (Enterotoxins A through E, Toxic shock syndrome toxin-1 (TSST-1), ETs-ETA, B, and D) and cytoxins (α-, β-, δ-, γ-haemolysin, Panton-Valentine Leukocidin, Leukocidin E-D, *S. aureus* exotoxin).[47,48,62] Extracellular enzymes secreted by *S. aureus* that may contribute to tissue damage include proteases, lipases, hyaluronidase, and collagenase.[48,63] *S. aureus* α-hemolysis secretion leads to pore formation in target cell membranes and subsequent activation of NF-κB inflammatory pathway.[64]

S. aureus is relatively resistant to killing by cationic antimicrobial peptides produced by host epithelial cell and phagocytes. One key underlying mechanism for this resistance involves alterations in the charge of the bacterial cell surface. The Dlt protein causes D-alanine substitutions in the ribitol techoic acids and lipoteichoic acids of the cell wall, slightly neutralizing the negatively charged cell surface to which cationic peptides usually bind.[65,66] The MprF enyme adds L-lysine to phosphatidyl glycerol, similarly neutralizing the negatively charged cell surface.[67] Mutants with defects in Dlt and MprF have been shown to be markedly more susceptible to human defensins.[65,68] *S. aureus'* staphylokinase binds and protects against defensins, while aureolysin cleaves human cathelicidin LL-37, offering further protection.[69,70]

S. aureus resists phagocyte killing at a number of different levels. Effective opsonization of the bacteria is inhibited by its polysaccharide capsule and the surface expressed clumping factor and protein A. The eponymous golden carotenoid pigment protects *S. aureus* against neutrophil killing *in vitro* by scavenging oxygen free radicals.[71]

Despite the usual classification of *S. aureus* as a transient pathogen, it may be better considered a normal component of the nasal microflora.[72,73] It is estimated that 86.9 million people (32.4% of the population) are colonized with *S. aureus*.[74] Other studies have suggested that among the population, 20% are persistently colonized, 60% of the population intermittently carry the bacteria, and 20% are never

colonized.[72] Colonization by *S. aureus* is certainly not synonymous with infection. Indeed, like *S. epidermidis*, healthy individuals rarely contract invasive infections caused by *S. aureus*.[57] *S. aureus* found on healthy human skin and in nasal passages are in effect acting as a commensal, rather than a pathogen. Certain strains of *S. aureus* have been shown to produce bacteriocins such as staphylococcin 462, a peptide responsible for growth inhibition of other *S. aureus* strains.[75] Since *S. aureus* has generally been regarded as a pathogen, little is known about its potential symbiotic effects.

Corynebacteria diphtheriae

*Coryneform*s are Gram-positive, non-motile, facultative anaerobic actinobacteria. These common members of the skin flora are divided into two species: *Corynebacterium diphtheriae* (*C. diphtheriae*) and nondiphtheriae Corynebacteria (diphtheriods). *C. diphtheriae* is categorized by biotype: *gravis*, *mitis*, *belfanti*, and *intermedius*, as defined by colony morphology and biochemical tests. *C. diphtheriae* is further divided into toxigenic and nontoxigenic strains. Toxinogenic *C. diphtheriae* produce the highly lethal diphtheria toxin, which can induce fatal global toxemia. Nontoxinogenic (nontoxin-producing) *C. diphtheriae* are capable of producing septicemia, septic arthritis, endocarditis, and osteomyelitis.[76-78] Both, nontoxigenic and toxigenic *C. diphtheriae* can be isolated from cutaneous ulcers of alcoholics, intravenous drug users and from hosts with poor hygiene standards, including endemic outbreaks in areas of low socioeconomic status.[79,80] Although immunization has successfully reduced the prevalence of diphtheria in most developed countries, the

disease has surfaced in individuals impacted by socioeconomic deprivation, as well as non-immunized and partially immunized individuals.[81]

C. diphtheriae virulence is mainly attributed to diphtheria toxin, a 62 kDa exotoxin. The crystal structure shows a disulfide-linked dimer with a catalytic, transmembrane, and receptor-binding domain.[82] Invasion of the exotoxin is a complex series of events that involves translocation into the cytosol and results in halted protein synthesis.

Corynebacteria jeikeium

The nondiphtheriae *Corynebacteria*, diphtheriods, are a diverse group, containing 17 different species, of which not all are present on human skin. Several species commonly colonize cattle, while others, such as *C. jeikeium* (formerly known as CDC group JK), are normal inhabitants of the human skin. Although many diphtheroids are found on human skin, *C. jeikeium* is the most frequently recovered and medically relevant member of the group. *C. jeikeium* is a pleomorphic, nonspore-forming, lipophilic, non-motile, Gram-positive rod.

In the last few years, *Corynebacteria* diphtheroids have gained interest due to the increasing number of publications on nosocomial infections. *C. jeikeium* causes infections in immune compromised patients, in conjunction with underlying malignancies, on implanted medical devices, and in skin barrier defects.[83] In addition, *C. jeikeium* has been suggested as the cause of papular eruption with histological

features of botryomycosis.[84] Once the bacterium has penetrated the skin's barrier, the bacterium can cause sepsis or endocarditis.[85]

C. jeikeium treatment varies from other Gram-positive organisms because it is resistant to multiple antibiotics. However it remains sensitive glycopeptides including vancomycin or teicoplanin. *C. jeikeium* antibiotic resistance stems from a variety of factors, ranging from the acquisition of antibiotic resistance genes to the polyketide synthesis of fadD enzymes and subsequent corynomycolic acid in the cell envelope. Iron and manganese acquisition by *C. jeikeium* may contribute to virulence. Sidephores produced by the bacterium allow for efficient iron sequestration in the host. Manganeses acquisition inhibits Mg-dependent superoxide dismutase, protecting the bacterium from superoxide production by the host or competing bacteria.[86] The C. *jeikeium* genome sequence reveals numerous putative proteins with homology to adhesion and invasion factors from other Gram-positive pathogens.[87] These include SurA and SurB (surface proteins similar to those of Group A *Streptococcus* and Group B *Streptococcus*), Sap proteins (surface anchored proteins resemble *C. diphtheriae* factors used in pili formation), CbpA protein (belongs to MSCRAMM family), and NanA protein (similar to neuraminidases from *Streptococcus pneumoniae*).[88-91]

C. jeikieum is considered part of the normal skin flora, similar to *S. epidermidis*. This bacterium species resides on most humans' skin and is commonly cultured from hospitalized patients.[83,92] In particular, colonization is seen in axillary, inguinal, and perineal areas.[93] Almost all infections caused by *C. jeikeium* are

nosocomial and in patients with pre-existing ailments. As with *S. epidermidis*, *C. jeikeium* is ubiquitous and largely innocuous, illustrating that the bacterium is commensal. In fact, *C. jeikeium* may offer epidermal protection, bolstering the argument that cutaneous microflora are mutualistic. Manganese acquisition effectively allows the bacteria to safeguard themselves from superoxide radicals. The enzyme superoxide dismutase may also function to prevent oxidative damage to epidermal tissue, a potential means by which bacteria protect the host. Moreover, iron and manganese are critical for organism survival, both pathogenic and non pathogenic. The act of scavenging these elements may prevent colonization by other microbes. Finally, *C. jeikeium* produces bacteriocin-like compounds used to ward off potential pathogens and competitors. Nisin, produced by *Lactococcus lactis,* and used as a food preservative, has a 66% homology to AucA, a hypothetical protein encoded in the *C. jeikeium* plasmid pA501.[94] Most likely, *C. jeikeium* produces other bacteriocins not yet identified. Since the study of virulence factors dominate the fields of microbiology and infectious disease, little is known about the potential mutualism of *C. jeikeium*. Given the prevalence of skin colonization, the relative rarity of *C. jeikeium* pathogenesis, and the yet unexplored benefits of the bacterium, *C. jeikeium* likely lives mutually on the skin.

Propionibacterium acnes

Commonly touted as the cause of acne vulgaris, *Propionibacterium acnes (P. acnes*) is an aerotolerant anaerobic, Gram-positive bacillus that produces propionic acid, as a metabolic byproduct. This bacterium resides in the sebaceous glands, derives

energy from the fatty acids of the sebum, and is susceptible to UV-light due to the presence of endogenous porphyrins.[95]

P. acnes is implicated in a variety of manifestations such as folliculitis, sarcoidosis and systemic infections resulting in endocarditis.[96,97] Occasionally, P. acnes causes SAPHO syndrome (synovitis, acne, pustulosis, hyperostosis, and osteitis), a chronic, inflammatory, systemic infection.[98] In the sebaceous gland, *P. acnes* produces free fatty acids as a result of triglyceride metabolism. These byproducts can irritate the follicular wall and induce inflammation through neutrophil chemotaxis to the site of residence.[99] Inflammation due to host tissue damage or production of immunogenic factors by *P. acnes* subsequently leads to cutaneous infections (Figure 1.4).[100,101]

The most well known ailment associated with *P. acnes* is the skin condition known as acne vulgaris, afflicting up to 80% of adolescents in the United States.[102] Several factors are thought to contribute to an individual's susceptibility. Androgens, medications (including steroids and oral contraceptives), the keratinization pattern of the hair follicle, stress, and genetic factors all contribute to acne predisposition.[103,104] Clinically, patients present with distended, inflamed, or scarred pilosebaceous units. Non-inflammatory acne lesions form either open or closed comedones, while inflammatory acne lesions develop into papules, pustules, nodules, or cysts.

Like *S. epidermidis*, *P. acnes* causes many post-operative infections. Prosthetic joints, catheters, and heart valves transport the cutaneous microflora into the body.[105] Sepsis and endocarditis result from systemic infections.[106] Another common port of entry for *P. acnes* is through ocular injury or operation. *P. acnes* causes endophthalmitis (inflammation of the interior of the eye causing blindness) weeks or months after trauma or eye surgery. The infection delay likely results from the low-virulence phenotype of *P. acnes*.[107]

Treatment for *P. acnes* infections varies depending on the presentation of disease. For acne, a variety of medications and prevention strategies are currently employed. Benzoyl peroxide and topical antibiotics are bactericidal and bacteriostatic, respectively, against *P. acnes* infections. Topical retinoids such as tretinoin and adapalene reduce inflammation of follicular keratinocytes and may interfere with Toll-like receptor 2 (TLR2) and *P. acnes* interactions.[108] A regiment of oral antibiotics is given to individuals with moderate acne. In addition to reducing the number of *P. acnes* on the skin, antibiotics provide an anti-inflammatory effect.[109] Oral isotretinoin, a compound related to retinol (vitamin A), is currently the only treatment that leads to permanent remission.[110] The cutaneous effects of isotretinoin and other vitamin A derivatives are currently being researched. Rare systemic infections, including endocarditis, that can develop postoperatively or in immune compromised patients, have been treated with effectively penicillin or vancomycin.[111-113]

Proposed *P. acnes* virulence factors include enzymes that aid in adherence and colonization of the follicle. In particular, hyaluronate lyase degrades hyaluronan in the extracellular matrix, potentially contributing to adherence and invasion.[114] The genome of *P. acnes* also encodes sialidases and endoglycoceramidases putatively involved in host-tissue degradation virulence.[102] *P. acnes* also produces biofilms, limiting antibiotic access to the site of infection.[99]

Studies have shown that Toll-like receptors (TLRs) play an important role in inflammation associated with *P. acnes* infection. *P. acnes* induces expression of TLR2 and TLR4 in keratinocytes;[115] and the bacterium can induce IL-6 release from TLR1-/-, TLR6-/-, and wild-type murine macrophages but not from TLR2-/- murine macrophages.[116] These combined data show that *P. acnes* interact with TLR2 to induce cell activation. *P. acnes* infection also stimulate production of pro-inflammatory cytokines such as IL-8 (involved in neutrophil chemotaxis), TNF-α, IL-1β, and IL-12.[117,118]

The major factors contributing to acne is the hypercornification of the outer root sheath and the pilosebaceous duct, increased sebum production and potentially, the overgrowth of *P. acnes*. Some have suggested that *P. acnes* involvement in inflammation is relatively minor and the abnormal bacterial growth in the sebaceous ducts may be a side effect of inflammation rather than a root cause (Figure 4). Although the bacterium is commonly associated with acne pathogenesis, healthy and acne prone patients alike are colonized.[11] Studies have also shown that antibiotics

primarily reduce inflammation and only secondarily inhibit *P. acnes* growth.[109] These data suggest that *P. acnes* has a low pathogenic potential with a minor role in the development of acne. The prevalence of *P. acnes* on healthy skin suggests a relationship of commensalism or mutualism rather than parasitism.

Together, the avirulence of *P. acnes* and the studies showing a beneficial effect on the host, suggest that the bacterium is mutualistic. In one study, mice, immunized with heat-killed *P. acnes* and subsequently challenged with LPS, showed increased TLR4 sensitivity and MD-2 up-regulation.[119] The authors suggested that the hyper-elevated cytokine levels indicated a detrimental effect by *P. acnes in vivo*. Alternatively, the data may suggest that *P. acnes* enables host cells to respond effectively to a pathogenic insult, in which case, *P. acnes* would serve a protective role. It is probable that a similar response could be seen with injections of other types of bacteria but these results serve to highlight potential mechanism for mutualism. *Propionibacteria* have also been shown to produce bacteriocins, or bacteriocin-like compounds. These include propionicin PLG-1, jenseniin G, propionicin SM1, SM2, T1,[120,121] and acnecin,[122] with activity against several strains of propionibacterium, several lactic acid bacteria, some Gram-negative bacteria, yeasts, and molds. Little is known about the production or role of bacteriocins in *P. acnes* oral or cutaneous survival. These bacteriocins may potentially secure the pilosebaceous niche and protect the duct from other pathogenic inhabitants. The supply of nutrient-rich sebum in exchange for protection against other microbes may be one mechanism by which *P. acnes* acts mutualistically.

Group A *Streptococcus* (GAS, *Streptococcus pyogenes*)

Known for causing superficial infections as well as invasive diseases, Group A *Streptococcus,* or GAS, forms chains of Gram-positive cocci. The bacterium is β-hemolytic on blood agar and catalase-negative. GAS are further sub-classified by their M protein and T antigen serotype.

The types of M protein and T antigen expressed indicate the strain's potential to cause superficial or invasive disease. GAS infections are diverse in their presentation, with 'strep throat', a mucosal infection, or impetigo of the skin being most common. Superficial GAS infections differ with age and cutaneous morphology. Non-bullous impetigo (pyoderma) prevails in infants and children. The post-infectious non-pyogenic syndromes rheumatic fever can follow throat infection and poststreptococcal glomerulonephritis can follow either skin or throat infection.[123] GAS is also associated with deeper-seated skin infections such as cellulitis (clostridial, nonclostridial, or synergistic necrotizing) and erysipelas, infections of connective tissue and underlying adipose tissue, respectively. These types of disease occur frequently in the elderly and in individuals residing in densely populated areas.[124] Bacterial infections generally occur in association with diabetes, alcoholism, immune deficiency, skin ulcers, and trauma. The invasive necrotizing fasciitis, or "flesh-eating" disease, carries a high degree of morbidity and mortality and is frequently complicated by streptococcal toxic shock syndrome (STSS). GAS can also cause

infections in many other organs including lung, bone and joint, muscle and heart valve, essentially mimicking the disease spectrum of *S. aureus*.

GAS disease treatment depends on location, severity, and type of infection. Superficial infections such as impetigo are easily eradicated with topical antibacterial ointments such as Bactroban (mupirocin) or Fusidic acid (fucidin). More extensive skin infections are treated with oral antibiotics such as penicillin, erythromycin, or clindamycin.[125] Invasive infections require systemic antibiotics and intensive support; surgical debridement of devitalized tissue is critical to management of necrotizing fasciitis.[126]

For the most part, GAS is sensitive to β-lactams (penicillin), but in severe infections the antibiotic fails due to large inoculum of bacteria and GAS' ability to down-regulate penicillin binding proteins during stationary growth phase.[127] In severe systemic infections, adjunctive therapy with intravenous gammaglobulin may provide neutralizing antibodies against streptococcal superantigens to prevent development of STSS.[128]

GAS is capable of subverting the host immune response in a variety of ways. Inhibiting phagocyte recruitment, GAS expresses the proteases ScpC, or SpyCEP, that cleave and inactivate the neutrophil chemokine IL-8.[129,130] GAS also produces a C5a peptidase that also cleaves and inactivates this chemoattractant byproduct of the host complement cascade.[131,132] Invasive strains of GAS produce DNAses (aka

streptodornases) that degrade the chromatin based- neutrophil extracellular traps (NETs) employed by the host innate immune system to ensnare circulating bacteria.[133,134] Hyaluronidase, secreted by GAS, allows for bacterial migration through the host extracellular matrix.[135] The surface expressed streptokinase, sequesters and activates host plasminogen on the bacterial surface, effectively coating the bacteria with plasmin that promotes tissue spread. The pore-forming toxins streptolysin O (SLO) and streptolysin S (SLS) are broadly cytolytic against host cells including phagocytes. A variety of streptococcal superantigens, e.g. SpeA, SpeC and SmeZ, can promote rapid clonal T cell expansion and trigger toxic shock-like syndrome.[136] GAS causes disease in compromised and healthy individuals alike, illustrative of a parasitic symbiosis between GAS and the host.

Potential host benefits of GAS may be deciphered in certain interactions of GAS with host epithelium. For example, several studies have shown that SLO promotes wound healing *in vitro* through stimulating keratinocyte migration.[137] Sublytic concentrations of SLO may induce CD44 expression, potentially modulating collagen, hyaluronate, and other extracellular matrix components in mouse skin. Both the tight skin mouse (Tsk) model of scleroderma and the bleomycin-induced mouse skin fibrosis model showed decreased levels of hydroxyproline after treatment with SLO.[138]

Plasminogen activation in the epidermis leads to keratinocyte chemotaxis, suppression of cell proliferation, and potential re-epithelialization of wounds.[139] Also,

streptokinase is now being used clinically for therapeutic fibrinolysis.[140,141] Thus, in a tissue-specific context, limited expression of certain GAS virulence factors may aid rather than harm the host.

Pseudomonas aeruginosa

This Gram-negative, rod shaped, aerobic bacterium is well known for its ability to produce fluorescent molecules, including pyocyanin (blue-green), pyoverdin or fluorescein (yellow-green), and pyorubin (red-brown). Fluorescence and the grape-like sweet odor allow for easy identification of *P. aeruginosa* from other Gram-negative bacteria.

P. aeruginosa is commonly found in non-sterile areas on healthy individuals, and much like *S. epidermidis*, is considered a normal constituent of human's natural microflora. The bacteria normally innocuously live on human skin and in the mouth, but are able to infect practically any tissue in which it comes into contact. Flexible, nonstringent metabolic requirements allow *P. aeruginosa* to occupy a variety of niches, making *P. aeruginosa* the epitome of an opportunistic pathogen. Due to the general harmlessness of the bacteria, infections occur primarily in compromised patients and in conjunction with hospital stays. Explicitly, immune-compromised individuals with AIDS, cystic fibrosis, bronchiectasis, neutropenia, hematologic, and malignant diseases develop systemic or localized *P. aeruginosa* infections. Transmission often occurs through contamination of inanimate objects and can result in ventilator-associated pneumonia and other device-related infections.

The main port of entry is through compromised skin, with burn victims commonly suffering from *P. aeruginosa* infections. Entry into the blood results in bone, joint, gastrointestinal, respiratory, and systemic infections. On the skin, *P. aeruginosa* occasionally causes dermatitis or deeper soft-tissue infections. Dermatitis occurs when skin contacts infected water, often in hot tubs. The infection is very mild and treated easily with topical antibiotics. Severe infections are treated with injectable antibiotics, such as aminoglycosides (gentamycin), quinolones, cephalosporins, ureidopencillins, carbapenems, polymyxins, and monobactams, though multidrug resistance is increasingly common in hospital settings and chronically infected individuals (e.g. cystic fibrosis patients).

During infection, the type IV pilus and non-pilus adhesins anchor the bacteria to the tissue. *P. aeruginosa* secretes alginate, protecting the bacterium from phagocytic killing and potentially from antibiotic access.[142] *P. aeruginosa* also produces a variety of toxins and enzymes including include lipopolysaccharide (LPS), elastase, alkaline protease, phospholipase C, rhamnolipids, and exotoxin A.[143] The regulation of these virulence factors is very complex and modulated by the host's response. It was found that *P. aeruginosa* is able to sense the immune response and up-regulate the virulence factor type I lectin (*lec*A).[144] Interferon-γ (IFN-γ) binds to the major outer-membrane protein OprF and the OprF-IFN-γ interaction induces the bacteria to express lectin and quorum sensing-related (bacterial communication system) virulence factors.[144,145] Many genes that encode porins and other virulence

factors are also being studied in correlation with quorum sensing and *P. aeruginosa* metabolism.

The medical significance of *P. aeruginosa* infections is heightened due to antibiotic resistance. *P. aeruginosa* expresses genes that encode enzymes that hydrolyze specific antibiotics. Specifically, the bacteria produce AmpC cephalosporinase, β-lactamases (PSE, OXA, TEM, SHV, and other class A-type), and metallo-carbapenemases.[146] Antibiotic resistance also results from mutations in the porin OMP, which normally encodes the D2 porin, OprD.[147] Subsequent inactivation of OprD leads to imipenem resistance. Aminoglycoside resistance due to a variety of mechanisms occurs through acquisition of gene resistance cassettes occasionally present in integrons simultaneously encoding metallo-β-lactamases.[148] Other antibiotic resistant mechanisms are attributed to up-regulation of efflux pumps, such as the MexAB-OprM system, and to mutations in topoisomerases II and IV.[149,150]

In spite of intermittent disease caused by *P. aeruginosa*, the bacteria have been shown to protect the human host from a variety of infections. The byproducts of *Pseudomonas* are so potent that several have been turned into commercial medications. One of the most well known products of a *Pseudomonas* (particularly *P. fluorescens*) is pseudomonic acid A, also called mupirocin or Bactroban.[151] Mupirocin is one of the only topical antibiotics used in treatment of topical infections caused by staphylococcal and streptococcal pathogens. *S. aureus* with resistance to multiple antibiotics often show sensitivity to mupirocin. *P. aeruginosa* also produces

compounds with similar antimicrobial activity. A peptide called PsVP-10, produced by *P. aeruginosa*, was shown to have antibacterial activity against *Streptococcus mutans* and *Streptococcus sobrinus*.[152] Additionally, *P. aeruginosa* suppresses fungal growth (Figure 5). Species of fungus that the bacteria fully or partially inhibit include *C. krusei, C. keyfr, C. guillermondii, C. tropicalis, C. lusitaniae, C. parapsilosis, C. pseudotropicalis, C. albicans, T. glabrata, S. cerevisiae,* and *A. fumigatus*.[153] Studies have shown that *P. aeruginosa* and *C. albicans* coexist in the host and the attenuation of *P. aeruginosa* results in *C. albicans* growth. The mechanism by which *P. aeruginosa* inhibits *C. albicans* may be due to the quorum-sensing molecule 3-oxo-C12 homoserine lactone (3OC12HSL).[154] This and other molecules, such has 1-hydroxyphenazine or pyocyanin, are shown to suppress the filamentous, or virulent, phase of *C. albicans* growth (Figure 1.5).[155] Thus, the presence of *P. aeruginosa* likely attenuates *C. albicans* and possibly other yeasts, thereby preventing infection. The repression of microbial growth by *P. aeruginosa* is not restricted to yeasts. Inhibition is also seen with *Heliobacter pylori*.[156]

The obvious benefit of *P. aeruginosa* lends toward the classification of this microbe as a mutual. The rarity of *P. aeruginosa*-related disease and the impedance of pathogenic organisms suggest that this bacterium maintains homeostasis between host and microbe, preventing disease. Its ubiquitous presence likely protects against pathogenic infection, making *P. aeruginosa* a participant in the host's cutaneous innate immune system.

CONCLUSIONS

Current research related to infectious diseases of the skin target microbial virulence factors and aim to eliminate harmful organisms. Some of these same microbes potentially also play an opposite role by protecting the host. The complex host:microbe and microbe:microbe interactions that exist on the surface of human skin illustrate that the microbiota have a beneficial role, much like that of the gut microflora. Microbes participate in inflammatory diseases yet may not cause infections. For the clinician, understanding these principles should guide appropriate use of currently available systemic and topical antibiotics. Future advances in our understanding of microbial pathogens as well as an increase in the appreciation of the complex relationship that humans have with the resident microbes promise to lead to novel diagnostic and therapeutic approaches to dermatologic disease.

ACKNOWLEDGEMENTS

The text of this chapter, in full, is modified from the material in Cogen AL, Nizet V, Gallo RL. Skin Microbiota: A source of disease or defence? *British Journal of Dermatology*. 2008 Mar; 158(3):442-55. I was the primary researcher and author and the co-authors listed in this publication directed and supervised the research which forms the basis for this chapter.

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Chapter II.

Staphylococcus epidermidis inhibits Group A *Streptococcus*; Purification and Identification of Phenol Soluble Modulins

ABSTRACT

Staphylococcus epidermidis (S. epidermidis) has long been regarded as a member of the skin's commensal flora. The microbe was thought to benefit from the cutaneous niche while leaving the host unaffected. Recent studies on the resident gut flora suggest that similar host-microbial relationships may not be that of commensalism, but rather mutualism where the microbe provides benefit to the host. Here, we hypothesized that *S. epidermidis* benefits the host by augmenting the antimicrobial barrier. We found that *S. epidermidis* was able to inhibit Group A *Streptococcus* (GAS) growth *in vitro* and *in vivo*. C_{18} column purification, using HPLC, identified an active fraction, eluting at 72% acetonitrile. MALDI TOF-TOF sequencing revealed the presence of 2 peptides in the active fraction: Phenol Soluble Modulin-δ and Phenol Soluble Modulin-γ. These data indicate that *S. epidermidis* inhibits GAS growth on the skin by producing antimicrobial peptides. Thus, *S. epidermidis* may play an important role in host defense, with the cutaneous innate system being redefined to include the resident flora.

INTRODUCTION

The skin has been shown to harbor a large quantity and diversity of microflora.[1] Of the most well known and commonly isolated are the members of the Firmicutes, including *Staphylococcus sp.* To date, there have been 23 species of *Staphylococcus* identified with *Staphylococcus aureus* (*S. aureus*) and *Staphylococcus epidermidis* (*S. epidermidis*) being the most characterized. Unlike *S. aureus,* which causes skin lesions on otherwise normal individuals, *S. epidermidis* typically innocuously resides on the cutaneous surface. *S. epidermidis*, a strict opportunistic pathogen, causes disease under particular circumstances: when the host is immuno-compromised or there is a disruption in the cutaneous barrier.

Such a relationship with the human host has been termed commensal, as the bacteria were thought to gain protection and nutrients from the skin's niche, while leaving the host generally unaffected. At one point, the gut flora were thought to possess a similar commensal relationship. Yet, new studies demonstrate the beneficial role of the intestinal flora, lending to the symbiotic classification to mutualism, where both organisms benefit.

Since many of the skin flora appear benign, like many of those in the gut, we hypothesized that the bacteria play a similarly beneficial role. One way in which the bacteria, such as *S. epidermidis,* may benefit the host is through production of bacteriocins or antimicrobial peptides (AMPs). AMPs, such as cathelicidin, have been

shown to play a critical role in host defense against pathogens.[2] The bacteriocins, produced by bacterial species, have been considered as a survival mechanism during intra and inter-species competition. Several bacteriocins have been identified from *S. epidermidis* isolates, including Pep5, epidermin, epilancin K7, and epicidin 280.[3-6] Despite the discovery of these novel bacteriocins, their potential role on the skin has yet to be elucidated.

Thus, we sought to determine whether the commensal skin isolate of *S. epidermidis*, ATCC 12228, has antimicrobial activity that could benefit the skin by inhibiting pathogen survival. First, we found that *S. epidermidis* and supernatants could inhibit a common skin pathogen Group A *Streptococcus* (GAS) on human skin. In addition, cell-free culture supernatant was able to prevent growth of GAS. The antimicrobial agents were found to be susceptible to trypsin degradation but not heat inactivation, or freezing. Using reversed-phase C_{18} column purification and HPLC, the supernatant was fractionated according to hydrophobicity. The fractions were assayed for antimicrobial activity by radial diffusion assay and the peptides in the active fraction were sequenced using MALDI TOF-TOF. Sequencing results identified 2 molecules: Phenol Soluble Modulin-γ (PSMγ) and Phenol Soluble Modulin-δ (PSMδ).

RESULTS

Staphylococcus epidermidis inhibits Group A *Streptococcus* on the skin

In order to determine if *S. epidermidis*, ATCC 12228, exhibited antimicrobial activity over GAS on the skin's surface, cleaned human fingers were painted with *S. epidermidis* (SE), GAS, or in combination (GAS+SE). After 10 minutes, fingers were printed onto blood agar plates. Hemolysis of blood agar demonstrated the ability of GAS to survive on the skin. *S. epidermidis*, a non-hemolytic bacterium, exhibited no hemolysis. Yet, Figure 2.1 shows that the presence of *S. epidermidis* reduced the survival of GAS (GAS+SE), as seen by the minimal hemolytic areas. As a negative control, *Lactococcus lactis* (*L. lactis*, LL) was painted on cleaned fingers alone or in combination with GAS (GAS+LL). Unlike *S. epidermidis*, *L. lactis* was unable to reduce the ability of GAS to survive as demonstrated by the large amount of hemolysis (Figure 2.1).

