SEXUAL DIMORPHISM IN HOST INNATE RESPONSE TO STAPHYLOCOCCUS AUREUS: MECHANISMS DRIVING SEX DIFFERENCES IN IMMUNE CELL FUNCTION AND HOST-DIRECTED INTERVENTIONS

Srijana Pokhrel
University of New Mexico

Follow this and additional works at: https://digitalrepository.unm.edu/biom_etds

Part of the Immunology and Infectious Disease Commons, and the Medicine and Health Sciences Commons

Recommended Citation
Pokhrel, Srijana. "SEXUAL DIMORPHISM IN HOST INNATE RESPONSE TO STAPHYLOCOCCUS AUREUS: MECHANISMS DRIVING SEX DIFFERENCES IN IMMUNE CELL FUNCTION AND HOST-DIRECTED INTERVENTIONS." (2020). https://digitalrepository.unm.edu/biom_etds/215

This Dissertation is brought to you for free and open access by the Electronic Theses and Dissertations at UNM Digital Repository. It has been accepted for inclusion in Biomedical Sciences ETDs by an authorized administrator of UNM Digital Repository. For more information, please contact amywinter@unm.edu, Isloane@salud.unm.edu, sarahrk@unm.edu.
Srijana Pokhrel  
Candidate  
Pharmaceutical Sciences  
Department

This dissertation is approved, and it is acceptable in quality and form for publication:

Approved by the Dissertation Committee:

Dr. Pamela R Hall, Chairperson

Dr. Eric R Prossnitz

Dr. Helen J Hathaway

Dr. Laurie G Hudson
SEXUAL DIMORPHISM IN INNATE HOST RESPONSE TO
STAPHYLOCOCCUS AUREUS: MECHANISMS DRIVING SEX
DIFFERENCES IN IMMUNE CELL FUNCTION AND HOST-
DIRECTED INTERVENTIONS

by

SRIJANA POKHREL

B.S., Microbiology, Tribhuvan University, 2009
B.S., Microbiology, Idaho State University, 2013

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy
Biomedical Sciences

The University of New Mexico
Albuquerque, New Mexico

July 2020

ii
DEDICATION

I would like to dedicate this dissertation to my lovely mother Mrs. Urmila Pokhrel and in the loving memory to my father Mr. Batu Kendra Pokhrel.
ACKNOWLEDGEMENTS

I would like to express a sincere gratitude to my mentor, Dr. Pamela R Hall. Her continuous support, encouragement, patience and helpful demeanor has made my graduate school journey a pleasant experience. I will forever be grateful to her for making me the PhD scholar I am today.

I would like to thank my committee members, Dr. Eric R. Prossnitz, Dr. Helen J. Hathaway and Dr. Laurie G. Hudson for their valuable insights and advice which has helped me with the research and my development as a scientist. I would also like to thank all my previous mentors who have encouraged me to pursue my interest in science.

I will forever remain indebted to my lab members, Kathleen D. Triplett, Jason A. Joyner, Dr. Bradley O. Elmore and Dr. Seth M. Daly, without whom working in the lab would not have been a fun filled experience.

A big thanks to the Biomedical Sciences Graduate Program, COP Department of Pharmaceutical Sciences, SOMREO, faculty, staff and friends I made during my stay at the University of New Mexico. Thank you! Graduate school would not have been such a great experience without my friends, Melanie R. Rivera, Muskan Floren and Kymberly C. Gustus. Thank you for always being kind, fun, supportive and motivating.

Last but certainly not the least, I would like to recognize my mother Urmila Pokhrel, my husband Nischal Rimal, my sisters, Rachana and Archana, brother in laws, Dr. Dadhi R. Adhikari and Samir Dhakal, nephew and niece (Ayush, Aarushi, Neal), my in-laws, Lila N. Rimal and Padma Rimal, Dr. Ujjwal Rimal and Dr. Binita Dhungel, and the rest of my family for their patience, belief, support and love throughout my journey.
SEXUAL DIMORPHISM IN HOST INNATE RESPONSE TO
STAPHYLOCOCCUS AUREUS: MECHANISMS DRIVING SEX DIFFERENCES
IN IMMUNE CELL FUNCTION AND HOST-DIRECTED INTERVENTIONS

by

SRIJANA POKHREL

B.S., Microbiology, Tribhuvan University, 2009
B.S., Microbiology, Idaho State University, 2013
Ph.D., Biomedical Sciences, University of New Mexico, 2020

ABSTRACT

With the emerging antibiotic resistance, Staphylococcus aureus, primary causative agent of skin and soft tissue infections (SSTIs) as well as other life-threatening conditions has become a global health concern. While factors contributing to host defense against S. aureus SSTIs has been well studied, impact of sex as a variable has not been reported. In Chapter 2, we uncover the sex-specific differences in host response to S. aureus SSTIs and the contribution of female sex hormone, estrogen (E2) in providing protection in females. In Chapter 3, we identify mechanisms of innate differences in neutrophils bactericidal efficacy between sexes and in Chapter 4, we report the protective role of G protein-coupled receptor (GPER) agonist, G-1 against S. aureus SSTIs. Because no vaccines against S. aureus has been approved yet, understanding the host defense mechanisms is important to identify potential targets for host-directed therapeutics to combat the ongoing antibiotic crisis.
TABLE OF CONTENTS

DEDICATION........................................................................................................................................... iii

ACKNOWLEDGEMENTS ......................................................................................................................... iv

ABSTRACT.................................................................................................................................................. v

LIST OF FIGURES .................................................................................................................................... ix

LIST OF TABLES ....................................................................................................................................... xii

CHAPTER 1: INTRODUCTION .................................................................................................................. 1

1.1. Background on *S. aureus* .............................................................................................................. 1

   *S. aureus* and antibiotic resistance................................................................................................. 1

   Types of MRSA ..................................................................................................................................... 2

   *S. aureus* Skin and Soft Tissue Infections (SSTIs)........................................................................ 3

   Pathogenesis of *S. aureus* ............................................................................................................... 5

   Current Strategies for Treatment of *S. aureus* Infections ............................................................. 8

1.2. Skin, the protective barrier against *S. aureus* SSTIs ................................................................. 9

1.3. Sex bias in host response to diseases ............................................................................................. 14

   Hormonal role in the immune response ......................................................................................... 17

   Sex-specific differences in *S. aureus* infections ............................................................................ 23

1.4. Neutrophils in host defense against *S. aureus* ........................................................................... 24

   Oxygen-dependent and independent killing by neutrophils ....................................................... 27

   *S. aureus* and evasion of neutrophil clearance .......................................................................... 29

   Sex hormones and neutrophils ...................................................................................................... 30
DISUSSION .................................................................................................................. 132

ACKNOWLEDGEMENTS .......................................................................................... 136

CHAPTER 5: DISCUSSION ......................................................................................... 138

SUMMARY .................................................................................................................. 138

FUTURE CONSIDERATIONS ..................................................................................... 144

5.1.1. Sex bias in skin cells composition and function against S. aureus .......... 147
5.1.2. Role of E2 and ERs in skin cells function and sex bias in S. aureus SSTI . 153
5.2.1. Innate differences in neutrophils between sexes...................................... 154
5.2.2. E2 and ERs in enhanced bactericidal capacity of female neutrophils ...... 157
5.2.3. Oxygen-independent clearance of S. aureus by female neutrophils ....... 159
5.3. Protective role of G-1 in male mice against S. aureus pneumonia............... 160

CONCLUSIONS ........................................................................................................... 164

APPENDICES ............................................................................................................ 166

APPENDIX A: ABBREVIATIONS ............................................................................. 166

APPENDIX B: REFERENCES ....................................................................................... 171
LIST OF FIGURES

Figure 1.1. Hla activates NLRP3 inflammasome leading to IL-1β production............ 7
Figure 1.2. The layers of skin. .................................................................................. 11
Figure 1.3. Estrogen and estrogen receptors signaling. ............................................. 19
Figure 1.4. Oxygen-dependent and independent killing mechanisms of neutrophils...... 26
Figure 1.5. Schematic of gaps addressed in Chapters 2, 3 and 4................................. 35
Figure 2.1. Female mice are resistant to Staphylococcus aureus skin infection compared with males. 51
Figure 2.2. BALB/c female mice are better protected against Staphylococcus aureus skin infection compared to males. ................................................................................ 52
Figure 2.3. Male and female mice respond similarly to skin infection with Staphylococcus aureus lacking Hla. .................................................................................. 54
Figure 2.4. Male and female mice differentially respond to skin infection with Staphylococcus aureus lacking Hla. .................................................................................. 55
Figure 2.5. Markers of NLRP3 inflammasome activation are reduced in female mice during Staphylococcus aureus skin infection compared with males. ........................................ 58
Figure 2.6. Expression of inflammasome related genes at the site of Staphylococcus aureus SSTI in male and female mice. .............................................................................. 59
Figure 2.7. Hla-induced dermonecrosis and inflammatory cytokine production is reduced in female mice compared with males................................................................. 61
Figure 2.8. Ovariectomy impairs and estrogen restores female innate resistance to Staphylococcus aureus dermonecrosis. ................................................................. 63
Figure 2.9. 17β-estradiol administration to male mice does not enhance protection against Staphylococcus aureus SSTI and does not directly alter bacterial growth....................... 64
Figure 2.10. Neutrophils from female mice have increased *S. aureus* bactericidal capacity versus neutrophils from males. .......................................................... 67

Figure 3.1. Female murine sera has higher C3 levels compared to males.................. 86

Figure 3.2. Female murine BMN have increased CR3 surface expression compared to males. ........................................................................................................... 89

Figure 3.3. Enhanced killing of *S. aureus* by female versus male murine BMNs is both serum- and cell-dependent. ................................................................. 92

Figure 3.4. CR3 blocking differentially reduces the bactericidal capacity of murine neutrophils *ex vivo*. .................................................................................. 93

Figure 3.5. ROS production is increased with female murine BMNs compared to males and partially relies on surface CR3 expression........................................ 96

Figure 4.1. G-1 promotes protection against *S. aureus* SSTI in male mice.............. 119

Figure 4.2. G-1 promotes protection against *S. aureus* SSTI in female mice.......... 120

Figure 4.3. G-1 does not inhibit *S. aureus* growth.............................................. 121

Figure 4.4. G-1-mediated protection against SSTI is GPER-dependent. ................. 123

Figure 4.5. G-1 reduces Hla-mediated lesion formation and inflammation. .......... 125

Figure 4.6. G-1 does not alter SSTI outcomes in the absence of Hla and does not inhibit Hla production or activity............................................................... 126

Figure 4.7. GPER is expressed in HaCaT cells and G-1 does not alter HaCaT cell growth or viability................................................................. 128

Figure 4.8. G-1 reduces Hla-mediated keratinocyte permeability barrier disruption. .... 131

Figure 5.1. Summary of the findings of Chapters 2, 3 and 4.................................. 143
Figure 5.2. Summary of gaps to be addressed based on the findings of Chapter 2, 3 and 4. .......................................................... 146

Figure 5.3. Sex bias in skin cells composition and function, role of E2 and ERs. .......... 149

Figure 5.4. Contribution of Langerhans cells (LCs) and γδ T cells to sex bias in host defense to S. aureus SSTI................................................................. 152

Figure 5.5. E2 and ERs in enhanced bactericidal capacity of female murine neutrophils. .............................................................................................................. 156

Figure 5.6. Role of G-1 in protection against S. aureus pneumonia............................... 163
LIST OF TABLES

Table 1.1. Sex bias in susceptibility to infections.......................................................... 16
Table 1.2. Sex-differences in immune cells response and effect of hormones.............. 22
Table 1.3. Effect of hormones on neutrophils................................................................. 32
Table 2. Sex Bias in incidence of S. aureus SSTI* .......................................................... 48
CHAPTER 1: INTRODUCTION

1.1. Background on S. aureus

_S. aureus_ and antibiotic resistance

_Staphylococcus aureus_ (_S. aureus_), a gram-positive bacteria, is a commensal organism usually found in the nares and on skin surfaces of humans, its natural reservoir (1). About thirty to fifty percent of healthy adults are colonized by _S. aureus_ and are at higher risk of subsequent infections because this commensal can act as an opportunistic pathogen under immunosuppressed conditions (2). Since its discovery in 1880 in the abscess of a patient, _S. aureus_ has been associated with many life-threatening conditions and the emergence of antibiotic resistant strains, such as methicillin-resistant _S. aureus_ (MRSA) makes it a serious health concern. Not only is _S. aureus_ the leading cause of skin and soft tissue infection (SSTI) (3-6), but it can also cause bacteremia, sepsis, endocarditis, and pneumonia (7-9). Challenges in treating _S. aureus_ infections were encountered as early as 1942 when the bacteria acquired resistance to the β-lactam antibiotic penicillin, which was discovered in 1940 (10). Resistance was due to the acquisition of _blaZ_, that encodes for the β-lactamase enzyme, which leads to the hydrolysis of the β-lactam ring of penicillin rendering the antibiotic inactive (10). Soon after the bacteria demonstrated resistance to methicillin, a semisynthetic penicillinase-resistant penicillin, leading to the emergence of MRSA (10, 11). The resistance of _S. aureus_ to methicillin is attributed to the _mecA_ gene that encodes for penicillin-binding protein 2a (PBP2a) (10, 11). Penicillin-binding protein (PBP) catalyzes the crosslinking of peptidoglycan chains during bacterial cell wall synthesis and β-lactams inhibit this activity of PBP (10, 11). PBP2a hinders this inhibitory
action of β-lactams by lowering the affinity of the bacteria to the antibiotic and thus confers resistance to most available β-lactam antibiotics (10, 11). The emergence of MRSA has resulted in a dearth of therapeutics against *S. aureus* infections and increased mortality rates (10, 12). In fact, the Center for Disease Control (CDC) listed MRSA, causing an estimated 80,461 invasive infections and 11,285 deaths per year, as a serious threat in its 2013 landmark report “Antibiotic Resistance Threats in the United States” (13).

### Types of MRSA

MRSA causes both health care-associated (HA) and community-associated (CA) infections (14). Hence, it has been classified into HA-MRSA and CA-MRSA depending on the site of onset (14). MRSA infections in patients with ongoing or previous healthcare exposure is further classified into healthcare-associated hospital onset (HAHO) or healthcare-associated community onset (HACO) depending on where the infections develop (hospital or community) (14). Both HA- and CA-MRSA cause similar infections however, HA-MRSA is the leading cause of lower respiratory tract and surgical site infections, as well as nosocomial pneumonia and bacteremia, whereas CA-MRSA is predominantly isolated from skin and soft tissue infections (15, 16). The emergence of CA-MRSA poses a serious health threat as this strain affects individuals without prior risk factors unlike HA-MRSA that mainly causes infection in patients with a history of hospitalization, surgery or long-term care patients, thus increasing disease burden among the general population (6, 17, 18).
The genetic differences in HA- versus CA-MRSA lie in the staphylococcal chromosome cassette *mec* (*SCCmec*) alleles that harbors genes for methicillin resistance (*mecA*) and the presence of the gene encoding the Panton–Valentine leucocidin (PVL) virulence factor (15, 18). HA-MRSA usually lacks genes for PVL and contains *SCCmec* type I, II or III alleles, whereas CA-MRSA has PVL genes and *SCCmec* type IV or V alleles (15, 18). These strains demonstrate differences in antibiotic susceptibility as well. CA-MRSA while highly virulent is less resistant to antibiotics compared to HA-MRSA (17). Differences in the rate of HA- versus CA-MRSA was investigated in a 2004-2005 demographic study in San Francisco, which found 243 cases of CA-MRSA infection/100,000 people compared to 31/100,000 for cases of infection with HA-MRSA (18). Similarly, of the 80,461 invasive infections in 2011 reported in the antibiotic threats by CDC, 18% infection was caused by HAHO-MRSA, 60% by HACO-MRSA and 21% by CA-MRSA (13, 14). Another study that investigated HA-and CA-MRSA related infections reported SSTI as the most common *S. aureus* infection and CA-MRSA caused about 86% of the SSTI (17). In this same study, HA-MRSA was isolated from 11% of bloodstream infection and 32% respiratory tract infections. Thus, both these strains can cause life-threatening infections and are responsible for the increased morbidity and mortality by *S. aureus* infections.

**S. aureus Skin and Soft Tissue Infections (SSTIs)**

SSTI accounts for approximately 90% of all *S. aureus* infections (19). As the name suggests, SSTIs are a result of microbial invasion of the skin and the underlying soft tissues (6, 20), encompassing conditions ranging from cellulitis to life-threatening necrotizing fasciitis (21). They have been categorized into superficial, uncomplicated SSTIs (impetigo,
abscess, furuncle, erysipelas, and cellulitis) or complicated SSTIs (necrotizing infection, infections associated with bites and animal contact, surgical site infections, and infections in the immunocompromised host) (8, 22, 23). Immunocompromised individuals include those with diabetes mellitus, HIV patients, individuals of advanced age as well as individuals with dysfunctional neutrophils (20, 24-26). Among SSTIs, abscesses and cellulitis are most common with about 50-75% of patients presenting with abscesses while 25-50% of patients present with cellulitis (21).

A study conducted by Suaya et al., investigating the incidence of *S. aureus* SSTI from 2001 through 2009 reported a 70% increase in all *S. aureus* associated hospitalization (27). Among these, *S. aureus* SSTIs accounted for 51% hospitalization in 2009, a 12% increase from 2001. This resulted in a significant increase in the total annual cost of *S. aureus* SSTI hospitalizations (44% increase from 2001) (27). Another study covering SSTI incidence from 2000 to 2012 reported a 40% increase in the overall incidence of SSTIs, resulting in an increase in treatment costs from 4.4 billion in 2000 to $13.8 billion in 2012 (28). This accounted for costs for ambulatory visits, emergency care as well as hospitalizations and prescriptions (28). In August 2004, Moran et al., enrolled patients presenting SSTIs in 11 university-affiliated emergency departments for a geographically diverse study to determine the prevalence of MRSA with SSTIs. Of the 422 patients enrolled, 76% were *S. aureus* SSTIs and 78% of these *S. aureus* strains were MRSA while 22% was methicillin-sensitive *S. aureus* (MSSA) (5). Similarly, Ray et al. reported that about 50% of *S. aureus* isolated from SSTIs were MRSA for the timeline they investigated (2006 to 2009) (21). These studies collectively demonstrate that increase in incidence of *S. aureus* SSTI, and
the prevalence of MRSA has resulted in an increase in the treatment cost, hospital stay and thus the economic burden by this pathogen.

**Pathogenesis of *S. aureus***

The pathogenesis of *S. aureus* is largely associated with its virulence factors regulated by the accessory gene regulator (*agr*) operon (29, 30). The *agr* operon regulates over 200 virulence genes responsible for invasion as well as adherence and biofilm formation (29, 30). The virulence and invasive phenotype of *S. aureus* infections is mainly attributed to secretion of alpha toxin or alpha hemolysin (Hla), a 33kDa protein, known to cause pore formation and promote inflammation (31-33). Studies on the role of Hla in skin infections in animal models attribute the ulcer (dermonecrosis) phenotype to Hla (34, 35). Hla activates the NLRP3 inflammasome complex leading to the production of active IL-1β, a pro-inflammatory cytokine ([Figure 1.1](#)) (33, 36). This toxin is secreted as a monomer and assembles on the cell surface to form a heptamer leading to pore formation (37). Hla interacts with a receptor, ADAM10 (a disintegrin and metalloproteinase 10) on the host cell surface as well as non-specifically with the cholesterol and sphingomyelin in the lipid bilayers to mediate this effect (38). At sub-lytic concentrations, Hla upregulates ADAM10 activity in epithelial cells and promotes the cleavage of E-cadherin, injuring the barrier integrity of epithelial cells (39, 40). This disruption of barrier integrity and the downstream signaling cascade leading to proinflammatory cytokine production (such as IL-1β, IL-6) causes immune cell recruitment and infiltration resulting in tissue damage (32, 36). This process contributes to the dermonecrotic lesions in animal models of *S. aureus* skin infection (35, 37). The significance of Hla in disease is highlighted in animal models using
an isogenic *hla* mutant strain where loss or mutation in Hla leads to reduced disease severity in SSTI as well as in a pneumonia model (34, 35, 37, 41-44).

Other pore forming toxins regulated by *agr* include the leukocidins: Panton-Valentine Leukocidin (PVL), γ-hemolysin, LukDE, and LukGH (LukAB) (29). These toxins also contribute to *S. aureus* pathogenicity. PVL is composed of two secreted components, LukS-PV and LukF-PV that assemble into pore-forming octamers on the membranes (29, 45). This toxin interacts with its receptors, complement C5a anaphylatoxin chemotactic receptor 1 (C5aR1) and C5a anaphylatoxin chemotactic receptor 2 (C5aR2) on the cell membrane leading to cell lysis (46). Neutrophils, critical for *S. aureus* clearance, are lysed by this toxin (19, 29, 45). However, their susceptibility to the toxin differs based on the host. Human and rabbit neutrophils are more susceptible to this toxin than mouse neutrophils (19). There are conflicting evidence on the role of PVL in disease severity in the case of *S. aureus* SSTI as well as other *S. aureus* infections such as pneumonia, and bone and joint infections (19, 45). Differences in outcomes are likely due to the varying susceptibility to the toxin because of differences in receptor expression between cells and between study subjects (human versus animals) (45, 46). LukDE and LukGH also contribute to the pathogenesis of *S. aureus* through lysis of phagocytic cells (29, 45). Specifically, LukGH, along with another peptide toxin phenol soluble modulin α (PSMα), help *S. aureus* evade phagocytic clearance by lysing the cells post phagocytosis (29, 45, 47). Thus, due to their pore forming as well as lytic ability, these toxins contribute to *S. aureus* pathogenesis (29, 46).
Figure 1.1. Hla activates NLRP3 inflammasome leading to IL-1β production.
Hla toxin binds to ADAM10 on the cell surface or to the lipid bilayers and leads to the activation of NLRP3 inflammasome complex. This leads to the generation of Caspase-1 through the cleavage of pro-caspase -1. The active caspase-1 then cleaves pro- IL-1β to active IL-1β. The pro-IL-1β and NLRP3 are synthesized by NF-κB, activated via binding of PAMPs to the TLRs on the surface. Adapted from: Ezekwe et al. (2016) (48) and Shao et al. (2015) (49).
Current Strategies for Treatment of S. aureus Infections

With the emergence of antibiotic resistant strains, limited antibiotics are available for treatment of S. aureus infections (4, 15, 20, 50). Infections caused by MSSA are treated with semi-synthetic penicillins such as nafcillin, and dicloxacillin, as well as first-generation cephalosporins (cephalexin, cefazolin), whereas to prevent resistance development, vancomycin is only prescribed for patients allergic to penicillin (23). However, for treatment of MRSA, vancomycin, clindamycin, linezolid, daptomycin, and trimethoprim/sulfamethoxazole can be used (23). While vancomycin is used for treatment of severe MRSA infections such as pneumonia, osteomyelitis, bacteremia and complicated SSTIs, as well as being an alternative for the corresponding MSSA infections, the emergence of vancomycin-intermediate (VISA) and the much less prevalent vancomycin-resistant S. aureus (VRSA) highlight the urgency of the current antibiotic crisis and the need for alternative therapeutic options (23). Besides antibiotic treatment for non-complicated SSTIs such as impetigo and cellulitis, drainage alone or drainage along with oral or topical antibiotics administration is recommended (6, 20, 23). However, for complicated SSTIs, hospitalization and intravenous therapy is required (6, 20, 23). Despite the availability of some antibiotics for treatment, the rising minimum inhibitory concentration (MIC) for these antibiotics, the treatment cost and mode of treatment highlight the need for susceptibility testing to prevent resistance development, and the necessity for the development of alternative therapeutic approaches, such as therapies that interfere with virulence factor function (22, 23). In line with this, there are multiple ongoing studies and clinical trials to develop efficacious vaccine or antibody therapies directed towards S. aureus virulence. Importantly, therapies aimed at secreted virulence factors are
expected to induce less or no resistance to therapeutic targeting (7, 42, 43, 51-62). However, these vaccines are yet to be approved. Besides identifying bacterial targets and developing vaccines, studies have also focused on identifying host factors contributing to defense against *S. aureus* (63, 64). Neutrophils are a key player in *S. aureus* susceptibility because of their role in bacterial clearance (63-66). Studies have also uncovered roles for different skin cells, such as keratinocytes and Langerhans cells, in protection against *S. aureus* skin infections (67-75). With the increasing antibiotic crisis, understanding mechanisms of host defense against *S. aureus* pathogenesis is especially important as therapeutics targeted towards enhancing host defense could prove essential in combating *S. aureus* infections.

1.2. Skin, the protective barrier against *S. aureus* SSTIs

Skin, the largest organ of a human body, is the first layer of defense against external insult (67-69, 76-78). It is composed of layers of resident skin cells and immune cells (Figure 1.2) that provide protection against breaches (67, 68). Thus, invading bacteria has to employ multiple mechanisms to breach this layer to cause infection (67-70, 78-80). Commensal bacteria, secreted antimicrobial peptides and a low pH of the skin are the first barriers against pathogenic bacteria colonizing the skin surface (67-70). The outer layer of skin, the epidermis, is primarily composed of keratinocytes, linked by intercellular junctions, and dead cells (mature keratinocytes) that form the hard barrier of the skin (67-71, 77, 78, 81). The epidermis also consists of melanocytes that produce melanin for color pigmentation as well as Langerhans cells and other resident immune cells (i.e. lymphocytes) (67, 68, 72, 77, 78, 82). The middle layer of skin, the dermis, contains many
other immune cells such as macrophages, T cells, B cells and fibroblasts, but primarily consists of collagen fibers (67, 68, 70, 77, 78, 83). The deepest layer of the skin is the hypodermis, made up of fats and connective tissue (67, 68, 70, 77, 78, 84). The dermal and hypodermal layers also house skin appendages such as hair follicles, sweat glands and sebaceous glands, as well as blood and lymph vessels that allow for neutrophil and monocyte recruitment during infection (67, 68, 77). The integrity of these layers and their resident immune cells contribute to the barrier function of the skin.

Skin keratinocytes have been widely studied for their role in *S. aureus* infections (67, 70, 71). These cells produce antimicrobial peptides, such as β-defensins and cathelicidins, which kill the bacteria. Keratinocytes are sensitive to alpha toxin (Hla), which can permeabilize them leading to cell death (85). This process is concentration dependent [low (0.1ug/mL) to high concentration (100ug/mL)] and includes cellular ATP depletion, calcium (Ca²⁺) and potassium (K⁺) efflux, and reduced oxygen consumption (85). Interaction of Hla with ADAM10 on keratinocytes promotes the cleavage of E-cadherin, an adherens junctions protein, injuring epithelial cells and skin barrier integrity (39, 40).
Figure 1.2. The layers of skin.
The skin consists of epidermal, dermal, and hypodermal layers that house different resident skin cells. The outermost layer, the epidermis, consists of commensals, antimicrobial peptides, low temperature and low pH, and dead keratinocytes (corneal layer) followed by layers of live keratinocytes and Langerhans cells that create a protective barrier against insult. The dermal layer contains immune cells such as macrophages, T cells, NK cells, as well as blood vessels and skin appendages (hair follicles). Immune cells such as neutrophils and monocytes get recruited to skin via chemoattractants secreted by the resident cells. The hypodermis mainly consists of adipose tissues, blood and lymph vessels. Adapted from: Miller and Cho (2011) (67) and Pasparakis et al. (2014) (69).
Upon the breach of the epithelial cells barrier, *S. aureus* is recognized by the pattern recognition receptors (PRRs) on keratinocytes and other resident immune cells (71, 73). Recognition of pathogen-associated molecular patterns (PAMPs) such as lipopeptides and peptidoglycan by PRRs [including toll-like receptors (TLRs) and nod-like receptors (NOD1 and NOD2)] leads to downstream activation of transcription factors including NF-κB and AP-1 that aid in the production of pro-inflammatory cytokines and chemokines such as IL-1β, IL-6, TNFα, CXCL1, CXCL2, and numerous anti-microbial peptides (AMPs) (71, 73). Genetic knockout mouse models have shown that these PRRs and downstream signaling is important to limit *S. aureus* SSTI infection [reviewed in (73)]. Scavenger receptors (CD36) also plays a role in host-defense against *S. aureus* (86).

