THE STAPHYLOCOCCUS AUREUS TWO-COMPONENT SYSTEM, SAER/S, MODULATES HOST MEDIATORS OF INFLAMMATION TO ENHANCE PATHOGENESIS

by

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an opportunistic pathogen that causes diseases ranging from superficial skin infections to life-threatening invasive disease. The emergence of community-associated MRSA has caused concern, as these infections appear in healthy individuals. Increasing prevalence of antimicrobial resistance warrants the development of alternative methods of combating infections. Modulating the host immune response during infection has shown promise in research settings, but has fallen short of application in the clinic. Characterizing how *Staphylococcus aureus* influences host mediators of inflammation is an essential step for understanding *S. aureus* pathogenesis and could lead to the development of novel therapies, including immuno-modulation. Previous studies demonstrated the SaeR/S two-component system of *S. aureus* strongly regulates exoprotein production and is essential for full virulence of this pathogen. However, the mechanisms behind the role(s) of SaeR/S in mediating pathogenesis are incompletely defined. To that end, this investigation examined the role SaeR/S in impacting host inflammatory responses during both invasive and superficial *S. aureus* infections. Using mutant *S. aureus* strains with deleted saeR/S (ΔsaeR/S), these studies show that SaeR/S is critical for promoting pathogen survival, dissemination and host mortality during peritonitis. Pro-inflammatory cytokines, such as interferon-gamma (IFNγ), tumor necrosis factor-alpha and interleukin (IL)-6 were significantly reduced in mice infected with ΔsaeR/S. IFNγ transcriptional activation and protein expression were significantly induced by saeR/S during skin and invasive infections. Interestingly, neutrophils were identified as the predominant source of saeR/S-induced IFNγ expression during early *S. aureus* peritonitis. Robust saeR/S-influenced IFNγ production prompted further studies investigating the role of this cytokine during *S. aureus* infections. IFNγ-deficient (GKO) mice were protected during invasive infection with wild-type *S. aureus* as reduced bacterial burdens and reduced host cellular cytotoxicity were observed. GKO mice were protected against wild-type *S. aureus* skin challenge in an IL-17-dependent manner. Interestingly, both normal and GKO mice exhibited similar pathologies when infected with ΔsaeR/S during invasive and superficial infections, suggesting that IFNγ and IL-17 impact saeR/S-mediated pathogenesis and that in the absence of saeR/S, these cytokines are inconsequential in mediating immunity. Collectively, these studies suggest that saeR/S promotes a deleterious IFNγ response that enhances *S. aureus* pathogenesis.
CHAPTER ONE

INTRODUCTION TO DISSERTATION

Pathogenic *Staphylococcus aureus* (*S. aureus*) and methicillin-resistant *S. aureus* (MRSA) infections account for approximately 478,000 hospitalizations in the United States, annually, thus representing a prominent concern.

Contemporary antimicrobials are becoming increasingly ineffective, as the majority of *S. aureus* infections display broad-spectrum resistance to traditional antibiotics (CDC; http://www.cdc.gov/mrsa/statistics/mrsa-surveillance-summary.html). Historically, *S. aureus* infections were linked to predisposed patients within hospital settings. However, over the past decade, *S. aureus* infections have occurred in otherwise healthy individuals independent of clinical environments\(^1\). The increasing prevalence of antibiotic-resistant *S. aureus* warrants the development of innovative prophylactic and therapeutic approaches. Currently, two novel strategies are gaining popularity: 1) Directly targeting the virulence imparted by *S. aureus*, and 2) Enhancing (or dampening) host immune responses via immunomodulation. In order for these approaches to be developed, comprehensive elucidation of both pathogen-mediated virulence as well as host inflammatory responses are necessary.

Traditionally, antibiotics have directly targeted the *S. aureus* bacterium, typically through inhibition of essential cell wall synthesis mechanisms. However, *S. aureus* has developed resistances to these antimicrobials either
through genetic mutation or acquisition of mobile genetic elements encoding for enzymes/proteins that neutralize antibiotic activity$^2$. *S. aureus* virulence, specifically the role of individual pathogen-derived determinants, is well-studied in the context of conferred pathogenicity. Much effort has been put into characterizing host-pathogen interactions to identify which specific virulence factors are essential for *S. aureus* to cause disease. It is thought that by targeting individual virulence determinants (or a combination thereof) with vaccination and/or therapeutic treatment, this would allow the host’s own immune system the opportunity to “catch-up” and clear the pathogen. Advantages to this approach include preserving the host microbiome, less selective pressure on both pathogenic and nonpathogenic microbes and expanding pathogenic targets$^3$. Included within this approach is the characterization of global regulators of pathogen virulence, which are typically activated through receptor-mediated signaling from within the pathogen’s cell membrane in response to environmental cues and influence the expression of diverse virulence factors$^4$. This approach has one significant advantage in that by directly targeting a single global regulator for inhibition, this could effectively “blind” the pathogen, cause a reduction in the expression of multiple virulence factors and, again, afford the immune system the upper hand.

Immuno-modulatory approaches are not exclusive to “targeting virulence” and, in fact, a combination of both would likely allow for the best possible outcome. However, exclusive immuno-modulatory therapies pose the most
promise for individuals with either acquired or genetic defects that compromise immunity. Individuals with defects in both innate and adaptive immune mechanisms display increased susceptibility to *S. aureus* infections. Thus, in addressing both “targeting virulence” and immuno-modulation, we herein characterize how one individual global regulator of virulence, the *S. aureus* SaeR/S system, is both essential for staphylococcal virulence as well as promoting robust inflammation during invasive and superficial infections. The studies described in this dissertation provide a unique perspective into *S. aureus* virulence in the context of host inflammation.

**Overview of Dissertation**

Herein, we evaluated the importance of the staphylococcal two-component regulator of gene expression, SaeR/S, in the context of both virulence and host inflammation. Using comparative analysis of wild-type *S. aureus* and isogenic mutant *S. aureus* strains with deleted *saeR/S* (ΔsaeR/S) we determined that this global regulator was critical for full pathogenicity and for inducing the production of host pro-inflammatory mediators. Using both invasive and superficial skin models of *S. aureus* infection, we established a link between the expression of host-derived pro-inflammatory cytokines and pathogen-derived factors regulated by SaeR/S. Specifically, *saeR/S*-regulated factors induced the robust expression of pro-inflammatory interferon-gamma (IFNγ). We further explore the role of this specific cytokine in the context of *saeR/S*-mediated pathogenesis during both staphylococcal
peritonitis and staphylococcal skin infection. The following paragraphs provide a brief summary of all chapters included within this dissertation and an overview of the collective findings originating from the studies described herein.

In chapter two, a review comprising both historical and contemporary relevant literature is provided to lay the foundation for the studies performed. In chapter three, we demonstrate how SaeR/S is essential for pathogen persistence, dissemination and for mediating mortality during lethal staphylococcal peritonitis. We further highlight how SaeR/S promotes both localized and systemic pro-inflammatory cytokines during invasive *S. aureus* infection, suggesting SaeR/S is critical for inducing sepsis. Additionally, we used a model of *S. aureus* skin infection to determine the impacts of SaeR/S on localized cytokine production. One shared feature between invasive and superficial skin infection was a link between the expression of host-derived pro-inflammatory IFNγ and factors regulated by SaeR/S.

In chapter four, we identify which host cell-type(s) produce IFNγ in response to SaeR/S during *S. aureus* peritonitis. We find that neutrophils are both the predominant cell type (~90%) at the infectious foci and the predominant source of IFNγ. We show this phenotype using two methods for intracellular cytokine accumulation, two distinct staining antibodies for murine neutrophil identification and using two distinct strains of *S. aureus*. Using IFNγ-deficient mice (GKO), we further characterize a role for IFNγ during saeR/S-mediated pathogenesis and find that GKO mice are partially
protected against infection with wild-type \textit{S. aureus}, but not when infected with $\Delta \text{saeR/S}$.

In chapter five, we again use GKO mice to characterize a role for IFN\(\gamma\) in impacting immunity against \textit{saeR/S}-mediated pathogenesis during staphylococcal skin disease. We find that GKO mice are protected against infection with wild-type \textit{S. aureus} and develop smaller dermonecrotic lesions relative to their wild-type BALB/c counterparts. We further determined that GKO mice are protected because they produce elevated protective interleukin (IL)-17 in response to \textit{saeR/S}-regulated factors. The protection imparted by IL-17 observed in GKO mice is exclusive to \textit{saeR/S}-mediated pathogenesis, as none of the strains of mice or treatment groups (recombinant IL-17 in BALB/c mice or neutralization of IL-17 in GKO mice) display differences in abscess development during infection with $\Delta \text{saeR/S}$, again linking IFN\(\gamma\) with \textit{saeR/S}-mediated pathogenesis.

In chapter six is a summary of the results from chapters three through five and includes a hypothetical model for the relationship between SaeR/S and IFN\(\gamma\) during staphylococcal disease. This chapter also contains additional findings that provide preliminary data for future studies. References for all chapters can be found on Page 125.
CHAPTER TWO

BACKGROUND

*Staphylococcus aureus* Background

*Staphylococcus aureus* (*S. aureus*) is a common Gram-positive bacterium that persists commensally among ~32% of the population\(^6\). However, *S. aureus* can also cause a diverse range of disease in humans. Being a primary cause of superficial skin infections, *S. aureus* can lead to rapidly fatal invasive diseases, such as necrotizing pneumonia, endocarditis and sepsis\(^7\). These life-threatening infections are worsened by the emergence of antibiotic-resistant *S. aureus* strains, such as methicillin-resistant *S. aureus* (MRSA). Historically, *S. aureus* was associated with hospital settings or individuals that possess immuno-compromising conditions. However, over the past two decades, an increasing prevalence of *S. aureus* infections have been acquired independently of medical environments\(^1\). Termed community-associated MRSA (CA-MRSA), these infections can cause disease in otherwise healthy individuals. Athletes, children, prisoners and military personnel demonstrate an increased prevalence of CA-MRSA infections, likely stemming from close person-to-person interactions\(^8\). CA-MRSA strains are distinguished from their hospital-associated MRSA (HA-MRSA) counterparts by genomic sequences encoding for the leukotoxin, Panton-Valentine leukocidin (PVL), and a type IV or V SCC\textit{mec} antibiotic-resistance encoding cassette, affording CA-MRSA resistance to all β-lactam antibiotics\(^9\).
Current studies are aimed at further defining the mechanisms behind CA-MRSA’s enhanced virulence; however, some studies have already shown that CA-MRSA strains evade mechanisms of innate immunity more efficiently and express elevated virulence factors, relative to HA-MRSA strains\textsuperscript{10,11}. The ability to infect healthy people, compounded by high resistance to antibiotics necessitates novel treatment approaches to address this increasing concern. Targeting specific virulence factors with vaccines and/or prophylactic therapies has been a proposed method for addressing the antibiotic-resistant phenomena. Alternatively (but not exclusively), modulation of host immunity has been an increasingly advocated strategy. Collectively, the studies described in this dissertation characterize an inflammatory role for the staphylococcal two-component system, SaeR/S, and go further to identify the impacts of pro-inflammatory cytokines in the context of saeR/S-mediated pathogenesis. The SaeR/S system is an essential global regulator of individual virulence determinant expression and several lines of evidence suggest that this system is instrumental in facilitating multiple immune evasion mechanisms that are displayed by \textit{S. aureus}\textsuperscript{12}. It is widely thought that \textit{S. aureus} is a successful pathogen, in large part, due to it’s diverse arsenal of factors aimed at disrupting functions in the primary host leukocyte responsible for bacterial clearance, the neutrophil.
Neutrophils are the most important leukocyte for extracellular pathogenic bacterial clearance and represent the primary recruited phagocytes during *S. aureus* infection\(^1\). The importance of these phagocytes in combating *S. aureus* is strongly highlighted by the increased susceptibility to *S. aureus* infections in populations with genetic neutrophil functional defects\(^14\text{-}16\). During infection, neutrophils are recruited to the site of infection via conserved pattern-recognition receptors (PRRs) expressed on tissue-resident sentinel cells that recognize microbial motifs and produce pro-inflammatory cytokines and chemokines that are detected either directly or indirectly by circulating phagocytes. Upon recruitment from circulation and entry into the infectious foci, a battle ensues whereby both neutrophils and *S. aureus* utilize diverse cellular, protein and molecular repertoires aimed at thwarting each other’s efforts. To fully understand how *S. aureus* mediates dysfunctional neutrophil activity, it is important to delineate basic neutrophil mechanics during inflammation and/or infection. The following sections describe current knowledge of neutrophil function during inflammation, *S. auerus*-neutrophil interactions and how each of these cells tries to eliminate the other during said-interactions.

**Neutrophil Recruitment from Circulation**

Neutrophils are short-lived terminally differentiated phagocytes originating from hematopoietic stem cells in the bone marrow\(^17\). Primary granule synthesis, including granules containing myeloperoxidase, proteases and antimicrobial
defensins, occurs during neutrophil hematopoeisis\textsuperscript{18}. Secondary granule synthesis, including granules containing lactoferrin, flavocytochrome b and collagenase occurs during myelocyte-stage neutrophil development\textsuperscript{18}. Late-stage hematopoeisis yields banded/mature neutrophils in addition to synthesis of tertiary granules containing arginase-1 and gelatinase, as well as secretory vesicles containing formylated peptide receptors (FPR) and FcγRIII receptors\textsuperscript{18}. Following maturation, neutrophils migrate from the bone marrow into the bloodstream and circulate for approximately seven to twelve hours, undergo apoptosis and are cleared from circulation if recruitment (or activation) to an inflamed area is not warranted\textsuperscript{19}. Approximately $10^{11}$ mature neutrophils are produced daily from the bone marrow to manage the rapid turnover\textsuperscript{18}. Once in circulation, neutrophils transiently interact with the endothelium, surveying the membrane for signs of tissue damage and inflammation in the adjacent tissue\textsuperscript{20}. During infection, pro-inflammatory cytokines and chemokines, originating from the inflamed/infected tissue, attract neutrophils that are surveying the adjacent endothelial barriers. Many of these inflammatory mediators, including G-CSF and GM-CSF, stimulate additional granulopoeisis in the bone marrow. Additionally, these inflammatory mediators activate the endothelium and signal to surveying neutrophils that an infection is ongoing\textsuperscript{21}. During acute localized inflammation, neutrophils are recruited from the circulating blood by a complex, yet highly efficient cascade of events. Pro-inflammatory mediators originating from impacted tissue-resident cells serve to activate the vascular endothelium and these endothelial cells rapidly express
adhesion molecules, such as P-selectin and E-selectin. Neutrophils that are moving through the impacted vasculature bind endothelial P- and E-selectins via their own surface adhesion molecules, including L-selectin and E-selectin ligand. These transient interactions are the first steps of neutrophil recruitment, termed tethering and rolling. Tight adhesion of neutrophils to the endothelium occurs via the binding of integrins (LFA-1, for example), expressed on neutrophils, to ICAM-1 that is expressed by endothelial cells. Activated neutrophil expression of Mac-1 (CD11b) facilitates intraluminal crawling and transmigration across the endothelial barrier. These endothelial ICAM-1/2 and VCAM-1 expressions are critical for the described transmigration events. The above stated molecules are examples of well-characterized adhesion molecules; however, both neutrophils and endothelial cells express numerous additional molecules with redundant functions to mediate efficient neutrophil-vascular extravasation.

Following transmigration across the endothelium, the neutrophils further hone in on the inflamed/infected tissue in a chemokine density gradient manner. The process of endothelial-neutrophil adherence, transmigration and chemotaxis is highly efficient under normal conditions; however, S. aureus expresses multiple factors capable of disrupting this process.

S. aureus secretes factors that inhibit neutrophil recruitment to the site(s) of infection. The chemotaxis inhibitor protein of staphylococci (CHIPS) binds with both the C5a-receptor (C5aR) and the formylated peptide receptor (FPR) on neutrophils to inhibit complement-mediated chemotaxis and formylated peptide-mediated chemotaxis, respectively. Additionally, extracellular adherence
protein (Eap) binds ICAM-1 on neutrophils and prevents adhesion to the endothelium and subsequent extravasation\textsuperscript{26}. However, despite secreting factors that can diminish neutrophil recruitment function, these phagocytes still accumulate at remote sites of \textit{S. aureus} infected tissues.

**Neutrophil Recognition and Phagocytosis**

Once at the infectious foci, the primary goal of the neutrophil is to engulf microbes, kill the pathogen, undergo apoptosis and be removed by macrophages with no further inflammation being elicited\textsuperscript{27}. To begin these events, the neutrophil must recognize the invading microbe to initiate the process of phagocytosis. Neutrophil surface expression of diverse pattern recognition receptors (PRRs), including toll-like receptors (TLRs) and TNF-receptor1 (TNFR1) recognize conserved staphylococcal surface motifs, including peptidoglycan, lipoproteins and protein A as foreign antigens\textsuperscript{27}. Mice deficient in TLR2, an essential PRR of peptidoglycan and lipoteichoic acid, display enhanced susceptibility to Gram-positive bacterial infections, such as \textit{S. aureus}\textsuperscript{28}. The activation of PRRs initiates phagocytosis, release of cytokines (IL-8, IL-1\textbeta) and the production of reactive oxygen species (ROS)\textsuperscript{27}. Phagocytosis of most bacteria, including \textit{S. aureus}, is enhanced by opsonin-coating factors, such as complement and serum antibodies deposited on the microbial surface\textsuperscript{27}. For this reason, neutrophils express several receptors for identifying opsonins, including Fc receptors for the binding of immunoglobulin and receptors for identifying deposited complement factors on the pathogen\textsuperscript{27}. Once engulfment of \textit{S. aureus}
into the intracellular phagosome occurs, phagosomal maturation results in fusion of both primary and secondary granules to the phagosome and assembly of the NADPH oxidase complex within the phagosomal membrane\textsuperscript{29}. During most bacterial infections, these recognition and phagocytosis mechanisms are highly effective at engulfing microbes; however, \textit{S. aureus} expresses factors that can interfere with these processes.

\textit{S. aureus} expresses (on its surface) and secretes several proteins that inhibit both neutrophil recognition and phagocytosis. Protein A and the second binder of immunoglobulin (Sbi) both bind the Fc region of immunoglobulin, effectively coating this pathogen in the host’s own nonspecific serum Abs (incorrectly orientated) and affords \textit{S. aureus} a “disguise” to inhibit both opsonization and phagocytosis\textsuperscript{30-32}. Sbi can also promote fluid-phase consumption of C3, resulting in reduced complement-mediated opsonization and complement-mediated lysis\textsuperscript{30}. Passive expression of surface polysaccharides also inhibits phagocytosis\textsuperscript{33}. Anchored into the peptidoglycan, clumping factor A (ClfA) forms strong links with fibrinogen that leads to reduced phagocytosis efficiency\textsuperscript{34}. Again, even with numerous mechanisms for preventing phagocytosis, \textit{S. aureus} is still engulfed by neutrophils and exposed to ROS and antimicrobial peptides contained within neutrophil granules that are introduced into the phagosome.

