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MECHANISMS OF INNATE IMMUNE REGULATION OF DERMONECROSIS DURING *STAPHYLOCOCCUS AUREUS* SKIN INFECTIONS

BY

MORIAH J. CASTLEMAN

B.S. Biological Sciences, University of Notre Dame, 2011

DISSERTATION

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

Biomedical Sciences

University of New Mexico

Albuquerque, New Mexico

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DEDICATION

I dedicate this dissertation to my father, Donald John Allen Gerrish. He taught me the value of education; to cherish it as a gift that not many receive and to always continue to learn in order to better myself and the lives of my family.

ACKNOWLEDGMENTS

I would like to acknowledge my mentor, Dr. Pamela R. Hall, for her continued encouragement and guidance. She is extremely supportive of my growth and maturation as a scientist and encourages my passion for discovery. Not only has she been a fantastic mentor, but she is also a great role model for women in science. Joining her lab and conducting research under her guidance has been a blessing for me.

I would also like to thank my committee members, Drs. Matthew Campen, Laurie Hudson, and Judy Cannon, for their valuable input and suggestions on this study. They have facilitated my development as a scientist. I am grateful for their time spent in discussion about research techniques and broader approaches to answering scientific questions.

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ABSTRACT

S. aureus is the dominant cause of skin and soft tissue infections (SSTIs) in humans. The importance of *S. aureus* as the primary cause of skin infections has increased exponentially over the last few decades since the emergence of resistance to β-lactam antibiotics. Furthermore, the rise of methicillin-resistant *S. aureus* (MRSA) strains has led to hospitals stays, increased financial burden and increased mortality. A significant proportion of MRSA infections have been attributed to community-acquired strains (CA-MRSA), specifically the USA300 isolates, which can cause deadly disease in otherwise healthy individuals. Due to the increase in antibiotic resistance and the lack of an effective vaccine against *S. aureus*, there is a limit in current treatment options available for infected patients and a need for better therapeutics that limit resistance while fighting infection. In order to design better therapeutics to combat *S. aureus*, it is imperative to understand the host innate immune factors that are protective against *S. aureus*. Our work focuses on the role of the scavenger receptor CD36 in regulation of the host inflammatory response during *S. aureus* skin infection, sex bias in susceptibility to infection with *S. aureus,* and the role of estrogen in host defense during infection. This work is especially important for patient populations which show increased susceptibility to infection due to dysfunctional innate immune mechanisms.

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Chapter 1 Introduction

This introduction chapter is divided into four parts. Part 1 will discuss the discovery, isolation and epidemiology of the bacterial pathogen *Staphylococcus aureus*, as well as current treatment options. Additionally, this section will focus on virulence factors used by *S. aureus* to cause infections, how the skin functions as a general barrier against infection and how the host immune system functions to combat skin infections caused by *S. aureus*. Part 2 will discuss the known functional roles of the scavenger receptor cluster of differentiation 36 (CD36), including roles of this receptor in the host immune response to *S. aureus* infection. Part 3 will focus on epidemiological data and *in vitro* and *in vivo* studies supporting a sex bias in susceptibility to bacterial infections, as well as providing an overview of the cellular response to estrogen signaling. Part 4 contains concluding remarks and hypothesis for the work in this dissertation.

Part 1: Combatting the bacterial pathogen *Staphylococcus aureus*

Discovery and isolation of *S. aureus*

The *Staphylococcus* genus is composed of at least 40 different species of bacteria, many of which impact human health. *Staphylococcus* was first isolated in 1880 by the Scottish surgeon Alexander Ogston from the abscess of a patient (Ogston, 1881). He lanced the abscess and smeared the pus onto a microscope slide for analysis and observed the presence of clusters of bacteria within the pus debris, which he termed "staphylococci" from the Greek word staphyle referring to bunches of grapes. Ogston then experimented by injecting pus from his human patients into the skin of mice and observed the development of an abscess (Ogston, 1882). When he heat-treated the pus samples, the mice did not develop an abscess, indicating to Ogston that acute abscesses were caused by a bacterial source and demonstrating the importance of aseptic practices during surgical operations to prevent post-operative mortality. A few years later, in 1884, the German surgeon Anton Rosenbach, isolated two different strains of staphylococci, which he named based on the colonies' pigmented appearance, *Staphylococcus aureus* for the Latin word aurum for "gold" and *Staphylococcus albus* for the Latin word albus or "white" (now referred to as *S. epidermidis*) (Rosenbach, 1884). Since its isolation, it has been shown that *S. aureus* is responsible for a wide range of infections in humans including osteomyelitis, endocarditis, pneumonia, bacteremia, and skin infections (Lowry, 1998). Despite its identification over 100 years ago, *S. aureus* is still regarded as a serious public health threat to humans.

Epidemiology of *S. aureus* infections

S. aureus is the dominant cause of skin and soft tissue infections (SSTIs) (Moran GJ and LK, 2006). *S. aureus* SSTIs in the USA account for 11.6 million outpatient and emergency room visits, as well as 500,000 hospital admissions per year (McCaig et al., 2006). The importance of *S. aureus* as the primary cause of skin infections has increased exponentially over the last few decades since the emergence of resistance to β-lactam antibiotics, such as penicillin and ampicillin. Resistance to these antibiotics is due to the acquisition of *blaZ*, which is translated into the β-lactamase enzyme that hydrolyzes the β-lactam ring rendering the antibiotic inactive (Lowry, 2003). In 1961, the anti-staphylococcal antibiotic methicillin was introduced as an antibiotic resistant to the effects of β-lactamase. Shortly after the introduction of methicillin, methicillin-resistant *S. aureus* (MRSA) isolates were reported (Lowry, 2003). MRSA antibiotic resistance is due to the acquisition of the chromosomally-inserted staphylococcal cassette chromosome mec (SCC*mec*), which contains the *mecA* gene, that codes for penicillin-binding protein 2a (PBP2a). Penicillin binding proteins (PBPs) catalyze transpeptidation for peptidoglycan cross-linking during bacterial cell wall formation, and are the target of β-lactam antibiotics. PBP2a is structurally unique, thereby conferring resistance to not only the anti-staphylococcal β-lactam antibiotics, methicillin, nafcillin, and oxacillin, but also to all penicillins, carbapenems, and most generations of the cephalosporins (Chambers and Deleo, 2009). In a recent report, 76% of bacterial skin infections presenting to the emergency room were due to *S. aureus* infection, and of those, 78% were caused by MRSA (Moran GJ and LK, 2006). Compared to infections caused by methicillin-sensitive *S. aureus* (MSSA), MRSA infections are associated with increased

mortality rates, longer hospitals stays and increased financial burden (Lodise and McKinnon, 2007). Even more concerning, a significant proportion of MRSA infections are due to community-acquired strains (CA-MRSA), specifically isolates of the pulsed-field gel electrophoresis (PFGE) type USA300, which can cause deadly disease in otherwise healthy individuals (Otto, 2013). In addition, MRSA has been labeled a serious threat by the CDC (CDC, 2013). From this epidemiological data, it is clear that *S. aureus* is a deadly pathogen responsible for a major burden on the health care system.

Current treatment regimens for *S. aureus* skin infections are dictated by the type and severity of infection. Skin infections can be categorized as uncomplicated or complicated (U.S.DHHS, 1998). Uncomplicated skin infections include impetigo (superficial sores), folliculitis (inflammation of hair follicles) and infected abrasions, and are often treated with incision and drainage in an outpatient setting (Rajan, 2012; Schmitz, 2011). Complicated skin infections include major abscesses, infected burns, and deep tissue infection called necrotizing fasciitis. These infections often require hospitalization, wound debridement via surgery, and antibiotics (Dryden, 2010; May, 2011). Gram stain and culture of pus or exudate is recommended for pathogen identification, along with susceptibility testing to identify the best method of antibiotic treatment. To treat moderate MSSA infections, patients are prescribed dicloxacillin or cephalexin, and for more severe MSSA infections nafcillin or clindamycin is prescribed (Stevens et al., 2014). To treat MRSA infections, patients may be prescribed trimethoprim/sulfamethoxazole, linezolid or ceftaroline (Stevens et al., 2014). Since there is now a limit in current treatment options as antibiotic resistance grows, and no protective vaccine exists, it is imperative to better understand not only the virulence

factors that *S. aureus* uses to cause invasive infection, but also how the immune system combats *S. aureus* skin infection. This knowledge could facilitate the design of therapeutics to bolster the immune system's ability to fight *S. aureus* infection (DeLeo et al., 2010).

Virulence Factors Arsenal of *S. aureus*

S. aureus is a human commensal of the skin and it is estimated that at least 30% of the human population is colonized with *S. aureus*, and up to 60% of the population is transiently colonized (Klevens et al., 2007). Humans are most often colonized by *S. aureus* within the nares or inguinal region of the body (Yang et al., 2010). *S. aureus* has an arsenal of factors that enable it to successfully colonize humans and to breach the innate skin barrier (described below). It is important to understand the mechanisms of colonization because colonization is a risk factor for subsequent *S. aureus* infection (Kluytmans et al., 1997). The factors that promote colonization include *S. aureus* surface components that increase adherence to host cell surfaces. These colonization factors are called microbial surface components recognizing adhesive matrix molecules, or MSCRAMMs, and include fibronectin-binding protein A and B (FnbpA, Fnbp B), clumping factor A and B (ClfA, ClfB), iron-regulated surface determinant A (IsdA) and wall teichoic acid (WTA) (Krishna and Miller, 2012a). FnbpA and Fnbp B are adhesins that function to create a bridge between the host cell membranes and *S. aureus* via fibronectin in the extracellular matrix (Cho et al., 2001). ClfA, ClfB and IsdA enable *S. aureus* to adhere to fibrinogen, a glycoprotein converted into fibrin during blood clot

formation. These proteins guide bacterial clumping to prevent phagocytosis by host immune cells (Corrigan et al., 2009). WTA is a surface exposed polymer covalently attached to peptidoglycan and has been known to be integral for *S. aureus* adherence to human epithelial cells (Weidenmaier et al., 2004). In the event of successful colonization, the risk of *S. aureus* switching from a colonization phenotype to an invasive phenotype increases.

Like many bacteria, *S. aureus* expresses different proteins at different stages of growth and in different niches, including during the switch from a colonizing to an invading phenotype. It is essential to understand how *S. aureus* switches from colonization to invasion, in order to design better treatment options that target these distinct phenotypes. Quorum sensing is a density-dependent sensing mechanism used by many pathogens to globally regulate the appropriate response to changing environmental conditions (Rutherford and Bassler, 2012). Quorum sensing in *S. aureus* regulates the phenotype switch from colonizing commensal to invasive pathogen using the accessory gene regulator (*agr*), which is a four gene operon consisting of *agr*A-D (Figure 1.1) (Novick and Geisinger, 2008; Parker and Sperandio, 2009). The components of this operon encode the linear peptide AgrD, the transmembrane protein AgrB, the receptor histidine kinase AgrC and the intracellular response regulator AgrA. AgrB is responsible for the processing of AgrD into a cyclized auto-inducing peptide (AIP), as well as exporting it into the extracellular space. Secreted AIP binds its cognate receptor, AgrC, which phosphorylates and activates AgrA that acts as a transcription factor to promote transcription from two divergent promoters, P2 and P3 (Novick and Geisinger, 2008; Thoendel et al., 2011). Activation of the P2 promoter drives transcription of the *agr*

operon in a positive feed-forward signal, while activation of the P3 promoter leads to RNAIII production (Painter et al., 2014). RNAIII in turn regulates expression of over 200 virulence factors (Novick, 2003; Queck et al., 2008). Understandably, the *agr* operon is often studied for its contribution to infection and disease.

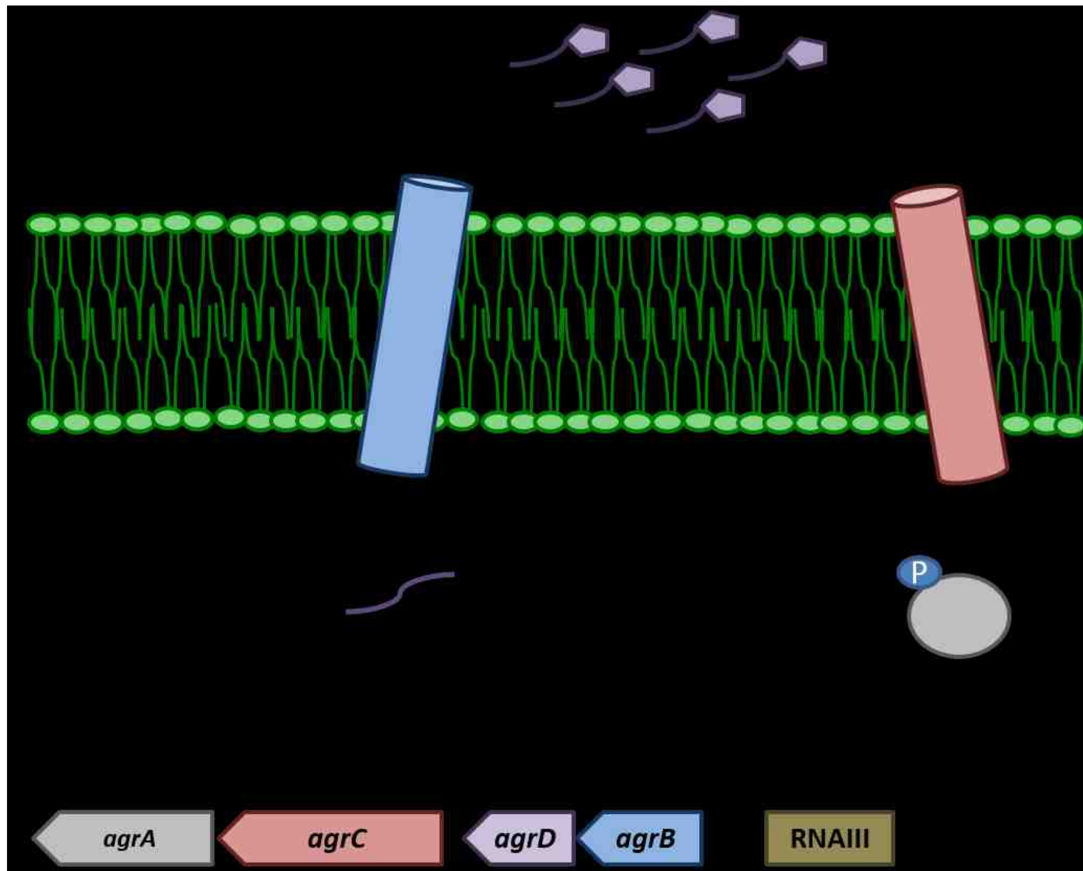

Figure 1.1 *S. aureus* accessory gene regulator (agr) operon.
The *agr* P2 promoter drives expression of the genes encoding the four proteins required for *agr* signaling. AgrD is the linear precursor of the *agr* auto-inducting peptide (AIP). AgrB secretes AIP from the bacteria where it binds to its cognate receptor, AgrC, on the bacterial membrane, which leads to phosphorylation of AgrA and activation of the P2 and P3 promoters. P3 promoter activation generates RNAIII which regulates expression of virulence factors which drive invasive infection. Adapted from (Novick and Geisinger, 2008).

Some of the virulence factors regulated by the *agr* operon that are important for invasive skin infections include alpha-hemolysin and the phenol soluble modulins. Alpha hemolysin (Hla) is one of the major virulence factors responsible for dermonecrosis during skin infection (Kennedy et al., 2010), and its production is, in large part, post-transcriptionally regulated by RNAIII under the *agr* operon (Xiong et al., 2006). Hla is secreted as a monomer and binds disintegrin and metalloprotease 10 (ADAM-10) on host cells. ADAM-10 is localized to the cell membrane and its functional role is to cleave E-cadherin to untether the adherens junctions between adjacent epithelial cells (Maretzky et al., 2005). ADAM10 provides a docking site for a monomer of Hla, which then oligomerizes to form a pore within the host cell membrane (Wilke and Bubeck Wardenburg, 2010). Pore formation leads to loss of intracellular potassium from the host cell, eventually leading to cell death (Walev et al., 1993). In addition, upon Hla binding, ADAM-10 relocalizes to lipid rafts enriched in caveolin. (Wilke and Bubeck Wardenburg, 2010). ADAM-10 and Hla complex formation leads to disruption of endothelial cell-cell focal adhesion to perturb cellular barriers for more invasive disease (Hocke et al., 2006; Wilke and Bubeck Wardenburg, 2010), such as providing a gateway for *S. aureus* superantigens to pass the host mucosal membrane (Brosnahan et al., 2009). Despite knowing that infection with Hla-expressing *S. aureus* leads to severe damage to the epidermis and dermis of the skin (Kennedy et al., 2010), it has not been thoroughly investigated as to how the host immune system regulates the subsequent inflammation and limits the damage caused by both Hla and the immune response to Hla.

Phenol soluble modulin (PSM) expression is regulated by the *agr* operon and has also been shown to be a major determinant of *S. aureus* virulence (Wang et al., 2007a).

PSMs are amphipathic peptides of which there are 7 types classified into two groups based on their length. The α-type PSMs (PSM α1-PSM α4) are half the size of the β-type PSMs (PSMβ1-PSMβ2, and δ toxin) (Cheung et al., 2014). PSMs have been shown to contribute significantly to *S. aureus* skin infection (Kobayashi et al., 2011a; Wang et al., 2007a). Furthermore, it was recently demonstrated that *psmα* expression modulates production of Hla, suggesting that coordinated action of the two toxins is necessary to achieve epithelial injury during *S. aureus* infections (Bryan J. Berube et al., 2014).

The Skin as a Barrier to *S. aureus* Infection

The surface of the skin is the first layer of defense against infection by potentially pathogenic bacteria. The skin surface is not hospitable to many types of bacteria due to its low temperature of 32-34°C, acidic pH, secreted antimicrobial peptides, and other resident commensals (Krishna and Miller, 2012b). The low pH of the skin is due to the breakdown of filaggrin, which binds keratin fibers in epithelial cells during keratinocyte differentiation. This leads to production of pyrrolidone carboxylic acid and urocanic acid, which have been shown to directly inhibit the growth of *S. aureus* (Miajlovic H, 2012). Another barrier to skin colonization includes the large class of antimicrobial peptides that are secreted by keratinocytes in the human epidermis, including β-defensins (hBD2, hBD3) and cathelicidin (LL-37) (Krishna and Miller, 2012a; Peschel and Sahl, 2006). In the murine epidermis, cathelicidin-related AMP (CRAMP), a homolog of LL-37, and mouse β-defensin 4 (mBD4), an ortholog of hBD2 are expressed (Bauer and Shafer, 2015). β-defensins are cationic and as such can interact with the negatively

charged membrane components lipoteichoic acid (LTA) for Gram positive bacteria and lipopolysaccharides (LPS) of Gram negative bacteria. This interaction causes a reduction in membrane stability, pore formation, and eventual cell lysis (Ryu et al., 2014). Cathelicidin functions within the lysosome of keratinocytes and directly targets the *S. aureus* lipoprotein membrane to destroy the bacteria (Braff MH, 2005). In addition, resident commensals such as *S. epidermis* and *P. acnes* inhabit the skin niche, thereby occupying the space that would otherwise be available for *S. aureus* to bind the skin (Lina et al., 2003). Furthermore, *S. epidermis* secretes a serine protease called Esp that degrades specific *S. aureus* proteins known to be crucial for biofilm formation and architecture, thereby hindering *S. aureus* growth (Sugimoto et al., 2013). Despite this inhospitable environment, *S. aureus* is still able to colonize a large portion of the human population, typically with little to no clinical concern, as long as the skin barrier is not compromised.

Much of the ability of the skin to provide a barrier against pathogens is based on the architecture of the tissue (Krishna and Miller, 2012a). The skin is divided into the epidermis and the dermis (Figure 1.2). The surface layer of the epidermis is called the corneal layer, which provides special protection against the environment. This layer is developed though the process of keratinization, which is the formation of terminally differentiated or dying keratinocytes filled with keratin and held tightly together by cross-linked fibrils. The corneal layer is not present in other epithelia exposed to the environment such as in the lungs or the gut. Below the corneal layer are the granular, spinous, and basal layers of the epidermis (Krishna and Miller, 2012a). As keratinocytes originate in the basal layer and migrate upward to the corneal layer, they continuously

reform the surface of skin, providing maintenance of this defense system. Below the epidermis is the dermis, which is composed of collagen and elastin fibers as well as many cell types that are important for innate immune defense (addressed below) (Kupper and Fuhlbrigge, 2004). The dermis can be further subdivided into the papillary layer, which connects to the epidermis and the deeper reticular layer, which in turn connects to adipose and muscle tissue. The epidermis and the dermis layers are spanned by hair follicles, sweat glands and sebaceous glands, which are called skin appendages and are open to the surface (Nestle et al., 2009). These appendages excrete free fatty acids which lead to acidification of the skin. Another important component of the skin includes the capillary and lymphatic vessels. These vessels are portals for immune cell recruitment and antigen surveillance (Tay et al., 2014). Therefore, the skin has a complex architecture providing an important barrier against pathogens.

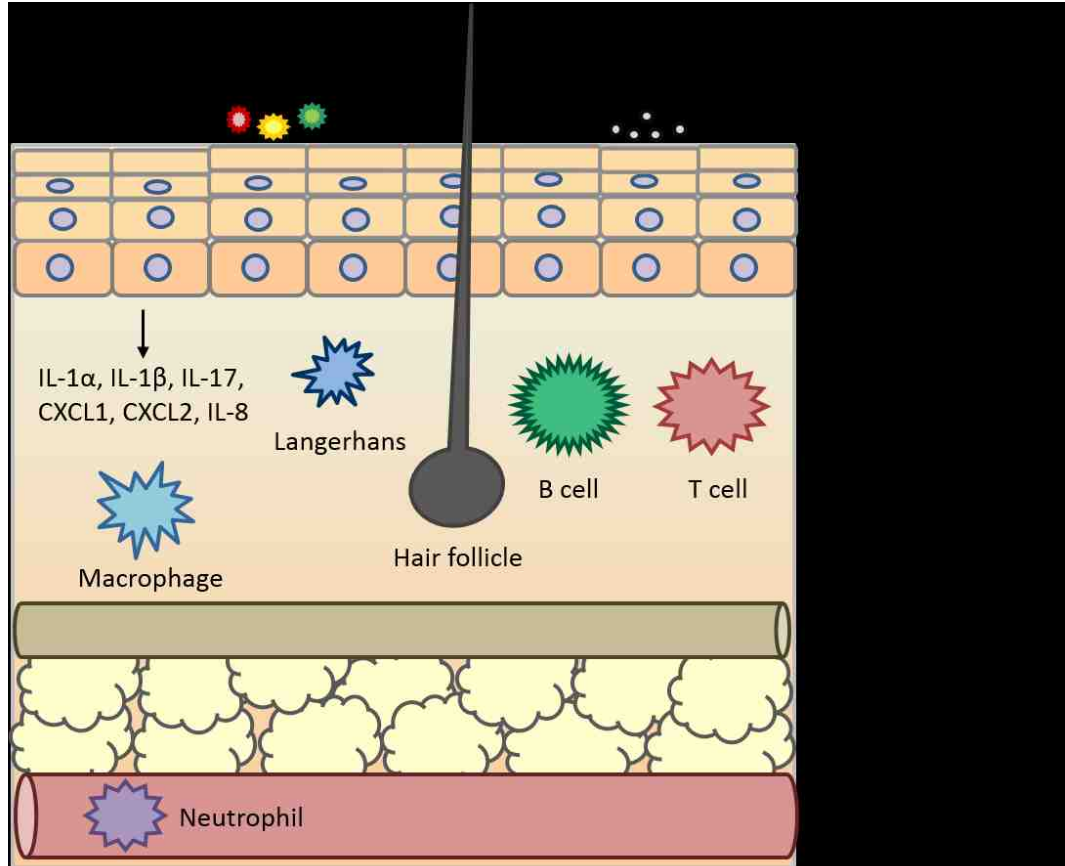

Figure 1.2. Structure of the Skin.
The surface of the skin has mechanisms to prevent infection such as low pH and low temperature, production of antimicrobial peptides by keratinocytes, resident commensals, and the physical barrier of the epidermis. Within the dermis reside skin and immune cells, such as resident macrophages, Langerhans, and B and T cells. During an infection, keratinocytes and immune cells produce inflammatory cytokines to promote the recruitment of neutrophils from the bloodstream. Also, lymphatic vessels innervate the skin to allow for antigen surveillance. Adapted from (Miller and Cho, 2011).

Innate immune recognition and response to *S. aureus* skin infection

The innate immune response to *S. aureus* is a multi-faceted response to promote clearance of the bacteria from the skin (Miller and Cho, 2011). The response includes cytokine production to activate local immune cells and chemokine production to recruit and activate neutrophils and other professional phagocytic cells. In addition to the physical barrier of the skin described above, the dermis also contains within it an immunological barrier (Kupper and Fuhlbrigge, 2004). This barrier is composed of immune cells, such as Langerhans dendritic cells, resident tissue macrophages and B and T lymphocytes, that conduct surveillance for pathogens by detecting pathogen-associated molecular patterns (PAMPs) (Nestle et al., 2009). PAMP motifs include bacterial surface components such as lipoteichoic acid (LPS) and LTA, as well as flagellin, each of which the host immune system recognizes as "non-self" (Akira and Takeda, 2004). These PAMPS are detected by pattern recognition receptors (PRR) which can be categorized by location and ligand specificity. For sensing *S. aureus* in the extracellular environment PRRs called Toll-Like Receptors (TLR), specifically TLR1 and TLR2, are present on the host cell surface and recognize components of the bacterial cell wall (Akira and Takeda, 2004). During infection with *S. aureus*, keratinocytes recognize LTA as it binds to TLR2 triggering the MyD88 signaling pathway, which signals internally via TRAF6 to activate transcription of cytokines via NF-κB (Newton and Dixit, 2012). For sensing *S. aureus* in the intracellular environment, keratinocytes also express cytoplasmic nod-like receptor 2 (NOD2) which detects the breakdown product of *S. aureus* peptidoglycan, called muramyl-dipeptide (Girardin et al., 2003). Activation of these PRRs leads to initiation of

the host innate immune response, which begins with cytokine and chemokine production (Miller and Cho, 2011).