Alongside keratinocytes, resident skin macrophages act as the first responders to the insult. Resident and perivascular macrophages phagocytose the bacteria as well as produce chemoattractants to recruit monocytes and neutrophils to the site of infection (73). Similar to macrophages, neutrophils clear the bacteria via phagocytosis and production of antimicrobial agents such as reactive oxygen species (ROS) and nitric oxide (NO) (73, 79).

In addition, neutrophils undergo degranulation and form neutrophil extracellular traps (NETs) to restrain the bacteria from spreading (73). However, because of the short life of neutrophils, the apoptotic neutrophils must be cleared by macrophages to avoid tissue damage, which is important for the resolution of infection (73).

In an attempt to confine the pathogen and resolve the infection, an abscess is formed which consists of viable and necrotic neutrophils, live bacteria, tissue debris and fibrin in a fibrous enclosed capsule surrounded by macrophages (73, 79). These abscesses either resolve
spontaneously after the elimination of the bacteria or require treatment (79) as described in the section (Current Strategies for Treatment of S. aureus Infections) above.

Skin Langerhans cells (LCs) in epidermal layers also contribute to the skin defense mechanism (72, 73, 87). These dendritic cell family members sample their surroundings for antigen and transport them to regional lymph nodes for presentation to naïve T cells to elicit an adaptive response (72, 73). These cells contribute to skin homeostasis by promoting proliferation and activation of regulatory T-cells in the skin under normal conditions. Whereas, upon infection or any other insult compromising the skin barrier, they participate in antimicrobial response and activate the adaptive T cells response (72, 82, 88). Release of cytokines from epidermal cells has been shown to modify LCs function and activation. For example, TNFα activates LCs, whereas thymic stromal lymphopoietin (TSLP) released by keratinocytes during atopic dermatitis inhibits its ability to induce adaptive response (reviewed in (72)). During a S. aureus skin infection, these cells sense the pathogen via their PRRs (langerin) that binds to WTA β-GlcNAc (N-acetylglucosamine modifications on wall teichoic acid) on S. aureus and activates the T cells (Th17) response (75).

Skin resident T cells, the alpha beta (αβ) and gamma delta (γδ) T cells, usually found in both epidermis and the dermis layers of the skin, also contribute to the skin homeostasis and host defense (83). In response to infection, these cells secrete cytokines and growth factors leading to cell proliferation, cytolysis, and recruitment of other immune cells to
infiltrate the damaged site (83). While about 20 billion T cells populate the human skin, only about 10% or less of these cells are γδ T cells [reviewed in (83)]. However, recent studies have demonstrated significant role of these resident γδ T cells, also called DETC(dendritic epidermal T cells) in mice, in maintaining keratinocytes production and proliferation as well as wound healing (83, 89). These cells produce IL-17 that plays a crucial role in bacterial clearance during *S. aureus* SSTI (90). T cells also produce IFN-γ that recruits other lymphocytes as well as activate the innate cells for bacterial clearance (74, 83, 90). These cells are also important for cell-mediated protection against reinfections (74, 91-93). Their importance in limiting recurrent SSTIs is evident in patients with low CD4+ T cells counts such as in HIV patients (24, 91). A recent study by Dillen et.al, highlights the possibility of using γδ T cells for therapeutic purposes against *S. aureus* infections (74) demonstrating the crucial role these cells play in host defense against *S. aureus* SSTI. Thus, the synergistic actions of the resident skin cells along with immune cells recruited to the site provides protection against *S. aureus* SSTIs.

**1.3. Sex bias in host response to diseases**

Many factors contribute to differences in disease outcomes and one such variable that is being widely recognized for driving this difference is sex of the species (94-101). Biological (sex hormones and chromosomes) as well as behavioral/environmental (habits/exposure to infectious agents) factors contribute to the sex differences in host response (94-108). Studies on the role of sex hormones suggest their effect on immune cells differs. Male sex hormone, testosterone, generally has an immunosuppressive effect while the female sex hormone, estrogen, plays an immunostimulatory role (97, 98, 100-
Sex chromosomes X and Y also contribute to sex differences in host response. X chromosome contains genes for PRRs, cytokine receptors as well as transcriptional factors, and Y chromosome contains regulatory genes that modulate immune cells function affecting the susceptibility of host to various immune-related diseases (100, 101). These factors modulate differences in host response along with the type of pathogen for infectious diseases (94-103, 105, 106, 108, 109, 111). Multiple epidemiological and animal model-based studies have investigated the contribution of these stimuli on disease outcomes. The enhanced immune response due to these stimuli in females alters the infection outcomes, such as in case of tuberculosis and aspergillosis, to be favorable in females compared to males (100, 101). While the prevalence of infections might be lower in females, they might experience increased disease severity and have fatal outcomes compared to males as seen with influenza or toxoplasmosis (100, 101). Also, the ability to mount a strong innate and adaptive response while still being protective can also make females prone to autoimmune diseases with increased inflammation (100). In terms of autoimmune diseases, it has been reported that about 80% of all the autoimmune cases reported in the US are in women (100, 112). However, there are some autoimmune conditions that are more prevalent in males such as myocarditis and idiopathic pulmonary fibrosis (100). Hence, based on the type of condition and the sex of the host disease outcomes vary and this could be modulated by chromosomal, hormonal, environmental and behavioral factors. Table 1.1 summarizes some of the epidemiological as well as experimental findings on the type of infection, pathogen (bacteria, virus, fungi and parasites) and differences in infection outcomes between sexes.
Table 1.1. Sex bias in susceptibility to infections

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Species</th>
<th>Type of Disease</th>
<th>Greater Incidence/Susceptible Sex</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Human, Mice</td>
<td>Bacteremia</td>
<td>Male</td>
<td>(109)</td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em></td>
<td>Human, Mice</td>
<td>Campylobacteriosis</td>
<td>Male</td>
<td>(113)</td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em></td>
<td>Human, Mice</td>
<td>Tuberculosis</td>
<td>Male</td>
<td>(114, 115)</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>Mice</td>
<td>Listeriosis</td>
<td>Female</td>
<td>(116)</td>
</tr>
<tr>
<td><em>Borrelia burgdorferi</em></td>
<td>Human</td>
<td>Lyme disease</td>
<td>Female</td>
<td>(117)</td>
</tr>
<tr>
<td>Herpes simplex virus type 2(HSV-2)</td>
<td>Human</td>
<td>Herpes</td>
<td>Female</td>
<td>(118, 119)</td>
</tr>
<tr>
<td>Human immunodeficiency virus (HIV)</td>
<td>Human</td>
<td>AIDS</td>
<td>Male</td>
<td>(120, 121)</td>
</tr>
<tr>
<td>Hepatitis B virus</td>
<td>Human</td>
<td>Hepatitis B (liver infection)</td>
<td>Male</td>
<td>(122, 123)</td>
</tr>
<tr>
<td>Middle East respiratory syndrome coronavirus (MERS-CoV)</td>
<td>Human, Mice</td>
<td>MERS</td>
<td>Male</td>
<td>(124, 125)</td>
</tr>
<tr>
<td>Cytomegalovirus</td>
<td>Human</td>
<td></td>
<td>Female</td>
<td>(126)</td>
</tr>
<tr>
<td><em>Aspergillus fumigatus</em></td>
<td>Human</td>
<td>Aspergilosis</td>
<td>Male</td>
<td>(127)</td>
</tr>
<tr>
<td><em>Cryptococcus neoformans</em></td>
<td>Human, Mice</td>
<td>Cryptococcusosis</td>
<td>Male</td>
<td>(128, 129)</td>
</tr>
<tr>
<td><em>Toxoplasma gondii</em></td>
<td>Human, Mice</td>
<td>Toxoplasmosis</td>
<td>Female</td>
<td>(130)</td>
</tr>
<tr>
<td><em>Plasmodium species</em></td>
<td>Human, Mice</td>
<td>Malaria</td>
<td>Male</td>
<td>(130)</td>
</tr>
<tr>
<td><em>Taenia solium</em></td>
<td>Mice</td>
<td>Cysticercosis</td>
<td>Female</td>
<td>(131)</td>
</tr>
</tbody>
</table>
Hormonal role in the immune response

Sex hormones such as progesterone (P4), estradiol (E2) and testosterone has been shown to be key players in modulating host immune responses, both innate and adaptive; and therefore, infection outcomes. Both males and females express these hormones. Female hormones, estrogen and progesterone, are expressed at high levels in females and in low levels in males while testosterone is expressed in low levels in females compared to males. There are three types of estrogen: estrone (E1), 17-β estradiol (E2) and estriol (E3). E2 is the most common form, predominantly expressed in women of childbearing age while E1 and E3 are produced after menopause and mainly during pregnancy, respectively. These hormones modulate their effects through different direct and indirect pathways regulated by their specific receptors (progesterone receptor- PR, testosterone or androgen receptor (AR) and estrogen receptors (ERα, ERβ and G-protein Coupled Receptor, GPER)). Both male and female cells express these receptors mediating the functionality of these hormones.

Estrogen signals through these receptors in a genomic and non-genomic action (Figure 1.3). The genomic pathway involves E2-ER dependent gene activation regulated by its direct interaction with the estrogen response elements (ERE) in the promoter of target genes and through indirect interaction with transcription factors, AP-1, SP-1 (146, 148). ERα and ERβ post E2 binding get translocated to the nucleus to mediate the genomic pathways (146, 148). The rapid, non-genomic pathway is regulated by the membrane ER and GPER mainly located in the endoplasmic reticulum (146, 148-150). The non-genomic
pathway involves the activation of PI3K/AKT and MAPK pathways and the production of cAMP, as well as intracellular calcium mobilization (146, 148-150).
Figure 1.3. Estrogen and estrogen receptors signaling.

Estrogen binds to the estrogen receptors on the surface, cytoplasm or the endoplasmic reticulum to initiate genomic and non-genomic signaling. Genomic actions involve gene activation via direct binding of E2-ERs to the estrogen response element (ERE), indirect binding to the transcription factors (TFs), or activation of TFs. The non-genomic, rapid, action involves PI3K/MAPK signaling and activation of downstream signals. Adapted from: Prossnitz and Barton (2014) (151).
Progesterone is known to regulate cellular functions genomically via nuclear PR and non-genomically via membrane PR (103, 153). In case of testosterone, it binds to the androgen receptor causing the receptors to dimerize and translocate to the nucleus to bind to the androgen response element (ARE) in the promoter regions of the genes (103, 154). Hence, these sex steroids bind to their respective receptors to regulate cellular function via genomic and non-genomic pathways. For example, testosterone has been shown to suppress the inflammatory response in macrophages and monocytes via decrease in expression of PRR such as TLR4, whereas estrogen enhances cell-mediated immune responses with upregulation in pro-inflammatory response and cytotoxicity as in the case of NK cells (103, 155). Similarly, estrogen and progesterone downregulate Th17 cell numbers as well as IL-17 production, while testosterone increases IL-17 production (100, 156). Besides regulating inflammatory responses, estrogen promotes antibody responses and vaccine efficacy, whereas testosterone decreases the response (100, 157, 158). The importance of estrogen in providing protection was further demonstrated in menopausal women where reduction in estrogen production made them susceptible to infections (158).

Estrogen contributes to skin thickness. The epidermal layer of the skin is thicker in females compared to males, however the male skin is 40% thicker in mice due to thicker dermis (159-161). Similar role of E2 is demonstrated in females with estrogen therapy (162). Estrogen also regulates collagen, elasticity, dryness, vascularity and pH of skin (159, 161, 162). In addition, it also plays a role in wound healing and skin pigmentation (163, 164). E2 accelerates wound healing in an ERβ-dependent manner by decreasing neutrophil infiltration at the site of inflammation while promoting re-epithelialization whereas
testosterone inhibits wound healing while enhancing the inflammatory response (135, 161, 162, 164-166). Similarly, E2 via GPER regulates melanin synthesis and contributes to skin pigmentation (161-163). Estrogen also modulates B cells and T cells activation for protective immune response and to maintain homeostasis (167). However, stimulated immune response by estrogen can also promote autoimmune diseases due to increased autoantibodies and inflammatory immune cells as in the case of Systemic Lupus Erythematosus (SLE) (168). Testosterone supplementation on the other hand improves SLE in female mice (168). Estrogen also modulates macrophages as well as neutrophils phagocytic and oxidative response (134, 140, 142, 169-178). Multiple studies have utilized specific agonists/antagonists to demonstrate the effect of estrogen and their receptors as well. Some known agonists are 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT) for ERα, 2,3-bis (4-hydroxyphenyl)-propionitrile (DPN) for ERβ and G-1 for GPER that work in lieu of estrogen to mediate receptor signaling (149, 179, 180). Whereas some known antagonists are 1,3-Bis (4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazoledihydrochloride (MPP) for ERα, -[2-phenyl-5,7-bis (trifluoromethyl) pyrazolo [1,5-a] pyrimidin-3-yl] phenol (PHTPP) for ERβ and G-15 and G-36 for GPER that inhibit the respective receptors signaling (149, 179, 180). However, some known ERα/β agonists and antagonists (PPT, tamoxifen, genistein, and ICI182,780) are known to activate GPER (151). Table 1.2 summarizes some known sex differences on immune cells response and the effects of hormones on these immune cells. Thus, through the regulation of downstream effectors sex hormones modulates innate and adaptive immune cells function contributing to the sex bias in host response.
Table 1.2. Sex-differences in immune cells response and effect of hormones

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Females</th>
<th>Effect of female hormones</th>
<th>Males</th>
<th>Effect of male hormone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages</td>
<td>Increased phagocytic activity and IL-10 production</td>
<td>Increased TLR4 expression</td>
<td>Increased pro-inflammatory cytokines production</td>
<td>Decreased nitric oxide (NO) and TNF</td>
</tr>
<tr>
<td></td>
<td>Increased TLR4 expression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>Increased phagocytic activity</td>
<td>Increased number and degranulation</td>
<td>Increased number and mobility</td>
<td>Decreased kinases and leukotriene formation</td>
</tr>
<tr>
<td>Dendritic Cells</td>
<td>Increased antigen production</td>
<td>Increased activation and TLR7 and 9</td>
<td>Decreased CXC10 and IFNα</td>
<td>No difference</td>
</tr>
<tr>
<td></td>
<td>Increased IFNγ production</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NK Cells</td>
<td>Increased granule response</td>
<td>Increased Granzyme B and IFNγ</td>
<td>Increased counts</td>
<td>Decreased number and proliferation</td>
</tr>
<tr>
<td></td>
<td>Increased IFNγ production</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Increased numbers and apoptosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T Cells</td>
<td>Higher CD4+ T count, CD4/CD8 T cell ratio, number of activated cells, CD8+ T cell cytotoxicity and Th2 bias</td>
<td>Increased CD8+ T cells response, and decreased Th17 cells numbers and IL-17 production, increased Th1 activity with low E2 and Th2 with high E2.</td>
<td>Higher CD8+ T cells, Treg cell numbers, Th1 bias</td>
<td>Decreased IFNγ, IL-4, IL-5, and increased IL-17 production, Decreased CD8+ T cells number and activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B Cells</td>
<td>Increased numbers</td>
<td>Increased IgG and IgM levels and decreased CD80 and CD86</td>
<td>No Difference</td>
<td></td>
</tr>
</tbody>
</table>

Adapted from: Klein et al. (2016) (100) and Capone et al. (2018) (181).
Sex-specific differences in *S. aureus* infections

In the case of *S. aureus* infections, sex specific differences in host response have been reported with differences in nasal carriage and as well as bloodstream infection outcomes in human (109, 182). Use of hormonal contraceptives has been shown to affect nasal *S. aureus* carriage with increased colonization for its users compared to females not on hormonal contraceptives. Also, nasal colonization is higher in males compared to females (182). In terms of bacteremia, while males are highly susceptible, mortality is higher in females. Moreover, exogenous estrogen administration increases disease severity. However, estrogen has protective effect on bone loss in mouse model of *S. aureus* arthritis (183). Sex differences were observed in rabbits infected with toxic shock syndrome toxin-1 (TSST-1) producing strain of *S. aureus* in the artificial chambers implanted subcutaneously. In this study, the authors found males to be more susceptible to infection, and castration reduced susceptibility in males (184). Also, ovariectomy rendered females susceptible to infection and estrogen supplementation provided protection in male rabbits, suggesting a protective role of estrogen (184, 185). Thus, there appears to be a female bias in *S. aureus* infection outcomes with increased susceptibility in males and a protective role of estrogen. While sex differences in the host response to *S. aureus* and the role of estrogen has been studied for some conditions, sex bias in host response to the most common *S. aureus* infection, SSTI, has not been reported.
1.4. Neutrophils in host defense against *S. aureus*

Neutrophils have been shown to be essential in clearance of *S. aureus* during infection (26, 63, 65, 67, 186-192). Using a mouse model for cutaneous infection, Mölne et al., reported increased severity of skin lesions and increased risk of bacteremia in neutrophil depleted animals (193). The importance of functional neutrophils for bacterial clearance is mainly observed in patients with neutropenia or conditions such as chronic granulomatous disease (CGD) that are at high risk for recurrent infections (66, 194-196). Dysfunction in neutrophils activity due to defects in phagolysosome formation as in Chediak-Higashi syndrome or transendothelial migration observed in leucocyte adhesion deficiency type 1 (LAD1) patients render them susceptible to life threatening infections (195, 197, 198).

During infection, these cells are the first innate responders recruited to the site via chemokines and cytokines secreted by the resident cell types (73, 79). These phagocytic cells internalize the bacteria and have antimicrobial mechanisms to clear them (65, 186). Phagocytosis is initiated when the neutrophils bind to the bacteria via its pattern recognition receptors (PRRs) such as TLRs that recognize pattern-associated molecular patterns (PAMPs) on the bacterial surface such as peptidoglycan (PGN) or lipoteichoic acid (LTA) (65, 186). In addition, opsonization of bacteria by serum factors such as complement proteins or antibodies (Igs) also promote the uptake of bacteria and its clearance by neutrophils (64, 65, 187, 191, 192, 199). Neutrophils express complement receptors such as CR3 (CD11b/CD18) also known as Mac-1, αmβ2 integrin, that recognizes a major serum opsonin iC3b and Fc receptors (FcRs) that recognize the Igs aiding in this process (65, 186,
191, 200-203). These receptors allow the internalization of *S. aureus* limiting the bacteria inside the neutrophil during infection. Interaction of the serum complement proteins with these receptors is crucial for clearance of *S. aureus* as inactivation of these proteins by heat-treatment of the serum leads to minimal to no killing by neutrophils (191). Internalized bacteria are then cleared by the antimicrobial defense (oxygen-dependent and independent) mechanisms of neutrophils (*Figure 1.4*) (186, 189, 204).
Figure 1.4. Oxygen-dependent and independent killing mechanisms of neutrophils.
Neutrophils employ oxygen-dependent and -independent bactericidal mechanisms for the clearance of *S. aureus* its recognition and phagocytosis by complement and Fc receptors. Priming and activation of neutrophils leads to NADPH oxidase complex assembly for reactive oxygen species (ROS) production. Oxidative responses include ROS derivatives (H$_2$O$_2$, O$_2^-$, HOCl), and reactive nitrogen species (RNS) such as nitric oxide and its derivatives (OONO$^-$.). Granule components (MPO, antimicrobial peptides, proteases and enzymes) contribute to oxygen-independent killing of *S. aureus*. *S. aureus* gets exposed to these antimicrobial agents inside the phagosome leading to its death. Adapted from: Rigby and DeLeo (2012) (64) and Nguyen et al. (2017) (205).
Oxygen-dependent and independent killing by neutrophils

The well-studied oxygen-dependent mechanisms involve reactive oxygen species (ROS) – superoxide production by neutrophils as well as generation of hypochlorous acid through interaction with myeloperoxidase (MPO) or peroxynitrite (ONOO•) by reacting with nitric oxide (NO) (186, 189, 204). These ROS and NO are key oxidants known to aid bacterial killing by oxidizing substrates in the medium (189). ROS is generated by NADPH oxidase that assembles on the phagosomal membrane after the neutrophil is primed by cytokines such as TNFα, GM-CSF or gets activated post bacterial interaction (65, 205-210). The NADPH complex of the phagocytes consists of six different subunits assembled together to form the NOX complex; the membrane proteins gp91phox and p22phox, and the cytosolic proteins p40phox, p47phox and p67phox (205, 207, 211-213). Activation of neutrophils lead to translocation of these cytosolic proteins to the membrane allowing for assembly of the NADPH oxidase complex (205, 207, 211-214). Activated NADPH oxidase then catalyzes the transfer of electrons from NADPH to molecular oxygen generating superoxide anions (O2•−)(205, 207, 211-213). The anion is converted to other reactive oxygen species that can damage nucleic acids, proteins and cell membrane, leading to the lysis of the phagocytosed bacteria (65, 205, 207, 211-213). Moreover, ROS is also released extracellularly, directly from the plasma membrane, to kill the non-phagocytosed bacteria present outside the cells (205). Functional NADPH complex is important in limiting infections as loss of its activity caused by mutations in gene encoding components such as in CGD patients leads to a defect in ROS production by phagocytes (194, 196). This failure to mount a respiratory burst leads to increased susceptibility and recurrent infections in CGD patients to various pathogens including S. aureus (188, 194, 196, 205, 215).
Neutrophils oxygen-independent mechanisms involve the role of various degradative enzymes and peptides contained in its granules and secretory vesicles (64, 65, 186, 187, 192, 216-219). Neutrophils have three granules: primary, secondary and tertiary; that can fuse with the phagosome in the process called degranulation exposing the bacteria in the phagosome to the granule components (216, 217, 220, 221). The primary granule, also known as azurophilic granules consists of toxic agents such as elastase, myeloperoxidase (MPO), cathepsins and defensins (216, 217, 219-222). The secondary and tertiary granules are MPO negative and contain lactoferrin and matric-metalloprotease 9 (MMP9) (216, 217, 219, 221). These enzymes and proteases are toxic to the pathogen and aids in their clearance in the phagosome (216-219, 222, 223). Importantly, the secondary and tertiary granules also contain CR3 (CD11b/CD18) as do the secretory vesicle membranes (186, 205, 216, 217, 224-226). These complement receptors are important in bacterial recognition and clearance as well as motility and adhesion of neutrophils (191, 200, 201, 203, 224, 227-230). Post-priming of neutrophils by TNFα, these receptors gets translocated to the membrane via exocytosis of these granules and vesicles increasing their surface expression in a p38MAPK-dependent manner (206, 209, 210, 231, 232). TNFα priming also leads to activation of inside-out signaling in the neutrophils aiding in the conformational change of these receptors on the surface from bent/closed to open/active state (205, 233)). This active state then leads to outside-in signaling by these receptors via ligand binding (C3-opsonized S. aureus) which is turn activates downstream signaling events leading to ROS production and bacterial clearance (205, 233). In addition to these, neutrophils undergoes NETosis, a process of release of DNA from neutrophils to form an
extracellular trap that also contains histones and antimicrobial granule components to trap and kill the pathogen, and thus contribute to bacterial clearance by neutrophils (234, 235). Overall, after phagocytosis of \textit{S. aureus}, these microbicidal granule components and ROS derivatives creates a toxic environment for the bacteria resulting in DNA and protein damage, ultimately leading to its death. However, \textit{S. aureus} have evolved with mechanisms to evade neutrophil killing (66, 80, 186).

\textbf{S. aureus and evasion of neutrophil clearance}

\textit{S. aureus} as a commensal organism has mechanisms to survive and grow in the human body (236, 237). Similarly, as a pathogen, it has evolved mechanisms to evade host defense and adapt at harsh environments, utilizing the host machinery for its survival and growth (25, 64, 186). \textit{S. aureus} escapes neutrophil clearance through various intrinsic and extrinsic resistance pathways ranging from scavenging of ROS, DNA damage repair to preventing uptake by the neutrophils (47, 64, 66, 186). \textit{S. aureus} utilizes its surface proteins such as protein A that binds to the Fc region of IgG and coats the bacteria with non-specific antibodies to prevent recognition and phagocytosis by neutrophils (186). Similarly, complement (C3b) mediated uptake of the bacteria is prevented by staphylococcal complement inhibitor via its interaction with C3 convertases, interfering with C3b generation and complement pathway activation (186, 238). Besides these defenses, \textit{S. aureus} also has machinery to prevent cell death caused by the antimicrobial agents of neutrophils (ROS, HOCl and ONOO\textsuperscript{-}). The bacteria produces enzymes such as superoxide dismutase and catalase to neutralize the formation of these oxidative products by converting superoxide anions (O\textsubscript{2}\textsuperscript{-}) to a less potent H\textsubscript{2}O\textsubscript{2}, and further into H\textsubscript{2}O and O\textsubscript{2} (63,
Additionally, *S. aureus* pigment staphyloxanthin has also been shown to have antioxidant properties (239). *S. aureus* also has mechanisms to evade oxygen-independent clearance by neutrophils (64). They have a three-component gene-regulatory system that regulates downstream bacterial responses which ultimately leads to decrease in the negative charge of the bacterial surface in the presence of antimicrobial peptides (63). These pathogens also produce exoenzymes, nucleases, that mediates it escape from NETs via degradation of DNA traps (241). Moreover, *S. aureus* produces toxins that have lytic activity on these cells (63, 64). Overall, *S. aureus* uses these additional strategies to avoid its uptake and clearance by neutrophils, allowing for persistent infection by these pathogens.

