Insights into the mechanisms used by *Staphylococcus aureus* biofilms to evade neutrophil killing

Dissertation

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By

Mohini Bhattacharya

Graduate Program in Microbiology

The Ohio State University

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Dissertation Committee

Daniel J. Wozniak, Advisor

John S. Gunn

Samantha J. King

Natividad Ruiz
Abstract

Methicillin-resistant *Staphylococcus aureus* is a leading cause of morbidity and mortality with multiple chronic infections. The proficiency of *S. aureus* as a pathogen, coupled with its ability to acquire antibiotic resistance, makes it a formidable infectious agent. *S. aureus* owes a large part of its success as a pathogen to its ability to form robust biofilms that can withstand relatively higher levels of antimicrobials compared to a planktonic or single-celled population of bacteria. Biofilms are organized communities of bacteria encased in a protective matrix often composed of protein, DNA and polysaccharides, and are commonly found during chronic infection. Planktonic and biofilm bacteria grow in distinct metabolic states and therefore exhibit virulence traits that are characteristic to each mode of growth. Most studies to understand the mechanisms of *S. aureus* pathogenesis have been done with planktonic populations. However, owing to their frequent association with chronic infections, it is of great significance to understand the virulence mechanisms specific to biofilms. The unique virulome of *S. aureus* biofilms has only recently begun to be understood. The work in this dissertation unravels multiple novel mechanisms of virulence exhibited by *S. aureus* biofilms that are distinct from planktonic bacteria. This work contributes to our knowledge of *S. aureus* biofilm virulence and sets a foundation for development of anti-biofilm therapeutic strategies. We show that when grown as biofilms, *S. aureus* simultaneously utilizes multiple virulence factors to effectively evade being killed by host innate immune cells, specifically neutrophils.

Neutrophils play an important role in the elimination of *S. aureus* from the host. *S. aureus* is notorious for expressing an arsenal of virulence factors that target host cells, including neutrophils. Leukocidins are a group of bi-component toxins that target
and lyse neutrophils. Depending upon the strain, *S. aureus* can produce up to five leukocidins, namely Panton Valentine leukocidin (PVL/LukSF), LukAB, gamma hemolysin AB (HlgAB), gamma hemolysin CB (HlgCB) and LukED. While extensive work has established roles for these toxins under planktonic growth conditions, here we show for the first time that leukocidins expressed by biofilm *S. aureus* play important roles in killing human neutrophils. We show that while the leukocidins PVL (also known as LukSF) and HlgAB are released in biofilm spent media and induce the formation of neutrophil extracellular traps (NETs), LukAB helps biofilms evade contact-dependent neutrophil killing via phagocytosis. The induction of NETs is unique to biofilms and does not occur in planktonic populations. Here we show that as biofilms, *S. aureus* skews the response towards NETosis, a process which although tailored to kill bacteria, is inefficient at clearing biofilms. We find that the nuclease NucA is expressed by biofilms to degrade NET DNA, allowing biofilm bacteria to survive. DNA degraded by NETs induces the dispersal of biofilm bacteria, potentially allowing the infection to disseminate.

Collectively, the work in this dissertation uncovers novel roles for leukocidins during biofilm growth and chronic infection of *S. aureus*. Our knowledge of the host response to *S. aureus* biofilms is limited. This work contributes to our understanding of *S. aureus* biofilm pathogenesis by uncovering an important aspect of the host response to *S. aureus* biofilms, namely neutrophil-mediated bacterial clearance.
Dedication

This dissertation is dedicated to my grandparents, Rui and Margarida De Gouveia Pinto who instilled in me a love for science and a never-ending thirst for knowledge
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I would like to thank my mentor, Dr. Daniel Wozniak for his constant and unfailing support. I am forever grateful to have had a mentor that has trained me with patience, compassion and humility and provided me with immense opportunities to develop a challenging project into a graduate career that has given me a strong foundation to build my career as a scientist. I will always have the greatest respect for Dr. Wozniak both professionally and personally and I am confident that his mentorship will continue to influence my career.

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Vita

2011- present. Graduate Research Associate, Ohio State University

2009- 2011. M.S., St. Xavier’s College Mumbai, IN

2006- 2009. B.S., St. Xavier’s College, Mumbai, IN

Publications


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Chapter 1. Introduction

1.1 Staphylococcus aureus as a pathogen

1.1.1 Epidemiology and Significance

*S. aureus* is a Gram-positive commensal that colonizes human skin and mucosal surfaces such as the nose, throat, gastrointestinal tract and vaginal wall. 30% of healthy individuals in the United States are colonized with *S. aureus*, with 20% of this population being colonized in the nasal cavity. Transmission of *S. aureus* primarily occurs through direct contact- or fomite-mediated transfer. Air-borne transmission through individuals that carry *S. aureus* in their upper respiratory tract (“cloud” individuals), although less common, also occurs. The colonization of nasal passages predisposes individuals to community-acquired methicillin resistant *S. aureus* infections (CA-MRSA). The risk of infection in these individuals, as well as the bacterial burden during infection, correlates with the state of carriage. Persistent carriers were more likely to develop severe disease with higher bacterial burdens in comparison with transient or non-carriers. Persistent carriers are defined as individuals that have the same *S. aureus* pulse-field type isolated from at least three swab collections. Studies have shown that the introduction of new *S. aureus* strains to these individuals results in a selection for the original colonizing strain. In contrast, transient carriers are intermittently colonized with various strains of *S. aureus* and are thought to develop a low level of adaptive immunity against the colonizing strain, thereby providing a degree of resistance to subsequent infections.

Nosocomial infections are often associated with *S. aureus*, commonly transmitted either by direct contact with colonized healthcare workers, or as a result of invasive medical
procedures including surgeries and the introduction of medical implants\textsuperscript{7,8}. In addition to treating vulnerable patient populations, effective treatment of nosocomial infections is further complicated by the ability of the bacterium to acquire multiple drug resistance. Recent evidence indicates that \textit{S. aureus}-associated infections lead to an increase in the length of hospital stays as well as hospital associated mortality, likely due to infection with antibiotic-resistant \textit{S. aureus}. This results in a substantial economic burden on the medical industry, with total values of \textit{S. aureus} infection-related hospital costs estimated at $450 million in the past decade\textsuperscript{9}.

1.1.2 Antibiotic resistance.
A significant problem with \textit{S. aureus} infections is the rapid development of antibiotic resistance. This can occur as a result of exposure of \textit{S. aureus} to antibiotic-selection pressures during infection\textsuperscript{10}. During persistent \textit{S. aureus} infections, this may be compounded by phenotypic tolerance associated with an increase in antibiotic minimum inhibitory concentrations (MIC) by up to 10 fold \textsuperscript{11}. \textbeta-lactams such as penicillin and methicillin were introduced in the 1960’s and were highly bactericidal against \textit{S. aureus} infections. The development of resistance to these drugs however, followed shortly after and could be attributed to horizontal gene acquisition\textsuperscript{12}. Glycopeptides were the next most common class of antibiotics used to treat \textit{S. aureus} infections. While not as potent a bactericidal as the penicillins, this class of antibiotic was in use for decades, before the occurrence of glycopeptide-intermediate susceptible strains that eventually led to the rise of resistant strains. Vancomycin, one such glycopeptide, is ineffective against most pathogenic community-acquired methicillin-resistant \textit{S. aureus} strains (CA-MRSA)\textsuperscript{13}. Unlike penicillin resistance, a single gene or gene product could not be attributable to this
phenotype. Vancomycin-intermediate susceptible strains (VISA) were found to arise from cell wall adaptations that trap vancomycin before it can enter the cell. Similarly, vancomycin resistant *S. aureus* (VRSA) strains arose from modifications in the lipid II precursor of cell wall peptidoglycan, wherein D-Ala-D-Ala is modified to D-Ala-D-Lac, preventing the binding of vancomycin and subsequently its entry into the cell. Daptomycin, a cyclic lipopeptide molecule, is a novel antibiotic that has been used for vancomycin-unresponsive *S. aureus* infections. Daptomycin disrupts the cytoplasmic membrane of bacteria, resulting in rapid depolarization and cessation of DNA, RNA and protein synthesis. However, the efficacy of this drug during chronic infections, as well as the propensity for development of persistent sub-populations of bacteria during such infections remains inconclusive.

Rifampin remains the only antibiotic that shows a high degree of efficacy against persistent infections with staphylococci, and when used in combination with meropenem (β-lactam, peptidoglycan inhibitor) currently represents the best algorithm for treatment.

1.1.3 *S. aureus* infections

Device-associated infections. An area of primary concern with *S. aureus* infections is the rapid increase in the use of medical implants and prostheses and the concomitant rise in device-related infections. *S. aureus* is commonly associated with artificial surfaces including prosthetic orthopedic implants, heart valves, pacemakers and vascular catheters. These infections are facilitated by direct contact with infected individuals or carriers (such as during invasive procedures) in hospitals or introduction of bacteria from the skin surface due to surgical incision. The surgical wound site of an implant is rich in proteins such as fibronectin, which are recognized by microbial surface components
recognizing adhesive matrix molecules (or MSCRAMMs), providing a niche for bacteria to form a biofilm\textsuperscript{21}. Biofilms are structured communities of bacteria often found during chronic infections and masked by an extracellular polymeric matrix often composed of DNA, polysaccharides and proteins (described in detail in Section 1.2). For orthopedic devices, biofilms may be present on the hardware itself, bone cement and/or the surrounding fibrous tissue\textsuperscript{17}. Clumps of detached bacteria from the surface are often also found in the joint fluid. If not cleared by host immune responses or antibiotic therapy, these bacteria can disperse and enter circulation, thereby resulting in bacteremia\textsuperscript{17}.

Since many clinical isolates of \textit{S. aureus} are either methicillin- or multiple drug-resistant, treatment of biofilm infections is amplified by the increased tolerance of these bacteria to the few antibiotics to which MRSA remains susceptible\textsuperscript{11}. Thus, the presence of device-associated infections may require revision surgery for replacement\textsuperscript{9} resulting in longer hospital stays, increased possibility of a secondary infection, and, potentially, removal of the implant if the infection is unabated, augmenting the burden on the patient as well as healthcare systems. Increasing average lifespan also correlates with a rise in the population of elderly individuals requiring such prostheses making MRSA or multiply drug resistant \textit{S. aureus} a particular concern\textsuperscript{17,22}. Novel approaches to develop materials and coatings that can prevent attachment and subsequent biofilm development by \textit{S. aureus} have therefore become an important aspect of therapeutic research\textsuperscript{23}.
**Tissue-associated infections.**

*Chronic wounds.* Chronic wounds include surgical site associated wounds, traumatic wounds, diabetic foot ulcers, pressure ulcers and venous foot ulcers. Their chronicity is defined by the inability to successfully complete the reparative process that allows for wound healing and a return to normal functional and anatomical integrity within a span of three months. These wounds are commonly characterized by arrest in the inflammatory phase of healing and are often associated with bacterial infections. Bacteria in chronic wounds are frequently present as biofilms.

Given that *S. aureus* is found on the surface of skin; it is not surprising that it is the most common pathogen associated with wound infections (93.5% of ulcers) in both Europe and the United States. Twenty to fifty percent of these infections however are due to MRSA, making them a clear problem in both inpatient care and wound clinics. The currently accepted regimen to prevent persistent infections in wounds includes a combination of physical and antimicrobial therapies. Debridement or removal of necrotic tissue and bacteria from the wound bed not only reduces the possibility of persistent infection but also exposes healthy tissue to functional immune cells that are important for clearance of bacteria from the site of infection. However, the ability of *S. aureus* to re-establish in the wound necessitates continued sharp debridement coupled with multiple antiseptic and antibiotic treatments. Although the development of antimicrobial wound dressings has greatly facilitated the treatment of *S. aureus* infections in acute wounds, it should be noted that these target the removal of susceptible (largely planktonic or non-biofilm) bacteria. There is therefore an urgent need to develop ‘anti-biofilm’ wound
dressings as well as other biofilm targeted strategies for the treatment of such infections\textsuperscript{34,35}.

\textit{Osteomyelitis.} Osteomyelitis is the infection of bone or bone marrow and can occur as a result of trauma with bone damage, insertion of a foreign body such as an implant, or if bacteria gain access to the bone via the blood stream (hematogenous osteomyelitis). Infections can also spread from adjacent soft tissue infections (contiguous- focus osteomyelitis) or as a result of adjacent vascular insufficiency (such as during diabetic foot infections). While bone is relatively resilient to infection, \textit{S. aureus} grows well in cultured osteoblasts and is able to successfully colonize and cause long-term infections in bone, making it a leading cause of these infections\textsuperscript{36,37}. Acute osteomyelitis is associated with inflammation and can often be cleared with a combination of antibiotics. However chronic infections involve the formation of sequestra, or dead bone foci, that are devoid of blood supply and are recalcitrant to most interventions, often requiring debridement in addition to antibiotic treatment\textsuperscript{38}.

\textit{Cystic Fibrosis.} One in every 25 children is born with a mutation in the gene encoding the cystic fibrosis transmembrane regulator (CFTR) protein making it the most common lethal genetic disorder associated with the Caucasian population. The most common of these mutations is a deletion in a phenylalanine (\textit{ΔF508})\textsuperscript{39,40}. The CFTR protein is required for efficient ion transport across the cell membrane. The resulting imbalance of ion transport and osmotic regulation leads to dehydration of cells lining the internal organs and causes the greatest impact on airway cells and ducts of pancreas\textsuperscript{41}. The lungs of CF patients are compromised in the ability to produce airway surface liquid necessary for efficient
mucociliary clearance of pathogens from the airways. Patients with CF are therefore susceptible to chronic infection by multiple opportunistic pathogens\textsuperscript{42}, but the major colonizers are \textit{Pseudomonas aeruginosa} and \textit{S. aureus}\textsuperscript{43}.

Studies indicate that CF lung infections are associated with the presence of biofilms, allowing long-term persistence in the host\textsuperscript{44}. Although \textit{P. aeruginosa} is the cause of most complications in CF adults, children (0-17 yrs) are prevalently colonized with \textit{S. aureus}\textsuperscript{44,45}. These infections are often caused by MRSA and correlate with worsened lung function, measured by forced expiratory volume (FEV1)\textsuperscript{46}. Persisting MRSA (> 2 years), as is the case for chronic infections, increases the risk of death in children with CF\textsuperscript{45}. Although the impact of \textit{S. aureus} infections in CF is evident, a lack of detailed understanding about the specific mechanisms of pathogenesis of \textit{S. aureus} during CF is currently impeding the progress of treatments specific to chronic \textit{S. aureus} infections in CF\textsuperscript{23}.

\textit{Endocarditis}. Infective endocarditis (IE) is an infection of the endocardium or of prosthetic surfaces in the heart, usually caused by bacteria. \textit{S. aureus} is a major cause of infective endocarditis in patients (40-50\% in the United States)\textsuperscript{47,48}. Surgical procedures involving implantation of prosthetic cardiovascular devices further increase the risk of such infections\textsuperscript{48}. Patients with mechanically injured (from previous valvular disease) or inflamed heart valves are particularly susceptible to \textit{S. aureus}-associated endocarditis\textsuperscript{48}. Injured valves provide a surface for attachment and biofilm formation as well as intracellular infection of inflamed endothelium, which induces further tissue destruction, higher turbulence and deposition of clotting factors\textsuperscript{48}. \textit{S. aureus} has multiple surface-
binding determinants to components of the coagulation cascade making it well adapted to the growing vegetation. Biofilm *S. aureus* (vegetation particles) can then replicate on damaged valvular endothelium and disseminate (embolization) to cause systemic disease resulting in complications such as congestive heart failure, sepsis, persistent bacteremia, and intracardiac abscess formation, which contribute to a higher rate of in-hospital mortality in these patients.

*S. aureus* endocarditis is increasingly observed with the use of cardiovascular implants such as prosthetic heart valves, grafts, hemodialysis catheters and pacemakers. For example, there has been a concurrent increase in cases of *S. aureus* endocarditis in patients who have undergone previous surgical implantation of cardiac prostheses, since *S. aureus* is easily disseminated with direct contact, during these procedures. Problematically, cases of IE are usually identified using the modified Duke criteria, which although proven effective for the diagnosis of IE, allow a diagnosis only after the infection has become sub-acute, progressing towards systemic infection. No protocols exist for the identification of infections in the early stages of vegetation growth, since these patients are usually asymptomatic. The ability of biofilms to evade host responses may factor into this lack of identifiable disease.

**Toxinoses.** *S. aureus* is notorious for causing several toxinoses, or infections resulting from toxin-induced pathologies. The most problematic of these are pneumonia, toxic shock syndrome and food poisoning. *S. aureus* can cause both ventilator-associated and community-acquired pneumonia, with antibiotics currently being the only means for treating these infections. The severity of pneumonia caused by *S. aureus* is attributed to
the expression of alpha toxin, with a strain lacking the toxin showing a significant loss in animal survival in a murine model of infection\textsuperscript{53,54}. The pathology associated with \textit{S. aureus} pneumonia is a result of alpha toxin mediated damage of the lung epithelium and vasculature, leading to inflammation and vascular leakage\textsuperscript{55}.

Staphylococcal food poisoning is caused by a group of pyrogenic toxins known as the staphylococcal enterotoxins, that cause varying degrees of diarrhea and emesis\textsuperscript{56}. Depending on the strain, \textit{S. aureus} expresses up to 20 enterotoxins that are acquired through horizontal gene transfer\textsuperscript{57}. These toxins can survive harsh conditions of pH, desiccation and heat and can remain active long after the death of the bacterium\textsuperscript{58}. Enterotoxins are superantigens or proteins that are able to by-pass antigen processing, causing T-cell activation and proliferation by non-specifically interacting with major histocompatibility complex II (MHC II) and T-cell receptors\textsuperscript{59–62}. Another such superantigen is the toxic shock syndrome toxin (TSST-1). Toxic shock syndrome can range in severity from symptoms similar to gastroenteritis to severe hypotension and potentially multiple organ system failure\textsuperscript{63}. The expression of numerous virulence factors is what gives \textit{S. aureus} the ability to successfully establish infections in the varied tissue environments described above\textsuperscript{64}. The work in this dissertation is aimed at understanding the role of virulence factors in an established \textit{S. aureus} infection. The main virulence mechanisms relevant to this work are therefore described below.

\textit{1.1.4 Virulence mechanisms}

\textit{S. aureus} is infamous for the expression of multiple virulence factors, which includes numerous toxins that cause host cell cytotoxicity as well as proteins that indirectly allow for the manipulation or evasion of host cell recognition\textsuperscript{62,65,66}. Some of the main
virulence factors that are mentioned in this dissertation are briefly reviewed in this section and summarized in Table 1.1. The toxins that are a focus of the work presented in the chapters that follow are described in further detail in Section 1.4. The ability of *S. aureus* to colonize varied host environments is owed in part to the ability of *S. aureus* to alter the expression of virulence factors relatively rapidly, depending upon the environment. The global regulatory systems that control the expression of major virulence factors pertinent to this work are also discussed below, with a summary of these networks depicted in Figure 1.1.

**Regulation of virulence.** The regulation of *S. aureus* virulence occurs via a large group of transcriptional regulators, two component systems and RNA effector molecules. Some of the main mechanisms that are involved in regulating the virulence factors pertinent to this dissertation are discussed below.

*The accessory gene regulator (Agr) and RNAIII:* Agr is a two-component signal transduction system that either directly or indirectly controls the expression of a majority of virulence genes in *S. aureus*. This system is also the main quorum sensing circuit in the bacterium and is therefore crucial for biofilm development. The *agr* operon itself is under the control of two promoters, P1 and P2. The P2 promoter drives the expression of the 4 genes that govern the signal transduction circuit (*agrA, agrB, agrC* and *agrD*) while the P3 promoter regulates the RNAIII transcript, which controls the expression of multiple virulence factors as well as the *saeRS* two component system, found to be essential for immune evasion by *S. aureus*. The expression of *agr* is temporal and changes, depending upon the growth phase. While *agr* expression is inactive in early exponential phase, it increases during late exponential phase, with stationary phase cells showing the highest
levels of gene expression. During post-exponential growth the \textit{agrD} gene encodes for a pro-peptide that is matured into an auto-inducing peptide (AIP) and secreted to the external environment through the transmembrane AgrB protein. This maturation of AgrD involves multiple proteolytic digestions and the addition of a thioester bond, critical for the interaction of secreted AIP with the transmembrane AgrC histidine kinase. This interaction drives the homodimerization and phosphorylation of the AgrC histidine domain, which in turn allows for the transfer of phosphorylation from AgrC to the cytoplasmic AgrA (response regulator). AgrA then binds to the \textit{agr} P2 and P3 to complete the circuit (Figure 1.1A)\textsuperscript{70,71}.

The expression of \textit{agr} results in a repression of surface-associated proteins and an up-regulation of secreted virulence factors via the activity of the pleiotropic effector RNA III transcript\textsuperscript{72,73}. RNAIII regulates virulence genes either directly or indirectly through the activation of other regulatory networks (SarT, SarS). Some of the virulence factors regulated by \textit{agr} include 7 enterotoxins, phenol soluble modulins, 5 leukotoxins as well as the three hemolysins \(\alpha\),\(\beta\) and \(\delta\), with the \(\delta\)-hemolysin being encoded for in the RNAIII transcript\textsuperscript{67}. In addition to its significant role in the regulation of toxins, the \textit{agr} regulatory network plays a role during every step of biofilm formation\textsuperscript{73–75}. This includes the regulation of proteins required for \textit{S. aureus} to successfully attach to abiotic and human tissue surfaces, including fibronectin- and collagen-binding proteins as well as the expression of the quorum sensing auto-inducing peptides and phenol soluble modulins essential for dispersal of cells into planktonic (free-floating) state.

\textit{The Staphylococcal accessory gene regulatory network (sar):} The \textit{sar} operon was discovered when a mutation in this locus caused a change in the expression of virulence
This operon consists of three overlapping transcripts under the control of separate promoters (sarP1, sarP2, sarP3) that are multi-σ factor-dependent and therefore can be expressed during all growth conditions (Figure 1.1 C). While sarP1 was expressed during mid-exponential growth, sarP3 was expressed during late exponential to stationary phase as well as during conditions of environmental stress. sarA encodes a DNA-binding protein that binds as a homodimer to target sequences in the -10 to -35 regions of promoters and causes a disruption in DNA superhelicity. This can either up-regulate (agrP2, agrP3) or repress (sspA, cna, spa) the transcription of genes depending upon the effect of this alteration on optimal promoter spacing required for transcription. SarR negatively regulates the expression of SarA by directly binding to the P1 and P2 promoter regions. The expression of sarR peaks during late-exponential growth and causes a decrease in the expression of SarA during this phase as well as the stationary phase. Additionally, SarS/SarH1, SarT and SarU are homologues of SarA that play roles in regulation of downstream virulence factors either independently of, or in conjunction with the agr/sarA regulatory circuits.

SaeRS two-component system: The S. aureus exoprotein expression (SaeRS) two-component system is a major virulence regulator that controls the transcription of virulence genes independently of agr. saeR and saeS are co-transcribed, separated by 2 nucleotides and show homology to numerous transcriptional regulators from other bacteria (Figure 1.1B). While the expression of this system was found to be essential for the expression of virulence factors including alpha-hemolysin, beta-hemolysin, coagulase, as well as the bi-component leukocidin proteins, this was independent of the expression of global regulators agr and sarA. The concerted activity of SaeR and the repressor of toxins (Rot)
has been implicated in causing the synergistic activation of promoters for the expression of superantigen-like immunomodulatory proteins (Ssl) in response to exposure to neutrophils, contributing to virulence in vivo. Furthermore, studies with mouse models of necrotizing pneumonia describe saeRS as being required for the survival of bacteria, with a saeRS mutant showing significantly lower bacterial burdens in comparison to a wild type infection. Therefore, while much remains to be understood about the mechanisms for target gene regulation by saeRS, these studies illustrate the broad impact of this two-component system.