Upon detection of *S. aureus* PAMPS, keratinocytes release the cytokines IL-1α, IL-1β and IL-17, and the chemokines CXCL1, CXCL2, and IL-8 (Krishna and Miller, 2012b). IL-1α is stored within keratinocytes and released during infection, whereas IL-1β secretion is induced by activation of the NLRP3 inflammasome after Hla forms a pore in the host cell membrane (depicted in Figure 1.3) (Craven et al., 2009; Munoz-Planillo et al., 2013). The IL-1 cytokines bind to the IL-1 receptor (IL-1R), leading to activation of the MyD88 signaling pathway (Miller and Cho, 2011). The importance of the IL-1 cytokine family has been demonstrated clinically, as humans with deficiencies in the IL-1R signaling pathway are susceptible to repeated *S. aureus* skin infections (van de Veerdonk and Netea, 2013) (Picard et al., 2010). In addition, IL-17 has also been shown to be important for protection against *S. aureus* skin infections, as mice deficient in the IL-17 receptor A (IL-17RA) have impaired clearance of *S. aureus* (Cho et al., 2010). Furthermore, IL-1α secretion and signaling causes CXCL1, CXCL2 and IL-8 production in order to recruit neutrophils from circulation to combat infection (Olaru and Jensen, 2010).

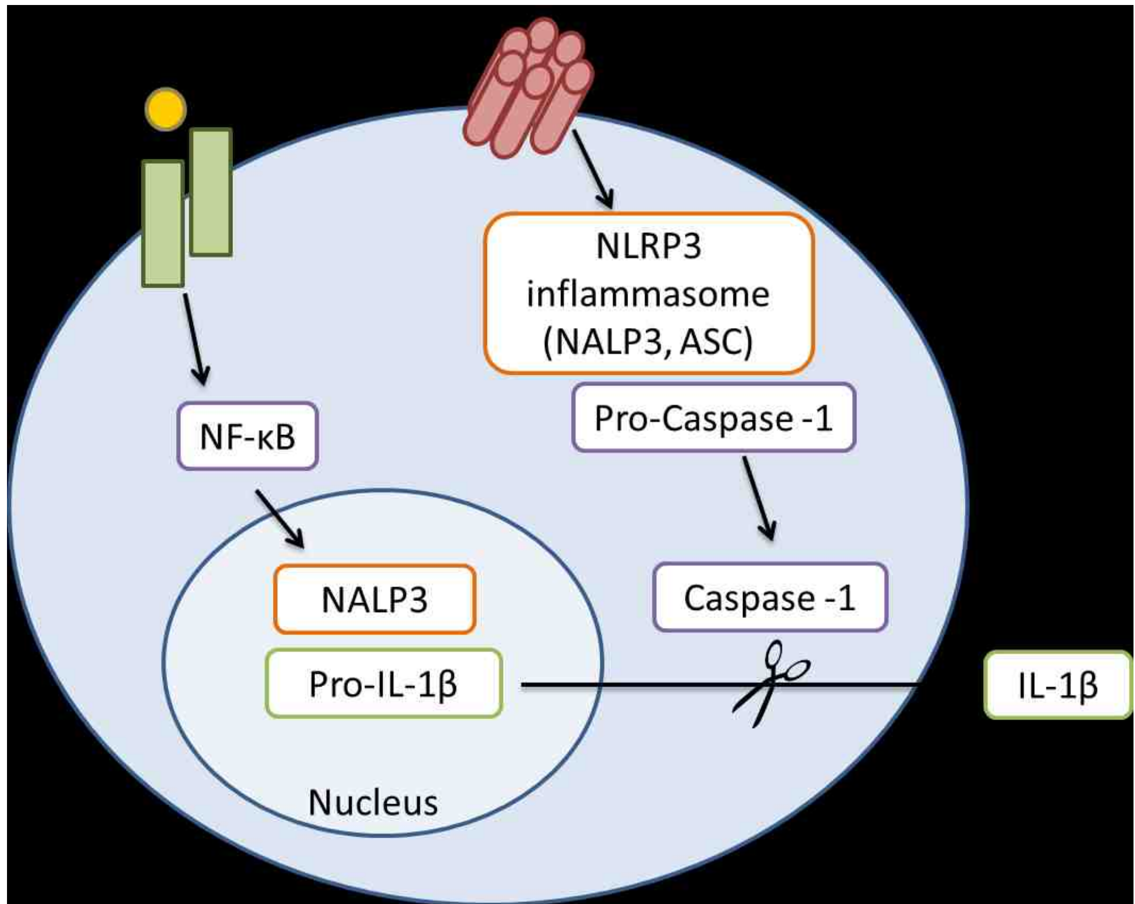

Figure 1.3. Production of IL-1β via activation of the NLRP3 inflammasome.
IL-1β is secreted following activation of two signaling pathways. Signal 1: TLR2 is activated by cell wall components of *S. aureus,* such as peptidoglycan (PGN) and lipoteichoic acid LTA), leading to pro-IL-1β production. Signal 2: Alpha hemolysin (Hla) forms a pore in the cell membrane, causing potassium efflux, leading to activation of the NLRP3 inflammasome. This results in cleavage and activation of caspase-1, which in turn cleaves pro-IL-1β, resulting in active IL-1β secretion. Adapted from (Gattorno and Martini, 2013).

Once the pro-inflammatory signaling cascades are activated, neutrophils are recruited to the site of infection to participate in abscess formations (Cho et al., 2012a). The abscess is primarily composed of neutrophils and bacteria surrounded by macrophages, creating a closed environment (Kobayashi et al., 2015). The abscess structure isolates the pathogen and noxious neutrophil killing components in one location, to prevent the spread of infection and to prevent damage to nearby healthy tissue (Kobayashi et al., 2015). Within the abscess, neutrophils use multiple mechanisms to kill bacteria including the release of neutrophil extracellular traps (NETs), secretion of antimicrobial peptides, proteinases, and acid hydrolases (Molne et al., 2000). NETs are composed of both webs of DNA that function to capture the bacteria as well as release a high concentration of antimicrobial effectors to kill the bacteria (Pilsczek et al., 2010). At the site of infection, neutrophils empty the contents of their azurophilic granules to release antimicrobial proteins, such as cathepsin G and neutrophil elastase to kill extracellular *S. aureus* (Segal, 2005). Bacteria engulfed by neutrophils are killed within the phagosome by antimicrobial peptides, degradative enzymes and reactive oxygen species (ROS). ROS such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), are generated by the NADPH oxidase in a mechanism called oxidative burst, (depicted in Figure 1.4) (Nauseef, 2007). Other ROS such as hyperchlorous acid (HOCl) are produced via the activity of myeloperoxidase (Kobayashi et al., 2015). The importance of the NADPH oxidase is highlighted clinically in that patients with chronic granulomatous disease, who have defective oxidative burst due to a genetic mutation causing defective subunits of the NADPH oxidase to be produced, have significantly increased

susceptibility to *S. aureus* infections (Bouma et al., 2010). Therefore, the rapid and potent neutrophil response to *S. aureus* is crucial to combating infection.

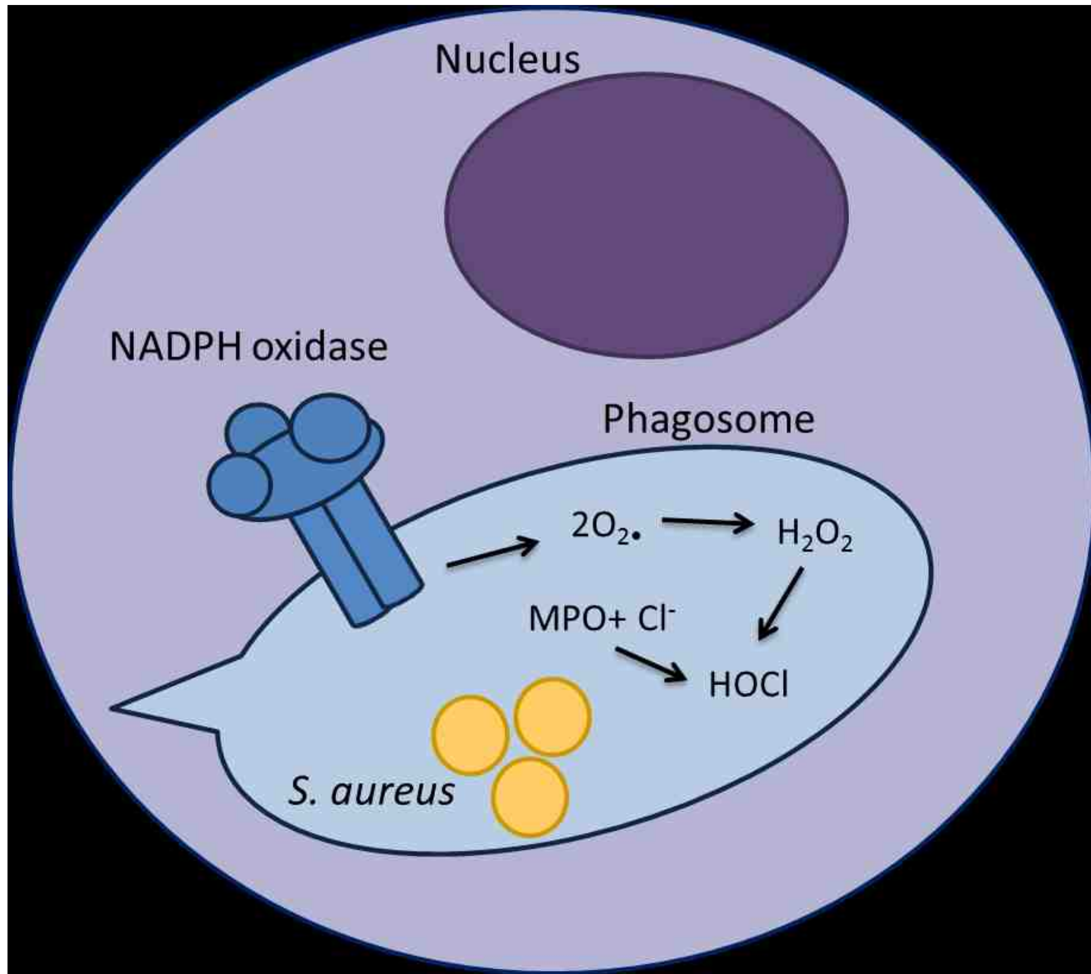

Figure 1.4. Neutrophil killing of *S. aureus*.
At the site of infection neutrophils phagocytose *S. aureus*. Within the phagosome, the neutrophils produce antimicrobial peptides, degradative enzymes, and reactive oxygen species (ROS) in order to kill the bacteria. Specifically, ROS are produced via the function of the NADPH oxidase. Adapted from (Kobayashi et al., 2015).

Regulation of the innate immune response to *S. aureus*

Once the initial assault against *S. aureus* is complete, regulation of the inflammatory immune response becomes crucial, as an unregulated response could lead to collateral damage to surrounding healthy tissue if the inflammation is allowed to persist (Wilgus et al., 2013). Although this has previously not been investigated with respect to skin infection, one plausible control mechanism is via recruitment of macrophages to the site of infection to clear apoptotic neutrophils before they become necrotic and release noxious intracellular components such as elastase, proteases, activated caspases, and signaling components such as danger associated molecular patters (DAMPs) (Silva, 2010, 2011). In general, neutrophil clearance occurs through macrophage recognition of phosphatidylserine (PS) on the surface of the apoptosing neutrophils (Fadok et al., 1998), followed by the formation of a thrombospondin bridge between CD36 on the macrophage and $\alpha_v\beta_3$ integrin on the neutrophil (Savill et al., 1992). Once the apoptosing neutrophils are cleared from the infection site, the environment surrounding the abscess could shift into a wound healing stage modulated by macrophages, and with new tissue formation and remodeling as keratinocytes proliferate (Rodero and Khosrotehrani, 2010). In Chapter 3 of this dissertation, we directly investigate the importance of CD36 in controlling the innate immune response to *S. aureus* skin infection.

Part 2: The role of CD36 in combating *S. aureus* infection and inflammation

Discovery and characterization of CD36

CD36 is a transmembrane cell surface protein and was first isolated from platelets (Clemetson et al., 1977). Since then, CD36 has been recognized as a multi-functional scavenger receptor expressed on the plasma membrane of many cells types, including microvascular endothelial cells, macrophages, and adipocytes (Silverstein and Febbraio, 2009). CD36 has many other names including glycoprotein IV (gpIV) and fatty acid translocase (FAT), and it is categorized as a member of the class B scavenger receptor family (Park, 2014). In humans, *cd36* is found on chromosome 7q11.2 and encodes a 53kD protein that undergoes extensive post-translational modification, including glycosylation, leading to a size of 88kD when expressed on the cell surface (Park, 2014; Silverstein and Febbraio, 2009). It has two transmembrane regions and a single extracellular region (depicted in Figure 1.5) with many ligand binding domains that bind numerous endogenous and exogenous ligands (Armesilla and Vega, 1994; Silverstein and Febbraio, 2009). For example, thrombospondin binds the extracellular amino acids 93-120, which has been shown to be important in macrophage recognition of apoptotic neutrophils (Savill et al., 1992). Oxidized low density lipoprotein (oxLDL) binding of amino acids 120-155 (Podrez et al., 2000) has been shown to be important for foam cell formation leading to atherosclerosis and cardiovascular disease (Silverstein, 2009). Long chain fatty acids bind CD36 in a small binding pocket and are engulfed by CD36 expressing adipocytes, implicating CD36 as a fatty acid transport receptor (Ibrahimi and Abumrad, 2002).

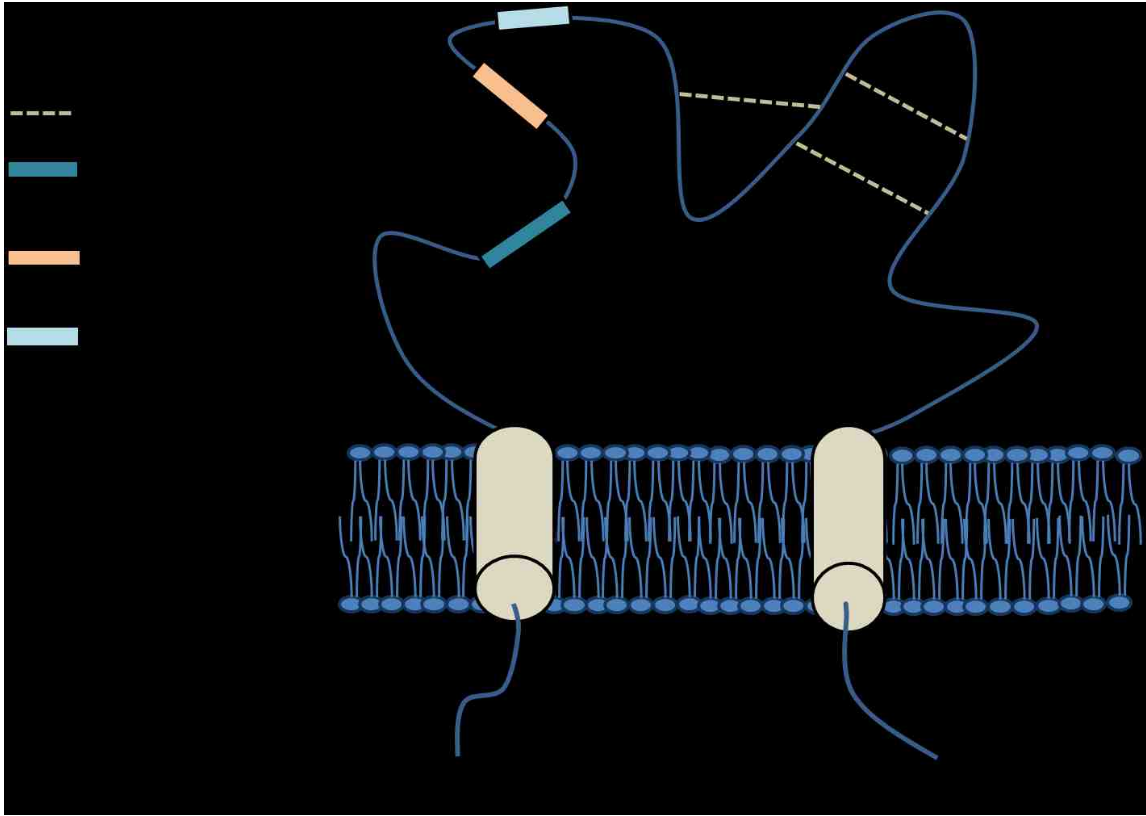

Figure 1.5. Structure of CD36.
CD36 is a transmembrane receptor with two short intracellular regions and one large extracellular domain with binding sites for thrombospondin, oxLDL and fatty acids. Adapted from (Park, 2014)

Anti-bacterial functions of CD36

In addition to the roles discussed above, CD36 also plays an important role in host defense. The first data showing that CD36 has a role in combating *S. aureus* infections came from the discovery that CD36 is a co-receptor for TLR2, and is required for detection of LTA from *S. aureus* (Hoebe et al., 2005b). Mice with genetic knockouts of CD36 are unable to contain *S. aureus* infection, as indicated by imaging showing increased dissemination of luminescent *S. aureus* after subcutaneous injection (Hoebe et al., 2005b). Therefore, it was concluded that CD36 plays an important function in host innate immune detection of *S. aureus*. This concept was expanded upon in another study where it was demonstrated that CD36 is not only important for sensing *S. aureus*, but also key to phagocytosis of *S. aureus* and LTA (Stuart et al., 2005). The C-terminal domain of CD36 signals in conjunction with TLR2 to initiate cytoskeleton rearrangement for engulfment of the bacteria, and triggers a TNF-α/IL-12 cytokine response to infection. Additionally, mice with genetic knockouts of CD36 had increased susceptibility to *S. aureus* bacteremia and reduced bacterial clearance (Stuart et al., 2005). However, the role of CD36 in combating *S. aureus* skin infections has not been directly investigated.

Part 3: The role of estrogen in combating *S. aureus*

Characterization of sex bias in susceptibility to infection

Epidemiological studies have shown that males are at an increased risk of many bacterial infections compared to females (McClelland and Smith, 2011; Silva, 2011). It is not known if this is due to differences in behavior, physiological differences in susceptibility to infection, or a combination of these and possibly other factors (Guerra-Silveira and Abad-Franch, 2013). If the sex bias is caused by a difference in behavior, then this may be due differences in hand washing (Johnson et al., 2003) or, in the case of skin infection, differences in physical activity that could lead to breaks in the skin barrier (Collins and O'Connell, 2012). If genetics and physiological differences also contribute to the sex bias in infection susceptibility, then this suggests that a dimorphic host immune response to bacterial infection exists.

Sexual dimorphism in infectious diseases has been noted in epidemiological studies and in animal models (McClelland and Smith, 2011), some of which are highlighted in Table 1. For example, it has been well documented that males are more susceptible to pulmonary *M. tuberculosis*, due in part to a reduced production of IgM antibodies and the influence of sex hormones (Nhamoyebonde and Leslie, 2014). Males are also more susceptible to *Treponema pallidum*, the causative agent of syphilis, due to a reduced CD3+, CD4+ and CD8+ T lymphocytes compared to females (Pope et al., 1994). Additionally, differing responses between the sexes to infection with fungi, parasites, and viruses have been documented indicating that sex plays an important large role in response to infection (Bernin and Lotter, 2014; McClelland and Smith, 2011). In

addition, recent studies have shown that compared to females, males of all ages exhibit an increased susceptibility to impetigo, which are blistering sores around the neck and face indicative of a contagious skin infection (Kim et al., 2011). Additionally studies have shown that males develop more methicillin-resistant *S. aureus* (MRSA) blood stream infections than females (Savill et al., 1989). Although basic research in this area is currently lacking, these clinical reports suggest that males may also have increased susceptibility to *S. aureus* skin infections compared to females.

Limited studies suggest that females may be innately protected against *S. aureus* infection. For example, during a study in rabbits with artificial chambers implanted subcutaneously within the lumbar region, scientists realized that male rabbits were more susceptible to *S. aureus* infection compared to female rabbits (Best et al., 1984). To determine if sex hormones were responsible for the difference in susceptibility to *S. aureus*, using the same artificial chamber model they infected ovariectomized female rabbits and castrated male rabbits and found that neutered rabbits of both sexes had comparable susceptibility to *S. aureus* infection, indicating that estrogen plays an important role in protection against *S. aureus* infection (Best et al., 1984). A more recent study *in vitro* demonstrated that alveolar macrophages from female mice were better able to kill internalized *S. aureus* compared to macrophages from males due to increased expression of nitric oxide synthase 3 (NOS3) (Yang et al., 2014), which generates nitric oxide that is toxic to pathogens (Burgner et al., 1999). However, these two studies do not address sex bias susceptibility during *S. aureus* skin infections, which is the most common infection caused by this pathogen (Moran GJ and LK, 2006). Taken together,

these data highlight the need to determine whether estrogen protects against *S. aureus* skin infection.

Mycobacterium marinum	Male	Mice	Lungs	(Yamamoto et al., 1991)
Cornebacterium kutscheri	Male	Mice	GI	(Komukai et al., 1999)
Listeria monocytogenes	Male	Mice	GI	(Pung et al., 1985)
Borrelia burgdorferi	Post-menopausal Female	Humans	Skin	(Jarefors et al., 2006)

Cellular responses to estrogen

To better understand the sex bias to infection, and the contribution of estrogen to the innate immune response, it is important to know how estrogen modulates gene expression and cellular function. Estrogen is a steroid sex hormone mainly studied for development of the female reproductive system (Jensen and DeSombre, 1972). Estrogen is synthesized from androstenedione, a cholesterol precursor (Cui et al., 2013). There are three types of estrogen produced depending on the stage of reproduction (Figure 1.6): estrone (E1) is produced during menopause, estradiol (E2), the predominant form, is produced during child-bearing years, and estriol (E3) is produced during pregnancy (Cui et al., 2013; Vrtacnik et al., 2014). In females, estradiol is produced in the ovaries, breasts, adrenal gland, adipocytes and the liver, and serum concentration ranges from 20-350 pg/mL during child-bearing years (Kratz et al., 2004). In males, estradiol is produced in the testis and sera concentration ranges from 0-20 pg/mL (Kratz et al., 2004). Estrogen induced signaling occurs when it binds to any of the three estrogen receptors ERα, ERβ, and GPR30 (Kovats, 2015). Estrogen receptors ERα and ERβ can form heterodimers or homodimers during ligand binding (Vrtacnik et al., 2014). Estrogen binding to these receptors leads to activation of either the classical or non-classical signaling pathway. The classical pathway is where ERα and/or ERβ directly trigger gene expression by binding to the Estrogen Response Element (ERE) found in the promoter of various genes. For the non-classical pathway, the receptors work as scaffolds for other transcription factors, such as AP-1, to bind promoter regions of DNA and trigger gene expression. The recently discovered G-protein coupled receptor 30 (GPR30/GPER) is found in the endoplasmic reticulum (Prossnitz et al., 2008). When estrogen binds this

receptor, it triggers non-genomic signaling pathways, such as activation of the second messengers calcium, cAMP and nitric oxide, as well as signaling cascades through PI3K and AKt (Revankar et al., 2005). Activation of these signaling pathways through may be important for host defense against *S. aureus*, since sex hormones have been demonstrated to have an important role in combating other bacterial infections (Garcia-Gomez et al., 2013; Kanda and Watanabe, 2005).

Figure 1.6. Structures of the three main types of estrogens.
Estrogen is a sex steroid hormone exhibiting the classical 4 ring steroid structure. Estrone has a ketone group attached to the D ring, while estradiol has one hydroxyl group attached to the D ring and estriol has two hydroxyl groups attached to the D ring.

Part 4: Concluding remarks and hypotheses

Given that *S. aureus* is the major cause of SSTI (Moran GJ and LK, 2006), the work described in this dissertation will focus on advancing our understanding of the innate immune response to these infections, specifically with respect to control of the inflammatory response (CD36) and natural resistance (estrogen). First, CD36 has been shown to aid in phagocytosis of *S. aureus* as a co-receptor for TLR2 (Hoebe et al., 2005b) and is also important for the general clearance of apoptotic neutrophils (Fadok et al., 1998). However, the contribution of this receptor to the regulation of the host immune response to *S. aureus* skin infection has not been investigated. We hypothesized that, independent of its role in bacterial phagocytosis, CD36 on macrophages would play a significant role in regulating local inflammation and dermonecrosis during *S. aureus* skin infection (depicted in Figure 1.7). The experimental studies to test this hypothesis are discussed in Chapter 3. Second, estrogen has been shown to be important for reduced susceptibility to some infectious diseases (Garcia-Gomez et al., 2013; Kanda and Watanabe, 2005), however, the contribution of this sex hormone to protection against *S. aureus* skin infection has not been investigated. We hypothesized that estrogen mediates protection against *S. aureus* skin and soft tissue infections by increasing bacterial clearance leading to reduced pathogenesis (depicted in Figure 1.8). The experimental studies to test this hypothesis are discussed in Chapter 4.