**Sex hormones and neutrophils**

In case of neutrophils, sex hormones, specifically estrogen (E2), can modulate different functionalities ranging from antimicrobial agents (ROS, NO, MPO) production to apoptosis of these cells in a dose dependent manner (136). E2 has also been shown to enhance neutrophil extracellular trap (NETs) formation through the G-protein coupled receptor (GPER) in neutrophil-like HL-60 cells (242). In a study conducted using human male and female neutrophils, significant difference in spontaneous apoptosis was observed (136). Moreover, when male neutrophils were treated with E2, apoptotic outcomes were delayed, suggesting a role for E2 in delayed apoptosis (136). Using human neutrophils, E2 has been shown to increase respiratory burst, degranulation and MPO activity in neutrophils (132, 243, 244). Similarly, studies have also demonstrated E2-dependent inhibition of superoxide (O$_2^-$) production as well as degranulation and lysosome release in
a dose- and time-dependent manner (132, 134, 169, 171). Likewise, testosterone dependent suppression of respiratory burst has been reported (134, 245). Estrogen stimulates nNOS expression in a concentration dependent manner as well, with low E2 levels decreasing nNOS expression at follicular phase compared to its expression at high E2 levels (ovulatory phase) of the female menstrual cycle (246, 247). Moreover, E2 treatment of male cells in vitro increases nNOS protein expression (246, 247). Thus, sex hormones modulate the functionality of neutrophils via regulation of its bactericidal mechanisms and survival. Table 1.3 summarizes some of these findings.
### Table 1.3. Effect of hormones on neutrophils

<table>
<thead>
<tr>
<th>Neutrophils from:</th>
<th>Hormone treatment (ex vivo)</th>
<th>Dose</th>
<th>Effects</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male and Female</td>
<td>Estrogen</td>
<td>Physiological</td>
<td>Delay in apoptosis</td>
<td>(136)</td>
</tr>
<tr>
<td>Female</td>
<td>Estrogen</td>
<td>Pharmacological</td>
<td>Reduction in superoxide generation, degranulation and lysosome release</td>
<td>(132)</td>
</tr>
<tr>
<td>Male</td>
<td>Estrogen</td>
<td>Physiological</td>
<td>Reduction in superoxide production</td>
<td>(169)</td>
</tr>
<tr>
<td>Male and Female</td>
<td>Estrogen</td>
<td>Physiological</td>
<td>Reduction in superoxide production</td>
<td>(134)</td>
</tr>
<tr>
<td>Combined</td>
<td>Testosterone</td>
<td>Physiological</td>
<td>Reduction in superoxide production</td>
<td></td>
</tr>
<tr>
<td>Sex Not Defined</td>
<td>Estrogen</td>
<td>Physiological</td>
<td>Increased superoxide production, degranulation and MPO activity</td>
<td>(173)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pharmacological</td>
<td>Inhibitory effect</td>
<td></td>
</tr>
<tr>
<td>Female mice</td>
<td>Estrogen</td>
<td>Physiological</td>
<td>Increased superoxide production</td>
<td>(140)</td>
</tr>
<tr>
<td>Male</td>
<td>Testosterone</td>
<td>Physiological</td>
<td>Reduction in superoxide production</td>
<td>(245)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pharmacological</td>
<td>Increased nitric oxide production</td>
<td></td>
</tr>
<tr>
<td>Female (premenopausal)</td>
<td>Estrogen</td>
<td>No treatment</td>
<td>Enhanced nNOS protein expression during ovulatory phase compared to follicular phase</td>
<td>(246, 247)</td>
</tr>
<tr>
<td>Female (premenopausal)</td>
<td>Estrogen</td>
<td>Physiological</td>
<td>Increased nNOS protein expression after E2 treatment to cells isolated at follicular phase</td>
<td></td>
</tr>
<tr>
<td>Female (postmenopausal)</td>
<td>Estrogen treatment</td>
<td>In vivo</td>
<td>Increased nNOS protein expression after E2 treatment</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>Estrogen</td>
<td>Physiological</td>
<td>Increased nNOS protein expression</td>
<td></td>
</tr>
<tr>
<td>Male mice</td>
<td>In vivo E2 treatment post orchidectomy</td>
<td>Physiological</td>
<td>Increased neutrophil serine proteases, elastase expression, MPO and iNOS protein expression</td>
<td>(248)</td>
</tr>
</tbody>
</table>
1.5. Concluding remarks and Hypotheses

*S. aureus* life-threatening infections and the emergence of the antibiotic resistant strains has posed a global health concern with limited treatment options in the absence of prophylactic or therapeutic vaccines (8, 13, 19). Similarly, multiple studies reporting increased susceptibility in immunocompromised individuals to *S. aureus* infections highlights importance of host immune response and the necessity to understand the host defense mechanisms against *S. aureus* (194, 196, 249). While multiple factors contributing to host defense against *S. aureus* infections have been investigated, contribution of sex hormones in regulating these immune responses have not been fully uncovered. Thus, in our studies detailed below, we will address the sex-specific differences in host response to the most common *S. aureus* infection: SSTI. Given the protective role of estrogen in most infections and its contribution to skin physiology and homeostasis, we hypothesized there to be a sex bias in host response to SSTIs with estrogen-dependent differences in immune cells response contributing to the innate resistance in females. In Chapter 2, we test this hypothesis using a murine model of SSTI. To further investigate the sex differences in host response to *S. aureus*, we determined the mechanisms driving the bias in neutrophils bactericidal efficacy between sexes in Chapter 3 based on our findings from Chapter 2, which suggests increased bactericidal capacity of female murine neutrophils *ex vivo*. As female neutrophils are known to be highly phagocytic and estrogen regulates antimicrobial mechanisms of neutrophils (100, 173), we postulate differences in opsonophagocytic mechanisms (complement and complement receptors) and ROS production between male and female neutrophils. We test this hypothesis in Chapter 3 utilizing murine neutrophils *ex vivo*. Given the protective role of estrogen in females and their differential response to
*S. aureus* toxin, Hla, identified in Chapter 2, we investigated the contribution of G protein-coupled estrogen receptor (GPER) in the protection observed in females using its agonist, G-1. Since Hla disrupts epithelial barrier integrity and contributes to *S. aureus* pathogenesis, we hypothesized that G-1 mediated activation of GPER provides protection against barrier disruption and limits *S. aureus* SSTI. Using our murine SSTI model and human keratinocyte cell line, HaCaT, we tested our hypothesis and the details of the approach and findings is explained in Chapter 4.
Figure 1.5. Schematic of gaps addressed in Chapters 2, 3 and 4.

(A) Sex differences in host response to *S. aureus* SSTI is addressed in Chapter 2 using murine model of SSTI. Based on the findings of Chapter 2, (B) Chapter 3 addresses mechanism(s) driving differences in bactericidal capacity of murine neutrophils *ex vivo* and (C) Chapter 4 investigates potential usage of estrogen receptor targeted therapeutic to decrease infection severity in males using murine model of SSTI.
CHAPTER 2: Innate Sex Bias of *Staphylococcus aureus* Skin Infection is Driven by alpha-Hemolysin

Moriah J. Castleman¹⁺, Srijana Pokhrel¹⁺, Kathleen D. Triplett¹, Donna F. Kusewitt², Bradley O. Elmore¹, Jason A. Joyner¹, Jon K. Femling³, Geetanjali Sharma⁴, Helen J. Hathaway⁵, Eric R. Prossnitz⁴ and Pamela R. Hall¹⁺⁺

¹University of New Mexico College of Pharmacy, Department of Pharmaceutical Sciences, Albuquerque, NM 87131, USA
²University of New Mexico School of Medicine, Department of Pathology, Albuquerque, NM 87131, USA
³University of New Mexico School of Medicine, Department of Emergency Medicine, Albuquerque, NM 87131, USA
⁴University of New Mexico School of Medicine, Department of Internal Medicine, Albuquerque, NM 87131, USA
⁵University of New Mexico School of Medicine, Department of Cell Biology & Physiology, Albuquerque, NM 87131, USA

*phall@salud.unm.edu*

⁺these authors contributed equally to this work

J Immunol December 8, 2017, ji1700810; DOI: https://doi.org/10.4049/jimmunol.1700810
ABSTRACT

Numerous studies have reported sex bias in infectious diseases, with bias direction dependent on pathogen and site of infection. *Staphylococcus aureus* is the most common cause of skin and soft tissue infections (SSTI), yet sex bias in susceptibility to *S. aureus* SSTI has not been described. A search of electronic health records revealed an odds ratio of 2.4 for *S. aureus* SSTI in males versus females. To investigate the physiological basis of this bias, we compared outcomes between male and female mice in a model of *S. aureus* dermonecrosis. Consistent with the epidemiological data, female mice were better protected against SSTI, with reduced dermonecrosis followed later by increased bacterial clearance. Protection in females was disrupted by ovariectomy and restored by short-term estrogen administration. Importantly, this sex-bias was mediated by a sex-specific response to the *S. aureus*-secreted virulence factor, alpha-hemolysin (Hla). Infection with wild-type *S. aureus* suppressed inflammatory cytokine production in the skin of female, but not male, mice when compared to infection with an isogenic *hla* deletion mutant. This differential response was conserved following injection with Hla alone, demonstrating a direct response to Hla independent of bacterial burden. Additionally, neutrophils, essential for clearing *S. aureus*, demonstrated sex-specific *S. aureus* bactericidal capacity *ex vivo*. This work suggests that sex-specific skin innate responsiveness to Hla and neutrophil bactericidal capacity play important roles in limiting *S. aureus* SSTI in females. Understanding the molecular mechanisms controlling this sex bias may reveal novel targets to promote host innate defense against *S. aureus* skin infection.
INTRODUCTION

Sex steroid hormones, such as estrogen and testosterone, can differentially modulate host immune responses (102, 110, 250), yet the impact of sex and the associated steroid hormones on mechanisms of disease susceptibility has historically been understudied (251, 252). This is especially true for skin-related research (253). Skin is the body’s largest organ and serves as the first line of defense against exogenous insult (77, 78). Although women have a greater incidence of dermatological disorders (253), they also demonstrate accelerated wound healing compared to men (165, 254-256). Yet despite these differences, very few studies have directly addressed sex-dependent effects on skin disease (253).

Between 2000 and 2012, the incidence of skin and soft tissue infections (SSTIs) in the USA is reported to have increased 40% with treatment expenditures increasing from $4.4 billion to $13.8 billion (in 2012 dollars) (28). Although males are often considered to be at increased risk of infection, innate sex bias in infection susceptibility and severity varies by pathogen and site of infection (95, 101). The dominant cause of SSTIs is Staphylococcus aureus (5, 21, 257), and in the United States, more than half of these infections are caused by methicillin-resistant strains (MRSA) (5, 257). Invasive S. aureus SSTI is mediated in large part by alpha-hemolysin (Hla), a secreted pore-forming toxin that is a major virulence factor causing dermonecrosis and inflammation (34, 35, 40, 258). However, despite the prevalence of S. aureus in SSTI and the contribution of Hla to pathogenesis, sex bias in innate susceptibility to S. aureus SSTI or Hla has not been reported.
Given the significant impact of SSTIs on human health, identifying and understanding sex-dependent differences in susceptibility to *S. aureus* skin infections could lead to novel treatment options. A recent literature review revealed that, compared to females, males are more often carriers of *S. aureus*, which may predispose them to infection (259), and males are at increased risk of *S. aureus* bacteremia (95, 259). Given the superior wound healing capacity of females compared to males (165, 254, 255), the protection afforded by estrogen in other models of Gram-positive infection (260, 261) and the contribution of Hla to pathogenesis (34, 35, 40, 258), we investigated whether female sex provides innate resistance to *S. aureus* SSTI in an Hla-dependent manner.

Here, we used a mouse model of *S. aureus* SSTI (258) to demonstrate that female sex, via the sex hormone estrogen, provides innate resistance to *S. aureus* SSTI – supporting a physiological component behind the epidemiological data showing a greater than two-fold higher incidence of *S. aureus* SSTI in male versus female patients. Specifically, female mice had an estrogen-dependent reduction in dermonecrosis and local inflammatory cytokine levels compared to males, followed days later by significant reductions in bacterial burden. Importantly, this bias was largely driven by a sex-specific response to Hla. Inflammatory cytokine production at the site of *S. aureus* infection increased in females infected with an isogenic *hla* deletion strain (LACΔhla) compared to infection with wild-type *S. aureus* (LAC), consistent with previous studies using female mice (262). However, when comparing the sexes, the opposite was observed in infected male mice, with either no change or with a trend toward reduced inflammatory cytokine levels in the absence of Hla. These differences were conserved following injection of Hla alone,
demonstrating independence from bacterial burden. In addition, in _ex vivo_ studies, neutrophils from female mice demonstrated increased _S. aureus_ bactericidal capacity compared to neutrophils from male mice. Given that neutrophils are essential for clearing _S. aureus_ infections (263), this work suggests that sex-specific differences in both skin innate responsiveness to Hla and neutrophil bactericidal capacity play important roles in limiting _S. aureus_ SSTI in females.

**MATERIALS AND METHODS**

**Electric Health Records (EHR) query parameters**

Incidence of _S. aureus_ SSTI in male versus female patients was evaluated by searching the University of New Mexico Health Sciences Center Clinical and Translational Science Center (CTSC) Health Facts® database. The database is comprised of de-identified electronic health records (EHR) from more than 600 participating CERNER hospitals (UL1TR001449 NIH CTSA at UNM). We queried the database for patients of reproductive age (postpubescent to premenopausal; 18-45 yo) receiving treatment for uncomplicated SSTI between 2011 and 2014. Based on previous studies (3, 264), SSTIs were defined by International Classification of Diseases, 9th Revision (ICD-9) (265) diagnosis codes 680.x (carbuncle and furuncle) and 681.x-682.x (cellulitis and abscess). SSTIs codes were matched with same encounter ICD-9 codes for methicillin-sensitive _S. aureus_ (MSSA) (41.11) or MRSA (41.12). Population statistics are based on the number of 18-45 yo (males or females) included in the database during the same time frame.
Bacterial strains and growth conditions

*S. aureus* USA300 LAC and the LAC *hla* deletion mutant (LACΔhla) were provided by Dr. F. DeLeo (Rocky Mountain Laboratories, National Institutes of Health/National Institute of Allergy and Infectious Diseases, Hamilton, MT) and Dr. J. Bubeck-Wardenburg (University of Chicago, Chicago, IL), respectively. Bacteria were cultured at 37°C in trypticase soy broth (TSB) to early exponential phase as described previously (266). CFUs were determined by plating serial dilutions on sheep blood agar (BD Biosciences, Franklin Lakes, NJ), and stocks maintained at -80°C in TSB with 10 % glycerol.

Mouse model of dermonecrosis

Animal work protocols were approved by the Institutional Animal Care and Use Committee of the University of New Mexico Health Sciences Center (UNM HSC) and carried out in the American Association for Accreditation of Laboratory Animal Care-accredited UNM HSC Animal Research Facility. All animal work was performed in strict accordance with recommendations in the Guide for the Care and Use of Laboratory Animals (267) and the US Animal Welfare Act (268). C57BL/6J and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were acclimated for a minimum of seven days prior to use. Ovariectomized C57BL/6J mice and sham surgery controls were purchased from The Jackson Laboratory. These mice were allowed to recover over several weeks (≥ 10 as previously described (261, 269)) prior to exogenous administration of vehicle or 17β-estradiol (E2).
The mouse model of dermonecrosis was performed according to previously published protocol (258), with no attempt to target a specific phase of the estrous cycle. Twenty-four hours before infection, 8-12 week-old male and female mice were anesthetized by isoflurane inhalation, and Nair™ (Church and Dwight, Ewing, NJ) used to remove hair from the right flank. On the day of infection, early exponential-phase *S. aureus* at the indicated inoculum was delivered in 50 µl of USP-grade saline (Braun, Irvine, CA) by subcutaneous injection into the right flank. Mice were weighed prior to infection and daily thereafter. Injection sites were photographed daily and area of dermonecrosis determined by ImageJ analysis (270). At the time of sacrifice, mice were euthanized by CO₂ asphyxiation and skin surrounding the injection site (2.25 cm²) excised and homogenized prior to serial dilution and plating on sheep blood agar for CFU determination. Homogenate was centrifuged at 12,500 x g for 10 min and supernatant (clarified homogenate) stored at -80°C for later cytokine analysis. Stock solutions of 17β-estradiol (E2) (Sigma-Aldrich®, St. Louis, MO) were prepared in ethanol then diluted 10-fold in 0.9% NaCl, 0.1% BSA, 0.01% Tween 20 for injection. Where indicated, mice received intraperitoneal injections of 10 µg/kg E2 or vehicle control on day -2 through day +2 relative to infection. Recombinant Hla for dermonecrosis studies was prepared as previously described (86), and 1 µg of purified Hla in 50 µl USP-grade saline was injected subcutaneously into the right flank as indicated.

**Cytokine and myeloperoxidase quantification from tissue homogenate**
Cytokines and myeloperoxidase (MPO) were measured in clarified supernatant from homogenized skin prepared as described above. Cytokines levels were determined using a custom designed multiplex assay (Millipore, Billerica, MA), and MPO levels measured using the ELISA Mouse Myeloperoxidase DuoSet kit (R&D Systems, Minneapolis, MN), each according to manufacturer’s directions. For cytokine levels below the limit of detection, one-half of the lowest standard concentration was utilized.

**Histology**

For histologic examination, skin samples were collected on day 3 post-infection or Hla-injection and a total of 6 lesions were evaluated for each sex and treatment group. Briefly, a 1-cm² sample of skin containing a lesion or an inoculation site was flattened on thin cardboard, fixed 24-48 hours in neutral buffered formalin, then stored in 70% ethanol. The skin sample was bisected through the center of the lesion or site of inoculation and processed routinely for embedding in paraffin, sectioning at 5 μm thickness, and staining with hematoxylin and eosin (H&E). H&E-stained slides were scanned by an Aperio CS2 scanner (Leica Biosystems, Buffalo Grove, IL) and morphometry was performed using the HALO image analysis platform (Indica Labs, Albuquerque, NM) to determine the length of necrotic epidermis and the area of dermal necrosis in each lesion. Because the necrotic material in abscesses adhered poorly to slides and was often lost during tissue processing, quantification of abscess size is not included.

**Quantitative RT-PCR to measure inflammasome gene expression**
Infection site tissue (2.25-cm²) was collected in RNALater (Qiagen, Valencia, CA). RNA was isolated and purified using Qiazol and RNeasy Kits (Qiagen), respectively, according to manufacturer’s directions. High-capacity cDNA RT kits with RNAsen inhibitor, random hexamer primers (Applied Biosystems, Foster City, CA) and a PTC-200 Peltier thermocycler (Bio-Rad, Hercules, CA) were used to generate cDNA. Quantitative PCR (qPCR) was performed on a ViiA 7 Real-Time PCR system (Applied Biosystems) using Taqman® Gene Expression master mix (Applied Biosystems). Gene expression was quantified using QuantStudio software (Applied Biosystems, Foster City, CA) relative to hprt using Prime Time Predesigned qPCR assays for nlrp3, asc, casp1 or il-1b (Integrated DNA Technologies, Coralville, IA).

**Western blot analysis of caspase-1 and IL-1β**

Frozen clarified abscess homogenates were thawed rapidly at 37°C and protein concentration measured by A_{280} (Nanodrop 1000 Spectrophotometer; ThermoFisher Scientific, Wilmington, DE). Equal amounts of total protein were separated by SDS-PAGE on a 4-12% Bis-Tris BOLT gel in MES-SDS running buffer (Life Technologies, Grand Island, NY), prior to transfer to nitrocellulose membranes. Membranes were blocked with 3% non-fat milk (NFM) in TBS (20 mM Tris, pH 7.5 and 150 mM NaCl) for 1.5 h at 22°C, then probed overnight at 4°C with rabbit anti-mouse anti-pro Caspase 1 + p10 + p12 antibody (Abcam, Cambridge, MA) or rabbit anti-mouse IL-1β (Abcam) in TBS plus 1% NFM. After washing with TBST (TBS with 0.1% Tween 20), membranes were developed using SuperSignal™ Maximum Sensitivity Substrate (ThermoFisher Scientific) following incubation with goat anti-rabbit IgG poly-horseradish peroxide–conjugated secondary.
antibody (ThermoFisher Scientific). Imaging was performed using a Protein Simple FluorChem R imaging system (Protein Simple, Santa Clara, CA) and band intensity determined using Image Studio Lite software (LI-COR Biosciences, Lincoln, NE) relative to total protein loaded based on SYPRO® Ruby staining (Lonza, Allendale, NJ).

Mouse bone-marrow neutrophil ex vivo killing of S. aureus

The indicated S. aureus isolates were prepared for ex vivo killing assays as follows: one day prior to the assay, 2 x 10^7 CFU/mL of S. aureus was cultured (37°C, 220 rpm) in TSB along with 50 nM autoinducing peptide 1 (AIP1) (Biopeptide Co., Inc, San Diego, CA) to stimulate quorum sensing. After 5 h, bacteria were washed twice with phenol red free HBSS (Gibco™, ThermoFisher Scientific, Waltham, MA) by centrifugation at 1800 x g at 4°C for 4 min. Bacteria were briefly vortexed and sonicated, suspended in HBSS to 6 x 10^6 CFU/mL and stored on ice at 4°C until use. On the day of the assay, bacteria were opsonized in 10 % autologous mouse serum for 20 min at 37 °C with gentle rotation (10 rpm). Bacteria were then plated for CFU determination.

On the day of the assay, bone marrow was collected by flushing mouse femurs and tibias with ice cold HBSS containing 2 mM EDTA using a 26-gauge needle. Red blood cells in bone marrow were lysed in 0.2 % sodium chloride, after which neutrophils were isolated by density gradient centrifugation using Histopaque® 1119 and 1077 (Sigma-Aldrich®). Isolated neutrophils were suspended in Assay buffer (phenol red free HBSS with calcium/magnesium (Lonza, Walkersville, MD), 20 mM HEPES, 1 % charcoal stripped FBS (J
Scientific, Woodland, CA)) and primed with 100 ng/mL mouse TNFα (BioLegend, San Diego, CA) for 30 min at 37 °C with 5 % CO₂.

To allow for phagocytosis, TNFα-primed neutrophils and opsonized S. aureus were combined at an MOI of 1 and incubated for 15 min at 37 °C with gentle end-over-end rotation. The mixture was then centrifuged at 200 x g (7 min) to remove non-phagocytosed bacteria, before resuspending the pellet in room temperature Assay buffer. A sample was sonicated in PBS containing 1 % Triton X-100 before serial dilution and plating on sheep blood agar to determine the number of bacteria phagocytosed (t0 CFUs). Percent phagocytosed bacteria was determined relative to the number of opsonized bacteria. The remaining sample was incubated for 1 h at 37 °C with gentle end-over-end rotation, before serial dilution and plating for CFU determination (t60). Percent killing was calculated as follows based on CFUs at t0 and t60: \(((t0–t60)/t0)*100\).

**S. aureus growth in the presence of E2**

*S. aureus* USA300 LAC at 1 x 10⁵ CFU/mL was grown overnight in TSB at 37°C with shaking in the presence of vehicle control or the indicated concentrations of E2. Growth was measured by optical density at 600 nm (OD₆₀₀) recorded at 15-min increments using a Tecan Systems (San Jose, CA) Infinite M200 plate reader.

**Statistical Analysis**
Statistical analyses were performed using Prism 7 software (Graph Pad Software, La Jolla, CA). Variance was determined by F-test and data analyzed as appropriate by Unpaired t-test or Mann-Whitney test for non-parametric data. Necrosis data were analyzed by Unpaired t-test with Welch’s correction for unequal variance. Multiple comparison analyses were performed by ANOVA with post-hoc tests as indicated.
RESULTS

Female sex confers innate resistance to S. aureus skin infections

To gain insight into potential sex bias in the incidence of S. aureus SSTI in humans, we queried the University of New Mexico Health Sciences Center Clinical and Translational Science Center (CTSC) Health Facts® database of electronic health records (EHR) for patients of reproductive age (postpubescent to premenopausal; 18–45 yo) receiving treatment for methicillin-sensitive S. aureus (MSSA) or MRSA SSTI between 2011 and 2014. We found a significantly increased incidence of infection in males compared to females (Table 2), resulting in an odds ratio of greater than 2.3 for male infection. Given that these findings likely result from both behavioral and biological factors (102), we focused on the biological contribution of sex to S. aureus SSTI.

Table 2. Sex Bias in incidence of S. aureus SSTI*

<table>
<thead>
<tr>
<th>Sex</th>
<th>SA SSTI (MSSA/MRSA)</th>
<th>Population</th>
<th>Incidence/100,000</th>
<th>X²</th>
<th>Odds Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>8263 (4022/4241)</td>
<td>11,611,077</td>
<td>71</td>
<td>p&lt;0.0001</td>
<td>2.375 (95% CI 2.30-2.45)</td>
</tr>
<tr>
<td>Female</td>
<td>6580 (2864/3716)</td>
<td>21,957,204</td>
<td>30</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Data from the Cerner Health Facts database, 18–45 y, 2011–2014. CI, confidence interval.

Using an established mouse model of S. aureus SSTI (258), we compared outcomes in male and female mice on days 1 and 3 after subcutaneous infection with the well-characterized, highly-virulent, MRSA isolate USA300 LAC (271). While differences in dermonecrosis did not reach significance on day 1 post-infection, by day 3, near the peak
of pathogenesis (272), female C57BL/6J mice showed significantly reduced dermonecrosis (>2.5-fold reduction) compared to males (Figure 2.1A-B). This effect was not restricted to C57BL/6J mice as infected female BALB/c mice also displayed reduced day 3 dermonecrosis versus males (>1.8-fold reduction) (Figure 2.2A-B). Histologically, the lesions in C57/BL6J males and females had a similar appearance, with a clearly delineated central region of epidermal necrosis overlying an area of dermal necrosis which extended into the panniculus carnosus. An intense inflammatory cell infiltrate, consisting predominantly of neutrophils, occupied the dermis adjacent to the central area of epidermal and dermal necrosis. An abscess consisting of degenerate neutrophils filled the subcutis at the base of the lesion (Figure 2.1C). Although similar in character, lesions in male and female mice varied significantly in extent, with males having greater lengths of epidermal necrosis (9.57±0.98 vs 6.35±0.75, p=0.0277) and greater areas of dermal necrosis (1.49±0.57 vs 0.74±0.36 mm², p=0.0238) than females.

Differences in dermonecrosis between the sexes appeared to be largely independent of bacterial burden at the site of infection, which differed only slightly, but significantly, on day 1 between C57BL/6J males and females (log CFUs of 8.9 and 8.6, respectively) (Figure 2.1D) and on day 3 between male and female BALB/c mice (8.5 vs 8.2 log CFUs, respectively) (Figure 2.2C). Similarly, weight loss, a measure of overall morbidity, was reduced on day 1, but not day 3, post-infection in female C57BL/6J mice versus male controls (Figure 2.1E, for BALB/c Figure 2.2D). We next asked if there were differences between the sexes in the cytokine response at the site of infection. Although inflammatory cytokines can drive bacterial clearance (272, 273), inflammation at the site of S. aureus
SSTI can also contribute to disease severity without improving bacterial clearance (274). Here, however, as might be expected from reduced dermonecrosis, on both days 1 and 3 post-infection female C57BL/6J mice displayed reduced levels of the pro-inflammatory cytokines IL-1β (2.6-fold, 2.9-fold, respectively), TNFα (2.0-fold, 2.9 fold), IL-6 (6.9-fold, 2.5-fold) and CXCL1 (3.1-fold, 3.1-fold) at the site of infection compared to males (Figure 2.1F). However, IL-10 levels did not significantly differ between the sexes at these time points, suggesting that the differential regulation is cytokine specific. A similar trend was observed for BALB/c mice (Figure 2.2E). Together, these results demonstrate an innate sex bias in the severity of S. aureus SSTI, with female sex providing a protective benefit against pathogenesis and inflammation.
Figure 2.1. Female mice are resistant to *S. aureus* skin infection compared with males.

C57BL/6J mice were infected s.c. with 2.3 10^7 USA300 LAC. (A) Representative images of infection sites (scale bar, 5 mm) and (B) area of dermonecrosis on days 1 and 3 postinfection (n = 12 mice per group from three independent experiments). (C) Histologic appearance of normal skin and infection site lesions on day 3 postinfection (top, uninfected; bottom, infected). Arrowheads mark the junction between viable and necrotic epidermis and asterisks indicate areas of dermal necrosis; areas of abscessation are identified by the letter A. Scale bar, 0.5 mm. (D) Bacterial burden (CFU) at the site of infection and (E) percent weight change on days 1 and 3 postinfection. (F) Day 1 and day 3 inflammatory cytokine levels in clarified infection site homogenate. Day 1, n = 4 mice per group; day 3, n = 6–8 mice per group from two independent experiments. Histology only, n = 6 mice per group. Data are mean ± SEM. Mann–Whitney U test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. ns, not significant.
Figure 2.2. BALB/c female mice are better protected against *S. aureus* skin infection compared to males.

BALB/c mice were infected subcutaneously with 3 x 10^7 LAC. (A) Representative images of infection sites (bar=5 mm), (B) area of dermonecrosis and (C) bacterial at the site of infection on day 3 post-infection. (D) Percent weight change on days 1 and 3 post-infection. (E) Day 3 inflammatory cytokine levels in clarified infection site homogenate. n=8 mice per group from two independent experiments. Data are mean ± SEM. Unpaired t-test, * p<0.05; ** p<0.01; **** p<0.0001. ND, not detected.
**Hla contributes to sex bias in *S. aureus* SSTI**

Sex-specific responses to *S. aureus*-secreted virulence factors such as Hla could account for the pronounced differences in dermonecrosis and inflammatory cytokine production in infected mice. To test this, we subcutaneously infected male and female C57BL/6J mice with wild-type *S. aureus* (LAC) or an isogenic hla deletion mutant (LACΔhla) (32). Due to the trend toward increased bacterial clearance in LAC-infected female versus male mice on day 3 post-infection (Figure 2.1D, Figure 2.2C), we postulated there would be a significant difference in bacterial clearance later post-infection. We compared outcomes over the course of a 7-day infection (see Figure 2.3 for data from days 1 and 3 post-infection with LACΔhla). By day 7 post-infection, female mice infected with LAC again showed significantly reduced dermonecrosis, but also significantly increased bacterial clearance, compared to infected males (Figure 2.4A-C). During LACΔhla infection, male mice showed small areas of dermonecrosis at the site of infection on day 7 (Figure 2.4A, B), suggesting that males may be more susceptible than females to *S. aureus* virulence factors other than Hla. Importantly, in contrast to LAC-infected mice, there was no significant difference in day 7 bacterial clearance between male and female C57BL/6J mice infected with LACΔhla (Figure 2.4C). These findings suggest that sex-specific differences in innate immune control of *S. aureus* infection are dependent on the ability of the pathogen to express Hla.
Figure 2.3. Male and female mice respond similarly to skin infection with \textit{S. aureus} lacking Hla.