To determine if *S. epidermidis* could similarly inhibit GAS on mouse skin, cleaned excised 8mm punch biopsies of mouse skin were prepopulated with *S. epidermidis* (SE) for 6 hours. GAS was added to the mouse skin and incubated for an additional 4 hours. Bacterial survival was then quantified. The data, displayed in Figure 2.2, illustrate that GAS survival was significantly reduced in the presence of *S. epidermidis*, but *S. epidermidis* survival was unaffected by the presence of GAS. The X-axis labels denote the bacteria enumerated and the table, below the graph, denotes the bacteria applied to the skin.

Since live *S. epidermidis* was able to inhibit survival of GAS on human and mouse skin, we sought to determine if cell-free supernatants would have a similar effect. Human forearm skin was cleaned and then treated for 10 minutes with *S. epidermidis* cell-free supernatant. After 20 minutes, the supernatant was removed and GAS was applied to the same surface immediately (t=0) or 1 hour after supernatant removal (t=1). GAS was incubated on the treated skin for 10 minutes, removed from the area and plated on blood agar to determine survival. Thus, the supernatant provided protection effectively against GAS survival even one hour after the supernatant was removed (Figure 2.3). These data demonstrate that *S. epidermidis* and cell-free *S. epidermidis* supernatant is able to inhibit GAS survival on human and mouse skin.

Staphylococcus epidermidis **inhibits Group A** ***Streptococcus in vitro***

Since the *S. epidermidis* cell-free supernatant was able to reduce GAS survival on the skin's surface, we sought to determine whether *S. epidermidis* and supernatant were able to inhibit bacterial growth similarly *in vitro*. To determine if *S. epidermidis* could inhibit GAS survival on an agar plate, stationary-phase GAS was spread on an agar plate. *S. epidermidis* or *L. lactis* were plated on top of the GAS lawn. After 24 hours, bacterial growth exhibited that *S. epidermidis* (SE), but not *L. lactis* (LL), was surrounded by a zone of inhibition (Figure 2.4). GAS was able to grow elsewhere on the plate indicating that *S. epidermidis* produces a diffusible antimicrobial that directly inhibits GAS. To confirm that the antimicrobial produced by *S. epidermidis* was

soluble and secreted, GAS was grown in stationary phase cell-free sterile supernatants. To account for nutrient depletion, GAS was also grown in *Propionibacterium acnes* (*P. acnes*) and GAS supernatants. GAS growth was inhibited by only the *S. epidermidis* supernatants indicating that inhibitory activity was due to secretion of an antimicrobial and not due to nutrient depletion (Figure 2.5).

Finally, we investigated the nature of the antimicrobial activity. The *S. epidermidis* supernatant was subjected to heat inactivation, a cycle of freeze/thaw, and trypsin digestion. GAS survival was then monitored in the treated supernatant to determine if antimicrobial activity remained after treatment. Freezing had no effect on the antimicrobial activity, as the frozen supernatants retained complete antimicrobial activity (Figure 2.6). Heat inactivation was able to reduce antimicrobial activity, yet the supernatants still reduced GAS survival significantly (Figure 2.6). These data suggest that either there are multiple antimicrobial agents in the supernatant or that the agent is partially susceptible to heat inactivation.

To determine if the antimicrobial is proteinaceous, *S. epidermidis* supernatants were treated with trypsin. After treatment, the trypsin was heat inactivated. GAS was then incubated in the treated supernatants, trypsin-treated LL-37 (positive control for trypsin digestion), or in PBS (negative control). Trypsin treatment completely inactivated all antimicrobial activity in the supernatants suggesting that the antimicrobials were proteinaceous (Figure 2.7). GAS survived in trysin-treated LL-37 and heat inactivated trypsin in PBS (Figure 2.7). Thus, these data suggest that the

antimicrobials produced by *S. epidermidis* are proteinaceous and may be due to multiple compounds, some of which are susceptible to heat inactivation.

Purification and Identification of antimicrobial compounds from *S. epidermidis*

The susceptibility of the supernatant to trypsin but only partially to heat suggests that the antimicrobial activity may be attributed to antimicrobial peptides (AMPs). AMPs being short peptides will similarly be susceptible to protein degradation, but relatively unsusceptible to heat, due to their lack of a complex tertiary structure. Since AMPs have a characteristic amphipathic nature, we utilized a hydrophobic reversed-phase C_{18} column to partially purify hydrophobic compounds from the *S. epidermidis* supernatant. Following elution off of the C_{18} column with acetonitrile, the hydrophobic eluent was lyophilized to concentrate the potential antimicrobial peptides. The eluent was subsequently fractionated from a C_{18} column using HPLC (Figure 2.8). Fractions were lyophilized, resuspended in water and assayed for antimicrobial activity in a radial diffusion assay (Figure 2.9). Fraction 37 contained antimicrobial activity as evidenced by the zone of inhibition (Figure 2.9).

To determine the sequence of the compound(s) present, the active fraction was submitted for MALDI TOF-TOF sequencing. Two sequences were identified: Phenol Soluble Modulin-δ (PSMδ) (Figure 2.10) and Phenol Soluble Modulin-γ (PSMγ) (Figure 2.11). Thus, purification and sequencing resulted in the identification and isolation of two putative *S. epidermidis* antimicrobial peptides, PSMδ and PSMγ.

DISCUSSION

Antimicrobial peptides have been previously shown to be critically important in host defense. Cathelicidins and defensins are examples of two classes of AMPs known to protect the host from infection. For example, mouse lacking cathelicidin are more susceptible to skin infections by GAS.[2] Other studies indicate the importance of the human β-defensins in defense against *Staphylococcus aureus* (*S. aureus*).[7] Since *S. epidermidis* lives on the skin, the production and secretion of antimicrobial peptides may play a similarly important role in preventing infection by pathogens such as GAS.

Rather than being described as antimicrobial peptides, Phenol Soluble Modulins (PSMs) from *S. epidermidis* and *S. aureus* have been generally characterized as virulence factors. These peptides have been previously shown to lyse neutrophils and are suggested to contribute to virulence of MRSA.[8] Although this study proposes that PSMs are cytolytic, this supports, rather than contradicts our hypothesis that these peptides have bacteriolytic properties. Membrane-disruption, the proposed mechanism by which the peptides act, is not necessarily limited to eukaryotic membranes (i.e. neutrophils), but may also occur in prokaryotic membranes (i.e. GAS).[9]

S. epidermidis PSMγ, also known as δ-haemolysin, δ-toxin, and δ-lysin, is encoded by the *hld* gene within the sequence coding for RNAIII. PSMγ expression has also been shown to be under control of the *agr* locus.[10] Although PSMγ is hypothesized to play a role in *S. epidermidis* virulence, there is limited data to confirm

this hypothesis. In contrast, δ-lysin produced by *S. aureus* has been shown to play an important role in virulence and has been the focus of physical and chemical analysis.[9,11-13] The differences in the peptides produced by *S. epidermidis* and *S. aureus* are seemingly minimal (Table 2.1), but may result in a previously undetermined variance. One particular disparity appears to be the antimicrobial activity. In our studies, we found *S. epidermidis* PSMγ to be present in an inhibitory active fraction after HPLC purification, indicative of antimicrobial activity. Yet, previous studies demonstrated that the *S. aureus* peptide lacked such activity.[11] Based on the previous studies suggesting that δ-lysin from *S. aureus* forms complexes, PSMγ likely acts through the barrel-stave mechanism to disrupt membranes.

Unlike PSMγ, PSMδ has not been extensively studied. In fact, the PSMδ was only first reported in 2004.[14] Other than the study reporting its proinflammatory activity, little is known about PSMδ.

Most studies evaluate *S. epidermidis* as a pathogen and the myriad of virulence factors used to promote disease. Yet, we believe that the resident flora are not inherently pathogenic, but may benefit the host. Our data illustrate that *S. epidermidis* and secreted peptides, prevent survival of GAS on the skin, thus acting as natural antibiotic barriers. In the subsequent chapters, the role of the *S. epidermidis* PSMs will be discussed in greater detail, relevance, and contribution to innate immune function of the host.

MATERIALS AND METHODS

Human skin studies

Finger experiments: *Staphylococcus epidermidis (S. epidermidis)*, ATCC 12228, *Lactococcus lactis*, and Group A *Streptococcus* NZ131 were grown to stationary phase in Todd-Hewitt Broth (THB, Sigma) at 37°C under shaking, with the exception of GAS, which was grown in a stationary culture. Fingers were cleaned with 70% ethanol and air-dried. 10μL of *S. epidermidis* or *L. lactis* were first spread along the length of each finger, and incubated for 5 minutes. GAS was added atop the treated fingers and incubated for 10 minutes. *S. epidermidis*, *L. lactis*, and GAS alone were also added to cleaned fingers as controls. Fingers were touched to blood agar plates (Hardy) and grown overnight at 37°C. GAS survival was determined by hemolysis. Forearm experiment: *S. epidermidis* stationary phase supernatants were sterilized using a 0.22μm filter. 1ml of supernatant or THB was placed in a circular tube on the forearm for 20 minutes. Supernatant was removed and the forearm was allowed to dry. 10μL GAS at 10^6 CFU/ml was spread on treated forearm directly after removal of supernatant (or THB) or 1 hour after supernatant (or THB) removal. GAS was incubated on forearm site for 10 minutes and removed by replacing the circular tube and aggressively washing area with 1x phosphate buffered saline (PBS). PBS containing GAS was plated for CFU. All human studies were approved by the Institutional Review Board.

***Ex vivo* mouse skin infections**

The backs of 10-12 week old wild-type C57BL/6 mice (Charles River) were shaved. Nair (a depilating agent) was added for 2 minutes and removed with a wet towel. 18 hours after hair removal, mice were euthanized using CO_2. The skin was cleaned with 70% ethanol. 8mm punch full-thickness punch biopsies were floated on Epilife media containing 0.06mM calcium and epidermal growth factor supplement (Cascade Biologics, Portland, Oregon). 5μL of mid-log phase *S. epidermidis* at 2 $x10^8$ CFU/ml or PBS in 1x dPBS was added to the punch biopsies for 6 hours at 37°C. 5μL of GAS was then added to the previously treated skin punches. Biopsies were incubated at 37°C for 4 hours. Colony forming units (CFU) were recovered by bead beating with 1mm zirconia beads in 1ml 1x dPBS for 1 minute. Samples were placed on ice for 5 minutes and then bead-beated for 1 minute. Supernatant was serially diluted onto blood agar for CFU enumeration. All experiments using mice were conducted according to institutional guidelines for animal experiments.

***In vitro* inhibition assays**

S. epidermidis, GAS, *L. lactis*, and *Propionibacterium acnes* (*P. acnes*) were grown to stationary phase in THB. All bacteria were shaken at 37°C except for GAS, which was grown in a stationary manner at 37°C. For agar plate assay, 30 μL of stationary phase GAS was spread on Todd Hewitt agar (THA) and allowed to dry. 5 μL of stationary phase *S. epidermidis* or *L. lactis* was plated on top of GAS lawn. Plates were incubated at 37°C overnight. For liquid culture assay, stationary phase supernatants of *P. acnes*, GAS, and *S. epidermidis* were filtered through 0.22μm PES

filter. To each supernatant, GAS was added at 10^5 CFU/ml. GAS growth was monitored by determining optical density at 600nm (OD_{600}). For supernatant treatment assays, *S. epidermidis* stationary phase supernatant was filtered through 0.22μm PES filter. Supernatant, LL-37, or PBS was frozen for 1 hour, heat inactivated at 65°C for 30 minutes, or treated with trypsin for 2 hours at 37°C and then heat inactivated. GAS was inoculated into treated supernatants at a concentration of 10^5 CFU/ml. Supernatants were serially diluted and plated at 1.5 and 3 hours for GAS enumeration.

Purification and Identification

S. epidermidis 18 hr supernatants containing 0.1% trifluoroacetic acid (TFA) were partially purified using a reversed phase C_{18} Sep Pak column (Waters Corporation). The eluted fraction between 30% and 80% acetonitrile (ACN)/0.1% TFA was lyophilized, resuspended in 30% ACN/0.1%TFA, and applied to a 4ml C_{18} column on HPLC. 1 ml fractions were collected, lyophilized, resuspended in 10μL water and analyzed via radial diffusion assay. This was performed by seeding GAS into liquid THA at 10^5 CFU/ml and allowing the bacteria-agar to solidify in 10mm square dishes. 1μL of each fraction was added to1mm holes punched into agar, and incubated for 18 hr at 37°C. The active fraction, #37, eluting at 72% ACN was submitted for MALDI TOF-TOF sequencing at UCSD Biochemistry Core.

ACKNOWLEDGMENTS

I would like to thank Robert Dorschner and Kenshi Yamasaki in the Gallo lab for their scientific, intellectual, and technical contributions to the purification of the PSMs. In addition, I would like to thank Justin Torpey for performing the MALDI TOF-TOF sequencing and Dr. Youngxuan Su for performing mass spectrometry on various samples during the purification.

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Chapter III.

Selective antimicrobial action is provided by Phenol Soluble Modulins derived from *Staphylococcus epidermidis,* a normal resident of the skin

ABSTRACT

Antimicrobial peptides serve as a first line of innate immune defense against invading organisms such as bacteria and viruses. In this study we hypothesized that peptides produced by a normal microbial resident of human skin, *Staphylococcus epidermidis*, might also act as an antimicrobial shield and contribute to normal defense at the epidermal interface. We show by circular dichroism and tryptophan spectroscopy that phenol soluble modulins (PSMs) γ and δ produced by *Staphylococcus epidermidis* have alpha-helical character and strong lipid membrane interaction similar to mammalian AMPs such as LL-37. Both PSMs directly induced lipid vesicle leakage and exerted selective antimicrobial against skin pathogens such as *Staphylococcus aureus*. PSMs functionally cooperated with each other and LL-37 to enhance antimicrobial action. Moreover, PSMs were able to reduce GAS but not *S. epidermidis* survival on mouse skin. Thus, these data suggest that the production of PSMγ and PSMδ by *S. epidermidis* can benefit cutaneous immune defense by selectively inhibiting survival of skin pathogens while maintaining the normal skin microbiome.

INTRODUCTION

Infections from organisms such as Group A *Streptococcus* (GAS, *Streptococcus pyogenes*) or *Staphylococcus aureus* range from superficial to invasive and collectively represent a severe societal burden, only escalating with the increase of resistance to pharmaceutically-derived antibiotics.[1-3] Increasing our understanding of innate host-derived antimicrobial peptides (AMPs) offers an alternative to the development of treatment of such infections since AMPs have retained the capacity to provide protection against infections by GAS, *S. aureus* and other microbes,[4-6] and have not lost their antimicrobial relevance as in the case of many pharmaceutical antibiotics.

Although our understanding of the AMPs remains incomplete, several classes of these antibiotic peptides have been described. AMPs such as the cathelicidins CRAMP in mice and LL-37 in humans are small cationic alpha-helical peptides that act through strong membrane activity. These helical peptides associate with lipid membranes[7,8] and are thought to kill microbes by their capacity to disrupt the normal structure of the lipid membrane[9]. Interestingly, several bacteria have also been shown to produce membrane-disruptive peptides. For example, delta-lysin (delta-toxin or hld gene), also known as phenol soluble modulin-γ (PSMγ), from *S. aureus* has been shown to cause lysis of membranes and displays strong lipid interactions.[10] This peptide causes disease through the destruction of red blood cells and neutrophils.[11,12] The mechanism of action for this member of the PSM group of peptides is similar to

some AMPs as NMR studies have shown that *S. aureus* delta-toxin forms an alpha-helix in lipid micelles.[13] A few prior studies have also found that delta-toxin from *S. aureus* can exert antibacterial activity when it is chemically modified by amino acid substitution and truncation, but the *S. aureus* the native peptide was unable to inhibit *E. coli* or *S. aureus*.[11,14] Despite these hints that PSMs could be antimicrobial, the action of these molecules as AMPs has not been extensively studied.

Since the related staphyloccal species, *S. epidermidis*, normally resides in abundance on the surface of healthy human skin, we sought here to investigate if the unique peptides PSMγ and PSMδ found in *S. epidermidis* could be beneficial to the host and thus serve as an additional AMP on the normal skin surface. Based on the structure, biophysical properties, and antimicrobial activity of PSMγ and PSMδ, this study suggests *S. epidermidis* plays a beneficial role to skin immune defense by producing innate, yet non-host-derived, AMPs on the skin surface.

RESULTS

Phenol Soluble Modulins have structural similarities to AMPs and strongly interact with synthetic lipid membranes

We observed that helical wheel plots of two peptides (PSMγ and PSMδ), produced by *S. epidermidis* predicted segregation of their hydrophobic and cationic amino acids in a manner with a 5 amino acid periodicity that resembles that of classic AMPs such as LL-37 (Figure 3.1). Since *S. epidermidis* is a major normal resident microbe on the surface of human skin, a structural similarity to native human AMPs suggested the potential for these peptides to act in a similar fashion and contribute to the normal antimicrobial defense of the skin. To evaluate the validity of this structural prediction by wheel plot, and test the capacity of the PSMs to adopt the charge distribution predicted by Figure 3.1, circular dichroism (CD) was performed on synthetically produced PSMγ and PSMδ. CD spectral analysis showed alpha-helical tendencies for both peptides in buffer alone (Figure 3.2). In the presence of anionic POPC/POPG lipid vesicles, PSMδ, but not PSMγ, became more alpha-helical (Figure 3.2).

Next, spectroscopic analysis of tryptophan in PSMγ was performed to evaluate the capacity of the peptide to associate with lipid membranes, a characteristic consistent with the amphipathic structure predicted by the wheel plot. Since tryptophan was present only in PSMγ, only this peptide was amenable to this form of spectroscopic analysis. In buffer alone, PSMγ's tryptophan emitted maximally at

339nm, while in the presence of POPC/POPG lipid vesicles the maximal emission shifted to 332nm, indicating a more buried state (Figure 3.3). The addition of urea successfully unfolded and dissociated peptide oligomers of PSMγ, as shown by a red shift of the maximal emission to 355nm. However, in the presence of lipid vesicles the maximal emission in urea remained low (335nm), indicating a continued strong membrane association (Figure 3.3). These data confirmed that PSMγ strongly associates with lipid membranes even under the strongly dissociating condition of 5.5 M urea. Thus, the combined observations of being alpha-helical, a secondary structural change in the presence of membranes, and a strong interaction with lipid vesicles, confirmed that the PSMs have similarities to AMPs in terms of secondary structure and membrane affinity.

S. epidermidis PSMs form multimeric complexes in solution

Another common characteristic of antimicrobial peptides is their ability to form complexes. To determine if PSMγ forms multimeric complexes, we generated unfolding curves of PSMγ as a function of peptide concentration in urea. In this two-state model, we considered 'folded' to be that of a multimeric state that allows for stabilization and embedding of the tryptophan. In the 'unfolded' state the peptides are monomeric in solution and the tryptophan residue will be solvent-exposed. PSMγ is shown to be in a multimeric state at 0M urea and a monomeric stated by 4M (Figure 3.4). The unfolding curve of 5μM PSMγ had a midpoint (C_m) of 2.14M urea, a slope of 0.67 kcal*M^{-1}*mol^{-1} at the midpoint, and a $\Delta G°_{(H20)}$ of 1.43kcal/mol (Figure 3.4). Increasing the concentration of PSMγ to 25μM caused a shift in the midpoint to 3.2M

urea, a slope of 0.49 kcal*M^{-1}*mol^{-1} at the midpoint, and a $\Delta G°_{(H20)}$ to 1.57kcal/mol. This result indicated that a greater concentration of urea was required to unfold PSMγ in the presence of more peptide, indicating formation of stable multimeric complexes.

In addition to multimeric complexes, we probed PSMδ and PSMγ for their ability to form hetero-multimeric complexes using CD. CD spectra of PSMγ were measured in the presence of increasing concentrations of PSMδ. The PSMδ spectrum alone was subtracted from the combined spectrum, so the output displays only the changes in secondary structure of PSMγ. Upon addition of PSMδ, the PSMγ signal increased in intensity, suggesting greater alpha-helical character (Figure 3.5). Thus, the alteration of PSMγ's spectrum by PSMδ suggests that the peptides are interacting.

Phenol Soluble Modulins disrupt artificial membrane vesicles and kill skin pathogens

Next, in order to determine if the physical similarities of PSMs to AMPs extends to the functional capacity to perturb lipid membrane vesicles, we tested their ability to perforate POPC/POPG vesicles. The vesicles evaluated were generated such that they encapsulated the fluorophore ANTS and a quencher DPX. As shown upon the addition of Triton X, release of ANTS from the vesicle resulted in increased fluorescence due to dissociation from the quencher (Figure 3.6). Incubation of these vesicles with increasing concentrations of PSMγ or PSMδ induced greater fluorescence, therefore demonstrating leakage of ANTS from the vesicles (Figure 3.6). Thus, PSMs are directly membrane active and can perforate POPC/POPG vesicles, a

functional characteristic similar to mammalian AMPs such as LL-37.

To directly determine if the capacity of the PSMs to disrupt membranes would extend to the ability of these peptides to kill or inhibit the growth of potential skin pathogens, Group A *Streptococcus* (GAS), *S. aureus*, and *S. epidermidis* were incubated with PSMs at various concentrations for 24 hours, then plated and colonies enumerated to determine bacterial survival. Growth of both GAS and *S. aureus* was inhibited, and bacteria were killed by either PSMγ or PSMδ at concentrations greater 16uM, while *S. epidermidis* was resistant and survived at the highest concentration tested, (64uM) (Figure 3.7). Similarly, Table 3.1 illustrates that these peptides produced by *S. epidermidis* showed significant bactericidal activity toward other pathogens, but were somewhat selective in their potency. *S. aureus* (including MRSA), *S. pyogenes*, and *E. coli* were unable to survive at PSMδ concentrations of 32μM and above while *S. epidermidis* survived at the highest concentration assayed.

To further evaluate the mechanisms responsible for the antimicrobial action of PSMs, and to support the data shown earlier, the direct action of PSM on bacterial membranes was evaluated. S *epidermidis*, GAS or S. *aureus* was incubated with PSMγ and cells fixed within 20 minutes for electron microscopy. This analysis showed that membrane blebbing of both GAS and *S. aureus* was induced by PSMγ (Figure 3.8). This effect was not seen in *S epidermidis* treated similarly. The degree of membrane blebbing seen in GAS and S aureus was similar to that seen when bacteria were treated with the mammalian AMP CRAMP (Figure 3.8). PSMγ and PSMδ also

induced dose-dependent membrane leakage in cultured mammalian cells as measured by cell propidium iodide (PI) uptake in cultured normal human keratinocytes (Figure 3.9). This capacity to disrupt mammalian cell membranes is a property previously reported for PSMs[12] and similar to AMPs also found on the skin such as LL-37 . Cell permeability was measured by PI uptake in cells exposed to the peptides in culture. The number of PI positive fluorescent cells per field indicates relative permeability effects on the cell population. Maximal PI uptake in the population occurred at 8μM for both LL-37 and PSMγ. Thus, PSMs cause membrane leakage and membrane perturbation in bacteria and mammalian cells and this function is similar to innate cutaneous AMPs.

Next, based on the spectroscopic observations suggesting interactions of PSMγ and PSMδ, we determined if the PSMδ and PSMγ would show increased antimicrobial action when combined. GAS was incubated with increasing concentrations of both PSMδ and PSMγ. As shown previously, at 8μM of PSMγ alone completely inhibited GAS. The addition of 2uM of PSMδ reduced the concentration of PSMγ needed to completely inhibit GAS growth to 4 μM (Figure 3.10). Similarly, PSMs were also able to act cooperatively with LL-37 to inhibit GAS survival (Figure 3.10). These data show that the PSMs function better together, and will further increase the potency of existing host AMPs on the skin such as LL-37.

Phenol Soluble Modulins are present in human skin and act *ex vivo* to selectively kill Group A *Streptococcus*

Since *S. epidermidis* is located on the epidermis, we sought to determine whether the presence of PSMs could prevent pathogen survival on the skin's surface. Sterilized skin explants from wild-type mice were treated with PSMs and then challenged with GAS or *S. epidermidis*. Similar to the *in vitro* MBC data that illustrates selective antimicrobial activity, PSMγ and PSMδ reduced GAS survival but not *S. epidermidis* on the skin's surface (Figure 3.11).

DISCUSSION

In this study we tested the hypothesis that *S. epidermidis* may have a beneficial or mutualistic relationship with human skin. Such findings have precedence with microbiota of the gut,[15-18] but has not been shown for cutaneous epithelia. Our findings here show that *S. epidermidis* produces two PSM peptides that have antimicrobial properties similar to that of host AMPs. Biophysical properties of the PSMs supporting this conclusion include observations that PSMγ forms multimeric complexes and exhibits strong α-helical structure in solution that is modified by the presence of lipid membranes, thus disrupting the aggregates in solution. These findings suggest that PSMγ acts through the barrel-stave mechanism to disrupt microbial membranes and kill the organism, a mechanism of action similar to some other AMPs. Furthermore, the antimicrobial properties of these PSMs directly coincide with the predictions based on biophysical measurements. Importantly, PSMs appear to provide a selective advantage for *S. epidermidis* as they inhibit several common skin microbes but do not inhibit its own growth. Thus, the observations that *S. epidermidis* typically resides harmlessly on the human skin surface and the potent antimicrobial properties of PSMγ and PSMδ, suggest that the peptides could provide benefit to the host as additional epithelial AMPs.

There is clear evidence that the presence of antimicrobial peptides at epithelial surfaces is beneficial. For example, we have previously shown that mice lacking cathelicidin (*Camp*$^{-/-}$) become much more susceptible to invasive GAS infections of

the skin.[5] Similarly, transgenic expression of an additional antimicrobial peptide in the small intestine provides protection against fatal infections by salmonella,[19] and expression in keratinocytes protects against skin infection.[20] However, there has yet to be evidence that cutaneous microbes offer similar benefit to host immunity. Despite the many reports of microbial-produced antimicrobial molecules such as bacteriocins,[21-23] the consequences of antimicrobial peptide production by resident microbes has not yet been investigated on skin. In contrast, microbes living on epithelial surfaces within the gut have been suggested to provide immune education and reciprocal benefit for the nutrient rich niche.[17] Based on the mounting data on the benefits of resident microbes, combined with the clear evidence of an important role for antimicrobial peptides in skin innate immunity, it is reasonable to conclude that AMPs produced by microbes on the skin can be beneficial.

The properties described here for the PSMs as AMPs are not inconsistent with the previously described properties of these peptides as virulence factors.[12] In the setting of an immocompromised host, and often in conjunction a broken skin barrier with catheters, the production of PSMs by *S epidermidis* can lead to tissue damage and cell lysis that contributes to virulence.[24-28] Similarly, host AMPs such as LL-37 can also lead to disease when abnormally expressed.[29-31] Thus, membrane active peptides often present a double-edged sword, providing defense but also potentially causing harm. In the situation with *S. epidermidis* PSMs it is possible that they are beneficial when present on the surface of intact skin, but become potentially dangerous to the host when the barrier is deeply invaded by live *S. epidermidis* and these bacteria then

inhibit leukocytes attempting to repel their invasion. Based upon the rare occurrence of this phenomenon in normal skin, it is unlikely that PSMs do more harm than good.

An important aspect of our data is that it shows the PSMs have a unique and highly desirable function for selective removal of pathogenic organisms on the skin such as *S. aureus* and GAS. Importantly, PSMs do this while retaining normal flora such as *S. epidermidis*, thus enabling the normal flora to maintain their proposed beneficial effects while eliminating the pathogens. The mechanism of action for selective killing by PSMs likely involves cooperative interaction with other native AMPs released by the host, thus boosting innate immune defense in an immediate and selective way. This finding presents the possibility for a topical antimicrobial strategy to kill common pathogens while the microbiome is preserved, an approach that would be likely to extend the duration of maximal immune defense and prevent repopulation by pathogens. This selective activity could become an important part of a normal microbial defense strategy against colonization and transmission of hospital acquired bacterial pathogens, and also could be exploited for a role in future anti-infective therapeutics.

MATERIALS AND METHODS

Peptides

PSMδ and PSMγ were commercially synthesized and purified by HPLC (Quality controlled biochemicals). PSMγ purity was >95% and PSMδ purity was >70%. mCRAMP and LL-37 were commercially synthesized and purified by HPLC to a purity >95% as previously described.[32]

Cell culture

Normal human epidermal keratinocytes (NHEK; Cascade Biologics) were grown in EpiLife medium (Cascade Biologics) supplemented with 0.06 mM $CaCl_2$, 1% EpiLife defined growth supplement, and penicillin/streptomycin (100 U/ml and 50μg/ml, respectively). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Vesicle preparation and leakage assay

POPC (1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine, Avanti Polar Lipids) and POPG (1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)], Avanti) were combined at a 2:1 molar ratio of POPC:POPG, dried under argon gas, resuspended by bath sonication in 20mM potassium phosphate buffer, pH 7.3 or in 20mM potassium phosphate buffer, pH 7.3, 50mM DPX (*p*-xylene-bis-pyridinium bromide) and 50 mM ANTS (8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt). Vesicles were extruded through 0.2 um polycarbonate film and run over a size

exclusion column to remove unencapsulated dye. Leakage assays were performed by incubating vesicles (1:5 dilution) with desired concentration of peptide for 1 h. Conditions for ANTS fluorescence were: excitation wavelength of 385 nm, emission wavelength of 400-700 nm, excitation and emission bandpass of 5 nm, 1 nm/step scan speed, and integration time of 0.2 s.