**Sigma factor B (σ^B) and Rot:** The association of σ^B, encoded by the *rpoF* gene, with the RNA polymerase core enzyme (αββ') occurs in response to environmental perturbations (heat/cold shock, salt, starvation). *rpoF* is part of a chromosomal cluster encoding RsbW, an anti-sigma factor that is also a phosphorylase, RsbV, an anti-anti σ^B factor and the phosphatase, RsbU (Figure 1.1D). The binding of RsbW to σ^B prevents its incorporation into the core enzyme and therefore transcription of target genes. This occurs during exponential phase and is associated with the phosphorylation of RsbV by RsbW (Figure 1.1). During conditions of stress, however, the activity of RsbU allows for dephosphorylation of RsbV and the formation of a strong RsbV-RsbW interaction, allowing for the release of σ^B and activation of downstream transcription. SigB allows for bacterial aggregation and adhesion to surfaces by positively regulating the transcription of genes for adhesin proteins as well as other factors required for biofilm formation. Additionally, SigB is also known to be required for the resistance of bacteria to superoxide stress such as that involved in the interaction with host cells. This likely occurs via the up
regulation of at least one catalase protein. SigB also controls the expression of the carotenoid proteins, further contributing to resistance to oxidants \textsuperscript{85–87}.

The repressor of toxins, Rot is a SarA homologue that was first described as a gene that directly down-regulates the expression of multiple toxins including leukocidins and alpha hemolysin \textsuperscript{88}. Recent studies have shown that Rot can positively regulate the expression of genes, such as the genes encoding for superantigen-like proteins in addition to numerous cell surface associated proteins \textsuperscript{81,89}. While 60 genes were found to be negatively regulated by Rot, 86 genes were up-regulated when a wild-type and \textit{Δrot} strain were compared. Whereas \textit{agr} negatively regulates the expression of cell surface-associated virulence proteins and up-regulates the expression of secreted virulence factors, Rot seems to act in an opposite role, with cell surface proteins being a large group of positively regulated proteins \textsuperscript{90}. Rot is thus thought to be an important global virulence regulator that functions during early infection/exponential phase growth when \textit{agr} is not expressed, to assists the colonization and adhesion of bacteria as well as early immune evasion \textsuperscript{89}. 
Summary of the major global regulatory networks that control the expression of virulence factors in *S. aureus*. The *agr* operon encodes for proteins that form the main, self-regulating quorum sensing circuit in *S. aureus*. The operon consists of two transcripts, RNAII and RNAIII. RNAIII positively regulates the expression of multiple toxins, indirectly through the downregulation of the repressor of toxins (Rot). RNAIII also negatively regulates the expression of cell-wall associated proteins (A). SaeRS is a two-component system that positively regulates the expression of toxins independent of Agr (B). The expression of SarA from three promoters, P1, P2 and P3 is dependent on the activity of multiple sigma factors. SarR negatively affects the expression of SarA by binding at the P2 promoter and upstream of P1. SarA has a positive effect on the expression of the *agr* RNAIII transcript(C). The sigma factor $\sigma^B$ positively regulates the expression of SarA and is sequestered by the anti-sigma factor RsbW during the exponential phase of growth. During conditions of stress RsbV selectively binds RsbW and allows for $\sigma^B$ to be active (D).


**Figure 1.1 Global virulence regulators in *S. aureus***

Summary of the major regulatory networks that control the expression of virulence factors in *S. aureus*. The *agr* operon encodes for proteins that form the main, self-regulating quorum sensing circuit in *S. aureus*. The operon consists of two transcripts, RNAII and RNAIII. RNAIII positively regulates the expression of multiple toxins, indirectly through the downregulation of the repressor of toxins (Rot). RNAIII also negatively regulates the expression of cell-wall associated proteins (A). SaeRS is a two-component system that positively regulates the expression of toxins independent of Agr (B). The expression of SarA from three promoters, P1, P2 and P3 is dependent on the activity of multiple sigma factors. SarR negatively affects the expression of SarA by binding at the P2 promoter and upstream of P1. SarA has a positive effect on the expression of the *agr* RNAIII transcript(C). The sigma factor $\sigma^B$ positively regulates the expression of SarA and is sequestered by the anti-sigma factor RsbW during the exponential phase of growth. During conditions of stress RsbV selectively binds RsbW and allows for $\sigma^B$ to be active (D). Adapted from Bronner et al, *FEMS Microbiol. Review*, 2004.
Secreted Toxins. Notorious for its arsenal of toxins, *S. aureus* tightly controls the expression of 25-30 proteins that directly target host cell membranes. Depending upon the strain, *S. aureus* can secrete up to 5 bi-component leukocidins that target and kill leukocytes. These proteins are the focus of the work presented in this dissertation and are therefore described in greater detail in the sections that follow. Discussed below are additional secreted toxins that are considered important virulence factors and are also relevant to this dissertation.

Phenol soluble modulins (PSMs) are small amphipathic surfactant peptides able to lyse leukocyte membranes and are therefore also classified as ‘leukocidins’ even though the mechanisms of pore formation and cell lysis are different from classic leukocidins described below. These alpha helical peptides are thought to cause cell lysis using both receptor-mediated and receptor-independent mechanisms, with pores being short lived and caused potentially due to membrane perturbations. An interesting function of these toxins is their ability to induce host cell lysis from within phagosomes. Following phagocytosis, PSM expression was found to be increased and was associated with the induction of the stringent response system in *S. aureus*. While PSMs have been shown to puncture neutrophils after escape from phagosomes, the mechanisms behind phagosomal escape are not fully understood. Although most commonly studied for their roles neutrophil lysis, PSMs are also able to lyse epithelial cells, endothelial cells, bone cells, monocytes and erythrocytes. Apart from their potent cytolytic activities, PSMs also play an important role in allowing for dispersal of biofilms. During growth as biofilms, PSMs switch from their amphipathic nature to form amyloid fibers, thereby modulating their dispersal function, until conditions are conducive to dispersal and a switch back to
the amphipathic peptide form\textsuperscript{98}. The mechanisms governing this switch however remain unknown.

Additionally, most \textit{S. aureus} strains can express 4 hemolysins, alpha, beta, gamma and delta. Alpha hemolysin is one of the earliest and best-known virulence factors to date. Unlike the octameric pores formed by leukocidins, alpha hemolysin forms a heptameric pore from a monomeric protein (HemA) and exclusively targets RBCs, with a high affinity for rabbit cells. Alpha hemolysin acts in concert with leukocidins to mediate the lysis of macrophages \textit{in vitro}. As described above, gamma hemolysin is in fact a member of the leukocidin family of proteins. Although the cell specificities of this toxin remain unclear, it is known to lyse both immune cells and RBCs from rabbits and humans\textsuperscript{99–102}. Similarly, although delta hemolysin was first characterized as a hemolysin based on its ability to lyse red blood cells, the discovery that this toxin can also lyse immune cells with mechanisms similar to PSMs, has classified it as a PSM toxin. In fact, most early work to describe the roles for PSMs were done using delta hemolysin\textsuperscript{93}.

\textit{Surface-bound proteins}. In addition to factors that are secreted to directly target host cells, \textit{S. aureus} expresses numerous proteins that are covalently and non-covalently bound to the cell surface\textsuperscript{103}. These include proteins that are required for adhesion to both biotic and abiotic surfaces. The expression of these proteins is tightly regulated by some of the regulatory networks described above and is tailored to the infection environment. Some of the most important covalently bound MSCRAMM proteins include the proteins required for adhesion to host tissue including epithelial cells (ClfB, IsdA, SasG), collagen (Cna), fibrin (FnBPA, FnBPB) and bone (BpB). Regardless of the protein, these cell-surface anchored molecules have an N-terminal signal peptide and a characteristic C-terminal
LPTXG motif that anchors the protein to the cell wall, often via the activity of a transpeptidase sortase (SrtA) that allows linking with the peptidoglycan layer. Protein A (described in detail below) is covalently linked to the cell surface and mediates the attachment of *S. aureus* to the Fc portion of immunoglobulins, resulting in an inhibition of phagocytosis. *S. aureus* also expresses 4 surface-associated heme iron-scavenging proteins (IsdA-IsdC, IsdH) that are controlled by the iron responsive regulator Fur, and cause platelet aggregation. Iron is a limited resource and is important for the growth and virulence of *S. aureus*. These proteins are known to play an important role in vivo, with IsdB allowing for significantly better rates of bacterial survival in a murine model of abscess formation.

*S. aureus* also expresses a number of proteins that are non-covalently linked to the surface via ionic bonds. Of note is the autolysin Atl, a peptidoglycan hydrolase involved in cell division, cell wall separation, peptidoglycan turnover and tolerance to penicillins. While the contribution of this enzyme to virulence is not fully understood, it is known to bind to fibrin and fibrinogen as well as facilitate the internalization of *S. aureus* by endothelial cells. A similar protein isolated from *S. epidermidis* was found to mediate binding to polystyrene surfaces as well as the release of bacterial DNA that forms a part of the EPS of *S. epidermidis* biofilms. Atl might therefore also play an important role in *S. aureus* biofilm establishment.

*Polysaccharides.* The polysaccharide intercellular adhesin (PIA) is the main polysaccharide associated with *S. aureus* and is composed of repeats of poly N-acetyl glucosamine. The proteins required for the synthesis and transport of PIA are encoded in the *icaACDB* operon. 15-20% of the N-acetylglucosamine residues are deacetylated,
giving PIA a positive charge\textsuperscript{114}. While studies show that PIA is important for biofilm development and persistence during implant-associated infections, the role of PIA in pathogenesis of \textit{S. aureus} remains controversial, owing to the identification of numerous clinical isolates, including the widespread USA300 CA-MRSA strain, that can proficiently form biofilms and cause infections independent of PIA\textsuperscript{115}.

Additionally, \textit{S. aureus} strains produce two capsular polysaccharides, namely Cap5 and Cap8, that have been described to be important factors in impeding phagocytosis of \textit{S. aureus} and allowing persistence \textit{in vivo}\textsuperscript{116}. Similar to studies with PIA however, numerous strains isolated from pediatric patients show that 82\% of MRSA isolated were unencapsulated, with over half of the isolates obtained from invasive dermatological infections being identified as unencapsulated USA300. The relative contribution of capsular polysaccharides to persistence \textit{in vivo} therefore remains poorly understood\textsuperscript{117}.

Lastly, \textit{S. aureus} also displays teichoic acids (TA) and lipoteichoic acids (LTA) on the cell surface. Teichoic acids consist of alternating phosphate and ribitol while lipoteichoic acids are composed of alternating phosphates and glycerol\textsuperscript{118}. Both molecules are highly charged with substitutions of D-alanine and N-acetylglucosamine. The positive charge of TA and LTA determine the binding of \textit{S. aureus} to abiotic surfaces with a \textit{dltA} mutant of TA being unable to form biofilms \textit{in vitro}. This is due to a change in the overall charge to a highly negative state, similar to most abiotic surfaces, including polystyrene and glass. A TA mutant was also found to have decreased attachment to endothelial surfaces and showed a significantly lower rate of success in causing infective endocarditis in a rabbit model of infection, further suggesting a role for these molecules in attachment and biofilm formation \textit{in vivo}\textsuperscript{109,119}. 

1.1.5 Host response to *S. aureus*

*S. aureus* is an opportunistic pathogen that colonizes 30% of the population without causing infection\textsuperscript{120,121}. Once the body’s defense is compromised or the pathogen finds a port of entry, *S. aureus* can cause devastating infections in multiple tissues, often leading to biofilm formation and chronic infection\textsuperscript{23}. Coupled with the rapid acquisition of antibiotic resistance, this makes *S. aureus* a major infectious disease problem. Depending upon the mode of entry, *S. aureus* has multiple mechanisms available to evade the innate immune system, the first line of defense for the body to eliminate bacterial pathogens\textsuperscript{122}. This includes the activity of macrophages, neutrophils and dendritic cells, ultimately leading to the activation of the acquired immune system. Regardless, *S. aureus* has developed mechanisms to evade these immune defenses at every step and often establishes prolonged infections in the human host\textsuperscript{123,124}. The main cascade of events that lead to successful establishment of invading *S. aureus* are described below. The virulence properties of an established infection (biofilm) are distinct from planktonic bacteria and form the main focus of this dissertation. These are therefore described in greater detail in the Section 1.2.

Evasion of innate immune defenses.

Regardless of the port of entry, the first step in the establishment of a bacterial infection requires the pathogen to evade the innate immune defenses. Neutrophils are the most critical innate immune effectors in protecting the human host from *S. aureus* infections\textsuperscript{65}. Neutrophils are a key aspect of the work presented in this dissertation and are therefore described in greater detail in Section 1.3. The efficient recruitment and functioning of
neutrophils relies upon the effective communication between neutrophils and other innate immune cells including macrophages, monocytes and dendritic cells\textsuperscript{122}. While macrophages are always patrolling host tissue environments for the presence of ‘alarmins’ or non-host entities, monocytes are commonly found in the blood, heading towards a site of infection, similar to the process by which neutrophils are recruited. Once recruited, monocytes can differentiate into macrophages and dendritic cells, thereby providing a sufficient level of antigen-presenting cells for the elimination of bacteria. Alternatively, monocytes can remain undifferentiated at the site of infection and contribute to tissue repair. Tissue macrophages are responsible for alerting neutrophils by activating the expression of tumor necrosis factors and IL-1, which fosters the rolling and adhesion of neutrophils that follow an increasing gradient of cytokines. This requires the interaction of neutrophil ligands (PSGL-1, LFA-1, CD44) with endothelial receptors. \textit{S. aureus} can interfere with the process of neutrophil chemotaxis via the activity of multiple superantigen-like proteins including chemotaxis inhibiting proteins (CHIPS) and phenol soluble modulins (PSMs)\textsuperscript{124}. Phagocytes that are able to reach the site of infection utilize complement-mediated killing and phagocytosis to eliminate the infection. \textit{S. aureus} has similarly developed mechanisms to evade complement-dependent killing by inactivating complement components at various stages of the complement cascade. The first step of complement cascades involves the deposition of C1q on the bacterial surface. Staphylococcal binder of immunoglobulin (Sbi) is known to bind the Fc portion of C1q and inactivate this process. Aureolysin is a secreted protein that cleaves C3 into active forms of C3a and C3b. Subsequently, factors I&H cleave C3b before it deposits onto the bacterial surface. Similarly, the Staphylococcal complement inhibitor protein (SCIN),
expressed only from human isolates, inhibits the activity of the C3 convertase that is required for the cleavage of C3 into C3a and C3b\textsuperscript{125,126}. Fibrinogen binding protein and complement binding proteins also perform this function. The formation of C5b is the last step of the classical complement pathway and results in the formation of the membrane attack complex. This is ineffective at clearing \textit{S. aureus}, potentially due to the presence of a thick peptidoglycan layer and/or capsules\textsuperscript{122,124}. Agglutination of \textit{S. aureus} with fibrin (via fibrin binding proteins) also protects bacteria from phagocytosis by neutrophils.

Once phagocytosis occurs, leukocytes use various mechanisms to kill \textit{S. aureus}. This includes the release of reactive oxygen and nitrogen species and antimicrobial peptides. In addition to expressing toxins that allow \textit{S. aureus} to escape phagocytosis by neutrophils, monocytes and macrophages (described in Section 1.3)\textsuperscript{93,127–129}, \textit{S. aureus} modifies its cell surface to disable binding of antimicrobial peptides produced by these innate immune cells. This includes D-alanylation of teichoic acids (functions encoded in the \textit{dlt} operon) and acetylation of peptidoglycan. Staphyloxanthin, the golden yellow pigment associated with \textit{S. aureus}, neutralizes reactive oxygen species in addition to catalase and alkyl hydroperoxide reductase, while lactate dehydrogenase contributes to the maintenance of redox homeostasis. Additionally, the extracellular adherence protein and its homologues neutralize neutrophil proteases including elastase, Cathepsin G and proteinase 3\textsuperscript{124,130}.

\textbf{Evasion of adaptive immune responses}. One of the most important virulence factors involved in the subversion of adaptive immune responses is Protein A. Protein A is a superantigen-like protein that is anchored to the surface via the activity of a sortase, but can also be secreted and has a high affinity for immunoglobulins\textsuperscript{131}. This protein acts to
assist immune evasion in two ways. First, it can bind Fc regions of IgG antibodies to block phagocytosis. It can also bind to Fab regions, particularly the V_{H}3 regions of IgM molecules, to cause crosslinking and ultimately apoptosis of antibody-secreting B-cells\textsuperscript{131}. Secondly, Protein A acts as a superantigen by binding to MHC-II molecules and causing antigen-independent crosslinking with T-cell receptors on T_{H} cells\textsuperscript{131,132}. This results in a non-specific interaction, leading to the ineffective release of cytokines and failure of the antigen-processing mechanism. Additionally, the δ-hemolysin encoded in the RNAIII transcript is able to lyse T-cells and cause mast cell degranulation\textsuperscript{124}. Lastly, depending upon the strain, \textit{S. aureus} can produce up to 23 superantigens that manipulate the host response using mechanisms similar to Protein A\textsuperscript{132–134}.

While past research has undoubtedly provided us with extensive knowledge about the mechanisms of pathogenesis used by \textit{S. aureus}, most of these studies have failed to differentiate between acute and chronic \textit{S. aureus} infections. Biofilms are commonly found in association with chronic infections and respond to host defenses using mechanisms that are distinct from planktonic or single-celled populations, often associated with acute infections. The focus of this research is to understand the host response to \textit{S. aureus} biofilm infections, particularly the response of biofilms to neutrophil antimicrobial strategies.
Table 1.1 Main virulence factors in *S. aureus*

<table>
<thead>
<tr>
<th>Virulence Factor</th>
<th>Target cell/Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Toxins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leukocidins</td>
<td>Leukocyte, RBC lysis</td>
<td>135</td>
</tr>
<tr>
<td>Phenol soluble modulins</td>
<td>Leukocyte lysis, biofilm dispersal factor</td>
<td>93,136</td>
</tr>
<tr>
<td>Alpha hemolysin</td>
<td>RBC lysis, evasion of neutrophil killing</td>
<td>101,127</td>
</tr>
<tr>
<td>Beta hemolysin</td>
<td>RBC lysis, nutrient scavenging</td>
<td>137</td>
</tr>
<tr>
<td>TSST-1</td>
<td>Prevents antigen processing by immune cells</td>
<td>138</td>
</tr>
<tr>
<td><strong>Adhesins/Nutrient scavenging</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ClfB, SasG</td>
<td>Attachment to epithelial cells</td>
<td>139</td>
</tr>
<tr>
<td>FnBPA, FnBPB</td>
<td>Fibrinogen, elastin binding proteins</td>
<td>140,141</td>
</tr>
<tr>
<td>Cna</td>
<td>Collagen binding protein</td>
<td>142,143</td>
</tr>
<tr>
<td>Bpb</td>
<td>Binding to bone sialoprotein, fibrinogen</td>
<td>144,145</td>
</tr>
<tr>
<td>IsdA-IsdC, IsdH</td>
<td>Adhesion to epithelial cells, iron scavenging proteins</td>
<td>146,106</td>
</tr>
<tr>
<td>Autolysin</td>
<td>Host tissue binding, endothelial internalization of <em>S. aureus</em>, tolerance to penicillins</td>
<td>111,147,148</td>
</tr>
<tr>
<td><strong>Polysaccharides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polysaccharide intercellular adhesin</td>
<td>Biofilm matrix component</td>
<td>114</td>
</tr>
<tr>
<td>(PIA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Capsular polysaccharides (Cap5/8)</td>
<td>Masking of surface associated molecular patterns/ impeding phagocytosis</td>
<td>117,149</td>
</tr>
<tr>
<td>Teichoic/Lipoteichoic acids</td>
<td>Attachment to abiotic surfaces</td>
<td>109,119</td>
</tr>
<tr>
<td><strong>Immune evasion factors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein A</td>
<td>Blocks antibody mediated killing, antigen presentation by immune cells</td>
<td>150</td>
</tr>
<tr>
<td>Chemotaxis inhibiting protein (CHIPS)</td>
<td>Inhibits neutrophil chemotaxis</td>
<td>151,152</td>
</tr>
<tr>
<td>Staphylococcal complement inhibitor</td>
<td>Inhibits complement mediated killing</td>
<td>152</td>
</tr>
<tr>
<td>protein (SCIN)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuc</td>
<td>Evasion of neutrophil killing, biofilm dispersal</td>
<td>87,153,154</td>
</tr>
<tr>
<td>Proteases (Aur, SspAB, ScpA Spl)</td>
<td>Evasion of complement mediated killing, biofilm dispersal</td>
<td>155–157</td>
</tr>
</tbody>
</table>
1.2 Biofilms: A Lifestyle Distinct from Free-floating populations

1.2.1 General characteristics

Until recently, most research has focused on the planktonic or free-floating growth of bacteria, reproduced as test tube grown cultures in the laboratory. However, bacteria in their natural habitats are very rarely found as single celled entities. In fact, the first description of bacteria, made by Anthony Van Leeuwenhoek as ‘scuff’ or ‘plaque’ on the surface of teeth, was the demonstration of a biofilm. Since then, studies have shown that, most often, in nature as well as in infection environments, bacteria reside as biofilms. Biofilms are aggregated, structured communities of bacteria encased in an extracellular matrix composed of protein, DNA and polysaccharides, often referred to as extracellular polymeric substances or EPS. Biofilms are a social and physical congregation of bacteria and while biofilms are often implicated in detrimental roles such as biofouling and chronic infections, they can also play beneficial roles. For example, biofilms form a part of the normal microbiota that eliminate pathogenic bacteria through ‘colonization resistance.’ They have also been extensively used in processes such as water filtration, waste-water treatment and microbial leaching of precious metals.

The three most important characteristics that define a biofilm include the presence of an EPS or matrix, the decreased susceptibility to antimicrobials and phagocytes (such as neutrophils) and quorum sensing or inter-cell communication. While creating a barrier for the entry of some foreign molecules such as certain antibiotics into the biofilm, the matrix allows for physiological heterogeneity that is characteristic of biofilms. It plays an important role in the formation of nutrient, oxygen and water gradients through the biofilm, that ultimately dictate bacterial responses to environmental cues.
The EPS immobilizes biofilm bacteria and allows cells to be in constant proximity to each other, thus facilitating inter-cell signaling and horizontal gene transfer. Although the cues that dictate formation and composition of the matrix are not fully understood, the matrix forms a crucial interface between the biofilm and its environment, with the composition of the EPS dictating, to a large extent, the architecture of the biofilm.

The decreased susceptibility or increased ‘tolerance’ to antimicrobials associated with biofilm bacteria is a transient effect that is lost once bacteria transition into planktonic phase\(^{165}\). One mechanism for this tolerance is via the interaction and sequestration of antimicrobials by EPS components. This however, depends upon the antimicrobial, with some antibiotics being able to efficiently penetrate the matrix\(^{166}\). A second mechanism for tolerance in biofilm bacteria is the development of ‘persister’ or metabolically dormant cells. The mode of action of most antibiotics involves targeting processes such as transcription and cell wall biosynthesis, that occur in actively growing bacterial cells, thus inadvertently rendering persisters tolerant\(^{165,167}\).

When bacteria reach a critical concentration or ‘quorum,’ they communicate with other bacteria through the expression of signaling molecules or ‘autoinducers’ in a process known as quorum sensing. This process of communication occurs in order to induce a response (such as biofilm formation, bioluminescence) that would prove exponentially more advantageous to the bacterium if it were induced by a population, rather than a single cell\(^{168}\).

The three broad stages that define the lifecycle of most bacterial biofilms are attachment, maturation and dispersal. Biofilm formation begins either when bacteria attach to a surface or to each other, as is the case for aggregate formation\(^{169}\). The expression of
genes often changes during the lifecycle of a biofilm, with attachment and dispersal phases often being quite distinct from the maturation phases\textsuperscript{170}. The signals that elicit the dispersal of biofilms largely depend on the environment. Dispersion may occur as a result of active cues from bacteria; for example, in response to a host immune attack, a chemotactic response to a nutrient or substrate, or via passive mechanisms including shear stress, mechanical sloughing, etc.\textsuperscript{171}. While there is no common mechanism for biofilm dispersal, detachment of bacteria results in a switch, back to a planktonic lifestyle until another surface or environment conducive for biofilm formation is found\textsuperscript{172–174}.

1.2.2 Host Responses

While infections with planktonic bacteria are often resolved by components of the innate immune system, pathogens such as \textit{S. aureus} have developed mechanisms to evade the innate response and survive, thereby transitioning into growth as biofilms\textsuperscript{65}. Once established, a bacterial biofilm represents an exponentially bigger challenge for the immune system, and often remains uncleared. Thus, biofilms are found associated with persistent, chronic infections\textsuperscript{25,175,176}. During growth as biofilms, bacteria exhibit increased tolerance to host defenses and antimicrobials, withstanding concentrations that would eliminate single-celled populations and making biofilm infections particularly difficult to eradicate\textsuperscript{11,23,177}.