C57BL/6J mice were infected subcutaneously with $2 \times 10^7$ LACΔhla. (A) Representative images of infection sites (bar=5 mm) and (B) area of dermonecrosis on days 1 and 3 post-infection (n=17-24 mice per group from at least three independent experiments). ND, not detected. (C) Histologic appearance of day 3 post-infection lesions in male (upper panel) and female (lower panel) mice infected with LACΔhla. Areas of abscessation are indicated by A. In both sexes, there were mild to moderate predominantly neutrophilic infiltrates in the dermis, hypodermis, and subcutis and extensive areas of abscessation in the hypodermis and subcutis. Scale bar = 0.5 mm. (D) Percent weight change and (E) bacterial burden (colony forming units, CFU) at the site of infection on days 1 and 3 post-infection. (F) Day 1 inflammatory cytokine levels in clarified infection site homogenate. (E-F) Day 1, n=4 mice per group; Day 3, n=6-8 mice per group from two independent experiments. Data are mean ± SEM. Mann-Whitney test; ns, not significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.
Figure 2.4. Male and female mice differentially respond to skin infection with *S. aureus* lacking Hla.

C57BL/6J mice were infected s.c. with 2–3 × 10^7 LAC or LACΔhla. (A) Representative images of infection sites (scale bar, 5 mm), (B) dermonecrosis (ND, not detected), and (C) bacterial burden at the site of infection on day 7 postinfection. n = 12 mice per group (LAC) and 7–10 mice per group (LACΔhla) from at least two independent experiments. Data are mean ± SEM. Mann–Whitney U test. (D) Cytokine levels in infection site homogenate on day 7 postinfection (n = 7–10 mice per group from two independent experiments). Data are mean ± SEM. ANOVA: p = 0.0007 (IL-1β), p = 0.0008 (TNF-α), p = 0.0006 (IL-6), p = 0.0003 (CXCL1), p = 0.0404 (IL-10). Dunn multiple comparison test: *p < 0.05, **p < 0.01, ***p < 0.001. ns, not significant.
Interactions between sex and Hla mediate the pro-inflammatory response during SSTI

In a previous study of *S. aureus* skin infection using female BALB/c mice, Hla was shown to suppress local inflammatory cytokine production and neutrophil influx (262). However, by comparing both sexes, our findings suggest that the local inflammatory response during *S. aureus* SSTI is not only dependent on Hla, but on the sex of the host as well. To test this, we compared inflammatory cytokine levels in the skin of male and female C57BL/6J mice on day 7 after infection with LAC or LACΔhla. As expected, given the reduced dermonecrosis and bacterial burden compared to males (Figure 2.4A-C), female mice infected with LAC had lower local levels of IL-1β, TNFα, IL-6 and CXCL1 (Figure 2.4D). Interestingly, unlike earlier time points (Figure 2.1F), day 7 post-infection females also had reduced expression of the anti-inflammatory cytokine IL-10, suggesting that the sex differences in cytokine production are both time and cytokine specific. In contrast to LAC-infected mice, however, there was no difference in cytokine levels between male and female mice infected with LACΔhla. Consistent with findings in BALB/c females (262), local IL-1β, TNFα and CXCL1 levels were increased in *S. aureus* infected C57BL/6J female mice in the absence of Hla (Figure 2.4D). However, this was not the case with male mice, which showed a trend toward cytokine reduction in the absence of Hla, although this did not reach statistical significance. Statistical analysis (2-way ANOVA) of these findings revealed significant interactions between host sex and Hla for each cytokine (IL-1β, p=0.0235; TNFα, p= 0.0132; IL-6, p=0.0391, CXCL1, p=0.0088 and IL-10, p=0.0258). These findings, together with sex- and Hla-dependent differences in pathogenesis and
bacterial clearance, demonstrate that interactions between host sex and *S. aureus* expression of Hla mediate innate sex bias in murine models of *S. aureus* SSTI.

**Females are innately resistant to Hla-mediated inflammation and pathogenesis**

Hla pore formation in host cell membranes leads to NLRP3 inflammasome activation, resulting in caspase-1 cleavage and activation, and cleavage and secretion of the mature inflammatory cytokine IL-1β (275, 276). Notably, estrogen-mediated suppression of inflammasome activation in another disease model has been reported (277). To gain insight into the lower levels of IL-1β in the skin of LAC-infected female versus male mice, we measured local transcription of inflammasome component genes *nlrp3* and *asc*, as well as *casp1* and *il-1β*, at different time points post-infection. Transcription of *nlrp3* was significantly reduced at the site of infection on days 1 and 3, as was *il-1β* on day 3 post-infection, in females relative to males (Figure 2.5A, B, Figure 2.6). In contrast, there was no difference in transcript levels of *asc* and *casp1* between the sexes at either time point (Figure 2.6C). However, western blot analyses showed that although there was no difference in infection site levels of pro-caspase 1 or pro-IL-1β between LAC-infected males and females (Figure 2.5C-F), both active caspase-1 and mature IL-1β levels (36) were significantly reduced in the females. Together, differences in local transcription of *nlrp3* and *il-1β*, reduced cleavage of caspase-1 and IL-1β and reduced IL-1β secretion in the skin of LAC-infected female versus male mice, suggests the potential for sex-dependent regulation of NLRP3 inflammasome activation in response to *S. aureus* infection.
Figure 2.5. Markers of NLRP3 inflammasome activation are reduced in female mice during *S. aureus* skin infection compared with males.

C57BL/6J mice were infected as described in Fig. 1. (A) *nlrp3* and (B) *il-1β* gene induction at the site of infection relative to infected males. Day 1, n = 3–4; day 3, n = 7–8 mice per group. (C–F) Immunoblotting and quantification of band intensity relative to total protein for (C and D) pro–caspase-1 and active caspase-1 and (E and F) pro–IL-1β and mature IL-1β in day 3 infection site homogenate (n = 3 mice per group). Data are mean ± SEM. Unpaired t test. *p < 0.05, **p < 0.01. ns, not significant.
Figure 2.6. Expression of inflammasome related genes at the site of *S.aureus* SSTI in male and female mice.

C57BL/6J mice were infected as described in Figure 1. (A) *nlrp3* and (B) *il-1β* transcription at the site of infection relative to *hprt* and uninfected male controls. (C) *asc* and (D) *casp1* (left) gene induction relative to infected males on days 1 and 3 post infection and (right) transcription at the site of infection relative to *hprt* and uninfected male controls (day 1 n=3-4; day 3 n=7-8 mice per group). Data are mean ± SEM. Mann-Whitney test; ns, not significant; *p*<0.05; *** *p*<0.001.
The well-characterized role of Hla in NLRP3 inflammasome activation, together with potential differences in regulation of inflammasome activation, suggested that females would be innately resistant to Hla-mediated pathogenesis, independent of the presence of S. aureus. To address this, we subcutaneously injected male and female mice with purified Hla. Compared to males, female mice displayed a greater than 4-fold reduction in dermonecrosis on day 3 post-injection (Figure 2.7 A, B). Histologically, lesions consisted of clearly defined regions of epidermal and dermal necrosis, with a mild granulocytic infiltrate in the dermis; no abscessation was seen (Figure 2.7C). These lesions were observed in all male mice treated with Hla, but in only a single female mouse. Thus, there were marked differences between the two sexes in average length of epidermal necrosis (14.14±1.81 vs 0.38±0.92 mm, p<0.0001) and average area of dermal necrosis (2.83±0.83 vs 0.04±0.11 mm², p=0.0004). Compared to males, female mice also had significantly reduced local inflammatory cytokine levels on both days 1 and 3 post-injection (Figure 2.7D), whereas IL-10 levels were below the limit of detection (data not shown). Overall, our findings demonstrate sex-specific innate resistance to Hla-mediated pathogenesis.
Figure 2.7. Hla-induced dermonecrosis and inflammatory cytokine production is reduced in female mice compared with males.

C57BL/6J mice were s.c. injected with 1 μg Hla. Day 1 and 3 postinjection (A) representative injection site images, (B) dermonecrosis, and (C) histologic appearance of Hla-induced lesions in male (upper panel) and female (lower panel) skin on day 3 postinjection. The arrowhead marks the junction between viable and necrotic epidermis and the asterisk indicates the area of dermal necrosis in the male skin. Black material located above the epidermis in the female skin is a marker indicating the site of Hla injection; marking was performed because no gross lesions developed. Note the marked difference between the sexes in the severity of the lesions and the absence of abscessation in either sex. Scale bar, 0.5 mm. (D) Day 1 and 3 cytokine levels in clarified injection site homogenate. n = 10–14 mice per group from at least three independent experiments. Histology only, n = 6 mice per group. Data are mean ± SEM. Mann–Whitney U test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Estrogen promotes innate resistance to *S. aureus* SSTI in female mice

Estrogen is known to support wound healing as well as modulate trafficking and function of innate immune cells important for bacterial clearance (165, 171, 172, 254, 255, 278-280), suggesting that ovariectomy would reduce protection during *S. aureus* SSTI. To test this, female mice were ovariectomized (OVX) or given a sham operation (SO), and allowed to recover for approximately 10 weeks (261, 269) prior to subcutaneous infection with *S. aureus*. Consistent with a role for estrogen in innate resistance to *S. aureus* SSTI, OVX mice displayed increased dermonecrosis (1.7-fold) compared to SO mice, as well as slightly increased local bacterial burden (7.5 to 8.0 log CFUs) (Figure 2.8A, B). Importantly, short-term treatment of OVX mice with exogenous estrogen in the form of 17β-estradiol (E2) restored protection against *S. aureus* dermonecrosis and reduced weight loss (Figure 2.8C-E), although there was no significant difference in bacterial burden with short-term E2 administration (Figure 2.8F). Furthermore, as expected based on differences in dermonecrosis, local inflammatory cytokine levels were increased in OVX mice and reduced with E2 treatment (Figure 2.8G). In contrast to OVX mice, E2 treatment of SO mice did not further improve disease outcome (Figure 2.8C-F), suggesting a threshold for E2-mediated protection in females. Furthermore, short-term treatment of male mice with E2 did not limit the severity of *S. aureus* SSTI (Figure 2.9A-C), possibly due to the suppressive effects of testosterone on the immune response (104, 137). Therefore, together with the absence of direct effects of E2 on *S. aureus* growth (Figure 2.9D), these findings demonstrate that estrogen is an important regulator of the female innate response to *S. aureus* pathogenesis.
Figure 2.8. Ovariectomy impairs and estrogen restores female innate resistance to *S. aureus* dermonecrosis.

Female C57BL/6J mice were OVX or underwent SOs. After recovery, mice were s.c. infected with 2 × 10^7 CFUs USA300 LAC. Day 3 postinfection (A) dermonecrosis and (B) bacterial burden at the site of infection in untreated mice (n = 11 mice per group from three independent experiments). Data are mean ± SEM. Unpaired t test. (C–G) OVX and SO mice received 10 μg/kg E2 or vehicle control (i.p.) on day −2 through day +2 relative to infection with LAC. (C) Representative images of infection site, day 3 postinfection (D) dermonecrosis (ANOVA, p = 0.0164), (E) percent weight change (ANOVA, p < 0.0001), and (F) bacterial burden at the site of infection (ANOVA, not significant) (n = 3 mice per group representative of two independent experiments). Data are mean ± SEM. Tukey multiple comparison test. (G) Day 3 cytokine levels in clarified infection site homogenate; ANOVA: p = 0.0284 (IL-1β), p = 0.0044 (TNF-α), p = 0.0212 (IL-6), and p = 0.0001 (CXCL1) (n = 3 mice per group). Dunn multiple comparison test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure 2.9. 17β-estradiol administration to male mice does not enhance protection against *S. aureus* SSTI and does not directly alter bacterial growth.

C57BL/6J male mice received 10 µg/kg 17β-estradiol (E2) or vehicle (Veh) control (i.p.) on day -2 through day +2 relative to infection with LAC. Day 1, 3 and 7 post-infection (A) dermonecrosis, (B) percent weight change and (C) day 7 bacterial burden at the site of infection. n=8-9 mice per group from two independent experiments. (D) Growth of LAC in the presence of the indicated concentrations of E2 or vehicle control as measured by OD$_{600}$. Data are mean ± SEM of n=6 and representative of two independent experiments.
Neutrophil bactericidal capacity against *S. aureus* is sex-dependent

Neutrophils are critical for clearance of *S. aureus* (263), and both sex- and hormone-specific differences in neutrophil trafficking and responses to exogenous stimulation have been reported (133, 280-284). Excessive neutrophil accumulation, however, can contribute to inflammation resulting in pathogenic effects (86, 285, 286). Given the differences in day 7 bacterial burden between male and female mice infected with LAC (Figure 2.4C), but not with LACΔhla, we first asked whether this was associated with differences in neutrophil presence at the site of infection. To address this, we measured myeloperoxidase (MPO) levels at the site of infection, which is widely used as a marker for phagocyte, frequently neutrophil, presence (272). We found that MPO levels were reduced in the skin of LAC-infected females versus males on both days 3 and 7 post-infection (Figure 2.10A). However, the opposite was observed in LACΔhla-infected mice, with MPO significantly increased (day 7) in females compared to males. While these differences could result from varying rates of phagocyte influx or clearance between the sexes, they suggest that increased bacterial clearance in LAC-infected female mice is not dependent on a sustained, elevated phagocyte presence at the site of infection.

Given the importance of neutrophils for clearance of *S. aureus* (263), increased *S. aureus* clearance despite reduced MPO levels in LAC-infected female versus male mice could result from differences in neutrophil bactericidal capacity. To determine whether neutrophils from female mice are more efficient at clearing *S. aureus* compared to those from male mice, we isolated bone marrow neutrophils and assessed phagocytosis and killing of *S. aureus* ex vivo. Consistent with our *in vivo* data, female neutrophils showed
significantly increased phagocytosis (1.5-fold) and killing (percent phagocytosed bacteria killed) of LAC compared to males (Figure 2.10B), resulting in a 2.2-fold reduction in CFUs. Similar to findings in male versus female mice, OVX mice also had higher local MPO levels on day 3 post-infection compared to SO controls, and neutrophils collected from OVX females had reduced ex vivo bactericidal efficacy compared to those from SO mice (Figure 2.10C, D). Neutrophils from females also showed increased phagocytosis of LACΔhla (1.5-fold) and total CFUs killed (1.4-fold) compared to males, although the percent of phagocytosed bacteria killed did not vary (Figure 2.10E). However, neutrophils from both sexes were better able to kill S. aureus ex vivo in the absence of Hla (Figure 2.10B, D). This suggests that other factors, such as increased inflammatory cytokine production in LACΔhla- versus LAC-infected females, may have contributed to equivalent bacterial clearance in male and female mice infected with LACΔhla (Figure 2.3 E, Figure 2.4C). Alternatively, expression of Hla and similarly regulated virulence factors following phagocytosis of S. aureus by human neutrophils has been shown to contribute to cell lysis (287). Whether murine neutrophil function is impacted by these factors in vivo, and whether female neutrophils are uniquely resistant to such effects compared to male neutrophils, is unclear. Regardless, these results indicate that sex-dependent differences in neutrophil bactericidal capacity may also contribute to the innate sex bias in S. aureus SSTI.
Figure 2.10. Neutrophils from female mice have increased S. aureus bactericidal capacity versus neutrophils from males.

(A) MPO levels in infection site homogenate on day 3 (left) and day 7 (right) postinfection (n = 7–10 mice per group from two independent experiments). Day 3, unpaired t test; day 7, ANOVA (p < 0.0001) with Tukey multiple comparison test. (B) Phagocytosis and killing (1 h) of LAC by bone marrow neutrophils (MOI = 1) collected from male and female C57BL/6J mice. (C) Local MPO levels from OVX and SO C57BL/6J mice on day 3 post-LAC infection. (D) LAC phagocytosis and killing (1 h) by bone marrow neutrophils (MOI = 1) collected from OVX and SO C57BL/6J mice. (E) Phagocytosis and killing (1 h) of LACΔhla by bone marrow neutrophils collected from male and female C57BL/6J mice. The starting inoculum was set to 100% for phagocytosis assays (left). For percent killing, the number of bacteria phagocytosed was set to 100% (n ≥ 3 mice per group). Data are mean ± SEM. Unpaired t test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
DISCUSSION

Despite epidemiological evidence of sex differences in infectious diseases and the ability of sex steroids to differentially regulate immune cells (95, 102, 110, 250), the biological impact of sex and sex hormones on mechanisms of infection susceptibility and pathogenesis have been largely understudied. Here we used a murine infection model to show that female sex, via the sex hormone estrogen, provides innate resistance to SSTI caused by the important human pathogen, *Staphylococcus aureus*. Remarkably, this resistance is mediated by significant interactions between host sex and the *S. aureus*-secreted virulence factor, Hla. We found suppressed inflammation in the skin of female vs male mice infected with an Hla-producing isolate of *S. aureus* - suppression which was lost when infected with an isogenic Hla deletion mutant. This differential response was conserved following injection with Hla alone, demonstrating a direct, sex-specific response to Hla, independent of bacterial burden. Together, these findings suggest that sex-specific differences in skin innate responsiveness to Hla play an important role in limiting *S. aureus* SSTI in females. In this era of growing antibiotic resistance, a detailed understanding of the molecular mechanisms driving this sex bias may reveal novel therapeutic approaches to promote host innate defense against infection.

Epidemiological studies have shown pathogen-specific sex bias in susceptibility and severity of a variety of infectious diseases (95, 96, 98, 99, 101). However, such reports outnumber research studies aimed at defining the physiological mechanisms driving these differences in infection susceptibility. Regarding *S. aureus*, males, as well as females using hormonal contraception, show increased *S. aureus* nasal colonization rates compared to
females not on hormonal contraceptives (288). Given that colonization is associated with increased risk of infection, perhaps it is not surprising that males have an increased incidence of *S. aureus* bacteremia (SAB) versus females (259, 289-292). However, females have increased risk of mortality from SAB, with reports on MRSA and community-acquired SAB showing higher 7- and 30-day mortality rates, respectively, for female versus male patients (259, 293, 294). Consistent with this epidemiological data showing human females are more likely to die from SAB, female mice show reduced survival following intravenous *S. aureus* infection compared to males (295), and administration of exogenous estrogen to females further increased mortality compared to vehicle-treated controls (296). In contrast, male rabbits were more susceptible than females in a lethal subcutaneous implant infection model using a toxic shock syndrome toxin (TSST-1) producing *S. aureus* isolate (184). In this model, neutering or estrogen administration reduced lethality in male rabbits (184, 185), whereas spaying increased infection lethality in females (184), pointing to the powerful contributions of sex steroid hormones on infection susceptibility and disease progression. Similarly, compared to placebo-treated OVX controls, E2 administration to OVX mice protected against *S. aureus* arthritis and bone loss without affecting bacterial burden (297). More recently, Coridden et al. reported on a mouse model of systemic *S. aureus* infection in which tamoxifen, a selective estrogen receptor agonist/antagonist, increased survival and enhanced bacterial clearance (298). This outcome was attributed to tamoxifen’s effects on neutrophil migration and function, including enhanced formation of neutrophil extracellular traps (NETs). Interestingly, the ability of tamoxifen to enhance neutrophil function resulted from an increase in intracellular ceramide production and was independent of estrogen receptors. Here, we
demonstrate male sex bias in *S. aureus* SSTI and show that sex-specific and estrogen-dependent differences in skin innate responsiveness to Hla drive this bias. It will be important in future studies to investigate sex-specific regulation of the inflammatory response to Hla *ex vivo* in murine resident skin cells, as well as validating our findings using human neutrophils and skin from both sexes. To better characterize the role of E2 in defense against *S. aureus* pathogenesis, it will also be revealing to perform more extensive studies using SO and OVX mice, together with short-term E2 administration, at additional time points post-infection using LAC versus LACΔhla or injection with Hla. Also, given the sex difference in *ex vivo* killing of *S. aureus* by murine neutrophils and the findings of Corriden et al. (298), it will be important to explore sex-specific and estrogen-dependent differences in murine and human neutrophil chemotaxis and NET formation. Meanwhile, the work reported here, focused on SSTI as the most common form of *S. aureus* infection (19), expands our understanding of host-pathogen interactions in *S. aureus*-mediated disease. Furthermore, the male sex bias in susceptibility to *S. aureus* SSTI or bacteremia, together with the female bias towards mortality with SAB, may suggest that sex bias varies not only by pathogen, but also by the type and severity of infection.

As the first line of defense against exogenous insult, skin plays a crucial role in the host immune response (77, 78) and sex steroids regulate many aspects of skin physiology. These hormones, specifically androgens such as testosterone in males and estrogens in females, contribute to skin architecture, including differences in epidermal and dermal thickness, as well as to immune system function (159, 299, 300). With respect to SSTI, differences in both the resolution of infection and subsequent tissue restoration or wound healing likely
contribute to disease severity. Interestingly, innate sex differences in the skin are perhaps most apparent in the accelerated wound healing ability of females versus males (301). Inhibition of cutaneous wound healing and enhanced inflammation in males has been attributed to testosterone and the androgen receptor (AR) (135). In contrast, intact female or ovariectomized mice treated with estrogen show accelerated wound healing and reduced inflammation (165). Similarly, in humans, topical estrogen accelerates cutaneous wound healing in elderly male and female patients, a finding associated with reduced neutrophil influx and increased fibronectin levels (166). Here, we show that short-term administration of E2 to ovariectomized mice restores protection against S. aureus dermonecrosis, further demonstrating the protective role that estrogen plays in the skin. Notably, a recent investigation into the female prevalence of autoimmune diseases identified an associated female-biased transcriptomic signature in the skin that is independent of sex steroid levels (302, 303). Whether such a transcription system independently contributes to female protection against infectious diseases of the skin, and whether it can be exploited to enhance host defense against SSTI, warrants further investigation.

Sex steroids are known to differentially modulate host immune responses, with estrogens being broadly associated with immunoenhancement and androgens with immunosuppression (100, 110, 137, 304). However, a detailed understanding of the cells and signaling pathways contributing to innate sex bias in susceptibility to a range of infectious diseases remains lacking (95, 98, 250). Estradiol (E2), the predominant form of estrogen produced by females, signals by binding the cytosolic/nuclear estrogen receptors ERα and ERβ, as well as the non-canonical membrane bound G-protein coupled estrogen
receptor (GPER) (149, 176, 305, 306). Signaling through each receptor can result in rapid, non-genomic effects such as calcium mobilization and cAMP production, as well as slower transcriptional (genomic) effects mediated by ERα and ERβ binding to gene promoters containing estrogen response elements (ERE) or via downstream GPER activation of transcription factors. Estrogen receptor signaling is further complicated by far ranging but varying cell distributions, as well as differences in expression levels and the existence of different isoforms of ERα and ERβ (148, 149, 305-307). Similarly, androgens signal through the AR, a ligand-inducible transcription factor that regulates gene expression by binding to androgen response elements (ARE) (152). Given the complexity of hormone signaling in vivo, particularly in the context of an intact immune system, it is unlikely that protection against an invading pathogen can be attributed to a single hormone or receptor. Rather, it is likely that the estrogen-mediated innate protection against S. aureus SSTI in female mice reported here results from a complex interplay among these receptors and their signaling pathways. Therefore, future studies must seek to unravel the molecular mechanisms controlling innate protection in females.

The biological impact of sex and sex hormones on disease susceptibility and treatment has historically been understudied. Fortunately, recent years have witnessed a growing recognition of this gap (251, 252), culminating in the recent National Institutes of Health call that sex be addressed as a biological variable in NIH-funded research (308). In addition to expanding our understanding of host-pathogen interactions in S. aureus-mediated disease, this work further highlights the significant contributions of sex and sex hormones to infectious disease susceptibility and severity.
ACKNOWLEDGEMENTS

This work was supported by research grants from the National Institutes of Health to P.R.H. (AI091917, AI128159) and E.R.P. (CA127731, CA163890, and CA194496), and utilized services and facilities provided through the National Institutes of Health–funded University of New Mexico Clinical and Translational Science Center (UL1TR001449). Research in this paper was also supported by the Human Tissue Repository and Tissue Analysis Shared Resource, funded by the Department of Pathology, University of New Mexico Comprehensive Cancer Center (CA118100).
CHAPTER 3: Complement Receptor 3 Contributes to the Sexual Dimorphism in Neutrophil Killing of Staphylococcus aureus

Srijana Pokhrel\textsuperscript{1}, Kathleen D. Triplett\textsuperscript{1}, Seth M. Daly\textsuperscript{1}, Jason A. Joyner\textsuperscript{1}, Geetanjali Sharma\textsuperscript{2}, Helen J. Hathaway\textsuperscript{3}, Eric R. Prossnitz\textsuperscript{2} and Pamela R. Hall\textsuperscript{1,*}

\textsuperscript{1}University of New Mexico College of Pharmacy, Department of Pharmaceutical Sciences, Albuquerque, NM 87131, USA

\textsuperscript{2}University of New Mexico School of Medicine, Department of Internal Medicine, Albuquerque, NM 87131, USA

\textsuperscript{3}University of New Mexico School of Medicine, Department of Cell Biology & Physiology, Albuquerque, NM 87131, USA

*phall@salud.unm.edu

Running Title: CR3 and innate sex bias in neutrophil killing of S. aureus

“Under Review”, May 2020
ABSTRACT

We previously reported sex differences in innate susceptibility to *Staphylococcus aureus* skin infection, and that bone marrow neutrophils (BMN) from female mice have an enhanced ability to kill *S. aureus* ex vivo compared to those of male mice. However, the mechanism(s) driving this sex bias in neutrophil killing have not been reported. Given the role of opsonins such as complement, as well as their receptors, in *S. aureus* recognition and clearance, we investigated their contribution to the enhanced bactericidal capacity of female BMN. We found that levels of C3 in the serum and CR3 (CD11b/CD18) on the surface of BMN were higher in female compared to male mice. Consistent with increased CR3 expression following TNFα priming, production of reactive oxygen species (ROS), an important bactericidal effector, was also increased in female versus male BMN in response to serum-opsonized *S. aureus*. Furthermore, blocking CD11b reduced both ROS levels and *S. aureus* killing by murine BMN from both sexes. However, at the same concentration of CD11b blocking antibody, *S. aureus* killing by female BMN was greatly reduced compared to those from male mice, suggesting CR3-dependent differences in bacterial killing between sexes. Overall, this work highlights the contributions of CR3, C3 and ROS to innate sex bias in the neutrophil response to *S. aureus*. Given that neutrophils are crucial for *S. aureus* clearance, understanding the mechanism(s) driving the innate sex bias in neutrophil bactericidal capacity could identify novel host factors important for host defense against *S. aureus*.
INTRODUCTION

Neutrophils are innate immune cells essential for *S. aureus* clearance (64, 193, 309-311). We recently reported a sex bias in host response to *Staphylococcus aureus* skin and soft tissue infection (SSTI) and sex-specific differences in the *ex vivo* bactericidal capacity of murine neutrophils against *S. aureus* (312). Specifically, in a mouse model of *S. aureus* SSTI, female mice were better protected than males against tissue damage and showed enhanced bacterial clearance at day 7 post-infection (312). Our findings also revealed increased *ex vivo* clearance of *S. aureus* by murine female bone marrow neutrophils (BMN) compared to those from their male counterparts. This finding could have important *in vivo* implications to the innate sex bias in *S. aureus* SSTI. However, the mechanism(s) driving this sex-specific difference in *ex vivo* *S. aureus* killing by neutrophils are yet to be determined.

Recognition of *S. aureus* by neutrophil surface molecules such as toll-like receptors (TLRs), G protein-coupled receptors (GPCRs), or opsonic receptors, such as complement (CR) or Fc receptors (FcR), promotes phagocytosis of bacteria and exposes them to a repertoire of antimicrobial effectors [reviewed in (64, 65)]. Known antimicrobial effectors against *S. aureus* include reactive oxygen species (ROS), nitric oxide (NO), myeloperoxidase (MPO) and neutrophil granule components released after *S. aureus* phagocytosis (65). While many mechanisms for *S. aureus* clearance by neutrophils have been described [reviewed in (63-66)], optimal bactericidal capacity requires cytokine priming (191, 313, 314). For example, compared to unprimed neutrophils, TNFα priming activates p38 MAPK, upregulates CR3 expression, and initiates production of reactive
oxygen species (ROS), creating a toxic environment for pathogens (205, 232, 315). Given this and that inactivation of serum complement by heat treatment results in little to no neutrophil killing of bacteria (191, 199, 202, 316-318), in the present study we focused on neutrophil complement receptor-mediated bacterial recognition and phagocytosis.