Circular dichroism assay

The molar ellipticity ([θ], deg*cm2/dmol) of synthetic PSMγ and PSMδ was determined at 25°C. For vesicle studies 20μM synthetic peptide in 20mM potassium phosphate buffer, pH 7.3 was incubated with or without 1mM 2:1 POPC:POPG lipid vesicles for 1 hour at 25°C. For peptide interaction studies, 20μM PSMγ was incubated with 5 or 10μM PSMδ for 1 hour at 25°C. Spectra were collected over 190-260 nm in an AVIV model circular dichroism spectrometer (Aviv Biomedical, Lakewood, NJ) with a 0.1 cm pathlength, collecting data at 1nm intervals. 5 repeat scans were taken for each sample, and the averaged baseline spectrum was subtracted from the sample average. For the vesicle studies, 20mM potassium phosphate, pH 7.3, in the presence or absence of vesicles, was used as the baseline. For the peptide interaction studies, 5 or 10μM PSMδ was used as the baseline.

Spectroscopic measurements

Synthetic peptide at concentrations of 5 and 25μM were incubated in solutions containing 20mM potassium phosphate, pH 7.3. Urea concentrations were determined by refractive index measurements taken on an Abbe 3L Baucsh & Lomb

refractometer.[33] Fluorescence measurements were taken on a Jobin Yvon SPEX FL3-11 spectrofluorometer equipped with an R928 photomultiplier tube. Conditions for tryptophan fluorescence are as follows: excitation wavelength of 290 nm, emission excitation and emission bandpass of 8nm and 4nm, respectively, 1 nm steps, and an integration time of 1s. Unfolding curves assumed a 2-state system[34] and reversible unfolding, and were performed in 20mM potassium phosphate buffer, pH 7.3, in the absence of lipid vesicles. Emission maximum were determined for all protein samples and normalized to a scale of 0-1. Fraction unfolded, *f*, was plotted against urea concentration, and were fit to the following equation[34]:

$$f = \frac{\exp\left[-m\left(\frac{C_m - C}{RT}\right)\right]}{1 + \exp\left[-m\left(\frac{C_m - C}{RT}\right)\right]} \qquad (1)$$

The fit-determined values for the slope, *m*, and the midpoint urea concentration, C_m, were used to calculate the free energy of unfolding in the absence of denaturant, using[34,35]:

$$\Delta G^{\circ}_{H_2O} = mC_m \qquad (2)$$

***In vitro* antibacterial studies**

Synthetic peptide (Quality Controlled Biochemicals) minimal bactericidal concentrations (MBC) and bacterial killing curves were performed as before in the presence of carbonate, with the only modification being with GAS (in 25% THB, 75% 1x dulbecco's (d) PBS), as GAS would not grow in media containing carbonate.[36] As

a control for the survival curve, GAS and *S. epidermidis* were grown in the same medium. For EM analysis, GAS, *S. epidermidis*, and *S. aureus* were grown to midlog phase in Todd Hewitt Broth (THB). Cells were washed with 1x dPBS and resuspended at 10^8 CFU/ml in 1x dPBS. A final concentration of 16μM peptide was added to the cells and incubated for 20 minutes on ice. The cells were submitted for EM analysis.

Keratinoyte toxicity study

Normal human epidermal keratinocytes (Cascade Biologics, Portland Oregon), grown to 75% confluency in Epilife media containing 0.02mM calcium and epidermal growth factor supplement (Cascade Biologics, Portland Oregon), were incubated with 0, 1, 2, 4, 8, and 16μM PSMγ, PSMδ, or CRAMP for 18 hours. The cells were stained for permeability using 50μg/ml propidium iodide in 1x dPBS for 10 minutes. The number of PI positive fluorescent cells were counted per 400X field. Three fields per well were enumerated and averaged. Experiment was performed twice with triplicate wells. In representative experiment, error bars represent +/- SEM.

***Ex vivo* mouse skin infections**

The backs of 10-12 week old wild-type C57BL/6 mice (Charles River) were shaved. Nair (a depilating agent) was added for 2 minutes and removed with a wet towel. 18-24 hours after hair removal, mice were euthanized using CO_2. The skin was cleaned with 70% ethanol. 8mm punch full-thickness punch biospsies were floated on Epilife media containing 0.06mM calcium and epidermal growth factor supplement (Cascade Biologics, Portland, Oregon). 5uL of synthetic PSMγ or PSMδ at 64μM

(0.32nmol) or 32μM (0.16nmol) in 1x dPBS was added to the punch biopsies for 5-10 minutes. 5uL of GAS (*S. pyogenes* NZ131) or *S. epidermidis* (ATCC 12228) at 2x 10^5 CFU/ml was then added to skin punches treated with the peptides. Samples were incubated at 37°C for 4 hours. Colony forming units (CFU) were recovered by bead beating with 1mm zirconia beads (which do not disrupt bacteria) in 1ml 1x dPBS for 1 minute. Samples were placed on ice for 5 minutes and then bead-beated for 1 minute, again. Supernatant was serially diluted onto Todd-Hewitt agar for CFU enumeration. Experiment was performed twice in triplicate. In combined experiment, error bars represent +/- SEM. All experiments using mice were conducted according to institutional guidelines for animal experiments.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 4.0. One-way ANOVA and Bonferroni post hoc test were used to determine significance for experiments with 3 more groups. An upaired t-test was used to determine significance for experiments with only 2 groups. Values of $p < 0.05$ were considered significant.

Conflict of Interest

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Marilyn Farquhar, Timo Merloo, Ingrid Niesman, and Krystyna Kudlicka in the UCSD immunoelectron microscopy core for analysis of bacterial membranes.

The text of this chapter, in full, is modified from the material in Cogen AL, Yamasaki K, Sanchez KM, Dorschner RA, Lai Y, MacLeod DT, Torpey JW, Otto M, Nizet V, Kim JE, Gallo RL. Selective antimicrobial action is provided by Phenol Soluble Modulins derived from *Staphylococcus epidermidis,* a normal resident of the skin. Accepted to the *Journal of Investigative Dermatology*. I was the primary researcher and author and the co-authors listed in this publication directed and supervised the research which forms the basis for this chapter.

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Chapter IV.

Staphylococcus epidermidis antimicrobial Phenol Soluble Modulins cooperate with host antimicrobial peptides to kill Group A *Streptococcus*

ABSTRACT

Antimicrobial peptides play an important role in host defense against pathogens. Recently, Phenol Soluble Modulins (PSMs) from *Staphylococcus epidermidis* (*S. epidermidis*) have been shown to interact with lipid membranes, form complexes, and exert antimicrobial activity. Based on the abundance and innocuity of the cutaneous resident *S. epidermidis*, we hypothesized that the PSMs contribute to host defense. Here we show that *S. epidermidis* PSMγ was deposited in the epidermis and sparsely in the dermis of normal human skin. Synthetic PSMγ was also seen to interact with neutrophil extracellular traps (NETs) and co-localized with cathelicidin. PSMγ was also able to induce NET formation in human neutrophils. In antimicrobial assays with Group A *Streptococcus* (GAS), PSMγ cooperated with CRAMP, hBD2, and hBD3. In whole blood the addition of PSMγ rendered GAS bacteriostatic, while in NETs, PSMγ increased killing capacity. Co-immunoprecipitation and tryptophan spectroscopy demonstrated direct binding of PSMγ to host antimicrobial peptides LL-37, CRAMP, hBD2, and hBD3. Finally, in a mouse wound model, GAS survival was reduced (along with Mip-2 cytokine levels) when the wounds were pretreated with PSMγ. Thus, these data demonstrate that *S. epidermidis* derived PSMγ cooperates with the host derived antimicrobial peptides in the innate immune system to reduce GAS survival.

INTRODUCTION

Antimicrobial peptides, in keratinocytes as well as in other cells types, have been shown to be critically important in the host's defense against infections. These antimicrobial peptides include the cathelicidins and defensins. Cathelicidin-deficient mice showed enhanced susceptibility to GAS skin infections, providing evidence that antimicrobial peptides are critical to the innate immune defense.[1] Additional models of infections including the gut,[2] brain,[3] and kidney[4] provide further evidence of the importance of cathelicidins in innate immune defense. Antimicrobial peptides have been shown to be important in multiple cell types, including, but not limited to, macrophages, neutrophils, mast cells, and epithelial cells. Pathogens come in to contact with antimicrobial peptides via host secretory mechanisms, phagocytosis, as well as formation of mast cell or neutrophil extracellular traps.[5,6] It has been suggested that neutrophil extracellular traps (NETs) entwine and kill circulating pathogens in a web of DNA, histones, and antimicrobial peptides.

Functionally, cathelicidins are multi-faceted. The peptides provide protection through immuno-modulation, inducing chemotaxis, and through direct pore-formation of a broad range of infectious agents including fungi, viruses, and bacteria. LL-37, a truncated and active form of cathelicidin, is suggested to form an amphipathic α-helix that subsequently inserts into negatively charged membranes forming veritable holes.[7] Previous reports suggest that cellular perforation requires multiple LL-37 peptides which coalesce to form oligomeric complexes.[8] Although complexes have been shown

to form, it is not known as to whether LL-37 physically forms heterologous complexes with other antimicrobial α-helical peptides.

Staphylococcus epidermidis (*S. epidermidis*), one of many cutaneous residents, is largely innocuous and frequently isolated. Studies on skin microbiota have identified *S. epidermidis* as a common colonizer of healthy human skin[9] and mouse skin.[10] Much like the gut, the microbiota of the skin is hypothesized to play a beneficial role in the cutaneous niche. We have previously demonstrated that peptides produced by *S. epidermidis* exhibit antimicrobial properties, potentially acting as an additional antimicrobial shield. Several Phenol Soluble Modulins (PSMs) produced by *S. epidermidis* are shown to exhibit antimicrobial properties and activity. In addition, the peptides have been shown to enhance the antimicrobial activity of LL-37 on Group A *Streptococcus* (GAS).

Previous studies have similarly reported that host AMPs act in synergy to kill bacteria.[11] Specifically, LL-37 and hBD2 have been shown to synergistically kill Group B *Streptococcus in vitro.*[12] Moreover, host AMPs have been shown to act synergistically with an antimicrobial peptide produced by *L. lactis* to inhibit *E. coli.*[13] Thus, we sought to determine whether the antimicrobial peptide, Phenol Soluble Modulin-γ (PSMγ), produced by the resident cutaneous microbe, *S. epidermidis*, could interact with the host antimicrobials leading to greater pathogen inhibition and enhancement of the host's innate immune system.

Here, we show that PSMγ is deposited by *S. epidermidis* on normal human skin, can bind to and even induce NETs. Furthermore, we find that PSMγ cooperatively enhances killing of GAS with the host antimicrobial peptides LL-37, CRAMP, hBD2, and hBD3. PSMγ dose-dependently renders GAS bacteriostatic in blood at sub-MIC levels and amplifies NET antimicrobial activity against GAS. In addition, tryptophan spectroscopy and co-immunoprecipitation illustrate the interactions of PSMγ with host antimicrobial peptides LL-37, CRAMP, hBD2, and hBD3. In a mouse wound model, an occasion where the *S. epidermidis* and products may interact with neutrophils in the blood, the presence of PSMγ reduces GAS survival. These data illustrate a novel means by which PSMγ contributes to the innate immune system, pointing towards a beneficial role for *S. epidermidis* on the skin's surface.

RESULTS

Phenol Soluble Modulin-γ is deposited on the skin and binds to neutrophil extracellular traps.

Phenol Soluble Modulins are multifunctional and can act to enhance virulence when invasive,[15] or act as antimicrobials when in direct contact with pathogens such as GAS. The action of PSMs has been experimentally demonstrated by *in vitro* analysis of synthetic peptides and artificial inoculation of *S. epidermidis* under controlled conditions. To evaluate if PSMs are commonly detectable under normal conditions, random human skin samples obtained from surgical excisions were stained for the presence of PSMγ. Immunohistochemistry demonstrated that PSMγ is abundantly detectable in the epidermis, hair follicle and sparsely in the dermis of normal skin (Figure 4.1a). Similar staining was observed in a second skin sample from a separate individual (data not shown). Next, since injured skin rapidly accumulates neutrophils at sites of infection and injury, and these cells act in part to protect the skin through the formation of neutrophil extracellular traps (NETS) containing antimicrobial peptides,[6] we evaluated if PSMγ from *S. epidermidis* could interact with NETs and contribute to their activity. PSMγ was added to neutrophils induced by PMA to form NETs in culture. Addition of PSMγ to these cells showed that PSMγ bound to the NETs and colocalized with cathelicidin endogenously released from the neutrophil (Figure 4.1b). As the cationic charge of this peptide predicts that this association with NETs could be through DNA binding, PSMγ association with DNA was next directly evaluated using tryptophan spectroscopy. In buffer alone, PSMγ's

tryptophan emits maximally at 341 nm. In the presence of neutrophil DNA, the maximal emission shifted to 331 nm (Figure 4.1c). The blue shift caused by the presence of neutrophil DNA suggested direct association with PSMγ. Finally, in addition to interacting with NETs, we sought to determine if PSMγ could also induce formation of NETs *in vitro*. Here, freshly isolated human neutrophils were stimulated with PSMγ, or PMA as a positive control. Like PMA, PSMγ was able to induce NETs (Figure 4.2). Thus, these data demonstrate that PSMγ derived from *S. epidermidis* is deposited on the skin and induces and interacts with NETs.

Phenol Soluble Modulin-γ enhances endogenous antimicrobial activity

To determine whether PSMγ was able to cooperatively enhance bacterial killing of host AMPs, GAS survival was assessed in the presence of PSMγ and CRAMP, hBD2, and hBD3. The presence of PSMγ and low concentrations of host AMPs increased GAS killing in a cooperative manner (Figure 4.1b and 4.1c; Figure 4.3a).

Since PSMγ enhanced the activity of antimicrobial peptides, PSMγ induced NET formation and because wounding typically results in bleeding, we investigated whether PSMγ could enhance antimicrobial activity of blood. This interaction would be predicted to occur in the earliest time period after injury, when the wounds bleed and blood mixes with the skin surface. For this, sub-MIC concentrations of PSMγ were added to whole blood containing GAS. Over time, GAS was able to grow in

blood without PSMγ, but was rendered bacteriostatic in blood containing 2-4μM PSMγ (Figure 4.3d), As PSMγ's ability to increase bacterial killing by whole blood may in part be due to the contribution of neutrophil extracellular traps (NETs) that form after injury, and PSMγ and closely related peptides have been known to lyse red blood cells and neutrophils,[15] we next examined if the activity of PSM in the presence of neutrophils is aided by DNA forming the NET. Treatment of NETs and PSM with DNase eliminated the capacity of both the NETs alone, or NETs with PSM, to inhibit GAS survival (Figure 4.3e). Hence, the addition of PSMγ to whole blood or to NETs increases their killing capacity.

Phenol Soluble Modulin-γ physically binds to host derived AMPs.

Since previous studies suggest that AMPs form heterodimeric and homodimeric complexes,[16] and because we found that PSMγ colocalizes with LL-37 in NETs, we hypothesized that PSMγ physically binds to the host derived AMPs. In order to determine whether PSMγ could associate with LL-37 and CRAMP, synthetic host AMPs were added to *S. epidermidis* cell-free, stationary phase supernatant, naturally containing PSMγ. Precipitation of PSMγ followed by an immunoblot for LL-37 and CRAMP demonstrated that PSMγ physically bound to the host derived peptides (Figure 4.4a).

In order to further confirm interaction between PSMγ and the host AMPs, tryptophan spectroscopy was performed on PSMγ in the presence and absence of host

AMPs. Unlike PSMγ, that has a tryptophan at position 15, LL-37, CRAMP, hBD2, and hBD3 are spectroscopically silent because they lack a tryptophan. Due to the spectroscopic silence of the host AMPs, a maximal tryptophan emission shift in PSMγ during incubation with a host AMP would signify an interaction with and structural change of PSMγ. To determine the ability of the host AMPs to interact with PSMγ, the peptide was incubated with host AMPs and tryptophan emission was monitored upon excitation at 290nm (Figure 4.4b and 4.4c). We found that PSMγ maximal tryptophan emission shifted from 341nm to 344nm in the presence of LL-37 and CRAMP, indicating a greater exposure of the tryptophan to the aqueous environment. Conversely, in the presence of hBD2 and hBD3, PSMγ's maximal tryptophan emission shifted from 341nm to 339nm, indicating that the tryptophan resides in a more hydrophobic and embedded environment (Figure 4.4b and 4.4c). In addition, we observed that the emission spectra intensity increased upon incubation with LL-37 and CRAMP and decreased upon incubation with hBD2 and hBD3 (Figure 4.4b). Similarly, we observed that PSMγ undergoes a conformation change in the presence of genomic keratinocyte DNA. Overall, these data illustrate that PSMγ directly interacts with, and binds to, host AMPs.

Phenol soluble modulin-γ reduces GAS survival and inflammation in mouse wounds.

As we have shown that PSMs may contribute to whole blood and neutrophil killing of GAS, we sought to directly determine if PSMs present in a wound could have a similar protective effect against bacteria, we utilized a mouse wound model.

PSMγ or PBS control was added to 4mm full-thickness fresh mouse wounds. After only 30 minutes, GAS was added to the wounds to mimic an infected wound. After 18 hours, the infected wounds and surrounding fascia were harvested. GAS survival was found to be significantly decreased in mouse wounds pretreated with PSMγ but not PBS (Figure 4.5a). Paralleling the GAS infection, Mip-2 (CXCL2) was significantly decreased in mouse wounds treated with PSMγ (Figure 4.5b). These data suggest that PSMγ reduces GAS survival *in vivo* and may contribute to the innate immune system.

DISCUSSION

We have previously demonstrated that PSMγ and PSMδ from *S. epidermidis* exhibit antimicrobial activity over pathogens. This activity appeared to result from membrane disruption, a trait common among many antimicrobials.[16] These data support studies suggesting a role of *S. aureus* PSMs in virulence, as the peptides induce pore formation in cells such as neutrophils.[15] Although the PSMs produced by *Staphylococcal sp.* are clearly pore-forming, the impact on the host is partially contingent upon the nature of the bacterium and area of residence in the host.

The antimicrobial effect of PSMs on skin pathogens and enhancement of host derived AMPs suggests a role for *S. epidermidis* in the innate immune system of the skin. Antimicrobial peptides have been previously shown to be crucial components of the innate immune system, as illustrated by the susceptibility of *Camp*$^{-/-}$ mice to GAS.[1] In addition, bacteria have been shown to benefit the human host, as suggested by the hygiene hypothesis and other studies on the gut microflora. Here we show that *S. epidermidis* benefits the host and provides an additional layer of protection against skin pathogens. *S. epidermidis* rather than acting alone, is able to kill pathogens by complementing the host's innate immune system. This close interaction of PSMγ with the host AMPs insinuates the presence of a mutual relationship between host and bacteria.

We have shown that PSMγ is deposited in the epidermis and sparsely in the dermis in normal healthy human skin. During scenarios, such as injury PSMγ may then be able to interact with a variety of cell types including neutrophils. Neutrophils expressing AMPs and forming extracellular traps facilitate eradication of potentially dangerous bacteria.[6] In fact, it has also been shown that injury itself increases AMP production by the innate immune cells and it is not yet known if injury also induces NETs in circulating neutrophils. As *S. epid*ermidis lives on the skin, injury would bring the bacterium and products in close proximity to the innate immune cells. In this occasion, PSMγ increases antimicrobial NET activity and likelihood of pathogen eradication. The NETs illustrate that the host AMPs and PSMγ intertwine with host DNA. The DNA binding that occurs by AMPs to the NETs may be more than an artifact of charge attraction, but rather suggests a novel role for AMPs. Many studies demonstrate that AMPs cause transcriptional and translational changes in cells. Of course, AMPs may bind receptors, or affect signaling pathways, but these peptides, including PSMs, may directly affect transcription at the level of DNA binding. Such DNA binding capacity of PSMγ also suggests a potential role for PSMs in bacterial gene regulation.

Heterologous complex formation and synergy of AMPs from different species have been minimally explored. Yet AMP synergy has been well documented between AMPs from the same species.[11,12] Though, one study demonstrated that bacteriocin synergistically enhanced pleurocidin activity against *E. coli.*[13] Although these synergy studies suggest an important relationship between different peptides, a physical

interaction between the peptides has not yet been proven. Here, we have shown for the first time, that AMPs from the resident *S. epidermidis* interacts physically with the host AMPs. Co-immunoprecipitation of PSMγ and CRAMP (or LL-37), along with the tryptophan spectroscopy demonstrated a direct AMP binding. Interestingly, the precipitated CRAMP peptide, ran slightly higher than the synthetic peptide positive control. This may have occurred due to the binding of PSMγ to CRAMP causing a slower migration.

To show that *S. epidermidis* PSMγ contributes to the antimicrobial response *in vivo*, the peptide was placed in wounds of mice later challenged with GAS. Since little PSMγ was detected in mouse skin (data not shown), we added synthetic PSMγ to mimic a wound in human skin. Significantly less GAS survived in the mouse wounds that were pretreated with PSMγ as compared to the vehicle (PBS) control. We also evaluated the levels of a proinflammatory cytokine Mip-2, as a monitor of infection severity. MIP-2, like the number of GAS recovered, indicated that PSMγ aids in bacterial clearance and general reduction of an inflammatory infection. PSMγ has been shown to actually induce rather than suppress proinflammatory signaling,[17,18] thus indicating that the reduction in MIP-2 purely parallels the GAS burden.

Overall, we show here that PSMγ is able to bind to host AMPs. Moreover, PSMγ enhances blood and NET killing of GAS. Finally, the functional reduction of GAS survival in a mouse wound pretreated with PSMγ suggests a beneficial innate

immune role and mutual role for *S. epidermidis*, a common constituent of the skin microbiome.

MATERIALS AND METHODS

In vitro antibacterial studies

Group A *Streptococcus* (GAS) was grown to mid-log phase in Todd-Hewitt Broth (THB, Sigma). A final concentration of 10^5 CFU/ml were incubated with synthetic PSMγ at 0, 1, 2, 4, 8, and 16μM in the presence and absence of LL-37 (0, 4, and 8μM), CRAMP (0, 2, and 4μM), hBD2 (0, 2, and 4μM), and hBD3 (0, 2, and 4μM). GAS was incubated with peptides in 25% THB and 75% 1x dulbecco phosphate buffered saline (dPBS) for 4 hours at 37°C and then plated on Todd-Hewitt agar for 18 hours before colony enumeration. Graphs are representative of two independent experiments performed in duplicate.

Whole Blood assay

Group A *Streptococcus* (GAS) was grown to mid-log phase in Todd-Hewitt Broth (THB, Sigma). A final concentration of 10^5 CFU/ml was incubated with heparanized human whole blood containing synthetic PSMγ at 0, 1, 2, or 4μM. GAS was plated and colonies enumerated at 0.5, 1, 1.5, and 2 hours post inoculation. Graph is representative of two independent experiments performed in triplicate.

Spectroscopic measurements

Synthetic PSMγ (QCB) at 5μM, was incubated with 5μM hBD2 (Peptide International), hBD3 (Peptide International), CRAMP, LL-37, or 16μg/ml genomic neutrophil DNA in 20mM potassium phosphate buffer, pH 7.3. Fluorescence

measurements were taken on a Jobin Yvon SPEX FL3-11 spectrofluorometer equipped with an R928 photomultiplier tube. Conditions for tryptophan fluorescence were as follows: excitation wavelength of 290 nm, emission excitation and emission bandpass of 8 nm and 4nm, respectively, 1 nm steps, and an integration time of 1s. Spectroscopic curves of host peptide hBD2, hBD3, CRAMP, or LL-37 were subtracted from respective PSMγ + host peptide curves. Similarly, the spectroscopic curve of DNA was subtracted from the PSMγ + DNA curve.

Immunoblot and precipitation of antimicrobial peptides

Staphylococcus epidermidis ATCC 12228 was grown for 18 hours to stationary phase in Todd-Hewitt Broth (THB, Sigma). Cells were centrifuged for 5 mins at 5000xg. Supernatants were harvested and filtered through 0.22 μm PES Express Millex GP filter. 1 μM final concentration of synthetic CRAMP or LL-37 was added to 1ml of the filtered supernatant and incubated on nutator at room temperature for 7 hours. Supernatants containing peptides were precleared with 1μg control IgG antibody and 20μL Protein A/G-Plus agarose (Santa Cruz Biotechnology) for 1.5 hours at 4°C rotating. Beads were pelleted by centrifugation at 2,500xg for 5 minutes and supernatant was removed and placed in new tube. The 1ml precleared supernatant was divided into 2 tubes with 500μL each. To one tube, 1μg mouse anti-PSMγ (Abcam) was added. To the other tube 1μg IgG control was added. Tubes were incubated overnight at 4°C, rotating. 20μL Protein A/G-Plus agarose were added for 4 hours at 4°C, rotating. Beads were pelleted by centrifugation at 2,500xg for 5 minutes

and washed three time in ice cold 1x PBS, spinning down after each wash as described. After the last wash, the supernatant was removed, and beads were resuspended in sample buffer for direct immunoblot. Beads were boiled for 5 minutes and loaded into 16% SDS-PAGE gel along with synthetic peptide as positive control and biotinylated protein ladder (Amersham) and run at 100V for 1.5 hours. Proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad) for 2 hours at 4°C. The membrane was blocked for 1 hour in blocking buffer (PBS, 5% nonfat milk with 3% bovine serum albumin) at room temperature. Incubation in primary antibody rabbit anti-LL-37 at 1:1000 or rabbit anti-CRAMP at 1:5000 was performed in blocking buffer overnight at 4°C. Membranes were then incubated in the appropriate horseradish peroxidase-conjugated secondary antibody goat anti-rabbit IgG for 1 hour at 1:5,000 for 1 hour at room temperature, followed by enhanced chemiluminescence (ECL) detection with the ECL detection kit (Amersham Biosciences).

Cell Culture and NETs killing assay

Neutrophils were purified from healthy donors using the PolymorphPrep TM system (Axis-Shield, Fresnius). $4x10^4$ cells were added to chamber slides in 500uL RPMI (Biowhitaker). 2×10^5 cells were added to 96 well plates in RPMI and 5% heat inactivated plasma. NET killing assays were performed as previously described [14], with the exception of adding 6uM PSMγ to NETs after 4 hours of PMA stimulation. PSMγ was allowed to bind to NETs for 1 additional hour, so that the total PMA stimulation time was 5 hours. Media was replaced from NETs, GAS NZ131 was

added, spun down for 10 minutes at 800g and incubated for 30 minutes. GAS was recovered and plated for CFU enumeration.

Fluorescence immunohistochemistry of NETs

Freshly isolated neutrophils in chamber slides were incubated with 25nM PMA in DMEM for 4 hours at standard tissue culture conditions to induce neutrophil extracellular trap formation. Media was carefully removed and replaced with media containing 5μM PSMγ for 1 hour. NHEKs, grown on chamber slides, were incubated with 1μM PSMγ for 1 hour. Neutrophils and NHEKs were fixed with 4% PFA for 20 minutes at room temperature, washed three time with 1x phosphate-buffered saline (PBS), blocked for 1 hour at room temperature with 3% bovine serum albumin in 1x PBS, and stained with primary antibodies mouse anti-PSMγ (Abcam), rabbit anti-LL-37, rabbit anti-CRAMP (Quality controlled biochemicals, QCB), rabbit anti-hBD2, and goat anti-hBD3 in 3% bovine serum albumin in 1x PBS overnight at 4°C. After washing the cells 3 times with PBS, the cells were stained with secondary antibodies goat anti-mouse IgG (Sigma), anti-rabbit IgG, and anti-goat IgG in 3% bovine serum albumin in 1x PBS for 1 hour at room temperature. Slides were mounted in ProLong Anti-Fade reagent (Molecular Probes).

***In vivo* mouse experiment**

The dorsal skin of wild-type C57BL/6 mice, 8-10 weeks old, was removed by shaving and Nair (a depilating agent). 4mm full thickness punch biopsies were performed on the hair-free dorsal skin. 5μL PBS or 5μL 16μM PSMγ was added to

wounds for 30 minutes. 5μL of $2x10^6$ CFU/ml was added to treated wounds for an additional 30 minutes. Wounds were harvested using a 6mm punch biopsy 18 hours post-infection. Wounds were placed in 1mL PBS with 1mm zirconia beads. Samples were bead-beated for 1 minute, placed on ice, and bead-beated for an additional minute. Supernatant was serial diluted and plated for CFU on Todd-Hewitt Agar (THA) as well as assayed for Mip-2 levels using ELISA kit following manufacturer's instructions (R&D Systems).