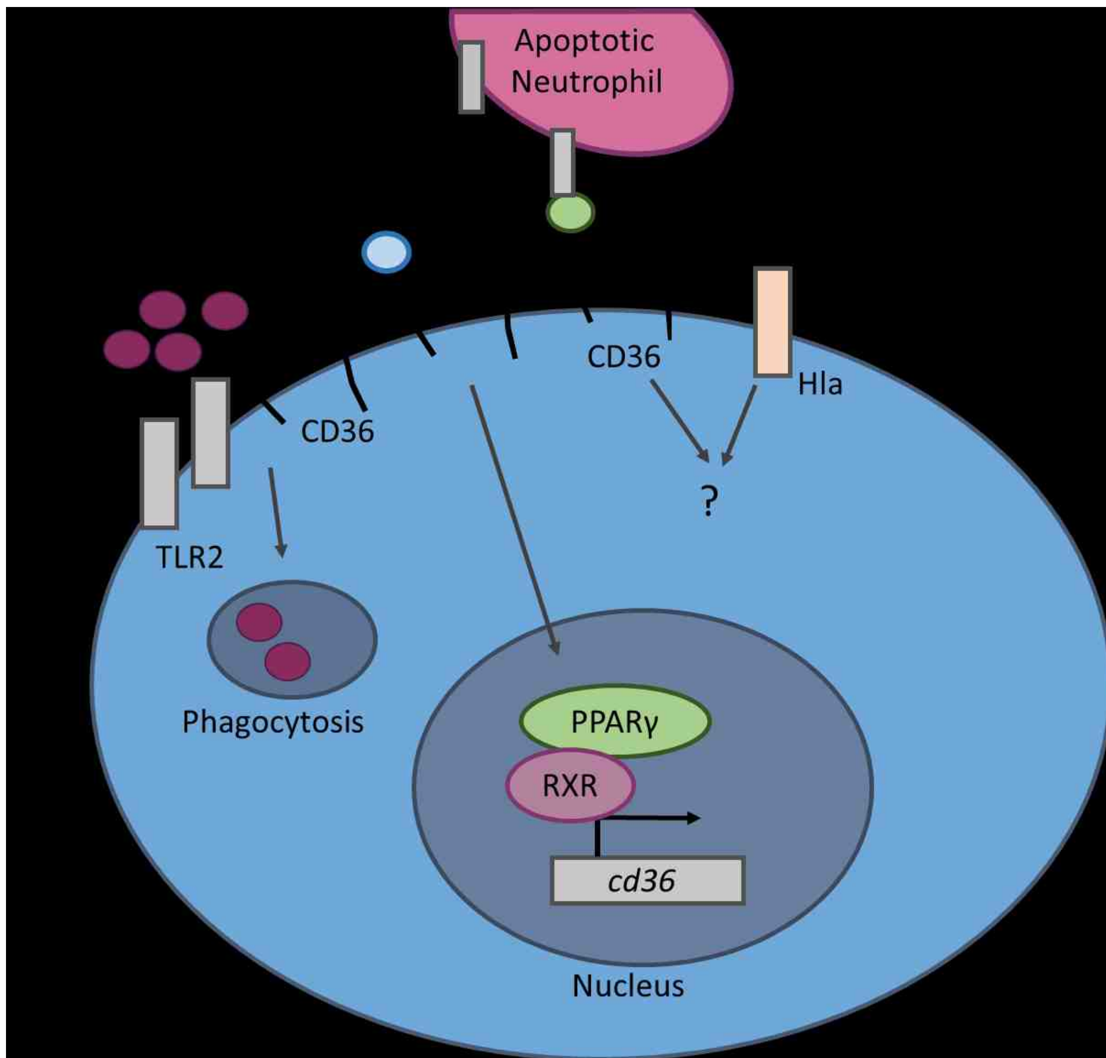

Figure 1.7. Roles of CD36 in host defense.
Cellular CD36 functions as a co-receptor with TLR2 to recognize lipoteichoic acid or peptidoglycan leading to phagocytosis of *S. aureus.* Expression of CD36 is regulated by binding of oxidized LDL or agonists for peroxisome proliferating activated receptor (PPARγ), which acts as a transcription factor binding to retinoid X receptor (RXR). CD36 binds to thrombospondin which functions as a bridge from the macrophage to apoptotic neutrophils expressing phosphatidylserine (PS), the "eat me" signal, resulting in clearance of dying neutrophils. It is unknown if CD36 contributes to regulation of the host inflammatory response to Hla producing *S. aureus.*

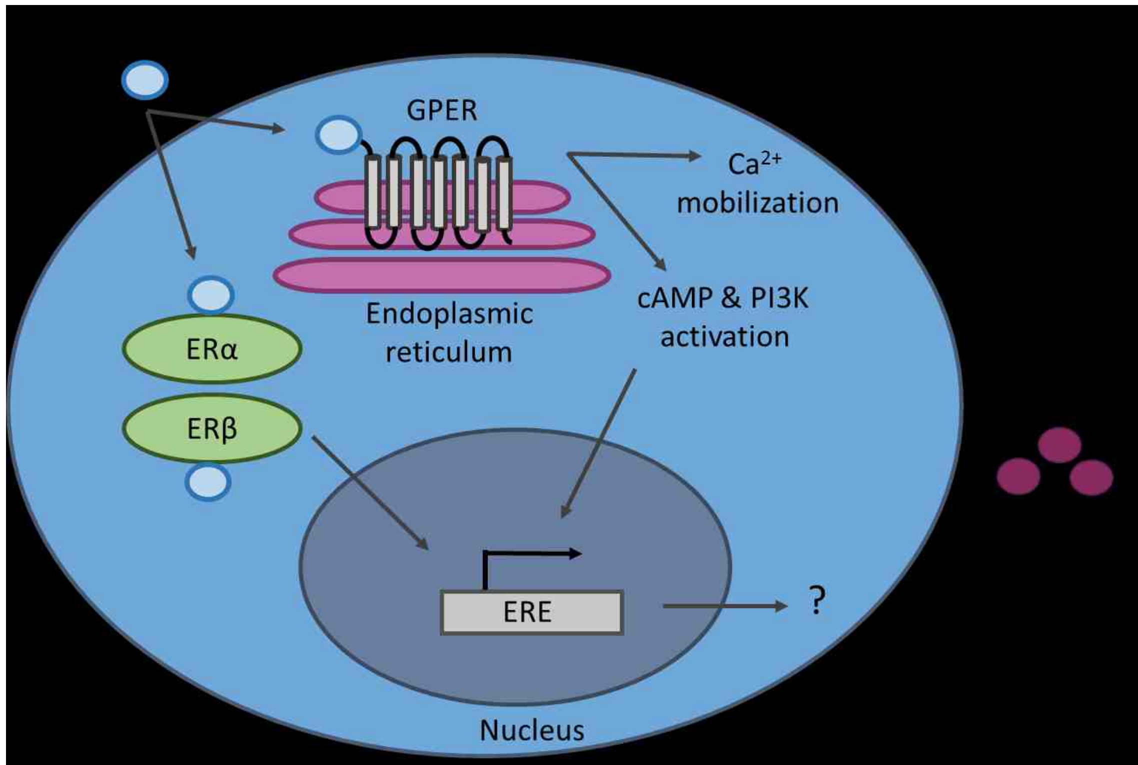

Figure 1.8. Roles of estrogen in host defense.
Estrogen leads to cell signaling when it binds to any of the three estrogen receptors ERα, ERβ, and GPR30 (Kovats, 2015). Estrogen receptors ERα and ERβ can form heterodimers or homodimers during ligand binding (Vrtacnik et al., 2014), which can then directly trigger gene expression by binding to the Estrogen Response Element (ERE) found in the promoter of various genes or by functioning as scaffolds for other transcription factors to bind promoter regions of DNA and trigger gene expression. GPER is found in the endoplasmic reticulum (Prossnitz et al., 2008), and estrogen binding leads to calcium mobilization, activation of cAMP and signaling cascades through PI3K pathways. It is unknown if estrogen through these signaling pathways aids in host defense against *S. aureus.*

Chapter 2 Methodology

The purpose of this chapter is to provide detailed protocols for the studies conducted in Chapter 3 and Chapter 4.

Bacterial Strains

S. aureus USA300 LAC and its isogenic *agr*-deletion mutant (LACΔ*agr*) were provided by Dr. Frank DeLeo (Rocky Mountain Laboratories, NIH/NIAID) and Dr. Michael Otto (NIH/NIAID). The isogenic *hla* deletion mutant (LAC was generously provided by Dr. Juliane Bubeck-Wardenburg (University of Chicago). The studies conducted in Chapter 3 use LAC, LACΔ*agr,* and LAC strains. The studies in Chapter 4 use the LAC strain.

Bacterial Growth Conditions

Bacteria for skin infection were used at early exponential phase and prepared as previously described with minor modifications (Rothfork et al., 2003). To generate bacteria at this phase, 25 mL cultures in trypticase soy broth (TSB) were inoculated and grown for 16-18 hours at 37°C with shaking at 240 rpm in a 50 mL conical tube. The bacteria were then pelleted by centrifugation at 1800x g for 4 minutes at 4°C. Supernatant was discarded and the pellet was washed twice with cold saline, then resuspended in 1 mL TSB and vortexed. Twenty-five microliters of this bacterial stock was used to inoculate a fresh 25 mL culture and grown for 2 hours at 37°C with shaking at 240 rpm in a 50 mL conical tube. Following incubation, bacteria were again pelleted

by centrifugation at 1800 x g for 4 minutes at 4°C. Supernatant was discarded and the pellet was washed three times with cold saline, then resuspended in 1 mL TSB and vortexed. Twenty-five microliters of this bacterial stock was used to inoculate a fresh 25 mL culture and grown for 2 hours at 37°C with shaking at 240 rpm in a 50mL conical tube. Following incubation, bacteria were again pelleted by centrifugation at 1800 x g for 4 minutes at 4°C. Supernatant was discarded and the pellet was washed three times with cold saline, then resuspended in 1 mL TSB and vortexed. Two hundred microliters of this bacterial solution was used to inoculate a fresh 25 mL culture and grown for another 2 hours at 37°C with shaking at 240 rpm in a 50 mL conical tube. After this incubation, bacteria were pelleted by centrifugation at 1800x g for 4 minutes at 4°C. Supernatant was discarded and the pellet was washed twice with cold saline, then resuspended in 1 mL TSB and vortexed. Three hundred microliters of bacteria were used to inoculate a fresh 25 mL culture and grown for a final 2 hours at 37°C with shaking at 240 rpm in a 50 mL conical tube. Bacteria were pelleted by centrifugation at 1800 x g for 4 minutes at 4°C and resuspended in 2 mL TSB/10% glycerol, sonicated for 30 seconds, and aliquoted in 100 µL volumes and frozen at -80°C until use.

To prepare bacteria for infection, the frozen 100 µL aliquot was resuspended in 900 µL saline, and bacteria were pelleted by centrifugation at 12,500 rpm for 4 minutes at 4°C to wash out the glycerol. The pellet was resuspended in 1 mL saline, vortexed for 30 seconds, sonicated for 30 seconds, and diluted to an appropriate OD_{600} for the desired inoculum. In the Chapter 3 studies, a low inoculum of $8x10^6$ CFUs/mouse or a higher inoculum of $2x10^7$ CFUs/mouse in 50 µL were used where indicated to infect mice. In

the Chapter 4 studies, $2x10^7$ CFUs/mouse in 50 μL were used to infect mice. CFUs were verified by plating serial dilutions on blood agar (Becton Dickinson, Franklin Lakes, NJ).

Mice

Animal work in this study was carried out at the AAALAAC accredited Animal Research Facility of the University of New Mexico Health Sciences Center in strict accordance with recommendations in the Eighth Edition of *The Guide for the Care and Use of Laboratory Animals* and the USA Animal Welfare Act. The protocol for these studies was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of New Mexico. C57BL/6, BALB/c, and ovariectomized and sham surgery C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). $CD36^{-/-}$ mice were bred within our facilities and breeders were provided by Dr. Maria Febbraio, Ph.D., of the University of Alberta. All rights, title, and interest in the $CD36^{-/-}$ mice are owned by Cornell University, where the $CD36^{-/-}$ mice were developed by Dr. Febbraio.

Skin Infection Model

We used a well-established model of *S. aureus* skin infection (Malachowa et al., 2013). Twenty-four hours before infection, age-matched mice (8-12 weeks, 22-28 g) were anesthetized by isoflurane and hair was removed from the right flank by first shaving then applying Nair (Church and Dwight Co., NJ) for 1 minute followed by rinsing with sterile water. For the studies conducted in Chapter 3, only male mice were used. For the studies in Chapter 4, both male and female mice were used as indicated.

On the day of infection, 50 . *aureus* strain at the described inoculum was injected subcutaneously into the right flank of anesthetized mice. Mice were weighed daily to assess weight loss as a marker of systemic morbidity, and photographed for abscess and dermonecrosis development, which was measured using ImageJ analysis (Schneider et al., 2012). This model is depicted in Figure 2.1.

For the intoxication experiments in Chapter 3, mice were subcutaneously injected with 50 µL of supernatant of the indicated strain of *S. aureus* (Wright et al., 2005). Supernatant was collected from a 5 mL culture grown in a 50 mL conical tube for 18 hours overnight at 37°C with shaking at 240 rpm. Cultures were normalized by OD_{600} and centrifuged at 1800 x g for 4 minutes at 4°C to collect supernatant, which was then passed through a 0.2 µM filter. The alpha-hemolysin (Hla) activity of the supernatant was verified in rabbit red blood cell assay described below. For some intoxication experiments, mice were subcutaneously injected with 1 µg of purified recombinant Hla or mutant Hla_{H35L} in 50 µL of sterile saline. Generation of recombinant Hla is described below.

For the neutrophil depletion experiments in Chapter 3, *CD36*$^{-/-}$ mice were given 100 µg of anti-mouse Ly6G (Clone 1A8, BioXCell, West Lebanon, NH) or isotype control (Rat IgG2a, BioXCell) by intraperitoneal injection on the day before and the day of infection. Antibodies were dialyzed in PBS at 4°C overnight before use and total protein concentration was confirmed by A_{280} using an extinction coefficient of 1.4 = 1mg/mL.

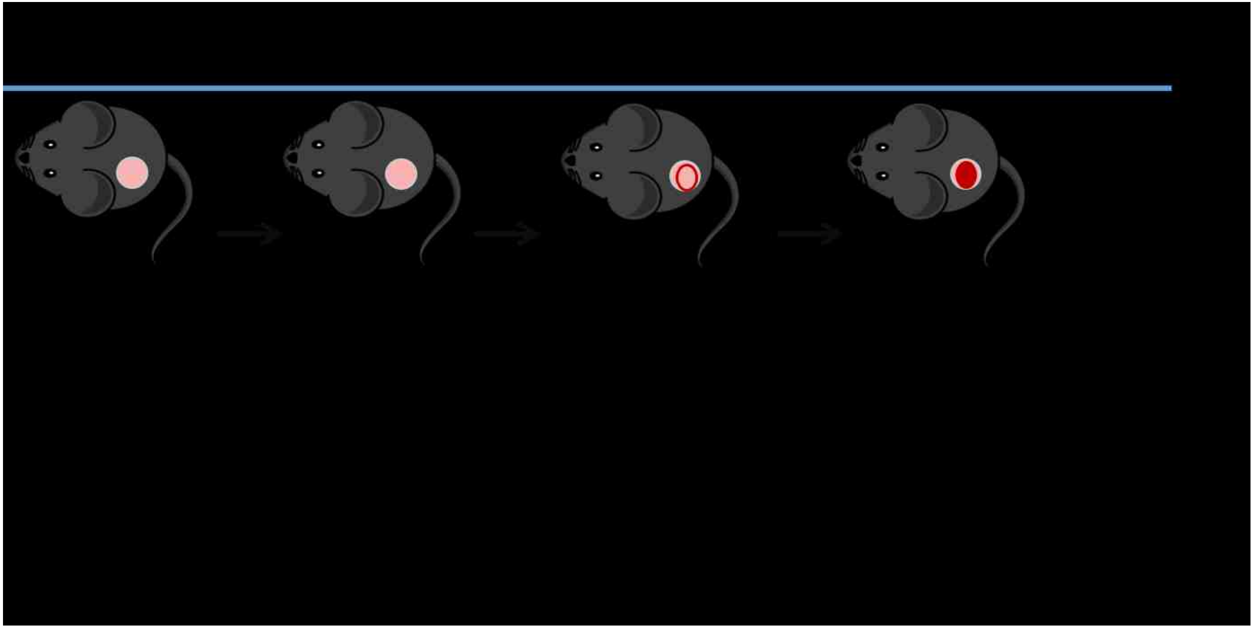

Figure 2.1 Skin infection model.
Fur is removed and mice are infected just under the skin. Disease progression is monitored, and samples are collected after mice are sacrificed (Malachowa et al., 2013).

Tissue Collection

At the indicated time points, mice were sacrificed by CO_2 asphyxiation, whole blood was collected by cardiac puncture, and the abscesses (area = 2.25 cm^2) and spleens were collected by excision as previously described (Sully et al., 2014). Whole blood sat at 4°C for 30 minutes before centrifugation at 10,000 rpm for 10 min at 22°C. Sera was collected and stored at -80°C until use (Hall et al., 2013). For CFU determinations, abscesses and spleens were collected in bead-beating tubes containing 2.3 mm Zirconia/Silica beads (BioSpec Products Inc., OK) and 1 mL of $HBSS^-$ (Gibco, Grand Island, NY) with 0.2% Human serum albumin (Sigma, St. Louis, MO) (Rothfork et al., 2003). Human serum albumin was used to prevent protein binding to the tube wall (Sully et al., 2014). Tissue was disrupted for 3 min in 1 min intervals using a Mini-Bead Beater-24 (BioSpec Products Inc., OK). During the intervals the tubes were placed on ice for cooling. Homogenates were diluted into PBS containing 0.1% Triton X-100 and sonicated, followed by plating serial dilutions on blood agar (Hall et al., 2013). For cytokine and western blot analyses, abscess homogenates were clarified by centrifugation at 12,500 x g and supernatant was stored at -80°C until use as described below. Preparation of collected abscesses for qPCR or single cell analysis by flow cytometry is described below.

Rabbit red blood cell lysis assay

Hemolysis activity of bacterial supernatant or recombinant Hla was determined using a rabbit red blood cell assay previously described (Bernheimer, 1988b). Sample

was diluted serially in PBS and incubated at 37°C for 1 hour in a 4% solution of rabbit red blood cells (Colorado Serum Co., Denver, CO). Red blood cell lysis was assessed spectrophotometrically by OD_{450}. The data were analyzed by nonlinear regression fit to a four-parameter logistic curve and represented as the HA_{50}, which equals 1/the dilution required for 50% complete lysis (Daly et. al., 2015).

Cytokine Analysis

Cytokines were measured in clarified supernatant from homogenized abscesses collected on days 1, 3, and 7 post-infection using custom designed multiplex assays (Millipore, Billerica, MA) according to the manufacturer's directions. For the assay, clarified supernatant from homogenized abscesses were pre-diluted 1:5 in assay buffer for the optimal range of measurement according to the standards provided. Cytokines measured include: IL-1β, TNF-α, IL-6, CXCL1, and MIP2. These cytokines were also measured in murine sera, and were nearly undetectable, so these data were not included in the chapters. The multiplex assays were ran at the UNM CTSC T1 lab on the Bio-Plex 200 (Biorad).

Quantitative RT-PCR

Abscess tissue was collected in RNALater (Qiagen, Valencia, CA), weighed and RNA was isolated using Qiazol according to manufacturer's instructions. RNA was then purified using RNeasy Kits (Qiagen) and stored at -80°C until use. cDNA was generated by high capacity cDNA RT kit with RNAse inhibitor and random hexamer primers

(Applied Biosystems, Foster City, CA) on a PTC-200 Peltier thermocycler (Biorad, Hercules, CA). cDNA was stored at -80°C until use. qPCR was performed using an ABI7000 Real Time PCR system with Taqman Gene Expression master mix (Applied Biosystems), Prime Time Predesigned qPCR Assays for *il-1beta, nlrp3, asc, caspase-1* (Integrated DNA technologies, Coralville, IA), and cDNA diluted 1:5 in RNase free water on. Gene expression was quantified using SDS RQ Manager Version 1.2.2 software (Applied Biosystems) relative to the housekeeping gene *hprt.*

MPO ELISA

Myeloperoxidase was measured in clarified supernatant from homogenized abscesses with the ELISA Mouse Myeloperoxidase DuoSet kit (R&D systems, Minneapolis, MN) according to manufacturer's directions. For the assay, clarified supernatant from homogenized abscesses were pre-diluted 1:100 in PBS for the optimal range of measurement according to the standards provided.

Western blot

Clarified abscess homogenates were rapidly thawed at 37°C and protein concentrations determined by A_{280} (Nanodrop 1000 Spectrophotometer, Thermo Fisher Scientific, Wilmington, DE). Equivalent amounts of total protein were separated by SDS-PAGE on 16% Tris-Glycine gels (Novex Life Technologies, Grand Island, NY) prior to transfer to polyvinylidene fluoride membranes for 1 hour at 4°C. Membranes were blocked with 1% BSA in Tris-buffered saline (TBS) (20 mM Tris pH 7.5, 150 mM

NaCl) for 30 min at 4°C, then probed with rabbit anti-mouse Caspase-1 p10 (Santa Cruz Biotechnology Inc., TX) or rabbit anti-mouse IL-1β (Abcam, Boston, MA) antibodies for 1 h at 22°C. Unbound primary antibody was removed by repeated washing with TBST (TBS with 0.1% Tween20). Membranes were developed using nitro-blue tetrazolium and 5-bromo-4-chloro-39-indolyphosphate (NBT/BCIP: Pierce Biotechnology Inc., Rockford, IL) following incubation for 1 h at 22°C with goat anti-rabbit IgG-alkaline phosphatase (AP) conjugated secondary antibody (Sigma, St. Louis, MO). Imaging was performed using a Protein Simple FluorChem R imaging system (Protein Simple, Santa Clara, CA).

Single Cell Suspension from Skin

Abscess tissue was collected in MACs storage solution (Miltenyi Biotec Inc., Auburn, CA) and enzymatically digested in gentle MACs C tubes in a 5 mL solution of 350 μg/mL Liberase TL (Roche, Indianapolis, IN), 2 mg/mL Hyaluronidase (Sigma), and 0.25 kU/mL DNase (Sigma) in RMPI 1640 Media (ATCC, Manassas, VA) for 2 hours while shaking at 37°C. Digestion was halted with addition of 2 mL of ice cold RPMI (Manassas, VA) supplemented with 10% FBS. Single cell suspensions were prepared by mechanical disruption in C tubes using the Gentle MACs Dissociator (Miltenyi Biotec) followed by f

counts were determined by trypan blue staining using the TC 20 Automated Cell Counter system (Biorad, Hercules, CA). Single cells suspension were used to identify cells of interest and/or isolate specific cell populations as described below.

Flow Cytometry

After generating a single cell suspension from skin as described above, cells were centrifuged at 800 x g for 3 minutes and resuspended in 500 µL of cold 0.075% sodium azide (Sigma), 0.5% BSA in PBS. The cells were then blocked for 30 minutes with 2% BSA at 4°C, followed by a 1 hour incubation at 4°C with anti-mouse Ly6G (BioXCell, West Lebanon, NH) conjugated to Alexa Fluor 488 (Protein labeling kit, Molecular Probes Inc., Eugene, OR), anti-mouse CD36-Alexa Fluor 647 (Biolegend, San Diego, CA), anti-mouse F4/80-PE (Abcam, Cambridge, MA), or isotype controls. Cells where washed with PBS prior to analysis by flow cytometry (Accuri C6, BD Biosciences, San Jose, CA). Data was analyzed using FlowJo vX software (FlowJo LCC, Ashland, OR).

Neutrophil Isolation

After generating a single cell suspension from skin as described above, $Ly6G^+$ cells were isolated by positive selection with an anti-Ly6G MicroBead Kit with the MiniMacs Seperator according to manufacturer's instructions (Miltenyi Biotec Inc.). Cells were eluted from the column in PBS, at pH 7.2 with 0.5% bovine serum albumin, and 2 mM EDTA. The isolated $Ly6G^+$ cells were then used on the neutrophil response assay described below. This kit was also used to isolate neutrophils from murine bone marrow.

Neutrophil Response Assay

Mice were sacrificed by CO_2 asphyxiation, femurs and tibias were exposed and bone marrow was recovered by flushing bones with warm Dulbecco's Modified Eagle's Medium (4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate) (ATCC) using a 26 gauge needle. Recovered cells were centrifuged at 800 x g for 3 min, red blood cells were lysed by incubation in cold water for 1 minute, and $Ly6G^+$ cells (neutrophils) were isolated as described above. Neutrophils were incubated in LAC supernatant for 1 h at 37°C and 5% CO_2. Cell supernatant was collected for cytokine analysis and stored at -80°C until use. Cells were lysed with RLT buffer (Qiagen) and RNA purified using an RNeasy Mini Kit (Qiagen). Purified RNA was stored at -80°C until use.

Peritoneal Macrophage Isolation

Mice were sacrificed by CO_2 asphyxiation, skin was cleaned with 70% EtOH and a large incision was made to expose the peritoneal lining. Mouse peritoneal macrophages were collected by peritoneal lavage with 5 mL warm Dulbecco's Modified Eagle's Medium (ATCC) followed by filtration through a 70 centrifuged at 800 x g for 3 min then resuspended in Dulbecco's Modified Eagle's Medium with 10% FBS, 10 mM Hepes, 2 mM L-glutamine, 1% penicillin/streptomycin prior to plating. Cells were incubated overnight at 37°C in 5% CO_2.

Macrophage Administration to Mice

Cultured peritoneal macrophages (collected above) were washed twice with Dulbecco's PBS to remove non-adherent cells. Macrophages were collected in Dulbecco's PBS via scraping and enumerated using the TC 20 Automated Cell Counter (Biorad). Macrophages (1.0 x 10^4 cells in 100

mice by subcutaneous injection near the site of intoxication. For phagocytosis inhibition, adherent cells were first incubated with 10 μM cytochalasin B or saline control for 1 hour at 37°C + 5% CO_2, and washed with Dulbecco's PBS before injection into mice.

Macrophage phagocytosis and killing of *S. aureus*

Raw 264.7 cells were grown in Dulbecco's Modified Eagle's Medium with 10% FBS, 10 mM Hepes, 2 mM L-glutamine, 100 U/mL penicillin and 100 ug/mL streptomycin at 37°C in 5% CO_2 (Peterson et. al., 2008). Twenty-four hours prior to the assay, cells were washed with PBS and the medium replaced with DMEM with 10 mM Hepes, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin and 10% Charcoal stripped FBS (to limit cell exposure to hormones) (JR Scientific, Woodland, CA). Prior to the assay, early exponential-phase LAC was cultured in 3 ml of TSB at $2x10^7$ CFU/ml with 50 nM exogenous AIP1 (Biopeptide Co., San Diego, CA) at 37°C while shaking for 5 hours. The bacteria were centrifuged, washed in PBS, sonicated, and suspended at $2x10^8$ CFUs/mL in DMEM with 10% Charcoal stripped FBS. The bacteria were opsonized overnight with rabbit anti- *S. aureus* IgG at 100 μg/ml (Accurate Chemical & Scientific Co., Westbury, NY) at 4°C on ice.