The effect of host immune cells on biofilms is significantly influenced by the nature of the matrix and the associations between host cells and matrix components\textsuperscript{178}. The EPS functions as a barrier, preventing host immune cells and antimicrobials from penetrating the biofilm. The EPS also masks the recognition of pathogen associated molecular patterns by host cells, a process crucial to the recruitment of innate immune cells and activation of
complement-mediated killing. Studies describe a role for matrix polysaccharides in impeding the innate immune response, by impairing pattern recognition receptor (PRR) activity, reducing complement sensitivity and dampening pro-inflammatory cytokine signaling. This is unlike the pro-inflammatory effect observed for matrix-associated DNA and protein components. Nevertheless, immune cells such as phagocytes are often able to penetrate the biofilm biomass and attempt to clear these infections. The mechanisms involved in host-biofilm interactions however, are poorly understood. The work in this dissertation attempts to understand some of the mechanisms associated with biofilm evasion of the host innate immune system, in the context of S. aureus infections.

Phagocytosis is an important mechanism by which innate immune cells such as neutrophils and macrophages eliminate bacteria. Engulfment of aggregates or biofilms however, represents an additional challenge for these cells. Phagocytes have recently been shown to engage neutrophil extracellular trap formation (NET formation), in response to biofilm infections. This mechanism of clearance is also known as a ‘beneficial suicide’ process that involves the release of the cell’s chromatin to trap a large object (such as a biofilm) and kill bacteria using neutrophil antimicrobial products laced on chromatin. However, our work as well as the work of others has shown that bacterial pathogens have developed mechanisms to evade killing via NETosis. Thus, the consequence of the innate immune response to pathogenic biofilms remains relatively unknown. Whether the host generates a specific immune response to a recurring biofilm infection, is uncertain. However, it is thought that both innate and adaptive immune responses might be simultaneously generated and that neither is successful at eliminating the biofilm, rather that these responses result in tissue damage and inflammation.
Of note, planktonic and biofilm bacteria often elicit very distinct responses in host cells, a difference that needs to be considered in order to fully understand the pathogenesis associated with an infection. This is a concept that is described in this dissertation, in terms of the neutrophil response to *S. aureus* biofilms, which in conjunction with previous studies, provides some broader implications for the host response to biofilm infections\textsuperscript{28,127,193–195}.

1.2.3 *S. aureus* biofilms

*S. aureus* is a proficient biofilm-forming pathogen. Combined with its ability to rapidly acquire antibiotic resistance, the increased antimicrobial tolerance of bacteria in biofilms makes the treatment of these infections a major healthcare concern\textsuperscript{23}. Like other pathogens, the biofilm lifecycle of *S. aureus* follows three main stages, attachment, maturation and dispersal (Figure 1.2). Recent studies describe the existence of an intermediate stage of dispersal dependent on extracellular matrix-associated DNA, which occurs after attachment and has been termed the ‘Exodus’ phase\textsuperscript{196}. Nevertheless, all stages are tightly controlled by the Agr regulatory network (described in Section 1.1). Agr forms the main quorum sensing circuit in *S. aureus* and is highly upregulated in stationary phase cells\textsuperscript{73}. This results in the expression of adhesion factors that allows attachment to host matrix components (ClfA, ClfB, FnBPA/B and Cna) or abiotic surfaces (lipotechoic acids, cytoplasmic proteins and DNA) such as glass and polystyrene\textsuperscript{16}. The contribution of polysaccharide, DNA and proteins to the matrix of an *S. aureus* biofilm depend in large upon the strain and the conditions of growth. Multiple reports describe the ability of *S. aureus* to form polysaccharide-independent biofilms, making the contribution of PIA (described in Section 1.1) controversial\textsuperscript{115,197,198}. Of note, the formation of PIA-dependent
biofilms was found to be common in methicillin-sensitive strains of *S. aureus* and might therefore be an important virulence factors in these less commonly studied strains\(^\text{198,199}\). Apart from the adhesion factors described above, multiple proteins are associated with the biofilm matrix. These are often passively derived from bacterial cells via the activity of autolysins. Autolysins are arguably one of the most important protein components in *S. aureus* biofilms. In addition to causing the release of cytosolic proteins, the expression of autolysins, regulated via the *cidABC/lrgAB* operons allows for the release of DNA that forms an important structural component of the biofilm\(^\text{196}\). While mature biofilms show a downregulation of *agr*, potentially to facilitate the conservation of energy and resources within a biofilm, the expression of the regulon is increased when dispersion occurs. This is likely in response to the re-availability of nutrition and/or conditions conducive to planktonic growth\(^\text{200,201}\). The process of dispersion in *S. aureus* biofilms involves the release of numerous exoenzymes that break down components of the biofilm matrix. This includes PSMs that act as surfactants, as well as proteases that catalyze the breakdown of matrix proteins, leading to the destabilization of biofilm structure\(^\text{97,155,202,203}\). PSMs were also recently found to switch to an amyloid fibril structure during the development of biofilms, thereby contributing to the matrix structure\(^\text{98}\).

The thermonuclease Nuc also plays a role in biofilm development. The expression of this nuclease is tailored to the later stages of biofilm development and allows for dispersal of biofilm bacteria by breaking extracellular DNA associated with the matrix\(^\text{87}\). This results in a loss of the biofilm scaffold structure and release of bacterial populations\(^\text{196}\). Nucleases are also known to play an important role in breaking down NET-associated DNA
released by neutrophils exposed to \textit{S. aureus}\textsuperscript{191}. Whether these two mechanisms are correlated during biofilm associated infections was so far unknown and is the basis for some of the work presented in this dissertation.
Model describing the main stages of the biofilm lifecycle of \textit{S. aureus}. Attachment involves the interaction of bacterial adhesion molecules with the surface, either host tissue or abiotic (A). Once attached, \textit{S. aureus} multiplies and reaches a critical number, quorum sensing ensues via the \textit{agr} regulatory network. This results in the expression of genes required for the synthesis of biofilm matrix components and auto inducing peptides, which continues the self-responding Agr quorum sensing circuit (B). A self-produced nuclease (NucA) causes the mass exodus of biofilm cells by degrading matrix associated DNA in an \textit{agr}-independent manner (C). Biofilm bacteria continue to multiply, and biomass increases, in an \textit{agr}-dependent process (D). Agr-dependent expression of dispersion factors including PSMs and proteases results in breakdown of matrix components and dispersal of biofilm bacteria to continue the cycle (E). \textit{Adapted from Bhattacharya et al, Exp. Review in Anti-Infective Therapy, 2015.}
1.3 Neutrophils as a defense against pathogens

1.3.1 Neutrophil characteristics and function

Neutrophils are the most abundant white blood cell in circulation (~70%) and were first described by Paul Ehrlich in 1880, as white blood cells with a ‘polymorphous nucleus’ that stained with neutral dyes. When neutrophils were first observed in large numbers during bacterial infections, they were thought to be the cause of infections. The observation of phagocytosis was concluded as neutrophils being a shuttle to transport bacteria and thereby protect them from the immune system during infection. The function of neutrophils as we know it today was described by Elie Metchnikoff, using a starfish embryo model of injury. Since then, researchers have developed tools to reveal that, indeed, neutrophils play a crucial role in the elimination of bacterial pathogens. Some of the basic characteristics of these cells, pertinent to this dissertation, are described below.

1.3.2 Neutrophil recruitment and activation

Neutrophils in blood circulation constantly probe the lining of vessels to detect the expression of ligands on the endothelial luminal surface. These include P- and E- selectins, expressed in response to bacterial exoproteins and ligands such as lipopolysaccharide and formyl methionine leucyl phenylalanine (fMLP) as well as host derived inflammatory cytokines (TNF-α, IL-1β and IL-17). This allows for neutrophils to ‘roll’ along the endothelial lining and a complex signaling cascade ensues, that causes neutrophils to adhere to the lining. This transition is characterized by a ‘firm adhesion’ where changes in neutrophil biology allow for endothelial transmigration. This process involves the clustering of β2-integrins and phagocytic receptors to the leading edge of the cell and movement into the site of infection in response to a chemo-attractive gradient set up by
cytokines\textsuperscript{124}. This is immediately followed by the commencement of the neutrophil oxidative burst with a combination of host and bacterial products dictating the response of neutrophils thereafter\textsuperscript{207}.

1.3.3 Microbial clearance mechanisms

While the primary function of neutrophils is to identify and eliminate pathogens, this is tightly balanced with a need to prevent the release of toxic neutrophil cytoplasmic contents that can cause inflammation and tissue damage\textsuperscript{208}. Some of the main mechanisms utilized by neutrophils to clear bacterial pathogens are outlined below and summarized in Figure 1.3.

Phagocytosis. The engulfment of bacteria into vacuoles known as ‘phagosomes’ is considered the main mechanism by which neutrophils attempt to eliminate bacterial pathogens. This process allows for exposure of bacteria to the neutrophil’s toxic cytoplasmic contents without the release of these potentially harmful molecules into the host environment. The process of phagocytosing bacteria can begin in one of two ways. The first is via specific interactions of neutrophil surface receptors with pathogen associated molecular patterns (PAMPs) such as bacterial exoproducts, or surface-associated molecules such as the lipopolysaccharide of Gram-negative bacteria called LPS\textsuperscript{209}. Phagocytosis can also ensue via non-specific interactions, including the complement cascade (C3bi binding to MAC1 receptors) and opsonin-mediated engulfment that involves the uptake of IgG-coated particles via an interaction with Fc-receptors\textsuperscript{210}. Studies have shown that while the end result is the formation of a phagosome, the processes involved in complement-mediated engulfment are distinct from opsonin-mediated uptake. While complement-mediated uptake involves a ‘sinking’ of the engulfed particle into the
phagocytic membrane, Fc-receptor-mediated uptake involves a clear entrapment of the particle by pseudopod extensions\textsuperscript{185,211}. Engulfment involves changes in the metabolic flux through neutrophils, with calcium ion gradients and the activity of gelsolin governing the movement of a leading actin edge that wraps around the object to be engulfed, to close off a phagosome in a zipper-like fashion\textsuperscript{212}. Nevertheless, once engulfed and contained in a phagosome, neutrophil granular contents as well as the NADPH oxidase are delivered to the phagosome via multiple fusion events with secretory vesicles and granules\textsuperscript{122,205}. The levels of cytosolic calcium are known to play an important role in the secretion of vesicles as well as the fusion of granules to the phagosome. Increased levels of cytosolic calcium are also known to accompany the uptake of particles during phagocytosis\textsuperscript{213}. This allows for the activity of granular antimicrobial serine proteases including elastases and cathepsin-G, as well as the release of reactive oxygen species\textsuperscript{214}. Unlike macrophages, the pH of the neutrophil phagosome starts off as alkaline and remains neutral through the process of phagocytosis. This is in part due to the release of reactive oxygen species and is important for maintaining the activity of granular proteins\textsuperscript{215,216}. The formation of an anti-microbial phagosome ultimately involves multiple fusion and fission events that result in the accumulation of levels of antimicrobial factors that are appropriate for the elimination of the pathogen in a process known as ‘phagosomal maturation’\textsuperscript{211}. Ultimately, the phagosome fuses with lysosomes to form a ‘phagolysosome’, a vacuole that is highly acidic and contains hydrolases for the efficient breakdown of luminal contents. Ultimately the phagolysosome, with its contained bacterial debris (residual body), is eliminated via exocytosis and processed by other immune cells including macrophages and monocytes\textsuperscript{124}. 
**Neutrophil extracellular trap (NET) formation.** The release of neutrophil extracellular traps was first described by Brinkmann *et al* in 2004. Neutrophils were found to release chromatin and granular components in response to bacterial infections, a phenotype that was later recapitulated *in vivo*. A relatively recent finding, much remains to be learnt about the process of NET formation or NETosis.

While most of the DNA associated with NETs is derived from the nucleus, NETs do contain mitochondrial DNA as well. Among the various functions of NETs, a major function particularly relevant to this work is the entrapment, neutralization and killing of bacterial pathogens. Additionally, NETs can also trap and kill fungi, viruses and parasites as well as trap non-biological triggers such as phorbol 12-myristate-13 acetate and crystals of monosodium urate. Multiple proteins have been found to be associated with the chromatin scaffold of NETs including neutrophil elastase (NE), myeloperoxidase (MPO), calprotectin, cathelicidins, defensins and actin. Additional proteins associate with NETs in a trigger-dependent manner. Regardless of the stimulus, the process of NETosis begins with the depolarization of cells associated with an arrest in actin dynamics. Next, the neutrophil membrane disassembles to allow for the decondensation of chromatin and inclusion of cytoplasmic and granular proteins into the DNA scaffold. The two most important proteins associated with these steps are NE and MPO. Reactive oxygen species (ROS) generated via the activity of NADPH oxidase triggers the activation and translocation of NE from granules. This is in part due to the interaction of NE with MPO. Once NE reaches the nucleus, it proteolytically processes histone proteins, leading to chromatin decondensation. MPO facilitates the activity of NE but is not required for NETosis to take place. Indeed, studies show that NET formation
occurs, albeit at a slower rate when MPO is inactivated\textsuperscript{223}. Another hallmark of NET formation is the deamination or citrullination of histones, wherein amino groups of histones are replaced by ketone groups via the enzymatic activity of protein arginine deiminase 4 (PAD4). Studies show that PAD-4 deficient mice are unable to release NETs in the presence of bacterial LPS, confirming the importance of this modification \textit{in vivo}\textsuperscript{224,225}. PAD-4 requires the presence of calcium ions and its activity is stimulated by hydrogen peroxide. The degree and specificity of citrullination depends upon the trigger and the presence of protein kinase C (PKC) isoforms that can either promote or inhibit PAD-4 activity. Whether the process of histone citrullination is required for the decondensation of chromatin however, remains controversial\textsuperscript{226–228}.

Similarly, while ROS production is associated with NETosis under most conditions, this depends on the stimulus, with some being able to induce NETosis in the absence of NADPH oxidase activity\textsuperscript{229–231}. This NADPH oxidase-independent mechanisms for rapid NET formation is thought to occur through a non-lytic pathway undertaken by the first neutrophils to arrive at the site of infection. Non-lytic NETosis is a form of neutrophil multitasking wherein anucleated cells that have released NETs continue to phagocytose bacteria\textsuperscript{190,232}.

A major factor influencing the progression of NETosis is microbial size. The detection of microbial size is crucial to the competition that occurs between the processes of phagocytosis and NETosis\textsuperscript{231}. This competition is primarily for the available neutrophil elastase, required for either process to occur. During the process of phagocytosis as described above, neutrophil elastase is sequestered in phagosomes and therefore becomes unavailable for the decondensation of chromatin required for NETosis. Cytosolic NE is
also involved in promoting the degradation of the actin cytoskeleton, inadvertently blocking phagocytosis and promoting NETosis. When a neutrophil is unable to engulf a large pathogen, this allows efforts to be directed towards NET formation and away from phagocytosis. Nevertheless, small microbes, able to escape phagocytosis have been found to trigger NETosis with a simultaneous increase in levels of phagocytosis as well and might be due to the process of antibody opsonization.

**Neutrophil antimicrobial products.** Regardless of the process employed to trap bacteria, neutrophils rely on a large arsenal of antimicrobial products to mediate the final killing of bacterial pathogens. A major group of compounds includes the generation of ROS including superoxide radicals, hydrogen peroxide, hydroxyl radicals and hypochlorite. These species directly target bacterial nucleic acid and proteins and are released in a process known as the ‘respiratory burst’. This process begins with the assembly of an NADPH oxidase complex at the plasma and phagosome membranes, where molecular oxygen is converted to superoxide. The activity of a superoxide dismutase catalyzes the conversion of superoxide to hydrogen peroxide, which can react with superoxide to produce hydroxyl radicals. Nitric oxide is commonly found in inflammatory sites and can react with superoxide to release peroxynitrite, which is also highly antimicrobial. The activity of MPO, especially in the phagosome, allows for the formation of the highly antimicrobial hypohalous acids including hypochlorous acid, which can react with amine groups to produce chloramines, another antimicrobial product. The importance of these reactive species is evident in patients with chronic granulomatous disease, who lack a functional NADPH oxidase, rendering these individuals highly susceptible to bacterial infections and prone to autoinflammatory conditions.
Neutrophils also have four types of granules with varying levels of antimicrobial components and proteins that facilitate the process of phagocytosis. These include the primary or azurophilic granules that contain MPO and NE as well as defensins, cathelicidins, cathepsins and lysozymes, the secondary or specific granules that contain lysozymes and lactoferrin, the tertiary/gelatinase granules and secretory vesicles that harbor gelatinase and complement receptor respectively. Primary granules contain the largest percentage of neutrophil antimicrobial compounds and are synthesized during the myeloblast stage of neutrophil development. The fusion of secondary granules to the phagosome membrane is important since b558, an important component of the NADPH oxidase machinery is contained in these granules. Secretory vesicles play an important role during the transmigration and activation processes.

Lastly, neutrophils granules also comprise various antimicrobial proteins including cationic peptides and enzymes that sequester essential bacterial nutrients. The cationic peptides target microbes either by inhibiting DNA and RNA synthesis and therefore expression of essential proteins, or by interfering with the charge of cell wall associated components such as negatively charged phospholipids. Cathelicidins such as LL-37 can form transmembrane pores to kill bacteria. Additionally, numerous neutrophil derived enzymes function to kill bacteria. While lysozymes target the bacterial cell wall for degradation, neutrophil elastase can cleave outer membrane proteins and bacterial virulence factors. Lastly, neutrophils also express metal-chelating proteins that starve bacteria of essential co-factors such as iron (lactoferrin), manganese and zinc (calprotectin). Armed with what is clearly an extensive repertoire of toxic compounds, it becomes crucial that neutrophils contain these cytoplasmic contents. Nevertheless, when
neutrophils lyse and expose these products to host tissues, this can cause a great degree of host tissue damage, at which point other innate immune cells such as macrophages, monocytes and dendritic cells play important roles in clearing host debris (including dead neutrophils) and resolving inflammation at the site of an infection\textsuperscript{122}. 
Two main mechanisms for microbial clearance are phagocytosis and neutrophil extracellular trap formation. Activation of neutrophils occurs via the interaction of microbes/microbial antigens with specific receptors including toll-like receptors, opsonization/complement receptor or antibody mediated Fc-receptor recognition. Phagocytosis involves formation of phagosomes, reactive oxygen burst associated with recruitment of the NADPH oxidase and myeloperoxidase released from multiple fusions with granules. This results in acidification and phagolysosomal formation, ultimately killing bacteria. Bacterial and neutrophil debris are cleared by exocytosis (A). Neutrophil extracellular trap formation (NETosis, (B)) involves the decondensation of nuclear chromatin and neutrophil elastase mediated breakdown of membranes resulting in the release of NETs laced with antimicrobial proteins to kill neutrophils. This can either result in neutrophil lysis (C) or involve phagocytosis by anuclear, non-lytic neutrophils (D).
1.4 Leukocidins: Bi-component pore-forming toxins.

1.4.1 Prevalence and Genomic organization

*S. aureus* strains can express up to 5 bi-component leukocidins that puncture and lyse leukocytes and erythrocytes\(^\text{241}\). The leukocidins expressed by clinical strains include LukSF(PVL), LukAB (also known as LukGH), HlgAB, HlgCB and LukED. Additionally, zoonotic strains of *S. aureus* express LukMF’ and LukPQ, not commonly expressed in human isolates\(^\text{242,243}\). Each toxin is comprised of two components, an S subunit and an F subunit, that form an octameric β-barrel pore in leukocyte plasma membranes, to induce ion imbalance leading to cell lysis\(^\text{244–246}\). Recognition of host cell surface receptors by each F subunit protein leads to the recruitment of S subunits that result in the formation a pore spanning the depth of the phospholipid bilayer. While genes for HlgACB and LukAB are encoded on the core genome and present in 99.5% human isolates, those for LukSF (PVL) are located on the temperate phage ΦSa2 (accessory genome) and are found in <2% of clinical isolates. A majority of circulating CA-MRSA strains however, encode for LukSF. Genes for LukED are found on a stable *S. aureus* pathogenicity island (vSaβ) and present in 70% of all clinical strains\(^\text{135}\).

Characteristically, the S and F subunits of leukocidins are co-transcribed from a common promoter that precedes the S- subunit gene. The only exception to this is the *hlgA* gene. While *hlgCB* are co-transcribed from a single promoter, *hlgA* is encoded upstream of *hlgC* and independently transcribed. *hlgB* however encodes for the common F- subunit that combines with either HlgA or HlgC to form HlgAB or HlgCB respectively\(^\text{247}\).
1.4.2 Structure and pore-formation.

Leukocidins are bi-component toxins that belong to the hemolysin family of β-barrel pore-forming toxins. The structure of leukocidin proteins remains highly conserved, with S and F subunits showing high degrees of amino acid identity between leukocidins. All leukocidin subunits have a molecular weight of approximately 33kDa and are released as independent, soluble monomers. LukAB is an exception to this rule and is secreted as an S-F dimer. LukA and LukB monomers form 3 salt bridges to exist as dimers in solution. This interaction also contributes to LukAB-receptor targeting. Each subunit structure comprises three parts namely the cap, the stem and the rim. The hydrophobic stem region remains embedded in the cap when a subunit is in its soluble form. When subunits interact with their respective host cell receptors and oligomerize, this is thought to induce a conformational change that allows the release of the stem domain that can be inserted into the phospholipid bilayer via the multimeric β-strand domain. Once formed, a leukocidin pore is 1-2nm in diameter and causes the release of potassium ions, critical to maintaining homeostasis in the cell. Apart from LukAB, S and F subunits are known to independently interact with cell membranes. These monomers can also interact with non-cognate subunits to form hybrid pores. While LukS can pair with LukD and hlgB to form pores, HlgA and HlgC can pair with LukF and LukD. This is likely due to the high degree of amino acid similarities between subunits.
1.4.3 Regulation.

Similar to other *S. aureus* pore-forming toxins, leukocidins are regulated by the accessory gene regulator (agr) system described previously. While agr is a self-sensing circuit that responds to bacterial numbers, nutritional and energy availability, regulation of leukocidins downstream of agr is yet to be fully understood. The agr locus controls virulence gene expression via the RNAIII transcript described previously\(^\text{72}\). Numerous transcriptional regulators, two-component systems and effector small RNAs are involved in the regulation of leukocidin genes downstream of RNAIII. These include the repressor of toxins (Rot), sigma factor B (\(\sigma^B\)), RpiRc (transcriptional regulators) and the SaeRS two-component system. A critical target of the RNA III transcript is the repressor of toxins (Rot), which directly targets promoters for the leukocidin genes\(^{89,251}\). The SaeRS two-component system has also been implicated in the regulation of leukocidin genes. This system responds to external signals including pH, osmolarity and host proteins to regulate promoters associated with various virulence genes including those encoding leukocidins.

The regulation of leukocidins through these systems is ultimately controlled in large part by the stage of growth and nutritional input available to cells. While cells in the early exponential phase have an inactive agr locus, the expression and regulation by agr increases and reaches maximum activity in stationary phase cells. Agr mediated expression of RNAIII allows for the inactivation of Rot and therefore an alleviation of the repression of leukocidin genes. The metabolic regulatory gene, RpiRc has recently been found to have a potent repressive effect on genes for the leukocidins LukSF-PV (PVL) and LukED. This was found to occur via a positive post transcriptional effect on Rot\(^\text{82}\). However, much
remains to be elucidated about the regulatory circuit that governs the regulation of these toxins.