ACKNOWLEDGEMENTS

The text of this chapter, in full, is based on the manuscript Cogen AL, Yamasaki K, Muto J, Sanchez KM, Crotty-Alexander L, Tanios J, Lai Y, Kim JE, Nizet V, Gallo RL. *Staphylococcus epidermidis* antimicrobial phenol soluble modulins cooperate with host antimicrobial peptides to kill Group A *Streptococcus,* currently in preparation for journal submission. I was the primary researcher and author and the co-authors listed in this publication directed and supervised the research which forms the basis for this chapter.

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Chapter V.

Structure-Function Relationship of Phenol Soluble Modulin-γ

ABSTRACT

Phenol Soluble Modulin-γ (PSMγ), secreted by *Staphylococcus epidermidis*, is a 25-residue antimicrobial peptide. Unlike δ-lysin, a closely related peptide from *Staphylococcus aureus*, PSMγ is antimicrobial. To dissect the structure-function relationship of complex formation, membrane binding, disruption, and antimicrobial activity, we generated the wild-type peptide (MAA-25) and 7 analogs. The analogs include c-terminal deletions (MAA-21 and MAA-17), n-terminal deletions (IIS-21 and IGD-17), helix disruption by insertion of proline (MAA-25P), charge neutralization by replacing the lysines with alanines (MAA-25K), and polarity disruption by replacing the valines with asparagines (MAA-25V). We observed that all the analogs, unlike MAA-25, were unable to form complexes as measured by tryptophan emission. C-terminal deletion analogs (MAA-21 and MAA-17), charge neutralization analog (MAA-25K), and polarity disruption analog (MAA-25V) were impaired in their ability to bind lipid membranes and cause vesicle leakage. Finally, minimal inhibitory concentrations of the peptides against Group A *Streptococcus* and *Staphylococcus aureus* suggest that the c-terminus, charge and polarity are important for bacterial killing. These combined data display the structure-function relationship of antimicrobial peptide, PSMγ.

INTRODUCTION

Antimicrobial peptides exert activity through a variety of means, with the most common being membrane disruption. Membrane disruption has been described to occur through one of several mechanisms including the torroidal pore, barrel stave, or the carpet mechanism.[1] We have previously demonstrated that Phenol Soluble Modulin-γ (PSMγ) forms multimeric complexes, binds lipid membranes, disrupts synthetic vesicles, and exerts selective antimicrobial activity over multiple pathogens. In part due to the formation of multimeric complexes, we hypothesized that PSMγ forms pores through the barrel-stave mechanism.

Several characteristics affect antimicrobial activity and specificity. These include size, sequence, charge, structure, hydrophobicity, and amphipathicity.[1] Many antimicrobial peptides, like the cathelicidins, are cationic, alpha-helical amphipathic peptides[2] while others such as dermcidin[3] and maximin H5[4] are anionic. Antimicrobial peptides may also exhibit specific amino acid enrichment, such as tryptophan-rich indolicidin.[5]

Previous structural studies have been performed on *Staphylococcus aureus* (*S. aureus*) δ-lysin, a peptide similar to *Staphylococcus epidermidis* (*S. epidermidis*) PSMγ. δ-lysin, containing 26 residues has a primary sequence of MAQDISTIGDLVKWIIDTVNKFTKK, in contrast to the 25 residue PSMγ, with the primary sequence MAADISTIGDLVKWIIDTVKFKK. The studies on δ-lysin

suggested that the peptide segment IISTIGDLVKWIIDTV could be made antimicrobial if either or both D resides were replace with a K.[6] δ-lysin adopted an alpha-helical conformation with a deletion of up to 4 n-terminal residues.[7] In addition, Dhople et. al found that almost all the n-terminal truncated mutants lost their ability to aggregate, as measured by tryptophan fluorescence. Insertion of P to disrupt conformation, similarly disrupted the peptide's ability to aggregate.[7]

Since PSMγ, from *S. epidermidis,* contains sequence variations from δ-lysin and in its native form, is antimicrobial, we sought to determine the role of structure in the capacity to aggregate (form complexes), bind membranes, and kill bacteria. In this study, we construct a series of PSMγ analogs with n-terminal, c-terminal, polarity, charge and helical disruption. All the analogs lost their ability to aggregate. C-terminal truncations, charge and polarity disruption reduced lipid binding ability and capacity to induce vesicle leakage. Minimal inhibitory concentrations of the analogs on *S. aureus* and Group A *Streptococcus* (GAS) demonstrated a similar trend. Hence these studies show that PSMγ c-terminus, charge and polarity are critical for membrane binding, disruption, and antimicrobial activity.

RESULTS

Alterations in Phenol Soluble Modulin-γ sequence impair multimeric complex formation

We have previously demonstrated that PSMγ exerts antimicrobial activity over a variety of pathogens, including *S. aureus* and GAS. To further characterize and understand the structural components necessary for this activity, PSMγ analogs were constructed (Table 5.1). These synthetic analogs include truncations in the c-terminus (c-term deletion), truncations in the n-terminus (n-term deletion), insertion of a proline (helix disruption), insertion of asparagines in place of the valines (polarity disruption), and finally, insertion of alanines in place of the lysines (charge neutralization). Table 5.1 lists the analog sequences and highlights the target of the mutation.

To determine the primary sequence and secondary structure requirements in PSMγ complex formation, tryptophan spectroscopy was performed on the PSMγ analogs. In 20mM potassium phosphate buffer, wild-type PSMγ's tryptophan emits maximally at 339nm. As previously demonstrated, in the presence of urea, PSMγ's tryptophan's maximal emission shifts to 356nm. The low maximal emission of PSMγ in buffer indicates complex formation and shielding of the tryptophan from the aqueous environment. Unlike wild-type PSMγ (MAA-25), the analogs all display a maximal tryptophan emission between 354 and 356nm. This results in a 14-16 nm red shift (+14 - +16) from the wild-type peptide (Figure 5.1). This red shift indicates that

in the analogs, the tryptophan is not embedded but exposed to the aqueous buffer. Thus, the analogs have lost the ability to form complexes.

C-terminus and polarity are critical for Phenol Soluble Modulin-γ membrane interaction

Since mutations in PSMγ abrogated the ability of the peptides to form complexes in solution, we sought to determine whether the mutations rendered the peptides unable to bind lipid membranes. The wild-type PSMγ and analogs were incubated with 1mM 2:1 POPC:POPG lipid vesicles in potassium phosphate buffer. The maximal tryptophan emission was determined and compared to the maximal emission in buffer alone (Kpi). Surprisingly, many of the analogs retained their ability to interact with lipid membranes, despite an inability to form complexes. Yet, the c-terminus deletion analogs, MAA-21 and MAA-17, still exhibited a maximal tryptophan emission at 353 and 354 nm, respectively, even in the presence of vesicles. This indicates that the tryptophan is not embedded in the lipid membrane, but interacting with the aqueous environment (Figure 5.2). Similar results were seen with MAA-25V (polarity disruption) and MAA-25K (charge neutralization), which had maximal tryptophan emissions of 355 and 351 nm, respectively, in the presence of lipid membranes. The inability of MAA-21, MAA17, MAA-25V, and MAA-25 to blue shift in the presence of lipid vesicles indicates weak membrane interaction.

On the contrary, IIS-21 and IGD-17 (both n-terminal truncations) exhibited -12 nm and -10 nm, respective blue shifts in tryptophan maximal emissions, in the

presence of vesicles (Figure 5.2). This indicates that mutations in the n-terminus did not significantly impair peptide-membrane interactions. Furthermore, MAA-25P, containing a proline to disrupt the alpha-helix, retained the ability to bind lipid membranes. This seems surprising considering the expected importance of secondary structure to membrane activity. Overall, these data suggest that the c-terminus (containing the charged positive lysines) and polarity of PSMγ are important for lipid membrane interactions.

Disruptions in c-terminus and polarity of Phenol Soluble Modulin-γ cause reduced vesicle leakage

Membrane binding of antimicrobial peptides is presumed imperative for membrane perforation. To verify the role of membrane interaction in pore formation, we investigated the ability of the PSMγ analogs to cause vesicle leakage. POPC:POPG vesicles, encapsulating a fluorophore (ANTS) and quencher (DPX) were incubated with PSMγ analogs or triton X, as a positive control. Leakage was determined by evaluating the maximal fluorescent emission of ANTS at 518nm. Figure 5.3 displays ANTS emission of vesicles incubated with 16μM PSMγ analogs after 1 hour. Maximal intensity (CPS) of ANTS emission at 518nm was determined for each sample. The intensities were normalized such that vesicles alone (without disruption) caused 0% leakage, while vesicles incubated with triton X caused 100% leakage. Vesicles were incubated with 16μM PSMγ analogs (Figures 5.3 and 5.4) or with 32μM PSMγ analogs (Figure 5.5).

At 16μM, MAA-21, MAA-17, MAA-25V, and MAA-25K caused 35.28%, 38.42%, 17.34%, and 38.42% leakage, respectively (Figure 5.4). Similarly, at 32μM, MAA-21, MAA-17, MAA-25V, and MAA-25K caused 70.73%, 61.22%, 59.16%, and 89.30% leakage, respectively (Figure 5.5). In contrast, IIS-21, IGD-17, and MAA-25P induced significant vesicle leakage, comparable to MAA-25 at both 16 and 32μM (Figure 5.4 and 5.5). These data demonstrate that the c-terminal deletions, charge disruptions and polarity reduce the ability of PSMγ to cause vesicle leakage.

Phenol Soluble Modulin-γ analogs have altered antimicrobial activities over pathogens

To evaluate the functionality of PSMγ's structure and sequence on antimicrobial activity, the PSMγ analogs were tested for their ability to kill pathogens GAS and *S. aureus*. GAS and *S. aureus* were incubated with increasing concentrations of PSMγ (MAA-25) or analogs for 24 hours. The minimal bactericidal concentration (MBC) was determined for each analog.

For GAS, the only PSMγ analogs that retained activity were IIS-21 and IGD-17, with MBCs of 16-32 and 32μM respectively (Table 5.2). These concentrations are similar to the MBC of the wild-type PSMγ (MAA-25), which was found to be 16μM. All the other analogs had no bactericidal activity at tested concentrations up to 64μM. In contrast to GAS, *S. aureus* was moderately susceptible to all the analogs. Yet, the MBCs of MAA-21, MAA-17, and MAA-25V were 32μM, a 4-fold increase over the

MBC of MAA-25 (8μM). IIS-21, IGD-17, MAA-25P, and MAA-25K had MBCs of 8μM, 16μM, 8-16μM, and 8μM, respectively. Hence, similar to the vesicle leakage data, the c-terminus, charge and polarity are important for antimicrobial activity.

DISCUSSION

The structures of antimicrobial peptides have been inevitably linked to their functions, antimicrobial or otherwise.[7,8] Here, we have similarly demonstrated that the membrane-interactive antimicrobial activity of PSMγ is dependent on several important structural features. Specifically, alterations in the c-terminus, charge, and polarity result in a lack of membrane binding, vesicle leakage, and reduction of antimicrobial activity.

Interestingly though, the pathogens GAS and *S. aureus* show marked differences in their susceptibilities to the PSMγ analogs. At concentrations up to 64μM, GAS showed susceptibility only to analogs IIS-21 and IGD-17, with n-terminal truncations. The order of MBCs, MAA-25<IIS-21<IGD-17, is potentially explained by the secondary structural differences. N-terminal truncations of *S. aureus* δ-lysin showed a correlation between the deleted number of n-terminal residues and alpha-helical character. The δ-lysin analog n-22 in Dhople et al, similar to PSMγ analog IIS-21, retained alpha-helical character, but lacked complex formation, as measured by circular dichroism and tryptophan emission, respectively. Yet, n-18, similar to IGD-17, showed only minimal alpha-helical character.[7] Although we have not measured the secondary structure of the PSMγ analogs, we hypothesize the occurrence of a similar trend. The increase in MBC of IGD-17 >IIS-21>MAA-25 is likely due to the lack of complex formation and reduced secondary structure formation of each analog. Similar increases in MBC were seen with *S. aureus*. These data indicate that alpha-helical

structure in solution and complex formation is only marginally influential on antimicrobial activity.

The other PSMγ analogs showed no effect on GAS survival, yet retained varied activity against *S. aureus*. The analogs with the highest MBCs (at 32μM) were MAA-21, MAA-17, and MAA-25V. Unlike, GAS, antimicrobial activity against *S. aureus*, was not significantly affected by alterations of charge (MAA-25K) or helix disruption (MAA-25P). This is interesting since MAA-21 deletes the last 4 amino acids: KKFK. The retention of antimicrobial activity of MAA-25K over MAA-21 suggests an important role of the phenylalanine. More surprising, though, was the reduction of activity of MAA-25V. This analog, with asparagines in place of the valines, resulted in polarity disruption and subsequent diminution of antimicrobial activity. The polarity disruption, as evidenced by tryptophan spectroscopy in Figure 5.2, abrogates the ability of the peptides to interact with membranes. This lack of interaction explains the reduced antimicrobial activity.

The mechanism by which the analogs exert antimicrobial activity is notably linked to membrane-binding capacity. The peptides with the highest MBCs (MAA-21, MAA-17, MAA-25V, and MAA-25K) do not exhibit significant, if any shifts in their maximal tryptophan emission. The lack of membrane interaction is mirrored in the vesicle leakage studies (Figure 5.3-5.5), where the same analogs minimally disrupt the synthetic vesicles. Intriguingly, but consistently, MAA-25P, the analog containing a

helical disruption through insertion of a proline, was able to bind membranes, cause vesicle leakage, and kill *S. aureus* (though not GAS).

The combined data demonstrate several phenomena. The first is that antimicrobial activity is pathogen-specific. Second, the ability of peptides to lyse membranes does not fully explain antimicrobial activity. In fact, membrane activity is not universal but due to more complex components, such as pathogen capsule, appendages, and antimicrobial peptide inactivators. Third, complex formation affects membrane binding and antimicrobial activity to a small degree. Although, occasionally variable, these studies do demonstrate that antimicrobial activity is closely and predictably linked to membrane binding. In the case of PSMγ, the c-terminus, charge, and polarity are the most important components for activity.

In the future, we hope to investigate the role of structure in formation of heterologous complex formation with host antimicrobial peptides, described in Chapter IV. Understanding structure-function relationships will provide novel insight into the mechanism of action and will ultimately aid in drug design.

MATERIALS AND METHODS

Peptides

Phenol Soluble Modulin-γ (MAA-25) was commercially synthesized and purified by HPLC (Quality controlled biochemicals). PSMγ purity was >95%. PSMγ analogs (MAA-21, MAA-17, IIS-21, IGD-17, MAA-25P, MAA-25V, and MAA-25K) were commercially synthesized and purified by HPLC (21st Century Biochemicals) to a purity of ~70%.

Vesicle preparation and leakage assay

POPC (1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine, Avanti Polar Lipids) and POPG (1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)], Avanti) were combined at a 2:1 molar ratio of POPC:POPG, dried under argon gas, resuspended by bath sonication in 20mM potassium phosphate buffer, pH 7.3 or in 20mM potassium phosphate buffer, pH 7.3, 50mM DPX (*p*-xylene-bis-pyridinium bromide) and 50 mM ANTS (8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt). Vesicles were extruded through 0.2 um polycarbonate film and run over a size exclusion column to remove unencapsulated dye. Leakage assays were performed by incubating vesicles (1:5 dilution) with desired concentration of peptide for 1 h. Conditions for ANTS fluorescence were: excitation wavelength of 385 nm, emission wavelength of 400-700 nm, excitation and emission bandpass of 5 nm, 1 nm/step scan speed, and integration time of 0.2 s.

Spectroscopic measurements

Synthetic peptide, at 5μM, were incubated in 20mM potassium phosphate, pH 7.3 in the presence or absence of 1mM POPC:POPG vesicles described above. Fluorescence measurements were taken on a Jobin Yvon SPEX FL3-11 spectrofluorometer equipped with an R928 photomultiplier tube. Conditions for tryptophan fluorescence are as follows: excitation wavelength of 290 nm, emission excitation and emission bandpass of 8nm and 4nm, respectively, 1 nm steps, and an integration time of 1s.

Antimicrobial assay

Minimal bactericidal concentration (MBC) assays for *Staphylococcus aureus* 113 were performed as before in the presence of carbonate.[9] For Group A *Streptococcus* (GAS) experiments were performed in 25% THB, 75% 1x dulbecco's phosphate buffered saline. Bacteria were incubated with peptides for 24 hours at 37°C and plated for CFU on Todd-Hewitt agar (THA). MBCs were performed twice in duplicate.

ACKNOWLEDGEMENTS

I would like to thank Katheryn Sanchez and Judy Kim for their experimental guidance, technical advice, and use of equipment for the tryptophan spectroscopy studies.

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Chapter VI.

Phenol Soluble Modulins from *Staphylococcus epidermidis* induce antimicrobial peptide production in keratinocytes

ABSTRACT

Antimicrobial peptides (AMPs) function to directly kill bacteria and alarm the immune system of injury or infection. AMPs induce cytokine expression and wound healing in the form of cell proliferation and migration. Phenol Soluble Modulins (PSMs) from *Staphylococcus epidermidis* (*S. epidermidis*) have also been shown to elicit a pro-inflammatory and in some cases TLR2 dependent response. Here, we investigate the ability of PSMs to stimulate normal human epidermal keratinocytes (NHEKs) to produce antimicrobial peptides hBD (human β-defensin) 2 and 3. Synthetic PSMγ and PSMδ induced dose-dependent hBD2 and hBD3 transcript. Immunohistochemistry of PSM-stimulated NHEKs similarly showed increased hBD3 levels of intracellular protein. Moreover, *S. epidermidis* WT supernatants induced significantly more hBD2 and hBD3 in NHEKs than *S. epidermidis Δpsmδ* supernatants. In the presence of toll-like receptor (TLR) 2 agonists, PSMγ synergistically enhanced hBD2 and hBD3 transcript levels in NHEKs. PSMs induced minimal TLR2 transcript levels in NHEKs, not accounting for the synergistic induction. These data demonstrate that *S. epidermidis* PSMs induce an antimicrobial response in keratinocytes, suggesting a role for PSMs in the cutaneous innate immune defense.

INTRODUCTION

Antimicrobial peptide (AMP) production by keratinocytes has been shown to be an important shield against pathogenic infections. Cathelicidins are key AMPs in combating Group A *Streptococcus* (GAS) infections,[1] while the hBDs are hypothesized to be critical antimicrobial agents against *Staphylococcus aureus* (*S. aureus*).[2] In addition to the host AMPs, we have previously demonstrated that the Phenol Soluble Modulins (PSMs) derived from *Staphylococcus epidermidis* (*S. epidermidis*) exert antimicrobial action over a variety of pathogens. Furthermore, we have also demonstrated in Chapter IV that PSMγ is deposited by *S. epidermidis* in the epidermis and dermis of normal human skin. Thus, we have hypothesized that *S. epidermidis* and its PSMs form an additional antimicrobial barrier in the skin.

In addition to exerting antimicrobial activity, AMPs act as "alarmins" through modulation of the host's response.[3] Specifically, human β-defensins (hBDs) 1-3 are chemotactic for cells such as T cells, mast cells, and neutrophils, whereas hBD3 and hBD4 are chemotactic for monocytes and macrophages.[4] In keratinocytes, AMPs also signal through G-protein coupled receptors[5] and activates EGFR resulting in cytokine production and cell proliferation, respectively.[6] Thus, AMPs are multifaceted, exhibiting both antimicrobial and immuno-modulatory activities.

PSMs, like host AMPs, elicit a host response and exert antimicrobial activity. Previous studies demonstrated that PSMs derived from *S. epidermidis* activated the

respiratory burst of human neutrophils and monocytes, stimulated neutrophil degranulation, and functioned as a chemoattractant factor for both neutrophils and monocytes.[7] Moreover, isolated PSM complexes were found to activate NF-κB in THP-1 cells and induce TNFα, IL-1β, and IL-6 release in THP-1 cells and human monocytes.[8] Finally, Hajjar AM, et. al., demonstrated that toll-like receptor (TLR) 2 confers PSM complex responsiveness in HEK293 cells.[9]

Since TLR2 mediates the epithelial antimicrobial response[10] and because the PSMs have been shown to elicit a pro-inflammatory response in the host through TLR2, we hypothesized that the *S. epidermidis* PSMs induce keratinocyte production of AMPs. Here, we find that both PSMγ and PSMδ induce hBD2 and hBD3 transcript in keratinocytes, dose-dependently. Immunohistochemistry also exhibited an increase in the level of intracellular protein upon stimulation with the PSMs. Moreover, *S. epidermidis* WT supernatants induced significantly more hBD2 and hBD3 in NHEKs than *S. epidermidis Δpsmδ* supernatants. In the presence of TLR2 agonists, PSMγ synergistically induced hBD2 and hBD3 transcript levels. Yet, PSM-stimulated NHEKs showed only minimal increases in TLR2 transcript levels. Hence, PSMs increase the antimicrobial barrier through increased production of AMPs in keratinocytes.

RESULTS

Synthetic Phenol Soluble Modulins increase keratinocyte production of antimicrobial peptides.

Previous work demonstrated that *S. epidermidis* is able to induce keratinocyte production of antimicrobial peptides hBD2 and hBD3 through TLR2 and p38 MAPK. In addition, PSMs have been shown to induce TNFα through TLR2.[9] Thus, we sought to determine if PSMs were able to increase expression of hBD2 and hBD3 in normal human epidermal keratinocytes (NHEKs).

NHEKs, stimulated for 24 hours with increasing concentrations of PSMs, were evaluated by qPCR for hBD2 and hBD3 transcript level, as compared to the housekeeping gene, GAPDH. Both PSMγ and PSMδ were able to dose-dependently increase hBD2 and hBD3 mRNA transcript levels (Figure 6.1). hBD2 was maximally induced by the PSMs at a concentration of 0.25μM (250nM). In contrast, hBD3 transcript was dose-dependently increased by both PSMs, with maximal induction occurring at the highest concentration evaluated, 1μM.

To determine if the synthetic PSMs could increase hBD3 protein in keratinocytes, NHEKs, in chamber slides, were stimulated for 24 hours with 500nM PSMδ or 1μM PSMγ. Both PSMs were able to increase intracellular staining of hBD3, as compared to the untreated control (Figure 6.2).

To confirm the role of PSMδ from *S. epidermidis* in hBD2 and hBD3 induction in NHEKs, an allelic exchange mutant of PSMδ was created. In the *S. epidermidis* chromosome, *psmδ* was directly replaced by *cat* (gene encoding chloramphenicol acetyl transferase), resulting in Δ*psmδ* (Figure 6.3). NHEKs were stimulated with vehicle (THB), *S. epidermidis* WT, or Δ*psmδ*. For hBD2 and hBD3, *S. epidermidis* WT supernatant was able to induce expression by roughly 8.2 and 4.5 fold, respectively. *S. epidermidis* Δ*psmδ* supernatant induced significantly less hBD expression (Figure 6.3). These combined data indicate that PSMs are able to induce an antimicrobial response in keratinocytes. Hence, *S. epidermidis* and PSMs may play a role in host defense through induction of antimicrobial peptides.

Antimicrobial peptide induction was enhanced by toll-like receptor 2 activation.

It has been previously reported that the PSM complex from *S. epidermidis* signals through TLR2 and induces TNFα in RAW cells.[9] To determine whether TLR signaling plays a role in hBD induction in NHEKs by PSMs, NHEKs were stimulated with PSMγ in the presence or absence of TLR agonists. qPCR for hBD2 and hBD3 revealed that a substimulatory concentration of PSMγ (100nM) synergistically increased expression in the presence of the TLR2 ligands (Figure 6.4). Specifically, PSMγ enhanced hBD2 and hBD3 induction by Pam3csk4 (TLR 2/1 agonist) and Malp-2 (TLR2/6 agonist) (Figure 6.5). This indicates that PSMγ amplifies NHEK sensitivity to TLR2 ligands Malp-2 and Pam3csk4 and allows for a greater innate

immune response to bacterial products. Thus, PSMγ may sensitize, enable, and "prime" the skin to respond to potential pathogens.

Phenol Soluble Modulins modestly increase TLR2 transcript levels in keratinocytes.

One potential mechanism by which PSMγ increases TLR2 responsiveness in NHEKs is through increasing the amount of TLR2 receptor, allowing for greater TLR signaling. To determine if PSMs are able to upregulate TLR2, NHEKs stimulated with increasing concentrations of the PSMs were evaluated for TLR2 transcript level by qPCR. The data demonstrate a small increase in TLR2 transcript as compared to GAPDH for both PSMs (Figure 6.6). Similarly, *S. epidermidis* WT supernatants increased TLR2 transcript level to a greater degree than *S. epidermidis* Δ*psmδ* supernatant (Figure 6.7). Although the PSMs did affect TLR2 transcript levels, the increases were modest. PSM induction of TLR2 is likely not the mechanism by which the peptides synergistically induce the hBDs in the presence of TLR agonists.

DISCUSSION

AMPs are known to play an important role in host defense against pathogens. In this study, our aim was to determine if the PSMs produced by *S. epidermidis*, previously shown to be present in the epidermis and dermis, are able to amplify the cutaneous innate antimicrobial response through AMP induction. Here we showed that synthetic PSMs were able to induce hBD2 and hBD3 in a dose-dependent manner. Simulations of NHEKs with bacterial supernatants (WT and Δ*psmδ*) confirmed the importance of PSMδ in *S. epidermidis'* ability to induce AMPs. In the presence of TLR agonists, PSMγ synergistically induced expression of hBD2 and hBD3, while only minimally increasing TLR2 transcript abundance. Thus, PSM induction of TLR2 transcript is likely not the mechanism by which the synergy occurs.

Other potential mechanisms through which PSMγ synergistically activates the hBDs include upregulation of TLR2 adapter proteins. Vitamin D, shown to exert a similar synergist effect, has been found to increase expression of CD14.[11] Another means by which PSMγ synergistically acts is through activation of downstream events in the TLR or other related pathways. A final possibility is PSMγ membrane interactions allowing for activation and potential migration of TLR complexes.

We hypothesize that *S. epidermidis* and products, such as the PSMs, allow for enhanced keratinocyte recognition and response during a pathogen insult. Though, it is not known whether hBD2 and hBD3 are able to kill *S. epidermidis*. Unlike GAS and

S. aureus, *S. epidermidis* is not susceptible to the PSMs at concentrations assayed. Thus, we similarly hypothesize that *S. epidermidis* resists killing by the hBDs. In the future, we hope to determine the relative susceptibility of *S. epidermidis* to host AMPs, which if resistant, partially describes the bacteria's continued colonization on the skin's surface.

This study illustrates an additional mechanism by which *S. epidermidis* protects the skin and functions as a component of the cutaneous innate immune system. In addition to providing an intrinsic antimicrobial barrier, the PSMs are able to activate the innate immune system to further ward off invasive pathogens.

MATERIALS AND METHODS

Peptides

PSMδ and PSMγ were commercially synthesized and purified by HPLC (Quality controlled biochemicals). PSMγ purity was >95% and PSMδ purity was >70%. mCRAMP and LL-37 were commercially synthesized and purified by HPLC to a purity >95% as previously described. [5]

Allelic replacement

PSMδ deletion in *S. epidermidis* 1457 was performed by allelic replacement using the procedure described, with the only significant modifications being incorporation of the Gateway cloning system (Invitrogen).[12] ~800bp upstream and downstream segments were amplified using chromosome specific primers. In-frame deletion of *psmδ* and incorporation of *cat* was confirmed by PCR.

Cell Culture

Normal human epidermal keratinocytes (NHEK; Cascade Biologics) were grown in EpiLife medium (Cascade Biologics) supplemented with 0.06 mM $CaCl_2$, 1% EpiLife defined growth supplement, and penicillin/streptomycin (100 U/ml and 50μg/ml, respectively). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. Cells were stimulated at 60-70% confluency for 24 hours with PSMs or 10KDa ultra-filtrate of stationary phase *S. epidermidis* supernatants.

Quantitative Real-time PCR

After cell stimulation, total RNA was extracted using TRIzol (Invitrogen), and 1μg of RNA was reverse transcribed using iScript (Bio-Rad). The transcript abundance of hBD2, hBD3, and cathelicidin was evaluated on an ABI 7300. For hBD2 and hBD3, predeveloped Taqman assay probes were used (Applied Biosystems, ABI). For GAPDH (glyceraldehydes-3-phosphate dehydrogenase), used as housekeeping gene, primers and probes were used as previously described.[13] All analyses were performed in triplicate in at least 2 independent cell stimulation experiments. Fold induction relative to the vehicle-treated control was calculated as previously described.[11]

Fluorescent Immunohistochemistry

NHEKs were seeded in chamber slides at 40-50% confluency. Cells were stimulated with 500nM PSMδ or 1uM PSMγ for 24 hours. Cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature. After subsequent washings in phosphate-buffered saline (PBS), slides were blocked for 1 hour at room temperature with 3% bovine serum albumin (BSA) in PBS. Slides were stained with rabbit anti-hBD3 antibody (Orbigen) or rabbit IgG in blocking solution at 4°C overnight. After multiple washings with PBS, slides were incubated with Alexa-fluor 568 labelled rabbit anti-mouse antibody in blocking solution for 1 hour at room temperature in the dark. Slides were washed with PBS and mounted in ProLong anti-fade reagent containing 4', 6-diamidino-2-phenylindole (Molecular Probes, Eugene OR) and evaluated with an Olympus BX41 microscope (Olympus, Melville, NY).