Specific to *S. aureus*, serum opsonin iC3b, generated after cleavage of complement protein C3, is important for neutrophil-mediated recognition and clearance of *S. aureus* (191, 199, 202, 318). Phagocytosis is initiated when iC3b deposited on *S. aureus* is recognized by complement receptor 3 (CR3), also known as Mac-1, integrin αMβ2 or CD11b/CD18, on the neutrophil surface (200, 201, 319). Not surprisingly, *in vivo* C3 deficiency impairs clearance of *S. aureus* in a mouse model of septic arthritis (320). However, the roles of CR3 and C3, and how they may regulate sex-specific neutrophil bactericidal capacity against *S. aureus*, have not been addressed. Given the importance of complement-complement receptor interactions and ROS production in *S. aureus* clearance, we postulated that increases in CR3 and C3 in females contributes to the sex bias in murine neutrophil killing of *S. aureus*.

As expected, we found increased C3 fragments in the serum of female versus male mice and increased CR3 expression on the surface of female murine BMN compared to those from males, independent of priming conditions. Surprisingly, no differences were observed in total CR3 protein levels, possibly suggesting differences in receptor trafficking to the cell surface or in p38 MAPK activation. We also found that female murine BMN treated
with serum-opsonized *S. aureus* produced increased ROS compared to male BMN. Importantly, at the same concentrations and compared to isotype control, CR3 blocking antibody treatment of female murine BMN reduced *S. aureus* killing to a much greater extent than male BMN, whereas CR3 blocking reduced ROS production by BMN of both sexes. This suggests that increased CR3-mediated *S. aureus* killing by female murine BMN may be dependent on non-ROS effector mechanism(s). Although the exact bactericidal effectors are yet to be identified, together, our findings support a role for CR3 and possibly C3 in driving the *ex vivo* sex-bias in murine neutrophil bactericidal capacity against *S. aureus*.

**MATERIALS AND METHODS**

**Ethical Statement and animals**

All animal work was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (267) and the U.S. Animal Welfare Act (321) and with the approval of Institutional Animal Care and Use Committee of the University of New Mexico Health Sciences Center. Age matched, eight- to twelve-week old male and female C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA) and acclimated for a minimum of seven days prior to use. No attempt was made to target a specific phase of the female estrous cycle. Furthermore, to avoid potential sex differences in the adaptive immune response resulting from potential prior *S. aureus* exposure, we used specific-pathogen free (SPF) mice for our studies (312) and focused on non-FcR-mediated bacterial clearance mechanisms.
**Bacterial strains and growth conditions**

MRSA USA300 isolate LAC (271) was provided by Dr. F. DeLeo (Rocky Mountain Laboratories, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Hamilton, MT). Bacteria were cultured in Trypticase Soy Broth (TSB) at 37°C to early exponential phase as described previously (312, 322). Stocks were prepared in TSB with 10% glycerol and maintained at -80°C. Stock titers (colony forming units, CFUs) were determined by plating serial dilutions on sheep blood agar (BD Biosciences, Franklin Lakes, NJ, USA).

**Isolation of murine BMN**

Mice were euthanized by CO2-inhalation according to approved methods prior to collection of bone marrow. BMN were isolated from age-matched, male and female mice as previously described (312). Briefly, femurs and tibias of the mice were flushed with ice cold phenol red-free Hank’s Balanced Salt Solution (HBSS) (Gibco™, ThermoFisher Scientific, Waltham, MA) plus 2 mM EDTA. Red blood cells (RBCs) were lysed using Ammonium-Chloride-Potassium (ACK) lysis buffer (Lonza, Walkersville, MD) and BMN isolated by density gradient centrifugation using Histopaque 1119 and 1077 (Sigma-Aldrich, St. Louis, MO).

**Western blot analyses**

Western blot analyses were conducted to determine levels of C3 in mouse serum as well as CD11b and p38 versus phospho-p38 levels in BMN. For complement protein C3
determination, murine male and female blood was collected in serum Z/1.3 microtubes (SARSTEDT AG & Co. KG, Germany) by cardiac puncture and serum clarified by centrifugation at 10,000 x g for 5 min. Serum samples were separated on a 4-12% Bis-Tris Bolt™ gel in MES buffer (Life Technologies, Grand Island, NY) before being transferred to 0.45 µm nitrocellulose membranes (Bio-Rad, Hercules, CA) for staining. Five percent non-fat milk in TBS/T buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20) was used for antibody dilutions as well as for blocking the membrane. After blocking for 1.5 h at 22°C, blots were probed at 4°C overnight using rabbit anti-mouse C3 antibody (ab200999) (Abcam, Cambridge, MA) followed by secondary goat anti-rabbit IgG (H+L) poly-HRP antibody (32260) (Thermo Fisher Scientific).

CD11b levels in unprimed or TNFα-primed (100 ng/mL, 30 min at 37°C) BMN, were determined following BMN lysis using Bolt™ LDS sample buffer (Thermo Fisher Scientific). For the quantification of phospho- and total-p38 MAPK, unprimed or TNFα-primed (10 ng/mL for 5 min at 37°C) BMN were lysed using 1X RIPA buffer (J62524, Alfa Aesar, Tewksbury, MA) containing protease and phosphatase inhibitors and EDTA (Halt™ Protease and Phosphatase Inhibitor Cocktail and EDTA solution (Thermo Fisher Scientific)) before addition to Bolt™ LDS sample buffer. For CD11b and p38 MAPK, equal amounts of protein (based on the cell number or as determined by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific), respectively) in Bolt™ LDS buffer were incubated at 95°C for 10 min before separation on a 4-12% Bis-Tris Bolt™ gel in MES buffer; transferred to a 0.45 µm nitrocellulose membrane and blocked using 5% non-fat milk in 1X TBS/T. Rabbit anti-mouse CD11b antibody (ab133357) (Abcam) and Goat
anti-rabbit IgG (H+L) poly-HRP (32260, Thermo Fisher Scientific) were used for CD11b detection, while mouse-anti-mouse/human p38 MAPK Phospho (Thr180/Tyr182) antibody (clone A16016A, Biolegend, San Diego, CA) and goat-anti-mouse IgG (H+L) poly-HRP antibody (32230, Thermo Fisher Scientific) was used for the detection of phospho-p38 MAPK. For detection of total p38 MAPK, the membrane was stripped using WB stripping buffer (62.5 mM Tris-HCl, 2% SDS, 100 mM β-mercaptoethanol) at 50°C for 30 min with gentle shaking. The blot was then reprobed with rabbit anti-mouse p38 MAPK antibody (Poly6224, Biolegend), followed by goat anti-rabbit IgG (H+L) poly-HRP.

For membrane development, either SuperSignal™ West Femto Maximum Sensitivity Substrate (C3, phospho-p38 and p38) or SuperSignal™ West Pico PLUS Chemiluminescent Substrate (for CD11b) (Thermo Fisher Scientific) was used and bands imaged using a Protein Simple FluorChem R imaging system (ProteinSimple, Santa Clara, CA). Band intensity was quantitated using Image Studio Lite (LI-COR Biosciences, Lincoln, NE). Signal intensity for C3 was quantified relative to total protein determined using Revert™ total protein stain (LI-COR Biosciences). For CD11b, β-actin was used as loading control (clone AC-15, Santa Cruz Biotechnology, Dallas, TX). The ratio of phospho-p38 to total p38 was calculated based on band intensity quantified as mentioned above.

**CR3 surface expression**
BMN (1 X 10^7 cells/mL) were TNFα primed (100 ng/mL for 30 min, 37°C) or left untreated (control group), then washed and buffer exchanged to flow buffer (PBS, 2% FBS, and 0.1% NaN₃) for staining. Cells were blocked with CD16/CD32 blocking antibody on ice for 10 min, followed by antibody staining on ice in the dark for 30 min. FITC anti-mouse Ly-6G (Biolegend), PE-Cy7 anti-mouse CD11b (eBioscience, Thermo Fisher Scientific), APC anti-mouse CD18 (BD Pharmingen, San Jose, CA) and their respective isotypes were used for staining. Post-staining, cells were fixed with 1% paraformaldehyde (PFA) for 5 min at 22°C, after which 10,000 events were recorded on an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA) for the Ly-6G positive population. For analysis, the median fluorescence intensity (MFI) in the Ly-6G positive population was determined for CD11b and CD18. Relative fluorescence units (FLU) was calculated by normalization to male untreated controls.

**Ex vivo killing of S. aureus by mouse BMN (bactericidal assay)**

One day prior to the neutrophil bactericidal assay, 2 x 10^7 CFUs/mL of stock culture was grown in TSB at 37°C with constant shaking (220 rpm) for 5 h in the presence of 50 nM autoinducing peptide 1 (AIP1) (Biopeptide Co., Inc, San Diego, CA). Following growth, bacteria were pelleted at 1800 x g for 4 min, washed with phenol red-free HBSS, vortexed, sonicated and resuspended in fresh HBSS at 6 x 10^6 CFUs/mL. Resuspended bacteria were stored on ice at 4°C until use. The final number of CFUs in this diluted culture was determined as described above. The day of use, bacteria were opsonized with 10% autologous (same sex) or heterologous (opposite sex) serum for 20 min at 37°C with gentle end-over-end rotation at 10 rpm.
BMN isolated as described above were primed with 100 ng/mL mouse TNFα (BioLegend) for 30 min (37°C with 5% CO₂) in assay buffer (phenol red-free HBSS with calcium/magnesium (Corning Inc., Corning, NY), 20 mM HEPES, 1% charcoal stripped FBS (JR Scientific, Woodland, CA)). To allow bacterial uptake and killing, TNFα-primed male and female murine BMN were incubated with serum-opsonized LAC at an MOI of 1 for 15 min at 37°C with rotation. After centrifugal removal of unbound bacteria, an aliquot of the remaining cells was lysed by sonication in PBS containing 1% Triton X-100 (t0). The number of bacteria present at t0 was determined by serial plating on blood agar. The remaining cells were incubated for 1 h at 37°C (10 rpm) after which CFUs were measured (t60). CFUs killed and S. aureus killing were calculated based on CFUs at t0 and t60 as: t0-t60 and (t0-t60)/t0 respectively. For relative S. aureus killing, killing for the controls was normalized to 1 and treatment groups compared accordingly.

**CR3 blocking of BMN**

To determine the impact of CR3 blocking on bacterial killing, BMN were treated with CD16/CD32 blocking antibody (BD Pharmingen Inc) 20 min after TNFα addition. Five minutes later, anti-CD11b antibody (clone M1/70, Biolegend) or isotype control (IgG2b, κ, Biolegend) were added in the concentrations described in the text and incubated for 5 min. Post-antibody treatment, BMN were incubated with autologous serum-opsonized LAC and bacterial killing was determined as described above.
**ROS detection**

For measurement of ROS production, TNFα-primed (10 ng/mL for 5 min, 37°C) BMN (9 x 10⁵ cells) were added to white 96-well tissue culture plates. Ten percent autologous serum-opsonized LAC was centrifuged to remove unbound serum, after which 9 x 10⁵ bacteria were added to an equal number of primed BMN. Luminol (50µM) (Sigma-Aldrich) and horseradish peroxidase (HRP) (1.2U/mL) (EMD Millipore, Burlington, MA) were used for detecting BMN production of hydrogen peroxide (H₂O₂). Chemiluminescence (Relative Luminescence Units, RLU) was measured at 37°C for 1 h with reads taken every minute with 1000 ms integration time using a SpectraMax i3x (Molecular Devices, LLC, San Jose, CA). The area under the curve (AUC) for ROS production was calculated and ROS levels normalized to male BMN. For ROS production with CR3 blocking, TNFα-primed BMN were treated with CD16/32 blocking antibody followed by 5 min with anti-CD11b (M1/70) antibody or isotype control then incubated with serum-opsonized LAC and luminol plus HRP. Chemiluminescence was then measured for an hour as described above.

**Statistical analysis**

Statistical analyses were performed using Prism 8.2.1 software (GraphPad Software, La Jolla, CA). Unpaired t-test or two-way ANOVA with post-hoc analyses were utilized as indicated in the figure legends. Significance is reported as *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001; ns, not significant.
RESULTS

Female mice have higher C3 serum levels compared to males

To begin to address the mechanism(s) of enhanced bactericidal capacity of female murine BMN against *S. aureus* *ex vivo*, we measured the levels of complement protein C3 and its fragments in the serum of male and female mice. Complement C3 plays an essential role in recognition and clearance of *S. aureus* by phagocytes (191, 320, 323), due at least in part to its role in opsonization and phagocytosis, as well as its ability to activate downstream antimicrobial pathways (324). Specifically, C3 is cleaved to generate C3b and iC3b, opsonins that both bind to the surface of *S. aureus* and to complement receptors on the neutrophil surface, thus mediating phagocytosis and clearance. Western blot analyses revealed increased levels of the C3 fragments C3b, αiC3b and C3ca2 in female compared to male murine serum (Figure 3.1). Therefore, increased levels of serum C3 fragments in female versus male serum may support a role for C3 in the enhanced bactericidal capacity of female BMN.
Figure 3.1. Female murine sera has higher C3 levels compared to males.

C3 fragment levels were measured by Western Blot in sera from age-matched male and female mice. (A) C3 immunoblot and (B) quantification of C3 fragments relative to total protein and normalized to males. Total C3 was calculated based on the average intensity of the fragments. Data are mean ± SEM. Student’s t-test; *p<0.05 **p<0.01, ***p<0.001.
BMNs from female mice have higher CR3 surface levels compared to those from males

C3 fragments bind to complement receptors expressed on the surface of neutrophils (200, 201, 319, 325). In particular, complement receptor 3 (CR3 or CD11b/CD18) has high affinity for the opsonin iC3b (200, 229, 319, 325, 326). To determine whether higher iC3b levels in the serum of female versus male mice correlated with increased CR3 expression on their BMN, we used immunostaining to measure expression of CD11b and CD18 on the surface of male and female murine BMN. Flow cytometric analysis revealed significantly higher cell surface expression of CD11b on female versus male BMN both before and after TNFα priming (Figure 3.2A). In contrast, CD18 surface expression on BMNs was greater for female versus male BMN only after TNFα priming (Figure 3.2B). CR3 is typically located in neutrophil secretory vesicles and granules and translocated to the surface post-priming and -activation by cytokines, bacteria or other neutrophil activating agents (206, 208, 209, 225, 232, 327). This suggested that increased CR3 surface expression on female versus male murine BMNs could result from overall higher CR3 protein expression by females. However, western blot analysis showed that overall CD11b levels did not differ between male and female murine BMNs (Figure 3.2C, D). This indicates that higher CD11b surface expression by female BMNs is not the result of an overall increase in CD11b protein levels.

TNFα-mediated upregulation of CR3 is dependent upon p38 MAPK (232, 328). Therefore, we next examined whether p38 MAPK was more readily activated, indicated by phosphorylation, in female BMN following TNFα priming. Using Western blot analysis,
we found no sex-specific differences in murine BMN p38 MAPK activation either pre- or post-TNFα priming (data not shown). These findings suggest that increased CR3 expression on the surface of female versus male murine BMN is independent of both overall CR3 expression levels and p38 MAPK-mediated translocation.
Figure 3.2. Female murine BMN have increased CR3 surface expression compared to males.
Flow cytometry analysis of male and female murine BMN immunostained for (A) CD11b and (B) CD18 pre- and post-TNFα priming. Data are based on fluorescence units (FLU) relative to male BMN pre-priming. Data are mean ± SEM from five independent experiments, N=5. (A-B) Two-way ANOVA; Tukey’s Multiple Comparison test. (C) Western blot analysis of male and female murine BMN showing total CD11b expression and (D) quantification of total CD11b relative to actin by Western blot. N=2-3 mice/group. Unpaired t-test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.
**S. aureus** killing by female murine BMN is enhanced by opsonization with autologous serum

Neutrophil bactericidal capacity can depend on both serum and cellular factors that contribute to the recognition of bacteria, as well as cellular bactericidal effectors (191, 199, 229, 232, 319, 324). Given our findings of increased C3 fragments in serum from female versus male mice, along with increased CR3 expression on the surface of female BMNs, we postulated that both serum and cellular components were necessary for the sex-bias in BMN killing of *S. aureus*. To test this, we measured *S. aureus* killing *ex vivo* by male and female murine BMNs using *S. aureus* opsonized with either same sex (here termed autologous) or opposite sex (heterologous) mouse serum. If BMN killing of *S. aureus* is solely a serum-dependent process, then we might expect an increase in killing by male BMN with heterologous serum opsonization and a reduction in the killing by female BMN when incubated with heterologous serum opsonized bacteria. However, if differences in BMN killing of *S. aureus* are solely dependent on cellular components, then we would expect the bactericidal capacities of BMN from mice of either sex to remain unchanged regardless of the sex of the serum used for opsonization. As shown in Figure 3.3, *S. aureus* killing by BMN from male mice did not increase regardless of the sex of serum used for opsonization. In contrast, BMN from female mice showed increased *S. aureus* killing compared to males, only when bacteria were opsonized with autologous serum. Given our earlier findings, this may suggest that the *S. aureus* bactericidal capacity of BMN from male mice is limited by their surface expression of CR3 and this cannot be overcome by additional C3 in the serum. This suggests a cell-dependent process of *S. aureus* killing by
male murine BMN, whereas, as expected, killing by female BMN is dependent upon both the sex of the cell and the sex of the serum.

**CR3 blocking reduces *ex vivo* *S. aureus* killing by murine BMNs in a sex-specific manner**

Consistent with our findings using murine BMN and serum, C3 and CR3 are important for protection against *S. aureus* infection (320, 329) and C3 expression has been shown to be regulated by estrogen (330-334). Given our finding that female murine BMN show increased surface expression of CR3 (CD11b) compared to males, we predicted a higher concentration of CD11b blocking antibody would be required to disrupt *S. aureus* killing by female compared to male murine BMN. To test this, we performed *ex vivo* *S. aureus* killing assays using male and female murine BMN in the presence of CD11b blocking antibody or isotype control. As expected, the bactericidal capacity of both male and female BMN was reduced by blocking CD11b ([Figure 3.4](#)). However, at the two highest antibody concentrations tested, blocking of CD11b significantly reduced *S. aureus* killing by female BMN compared to males, in direct contrast to predictions. This suggests that the bactericidal capacity of female BMN is more CR3-dependent compared to males and that primary mechanisms of male BMN bactericidal capacity are largely CR3-independent. Whether this is the result of sex-specific differences in CR3 signaling (cross-linking/clustering), CR3-specific downstream effectors, or other CR3-dependent mechanism(s) remains unknown. Regardless, these results clearly indicate a sex-specific, CR3-dependent difference in the bactericidal capacity of murine BMN.
Figure 3.3. Enhanced killing of *S. aureus* by female versus male murine BMNs is both serum- and cell-dependent.

Male and female murine BMNs were incubated with *S. aureus* opsonized with autologous (same sex) or heterologous (opposite sex) serum at an MOI =1 and bacterial killing determined after 1 h. (A) Total CFUs killed and (B) relative *S. aureus* killing compared to male cells with male serum opsonized bacteria. Data are mean ± SEM from three independent experiments, N=3. Two-way ANOVA; Tukey’s Multiple Comparison test, **p< 0.01; ***p<0.001; ****p<0.0001.
Figure 3.4. CR3 blocking differentially reduces the bactericidal capacity of murine neutrophils ex vivo.

BMNs from male and female mice were treated ex vivo with increasing concentrations of CD11b blocking antibody or isotype control and S. aureus killing measured after 60 min. (A) Total CFUs killed and (B) relative S. aureus killing by male versus female murine BMNs with CD11b blocking compared to isotype control set to 1.0. Data are mean ± SEM from 2-3 independent experiments each with 3 technical replicates. Unpaired t-test; ns, not significant; *p<0.05; **p<0.01; ***p<0.001.
Higher ROS production in female murine BMNs is largely independent of CR3

ROS are well known effectors of phagocyte killing of *S. aureus* (315, 335). Notably, TNFα priming upregulates neutrophil CR3 expression, thus potentiating NADPH oxidase complex formation and production of ROS (232, 328, 336). Given increased expression of CR3 on the surface of female versus male murine BMNs, we postulated that TNFα-primed female BMN would produce enhances ROS levels when exposed to serum opsonized *S. aureus* compared to male murine BMNs. Consistent with increased CR3 levels, female murine BMN produced significantly more ROS compared to those from male mice (Figure 3.5A-B).

As described above, female BMN were better able to kill *S. aureus* versus male murine BMN, and less anti-CD11b antibody was required to block *S. aureus* killing by female BMN. Therefore, we predicted that blocking with anti-CD11b antibody, at concentrations that more significantly reduced *S. aureus* killing by female versus male BMN, would also result in greater reductions in ROS production by these BMN. We found that blocking CD11b at antibody concentrations which significantly reduced the bactericidal capacity of female murine BMN, also significantly reduced ROS production compared to isotype-treated controls (Figure 3.5C). However, with female BMN, in contrast to our prediction, this response was not dose-dependent at the concentrations of CD11b blocking antibody tested, and only reduced ROS production to levels seen with male BMN treated with isotype control antibody. In contrast, reductions in ROS production by male BMN upon CD11b blocking appeared dose-dependent, showing a greater impact of blocking at the higher concentration. Clearly, blocking CD11b results in a more significant reduction in *S.
*aureus* killing by female versus male murine BMN, but with a reduced impact on ROS production compared to male BMN. This may suggest that female murine BMN killing of *S. aureus* is mediated by a mechanism involving CR3 but largely independent of ROS. Together, these findings indicate that CR3, C3 and potentially ROS contribute to the innate sex bias in the neutrophil response to *S. aureus ex vivo.*
Figure 3.5. ROS production is increased with female murine BMNs compared to males and partially relies on surface CR3 expression.

(A) Representative chemiluminescence assay comparing ROS production by male and female murine BMNs primed with TNFα and incubated with serum-opsonized *S. aureus*. (B) Calculated area under the curve for female BMN ROS production relative to male BMNs. (C) Relative ROS production by male and female BMNs post-treatment with 62.5 or 125 ng of CD11b blocking antibody versus isotype control. Data are mean ± SEM from three independent experiments, N=3 mice/group. (B) Analyzed by unpaired t-test and (C) two-way ANOVA with Tukey’s Multiple Comparison test. ns, not significant; *p<0.05; **p< 0.01; ***p<0.001; ****p<0.0001.
DISCUSSION

Neutrophils are crucial early responders to *S. aureus* infection, as numerous studies have shown increased susceptibility to infection and severe outcomes in neutropenic patients, patients with dysfunctional neutrophils, or mouse models of neutropenia (64, 193-196, 337, 338). Neutrophils play a critical role in confinement and clearance of the pathogen leading to resolution of the infection (63, 64, 79, 193). We recently reported sex-specific differences in *S. aureus* killing by murine BMN *ex vivo* (312), with BMN from female mice showing enhanced bacterial killing compared to those from males. However, the factor(s) driving this sex-specific difference remained unclear. Here we show evidence that serum C3 and cellular CR3 contribute to the sex-bias in murine BMN killing of *S. aureus*. Specifically, we measured increased C3 levels in serum from female mice and increased CR3 expression on the surface of female murine BMN, both pre- and post-TNFα priming, compared to males. Surprisingly, differences in CR3/CD11b BMN surface expression between the sexes did not result from overall differences in cellular CD11b expression or TNFα-mediated activation of p38 MAPK. Consistent with increased CR3, ROS production was greater for female BMN compared to those from male mice. However, at equal antibody concentrations, blocking CD11b reduced *S. aureus* killing by female BMN to a greater extent than male BMN, whereas it had a much-reduced effect on ROS production. Therefore, our results point to sex-specific mechanism of *S. aureus* killing by murine BMN, with female BMN killing of *S. aureus* largely mediated by a mechanism(s) involving CR3, but largely independent of ROS. Given the importance of the iC3b-CR3 interaction in the bactericidal efficacy of neutrophils, it is perhaps not surprising that their increased
expression by female mice could be a key factor contributing to the innate sex bias in murine BMN bactericidal capacity against *S. aureus*.

Serum opsonins and phagocyte (cellular) receptors each contribute to neutrophil bactericidal capacity (191, 318-320). For example, opsonization of *S. aureus* with serum heat-treated to inactive complement results in reduced to no killing of the bacteria by human neutrophils (191, 199, 316-318). Also, the importance of pattern recognition receptors (PRRs) and opsonic receptors (CRs or FcRs) for bacterial recognition is well documented (63, 64). Complement receptors are crucial for neutrophil functions, such as migration, as well as for recognition and clearance of bacteria (200, 201, 229, 319, 339). Notably, CR3 is the predominant complement receptor for *S. aureus* uptake by neutrophils (200, 201). Here we show that both serum and cellular factors contribute to the sex-specific differences in murine neutrophil bactericidal capacity. Specifically, incubation of female neutrophils with male serum-opsonized *S. aureus* significantly reduced bactericidal capacity compared to incubation with female serum-opsonized *S. aureus*. However, when male neutrophils were incubated with female serum-opsonized *S. aureus*, their bactericidal capacity remained unchanged relative to bacterial opsonization with autologous serum, suggesting a cell-dependent reduction in clearance ability of male murine BMN compared to females. The contribution of C3 to this sex difference will be more thoroughly investigated in future studies using C3-binding antibodies or C3-deficient serum for bacterial opsonization.
Blocking of CR3 hinders bacterial killing by human neutrophils, although sex of the neutrophils was not reported (191, 201). Similarly, blocking Cd11b in male and female murine BMN in our study resulted in a dose-dependent decrease in their bactericidal capacity. The effect was drastic for female murine BMN relative to males, despite increased CR3 expression on their surface. Independent of TNFα priming, we measured an increase in CR3 surface expression on female versus male BMN, which could be in part due to differences in receptor clustering and activation. TNFα priming of neutrophils can activate inside-out signaling that leads to a conformational change in integrins from a closed/bent to open/active state, thus stimulating clustering and activation resulting in increased avidity as well as ligand affinity (205, 233, 340-342). This allows the cells to respond to lower ligand concentrations (340, 343). Although this awaits empirical determination, the significant blocking effect of anti-CD11b antibody on the bactericidal capacity of female BMN could be partly explained by differences in CR3 clustering between the sexes.

In primed and activated neutrophils, upregulated CR3 induces ROS production in a p38 MAPK-dependent manner (232, 328). While we detected no difference in p38 MAPK activation between sexes, there were clear sex-specific differences in CR3 expression and ROS production. Notably, ROS production was significantly higher by female versus male BMN following activation with serum-opsonized S. aureus. Consistent with the role of CR3 in ROS production, CD11b blocking reduced ROS production by both male and female murine BMN. Given its involvement in the regulation of other antimicrobial mechanisms, the differences in ROS levels observed between the sexes could have
differential implications for the sex-specific bactericidal efficacy of neutrophils. Therefore, the potential differential impact of ROS production by male and female murine BMN on overall bactericidal capacity should be addressed in further studies focused on the sex-specific role of ROS in mediating these other mechanisms of bacterial killing by phagocytes.