1.4.4 Receptor interactions.

The differing roles that leukocidins play during infection can be attributed in large part to the specific protein-receptor interactions that occur between leukocidins and host cells. While all 5 leukocidins can target human neutrophils, apart from LukED, the leukocidins show greater tropism towards human neutrophils than the other species tested so far. In contrast, LukED was found to show equal affinity towards neutrophils from all species tested so far. The specificity of these interactions also governs leukocidin cell-type tropisms. While this brings animal models for leukocidin virulence into question, it provides important information regarding the specificity of leukocidin-host cell interactions. All leukocidin receptors belong to the Class A rhodopsin-like G-protein coupled receptors that have 7 transmembrane domains and are highly expressed on the surface of leukocytes. The interaction of the S-subunit with the receptor occurs at very high affinity (nM). While S-subunits of various leukocidins have a high degree of amino acid identity (70-90%), the rim regions of these monomers have been described to have a divergent region (DR) that might attribute a level of promiscuity to the host cell receptors. For example, while DR1 skews LukE towards binding to the CCR5 receptors, DR4 targets LukE to CXCR-1/CXCR-2 receptors. While the receptor for HlgAB remains unknown, LukSF(PVL) and HlgCB share common receptors (C5aR1, C5aR2) likely attributed to the high percentage of amino acid similarity between HlgC and LukS. However, while specific interactions between the 1st and 3rd extracellular transmembrane domains of C5aR1 were found to mediated binding of HlgC, the 2nd extracellular transmembrane seems to have a
specific affinity for LukS, thus differentiating the binding specificities of these monomers. Nevertheless, HlgC and LukS are thought to compete for binding to leukocyte receptors. Interestingly, the specificity of LukAB towards its receptor, CD11b is governed by the interaction of a single glutamic acid residue at the C-terminal extension of the protein (position 323). This leukocidin unlike others, is secreted as a dimer. The salt bridge that contributes to dimer formation also seems to be required for the interaction of LukAB with its host cell receptor.

1.4.5 Mechanisms of host cell killing.

The effects caused by leukocidins on host cells are governed by multiple factors including toxin concentrations, host cell receptor availability and synergistic or antagonistic interactions between toxins. Regardless, leukocidins can kill cells at low concentrations (~1 nM) with the activity of leukocidins resulting in the release of divalent cations required for homeostasis. The formation of β-barrel pores by leukocidins results in lytic cell death associated with the activation of NLRP-3/IL-1β and inflammasome formation through Caspase-1 (Figure 1.4). LukED causes inflammation and lethality in a mouse model of bacteremia, while LukAB and PVL have similarly been associated with the induction of an inflammatory response in a rabbit model of skin infection. It is thought that the release of divalent cations is what signals the activation of the inflammasome cascade. Concurrent with this, the formation of pores was found to result in downstream Caspase-1 associated signaling regardless of the type of toxin, suggesting that all leukocidins cause inflammasome activation and that the specific response induced by a leukocidin might depend on other factors such as the species of cation released and/or the
concentration of toxin available, as well as the inter-toxin interactions that occur when multiple leukocidins are secreted\textsuperscript{256}.

Additionally, individual leukocidin subunits can prime neutrophils to cause an increase in phagocytic activity, generate ROS and induce granule exocytosis\textsuperscript{135}. For example, sub-lytic concentrations of PVL can prime neutrophils for enhanced phagocytosis and ROS production in response to a secondary stimulus, as well as induce the release of neutrophil extracellular traps\textsuperscript{232,257}. Thus, although these toxins are capable of puncturing leukocyte lipid bilayers, there are other effects that might be caused by the attachment of subunit proteins to the membrane as well as inter-toxin interactions that remain poorly understood.
Figure 1.4. Mechanism for leucocidin-mediated host cell lysis.

The first step in leucocidin mediated host cell killing is the recognition of cognate receptors by leucocidin S-subunits (A). This results in the recruitment of F-subunit proteins (B). 4 such pairs form on the phospholipid bilayer to form a pre-pore complex (C). Conformational changes allow for the release of subunit stem loops to penetrate the membrane and form a pore (D). This results in an ion imbalance with increased levels of cytoplasmic calcium, activating the inflammasome and ultimately leading to host cell lysis (E). Adapted from Spaan et al, Nature Microbiology Reviews, 2017 and Yoong and Torres, Curr Opin Microbiol, 2014.
1.4.6 Contributions to Virulence.

While the redundancy of leukocidin function is still disputed, various studies with in vitro and in vivo models describe roles for each leukocidin under different infection conditions.\(^{249,250}\). Which leukocidin plays the pivotal role depends to a large extent, upon the infection environment and therefore nutritional availability, the expression of each leukocidin as well as their relative levels of expression. Described here are some of the main contributions of each leukocidin, to infections by *S. aureus*.

**PVL.** The first and probably most well documented leukocidin, the Panton Valentine leukocidin has been described as having varying roles depending on the type of infection, as well as the levels of toxin present\(^ {244}\). The significance of this toxin is still being debated due to conflicting evidence obtained from various animal models. This is in part due to the species specificity associated with PVL. PVL has been found to inefficiently find its cognate C5aR receptor on murine neutrophils, thus confounding the results associated with mouse models of infection\(^ {258}\). Nevertheless, PVL has been found to play an important role in the survival of bacteria in a rabbit model of necrotizing pneumonia, associated with increased inflammation and bacterial burdens in the lung\(^ {259,260}\). Similar results were obtained in a rabbit model of osteomyelitis\(^ {261}\). Furthermore, while only 5% of *S. aureus* strains encode for PVL, 95% of these strains are CA-MRSA, making PVL a virulence factor of significance to the majority of nosocomial and community-acquired infections\(^ {262}\).

While relatively high levels are expressed during acute infections and is known to induce significant inflammation, lower levels are released during chronic infections\(^ {232,263}\). Studies with a rabbit model of skin infection show that while a strain capable of expressing PVL showed significant levels of inflammation, the degree of disease severity caused by this
strain was less severe in comparison to an isogenic deletion strain. Consistent with these finding, our work provides evidence indicating that expression of PVL from biofilms, associated with chronic infections, results in a disadvantage to the host.

HlgAB/HlgCB. The second leukocidin to be discovered, gamma hemolysin was the first leukocidin found to lyse erythrocytes\textsuperscript{99,100}. It was later found that these proteins could also lyse leukocytes and lymphoblasts and required a dimer of either HlgA/HlgB or HlgC/HlgB\textsuperscript{264}. In comparison to PVL, the role of gamma hemolysin during infection is relatively poorly characterized. The major areas of research for gamma hemolysin have been during sepsis, septic arthritis and ocular infections. While a deletion of gamma hemolysin showed modest reductions in rates of mortality using a mouse model of sepsis, it was found to play an important role in conjunction with alpha hemolysin, during septic arthritis, with rates if arthritis being significantly lower in a mutant lacking both toxins\textsuperscript{265–267}. The intraocular injection of gamma hemolysin in a rabbit model of eye infection showed higher levels of retinal necrosis and heightened inflammation. The contribution of gamma hemolysins to chronic infections remains to be discerned. However, these studies show the importance of considering the role of this leukocidin in association with other toxins.

LukAB. Also known as LukGH, LukAB is a unique leukocidin, in that it is secreted as a dimer and does not share receptors with other leukocidins\textsuperscript{135}. Similar to PVL, LukAB kills human neutrophils preferentially to murine neutrophils but can also target macrophages and monocytes\textsuperscript{268,269}. In addition to its role in allowing the survival of \textit{S. aureus} exposed to neutrophils, LukAB has been found to be required for survival of \textit{S. aureus} during bloodstream infections. Recent studies show that LukAB is upregulated in response to
neutrophils and allows for the survival and release of phagocytosed S. aureus from the phagosome. LukAB was found to have a more potent PMN membrane-damaging effect once phagocytosed, potentially due to higher localized concentrations of LukAB available in the phagosome. LukAB also acts in concert with alpha hemolysin to mediate the escape of S. aureus engulfed by macrophages in an orthopedic implant model of biofilm infection\textsuperscript{127}. Like PVL however, the role of LukAB during infection remains debated. While either LukAB or PVL are sufficient to induce moderate levels of inflammation in a rabbit model of skin infection, this was not significantly higher than inflammation caused by S. aureus in the absence of these proteins\textsuperscript{265}. The role of LukAB (and PVL) therefore, might be in manipulating the host immune system and allowing prolonged colonization of the bacterium, rather than causing a dramatic inflammatory response and potential clearance of infection, a notion that is supported by the data presented in this dissertation\textsuperscript{253}.

\textit{LukED.} Amongst the leukocidins, LukED is relatively poorly characterized. Mouse models of infections describe a role for LukED in ultimately causing lethality of mice by playing a crucial role in the killing of phagocytes during blood stream infections with S. aureus. With a relatively wide range of target species, LukED can and has been studied in human, mouse and rabbit models of infection\textsuperscript{254,259,271}. LukED is a hemolytic leukocyte that has potent activity against human erythrocytes and is thought to play a role in the scavenging of iron, an element essential for the growth and virulence of S. aureus. Additional roles for LukED, such as competition against host molecules for host cell receptors as well as synergistic interactions with other leukocidins or toxins, remains to be understood. For example, recent studies have shown that LukSF (PVL) inhibits the activity of LukED both
in vitro and in vivo by forming ‘hybrid’ or mixed pores. This might contribute to the inconclusiveness of some of the animal models used to study this toxin\textsuperscript{272}.

1.5 Overall summary of thesis

The overarching goal for the work discussed in this dissertation is to gain insight into virulence mechanisms that are unique to \textit{S. aureus} when grown as biofilms. Biofilms are populations that are metabolically distinct from planktonic bacteria and therefore exhibit virulence traits that are unique to this mode of growth\textsuperscript{273}. Our knowledge of \textit{S. aureus} biofilm pathogenesis is relatively limited\textsuperscript{127,274}. The work described here contributes to this by uncovering the nature of the interaction between \textit{S. aureus} biofilms and neutrophils. Neutrophils are the most abundant white blood cell in circulation that play a significant role in the elimination of bacterial pathogens\textsuperscript{122}. Here we identify a role for 3 leukocidin toxins (PVL, HlgAB and LukAB) and a nuclease (NucA) secreted by \textit{S. aureus} biofilms to evade killing by neutrophils.

\textit{S. aureus} secretes numerous toxins to target host cells including neutrophils, with each protein playing a role in virulence under varying infection conditions. Studies have made it evident that the interplay between various toxins has significant implications to the outcome of the host cell response, as well as the survival benefit granted to \textit{S. aureus} by these toxins\textsuperscript{249,272}. This work describes one example of such an interaction, where the concerted effect of proteins released from biofilms provides \textit{S. aureus} with an advantage that allows for its survival in the face of the neutrophil antimicrobial response. As described above, the efficiency of the neutrophil response affects the downstream response of other immune cells and is therefore crucial for the elimination of bacterial pathogens\textsuperscript{124}. This work provides some explanations for the recalcitrance that \textit{S. aureus} displays against the
innate immune defenses. *S. aureus* biofilms are commonly associated with chronic infections²⁸,¹⁹³,¹⁹⁴,²⁷⁵,²⁷⁶. This work is a first report of a major role for these toxins under biofilm growth conditions and therefore provides important insights about the mechanisms of host-pathogen interactions during chronic infections, a response that is distinct from planktonic *S. aureus*. Owing to its large arsenal of virulence factors, it is likely that *S. aureus* utilizes other virulence factors to persist during chronic infections *in vivo*. Therefore, while these studies uncover some important aspects of persistent *S. aureus* infections caused by biofilms, much remains to be understood about the unique response of *S. aureus* biofilms to various facets of the host immune system.
Chapter 2. *Staphylococcus aureus* releases leukocidins to elicit neutrophil extracellular trap formation and allow persistence during chronic infection

Mohini Bhattacharya, Evelien T. M. Berends, Rita Chan, Elizabeth Schwab, Sashwati Roy, Chandan K. Sen, Victor J. Torres and Daniel J. Wozniak (*Under Revision at PNAS*)

2.1. Abstract

Bacterial biofilms efficiently evade immune defenses, greatly complicating the prognosis of chronic infections. The mechanisms that allow methicillin-resistant *S. aureus* (MRSA) biofilms to evade host immune defenses however, are largely unknown. Neutrophils are the most abundant white blood cell in circulation, playing key roles in the control and elimination of bacterial pathogens. Here we show that *S. aureus* biofilms kill neutrophils via neutrophil extracellular trap (NET) formation, through the activities of leukocidins Panton-Valentine leukocidin (PVL) and gamma hemolysin AB (HlgAB). This response was unique to biofilms and allowed for efficient and rapid immune evasion. Specifically, the antimicrobial activity of released NETs was ineffective at clearing biofilm bacteria. Rather, these studies suggest that NETs could inadvertently potentiate biofilm infections. Chronic infection in a porcine burn wound model clearly demonstrated that leukocidins are required for NETosis and bacterial survival *in vivo*. This study is a description of some of the major mechanisms that are required for *S. aureus* biofilms to evade the innate immune response and provides insight into the key virulence mechanisms that allow for survival and persistence of bacteria during chronic infections.

2.2. Introduction

*Staphylococcus aureus* is a Gram-positive opportunistic pathogen that causes numerous debilitating infections. In addition to secreting multiple virulence factors, *S. aureus* is
successful at persisting as robust biofilms, commonly found in chronic infections\textsuperscript{277}. Biofilms and single celled/planktonic populations often have distinct virulomes and therefore differentially interact with the host\textsuperscript{278,273,279,280}. With the continuing rise in antibiotic-resistant \textit{S. aureus}, biofilm formation therefore presents an additional hurdle to treating these infections\textsuperscript{12,23,281}.

As sessile, dormant groups of bacteria, biofilms are often thought to evade the host immune response by concealing their existence, rather than using active defense mechanisms typically associated with planktonic \textit{S. aureus}\textsuperscript{186,274}. Biofilms accomplish this by masking pathogen-associated molecular patterns, impeding the entry and function of phagocytes, as well as the expression and secretion of proteins that skew the immune system towards anti-inflammatory responses\textsuperscript{127,282}. Among the innate immune cells that respond to an infection, neutrophils play a crucial role in eliminating bacterial pathogens. This is attributed to multiple neutrophil functions including phagocytosis, release of antimicrobial proteins, reactive oxygen/nitrogen species and formation of neutrophil extracellular traps (NETs)\textsuperscript{122}.

Histopathological analyses of various animal models of infection reveal that host immune cells, including neutrophils, are able to migrate to the site of infection but that they are unable to clear infections associated with aggregates or biofilm bacteria\textsuperscript{30,193,283–285}. This is coupled with tissue necrosis and immune cell death, suggesting an active mechanism for biofilm-mediated host cell killing\textsuperscript{65,194,286}. Here we describe some of the major mechanisms used by biofilms to combat neutrophil mediated killing, providing an explanation for their persistence during chronic infections.

\textit{S. aureus} strains can produce up to 5 different bi-component leukocidins namely Panton-Valentine leukocidin (PVL), LukAB (also known as LukGH), HlgAB, HlgCB and
LukED\textsuperscript{135,241}. Each leukocidin is comprised of an S-subunit protein that targets a specific cellular receptor and pairs with a corresponding F subunit. Four S-F pairs assemble into octameric pore-forming complexes in cell membranes of immune cells, including neutrophils\textsuperscript{135}. While these toxins are known to contribute to virulence during acute infections, their role during chronic infections remains largely unknown\textsuperscript{127,287}. Here we show that \textit{S. aureus} biofilms actively target neutrophils by releasing PVL and HlgAB that kill neutrophils with the formation of NETs. NET formation is a controlled release of the neutrophil chromatin, laced with modified histones and granular proteins often occurring in response to infections with bacteria or large objects unable to be phagocytosed, such as a biofilm\textsuperscript{187,231,263,288,289}.

In this study we show that as biofilms, community-acquired methicillin-resistant \textit{S. aureus} (CA-MRSA) USA300, the predominant CA-MRSA in the United States\textsuperscript{290}, releases PVL and HlgAB to elicit NET formation. Specifically, the activity of either toxin results in neutrophil killing by USA300 biofilms, a response that was unique to biofilms and not elicited by planktonic populations. Conversely, biofilms that do not produce either toxin were unable to kill neutrophils. Rapid killing with NET formation was elicited by biofilm spent media and therefore did not require physical contact with bacteria. Upon direct contact, while neutrophils were unable to clear bacteria from wild-type biofilms, there was a significant decrease in biomass with biofilms unable to express PVL and HlgAB. These in vitro findings were recapitulated \textit{in vivo} in a porcine chronic burn wound model, with leukocidins being required for the induction of NETosis in the wound bed and significantly contributing to bacterial survival in wounds. Altogether our studies suggest that the
activities of PVL and HlgAB play major roles in preventing clearance of biofilms by neutrophils and allows S. aureus to persist during chronic infections.

2.3. Results

2.3.1. CA-MRSA biofilms release proteins that kill human neutrophils. To assess the effects of CA-MRSA biofilm-derived factors on neutrophils, we treated primary blood-derived human neutrophils with USA300 strain LAC (hereafter USA300) biofilm spent media and monitored neutrophil viability over 90 minutes using a LIVE-DEAD assay. Results indicate a significant reduction in the number of live neutrophils compared to protein concentration-matched planktonic spent media (Figure 2.1A).

Biofilm spent media treated with either proteinase K or heat (Figure 2.1B) had significantly lower neutrophil killing activity in comparison to an untreated control, suggesting a role for biofilm-released proteins. To assess the contribution of other EPS components (polysaccharide and DNA) to neutrophil killing, we used spent media from biofilms of an icaB- (transglycosylase) strain, since the intracellular adhesin (ica) locus is required for the production of polysaccharide intracellular adhesin, the major polysaccharide in USA300 biofilms. To evaluate a potential role for DNA, wildtype spent media was treated with DNase-I. In either case, there was no significant loss in killing activity (Figure 2.2A & B), confirming that killing of neutrophils was likely attributed to protein components.

To assess whether proteins secreted during planktonic growth were being degraded by proteases, indirectly reducing the cytotoxicity of these media, we tested the neutrophil killing activity of planktonic and biofilm spent media in an isogenic deletion mutant lacking five major proteases, namely aureolysin, SspAB, ScpA and Spl (Aprotease).
We found that *Aprotae* planktonic spent media exhibited levels of cytotoxicity similar to wild type (Figure 2.1C). To test for protease-independent factors that might be inhibiting the activity of planktonic toxins, we pre-incubated planktonic and biofilm spent media together (1:1) for 60 minutes prior to neutrophil treatment (Figure 2.1D). This mixture retained neutrophil killing activity, further supporting the presence of neutrophil killing proteins unique to biofilms.

Next, we used confocal laser scanning microscopy (CLSM) to visualize the fate of neutrophils that were treated with wild type biofilm spent media. DNA of membrane damaged and dead cells was stained with Ethidium homodimer-1 (EthHD-1/red). Esterase activity was used as an output for enzymatic activity in cells (Calcein AM/green). Neutrophils treated with biofilm spent media showed a large proportion of dying cells, with membrane damage and DNA release, as observed by staining with EthHD-1 as well as Calcein AM (Figure 2.1E) $^{263,292}$. This was in contrast to treatment with planktonic spent media (Figure 2.1F). We focused our efforts on identifying the factors required for this biofilm mediated neutrophil killing.
Figure 2.1 Proteins in S. aureus biofilm spent media kill neutrophils.

Primary human neutrophils incubated planktonic (WTP) or biofilm (WTBF) spent media from wild type USA300LAC S. aureus. Neutrophil viability monitored for 90 minutes using a LIVE-DEAD assay (A). LIVE-DEAD measurements similar to (A) for biofilm spent media treated with 0.1mg/mL proteinase K or heat (100°C) for 1 hour (WTBF + Heat) and assessed neutrophil killing activity after 30 minutes (B). Neutrophil killing of WTP and WTBF spent media compared to an isogenic Δprotease (Δaur, ΔsspAB, ΔscpA, Δspl:erm) strain(C). LIVE-DEAD analysis of wild type biofilm and planktonic spent media (WTP+WTBF 1:1 by volume/ 60mins) treated neutrophils(D). Confocal laser scanning microscopy images of Cell Tracker Blue labelled neutrophils treated with wild type planktonic or biofilm spent media for 30 minutes (600X) and stained with Calcein AM/EthD-1(E-F). Percentage cell death was calculated in comparison to neutrophils treated with 0.1% sodium dodecyl sulfate (SDS) for microplate assays and 1μg/mL phorbol myristate acetate (PMA) for microscopy. Hanks Balanced Salt Solution (HBSS) was used as a negative control. Results represent an average of three independent experiments.
performed in triplicate + standard error of means (s.e.m) **P<0.01, ***P<0.001 using one-way analysis of variance and a Tukey’s post hoc analysis.
Figure 2.2. Neutrophil killing requires *agr* regulated proteins.

LIVE-DEAD assays of primary human neutrophils (4X10^6/mL) incubated with biofilm spent media from overnight cultures of an *icaB* transglycosylase transposon mutant. Neutrophils treated with spent media from planktonic (WTP) or biofilm (WTBF) wild type USA300LAC *S. aureus*, are shown for comparison (A). LIVE-DEAD assay similar to that shown in A using wildtype biofilm spent media treated with DNase I for 1 hour (WTBF+DNase) before incubation with neutrophils (B). Crystal violet absorbance measured at 565nm as a readout of biofilm biomass generated after 24-hour growth of wildtype and an isogenic Δ*agr* strain. Δ*nucA* biofilms were used as a positive control whereas Δ*sarA* biofilms were used as a negative control (C). Percentage cell death for all neutrophil killing assays was calculated in comparison to neutrophils treated with 0.1% sodium dodecyl sulfate as a positive control, whereas neutrophils in Hank’s Balanced Salt Solution (HBSS) were used as a negative control (+HBSS). Results represent an average of 6 independent experiments ± s.e.m. **P<0.01, ***P<0.001 using one-way analysis of variance and a Tukey’s post hoc analysis.
2.3.2. Neutrophil killing is regulated by *agr/saeRS* and requires leukocidins.

Approximately 90% of the exoproteome, including most *S. aureus* toxins, are regulated by the accessory gene regulator (*agr*) network. While there was no loss of biomass in biofilms of an isogenic USA300 agr deletion strain, we did observe a significant decrease in neutrophil-killing activity from these spent media. (Figure 2.2C, Figure 2.3A). The *saeRS* two component system is required for relaying signals that activate the expression of multiple *agr*-dependent toxins. LIVE-DEAD measurements showed a significant loss in killing activity with spent media from biofilms of USA300 *saeR* and *saeS* mutants (Figure 2.3B). Together these data suggest a role for Agr/SaeRS regulated toxins in biofilm-mediated neutrophil killing.