ACKNOWLEDGEMENTS

I would like to acknowledge and thank Yuping Lai for her scientific and intellectual contributions to this work. This work stems from her previous studies on the role of *S. epidermidis* in inducing an antimicrobial response in the skin. I would also like to thank Jeffrey Locke for his guidance while creating the allelic exchange mutant.

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Chapter VII.

Conclusions and Perspectives

Introduction

Staphylococcus epidermidis (*S. epidermidis*) is a member of the commensal skin microflora that has been previously unexplored for its beneficial effects on the human host. Under normal circumstances, *S. epidermidis* resides innocuously on and within the stratum corneum. When the host is immunocompromised or receiving an implant, such as a catheter, *S. epidermidis* can cause superficial or invasive infections. Many virulence factors utilized by *S. epidermidis* to cause disease have been significantly studied, as detailed in Chapter I. In contrast, we aim to study the beneficial role of the resident *S. epidermidis*. The following summarizes the data from the previous Chapters II through VI.

Purification of antimicrobial peptides from *S. epidermidis*

It has previously been shown that antimicrobial peptides play an important role in host defense against pathogens.[1] To determine if *S. epidermidis* may provide a similar antimicrobial shield, we evaluated the bacteria's ability to inhibit pathogens such as Group A *Streptococcus* (GAS). We found that *S. epidermidis* was able to inhibit GAS *in vitro*: in liquid culture and on an agar plate. Furthermore, on human and mouse skin, pre-population with *S. epidermidis* significantly reduced GAS survival. These data demonstrate that the presence of *S. epidermidis* on the skin benefits the host by reducing GAS survival.

In order to determine which of the molecules produced by *S. epidermidis* are responsible for GAS inhibition, the *S. epidermidis* supernatant was fractionated and

purified by HPLC and a C_{18} column. Hydrophobic components of the *S. epidermidis* supernatant were first bound to the C_{18} column. A gradient of acetonitrile was used to elute and fractionate the bound molecules. Fractions were assayed for anti-GAS activity using a radial diffusion assay. The active fraction eluted at 72% acetonitrile. MALDI TOF-TOF sequencing revealed the presence of two *S. epidermidis* peptides in the active fraction: Phenol Soluble Modulin-γ (PSMγ) and Phenol Soluble Modulin-δ (PSMδ).

In summary, this work elucidated a unique function for *S. epidermidis* on the skin. Here, we found that the bacterium prevents survival of GAS and produces antimicrobial peptides PSMγ and PSMδ, which may protect the skin from infection.

Antimicrobial activity of Phenol Soluble Modulins

To determine whether the PSMs that were isolated from *S. epidermidis* displayed characteristics common to many antimicrobial peptides, synthetic peptides were evaluated for their secondary structure, ability to interact with membranes and form complexes. Both PSMγ and PSMδ were α-helical in the presence and absence of lipid membranes, as measured by circular dichroism. The peptides' α-helical character was altered slightly by the presence of vesicles indicating membrane interaction. Tryptophan spectroscopy of PSMγ demonstrated that the peptide forms complexes and strongly interacts with lipid membranes. Addition of PSMδ to PSMγ caused a secondary structure shift of PSMγ, indicating further heterologous complex formation.

To ascertain whether the PSMs have antimicrobial activity, vesicle leakage and minimal bactericidal concentration assays were performed on a variety of bacteria. The vesicle leakage assays confirmed the ability of the PSMs to lyse membranes in a dose-dependent manner. This was further confirmed by the antimicrobial assays. The PSMs were able to kill *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli)*, but not *S. epidermidis*. Electron microscopy illustrated selective disruption of *S. aureus* but not *S. epidermidis* membranes, after treatment with PSMγ. Since *S. epidermidis* normally lives on the skin, we determined whether keratinocytes were susceptible to PSM-induced membrane leakage, like the host peptide LL-37. We found that both PSMγ and LL-37 caused dose-dependent membrane perforation of normal human keratinocytes, as measured by propidium iodide uptake.

Since the skin contains antimicrobial peptides such as those in the cathelicidin family, we also found that the PSMs could cooperate with LL-37 to kill GAS. Finally, on mouse skin explants the PSMs were able to significantly reduce GAS survival. In conclusion, these combined data display a novel role for *S. epidermidis* PSMs as antimicrobial peptides able to cooperate with the host and kill pathogenic bacteria *in vitro* and *ex vivo*. Moreover, these data suggest that *S. epidermidis*, rather than an innocuous resident organism, participates in host defense and adds to the antimicrobial barrier of the skin.

Phenol Soluble Modulins cooperate with host antimicrobial peptides

Since we showed that the *S. epidermidis* PSMs were antimicrobial peptides, which could form complexes and cooperate with host antimicrobial peptide LL-37, we sought to explore the interaction of the PSMs with the host. *S. epidermidis* resides on the skin and presumably produces and secretes the PSMs. We hypothesized that the PSMs provide a protective antimicrobial layer and thus, we investigated whether normal human facial skin contained PSMγ. In fact, we found that PSMγ was deposited in the epidermis and sparsely in the dermis.

In the dermis, there exist resident fibroblasts as wells as circulating neutrophils and mast cells. Neutrophils, containing a large amount of antimicrobial peptides kill bacteria through phagocytosis, degranulation, and neutrophil extracellular trap (NET) formation.[2] Upon formation of NETs, the neutrophil DNA casts a web punctuated with antimicrobial peptides. With our previous data illustrating that PSMs cooperate with LL-37 and are capable of forming antimicrobial complexes we hypothesized that PSMγ would interact with the antimicrobial NETs, contributing to host defense. We found that PSMγ bound neutrophil DNA (using tryptophan spectroscopy) and also co-localized with LL-37 on NETs. Moreover, PSMγ was also able to induce NET formation, similar to PMA.

Addition of PSMγ to NETs resulted in greater GAS killing, which was subsequently eliminated by DNase. In whole blood, the addition of PSMγ rendered GAS bacteriostatic. Although multiple mechanisms are likely responsible for the

ability of PSMγ to reduce survival of GAS in these scenarios, we conjectured that PSMγ was combining with host AMPs to kill the bacteria. This was supported by the co-localization of PSMγ with LL-37 in the NETs and data from Chapter II showing that PSMγ could cooperate with host AMPs to kill GAS. Here, we further demonstrated that PSMγ could cooperate with other host AMPs, including human β-defensin (hBD) 2, 3, and CRAMP.

Antimicrobial peptide complex formation occurs between peptides of the same and different species. Such formation is thought to aid in antimicrobial synergy. Since PSMγ is deposited in the epidermis and dermis by *S. epidermidis*, co-localizes with LL-37 in NETs, and cooperates with host AMPs to kill GAS, we evaluated the physical interaction of PSMγ with the host AMPs. Co-immunoprecipitations and tryptophan spectroscopy confirmed that PSMγ binds to host AMPs LL-37, CRAMP, hBD2, and hBD3. Finally, to determine whether PSMγ could augment the killing of GAS in the host, PSMγ was added to mouse wounds that were later challenged with GAS. GAS recovery from mouse wounds treated with PSMγ was significantly reduced as compared to wounds treated with phosphate-buffered saline.

Here we demonstrate that PSMγ from *S. epidermidis* interacts with the antimicrobial peptides produced by the host. This interaction leads to an increase in antimicrobial defense and further supports the innate immune function of the resident cutaneous *S. epidermidis*.

Structure-function relationship of Phenol Soluble Modulin-γ

The amino acid sequence of antimicrobial peptides has been shown to play an important role in their ability to exert antimicrobial activity over pathogens.[3,4] Specific factors, such as secondary structure, polarity, charge, and length, influence peptide-membrane binding, complex formation, and membrane-disruption. Here, we created a small library of PSMγ analogs that include truncations in the n and c terminus, helix disruption via insertion of a proline, charge neutralization, and polarity disruption. We evaluated the ability of the peptides to form complexes, interact with lipid membranes, cause vesicle leakage, and finally, kill pathogens such as GAS and *S. aureus*.

Interestingly, using tryptophan spectroscopy we found that all the analogs lacked the ability to form multimeric complexes, as compared to the WT peptide, MAA-25. Yet, many of the peptides retained their ability to interact with membranes, with the exception of the c-terminal truncation analogs (MAA-21 and MAA-17), polarity disruption analog (MAA-25V), and to a lesser extent, the charge neutralization analog (MAA-25K). The vesicle leakage assay showed a similar trend, where MAA-21, MAA-17, MAA-25V, and MAA-25K caused significantly less leakage than the WT peptide, MAA-25, and the other analogs. Lastly, we evaluated the minimal bactericidal concentration (MBC) of the analogs against *S. aureus* and GAS. For GAS, the only analogs, other than the WT peptide (MAA-25), that were able to kill the bacteria at the concentrations tested, were IIS-21 and IGD-17, with n-terminal deletions. For *S. aureus*, all the peptides retained at least some activity, with

the most bactericidal being MAA-25, IIS-21, IGD-17, MAA-25K, and MAA-25P. MAA-21, MAA-17, and MAA-25V were the least potent with MBCs of 32μM.

These data demonstrate that antimicrobial activity directly correlates with membrane binding and disruption, rather than bacterial inhibition at the genetic level. PSMγ's antimicrobial activity depends on polarity and the c-terminal residues, rather than on complex formation and α-helical character. Further experiments need to be performed to validate the analogs' structural characteristics. Overall, here we show that the structure is imperative for PSMγ's antimicrobial activity.

Induction of host antimicrobial peptides by Phenol Soluble Modulins

Antimicrobial peptides (AMPs) are known to function as both bactericidal agents as well as immunomodulators. For example, cathelicidins act through the G-coupled protein receptor[3] and potentially others, which have not been discovered as of yet. *S. epidermidis* PSMs have similarly been shown to induce NF-κB signaling and cytokine release from monocytes.[5,6] Since NF-κB signaling can result in human β-defensin (hBD) induction, we sought to determine if the PSMs could further enhance the cutaneous innate immune response by inducing keratinocyte production of AMPs.

To determine if the PSMs could induce AMP expression in keratinocytes, cells were stimulated with increasing doses of the PSMs. In fact, hBD2 and hBD3 were dose-dependently up-regulated upon stimulation. Keratinocytes stimulated with *S. epidermidis* WT supernatants but not *S. epidermidis Δpsmδ* supernatants showed a

significant increase in hBD2 and hBD3 expression, confirming that PSMδ was in part responsible for *S. epidermidis* hBD induction.

Previous studies have implicated a role for toll-like receptor (TLR) 2 in hBD induction as well as PSM-induced cytokine production. Thus, we sought to determine the role of TLR2 in hBD induction by PSMγ. Keratinocytes were stimulated with TLR ligands in the presence or absence of PSMγ. PSMγ synergistically enhanced hBD induction in the presence of TLR2 ligands, Malp2 and Pam3csk4. Yet, PSMγ only marginally increased TLR2 expression, suggesting that PSMγ amplified the TLR2 signal through an unknown mechanism.

In summary, *S. epidermidis* PSMs induce an antimicrobial peptide response in human keratinocytes. PSMγ is also able to synergistically enhance hBD production in the presence of a TLR2 ligand. This suggests that the presence of PSMs on the skin allows the keratinocytes to respond more robustly to a pathogen, inducing the TLR2 pathway. Thus, the PSMs contribute to the innate immune response of the skin by amplifying the cutaneous innate immune response.

Conclusions

In recent years, our understanding of bacteria:host relationships has evolved. Historically, bacteria and other microorganisms have been thought commensal or parasitic by nature. Yet, evidence of the gut microflora substantiates the mutually beneficial relationship that some organisms have with the human host. Like the gut,

the skin harbors a diverse and abundant colony of microorganisms, which include viruses and bacteria, among others. Here, we have investigated the beneficial role of *S. epidermidis,* yet this bacterium is merely one of many cutaneous microbes that likely provide benefit. The understanding gleaned from these and other studies will hopefully advance our global appreciation of the complex cutaneous niche. Additionally, such research will ideally lead to novel therapies for not only infection but also for aberrant immune responses, as microorganisms are now thought to educate and regulate the host's immune system. Finally, this mutual relationship between the host and microbe redefines the innate immune system to include the resident cutaneous beneficial flora.

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APPENDIX

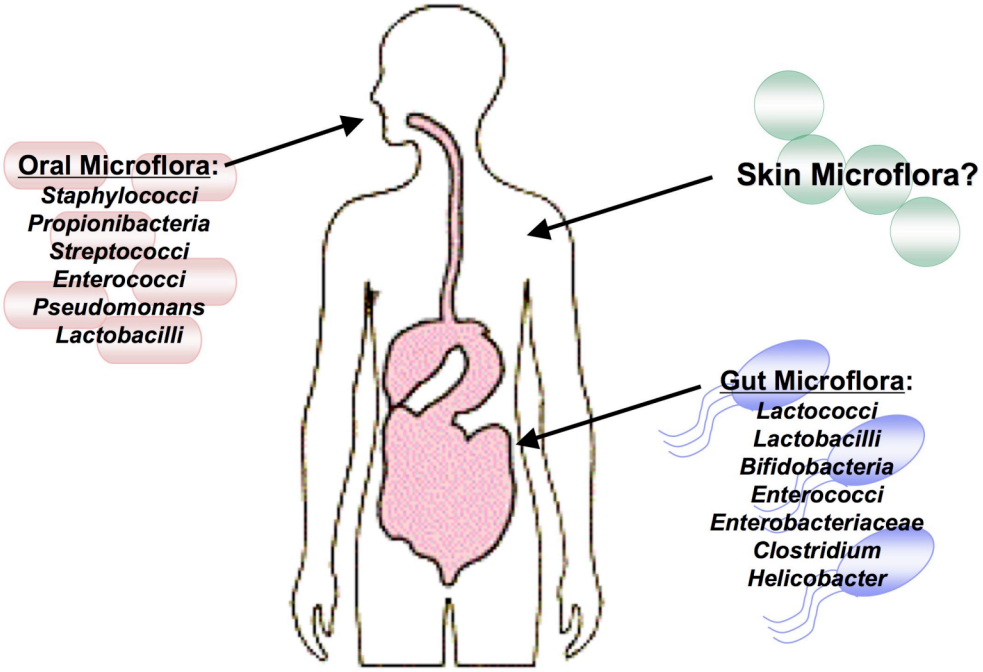


Figure 1.1. Resident microflora that are beneficial to the host. The gut and mouth contain many species of microflora. Microbiota in the intestines protect the host by educating the immune system and preventing pathogenic infections. These microflora benefit the systemic immune system of the host and positively affect other organs, such as the lung. In the mouth, over 500 species of bacteria protect the mucosa from infections by preventing colonization of dangerous yeasts and other bacteria. It is yet unclear if the microflora of the skin play a similar role in protecting the host. Image from http://www.giconsults.com with permission.

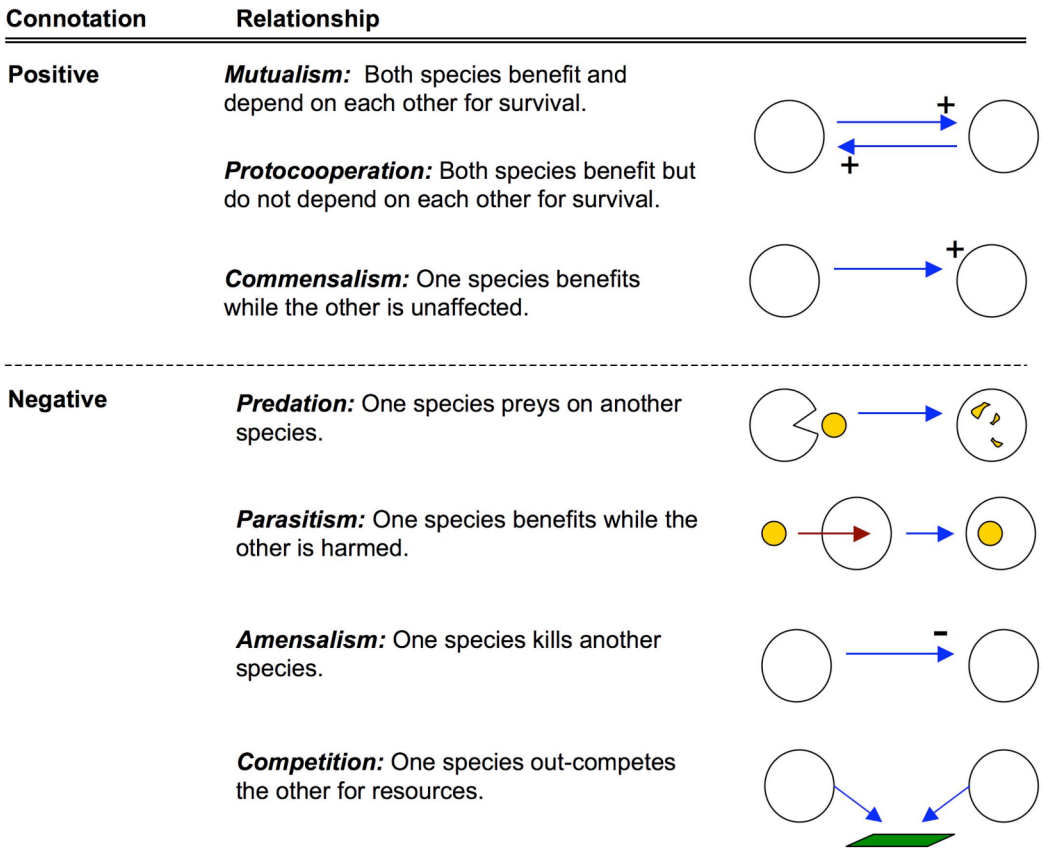


Figure 1.2. Types of symbiotic relationships.

Table 1.1. Frequency of microbial colonization through clinical and molecular detection methods. Data obtained from Blaser et. al., and Dekio et. al.

Organism	Clinical Isolate Observations	Molecular Detection
Staphylococcus epidermidis	Common, occasionally pathogenic	Frequent
Staphylococcus aureus	Infrequent, usually pathogenic	Frequent
Staphylococcus warneri	Infrequent, occasionally pathogenic	Occasional
Streptococcus pyogenes	Infrequent, usually pathogenic	Occasional
Streptococcus mitis	Frequent, occasionally pathogenic	Frequent
Propionibacteria acnes	Frequent, occasionally pathogenic	Frequent
Corynebacteria sp.	Frequent, occasionally pathogenic	Frequent
Acinetobacter johnsonii	Frequent, occasionally pathogenic	Frequent
Pseudomonas aeruginosa	Infrequent, occasionally pathogenic	Frequent

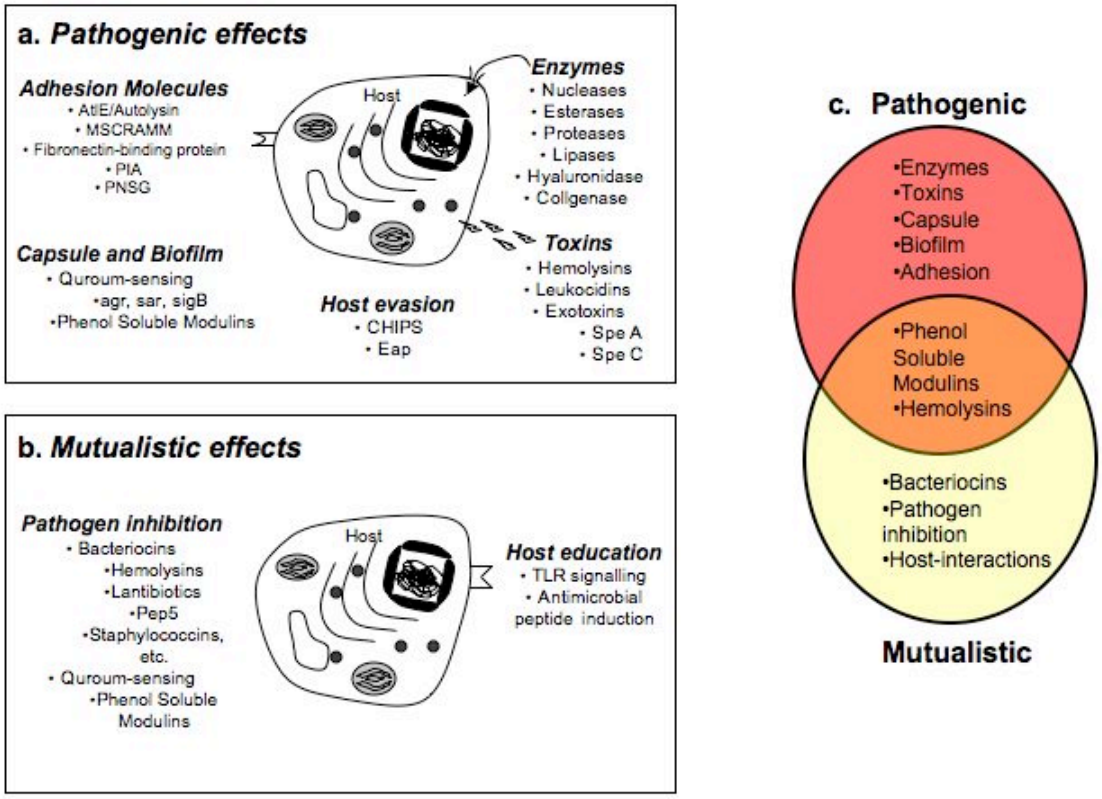


Figure 1.3. *Staphylococci* are pathogenic and mutualistic. a, Virulence factors and molecules produced by *Staphylococci* that aid in pathogenesis. b, *Staphylococci* act mutually by inhibiting pathogens and priming the immune response. c, Molecules from *Staphylococci* molecules that have dual functions.

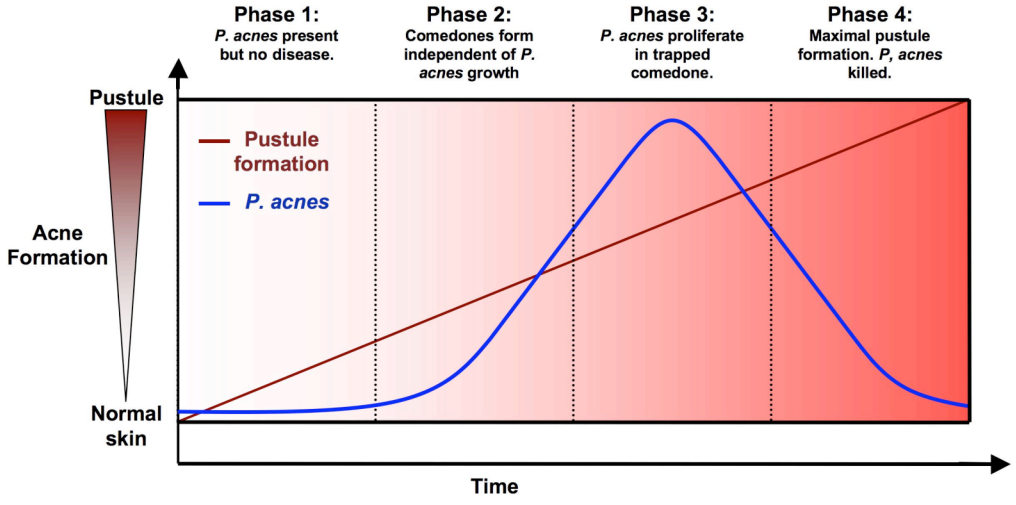


Figure 1.4. Hypothetical model for relationship between *Propionibacteria acnes* and pustule formation. Graph depicts pustule formation and *P. acnes* growth over time. In phase 1, *P. acnes* is present, but comedones are not. In phase 2, comedone formation begins, independently of *P. acnes* growth. *P. acnes* begin to proliferate only after condone forms. In phase 3, *P. acnes* proliferate in trapped comedone. In phase 4, *P. acnes* is killed by inflammatory response. Disease and pustule formation is maximal despite eradication of *P. acnes*. This model illustrates that acne formation is not triggered by the ubiquitous and resident *P. acnes* and at the maximal disease stage, *P. acnes* has already been eliminated.

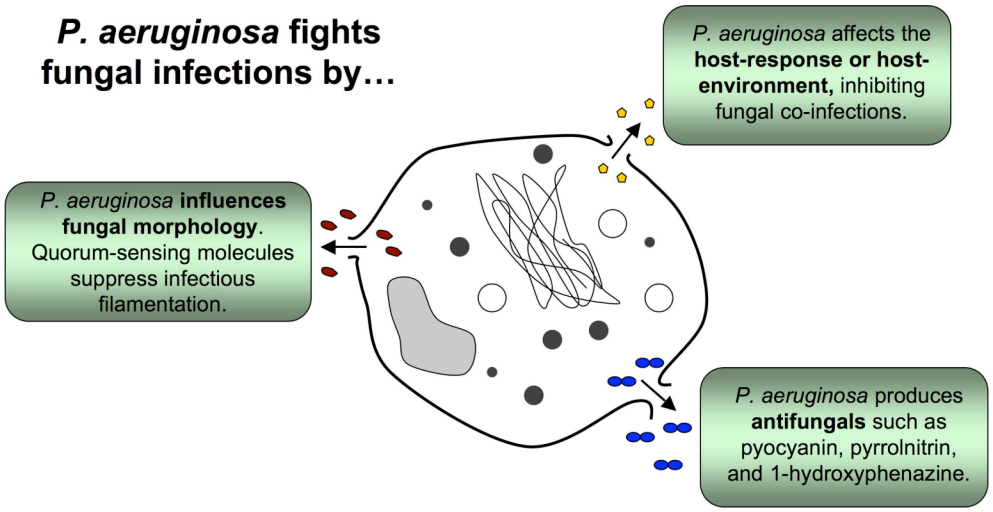


Figure 1.5. *Pseudomonas aeruginosa* fights fungal infections. *P. aeruginosa* produces compounds such as pyocyanin, pyrrolnitrin, and 1-hydroxyphenazine that kill and inhibit fungal growth. *P. aeruginosa* also prevents the morphological transition of fungi from yeast-form cells to virulent filamentous cells. Filamentation of *Candida albicans* is associated with pathogenesis, adhesion, invasion, and virulence-related products. *P. aeruginosa* interact with the host creating an environment inhospitable to fungi.

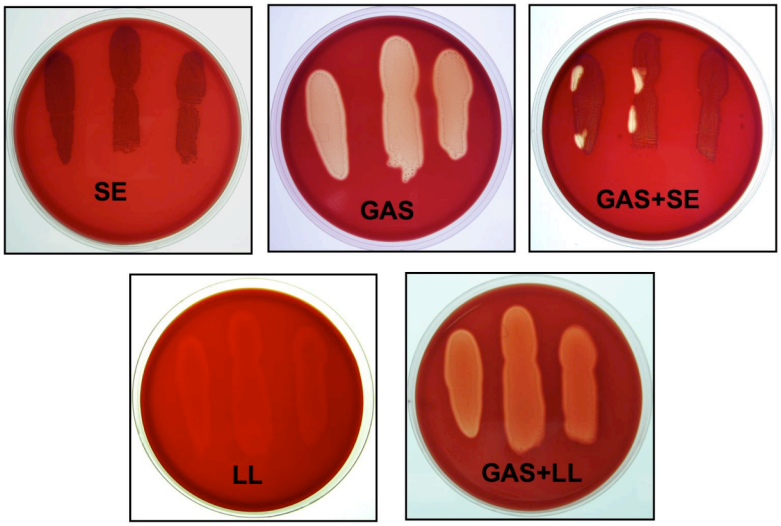


Figure 2.1. *Staphylococcus epidermidis* prevents Group A *Streptococcus* survival on human fingers. Cleaned human fingers were treated with *S. epidermidis* (SE), Group A *Streptococcus* (GAS), GAS+SE, *L. lactis* (LL) or GAS+LL. SE reduced GAS survival on human fingers.