Together with differences in CR3 expression on the surface of BMN from female mice, we also report an increase in C3 in their serum. As mentioned previously, estrogen (E2) can modulate C3 expression (330-334). In addition, E2 can regulate neutrophil functions ranging from ROS production and degranulation to NET formation (132, 134, 139, 140, 169, 171-173, 175, 244, 248, 344, 345). However, the contribution of E2 to any innate immune function depends on the cell type, doses used (physiological or pharmacological), as well as the specific parameters being examined (100, 102, 132, 134, 142, 171, 172, 181). Although here we made no attempt to target a specific phase of the estrous cycle, in an attempt to reflect the variety of estrous stages human females with S. aureus SSTI likely face, our previous findings using BMN from ovariectomized (OVX) female mice showed reduced bactericidal efficacy compared to BMN from sham surgery (SO) controls. This supports a role for E2 in the increased bactericidal capacity of female murine BMN (312). In addition to its role in ROS production, degranulation and NET formation, E2 can regulate neutrophil number, chemotaxis and apoptosis (133, 136, 248, 346, 347). In fact, sex differences in spontaneous apoptosis between male and female neutrophils has been reported with female neutrophils showing delayed apoptosis compared to males (136). TNFα, in a dose- and time-dependent manner, is also known to modulate neutrophil
apoptosis (348-352). Similarly, interaction of MPO with CR3 can delay neutrophil apoptosis (352, 353). In contrast, CR3-mediated phagocytosis of iC3b opsonized bacteria induces apoptotic cell death (352, 354, 355). Therefore, both E2 and CR3 could drive differences in neutrophil viability thus contributing to differences in bactericidal outcomes. The exact impact of E2 and increased CR3 expression on murine BMN viability and longevity will require empiric determination.

Even though the contribution of sex hormones in modulation of neutrophil functions has been reported, studies on sex differences in neutrophil function are extremely limited. Differences in the number of neutrophils between sexes have been differentially reported for both human and mouse (356-358). Similarly, differences in ROS production by rat neutrophils were reported, with suppressed ROS production by male neutrophils in response to sound stress (359). Furthermore, sex differences in phagocytosis by rat neutrophils were observed and the outcome varied based on the stimulus used (284). Our findings with murine neutrophils add to the mechanisms contributing to the sex differences in neutrophils.

Overall, our findings suggest that differences in serum C3, BMN expression of its receptor, CR3, and potential differences in respiratory burst, each contributes to the sex bias in murine neutrophil bactericidal efficacy against S. aureus. Although our observations must be reproduced in future studies using neutrophils from human males and females, this knowledge of potential contributors to the innate sex bias in neutrophil killing of S. aureus
expands our knowledge of the mechanistic aspects driving the innate protection in females against this important human pathogen.

AKNOWLEDGEMENTS

This work was supported in part by research grants from the National Institutes of Health to PRH (AI128159, AI145324) and ERP (CA163890 and CA194496), and utilized services and facilities provided through the NIH-funded University of New Mexico Clinical and Translational Science Center (UL1_TR001449). Support was also provided by the UNM Comprehensive Cancer Center (P30 CA118100) and the Flow Cytometry shared resource, as well as the Autophagy, Inflammation and Metabolism (AIM) Center of Biomedical Research Excellence (CoBRE) supported by NIH P20 GM121176.
CHAPTER 4: GPER Activation Protects against Epithelial Barrier Disruption by Staphylococcus aureus α-toxin

Kathleen D. Triplett\textsuperscript{1+}, Srijana Pokhrel\textsuperscript{1+}, Moriah J. Castleman\textsuperscript{1}, Seth M. Daly\textsuperscript{1}, Bradley O. Elmore\textsuperscript{1}, Jason A. Joyner\textsuperscript{1}, Geetanjali Sharma\textsuperscript{2}, Guy Herbert\textsuperscript{1}, Matthew J. Campen\textsuperscript{1}, Helen J. Hathaway\textsuperscript{3}, Eric R. Prossnitz\textsuperscript{2} and Pamela R. Hall\textsuperscript{1,*}

\textsuperscript{1}University of New Mexico College of Pharmacy, Department of Pharmaceutical Sciences, Albuquerque, NM 87131, USA

\textsuperscript{2}University of New Mexico School of Medicine, Department of Internal Medicine, Albuquerque, NM 87131, USA

\textsuperscript{3}University of New Mexico School of Medicine, Department of Cell Biology & Physiology, Albuquerque, NM 87131, USA

*phall@salud.unm.edu

+these authors contributed equally to this work

Scientific Reports | (2019) 9:1343 | https://doi.org/10.1038/s41598-018-37951-3
ABSTRACT

Sex bias in innate defense against *Staphylococcus aureus* skin and soft tissue infection (SSTI) is dependent on both estrogen production by the host and *S. aureus* secretion of the virulence factor, α-hemolysin (Hla). The impact of estrogen signaling on the immune system is most often studied in terms of the nuclear estrogen receptors ERα and ERβ. However, the potential contribution of the G protein-coupled estrogen receptor (GPER) to innate defense against infectious disease, particularly with respect to skin infection, has not been addressed. Using a murine model of SSTI, we found that GPER activation with the highly selective agonist G-1 limits *S. aureus* SSTI and Hla-mediated pathogenesis, effects that were absent in GPER knockout mice. Specifically, G-1 reduced Hla-mediated skin lesion formation and pro-inflammatory cytokine production, while increasing bacterial clearance. *In vitro*, G-1 reduced surface expression of the Hla receptor, ADAM10, in a human keratinocyte cell line and increased resistance to Hla-mediated permeability barrier disruption. This novel role for GPER activation in skin innate defense against infectious disease suggests that G-1 may have clinical utility in patients with epithelial permeability barrier dysfunction or who are otherwise at increased risk of *S. aureus* infection, including those with atopic dermatitis or cancer.
INTRODUCTION

*Staphylococcus aureus* is the primary cause of skin and soft tissue infection (SSTI) worldwide (5, 12, 257). In the U.S., more than half of the isolates are methicillin-resistant (MRSA) strains, limiting antibiotic treatment strategies (5, 257). The skin permeability barrier serves as the first line of defense against external insults such as bacterial pathogens(77, 78), still the cost of treating SSTI reaches billions of dollars annually (360).

To breach epithelial barriers, the majority of *S. aureus* isolates secrete the pore-forming toxin, alpha-hemolysin (Hla) (361). Hla facilitates invasive infection by hijacking the host molecule ADAM10 (a disintegrin and metalloprotease 10) to disrupt cell junctions and thus host permeability barriers (31, 32, 34, 35, 39, 40, 258, 361-363). Since Hla-mediated epithelial injury controls infection outcome (364), numerous prophylactic and therapeutic strategies to directly target Hla are being pursued as treatment options (34, 51-55, 57, 59, 61). Interestingly, we recently reported a sex bias in SA SSTI in male versus female patients (312), and showed in a murine SSTI model that sex bias in *S. aureus* SSTI is driven by a sex- and estrogen-specific response to Hla (258, 312). This suggests that host-directed therapies (HDT) might be developed to limit invasive disease by protecting barrier integrity in the face of Hla-challenge.

Historically, estrogen has been known to exert its numerous effects on the immune response by signaling through the classical nuclear estrogen receptors ERα and ERβ (100). More recently, the G protein-coupled estrogen receptor (GPER) has been recognized as mediating many of the rapid and even long-term effects of estrogen (150, 365). GPER activation has been shown to modulate macrophage cytokine production and neutrophil
function (244, 344, 366), as well as to reverse stroke-induced peripheral immunosuppression in ovariectomized mice (367). Interestingly, GPER activation by the highly selective GPER agonist G-1 (368) has also been reported to block disruption of endothelial barrier integrity as shown by its ability to limit blood-brain barrier (BBB) disruption following global cerebral ischemia (GCI) (369). In addition to endothelial cells, GPER is also expressed in numerous types of skin cells including keratinocytes, melanocytes and dermal fibroblasts (84, 370-372). However, the potential contribution of GPER activation to skin immunity, particularly with respect to innate defense against bacterial infection, has not been addressed. Therefore, given the role of *S. aureus* Hla in SSTI and disruption of epithelial cell junctions, we hypothesized that G-1-mediated activation of GPER would limit Hla-mediated epithelial permeability barrier disruption and reduce *S. aureus* pathogenesis.

To test this hypothesis, we used a murine model of SSTI(258) to test whether G-1 limits *S. aureus* SSTI and Hla-mediated pathogenesis in a GPER-dependent manner. Specifically, G-1 treatment reduces Hla-mediated skin lesion formation and production of pro-inflammatory cytokines *in vivo*. Consistent with its ability to support BBB integrity following GCI (369), G-1 treatment of a human keratinocyte cell line increased intercellular junction integrity in the face of Hla-mediated permeability barrier disruption. Furthermore, G-1 reduced keratinocyte surface expression of the Hla receptor, ADAM10, as well as E-cadherin cleavage with Hla-challenge, a mechanism that may contribute to the overall increase in permeability barrier integrity. Together, these studies clearly demonstrate a novel role for GPER activation in skin innate defense against *S. aureus*
infection and the important virulence factor, Hla, as well as the potential of G-1 as an HDT to limit infectious disease.

MATERIALS AND METHODS

Reagents and cell culture

G-1 was synthesized as previously described (368). G-1 was dissolved in absolute ethanol to make a 1 mg ml\(^{-1}\) stock and stored at -20°C until use. HaCaT cells were generously provided by Dr. Laurie Hudson (University of New Mexico Health Sciences Center, Albuquerque, NM, USA). Prior to use as described below, HaCaT cells were cultured at 37°C, 5% CO\(_2\) in HyClone™ Dulbecco’s Modified Eagle Medium with low glucose (DMEM low), sodium pyruvate and without phenol red (GE Healthcare, Pittsburgh, PA, USA), plus L-glutamine, 1% HyClone™ Minimal Essential Media with Non-Essential Amino Acids (MEM NEAA) and 10% FBS (Gibco®, ThermoFisher Scientific, Grand Island, NY, USA).

Bacterial strains and growth conditions

The MRSA USA300 isolate LAC(271) the LAC \(hla\) deletion mutant (LAC\(\Delta hla\)) were provided by Dr. F. DeLeo (Rocky Mountain Laboratories, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Hamilton, MT) and Dr. J. Bubeck-Wardenburg (Washington University School of Medicine, St. Louis, MO), respectively. For infection, bacteria were cultured in trypticase soy broth (TSB) at 37°C to early exponential phase as described previously (266). Stocks were prepared in TSB with
10% glycerol and maintained at -80°C for no more than two weeks prior to use. The number of CFU per ml of frozen stock was determined by plating ten-fold serial dilutions onto trypticase soy agar containing 5% sheep blood (Becton, Dickinson and Company; Franklin Lakes, NJ, USA). On the day of infection, bacteria were diluted to the indicated concentration in USP-grade saline (B. Braun Medical, Irvine, CA, USA).

**Mouse skin infection model**

Male and female C57BL/6J were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). Mice were acclimated for a minimum of seven days prior to use. Gper−/− mice (GPER KO) (Procter & Gamble, Cincinnati, OH, provided by Jan S. Rosenbaum) were backcrossed onto the C57BL/6 background (mice originally purchased from Harlan Laboratories, currently Envigo, Indianapolis, IN, USA) as previously described[373]. Gper−/− and corresponding wild-type (WT) mice (C57BL/6) were bred in-house at the University of New Mexico Animal Resources Facility. The mouse model of SSTI was utilized as previously described[258]. Briefly, four to six days before infection we used Nair™ to depilate the right flank of the mice. The day of use, G-1 stock was diluted in absolute ethanol to 20 µg ml−1. Immediately prior to injection, 100 µl of G-1 at 20 µg ml−1 in absolute ethanol or 100 µl absolute ethanol control was added to 900 µl diluent (0.9% NaCl with 0.1% bovine serum albumin and 0.1% Tween-20). On days -2 through +2 relative to infection or Hla challenge, mice were treated by intraperitoneal (IP) injection of 100 µl of vehicle or G-1 (200 ng, ~ 10 µg kg−1). On the day of infection (d0), mice (8 to 12 weeks of age) were anesthetized by isoflurane inhalation and infected by subcutaneous (SQ) injection of 50 µl of saline containing 2-3 x 10⁷ CFU of LAC or LACΔhla. Similarly,
mice used for Hla challenge studies were injected SQ with 1 µg recombinant Hla (86). Mice were weighed prior to infection and every 24 hours until sacrifice, and percent weight loss is relative to pre-infection weight at 100%. Challenge sites (infection or Hla injection) were photographed daily and lesion areas determined by ImageJ analysis (270). Lesion area and weight loss over the course of infection was integrated (Prism 8.0.0, GraphPad Software, La Jolla, CA, USA) and presented as area under curve (AUC) in mm². On day 3 post-infection, mice were sacrificed by CO₂ asphyxiation and a 2.25-cm² section of skin surrounding the infection site was excised for mechanical disruption. As appropriate, skin homogenate was serially diluted and plated on sheep blood agar to determine infection site CFUs. Remaining skin homogenate was clarified by centrifugation and the clarified fraction stored at -80°C for subsequent analysis.

**Cytokine and MPO analysis**

The concentration of the indicated cytokines in clarified skin homogenate was determined using a BioPlex 200 system, BioPlex manager software (Bio-Rad, Hercules, CA, USA) and a custom-designed mouse multiplex assay (EMD Millipore, Billerica, MA, USA) according to manufacturer’s directions. Where cytokine levels were below the limit of detection, one-half of the lowest standard concentration was utilized. Myeloperoxidase (MPO) levels in clarified homogenate were measured using the ELISA Mouse Myeloperoxidase DuoSet kit (R&D Systems, Minneapolis, MN, USA) according to manufacturer’s directions.
ECIS

TER of HaCaT cell monolayers was measured using an ECIS® Zθ instrument (Applied Biophysics, Troy, NY, USA). Cells were maintained at 37°C, 5% CO₂ ECIS 96W10idf disposable electrode arrays (Applied Biophysics) were coated with 25 µl of 0.01% poly-L-lysine (Millipore Sigma, Burlington, MA, USA) and incubated at least 15 min. Poly-L-lysine was then removed and replaced with media A (250 µl DMEM low without phenol red, with sodium pyruvate, L-glutamine, 1% MEM-NEAA and 10% charcoal-stripped FBS (JR Scientific, Woodland, CA, USA)) and TER was measured every 10 min at 1000 Hz. After resistance stabilization, media A was removed and replaced with 300 µl of HaCaT cells (6.3 x 10⁴) in prewarmed media A containing 100 nM G-1, 1 µM G15, 100 nM G-1 plus 1 µM G15 or vehicle control (+G-1/G15/Veh). Cells were allowed to stabilize for six days with media replaced on day 3. On day 5, media was changed to media B (DMEM high glucose, without phenol red, with sodium pyruvate, L-glutamine and 1 % MEM-NEAA) + G-1/G15/Veh. On day 6, media was replaced with media B+ G-1/G15/Veh containing either 1 µg Hla, HlaH35A or PBS (phosphate buffered saline) control, and TER measures continued for 24 hours.

Quantitative RT-PCR

Infection site tissue (2.25-cm²) was collected in RNALater (Qiagen, Valencia, CA), RNA isolated using Qiazol and purified using RNeasy Kits (Qiagen, Germantown, MD, USA) according to manufacturer’s directions. cDNA was generated from RNA using a PTC-200 Peltier Thermocycler (Bio-Rad, Hercules, CA, USA) with High-capacity cDNA RT kits.
with RNase inhibitor and random hexamer primers (Applied Biosystems, Foster City, CA, USA). Quantitative PCR (qPCR) was performed on a ViiA 7 Real-Time PCR system (Applied Biosystems) using Taqman® Gene Expression master mix (Applied Biosystems). Gene expression was quantified using QuantStudio software (Applied Biosystems) relative to GAPDH using a PrimeTime Predesigned qPCR assays for CDH1 and ADAM10 (Integrated DNA Technologies, Coralville, IA, USA).

**Immunofluorescent staining and flow cytometry**

HaCaTs were seeded in 24-well plates (4 x 10^5 cells in one ml in media A with G-1/Veh) and replaced as needed until confluent. Media was changed to media B + G-1/Veh 24 hours prior to flow analysis. HaCaTs were trypsinized (0.25% Trypsin-EDTA, Gibco) and cells pooled from either G-1- or Veh-treated wells. Pooled cells were subsequently washed and exchanged into flow buffer (PBS, 2% FBS, and 0.1% NaN₃) then diluted to equal cell concentrations (~3 x 10^6 cells ml⁻¹). Fc inhibitor antibody (14-9161-71, ThermoFisher) was added at 20 µl ml⁻¹ and cells incubated at room temperature (RT) for 20 min. Cells were then stained on ice in the dark for 30 min with either isotype control (12-4714-81, ThermoFisher) or anti-ADAM-10 (352703, BioLegend, San Diego, CA) antibody at 10 µg ml⁻¹. Cells were washed three times with flow buffer and 20,000 events were recorded on an Accuri C6 flow cytometer (BD Biosciences, San Diego, CA) with isotype corrected mean fluorescence normalized to Veh-treated controls.

**Western blot analysis of E-cadherin cleavage**
Throughout this assay, all media included either 100 nM G-1 or vehicle control (G-1/Veh) as described above and cells were maintained at 37°C, 5% CO₂. HaCaTs were seeded in 24-well plates (4 x 10⁵ cells in one ml of media A + G-1/Veh) and media was replaced every 48 hours until cells were confluent. Cells were then grown for 24 hours in media B + G-1/Veh. After 24 hours, cells were treated for eight hours with 0.5 µg ml⁻¹ Hla (or PBS) in media B + G-1/Veh. To collect both cleaved E-cadherin (CTF), which is released into the media, and full-length E-cadherin (FL), which is cell-associated, an equal volume of 2X RIPA lysis buffer (374) (Triton X-100 was substituted for Nonidet P-40) was added to each well and the plate incubated on ice for five min. Equal volumes of lysate were resolved on 4-12% Bis-Tris Plus gels in MES buffer (ThermoFisher) and transferred to 0.45 µm nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were blocked with 5% nonfat milk in TBST (20 mM Tris, pH 7.6, 150 mM NaCl, and 0.1% Tween 20) for 90 min at RT. E-cadherin was detected using mouse anti-human E-cadherin antibodies (CL-sc-8426, Santa Cruz Biotechnology, Dallas, TX; FL-610181, BD Biosciences, San Diego, CA) at 1:1000 followed by goat anti-mouse poly-HRP antibody (32230, ThermoFisher), both in 1% milk in TBST. Membranes were developed using SuperSignal West Femto Substrate (ThermoFisher), and imaged on a Protein Simple FluorChem R system (ProteinSimple, Santa Jose, CA). Band intensity was quantitated using Image Studio Lite (v5.2, LI-COR, Lincoln, NE) and normalized to vehicle-treated, no Hla lysates.

**S. aureus growth assay**

An overnight culture of LAC was diluted to 5 x 10⁵ CFU ml⁻¹ and treated with 1 µM or 100 nM G-1 or vehicle control. Treated bacteria were aliquoted to a 96-well plate sealed
with a breathable membrane (USA Scientific, Ocala, FL). Plates were incubated at 37°C with shaking, and the OD$_{600}$ was measured every 15 min for 16 hours using a Synergy HT plate reader (BioTek, Winooski, VT).

**Hla Western blot and relative quantification**

Overnight cultures of LAC and LACΔhla were diluted 1:100, treated with 100 nM G-1 or vehicle and incubated at 37°C with shaking for 18 hours. The bacteria were pelleted and the supernatant filtered through a 0.2 µm polyethersulfone filter (VWR, Radnor, PA). Protein concentrations were determined by bicinchoninic acid assay (BCA) (ThermoFisher) and equal protein volumes were resolved on a 4-12% Bis-Tris Plus gel in MES buffer (ThermoFisher) before transfer to a 0.45 µm nitrocellulose membrane (Bio-Rad, Hercules, CA). Membranes were blocked with 5% nonfat milk in TBS (20 mM Tris, pH 7.6, and 150 mM NaCl) for 2 hours at RT. Hla was detected with mouse anti-HLA antibody (6C12, IBT Bioservices, Rockville, MD) at 1:1000 and goat anti-mouse poly-HRP antibody (32230, ThermoFisher) at 1:10,000, both in 1% milk in TBS. TBST (TBS plus 0.1% Tween 20) was used for membrane washes between incubations. Membranes were developed in SuperSignal West Femto Substrate (ThermoFisher), and imaged using a Protein Simple FluorChem R (ProteinSimple, Santa Jose, CA). Band intensity was quantitated using ImageStudio Lite (v5.2, LI-COR, Lincoln, NE) and normalized to vehicle-treated, no Hla lysates.

**Rabbit red blood cell lysis assays**
To assess G-1 effects on Hla production and activity, glycerol stocks of LAC were diluted to $2 \times 10^7$ CFU ml$^{-1}$ and either left untreated or treated with 1 µM and 100 nM G-1 or vehicle then incubated at 37°C with shaking for 5 hours. Bacteria were pelleted by centrifugation and the supernatant filtered through a 0.2 µm polyethersulfone filter (VWR). To determine the effects of G-1 on Hla function, G-1 or vehicle was added to previously untreated cultures just prior to supernatant harvest as described above. Supernatants were diluted 2-fold in PBS and incubated statically with an equal volume of PBS-washed, 4% rabbit red blood cells (31081, Colorado Serum Company, Denver, CO) at 37°C. Lysis was measured as a decrease in turbidity at OD$_{650}$ using a SpectraMax 340 plate reader (Molecular Devices, San Jose, CA).

**HaCaT cell count and viability**

HaCaTs (300 µl with $6.3 \times 10^4$ total cells) were seeded into a 96-well plate in media A + G-1/Veh and media replaced every 48 hours until the cells were confluent. Cells were then grown for 24 hours in media B +G-1/Veh before being trypsinized (0.25% Trypsin-EDTA, Gibco) and diluted 1:1 with 0.4% trypan blue (Gibco). Cells were counted using a TC20 automated cell counter (Bio-Rad). Viable cells were those that excluded trypan blue.

**Immunofluorescence and siRNA for GPER expression**

Human keratinocyte HaCaT cells were seeded on coverslips for 2-3 days, washed and fixed in 2% paraformaldehyde. For some experiments, cells on coverslips were transfected with human GPER siRNA (Dharmacon L-005563-00) or non-targeting control siRNA
(Dharmacon D-001810-10-05) using Lipofectamine 3000 (Thermo Fisher Scientific) as per manufacturer’s instructions 48 hours before fixing. For staining GPER, cells were permeabilized and blocked with 3% BSA in PBST (PBS containing 0.1% Triton X-100) for 1 h and then incubated with rabbit anti-human GPER antibody (SAB 2700363, Sigma at 1:250), using 3% normal goat serum (NGS) in PBS as blocker for 3 h. Cells were washed with PBS and incubated with goat anti-rabbit IgG conjugated to Alexa Fluor 568 in PBS containing 3% NGS for 1 h. Subsequently, cells were washed, mounted in Vectashield, and imaged by confocal microscopy using a Zeiss LSM800 Airyscan confocal microscope (Zeiss, Oberkochen, Germany).

**Statistical analysis**

GraphPad Prism version 7.03 (GraphPad Software, San Diego California) was used for all statistical evaluations. Statistical analyses of two groups was performed using an Unpaired Students $t$-test or Mann Whitney $U$ test for non-parametrics as appropriate based on variance (F test). For comparisons between more than two groups, one-way ANOVA was used with parameters based on D’Agostino & Pearson omnibus or Shapiro-Wilk normality tests, and with Tukey’s or Bonferroni’s (ANOVA) or Dunn’s (Kruskal-Wallis test, non-parametrics) post-hoc multiple comparison analyses as indicated. Results were considered statistically significant at $p<0.05$.

**Ethics approval**

All animal studies were conducted in adherence with the recommendations in the Guide
for the Care and Use of Laboratory Animals (267) and the Animal Welfare Act, and were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of New Mexico Health Sciences Center (Animal Welfare Assurance number D16-00228).
RESULTS

**GPER activation reduces pathogenesis in a mouse model of *S. aureus* SSTI**

GPER activation has a variety of effects on innate immune function, including modulation of macrophage cytokine production and neutrophil function (244, 344, 366), as well as reversing stroke-induced immunosuppression (367). To determine whether GPER activation would support innate immune defense against infectious disease, we evaluated the effects of GPER activation on the outcomes of *S. aureus* infection using a well-characterized murine model of SSTI (258). Male mice were treated with the GPER-selective agonist G-1 (368, 375) or vehicle control prior to subcutaneous (SQ) infection with the community-acquired MRSA isolate LAC (271) (Figure 4.1a). Over the course of a three-day infection, G-1-treated mice showed significantly reduced lesion area (neutrophil-filled abscesses with subsequent dermonecrosis) (p<0.001) and weight loss (p<0.05) (a general measure of morbidity) compared to vehicle-treated controls (Figure 4.1b-c). On day 3 post-infection (typically the peak of lesion formation (272)), G-1-treated males also had reduced bacterial burden compared to control-treated mice (Figure 4.1d). Consistent with reduced lesion area, bacterial burden, and the demonstrated anti-inflammatory effects of G-1 (366), G-1-treated mice also had lower local levels of the inflammatory cytokines IL-1β, TNFα, IL-6 and CXCL1 (Figure 4.1e). As expected, given lower levels of the neutrophil-recruiting chemokine CXCL1, local levels of myeloperoxidase (MPO), often used as a surrogate marker for neutrophil presence (376), were reduced in G-1-treated mice (Figure 4.1f) suggesting a potential association between reduced lesion size with G-1-treatment and reduced neutrophil accumulation. In contrast to reduced levels of pro-inflammatory cytokines, levels of the anti-inflammatory cytokine
IL-10 did not significantly differ between groups (p=0.0884). This indicates that while G-1 reduces inflammation, it is not a general suppressor of cytokine production (Figure 4.1e).

Female mice are innately better protected than males against *S. aureus* SSTI (312), so we asked whether G-1 would further limit pathogenesis in female mice. At the male infectious dose of $2 \times 10^7$ LAC CFU, females develop much smaller lesions than males. Therefore, to assess additional G-1 protection in females, the infectious dose was increased to ~$3 \times 10^7$ CFU. Compared to vehicle-treated controls, G-1-treated female mice showed significant reductions in lesion area (p<0.01), whereas differences in weight loss and bacterial burden did not reach statistical significance (Figure 4.2). G-1-treated female mice also had reduced levels of the inflammatory cytokine TNFa at the site of infection, with no significant reduction in levels of the other cytokines tested or MPO compared to vehicle-treated mice (Figure 4.2). Given that G-1 does not directly inhibit bacterial growth (Figure 4.3), these results suggest that GPER activation limits the severity of *S. aureus* SSTI by a host-dependent mechanism.
Figure 4.1. G-1 promotes protection against *S. aureus* SSTI in male mice.