**Neutrophil Bactericidal Mechanisms**

Activation of the neutrophil results in NADPH oxidase assembly, which is required for ROS production\textsuperscript{35}. NADPH oxidase assembly on the phagosomal
membrane transfers electrons across the membrane and results in the production of superoxide within the phagosome. Superoxide reacts directly with the microbial membrane in addition to generating secondary ROS, including hypochlorous acid, hydroxyl radical and singlet oxygen, all of which are potent microbicides that directly attack microbial membranes. In addition to ROS, neutrophils possess diverse potent oxygen-independent microbicidal agents, including α-defensins, cathelicidins, lactoferrin (sequestration of iron), lysozyme and elastase, which are contained within primary, secondary and tertiary granules that fuse with the pathogen-containing phagosome. Recently identified, novel structures termed neutrophil extracellular traps (NETs) consist of decondensed chromatin and histones and have exhibited direct anti-S. aureus activity. ROS production and NETs are intimately linked, as neutrophils derived from CGD patients are unable to form NETs. NETs are released from neutrophils into the extracellular space and are thought to prevent S. aureus spread in addition to direct killing of microbes. However, it is still to be determined if NETs play a significant role in impacting protection against S. aureus or simply enhance inflammation by a damage-associated molecular pattern (DAMP)-mediated mechanism. Additionally, a cell-programmed mechanism for NET formation is yet to be defined and some postulate that these structures are a spontaneous bi-product of a dying or distressed neutrophil. These potent factors are very successful at degrading most bacterial species; however, S. aureus produces factors that reduce and/or neutralize the efficiency of these antibacterial mechanisms.
S. aureus expresses additional mediators that can effectively neutralize the activity of ROS and antimicrobial peptides. The S. aureus carotenoid pigment not only gives this pathogen its golden color, but displays antioxidant activity that neutralizes oxygen-dependent antibacterial molecules. S. aureus catalase and superoxide dismutase also exhibit neutralization of ROS. This pathogen expresses additional factors for resistance against the oxygen-independent bactericidal mechanisms, particularly the antimicrobial peptides (AMP) contained within neutrophil granules. S. aureus secretes non-specific proteases, as well as possessing the ability to sense the presence of AMPs to up-regulate its own “defense” mechanisms. AMPs characteristically display cationic activity and are attracted to the overall negative charge on the surface membranes of microbes. Upon sensing the presence of AMPs via a three-component system, the S. aureus membrane undergoes D-alanylation substitutions in the teichoic acids within the peptidoglycan. This process reduces the overall negative charge in the membrane and reduces the affinity of AMPs for the bacterial surface. Additionally, the S. aureus VraFG transporter removes AMPs from the cytoplasm and plasma membrane. These mechanisms allow S. aureus to resist killing by the neutrophil and likely allow this pathogen to eventually “break free”, lyse the surrounding neutrophil and continue to survive.

Neutrophil Apoptosis

Following activation, phagocytosis, ROS synthesis and degranulation, all aimed at degrading ingested microbes, neutrophils undergo programmed cell
death (apoptosis)\textsuperscript{48}. If neutrophils do not undergo apoptosis following activation and are not cleared, their ROS and granule components become a biological liability that can lead to damage of host bystander cells/tissue\textsuperscript{27}. In the absence of stimulus, circulating neutrophils will undergo spontaneous apoptosis and be removed within approximately 24 hours following release from the bone marrow\textsuperscript{49}. Regulation of neutrophil activation and subsequent apoptosis is essential to resolution of inflammation following insult and/or infection. Interestingly, some studies have indicated that the neutrophil microbicides induce a survival response in \textit{S. aureus}. This response has been associated with prolonged neutrophil survival even following phagocytosis and degranulation, a point at which apoptosis mechanisms would optimally begin\textsuperscript{50}. This observation was supported by other studies suggesting that prolonged \textit{S. aureus} survival within the neutrophil leads to delayed apoptosis and enhanced bacterial replication from within the phagocyte\textsuperscript{51,52}. Put together, these studies did not negate the essential role for neutrophils, but do suggest that these phagocytes can provide a safe haven for \textit{S. aureus} and allow infections to persist.

Finally, \textit{S. aureus} expresses multiple toxins with varying abilities to lyse neutrophils. These leukocidins display cytotoxic impacts against neutrophils, other immune cells and red blood cells. These neutrophil-lysing toxins include: Panton-Valentine leukocidin (PVL), \textgamma-hemolysin, \alpha-type phenol-soluble modulins and a newly characterized toxin, LukAB (GH)\textsuperscript{53-57}. Although these toxins have demonstrated potential for lysing neutrophils through disruption of surface
membranes, it is unclear if these factors are essential for *S. aureus* to exit the phagosome following engulfment.

Put together, the neutrophil retains highly specific, rapid-responding functions aimed at clearing the host of microbial pathogens and promoting inflammatory resolution. However, *S. aureus* occupies a relatively unique pathogenic niche, in that it expresses factors that can modulate, inhibit or neutralize virtually all of the neutrophil processes described above. These virulence components directly impact host innate defenses and can allow *S. aureus* infections to persist. Ultimately, these *S. aureus* resistances to neutrophil antibacterial mechanisms only confer some protection to the pathogen, as these phagocytes are still recruited and able to phagocytose and kill *S. aureus* with more or less efficiency. It is likely that the pathogen burden, which specific *S. aureus* strain is encountered (different strains of *S. aureus* express these resistance factors at different levels\textsuperscript{11}), and how many neutrophils are present will dictate outcome and determine if the neutrophil or *S. aureus* will prevail during these interactions.

The majority of the individual *S. aureus* virulence determinants described above (and others not described) are under the regulation of global sensors/regulators that are expressed by this pathogen. The following section describes current knowledge of the SaeR/S two-component system, an essential regulator of staphylococcal virulence.
Interestingly, *S. aureus* can exist as both a commensal colonizer in humans, as well as a destructive pathogen capable of diverse infections. It is thought that the differential expression of virulence determinants mediates pathogenicity and infection versus colonization. Bacterial two-component gene regulatory systems mediate responses to changing biological environments and coordinate downstream changes in transcriptional activity\(^\text{58}\). Two-component systems consist of a transmembrane histidine kinase (sensor) and an intracellular response regulator\(^\text{59,60}\). After sensing an environmental signal, the sensor-kinase (SaeS, for example) autophosphorylates at a transmembrane histidine residue. The phosphate group is then transferred to the response regulator (SaeR, for example) at an aspartic acid residue, after which the phosphorylated regulator binds to a specific DNA sequence to alter the transcriptional profile of target genes. Currently, 16 putative *S. aureus* two-component systems have been identified\(^\text{61}\). The studies in this dissertation focus on characterizing overall pathogenesis and host inflammatory responses in the context of the two-component *Staphylococcus aureus* exoprotein system, SaeR/S.

SaeR/S is a major global regulator of staphylococcal virulence and directly (or indirectly) influences *S. aureus* exoprotein production\(^\text{12,62,63}\). Serine proteases, \(\alpha\)-, \(\beta\)- and \(\gamma\)-hemolysins (*hla, hlb, hlg*), exotoxins/superantigens (*seb, sec, ssl(s)*), leukotoxins (PVL, LukAB (GH)) nuclease, staphylococcal binder of immunoglobulin (Sbi) and adhesins, including: coagulase, fibronectin-binding
protein A (FnBPA), Eap and an extracellular matrix protein (Emp) exhibit regulation by SaeR/S\textsuperscript{12,63-72}. Upon early interactions with the human neutrophil, saeR/S is rapidly up-regulated, suggesting \textit{S. aureus} utilizes this system for responding to innate immunity effector cells\textsuperscript{11}. This early observation prompted additional studies to characterize the role of saeR/S during \textit{S. aureus} infections, specifically its role in impacting mechanisms involved in the evasion of innate immunity.

Using a \textit{S. aureus} isogenic deletion mutant of saeR/S, it was shown that SaeR/S was critical for evasion from neutrophils\textsuperscript{12}. \textit{S. aureus} survival in human whole blood and following human neutrophil interactions is significantly reduced in the absence of saeR/S\textsuperscript{12}. Additionally, saeR/S-deficient \textit{S. aureus} causes significantly reduced neutrophil cytotoxicity, suggesting saeR/S-regulated leukotoxins are promoting neutrophil lysis\textsuperscript{12}. Using distinct strains of \textit{S. aureus}, SaeR/S has been shown to be essential for promoting mouse mortality during both bloodstream and peritoneal infections\textsuperscript{12,63,73}. Moreover, during skin infection, saeR/S is necessary for the development of dermonecrotic lesions\textsuperscript{63}. These studies delineated a role for saeR/S and the attenuated virulence is likely a combined result from the reduced expression of multiple virulence determinants during infection. However, the exact mechanisms of \textit{S. aureus} pathogenesis mediated by saeR/S remain incompletely defined.
SaeR/S-Regulated Factors Impact Pathogenesis and Inflammation

As mentioned above, saeR/S regulates a diverse set of *S. aureus* virulence factors. These mediators directly and/or indirectly influence host inflammatory responses during *S. aureus* infection. The following sections describe current knowledge about individual saeR/S-regulated mediators of *S. aureus* pathogenesis and their associated impacts on inflammation and host cells during infection.

**Membrane-Damaging Toxins.** SaeR/S regulates *S. aureus* expression of α-, β- and γ-hemolysins\(^{12,62,63,65,74}\). Lysis of host red blood cells is predominantly mediated by this class of toxins\(^{75}\). However, these pathogen-derived factors also impact additional inflammatory pathways and mediate pathogenesis during *S. aureus* infection.

β-hemolysin has a direct impact during *S. aureus* pulmonary and corneal infections through altering host cellular function. This toxin abrogates the ciliary activity of nasal epithelial cells and induces scleral inflammation, in the form of neutrophil recruitment\(^{76}\). During pulmonary *S. aureus* infection, β-hemolysin promotes neutrophil infiltration and vascular leakage of serum proteins into airway passages\(^{77}\). Rabbit nasal epithelial cells exhibit reduced ciliary activity following treatment with β-hemolysin, further exacerbating a sinusitis condition\(^{78}\). Furthermore, β-hemolysin enhances the killing of proliferating human lymphocytes\(^{79}\).
Staphylococcal α-hemolysin (toxin) has gained recent attention for both its contribution to *S. aureus* pathogenesis as well as being a candidate for vaccine development\(^8^0\). This toxin is a critical virulence factor in promoting *S. aureus* pneumonia, sepsis, septic arthritis, brain abscess development and corneal infections\(^8^1\)-\(^8^6\). Alpha-hemolysin induces host production of cytokines and chemokines, including interleukin (IL)-6, IL-8, IL-1β, IL-1α, tumor necrosis factor-alpha (TNFα), KC and MIP-2\(^8^2,8^7\)-\(^9^1\). Moreover, α-hemolysin promotes the early induction of plasma cell membrane permeability and programmed cell death in human T cells, B cells and monocytes\(^9^2\), an observation that was supported by earlier studies demonstrating that α-toxin promotes apoptosis in human T cells and endothelial cells\(^9^3,9^4\).

**Leukotoxins.** SaeR/S regulates the expression of *S. aureus* leukotoxins, including PVL and a newly described cytotoxin encoded by *lukAB* (*lukGH*)\(^1^2,6^3\). Both PVL and LukAB are bi-component toxins, ineffective as individual subunits, but oligomerize to form β-barrel pores in the membrane of host cells\(^9^5,9^6\). Both exhibit cytolytic activity against leukocytes, most notably neutrophils\(^5^6,9^7,9^8\).

The role of PVL during *S. aureus* disease is well-characterized, but often disputed with studies demonstrating significant roles in mediating pathogenesis and other studies suggesting a negligible role. Using deletion mutant strains of CA-MRSA, lacking *pvl*, it was shown that this leukotoxin was not essential for inducing mortality in murine models of *S. aureus* sepsis\(^9^9,1^0^0\). Additionally, PVL was not necessary for lesion development in a murine model of *S. aureus* skin
disease\textsuperscript{99,100}. However, purified PVL does exhibit cytotoxic activity against human neutrophils\textsuperscript{97,98}. Purified PVL causes dermonecrotic lesions in the skin of rabbits and lethal necrotic lesions in the lungs of mice\textsuperscript{55,101}. Wild-type \textit{S. aureus}-infected mice exhibit elevated muscle tissue destruction compared to \textit{pvl}-deficient mice, and this is linked to the production of KC, MIP-2, RANTES and neutrophil influx\textsuperscript{102}. When cultured with human neutrophils, purified PVL induces the secretion of leukotriene B4 and oxygen metabolites\textsuperscript{103}. Although the role for PVL in \textit{S. aureus} pathogenicity remains unclear, this leukotoxin demonstrates clear pro-inflammatory capabilities in the form of neutrophil recruitment and by inducing the expression of pro-inflammatory cytokines and chemokines.

The role of LukAB is relatively undefined, to-date, as this factor was identified very recently. However, early studies have shown this toxin to promote the killing of human neutrophils, macrophages and dendritic cells\textsuperscript{56,97}. Additionally, purified LukAB induces abscess development in the skin of rabbits\textsuperscript{97}. Although specific cytokine and chemokine induction mediated by \textit{lukAB} are yet to be characterized, the studies performed by DuMont et al and Malachowa et al\textsuperscript{56,97} would suggest a role for this leukotoxin in neutrophil recruitment, likely through a DAMP-mediated mechanism.

Cellular cytotoxicity has been a long-standing inducer of inflammatory pathways, as the release of host intracellular components are recognized by bystander leukocytes as DAMPs\textsuperscript{104}. It is likely that leukotoxins promote inflammation through both DAMP-mediated mechanisms under high toxin concentrations that promote direct cellular lysis and through low toxin
concentration-mediated mechanisms that modulate inflammatory responses through DAMP-independent processes. However, specific mechanisms by which staphylococcal leukotoxins promote inflammation are undefined, although a recent study suggests that inflammation resulting from these toxins is receptor-mediated\textsuperscript{105}.

**Exotoxins/Superantigens.** SaeR/S regulates the expression of several enterotoxins, including *seb*, *sec* and superantigen-like proteins (*ssl(s)*), which are exoproteins with analogous superantigenic-like peptide sequences\textsuperscript{12,70,71}. Superantigens nonspecifically bind the MHC class II molecule of antigen-presenting cells with T-cell receptors (TCRs), leading to T lymphocyte activation and massive pro-inflammatory cytokine production\textsuperscript{106}. These toxins are significant mediators of staphylococcal toxic shock syndromes (TSS) and sepsis.

Staphylococcal enterotoxin (SE) B (SEB; encoded by *seb*) is a toxin most commonly associated with food poisoning, nonmenstrual TSS and for its potential application as an agent of biowarfare/bioterrorism\textsuperscript{107,108}. Mice treated with SEB succumb to toxic-shock syndrome (TSS) in an MHC class II-dependent manner and this is associated with dramatically elevated TNF\(\alpha\), IL-6 and interferon-gamma (IFN\(\gamma\))\textsuperscript{109,110}. MCP-1 and IL-2 are also directly correlated with lethality in SEB murine models of TSS\textsuperscript{110}. Cultured human PBMCs produce IL-1, IL-2, IL-6, TNF and IFN\(\gamma\) in response to treatment with SEB and SEC\textsuperscript{111}. Additionally, mice immunized with a non-reactive mutated SEC protein were protected following challenge with live *S. aureus*, implicating this toxin in potential
vaccine therapies\textsuperscript{112}. Moreover, immunized mice produce lower IFN\gamma and exhibited a polarized Th2 cytokine response, in the form of elevated IL-10 that correlates with decreased overall pathogenicity\textsuperscript{112}.

Fourteen genes encoding superantigen-like proteins (SSL) have been identified in \textit{S. aureus}\textsuperscript{113-116}. SaeR/S shows direct regulation of \textit{ssl}5, \textit{ssl}7, \textit{ssl}8, \textit{ssl}9 and \textit{ssl}11\textsuperscript{70,71}. Interestingly, SSLs are not mitogenic for T cells and do not bind MHC class II, despite sharing sequence homology with classical superantigens\textsuperscript{117}. However, SSLs have been shown to impact effectors of innate immunity\textsuperscript{116}. SSL5 and SSL11 target neutrophils by binding P-selectin and inhibiting neutrophil-endothelial cell adherence\textsuperscript{118}. SSL7 binds both complement component C5 and monomeric and secreted IgA\textsuperscript{117}. Binding of C5 reduces complement-mediated hemolytic activity in addition to reduced cleavage of chemotactic C5a and decreased IgA function is predicted to allow bacterial persistence at mucosal surfaces\textsuperscript{117}. Put together, the staphylococcal classes of exotoxins and superantigens are capable of both promoting robust inflammation (locally and systemically), as well as inhibiting effective immune responses during \textit{S. aureus} disease.

\textbf{Complement Inhibitors and Adhesins.} \textit{S. aureus} SaeR/S influences the expression of multiple complement inhibitors and adhesins with diverse but overlapping functions\textsuperscript{12,63}. A functional complement system is essential for both leukocyte chemotaxis and complement-mediated lysis of pathogens. Staphylococcal adhesins, commonly referred to as MSCRAMMs (microbial
surface components recognizing adhesive matrix molecules), afford the bacteria a binding niche, whereby this pathogen can strongly adhere to proteins in the extracellular matrix. Extracellular matrix proteins include: fibronectin (Fn), fibrinogen (Fg), vitronectin (Vn), thrombospondin, bone-sialoprotein, elastin, collagen, and von Willebrand factor binding proteins. MSCRAMM binding of these proteins is implicated in primary adherence, persistent infections, and contribute to staphylococcal biofilm formation\textsuperscript{119}.

Sbi is a saeR/S-regulated inhibitor of complement that binds host Factor H and complement factor C3b to inhibit alternative complement pathway activation\textsuperscript{120}. Additionally, Sbi binds the Fc region of IgG, effectively coating the pathogen in the host’s nonspecific antibodies (in the wrong orientation) and reducing neutrophil recognition of \textit{S. aureus}\textsuperscript{121}. Both surface-bound and secreted Sbi aide \textit{S. aureus} to evade neutrophils by diminishing opsonophagocytosis capabilities\textsuperscript{30}.

\textit{S. aureus} expresses several microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) which are anchored into the surface-bound peptidoglycan\textsuperscript{122}. SaeR/S-regulated FnBPA strongly binds with Fn, Fg, elastin and is important for the pathogen in mediating intercellular adhesion\textsuperscript{12,123-126}. FnBPA has been shown to be essential for internalization of \textit{S. aureus} into host cells, a postulated pathogen strategy for evading innate immunity by “hiding”\textsuperscript{127}. Additionally, systemic inflammation is induced by FnBPA during invasive disease\textsuperscript{122}. Specifically, FnBPA has been demonstrated to mediate sepsis in murine models and this is associated with systemic platelet
activation\textsuperscript{127,128}. Long-studied and also regulated by \textit{saeR/S}, coagulase converts fibrinogen to fibrin and promotes blood clotting during infection\textsuperscript{12,129}. Consistent with this, fibrin is an important factor for abscess development during \textit{S. aureus} tissue infection\textsuperscript{129}. Put together, \textit{S. aureus} possesses a diverse repertoire of extracellular matrix-modifying proteins aimed at both establishing successful infection and evading recognition by host innate immunity.

Finally, \textit{S. aureus} also produces numerous secretable expanded repertoire adhesive molecules (SERAMs), of which Eap and Emp are under the direct regulation of \textit{saeR/S}\textsuperscript{12,127}. Eap and Emp are essential for \textit{S. aureus} to bind to Fg, Fn and Vn\textsuperscript{127}. Additionally, Eap interacts with ICAM-1, inhibiting neutrophil binding to the endothelium and subsequent phagocyte transmigration\textsuperscript{127}. Interestingly, Eap has also been demonstrated to reduce angiogenesis, suggesting an inhibitory role for tissue reconstruction following damage\textsuperscript{130}. Recently, Eap was shown to promote \textit{S. aureus}-endothelial interactions in a TNF\textgreek{a}-dependent manner\textsuperscript{131}. The authors showed that Eap directly induces TNF\textgreek{a} production in human whole blood and that both TNF\textgreek{a} and Eap were essential for pathogen-endothelial adherence\textsuperscript{131}. This latter study is a good demonstration of how a \textit{saeR/S}-regulated virulence factor (Eap) modulates the host immune response (increased TNF\textgreek{a} production) to promote \textit{S. aureus} pathogenesis (enhanced \textit{S. aureus}-endothelial interactions). It is likely that TNF\textgreek{a}-mediated activation of the endothelium facilitates uptake (or invasion) by this pathogen.
Clearly, the factors regulated by SaeR/S exhibit both damaging and pro-inflammatory impacts during disease and often these two are related. However, there are additional virulence factors, independent of saeR/S-mediated expression, that also contribute to the pathogenesis of this versatile pathogen. Interestingly, many of these factors (both saeR/S-dependent and independent) are aimed at inhibiting the primary functions of the neutrophil, as well as causing robust inflammation. Consistent with this idea, it is likely that several saeR/S-regulated factors contribute to both localized inflammation, in addition to systemic inflammation, a frequent observation in bacterially-derived sepsis syndromes.