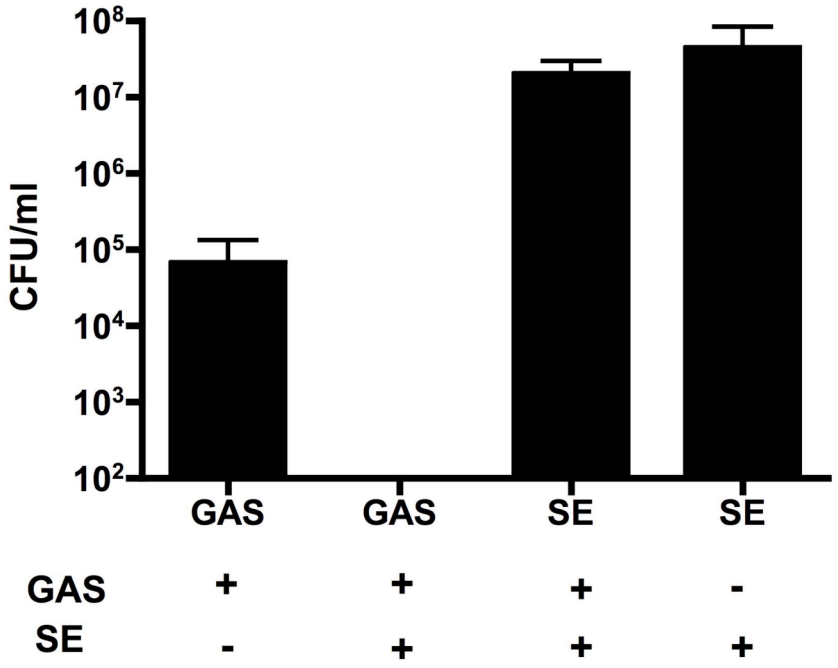


Figure 2.2. *S. epidermidis* reduces GAS survival on mouse skin. Mouse explants treated with GAS or SE as shown in table below graph. X-axis denotes microbe evaluated for CFU/ml.

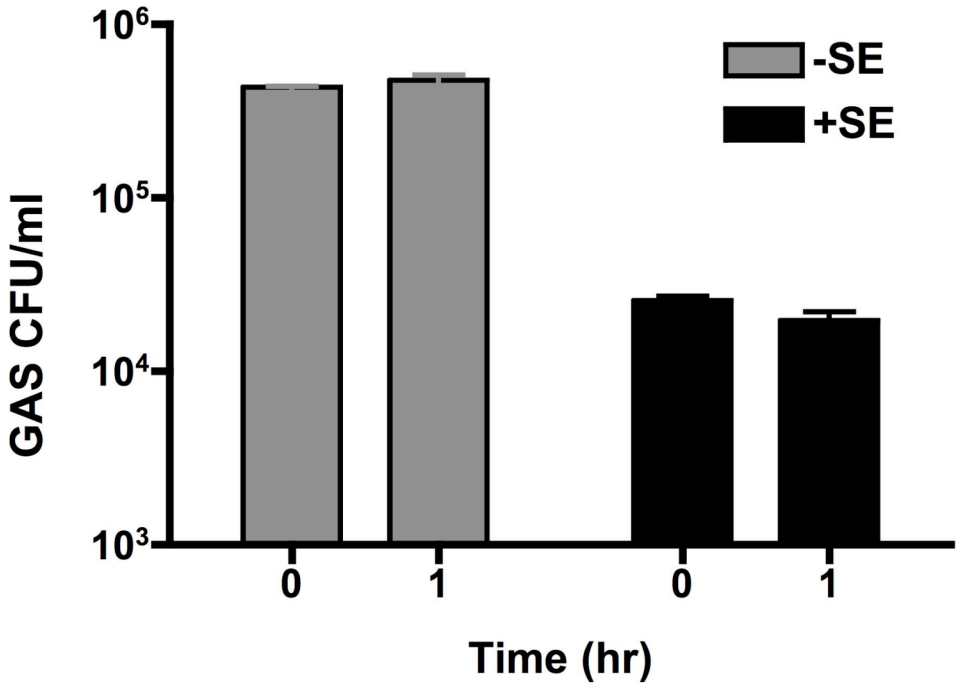


Figure 2.3. *S. epidermidis* supernatant reduces GAS survival on human forearm skin. Human forearm skin was treated with *S. epidermidis* (+SE) supernatant or media control (-SE). Supernatant or media control was removed and GAS added to skin at 0 or 1 hour after treatment. GAS was recovered and enumerated for CFU/ml.

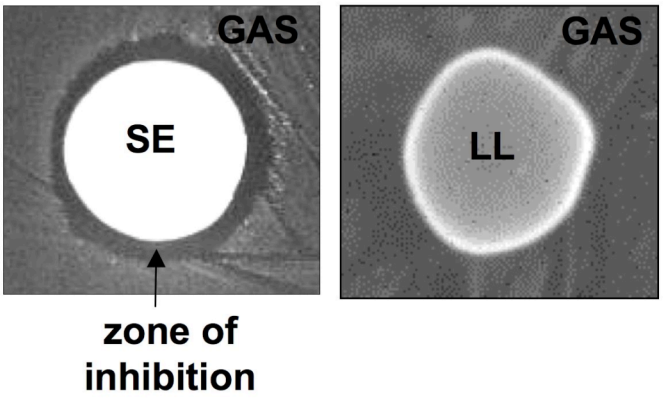


Figure 2.4. *S. epidermidis* inhibits GAS on agar. *S. epidermidis* (SE) or *L. lactis* (LL) was plated on top of a GAS lawn on THA.

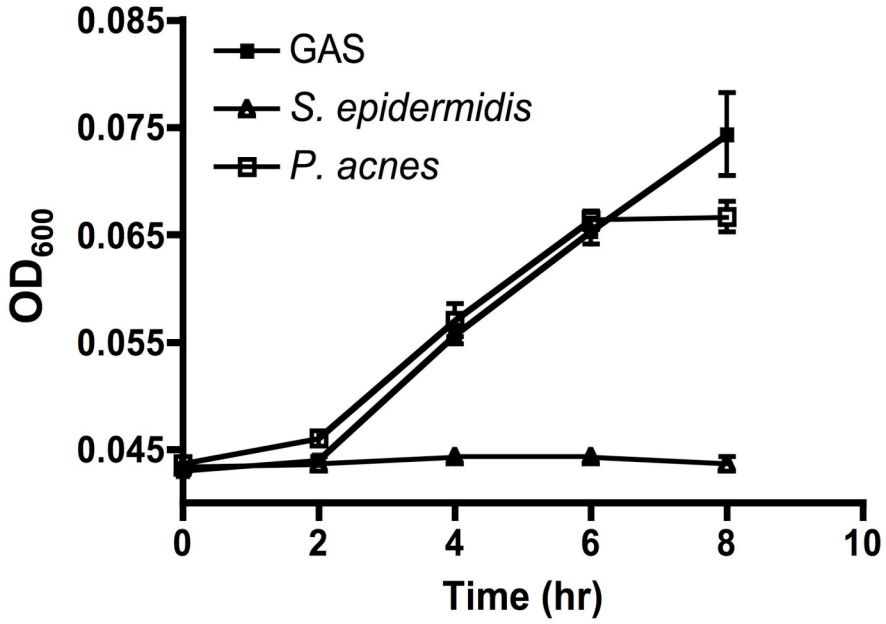


Figure 2.5. *S. epidermidis* supernatant inhibits GAS *in vitro*. GAS was grown in stationary phase cell-free culture supernatants of *S. epidermidis, P. acnes,* and GAS. GAS growth was measured for OD_{600}.

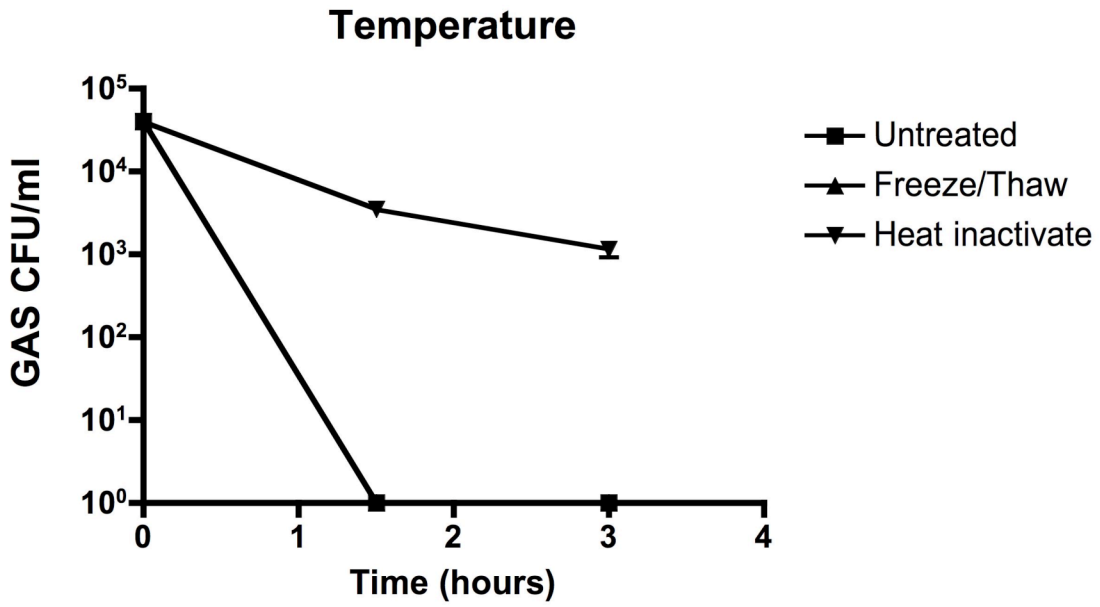


Figure 2.6. *S. epidermidis* supernatant antimicrobial activity was minimally by termperature. *S. epidermidis* supernatants retained anti-GAS activity after freeze/thaw cycle but lost partial activity activity after heat inactivation at 65°C for 30 minutes.

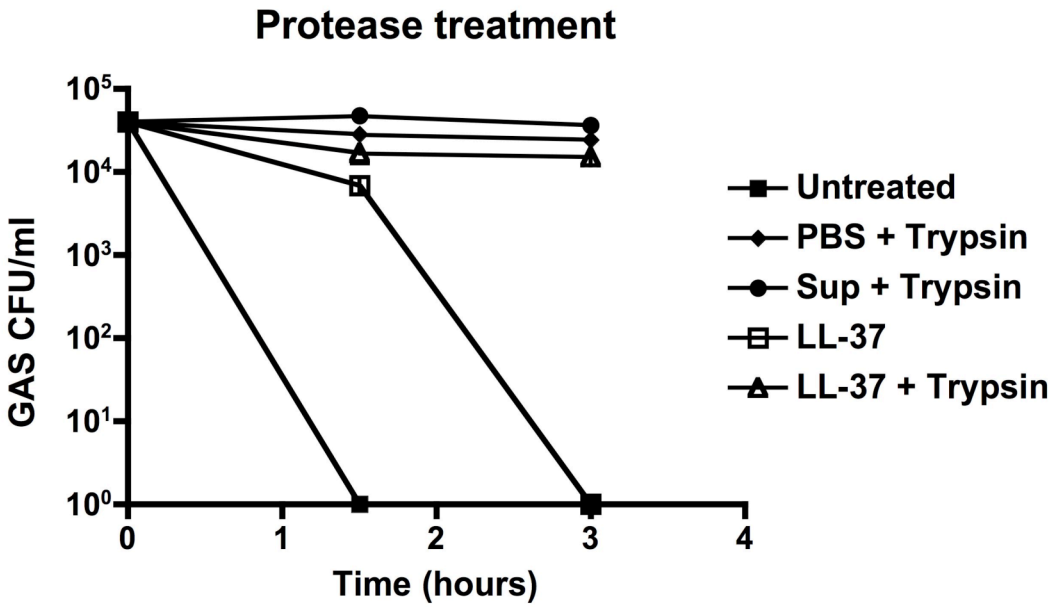


Figure 2.7. *S. epidermidis* supernatant antimicrobial activity was susceptible to trypsin digestion. *S. epidermidis* supernatants lost activity after treatment with trypsin. Similar results were seen with LL-37.

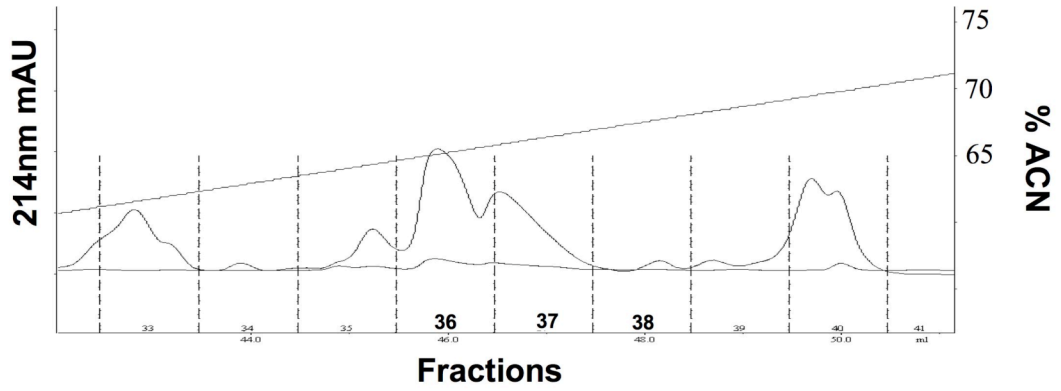


Figure 2.8. 214nm trace after HPLC purification of *S. epidermidis* supernatants. Supernatants were fractionated using HPLC. Protein content was determined by absorption at 214nm. The % acetonitrile (ACN) is labelled on the right hand side of graph.

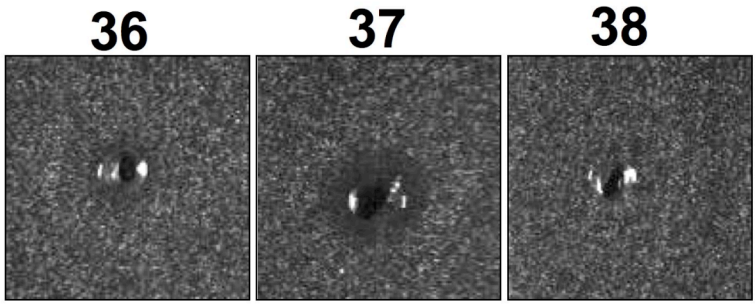


Figure 2.9. Radial diffusion assay of fractions 36, 37, and 38. Inhibitory zone in fraction 37 demonstrates presence of antimicrobial molecules.

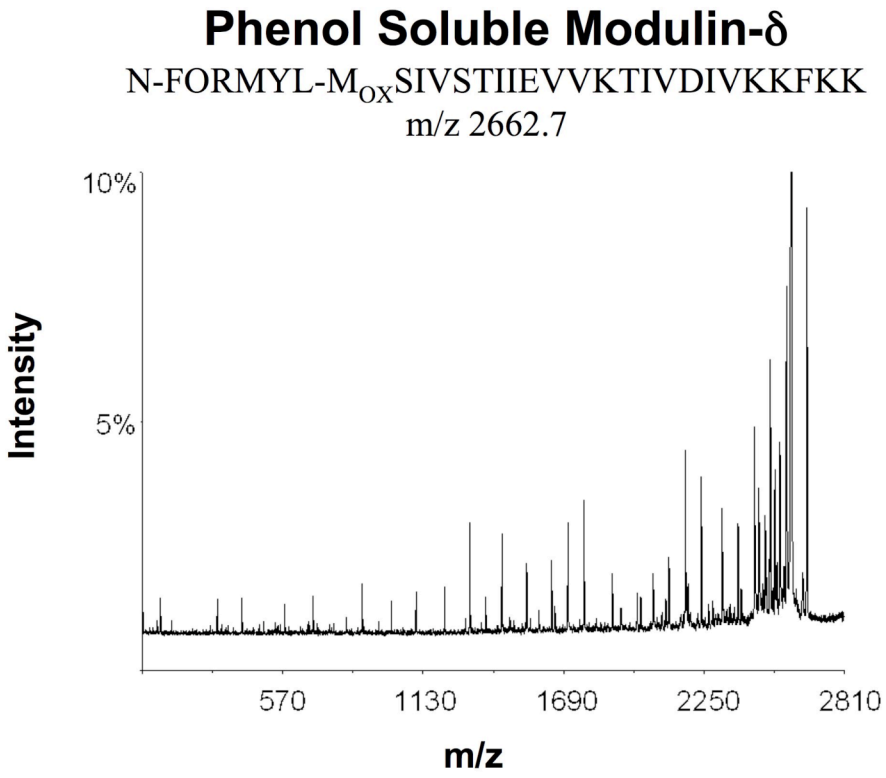


Figure 2.10. Phenol Soluble Modulin-δ was present in active fraction 37. The presence of PSMδ in fraction 37 was determined by MALDI TOF-TOF sequencing.

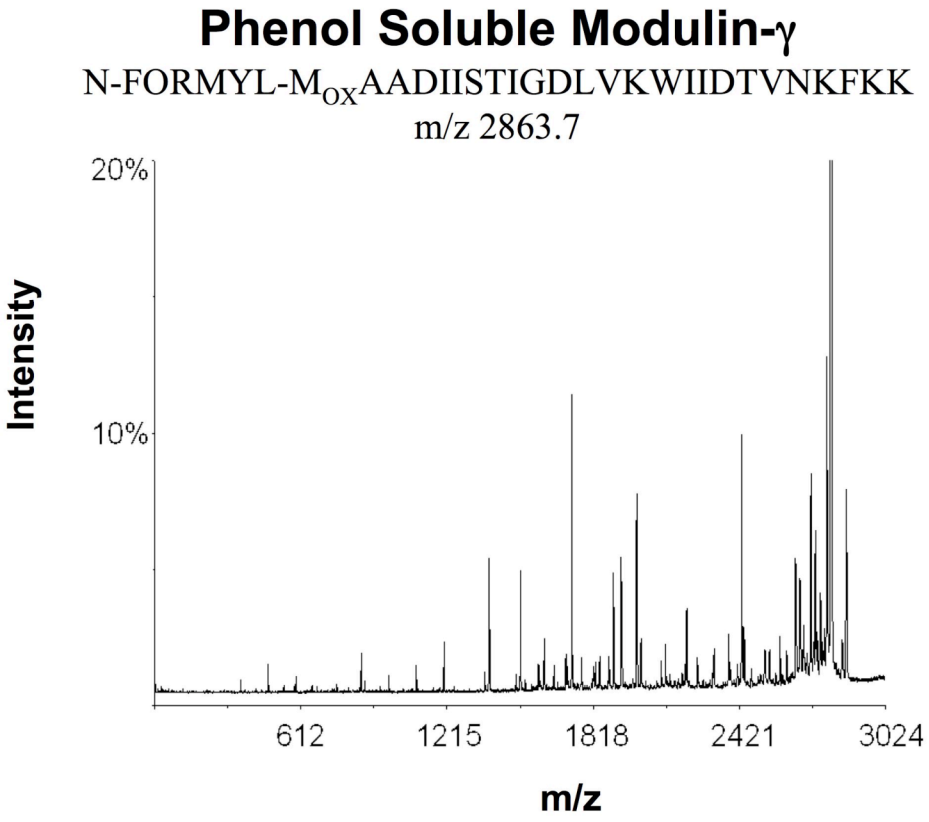


Figure 2.11. Phenol Soluble Modulin-γ was present in active fraction 37. The presence of PSMγ in fraction 37 was determined by MALDI TOF-TOF sequencing.

Table 2.1 Sequences of δ-lysin from *S. epidermidis* and *S. aureus.*

	Sequence
S. epidermidis	MAADIISTIGDLVKWIIDTVNKFKK
S. aureus	MAQDIISTIGDLVKWIIDTVNKFTKK

a

Peptide	Sequence
Phenol Soluble Modulin γ	5'-MAADIISTIGDLVK**W**IIDTVNKFKK
Phenol Soluble Modulin δ	5'-MSIVSTIIEVVKTIVDIVKKFKK

b

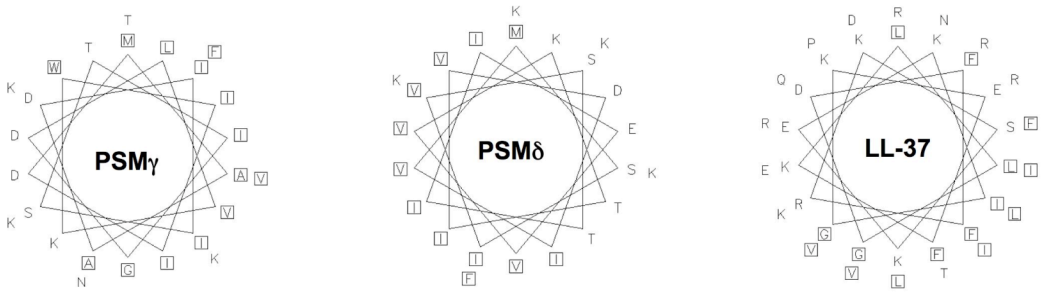


Figure 3.1. Phenol Soluble Modulins have structural similarities to host antimicrobial peptides. a, Sequences of PSMγ and PSMδ, highlighting the tryptophan in PSMγ. b, Helical wheel plots show sequestration of hydrophobic residues for PSMγ, PSMδ, and LL-37.

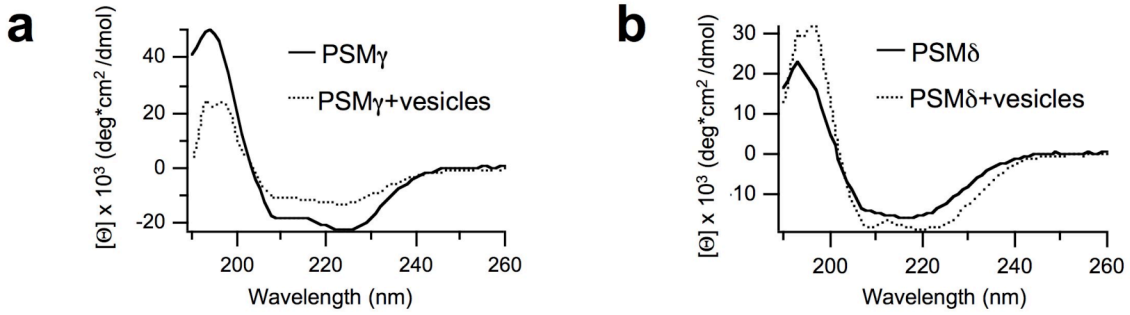
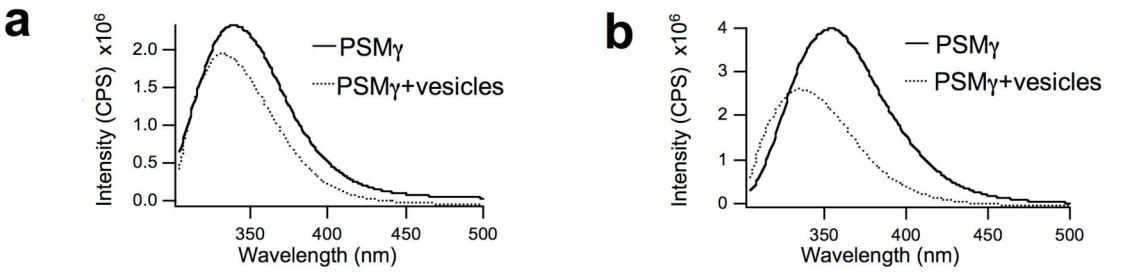


Figure 3.2. The secondary structure of Phenol Soluble Modulins change in the presence of lipid membranes. Circular dichroism spectra of 20mM PSMδ (a) or PSMγ (b) in the presence and absence of 1mM 2:1 POPC:POPG lipid vesicles show α-helical structure and structural changes of PSMδ and PSMγ in the presence of lipid vesicles.

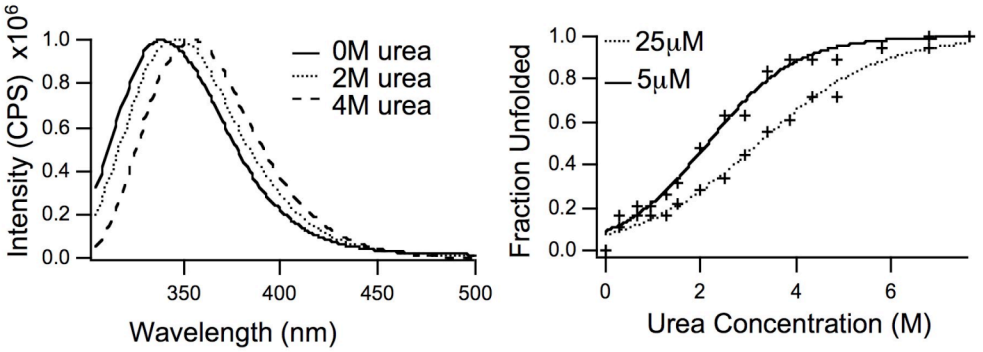


c

	Maximum Wavelength	Shift
PSMγ, 20mM Kpi	339 nm	-
PSMγ + POPC:POPG vesicles	332 nm	-7 nm
PSMγ, 5.5M Urea	355 nm	+16 nm
PSMγ + POPC:POPG vesicles, 5.5M Urea	335 nm	-4 nm

Figure 3.3. Phenol Soluble Modulin-γ interacts with lipid membranes. Tryptophan fluorescence spectra of PSMγ in the presence and absence of 1mM 2:1 POPC:POPG vesicles Kpi (a) or in the presence of 5.5M urea (b). c, Table displaying the maximum emission wavelength of PSMγ's tryptophan. POPC:POPG vesicles in Kpi cause a blue shift in the tryptophan's maximal emission indicating embedding of PSMγ in the lipid membrane.

a



b

PSMγ (μM)	Slope, m (kcal*M^{-1}*mol^{-1})	Midpoint, C_m (M)	$\Delta G^{o}_{(H2O)}$ (kcal*M^{-1})
5	0.67	2.14	1.43
25	0.49	3.20	1.57

Figure 3.4. Phenol Soluble Modulin-γ forms multimeric complexes. a,Tryptophan spectra of 5, 10 and 25mM PSMγ Kpi. Tryptophan shift in PSMγ in the presence and absence of vesicles and urea. b, Table of slope, midpoint, and ΔG°(H20) from unfolding curves of PSMγ at 5 and 25mM. Increased concentrations of PSMγ results in a shift of the ΔG°(H20) from 1.43 to 1.57 kCal/mol which indicates greater stability and complex formation.

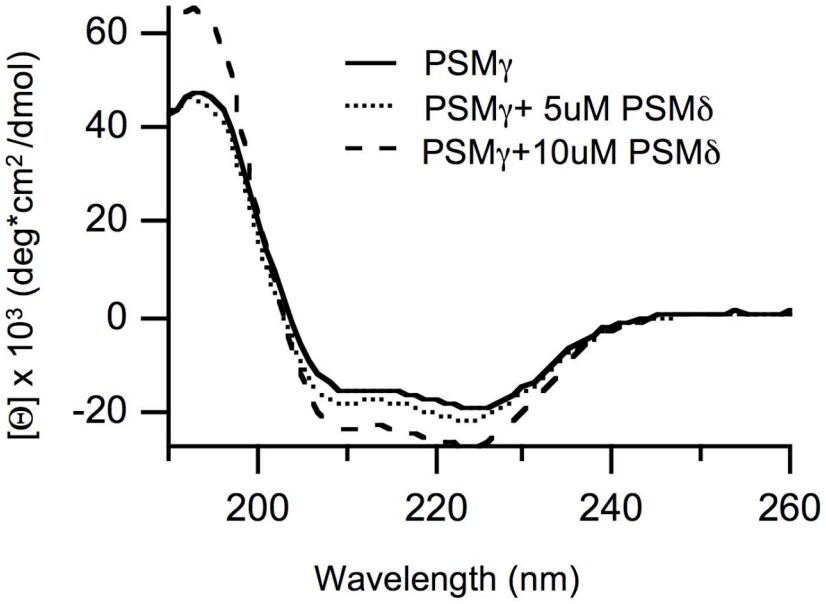


Figure 3.5. Phenol Soluble Modulin-γ interacts with Phenol Soluble Modulin-δ. Circular dichroism spectra of 20mM PSMγ in the presence and absence of 5 or 10mM PSMδ. PSMδ curves were subtracted from PSMγ curves.

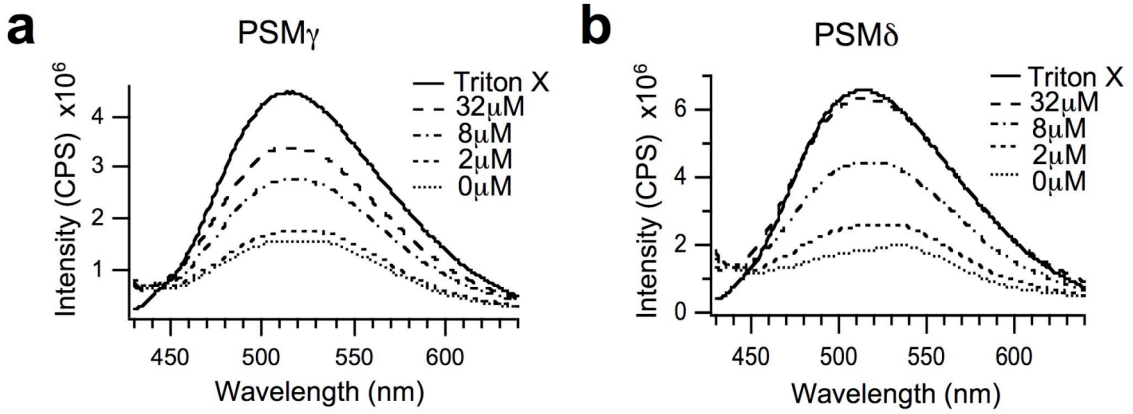


Figure 3.6. Phenol Soluble Modulin-γ interacts with Phenol Soluble Modulin-δ induce vesicle leakage. Synthetic vesicles encapsulating ANTS (fluorophore) and DPX (quencher) show dose-dependent fluorescence (leakage) in the presence of increasing concentrations of a, PSMγ or b, PSMδ.