On the day of the assay, RAW 264.7 cells were detached with Cell Stripper (Corning, Manassas, VA), and enumerated using the TC 20 Automated Cell Counter (Bio-rad) (Peterson et. al., 2008). Cells were resuspended at $2x10^7$ cells/ml in DMEM with 10% Charcoal stripped FBS and incubated with 500 nM or 5 µM 17-β Estradiol (Sigma-Aldrich, St. Louis, MO) or vehicle control for 2 hours at 37°C in 5% CO_2. The cells were then combined with opsonized bacteria at a multiplicity of infection (MOI) of 10. The cells and bacteria were centrifuged at 500 x g for 3 min to initiate contact, followed by incubation at 37°C in 5% CO_2 for 1 hour to allow for phagocytosis. At 1 hour, lysostaphin (Sigma-Aldrich) was added at 2 µg/ml for 15 min to kill extracellular bacteria and then removed by centrifugation and replaced with fresh medium. Half of the samples were immediately processed for CFU determination to determine percent phagocytosis relative to initial bacterial concentration, and the other half were incubated for an additional 4 hours at 37°C in 5% CO_2 before CFU enumeration to determine percent killing. Bacteria were enumerated by dilution into PBS with 0.1% Triton X-100, followed by sonication and plating onto blood agar.

Expression and purification of recombinant Hla and mutant H35L

The coding sequence for Hla was PCR amplified from USA300 LAC genomic DNA using the following primers for insertion into the expression vector pET-28a(+) (Novagen, EMD Millipore, Billerica, MA) using the 5' *Nco*I and a 3' *Xho*I restriction sites: *HylA* F, 5'- GAGATACCATGGCAGATTCTGATATTAATATTAAAACCGG-3' and *HylA* R, 5'- CATTATCTCGAGATTTGTCATTTCTTCTTTTTCCCAATCGAT

TTTATATC-3’ (Integrated DNA Technologies, Coralville, IA). The resulting construct includes an N-terminal methionine and a C-terminal poly-histidine tag (LEHHHHHH). This construct was transformed into the cloning strain 5-alpha F’ I^q (New England BioLabs, Ipswich, MA). The H35L variant (Hla_{H35L}) (Menzies et. al., 1994) was constructed using Q5 Site Directed Mutagenesis Kit (New England Biolabs) according to manufacturer’s instructions and using the following primers: *Q5SDM* F, 5'- ATGGATA GAACTGAGCATCCAAACAACAAAC-3’ and *Q5SDM* R, 5'-AGTAATAACTGTAGC GAAG-3’. The Hla_{H35L} variant construct was inserted into pET-28a(+) (Novagen).

Both Hla constructs were transformed into BL21 Star (DE3) Competent *E. coli* (Life Technologies, Grand Island, NY) for expression. Bacteria were grown in Terrific Broth with 30 µg/mL kanamycin at 37 °C, 220 rpm and expression induced at an OD_{600} of 0.7 by 400 µM IPTG. The biomass was harvested 3 hrs post-induction by centrifugation and the cell pellet was frozen at -80 °C. The pellet was thawed in 5 volumes of lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 0.5 mg/mL lysozyme, 2 mM EDTA, Pierce Universal Nuclease for Cell Lysis (Life Technologies), pH 8.0) and incubated for one hour at 37°C with rotation. Remaining intact cells were lysed by sonication and cellular debris removed by centrifugation (14000 x g for 30 min at 4 °C). Clarified lysate was loaded on a GE Healthcare C 10/200 column containing Clontech Talon Metal Affinity Resin (Mountain View, CA) pre-equilibrated in binding buffer (50 mM Tris-Cl, 150 mM NaCl, pH 8.0). After washing, bound protein was eluted with 50 mM Tris-Cl, 150 mM NaCl and 300 mM imidazole, pH 7.0. The eluted protein was then further purified and buffer exchanged using a GE Healthcare Sephacryl S-400 16/60 prepacked column (Pittsburgh, PA) equilibrated in PBS. The final protein ran as a single

band on SDS/PAGE (data not shown). Recombinant protein was filtered for endotoxin removal (0.2 μm Acrodisc® Unit with Mustang™ E membrane, Pall Life Science, Ann Arbor, MI) and tested for endotoxin according to manufacturer's directions (ToxinSensor™ Chromogenic LAL Endotoxin Assay, GenScript, Piscataway, NJ) before use. Total protein concentration was confirmed by A_{280} with extinction coefficient = 1.1. The activity of Hla and the inactivity of Hla_{H35L} were verified using the rabbit red blood cell lysis assay as previously described (Bernheimer, 1988b).

Statistical Analysis

Statistical analyses were performed using Graph Pad Prism 6.0 software (Graph Pad Software, Inc., La Jolla, CA). *In vitro* data were analyzed by Student's *t* test and *in vivo* results by the Mann-Whitney U test for non-parametric data.

Chapter 3 CD36 is essential for regulation of the host innate response to *Staphylococcus aureus* alpha-toxin-mediated dermonecrosis

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Abstract

Staphylococcus aureus is the primary cause of skin and skin structure infections (SSSI) in the USA. Alpha-hemolysin (Hla), a pore-forming toxin secreted by *S. aureus* and a major contributor to tissue necrosis, prompts recruitment of neutrophils critical for host defense against *S. aureus* infections. However, the failure to clear apoptotic neutrophils can result in damage to host tissues, suggesting that mechanisms of neutrophil clearance are essential to limiting Hla-mediated dermonecrosis. We hypothesized that CD36, a scavenger receptor which facilitates recognition of apoptosing cells, would play a significant role in regulating Hla-mediated inflammation and tissue injury during *S. aureus* SSSI. Here we show that CD36 on macrophages negatively regulates dermonecrosis caused by Hla-producing *S. aureus*. This regulation is independent of bacterial burden, as CD36 also limits dermonecrosis caused by intoxication with sterile bacterial supernatant or purified Hla. Dermonecrotic lesions of supernatant intoxicated *CD36*$^{-/-}$ mice are significantly larger, with increased neutrophil accumulation and IL- *D36*$^{+/+}$ (wild-type) mice. Neutrophil depletion of *CD36*$^{-/-}$ mice prevents this phenotype, demonstrating the contribution of neutrophils to tissue injury in this model. Furthermore, administration of *CD36*$^{+/+}$*,* but not *CD36*$^{-/-}$*,* macrophages near the site of intoxication reduces dermonecrosis, IL- production and neutrophil accumulation to levels seen in wild-type mice. This therapeutic effect is reversed by inhibiting actin polymerization in the *CD36*$^{+/+}$ macrophages, supporting a mechanism of action whereby CD36-dependent macrophage phagocytosis of apoptotic neutrophils regulates Hla-mediated dermonecrosis. Together, these data

demonstrate that CD36 is essential for controlling the host innate response to *S. aureus* skin infection.

Introduction

Staphylococcus aureus is the primary cause of skin and skin structure infections (SSSI) presenting to emergency departments throughout the US (Moran et al., 2006; Talan et al., 2011). SSSIs include an intense local inflammatory response, which often precedes the formation of necrotic lesions. Although *S. aureus* secretes numerous virulence factors, the formation of necrotic lesions is largely due to the action of the pore-forming toxin alpha-hemolysin (Hla) (Inoshima et al., 2012; Kennedy et al., 2010; Kobayashi et al., 2011b; Sampedro et al., 2014; Tkaczyk et al., 2013). In recent years, the cellular receptor for Hla, the metalloprotease ADAM10, and its contribution to dermonecrosis, has been well-characterized (Becker et al., 2014; Berube and Wardenburg, 2013; Inoshima et al., 2011; Inoshima et al., 2012; Powers et al., 2012; Sampedro et al., 2014; Wilke and Wardenburg, 2010). In contrast, little is known regarding mechanisms used by the host to control local inflammation and limit Hla-mediated tissue injury. Insight into these mechanisms of host control could inform novel approaches to limit the pathogenesis of *S. aureus* SSSIs.

Neutrophils are critical for host defense against *S. aureus* and utilize a variety of mechanisms to kill the bacteria to limit invasive infection (reviewed in (Rigby and DeLeo, 2012)). However, neutrophils are short-lived and contain many noxious substances which, if released, are toxic to host tissues (Soehnlein and Lindbom, 2010; Weiss, 1989). Therefore, the clearance of apoptotic neutrophils, prior to the loss of

membrane integrity during secondary necrosis, is essential to limiting tissue damage. Importantly, Hla contributes to the recruitment of circulating neutrophils (Bartlett et al., 2008), suggesting that mechanisms of neutrophil clearance are essential to limiting Hla-mediated dermonecrosis.

The scavenger receptor CD36 is a membrane glycoprotein present on many mammalian cells, in particular monocytes and macrophages (reviewed in (Silverstein and Febbraio, 2009)), which is primarily known for its contribution to atherosclerosis (Collot-Teixeira et al., 2007; Park, 2014). However, CD36 also contributes to host innate defense against *S. aureus*. In conjunction with Toll-like receptor 2 (TLR2), CD36 recognizes *S. aureus* cell wall diacylglycerides, facilitating bacterial phagocytosis and cytokine production (Hoebe et al., 2005a; Stuart et al., 2005). Importantly, CD36 also enables macrophage recognition and clearance of apoptotic neutrophils (Navazo et al., 1996; Ren et al., 1995; Savill et al., 1991; Savill et al., 1992), suggesting an important role for CD36 in controlling the host response to skin infection; however the contribution of this receptor to the regulation of Hla-mediated dermonecrosis has not been investigated. We hypothesized that CD36 on macrophages would play a significant role in regulating local Hla-mediated inflammation and dermonecrosis, independent of its role in bacterial phagocytosis.

Using a mouse model of *S. aureus* dermonecrosis, here we show that CD36 negatively regulates dermonecrosis following infection with Hla-producing *S. aureus*. At early time points post-infection, this regulation is independent of bacterial clearance, as CD36 also limits dermonecrosis following subcutaneous intoxication with *S. aureus* secreted virulence factors and purified Hla. *CD36*$^{-/-}$ mice intoxicated with sterile *S.*

aureus supernatant show significantly increased dermonecrosis, with increased neutrophil accumulation and local IL- [redacted] *CD36*$^{+/+}$ (wild-type) mice. This phenotype is prevented by neutrophil depletion, pointing to the contribution of neutrophils to tissue injury in this model. Importantly, therapeutic administration of *CD36*$^{+/+}$, but not *CD36*$^{-/-}$, macrophages near the site of intoxication limits dermonecrosis, IL- [redacted] administration of *CD36*$^{+/+}$ macrophages significantly reduces the presence of necrotic neutrophils at the site of intoxication, this is reversed by pharmacological blockade of macrophage actin polymerization. This supports a mechanism of action whereby CD36-mediated macrophage phagocytosis of apoptotic neutrophils plays a significant role in host regulation of Hla-mediated dermonecrosis. Together, these data demonstrate for the first time that, independent of its role in bacterial clearance (Stuart et al., 2005), CD36 expression on macrophages plays an important role in host control of inflammation and skin injury during *S. aureus* skin infection.

Materials and Methods

Bacterial strains and growth conditions

S. aureus USA300 LAC and its isogenic *agr*-deletion mutant (LACΔ*agr*) were provided by Dr. Frank DeLeo (Rocky Mountain Laboratories, NIH/NIAID) and Dr. Michael Otto (NIH/NIAID). The isogenic *hla* deletion mutant (LAC was generously provided by Dr. Juliane Bubeck-Wardenburg (University of Chicago). Bacteria were grown in trypticase soy broth (TSB) at 37°C to early exponential phase as previously described (Rothfork et al., 2004) and CFUs determined by plating serial dilutions on blood agar (Becton Dickinson, Franklin Lakes, NJ).

Mouse model of S. aureus skin and soft tissue infection

Animal work in this study was carried out at the AAALAC accredited Animal Research Facility of the University of New Mexico Health Sciences Center in strict accordance with recommendations in the Eighth Edition of *The Guide for the Care and Use of Laboratory Animals* and the USA Animal Welfare Act. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of New Mexico. C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and *CD36*$^{-/-}$ mice, provided by Dr. Maria Febbraio, were bred within our facilities. The mouse model of dermonecrosis was performed as previously described (Malachowa et al., 2013). Briefly, twenty-four hours before infection, male, age-matched mice (8-12 weeks, 22-28 g) were anesthetized by isoflurane and hair was removed from the right flank using Nair (Church and Dwight Co., Ewing, NJ). On the day of infection, 50

sterile saline containing 8 x 10^6 CFUs of the indicated *S. aureus* strain was injected subcutaneously into the right flank of anesthetized mice. Mice were weighed daily and photographed for abscess and ulcer area which was measured using ImageJ analysis (Schneider et al., 2012). At the indicated time points post-infection, mice were sacrificed by CO_2 asphyxiation and the abscesses (area = 2.25 cm^2) and spleens were collected. For CFU determinations, abscesses and spleens were collected in bead-beating tubes containing 2.3 mm Zirconia/Silica beads (BioSpec Products Inc., Bartlesville, OK) and 1 mL of $HBSS^-$ (Gibco, Grand Island, NY) with 0.2% Human serum albumin (Sigma-Aldrich, St. Louis, MO). Tissue was disrupted for 3 min in 1 min intervals using a Mini-Bead Beater-24 (BioSpec Products Inc.). Homogenates were diluted into PBS containing 0.1% Triton X-100 and sonicated, followed by plating serial dilutions on blood agar. For cytokine and western blot analyses, abscess homogenates were clarified by centrifugation at 12,500 × g and supernatant was stored at -80°C until use as described below. For intoxication experiments, mice were subcutaneously injected with 50 µL of 0.2 micron filtered supernatant from an 18-hour culture of the indicated strain of *S. aureus* or with 1 µg of purified Hla in 50 µL of sterile saline. For neutrophil depletion experiments, *$CD36^{-/-}$* mice were given 100 µg of anti-mouse Ly6G (Clone 1A8, BioXCell, West Lebanon, NH) (Daley et al., 2008) or isotype control (Rat IgG2a, BioXCell) by intraperitoneal injection on the day before and the day of infection.

Quantification of cytokines and MPO

Cytokines were measured in clarified supernatant from homogenized abscesses using custom designed multiplex assays (Millipore, Billerica, MA) according to the

manufacturer's directions. Myeloperoxidase was measured in clarified supernatant from homogenized abscesses with the ELISA Mouse Myeloperoxidase DuoSet kit (R&D systems, Minneapolis, MN) according to manufacturer's directions.

Expression and purification of recombinant Hla and mutant H35L

The coding sequence for the mature Hla was PCR amplified from USA300 LAC genomic DNA using the following primers for insertion into the expression vector pET-28a(+) (Novagen, EMD Millipore, Billerica, MA) using the 5' *Nco*I and a 3' *Xho*I restriction sites: *HylA* F, 5'- GAGATACCATGGCAGATTCTGATATTAATATTAAAACCGG and *HylA* R, 5'- CATTATCTCGAGATTTGTCATTTCTTCTTTTTCCCAATCGA TTTTATATC (Integrated DNA Technologies, Coralville, IA). The resulting construct includes an N-terminal methionine and a C-terminal poly-histidine tag (LEHHHHHH). This construct was transformed into the cloning strain 5-alpha F' I^q (New England BioLabs, Ipswich, MA). The H35L variant (Hla_{H35L}) was constructed using Q5 Site Directed Mutagenesis Kit (New England Biolabs) according to manufacturer's instructions and using the following primers: *Q5SDM* F, 5'-ATGGATAGAActgAGCATCCAAACAACAAAC and *Q5SDM* R, 5'-AGTAATAACTGTAGCGAAG. The Hla_{H35L} variant construct was again inserted into pET-28a(+) (Novagen). Both Hla constructs were transformed into BL21 Star (DE3) Competent *E. coli* (Life Technologies, Grand Island, NY) for expression. Bacteria were grown in Terrific Broth with 30 µg/mL kanamycin at 37 °C, 220 rpm and expression induced by 400 µM IPTG at an OD_{600} of 0.7. The biomass was harvested 3 hrs post-induction by centrifugation and the cell pellet was frozen at -80 °C. The pellet thawed in

5 volumes of lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 0.5 mg/mL lysozyme, 2 mM EDTA, Pierce Universal Nuclease for Cell Lysis (Life Technologies), pH 8.0) and incubated for one hour at 37°C with rotation. Remaining intact cells were lysed by sonication and cellular debris removed by centrifugation (14000 x g for 30 min at 4 °C). Clarified lysate was loaded on a GE Healthcare C 10/200 column containing Clontech Talon Metal Affinity Resin (Mountain View, CA) pre-equilibrated in binding buffer (50 mM Tris-Cl, 150 mM NaCl, pH 8.0). After washing, bound protein was eluted with 50 mM Tris-Cl, 150 mM NaCl and 300 mM imidazole, pH 7.0. The eluted protein was then further purified and buffer exchanged using a GE Healthcare Sephacryl S-400 16/60 prepacked column (Pittsburgh, PA) equilibrated in PBS. The final protein ran as a single band on SDS/PAGE (data not shown). Recombinant protein was filtered for endotoxin removal (0.2 μm Acrodisc® Unit with Mustang™ E membrane, Pall Life Science, Ann Arbor, MI) and tested for endotoxin according to manufacturer's directions (ToxinSensor™ Chromogenic LAL Endotoxin Assay, GenScript, Piscataway, NJ) before use. The activity of Hla and the inactivity of Hla_{H35L} were verified using the rabbit red blood cell lysis assay as previously described (Bernheimer, 1988a).

Western blot analyses

Clarified abscess homogenates were rapidly thawed at 37°C and protein concentrations determined by A_{280nm} absorbance (Nanodrop 1000 Spectrophotometer, Thermo Fisher Scientific, Wilmington, DE). Equivalent amounts of total protein were separated by SDS-PAGE on 16% Tris-Glycine gels (Novex Life Technologies, Grand Island, NY) prior to transfer to polyvinylidene fluoride membranes. Membranes were blocked with 1% BSA

in Tris-buffered saline (20 mM Tris pH 7.5, 150 mM NaCl) for 30 min at 4°C, then probed with rabbit anti-mouse Caspase-1 p10 (Santa Cruz Biotechnology Inc., Dallas, TX) or rabbit anti-mouse IL-1β (Abcam, Cambridge, MA) antibodies for 1 h at 22°C. Unbound primary antibody was removed by repeated washing with TBST (TBS with 0.1% Tween20). Membranes were developed using nitro-blue tetrazolium and 5-bromo-4-chloro-39-indolyphosphate (NBT/BCIP: Pierce Biotechnology Inc., Rockford, IL) following incubation with goat anti-rabbit IgG-alkaline phosphatase (AP) conjugated secondary antibody (Sigma-Aldrich, St. Louis, MO). Imaging was performed using a Protein Simple FluorChem R imaging system (Protein Simple, Santa Clara, CA).

Quantitative RT-PCR

Abscess tissue (area = 2.25 cm^2) was collected in RNALater and RNA isolated using Qiazol according to manufacturer's instructions (Qiagen, Valencia, CA). RNA was purified using RNeasy Kits (Qiagen) and stored at -80°C until use. cDNA was generated by high capacity cDNA RT kit with RNAse inhibitor and random hexamer primers (Applied Biosystems, Foster City, CA) on a PTC-200 Peltier thermocycler (Bio-Rad, Hercules, CA). qPCR was performed using Taqman Gene Expression master mix (Applied Biosystems) and an ABI7000 Real Time PCR system. Gene expression was quantified using SDS RQ Manager Version 1.2.2 software (Applied Biosystems) relative to *hprt* or *gapdh* using Prime Time Predesigned qPCR Assays for *il-1beta, tnfα, cxcl1, nlrp3, asc and caspase-1* (Integrated DNA technologies, Coralville, IA).

Flow cytometry

Abscess sections were collected in MACs storage solution (Miltenyi Biotec Inc., Auburn, CA) and enzymatically digested in gentle MACs C tubes using 350ug/mL Liberase TL (Roche, Indianapolis, IN), 2mg/mL Hyaluronidase (Sigma-Aldrich), and 0.25 kU/mL DNase (Sigma-Aldrich) in RMPI 1640 Media (ATCC, Manassas, VA) for 2 hours while shaking at 37°C. Digestion was halted with ice cold RPMI (Manassas, VA) supplemented with 10% FBS. Single cell suspensions were prepared by mechanical disruption in C tubes using the Gentle MACs Dissociator (Miltenyi Biotec) followed by filtration [illegible]ad counts were determined by trypan blue staining using the TC 20 Automated Cell Counter system (Bio-Rad, Hercules, CA). Cells were centrifuged 800 × g for 3 minutes and resuspended in cold 0.075% sodium azide (Sigma-Aldrich), 0.5% BSA in PBS and blocked for 30 minutes with 2% BSA, followed by a 1 hour incubation at 4°C with anti-mouse Ly6G (BioXCell, West Lebanon, NH) conjugated to Alexa Fluor 488 (Protein Labeling Kit, Molecular Probes Inc., Eugene, OR), anti-mouse CD36-Alexa Fluor 647 (Biolegend, San Diego, CA), anti-mouse F4/80-PE (Abcam), or isotype controls. Where indicated, $Ly6G^{+}$ cells were isolated with an anti-Ly6G MicroBead Kit according to manufacturer's instructions (Miltenyi Biotec Inc.). Cells were washed with PBS prior to analysis by flow cytometry (Accuri C6, BD Biosciences, San Jose, CA). Data was further analyzed using FlowJo vX software (FlowJo LCC, Ashland, OR).

Bone marrow-derived neutrophils

Mouse femurs and tibias were collected and bone marrow recovered by flushing with warm Dulbecco's Modified Eagle's Medium (4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate) (ATCC) using a 26-gauge needle. Recovered cells were centrifuged at 800 × g for 3 min and Ly6G+ cells (neutrophils) isolated as described above. Neutrophils were incubated in LAC supernatant for 1 h at 37°C and 5% CO_2. Cell supernatant was collected for cytokine analysis or cells were lysed with RLT buffer (Qiagen) and RNA purified using an RNeasy Protect Mini Kit (Qiagen). Purified RNA was stored at -80°C until use for qPCR.

Peritoneal macrophages

Mouse peritoneal macrophages were collected by peritoneal lavage with 5 mL warm

cell strainer. Cells were centrifuged at 800 × g for 3 min then resuspended in Dulbecco's Modified Eagle's Medium with 10% FBS, 10mM Hepes, 2mM L-glutamine, 1% penicillin/streptomycin prior to plating and overnight incubation at 37°C in 5% CO_2. Cells were washed twice with Dulbecco's PBS to remove non-adherent cells. Macrophages were collected in Dulbecco's PBS via scraping and enumerated using the TC 20 Automated Cell Counter (Bio-Rad). Macrophages (1.0 × 10^4

were administered to LAC intoxicated mice by subcutaneous injection adjacent to the site of intoxication. For phagocytosis inhibition, adherent cells were incubated with 10 μM cytochalasin B or saline control for 1 hour at 37°C + 5% CO_2, and washed with Dulbecco's PBS before injection into mice.

Statistical analysis

Statistical analyses were performed using Prism 6.0 software (Graph Pad Software, Inc., La Jolla, CA). *In vitro* data were analyzed by Student's t test and *in vivo* results by the Mann-Whitney U test for non-parametric data.

Results

CD36 limits dermonecrosis during *S. aureus* SSSI

To determine whether CD36 contributes to limiting pathogenesis during *S. aureus* SSSI, we infected C57BL/6 (wild-type) and *CD36*$^{-/-}$ mice by subcutaneous injection with the CA-MRSA USA300 isolate LAC. Compared to wild-type mice, *CD36*$^{-/-}$ mice had significantly larger abscesses and increased dermonecrosis on days 1-7 post-infection (Fig. 3.1A). Importantly, at the apex of dermonecrosis on day three post-infection, there was no difference in local bacterial burden between *CD36*$^{-/-}$ and wild-type mice (Fig. 3.1B), suggesting that the dermonecrotic phenotype at this early time point was independent of bacterial burden. However, by day 7 post-infection, *CD36*$^{-/-}$ mice showed increased bacterial burden at the site of infection compared to wild-type mice, consistent with the previously demonstrated role of CD36 in *S. aureus* phagocytosis (Stuart et al., 2005). Therefore, these data suggest that CD36 plays a protective role against *S. aureus* dermonecrosis and, at early time points post-infection, this protection is independent of its role in bacterial clearance.

CD36 is a negative regulator of alpha-hemolysin mediated dermonecrosis

Hla is a secreted, pore-forming toxin which is a major contributor to dermonecrosis during *S. aureus* SSSI (Inoshima et al., 2012; Kennedy et al., 2010; Kobayashi et al., 2011b; Sampedro et al., 2014). To determine whether Hla is necessary for increased dermonecrosis in the absence of CD36, we infected wild-type and *CD36*$^{-/-}$ mice with a LAC isogenic *hla*-deletion mutant (LAC*Δhla*) (Bubeck Wardenburg et al., 2007). At the same inoculum used for LAC (8×10^6 CFU), mice infected with LAC*Δhla* failed to

develop dermonecrosis, consistent with previous reports (data not shown) (Berube et al., 2014; Kennedy et al., 2010). However, at a higher inoculum (1.1 x10^7 CFU), LACΔ*hla* infected *CD36*$^{-/-}$ mice showed small, but increased abscess formation and dermonecrosis on day three post-infection compared to wild-type mice (Fig. 3.1C, D). Notably, both measures were significantly reduced compared to *CD36*$^{-/-}$ mice infected with LAC, demonstrating that Hla is a major contributor to the dermonecrotic phenotype in the knockout mice. Furthermore, mice infected with a LAC isogenic *agr* deletion mutant (LACΔ*agr*) (1.1 x10^7 CFU) did not develop dermonecrosis (Cheung et al., 2011; Daly et al., 2015; Montgomery et al., 2010; Sully et al., 2014) (data not shown), supporting the role of other *agr*-regulated factors in dermonecrosis (Cheung et al., 2011; Montgomery et al., 2010; Wang et al., 2007b). Importantly, in contrast to infection with LAC, there was no significant difference in day seven bacterial burden in mice infected with LAC*Δhla* (1.1 x10^7 CFU) (Fig. 3.1E). This demonstrates that CD36 is important for host clearance of Hla-expressing *S. aureus* at later time points during SSSI. Together, these data demonstrate that while CD36 largely affords protection against Hla-mediated dermonecrosis, its protection can extend to dermonecrosis caused by other *agr*-regulated virulence factors.