**Invasive Staphylococcal Disease and Sepsis**

Staphylococcal-derived bacteremia can frequently be complicated with the presentation of metastatic infection, endocarditis and sepsis syndromes\(^7\). Vascular endothelial cells play a central role in influencing these conditions. Binding of *S. aureus* to the endothelium, via adhesin-receptor interactions in an integral aspect of staphylococcal endovascular disease\(^{132-134}\). *S. aureus* exhibits a tropism for the intracellular environment of endothelial cells, whereby the pathogen is protected from innate immune effectors and antibiotics\(^{135,136}\). In addition to providing protection for the pathogen, *S. aureus*-containing endothelial cells release pro-inflammatory cytokines, including IL-1β, IL-6 and IL-8\(^{137}\). Systemic vascular activation and chemokine production can lead to additional systemic inflammation and also reduce migration of neutrophils to distant areas of infection\(^{138}\).
Molecular and cellular events leading to the induction of staphylococcal sepsis are similar among most bacterially-derived sepsis conditions\textsuperscript{7}. Upon interacting with \textit{S. aureus}, monocytes and macrophages produce TNFα, IL-1β, IL-6 and IL-8\textsuperscript{139,140}. Levels of IL-1, TNFα and IL-6 are used as predictive markers to gauge sepsis outcomes/prognoses\textsuperscript{141}. Both pro-inflammatory cytokine production and activation of the endothelium can induce coagulation pathways. These events cause fever, hypotension, capillary leak, disseminated intravascular coagulopathy, depression of myocardial function, and multi-organ dysfunction\textsuperscript{7}. As discussed above, several \textit{S. aureus} virulence factors regulated by \textit{saeR/S} are capable of driving these pro-inflammatory responses\textsuperscript{142}. For example, using animal models it has been shown that exposure to purified α-hemolysin can mimic several responses associated with sepsis, including hypotension, thrombocytopenia, and reduced oxygenation\textsuperscript{143}. \textit{S. aureus} toxins, specifically superantigenic proteins, lead to systemic T cell activation and excessive cytokine production. These pro-inflammatory mediators can contribute to tissue destruction observed during systemic inflammation\textsuperscript{142,144,145}. One hallmark of systemic vascular activation and systemic cytokine/chemokine production is the reduced migration of neutrophils to the distal infected tissues and this is frequently observed in septic individuals\textsuperscript{138}.

\textbf{Neutrophil Function During Sepsis}

During acute localized inflammation, neutrophils are very efficient at being recruited from circulation to specifically hone in on an impacted area. However,
during systemic inflammation (i.e. sepsis), neutrophils exhibit severe deficiencies in the context of migration to distal inflamed/infected sites\textsuperscript{146}. Studies characterizing systemic inflammatory responses and systemic vasculature activation have pointed to the neutrophil as being a prime suspect in mediating organ failure, a frequent observation in septic patients\textsuperscript{147}. Neutrophil granule components and ROS are highly effective at clearing microbial pathogens; however, these factors can also cause significant host tissue damage\textsuperscript{148}. This concept is exemplified in septic patients who succumb to lung and/or kidney failure and display high numbers of neutrophils in these failed organs in addition to having higher than average neutrophil numbers in circulation (neutrophilia)\textsuperscript{147,149,150}. To understand why neutrophils accumulate in non-infected organs during sepsis, it is important to consider the impacts of systemic pro-inflammatory cytokines and chemokines on these circulating phagocytes.

Septic patients frequently display with neutrophils that express increased CD11b and decreased L-selectin, the classic phenotype of an activated neutrophil, and it is widely thought that these cells are committed to undergo firm adhesion to the endothelium\textsuperscript{147}. CD11b strongly binds to ICAM-1 on endothelial cells and both of these surface receptors are systemically up-regulated during sepsis, likely through the wide-spread production of pro-inflammatory cytokines, including TNFα and IL-1\textsuperscript{147}. These mechanisms suggest that neutrophils are being activated and strongly binding the endothelium in a systemic manner, independently of an area of localized infection. These neutrophils also display reduced CXCR1 and CXCR2 receptors and exhibit reduced chemotactic
responsiveness to IL-8\textsuperscript{151}. The attenuated responsiveness to chemotactic factors is highlighted in models demonstrating reduced neutrophil migration to distal areas of inflammation during sepsis\textsuperscript{152}. Interestingly, blockage of CXCR1/2 inhibited multi-organ failure and disseminated intravascular coagulation in an experimental model of murine sepsis, indicating an important pathological role for these receptors\textsuperscript{153}. Additionally, circulating neutrophils from septic patients are in a primed state, exhibiting increased oxidative activity and elevated expression of NF-\kappa B\textsuperscript{154,155}. Conversely, reduced NF-\kappa B activity in neutrophils is associated with an improved outcome during sepsis\textsuperscript{156}. Finally, apoptosis is significantly reduced (or delayed) in septic patients, likely stemming from pro-inflammatory cytokines and/or circulating PAMPs\textsuperscript{157}.

Collectively, neutrophil function is dramatically impaired during systemic inflammatory conditions. It is widely thought that the universal activation of circulating neutrophils, combined with the systemic neutrophil-endothelium interactions lead to increased vascular damage and vascular permeability, likely stemming from neutrophil-derived proteolytic enzymes and ROS. Exactly why these neutrophils accumulate in such sensitive organs as the lungs and kidneys is not known. However, observations of vascular permeability and additional inflammation is closely linked to very serious conditions, such as disseminated intravascular coagulation, multi-organ failure and death.
Upon presentation with an uncomplicated *S. aureus* skin infection, such as folliculitis, impetigo and/or infected abrasions, outpatient treatment with topical and/or oral antibiotics is frequently successful\(^{158}\). Conversely, complicated *S. aureus* skin disease, such as cellulitis or infected wounds, often requires hospitalization, debridement (including amputation) of infected tissue and intravenous antibiotics\(^{159,160}\). An epidemiological survey conducted in 2005 found that *S. aureus* skin and soft-tissue infections (SSTIs) result in 11.6 million clinical visits in the United States, annually\(^{161}\). These conditions are further compounded by antibiotic-resistant strains of *S. aureus*, including both HA-MRSA and CA-MRSA\(^{158}\). Epidemiological findings indicate that 76% of all SSTIs are *S. aureus*-derived and that 78% of *S. aureus* SSTIs display methicillin resistance, indicating MRSA comprises more than half of all SSTIs clinically reported\(^{162}\). Human staphylococcal colonization is a risk factor for developing SSTIs and it is estimated that 30% of adults, in the United States, are transiently colonized with *S. aureus*\(^{163,164}\).

Much of the current research focuses on *S. aureus*-derived virulence factors and their respective roles in mediating pathogenesis, with a relatively smaller (but increasing) focus on host immunity against staphylococcal skin disease. To this end, an in-depth understanding of both pathogen-mediated virulence and its impact on skin immunity during *S. aureus* infection is essential if novel therapies, including immuno-modulation and prophylactic vaccination, are to be
successful\textsuperscript{5,165}. The following sections describe current knowledge of innate immune responses during \textit{S. aureus} skin infection.

**Primary Physical Defense**

The skin is the most important barrier for protecting humans from invasion by microorganisms in the environment. Firstly, the skin surface has a low pH and low temperature that is not conducive for the growth/survival of most bacterial species\textsuperscript{166}. The outer layer of the epidermis consists of dead keratinocytes, tightly linked by keratin bridges\textsuperscript{167}. Beneath the outer layer, the basal layers are made up of live keratinocytes, which eventually die, migrate to the outer epidermis and are shed. This process is continuous with new keratinocytes migrating into the basal layers as dead ones move outward\textsuperscript{167,168}. Within both the epidermis and basal layers, numerous immune cell-types make up the repertoire of first responders during pathogen invasion. These cells include: dendritic cell (DC) subsets, NK cells, T cells, macrophages, B cells and mast cells\textsuperscript{167,168}. These skin sentinels, including keratinocytes, recognize bacterial PAMPs to initiate the production of direct antimicrobial factors and the production of inflammatory mediators to signal the body that an invasion has occurred. However, in addition to the skin sentinel cells that recognize \textit{S. aureus}, commensal micro-organisms, such as \textit{Staphylococcus epidermidis}, \textit{Matassezia} spp., \textit{Corynbacterium} spp. and \textit{Propionibacterium acnes} occupy microbial niches and provide protection by inhibiting \textit{S. aureus} colonization and invasion\textsuperscript{166}. 
Keratinocytes

Keratinocytes possess multiple receptors and cellular pathways for mounting both direct attacks against invading *S. aureus*, as well as initiating inflammatory responses. Keratinocytes both constitutively express and can increase the expression of antimicrobial peptides that have direct bactericidal and/or bacteriostatic impacts against *S. aureus*, including LL-37, RNase 7, β-defensin and hBD3. The importance of these antimicrobial peptides in mediating defense against *S. aureus* is highlighted by the increased susceptibility of *S. aureus* colonization in patients with deficient β-defensin and cathelicidin production, as seen in individuals with atopic dermatitis. Keratinocyte recognition of invading *S. aureus* is performed through PRRs, consisting of TLRs and NOD-like receptors. These PRRs recognize staphylococcal PAMPs, including peptidoglycan, lipoteichoic acid and muramyl peptides to initiate both production of antimicrobial peptides and inflammatory pathways.

Keratinocytes are an important source of chemokines for recruiting immune effector cells to the skin during pathogenic infection. Recognition of *S. aureus* induces keratinocytes to express CC-chemokine ligand (CCL) 20, CXCL9, CXCL10 and CXCL11 to selectively attract effector T cells. Neutrophils are recruited to the skin via keratinocyte-derived IL-1β, IL-6, TNFα, IL-18, CXCL1/KC and CXCL8/IL-8. Keratinocytes can recruit precursor Langerhans cells (epidermal-resident DCs) through expression of CCL20 via a positive-feedback mechanism. Keratinocyte-derived IL-1β, IL-6, IL-18 and TNFα lead to the activation of dermal DCs in the presence or absence of antigen. Additionally,
keratinocyte-mediated activation of plasmacytoid DCs leads to subsequent activation of dermal DCs via interferon-α, which leads to further inflammatory mediator production\textsuperscript{168}. These processes can be initiated by \textit{S. aureus} protein A that binds TNFR1, expressed on keratinocytes, leading to NF-κB activation and robust cytokine and chemokine expression\textsuperscript{177}. Put together, keratinocytes not only serve as a solid barrier against invading \textit{S. aureus} but are also instrumental in creating an inflammatory milieu to facilitate the activation and recruitment of additional immune cells aimed at mounting a robust effective response aimed at pathogen clearance.

**Neutrophil Recruitment to the Skin**

As described above, neutrophils are the primary phagocyte responsible for clearance of \textit{S. aureus} during infection\textsuperscript{178}. The importance of neutrophils in maintaining protection against \textit{S. aureus} infections, specifically skin infections, is highlighted by the increased susceptibility to staphylococcal skin infection displayed by humans with defects in neutrophil development and neutrophil function. Most notably, individuals with chronic granulomatous disease (CGD) present with recurrent \textit{S. aureus} skin infections\textsuperscript{14}. CGD patients have a genetic defect that causes a loss of function in neutrophil NADPH oxidase assembly\textsuperscript{14}. The phagosomal membrane-bound NADPH oxidase complex produces high levels of superoxide anions and secondarily-derived reactive products which are highly potent staphylocidal molecules\textsuperscript{179-182}. Additionally, patients with neutrophil myeloperoxidase deficiency, neutrophil-specific granule deficiency, leukocyte
adhesion deficiency and individuals with acquired neutropenic conditions, such as chemotherapy patients, all display increased susceptibility to \(S.\ aureus\) skin infections\(^{15,16}\). These human subpopulations display defects in one of the three (or combination thereof) primary neutrophil-specific events that are required for the maintenance and resolution of \(S.\ aureus\) skin infections: 1) neutrophil recruitment from blood circulation, 2) neutrophil survival at the infectious foci and 3) homing of c-kit\(^+\) progenitor cells that locally proliferate to form mature neutrophils\(^{164,183}\).

Upon recognition of \(S.\ aureus\) by cells in the epithelium, skin sentinel cells will produce pro-inflammatory cytokines and chemokines that promote neutrophil recruitment. Tissue-resident macrophages, epithelial and endothelial cells produce high amounts of IL-8, a potent neutrophil chemokine in response to conserved bacterial PAMPs, such as lipoteichoic acid and capsular polysaccharide\(^{184-186}\). In mice, localized production of CXCL5, MIP2 and Gro-\(\alpha/KC\) attract neutrophils in a density-gradient manner to the origin of infection by activating CXCR2 on the surface of phagocytes in circulation and those undergoing transmigration across the endothelial barrier\(^{21}\). Efficient recruitment of neutrophils to the site of infection is dependent upon a multitude of chemotactic and pro-inflammatory mediators; however, recent studies have characterized the roles of two cytokines, IL-1\(\beta\) and IL-17, as being critical for orchestrating neutrophil chemotactic factor production and abscess development, two critical events necessary for the sequestration and clearance of a \(S.\ aureus\) infection.
IL-1β production is observed during early and ongoing stages of S. aureus skin infection and is essential for efficient neutrophil recruitment during cutaneous infection with diverse pathogens\textsuperscript{164,168,187}. S. aureus lipoproteins and hemolysins activate intracellular Nlrp3 inflammasomes and caspase-1, leading to cleavage of pro-IL-1β and secretion of mature IL-1β\textsuperscript{188}. Additionally, S. aureus PAMPs elicit activation of TLR2 and NOD2, which synergistically activate the neutrophil inflammasome and lead to mature IL-1β cleavage and secretion\textsuperscript{187}. IL-1β binds IL-1R and signals through MyD88 and IRAK4 to activate NF-κB, resulting in additional pro-inflammatory cytokine and chemokine expression\textsuperscript{5}. The importance of these signaling pathways in mediating defense is demonstrated by patients with deficiencies in MyD88 and/or IRAK4 signaling and their increased susceptibility to recurrent S. aureus skin infections\textsuperscript{189}. Genetically-defined mice deficient for TLR2, NOD2, formylated peptide receptor (FPR), IL-1β or IL-1R all display reduced neutrophil recruitment, increased dermonecrotic lesion development and elevated bacterial burdens during S. aureus skin disease\textsuperscript{187,190}. Although IL-1β expression originates from within tissue-resident skin sentinels, it has been recently shown that neutrophil-derived IL-1β is sufficient for maintaining neutrophil recruitment, abscess formation and pathogen clearance during skin infection in mice\textsuperscript{168,187}. Put together, it is likely that neutrophil-independent mechanisms of IL-1β expression help to initiate inflammation, but that recruited neutrophils provide a sustained source of IL-1β throughout the course of infection and facilitate the continuous influx of these critical phagocytes.
In addition to the important role of IL-1β, recent reports have indicated an important role for IL-17, specifically IL-17A and to a lesser extent IL-17F, in promoting innate defense mechanisms against *S. aureus* cutaneous infections. Th17 cells, γδ T cells, NKT cells and NK cells are the primary producers of IL-17 during inflammation\textsuperscript{191}. IL-17 induces the expression of neutrophil-attracting chemokines, including CXCL1, CXCL2 and IL-8, in addition to granulopoeisis factors, including G-CSF and GM-CSF\textsuperscript{191,192}. Mice deficient in γδ T cells, IL-17 and/or IL-17R expression display reduced neutrophil recruitment, enhanced dermonecrosis and elevated bacterial burdens during infection\textsuperscript{193}. In the context of IL-17-mediated events, signaling through IL-1R was necessary to induce expression during infection, again suggesting IL-1 production is critical\textsuperscript{193}. This observation was supported by earlier studies demonstrating that IL-1β was important for the development of Th17 cells\textsuperscript{194,195}. Put together, it is likely that tissue-resident and neutrophil-derived IL-1β combine with T cell-derived IL-17 to synergistically promote a robust innate response in the form of neutrophil recruitment.

\textbf{T Cells.} T cells are a potent source of pro-inflammatory mediators during infections with diverse bacterial and viral pathogens. The role of T cells in mediating immunity against *S. aureus* is conflicted, with studies demonstrating both protective and deleterious roles for these lymphocytes. However, the importance of T cells, particularly T helper cells, in conferring protection against staphylococcal disease is clinically highlighted by individuals with T cell defects,
notably HIV/AIDS patients, and their increased susceptibility to S. aureus skin colonization and infection\textsuperscript{196-198}. Two subsets of T cells, Th1 and Th17 cells, have gained recent attention for their critical, yet sometimes conflicting roles in mediating immunity against S. aureus cutaneous disease.

**Th1 Cells.** The classic hallmark of Th1 cell phenotypes is the production of pro-inflammatory IFN\(\gamma\)\textsuperscript{199}. Infection with virulent S. aureus is associated with elevated IFN\(\gamma\) expression compared to infection with avirulent S. aureus\textsuperscript{200}. Using a surgical wound model of S. aureus disease, Th1 cell-derived IFN\(\gamma\) was shown to elicit the production of CXC chemokines and promote a robust neutrophil presence\textsuperscript{51,201}. Interestingly, the investigators noted that the IFN\(\gamma\)-mediated increased neutrophil recruitment was associated with elevated bacterial burdens\textsuperscript{51}. These interesting results are consistent with an earlier study demonstrating that S. aureus can survive within neutrophils for extended periods of time and prolong infection\textsuperscript{52}. Put together, these findings suggest that although IFN\(\gamma\)-induced expression of chemokines leads to robust neutrophil recruitment, the presence of these phagocytes did not result in clearance of S. aureus, however, a conclusive link between IFN\(\gamma\) expression and survival of S. aureus within the neutrophil-rich environment was not defined.

**IL-17-Producing Cells.** During pathogenic infection, IL-17-producing cells express IL-17A and IL-17F and these two cytokines induce other cells to produce neutrophil chemokines and granulopoeisis factors\textsuperscript{191,192}. Specifically, \(\gamma\)\(\delta\)T cells are required for expression of IL-17A during S. aureus cutaneous infection in
mice^{193}. Patients with hyper-IgE syndrome possess a mutation in STAT3, are
deficient in IL-17-producing cells and display increased susceptibility to *S. aureus*
cutaneous infection^{202}. Moreover, mice deficient in either IL-17A or IL-17F
develop spontaneous *S. aureus* skin infections^{203}. Additionally, IL-17 contributes
to the primary antimicrobial defenses of keratinocytes by enhancing their
expression of LL-37 and hBD2^{204,205}.

It is interesting to note that IFNγ-mediated neutrophil recruitment correlated
with elevated *S. aureus* burdens, but that IL-17-mediated neutrophil recruitment
correlated with reduced bacterial burdens^{51,193}. These opposing findings suggest
that IFNγ may impact neutrophil function at the infectious foci in an as-yet
undefined deleterious manner, resulting in reduced pathogen clearance.
However, it has been shown that IFNγ and IL-17 have an antagonistic
relationship in the context of Th1 and Th17 cell development and this may
provide insights into how these two cytokines impact innate antibacterial
mechanisms during *S. aureus* infection^{199}.