Among the proteins regulated by the Agr/SaeRS network are 5 bi-component leukocidins that can puncture phagocyte membranes. The S subunits of PVL (LukSF-PV), LukED, LukAB, and gamma hemolysins HlgAB and HlgCB bind to cell-specific receptors, after which their corresponding F subunit is recruited to assemble into octameric pore-forming complexes within the target cell membrane. We found that biofilm spent media from an isogenic USA300 deletion mutant lacking all bi-component leukocidin genes (Δ5) showed a drastic decrease in neutrophil killing activity, strongly suggesting a role for leukocidins in this process (Figure 2.3C). Upon testing the effect of biofilm spent media from mutants lacking individual leukocidins we found that none of these strains showed significant reductions in neutrophil killing activity in comparison to the wild type (Figure 2.3C). These data suggest the involvement of multiple biofilm released leukocidin proteins in neutrophil killing.
PVL and HlgAB are required for biofilm mediated neutrophil killing activity. LIVE-DEAD assay similar to those described in Figure 2.1(B-D), showing neutrophil death after incubation with biofilm spent media from wild type and an isogenic Δagr strain (A). LIVE-DEAD assays similar to (A) showing neutrophil death after incubation with sacR- and sacS- transposon mutants (B). LIVE-DEAD assays with biofilm spent media from isogenic mutants lacking the five leucocidins(Δ5), each of 5 leukocidins PVL(ΔlukSF), HlgAB, HlgCB (ΔhlgACB), LukED (ΔlukED) and LukAB (ΔlukAB), both PVL and LukED (ΔlukSF ΔlukED) as well as both PVL and HlgACB (ΔlukSFΔhlgACB). Neutrophil cytotoxicity was monitored for 30 minutes with a LIVE-DEAD viability assay, using biofilm spent media from the wildtype USA300LAC strain as a comparison (C). LIVE-DEAD analysis of neutrophils treated with spent media from strains in a ΔlukSF background with deletion of each of three gamma hemolysin subunits (ΔlukSF ΔhlgA, ΔlukSFΔhlgC, ΔlukSF ΔhlgB). Wild type and ΔlukSFΔhlgACB treated neutrophils are shown for comparison (D). Spent media shown in D were mixed 1:1 by volume with those from biofilms of a ΔhlgACB strain. LIVE-DEAD measurements were made as described above (E). Percentage death was calculated in comparison to neutrophils treated with 0.1% SDS for all assays. Neutrophils in HBSS were used as a negative control. Statistical significance shown in all panels is in comparison to neutrophils treated with wild type.
biofilm derived spent media. Results represent an average of three independent experiments performed in triplicate ± s.e.m. **P<0.01, ***P<0.001 using one-way analysis of variance and a Tukey’s post hoc analysis.
2.3.3. PVL and HlgAB are necessary for biofilm mediated neutrophil killing. To identify which leukocidins were being released by USA300 biofilms, we performed mass spectrometry analysis of proteins in spent media from planktonic or biofilm-grown cultures. These results, as well as western blot analyses, identified PVL subunits in biofilm spent media (Table 2.1, Figure 2.4A). However, since single leukocidin deletions did not reduce neutrophil killing activity (Figure 2.3C), we reasoned that PVL might be functioning independently of other leukocidins. This notion is supported by previous observations that bi-component leukocidins can form non-cognate active toxins and can also potentiate each other\textsuperscript{102,249,250,293}. Accordingly, we tested a series of isogenic strains that lacked lukSF in combination with the others leukocidins, for the neutrophil killing activity of spent media from biofilms. We found no appreciable loss in killing activity in the ΔlukSFΔlukED strain, suggesting that LukED was not playing a role. In contrast, spent media from a ΔlukSFΔhlgACB strain showed a significant loss in killing activity (Figure 2.3C). This abrogation was comparable to the isogenic deletion of all 5 leukocidins, indicating that PVL and HlgACB cause biofilm-mediated killing. Although none of the single deletion mutants showed an appreciable loss in killing activity after 30 minutes, a LIVE-DEAD assay carried out to 60 minutes shows a partial loss of killing activity with spent media from a ΔlukSF strain, suggesting a more substantial role for PVL over HlgACB (Figure 2.4B). These results indicate that the expression of either PVL or HlgACB is sufficient to induce NETs.
We next sought to understand whether HlgAB or HlgCB was playing a role in neutrophil killing activity, in addition to PVL. To do this, we created double mutant strains of lukSF and each of the three genes, hlgA, hlgC and hlgB. Since HlgB is the common F subunit for both HlgA and HlgC\(^{293}\), the ΔlukSFΔhlgB::bursa strain showed a substantial loss of killing activity comparable to the Δ5 strain (Figure 2.3D). In contrast to the ΔlukSFΔhlgC::bursa strain, deletion of hlgA (hlgA::bursa) in the ΔlukSF background resulted in a loss of activity comparable to the ΔlukSFΔhlgB::bursa strain (Figure 2.3D). Furthermore, addition of spent media from biofilms of the ΔhlgACB strain (containing PVL) to those from the strains ΔlukSFΔhlgA::bursa, ΔlukSFΔhlgC::bursa or ΔlukSFΔhlgB::bursa significantly restores killing activity, comparable to wild type (Figure 2.3E). Altogether, these results implicate a role for PVL and HlgAB in biofilm-mediated neutrophil killing.
Table 2.1 Proteins identified in biofilm spent media

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<th>Biofilm protein</th>
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<th>Molecular Weight (kDa)</th>
<th>Planktonic protein</th>
<th>Accession number</th>
<th>Molecular weight (kDa)</th>
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<td>Triacylglycerol lipase</td>
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<td>A0A0H2XG41_STAA3</td>
<td>77</td>
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<td>Immunoglobulin G binding protein A (spa)</td>
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<td>Uncharacterized protein</td>
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<td>Lipoteichoic acid synthase (ltAS)</td>
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<td>Alpha hemolysin</td>
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<td>Probable transglycosylase (isaA)</td>
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<td>Enolase</td>
<td>ENO_STAA3</td>
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<td>V8 protease (sspA)</td>
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<td>27</td>
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<td>ISAB_STAA3</td>
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Figure 2.4. PVL and HlgAB are released from biofilm spent media and are sufficient to induce neutrophil killing.

Western blot analysis of PVL LukF-PV subunit protein released in biofilm spent media. Membranes were probed with a rabbit polyclonal antibody against LukF-PV and an AlexaFluor-680-conjugated anti-rabbit antibody was used as a secondary. Molecular marker shows 56kDa = LukS, 43kDa = LukF (A). Kinetic assay showing percentage dead neutrophils every 3 minutes for a 60-minute incubation with biofilm spent media from strains wild type (WTBF), ΔhlgACB, ΔlukSF, ΔlukSFΔhlgACB or a deletion of all 5 leukotoxins (Δ5) (B). Percentage cell death for neutrophil killing assays was calculated in comparison to neutrophils treated with 0.1% sodium dodecyl sulfate as a positive control for all assays, whereas neutrophils in Hank’s Balanced Salt Solution were used as a negative control (+HBSS). Results represent an average of 3 independent experiments +s.e.m.
2.3.4. **PVL and HlgAB induce formation of NETs.** To verify the fate of neutrophils by microscopy, Calcein AM and EthHD-1 were used to stain neutrophils treated with biofilm spent media from USA300 strains ΔlukSF, ΔhlgACB, ΔlukSFΔhlgACB and Δ5 (Figure 2.5 A-D). Similar to quantitative LIVE-DEAD analyses, only the ΔlukSFΔhlgACB and Δ5 mutants lost neutrophil-killing activity, comparable to a negative control (Figure 2.5E). This was evident by a loss of staining with EthHD-1, supporting a role for both toxins. Furthermore, deletion of either hlgA or hlgB in the ΔlukSF mutant resulted in a loss of cell damage (Figure 2.6B, 2.6C), whereas a deletion of hlgC had no such effect (Figure 2.6D).

A striking feature observed in neutrophils incubated with USA300 biofilm spent media, was the ability of media containing either PVL or HlgAB to induce the release of nuclear DNA, similar to wild type conditions. This was evident from staining with EthHD-1, specific for DNA of damaged or dying mammalian cells\(^\text{187}\). Staining for DNA with EthHD-1 was concomitant with enzymatic activity as seen by staining with Calcein AM, suggesting a controlled mechanism for neutrophil death associated with DNA release. We therefore hypothesized the induction of neutrophil death associated with the release of neutrophil extracellular traps or ‘NETosis’\(^\text{187}\).

A hallmark of NETosis is the modification of arginines in chromatin histone proteins to the non-conventional amino acid citrulline, by peptidyl arginine deiminases (PADs)\(^\text{226}\). To test for NET formation, neutrophils were incubated with biofilm spent media from wild type USA300 and isogenic mutants ΔlukSF, ΔhlgACB, ΔlukSFΔhlgACB or Δ5, and stained with antibodies against citrullinated H3 histone proteins. In comparison to wild type and single deletion strains (Figure 2.7A-C), incubation with spent media from biofilms of a double mutant (ΔlukSFΔhlgACB) or Δ5 resulted in a loss of staining with anti-citrullinated
H3 antibody, comparable to a negative control (Figure 2.7D-F). Either LukSF or HlgACB was sufficient and necessary to show significant levels of histone citrullination staining. To understand the contribution of either HlgAB or HlgCB to NET formation, we once again utilized mutants in each of the three genes hlgA, hlgB or hlgC, in the ΔlukSF background. Indeed, as seen by staining with antibodies against histone citrullination, deletion of hlgA and hlgB but not hlgC significantly reduced the induction of NETs (Figure 2.8).

Release of myeloperoxidase (MPO) and neutrophil elastase (NE) during degranulation is also associated with NET formation. In support of results in Figure 2.7, neutrophils treated with biofilm spent media stained with antibodies against both MPO (Figure 2.9) and NE (Figure 2.10). Quantification of mean fluorescence intensities from 10 independent fields showed that strains producing either PVL or HlgACB had significantly higher staining with antibodies against citrullinated histones (Figure 2.7G) and MPO (Figure 2.7H) as well as higher levels of staining with antibodies against NE (Figure 2.7I). In contrast, mutants lacking both toxins showed negligible levels of fluorescence. We therefore concluded that PVL and HlgACB were required for biofilm-mediated NET formation.
Figure 2.5. PVL and HlgACB induce neutrophil membrane damage and DNA release.

Confocal laser scanning microscopy images of primary human neutrophils (4X10⁶ cells/mL) treated with spent media from biofilms of strains ΔlukSF (A) ΔhlgACB (B), ΔlukSFΔhlgACB (C) and Δ5(D). Neutrophils were pre-stained with Cell Tracker Blue (cytosol) for 30 minutes. After a 30-minute incubation with spent media, Calcein AM (metabolic activity/live) and ethidium homodimer-1(DNA/dead) were used to image neutrophil viability. Images were captured at a 600X total magnification and represent the majority population phenotype of six independent experiments performed in triplicate. Neutrophils incubated with HBSS buffer were used as a negative control (E).
Figure 2.6. HlgAB and not HlgCB is required for biofilm mediated neutrophil killing.

Confocal laser scanning microscopy images of primary human neutrophils (4X10⁶ cells/mL) treated with spent media from biofilms of strains ΔlukSF (A), ΔlukSFΔhlgA::bursa (B), ΔlukSFΔhlgC::bursa (C), ΔlukSFΔhlgB::bursa (D) and ΔlukSFΔhlgACB (E). Neutrophils were pre-stained with Cell Tracker Blue (cytosol). After a 30-minute incubation with spent media, Calcein AM (metabolic activity/live) and ethidium homodimer-1 (DNA/dead) were used to image neutrophils. Images were captured at a 600X magnification and represent the majority population phenotype of six independent experiments performed in triplicate.
Figure 2.7. Biofilm spent media containing either PVL or HlgACB is sufficient for release of neutrophil extracellular traps.

Confocal laser scanning microscopy of primary human neutrophils (4X10^6 cells/mL) treated with spent media from biofilms of wild type (A), ΔlukSF (B), ΔhlgACB (C), ΔlukSFΔhlgACB (D) and Δ5 (E) strains. Neutrophils were pre-stained with Cell Tracker Blue (cytosol). After a 30-minute incubation with spent media, cells were stained with ethidium homodimer-1(DNA/red), fixed with 2% paraformaldehyde and incubated with an anti-histone H3 (citrulline R2) antibody. Cells were then stained with Alexa Fluor 488 (secondary antibody/green). Images were captured at a 600X total magnification and represent the majority population phenotype of six independent experiments performed in triplicate. Neutrophils incubated with HBSS were used as a negative control (F).

Quantification of neutrophil staining with anti-citrullinated histone (G), anti-MPO (H) and anti-NE (I) antibodies after incubation with biofilm spent media of WT, ΔlukSF, ΔhlgACB, ΔlukSFΔhlgACB and Δ5 strains. Image J software Ver 5.3 was used to set the same threshold for all conditions and mean fluorescence intensity (MFI) was calculated per 100 cells for 10 fields from 6 independent experiments +s.e.m. **P<0.01, ***P<0.001 using one-way analysis of variance and a Tukey’s post hoc analysis.
Figure 2.8. HlgAB and not HlgCB contributes to neutrophil extracellular trap formation.

Confocal laser scanning microscopy of primary human neutrophils (4X10^6 cells/mL) treated with spent media from biofilms of strains ΔlukSF, ΔlukSFΔhlgA::bursa, ΔlukSFΔhlgC::bursa and ΔlukSFΔhlgB::bursa. Neutrophils were pre-stained with Cell Tracker Blue (cytosol). After a 30-minute incubation with spent media, cells were fixed with 2% paraformaldehyde and incubated with an anti-histone H3 (citrulline R2) antibody. Alexa Fluor 488 (secondary antibody/ green) and ethidium homodimer-1(DNA/ red) were then used to visualize cells. Images were captured at a 600X magnification and represent the majority population phenotype of six independent experiments performed in triplicate.
Figure 2.9. Neutrophils treated with biofilm spent media release myeloperoxidase.

Confocal laser scanning microscopy of primary human neutrophils (4X10^6 cells/mL) treated with spent media from biofilms of ΔlukSF, ΔhlgACB, ΔlukSFΔhlgACB strains and a knockout of all 5 leukocidins (Δ5) in comparison to the wild type USA300LAC strain. Neutrophils pre-stained with Cell Tracker Blue were incubated with spent media for 30 minutes, and fixed (2% paraformaldehyde). Cells were treated with anti-neutrophil myeloperoxidase antibody and stained with an Alexa Fluor 488 secondary (green). Ethidium homodimer-1(DNA/dead) was used to stain DNA of dead/damaged neutrophils. Images were captured at a 600X magnification and represent the majority population phenotype of 6 independent experiments. Neutrophils treated with 1µg/mL phorbol myristate acetate (PMA) are shown as a positive control.
Figure 2.10. Neutrophils treated with biofilm spent media release neutrophil elastase.

Confocal laser scanning microscopy of primary human neutrophils (4X10⁶ cells/mL) treated with spent media from biofilms of ΔlukSF, ΔhlgACB, ΔlukSFΔhlgACB strains and a knockout of all 5 leukocidins (Δ5) in comparison to the wild type USA300LAC strain. Neutrophils pre-stained with Cell Tracker Blue were incubated with spent media for 30 minutes before being fixed (2% paraformaldehyde) and treated with an antibody against neutrophil elastase. Cells were stained using an Alexa Fluor 488 (green) secondary antibody. Ethidium homodimer-1 was used to stain for DNA of dead/damaged neutrophils. Images were captured at a 600X total magnification and represent the majority population phenotype of 6 independent experiments. Neutrophils treated with 1µg/mL phorbol myristate acetate (PMA) are shown as a positive control.
2.3.5. Leukocidins prevent bacterial killing and biofilm clearance by neutrophils. Our studies indicate that PVL and HlgAB released from USA300 biofilms, kill neutrophils via NETosis. However, neutrophils adhere and penetrate *S. aureus* biofilms under conditions mimicking physiological shear stress\(^{184}\). We therefore sought to understand what the consequence of direct neutrophil-biofilm contact would be, to bacteria in a biofilm.

Wild-type (24 hour) USA300 biofilms were incubated with Cell Tracker Blue-labelled neutrophils for 2 hours. Bacterial viability was assessed using Syto 9 (green), neutrophil death and DNA release was visualized with EthHD-1 (red). After a 30-minute incubation with biofilms, we found that neutrophils were indeed able to adhere and penetrate the biofilm and show membrane damage, as assessed by EthHD-1 staining (Figure 2.11A, 2.11B). Remarkably, after a 1-hour incubation with neutrophils, levels of Syto-9 staining suggested that bacteria remain viable while neutrophils release DNA (Figure 2.11C, 2.11D white arrows/inset show DNA). Indeed, at 2-hours post incubation with neutrophils, levels of Syto 9 staining are comparable to an untreated biofilm control (Figure 2.11E, 2.11F/ Figure 2.12D). Concurrent with bacterial viability at 2-hours post incubation, EthHD-1 stained neutrophils can be found at the bottom of the biofilm, as seen in a section taken close to the base (Figure 2.11F). These results suggest that USA300 biofilms kill neutrophils and continue to grow. Similar experiments performed with a USA300LAC13C:*PftsAZ:gfp* strain, constructed with a chromosomal gfp fusion showed GFP activity up to 2 hours after incubation with neutrophils, confirming retention of biofilm viability (Figure 2.12 A-C).
To understand the contribution of leukocidins to bacterial survival upon direct contact with neutrophils, we performed similar experiments using biofilms of the Δ5 strain, lacking all leukocidin genes. In contrast to observations with wild type USA300, the Δ5 strain showed higher levels of biomass clearance at 30 (Figure 2.11 G, H) and 60 (Figure 2.11 I, J) minutes post incubation with neutrophils, in comparison to an untreated control (Fig 2.12E). In stark contrast to DNA release observed with wild type biofilms, neutrophils treated with Δ5 biofilms retained nuclear structure (Fig 2.11 J, white arrows/inset). Biomass of Δ5 biofilms after a 2-hour incubation with neutrophils was comparable to similarly treated ΔlukSFΔhlgAB biofilms and was significantly lower than the wild type (Fig 2.11K, 2.11 L/ Fig 2.12F, 2.12G).
Figure 2.11. NET formation does not affect biofilm biomass and viability.

Neutrophils were stained with Cell Tracker Blue for 30 minutes and then incubated with 24-hour wild type *S. aureus* biofilms for 30 minutes (A, B) 1 hour (C, D) and 2 hours (E, F). Viable bacteria were stained with Syto-9 (green), DNA of membrane damaged or dead cells were stained with ethidium homodimer-1 (red). Images were collected as a Z-stack, representing the thickness of the biofilm (left panels). Sections through the biofilm were imaged to assess penetration of neutrophils into the biofilm biomass (right panels). Similar experiments performed with the isogenic Δ5 strain, lacking all 5 leukocidins (G-L). White arrows are used to highlight nuclear DNA of neutrophils. Images are representative of six independent experiments performed in triplicate.
Figure 2.12. NET induction does not affect biofilm bacterial survival.

Experiments were performed as described for Figure 2.11. Biofilms described here are with a USA300LAC13C::PftsAZ::gfp strain grown for 24 hours and incubated for 30 mins (A), 1 hour (B) or 2 hours (C) with 4x10⁶ neutrophils/mL. Neutrophils were pre-incubated with Cell Tracker Blue and fixed with 2% paraformaldehyde after incubation periods. Ethidium homodimer-1 was used to stain for DNA of dead/damaged neutrophils. Images were collected as a Z-stack, representing the thickness of the biofilm (left) growing in respective flow cells. Sections through the biofilm were imaged to assess penetration of neutrophils into the biofilm (right). Images are representative of three independent experiments. Negative control showing biofilms of WT USA300LAC (D) and Δ5 strains (E) incubated without neutrophils and stained with Syto 9 (green) and EthHD-1 (red). Experiments similar to those performed in A-C, with the ΔlukSFΔhlgAB strain (F). Volume quantification of WT, ΔlukSFΔhlgAB and Δ5 biofilms, after treatment with neutrophils for 2 hours (G). Imaris software version x 64 8.4.1 was used for quantification of biofilm volume. Results represent an average of quantification from 5 independent experiments ± s.e.m. **P<0.01, ***P<0.001 using one-way analysis of variance and a Tukey’s post hoc test.
2.3.6. Leukocidins released during chronic infections induce NET formation and aid bacterial survival. While our studies suggest that either PVL or HlgAB are sufficient to cause NETosis, it is likely that other leukocidins might be playing a role during chronic infections in vivo. In order to understand the relevance of NET induction by S. aureus biofilms in vivo, we therefore compared infections caused by USA300LAC and Δ5 strains, in a porcine full thickness burn wound model of chronic infection. Longitudinal sections collected from wounds at 7 days post infection were stained with the anti-citrullinated histone antibody described above (green) to determine whether NETs were released by host cells in a leucocidin-dependent manner. To assess the presence of bacteria, an anti-S. aureus antibody (red) was utilized. In comparison to animals infected with the wild-type strain (Figure 2.13A), those infected with the Δ5 strain (Figure 2.13 B) showed significantly lower levels of anti-histone staining, comparable to an uninfected control section (Figure 2.13C, D). Concurrently, pigs infected with the Δ5 strain showed significantly lower bacterial burdens as assessed by immunofluorescence and counting colony forming units (cfu) enumeration (Figure 2.13 E, F). Lastly, staining with the anti-histone antibody co-localized with S. aureus and was higher in pigs infected with wild type bacteria (Figure 2.13 G) compared to pigs infected with the Δ5 strain (Figure 2.13 H). These results demonstrate that leukocidins induce NETosis and are required for persistence of S. aureus during chronic infections in vivo.
Figure 2.13. Leukocidins cause NET formation and aid bacterial survival in vivo

Longitudinal sections taken across the wound bed of pigs infected with wild type S. aureus USA300LAC (A) and the isogenic Δ5 strain, lacking all 5 leukocidins (B) at day 7 post inoculation. Uninfected control section (C). Sections were stained with an anti-citrullinated histone antibody (green) and an anti- S. aureus antibody (red). Tissue cells were stained with 4’,6’-diamidino-2-phenylindole (DAPI) and visualized using confocal microscopy at 100X total magnification. Images taken at 600X magnification from surface (white arrows) of corresponding wounds A-C (inset). Mean fluorescence intensities of cells stained with antibodies against citrullinated histone and anti-S. aureus antibodies calculated for 10 independent fields taken across wild type (D) and Δ5 (E) infected wounds and represented graphically. Colony forming units (cfu) per gram of tissue biopsied from wild type and the isogenic Δ5 strain infected animals (F). Pearson’s coefficients quantifying the degree of co-localization for anti-histone and anti-S. aureus antibodies for ten fields of view, taken across wildtype (G) and Δ5 infected wounds (H). Results represent an average of two independent infections per strain, performed in triplicate +s.e.m. **P<0.01, ***P<0.001 using one-way analysis of variance and a Tukey’s post hoc analysis.
2.4. Discussion

*S. aureus* tightly controls the expression of >100 virulence factors, tailored to the host environment and mode of growth (planktonic vs biofilm), with simultaneous expression of multiple virulence factors allowing for rapid evasion of immune defenses\textsuperscript{273,279,280}. The virulence mechanisms that play key roles during biofilm infections are relatively unknown\textsuperscript{127,184}. Here we show that PVL and HlgAB released from biofilms, induce NETosis. Amongst the leukocidins, PVL has a concentration dependent effect on neutrophils. While low concentrations induce NETs, high concentrations of the toxin cause necrosis\textsuperscript{232}. Such low levels could be the reason for PVL associated NET induction under biofilm growth conditions.

There are very few studies that clearly ascribe roles to gamma hemolysins HlgAB and HlgCB\textsuperscript{293,295}. The lack of evidence so far might be due to low expression levels of the toxin under planktonic conditions, used to perform these studies\textsuperscript{82}. We show that when grown as a biofilm, USA300 produces sufficient HlgAB to kill neutrophils. While USA300 produces PVL and HlgACB, epidemiological studies report a majority *S. aureus* clinical strains as being PVL negative, with 99.5% encoding for HlgACB\textsuperscript{296,297}. Our studies show that either PVL or HlgAB induces neutrophil death, which might account for the ability of PVL negative strains to successfully persist as chronic infections.

When placed in contact with a biofilm, although neutrophils are able to penetrate and engulf bacteria, a large population of bacteria remain viable. After two hours of incubation with biofilms, neutrophils with intact cell membranes were found close to the base of the biofilms. This is reminiscent of studies describing the ability of neutrophils to undergo ‘non-lytic’ NET formation, where release of the chromatin leaves anuclear cells that are
still capable of phagocytosis. In an attempt to restrict the spread of large numbers of bacteria, ‘non-lytic’ NET formation becomes an effective means for neutrophils to engulf and contain/kill microbes while releasing its anti-microbial arsenal as NETs to eliminate extracellular bacteria. In fact, previous work describes the ability of neutrophils to remain intact after NET induction by PVL, similar to results obtained here.

Our study describes a new and important role for released leukocidins in neutrophil-mediated *S. aureus* biofilm clearance. Specifically, our data support the idea that leukocidins exhibit potent activity as independent toxins, to impact host-pathogen interactions. Together with recently published work by us and others, it is clear that these toxins need to be evaluated as a whole, as they can interact to synergize or antagonize their effects towards host cells. Moreover, these toxins also work in concert with other virulence factors. For instance, studies with murine macrophages describe a role for LukAB and alpha-toxin in killing and preventing phagocytosis of 6-day old biofilms. Similar to our work, these toxins were found exclusively in spent media of biofilms, with planktonic cultures remaining non-cytotoxic. It is important to note that other virulence factors likely also contribute to bacterial survival during biofilm infection, especially during cell-contact dependent killing. For instance, PSMα peptides are surfactant molecules released during the later stages of biofilm maturation and have previously been found to lyse neutrophils from within phagosomes, under planktonic conditions.

While NET release by neutrophils is a focus of these studies, other host cells such as macrophages and eosinophils undergo similar release of chromatin. This could contribute to the NET formation we observe *in vivo* and might be attributed to other virulence factors expressed by *S. aureus*. 