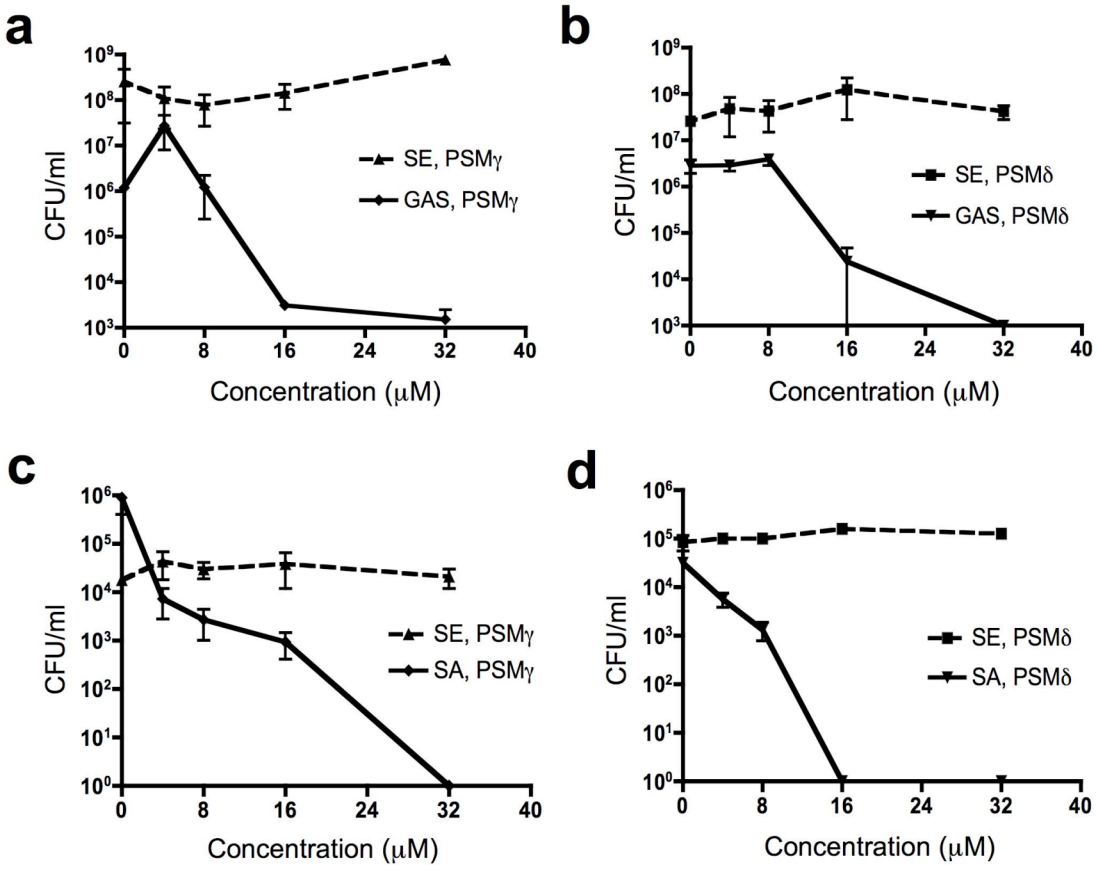


Figure 3.7. Phenol Soluble Modulins selectively kill pathogens. Both PSMs exhibit selective dose-dependent inhibition. GAS, but not *S. epidermidis* (SE) is susceptible to a, PSMγ and b, PSMδ. Similarly, c, PSMγ and d, PSMδ exert selective antimicrobial killing with *S. aureus* (SA) but not SE.

Table 3.1. Minimal bactericidal concentrations (MBCs) of Phenol Soluble Modulins.

Phenol Soluble Modulin γ	**MBC**
Staphylococcus epidermidis, 12228	>64μM
Staphylococcus epidermidis, 1457	>64μM
MRSA, Sanger 252	>64μM
MRSA, USA 300	>64μM
Staphylococcus aureus, 113	4-8μM
Streptococcus pyogenes, NZ131	16μM
Escherichia coli	8-16μM
Phenol Soluble Modulin δ	
Staphylococcus epidermidis, 12228	>64μM
Staphylococcus epidermidis, 1457	>64μM
MRSA, Sanger 252	16-32μM
MRSA, USA 300	32μM
Staphylococcus aureus, 113	8μM
Streptococcus pyogenes, NZ131	16μM
Escherichia coli	8μM

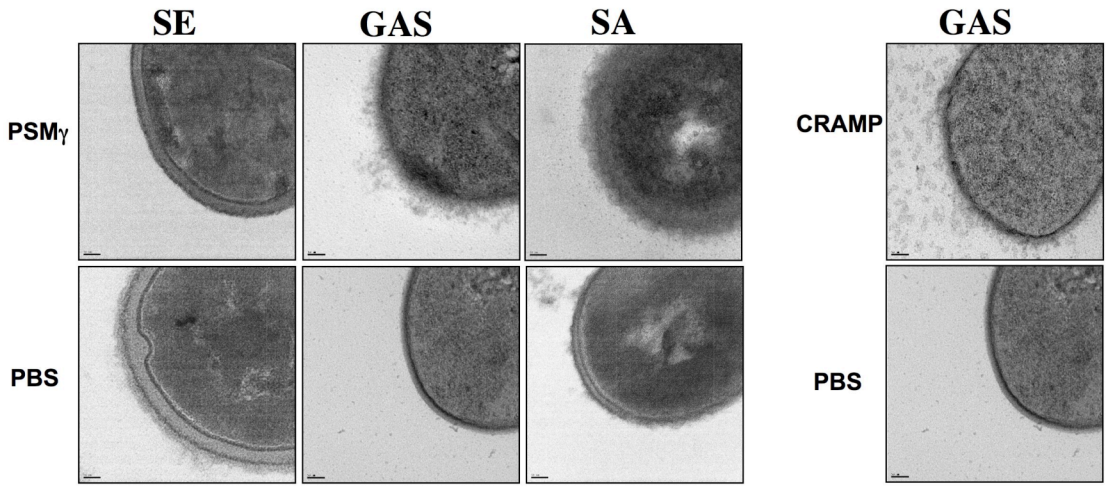


Figure 3.8. Phenol Soluble Modulin-γ causes selective membrane disruption. TEM analysis of SE, GAS, and SA membranes after incubation with PBS, PSMγ, or CRAMP (with GAS only). GAS and SA, but not SE showed membrane blebbing after incubation with PSMγ. The effect of PSMγ on GAS membranes was similar to blebbing that occurred after incubation with CRAMP. Images at 30,000x and scale bars represent 50nm.

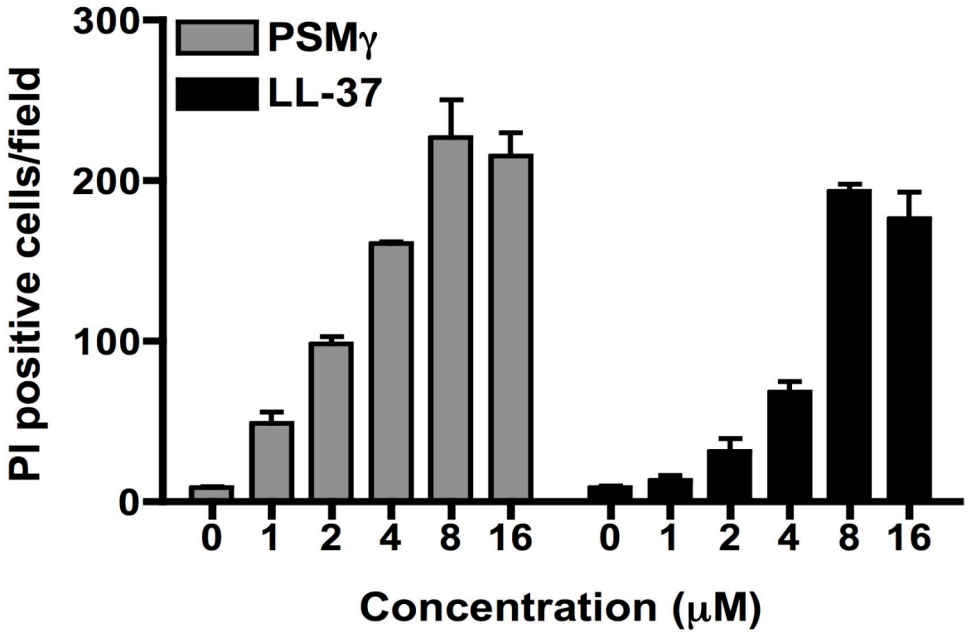


Figure 3.9. LL-37 and Phenol Soluble Modulin-γ induce similar dose-dependent changes in keratinocyte membrane permeability. Normal human epidermal keratinocytes, incubated with increasing concentrations of LL-37 or PSMγ were stained with propidium iodide (PI) to evaluate disruption in membrane permeability. PI uptake was measured by counting PI-positive fluorescent cells per microscopic field. Data represent mean ±SEM of three random fields and are representative of two independent experiments.

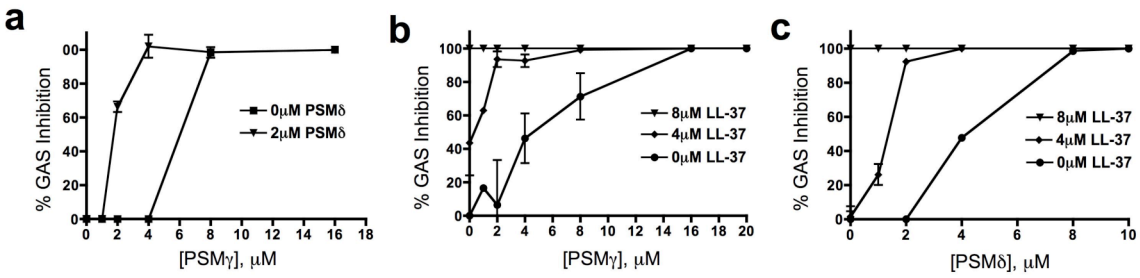


Figure 3.10. Phenol Soluble Modulins cooperate with each other and host AMPs to kill GAS. a, Co-incubation of GAS with PSMγ and PSMδ shows cooperative antimicrobial effect. Co-incubation of GAS with LL-37 and PSMγ (b) or PSMδ (c) shows cooperative antimicrobial effect. Data representative of 2 individual experiments performed in triplicate. Data are mean ± SEM of a single experiment performed in duplicate and are representative of 2 independent experiments.

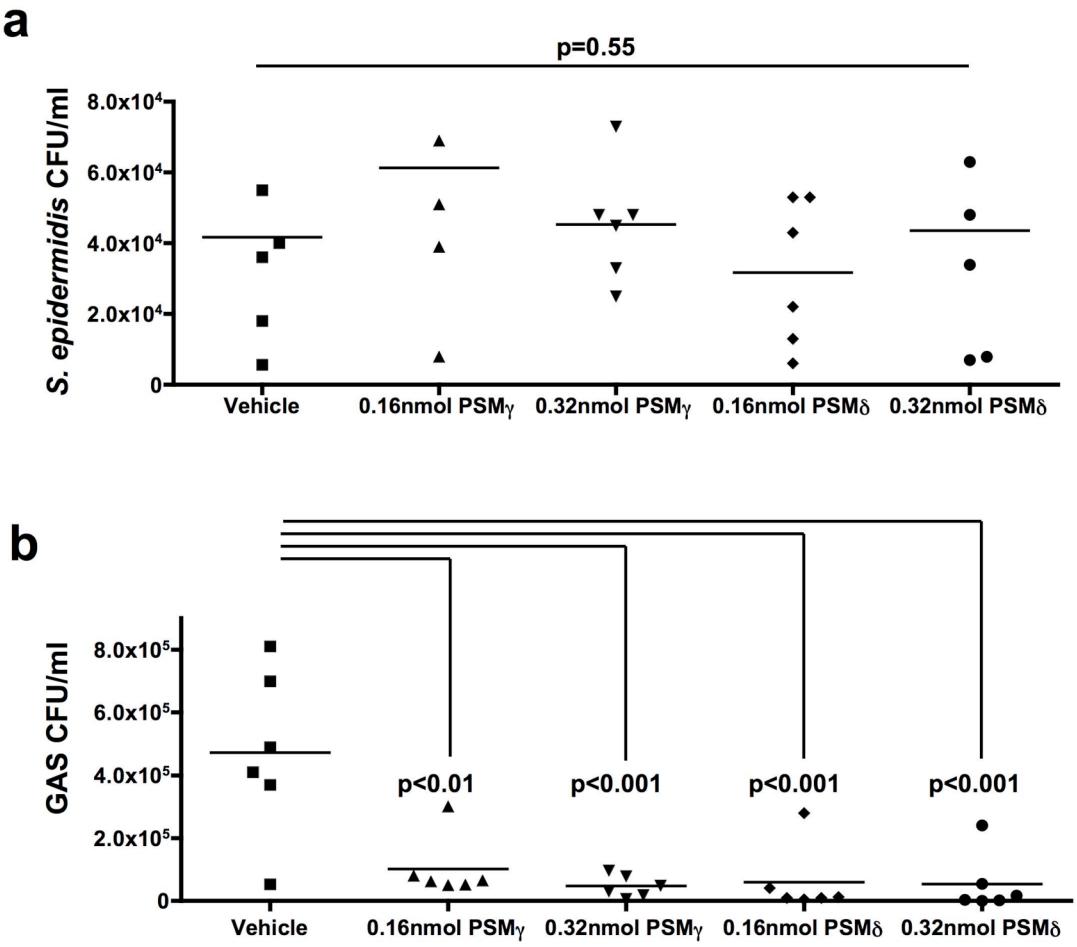


Figure 3.11. Phenol Soluble Modulins selectively kill GAS but not *S. epidermidis* on the skin's surface. a, Survival of *S. epidermidis* on mouse skin treated with PSMs at 0.16nmol or 0.32nmol. Addition of PSMs on skin did not affect colonization of *S. epidermidis*. b, GAS survival on skin was greatly reduced by addition of PSMs at 0.16nmol or 0.32nmol. Data are means of two independent experiments performed in triplicate. p values were calculated using ANOVA with Bonferroni post-hoc test.

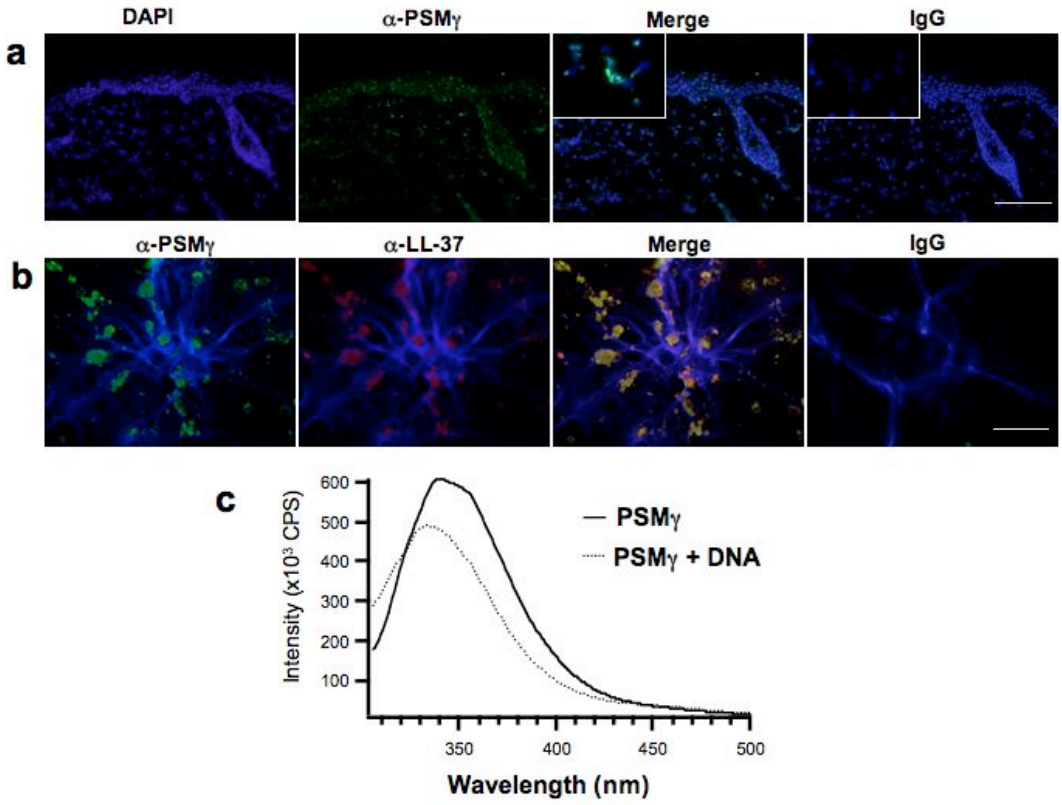


Figure 4.1. Phenol Soluble Modulin-γ is deposited in the skin by *S. epidermidis* and binds neutrophil extracellular traps. a, normal healthy human skin stained for PSMγ showed deposition in the epidermis and dermis at 40X magnification. Inset is 100X magnification of PSMγ in dermis. Bar represents 50μm. This is a single specimen representative of 2 patients. b, PSMγ was added to neutrophil extracellular traps (NETs) and subsequently stained for LL-37 and PSMγ. Staining shows colocalization of antimicrobial peptides along DNA strands. Bar represents 20μm. c, tryptophan spectroscopy of PSMγ in the presence and absence of neutrophil DNA.

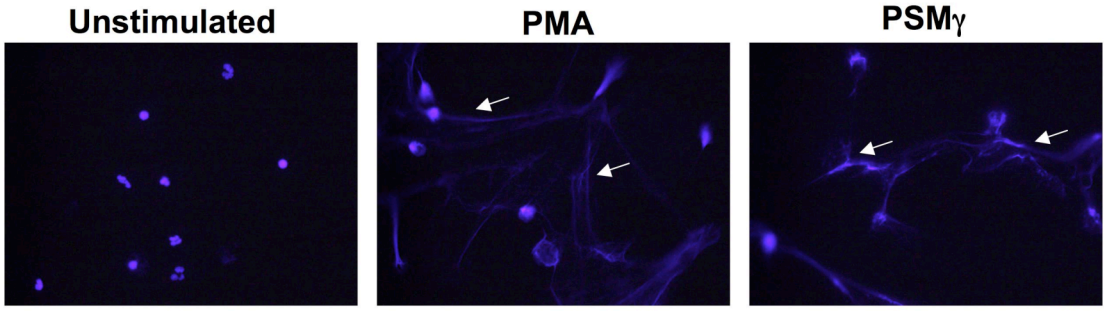


Figure 4.2. Phenol Soluble Modulin-γ induces NET formation. Freshly isolated human neutrophils were cultured with media only (unstimulated), 25nM PMA, or 6μM PSMγ for 4 hours. Like PMA, PSMγ induced NET formation as seen by the DNA strands (white arrows).

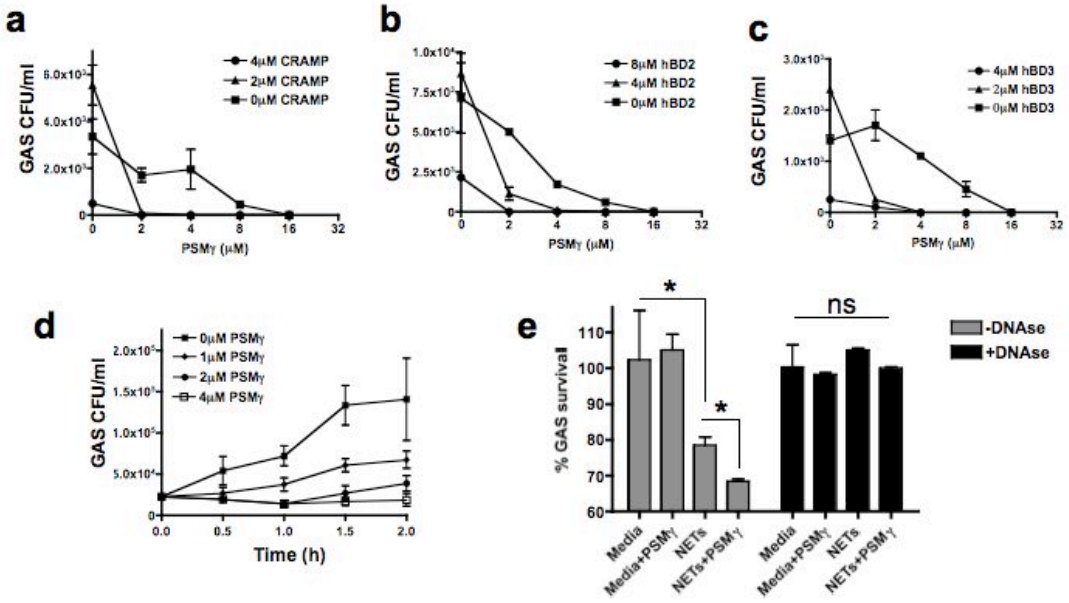
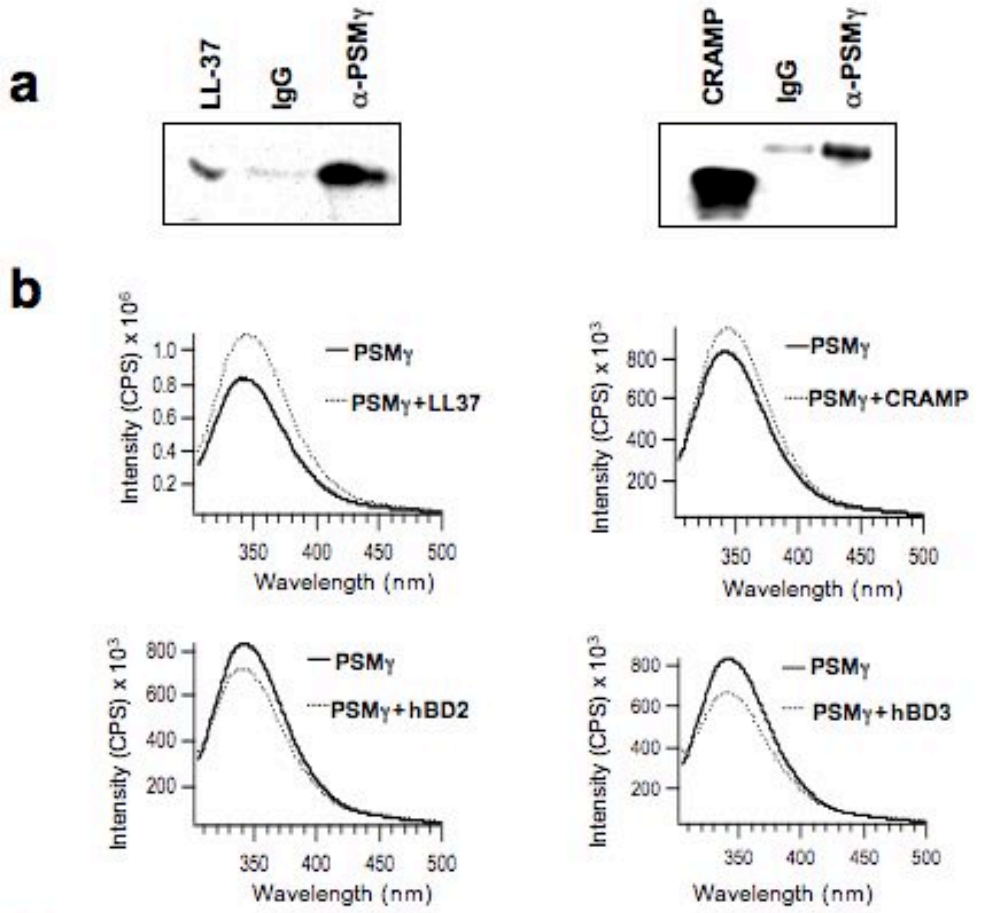


Figure 4.3. Phenol Soluble Modulin-γ cooperates with host antimicrobial peptides and enhances whole blood and NET killing of GAS. PSMγ cooperates with host antimicrobial peptides CRAMP (a), hBD2 (b), and hBD3 (c) to kill GAS. d, in whole blood, increasing concentrations of synthetic PSMγ rendered GAS bacteriostatic. e, PSMγ added to NETs showed greater GAS killing than NET killing alone. The effect was abrogated by DNase. Data are representative of 2 individual experiments performed in triplicate. *$p<0.05$. Student's t-test.



c

	Maximum Wavelength (nm)	Shift (nm)
PSMγ	341	-
PSMγ + LL-37	344	+3
PSMγ + CRAMP	344	+3
PSMγ + hBD2	339	-2
PSMγ + hBD3	339	-2

Figure 4.4. Phenol Soluble Modulin-γ physically binds to host derived antimicrobial peptides. Synthetic 1μM LL-37 or 1μM CRAMP was added to *S. epidermidis* supernatants. PSMγ was precipitated and immunoblotted for LL-37 (a) or CRAMP. Immunoblots show co-precipitation of LL-37 or CRAMP with PSMγ. b, emission spectra of PSMγ's tryptophan in buffer or in the presence of LL-37, CRAMP, hBD2, or hBD3. c, table of maximal wavelength emission and shift in wavelength upon addition of host antimicrobial peptide. Data are representative of 2 individual experiments.

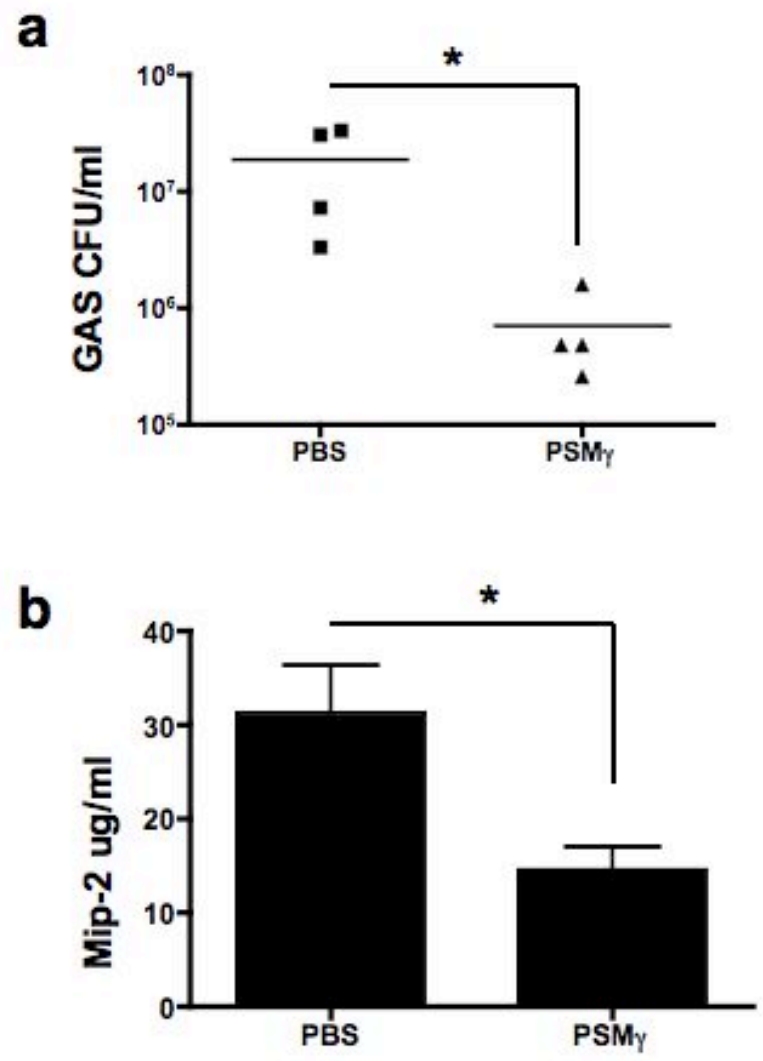
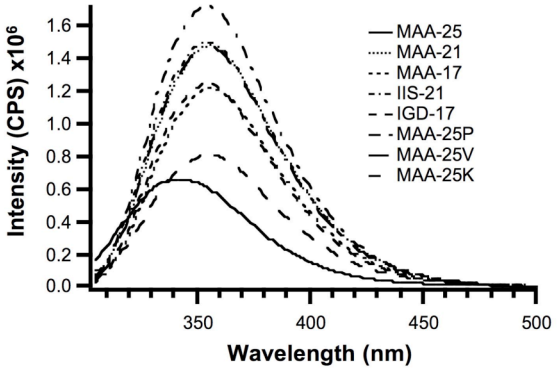


Figure 4.5. Phenol Soluble Modulin-γ reduces GAS survival and inflammation in mouse wounds. PSMγ or PBS was added to mouse wounds. After 30 minutes, the treated wounds were challenged with GAS. Wounds were excised, homogenized and assayed for GAS CFU/ml (a) or Mip-2 protein levels by ELISA (b). Data are representative of 3 individual experiments performed with n=3-4. *$p<0.05$, Student's t-test.

Table 5.1. Phenol Soluble Modulin-γ analogs.