**Th1-Th17 Antagonism.** The type of infection (bacterial, viral, etc) is a strong
factor in mediating specific T cell subset differentiation. When T cells commit to
a specific subset, they will often produce factors that inhibit the production of
different subsets^{199}. For example, during viral infection, Th1 IFNγ expression has
been shown to reduce Th17 cell populations^{206,207}. Additionally, IFNγ reduces
Th17 cellular differentiation and induces apoptosis of Th17 cells, *in vitro*^{208,209}.
Interestingly, *in vitro* studies have indicated that *Candida albicans*-specific Th17
cells co-produce IL-17 and IFNγ, but that S. aureus-specific Th17 cells could only produce IL-17 or IFNγ. To date, the potentially antagonistic relationship between IL-17 and IFNγ have not been studied in the context of S. aureus infection, in vivo, but studies would suggest that their respective roles would be overlapping in the context of mediating neutrophil recruitment.

Neutrophils as a Source of IFNγ

IFNγ impacts several neutrophil-specific antibacterial mechanisms, including ROS production and degranulation mechanisms, in addition to the neutrophil’s capacity for expression of chemotactic factors, such as IL-8. The source of IFNγ was long accepted to be lymphocyte-derived, specifically, αβ T cells, γδ T cells, NKT cells and NK cells were thought to be the primary sources of early IFNγ production during inflammation and/or infection. However, it became apparent that an alternative lymphocyte-independent cellular source of IFNγ exists, when it was observed that IFNγ was being produced even when T cells were scarcely present. These early observations suggested a myeloid-derived source of IFNγ during early innate inflammation.

Human Neutrophils Produce IFNγ

Neutrophils were long thought to be primary innate effector cells only, with a relatively inert capacity for dynamic transcriptional and translational responses. However, more recent studies have determined that neutrophils
respond with robust inflammatory transcriptional changes that influence several inflammatory pathways\textsuperscript{210}. Neutrophils as a source of IFN\(\gamma\) has been historically overlooked and severely underestimated\textsuperscript{218}; however, more recent studies have indicated that human neutrophils are capable of producing this pro-inflammatory mediator. Using \textit{in situ} methods in addition to highly purified peripheral blood neutrophils (> 99\% purity), human neutrophils were shown to be capable of expressing IFN\(\gamma\) in response to several factors, including LPS, TNF\(\alpha\) and IL-12\textsuperscript{219}. Additionally, human neutrophils express preformed IFN\(\gamma\) that can be rapidly released in response to inflammatory stimuli\textsuperscript{220}. The amount of IFN\(\gamma\) produced by an individual human neutrophil has been quantified and ranges in the attograms (10\textsuperscript{-18} g/neutrophil) under IL-12 and LPS-stimulating \textit{in vitro} conditions\textsuperscript{219}; however, when considering the extremely high number of these phagocytes that are present during inflammation and/or infection, the biological relevance/function of neutrophil-derived IFN\(\gamma\) cannot be underestimated. Moreover, human neutrophil-derived IFN\(\gamma\) has not been characterized in the context of host-pathogen interactions, specifically pathogenic bacteria such as \textit{S. aureus}. This idea of elevated neutrophil-derived IFN\(\gamma\) during host-pathogen interactions is highlighted by multiple studies demonstrating that mouse neutrophils produce IFN\(\gamma\) during infection with diverse bacterial pathogens.
Murine Neutrophils Produce IFNy During Bacterial Disease

Mouse neutrophils began to be suspected as a source of IFNy, when it was observed that IFNy was being robustly produced during early bacterial infection and this correlated with peak neutrophil recruitment\textsuperscript{221}. Similar observations have been made during infection with diverse bacterial pathogens and several studies have strongly demonstrated that mouse neutrophils produce IFNy during early inflammation\textsuperscript{221-225}. Using a mouse pulmonary model of Nocardia asteroides disease, Ellis et al showed that recruited neutrophils express IFNy within 24 hours of infection\textsuperscript{221}. This observation was supported by a later study demonstrating that neutrophils recruited to the lungs produce IFNy within 24 hours following pulmonary infection with Streptococcus pneumoniae or S. aureus\textsuperscript{225}. Mouse neutrophil-derived IFNy is not exclusive to bacterial infections of the lungs, as Kirby et al demonstrated neutrophils as an early source of IFNy following oral challenge with Salmonella typhimurium\textsuperscript{223}. Additionally, mouse intradermal infection with Francisella tularensis showed that neutrophils are capable of producing IFNy at distal sites to infection, including the spleen and liver\textsuperscript{224}. Finally, an intravenous infection model of Listeria monocytogenes disease yielded neutrophils as the primary source of early IFNy\textsuperscript{222}. Taken together, these studies clearly show that neutrophils have been overlooked as being mediators of additional immune responses and pathways, specifically retaining a capacity for IFNy expression, and are not end-point phagocytes
with restricted inflammatory function. However, the influence of neutrophils producing IFNγ on immunity and overall pathogenesis during infection remains to be elucidated.

A Conflicting Role for IFNγ in *Staphylococcus aureus* Disease

IFNγ has been used as an immuno-modulatory agent for combating *S. aureus* disease for many years. Currently, CGD patients receive IFNγ treatment and these individuals show an increase in protection against several bacterial pathogens\(^2\). However, a role for IFNγ in treating acute stage *S. aureus* infections in healthy individuals has not been clearly defined, either in humans or in mouse models of disease. The following sections outline current knowledge regarding IFNγ in impacting immunity against *S. aureus* infections.

**CGD Patients and Human Cell Line Studies**

Most notably, from a clinical standpoint, CGD patients receiving recombinant human IFNγ protein display a marked decrease in *S. aureus* infections\(^2\). It was initially thought that recombinant IFNγ treatment enhanced the neutrophil NADPH oxidase function in CGD patients; however, a later study showed no change in NADPH oxidase activity in CGD patient-derived neutrophils treated with IFNγ\(^2\). The defined mechanism behind the protection conferred to CGD patients, via IFNγ treatment, is currently unknown. However, additional *in vitro* studies using human cell lines point to a beneficial role for IFNγ. Cultured human endothelial cells show reduced intracellular bacterial loads and increased overall
cellular integrity following treatment with IFNγ and subsequent S. aureus challenge\textsuperscript{228}. Additionally, cultured human lung bronchus epithelial cells and human type II alveolar cells restricted the growth of S. aureus following IFNγ treatment\textsuperscript{229}. IFNγ enhances human monocyte-derived macrophage killing of intracellular S. aureus when combined with triple antibiotic treatment\textsuperscript{230}. Human lung epithelial cell line A549 and monocytic cell line THP-1 treated with IFNγ increased these cells resistance to α-hemolysin-mediated cell membrane permeability and death, suggesting IFNγ enhances cellular defenses against S. aureus pore-forming toxins\textsuperscript{231}. Using a human whole blood model, IFNγ increased S. aureus killing\textsuperscript{232}. Moreover, priming of primary human neutrophils with IFNγ increased the killing of S. aureus\textsuperscript{233}. Taken together, IFNγ treatment/priming increases the anti-S. aureus activity of numerous human innate effector cells as well as endothelial cells. However, using mouse models of S. aureus infection and toxin-mediated pathogenesis, a role for IFNγ in mediating immunity is more controversial.

**Mouse Studies**

Using mouse models of disease can provide key insights into both immunological mechanisms as well as characterizing pathogen-mediated virulence. However, direct analogous comparison with these defined mechanisms to humans can fall substantially short, as the two mammalian species can differ dramatically in their respective immune responses. Such differences include leukocyte subset balance, defensin expression, TLR
expression, nitric oxide (NO) synthesis and overall cytokine profiles during inflammation\textsuperscript{234}. However, regardless of differences, mouse models of disease repeatedly yield foundational knowledge, and often times, analogous pathways in humans are still identified. The following describes current knowledge of IFN\textgreek{y} in mediating immunity during murine \textit{S. aureus} disease. Interestingly, these findings using mouse models have indicated both protective and detrimental roles for this pro-inflammatory cytokine.

As described above, \textit{S. aureus}-derived enterotoxins/superantigens play an important role during staphylococcal disease and many studies demonstrate that IFN\textgreek{y} significantly impacts these pathologies. Exposure to the \textit{S. aureus} enterotoxin SEB causes weight loss and the onset of hypoglycemia\textsuperscript{235}. However, neutralization of IFN\textgreek{y} is shown to abrogate both of these conditions\textsuperscript{235}. This is supported by another study demonstrating that IFN\textgreek{y}-deficient mice are protected against lethal challenge with SEB\textsuperscript{236}. SEA elicits CD8 T cells to produce IFN\textgreek{y} during pulmonary challenge and this is associated with severe lung pathology\textsuperscript{237}. However, in this same model, depletion of CD8 T cells reduced IFN\textgreek{y} levels and attenuated the toxin-mediated pathology\textsuperscript{237}. Again, this is supported using IFN\textgreek{y}-deficient mice that display marked protection during pulmonary challenge with SEA\textsuperscript{237}. Put together, these studies strongly indicate that IFN\textgreek{y} exacerbates staphylococcal enterotoxin-mediated pathogenesis, however, when using live \textit{S. aureus}, the role for IFN\textgreek{y} is more conflicted.
Consistent with findings demonstrating that IFNγ enhances toxin-mediated virulence, numerous reports have suggested IFNγ plays a deleterious role during infection with live *S. aureus*. Neutralization of IFNγ is shown to increase mouse survival rates in a lethal *S. aureus* bacteremia model\(^{238}\). This observation is supported by another study showing that IFNγ receptor-deficient mice are protected against late-stage staphylococcal-mediated lethality\(^{239}\). Additionally, during *S. aureus* bacteremia, IFNγ-deficient mice exhibit increased survival, reduced bacterial loads and attenuated overall tissue pathology\(^{240}\). Other groups have suggested that IFNγ also plays a deleterious role by showing increased *S. aureus* colonization rates, increased bacterial burdens and increased mortality that correlated strongly with elevated IFNγ expression\(^{200,241,242}\). Finally, using a murine surgical wound model of *S. aureus* infection, IFNγ-deficient mice display reduced *S. aureus* burdens at the infectious foci\(^{51}\). Put together, these reports elucidate a negative, seemingly deleterious role for IFNγ in mediating immunity, specifically in the context of pathogen clearance, promoting sepsis and toxin-mediated pathogenesis. However, other studies have suggested a protective role for IFNγ in mediating protection against *S. aureus* disease.

Consistent with studies using human cell lines demonstrating a protective role for IFNγ, leukocytes from mice treated with recombinant IFNγ displayed enhanced rolling and adhesion qualities, suggesting IFNγ mediates leukocyte trafficking and transmigration to infected tissues\(^{243}\). IFNγ has been shown to induce nitric oxide (NO) production in mice and that IFNγ-mediated NO
production is protective against lethal *S. aureus* challenge\textsuperscript{244}. Additionally, a role for IFN\(\gamma\) in facilitating adaptive immunity against *S. aureus* had been proposed. Investigators administered a nonlethal *S. aureus* challenge to mice followed later by a lethal challenge, in which normal healthy mice survived, but IFN\(\gamma\)-deficient mice did not survive\textsuperscript{245}. Consistent with this, recombinant IFN\(\gamma\) treatment reduced murine lethality and reduced bacterial burdens during *S. aureus* bacteremia\textsuperscript{246}. This same study also showed that mAb neutralization did not impact mouse survival, but did abrogate IFN\(\gamma\)-mediated *S. aureus*-derived arthritis\textsuperscript{246}. Put together, these studies indicate a protective role for IFN\(\gamma\) for mediating immunity against *S. aureus* invasive disease.

Collectively, using mice to characterize the impact of IFN\(\gamma\) in mediating *S. aureus* pathogenesis and immunity produces conflicting observations, even in redundant models. Using *S. aureus* toxins, IFN\(\gamma\) appears to play a deleterious role and exacerbate conditions associated with toxin-mediated pathology\textsuperscript{235,236}. However, using viable (live) *S. aureus* in murine models of disease, characterization of IFN\(\gamma\) produces inconsistent and sometimes contradicting observations. IFN\(\gamma\) and its role in mediating *S. aureus* disease is likely dependent on multiple factors, including strain of *S. aureus* studied (some strains produce more and/or different toxins than others), mouse strain used (inflammatory responses vary between mouse strains), infectious dose, type of infection (i.e. skin, pulmonary, etc), and the parameters measured to indicate the degree of pathogenesis. It is apparent that further comprehensive studies are warranted to clearly outline a role for IFN\(\gamma\) in *S.*
*aureus* disease, although defining an over-arching universal role may remain elusive.
CHAPTER THREE

THE SAER/S GENE REGULATORY SYSTEM INDUCES A PRO-INFLAMMATORY CYTOKINE RESPONSE DURING STAPHYLOCOCCUS AUREUS INFECTION

Contribution of Authors and Co-Authors

Manuscripts in Chapters 3, 4, and 5

Author: Robert L Watkins
Contributions: Conceived the study, designed and performed experiments, collected and analyzed data, and wrote manuscript.

Co-author: Kyler B Pallister
Contributions: Assisted with experiments and analyzed data.

Senior Co-author: Jovanka M Voyich
Contributions: Conceived the study, edited manuscript and provided financial support.
Abstract

Community-associated methicillin-resistant \textit{Staphylococcus aureus} accounts for a large portion of the increased staphylococcal disease incidence and can cause illness ranging from mild skin infections to rapidly fatal sepsis syndromes. Currently, we have limited understanding of \textit{S. aureus}-derived mechanisms contributing to bacterial pathogenesis and host inflammation during invasive staphylococcal disease. Herein, we characterize an influential role for the \textit{saeR/S} two-component gene regulatory system in mediating cytokine induction using mouse models of \textit{S. aureus} pathogenesis. Invasive \textit{S. aureus} infection induced the production of localized and systemic pro-inflammatory cytokines, including tumor necrosis factor alpha (TNF-\(\alpha\)), interferon gamma (IFN-\(\gamma\)), interleukin (IL)-6 and IL-2. In contrast, mice infected with an isogenic \textit{saeR/S} deletion mutant demonstrated significantly reduced pro-inflammatory cytokine levels. Additionally, secreted factors influenced by \textit{saeR/S} elicited pro-inflammatory cytokines in human blood \textit{ex vivo}. Our study demonstrated robust \textit{saeR/S}-mediated IFN-\(\gamma\) production during both invasive and subcutaneous skin infections. Results also indicated a critical role for \textit{saeR/S} in promoting bacterial survival and enhancing host mortality during \textit{S. aureus} peritonitis. Taken together, this study provides insight into specific mechanisms used by \textit{S. aureus} during staphylococcal disease and characterizes a relationship between a bacterial regulator of virulence and the production of pro-inflammatory mediators.
**Introduction**

*Staphylococcus aureus* predominates as a global cause of bacterial infections, which can range from mild skin irritations to severe life-threatening invasive disease\(^7\). Increases in reported cases of methicillin-resistant *S. aureus* infections (MRSA) including community-associated MRSA (CA-MRSA) infections that occur in otherwise healthy individuals, independent of hospital settings\(^{162,247,248}\), are an important public health concern. Generally associated with soft tissue infections, *S. aureus* disease is also associated with such severe conditions as septicemia, necrotizing pneumonia and necrotizing fasciitis\(^{247,249-252}\). In 2005, the United States reported over 18,000 deaths resulting from invasive MRSA disease, a number surpassing the annual fatalities associated with HIV/AIDS\(^{253,254}\).

Gram-positive bacterial infections account for \(\sim 50\%\) of all reported sepsis cases and are associated with the dysfunctional production of pro-inflammatory cytokines\(^{255-258}\). Systemic *S. aureus* infections are associated with the endogenous production of interferon gamma (IFN-\(\gamma\)), tumor necrosis factor alpha (TNF-\(\alpha\)) and interleukin (IL)-6\(^{238,244}\). Additionally, innate immune effectors exhibit severely diminished anti-bacterial function during sepsis and *S. aureus* infections. However, studies characterizing pathogen-derived mediators of the host inflammatory response have predominately focused on single toxins and proteins, rendering the inflammatory modulating impacts of global virulence regulators relatively undefined.
S. aureus possesses 16 two-component gene-regulatory systems that monitor changing environmental conditions to influence gene transcription. Numerous studies have indicated a regulatory role for the S. aureus two-component system SaeR/S in the expression of secreted virulence factors. Previous findings have demonstrated a critical role for saeR/S in evading destruction by neutrophils and enhancing mortality in murine bacteremic models. However, significant gaps in our understanding of how saeR/S contributes to S. aureus pathogenesis exist. To that end, we investigated the influence of saeR/S on pathogen survival and the host response during invasive disease and demonstrated that saeR/S strongly influenced the production of inflammatory cytokines during S. aureus infection. These current findings further support the hypothesis that saeR/S is a critical mediator of pathogenesis during staphylococcal disease.

Results

SaeR/S Significantly Enhances S. aureus Survival, Dissemination and Mortality During Invasive Disease

Invasive staphylococcal disease is associated with bacterial persistence and dissemination to deep tissues. To investigate the effects of saeR/S on bacterial survival and dissemination during invasive infection, we used a mouse model of S. aureus peritonitis via intraperitoneal (i.p.) inoculation with wild-type (MW2) or S. aureus with deleted saeR/S (MW2ΔsaeR/S) (figure 1). At 4 hours post-infection, saeR/S did not impact S. aureus survival in the
peritoneum, as both MW2 and MW2ΔsaeR/S-infected mice exhibited similar bacterial loads ($P > 0.05$; figure 1A). However, at 10 hours post-infection, saeR/S-regulated factors significantly increased $S. aureus$ survival in the peritoneum of MW2-infected mice ($P < 0.0001$; figure 1B). To further investigate the contributions of saeR/S on $S. aureus$ tissue infiltration, we harvested both kidneys and enumerated $S. aureus$ burdens at 10 hours post-
infection. Mice infected with MW2ΔsaeR/S had significantly less colony forming units (cfu) in the kidneys (P < 0.0001; figure 1C), suggesting a role for saeR/S in pathogen dissemination. To characterize the influence of saeR/S on S. aureus dissemination from the infectious foci, we exsanguinated mice and harvested hearts for additional S. aureus quantification. Consistent with peritoneal cavity and kidney burdens, the absence of saeR/S significantly reduced bacterial loads in the hearts from MW2ΔsaeR/S-infected mice (P < 0.01; figure 1D). Surprisingly, both groups exhibited similar S. aureus burdens in the blood (P > 0.05; figure 1E). This suggests a transient presence of S. aureus in the blood following peritonitis induction. Consistent with observations of the influence of saeR/S on murine mortality during S. aureus bacteremia\textsuperscript{12,63}, saeR/S-regulated factors significantly enhanced morbidity and mortality in the peritonitis model (1 mouse infected with MW2 survived the 72 hours time course, compared to 8 mice infected with MW2ΔsaeR/S; P < 0.001; figure 1F). These results are congruent with previous observations that saeR/S contributes to host mortality\textsuperscript{12,63} and demonstrates saeR/S is essential for S. aureus survival and dissemination following invasive infection.