To confirm that the role of CD36 in limiting Hla-mediated dermonecrosis at early time points is independent of its contribution to bacterial clearance and, based on the results above, also independent of any potential contribution to *agr* regulation (Peterson et al., 2008), we intoxicated mice by subcutaneous injection of sterile filtered supernatant from overnight cultures (Wright et al., 2005) of LAC or LAC*Δhla*.

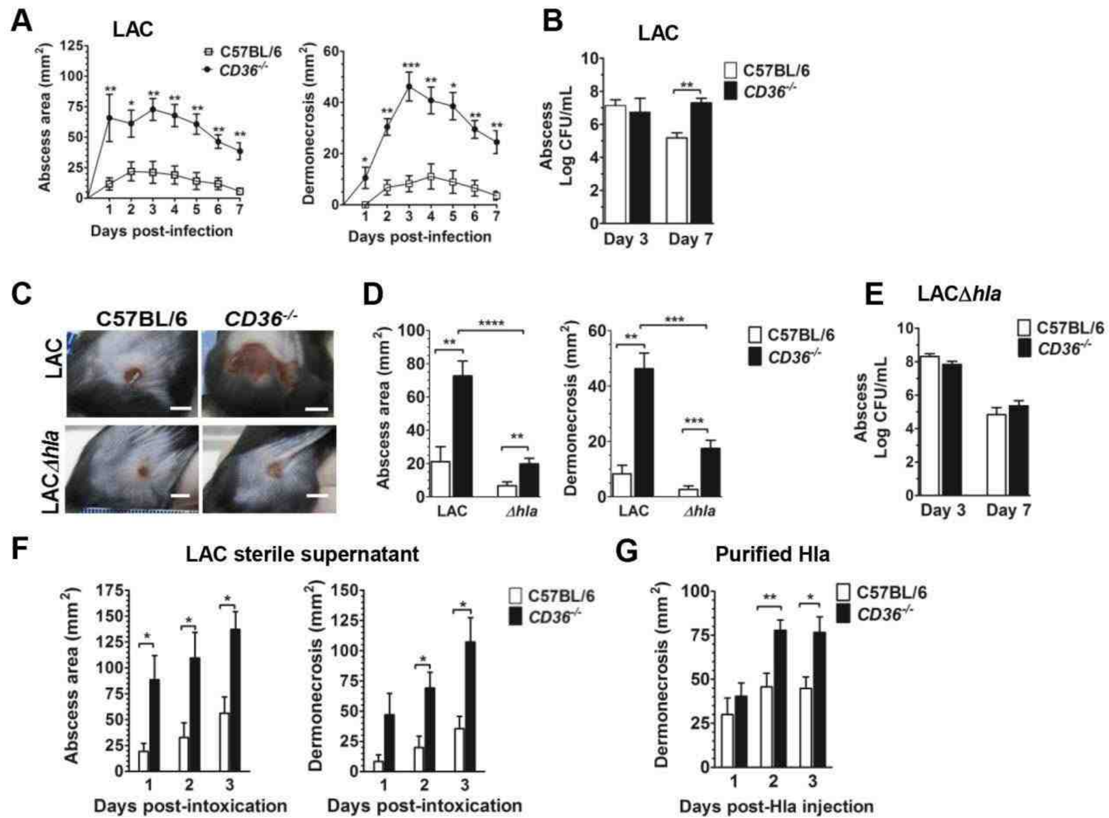
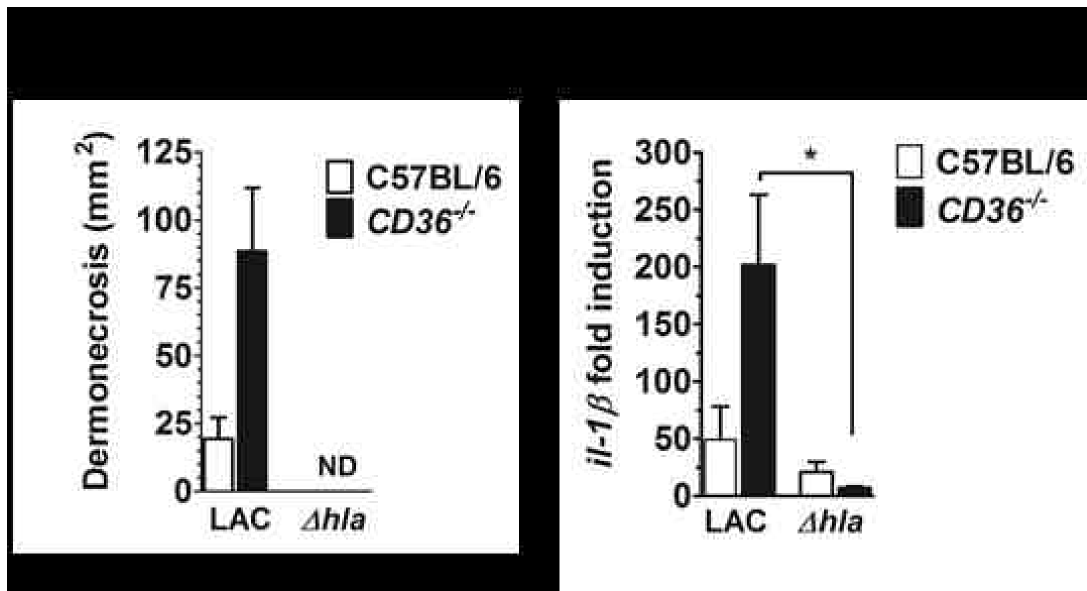


Figure 3.1 *CD36$^{-/-}$* mice have increased dermonecrosis in response to *S. aureus* infection and alpha hemolysin.

(A) Area of abscess (left), dermonecrosis (right) and (B) bacterial burden of mice subcutaneously infected with LAC (8 x 10^6 CFU). N=8 mice per group from two independent experiments. (C) Representative images of infection sites taken on day 3 post-infection (LAC, 8 x 10^6 CFU; LACΔ*hla* 1.1 x 10^7 CFU) (scale bar = 5 mm). (D) Abscess area and dermonecrosis on day 3 post-infection with LAC or LAC[illegible]*a*. N=8 (LAC) and 20 (LACΔ*hla*) mice per group from two and four independent experiments, respectively. (E) Bacterial burden at the site of infection 3 and 7 days post-infection with LACΔ*hla* (1.1 x 10^7 CFU). N=8 mice per group from two independent experiments. (F) Mice were intoxicated by subcutaneous injection with LAC sterile supernatant (18 hour culture). Area of abscess and dermonecrosis were measured daily. N=6 mice per group. (G) Area of dermonecrosis of mice injected subcutaneously with 1μg of purified Hla. N=8 mice per group from two independent experiments. Data shown as mean + SEM. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.

Whereas LAC*Δhla* intoxicated mice did not develop dermonecrosis (Supplemental Fig. 1A), *CD36*$^{-/-}$ mice intoxicated with LAC supernatant showed significantly increased abscess size and dermonecrosis (Fig. 3.1F), compared to wild-type controls. Furthermore, *CD36*$^{-/-}$ mice subcutaneously injected with purified recombinant Hla (Fig. 3.1G) also showed significantly increased dermonecrosis compared to wild-type mice, the timing of which was indistinguishable from abscess formation. As expected, mice injected with the purified inactive Hla_{H35L} mutant (Jursch et al., 1994) did not develop dermonecrosis (data not shown). Together, these results confirm that CD36 is a negative regulator of Hla-mediated dermonecrosis, independent of its role in phagocytosis and bacterial clearance.



Supplemental Figure 1. *CD36*$^{-/-}$ mice do not develop dermonecrosis in response to *S. aureus* lacking alpha hemolysin.

(A) Area of dermonecrosis on day 1 following subcutaneous injection of mice with sterile supernatant from the indicated strains. (B) Local *il-1β* transcription from experiments shown in A. *, $p<0.05$; ND, not detected.

CD36 limits inflammatory cytokine production associated with dermonecrosis

Given that Hla contributes to activation of the NLRP3 inflammasome and production of IL-1β (Bubeck Wardenburg and Schneewind, 2008; Craven et al., 2009; Munoz-Planillo et al., 2009), we predicted that the increased dermonecrosis in LAC infected *CD36*$^{-/-}$ mice would be associated with increased local levels of IL-1β compared to wild-type controls. As expected, *CD36*$^{-/-}$ mice infected with LAC, but not with LAC*Δhla*, showed significantly increased local pro- and active IL-1β on day 3 post-infection (Fig. 3.2A,B) compared to wild-type mice (~ increase 7.4-fold). In addition, both TNFα and CXCL1 were increased at the site of infection in LAC, but not LAC*Δhla*, infected *CD36*$^{-/-}$ mice compared to controls (Fig. 3.2C). These results suggest that CD36 regulates inflammatory cytokine expression in the skin of *S. aureus* infected mice in a Hla-dependent manner.

To confirm that CD36-mediated regulation of inflammatory cytokine production is also independent of bacterial burden, we measured cytokines in the skin of mice on days 1 and 3 following intoxication with LAC sterile supernatant. As expected, local IL-1β, TNFα and CXCL1 levels increased for both mouse groups between days 1 and 3 post-intoxication. LAC intoxicated *CD36*$^{-/-}$ mice showed significantly increased local IL-1β expression on day 3 post-intoxication compared to wild-type controls (~ increase 3.5-fold), while TNFα and CXCL1 levels trended higher in the knockout mice with differences in day 1 CXCL1 levels reaching statistical significance (Fig. 3.2D). Additionally, local transcription of these cytokines was higher in LAC intoxicated *CD36*$^{-/-}$ versus wild-type mice, with significant increases in *il-1β* and *cxcl1* transcription on day

3, and in *tnfα* transcription on day 1, post-intoxication (Fig. 3.2E). Importantly, consistent with the lack of differential IL-1β production in LACΔ*hla* infected mice (Fig. 3.2A), *CD36*$^{-/-}$ mice intoxicated with LACΔ*hla* supernatant showed significantly less local *il-1β* transcription on day 1 post-intoxication compared to *CD36*$^{-/-}$ mice intoxicated with LAC supernatant (Supplemental Fig. 1B). Together, these data demonstrate that, independent of bacterial burden, CD36 regulates IL-1β expression in the skin in response to Hla within the *S. aureus* virulence secretome.

Since local IL-1β levels were elevated in both LAC infected and supernatant intoxicated *CD36*$^{-/-}$ mice, we postulated that these mice would also show increased local transcription of components of the NLRP3 inflammasome, including *nlrp3*, *asc* and *caspase-1* (reviewed in (Sutterwala et al., 2014)). As predicted, *CD36*$^{-/-}$ mice subcutaneously intoxicated with LAC supernatant showed significantly increased local *nlrp3* transcription on day 3 post-intoxication compared to wild-type controls (Fig. 3.2F). Furthermore, while there was no difference in local transcript levels of *asc* or *capase-1* (data not shown), there was increased active caspase-1 (p10) in the skin of LAC intoxicated *CD36*$^{-/-}$ mice compared to controls (Fig. 3.2F), indicating increased local NLRP3 inflammasome activation in the absence of CD36.

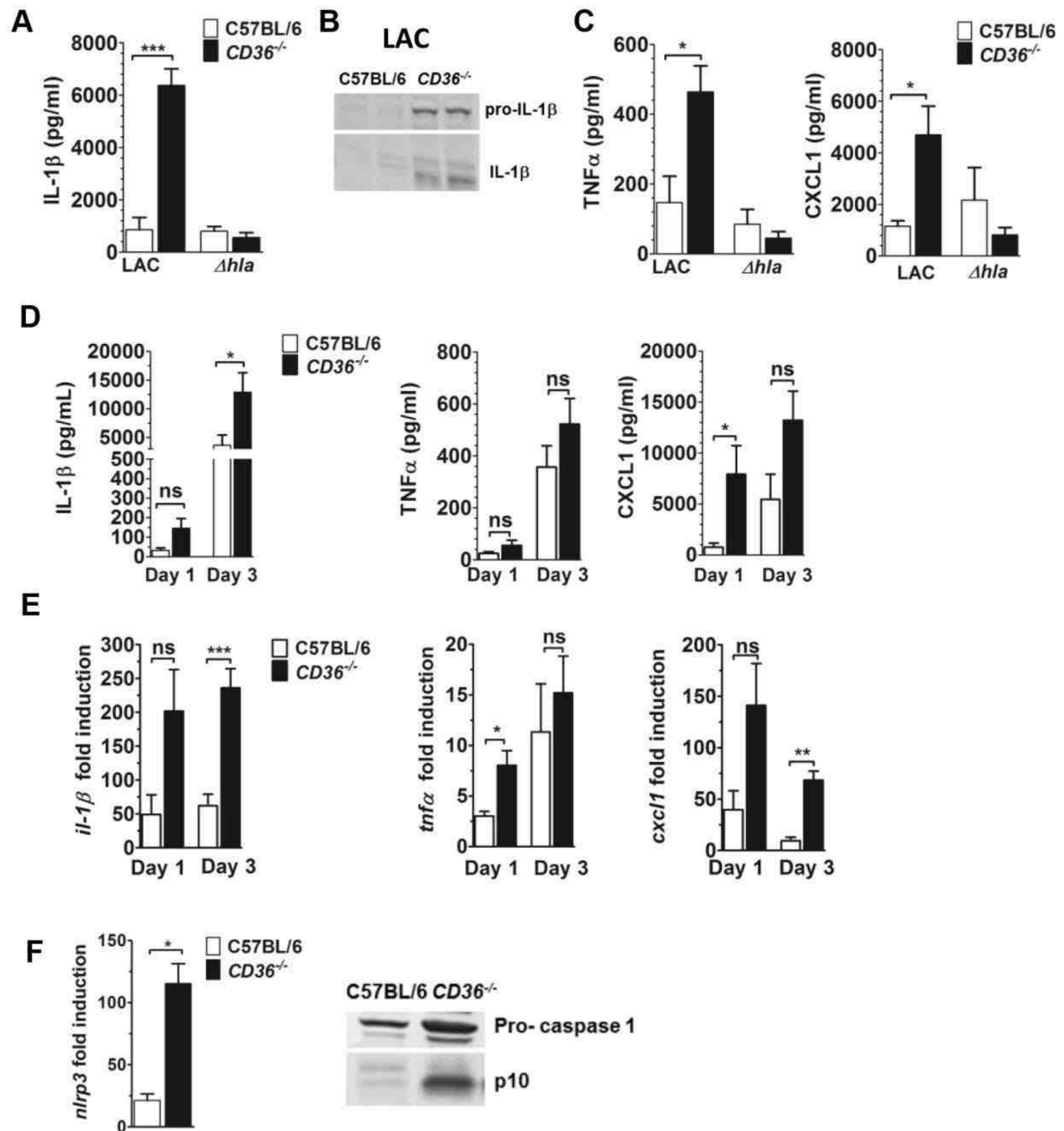


Figure 3.2 *CD36$^{-/-}$* mice have increased local pro-inflammatory cytokine production and NLRP3 inflammasome activation in response to *S. aureus* secreted virulence factors.

(A-C) Mice were subcutaneously infected with LAC (8 x 10^6 CFU) or LACΔ*hla* (1.1 x 10^7 CFU). The following were measured in clarified abscess tissue homogenate on day 3 post-infection: (A) IL-1β, (B) Western blot for pro- and active-IL-1β. (C) TNFα and CXCL1 expression. N=4-5 mice per group. (D-F) Mice were intoxicated by subcutaneous injection with LAC sterile supernatant (18 hour culture) and the following measured in injection site tissue homogenate on days 1 and 3 post-intoxication:(D) Cytokine expression and (E) *il-1b*, *tnfα* and *cxcl1* transcription. (F) *nlrp3* transcription and caspase-1 expression and activation (Western blot), in LAC intoxicated mice on day 3 post-intoxication. N=4-8 mice per group. Data reported as mean ± SEM. ns, not significant; *, p<0.05; **, p<0.01; ***, p<0.001.

CD36 regulates neutrophil accumulation driven by *S. aureus* secreted virulence factors

Neutrophil-derived IL-1β is sufficient for abscess formation during *S. aureus* SSSI, and neutrophils have been shown to produce IL- Cho et al., 2012b). Therefore, we predicted that the increased dermonecrosis and IL- in LAC supernatant-intoxicated $CD36^{-/-}$ mice would be associated with increased local neutrophil accumulation. To address this, we subcutaneously intoxicated mice with LAC supernatant and prepared single cell suspensions of injection site tissue on day 1 post-intoxication. As predicted, $CD36^{-/-}$ mice had significantly increased neutrophil (Ly6G^{+}) accumulation at the site of intoxication compared to wild-type mice (Fig. 3.3A,B). Furthermore, although the total number of cells present did not differ, $CD36^{-/-}$ mice had fewer macrophages (F4/80^{+}) at the site of intoxication, compared to controls (Fig. 3.3C,D). Importantly, to our knowledge, no deficiencies in macrophage levels in $CD36^{-/-}$ mice have been reported. Therefore, these data suggest a role for CD36 in regulating immune cell accumulation in the skin in response to *S. aureus* secreted virulence factors.

To begin to address the mechanism of increased dermonecrosis, immune cell accumulation and IL-1β production in the skin of LAC intoxicated $CD36^{-/-}$ mice, we first asked whether neutrophils from $CD36^{-/-}$ mice produced increased IL- Hla, compared to neutrophils from wild-type controls. Using flow cytometry, we were unable to detect CD36 on neutrophils isolated from wild-type mice (data not shown), suggesting that the presence or absence of this protein on neutrophils themselves did not contribute to the dermonecrotic phenotype in $CD36^{-/-}$ mice.

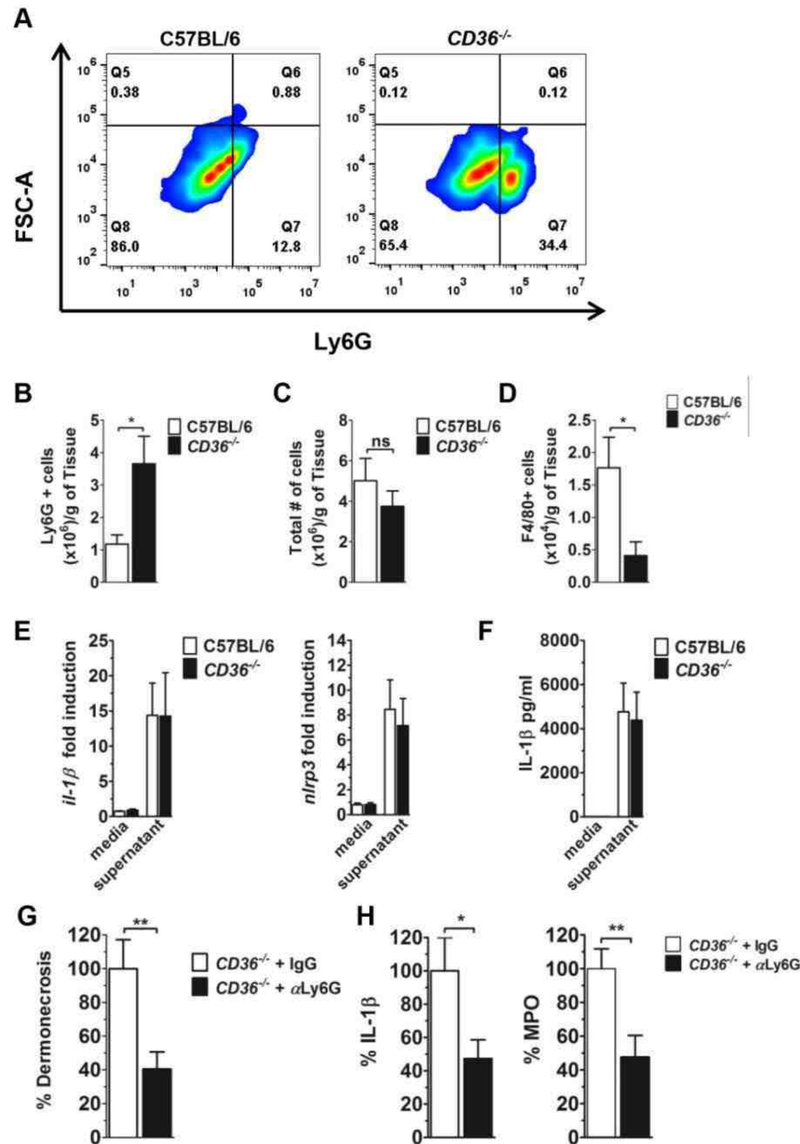


Figure 3.3. *CD36$^{-/-}$* mice have increased local neutrophil accumulation compared to wild-type mice following LAC supernatant injection.

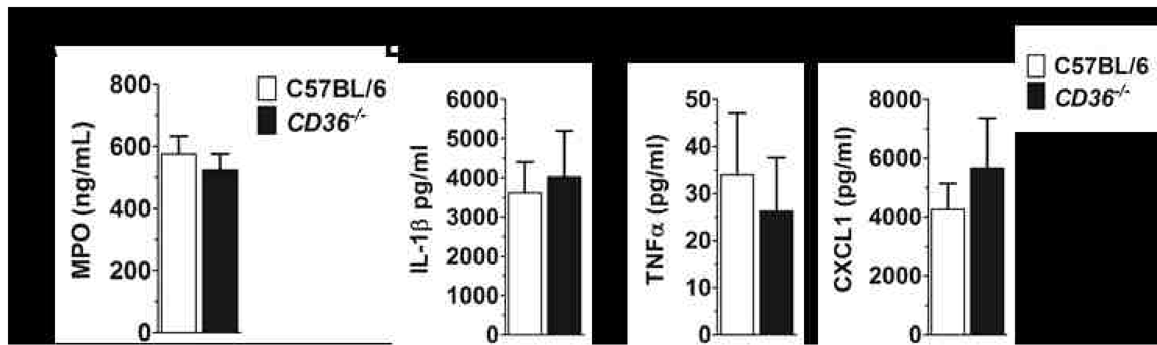
Flow cytometry analysis of single cells suspensions from injection site tissue on day 1 post-LAC intoxication of C57BL6 and *CD36$^{-/-}$* mice. (A,B) Presence of Ly6G+ cells, (C) total cell count and (D) F4/80+ cells at the site of intoxication. N=6 mice per group from two independent experiments. (E) *il-1b* and *nlrp3* transcription and (F) IL-1β expression by bone marrow derived neutrophils in response to 1 h incubation with LAC sterile supernatant or media control. N=6 from two independent experiments. (G) Day 1 dermonecrosis, (H) along with MPO and local IL-1β levels, from supernatant intoxicated *CD36$^{-/-}$* mice following treatment with anti-Ly6G or isotype control. Values are expressed as percent of isotype control. N=12-15 mice/group from four independent experiments. Data are mean ± SEM. ns, not significant; *, $p<0.05$; **, $p<0.01$.

In addition, bone marrow-derived neutrophils from wild-type and *CD36*$^{-/-}$ mice showed similar *il-1β* and *nlrp3* transcription, and IL-1β production, upon exposure to LAC supernatant (Fig. 3.3E,F). Therefore, these results point to a mechanism whereby CD36 negatively regulates dermonecrosis and IL-1β production in the skin by controlling neutrophil accumulation in response to *S. aureus* secreted virulence factors, rather than by directly affecting the neutrophil response to Hla.

If neutrophil accumulation at the site of *S. aureus* intoxication is responsible for the increased dermonecrosis in *CD36*$^{-/-}$ mice, then depletion of neutrophils prior to intoxication should prevent the enhanced dermonecrotic phenotype. As expected, on day 1 post-intoxication, *CD36*$^{-/-}$ mice treated with antibody targeting Ly6G to deplete neutrophils, showed significantly reduced dermonecrosis, local IL-1β production and local myeloperoxidase (MPO) levels, a surrogate for neutrophil accumulation (Cho et al., 2012b), compared to isotype treated controls (Fig. 3.3G). Together, these data demonstrate that CD36 protects against neutrophil-dependent skin injury induced by *S. aureus* secreted virulence factors.

We next asked whether increased dermonecrosis and neutrophil accumulation in *CD36*$^{-/-}$ mice resulted from increased neutrophil influx. Maximum neutrophil accumulation in the developing lesion has been reported to occur at six hours following *S. aureus* infection (Ford et al., 1989; Wright et al., 2005). Therefore, we assessed local MPO levels, as an indicator of neutrophil accumulation, along with IL-1β, TNFα and CXCL1 levels at six hours following LAC infection of wild-type and *CD36*$^{-/-}$ mice. At this time point, there was no significant difference in MPO, IL-1β, TNFα or CXCL1 expression at the site intoxication between the two mouse groups (Supplement Fig. 2),

suggesting that differences in neutrophil accumulation in the absence of CD36 do not result from increased neutrophil influx early post-infection.



Supplemental Figure 2. Local inflammation hours post intoxication.

Mice were infected with LAC (8 x 10^6 CFU) and tissues collected at six hours post-infection. (A) MPO, (B) IL-1β, TNFα and CXCL1 levels at the site of infection.

Macrophage CD36 is sufficient to limit dermonecrosis in *CD36*$^{-/-}$ mice

CD36 on macrophages facilitates clearance of apoptotic neutrophils, thus limiting tissue damage which follows loss of membrane integrity in dying cells and the subsequent release of noxious cell contents (Navazo et al., 1996; Ren et al., 1995; Savill et al., 1991; Savill et al., 1992; Weiss, 1989). If CD36 on macrophages is necessary to limit tissue damage caused by *S. aureus* secreted virulence factors, then administration of macrophages from wild-type (*CD36*$^{+/+}$) mice, but not from *CD36*$^{-/-}$ mice, should prevent the excessive dermonecrotic phenotype in *CD36*$^{-/-}$ mice following *S. aureus* intoxication. As expected, compared to untreated *CD36*$^{-/-}$ mice, local subcutaneous administration of wild-type macrophages to *CD36*$^{-/-}$ mice four hours post-intoxication was sufficient to significantly reduce day one dermonecrosis, whereas administration of macrophages from *CD36*$^{-/-}$ mice had no effect (Fig. 3.4A). Administration of *CD36*$^{+/+}$ macrophages to *CD36*$^{-/-}$ mice also reduced local IL-1β and myeloperoxidase (MPO) levels (Fig. 3.4B). Furthermore, administration of *CD36*$^{+/+}$, but not *CD36*$^{-/-}$, macrophages to wild-type mice prevented dermonecrosis, with further reductions in local IL-1β and MPO levels (Fig. 3.4A,B). Therefore, the ability to prevent excessive dermonecrosis in the *CD36*$^{-/-}$ mice by administration of *CD36*$^{+/+}$, but not the same number of *CD36*$^{-/-}$ macrophages, suggests that the protective mechanism of action is dependent on the presence of CD36 on the macrophages after reaching the site of inflammation.