Phenol Soluble Modulin-γ Analogs	Peptide name	Target
MAADIISTIGDLVKWIIDTVNKFKK	MAA-25	wild-type
MAADIISTIGDLVKWIIDTVN - - - -	MAA-21	c-term deletion
MAADIISTIGDLVKWII - - - - - - - -	MAA-17	c-term deletion
- - - - IISTIGDLVKWIIDTVNKFKK	IIS-21	n-term deletion
- - - - - - - -IGDLVKWIIDTVNKFKK	IGD-17	n-term deletion
MAADIIST**P**GDLVKWIIDTVNKFKK	MAA-25P	helix disruption
MAADIISTIGDL**N**KWIIDT**N**NKFKK	MAA-25V	polarity
MAADIISTIGDLV**A**WIIDTVN**A**F**AA**	MAA-25K	charge neutralization

a

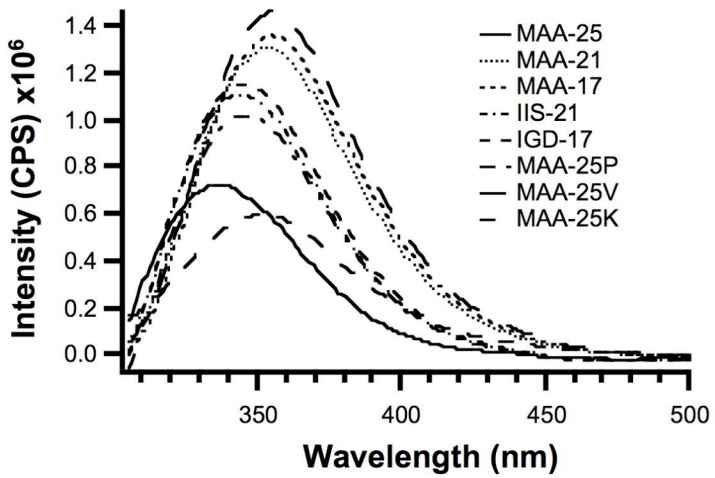


b

Peptides	Maximal Emission, λ_{max} (nm)	Change in λ_{max} from MAA-25
MAA-25	339	0
MAA-21	356	+16
MAA-17	355	+15
IIS-21	355	+15
IGD-17	355	+15
MAA-25P	355	+15
MAA-25V	354	+14
MAA-25K	355	+15

Figure 5.1. Phenol Soluble Modulin-γ analogs do not form complexes. a, tryptophan emission curves of PSMγ and analogs in Kpi. b, table listing tryptophan maximal emission (λ_{max}) in center column and change in λ_{max} from wild-type (MAA-25) PSMγ. PSMγ analogs showed red-shifted λ_{max}, indicating lack of complex formation.

a



b

Peptides	Kpi: Maximal Emission, λ_{max} (nm)	Vesicles: Maximal Emission, λ_{max} (nm)	Vesicles: Change in λ_{max} from MAA-25	Change in λ_{max}: Vesicles-Kpi
MAA-25	339	336	0	-3
MAA-21	356	353	+16	-3
MAA-17	355	354	+17	-1
IIS-21	355	343	+6	-12
IGD-17	355	345	+8	-10
MAA-25P	355	344	+7	-11
MAA-25V	354	355	+18	+1
MAA-25K	355	351	+14	-4

Figure 5.2. Phenol Soluble Modulin-γ analogs variably interact with lipid membranes vesicles. a, tryptophan emission curves of PSMγ and analogs in presence of POPC:POPG vesicles. b, table listing tryptophan maximal emission (λ_{max}) in columns 2 and 3, change in λ_{max} of analogs with vesicles from wild-type with vesicles (MAA-25) PSMγ in column 4, and change in λ_{max} of analogs vesicles from Kpi (λ_{max} with vesicles- λ_{max} Kpi).

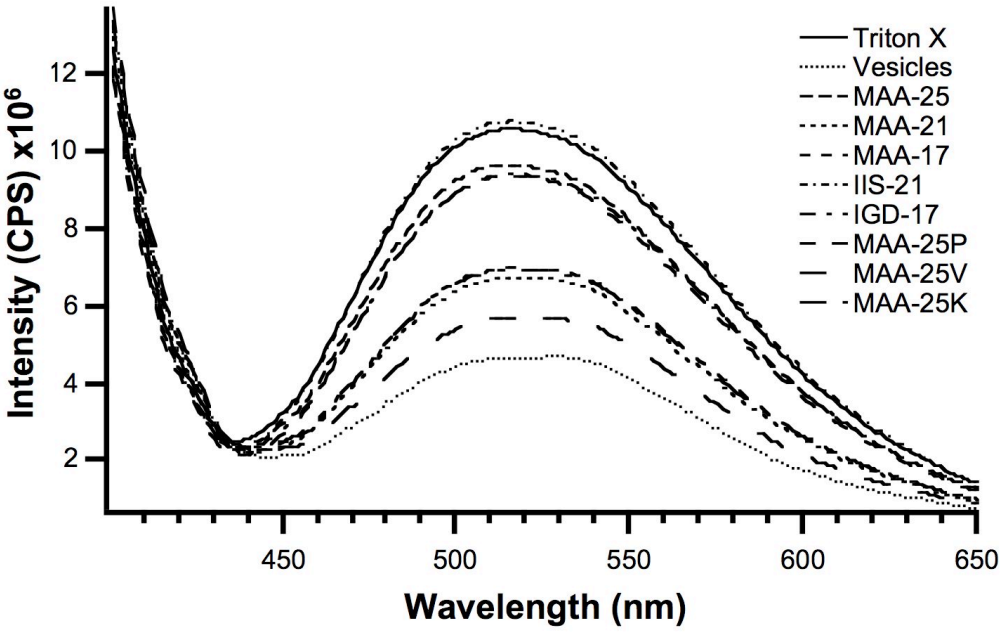


Figure 5.3. Phenol Soluble Modulin-γ analogs induce differential vesicle leakage. Emission spectra of ANTS and DPX encapsulated vesicles after incubation with triton X or 16μM PSMγ analogs.

a

	Normalized Intensity (CPS)	Normalized % of Triton X
Vesicles	0.00E+00	0.00 %
Triton X	5.95E+06	100.00 %
MAA-25	5.03E+06	84.43 %
MAA-21	2.10E+06	35.28 %
MAA-17	2.29E+06	38.42 %
IIS-21	6.15E+06	103.29 %
IGD-17	4.74E+06	79.63 %
MAA-25P	4.74E+06	79.63 %
MAA-25V	1.03E+06	17.34 %
MAA-25K	2.29E+06	38.42 %

b

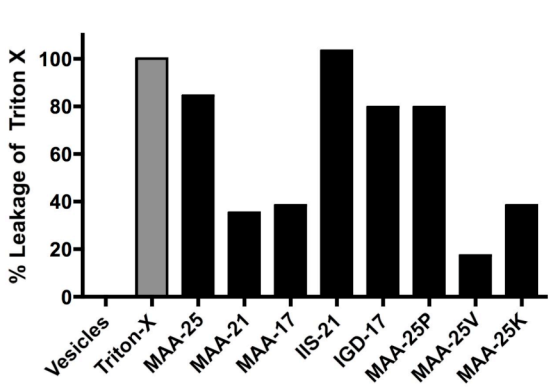


Figure 5.4. Quantitative analysis of vesicle leakage by 16μM Phenol Soluble Modulin-γ analogs. a, table of normalized maximal emission intensity (column 2) and normalized percent of triton X induced leakage (% leakage). b, graphical representation of % leakage induced by 16μM PSMγ analogs compared to triton X.

a

	Normalized Intensity (CPS)	Normalized % of Triton X
Vesicles	0.00E+00	0.00 %
Triton X	6.56E+06	100.00 %
MAA-25	5.54E+06	84.51 %
MAA-21	4.64E+06	70.73 %
MAA-17	4.01E+06	61.22 %
IIS-21	6.24E+06	95.22 %
IGD-17	6.24E+06	95.22 %
MAA-25P	6.24E+06	95.22 %
MAA-25V	3.88E+06	59.16 %
MAA-25K	5.85E+06	89.30 %

b

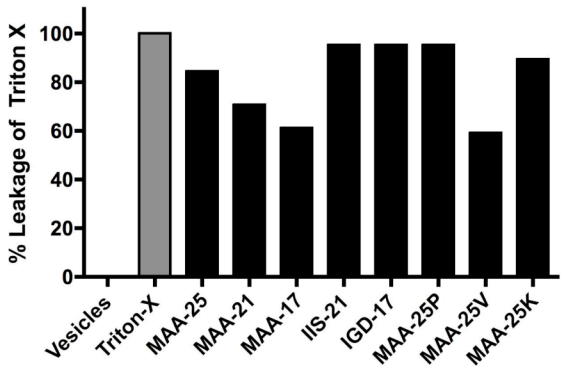


Figure 5.5. Quantitative analysis of vesicle leakage by 32μM Phenol Soluble Modulin-γ analogs. a, table of normalized maximal emission intensity (column 2) and normalized percent of triton X induced leakage (% leakage). b, graphical representation of % leakage induced by 32μM PSMγ analogs compared to triton X.

Table 5.2. Minimal Bactericidal Concentrations of Phenol Soluble Modulin-γ analogs.

	Minimal Bactericidal Concentrations	
Peptide	**Group A *Streptococcus***	***Staphylococcus aureus***
MAA-25	16μM	4-8μM
MAA-21	>64μM	32μM
MAA-17	>64μM	32μM
IIS-21	16-32μM	8μM
IGD-17	32μM	16μM
MAA-25P	>64μM	8-16μM
MAA-25V	>64μM	32μM
MAA-25K	>64μM	8μM

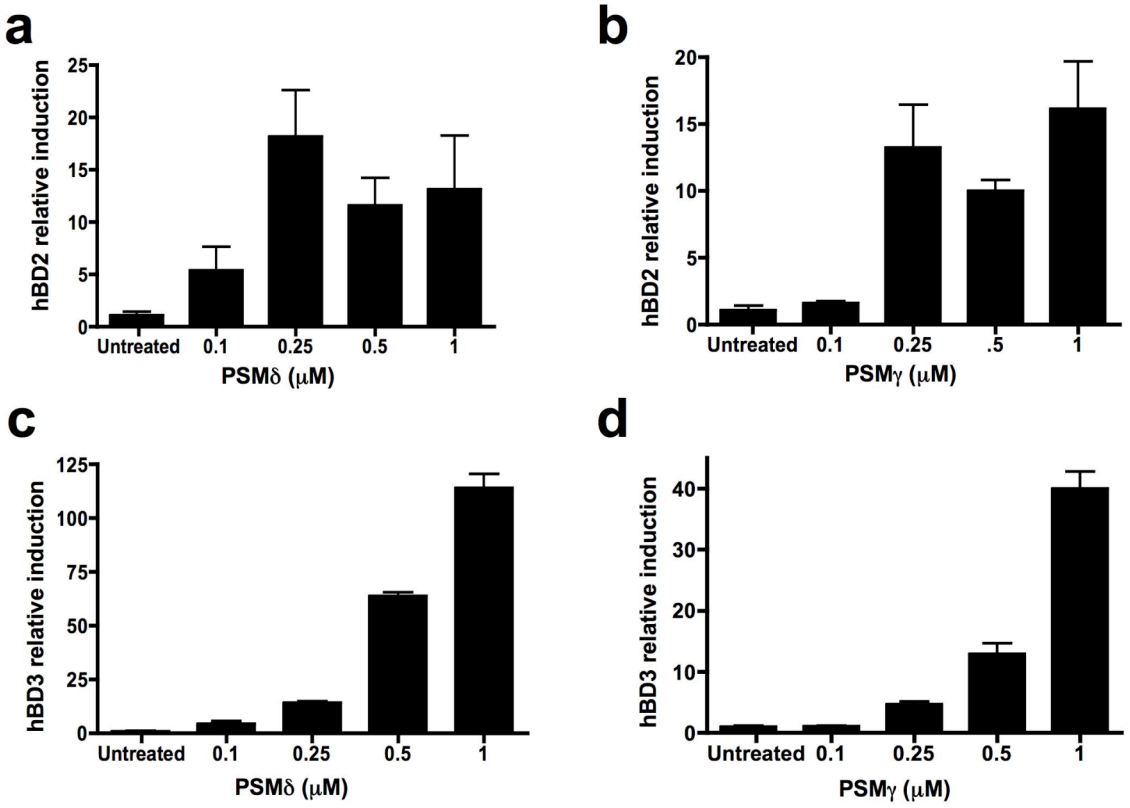


Figure 6.1. Phenol Soluble Modulin-γ and -δ induce human keratinocyte expression of hBD2 and hBD3. Normal human epidermal keratinocytes (NHEKs) were evaluated for hBD2 relative induction (a and b) or hBD3 relative induction (c and d) after stimulation with PSMδ (a and c) or PSMγ (b and d).

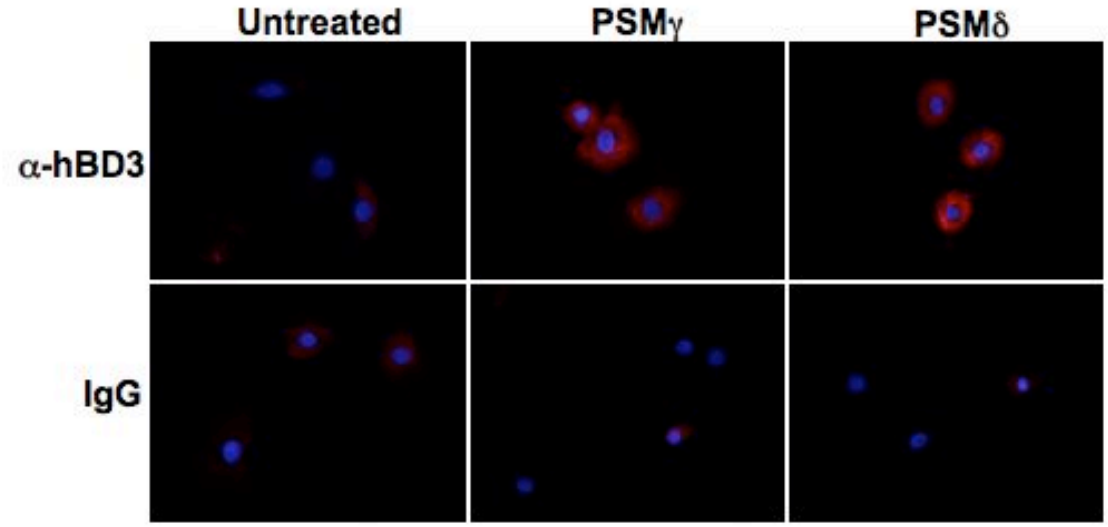


Figure 6.2. Immunohistochemistry for hBD3 of human keratinocytes stimulated with Phenol Soluble Modulins. NHEKs were stimulated with 1μM PSMγ or 500nM PSMδ for 24 hours. Cells showed increased hBD3 protein expression intracellularly after stimulation.

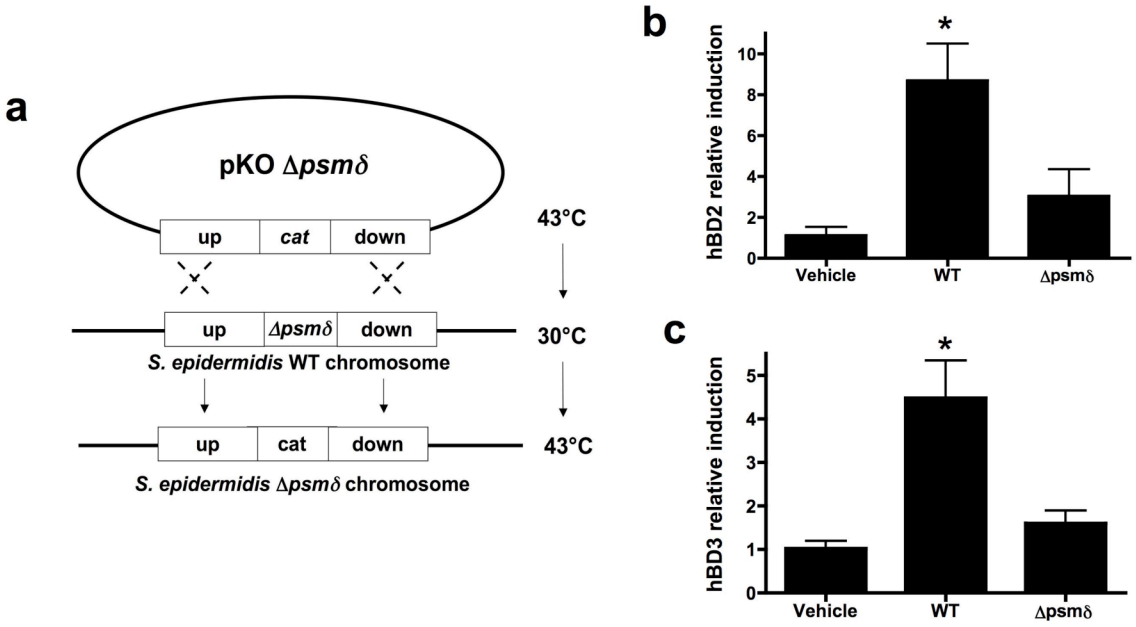


Figure 6.3. Allelic exchange mutagenesis of PSMδ. a, allelic exchange mutagenesis scheme for replacing *psmδ* with *cat* in *S. epidermidis.* hBD2 (b) and hBD3 (c) relative induction in NHEKs after stimulation with supernatants from *S. epidermidis* WT or *S. epidermidis Δpsmδ.* *p<0.05, Student's t-test, compaired to control.

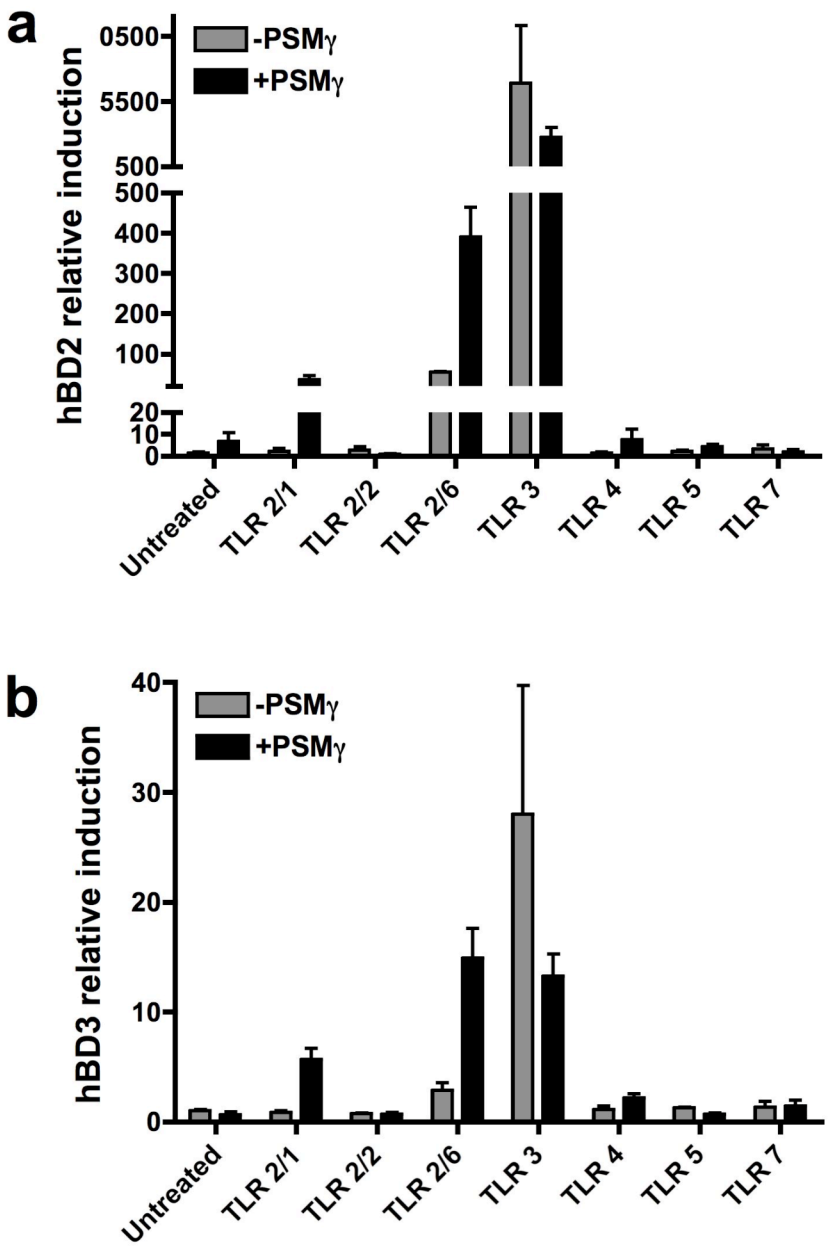


Figure 6.4. hBD2 and hBD3 induction in keratinocytes by TLR ligands and Phenol Soluble Modulin-γ. Keratinocytes were stimulation with TLR ligands in the presence or absence of PSMγ. PSMγ synergistically induced hBD2 (a) and hBD3 (b) in the presence of TLR2/1 and TLR 2/6 ligands.

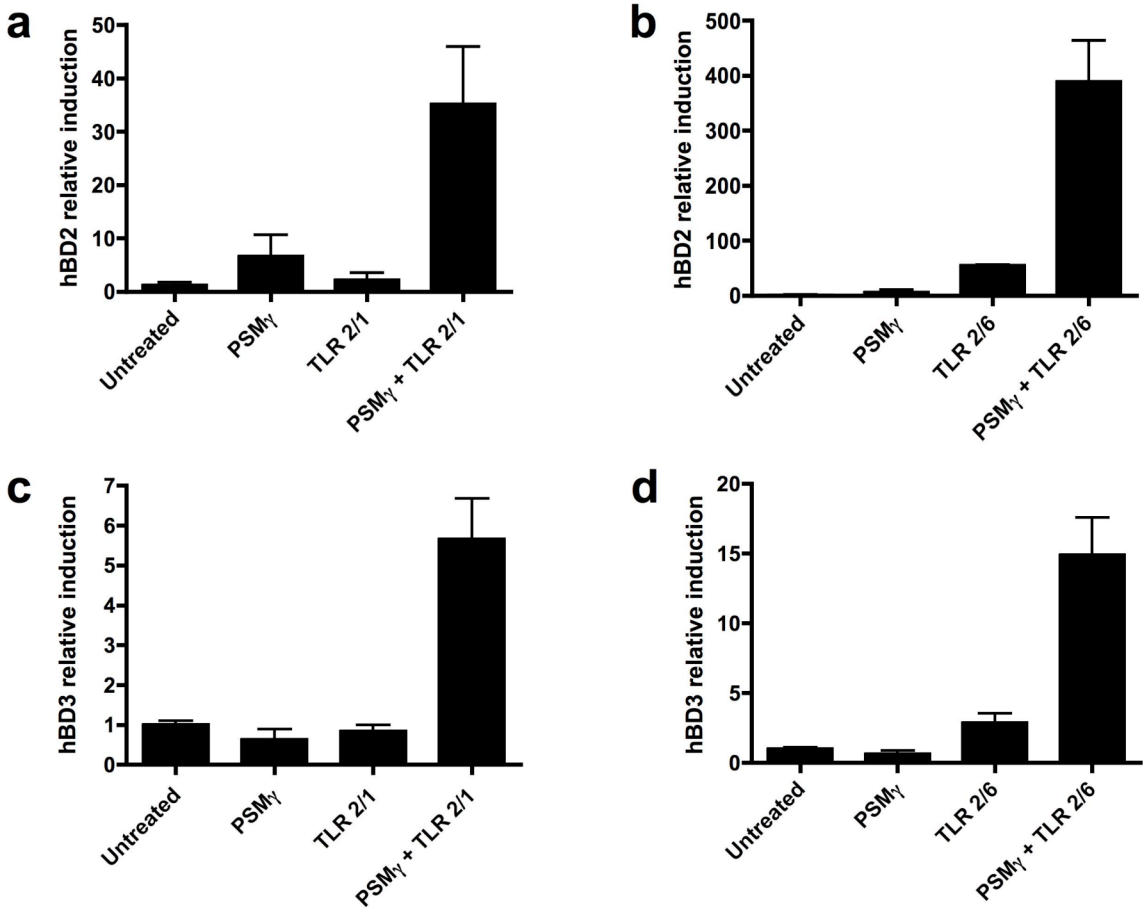


Figure 6.5. Phenol Soluble Modulin-γ synergistically induced hBDs in presence of TLR2 ligands. Keratinocytes were stimulation with TLR2/1 (Pam3csk4) or TLR 2/6 (Malp-2) ligands in the presence or absence of PSMγ. PSMγ synergistically induced hBD2 (a,b) and hBD3 (c,d) in the presence of TLR2/1 and TLR 2/6 ligands.

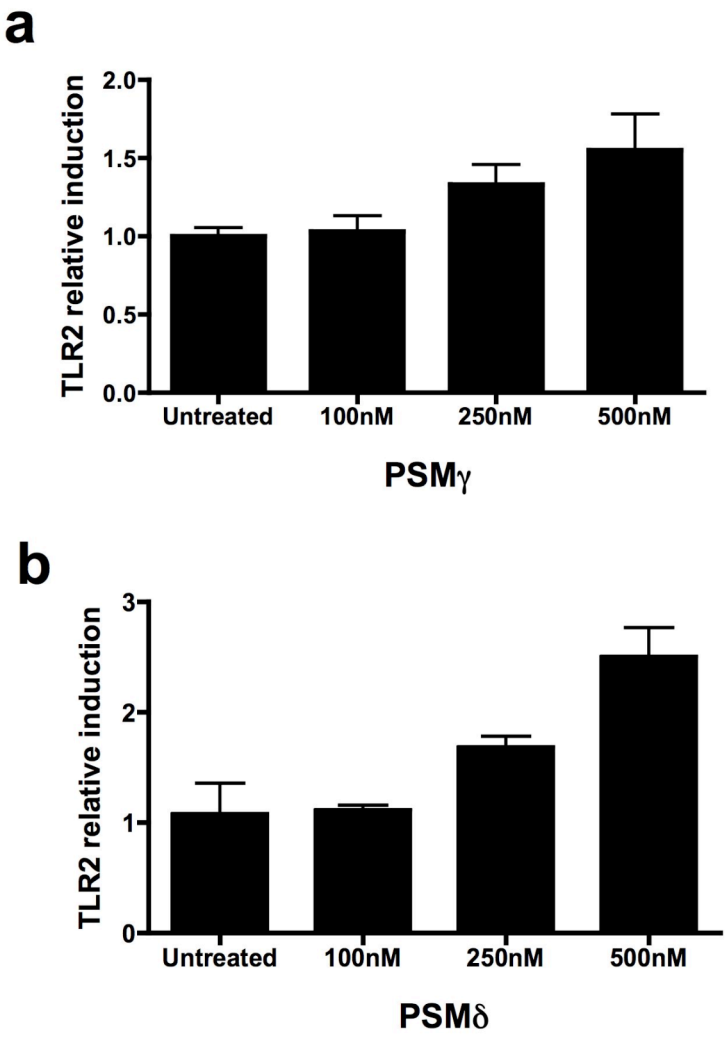


Figure 6.6. Phenol Soluble Modulins minimally induce TLR2 expression in keratinocytes. Keratinocytes were stimulation with PSMγ (a) or PSMδ (b). TLR2 transcript abundance was increased by roughly 1.5 fold by PSMγ and 2.5 fold by PSMδ.

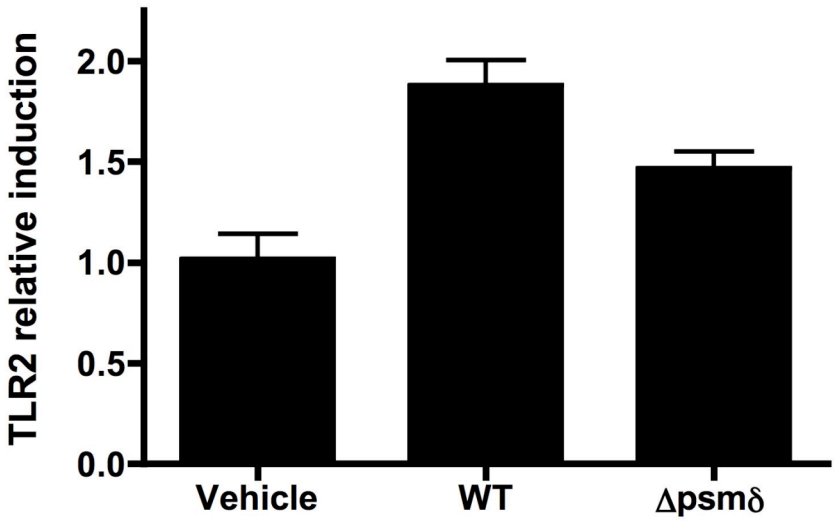


Figure 6.7. *S. epidermidis* WT induced TLR2 to a greater extent than *S. epidermidis Δpsmδ*. *S. epidermidis* WT supernatants induced a slightly greater increase in TLR2 in keratinocytes as compared to S. *epidermidis Δpsmδ* supernatants.