SaeR/S Promotes Pro-Inflammatory Cytokine Gene Transcription

Sepsis syndromes are commonly associated with an early rapid induction of pro-inflammatory cytokines\textsuperscript{257}. Following i.p. infection with MW2 and MW2ΔsaeR/S, we investigated the influence of saeR/S-regulated factors on the transcriptional regulation of 84 host-derived inflammation-associated
genes in leukocytes isolated from the peritoneal cavity (Tables 1, S1 and S2), 4 hours post-inoculation, when the bacterial recoveries between MW2 and MW2ΔsaeR/S were virtually equal (figure 1A). Deletion of saeR/S resulted in a down-regulation of key inflammatory cytokines (Tables 1 and S1). Of the 84 assayed genes, 47 were down-regulated ≥ 3-fold with only 6 genes up-regulated ≥ 3-fold, MW2ΔsaeR/S relative to MW2 (Tables 1 and S1). Several pro-inflammatory genes commonly expressed during early sepsis were down-regulated in MW2ΔsaeR/S-infected mice compared to MW2-infected mice, including: IFN-γ: -11.27-fold (P ≤ 0.05), TNF: -9.37-fold (P > 0.10), IL-18: -7.17 (P ≤ 0.01), CD40 ligand: -4.92 (P ≤ 0.01), IL-1β: -4.07 (P ≤ 0.05) and C-reactive protein: -4.43 (P ≤ 0.05) (Tables 1 and S1). Interestingly, transcription of anti-inflammatory cytokine genes were relatively unaffected with the exception of IL1R1I, which was up-regulated 21.38-fold, MW2ΔsaeR/S

<table>
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Table 1. SaeR/S-mediated factors elicit host pro-inflammatory transcription during invasive disease. NOTE: Genes listed display fold-regulation values of MW2ΔsaeR/S-infected mice relative to MW2-infected mice (3 per group). RNA was collected from all leukocytes isolated from the peritoneum, 4 hours post-infection. Fold-regulation and P values calculated using SA Biosciences™ web-based software utilizing the ΔΔCt method.
relative to MW2 (Tables S1). Both MW2 and MW2ΔsaeR/S-infected mice displayed up-regulation of cytokine transcription compared to PBS-inoculated control mice (Table S2). These results suggest differences in transcriptional activity stem from saeR/S-regulated factors, and indicate a saeR/S-mediated pro-inflammatory cytokine response during S. aureus infection.

SaeR/S-Regulated Factors Promote Pro-Inflammatory Cytokines in the Blood During Invasive Disease

Sepsis and septic shock are associated with the systemic production of inflammatory cytokines\(^\text{255-258}\). To determine if the saeR/S-mediated inflammatory response was systemic during S. aureus peritonitis, we infected mice i.p. with 5 x 10\(^7\) cfu of MW2 or MW2ΔsaeR/S for 10 hours and measured protein levels of several pro- and anti-inflammatory cytokines in the serum by cytometric bead array (figure 2). Of note, serum IFN-γ and TNF levels were significantly reduced in mice infected with MW2ΔsaeR/S (\(P < 0.05\) and \(P < 0.01\), respectively; figures 2A and 2B). Additional pro-inflammatory cytokines associated with sepsis syndromes were influenced by saeR/S as serum IL-6 and IL-2 concentrations were significantly lower in MW2ΔsaeR/S-infected mice (\(P < 0.01\); figure 2C and 2D). Serum IL-17A levels were also significantly reduced in mice infected with MW2ΔsaeR/S (\(P < 0.01\); figure 2E). In contrast, IL-4 and IL-10 serum concentrations did not exhibit differences between MW2 and MW2ΔsaeR/S-infected groups (\(P > 0.05\); figures 2F and 2G). These results are consistent with previous sepsis syndrome observations, in which an
early pro-inflammatory cytokine response (IFN-γ, TNF, IL-2, and IL-6) was concomitant with a relatively subdued anti-inflammatory cytokine (IL-4 and IL-10) response\textsuperscript{257,258}. The differentially regulated transcriptional activity in the peritoneum (Tables 1 and S1) and the polarized pro-inflammatory cytokine response in the blood (figure 2) indicate that saeR/S-regulated factors promote localized and systemic inflammation during invasive disease.
Secreted Factors, Regulated by \textit{saeR/S}, Significantly Enhance the Production of Pro-Inflammatory Proteins in Human Whole Blood

Numerous secreted \textit{S. aureus} virulence factors are regulated by \textit{saeR/S} \cite{62,63,66-68,260-263}. To investigate the concerted role of these exoproteins on the production of cytokines in human blood and to confirm the \textit{saeR/S}-mediated production of IFN-\(\gamma\), TNF, IL-6, IL-2 and IL-17A observed in mice (figure 2 and Table 1), we treated human whole blood with supernatants from cultures of MW2, MW2\textDelta{saeR/S} and a \textit{saeR/S}-complemented MW2\textDelta{saeR/S} strain (MW2\textDelta{saeR/S}\text{comp}) and compared plasma cytokine levels (figure 3). The data shown are normalized for each individual donor to account for large variations in the magnitude of cytokine expression between individuals. Consistent with cytokine profiles observed in our mouse studies, MW2-treated human blood produced significantly higher levels of IFN-\(\gamma\) and TNF compared to MW2\textDelta{saeR/S}-treated blood \((P < 0.05; \text{figures } 3A \text{ and } 3B)\). Treatment with MW2\textDelta{saeR/S}\text{comp} supernatant restored the observed IFN-\(\gamma\) phenotype and partially restored the TNF phenotype (figures 3A and 3B, respectively). IL-2 and IL-6 levels were also significantly elevated in the MW2 treatment groups \((P < 0.05; \text{figures } 3C \text{ and } 4D, \text{respectively})\), whereas IL-17A was not significantly influenced by supernatants from treatment groups (data not shown). Control groups (media-treated and no treatment) did not result in substantial production of the assayed proteins (data not shown). These
results demonstrate that saeR/S-regulated secreted factors stimulate the production of pro-inflammatory cytokines in human blood.
The Absence of SaeR/S Significantly Attenuates Production of Localized IFN-γ and TNF-α

To further investigate the role of saeR/S-mediated factors on the production of IFN-γ and TNF-α at the infectious foci, we measured protein levels in the peritoneal exudates following i.p. inoculation with MW2 or MW2ΔsaeR/S. At 10 hours post-infection, IFN-γ was significantly reduced in mice infected with MW2ΔsaeR/S compared to MW2, following i.p. inoculation with 5 x 10^7 cfu (P < 0.001; figure 4B). Significant increases in IFN-γ production in MW2 compared to MW2ΔsaeR/S were also observed using a ten-fold decrease in i.p. inoculum (5 x 10^6 cfu; P < 0.05; figure 4B). Of note, mice infected with 5 x 10^6 MW2 cfu produced significantly higher IFN-γ concentrations compared to mice infected with 5 x 10^7 MW2ΔsaeR/S cfu (P <
0.01; figure 4B). This demonstrates that differences in bacterial burden (figure 4A) do not account for the observed decrease in IFN-γ in MW2ΔsaeR/S-infected mice and that the robust IFN-γ production is saeR/S-mediated. TNF-α protein was also significantly elevated in mice infected with MW2 compared to MW2ΔsaeR/S, for both $5 \times 10^7$ and $5 \times 10^6$ cfu inoculums ($P < 0.01$; figure 2C).

Collectively, these findings demonstrate that saeR/S-regulated factors elicit the production of IFN-γ and TNF-α at the infectious foci during invasive *S. aureus* infection.

SaeR/S Promotes IFN-γ During USA300 Skin Infection

*S. aureus* pulsed-field gel electrophoresis type USA300 (LAC) is a major cause of CA-MRSA skin infections. To investigate if saeR/S promotes IFN-γ during skin infection and to confirm our previous observation that IFN-γ production is influenced by saeR/S-regulated factors, we infected mice subcutaneously with LAC and an isogenic deletion mutant of saeR/S (LACΔsaeR/S). At 8 hours post-infection, the deletion of saeR/S did not impact bacterial load at the site of infection (Figure 5A). These findings are consistent with our previous studies demonstrating saeR/S does not significantly influence abscess size or *S. aureus* burden during early skin infection (i.e. less than two days). However, saeR/S did promote a significant increase in IFN-γ in LAC-infected mice compared to LACΔsaeR/S-infected mice ($P < 0.05$; Figure 5B). IFN-γ concentrations in un-infected mouse skin tissues were undetectable (Figure 5B). These data are consistent
with our observations of enhanced IFN-γ during peritonitis (figure 4) and are in further support that robust IFN-γ production observed during S. aureus disease is saeR/S-mediated.

**Discussion**

In the current study, we found that absence of saeR/S significantly decreased the localized and systemic production of pro-inflammatory mediators (i.e. TNF-α, IFN-γ, IL-1β, IL-2, IL-6 and IL-17A) during invasive staphylococcal disease (Tables 1, S1 and figures 2-4). We also observed that robust IFN-γ production was saeR/S-mediated during both S. aureus peritonitis and superficial skin infections (Table 1 and figures 4 and 5). Significantly elevated systemic pro-inflammatory cytokines coupled with the
onset of mortality in wild-type-infected mice strongly indicate that saeR/S is critical for mediating sepsis, a phenotype absent in mutant-infected groups. This conclusion is supported by the ‘cytokine storm’ hypothesis, a phenomena characterized by a rapid pro-inflammatory cytokine response that correlates very strongly with coagulation dysfunction, organ failure and death\textsuperscript{264,265}. Our observation demonstrating both pro-inflammatory transcript abundance and protein concentration as significantly elevated in MW2-infected groups (figures 2-4 and Tables 1 and S1), when bacterial burdens are virtually equal at the sites of inflammation (figures 1A, 1E and 4A), suggest pro-inflammatory cytokine production stems from saeR/S-regulated factors. This idea is further supported by our data demonstrating IFN-γ production is significantly reduced in mice infected with MW2ΔsaeR/S compared to MW2, even when the bacterial burden in mutant S. aureus-infected mice is significantly increased (~10-fold) over wild-type S. aureus-infected mice (figures 4A and 4B). These data suggest factors regulated by saeR/S are responsible for the robust IFN-γ response observed during S. aureus infection. However, additional studies are needed to define the contribution of other S. aureus global regulators of virulence in mediating the production of IFN-γ and other pro-inflammatory cytokines.

During S. aureus infection, the role of IFN-γ has been disputed with studies demonstrating that this inflammatory mediator plays either protective or deleterious roles. For example, using a surgical wound model, McLoughlin et al\textsuperscript{51} reported that in the absence of IFN-γ, a decreased S. aureus burden
was observed at the site of infection. Zhao et al\textsuperscript{246} observed that monoclonal antibody-neutralization of IFN-γ decreased the frequency and severity of \textit{S. aureus}-mediated arthritis. Using IFN-γ-deficient mice, Sasaki et al\textsuperscript{240} reported a decrease in \textit{S. aureus} burden and an increase in survival rates using a bacteremic model of infection. Conversely, others have demonstrated that administration of exogenous IFN-γ decreased mouse mortality and reduced bacterial loads following \textit{S. aureus} bacteremia\textsuperscript{246,266}. Our current findings correlate increased IFN-γ with elevated bacterial burdens and increased morbidity and mortality. However, it is likely that the observed effects of IFN-γ during \textit{S. aureus} disease are dependent upon multiple factors, including strain of \textit{S. aureus} studied and type/route of infection. Clearly, additional studies are necessary to characterize the precise role of IFN-γ as a mediator of \textit{S. aureus} pathogenesis.

\textit{S. aureus} produces several factors that have been implicated in pro-inflammation, including superantigens and exotoxins\textsuperscript{64}. Superantigens non-specifically bind the major histocompatibility complex type II (MHCII) of antigen-presenting cells to T-cell receptors, causing massive T-cell activation and release of pro-inflammatory cytokines\textsuperscript{265}. SaeR/S regulates several of these factors\textsuperscript{12,63,71}. For example, Pantrangi et al\textsuperscript{71} showed saeR/S to positively regulate staphylococcal superantigen-like genes ssl5 and ssl8. Staphylococcal enterotoxin C exhibits a superantigenic phenotype and is also regulated by saeR/S\textsuperscript{12,267}. SaeR/S also regulates exotoxin/cytolysin production (i.e. \textit{hla} and \textit{hlg}) and these factors elicit pro-inflammatory cytokine
Finally, our previous study showed that *saeR/S*-regulated factors significantly enhanced the lysis of neutrophils\textsuperscript{12}. Cellular lysis promotes the release of host-derived intracellular components and peripheral leukocytes may recognize these danger-associated molecular patterns (DAMPs) to further perpetuate the pro-inflammatory response\textsuperscript{268}. Thus, *saeR/S* regulates a full repertoire of factors capable of eliciting a dysfunctional pro-inflammatory cytokine response, an essential mechanism of sepsis.

In addition to *saeR/S*, other *S. aureus* regulatory systems play pivotal roles in virulence and inflammation during infection. Both *agr* and *sarA* are essential for full virulence during invasive *S. aureus* infection\textsuperscript{269,270}. Heyer et al\textsuperscript{271} explored the role of *agr*- and *sarA*-mediated cytokine production in a murine model of *S. aureus* pneumonia and reported neither regulator was required for the production of IL-8, a potent pro-inflammatory mediator of neutrophil recruitment. However, both *agr* and *sarA* did promote granulocyte-macrophage colony-stimulating factor (GM-CSF), an inflammatory factor implicated in global pro-inflammatory responses during invasive infection\textsuperscript{271}. Furthermore, *agr* has been shown to elicit cytokines and chemokines, important in leukocyte trafficking, from endothelial cells\textsuperscript{272}. Taken together with our current report, these studies support the hypothesis that a robust host inflammatory response results from pathogen-derived factors under the influence of global regulators. However, more studies are needed to indentify the specific *S. aureus* factors responsible for the pro-inflammatory response.
In the current study, we report key pro-inflammatory cytokines, associated with sepsis syndromes, as being induced in response to factors regulated by the *S. aureus* two-component gene regulatory system, *saeR/S*. We hypothesize that *saeR/S* plays a critical role in *S. aureus* pathogenesis during invasive disease, likely through a synergistic mechanism of innate immune evasion\textsuperscript{12} and the initiation of potentially dysfunctional host inflammatory pathways. This study provides a foundation for future work to identify the individual contributions of specific *saeR/S*-regulated factors to the host inflammatory response during staphylococcal disease.

**Materials and Methods**

**Bacterial Strains and Culture**

*S. aureus* isolates, pulsed-field gel electrophoresis type USA400 (MW2) and pulsed-field gel electrophoresis type USA300 (LAC), were selected based on clinical relevance\textsuperscript{11,12,63,249-255,273-276}. *S. aureus* was cultured in tryptic soy broth (TSB) supplemented with 0.5% glucose and harvested as described elsewhere\textsuperscript{11}. MW2, MW2\textit{saeR/S} and MW2\textit{ΔsaeR/S}comp were generated in prior investigations\textsuperscript{12}. LAC and LAC\textit{ΔsaeR/S} were generated in prior investigations\textsuperscript{63}.

**Mouse Infection Models**

All animal studies were performed in accordance with the National Institutes of Health guidelines and approved by the Animal Care and Use
Committee at Montana State University-Bozeman. For the peritonitis model, male and female BALB/c mice (aged 8-10 weeks) were purchased from commercial sources and the Montana State University Animal Resource Center. *S. aureus* was harvested at mid-exponential growth phase, washed in sterile Dulbecco’s phosphate buffered saline (DPBS) and re-suspended in sterile DPBS at a concentration of $5 \times 10^6$ or $5 \times 10^7$ cells per 100 µl. All mice were inoculated via intraperitoneal route (i.p.) with MW2 or MW2ΔsaeR/S and control mice received sterile DPBS.

Bacterial burdens were determined as follows: to enumerate *S. aureus* in the peritoneum, the peritoneal cavity was washed with 10 ml sterile HANKs’ balanced salt solution using an 18 gauge needle and 10 ml syringe. Exudate was diluted in distilled water (dH$_2$O) and plated on tryptic soy agar (TSA) plates for enumeration of colony forming units (cfu). To determine *S. aureus* load in the blood, mice were anesthetized in isoflurane then exsanguinated via the axillary vessels. Blood was diluted in dH$_2$O and plated on TSA. To determine *S. aureus* burden in selected organs, hearts and both kidneys were aseptically removed, washed in dH$_2$O and then homogenized in dH$_2$O. Homogenates were diluted in dH$_2$O and plated on TSA. TSA plates were incubated overnight in 37°C; 5% CO$_2$ and cfu counted the following day.

The survival study was performed by inoculating mice i.p. with $5 \times 10^7$ *S. aureus*. Mice were monitored every 2 hours for 48 hours and then every 4 hours for an additional 24 hours. Mice were euthanized if they became
immobile, exhibited labored breathing or were unable to eat or drink. Survival statistics were performed using a log-rank test.

Skin infection models were performed as described elsewhere\textsuperscript{12,63}. Crl;SKH1-hrBR hairless mice (Charles River) were inoculated subcutaneously with $1 \times 10^7$ bacteria. Eight hours post-bacterial inoculation, the infected skin area was excised using a 9 mm diameter “punch.” Tissues were homogenized in sterile DPBS for bacterial enumeration and cytokine measurements by ELISA per the manufacturer’s instructions (R&D Systems).

**Mouse Inflammatory Gene Expression**

To compare host inflammatory transcript levels, mice were inoculated i.p. with $5 \times 10^7$ MW2 or MW2ΔsaeR/S or control DPBS for 4 hours. Peritoneal cavities were washed as described above, and the exudate was centrifuged for 5 min at 600 x g. Pellets were resuspended in RLT lysing buffer (Qiagen) and RNA was purified using RNeasy kits as described by the manufacturer (Qiagen). Contaminating DNA was digested on-column using DNase (Qiagen). Complementary DNA (cDNA) was synthesized using ~200 ng purified RNA and C-03 RT\textsuperscript{2} First Strand Kit (SA Biosciences). Detection of cDNA was performed using RT\textsuperscript{2} Real-time\textsuperscript{TM} SYBR Green/ROX PCR master mix (SA Biosciences). Master mix with cDNA was loaded onto the Mouse Inflammatory Cytokines and Receptors RT\textsuperscript{2} Profiler\textsuperscript{TM} PCR Array (SA Biosciences). Real-time PCR was performed using a 7500 Fast Real-time PCR system with Fast Real-time PCR system software v1.4.0 (Applied
Biosystems). Calculated threshold ($C_t$) values were uploaded to the manufacturer’s website (SA Biosciences; http://www.sabiosciences.com/pcr/arrayanalysis.php) for analysis using the $\Delta\Delta C_t$ method. Fold-regulation and $P$ values were calculated by SA Biosciences software (SA Biosciences; http://www.sabiosciences.com). Gene analyses are displayed as fold-regulation values of MW2ΔsaeR/S-infected mice relative to MW2-infected mice (Tables 1 and S1) and MW2 or MW2ΔsaeR/S-infected mice relative to DPBS-treated mice (Table S2).

**Mouse Cytokine Assays**

TNF-$\alpha$ and IFN-$\gamma$ concentrations in peritoneal exudates were measured using sandwich ELISA per the manufacturer’s instructions (R&D Systems). Mouse blood was collected as described above, and mouse serum was separated from whole blood by centrifugation. Protein levels of IFN-$\gamma$, TNF, IL-6, IL-2, IL-17a, IL-4 and IL-10 were measured using mouse Th1/Th2/Th17 Cytometric Bead Arrays per the manufacturer’s instructions (BD Biosciences). All results are displayed as mean concentrations ± SEM (pg/ml).