WT (C57BL/6J) male mice were treated IP with vehicle (Veh) or 200 ng G-1 on days −2, −1, 0, +1 and +2 relative to SQ infection with $2 \times 10^7$ CFU of USA300 MRSA isolate LAC. Mice were weighed and lesion size measured daily before mice were sacrificed on day 3 post-infection. (a) Illustration of the G-1 treatment and infection timeline. (b) Representative day 3 post-infection images and area under the curve (AUC) for lesion size (mm²) and (c) percent weight change (relative to pre-infection weight) over the 3-day infection. (d) Day 3 post-infection bacterial burden. (e) IL-1β, TNFα, IL-6, CXCL1 and IL-10 levels and (f) myeloperoxidase levels in clarified injection site homogenate collected on day 3 from the site of infection. Data are mean ± SEM, n = 6–7 mice per group from two independent experiments. Unpaired t-test: ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001.
**Figure 4.2. G-1 promotes protection against *S. aureus* SSTI in female mice.**

WT (C57BL/6J) female mice were treated IP with vehicle (Veh) or 200 ng G-1 on days -2, -1, 0, +1 and +2 relative to SQ infection with 3 x 10^7 CFU of USA300 MRSA isolate LAC. Mice were weighed and lesion size measured daily before mice were sacrificed on day 3 post-infection. (a) AUC for lesion size (mm^2) and (b) percent weight change over the 3-day infection. (c) Day 3 post-infection bacterial burden. (d) IL-1β, TNFα, IL-6, CXCL1 and IL-10 levels in clarified injection site homogenate collected on day 3 from the site of infection. (e) Day 3 post-infection myeloperoxidase levels in clarified infection site homogenate. n=7-8 mice per group from two independent experiments. Data are mean ± SEM Unpaired t-test: ns, not significant; *, p<0.05; **, p<0.01.
Figure 4.3. G-1 does not inhibit *S. aureus* growth.
Growth of LAC in the presence of 100 nM and 1 μM G-1 or vehicle control as measured by OD$_{600}$. Data are mean ±SEM.
G-1 limits the severity of *S. aureus* SSTI in a GPER-dependent manner

G-1 is a highly selective GPER ligand with respect to both classical estrogen receptors and other GPCRs (366, 368), suggesting that the benefits of G-1-treatment against *S. aureus* SSTI should depend on host expression of GPER. To test this, we compared outcomes between male GPER knockout (GPER KO) mice and corresponding wild-type (WT) C57BL/6 controls treated with G-1 or vehicle and infected with LAC. Compared to vehicle-treated controls, G-1-treatment significantly reduced lesion size (p<0.001) and increased bacterial clearance (p<0.01) without affecting weight loss in infected WT mice, but had no effect on lesion size or bacterial clearance in GPER KO mice (Figure 4.4a-c). Notably, infection outcomes in the absence of G-1 did not significantly differ between WT and GPER KO male (Figure 4.4a-c) or female (Figure 4.4d-f) mice. This indicates that innate defense in this model is normally GPER-independent and suggests that increased estrogen-dependent, innate protection in female mice (312) is mediated through classical estrogen receptors. However, our findings clearly show that G-1 limits pathogenesis in both males and females and that GPER could be a promising target for HDT. Most importantly, these results demonstrate that the efficacy of G-1 in limiting *S. aureus* SSTI is host- and GPER-dependent.
Figure 4.4. G-1-mediated protection against SSTI is GPER-dependent.
(a–c) WT (C57BL/6) and GPER KO male mice were treated IP with vehicle or 200 ng G-1 on days −2, −1, 0, +1 and +2 relative to SQ infection with ~3 × 10^7 CFU of USA300 MRSA isolate LAC. Shown are (a) lesion area (AUC in mm^2) over the course of a 3-day infection, (b) day 3 post-infection bacterial burden at the site of infection and (c) percent weight change (AUC) post-infection. n = 6–9 mice per group from two independent experiments. (d–f) Female WT and GPER KO mice were infected as described above. Shown are (d) lesion AUC (mm^2) over the course of a 3-day infection, (e) day 3 post-infection bacterial burden at the site of infection and (f) percent weight change (AUC) post-infection. n = 14–21 mice per group from three independent experiments. Data are mean ± SEM. ANOVA (a) p = 0.0002, (b) p = 0.0024 and (c) not significant. Tukey’s multiple comparison test (a–c) or Unpaired t-test (d-f): ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001.
G-1 limits Hla-mediated pathogenesis in a murine dermonecrosis model

In animal models of *S. aureus* SSTI, the secreted virulence factor alpha-hemolysin (Hla) drives lesion formation at the site of infection (34, 35, 40). Given the host-dependent protective effects of G-1 against *S. aureus* SSTI, we postulated that G-1-treatment would limit pathogenesis in male mice directly challenged with Hla. Consistent with results in mice infected with *S. aureus*, G-1 treatment significantly reduced lesion formation, inflammatory cytokine production (IL-1β, TNFα, IL-6 and CXCL1) and MPO levels at the site of subcutaneous Hla-injection compared to vehicle-treated controls (Figure 4.5). To corroborate the role of Hla in G-1-mediated protection in the skin, we infected male mice with an isogenic Hla deletion mutant of *S. aureus* (LACΔhla). In the absence of Hla expression, skin lesion formation is minimal or absent, so outcomes are based largely on bacterial burden and weight loss. Importantly, G-1-treatment did not significantly alter LACΔhla infection outcomes based on day three post-infection bacterial clearance and overall weight loss (Figure 4.6). Given that G-1 does not impact bacterial Hla expression or Hla activity (Figure 4.6), these results provide support for a mechanism in which G-1 protects against Hla-mediated pathogenesis by altering the host response.
Figure 4.5. G-1 reduces Hla-mediated lesion formation and inflammation.

WT (C57BL/6 J) male mice were treated IP with vehicle or 200 ng G-1 on days −2 through +2 relative to SQ injection of 1 µg Hla. (a) Representative day 3 post-injection images (scale bar = 5 mm) and lesion size (AUC in mm2) over the 3 days post-injection. (b) IL-1β, TNFα, IL-6, CXCL1 and (c) myeloperoxidase levels measured in clarified injection site homogenate collected on day 3 post-injection. Data are mean ± SEM, n = 8 mice per group from two independent experiments. Mann–Whitney U test: *p < 0.05; **p < 0.01; ***p < 0.001.
Figure 4.6. G-1 does not alter SSTI outcomes in the absence of Hla and does not inhibit Hla production or activity.

(a-b) WT (C57BL/6J) male mice were treated IP with vehicle or 200 ng G-1 on days -2, -1, 0, +1 and +2 relative to SQ infection with 3 x 10^7 CFUs LACΔhla. (a) Day 3 post-infection bacterial burden and (b) percent weight change (AUC) over the 3 day infection. Data are mean ± SEM, n=13 mice per group from two independent experiments. (c) Western blot (left) and quantification (right) of Hla in sterile supernatant from LAC and LACΔhla grown for 18 h in the presence of vehicle control or 100 nM G-1. Three samples per group are shown (left). ND, not detected. (d-e) Analysis of Hla-mediated rabbit red blood cell (rRBC) lysis using sterile LAC supernatant collected after 5 h growth in the presence of the indicated concentrations of G-1 or vehicle control (d) or sterile 5 h LAC supernatant with the indicated concentrations of G-1 or vehicle control added at the time of the assay (e). Percent protection against rRBC lysis relative to PBS at 100%. Triton control shows total lysis. Data are mean ± SD. Unpaired t-test; ns, not significant.
G-1 limits keratinocyte permeability barrier disruption by Hla

Hla is a major contributor to the pathogenesis of *S. aureus* infections involving epithelial cells, including SSTI and pneumonia (PNA) (31, 32, 34, 35, 40, 42-44). During these infections, Hla disrupts host permeability barriers to facilitate invasive infection (31, 32, 34, 35, 39, 40, 258). Recently, GPER activation was reported to limit disruption of BBB integrity in a rodent model of GCI (369). Furthermore, GPER is expressed in numerous types of skin cells including melanocytes, dermal fibroblasts and the most prominent skin cell type, keratinocytes (84, 370-372). Given our in vivo data showing that G-1 limits skin damage caused by Hla, and the role of *S. aureus* Hla in disrupting epithelial cell junctions, we hypothesized that activation of GPER with G-1 would limit Hla-mediated epithelial permeability barrier disruption. One measure of permeability barrier integrity is resistance of cell monolayers to passage of an electric current (electrical cell-substrate impedance sensing (ECIS)) (377). As keratinocytes are the major skin cell type, we used the HaCaT human keratinocyte cell line (378) to determine whether G-1 limits Hla-mediated disruption of epithelial barrier integrity. GPER expression in HaCaT cells was first verified by immunofluorescent staining with and without siRNA knockdown of GPER, after which we demonstrated that G-1 did not affect cell growth or viability during culture (Figure 4.7).
Figure 4.7. GPER is expressed in HaCaT cells and G-1 does not alter HaCaT cell growth or viability.
(a) Immunofluorescence microscopy showing GPER staining in siControl-treated HaCaT cells, but not in siGPER-treated cells. (b) Cell count and viability of HaCaT cells grown to confluence in the presence of 100 nM G-1 or vehicle. Data are mean ± SEM. Unpaired t-test. ns, not significant.
Next, HaCaT cells were grown to confluence in the presence of vehicle or G-1 and changes in transepithelial electrical resistance (TER) were measured by ECIS. After reaching stable resistance, monolayers were exposed to Hla or the inactivate Hla mutant, HlaH35A (41, 379). As previously reported, there was no significant reduction in TER between control cells and those challenged with HlaH35A (40) regardless of G-1-treatment (Figure 4.8a and data not shown). In contrast, whereas Hla rapidly reduced keratinocyte barrier integrity (decreased TER) in vehicle-treated cells, cells grown in the presence of G-1 were significantly more resistant (p<0.01) to permeability barrier disruption, with an average 33% increase (p=0.0079) in TER compared to vehicle control (Figure 4.8a-b). The G-1 mediated reduction in permeability barrier disruption was reversed in the presence of the GPER-antagonist, G15 (375) (Figure 4.8c), consistent with the requirement for GPER for G-1 efficacy in vivo (Figure 4.4a-c). Notably, G-1 did not alter HaCaT cell numbers (Figure 4.7), suggesting that increased barrier integrity is independent of potential G-1-mediated effects on cell growth or viability.

Hla disrupts epithelial barriers by binding its cell surface receptor, ADAM10 (39, 40, 380), which in turn cleaves E-cadherin. Given that E-cadherin is a component of the adherens junctions responsible for epithelial permeability barrier integrity (40, 380), and that G-1 limited keratinocyte permeability barrier disruption by Hla (Figure 4.8a-b) and reduced skin damage (lesion area) in Hla-injected mice (Figure 4.5a), we predicted that G-1 would reduce E-cadherin cleavage following Hla challenge. To test this, we used immunoblotting to measure full-length E-cadherin (FL) and the cleaved C-terminal fragment (CTF) from HaCaT cell monolayers grown in the presence of vehicle or G-1 and exposed to Hla.
In the absence of Hla, G-1 significantly increased expression of FL E-cadherin (Figure 4.8e, left) while decreasing baseline cleavage (CTF) (Figure 4.4e, right). As expected, following an eight-hour culture with Hla, FL E-cadherin was significantly decreased (Figure 4.8e, left) and cleavage (CTF) was increased independent of treatment (Figure 4.8e, right). However, whereas FL E-cadherin levels were equivalent between vehicle- and G-1-treated cells in the presence of Hla (Figure 4.8e, left), G-1-treatment reduced E-cadherin cleavage (CTF) (Figure 4.8e, right). Given reduced Hla-mediated E-cadherin cleavage with G-1, we asked whether G-1 altered expression of E-cadherin or the Hla receptor ADAM10. Although transcription of CDH1, which encodes E-cadherin, was not altered by G-1 alone, CDH1 transcription was increased in G-1-treated keratinocytes exposed to Hla (Figure 4.8f). Also, whereas G-1 alone increased ADAM10 transcription approximately 15% (p<0.05) (Figure 4.8g), HaCaT cells treated with G-1 displayed 23% less ADAM10 (p<0.0001) on the cell surface compared to control-treated cells (Figure 4.8h). Together, these findings suggest that G-1 may contribute to transcriptional regulation of CDH1 when Hla is present, as well as post-transcriptional regulation of ADAM10 expression or trafficking to the keratinocyte cell surface. This in turn may contribute to maintenance of epithelial permeability integrity in the face of Hla-challenge (Figure 4.8a-b).
Figure 4.8. G-1 reduces Hla-mediated keratinocyte permeability barrier disruption.

HaCaTs were grown to confluence in the presence of vehicle or 100 nM G-1 prior to challenge (t = 0) with 1 µg/mL Hla or HlaH35A (indicated by arrow in (a)). Changes in permeability barrier resistance at 1000 Hz were measured by ECIS. (a) Representative ECIS recording of HaCaT monolayer treated as described above. (b) Change in HaCaT permeability barrier resistance at 12 h after Hla exposure. Data are mean ± SEM from two (HlaH35A) to six (Control and Hla) independent experiments each with 6–8 technical replicates per condition. Mann-Whitney U test. (c) HaCaT cells were grown in G-1, G15, G-1 + G15 or vehicle control. Shown is the change in permeability barrier resistance at 12 h after Hla exposure. Data are mean ± SEM of a representative experiment of two independent experiments each with a minimum of 8 technical replicates. ANOVA < 0.0001. (d) Western blot analysis of full-length (FL) E-cadherin and the cleaved C-terminal fragment (CTF) in vehicle- and G-1-treated (100 nM) HaCaT cell monolayers after eight hours incubation with 0 or 0.5 µg/mL Hla. MW, molecular weight markers. Four replicates for each group are shown. Image Studio Lite was used to invert luminescent image to that shown to uniformly enhance contrast for quantification of CTF. (e) Relative quantification of E-cadherin FL (left) and CTF (right) of samples in (c) and based on band intensity relative to vehicle-treated cells in the absence of Hla. (f) Quantitative PCR analysis of CDH1 and (g) ADAM10 transcription by vehicle- and G-1-treated (100 nM) HaCaT cells. Expression is shown relative to GAPDH and normalized to vehicle-treated control cells. (h) Surface expression of ADAM10 on vehicle- and G-1-treated (100 nM) HaCaT cells measured by immunofluorescent staining and flow cytometry. Shown is percent expression relative to vehicle-treated controls. Data are mean ± SEM from two independent experiments each with three technical replicates per condition. Unpaired t-test: ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.
DISCUSSION

The skin permeability barrier provides protection against transcutaneous water loss, invasion by microbial pathogens and access of environmental toxins to underlying sensitive tissues (77, 78). However, bacteria have had countless generations to evolve powerful tools to disrupt this barrier and cause SSTIs resulting in annual treatment costs of billions of dollars (360). As the most common cause of SSTI, *S. aureus* secretes Hla to disrupt epithelial barriers and facilitate invasive infection. Specifically, Hla binds ADAM10 on host cells, resulting in cleavage of the cell junction protein E-cadherin and loss of permeability barrier integrity (31, 32, 34, 35, 39, 40, 258, 362, 363). Here we show that G-1, the highly selective ligand of the non-classical estrogen receptor GPER, limits the severity of *S. aureus* SSTI and production of pro-inflammatory cytokines in a murine challenge model. The effects of G-1 are dependent on *S. aureus* expression of Hla, a finding supported by reduced skin pathogenesis (lesion formation) in G-1-treated mice compared to controls following direct Hla challenge. Not surprisingly, G-1 efficacy is dependent upon host expression of GPER, as protection against *S. aureus* SSTI is lost in GPER KO mice. Furthermore, G-1 reduces keratinocyte surface expression of the Hla receptor ADAM10 and limits Hla-mediated disruption of epithelial barrier integrity *in vitro*. Therefore, along with supporting endothelial permeability barrier integrity following ischemic injury (369), our findings show that G-1 promotes epithelial barrier integrity and host innate defense against a major bacterial toxin. Given that Hla-mediated epithelial injury controls infection outcome (364), and that G-1 lacks the feminizing effects seen with estrogen treatment (373), this work demonstrates the potential efficacy of G-1 as an HDT to promote skin innate defense and reduce the burden of *S. aureus* SSTI.
The role of estrogen signaling in immune regulation has historically been studied in terms of the classical estrogen receptors ERα and ERβ (100). In contrast, although GPER is expressed by a variety of immune cells, including monocytes, macrophages, neutrophils and lymphocytes (147, 167, 244, 344, 381-386), the impact of GPER signaling on immune function is in the early stages of investigation. Interestingly, GPER activation can result in both pro- and anti-inflammatory responses. For example, GPER signaling can modulate neutrophil function (244, 344), regulate both pro- and anti-inflammatory cytokine production (366, 384) and promote regulatory T-cell responses (383). GPER has also been shown to provide protection in a mouse model of multiple sclerosis (366), to contribute to monocyte-dependent skin inflammation in response to serum from lupus patients, and to reverse peripheral immunosuppression in an ovariectomized mouse model of stroke (367). GPER is also expressed in skin cells (84, 370-372), where it contributes to cytoskeletal organization (371, 387) and melanin synthesis (370). Here we demonstrate the contribution of GPER activation to host innate defense against S. aureus skin infection and to increasing epithelial barrier integrity in the face of Hla challenge. These findings not only significantly expand our understanding of GPER signaling in innate immune defense, but also demonstrate a novel role for G-1 in the maintenance of barrier integrity in human keratinocytes.

Hla plays a major role in the pathogenesis of S. aureus infection in numerous animal models, particularly models of SSTI and PNA (34, 35, 39, 40, 362, 363). Its importance is further evidenced by ongoing efforts to develop prophylactic and therapeutic strategies targeting Hla expression or function (34, 51-55, 57, 59, 61). For example, recombinant Hla
toxoid was recently shown to be a safe and immunogenic candidate vaccine antigen in healthy adults in Phase 1-2 clinical trials (NCT01011335) (57). In addition, Suvratoxumab (formerly known as MEDI4893), an anti-Hla monoclonal antibody, has successfully completed Phase 1 safety trials (NCT01769417) (388) and is currently in a Phase 2 safety and efficacy trial (NCT02296320) (389) to prevent or limit S. aureus pneumonia in mechanically ventilated adults. Along with these approaches, the use of G-1, which also protects against Hla-mediated pathogenesis, could provide an additional therapeutic benefit to patients. Interestingly, combination treatment with the Hla neutralizing antibody MEDI4893, and either vancomycin or linezolid, two clinically important antibiotics, improved outcomes in mouse models of S. aureus SSTI (59) and PNA (60, 61). Whether G-1 will have similar adjunctive efficacy with antibiotic therapy against S. aureus infection is an important point for future investigation. Further studies are also required to determine the mechanism by which G-1 increases keratinocyte transcription of the gene encoding the Hla receptor ADAM10 while also reducing its surface expression (Figure 4.8f-g). Although speculative, this mechanism may involve G-1-mediated effects on post-translational regulation of ADAM10, including suppression of ADAM10 trafficking to the cell surface. In any case, the use of G-1 as an HDT to promote skin innate defense may prove a valuable component of a multifaceted approach to reduce the burden of S. aureus infection.

The ability of G-1 to promote epithelial barrier integrity and to limit S. aureus infection supports its potential clinical utility in patient populations at increased risk for infection. For example, S. aureus colonization is frequently associated with atopic dermatitis and
psoriasis, skin diseases that feature epidermal barrier dysfunction (390-393), and S. aureus may actually contribute to skin inflammation in these patients (394). Recurrent S. aureus SSTI is also a hallmark of autosomal dominant hyper IgE syndrome (AD-HIES) (91) and it has recently been shown that the impaired epithelial response to infection in these patients results from overproduction of the pro-inflammatory cytokine TNFα (395). TNFα also contributes to the severity of atopic dermatitis, though TNF blockade by anti-TNF biologicals has thus far failed to improve outcomes in these patients (62). Here we show that whereas G-1 provides greater overall improvement in S. aureus skin infection outcomes in the more susceptible male (312) versus relatively resistant female mouse population, G-1-treatment significantly reduces lesion size as well as TNFα production in S. aureus infected mice of both sexes. This suggests that G-1, which reduces but does not completely prevent TNFα production and signaling, may be efficacious in limiting the severity of S. aureus skin infection in highly susceptible AD-HIES and atopic dermatitis patients.

Here we used a short-term skin infection model to capture the innate immune response to S. aureus infection in advance of the development of any adaptive immunity. Therefore, our findings indicate that G-1 enhances innate immune defense against infection. This suggests the potential of G-1-treatment for limiting infection in groups with increased risk of S. aureus infection due to impaired innate immunity such as cancer patients undergoing chemotherapy or surgery (396-399). Given that what begins as an SSTI can lead to S. aureus pneumonia, sepsis or other life threatening infection (400), it will be essential to experimentally determine the efficacy of G-1 in limiting S. aureus skin infection in disease
models of patients with increased susceptibility to *S. aureus*. Furthermore, since a single dosing regimen was utilized for the current studies, additional investigations will be needed to determine the optimal dosing strategy for efficacy against *S. aureus* SSTI in immunocompetent mice as well as disease models of susceptible patient populations. Overall, developing an HDT that limits infection in diverse patient populations could positively influence clinical practice and improve human health.

Although our findings suggest the potential utility of G-1 to limit pathogenesis during *S. aureus* skin infection, it could have much broader clinical utility. For example, since many innate defense mechanisms are effective against a variety of bacterial pathogens, therapies aimed at GPER activation may also prove efficacious for treating a variety of infections. In addition, given that a monoclonal antibody targeting Hla has shown synergy with antibiotic therapy in animal models of *S. aureus* infection (59-61), G-1 may likewise have adjunctive efficacy. Furthermore, the ability of G-1 to promote endothelial (369) and epithelial barrier integrity may prove useful in treating AD, psoriasis, or other diseases involving dysfunctional permeability barriers. Finally, regardless of its therapeutic potential, G-1 may provide a powerful tool for identifying other GPER-dependent host targets to improve epithelial barrier integrity and promote host innate immune defense.

**ACKNOWLEDGEMENTS**

This work was supported by the National Institutes of Health grant AI128159 (P.R.H.), CA163890 and CA194496 (E.R.P.) and ES014639 (M.J. Campen). Support was also
provided by the UNM Comprehensive Cancer Center (P30 CA118100) and the Autophagy, Inflammation and Metabolism (AIM) Center of Biomedical Research Excellence (CoBRE) supported by NIH P20 GM121176. We thank Dr. H.D. Gresham for valuable insight.
CHAPTER 5: DISCUSSION

SUMMARY

*Staphylococcus aureus*, the primary cause of SSTI, is a global health concern possessing mild to life threatening risks (5, 8, 21, 257, 264). The incidence of *S. aureus* SSTI has increased over the years with a 40% increase in the SSTIs incidence in the US between 2000 to 2012 (28, 50). Another study conducted by Miller and colleagues have reported increased frequency of SSTIs than other conditions such as urinary tract infections or pneumonia from 2005 to 2010 in ambulatory and inpatient settings (50, 264). This has increased the number of hospital visits and treatment costs significantly (28, 50). With about 50% of the SSTI cases reported caused by MRSA (5, 50, 257), the rising antibiotic crisis points towards a need for an alternative therapeutic approach (9, 13).

Numerous studies have identified bacterial factors responsible for *S. aureus* pathogenesis and multiple antibodies and vaccines developed against these targets are being tested for their ability to limit *S. aureus* infections (34, 52, 54, 55, 59-61, 262). However, with the ever-evolving survival mechanisms of the bacteria and multiple systems regulating their virulence, developing a vaccine that encompasses all these targets is challenging (401, 402). Hence, the necessity to utilize multiple approaches to combat *S. aureus* infection. One such approach would be to boost the host immunity against the pathogen. For the identification of host targets for therapeutic purposes, understanding the mechanism of host defense against *S. aureus* is important. Multiple studies have highlighted the importance of innate immune cells, neutrophils and macrophages as well as resident skin cells in
combating *S. aureus* skin infections (20, 68). Also, research focused on the roles of hormones on regulating skin physiology as well as immune cells function have been conducted (403, 404). However, *S. aureus* SSTI outcomes have not been examined from a sex-bias perspective. Sex-specific differences in infectious diseases has been reported with variation in outcomes depending on the type of the pathogen, infection, and the host factors (behavioral, biological or physiological) (94-107). Importance of including both sexes in research studies was demonstrated by the differences in clinical trials outcomes based on findings from one sex (405, 406) and thus, NIH has mandated the inclusion of both sexes in the research funded by the institute and that the data be reported including sex as a biological variable (308). Our findings relative to this mandate that are reported here are outlined in Figure 5.1 and briefly highlighted below.

Sex differences in *S. aureus* nasal colonization as well as susceptibility to *S. aureus* bacteremia has been reported for humans (109). However, sex-specific differences in host response to *S. aureus* SSTI that accounts for about 90% of all *S. aureus* infections (19) has not been reported. Hence, in Chapter 2 we investigated the sex bias in host response to *S. aureus* SSTI using a murine infection model. Based on the increased nasal carriage and susceptibility to *S. aureus* bacteremia in males (109) and the effect of female hormone estrogen in wound healing (164), we hypothesized there to be a female bias in *S. aureus* SSTI outcomes with estrogen contributing to the protection in females. Using a murine SSTI model, we were the first to report sex-specific differences in innate response to *S. aureus* SSTI. Our findings show female mice to be innately resistant to *S. aureus* SSTI and that the resistance is E2-dependent. Our animal study results were in line with the UNM
CTSC database search results that showed increased incidence of *S. aureus* SSTI in males compared to females for the age group (18-45 years) and the timeline (2011-2014) queried with an odds ratio of greater than 2 for male infection. While the database search results could vary due to differences in hospital visits, as well as biological and behavioral differences between sexes, the animal study suggests physiological differences in host response to infection. The work presented in Chapter 2 also uncovers a key role for host cell, neutrophils and bacterial toxin, Hla in driving the differences in infection outcomes, suggesting that multiple variables contribute to the sex bias. We found differences in neutrophils bactericidal capacity *ex vivo* with increased *S aureus* clearance by female murine neutrophils compared to males. Also, males and females respond differentially to Hla, a key contributor to dermonecrosis and invasive *S. aureus* infection (31-33). These findings highlight the importance of investigating both sexes for infection outcomes. Taking this work further, we investigated the mechanisms driving the sex-specific differences in bactericidal capacity of murine neutrophils in Chapter 3. We examined the contribution of complement system (C3 and CR3) and antimicrobial agents (ROS) in driving the enhanced bactericidal capacity of female neutrophils. In Chapter 3, we report increased complement protein C3 levels in the female serum, CR3 expression on neutrophils surface and ROS production by female murine neutrophils. Based on our findings on Chapter 2 where we saw E2-dependent protection in females we explored the role of estrogen receptor, GPER and its agonist G-1 in driving the sex bias and in providing protection against *S. aureus* SSTI in Chapter 4. We show GPER activation limits *S. aureus* pathogenesis, uncovering a novel role for this receptor in skin innate defense mechanism. Importantly, G-1 treatment reduced disease severity in males. Mechanistically, we show
reduction in ADAM10 expression and barrier permeability with G-1 administration to the human keratinocyte cell line HaCaTs. This effect of G-1 on S. aureus pathogenesis and infection outcome suggests a potential therapeutic role for G-1 in limiting S. aureus SSTI as well as other conditions involving barrier dysfunctions.

Overall, we have identified: (A) sex bias in host innate response to S. aureus SSTI, differences in murine neutrophils bactericidal capacity ex vivo and differential response to Hla between sexes in Chapter 2, (B) factors contributing to enhanced S. aureus clearance ability of female murine neutrophils ex vivo in Chapter 3 and (C) protective role of G-1 and GPER in barrier integrity and against S. aureus pathogenesis in Chapter 4 (summarized in Figure 5.1). Thus, our findings were the first to show that sex bias exists in host innate response to S. aureus SSTI and that female mice are innately resistant to it compared to males. We also found differences in bactericidal capacity of murine neutrophils between sexes ex vivo and demonstrated that differences are potentially due to differences in serum complement protein C3, cellular CR3 expression and ROS production with their levels being higher in females compared to males. In addition, we demonstrated a protective role for estrogen in providing protection in female mice against S. aureus SSTI, and found a novel role for GPER agonist, G-1, in reducing disease severity, especially in male mice. Mechanistically, we found a protective role of G-1 in barrier integrity in vitro with G-1 treatment reducing epithelial cells (HaCaTs) barrier disruption by S. aureus toxin, Hla. Our work highlights the importance of investigating sex differences in host response and provides insight into mechanisms driving sex bias in host response to S. aureus SSTI.
These findings could be useful for the identification and development of potential host targets to combat the antibiotics crisis.
Figure 5.1. Summary of the findings of Chapters 2, 3 and 4. See text for details.
FUTURE CONSIDERATIONS

Although we reported differences in host response to *S. aureus* SSTI and a role for host neutrophils and differential response to the bacterial toxin, Hla, in driving the difference, several questions remain. One of the first gaps that needs to be addressed is the mechanistic differences in skin resident cells function between sexes. Also, it must be determined how these cells respond differentially to Hla and contribute to the sex bias. We reported a role for E2 in providing protection in females against SSTI. Estrogen’s role in skin thickness as well as regulation of skin immune cells function is known (403, 404). Thus, understanding the mechanism of E2 mediated protection against *S. aureus* pathogenesis and the contribution of its receptors (ERα, ERβ and GPER) in driving the sex bias is important to identify their potential as therapeutic targets. We have already shown a role for GPER and its agonist G-1 in providing protection against *S. aureus* SSTI. Taking this further, identifying the role for other ERs (ERα, ERβ) will be important in devising strategies to enhance host response.