To determine whether inhibition of dermonecrosis by administration of *CD36*$^{+/+}$ macrophages is associated with a local reduction in dying neutrophils, we used propidium iodide staining (Nygaard et al., 2012) to assess the loss of membrane integrity in neutrophils isolated from the site of intoxication. As expected, neutrophils isolated from

the site of intoxication of *CD36*$^{-/-}$ mice showed reduced propidium iodide (PI) staining following *CD36*$^{+/+}$ macrophage treatment compared to untreated controls (Fig 3.4C,D). Furthermore the reduction in PI uptake was reversed by pre-incubating macrophages with cytochalasin to inhibit phagocytosis, consistent with the previously described role of CD36 in macrophage phagocytosis and clearance of apoptotic neutrophils (Navazo et al., 1996; Ren et al., 1995; Savill et al., 1991; Savill et al., 1992). Together, these data demonstrate that CD36 expression on macrophages is necessary to limit skin injury associated with inappropriate neutrophil accumulation in response *S. aureus* secreted virulence factors, and suggest that the mechanism of action is via CD36-mediated phagocytosis and clearance of apoptotic neutrophils.

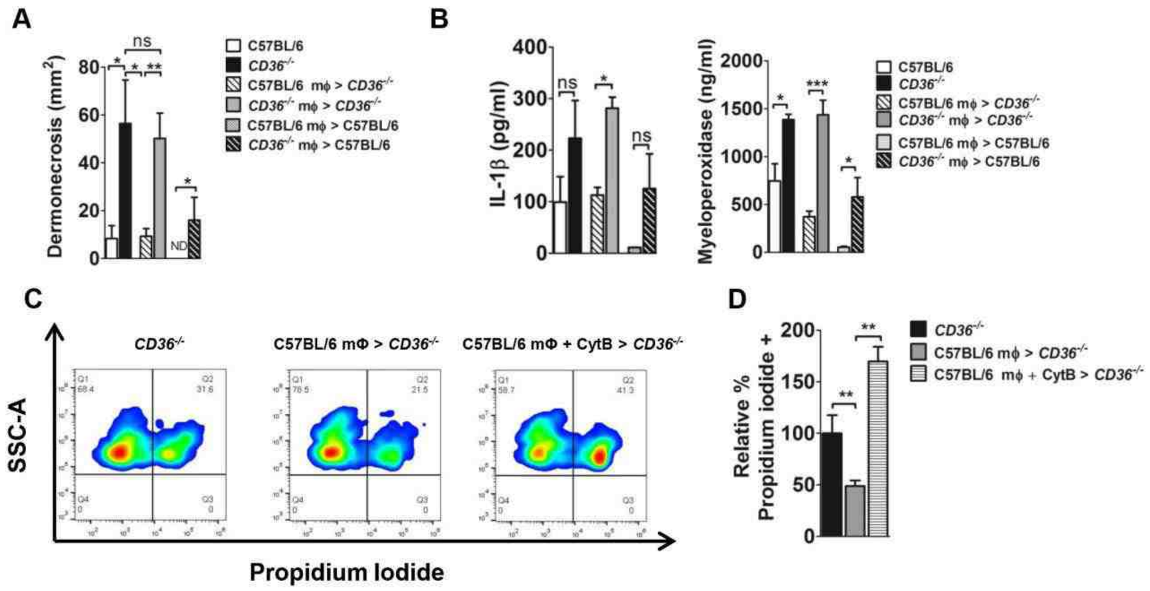


Figure 3.4 CD36$^{+/+}$ macrophages negatively regulate the dermonecrotic phenotype of LAC intoxicated *CD36*$^{-/-}$ mice.

Mice were intoxicated by subcutaneous injection with LAC sterile supernatant. 4 h later, mice were either left untreated or peritoneal macrophages were injected adjacent to the site of intoxication. (A) Area of dermonecrosis and local (B) IL-1β and MPO levels measured day 1 post-intoxication. Data reported as mean± SEM. N=4-6 mice per group from two independent experiments. (C,D) Membrane integrity of Ly6G+ cells from intoxication site skin sections measured by flow cytometry as percent propidium iodide uptake relative to *CD36*$^{-/-}$ controls. N=3-4 mice/group. ns, not significant; ND, not detected; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Discussion

Host innate defense against *S. aureus* skin infections is a highly orchestrated process aimed at clearing the infection and minimizing tissue damage. If left unchecked, however, the innate immune response to *S. aureus* infection, and to virulence factors such as Hla, can itself contribute to tissue injury. Here we report that CD36, a scavenger receptor that facilitates macrophage clearance of apoptotic neutrophils (Navazo et al., 1996; Ren et al., 1995; Savill et al., 1991; Savill et al., 1992), plays a major role in regulating Hla-mediated skin injury. In the absence of CD36, neutrophils recruited to the skin, in large part by Hla (Bartlett et al., 2008), can accumulate, become necrotic and ultimately cause tissue damage likely due to the release of their toxic cellular contents. Importantly, the administration of CD36-bearing macrophages is sufficient to limit dermonecrosis in mice lacking CD36, and to prevent dermonecrosis in wild-type mice. *CD36*$^{+/+}$ macrophage administration significantly reduces necrotic neutrophil accumulation at the site of intoxication, a benefit which is negated by obstructing macrophage actin polymerization. Therefore, these data support a mechanism of action whereby CD36-mediated macrophage phagocytosis of apoptotic neutrophils plays a significant role in host regulation of Hla-mediated dermonecrosis. Although further research is needed, these results may suggest that therapies targeted at local upregulation of CD36 on macrophages could prove efficacious in ameliorating *S. aureus*-mediated skin injury.

The formation of neutrophil-rich abscesses is considered a host defense mechanism for preventing bacterial dissemination and pathogenesis in response to *S. aureus* skin infection. However, this innate neutrophil response must be tightly controlled to limit

self-damage to host tissues. The potential damage resulting from unregulated neutrophil accumulation is suggested by the following: (i) neutrophils produce IL-1β to propagate their own recruitment (Cho et al., 2012b; Miller et al., 2006; Miller et al., 2007), (ii) they are short-lived and subject to lysis by *S. aureus*-secreted virulence factors (Alonzo and Torres, 2013; Rigby and DeLeo, 2012; Surewaard et al., 2013), and (iii) necrotic neutrophils release toxic substances, such as proteases and elastase (Henson and Johnston, 1987), as well as DAMPS (danger-associated molecular patterns) such as High-mobility group box 1 protein (HMGB1) (Raucci et al., 2007), which all contribute to tissue damage. Here we report that CD36 contributes to regulation of the host innate response to virulence factors secreted by *S. aureus* by limiting accumulation of dead or dying neutrophils during SSSI. It is important to note that, in addition to having more neutrophils at the site of inflammation compared to controls, *S. aureus* intoxicated *CD36*$^{-/-}$ mice also had fewer macrophages, suggesting a potential role for CD36 in macrophage accumulation in this model. However, local administration of *CD36*$^{+/+}$, but not an equivalent number of *CD36*$^{-/-}$ macrophages, prevented the enhanced dermonecrotic phenotype of *CD36*$^{-/-}$ mice. Therefore, the primary role of CD36 in regulating the host innate response in this model is in preventing excessive neutrophil accumulation once macrophages are at the site of infection or intoxication.

CD36 is a multifunctional protein with numerous and diverse ligands, co-receptors and functions (Park, 2014; Silverstein and Febbraio, 2009). Whether the downstream effects of CD36 signaling are pro- or anti-inflammatory depend on the nature of the ligand and the accompanying co-receptors. CD36 was first classified as a scavenger receptor and best studied for its role in sterile inflammation and atherosclerosis. In conjunction with

TLR4 and TLR6, CD36-mediated uptake of oxidized low density lipoprotein (oxLDL) results in the formation of intracellular cholesterol crystals, which drive activation of the NLRP3 inflammasome and secretion of the pro-inflammatory cytokine IL-1β (Sheedy et al., 2013; Stewart et al., 2010). This in turn leads to foam cell formation and atherosclerosis (reviewed in (Park, 2014)). CD36 also contributes to the host inflammatory response to Gram-positive pathogens. In this respect, CD36 mediates production of reactive oxygen species in response to *Propionibacterium acnes* (Grange et al., 2009), phagocytosis of *S. aureus* (Stuart et al., 2005), and, together with TLR2, recognition of *S. aureus* cell wall components (Hoebe et al., 2005a). Importantly, each of these functions results in a pro-inflammatory cytokine response (Grange et al., 2009; Hoebe et al., 2005a; Stuart et al., 2005). In contrast, CD36 suppresses early pro-inflammatory cytokine production by both macrophages and epithelial cells in response to lipotechoic acid-phosphocholine complexes from *Streptococcus pneumoniae* (Sharif et al., 2013). Our findings extend the known anti-inflammatory contributions of CD36 to protection against Hla-mediated *S. aureus* dermonecrosis, and suggest that therapeutic modulation of CD36 expression during skin infection could both enhance bacterial clearance (Stuart et al., 2005) and limit tissue damage caused by the host response to *S. aureus* virulence factors.

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Chapter 4 Sex bias during *Staphylococcus aureus skin infections*

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Abstract

Staphylococcus aureus is the primary cause of skin and soft tissue infections (SSTIs) in the USA. Between January 2013 and June 2015 at the University of New Mexico Hospital, almost twice as many males were diagnosed with *S. aureus* SSTI compared to females. Males and females differ in susceptibility to infection by various pathogens, with males frequently showing increased susceptibility and morbidity. However, the contributions of innate differences in the physiological response to infection, versus behavioral differences, to this bias remain largely unexplored. We hypothesized that estrogen mediates protection against *S. aureus* SSTI by increasing bacterial clearance leading to reduced pathogenesis. Here we show that male mice are more susceptible to *S. aureus* skin infection as evidenced by reduced bacterial clearance and increased inflammation compared to female mice. Furthermore, ovariectomized mice had reduced bacterial clearance compared to sham control surgery mice, further indicating the importance of estrogen in controlling *S. aureus* clearance. Importantly, estrogen treated macrophages were better able to phagocytize and kill *S. aureus*, and had reduced pro-inflammatory cytokine production, compared to vehicle-treated controls. Together, these data demonstrate that estrogen positively regulates host innate defense against *S. aureus* SSTI.

Introduction

Recognizing that sex is a major contributor to disease susceptibility, the National Institutes of Health (NIH) recently enacted a policy (NOT-OD-15-102) that NIH-funded basic biomedical research must be conducted in both male and female animals, and the resulting data stratified in order to expose sex differences in disease. However, sex has long been recognized as an important variable in susceptibility to infectious disease, and epidemiological studies show that males are at an increased risk of many bacterial infections compared to females (McClelland and Smith, 2011; Silva, 2011). However, it is largely unclear if increased infection rates in males are due to sex differences in behavior, such as handwashing (Johnson et al., 2003), or to innate physiological differences between the sexes, such as hormones, or a combination thereof (Klein, 2000).

Staphylococcus aureus is the dominant cause of skin and soft tissue infections (SSTI) presenting to emergency departments throughout the US (Moran et al., 2006; Talan et al., 2011). Although clinical reports indicate that males more often present with impetigo, caused by *S. aureus* (Kim et al., 2011), as well as methicillin-resistant *S. aureus* (MRSA) blood stream infections (Savill et al., 1989), compared to females, the potential for sex bias in *S. aureus* SSTI has not been directly assessed. However, related studies suggest that females would be innately protected. For example, in an artificial implant infection model, female rabbits showed increased survival following infection with *S. aureus* (Best et al., 1984), and the protective effect was lost with ovariectomy (Best et al., 1984). Additionally, estradiol as shown to reduce pathogenesis in a mouse model of *S. aureus* induced arthritis (Gjertsson et al., 2012), and alveolar macrophages from female versus male mice were shown to be better able to kill internalized *S. aureus* (Yang et al., 2014).

However, whether estrogen protects against *S. aureus* skin infection has not been investigated.

Here we utilized a murine model of *S. aureus* dermonecrosis to test the hypothesis that estrogen mediates protection against *S. aureus* SSTI by increasing bacterial clearance and reducing pathogenesis. As expected, female mice showed reduced dermonecrosis and increased bacterial clearance over the course of a seven day infection compared to male controls. The female protective advantage against *S. aureus* skin infection was observed in two strains of mice, C57BL/6 and BALB/c, suggesting that protection is independent of genetic background. Additionally, ovariectomized mice demonstrated a reduction in bacterial clearance compared to sham surgery control mice, indicating the importance of estrogen producing ovaries for bacterial clearance. Importantly, compared to vehicle treated control cells, treatment of a male mouse macrophage cell line (Raw 264.7 cells) with increasing concentrations of 17β-estradiol (E2) significantly increased both phagocytosis and intracellular killing of *S. aureus*. Therefore, these data suggest that estrogen positively regulates host innate defense against *S. aureus* SSTI

Materials and Methods

Bacterial growth conditions

The strain used for these studies, *S. aureus* USA300 LAC, was provided by Dr. Frank DeLeo (NIH/NIAID) and was grown in trypticase soy broth (TSB) at 37°C to early exponential phase (previously described) (Rothfork et al., 2004). Bacterial colony forming units (CFUs) were determined by plating serial dilutions on sheep blood agar (Becton Dickinson, Franklin Lakes, NJ).

Mouse model of S. aureus skin and soft tissue infection

The animal work in this study was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of New Mexico and was carried out at the AAALAC accredited Animal Research Facility of the University of New Mexico Health Sciences Center in strict accordance with recommendations in the Eighth Edition of *The Guide for the Care and Use of Laboratory Animals* and the USA Animal Welfare Act. C57BL/6 and BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice used in these studies were age and size matched (8-12 weeks, 22-28 g). The mouse model of *S. aureus* skin infection was performed as previously described (Malachowa et al., 2013). In brief, twenty-four hours before infection, mice were anesthetized by isoflurane and hair was removed from the right flank using Nair (Church

CFUs

of LAC was injected subcutaneously into the right flank of anesthetized mice. Mice were

weighed daily as a marker of systemic morbidity, and photographed for abscess and dermonecrosis area, which was measured using ImageJ analysis (Schneider et al., 2012).

Tissue collection

On day 3 or day 7 post-infection, mice were sacrificed by CO_2 asphyxiation. Skin abscesses (area = 2.25 cm^2) were collected for CFU determinations or cytokine measurements. To determine CFUs, abscesses were collected in bead-beating tubes containing 2.3 mm Zirconia/Silica beads (BioSpec Products Inc., OK) and 1 mL of $HBSS^-$ (Gibco, Grand Island, NY) with 0.2% Human serum albumin (Sigma, St. Louis, MO). Tissue was disrupted for 3 min in 1 min intervals using a Mini-Bead Beater-24 (BioSpec Products Inc., OK). Homogenates were diluted into PBS containing 0.1% Triton X-100 and sonicated. This was followed by plating serial dilutions on blood agar to enumerate CFUs. For cytokine analyses, abscess homogenates were clarified by centrifugation at 12,500 x g and supernatant was stored at -80°C until use.

Quantification of cytokines

Cytokines were measured in clarified supernatant from homogenized abscesses using custom designed multiplex assays (Millipore, Billerica, MA) according to the manufacturer's directions.

Macrophage phagocytosis and killing of S. aureus

Raw 264.7 cells were grown in Dulbecco's Modified Eagle's Medium with 10% FBS, 10mM Hepes, 2mM L-glutamine, 100U/mL penicillin and 100ug/mL streptomycin at 37°C in 5% CO_2. Twenty-four hours prior to the assay, cells were washed with PBS and

the medium replaced with DMEM with 10mM Hepes, 2mM L-glutamine, 100U/mL penicillin and 100ug/mL streptomycin and 10% Charcoal stripped FBS (to limit cell exposure to hormones) (JR Scientific, Woodland, CA). Prior to the assay, early exponential-phase LAC was cultured in 3 ml of TSB at $2x10^7$ CFU/ml with 50 nM exogenous AIP1 (Biopeptide Co., San Diego, CA) at 37°C while shaking for 5 hours. The bacteria were centrifuged, washed in PBS, sonicated, and suspended at $2x10^8$ CFUs/mL in DMEM with 10% Charcoal stripped FBS. The bacteria were opsonized overnight with rabbit anti- *S. aureus* IgG at 100 ug/ml (Accurate Chemical & Scientific Co., Westbury, NY) at 4°C on ice. On the day of the assay, RAW 264.7 cells were detached with Cell Stripper (Corning, Manassas, VA), and enumerated using the TC 20 Automated Cell Counter (Bio-rad). Cell were resuspended at $2x10^7$ cells/ml in DMEM with 10% Charcoal stripped FBS and incubated with 500nM 17-β Estradiol or 5µM 17-β Estradiol (Sigma-Aldrich, St. Louis, MO) or vehicle control for 2 hours at 37°C in 5% CO_2. The cells were then combined with opsonized bacteria at a multiplicity of infection (MOI) of 10. The cells and bacteria were centrifuged at 500 x g for 3 min to initiate contact, followed by incubation at 37°C in 5% CO_2 for 1 hour to allow for phagocytosis. At 1 hour, lysostaphin (Sigma-Aldrich) was added at 2ug/ml for 15 min to kill extracellular bacteria and then removed by centrifugation and replaced with fresh medium. Half of the samples were immediately processed for CFU determination to determine percent phagocytosis relative to initial bacteria concentration, and the other half were incubated for an additional 4 hours at 37°C in 5% CO_2 before CFU enumeration to determine percent killing. Bacteria were enumerated by dilution into PBS with 0.1% Triton X-100, followed by sonication and plating onto blood agar.

Statistical analysis

Statistical analyses were performed using Prism 6.0 software (Graph Pad Software, Inc., La Jolla, CA). *In vitro* data were analyzed by Student's t test and *in vivo* results by the Mann-Whitney U test for non-parametric data.

Results

Females are diagnosed with *S. aureus* skin infection at UNM Hospital less frequently than males

To determine if males have increased rates of *S. aureus* skin infections compared to females locally, we queried the UNM Clinical and Translation Science Center (CTSC) Informatics Integrating Biology and the Bedside (i2b2) Database. The database was searched for patients that were seen between 1/1/2013 and 6/30/15, and were of child-bearing age (18-44), and had a diagnosis of either MSSA or MRSA skin infection. Of 84 patients diagnosed with an MSSA skin infection, 60 (71.43%) were male and 24 (28.57%) were female (Fig. 4.1). Of 176 patients diagnosed with an MRSA skin infection, 106 (60.23%) were male and 70 (39.77%) were female (Fig. 4.1). Although this is a limited group of patients, this epidemiological data suggests that males are more likely to develop clinical *S. aureus* skin infections than females.

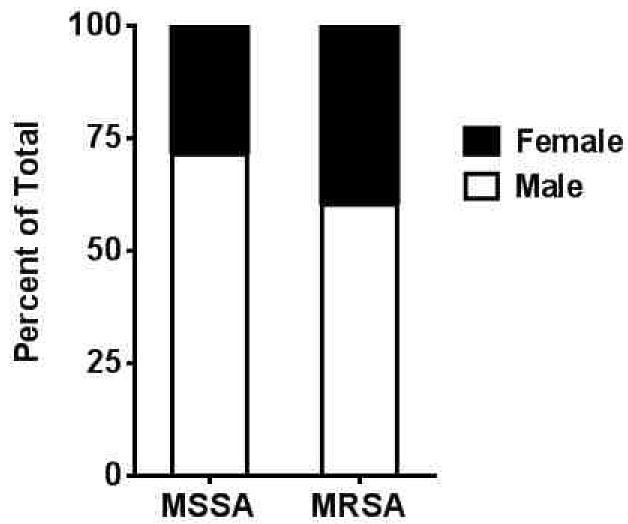

Figure 4.1 More males present to the UNM Hospital with MRSA or MSSA skin infections than females.
CTSC i2b2 Database was queried for patients that were seen between 1/1/2013 and 6/30/15, and were in the age range of 18-44, with a diagnosis of MSSA (N=84) or MRSA (N=176) skin infection. Data are shown as percent of total stratified by sex.

Female mice are less susceptible to *S. aureus* skin infections compared to males

To begin to address whether physiological factors contribute to the sex bias observed in human male susceptibility to *S. aureus* SSTI, we used a well-established mouse model of *S. aureus* skin infection (Malachowa et al., 2013). Specifically, we infected C57BL/6 male and female mice by subcutaneous injection with MRSA USA300 LAC. Compared to male mice, female mice had significantly smaller abscesses and reduced dermonecrosis on days 1-7 post-infection (Fig. 4.2A-E). At the apex of dermonecrosis on day 3 post-infection, there was a slight, but non-significant reduction in bacterial burden in female versus male mice (Fig. 4.2F), suggesting that differences in inflammatory response, rather than bacterial clearance, largely contributed to disease severity in males at this early time point. However, by day 7 post-infection, female mice showed significantly reduced bacterial burden at the site of infection compared to males. Therefore, these data demonstrate that, compared to males, C57BL/6 female mice are innately able to limit *S. aureus* SSTI.

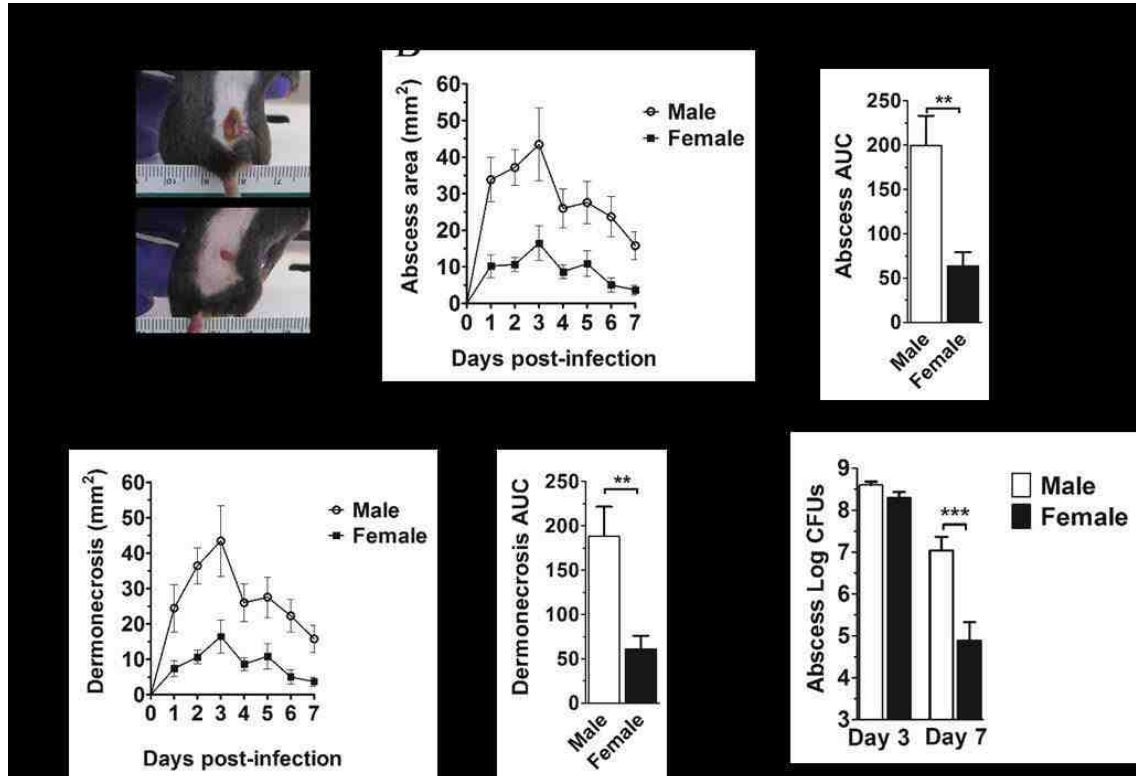

Figure 4.2 Male C57BL/6 mice have increased dermonecrosis and reduced bacterial clearance compared to female C57BL/6 mice during *S. aureus* infection.
C57BL/6 male and female mice were infected subcutaneously with *S. aureus* USA 300 LAC. (A) Representative images of the site of infection on day 3 post-infection. (B) Abscess area measured daily. (C) Quantification of area under the curve for abscess size over the course of infection. (D) Dermonecrosis measured daily. (E) Quantification of area under the curve for dermonecrosis formation over the course of infection. (F) Bacterial burden at the site of infection on days 3 and 7 post-infection. N=8 mice/group from two independent experiments. Data shown as mean $\pm$ SEM. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

To verify that female protection against *S. aureus* skin infections is not limited to a single murine genetic background, we repeated these studies in BALB/c male and female mice. Again, female mice had significantly smaller abscesses and reduced dermonecrosis on days 1-3 post-infection compared to males (Fig. 4.3A-C). Female BALB/c mice showed a significant reduction in local bacterial burden compared to males on day 3 post-infection (Fig. 4.3D). These data suggest that the sex bias in susceptibility to *S. aureus* skin infection is independent of murine genetic background.