**Cytokine Expression in Human Whole Blood**

Heparinzed venous human whole blood was collected from healthy individuals in accordance with an approved protocol by the Montana State University Institutional Review Board. Donors provided written consent to participate in the study. Overnight S. aureus cultures were inoculated in TSB at a ratio of 1:100 and allowed to incubate for 6 hours to early stationary
growth phase. At this time, no differences in bacterial growth exist between S. 
_aureus_ strains (data not shown)\(^\text{12}\). Ten ml of _S. aureus_ cell suspension was
pelleted by centrifugation, and 1 ml of supernatant was sterile filtered using
0.22 µm syringe filters. Filtrate was plated to ensure the absence of _S. aureus_
(data not shown). Filtered _S. aureus_ supernatant was incubated with 1 ml
human blood at final ratios of 1:8, 1:16 and 1:32 using an end-over-end
apparatus (Heto Rotamix RK) at 20 RPM for 3 hours at 37°C and 10% CO\(_2\).
Plasma was isolated by centrifugation and stored at -80°C until assayed for
protein concentration using human Th1/Th2/Th17 Cytometric Bead Arrays per
the manufacturer’s instructions (BD Biosciences).

**Statistical Analyses**

All data sets were analyzed using GraphPad Prism, version 4.0c for
Macintosh (GraphPad Software, San Diego, CA). All mouse data sets were
analyzed using paired t tests (see figure legends). Human blood data sets
were analyzed using one-way analysis of variance (ANOVA) with Tukey’s
post-test. For bar graphs, error bars represent the standard error of the mean.
Mouse survival statistics were analyzed using the log-rank test.

**Supplemental Material**

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### Table S1. SaeR/S promotes inflammatory cytokine gene transcription.

**NOTE.** Genes listed display fold-regulation values of MW2ΔsaeR/S-infected mice relative to MW2-infected mice (3 per group). RNA was collected from all cells washed from the peritoneum, 4 hrs post-infection. Fold-regulation values and *P* values calculated using SA Biosciences™ web-based software utilizing the ΔΔCt method.

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<tr>
<th>Gene Symbol</th>
<th>Encoded protein</th>
<th>MW2 Fold-regulation change</th>
<th>MW2ΔsaeR/S Fold-regulation change</th>
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<th><em>P</em> value</th>
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<td>Chemokine (C motif) receptor 1</td>
<td>8.9 &gt;0.10</td>
<td>3.07 &gt;0.10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table S2. Invasive S. aureus infection promotes inflammatory gene transcription. NOTE. Genes listed display fold-regulation values of MW2 and MW2ΔsaeR/S-infected mice relative to PBS-treated mice (3 per group). RNA was collected from all cells washed from the peritoneum, 4 hrs post-infection. Fold-regulation values and P values calculated using SA Biosciences™ web-based software utilizing the ΔΔCt method.
Acknowledgements

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CHAPTER FOUR

THE SAER/S TWO-COMPONENT SYSTEM PROMOTES INTERFERON-GAMMA IN NEUTROPHILS DURING INVASIVE STAPHYLOCOCCUS AUREUS INFECTION

Contribution of Authors and Co-Authors

Manuscripts in Chapters 3, 4, and 5

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Contributions: Conceived the study, designed and performed experiments, collected and analyzed data, and wrote manuscript.

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Manuscript Information Page

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*Microbes and Infection*

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Abstract

*Staphylococcus aureus* (*S. aureus*) is a major pathogen that causes diverse infections. Invasive disease is associated with neutrophil activity and production of pro-inflammatory cytokines, including interferon-gamma (IFN\(\gamma\)). Herein, we identify neutrophils as the predominant source of IFN\(\gamma\) during *S. aureus* peritonitis and demonstrate the SaeR/S two-component system as a significant inducer of IFN\(\gamma\) in these phagocytes. We further show IFN\(\gamma\)-deficient mice exhibit enhanced bacterial clearance and reduced host cell cytotoxicity against wild-type *S. aureus* infection, but not against infection with mutant *S. aureus* lacking saeR/S. Collectively, our findings suggest that saeR/S-mediated IFN\(\gamma\) expression in neutrophils diminishes innate anti-bacterial mechanisms against *S. aureus*. 
**Introduction**

*Staphylococcus aureus* is a common Gram-positive pathogen that causes disease in humans ranging from mild skin infections to severe invasive diseases including necrotizing pneumonia, endocarditis and sepsis\(^7\). Staphylococcal peritonitis is a serious threat among patients receiving peritoneal dialysis and can lead to hospitalization and death\(^{277,278}\). Invasive infection commonly manifests with massive pro-inflammatory cytokine production. During severe complicated infections, such as toxic shock syndrome and sepsis, excessive cytokine production is associated with severe pathologies, such as systemic neutrophil dysfunction, disseminated intravascular coagulation and multi-organ failure\(^{146,258,279}\). These life-threatening conditions are compounded by the emergence of antibiotic-resistant *S. aureus* strains, including methicillin-resistant *S. aureus* (MRSA). Broad-spectrum antibiotic resistance displayed by this pathogen necessitates the development of alternative approaches, such as immuno-modulatory therapies.

The *S. aureus* two-component system, SaeR/S, is a potent regulator of virulence factors, including cytotoxins, superantigens, immune-modulatory proteins and adhesins\(^{12,63}\). *S. aureus* mutant strains lacking saeR/S exhibit dramatically reduced virulence during human-neutrophil interactions and in murine models of superficial and invasive infections\(^{12,63,200}\). Previously, we reported that saeR/S is a significant mediator of bacterial persistence,
dissemination and mortality during S. aureus peritonitis\textsuperscript{200}. These studies demonstrated that SaeR/S is necessary for the rapid induction of pro-inflammatory cytokines and strongly promotes interferon-gamma (IFN\gamma) production in mice and human leukocytes\textsuperscript{200}. However, a source of saeR/S-dependent IFN\gamma production and a role for IFN\gamma during saeR/S-mediated S. aureus pathogenesis has yet to be defined.

In this study, using a mouse model of staphylococcal peritonitis, we identified neutrophils as the predominant cell type that produces IFN\gamma in response to saeR/S-regulated factors. We further characterize a role for IFN\gamma in impacting saeR/S-mediated pathogenesis using IFN\gamma-deficient mice (GKO). Collectively, our study demonstrates neutrophils as a source of IFN\gamma and suggests this response is deleterious to the outcome of infection.

**Results**

**SaeR/S Promotes IFN\gamma During Early S. aureus Peritonitis**

IFN\gamma is a potent activator of innate immune effector cells, including macrophages and neutrophils. Previously, we showed that saeR/S promotes IFN\gamma production during S. aureus peritonitis at 10 hours post-infection\textsuperscript{200}. To determine if this phenotype is present during earlier infection, BALB/c mice were inoculated i.p. with wild-type S. aureus (MW2) or MW2 with deleted saeR/S (MW2\Delta saeR/S) and bacterial loads and IFN\gamma protein were measured at 7 hours post-infection (Figs. 1A and 1B). SaeR/S did not impact bacterial
survival at 7 hours, as both MW2 and MW2ΔsaeR/S-infected mice exhibited similar S. aureus burdens (Fig. 1A). Consistent with our previous observation at 10 hours post-infection\(^{200}\), MW2-infected mice exhibited significantly elevated IFN\(\gamma\) protein levels in the cell-free peritoneal exudates (Fig. 1B).

To confirm that saeR/S induces IFN\(\gamma\) at this early time point and to identify the cellular source(s) of IFN\(\gamma\) in response to saeR/S-regulated factors,
we used an *in vivo* approach for intracellular cytokine accumulation. At 4 hours post-infection, mice were inoculated i.p. with 5 µg of brefeldin A. At 7 hours, peritoneal cells were extracted and stained for intracellular IFNγ (Fig. 1C). Consistent with IFNγ protein concentrations in the cell-free exudates (Fig. 1B), cells from MW2-infected mice displayed significantly elevated IFNγ expression relative to cells from MW2ΔsaeR/S-infected mice, further demonstrating that IFNγ production is promoted by saeR/S (Fig. 1C).

Complementation of MW2ΔsaeR/S with saeR/S (MW2ΔsaeR/Scomp) partially restored the wild-type phenotype, further supporting the observation that IFNγ production is saeR/S-mediated (Fig. 1C).

Historically, T cells and natural killer (NK) cells were thought to be the predominant sources of IFNγ during inflammatory conditions. To identify which cell type(s) produce IFNγ during *S. aureus* peritonitis, we stained for T cells (CD3^+^), NK cells (CD49b^+^), dendritic cells (CD11c^+^) or macrophages (F4/80^+^) in combination with IFNγ and measured for IFNγ expression by dual-color FACS analysis (Fig. 1D). Surprisingly, these cell types displayed only negligible IFNγ expression (Fig. 1D).

**SaeR/S-Regulated Factors Promote IFNγ Production in Neutrophils During *S. aureus* Peritonitis**

In our model of *S. aureus* peritonitis, saeR/S does not mediate IFNγ expression in T cells, dendritic cells, NK cells, or macrophages, as these cells were scarcely present at the time of harvest (Fig. 1D). To determine if
SaeR/S impacts IFNγ production in neutrophils during *S. aureus* peritonitis, we infected mice with MW2, MW2ΔsaeR/S or MW2ΔsaeR/Scomp and measured IFNγ expression in GR-1\(^{high}\) or Ly6G\(^{high}\) cells (Fig. 2). Inoculation

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**Figure 2.** SaeR/S promotes IFNγ expression in neutrophils recruited to the peritoneum following i.p. infection with *S. aureus* strains MW2 and LAC. A. Average percentage of Ly6G\(^{high}\) cells recovered from the peritoneum at 7 hours post-infection. B. Representative FACS plots for Ly6G/IFNγ expression and a bar graph displaying the average percentage of Ly6G\(^{high}\)/IFNγ\(^{+}\) cells using the *in vivo* assay. C. Representative FACS plots for GR-1/IFNγ expression and a bar graph displaying the average percentage of GR-1\(^{high}\)/IFNγ\(^{+}\) cells using the *in vivo* assay. D, E. Bar graphs displaying average percentage of Ly6G\(^{high}\)/IFNγ\(^{+}\) cells (D) or average percentage of GR-1\(^{high}\)/IFNγ\(^{+}\) cells (E) following i.p. infection with LAC or LACΔsaeR/S using the *in vivo* assay. *P* < 0.05 as measured by Student’s *t* test. Data in (A/B), (C) and (D, E) are from 3 separate experiments of 3 mice/group, with the exception of (B), for which there are 2 mice in the MW2ΔsaeR/Scomp group. NS = not significant.
with MW2, MW2ΔsaeR/S or MW2ΔsaeR/Scomp did not appear to differentially impact neutrophil recruitment at the time of harvest, as approximately 90% of all cells were Ly6G\textsuperscript{high} in all infected groups at 7 hours (Fig. 2A). The robust neutrophil presence (\sim 90%) is congruent with the observation that other cell types were not recruited, nor present, at the time of investigation (Fig. 1D). SaeR/S did impact IFN\textgamma production in recruited neutrophils, as demonstrated by a significantly higher percentage of Ly6G\textsuperscript{high}/IFN\textgamma\textsuperscript{+} cells in MW2-infected mice relative to mice infected with MW2ΔsaeR/S (Fig. 2B). Infection with MW2ΔsaeR/Scomp restored the wild-type phenotype (Fig. 2B). Because neutrophils are not a traditional source of IFN\textgamma during inflammation, we used a separate ex vivo intracellular cytokine assay for measuring IFN\textgamma and an alternative staining mAb for neutrophil identification (GR-1). Cells were extracted at 4 hours post-infection, incubated ex vivo with brefeldin A for 3 hours and then analyzed for GR-1 and IFN\textgamma expression (Fig. 2C). Consistent with the higher percentage of Ly6G\textsuperscript{high} cells expressing IFN\textgamma in response to saeR/S using the in vivo assay, a significantly higher percentage of GR-1\textsuperscript{high} cells from mice infected with MW2 or MW2ΔsaeR/Scomp displayed IFN\textgamma expression relative to MW2ΔsaeR/S-infected mice (Fig. 2C). However, \sim 55% of cells from MW2-infected mice were Ly6G\textsuperscript{high}/IFN\textgamma\textsuperscript{+} using the in vivo assay and \sim 40% of GR-1\textsuperscript{high}/IFN\textgamma\textsuperscript{+} were observed using the ex vivo approach. This difference is likely a result from the time-points at which leukocytes were extracted from the peritoneum post-
infection. Using the in vivo assay, leukocytes were extracted at 7 hours post-infection, whereas in the ex vivo assay, leukocytes were taken at 4 hours.

*S. aureus* strains are extremely diverse regarding differential expression of virulence determinants\textsuperscript{11}. To verify the observation that *saeR/S* promotes IFN\textgamma expression in neutrophils was not exclusive to MW2, mice were inoculated i.p. with *S. aureus* pulsed-field gel electrophoresis type USA300 (LAC) or its isogenic *saeR/S* mutant (LAC\textdarr{saeR/S}) and production of IFN\textgamma was analyzed using the in vivo model. Consistent with our findings using MW2 strains, *saeR/S* induced elevated expression of IFN\textgamma in Ly6G\textsuperscript{high} cells and significantly higher IFN\textgamma in GR-1\textsuperscript{high} cells of LAC-infected mice relative to LAC\textdarr{saeR/S}-infected mice (Figs. 2D and 2E). Taken together, these data demonstrate that *saeR/S*-regulated factors promote IFN\textgamma expression in mouse neutrophils during early *S. aureus* peritonitis.

**IFN\textgamma-Deficient Mice Have Reduced Bacterial Burden and Decreased Cellular Cytotoxicity During Wild-type *S. aureus* Peritonitis**

In a previous study, using a peritonitis model, we showed that *saeR/S* promoted *S. aureus* survival 10 hours post-infection in the peritoneal cavity. This observation was associated with elevated IFN\textgamma in wild-type infected mice\textsuperscript{200}. To investigate a role for IFN\textgamma during *saeR/S*-mediated pathogenesis, we infected wild-type (BALB/c) and IFN\textgamma-deficient mice (GKO) i.p. with MW2 or MW2\textdarr{saeR/S} and quantified bacterial survival and host cell cytotoxicity (Fig. 3). GKO mice infected with MW2 had significantly reduced bacterial
loads relative to BALB/c mice infected with the same strain (Fig. 3A). This observation was consistent with a previous study demonstrating that GKO mice displayed reduced *S. aureus* burdens in a murine bacteremia model\(^{240}\).

Surprisingly, MW2-infected GKO mice exhibited similar *S. aureus* burdens compared to MW2Δsaer/S-infected BALB/c and MW2Δsaer/S-infected GKO mice (Fig. 3A). A similar trend was observed in characterizing host cell cytotoxicity. MW2-infected BALB/c mice displayed significantly higher LDH activity relative to all other groups (Fig. 3B). MW2Δsaer/S-infected GKO,
MW2ΔsaeR/S-infected BALB/c and MW2-infected GKO mice all exhibited similar LDH activity (Fig. 3B). Taken together, these data demonstrate that during *S. aureus* peritonitis, GKO mice have elevated resistance to saeR/S-mediated bacterial persistence and saeR/S-mediated cellular cytotoxicity.

**Discussion**

In this study, we identify neutrophils as the primary source of IFNγ in response to early *S. aureus* peritonitis. This observation was redundantly identified by cell surface staining with GR-1 and Ly6G mAbs and using two distinct clinically relevant strains of *S. aureus* (MW2 and LAC). We further showed that the SaeR/S two-component system significantly promoted IFNγ expression in these recruited neutrophils. To our knowledge, this is the first study demonstrating a pathogen-derived global regulator of virulence (*saeR/S*) as a potent inducer of neutrophil-derived IFNγ.

Highly regulated inflammatory responses to pathogenic microbes are necessary for both containment and resolution of infection. Innate and adaptive responses need to be tightly controlled otherwise systemic inflammatory conditions may arise from localized infections\(^{258}\). *S. aureus* is an opportunistic pathogen demonstrating both intracellular and extracellular tropisms. This microbe can cause cellular dysfunction of virtually every type of immune cell, via its diverse repertoire of toxins and leukocidins. During early infection, neutrophils are recruited and activated by chemokines and cytokines originating from tissue-resident sentinel cells. An inflammatory
response is necessary to properly recruit and activate neutrophils to eradicate infection but it must be balanced to avoid significant tissue and bystander cell destruction that would favor pathogen replication.

Historically, IFNγ was thought to primarily originate from T cells and NK cells during inflammatory conditions, with its downstream impacts targeted at myeloid cells for innate effector function. However, myeloid-lineage cells began to be identified as significant sources of IFNγ upon observing that when T cells were scarcely present within the inflamed environment, IFNγ was still being produced\(^\text{217}\). Recently, it has been shown that purified and peripheral blood cultured human neutrophils both produce and contain preformed IFNγ\(^\text{281}\). Using different mouse models of infection (route of infection indicated in parentheses) and staining for GR-1 and/or Ly6G cells, IFNγ-producing neutrophils have been identified following infection with diverse pathogenic bacteria including: *Listeria monocytogenes* (intravenous), *Salmonella typhimurium* (oral), *Francisella tularensis* (dermal), *Nocardia asteroides* (pulmonary) and *Streptococcus pneumonia* (pulmonary)\(^\text{221-225}\). Most recently, *S. aureus* was shown to induce IFNγ expression in recruited neutrophils during bacterial pneumonia\(^\text{225}\). Taken together, these recent reports clearly indicate that neutrophils have been historically overlooked as being potent sources of IFNγ.

IFNγ impacts neutrophil function in many ways, including priming of oxidative burst and degranulation mechanisms\(^\text{210}\). However, influence on the outcome of infection by neutrophils producing IFNγ remains to be defined in
the aforementioned models with the exception of adoptive transfer of IFNγ-producing neutrophils that conferred protection in GKO mice following intravenous infection with *L. monocytogenes*. This observation is consistent with knowledge of obligate intracellular *L. monocytogenes*’ tropism for macrophages and the macrophage-activating impacts of IFNγ that leads to protection against this pathogen. Using a surgical wound model of *S. aureus* infection, it was reported that IFNγ-mediated CXC chemokine production by T cells promoted a robust neutrophil presence, but that *S. aureus* burdens were elevated at the infectious foci. This observation was supported by an earlier study demonstrating that intracellular *S. aureus*, contained within the neutrophil, can remain viable and exacerbate infection. These studies suggested that an excessively neutrophil-rich environment is deleterious for clearance of *S. aureus*. Our findings suggest that saeR/S-induced IFNγ production in neutrophils is deleterious to the resolution of infection, as GKO mice are protected from saeR/S-mediated bacterial persistence and host cell cytotoxicity. We further propose that in the absence of saeR/S, a role for IFNγ in impacting immunity is relatively inconsequential, as neither BALB/c nor GKO mice infected with MW2ΔsaeR/S displayed differences in *S. aureus* burdens or cellular cytotoxicity. However, additional studies are necessary to clearly define the contribution of saeR/S-mediated IFNγ-producing neutrophils to *S. aureus* pathogenesis and to define how production of IFNγ by neutrophils influences neutrophil recruitment and
function. Overall, this report provides a unique observation into how \textit{S. aureus} can modulate host inflammatory mechanisms.

**Materials and Methods**

**Bacterial Strains and Culture**

Community-associated MRSA strains USA400 (MW2) and USA300 (LAC) were selected based on clinical relevance and available genomic information\cite{11,12,63,249-255,273-276}. Mutant MW2 and LAC strains with deleted \textit{saeR/S} (MW2\textDelta s\textit{aeR/S} and LAC\textDelta s\textit{aeR/S}, respectively) and MW2\textDelta s\textit{aeR/S} with complemented \textit{saeR/S} (MW2\textDelta s\textit{aeR/Scomp}) were generated in prior studies\cite{12,63}. Bacteria were grown in Tryptic Soy Broth (TSB) and harvested at mid-exponential growth phase as previously described\cite{12,63}. Enumeration of \textit{S. aureus} in the peritoneum post-infection was done as previously described\cite{200}.