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Our results indicate that biofilms retain viable cells and biomass even after exposure to neutrophils. This suggests that S. aureus trapped in NETs are potentially able to circumvent the bactericidal activity of antimicrobial proteins. The staphylococcal thermonuclease NucA, digests NET DNA which is converted to deoxyadenosine via adenosine synthase produced by S. aureus\textsuperscript{191,302}. Indeed, our mass spectrometry identified thermonuclease NucA in biofilm spent media, suggesting a contribution to survival of bacteria trapped in NETs under these conditions (Table 2.1). The activity of this nuclease however, can promote biofilm dispersal\textsuperscript{196}. Conversely therefore, the activity of the thermonuclease on NET DNA might result in biofilm dispersal and dissemination of bacteria, leaving viable, phagocytosed bacteria to eventually perpetuate the biofilm. NET DNA that has been digested by DNases has diminished antibacterial activity, which could contribute to bacterial survival observed in these biofilms\textsuperscript{187}.

All together this study provides evidence of an active mechanism for biofilm-mediated neutrophil killing, dependent on leukocidins PVL and HlgAB. More importantly, regardless of physical contact with bacteria, both toxins induce NET formation, a controlled, non-inflammatory response. The drastic reduction in biofilm biomass after neutrophil treatment of the Δ5 strain in comparison to wild type, as well as the reductions in cell numbers \textit{in vivo}, suggest that the use of a leukocidin antibody cocktail with antibiotics could provide a viable therapeutic option to boost the host’s ability to eliminate biofilms.

2.5. Materials and Methods

\textit{Ethics Statement.} All experiments with animals were reviewed and approved by the Institutional Animal Care and Use Committee at The Ohio State University (2008A0012-
Primary human neutrophils were obtained from healthy adult donors according to the protocol approved by The Ohio State University Biomedical Sciences Institutional Review Board (2009H0314), where informed, written consent was obtained from all donors.

**Bacterial strains and growth conditions.** Unless otherwise specified, all studies were performed in the USA300LAC background (AH1263, provided by Dr. Alex Horswill Carver College of Medicine, University of Iowa, Iowa) and strains were grown in trypticase soy broth (TSB) supplemented with dextrose (2.5g/L, Becton Dickinson). The previously published AHLAC (USA300LAC) protease knockout strain was also generously provided by Dr. Horswill. Transposon mutants used in these studies were obtained from the Network for Antimicrobial Research in *Staphylococcus aureus* at the University of Nebraska, Medical Center. The presence of transposon insertions for these mutants was verified by plating onto TSA + 5 µg/mL erythromycin before use and performing PCR reactions with primers specific for the transposon as well as the respective genes (Table 2.2). Double mutants of lukSF and either hlgA, hlgB, or hlgC were constructed by transducing the USA300LAC (JE2) hlgA::bursa, hlgB::bursa, or hlgC::bursa strains from the Nebraska Transposon Mutant Library into the AH1263 LACΔpvl strain using phage 80α. Mutants were confirmed by PCR and Western blot. For detailed information on various mutants and how they were generated please refer to Table 2.3.

**Spent media collection.** To generate spent media for neutrophil killing assays, isolated colonies were used to inoculate 5 mL TSB and grown for 16-18 hours at 37°C under shaking (200 RPM) conditions. These were then allowed to grow to an optical density (OD at 600 nm) of 0.5-0.7, equivalent to an exponentially growing *S. aureus* culture. These cultures were then diluted to an OD600 of 0.1 and used to inoculate TSB either as
planktonic (5 mL/15mL test tube) or biofilm cultures (tissue culture treated 96-well plates, 200 µL/well)\textsuperscript{304}. Planktonic (shaking/200 RPM) and biofilm (static) cultures were then allowed to grow for 24 hours, centrifuged and filter sterilized using a 0.22 µm filter. Protein concentrations were measured using a Bradford assay.

Neutrophil isolation. Neutrophils were isolated from whole blood using previously published methods\textsuperscript{305}.

Neutrophil viability assays. Neutrophils were incubated with bacterial spent media for 30 minutes and stained with a 1:1 mixture (by volume) of Syto-9 (3.34 mM) and propidium iodide (20 mM) for 15 minutes (Thermo Fisher), after which the ratio of fluorescence generated by Syto -9 (Ex 485nm/ Em 525nm) and propidium iodide (Ex 525nm/ Em 630nm) was measured using a standard fluorometer. Numbers of neutrophils were calculated as a percentage of the positive (0.1% SDS treated) control (dead). For microscopy analyses, 4 X 10\textsuperscript{6} cells/mL were stained for 30 minutes with 100 µM Cell Tracker Blue (Thermo Fisher) followed by a 15-minute incubation with spent media. 2 µM Calcein AM (Invitrogen) and 4 µM Ethidium homodimer-1 (Invitrogen) were then used to stain live and dead cells respectively for an additional 15 minutes. Neutrophils treated with 1 µg/mL phorbol 12-myristate 13-acetate (PMA) were used as a positive control for microscopy. Cell suspensions were centrifuged and mounted onto glass slides using Prolong Gold Antifade mounting medium (Thermo Fisher). Slides were then imaged at a total magnification of 600X using confocal laser scanning microscopy (Olympus FluoView).

Porcine full thickness burn wound model. White female Yorkshire pigs (70-80 lb, ~11 weeks old) were obtained from Hartley Farms, Circleville, Ohio. (Male pigs were excluded
to prevent potential uro-genital obstruction caused by dressings. The use of female pigs maximizes the area available for creating burns, as the dressing can be wrapped farther caudal). Six 2X2 inch burn wounds were created on the backs of two pigs, using previously established methods. To prevent cross contamination wild type and Δ5 strains were infected on separate pigs (n=2 each) and housed individually. Briefly, 3 days after wounding pigs using an electrically heated burning device, 10⁷ bacteria were spread over the surface of each wound (n=6 per animal). Wounds were then covered using a semi-occlusive dressing as previously described. 8mm punch biopsies (n=3 per wound) and a longitudinal full thickness section were collected from each wound at 7, 14 and 35 days after infection. Each of these wounds healed separately and were considered as experimental replicates, with biopsies for cfu analysis carried out in triplicate. Punch biopsies were homogenized and resuspended in 1 mL of phosphate buffered saline to calculate cfu/g of tissue. Longitudinal sections were utilized for all antibody staining procedures. Samples were coded, and data collection performed in a blinded fashion.

Citrullinated histone staining. For imaging of neutrophils with antibodies against citrullinated histone proteins, 4 X 10⁶ cells/mL were stained using the LIVE-DEAD protocol described above, after which cells were centrifuged and fixed using 2% paraformaldehyde. Fixed cells were then blocked using 1% bovine serum albumin for 1 hour at room temperature (or overnight at 4°C). Blocked cell suspensions were centrifuged at 149 x g and washed with phosphate buffered saline containing 0.1% Tween-20 for a total of 3 washes. Cells were then centrifuged and re-suspended in rabbit polyclonal primary antibody against the histone H3 (citrulline R2) protein modification (abcam ab174992). Cells were then incubated for 2 hours at room temperature (or overnight at 4°C)
and washed as described above. Alexa Fluor 488 goat anti-rabbit protein was used as a fluorescent secondary antibody (Invitrogen). Cells were incubated for an additional 2 hours at room temperature (or overnight at 4°C) washed and mounted onto glass slides using Prolong Gold Antifade mounting medium (Thermo Fisher). Slides were then imaged at 600X using confocal laser scanning microscopy.

Polyacrylamide Gel Electrophoresis and Liquid Chromatography-Mass Spectrometry. To visualize proteins, present in biofilm supernatants, cultures were grown as previously described. Supernatants from planktonic and biofilm cultures were then filter sterilized and desalted in HBSS using Bio-Rad 10DG desalting columns. Desalted supernatants were then concentrated by lyophilization and amount of proteins was estimated before and after desalting, as well as after lyophilization was estimated using a Bradford assay. Proteins were applied to SDS-PAGE using a 12% Tris-Glycine SDS-Polyacrylamide gels under denaturing conditions. Detection and staining of proteins in polyacrylamide gels was done with Coomassie Brilliant Blue R250. Following staining, with Coomasie blue reagent bands were cut out from gels in matched pairs (WTP and WTBF) de-stained and trypsin digested. The peptides were then extracted for mass spectrometric analyses. Mass spectrometry analysis was carried out using a Thermo Fusion Orbitrap HPLC MS-MS system, by the Campus Chemical Instrument Center at The Ohio State University.

Western Blot Analysis. All western blot analyses were carried out by the Torres Lab at the Langone Medical Center, New York University, New York. Briefly, Freeze-dried pellets were reconstituted in 1x SDS loading buffer and boiled for 10 minutes. Samples were loaded on a 12% SDS-PAGE gel and subsequently transferred to a nitrocellulose membrane. The membranes were probed with a rabbit polyclonal antibody against LukF-
PV, previously produced\textsuperscript{306}. As a secondary antibody, AlexaFluor-680-conjugated anti-rabbit (Invitrogen) was used after which membranes were imaged using an Odyssey CLx Imaging System (LI-COR Biosciences).

\textit{Antibody staining of porcine tissue sections.} To stain for the presence of bacteria as well as for citrullinated histone proteins, biopsies taken across the length of the wound were embedded in paraffin and sectioned (100 µm). These sections were mounted onto glass slides and blocked using 1\% bovine serum albumin for 16-18 hours (4°C). Slides were then incubated with primary antibodies against citrullinated histones (abcam 174992) and \textit{S. aureus} (abcam 37644) for 2 hours at room temperature. Slides were then washed with phosphate buffered saline containing 0.1\% Tween 20. Goat anti-rabbit Alexa Fluor 488 (Invitrogen) was used as a secondary fluorescent antibody for citrullinated histones while a goat anti-mouse IgG Alexa Fluor 647 (abcam 150115) was used to visualize \textit{S. aureus}. Finally, slides were stained with 1µg/mL DAPI for 30 minutes and visualized using confocal microscopy. Colocalization analyses and calculation of Pearson’s coefficients was performed using Imaris software version x 64 8.4.1 using ten fields of view from 3 independent longitudinal sections. Lastly, results from immunofluorescence were corroborated with a blinded gross analysis of the differences in levels of bacterial and histone staining by six independent individuals, using triplicate, stained longitudinal sections.

\textit{Construction of the USA300-ftsAZ::gfp reporter strain.} For generation of the USA300-\textit{ftsAZ::gfp} reporter strain the pWSE-1 plasmid was used, a derivative of the plasmid pCL25\textsuperscript{307}. The \textit{ftsAZ::gfp} reporter fusion was made by replacement of the cadmium-inducible promoter in front of the gene encoding the short half-life GFPaav in the plasmid
pEM64\textsuperscript{308}, a derivative of the pBK123\textsuperscript{309}, with 0.6 kb SphI-BamHI DNA fragment containing \textit{ftsAZ} promoter. The resulting plasmid was designated as pEM69. Then, a 1.8-kb PCR fragment of the pEM69 containing the \textit{ftsAZ::gfp} reporter fusion was amplified using primers FTSZ-f (5’- CATGCTAATCAAAAAGTTGAGATACACAAAGC) and SacI-blaZ-r (5’-CAAGAGCTCGCCTGTCACTTTGCTTGATATATGAG). Following digestion with the restriction endonuclease SacI, the PCR product was ligated into the plasmid pCL25 hydrolyzed with restriction endonuclease SacI and SmaI. The resulting pWSE-1 plasmid was introduced into the strain CYL316 with following integration at the L54a \textit{attB} site located within the \textit{geh} gene in \textit{S. aureus} chromosome as previously described\textsuperscript{307}. The USA300-\textit{ftsAZ::gfp} strain was constructed by bacteriophage \textit{Φ11}-mediated transduction of the integrated pWSE-1 plasmid as described.

\textit{Myeloperoxidase/ Elastase antibody staining}. For imaging of neutrophils with antibodies against MPO and NE, 4X10\textsuperscript{6}cells/mL were stained using the LIVE-DEAD protocol described above, after which cells were centrifuged and fixed using 2\% paraformaldehyde. Fixed cells were then blocked using 1\% bovine serum albumin for 1 hour at room temperature (or overnight at 4°C). Blocked cell suspensions were centrifuged at 233 x g and washed with phosphate buffered saline containing 0.1\% Tween-20 for a total of 3 washes. Cells were then centrifuged and re-suspended in primary antibody against either myeloperoxidase or neutrophil elastase (abcam ab9535/ ab21595). Cells were incubated for 2 hours at room temperature (or overnight at 4°C) and washed as described above. Alexa Fluor 488 goat anti-rabbit protein was used as a fluorescent secondary antibody. Cells were incubated for an additional 2 hours at room temperature (or overnight at 4°C)
washed and mounted onto glass slides using prolong golf antifade reagent. Slides were then imaged at 600X using confocal laser scanning microscopy.

*Biofilm formation assays.* To test the ability of strains to form biofilms, cultures were grown for 16-18 hours at 37°C under shaking conditions (200RPM) and diluted in fresh media (TSB+glucose (2.5g/L, Becton Dickinson)). Once cultures reached exponential phase (O.D of 0.5-0.7 @600nm) these were seeded into 96-well tissue culture plates and grown for another 16-18 hours under static conditions (37°C) to facilitate biofilm formation. Supernatants were then removed, and biomass attached to the bottom of the well was measured using a previously published crystal violet staining assay. Briefly, biomass was incubated with 0.1% crystal violet and incubated for 20 minutes. Cells were then washed with water and incubated with 95% ethanol with shaking, for an additional 20 minutes to slough biomass off the walls of the wells. Optical density of biomass was then measured at 565nm.

*Quantification of antibody staining.* Neutrophils were stained with respective antibodies as described above. Image J software Ver 5.3 was used to set the same threshold for all conditions and mean fluorescence intensity (MFI) was calculated per 100 cells for 10 fields from 6 independent experiments.

*Neutrophil-biofilm interaction studies.* For studies involving contact dependent interactions of neutrophils with biofilms, cultures were grown as described for spent media collection and seeded in chambers of a 6-channel μ-slide (ibidi). These were incubated for 16-18 hours at 37°C under static conditions and treated with neutrophils as described for microscopy analyses.
Quantification and Statistical Analysis. All statistical analysis was carried out using Graph Pad Prism Ver 5.0b. One-way analysis of variance with Tukey’s least significant difference was a post-hoc test where appropriate.

2.6. Acknowledgments

We are grateful to Drs. Kenneth Bayles, Marat Sadykov and Todd Wildhem for generating the GFP-labelled USA300LAC strain and Dr. Alex Horswill for providing the Δprotease strain. We would also like to thank Brian Rhea and Dr. Shomita S. Mathew-Steiner processing of porcine burn wound sections. Microscopy was performed at the Campus Microscopy and Imaging Facility at The Ohio State University. Mass spectrometry was performed by the mass spectrometry and proteomics facility at The Ohio State University.
### Table 2.2. Primers used in this study

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Table 2.3. Bacterial strains used in Chapter 2

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<td>AH LAC transduced with the pvl::spec mutation</td>
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Chapter 3. Leukocidins act in concert with the nuclease NucA to prevent neutrophil-mediated killing of *Staphylococcus aureus* biofilms

Mohini Bhattacharya, Evelien T. M. Berends, Rita Chan, Victor J. Torres, Daniel J. Wozniak (*In preparation*)

3.1. Abstract.

Bacterial biofilms are linked with chronic infections and have properties distinct from single-celled populations. The virulence mechanisms associated with *Staphylococcus aureus* biofilms are only recently being established. Neutrophils play a crucial role in the innate immune response to bacterial infections, including *S. aureus*. Here we describe two novel virulence mechanisms that contribute to the ability of *S. aureus* biofilms to evade killing by neutrophils. Specifically, we show that while neutrophils exposed to *S. aureus* biofilms produce extracellular traps (NETs) and phagocyte bacteria, both mechanisms are inefficient at clearance of biofilm biomass. This is attributed to the leukocidin LukAB, which allows for *S. aureus* survival during phagocytosis. We also show that the persistence of biofilm bacteria trapped in NETs is facilitated by *S. aureus* nuclease (NucA)-mediated NET DNA degradation. This study therefore describes key aspects of the interaction between neutrophils and *S. aureus* biofilms and provides an explanation for how *S. aureus* evades the neutrophil response to cause persistent infections.
3.2. Introduction

*Staphylococcus aureus* is a leading cause of multiple devastating chronic infections\(^{312}\). This is attributed in part to a large repertoire of toxins simultaneously expressed by the pathogen, allowing rapid evasion of host immune defenses\(^{64}\). *S. aureus* is proficient at forming biofilms, a characteristic that contributes greatly to its success as a pathogen\(^{136}\). Bacterial biofilms are structured communities with properties that are distinct from single-celled (planktonic) populations\(^{161}\). This often includes a change in the virulence mechanisms utilized by the pathogen to survive host defenses\(^{273,280}\). Neutrophils are the most abundant white blood cell in circulation and are a crucial aspect of the innate immune response to *S. aureus*\(^{313}\). Some of the main mechanisms used by neutrophils to clear bacteria are phagocytosis, release of antimicrobial peptides, generation of antimicrobial reactive oxygen and nitrogen species, as well as the formation of antibacterial neutrophil extracellular traps (NETs)\(^{122,187}\). Understanding the responses that govern the interactions of *S. aureus* with neutrophils is vital to the development of interventions for these infections. While *S. aureus* biofilms are often associated with chronic infections, our knowledge of the response of biofilms to neutrophil defenses is somewhat limited\(^{274,314}\) (See Chapter 2). Here we describe two of these mechanisms and provide insights as to why neutrophils fail to eradicate *S. aureus* biofilms.

Leukocidins are a group of toxins that lyse leukocytes and red blood cells and play important roles in *S. aureus* pathogenesis. *S. aureus* strains can express up to 5 leukocidins, namely PVL (LukSF-PV), LukAB (also known as LukGH), LukED, HlgCB and HlgAB\(^{135,241}\). Each leukocidin is comprised of an S- subunit that recognizes a receptor on the host cell to recruit the corresponding F- subunit. 4 such S-F pairs result in the formation...
of an octameric pore in the cell membrane, ultimately causing cell death\(^{135}\). Most studies have evaluated roles for leukocidins during single celled (planktonic) \textit{S. aureus} growth\(^{82,246,253,315}\). Chronic infections however, are commonly associated with aggregate or biofilm bacteria that often utilize virulence mechanisms distinct from planktonic populations\(^{279,280,316}\). The roles for leukocidins during growth as biofilms remain largely uncharacterized\(^{127}\). Recent work by us and others have shown that leukocidins can synergize or antagonize each other’s activities\(^{249,250,317}\). We found that unlike necrotic cell death associated with planktonic populations, PVL and HlgAB released during growth as biofilms, skew neutrophils towards NETosis (See Chapter 2). NET formation is the controlled release of neutrophil chromatin laced with antimicrobial proteins, designed to physically trap and kill bacteria respectively\(^{318}\). Interestingly however, we found that NETs were not effective at clearing bacteria from biofilms.

In the present study we describe the virulence mechanisms that allow for \textit{S. aureus} biofilm survival during the induction of NETs by biofilm-released PVL and HlgAB. Specifically, we show that two mechanisms function to allow for \textit{S. aureus} biofilm survival. First, LukAB enhances persistence of bacteria that are phagocytosed by neutrophils, since the absence of LukAB results in a distinct survival disadvantage for \textit{S. aureus} in the presence of neutrophils. This survival phenotype could be mitigated when phagocytosis was blocked, thus solidifying a role for LukAB during phagocytosis. Furthermore, we show that neutrophil lysis occurs as a consequence of LukAB activity, and that the process of NETosis does not result in neutrophil lysis\(^{128,311}\). Second, we describe a role for the nuclease NucA, in facilitating the break-down of NET DNA and allowing for survival of trapped \textit{S. aureus}, as a biofilm-grown \textit{nuc::bursa} transposon
mutant showed reduced levels of survival, when compared to survival of wild type *S. aureus*. However, when a *nuc::bursa* biofilm was exposed to neutrophils and treated with DNase-I, this restored *S. aureus* survival. Lastly, we show that degradation of NET DNA by NucA causes a dispersal event, potentially allowing dissemination of biofilm infections.

Studies with planktonic *S. aureus* describe an upregulation of LukAB from *S. aureus* that is exposed to neutrophils. The pore-forming activity of LukAB was further found to facilitate the escape of phagocytosed *S. aureus*\textsuperscript{311}. LukAB was also found to contribute to the survival of biofilm *S. aureus* exposed to macrophages\textsuperscript{127}. The present study builds on these findings by describing a role for LukAB in mediating the escape of biofilm *S. aureus* that is phagocytosed by neutrophils.

In the absence of immune cells, NucA degrades bacterial DNA associated with the biofilm matrix, resulting in a dispersal event\textsuperscript{196}. Additionally, during planktonic growth, NucA degrades NETs that are released in response to *S. aureus*\textsuperscript{153,319}. Here we show that both functions of NucA are important when *S. aureus* biofilms are exposed to neutrophils. NucA degrades NET DNA that is released due to the activity of PVL and HlgAB. This degraded NET DNA causes a dispersal event, contributing to *S. aureus* evasion of neutrophil killing via NET-associated antimicrobial proteins\textsuperscript{318}. Altogether this study provides an understanding of some of the mechanisms by which biofilm-grown *S. aureus* survives upon exposure to neutrophils.

3.3. Results

**Leukocidins are required for biofilm bacterial survival.** To understand the fate of *S. aureus* biofilms exposed to neutrophils, we incubated wild type USA300LAC (widespread community acquired MRSA strain) *S. aureus* biofilms with Cell Tracker Blue labelled
neutrophils for 2 hours. Syto-9 was used to assess the levels of viable bacteria remaining after this incubation and ethidium homodimer-1 (EthHD-1) was used to evaluate the release of neutrophil chromatin material, associated with neutrophil death via the release of extracellular traps\textsuperscript{318}. Similar to previous reports, we found that neutrophils penetrate the biofilm biomass but are unable to clear wild type bacteria (Figure 3.1A)\textsuperscript{184}. Intact neutrophils were found at the base of the biofilm, releasing DNA (Figure 3.1B/ white arrows). When similar experiments were performed with a $\Delta$lukSF$\Delta$hlgACB strain, impaired in the ability to induce NETs, bacterial survival and biofilm thickness was greatly diminished (Figure 3.1C, D). Biofilms from a $\Delta$S strain (Figure 3.1E, F) lacking all leukocidins showed an even lower biomass than the $\Delta$lukSF$\Delta$hlgACB strain upon exposure to neutrophils (Figure 3.1G), confirming previous reports (See Chapter 2) and indicating that other leukocidins were playing roles in facilitating the survival of $S$. aureus biofilms exposed to neutrophils.
Figure 3.1. Leukocidins are required for survival of biofilms exposed to neutrophils.

Wild type USA300LAC biofilms were incubated with Cell Tracker Blue labelled primary human neutrophils for 2 hours, stained with Syto-9 (live bacterial cells) and Ethidium homodimer-1 (DNA/ Dead mammalian) and imaged using confocal laser scanning microscopy. Image represents a section taken close to the base of the biofilm (A). 3-D image showing a wild type USA300LAC biofilm after a 2-hour incubation with primary human neutrophils. Arrow highlights DNA of dying neutrophil (B). Experiments similar to A and B, performed with biofilms of a ΔlukSFΔhlgACB strain. (C, D). Experiments similar to A and B, performed with biofilms of a Δ5 strain, lacking all leukocidin proteins (E, F). Volume quantification of WT, ΔlukSFΔhlgACB and Δ5 biofilms, after treatment with neutrophils for 2 hours (G). Images were taken from biofilms grown in µ-slides and captured at a 600X magnification. Images represent the majority population phenotype of six independent experiments performed in triplicate. Student t-tests were performed for pair-wise comparisons. P-values are as indicated.
**LukAB contributes to survival of biofilm bacteria during phagocytosis.** LukAB has been reported to play a role in the survival of biofilms exposed to macrophages\textsuperscript{127}. Additionally, the expression of LukAB is increased in planktonic *S. aureus* exposed to neutrophils and facilitates survival of bacteria in phagosomes\textsuperscript{311}. To understand if LukAB was contributing to the differences in biofilm biomass observed between the Δ*lukSFΔhlgACB* and Δ5 strains (Figure 3.1G), we therefore compared the survival of wild type USA300LAC and isogenic Δ*lukAB* biofilms, when exposed to neutrophils for 1 and 2 hours. We found that neutrophils phagocytosed wild type biofilms (Figure 3.2A) but were unable to clear the biomass after a 2-hour incubation period (Figure 3.2B). In comparison, phagocytosis was increased when a Δ*lukAB* strain was similarly treated (Figure 3.2C). However, biofilm biomass was still retained 2 hours post exposure to neutrophils (Figure 3.2D). Nevertheless, Δ*lukAB* biofilms treated with neutrophils had significantly lower levels of biomass retained, in comparison to the wild type strain (Figure 3.2E). Since Δ*lukAB* biofilms treated with neutrophils seemed to be more efficiently phagocytosed, we reasoned that LukAB might be playing a role in the survival of *S. aureus* during phagocytosis. To evaluate this, we measured the numbers of viable bacteria retained before and after treatment of wild type and Δ*lukAB* biofilms with neutrophils. While there was no significant decrease in wild type biofilm bacteria in comparison to an untreated control (Figure 3.2F), there was a significant (~4-log) decrease in Δ*lukAB* biofilms that were treated with neutrophils, in comparison to a similar control (Figure 3.2F).
Figure 3.2. LukAB contributes to bacterial survival when biofilms are treated with neutrophils.