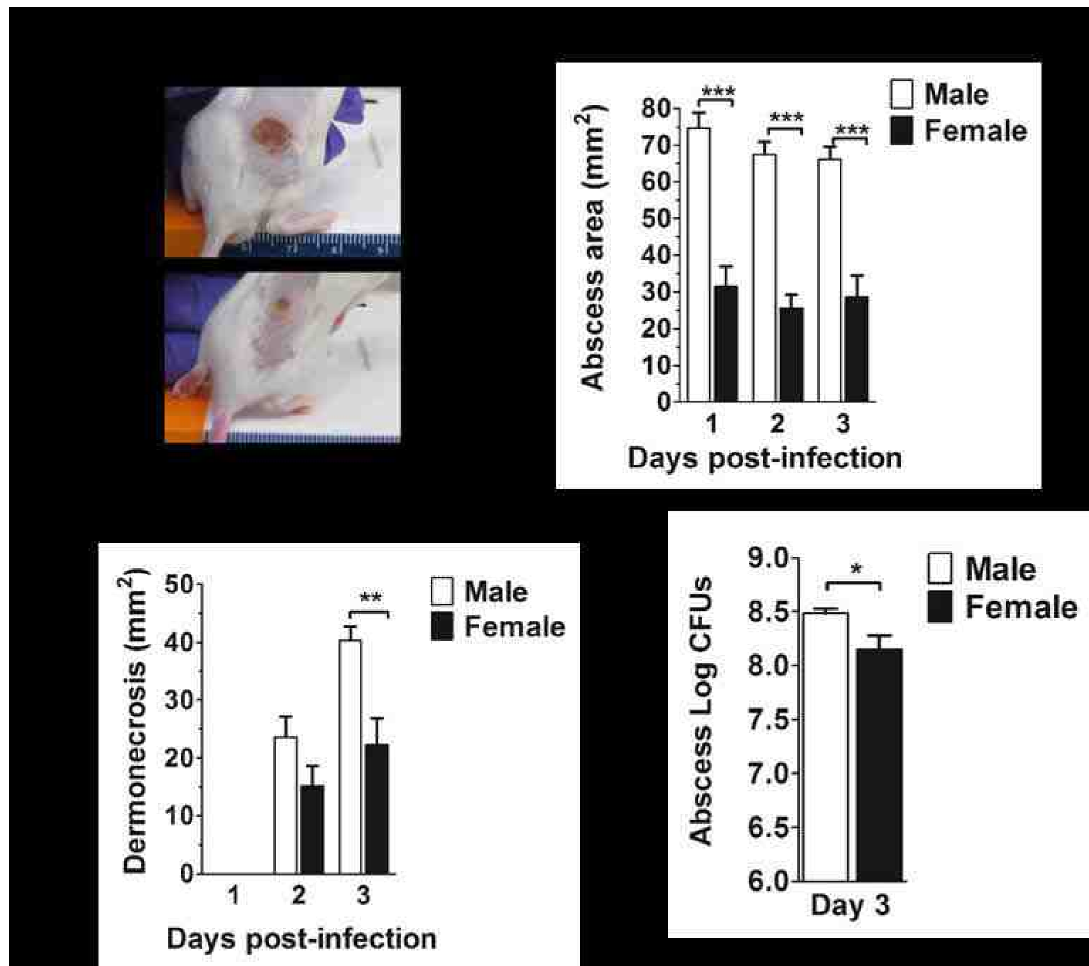

Figure 4.3 Male BALB/c mice have increased dermonecrosis and reduced bacterial clearance compared to female mice during *S. aureus* skin infection.

BALB/c male and female mice were infected subcutaneously with *S. aureus* USA 300 LAC. (A) Representative images of the site of infection on day 3 post-infection. (B) Abscess area and (C) dermonecrosis were measured daily. (D) Bacterial burden at the site of infection on day 3 post-infection. N=8 mice/group from two independent experiments. Data shown as mean $\pm$ SEM. *p< 0.05, ** p< 0.01, *** p< 0.001.

Female mice have reduced local inflammatory cytokine expression during *S. aureus* skin infection compared to male mice

Given that host innate immune cells produce local cytokines during *S. aureus* skin infection (Krishna and Miller, 2012b), we predicted that the reduced dermonecrosis in MRSA infected female mice would be associated with reduced local levels of inflammatory cytokines compared to male mice. As expected, although baseline cytokine levels in skin from uninfected mice did not differ between the groups (data not shown), female mice showed significantly decreased local production of IL-1β, IL-6, TNF-α and CXCL1 on day 3 post-infection compared to males in both C57BL/6 (Fig. 4.4A) and BALB/c mice (Fig. 4.4B). Together, these data support a mechanism whereby females may limit *S. aureus* SSTI, at least in part, through strict regulation of the skin inflammatory response.

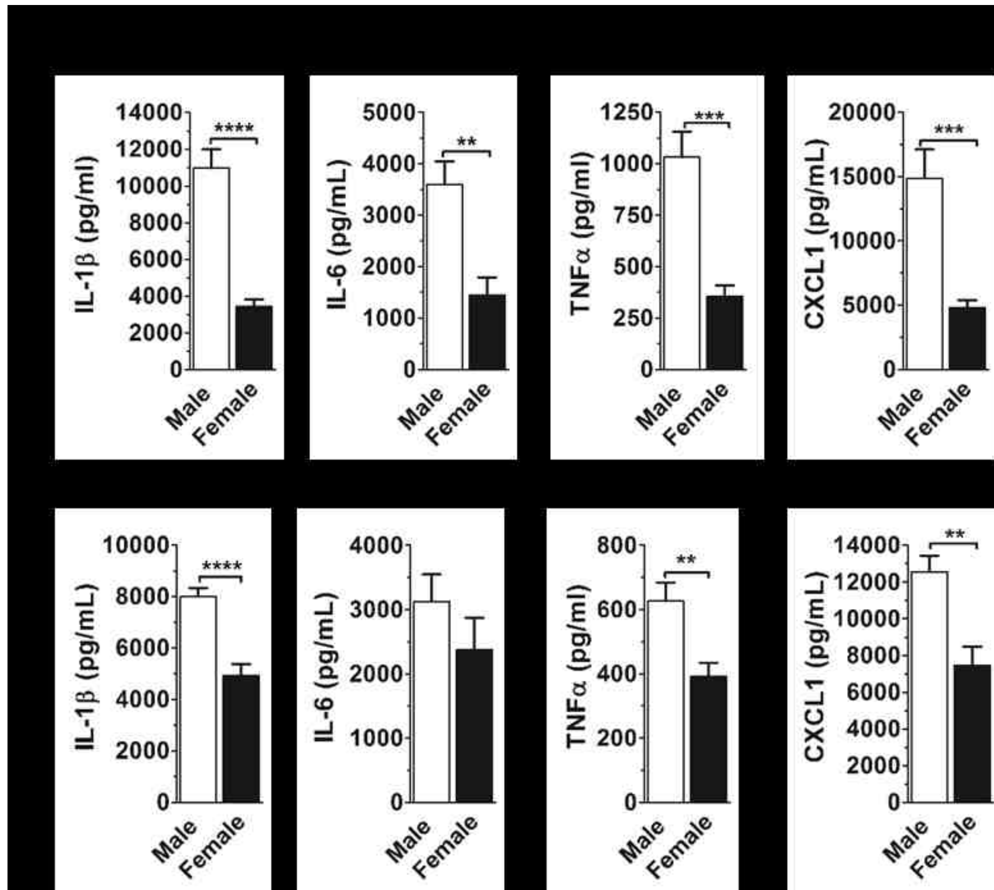

Figure 4.4 Male mice have increased local pro-inflammatory cytokine production during *S. aureus* skin infection compared to females.

(A) C57BL/6 male and female mice were infected subcutaneously with *S. aureus* USA 300 LAC. The following were measured in clarified abscess homogenate on day 3 post-infection: IL-1β, IL-6, TNF-α, CXCL1. (B) BALB/c male and female mice were infected subcutaneously with *S. aureus* USA 300 LAC. The following were measured in clarified abscess homogenate on day 3 post-infection: IL-1β, IL-6, TNF-α, CXCL1. N=8 mice/group from two independent experiments. Data shown as mean $\pm$ SEM. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Estrogen facilitates *S. aureus* clearance in vivo and in vitro

To more directly test whether estrogen mediates protection against *S. aureus* SSTI by increasing bacterial clearance, we infected ovariectomized and sham surgery control female mice by subcutaneous injection with LAC. Compared to sham surgery controls, ovariectomized mice had reduced clearance of bacteria at the site of infection (Fig. 4.5A-B), supporting a mechanism whereby estrogen is a positive regulator of host innate defense against *S. aureus* skin infection.

To ascertain whether estrogen treatment could enhance *S. aureus* clearance by innate immune cells, we treated Raw 264.7 murine macrophages with 17β-Estradiol (E2) or vehicle, followed by incubation with *S. aureus*. Compared to vehicle-treated controls, estradiol treated macrophages demonstrated increased phagocytosis and killing of *S. aureus* (Fig. 4.5C). Additionally, estradiol treated macrophages produced less IL-6, TNF-α, and CXCL1 compared to vehicle- treated cells (Fig. 4.5E-G), consistent with our *in vivo* observations (Fig. 4.4). To verify that enhanced bacterial killing with estradiol treatment was dependent on the macrophages, rather than possible antimicrobial effects of estradiol on *S. aureus*, we incubated *S. aureus* with estradiol and observed no direct killing (Fig. 4.5D). Therefore, these results support our hypothesis that estrogen mediates protection against *S. aureus* SSTI by increasing bacterial clearance and reducing pathogenesis.

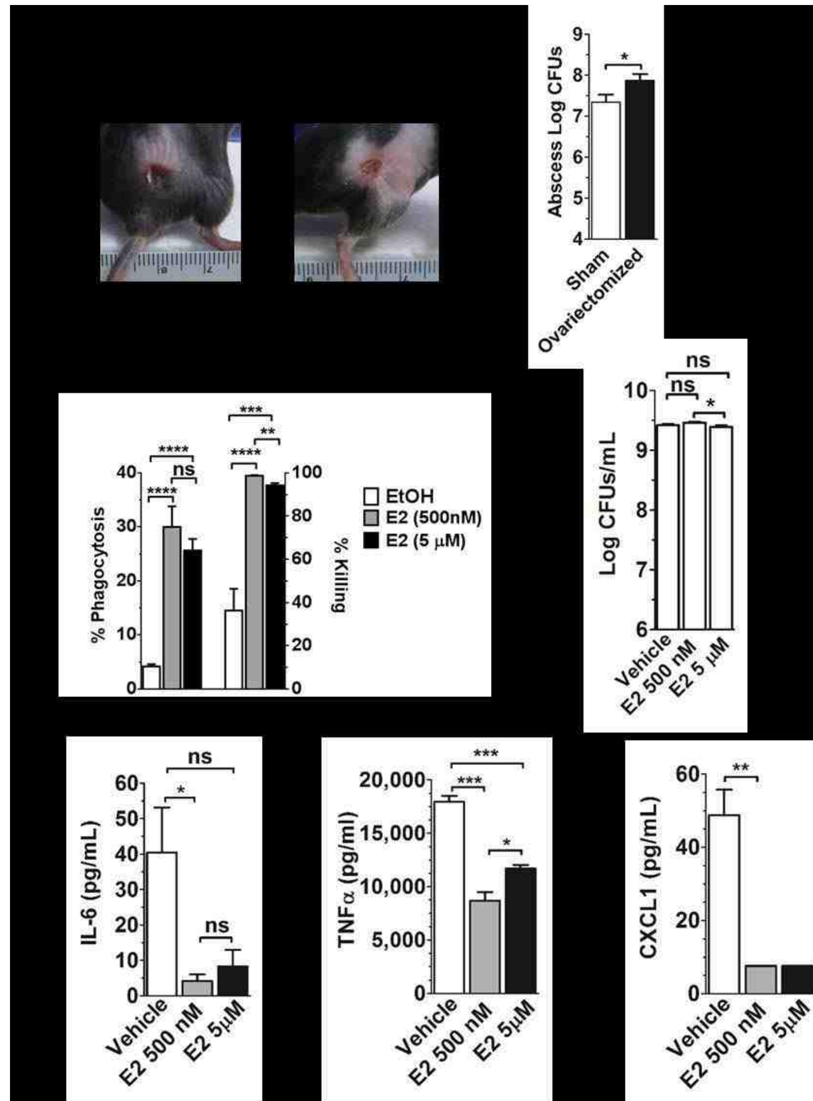

Figure 4.5 Ovariectomized mice have reduced bacterial clearance and 17β-Estradiol causes an increase in phagocytosis and killing of *S. aureus* by Raw 264.7 cells.
Ovariectomized and sham surgery control C57BL/6 female mice were infected subcutaneously with *S. aureus* USA 300 LAC. (A) Representative images of infection site, and (B) bacterial burden at the site of infection on day 3 post-infection. N=8 mice/group from two independent experiments. (C, E-G) Raw 264.7 macrophages were incubated for 2 hours with either vehicle control or 500nM E2 or 5μM E2, followed by incubation with opsonized *S. aureus.* (C) Percent of intracellular bacteria at 1 hour and 5 hours compared to starting CFUs. Percent of *S. aureus* killed at 5 hours, compared to bacteria phagocytosed by 1 hour. (D) CFUs of estradiol treated *S. aureus.* (E-G) Cytokines produced by Raw 264.7 cells measured at 5 hours post incubation with *S. aureus.* N=6 per group. Data shown as mean $\pm$ SEM. Ns, not significant; *p< 0.05, ** p< 0.01, *** p< 0.001.

Discussion

Host innate defense against *S. aureus* skin infections is a highly orchestrated process aimed at clearing the infection, thereby reducing pathogenesis. Here we report that estrogen plays a protective role against *S. aureus* skin infections. Our data demonstrate that female mice are less susceptible to *S. aureus* SSTI compared to male mice, as indicated by reduced abscess and dermonecrosis development, and female mice have increased bacterial clearance during infections compared to males. Raw 264.7 macrophages treated with estradiol are better able to phagocytose and kill *S. aureus* compared to vehicle control treated cells, supporting an estrogen dependent mechanism of host protection. Therefore, these data support a mechanism of action whereby estrogen promotes clearance of *S. aureus*. Furthermore, our data demonstrate that the epidemiological differences in *S. aureus* SSTI in males and females may be attributed, at least in part, to the innate physiological difference of the presence of the sex hormone estrogen.

Understanding the sex bias in susceptibility to *S. aureus* skin infections may aid in developing treatments to reduce pathogenesis and enhance bacterial clearance. Although further studies are needed, our data suggest that estrogen could have therapeutic potential to combat *S. aureus* skin infection. Determination of the specific targets for estrogen-mediated enhancement of the immune response to *S. aureus* could facilitate therapeutic development. At present, the role of the three estrogen receptors ERα, ERβ, and GPER in host innate defense against *S. aureus* undefined. However, many estrogen receptor specific synthetic agonists and antagonists are readily available, as well as, specific estrogen receptor knock out mice, to delineate relevant paths important for defense

against *S. aureus*. Activation of the appropriate estrogen receptor(s) could provide a therapeutic target to initialize the important anti-bacterial signaling pathways used in the innate immune response against *S. aureus* skin infection. Should estrogen be viable as a therapeutic against *S. aureus*, there could foreseeably be resistance to systemic administration of estrogen. In order to combat that, and prevent off-target effects, we propose that formulations of specific agonists against an estrogen receptor(s) targeted at the site of infection could prove efficacious in ameliorating *S. aureus*-mediated skin injury. Clinical utilization of these specific agonists via topical application may bolster the innate immune response against *S. aureus* skin infection.

The NIH officially recognized the importance of including sex as a potential variable in basic biomedical research, and released a policy stating that federally funded studies need to be conducted in cells or whole animal from both sexes. In support of this, our studies demonstrate that sex is a significant variable in susceptibility to *S. aureus* SSTI. The significance of the differing immune response between males and females to this clinically important pathogen suggests that limiting research to a single sex may bias research results and potentially negatively impact outcomes of clinical trials based on these studies. Therefore, future studies testing potential therapeutics or vaccines against *S. aureus* skin infections need to take into consideration the differing immune response between males and females in order to determine the best method of treatment.

Acknowledgments

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Chapter 5 Summary, Future Directions, and Conclusions

Summary

S. aureus is an important clinical pathogen as it is the dominant cause of skin and soft tissue infections (SSTIs) (Moran GJ and LK, 2006), which in the USA account for 11.6 million outpatient and emergency room visits, as well as ~500,000 hospital admissions per year (McCaig et al., 2006). The emergence of antibiotic resistant *S. aureus* over the last few decades and the widespread identification of methicillin-resistant *S. aureus* (MRSA) isolates highlight the dangers of infection with this bacterial pathogen. In a recent report, 76% of bacterial skin infections presenting to the emergency room were due to *S. aureus* infection, and of those, 78% were caused by MRSA (Moran GJ and LK, 2006). MRSA has been labeled a serious threat by the CDC due to its harmful impact on public health (CDC, 2013). MRSA infections are associated with increased mortality rates, longer hospitals stays and increased financial burden, compared to infections caused by methicillin-sensitive *S. aureus* (MSSA) (Lodise and McKinnon, 2007). Even more concerning, a significant proportion of MRSA infections are due to community-acquired strains (CA-MRSA), which can cause deadly disease in otherwise healthy individuals (Otto, 2013). From this epidemiological data, it is clear that *S. aureus* is a deadly pathogen responsible for a major burden on the health care system.

With the continued development of antibiotic resistance, and lack of a functional vaccine to prevent *S. aureus* infection, it becomes imperative to better understand the mechanisms of host defense against this pathogen in the hope of developing therapeutics to bolster host immunity. Furthermore, understanding how the protective innate immune response to *S. aureus* skin infection occurs could aid in development of prophylactic strategies to prevent infection. It is especially important to understand the mechanisms of

the host immune response to *S. aureus* for patient populations that are at risk for more serious forms of the disease or repeated infections, such as surgical patients, diabetics, and the immunocompromised.

In Chapter 3, we examined the role of the scavenger receptor CD36 in combating skin infections caused by *S. aureus*. Before these studies were conducted, it was unclear how the host limited inflammation during infection, because host detection of the *S. aureus* secreted pore-forming toxin alpha hemolysin (Hla) leads to an intense production of pro-inflammatory cytokines and the recruitment of the main responders, neutrophils (Kobayashi et al., 2015). If left unchecked or unregulated, the inflammatory response could cause collateral damage to healthy tissue due to neutrophil necrosis, which is the release of noxious components from azurophilic granules and DAMPs during neutrophil death, if the cells are not cleared by macrophages via phagocytosis (Savill et al., 1992). It was also not known if CD36 played a role in controlling the inflammatory response to *S. aureus.* Therefore, we postulated that the scavenger receptor CD36, which facilitates macrophage clearance of apoptotic neutrophils, would play a significant role in regulating local inflammation and dermonecrosis mediated by Hla. In Chapter 3, we identified a novel role for CD36 during *S. aureus* skin infection. We found that CD36 on macrophages is essential to regulation of the host innate immune response to *S. aureus* Hla-dependent invasive skin infection through its ability to recognize and direct macrophage phagocytosis of dying neutrophils. This work illuminates how CD36 functions to limit inflammation and clear dying neutrophils at the site of infection, preventing excessive Hla-mediated damage to surrounding healthy tissue during infection. Importantly, this innate effector role of CD36 is independent of its previously

reported role in bacterial phagocytosis (Hoebe et al., 2005b; Stuart et al., 2005). Taken together, the work in Chapter 3 significantly expands our understanding of the contribution of scavenger receptor CD36, best known for its role in atherosclerosis (Park, 2014; Silverstein, 2009), to host innate defense against *S. aureus* skin infection, specifically through regulation of the host inflammatory response. Furthermore, our work suggests that the role of CD36 in regulation of the skin inflammatory response may extend to other skin infections and dermatological conditions which cause dermonecrosis.

In Chapter 4, we examined sex bias during skin infection with *S. aureus*. Clinical reports indicate that males more often present with impetigo, a rash-like contagious infection (Kim et al., 2011), and blood stream infections caused by MRSA (Savill et al., 1989) compared to females. We queried the UNM CTSC database and found that between January 2013 and June 2015 at the University of New Mexico Hospital, almost twice as many males were diagnosed with *S. aureus* skin infections compared to females. However, the potential for sex bias in *S. aureus* skin infection has not been directly assessed. It is unknown if males presented with skin infections more often than females due to behavioral differences, such as occupation and hygiene (Collins and O'Connell, 2012; Johnson et al., 2003), innate physiological differences, such as sex hormones controlling signaling pathways, or a combination of the two components. Therefore, we postulated that estrogen, (Bernin and Lotter, 2014; McClelland and Smith, 2011), mediates protection against *S. aureus* skin infections by increasing bacterial clearance and reducing pathogenesis. We showed for the first time that female mice have innate resistance to *S. aureus* skin infection, therefore suggesting that physiological differences are a major contribution to sex bias susceptibility to *S. aureus*. Furthermore, we found

that estrogen enhanced macrophage phagocytosis and clearance of *S. aureus*, providing a potential mechanism by which estrogen limits pathogenesis during skin infection. Importantly, the studies in Chapter 4 support a biological basis for reduced clinical presentation of *S. aureus* skin infection in females compared to males, and may have clinical implications for treatment based on sex. This work further suggests that estrogen may be protective against other bacterial pathogens, and highlights the importance of considering sex bias in pre-clinical studies, a variable that is often overlooked in biomedical research.

Future Directions

Although in Chapter 3 we determined that CD36 is essential for regulation of the host innate immune response to *S. aureus* dermonecrosis mediated by Hla, and in Chapter 4 that sex bias during *S. aureus* skin infections is due, at least in part, to the functions of estrogen, many questions still remain from these studies. For the work completed in Chapter 3, the gaps in knowledge that still remain to be addressed include i) whether upregulation of CD36 using PPARγ agonists has therapeutic potential, ii) whether reduced CD36 expression in chronic granulomatous disease patients increases susceptibility to *S. aureus*, iii) whether soluble CD36 has a role in diabetic patients susceptibility to infection, and iv) whether administration of peptides from commensals that signal through CD36 can provide therapeutic benefit. A summary of these gaps in knowledge is depicted in Figure 5.1. For the work completed in Chapter 4, the gaps in knowledge that still remain to be addressed include i) which estrogen receptors contribute to sex bias during skin infection, ii) whether targeting specific estrogen receptors has therapeutic potential, and ii) whether estrogen is protective against other pathogens causing skin disease. A summary of these gaps in knowledge is depicted in Figure 5.2. The rationales behind these gaps and approaches to addressing them are discussed below.

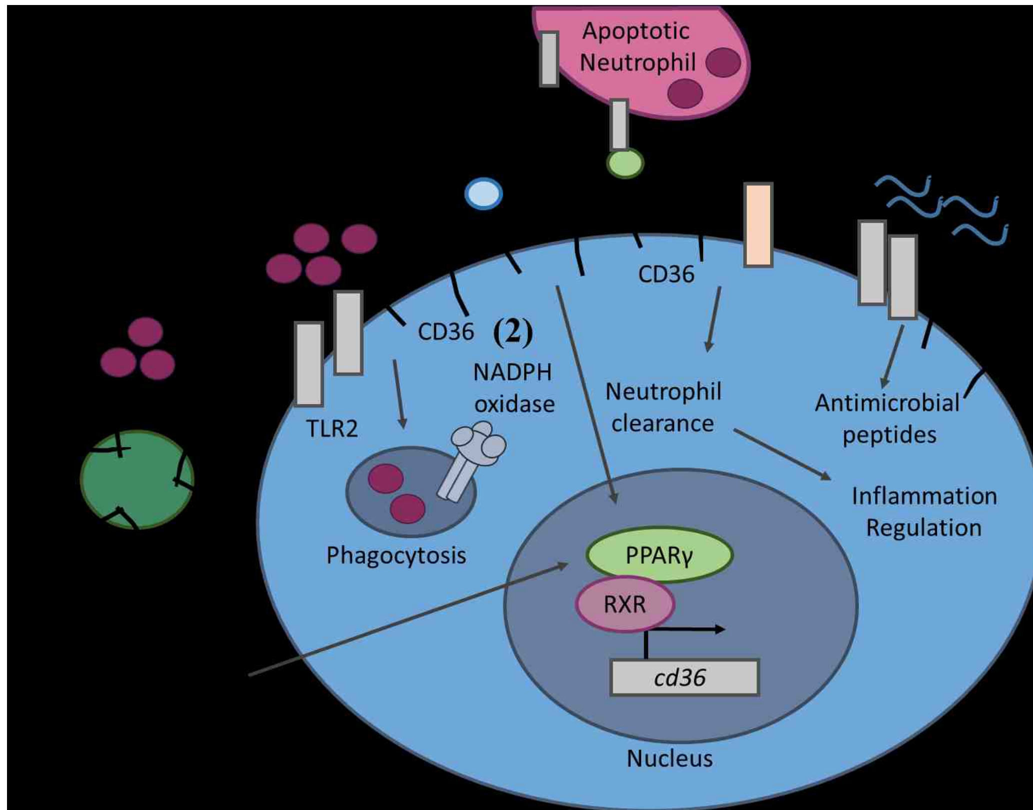

Figure 5.1 Multi-functional roles of CD36.
Future directions to investigate are enumerated. (1) Does upregulation of CD36 using PPARγ agonists have therapeutic potential in treatment of *S. aureus* skin infection? Expression of CD36 is regulated by binding of oxidized LDL or agonists for peroxisome proliferating activated receptor (PPARγ), which acts as a transcription factor binding to retinoid X receptor (RXR). (2) Will increased macrophage CD36 expression in CGD patients provide protection against *S. aureus* SSTI by facilitating clearance of bacteria and dying neutrophils? ROS is produced by the NADPH oxidase complex, which is impaired in CGD patients. (3) Does soluble CD36 have a role in diabetic patient susceptibility to infection by competitive binding to *S. aureus* ligands? Cellular CD36 functions as a co-receptor with TLR2 to recognize lipoteichoic acid or peptidoglycan leading to phagocytosis of *S. aureus.* (4) Will therapeutic administration of peptides from commensals (LP01) that signal through CD36 lead to upregulation of antimicrobial peptides such as β–defensins and provide therapeutic benefit against *S. aureus* SSTI? CD36 along with TLR2 recognizes LP01, produced by *S. epidermidis*, leading to upregulation of antimicrobial peptides such as β–defensins.