**Mouse Strains and Infection Model**

All studies conformed to National Institutes of Health guidelines and were approved by the Animal Care and Use Committee at Montana State University-Bozeman, MT. Wild-type BALB/c mice and IFN\gamma-deficient BALB/c mice (GKO; C.129S7(B6)-IFN\gamma\textsuperscript{tm1Ts}/J) were bred under pathogen-free conditions at the Animal Resources Center; MSU-Bozeman, or purchased from commercial sources. All mice were inoculated with 2 \times 10^7 \textit{S. aureus} colony forming units (cfu) via the intraperitoneal (i.p.) route.
IFNγ Protein Measurement

Mice were infected as described above, euthanized at 7 hours post-infection and the peritoneal cavities were washed with 10 mls PBS. IFNγ was measured in the cell-free peritoneal exudates using a TH1/TH2/TH17 cytometric bead array kit as described by the manufacturer (BD Biosciences).

Cellular Cytotoxicity

Extracellular lactate dehydrogenase (LDH) was measured as an indication of cellular cytotoxicity. At 10 hours post-infection, LDH activity was measured in the cell-free peritoneal exudates using a commercially available CytoTox-ONE kit per the manufacturer's instructions (Promega).

In vivo Cytokine Assay

At 4 hours post-infection, mice were inoculated i.p. with 5 µg of brefeldin A (GolgiPlug; BD Biosciences). Mice were euthanized at 7 hours post-infection and peritoneal cavities were washed with 10 mls PBS containing 2% horse serum and 1mM sodium azide (FACS buffer). Cells were washed in FACS buffer and then stained for surface receptors and intracellular IFNγ expression using commercially available Cytofix/Cytoperm kits per the manufacturer’s instructions (BD Biosciences).

Ex vivo Cytokine Assay

At 4 hours post-infection, mice were euthanized and the peritoneal cavities washed with 10 mls of RPMI + 5 mM HEPES + 1 µg/ml brefeldin A.
Cell suspensions were incubated for 3 hours in 6-well tissue culture plates at 37°C; 5% CO₂. Cells were washed in FACS buffer and then stained for surface receptors and intracellular IFNγ as described above.

Antibodies and Flow Cytometric Analysis

The following anti-mouse monoclonal antibodies (mAbs) were used: CD49b (DX5)-FITC, GR-1 (RB6-8C5)-FITC, CD3ε (145-2C11)-PE, CD11c (HC3)-PE, F4/80 (BM8)-PERCP/Cy5.5, Ly6G (1A8)-FITC, IFNγ (IgG1,κ)-APC and IgG1,κ isotype-APC (control). CD49b and GR-1 mAbs were generous gifts from Drs. David Pascual and Mark Jutila, respectively (MSU-Bozeman, MT). All other mAbs were obtained from commercial sources. Single and dual-parameter fluorescence-activated cell sorting (FACS) analysis was performed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

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CHAPTER FIVE

THE SAER/S VIRULENCE SYSTEM PROMOTES INTERFERON-GAMMA TO ENHANCE STAPHYLOCOCCUS AUREUS SKIN DISEASE IN AN INTERLEUKIN-17-DEPENDENT MANNER

Contribution of Authors and Co-Authors

Manuscripts in Chapters 3, 4, and 5

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Contributions: Conceived the study, designed and performed experiments, collected and analyzed data, and wrote manuscript.

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Manuscript Information Page

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Abstract

Cutaneous infections caused by *Staphylococcus aureus* (*S. aureus*) make up the majority of pathogen-derived skin and soft-tissue infections in the clinic. Previously, we established a link between interferon-gamma (IFNγ) and the staphylococcal SaeR/S two-component system in a mouse model of *S. aureus* skin infection. In the current study, we show that IFNγ-deficient (GKO) mice display significantly smaller dermonecrotic lesions when infected with wild-type *S. aureus* (USA300), compared to normal (BALB/c) mice infected with USA300. Compared to BALB/c mice, GKO mice produce elevated interleukin (IL)-17 when infected with USA300 and we further demonstrate that this elevated IL-17 response accounts for the enhanced immunity and reduced lesion sizes. Neutralization of IL-17 in USA300-infected GKO mice significantly increased lesion sizes and were similar to lesions observed in BALB/c mice. Furthermore, BALB/c mice given recombinant IL-17 showed reduced severity of skin infection when infected with USA300, confirming the observed protective role of IL-17 in GKO mice. Additionally, we investigated the influence of the SaeR/S regulatory system on IFNγ and IL-17. Interestingly, none of the treatment groups or strains of mice exhibited differences in abscess development when infected with a USA300 *saeR/S* deletion mutant. Taken together, these observations suggest increased production of IFNγ and decreased production of IL-17 is mediated by *saeR/S* to promote disease during staphylococcal skin infection.
Introduction

*Staphylococcus aureus* (*S. aureus*) is the leading cause of skin and soft tissue infections (SSTIs), including both superficial infections such as impetigo and more complicated infections such as cellulitis, subcutaneous abscesses and necrotizing fasciitis\textsuperscript{161}. *S. aureus* SSTIs account for approximately 12 million outpatient and emergency room visits in the United States, annually\textsuperscript{161}. Beyond common skin disease presentations, *S. aureus* can cause life-threatening invasive infections including bacteremia, meningitis, endocarditis and sepsis\textsuperscript{251}. Complications surrounding superficial and invasive *S. aureus* infections have been compounded by the rapid emergence of antibiotic strains, including methicillin-resistant *S. aureus* (MRSA)\textsuperscript{158}. Historically, infections arose within the clinics, but over the last decade, community-associated MRSA (CA-MRSA) isolates, such as the highly virulent *S. aureus* strain USA300 (LAC), have occurred independently of clinical environments\textsuperscript{283}.

*S. aureus* possesses a diverse repertoire of mediators aimed at thwarting an effective host immune response. The majority of these virulence factors are under the direct or indirect influence of global regulators\textsuperscript{61}. The staphylococcal two-component system, SaeR/S (encoded by saeR/S), is a global regulator of gene expression and impacts the expression of multiple virulence determinants, including leukocidins/cytotoxins, superantigens, chemotaxis inhibitors and adhesins\textsuperscript{12,63}. Previously, it was shown that saeR/S was essential for full virulence of *S. aureus* following human neutrophil phagocytosis and during
invasive disease in mice\textsuperscript{12,63,200}. Additionally, saeR/S has been shown to be critical for dermonecrotic lesion development during murine skin infection\textsuperscript{63}. However, the host mechanisms that contribute to the SaeR/S phenotype following \textit{S. aureus} skin infection are currently undefined.

In a previous study, we demonstrated that elevated host-derived interferon-gamma (IFN\textgreek{y}) was associated with the dermonecrotic phenotype observed in wild-type \textit{S. aureus}-infected mice, suggesting IFN\textgreek{y} may play a deleterious role in mediating the inflammatory response to staphylococcal skin infection\textsuperscript{200}. In support of this observation, McLoughlin et al used a surgical wound model of \textit{S. aureus} infection and demonstrated that T cell-derived IFN\textgreek{y} promoted a neutrophil-rich environment that resulted in increased bacterial burdens and exacerbation of infection\textsuperscript{51}. In the current study, we used GKO mice to define the impacts of SaeR/S and IFN\textgreek{y} in mediating pathogenesis during subcutaneous \textit{S. aureus} infection. Interestingly, our data suggests that IFN\textgreek{y} does not play a significant direct role in mediating immunity, but that saeR/S-mediated IFN\textgreek{y} induction reduces the production of interleukin (IL)-17, and the reduced production of IL-17 is responsible for the increased severity of skin infection observed in normal (BALB/c) mice.
Results

IFNy-deficient Mice are Protected Against saeR/S-Mediated Pathogenesis During *S. aureus* Skin Infection

IFNy is a potent mediator of inflammation via neutrophil chemotactic factor production during *S. aureus* infection\(^{51,201}\). Previously, we demonstrated increased IFNy production in response to saeR/S-mediated factors in the skin of wild-type *S. aureus* (LAC)-infected mice, which correlated with the development of open dermonecrotic lesions\(^{200}\). To determine if IFNy impacts immunity and pathogenesis during *S. aureus* skin infection, BALB/c and GKO mice were inoculated subcutaneously with LAC or LAC with deleted saeR/S (LACΔsaeR/S) and monitored daily for lesion and/or abscess development. Consistent with our previous findings, LACΔsaeR/S-infected BALB/c mice presented only with abscesses and no dermonecrotic lesion (Fig. 1)\(^{63}\). Both BALB/c and GKO mice infected with LAC developed open dermonecrotic lesions; however, BALB/c mice displayed significantly larger lesions (Figs. 1A and 1D). Additionally, the onset of dermonecrosis was delayed in LAC-infected GKO mice, as BALB/c mice began displaying necrosis on day 2, whereas GKO mice did not display necrosis until day 3 (Figs. 1B and 1D). Interestingly, there were no significant differences in abscess development or in abscess duration between BALB/c and GKO mice infected with LACΔsaeR/S (Figs. 1C/D). The observation that GKO mice display dramatically reduced lesion sizes compared to BALB/c mice that were both infected with wild-type *S. aureus*, but that neither GKO mice or BALB/c infected
with LACΔsaeR/S displayed differences in abscess development suggests that...
IFNγ reduces innate immunity mechanisms exclusively against saeR/S-mediated pathogenesis.

To determine if endogenous IFNγ production impacts immunity against saeR/S-mediated S. aureus pathogenesis, a neutralizing anti-mouse IFNγ mAb...
(50 µg) or an isotype-matching control mAb (50µg) was given to BALB/c mice simultaneously with a S. aureus inoculum (Fig. 2). LAC-infected mice that were treated with the anti-IFNγ mAb developed lesions that were notably smaller than mice that were treated with control antibody, but these differences were not statistically significant (Figs. 2A and 2C). Consistent with the observation that GKO and BALB/c mice infected with LACΔsaeR/S did not show differences in abscess growth, neutralization of IFNγ did not impact abscess development in mice infected with mutant S. aureus (Figs. 1C/D and 2B/C). GKO mice exhibit protection when infected with LAC, but neutralization of IFNγ in BALB/c mice did not confer the same protection. This observation implies alternative host-derived mediators of inflammation are playing a significant role in the enhanced immunity displayed by GKO mice in response to infection with LAC.

IFNγ-deficient Mice Produce Elevated IL-17 in Response to Infection with Wild-type S. aureus

IFNγ has been shown to antagonize the development and maintenance of Th17 cell populations. To determine if GKO mice differentially express IL-17 when in infected with S. aureus, we infected BALB/c and GKO mice with LAC or LACΔsaeR/S and measured S. aureus burdens and IL-17 in the skin tissue at 8 hours post-infection (Fig. 3). Consistent with our previous findings using hairless mice, saeR/S did not impact pathogen survival at 8 hours post-infection in LAC-infected BALB/c mice compared to LACΔsaeR/S-infected BALB/c mice, and these burdens were similar to those observed in both LAC-infected and LACΔsaeR/S-infected GKO mice (Fig. 3A). BALB/c mice and GKO mice
infected with LAC
saeR/S produced similar IL-17 concentrations (Fig. 3B).

However, GKO mice infected with LAC produced significantly elevated IL-17
compared to BALB/c mice infected with the same strain (Fig. 3B). This finding
indicates that compared to the BALB/c mice, GKO mice produce elevated IL-17
in response to saeR/S-regulated factors.

Recombinant IL-17 Protects BALB/c Mice
from Infection with Wild-type S. aureus

IL-17 has been recently demonstrated to be an essential mediator of
protection against S. aureus laboratory-derived strain SH1000 in C57BL/6
mice. To determine if IL-17 is protective against clinically-relevant S. aureus,
we administered recombinant(r) IL-17 (1,000 ng) with clinically-derived S. aureus

Figure 3. Wild-type S. aureus-infected GKO mice produce elevated IL-17
compared to BALB/c mice. A. S. aureus burdens in the skin of infected mice.
B. IL-17A protein concentrations in the skin of infected mice. *P < 0.05 as
measured by Student’s t test. Data expressed are from 3 mice/group, with the
exception of mock-infected control mice, for which there is only 1
mouse/group. NS = not significant.
strain LAC to BALB/c mice, monitored lesion development daily and compared this to infection in GKO mice, which exhibit marked protection (Fig. 4). BALB/c mice treated with rIL-17 exhibited significantly smaller lesions than BALB/c mice treated with vehicle only (Figs. 4A/B). Surprisingly, the rIL-17-treated mice displayed notably smaller lesions than GKO mice treated with vehicle only, but differences were not significant (Figs. 4A/B). These findings show that IL-17 is protective against clinically-derived S. aureus and this protection is similar to that observed in LAC-infected GKO mice (Figs. 1 and 4).
Neutralization of IL-17 Abrogates Protection Displayed by IFNγ-deficient Mice

GKO mice infected with LAC show reduced lesion sizes compared to BALB/c mice infected with LAC, and this is associated with elevated IL-17 production and the presence of the SaeR/S system (Figs. 1, 3 and 4). To determine if the elevated IL-17 response in GKO mice is the mechanism by which these mice are protected against S. aureus infection, a neutralizing IL-17A mAb (50 µg) or an isotype-matching control mAb (50 µg) was administered to GKO mice simultaneously with LAC or LACΔsaeR/S and the lesions/abscesses were compared to BALB/c mice (LAC- or LACΔsaeR/S-infected) treated with vehicle only (Fig. 5). LAC-infected GKO mice treated with the anti-IL-17 mAb developed similar lesions to BALB/c mice treated with vehicle only (Figs. 5A/C). GKO mice treated with the isotype mAb displayed significantly smaller lesions compared to GKO mice treated with neutralizing IL-17 mAbs and BALB/c mice treated with vehicle only (Figs. 5A/C). Neutralization of IL-17 did not impact abscess development in LACΔsaeR/S-infected GKO mice. Taken together, these findings show that GKO mice are protected against saeR/S-mediated pathogenesis in an IL-17-dependent manner and that in the absence of saeR/S, IL-17 is not a significant mediator of abscess development.

Discussion

IFNγ-regulated chemokine production mediates robust recruitment of neutrophils, the primary phagocytes necessary for clearance of pathogenic S.
Figure 5. Neutralization of IL-17 abolishes protection against saeR/S-mediated pathogenesis in GKO mice. A. Lesion sizes of LAC-infected BALB/c mice treated with vehicle only and GKO mice treated with anti-IL-17 mAbs or isotype-matching mAbs. B. Abscess sizes from LACΔsaeR/S-infected BALB/c mice treated with vehicle only and GKO mice treated with anti-IL-17 mAbs or isotype-matching mAbs. Representative photos of lesions and abscesses taken on day 2. Black arrows indicate measured abscesses. *P < 0.05 and **P < 0.01, BALB/c-PBS and GKO-anti-IL-17 relative to GKO-isotype, as measured by Student’s t test. Data expressed in (A) are from 2 biological replicates of 5 mice/group. Data in (B) are from 5 mice/group. NS = not significant.

*S. aureus*<sup>51,178,201</sup>. Consistent with this, using a surgical wound model of *S. aureus*
infection, IFNγ-deficient mice displayed a reduced neutrophil presence, however, these mice also had reduced bacterial burdens, suggesting that a neutrophil-rich environment is deleterious for pathogen-clearance\textsuperscript{201}. Using a murine model of \textit{S. aureus} skin disease, we've previously linked the endogenous production of IFNγ to the staphylococcal two-component global regulator of virulence, SaeR/S\textsuperscript{200}. In the current study, we demonstrate that GKO mice are protected against skin and soft-tissue infection with wild-type \textit{S. aureus}, as these mice had significantly reduced dermonecrosis compared to their wild-type BALB/c counterparts. GKO and BALB/c mice infected with mutant \textit{S. aureus}, lacking saeR/S, display no differences in abscess development, indicating a link between saeR/S-mediated IFNγ and the increased lesion sizes observed in BALB/c mice infected with wild-type \textit{S. aureus}.

Neutralization of IFNγ yielded only partial protection, as these mice did display a notable, albeit statistically insignificant reduction in dermonecrosis. This observation was consistently observed following biological replicates with increased mouse numbers (17 mice total). This observation is congruent with a previous study demonstrating that IFNγ-receptor-deficient mice did not display differences in lesion development or bacterial burdens when cutaneously infected with \textit{S. aureus}\textsuperscript{193}. Taken together, these findings indicate that IFNγ is deleterious for pathogen clearance, as suggested by significantly reduced lesion sizes in GKO mice, partially reduced lesion sizes in mice treated with anti-IFNγ mAbs and the aforementioned study showing reduced bacterial loads at the infectious foci in GKO mice\textsuperscript{51}. Recently, Cho et al demonstrated that γδT cell-
derived IL-17 is critical for an effective innate immune response to S. aureus skin infection\textsuperscript{193}. However, the investigators noted that when recombinant-IL-17 was administered to wild-type mice, there was no impact on lesion development or bacterial burden\textsuperscript{193}. In the current study, we observed that treatment of wild-type mice with recombinant-IL-17 yielded a significant reduction in lesion development. These discrepancies are likely a result from strain of mouse used and strain of S. aureus. In the previous study, Cho et al used C57BL/6 mice and laboratory-derived S. aureus strain SH1000\textsuperscript{193}. In the current study, we used BALB/c mice and a clinical S. aureus strain, USA300 (LAC). It is possible that C57BL/6 mice produce elevated IL-17, compared to BALB/c mice, during S. aureus skin infection. This idea is supported by a recent study comparing IL-17 responses during Candida albicans infection, in which the investigators showed that C57BL/6 mice produce elevated IL-17 and reduced IFN\gamma compared to BALB/c mice\textsuperscript{284}. However, a different study concluded that BALB/c mice produce increased IL-17, compared to C57BL/6 when infected with Leishmania major\textsuperscript{285}. Another study showed that a prosthetic implant model of S. aureus biofilm infection yielded no differences in IL-17 or IFN\gamma production between C57BL/6 or BALB/c mice\textsuperscript{286}. Clearly, further studies are necessary to continue defining the protective impacts of IL-17 during staphylococcal skin disease and our study indicates that GKO mice may provide a tool for studying IL-17-polarized responses during S. aureus skin infection.

Multiple studies have shown that IFN\gamma plays an antagonistic role for Th17 cell development and IL-17 expression\textsuperscript{206,287-289}. Interestingly, GKO mice infected
with LACΔsaeR/S did not produce elevated IL-17 compared to BALB/c mice infected with the same strain. These findings indicate that GKO mice, compared to BALB/c mice, produce increased IL-17 in response to saeR/S-mediated factors and are further supported by our previous study showing that LACΔsaeR/S-infected mice produce negligible IFNγ relative to LAC-infected mice\textsuperscript{200}. Taken together, these results suggest that IFNγ and IL-17 only play significant roles in mediating immunity against wild-type \textit{S. aureus}, as none of the mouse strains or treatment groups yielded differences in abscess development during infection with LACΔsaeR/S.

To our knowledge, this is the first study demonstrating a link between IL-17 and IFNγ during \textit{S. aureus} skin infection and the first to associate a pathogen-derived global regulator of virulence (saeR/S) to this response. Taken together, these findings strongly suggest that saeR/S-mediated IFNγ production reduces IL-17 expression and that this contributes to the increased dermonecrosis observed in LAC-infected BALB/c mice relative to GKO mice. Overall, this study provides a unique insight into how \textit{S. aureus} modulates the host inflammatory response to enhance pathogenesis.

**Materials and Methods**

**Bacterial Strains and Culture**

For this study, we used the CA-MRSA isolate USA300 (LAC), based on clinical relevance and available genomic information\textsuperscript{11,12,63,249-255,273-276}. Mutant \textit{S. aureus} LAC strain with deleted saeR/S (LACΔsaeR/S), was generated in a
prior study\textsuperscript{63}. Bacteria were grown in tryptic soy broth and harvested at mid-exponential growth phase by centrifugation, washed in phosphate-buffered saline (PBS) and then re-suspended in PBS at a final concentration of $1 \times 10^6$ colony-forming units per inoculum.