Wild type USA300LAC biofilms incubated with Cell Tracker Blue labelled primary human neutrophils for 1 (A) and 2 hours (B), stained with Syto-9 (live/ bacteria) and imaged using confocal laser scanning microscopy. Representative image of sections taken close to the base of biofilms (A-B). Experiments similar to those described in A and B, performed with an isogenic ΔlukAB strain (C, D). Biofilm biomass measured at 1-hour post incubation of wild type (WT) and ΔlukAB biofilms incubated with neutrophils for 1 hour(E). Total colony forming units per mL (cfu/mL) of biofilms from indicated strains, incubated with (+PMN) and without (-PMN) neutrophils for 1 hour. (F). Images were taken of biofilms grown in µ-slides and captured at a 600X total magnification. Results represent an average of six independent experiments performed in triplicate ± s.e.m. Images and measurements of volume were taken using Imaris Software Version X 6.4. CfU/mL enumerations were done independently, using biofilms grown in silicone tubing (as described in methods section). Student t-tests were performed for pair-wise comparisons. P-values are as indicated.
To confirm a role for LukAB in survival during phagocytosis, we tested the ability of ΔlukAB biofilms (Figure 3.3A, B) to survive in the absence of neutrophil phagocytosis. Neutrophils were treated with Cytochalasin-D to block actin polymerization and phagocytosis, before incubation with ΔlukAB biofilms (Figure 3.3C, D). We found that under these conditions there was a significant increase in the biofilm biomass retained after a 1-hour incubation with Cytochalasin-D treated neutrophils, compared to a ΔlukAB control (Figure 3.3E). There was also a significant increase in the total population (~2-log) of surviving bacteria from ΔlukAB biofilms incubated with Cytochalasin-D treated neutrophils, in comparison to an untreated control (Figure 3.3F). These results suggest that LukAB plays a role in the survival of biofilm bacteria during phagocytosis.
Biofilms from a ΔlukAB strain incubated with Cell Tracker Blue labelled primary human neutrophils for 1 hour and stained with Syto-9 (live bacteria) and Ethidium homodimer-1 (dead/dying neutrophils) (A). Representative image of section taken close to the base of the biofilm (B). Cell Tracker Blue labelled primary human neutrophils were incubated with Cytochalasin D for 1 hour before incubation with ΔlukAB biofilms for 1 hour and stained similar to A and B (C, D). Biofilm biomass measured at 1-hour post incubation with neutrophils, for the ΔlukAB strain treated with Cytochalasin D (+CCD) (E). Colony forming units per mL calculated for biomass retrieved from biofilms of the ΔlukAB strain and the ΔlukAB strain after treatment with Cytochalasin D (+CCD) (F). Images were captured at 600X total magnification and measurements were taken using Imaris Software Version X 6.4. Results represent an average of six independent experiments performed in triplicate ± s.e.m. Cfu/mL enumerations were done independently, using biofilms grown in silicone tubing (as described in methods section). Student t-tests were performed for pairwise comparisons. P-values are as indicated.
To determine if additional leukocidins were playing a role in biofilm survival, we evaluated the ability of a mutant unable to express PVL, HlgAB, and LukAB ($\Delta$lukSF$\Delta$hlgAB$\Delta$lukAB) to survive neutrophil killing. Biofilms of this strain were incubated with neutrophils and imaged as described above. In comparison to a wild type strain (Figure 3.4A, B) biofilms of the $\Delta$lukSF$\Delta$hlgAB$\Delta$lukAB mutant unable to express PVL, HlgAB and LukAB, were efficiently cleared by neutrophils (Figure 3.4C, D) at a level comparable to the $\Delta$5 strain (Figure 3.4E, F), with most of the biomass lost at 1-hour post infection (Figure 3.4G). This was corroborated by measurements of the total numbers of surviving bacteria (Figure 3.4H), indicating that the combined action of leukocidins PVL, HlgAB and LukAB was required for survival of $S.\ aureus$ biofilms treated with neutrophils.
Figure 3.4. LukAB, PVL and HlgAB are required for biofilms to survive neutrophil killing

Neutrophils were incubated with wild type biofilms and imaged after 1 hour. Staining of neutrophils and bacteria was done as described for Figure 1 (A). Representative image of section taken close to the base of wild type biofilms and imaged similar to A (B). Experiments performed similar to A, with an isogenic mutant of lukSF, hlgAB and lukAB (ΔlukSFΔhlgACBΔlukAB) (C, D). Experiments performed similar to A, with an isogenic mutant of all 5 leukocidins (Δ5) (E, F). Biofilm volume quantified after a 1-hour incubation of biofilms from indicated strains, with neutrophils (G). Cfu/mL measurements of total bacteria isolated from biofilms of indicated strains, after a 1-hour incubation with neutrophils (H). Results represent an average of six independent experiments performed in triplicate ±s.e.m. Cfu/mL enumerations were done independently, using biofilms grown in silicone tubing (as described in methods section). Multiple comparisons were done using one-way analysis of variance and a Tukey’s post hoc analysis where appropriate **P<0.01, ***P<0.001. Images were taken using Imaris Software Version X 6.4. MFI calculations were performed using Image J.
**LukAB and not NETosis, causes lysis of neutrophils exposed to S. aureus biofilms.** Neutrophils are able to release chromatin material via NETosis, while retaining cell membrane integrity (non-lytic NETosis). Additionally, anuclear neutrophils can continue to phagocytose bacteria, post NET-release\(^{190,263,319}\). Our observations indicate that neutrophils incubated with wild type biofilms remain intact after a 1-hour incubation, since they retain the cytosolic dye Cell Tracker Blue (Fig. 3.1A). We therefore reasoned that neutrophils treated with wild type biofilms were undergoing the process of non-lytic NETosis\(^{319}\). Indeed, when neutrophils were treated with wild type biofilms, they retained cytosolic staining with Cell Tracker Blue and anti-CD45 antibody, which recognizes the neutrophil surface protein CD45 (Figure 3.5A, white arrows). However, these cells lost staining for Ethidium Homodimer-1 (Figure 3.5B, white arrows) indicating the presence of cells with intact membranes, post-NETosis. Furthermore, examination \(\Delta{lukSF\Delta{hlgACB}}\) biofilms treated with neutrophils (unable to induce NETosis) at a higher resolution showed neutrophil lysis accompanying the release of large numbers of bacteria (Figure 3.5C/white arrow). Although this was associated with observable cell debris, these observations indicated that neutrophil lysis appears to be independent of NETosis. This was in sharp contrast to neutrophils treated with biofilms of the \(\Delta{5}\) strain lacking all leukocidins, which showed intact neutrophils (Figure 3.5D). This indicated that lysis of neutrophils was not due to NETosis, but potentially mediated by the activity of LukAB during phagocytosis. Indeed, lower levels of cell debris was observed when neutrophils were incubated with a \(\Delta{lukAB}\) strain, in comparison to the \(\Delta{lukSF\Delta{hlgACB}}\) strain (no NET formation) (Figure 3.6 B, C). Cell membrane integrity of neutrophils treated with \(\Delta{lukAB}\) biofilms was
comparable to the Δ5 strain. (Figure 3.6 D). These results provide evidence that lysis of neutrophils occurs due to the activity of LukAB and not NETosis.
Figure 3.5. LukAB and not NETosis induces neutrophil lysis.

Wild type biofilms incubated with Cell Tracker Blue- labelled primary human neutrophils for 1 hour, stained with Ethidium homodimer-1 (dead/dying neutrophils) and an FITC labelled anti-CD45 antibody (green). Arrows point to cells that retain staining with Cell Tracker Blue and anti-CD45 antibody but not Ethidium homodimer-1 (A, B). Biofilms from a ΔlukSFΔhlgACB (C) or Δ5 strain (D), incubated with Cell Tracker Blue labelled primary human neutrophils for 1 hour, followed by staining with Syto-9 (green/live bacteria) and Ethidium homodimer-1 (dead/dying neutrophils). Arrows point to neutrophils to show neutrophil lysis with release of intracellular bacteria (C) or lack thereof (D). Results represent an average of six independent experiments. Images were taken at 600X total magnification. Digital zoom (X1.2) was applied to images in A and B, using Imaris Software Version X 6.4.
Figure 3.6. LukAB and not NETosis causes lysis of neutrophils exposed to *S. aureus* biofilms.

Biofilms of indicated strains were treated with neutrophils for 1-hour. Neutrophils were then labelled with an anti-CD45 antibody and stained with an Alexa-Fluor 488 secondary antibody (green) (A-D). An anti-IgG antibody was used as an isotype control (E). Results represent an average of six independent experiments performed in triplicate. Images were taken using Imaris Software Version X 6.4. MFI calculations were performed using Image J.
**NucA contributes to survival of biofilm bacteria associated with NETs.** Our observations indicate a role for LukAB in aiding the survival of biofilm bacteria during phagocytosis. As described above, we found that although there was a significant reduction in survival of neutrophil-associated bacteria (~4 log) when a \( \Delta \text{lukAB} \) biofilm was treated with neutrophils, this strain was able to retain biofilm biomass after a 2-hour treatment with neutrophils (Figure 3.2D). This indicated that while LukAB was playing a role in survival of biofilm bacteria during phagocytosis, other factors were involved in the survival of extracellular bacterial populations. We therefore reasoned that these factors were contributing to the ability of *S. aureus* to escape killing mediated by the antimicrobial properties of NETs\(^{232,263,318}\). Planktonic *S. aureus* releases the nuclease NucA, that can cleave NET DNA, resulting in a loss of NET antimicrobial properties and allowing for bacterial survival during NETosis\(^{153,319}\). We therefore reasoned that NucA could be playing a similar role in the survival of biofilm bacteria during NETosis.

We tested the effect of neutrophils on biofilms of an isogenic *nuc::bursa* strain. Indeed, in comparison to a wild type strain (Figure 3.7A), biofilms of the *nuc::bursa* strain showed a higher level of staining with EthHD-1, 1-hour post incubation with neutrophils (Figure 3.7B). The levels of NETs observed could be reduced when *nuc::bursa* biofilms were treated with neutrophils in the presence of DNase-I or supernatants from wild type biofilms, containing NucA (Figure 3.7C, 3.8A). These observations were corroborated by measuring the mean fluorescence intensities for EthHD-1 to stain for DNA of dead or dying neutrophils. Indeed, *nuc::bursa* biofilms treated with neutrophils showed significantly higher levels of EthHD-1 staining in comparison to the wild type and *nuc::bursa*+DNase-
I conditions (Figure 3.7D). Additionally, spent media collected from wild type and *nuc::bursa* biofilms showed no significant differences in their ability to kill neutrophils, indicating that these results were not attributed to an increased expression of the NET-inducing toxins PVL and HlgAB from *nuc::bursa* biofilms (Figure 3.8C). Lastly, to understand whether the breakdown of NET DNA by NucA provided biofilms with a survival advantage, neutrophils were separated from biofilm bacteria after a 1-hour incubation. Bacterial survival was assessed in each population. We found that in *nuc::bursa* biofilms, there was a significant reduction in the numbers of non-phagocytosed (extracellular) biofilm bacteria in comparison to neutrophil associated bacterial populations (Figure 3.7E). A similar reduction was not observed in the wild type strain, indicating that NucA is required for the extracellular or phagocytosis-independent survival of biofilm bacteria.
Figure 3.7. NucA facilitates survival of biofilm *S. aureus* during NETosis

Cell Tracker Blue labelled neutrophils were incubated with biofilms of wild type (WT) or an isogenic *nuc::bursa* strain for 1 hour and stained similarly to Figure 1 (A, B). Sections taken close to the base of the biofilm are shown in panels on the right. Images similar to those described in A and B, with *nuc::bursa* biofilms that were treated with DNase-I for 1-hour post incubation with neutrophils (C). Mean fluorescence intensity measurements of Ethidium Homodimer-1 staining, comparing wild type (WT) biofilms treated with neutrophils, to *nuc::bursa* or *nuc::bursa*+DNase-I similarly treated (D). Cfu/mL calculated for biofilms grown in silicone tubing and treated with neutrophils for 1 hour. Neutrophil associated bacteria were enumerated and compared to biofilm associated extracellular populations (BF) for wild type and *nuc::bursa* strains (E). Comparisons of dispersed bacteria before (-PMN) and after (+PMN) treatment of wild type (WT) or *nuc::bursa* biofilms with neutrophils for 1-hour (F). Enumerations similar to F, comparing neutrophil-treated wild type biofilms with *nuc::bursa* biofilms +/- DNase-I treatment subsequent to incubation with neutrophils (G). Results represent an average of six independent experiments performed in triplicate ±s.e.m. Cfu/mL enumerations were done independently, using biofilms grown in silicone tubing (as described in methods section).
Student t-tests were performed for pair-wise comparisons. P-values are as indicated. Multiple comparisons were done using one-way analysis of variance and a Tukey’s post hoc analysis where appropriate **P<0.01. Images were taken using Imaris Software Version X 6.4. MFI calculations were performed using Image J.
Figure 3.8. NucA mediated breakdown of NET DNA contributes to biofilm bacterial dispersal

*nucA::bursa* biofilms were treated with neutrophils for 1 hour and subsequently with wild type (WT) biofilm supernatant for an additional hour. Staining was done as described for Figure 1 (A). Cross section of biofilm incubated with neutrophils as described for A, taken at the bottom of the biofilm (B). Neutrophil killing activity for spent media collected from wild type and *nucA::bursa* biofilms. Neutrophils were incubated with spent media for 30 minutes. LIVE-DEAD measurements show a ratio of Syto-9 (live) to propidium iodide (PI) fluorescence calculated as a percentage against a positive control (neutrophils+0.1%SDS/+). Neutrophils treated with Hanks Balanced Salt Solution (HBSS) were used as a negative control (C). Colony forming units per mL of dispersed bacterial populations collected after a 60-minute incubation of wild type and *nucA::bursa* biofilms incubated with primary human neutrophils, before and after treating with Cytochalasin-D +/- DNAse-I (D). Experiments similar to D, performed with neutrophils treated with WT and ΔlukAB biofilms (E). Cfu/mL measurements were done using biofilms grown in silicone tubing. Results represent an average of six independent experiments performed in triplicate ±s.e.m. **P<0.01, ***P<0.001 using one- way analysis of variance and a Tukey’s post hoc analysis.
NucA mediated breakdown of NET DNA induces biofilm dispersal. In the absence of immune cells, *S. aureus* biofilms release NucA to break down matrix-associated bacterial DNA. This results in the dispersal of a subpopulation of cells. Whether this function can contribute to the dispersal of *S. aureus* biofilms trapped in host DNA (such as NETs) has not yet been evaluated. To test whether the breakdown of NET DNA by NucA could cause dispersion, we compared the number of colony forming units per mL of effluent collected after a 1-hour incubation of neutrophils with either wild type or *nuc::bursa* biofilms. Treatment of neutrophils with wild type biofilms resulted in a significantly higher number of dispersed bacteria, in comparison to corresponding untreated controls (Figure 3.7F). A similar comparison did not show significant differences with a *nuc::bursa* biofilm. Rather, numbers of dispersed populations were significantly lower (~4 log) in *nuc::bursa* biofilms treated with neutrophils, in comparison to wild type biofilms that were similarly treated (Figure 3.7F, black bars). This indicated that indeed, NucA was contributing to the dispersal of *S. aureus* biofilms treated with neutrophils. Furthermore, we found that this dispersal could be abrogated by treating *nuc::bursa* biofilms with DNase-I after a 1-hour incubation with neutrophils (Figure 3.7G).

When *nuc::bursa* biofilms were treated with Cytochalasin-D to block phagocytosis, this did not affect the dispersal of bacteria. Treatment of neutrophils with both Cytochalasin-D and DNase-I however, showed significantly higher levels of dispersal, comparable to wild type biofilms. These results further indicated a role for NucA mediated dispersal of *S. aureus* biofilms exposed to neutrophils (Figure 3.8D). Lastly, when ΔlukAB biofilms were similarly incubated with Cytochalasin-D alone or in combination with DNase-I, we observed no change in the numbers of dispersed bacteria, indicating that the
observed reduction in bacterial numbers was not affected by phagocytosis/LukAB activity and was indeed attributed to the activity of NucA (Figure 3.8E).

Collectively these data indicate that both functions of NucA previously characterized for planktonic bacteria, occur when *S. aureus* biofilms are in the presence of neutrophils\(^87,153,319\). First, NucA degrades NET DNA that is released due to the activity of PVL and HlgAB, released from biofilms. Second, this degradation causes a dispersal event in the biofilm, potentially allowing the infection to disseminate\(^87\).
3.4. Discussion

*S. aureus* is a versatile pathogen that utilizes multiple virulence factors to evade host immune responses during infection\(^{64,67}\). Recent studies make it evident that delineating the collective response of these factors to a single infection environment is necessary for a proper understanding of *S. aureus* virulence mechanisms\(^{127,249,250,320}\). Most studies on *S. aureus* pathogenesis have focused on single-celled (planktonic) bacterial populations\(^{246,253,321}\). While these have proved crucial for establishing the fundamentals of *S. aureus* virulence, these populations may not represent those from persistent infections\(^{158,278}\). These bacteria are found as aggregates or biofilms, with properties that are often distinct from single-celled populations\(^{273,280,316}\). Studies by us and others have emphasized the importance of evaluating the unique response of biofilms to host defense mechanisms\(^{127}\). Specifically, studies show that while *S. aureus* biofilms express many of the same virulence factors that they do during planktonic growth, the mechanisms used for immune evasion by biofilms are markedly different from those used by the same factors during planktonic growth\(^{127}\).

While neutrophils play a crucial role in eliminating bacterial infections, very little is known about their response during chronic infections, associated with biofilms\(^{122,322}\). Here we reveal some novel mechanisms that facilitate the evasion of neutrophil antimicrobial responses by *S. aureus* biofilms. These studies build on our previous findings that describe the role of PVL and HlgAB released by *S. aureus* biofilms to induce NETosis during biofilm associated infections (See Chapter 2). Consistent with *in vivo* studies by others, we postulate that these neutrophils are functional during the process of biofilm triggered NETosis and can efficiently phagocytose bacteria\(^{232,298}\). These reports describe a
function for non-lytic NETosis post phagocytosis, as a mechanism for controlling the spread of bacteria, similar to the work described here. We show that neutrophils indeed undergo the antimicrobial processes of NETosis and phagocytosis, however neither of these mechanisms are effective at preventing *S. aureus* biofilm persistence. When genes encoding for both PVL and HlgAB are deleted, NETosis is impeded yet phagocytosis of biofilm bacteria continues. While NucA allows for the escape of bacteria trapped in NETs, we show that LukAB facilitates the survival of bacteria during phagocytosis.

LukAB is expressed when planktonic *S. aureus* are exposed to neutrophils and it facilitates bacterial survival within phagosomes. Phenol soluble modulins (PSMs) are also considered an important virulence factor mediating survival of bacteria after phagocytosis by neutrophils. Which proteins play the pivotal role depends in part on the nutrients and bacterial stressors of the infection environment. Similarly, after phagocytosis of *S. aureus* by macrophages, the concerted effect of LukAB and alpha hemolysin contribute to bacterial survival. Therefore, leukocidin independent mechanisms could contribute to survival of biofilms in vivo. However results from multiple studies including ours indicates that LukAB is a key contributor to survival upon exposure to immune cells.

Here we show that the activity of LukAB is responsible for ultimately lysing neutrophils, consistent with studies on planktonic *S. aureus*. However, while LukAB is known to puncture neutrophil membranes, whether LukAB triggers a host signaling cascade that leads to neutrophil lysis is still debated. Biofilm bacteria often adapt and evolve in response to environmental stresses and this might contribute to lower levels of LukAB expressed from extracellular biofilm bacteria that are exposed to neutrophils.
The intracellular environment of neutrophils however, may play a role in increased expression of LukAB from phagocytosed bacteria and this could contribute to the role of LukAB in biofilm mediated neutrophil killing.

In the absence of immune cells, NucA plays a role in mediating the dispersal of biofilm bacteria via its activity on biofilm matrix associated DNA\textsuperscript{154,196}. Consequently a *nuc::bursa* strain forms a significantly thicker biofilm than its corresponding wild type parent strain\textsuperscript{87}. NucA also degrades DNA derived from NETs\textsuperscript{153}. Here we show that NucA performs both functions when *S. aureus* biofilms are exposed to NET DNA and therefore may play a role in preventing bacterial killing by NET associated antimicrobial factors\textsuperscript{187,326}. Indeed, we find that consistent with observations on planktonic *S. aureus*, NucA cleaves NET DNA and this increases dispersion of bacteria\textsuperscript{153}. This dispersal event could contribute to bacterial dissemination during infection. However, other virulence factors such as PSMs are also involved in the dispersal of *S. aureus* biofilms and might be playing additional roles, especially at later time points such as those observed with chronic infections *in vivo*\textsuperscript{136}.

Studies with planktonic populations indicate a strong antimicrobial activity of NET-associated histone H4 proteins, towards *S. aureus*\textsuperscript{327}. Biofilms however, show enhanced tolerance to antimicrobials\textsuperscript{11}. Therefore, while we show that NucA allows for cleavage of NET DNA and dispersal of bacteria, the concentrations of antimicrobial NET components such as histones and granular proteins might significantly affect the viability of biofilm bacteria\textsuperscript{327}. Recent studies describe a loss in antimicrobial activity of NETs when DNA is cleaved\textsuperscript{328,329}. This could be contributing to the persistence of *S. aureus* biofilms observed here. Nevertheless, our studies unravel some of the mechanisms used by *S.
*aureus* biofilms to evade the neutrophil antimicrobial response, greatly adding to our knowledge of biofilm-neutrophil interactions. More importantly, these studies highlight the value of considering the contribution of multiple virulence factors to the outcome of bacterial infections and further potentiates the significance of leukocidins to evasion of neutrophil killing by *S. aureus* biofilms.
3.5. Materials and Methods

**Bacterial strains and biofilm growth.** Unless otherwise specified, all studies were performed in the USA300LAC background (AH1263, provided by Dr. Alex Horswill (University of Colorado, Denver). Bacterial cultures were grown in tryptic soy broth + dextrose for 16-18 hours with shaking (37°C/ 200RPM), diluted and allowed to reach exponential phase (O.D 600 0.5- 0.7). These cultures were diluted to an O.D. of 0.1 and seeded into chambers of a 6- channel μ-slide (ibidi) for biofilm formation. μ-slides were incubated overnight at 37°C under static conditions and biofilms were allowed to form for 16-18 hours. Biofilms were washed with Roswell Park Memorial Institute Medium (RPMI) without phenol red before performing neutrophil interaction studies. For details about the various mutants used in these studies please refer to Table 3.1.

Transposon mutants used in these studies were obtained from the Network for Antimicrobial Research in *Staphylococcus aureus* at the University of Nebraska, Medical Center. The presence of transposon insertions for these mutants was verified by plating onto TSA + 5 µg/mL erythromycin before use and performing PCR reactions with primers specific for the transposon as well as the respective genes.

**Neutrophil isolation.** Neutrophils were isolated from whole blood using previously published methods. Briefly, heparinized blood derived from healthy human donors by venipuncture was collected in saline and layered into a Ficoll-Paque gradient. Tubes were then centrifuged at 404 x g for 40 minutes at 23°C after which pellets were allowed to sediment in a 1:1 solution of 3% Dextran and 0.9% saline. Spent media was collected and centrifuged at 335 x g for 10 minutes. RBCs in pellets were then lysed with sterile water and 1.8% sodium chloride used to restore isotonicity. Tubes were centrifuged at 233 x g...
for 3 minutes. Pellets containing 95-97% neutrophils were re-suspended in HBSS and counted in a hemocytometer chamber, using trypan blue exclusion\textsuperscript{330}.