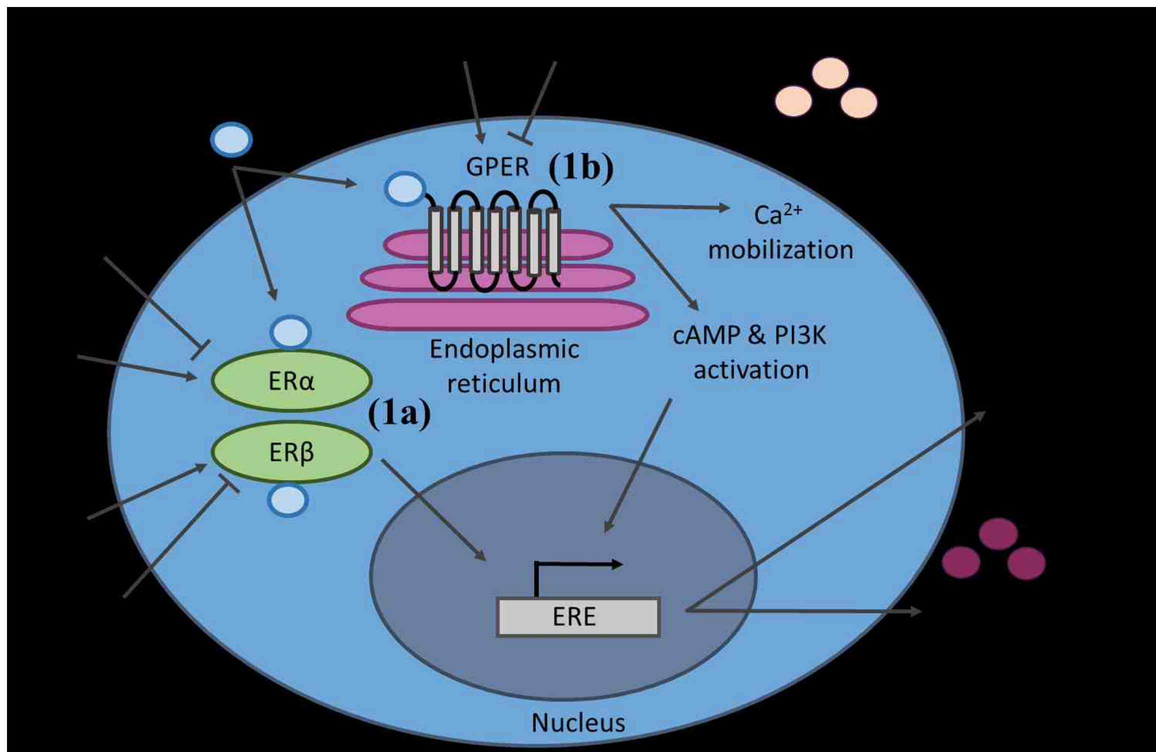

Figure 5.2 Role of estrogen in host defense.
Future directions to investigate are enumerated. (1) Which estrogen receptors (ERα, ERβ, GPER) contribute to sex bias during skin infection? Estrogen leads to cell signaling when it binds to any of the three estrogen receptors ERα, ERβ, and GPR30 (Kovats, 2015). Estrogen receptors ERα and ERβ (1a) can form heterodimers or homodimers during ligand binding (Vrtacnik et al., 2014), which can then directly trigger gene expression by binding to the Estrogen Response Element (ERE) found in the promoter of various genes or by functioning as scaffolds for other transcription factors to bind promoter regions of DNA and trigger gene expression. GPER (1b) is found in the endoplasmic reticulum (Prossnitz et al., 2008), and estrogen binding leads to calcium mobilization, activation of cAMP and signaling cascades through PI3K pathways (Revankar et al., 2005). (2) Does targeting specific estrogen receptors with agonists or antagonists have therapeutic potential? PPT and MPD are agonists and antagonists, respectively for ERα. DPN and PHTPP are agonists and antagonists, respectively, of ERβ. G1 and G36 are agonists and antagonists, respectively, of GPER. (3) Is estrogen protective against other skin pathogens such as *S. pyogenes*?

Part 1: Utilization of PPARγ agonists as therapeutics for *S. aureus* skin infections

The studies conducted in Chapter 3 suggest that, due to its multi-functional role in combatting *S. aureus* skin infections, upregulation of CD36 on macrophages is a potential therapeutic approach for protection against *S. aureus* skin infections. Due to its roles in bacterial phagocytosis (Baranova et al., 2008; Stuart et al., 2005) and in regulation of the innate immune response (Castleman et al., 2015), increased CD36 expression during skin infection could enhance bacterial clearance and limit tissue damage caused by the host response to virulence factors. Expression of CD36 is regulated by binding of the peroxisome proliferating activated receptor (PPARγ) transcription factor to the retinoid X receptor (RXR), which subsequently binds the PPAR response element in the promoter region of CD36 to activate gene expression (Hirano et al., 2003). PPARγ agonists have commonly been used in biomedical research to increase the expression of CD36 on various cells including macrophages *in vitro* and *in vivo* (Nicholson, 2004). For example, PPARγ agonists are known to limit inflammation in the central nervous system and attenuate microglial activation (Kielian and Drew, 2003). For this reason, PPARγ agonists have been used to treat *S. aureus* brain abscess development in mice by reducing activation of microglial cells thereby reducing inflammation and enhancing bacterial clearance (Kielian et al., 2008). However their utility in combatting the most common form of *S. aureus* infection, in the skin, has not been addressed.

To find clinical evidence that PPARγ agonists are protective *S. aureus* skin infections, we will use a national database to investigate the treatment of Type II Diabetes mellitus patients with the PPARγ agonist, pioglitazone. Diabetic patients are extremely susceptible to infection with *S. aureus* as it is present in up to 70% of diabetic

infections in the U.S. (Eleftheriadou et al. 2010). Type II diabetic patients are prescribed medications that increase insulin production by the pancreas, increase sensitivity of cells to insulin or decrease glucose absorption in the gastrointestinal track all in order to lower the levels of glucose in the blood (American Diabetes, 2009). Drugs commonly prescribed are in the classes of sensitizers (biguanides, thiazolidinediones), secretagogues (sulfonylureas), and glucosidase inhibitors. Thiazolidinediones (TZDs), which function to increase cell sensitivity to insulin, are agonists of PPARγ and one FDA approved drug pioglitazone is currently prescribed to patients (Gillies and Dunn, 2000). We hypothesize that diabetic patients treated with pioglitazone will display overall reduced incidence of *S. aureus* skin infection compared to patients treated with other diabetic drugs. To investigate this hypothesis we will obtain national estimates of drugs prescribed to diabetic patients in order to determine the percent of patients taking pioglitazone compared to other drugs. We will then query a national database containing diabetic patient records of *S. aureus* skin infection. The data collected would need to be stratified in order to eliminate comorbidities such as non-maintenance of diabetes or cardiovascular disease. We will use this data to determine the incidence of diabetic patients that present to the hospital with *S. aureus* skin infections and what drugs they are prescribed. If diabetic patients who are prescribed drugs other than piolglitazone present more often with a *S. aureus* skin infection, then that suggests that the PPARγ agonist piolglitazone is protective against infection and provides evidence that investigation of PPARγ agonists in combating *S. aureus* represents a viable research direction.

We hypothesize that using a PPARγ agonist to upregulate CD36 expression will lead to increased clearance of apoptotic neutrophils. First we need to determine if

increased CD36 expression on a *CD36*$^{+/+}$ macrophage increases neutrophil clearance. A preliminary assay to test this would be to overexpress CD36 in a macrophage cell line by transfection of a plasmid containing the CD36 gene sequence or control plasmid. We would verify increased CD36 expression using flow cytometry or western blot. We would use a co-culture method to incubate macrophages with apoptotic neutrophils. We expect that the cells overexpressing CD36 will be better able to clear the apoptotic neutrophils compared to cells transfected with the control plasmid or *CD36*$^{-/-}$ cells. We would follow this assay with another *in vitro* assay by treating macrophages with a PPARγ agonist or vehicle control before incubation with apoptotic neutrophils. The expected results for this assay are that the macrophages treated with the PPARγ agonist will have increased CD36 expression and increased phagocytosis of neutrophils control cells. These results would suggest that a PPARγ agonist could be protective in a murine model of *S. aureus* skin infection through the known role of CD36 clearance of apoptotic neutrophils (Castleman et al., 2015).

To test the hypothesis that a PPARγ agonist to upregulate CD36 expression will be protective in a mouse model of *S. aureus* skin infection, we would infect mice subcutaneously with *S. aureus,* then administer a PPARγ agonist therapeutically. However with the long term goal of patient treatment in mind, careful considerations of the utility of using PPARγ agonists as a treatment need to be taken into account. CD36 has mainly been studied for its contribution atherosclerosis (Park, 2014); it is a scavenger receptor for oxLDL and uptake of too much oxLDL leads to foam cell formation, which leads to development of atherosclerotic plaques (Febbraio et al., 2000). Despite CD36 having a demonstrated protective role against *S. aureus* infection, systemic enhancement

of CD36 expression could be detrimental to the cardiovascular system. For this reason, we propose that to modulate CD36 function against *S. aureus* skin infection, its expression would need to be enhanced locally at the site of infection rather than systemically to prevent undesirable side effects. Therefore, we recommend treatment with a PPARγ agonist by injection near the site of infection or within a topical cream formulated to trigger increased CD36 expression at the site of infection, to reduce or prevent off target effects such as increased foam cell formation if treatment is given systemically. In the *S. aureus* brain abscess study mentioned above (Kielian et al., 2008), mice were treated intraperitoneal with 10 or 50 mg/kg/day of pioglitazone (FDA App. No. NDA- 021073), an FDA approved PPARγ agonist used to treat Type II Diabetes, these concentrations limited inflammation without adverse effects such as extreme weight loss and would serve as a guide for our initial studies. It would be clinically relevant to determine if Type II diabetes patients prescribed pioglitazone present less often with *S. aureus* skin infection. We would also need to investigate a dosing strategy for local or topical administration of the PPARγ agonist to determine the following parameters; optimal dose, frequency of dosing, and time until therapeutic effect is observed. For these studies, we expect that mice given the PPARγ agonist will show reduced dermonecrosis and decreased inflammation in response to the *S. aureus* infection compared to vehicle control treated mice, due to the functions of CD36 identified by ourselves and others (Castleman et al., 2015; Savill et al., 1992; Stuart et al., 2005). These expected results would indicate that PPARγ agonists may have clinical utility in treatment against *S. aureus* skin infection via modulation of CD36 expression and enhancement of the innate immune response.

The increase in antibiotic resistant bacterial infections highlights the need for therapeutic strategies that reduce the need for antibiotics or extend the life of current antibiotics by reducing the dosage and frequency of use in the clinic. Should the use of PPARγ agonists provide therapeutic protection against *S. aureus* skin infection, then we propose an additional study to test the function of a PPARγ agonist as an adjunct to antibiotics commonly used to treat MRSA skin infections (Stevens et al., 2014). We hypothesize that therapeutic administration of a PPARγ agonist in conjunction with an anti-staphylococcal antibiotic at suboptimal concentrations will be as protective in a mouse model of *S. aureus* skin infection as treatment with antibiotic alone at standard concentrations. We expect that mice treated with the combination therapy will demonstrate similarly reduced dermonecrosis and increased bacterial clearance as mice given standard concentration monotherapy compared to no treatment. These results would suggest that PPARγ agonists can enhance the lifetime of antibiotics currently in use against *S. aureus*.

Part 2: Utilization of PPARγ agonists as a therapeutic for chronic granulomatous disease patients during *S. aureus* skin infections

Chronic granulomatous disease patients (CGD) are highly susceptible to *S. aureus* skin infection. CGD is caused by genetic mutations in the genes coding for the subunits of the NADPH oxidase, an enzymatic complex that generates ROS for oxidative killing of bacterial pathogens and activation of antimicrobial proteins within the phagolysosome (Holland, 2010; Segal et al., 2000). Patients with CGD are deficient in ROS production and display exaggerated inflammation thought to result, at least in part, from accumulation of apoptotic neutrophils (Fernandez-Boyanapalli et al., 2010). Using *gp91*$^{phox-/-}$ mice as a murine model of CGD, it was shown that these mice have reduced expression of CD36 and have reduced clearance of apoptotic neutrophils, however treatment with a PPARγ agonist reversed these phenotypes (Fernandez-Boyanapalli et al., 2010). Furthermore, treatment of *gp91*$^{phox-/-}$ mice with a PPARγ agonist lead to enhanced ROS production and significantly increased bacterial clearance compared to control treated mice in a model of *S. aureus* peritonitis (Fernandez-Boyanapalli et al., 2015). These studies suggest that upregulation of CD36 with a PPARγ agonist may provide benefit to CGD patients against *S. aureus* skin infection since they are predisposed to these infections. We hypothesize that *gp91*$^{phox-/-}$ mice treated with a PPARγ agonist would have increased protection against *S. aureus* skin infection compared to untreated mice. To test this we would infect *gp91*$^{phox-/-}$ mice subcutaneously with *S. aureus,* then locally and therapeutically administer a PPARγ agonist. We expect that *gp91*$^{phox-/-}$ mice given a PPARγ agonist would show reduced dermonecrosis, decreased inflammation and increased bacterial clearance in response to the *S. aureus* infection compared to vehicle

control treated mice, due to the increased expression of CD36. These expected results would suggest that modulation of local CD36 expression via PPARγ agonists may have clinical utility in treatment of *S. aureus* skin infections in CGD patients.

Part 3: Examination of diabetic patients' susceptibility to *S. aureus* infection

Diabetics are another patient population that is extremely susceptible to infection with *S. aureus*. The prevalence of Type II Diabetes mellitus is increasing with 1.7 million new cases each year and currently it affects 9.3% of the U.S population (American Diabetes, 2013). *S. aureus* is the most common pathogen affecting diabetic patients as it is present in up to 70% of diabetic infections in the U.S. and almost half of those are further attributed to infection with MRSA (Eleftheriadou et al. 2010). Patients with diabetes may have an up to 4-fold increase in circulating CD36, found on microvesicles in the blood, compared to non-diabetic controls (Alkhatatbeh et al., 2013). High levels of circulating CD36 in the form of microvesicles, historically referred to as soluble CD36 (sCD36) (Alkhatatbeh et al., 2011), is associated with increased risk of Type II diabetes and insulin resistance (Handberg et al., 2010). However, whether elevated levels of sCD36 impacts host innate defense against bacterial infection remains to be investigated.

We hypothesize that sCD36 will antagonize host defense against *S. aureus,* unlike its cellular form, because it lacks the ability to stimulate intracellular signaling to trigger bacterial phagocytosis and clearance of apoptotic neutrophils. To first determine if sCD36 recognizes and binds *S. aureus*, we would isolate microvesicles containing CD36 from the plasma of diabetic patients, incubate the microvesicles with a lipid intercalating dye, then add them to an ELISA plate coated with *S. aureus*. Binding of the microvesicles to *S. aureus* could be quantified on a plate reader set to the appropriate wavelength. We expect that sCD36 will still be able to bind *S. aureus* as it contains the

same extracellular ligand binding sites as the cellular form. To determine if sCD36 antagonizes defense against *S. aureus*, we would isolate microvesicles containing sCD36 from the plasma of mice and incubate the microvesicles in increasing concentrations with mouse macrophages and *S. aureus*. As a control, we would use microvesicles from *CD36*$^{-/-}$ mice. We would measure phagocytosis of *S. aureus* and production of cytokines by the macrophages. We expect that macrophages in the presence of increasing concentrations of *CD36*$^{+/+}$ microvesicles will phagocytose fewer bacteria compared to controls and have reduced cytokine expression. These results would suggest that sCD36 is a non-functional scavenger for LTA and PGN from *S. aureus,* thereby competing for binding (Stewart and Nagarajan, 2006), and preventing cellular CD36 from binding to the appropriate ligands needed for initiation of host defense. These expected results could provide some explanation for the enhanced susceptibility of diabetic patients to *S. aureus* skin infection. These studies could then be repeated using microvesicles purified from the plasma of diabetic patients.

Part 4: Utilization of LP01 as a therapeutic for *S. aureus* skin infections

In addition to the roles described above, CD36 has indirect roles in host defense against infection. For example, it was demonstrated that the commensal *S. epidermis* secretes a lipopeptide called LP01, which activates TLR2 and CD36 on keratinocytes leading to an increase in the production of antimicrobial peptides, including β-defensins (hBD2 and hBD3) (Li et al., 2013). β-defensins have been shown to directly cause *S. aureus* bacterial cell membrane lysis (Li et al., 2013; Ryu et al., 2014). Prophylactic treatment of mice with 2mg/kg of LP01 before *S. aureus* SSTI, reduced bacterial burden by increasing expression of mouse β-defensin 4 (Li et al., 2013). However, it is unknown if administration of LP01 could be used therapeutically treat *S. aureus* skin infection. We hypothesize that therapeutic administration of LP01 to *S. aureus* infected mice would aid in combating infection. We expect that mice given LP01 will produce more antimicrobial peptides, have reduced dermonecrosis, and enhanced bacterial clearance in response to skin infection. These expected results would indicate that therapeutic administration of LP01 enhances the natural defense mechanism of keratinocytes against *S. aureus* and that products from other commensals or peptide mimetics could be utilized to treat *S. aureus* skin infection. Furthermore, these results would suggest that previously unknown ligands of CD36 may be useful in combating *S. aureus* skin infections via enhancement of the host innate immune response, and that activation of CD36 with the appropriate ligand could provide therapeutic benefit.

Part 5: Investigation of intracellular signaling pathways important for CD36 protection against *S. aureus* skin infections

Macrophage identification of apoptotic neutrophils occurs through recognition of "eat me" signals, such as expression of phosphatidylserine on the surface of the neutrophils (Silva, 2011). This process is important to protection against tissue injury caused by neutrophils undergoing a form of apoptosis called necrosis in which they release toxic materials (Savill et al., 1989). Recognition of apoptotic neutrophils occurs through the formation of a thrombospondin bridge, which binds both the macrophage and neutrophil (Savill et al., 1992). On the macrophage, CD36 is the receptor for thrombospondin and works in conjunction with integrin $\alpha_v\beta_3$ to mediate clearance of apoptotic neutrophils (Savill et al., 1992). The receptor for thrombospondin on the apoptotic neutrophil has not yet been identified. Binding of the ligand oxLDL to CD36 leads to intracellular activation of Src family tyrosine kinases and serine/threonine kinases resulting in actin polymerization (Silverstein and Febbraio, 2009), however the intracellular pathways activated during thrombospondin binding have not been investigated to our knowledge.

We hypothesize that binding of thrombospondin to CD36 leads to activation of the Src family kinases which mediates actin polymerization for phagocytosis of apoptotic neutrophils. To investigate this we will use a co-culture method with healthy macrophages along with neutrophils induced to an apoptotic state. We will treat the macrophages with pharmacological Src family kinase inhibitor or vehicle control before co-incubation with neutrophils. We expect that the macrophages treated with the inhibitor will have reduced neutrophil uptake and reduced actin polymerization. This

assay will indicate that the macrophages treated with Src family kinases inhibitors will be unable to signal intracellularly and that these kinases are important for macrophage mediated of clearance of apoptotic neutrophils.

It is thought that the C-terminal domain of CD36 is integral for intracellular signaling activation upon ligand binding, as single point mutations of specific tyrosine or cytosine residues leads to CD36 unresponsiveness to ligand binding (Stuart et al., 2005). However, the functional role of the intracellular C-terminal domain of CC36 in intracellular signaling for clearance of apoptotic neutrophils has not been investigated. To test that the C-terminal domain of CD36 is important for this process, we would repeat the co-culture assay described above using macrophages that express CD36 containing point mutations in specific tyrosinse or cysteines in the C-terminal domain. We expect that macrophages with mutations in CD36 will have reduced clearance of apoptotic neutrophils. This data would suggest that the tyrosine or cytosine residues of the C-terminus of CD36 are integral for intracellular signaling and phagocytosis.

Part 6: Identification of the estrogen receptor(s) that mediate *S. aureus* clearance during infection

The studies in Chapter 4 suggest that estrogen may be a viable therapeutic for the treatment of *S. aureus* skin infection. Therefore it is important to determine which, or the combination of which, of the estrogen receptors, ERα/ERβ/GPER, mediates this anti-bacterial immune response. To test the impact of each estrogen receptor on defense against *S. aureus* we would compare the infection susceptibility of wild type versus estrogen receptor mutant mice (αERKO, βERKO, and GPERKO). We hypothesize that one or more of the estrogen receptor knockout mice will be more susceptible to *S. aureus* skin infection because of the studies conducted in Chapter 4 indicating that estrogen signaling is important for bacterial clearance. Due to the complexity of estrogen signaling during inflammation we expect that more than one murine knockout strain will demonstrate increased dermonecrosis and bacterial burden in response to *S. aureus* skin infection. These expected results will identify which of the estrogen receptors are important for intracellular signaling mechanisms to aid in bacterial clearance because in their absence there is a reduction in bacterial clearance.

Part 7: Utilization of estrogen receptor agonists as therapeutics for *S. aureus* skin infections

We would use the knowledge gained from the studies above with the estrogen receptor knockout mice to guide subsequent studies. To determine if activation of specific estrogen receptors identified above with synthetic agonists has therapeutic potential against *S. aureus* skin infection, we would infect C57BL/6 mice subcutaneously with *S. aureus* then, at various time points post-infection, treat with agonists for a specific receptor, then measure disease outcome. In this study specific attention should be paid to the method of agonist administration; it would be ideal to administer the agonist locally at the site of infection via injection or cream formulation in order to prevent unnecessary off target effects of other estrogen signaling pathways. Another important consideration for these studies is that the estrogen receptors may trigger opposing signaling pathways. In this instance, it may be necessary to combine an agonist of one receptor with an antagonist of another receptor to trigger beneficial anti-bacterial signaling and prevent conflicting signaling. We expect that mice treated with an agonist or combination of agonists for the specific receptors will display reduced dermonecrosis, enhanced bacterial clearance, and overall increased protection against *S. aureus* skin infection. These expected results will support a novel therapeutic strategy to bolster the innate immune response against *S. aureus* skin infections.

Part 8: Examination of intracellular signaling mechanism for estrogen protection against *S. aureus* skin infections

Investigation of the signaling pathways triggered by the estrogen receptors that lead to increased bacterial clearance is an important step in basic biomedical research. The estrogen receptors ERα and ERβ form heterodimers or homodimers during ligand binding (Vrtacnik et al., 2014). These receptor can directly trigger gene expression by binding to the Estrogen Response Element (ERE) found in the promoter of various genes or by functioning as scaffolds for other transcription factors to bind promoter regions of DNA and trigger gene expression. To narrow the focus and identify specific genes activated for host defense by the estrogen receptors we will use RNA sequencing on skin samples from male and female mice infected with *S. aureus* and control uninfected mice to establish baseline expression. We hypothesize that these estrogen response pathways trigger gene expression important for increased production of reactive oxygen species or antimicrobial peptides to kill phagocytosed bacteria. Genes that are upregulated or down regulated in the female infected skin samples compared to the male samples could indicate a contribution to host defense. These genes would be further investigated using *both in vitro* and *in vivo* studies to verify their contribution to sex-bias in *S. aureus* infection.

Part 9: Determination of the role of estrogen in innate host defense against other bacterial skin infections

Although, *S. aureus* is the main contributor to skin infections, it is not the only pathogen of clinical importance. *Streptococcus pyogenes* also contributes to the clinical burden of infectious skin disease (Fiedler et al., 2015; Walker et al., 2014). Despite the studies conducted in Chapter 4, in which a sex bias in susceptibility to *S. aureus* SSTI was defined, it is still unknown if a sex bias exists in susceptibility to other skin infecting pathogens such as *S. pyogenes.* We hypothesize that female mice will be resistant to other skin disease causing bacterial pathogens because of the manner in which estrogen enhances macrophage clearance of bacteria. To test this we would infect C57BL/6 male and female mice subcutaneously with *S. pyogenes* and measure disease outcome. We expect that female mice will demonstrate reduced dermonecrosis and increased bacterial clearance compared to male mice. These expected results would indicate that estrogen provides natural protection against skin disease caused by multiple pathogens.

Conclusions

Despite significant scientific effort, no useful vaccine has shown clinical potential against *S. aureus* infection. Furthermore, the ability of *S. aureus* to become resistant to antibiotics highlights the need for the development of novel therapeutic approaches. The long term goal of our studies investigating the host innate immune response to *S. aureus* is to better understand how the host fights infection. At the same time, we hope to identify approaches to promote host defense against infection. The relevance of the studies we conducted in Chapter 3 to understanding how the host innate immune system regulates the inflammatory response to infection is key to infection management. Any therapeutics aimed at bacterial clearance need to avoid inducing an exacerbated inflammatory response as it will damage surrounding tissue, while at the same time supporting anti-inflammatory responses integral for wound resolution and tissue healing. Our studies also underscore the importance of investigating receptors thought to have fully defined functions for their contribution to host defense against pathogens. The relevance of our studies in Chapter 4 to clinical treatment of *S. aureus* skin infections cannot be overstated. The significance of the differing immune response between males and females has long been overlooked and our data indicates that it may dictate the effectiveness of clinical therapeutics. Future studies testing potential therapeutics against *S. aureus* skin infections need to take into consideration the differing immune response between males and females in order to determine the best method of treatment.

Appendix A: List of Abbreviations

ADAM-10: a disintegrin and metalloprotease 10

AIP: auto-inducing peptide

Agr: accessory gene regulator

CA-MRSA: community acquired Methicillin-resistant *Staphylococcus aureus*

CD36: cluster of differentiation 36

ClfA: clumping factor A

DAMPS: danger-associated molecular patterns

E2: 17-β estradiol

ERα: estrogen receptor α

ERβ: estrogen receptor β

ERE: Estrogen Response Element

FnbpA: fibronectin-binding protein A

GPER: G-coupled protein estrogen receptor

GPR30: G-coupled protein receptor 30

hBD2: human β defensin 2

Hla: alpha hemolysin or alpha toxin

IsdA: iron surface determinant A

LPS: Lipopolysaccharide, or endotoxin

LTA: lipoteichoic acid

MPO: Myeloperoxidase

MSCRAMMS: microbial surface components recognizing adhesive matrix molecules

MSSA: Methicillin-susceptible *Staphylococcus aureus*

MRSA: Methicillin-resistant *Staphylococcus aureus*

NETs: neutrophil extracellular traps

NLRP3: NLR family, pyrin domain containing 3

NOD2: nucleotide binding oligomerization domain containing protein 2

LIMP2: lysosomal integral membrane protein 2

oxLDL: oxidized low density lipoprotein

PAMPS: pathogen-associated molecular patterns

PPARγ: peroxisome proliferating activated receptor

PRR: pattern recognition receptors

ROS: reactive oxygen species

RXR: retinoid X receptor

SSSI: skin and skin structure infections

SSTI: skin and soft tissue infections

TLR2: Toll like receptor 2

WTA: wall teichoic acid

Appendix B: References

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