**Mouse Strains and Infection Model**

All studies conformed to National Institutes of Health guidelines and were approved by the Animal Care and Use Committee at Montana State University-Bozeman, MT. Age and sex-matched wild-type BALB/c mice and IFN\textgamma-deficient BALB/c mice (GKO; C.129S7(B6)-IFNg\textsuperscript{tm1Ts/J}) were bred under pathogen-free conditions at the Animal Resources Center; MSU-Bozeman, or purchased from commercial sources. Mice were anesthetized using aerosolized isoflurane (3.5\% in oxygen). Backs of mice were shaved with electric clippers and remaining hair was removed with ectopic application of commercially available Nair\textsuperscript{®} for \~45 seconds. Mice were rested for 24 hours, re-anesthetized and then subcutaneously inoculated with $2 \times 10^6$ CFUs. Mice were monitored daily (or every other day) for the development of skin lesions/abscesses. Lesions and/or abscesses were measured using electronic calipers with precision to 0.01 mm. Abscess volumes were calculated using an equation for spherical ellipsoid \textsuperscript{290}:

$$V = \frac{\pi}{6}lw^2$$

**Bacterial Enumeration and IL-17 Measurement**

At 8 hours post-infection, mice were euthanized by CO\textsubscript{2} asphyxiation. Inoculated skin areas of mice were excised with surgical scissors and a 9 mm
“punch” biopsy sample removed. Skin “punch” samples were weighed, homogenized in 2 mls PBS and then serially diluted in distilled H₂O. Dilutions were plated on tryptic soy agar, incubated overnight at 37°C; 5% CO₂ and CFUs counted and expressed as S. aureus CFU/mg of tissue.

To measure IL-17, “punch” sample homogenates were pelleted by centrifugation. Supernatants were sterile filtered using 0.22 µm syringe filters and then frozen at -80°C until protein analysis. IL-17 protein was measured in the cell-free filtrates using a mouse Th1/Th2/Th17 cytometric bead array kit, per the manufacturer’s instructions (BD Biosciences). IL-17 concentrations were displayed as pg/g of tissue.

**In vivo Administration of Recombinant Proteins and Neutralizing mAbs**

To neutralize IFNγ, mice were given 50 µg of neutralizing anti-mouse IFNγ mAbs (suspended in sterile PBS; eBioscience) mixed with 2 x 10⁶ S. aureus CFUs and a single 100 µl inoculum was administered to BALB/c mice. Control BALB/c mice received 50 µg of the isotype-matching mAbs mixed with S. aureus (eBioscience).

Treatment with recombinant IL-17 (rIL-17) was done as previously described¹⁹³. Briefly, a single dose of rIL-17 (1,000 ng/100 µl; R&D systems) in sterile PBS was mixed with 2 x 10⁶ S. aureus CFUs and a single 100 µl inoculation was given to BALB/c mice. Control mice received PBS alone. RIL-17 does not display bactericidal or bacteriostatic activity against S. aureus, *in vitro*. ¹⁹³
In vivo neutralization of IL-17 was done as previously described\textsuperscript{193}. Briefly, 50 µg of neutralizing anti-mouse IL-17A mAbs (suspended in sterile PBS; R&D Systems) were mixed with $2 \times 10^6$ S. aureus CFUs and a single inoculum was administered to GKO mice. Control GKO mice received 50 µg of isotype-matching mAbs (R&D Systems).

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Tightly controlled inflammatory responses are critical for mediating immunity during *S. aureus* disease. This concept is highlighted in individuals with diverse genetic and acquired immune defects that display increased susceptibility to *S. aureus* infections \(^5\). Immuno-modulatory therapies are aimed at these individuals in hopes that clinicians can boost their immunity and increase their resistance to bacterial pathogens. In order for these approaches to be successful, in-depth characterization of both beneficial and deleterious inflammatory responses is required. The studies described in this dissertation shed some light on this subject by defining host and pathogen responses that are deleterious to pathogen clearance. From the pathogen’s perspective, we find that the SaeR/S global regulator is absolutely critical for full *S. aureus* pathogenicity. We also find that factors regulated by *saeR/S* are essential for inducing pro-inflammatory responses associated with sepsis during invasive disease and for promoting pro-inflammatory responses during skin infection.

In chapter three, we provide evidence that factors regulated by *saeR/S* are critical for *S. aureus* survival during invasive infection. Mice infected with mutant *S. aureus*, lacking *saeR/S* (*ΔsaeR/S*), displayed significantly reduced
bacterial burdens at the infectious foci, in the kidneys and in the hearts, suggesting *saeR/S* enhances *S. aureus* persistence and dissemination to deeper tissues. Moreover, Δ*saeR/S*-infected mice displayed marked improvement in survival compared with wild-type *S. aureus*-infected mice. The elevated mortality rate in wild-type *S. aureus*-infected mice was associated with a sepsis phenotype, evidenced by increased expression of pro-inflammatory cytokines (IL-6, TNFα, IL-2, IFNγ) both locally and systemically. Additionally, we established a link between the expression of IFNγ and SaeR/S. This *saeR/S*-mediated IFNγ expression was also demonstrated using a different strain of *S. aureus* during skin infection.

In chapter four, we elaborated on the findings described in chapter three by identifying the predominant cellular source of IFNγ and characterized a role for this cytokine during invasive *S. aureus* infection. We utilized both *in vivo* and *ex vivo* models of cytokine accumulation and identified recruited neutrophils as the predominant source of IFNγ during *S. aureus* peritonitis. Both MW2 and LAC strains induced the expression of IFNγ in Ly6G\textsuperscript{high} and GR-1\textsuperscript{high} cells in a *saeR/S*-dependent manner. IFNγ-producing neutrophils correlated with reduced bacterial clearance and elevated host cell cytotoxicity and we demonstrated that wild-type *S. aureus*-infected IFNγ-deficient mice (GKO) had reduced pathogen burdens and reduced cytotoxicity relative to wild-type *S. aureus*-infected IFNγ-competent mice (BALB/c). Interestingly, both GKO and BALB/c mice infected with Δ*saeR/S* displayed similar *S. aureus* burdens and cellular cytotoxicity. These latter findings showing no
differences in GKO and BALB/c mice infected with ΔsaeR/S are consistent with our data in chapter three showing that ΔsaeR/S-infected mice display negligible IFNγ production. Taken together, these data demonstrate the role of IFNγ in mediating immunity is only relevant in the context of saeR/S-mediated pathogenesis and suggest IFNγ deleteriously impacts innate antibacterial mechanisms during invasive disease.

In chapter five, we again expand on the findings in chapters three and four by characterizing a role for IFNγ in mediating immunity during S. aureus skin infection. These studies used BALB/c and GKO mice infected subcutaneously with wild-type S. aureus or ΔsaeR/S. As seen during invasive infection, GKO mice infected with wild-type S. aureus developed smaller dermonecrotic lesions relative to BALB/c mice. We found that these GKO mice expressed increased IL-17 in response to saeR/S-mediated factors and that this elevated IL-17 response conferred protection. We cannot completely rule out that IFNγ expression itself (or IFNγ signaling), directly reduces innate immune mechanisms, as neutralization of IFNγ did impart some protection, but this was not significant. Also consistent with our invasive studies, mice (BALB/c or GKO) infected with ΔsaeR/S displayed no differences in abscess development nor did they express different levels of IL-17. Neutralization of IL-17 or IFNγ had no impact on abscess development in ΔsaeR/S-infected mice. This finding supports our earlier hypothesis that IFNγ is only relevant in mediating immunity in response to saeR/S-mediated
pathogenesis and demonstrates this pro-inflammatory cytokine is deleterious in impacting immunity during superficial S. aureus skin infection.

Taken together, our work has shed insights into the mechanisms behind the enhanced virulence imparted by saeR/S. Collectively, these findings indicate that saeR/S hijacks the inflammatory response by promoting robust IFNγ expression that reduces mechanisms of innate defense. It is likely that S. aureus not only directly impacts innate effector cells, via its diverse repertoire of saeR/S-regulated virulence factors (leukotoxins, adhesins, etc), but also reduces innate responses by promoting dysfunctional inflammatory responses. These proposed concepts suggest that S. aureus orchestrates its own version of immuno-modulation during infection to enhance pathogenesis.

**Future Studies**

**Identify a Cellular Source of IL-17 During S. aureus Skin Infection**

In Chapter Five, we demonstrated that GKO mice exhibit protection relative to wild-type BALB/c mice, against saeR/S-mediated pathogenesis during S. aureus skin infection. We defined this further to show that GKO mice produce elevated IL-17 in response to saeR/S-mediated factors and that increased IL-17 affords this increased immunity. Our findings are consistent with a previous study showing that IL-17 is necessary for an efficient immune response during S. aureus skin infection\(^ {193}\). Although our findings are similar
to the published findings, showing ultimately that IL-17 is critical for immunity\textsuperscript{193}, our data suggests there may be alternative mechanisms for IL-17 production, specifically an alternative skin-resident (or rapidly recruited) cellular source of IL-17. This idea stems from the following differences between our data and the previous study\textsuperscript{193}: 1.) Using a skin model of \textit{S. aureus} infection in wild-type BALB/c mice, we demonstrated that recombinant IL-17 treatment significantly reduced the development of dermonecrotic lesions. This is in contrast to the previous study, in which the investigators noted that recombinant IL-17 treatment in wild-type C57BL/6 mice did not reduce lesion development\textsuperscript{193} and 2.) The previous study showed that C57BL/6 mice develop relatively small lesions when infected with \textit{S. aureus} and that IL-17 neutralization or removal of γδ T cells (the identified source of IL-17) was necessary to increase host susceptibility to infection. In contrast, we demonstrated that BALB/c mice develop relatively large dermonecrotic lesions and as stated above, exogenous IL-17 was necessary to achieve similar protection (smaller lesion size) that Cho et al observed in C57BL/6 mice.

To our knowledge, no studies have yet characterized the immune responsiveness of skin-resident γδ T cells, between BALB/c and C57BL/6 mice, specifically in the context of subcutaneous \textit{S. aureus} infection. However, an early study characterizing total percentage of γδ T cells in the dermis of BALB/c and C57BL/6 mice yielded no differences between these strains of mice\textsuperscript{291}. In this context, our ongoing studies will shed insight into
both saeR/S-mediated pathogenesis as well as γδ T cell function during host-pathogen interactions in the skin. Alternatively, these studies may identify a unique, as yet undefined source of IL-17. Consistent with this latter concept, CD8+ T cells, NKT cells and NK cells have demonstrated the ability to express IL-17 in the skin under certain inflammatory conditions\textsuperscript{292-294}, but to our knowledge, this has not been studied in the context of S. aureus skin disease.

**SaeR/S Impacts Neutrophil Function During Invasive Disease?**

We previously reported that saeR/S-regulated factors promote human neutrophil lysis, ex vivo\textsuperscript{12}; however, this phenotype has yet to be demonstrated, in vivo. In chapter four, we demonstrated that wild-type S. aureus (MW2)-infected BALB/c mice display elevated cellular cytotoxicity compared to MW2ΔsaeR/S-infected BALB/c mice, and we linked this to IFNγ, as wild-type S. aureus-infected GKO mice had reduced cytotoxicity. However, which host cell type(s) that are lysed has yet to be definitively identified. The findings in chapter four might suggest that neutrophils may be the primary cytotoxic cell, as they account for ~90% of all cells at 7 hrs post-infection in the infectious foci. To fill in these gaps in knowledge, we are further characterizing neutrophils in the context of saeR/S-mediated pathogenesis during lethal staphylococcal peritonitis. We propose that the pro-inflammatory milieu, combined with the direct virulence imparted by saeR/S-regulated virulence factors adversely impacts neutrophil function.
The following subsections describe some ongoing efforts to understand how SaeR/S and inflammatory mediators, specifically IFNγ, impact neutrophil function during invasive S. aureus infection.

**SaeR/S Mediates a Reduced Neutrophil Presence.** In chapter three, we provide evidence that SaeR/S enhances S. aureus survival during lethal peritonitis at 10 hours post-infection. Elevated bacterial burdens strongly suggest that the primary antibacterial phagocytes, the neutrophils, are not successfully clearing wild-type S. aureus as well as they clear MW2ΔsaeR/S. To begin to understand what impact SaeR/S has on neutrophil function, we employed a lethal model of S. aureus peritonitis (described in chapter three) and characterized the neutrophil presence during early infection (4 hours) and just prior to the onset of mortality (10 hours) (Fig. 1). During early infection, there were no differences in bacterial burdens (chapter three) and this correlated with equal numbers of neutrophils (GR-1^high^) between MW2- and MW2ΔsaeR/S-infected mice (Figs. 1A/C). However, at 10 hours, there were elevated bacterial burdens in MW2-infected mice (chapter three) and this correlated with a decreased neutrophil presence compared to MW2ΔsaeR/S-infected mice (Figs. 1B/D). Microscopic images from peritoneal exudates show reduced neutrophils and elevated extracellular S. aureus from MW2-infected mice (Fig. 1D). These observations strongly suggest that: 1) SaeR/S-regulated factors are inhibiting neutrophil recruitment, and/or 2) SaeR/S-regulated factors are causing neutrophils to be lysed and/or become
dysfunctional at the site of infection. The next subsection sheds some insight into the latter concept (saeR/S-mediated neutrophil lysis) and suggests that this mechanism contributes to the reduced neutrophils observed during invasive S. aureus infection.

**SaeR/S Mediates Neutrophil Membrane Permeability *In vivo.*** Previously, we demonstrated that saeR/S-regulated factors promote lysis of human neutrophils, *ex vivo*\(^ {12} \). To determine if SaeR/S has a similar cytotoxic impact

![Figure 1](image-url). Wild-type *S. aureus*-infected mice exhibit reduced neutrophils during peritonitis. A. Percentage of GR-1\(^ {\text{high}} \)-expressing cells in the peritoneum at 4 hours post-infection. B. Percentage of GR-1\(^ {\text{high}} \)-expressing cells in the peritoneum at 10 hours post-infection. Representative microscopic images of cytospin preparations from peritoneal exudates at 4 (C) and 10 (D) hours post-infection. For (A), n = 12 mice/group; for (B), n = 8 mice/group. *P < 0.05 as measured by Student’s t test. t = time; hrs = hours.
on neutrophils, *in vivo*, we again used a mouse model of staphylococcal peritonitis to characterize membrane permeability on recruited neutrophils by propidium iodide (PI) uptake. We also used GKO mice in this model to determine if IFNγ plays a role in these events. It should be noted that for these studies, $2 \times 10^7$ *S. aureus* CFUs was used, whereas the mice in data shown in Fig. 1 received $5 \times 10^7$ CFUs. At 10 hours post-infection, there were no differences in overall percentage of Ly6G$^{\text{high}}$ cells between all groups receiving the lower infectious dose, indicating a similar neutrophil presence at this time point (Fig. 2A). However, of those cells expressing Ly6G$^{\text{high}}$, a notably higher number of them were PI-positive in the MW2-infected BALB/c mice compared to all other groups (Fig. 2B). In the absence of saeR/S, PI uptake by Ly6G$^{\text{high}}$ cells was similar in both BALB/c and GKO mice and
comparable to the percentage of PI-positive cells from MW2-infected GKO mice (Fig. 2B). These findings suggest that SaeR/S-regulated factors promote neutrophil membrane disruption/permeability during invasive disease and this is consistent with findings from our previous human neutrophil studies\textsuperscript{12}. Additionally, IFNγ appears to play a role in promoting saeR/S-mediated neutrophil membrane permeability, in that neutrophils from MW2-infected GKO mice exhibited reduced PI uptake relative to BALB/c mice infected with the same strain (Fig. 2B). These findings support the cellular cytotoxicity studies described in chapter four and provide preliminary evidence that neutrophils are being damaged by saeR/S-regulated factors during invasive disease. Moreover, the data suggest that IFNγ plays a substantial role in neutrophil cytotoxicity during staphylococcal peritonitis.

Collectively, our observations indicate that SaeR/S mediates a reduced neutrophil presence by promoting a cytotoxic effect in neutrophils that is enhanced by saeR/S-mediated neutrophil-derived IFNγ. These findings are consistent with published studies outlined in chapter two, demonstrating that saeR/S regulates several factors, specifically leukotoxins, are capable of adversely impacting neutrophil function and stability.

Hypothetical Mechanism of saeR/S-Mediated S. aureus-Neutrophil Interactions During Invasive Disease (Fig. 3)

Published findings demonstrate that S. aureus is rapidly ingested by human neutrophils\textsuperscript{11} and that this process is not dependent on saeR/S\textsuperscript{12}. 
Sometime during early phagocytosis and degranulation, SaeR/S is activated and up-regulates the expression of multiple *S. aureus* virulence determinants, including: serine proteases, nuclease, leukotoxins, complement inhibitors and superantigenic proteins\(^{11,12,63}\). In turn, these factors induce the neutrophil to express IFN\(\gamma\) via an unknown mechanism (Watkins et al, Submitted). We hypothesize that IFN\(\gamma\) impacts neutrophil and other leukocyte function in both an autocrine and paracrine manner. Alternatively, but not exclusively, it is possible that neutrophil-derived IFN\(\gamma\) is a neutrophil “distress signal” indicating cellular damage is occurring. This idea stems from: 1.) SaeR/S is essential for mediating neutrophil lysis during interactions with *S. aureus*\(^{12}\) and 2.) It has been shown that neutrophils contain preformed IFN\(\gamma\)\(^{220}\), and we hypothesize this preformed IFN\(\gamma\) (as well as induced *saeR/S*-mediated neutrophil-derived IFN\(\gamma\)) is released during neutrophil cellular membrane damage.

Although the specific role of IFN\(\gamma\) in affecting neutrophil-mediated immunity against *S. aureus* is incompletely defined, IFN\(\gamma\) has been shown to impact neutrophil function in diverse ways that would likely influence interactions with this pathogen. Firstly, IFN\(\gamma\) promotes neutrophil antibacterial mechanisms, including degranulation, NADPH oxidase assembly and phagocytosis mechanisms\(^{210}\). We hypothesize that granule components and ROS are released into the extracellular space during *saeR/S*-mediated neutrophil cytotoxicity causing additional host tissue damage. Additionally, IFN\(\gamma\) reduces neutrophil-derived expression of IL-8/KC, MIP-1\(\alpha/\beta\) and
chemotactic responsiveness to formylated peptides (fMLP)$^{210}$ and it is possible these changes in expression of chemotactic factor production impact neutrophil recruitment. Moreover, IFN$\gamma$ promotes neutrophil-derived pro-inflammatory cytokine production, including TNF$\alpha$, IL-1$\beta$ and IL-6 as well as reducing apoptosis mechanisms$^{210}$, likely resulting in necrosis. The latter would suggest IFN$\gamma$ enhances neutrophil-mediated inflammation and it is possible that neutrophil-derived pro-inflammatory cytokines, specifically TNF$\alpha$ and IL-6, may play a role in mediating sepsis events, especially if systemic IFN$\gamma$ is promoting systemic TNF and IL-6 production. This idea is supported by the robust pro-inflammatory environment observed during peritonitis that is abolished in mice infected with a saeR/S-deletion mutant$^{200}$. Our findings,
combined with the previous studies characterizing the impacts of IFN\textgamma on neutrophil function, would suggest that IFN\textgamma is detrimental to the host during neutrophil-\textit{S. aureus} interactions, specifically in the context of saeR/S-mediated pathogenesis during invasive \textit{S. aureus} disease.
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