In terms of sex bias in immune cells response, we have demonstrated enhanced *S. aureus* bactericidal capacity of female murine neutrophils *ex vivo* with serum complement protein C3, its receptor CR3, and ROS levels contributing to this increased clearance ability of female neutrophils. Differences in neutrophils function between sexes might be a result of developmental differences, thus a gene expression analysis using RNA sequencing will help uncover innate differences between sexes. We know ROS is crucial for bacterial clearance by neutrophils, however, neutrophils employ other oxygen-independent mechanisms for bacterial clearance as well (63, 64). Thus, determining their role in
enhanced bactericidal capacity of female neutrophils is important to identify other factors driving the differences in neutrophils clearance ability besides ROS. In our study presented in Chapter 2, we also show estrogen dependent differences in clearance ability of female neutrophils, with decreased bactericidal efficacy of OVX neutrophils compared to sham controls. E2 is known to modulate neutrophils function (antimicrobial agents production, degranulation) as well as chemotaxis, hence the role of E2 and ERs in driving the differences in bactericidal efficacy between sexes needs to be determined. In addition, since G-1 via GPER contributes to host defense against S. aureus SSTI and barrier integrity, we will investigate its role in other S. aureus infections, such as pneumonia to expand the therapeutic potential of G-1 against other S. aureus infection.

The gaps that will be addressed are: (A) differences in resident skin cells composition between sexes, their response against S. aureus insult, and the role of E2 and ERs in regulating these functions and sex bias in S. aureus SSTI, (B) innate differences in neutrophils between sexes, contribution of oxygen-independent mechanisms to increased bactericidal capacity of female murine neutrophils and the role of E2 and ERs, and (C) efficacy of G-1 in protection against S. aureus pneumonia through regulation of barrier permeability. A summary of these gaps and experimental schematics are depicted in Figures 5.2. to 5.6. The discussion below will elaborate on these gaps and approaches to address them.
(A) Does resident skin cells composition and their function against *S. aureus* differ between sexes? What role do E2 and ERs play in regulating these function and sex bias in *S. aureus* SSTI?

(B) Do neutrophils between sexes innately differ? What role does oxygen-independent mechanisms play and how E2 and ERs contribute to the increased *S. aureus* clearance ability of female murine neutrophils?

(C) Does GPER agonist G-1 have a therapeutic potential against *S. aureus* pneumonia? What is the mechanism of protection conferred by G-1 in this model, if any?

Figure 5.2. Summary of gaps to be addressed based on the findings of Chapter 2, 3 and 4.
See text for details.
5.1.1. Sex bias in skin cells composition and function against *S. aureus*

The skin is composed of multiple layers of cells as described in Chapter 1. These resident cells contribute to protection against external insult. Specifically, keratinocytes, fibroblasts Langerhans cells, resident T cells, neutrophils and macrophages have been studied for their role in protection against *S. aureus* pathogenicity (20, 68). However, the difference in their functionality against *S. aureus* has not been assessed from a sex bias perspective. Male and female skin differ in thickness with male skin being thicker than females. However, the female epidermis is known to be thicker than males, likewise, the male dermis is known to be thicker than females (160, 407). In addition, a study by Koyama et al. have reported sex specific differences in Langerhans cells density in mice where they quantitively demonstrated increased LCs in females compared to males (408). Their findings suggest the suppressive effect of testosterone in LCs count as castration increased the LC numbers in male mice. Hence, we expect to find differences in the number of epidermal and dermal cells between the sexes. To determine this, first we will isolate the single cell populations from male and female skin following the protocols from Kashem et al. (409) and Benck et al. (410). Differences in the baseline number of these cells between the sexes will be assessed by utilizing cell specific markers [described in (409, 410)] and their analysis by flow cytometry.

To determine their role in protection against *S. aureus* pathogenesis between sexes, these single cells isolated as mentioned above will be treated *ex vivo* with *S. aureus* culture supernatants from wild-type (WT, containing all secreted toxins and PAMPs), as well isogenic Hla mutant, Δhla strain (lacking secreted Hla toxin) and recombinant purified Hla
given the significance of Hla in dermonecrosis. These cells will then be examined for NLRP3 inflammasome activation as well as production of pro-inflammatory cytokine using western blot analysis for active caspase-1 and IL-1β (Figure 5.3). We will also determine differences in other pro- (TNFα, IL-6) and anti-(IL-10) inflammatory cytokines produced as a result of NF-κB activation. Differences in the cytokines profile might provide insight into some key factors providing tolerance against Hla in females. These findings will shed some light onto whether it is the composition and absolute number of the cells or the difference in their response to S. aureus pathogenesis or both that dictates the sex bias in host response to S. aureus SSTI.
Figure 5.3. Sex bias in skin cells composition and function, role of E2 and ERs. Male and female skin differs in epidermal, dermal and overall thickness. E2 contributes to reduced skin thickness in females. Estrogen also modulates NF-κB activation that regulates NLRP3 inflammasome activation. Gaps to be addressed are: [I] Differences in skin layers composition between sexes and [IIa] their function (NLRP3 inflammasome activation) in response to *S. aureus* toxin, Hla and PAMPs. [IIb] effect of E2/ER on skin cells function and their contribution to *S. aureus* SSTI. Adapted from: Miller and Cho (2011) (67) and Pasparakis et al. (2014) (69); Ezekwe et al. (2016) (48) and Shao et al. (2015) (49).
Based on the outcomes, we will also determine their specific roles in the infection outcomes using the murine *S. aureus* SSTI model. We will specifically focus on Langerhans cells and the tissue resident γδ T cells as these cells are known to contribute to host defense against *S. aureus* infections (73, 82, 83, 87, 89). Langerhans cells are known to regulate proliferation as well as antigen presentation to T cells ((72, 82, 87)). T cells have also been shown to enhance recruitment of phagocytes, contribute to host defense via production of IL-17 as well as maintain keratinocyte production and proliferation (74, 83, 89-91). However, their contribution in *S. aureus* SSTI outcomes has not been investigated from a sex bias perspective. Hence, we will determine the contribution of these cells to females’ resistance to *S. aureus* SSTI. We will determine differences in lesion size and bacterial clearance. Based on Koyama Y, et.al (408) findings, we know LCs number differs in the skin of male and female mice. Given its role in host defense, we expect to see a significant loss of protection with LCs after depletion in the skin of female mice using a Langerin-DTR mouse model. These mice lack all Langerin+ cells after diphtheria toxin injection (411, 412). Similarly, with the depletion of resident γδ T cells using commercially-available anti–TCR γδ antibody as in (413), we expect to see exacerbated infection outcomes in γδ T cells-depleted female mice compared to isotype treated controls. With the males, we expect the infection condition to worsen given the protective roles of these cells. The schematic of infection is outlined in Figure 5.4. The outcomes of these infection studies will help determine whether these cells are the key players contributing to innate resistance to females. This information will further the therapeutic research by providing host targets to enhance immune response against *S. aureus* SSTI. This would be extremely
useful for the treatment of immunocompromised patients that are highly susceptible to *S. aureus* skin primary and recurring infections.
Figure 5.4. Contribution of Langerhans cells (LCs) and γδ T cells to sex bias in host defense to *S. aureus* SSTI.
LCs and γδ T cells, resident skin cells, are known to contribute to host defense against *S. aureus* infections. However, their contribution to sex bias in host response to *S. aureus SSTI* is not yet known. Using the murine dermonecrosis model, gaps to be addressed are:

[1] Contribution of LCs and [2] γδ T cells to the female innate resistance to *S. aureus* SSTI and effect of loss of these cells on male SSTI outcomes using LC depleted mice and antibody depletion of γδ T cells.
5.1.2. Role of E2 and ERs in skin cells function and sex bias in *S. aureus* SSTI

Our findings in Chapter 2 suggest a role for E2 in the protection against *S. aureus* SSTI in females. Similarly, in Chapter 4 we see protective effect of G-1 against *S. aureus* SSTI in both sexes. Hence, to determine the mechanisms behind this protection we will take two approaches. First, we will determine the expression of ERs in all the skin cell types mentioned above and treat both the male and female cells *ex vivo* with physiological concentrations of E2 prior to and during their exposure to *S. aureus* WT and Δhla supernatants as well as purified Hla toxin. Then we will assess the difference in outcomes (as mentioned as above) between E2 and vehicle treated male and female cells to determine the effect of E2 treatment on their response (Figure 5.3). E2 via ERs is known to promote NF-κB activation as an early response to infection yet inhibit the activation of NF-κB or increase the expression of anti-inflammatory genes during later response to prevent damage (108, 414, 415). Because NF-κB regulates NLRP3 inflammasome activation as well as inflammatory responses, we expect to see a time-dependent decrease in inflammasome activation as well as production of pro-inflammatory markers with E2 treatment, in line with our findings with decreased inflammasome activation in females. The second approach will be to use ER genetic knock out animal models to determine their role in *S. aureus* infection outcomes between sexes. We have already reported no difference in infection outcomes between wild-type (WT) and GPER<sup>−/−</sup> mice. This suggests a role for classical ERs (ERα and ERβ) in E2 mediated protection in females. ERα and ERβ are well studied for their role in prevention of skin flap necrosis as well as wound healing, respectively (164, 404, 416). Our preliminary findings as well as a published report (417) also suggests increased skin thickness in the absence of ERα. Thus, loss of these receptors
might exacerbate the infection outcomes. In the case of GPER, use of its agonist G-1 reduced *S. aureus* pathogenesis even though loss of GPER did not affect disease severity of *S. aureus* SSTI. This suggests activation of GPER-dependent pathways could confer protection in mice. Similar outcomes could be expected with the classical ER knockouts as well where loss of the receptor might not cause any difference in outcomes, but activation of their downstream pathways could enhance protection. This might explain the mechanism of E2-dependent protection in females. It is likely that more than one ER contributes to the protective role of E2 and therefore multiple agonists of these receptors need to be investigated for therapeutic purposes.

5.2.1. **Innate differences in neutrophils between sexes**

Neutrophils originate in the bone marrow and undergo a series of processes to become a mature and functional phagocyte (192, 418). These cells are then recruited to the site of infection via chemoattractants secreted by resident cells post-insult by a foreign substance, where these cells contribute to host defense via its phagocytic and bactericidal function (192, 418). Studies have focused on the role of sex hormones in regulation of neutrophil chemotaxis as well as antimicrobial functions (242, 244, 346, 419); however, the innate differences in murine neutrophils between sexes have not been examined. Dysfunctional neutrophil conditions such as Chédiak–Higashi syndrome (CHS), myeloperoxidase (MPO) deficiency, Leukocyte adhesion deficiency type 1 (LAD1) are linked to mutations in genes at specific chromosomal locations (215). Thus, we predict RNA sequencing for the gene expression analysis of male and female neutrophils could uncover factors that contribute to innate differences in neutrophils between sexes. The genes with greater than 2-fold
differences in their expression profiles between sexes can be further investigated to determine their role in neutrophils bactericidal function. The findings from this will help uncover innate versus induced differences in neutrophils between sexes.
Figure 5.5. E2 and ERs in enhanced bactericidal capacity of female murine neutrophils.

E2 is known to regulate oxygen-dependent and independent killing mechanisms of neutrophils as well as its apoptosis. Gaps to be addressed are: Innate differences in neutrophils between sexes and role of E2 and ERs (ERα, ERβ, and GPER) in: [1] enhanced oxygen-dependent clearance of S. aureus by female neutrophils, [2] degranulation and oxygen-independent clearance of S. aureus by female neutrophils, and [3] regulation of neutrophil apoptosis. Adapted from: Rigby and DeLeo (2012) (64) and Nguyen et al. (2017) (205).
5.2.2. E2 and ERs in enhanced bactericidal capacity of female neutrophils

In Chapter 2, we reported E2-dependent enhancement in the bactericidal capacity of female neutrophils using neutrophils from the ovariectomized (OVX) mice. However, the mechanism by which E2 regulates this is not yet known (Figure 5.5). The effect could be mediated by differences in ER-dependent functions that affect their bactericidal capacity. The effect of ERs could be genomic, due to the regulation of transcription factors, likely by classical ERs or rapid and transient via GPER. Rodenas et al. have highlighted GPER’s role in enhancing neutrophil function with increased superoxide production post activation with pharmacological concentrations of G-1 (244). Also, E2 has been shown to modulate neutrophil oxygen-dependent killing mechanisms (136, 172, 346). Thus, to determine the role of specific ERs in mediating E2’s function, we will isolate neutrophils from ER knockout mice and determine the bactericidal efficacy as well as ROS production by these cells. It is likely that more than one ER contributes to the enhanced clearance ability of female neutrophils, thus we might see reduction but not complete abolishment of the effect in these knockout cells. However, with the identification of receptors function and their downstream signals we could supplement estrogen or ER agonists to OVX female neutrophils or male neutrophils and determine their application to enhance murine neutrophils bactericidal capacity against S. aureus.

E2 is also known to regulate neutrophil apoptosis (136, 244, 420). In terms of E2’s role in neutrophil apoptosis, studies have reported dose-dependent differences with conflicting evidence on pro- versus anti-apoptotic effect of E2 on neutrophils (136, 244, 420). Using GPER-specific agonist G-1, Rodenas et al. have demonstrated increased viability and
functionality of neutrophils post-activation (244). The delayed apoptosis as well as decreased chemotaxis of neutrophils by E2 could compliment our findings that show enhanced bactericidal capacity of female neutrophils ex vivo as well as lower levels of MPO, a neutrophil surrogate marker, as early as 24 hours post-S. aureus subcutaneous administration at the site of infection. This suggests E2-dependent increase in bactericidal capacity and delayed apoptosis contributes to enhanced clearance of bacteria by female neutrophils despite fewer cells being recruited to the site of infection. However, recent studies have suggested a role for Annexin 1, an endogenous glucocorticoid protein in reducing neutrophil infiltration and activating apoptosis of these cells (192, 421). This protein is also known to promote clearance of apoptotic neutrophils by macrophages for resolution of inflammation (192, 421). A recent study by Nadkarni et al. reported increased Annexin 1 expression in female neutrophils compared to males and increased mobilization of this protein to the surface post E2 treatment of male human neutrophils (422). Based on E2’s role in Annexin 1 expression and the role of this protein in promoting apoptosis, the contribution of E2 in neutrophil apoptosis is unclear. This is likely due to differences in E2 dosage used in these studies as well as differences in E2 levels at different phases of menstrual cycle as well as pregnancy. Thus, the viability of male and female neutrophils in our studies need to be determined (Figure 5.5). The increased Annexin 1 expression in the study by Nadkarni et al. was shown to be mediated by the classical ERs via a non-genomic mechanism. Since G-1 via GPER has been shown to delay apoptosis of neutrophils, there is likely an ER-dependent balance on how E2 regulates the survival of neutrophils.
5.2.3. Oxygen-independent clearance of *S. aureus* by female neutrophils

In Chapter 3, we focused on the differences in ROS production by neutrophils and the increased ROS contributing to the bactericidal capacity of female neutrophils. Considering the role of E2 in regulating degranulation of neutrophils, we will investigate the contribution of this mechanism to enhanced bactericidal capacity of female neutrophils (Figure 5.5). Neutrophil antimicrobial peptides (AMPs), antimicrobial proteins such as α-defensins, elastase, cathelicidins, cathepsins, azurocidin, lactoferrin, lysozyme and proteinase-3 are the oxygen-dependent antimicrobial agents (63, 204, 216, 217). All these microbicidal agents are found in neutrophils granules and vesicles. Exocytosis and degranulation lead to the release of these oxygen-independent antimicrobial agents to the plasma membrane or the phagosomal membranes (64, 209, 423). As a marker of granule release, we will specifically determine differences in elastase (primary), lactoferrin (secondary) and MMP-9 or gelatinase (tertiary) by ELISA in male and female murine neutrophils using commercially-available kits. Another way of testing this would be to determine intracellular calcium level between sexes as Ca$^{2+}$ signaling is known to contribute to granule release with increased intracellular Ca$^{2+}$ promoting release of the granules (217, 424). Thus, post-TNFα priming and activation with serum-opsonized LAC, we will determine the differences in intracellular Ca$^{2+}$ levels in male and female neutrophils. Activation of GPER by E2 is known to rapidly increase Ca$^{2+}$ intracellular levels in cells (150, 365). Thus, we expect to find increased Ca$^{2+}$ levels as well as increased release of the granule components in females compared to male neutrophils. As reviewed in Rigby and Deleo (2012) (64), there is likely a synergistic action between oxygen-
dependent and independent antimicrobial agents in enhancing the bactericidal capacity of female neutrophils.

5.3. Protective role of G-1 in male mice against *S. aureus* pneumonia

Pneumonia is a life-threatening infection caused by *S. aureus*, with an estimated 50,000 cases per year in the US alone (56). *S. aureus* has been a leading cause of ventilator-associated pneumonia (VAP) and is now being recognized as a cause of community-acquired pneumonia (CAP) as well (56). As with SSTIs, more than 50% of the isolates are methicillin-resistant, resulting in increased mortality from MRSA pneumonia (56). Similarly, Hla regulates the virulence of *S. aureus* in pneumonia (32, 56). Hence, multiple Hla directed therapeutics are being tested for efficacy against *S. aureus* pneumonia, both alone and in conjunction with antibiotics (vancomycin and linezolid) (43, 56, 58, 425). Sexual dimorphism in bacterial pneumonia has been reported, with males having higher susceptibility than females. In terms of sex-specific differences in *S. aureus* pneumonia, the incidence was higher in males compared to females, with a 1.84 M/F ratio (111, 426). The protective role of E2 has also been demonstrated, with resistance to pneumococcal pneumonia reported in females and E2 treated male mice compared to male controls (261). This study highlighted the role of alveolar macrophages in bacterial killing of *Streptococcus pneumoniae* as well as *S. aureus*. Furthermore, this enhanced killing of *S. pneumoniae* by female macrophages was attributed to E2-dependent activation of endothelial NOS, NOS3 (261). Both G-1 and E2 are known to induce phosphorylation of eNOS and NO formation via GPER (427). Based on these and our findings on role of G-1 in protection against Hla pathogenesis, we hypothesize that G-1 could be used as a potential
therapeutic for protection against *S. aureus* pneumonia in males. To test the hypothesis, we will use a well-established mouse model of *S. aureus* pneumonia (274, 428), and administer G-1 following the same 5 day regimen as in Chapter 4, 2 days prior to intranasal inoculation of *S. aureus* to male mice to 2 days after the infection (outlined in Figure 5.6). Disease progression and severity will be assessed by monitoring for hunched posture, piloerection, labored breathing, immobility, and loss of resistance to handling (428). We expect to see differences in infection outcomes with increased survival, or less severe symptoms, in G-1 treated mice compared to controls. We will also examine differences in male alveolar macrophage response *ex vivo* after *in vivo* G-1 treatment (5-day regimen) and *in vitro* short-term treatment with *S. aureus* killing assay. The macrophages will be isolated from bronchoalveolar lavage (BAL) fluid following the protocol from Nayak et al. (429). Similar to the findings of Yang et al., we expect to see increased bacterial clearance and increased NOS3 expression in G-1 treated macrophages post-incubation with *S. aureus* and its culture supernatants, respectively. Also, we will examine for differences in ADAM10 expression and protein levels in G-1 treated or control macrophages after treatment with *S. aureus* culture supernatants. In addition, their levels will also be examined in G-1 treated lung epithelial cells, isolated following the protocol in Nakano et al. (430), based on our findings on skin epithelial cells (human keratinocytes cell line, HaCaT). We will perform these experiments using female mice as well to determine G-1’s role in enhancing protection in females. Since, pathogenicity of *S. aureus* pneumonia is associated with Hla, decrease in ADAM10 protein with G-1 would suggest dual role for G-1 in protection in *S. aureus* pneumonia along with NOS3 expression. The findings of
these experiments will provide insight into the broad role of G-1 as a therapeutic against *S. aureus* infections in males with no feministic effect.
Figure 5.6. Role of G-1 in protection against *S. aureus* pneumonia.

Males are more susceptible to *S. aureus* pneumonia. Female macrophages demonstrate enhanced clearance of *S. aureus* through E2 dependent increase in NOS3 expression. Gaps to be addressed are: [1] Protective effect of G-1 in *S. aureus* pneumonia model, and [2] Effect of G-1 treatment (short and long term) on alveolar macrophages *S. aureus* killing ability, NOS3 expression and ADAM10 expression and protein levels in macrophages and epithelial cells post incubation with *S. aureus* culture supernatants.
CONCLUSIONS

Despite the global health threat posed by *S. aureus* infections, no vaccines nor monoclonal antibodies have yet proven efficacious in clinical trials (58). However, there are multiple ongoing studies at different phases of clinical trials assessing the efficacy and safety of these vaccines and monoclonal antibodies (58). To add to this, the emergence of antibiotic-resistant strains and their increased association with infections makes treatment more challenging; hence the need for alternate therapeutic approaches (6, 13, 15, 18, 431). While there are ongoing studies to identify bacterial targets and clinical trials for antibacterial therapies, our studies highlight the importance of understanding the mechanism of host response to the pathogen. With differences observed in *S. aureus* SSTI outcomes between sexes, with females being innately resistant to infection, our findings demonstrate the necessity for inclusion of both sexes in future *S. aureus* research. The increased resistance of females to *S. aureus* SSTI was in part due to female sex hormone, estrogen as well as innate immune cells, neutrophils role in providing protection and differential response to Hla toxin. In **Chapter 4**, we demonstrated a novel, protective effect of GPER agonist, G-1 suggesting the potential therapeutic use of G-1 against *S. aureus* SSTI. Further studies need to be conducted to investigate the role of other estrogen receptors and their agonists for protection. Our findings in **Chapter 3** reveal the differences between male and female murine neutrophils *S. aureus* clearance mechanisms. The work in these chapters demonstrate the importance of considering the sex-specific differences in host response and the contribution of sex hormones in driving the infection outcomes. In addition, it highlights the importance of understanding how the host responds to an infection. This will be useful to identify targets in the host to promote host defense. Our findings on sex bias
in host response and potential therapeutic usage of G-1 could be expanded to other *S. aureus* infections to identify other host targets and to broaden the therapeutic potential of G-1. Based on these findings, G-1 in conjunction with anti-bacterial therapeutics could be used to enhance the host defense while inhibiting bacterial growth and virulence. Overall, differences in host immune response needs to be considered while designing therapeutic approaches to maximize their efficacy in disease prevention.
APPENDICES

APPENDIX A: ABBREVIATIONS

α: Alpha
β: Beta
β-GlcNAc: β N-acetylglucosamine
AD-HIES: Autosomal Dominant Hyper IgE Syndrome
ADAM10: A Disintegrin and Metalloproteinase 10
Agr: accessory gene regulator
AIDS: Acquired Immunodeficiency Syndrome
AIP: Auto Inducing Peptide
AKT: Protein Kinase B
AMP: Anti-microbial Peptides
AR: Androgen Receptor
ARE: Androgen Response Element
AUC: Area Under the Curve
ASC: Apoptosis-associated speck-like protein containing a CARD
BMNs: Bone marrow neutrophils
C3: Complement 3
CA-MRSA: Community-acquired Methicillin Resistant S. aureus
CFU: Colony Forming Unit
CGD: Chronic Granulomatous Disease
CHS: Chediak-Higashi Syndrome
CR3: Complement receptor 3
DETC: Dendritic Epidermal T Cells
DNA: Deoxyribonucleic acid
DPN: 2,3-bis (4-hydroxyphenyl)-propionitrile
E2: Estrogen/17β -estradiol
ECIS: Electrical Cell-substrate Impedance Sensing
EDTA: Ethylenediaminetetraacetic acid
ER: Estrogen Receptor
ER: Estrogen Response Elements
fMLP: N-formyl-L-methionyl-L-leucyl-phenylalanine
GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor
GPCR: G Protein-Coupled Receptor
GPER: G Protein-coupled Estrogen Receptor
HaCaT: Human keratinocyte cell line
HA-MRSA: Hospital-acquired Methicillin Resistant S. aureus
HBSS: Hanks’ Balanced Salt solution
HDT: Host-Directed Therapeutics
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV: Human Immunodeficiency Virus
HRP: Horseradish Peroxidase
HSV: Herpes Simplex Virus
LAC: Los Angeles County
LAD-1: Leucocyte Adhesion Deficiency-1
LCs: Langerhans Cells
LTA: Lipoteichoic Acid
Mac-1: Macrophage-1 antigen
MAPK: Mitogen-activated Protein Kinase
MERS: Middle East Respiratory Syndrome
MIC: Minimum Inhibitory Concentration
MOI: Multiplicity of Infection
MPO: Myeloperoxidase
MPP: 1,3-Bis (4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazoledihydrochloride
MRSA: Methicillin Resistant *S. aureus*
MSSA: Methicillin Sensitive *S. aureus*
NADPH: Nicotinamide Adenine Dinucleotide Phosphate
NET: Neutrophil Extracellular Trap
NLRP3: NOD-, LRR- and pyrin domain-containing protein 3
NO: Nitric Oxide
NOD: Nucleotide-binding and oligomerization domain
NOS: Nitric Oxide Synthase
NOX: NADPH Oxidase
OVX: Ovariectomized
PAMPs: Pathogen-Associated Molecular Patterns
PBP: Penicillin Binding Protein
PBS: Phosphate-Buffered Saline
PGN: Peptidoglycan
PHTPP: [2-phenyl-5,7-bis (trifluoromethyl) pyrazolo [1,5-a] pyrimidin-3-yl] phenol

PI3K: Phosphoinositide 3-kinase

PPT: 4,4’,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol

PR: Progesterone Receptor

PRR: Pattern-Recognition Receptors

PSMα: Phenol Soluble Modulin alpha

PVL: Panton-Valentine Leukocidin

RIPA buffer: Radioimmunoprecipitation assay buffer

RNA: Ribonucleic acid

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

SAB: S. aureus bacteremia

SDS-PAGE: Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

SLE: Systemic Lupus Erythematosus

SO: Sham

SSTI: Skin and Soft Tissue Infection

TER: Transepithelial Electrical Resistance

TF: Transcription Factor

TNF: Tumor-Necrosis Factor

TLR: Toll-like Receptor

TSB: Trypticase soy broth

TSLP: Thymic Stromal Lymphopoietin

TSST-1: Toxic Shock Syndrome Toxin-1
VISA: Vancomycin-Intermediate *S. aureus*

VRSA: Vancomycin-Resistant *S. aureus*

WTA: Wall Teichoic Acid
APPENDIX B: REFERENCES


124. Channappanavar R, Fett C, Mack M, Ten Eyck PP, Meyerholz DK, Perlman S. Sex-Based Differences in Susceptibility to Severe Acute Respiratory Syndrome


176. Kumar S, Lata K, Mukhopadhyay S, Mukherjee TK. Role of estrogen receptors in pro-oxidative and anti-oxidative actions of estrogens: a perspective. Biochim Biophys


187. El Kebir D, József L, Pan W, Wang L, Petasis NA, Serhan CN, Filep JG. 15-Epi-
lipoxin A4 Inhibits Myeloperoxidase Signaling and Enhances Resolution of Acute Lung
1601OC.

188. Segal AW. How Neutrophils Kill Microbes. Annu Rev Immunol. 2005;23:197-

189. Hampton MB, Kettle AJ, Winterbourn CC. Involvement of superoxide and
myeloperoxidase in oxygen-dependent killing of Staphylococcus aureus by neutrophils.

10.1002/0471142735.im0723s111.

191. Ferrante A, Martin AJ, Bates EJ, Goh DH, Harvey DP, Parsons D, Rathjen DA,
Russ G, Dayer JM. Killing of Staphylococcus aureus by tumor necrosis factor-alpha-
activated neutrophils. The role of serum opsonins, integrin receptors, respiratory burst,

192. Rosales C, Demaurex N, Lowell CA, Uribe-Querol E. Neutrophils: Their Role in

193. Molne L, Verdregh M, Tarkowski A. Role of neutrophil leukocytes in cutaneous
PMC97694.

19504359.

195. Leliefeld PH, Wessels CM, Leenen LP, Koenderman L, Pillay J. The role of
PMC4804478.

Staphylococcus aureus, phagocyte NADPH oxidase and chronic granulomatous disease.

197. Anderson DC, Springer TA. Leukocyte adhesion deficiency: an inherited defect in

198. Kjeldsen L, Calafat J, Borregaard N. Giant granules of neutrophils in Chediak-
Higashi syndrome are derived from azurophil granules but not from specific and


360. Lee GC, The University of Texas at Austin and UT Health Science Center at San Antonio SA, TX, USA, Boyd NK, The University of Texas at Austin and UT Health Science Center at San Antonio SA, TX, USA, Lawson KA, The University of Texas at Austin A, TX, USA, Frei CR, The University of Texas at Austin and UT Health Science Center at San Antonio SA, TX, USA. Incidence and Cost of Skin and soft Tissue