**Silicone tube biofilms and CFU enumerations.** For studies describing colony forming units (CFU) collected from biofilms treated with neutrophils, cultures were grown as described above and $2 \times 10^8$ cells (2mL) were seeded into 10cm long peroxide-cured silicone tubing (0.5cm inner diameter) that was plugged on either end. Tubes were incubated for 16-18 hours at 37°C under static conditions to allow for biofilm formation. 4X10\textsuperscript{6} neutrophils per mL (2mL) were stained with Cell Tracker Blue for microscopy (or used unstained for cfu/mL analyses) as described above and incubated for an hour. Dispersed populations of bacteria and unattached neutrophils were collected by unplugging one end of the tubing. Tubes were then cut longitudinally, and biofilms scraped off aseptically using a metal spatula and 2mL of phosphate buffered saline. Suspensions containing biofilms and attached neutrophils were lightly vortexed and centrifuged at 233Xg for 3 mins @ 4°C to pellet neutrophils. Extracellular populations were calculated from supernatants after spinning at 20,000Xg to pellet bacteria. Intracellular populations were enumerated from pelleted neutrophils by centrifuging at 20,000Xg. Neutrophils lyse at this speed allowing for intracellular bacteria to be collected from the pellet. Pellets were imaged before and after centrifugation to validate the separation of neutrophils from bacterial populations, using the LIVE-DEAD procedure describe above (Figure 3.9).
Figure 3.9. Separation and enumeration of intracellular and extracellular biofilm associated bacteria.

Neutrophils retrieved from biofilms of indicated strains before (left panels) and after (right panels) centrifugation (@233g) to separate biofilm bacteria from neutrophils. Neutrophils were stained with Cell Tracker Blue before incubation with biofilms, as described previously, followed by staining with Syto-9 (Live/bacterial) and Ethidium Homodimer-1 (dead, dying/ neutrophil). Images were captured at a 600X total magnification and represent the majority population of six independent experiments performed in triplicate.
### Table 3.1. Bacterial strains used in Chapter 3

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH1263 LAC</td>
<td>Erm^S USA300 parent strain</td>
<td>157</td>
</tr>
<tr>
<td>AH ΔlukSFDhlgACB (VJT 38.83)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AH LAC ΔhlgACB::tet transduced with the pvl::spec mutation</td>
<td>Ref Chapter 2 (Pauline Yoong)</td>
<td></td>
</tr>
<tr>
<td>AH LAC ΔΔΔΔΔΔ (VJT 47.15)</td>
<td>AH LAC ΔlukAB created using the pKOR1 ΔlukAB plasmid</td>
<td>311</td>
</tr>
<tr>
<td>AH LAC ΔΔΔΔΔΔ (VJT 47.15)</td>
<td>AH LAC ΔpvlΔlukEDΔhlgACB also harboring a clean deletion of lukAB using the pKOR1-ΔlukAB plasmid</td>
<td>Ref Chapter 2 (Ashira and Will)</td>
</tr>
<tr>
<td>AHLAC nuc::bursa</td>
<td>transposon insertion in the nuc gene Erm^R</td>
<td>303</td>
</tr>
<tr>
<td>AH ΔlukABΔlukSFDhlgAB</td>
<td>ΔlukAB pvl::spec hlgA::bursa</td>
<td>This study</td>
</tr>
</tbody>
</table>
**Biofilm-neutrophil interaction imaging.** To understand the role of biofilm released leukocidins during interaction with neutrophils, biofilms grown as described above were incubated with 4×10^6 PMNs/mL and incubated for either 1 or 2 hours, as required (37°C/ +CO_2). To visualize intact neutrophils, cells were incubated with 100 µM Cell Tracker Blue (Thermo Fisher) for 30 minutes before interaction with biofilms. After incubation, flow cells were washed with RPMI (without phenol red) to remove planktonic bacteria and fixed with 2% paraformaldehyde. Syto-9 was used to stain live cells while EthHD-1 stained for DNA of dead or damaged cells. Images were captured using a FluoView filter confocal microscopy at 600X total magnification and 3 dimensional images analyzed with Imaris software version x 64 8.4.1

**Cytochalasin-D and DNase-I treatments.** To block phagocytosis and actin polymerization, neutrophils were simultaneously treated with 10µg/mL Cytochalasin-D and 100µM Cell Tracker Blue for 30 minutes. DNase-I treatments were done at a concentration of 0.5µg/mL as described previously^{153}. LIVE-DEAD analyses were carried out as described above.

**CD45 labelling of neutrophils.** Neutrophils were incubated with biofilms in the 6-channel μ-slide (ibidi) model described above. Biofilms were then blocked with 1% bovine serum albumin overnight @ 4°C. Neutrophils were stained using an FITC labelled anti-CD45 antibody (Biolegend 368507)^{190}. Anti-mouse IgG-κ was used as a negative isotype control (Biolegend 400109) and Ethidium homodimer-1. Cells were visualized with confocal laser scanning microscopy at 600X total magnification.
Quantification of Ethidium Homodimer-1 staining. Neutrophils were stained with EthHD-1 as described above. Image J software Ver 5.3 was used to set the same threshold for all conditions and mean fluorescence intensity (MFI) was calculated from 6 independent experiments.

Quantification and Statistical Analysis. All statistical analysis was carried out using Graph Pad Prism Ver 5.0b. One-way analysis of variance with Tukey’s least significant difference was a post-hoc test where appropriate. Bartlett’s test was used to determine the extent of variability between sample populations. Variance between groups was found to be similar. Results for all experiments represent triplicate conditions performed in at least 3 independent experiments. Sample sizes for neutrophil killing assays were chosen based on the standard deviation of a pilot experiment performed in triplicate to achieve approximately a specified half width of the 95% confidence interval (α=0.05).

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Conflicts of Interest. VJT is an inventor on patents and patent applications filed by New York University School of Medicine, which are currently under commercial license to Janssen Biotech Inc.

Author contributions. Conceptualization, M.B, D.J.W, V.J.T; Writing – Original Draft, M.B. and D.J.W; Writing – Review & Editing, M.B, D.J.W, V.J.T, E.T.M.B; Funding Acquisition, D.J.W, V.J.T; Resources, D.J.W, V.J.T; Supervision, D.J.W, V.J.T.
Chapter 4. Overall discussion and future perspectives

Methicillin resistant *S. aureus* infections are responsible for 11,285 deaths per year in the United States\(^3\). With the ability to acquire antibiotic resistance and counteract host immunity, novel therapeutics for these infections is an emergent need. Biofilms are structured communities of microorganisms that persist, causing chronic infections. Biofilms can tolerate significantly higher antimicrobial concentrations than single cells (planktonic), greatly complicating the prognosis of *S. aureus* infections\(^3\). *S. aureus* is known to efficiently surpass the innate immune response to persist in the host\(^2\,6\,5\,190\,3\,3\,3\). However, our knowledge of the virulence mechanisms associated with the evasion of innate immunity by *S. aureus* biofilms is relatively scarce\(^12\,7\,18\,4\). This knowledge is important for the development of anti-infection therapies, since planktonic and biofilm populations are often metabolically distinct and are therefore likely to exhibit virulence traits unique to the mode of growth\(^2\,7\,9\,2\,8\). The work in this dissertation provides evidence for novel virulence mechanisms associated with *S. aureus* biofilm infections. Specifically, here we show that the evasion of neutrophil killing by *S. aureus* biofilms is largely attributed to the activity of biofilm-released leukocidins. Neutrophils are an innate immune cell-type essential to the elimination of bacterial pathogens\(^12\,2\,1\,0\). The studies described here show that leukocidin mediated neutrophil killing provides biofilm bacteria with a significant survival advantage both *in vitro* and *in vivo*. Chapter 2 describes a role for two leukocidins, PVL and HlgAB, in inducing neutrophil extracellular trap formation (NETs) when neutrophils are exposed to *S. aureus* biofilm derived spent media. Chapter 3 builds on the findings made in Chapter 2 and provides evidence to show that 2 additional virulence
factors play roles when neutrophils are placed in contact with *S. aureus* biofilms (as opposed to spent media). The significance of these findings is three-fold.

1. In Chapter 2 we show that PVL and HlgAB are both released from *S. aureus* biofilms, with the expression of either toxin being sufficient to induce NETosis in a large percentage of neutrophils (60-80%). Therefore, this is a process of bacteria-mediated neutrophil killing that is independent of bacterial contact. This could have important implications during infection, by hindering the ability of neutrophils to recognize bacterial antigens. The recognition of bacterial antigens is important for engaging the appropriate immune cell responses required to clear a pathogen. Secreting PVL and HlgAB to kill neutrophils before they make contact and recognize biofilm-associated molecules, could therefore provide *S. aureus* with a significant advantage over the host. Additionally, the accumulation of neutrophil debris around biofilms, resulting from leukocidin activity, could function as a shield to prevent further recognition of bacterial or ‘non-host’ molecules. Potentially, DNA, proteins and cell membrane fractions from dead neutrophils could be utilized as nutrition sources for the biofilm. These neutrophil components could also be incorporated into the biofilm matrix, further confounding the recognition of bacterial antigens by immune cells and allowing for biofilm persistence. Lastly, the non-inflammatory nature of NETosis could delay or prevent downstream immune cell recruitment and functioning. These aspects require further investigation and would provide better insight into the mechanisms for *S. aureus* immune evasion *in vivo*. Other immune cells including macrophages, eosinophils and monocytes are known to similarly release extracellular traps. Whether these cells release extracellular traps in response to
1. *S. aureus* biofilms and subsequently contribute to the pathogenesis observed *in vivo*, remains to be understood.

2. These studies assert a distinction between the virulence mechanisms of planktonic and biofilm populations. The induction of NETosis was unique to biofilm spent media and was not elicited by planktonic spent media. This emphasizes the need to study biofilms as populations that are distinct from their planktonic counterparts. PVL is expressed at high levels in planktonic populations. At these levels, PVL causes necrotic cell death in neutrophils. In contrast, when PVL is expressed at sub-lytic concentrations, this results in the induction of NETosis, as observed with biofilm grown *S. aureus*. The work in Chapter 2 shows that this switch to the induction of NETosis during biofilm growth, provides a survival advantage for bacteria (Ref). Additionally, *S. aureus* biofilms release HlgAB, a leukocidin that is expressed at low levels under planktonic growth. Thus, rather than a unique biofilm associated factor, *S. aureus* controls the levels of expression of toxins common to planktonic growth, to elicit a distinct response. The mechanisms that influence the levels of expression and secretion of these toxins upon transitioning from planktonic to biofilm growth could include host cell released factors as well as stress signals associated with the infection environment (oxygen, pH, nutrition). Further investigation of these regulatory mechanisms would provide a better understanding of what triggers an effective immune evasion response from *S. aureus* biofilms.

3. These studies highlight the importance of considering the contribution of multiple bacterial virulence factors, to a single aspect of the host immune response. Work in Chapter 2 and 3 shows that the concerted effect of 4 proteins (3 leukocidins and a nuclease) released by biofilms, allows *S. aureus* to efficiently evade neutrophil killing.
(Figure 4.1). These studies provide evidence to show that there are three main aspects to *S. aureus* biofilm evasion of neutrophil killing:

- In addition to requiring one of two NET-inducing toxins (PVL or HlgAB), biofilms require LukAB in order to evade phagocytosis. LukAB lyses neutrophils, allowing phagocytosed *S. aureus* to survive\(^{270}\).
- The nuclease NucA, degrades NETs and potentially prevents bacteria from being killed via the activity of NET-associated antimicrobial proteins\(^{318,337}\).

The loss of any one of these three aspects of biofilm virulence, results in a survival disadvantage to *S. aureus*, illustrating the significance of multiple virulence mechanisms simultaneously used by the pathogen.

In Chapter 3 we show that upon exposure to biofilm bacteria, the process of phagocytosis occurs simultaneous to NETosis and that the leukocidin LukAB mediates the survival of *S. aureus* during the process of phagocytosis. Previous studies show that LukAB is upregulated when planktonic *S. aureus* is exposed to neutrophils\(^{270}\). Here we show that the expression of LukAB is also important for the survival of phagocytosed biofilm *S. aureus*, independent of NETosis. Furthermore, we show that the process of NETosis does not result in neutrophil lysis. Rather, in accordance with reports on planktonic bacteria, it is the pore-forming activity of LukAB that results in lysis of neutrophils and release of intracellular bacteria post-phagocytosis\(^{311}\). LukAB also contributes to macrophage and monocyte lysis. The lytic activity of LukAB therefore likely contributes to *in vivo* inflammation associated with *S. aureus* infections\(^{127,129}\).
Additionally, we describe a role for the thermonuclease NucA, in breaking down NET DNA to allow for the survival of NET-associated biofilm bacteria. NucA is known to facilitate the degradation of NET DNA \textit{in vitro} and \textit{in vivo}\textsuperscript{153,338}. In the absence of immune cells, NucA is required for the breakdown of biofilm matrix associated DNA, leading to bacterial dispersion\textsuperscript{87,196}. Here we show, that both previously reported functions of NucA play roles in survival of biofilms exposed to neutrophils, in that degradation of NET DNA by NucA results in the dispersal of biofilm bacteria. Whether NucA contributes to the dissemination of \textit{S. aureus in vivo} however, remains to be understood. More importantly, this dispersal event may now make \textit{S. aureus} more vulnerable to host immune cell attack. The consequence of NucA mediated biofilm dispersal therefore requires further investigation.

Histones, released during the process of NETosis, have potent antimicrobial activity. NETs are laced with neutrophil elastase, myeloperoxidase and cathelicidins. The role of these and other neutrophil antimicrobial proteins in killing biofilm \textit{S. aureus}, remains to be evaluated\textsuperscript{122,339}. Additionally, neutrophils are rich in metal scavenging proteins including lactoferrin and calprotectin\textsuperscript{122}. Iron and manganese are required for the survival of \textit{S. aureus} and contribute to its success as a pathogen\textsuperscript{108,340}. Thus, these neutrophil proteins could negatively influence \textit{S. aureus} biofilm persistence by withholding metals essential for bacterial growth. Conversely, the role of biofilm released factors such as proteases, in the degradation of these neutrophil antimicrobial products could additionally contribute to bacterial evasion and requires further investigation\textsuperscript{341}.

Collectively the data presented here show that while neutrophils undergo both phagocytosis and NETosis in response to biofilm infections, neither is effective at clearing
the biofilm biomass. While PVL and HlgAB cause the release of NETs, LukAB facilitates the survival of biofilm bacteria during phagocytosis. The thermonuclease NucA, degrades released NETs thereby preventing NET-mediated bacterial killing. NucA mediated breakdown of NETs causes a dissemination of biofilms, allowing the biofilm lifecycle to continue. There is a relative insufficiency in our understanding of the host response to biofilms. This research provides fundamental mechanistic insights and significantly contributes to our knowledge of the pathogenesis of chronic *S. aureus* infections, one of the most common and problematic causes of persistent bacterial infections today\(^2\).

While this work focuses on *S. aureus* biofilms, it emphasizes the potential for bacterial toxins to elicit distinct host cell responses, depending on the type of infection i.e. planktonic (acute) v/s biofilm (chronic). This work also highlights the ability of biofilm bacteria to actively kill host cells in order to persist, as opposed to the generally accepted senescence that has been attributed to growth as biofilms\(^3\). Although much remains to be understood about the nature of host-biofilm interactions, especially with regards to the innate immune response, this work sets a foundation for the development of further studies in *S. aureus* biofilms. This area of research is pertinent to the development of intervention strategies for *S. aureus* infections but has only recently begun to be understood.
Figure 4.1. Proposed model for neutrophil response to *S. aureus* biofilms.

The activity of leukocidins PVL and gamma hemolysin AB (HlgAB) released from biofilms, results in the induction of neutrophil extracellular traps or NETosis. While anuclear neutrophils are still capable of penetrating the biofilm structure and potentially phagocytosing bacteria, antimicrobial activity of NETs is insufficient for clearing *S. aureus* biofilms. NET DNA is broken down by nucleases while the leukocidin, LukAB allows for survival of bacteria during phagocytosis, thus perpetuating chronic infection.


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Appendix A: Genome-wide screen for non-essential genes contributing to neutrophil killing activity of *Staphylococcus aureus* biofilm spent media

**Introduction**

*S. aureus* is a proficient pathogen that can target host cells using multiple virulence factors. Our knowledge of the virulence mechanisms used by *S. aureus* when grown as biofilms is somewhat limited\(^\text{127,184,287}\). In Chapters 2 and 3, we describe the identification of novel virulence traits associated with biofilm-mediated neutrophil killing. In Chapter 2, we show that the leukocidins PVL and HlgAB are released by *S. aureus* biofilms and cause the induction of neutrophil extracellular trap formation (NETosis). This was distinct from the response elicited by planktonic spent media (Figure 2.1B). HlgAB is expressed at low levels under planktonic growth conditions. PVL is highly expressed in planktonic cells and causes necrotic cell death. Sub-lytic levels of PVL causes NETosis, similar to the phenotype we report for biofilm released PVL. It is evident therefore, that biofilms regulate the expression of these toxins using mechanisms that are distinct from planktonic bacteria.

We therefore wanted to understand the factors that were influencing the expression and release of these toxins under biofilm grown conditions. To do this, we utilized a transposon library in non-essential genes of USA300LAC (Nebraska Transposon Mutant Library\(^303\)), a common clinically isolated strain of *S. aureus*. So far, 1,532 out of 1,952 mutants have been screened for neutrophil killing activity generated from biofilm spent media using methods described in Chapter 2. Described below are some of the main findings from this screen, with a discussion of their potential implications and future perspectives.
Results and Discussion

To gain an understanding of the genes contributing to the expression and secretion of factors (including PVL and HlgAB) that mediate neutrophil killing under biofilm growth conditions, biofilm spent media from mutants in the transposon library were assessed for their ability to kill neutrophils, using the LIVE-DEAD assay described in Chapter 2. Figure A1 shows an example of neutrophil killing data that was collected and assessed for statistical significance. Mutants with a significant loss of neutrophil killing activity in comparison to wild type biofilm spent media, are summarized in Table A1.

The agr regulatory network controls the expression of a majority virulence factors in USA300LAC, a common clinical strain of S. aureus. As described in Chapter 2, the neutrophil killing activity of biofilm spent media was due to the activity of PVL and HlgAB, resulting in the induction of neutrophil extracellular trap formation (NETs). The expression of PVL and HlgAB is indirectly controlled by agr. It was therefore not surprising that an agrB::bursa transposon mutant showed a significant loss of neutrophil killing activity (Figure 2.3 A, Figure A1). This mutant was therefore used as a control when screening for neutrophil killing activity of transposon mutants. Strains showing significantly higher levels of neutrophil killing in comparison to the wild type control are not discussed in the present screen, but these results would provide insights into the genetic mechanisms involved in downregulating the expression of S. aureus biofilm-released neutrophil killing factors. Of the 1,532 strains screened, mutants with a significant loss of neutrophil killing activity from biofilm spent media were grouped into 5 major categories of genes (Table A1). Each category is discussed in detail below, with a description of the potential implications of these findings for future work.
LIVE-DEAD assays performed similar to Figure 1. Percentage cell death for all neutrophil killing assays was calculated in comparison to neutrophils treated with 0.1% sodium dodecyl sulfate as a positive control, whereas neutrophils in Hank’s Balanced Salt Solution (HBSS) were used as a negative control (+HBSS). Results represent an average of 6 independent experiments ±s.e.m. **P<0.01, ***P<0.001 in comparison to wild type, using one-way analysis of variance and a Tukey’s post hoc analysis.

Figure A.1 Representative set of data for neutrophil killing activity of biofilm spent media collected from the USA300LAC transposon mutant library.
1. **Virulence.** So far, we have identified a loss of neutrophil killing from biofilm spent media of 3 transposon mutants with insertions in genes associated with virulence (Table A1). These did not include the genes encoding for leukocidin subunit proteins. This was not surprising because, as described in Chapter 2, the activity of either PVL or HlgAB is sufficient to cause neutrophil killing and result in the induction of NETs. Thus, a deletion of any of the 4 genes that encode for protein subunits of either toxin (\textit{lukS, lukF, hlgA, hlgB}) shows no difference in killing from spent media of transposon mutant.

Of note, a mutant in the KdpDE two-component system showed a significant loss of killing activity associated with biofilm spent media (\textit{kdpD}). The KdpDE two-component system is activated in response to salt stress and potassium limitation and is upregulated by Agr/RNAIII\textsuperscript{342}. KdpDE senses extracellular potassium to regulate the expression of multiple virulence factors\textsuperscript{342}. Indeed, previous studies have shown that a \textit{ΔkdpDE} strain shows significantly lower levels of expression of HlgB, the F subunit of HlgAB, one of two toxins that play major roles in biofilm-mediated neutrophil killing. A similar change in gene expression was not found for genes encoding PVL proteins (\textit{lukSF}), likely because these studies were performed in a PVL\textsuperscript{-} background\textsuperscript{342,343}. This makes KdpDE an exciting target for further studies to understand the stimuli that induce neutrophil cytotoxicity in biofilm spent media. Another two-component system SaeRS, is known to be required for the expression of both PVL (\textit{lukSF}) and HlgAB (\textit{hlgACB})\textsuperscript{344}. Indeed, we find that transposon mutants in these genes show a loss of biofilm associated neutrophil killing, corroborating previous reports (Figure 2.3B). Interestingly, both KdpDE and SaeRS are upregulated by the Agr regulatory network.
via the repressor of toxins, Rot. Thus, both systems could be involved in the expression of leukocidin genes, with the contribution of either system depending upon the leukocidin as well as the stress stimuli present in the infection environment. Clumping factor B (clfB) is a cell surface-associated molecule that binds fibrinogen and facilitates colonization of nasal passages by S. aureus\textsuperscript{141}. A decrease in neutrophil killing in biofilm spent media of a clfB transposon mutant might therefore be associated with a reduction in biofilm surface adhesion and/or bacterial aggregation and remains to be investigated.

2. Membrane-associated genes. We identified 15 mutants with insertions in genes encoding for known and putative membrane associated proteins, including transporters and ion pumps, as well as genes required for capsule and teichoic acid biosynthesis (Table A1). We also identified a gene encoding for a protein in the twin-arginine translocation machinery (tatC). Neither LukSF/PVL or HlgAB have signal peptides that are characteristic for Tat-mediated export (R-R-x-F-L-K) and are likely not secreted through this system\textsuperscript{345}. S. aureus utilizes a Sec-dependent machinery for the translocation of multiple secreted proteins. Mutants of genes encoding for the Sec-dependent pathway (secD::bursa, secF::bursa) did not show a loss of neutrophil killing, indicating that the leukocidins PVL and HlgAB are potentially not secreted via this pathway\textsuperscript{346}. The secretion machinery used to export these toxins therefore remains to be elucidated.

Interestingly however, studies with a related species of Staphylococcus have shown that the Tat system is important for the release of iron-scavenging proteins including an iron binding lipoprotein, an iron dependent peroxidase and a high affinity iron
permease$^{345}$. While the function of these genes has not been characterized in USA300LAC, the loss of this iron scavenging system might have adverse metabolic effects on the cells, and consequently affect the expression and secretion of virulence factors, as observed with a loss of neutrophil killing activity of these spent media$^{345}$. The role of capsular polysaccharides in pathogenesis of various S. aureus strains remains debated. Indeed, the USA300LAC does not present a capsule$^{117}$. The cap$^{5H}$ genes identified in this screen as having a loss of biofilm mediated neutrophil killing, while annotated as a capsular polysaccharide biosynthesis gene, is an O-acetyl transferase (UniProt ID: A0A0H2XE77_STAA3) which could be involved in protein folding and or modification. This might consequently contribute to the activity of leukocidins and/or other virulence factors that are important for neutrophil killing.

3. **Metabolism.** We identified multiple mutants with insertions in genes associated with the expression of proteins involved in metabolism (Table A1), that showed a significant loss of biofilm spent media derived neutrophil killing. Of note, a number of these mutants were of genes in the arginine metabolism pathway. We therefore performed a second screen to test for transposon mutants of genes known to be required for arginine metabolism. Indeed, we found a significant loss of biofilm-mediated neutrophil killing activity from mutants of multiple genes associated with arginine metabolism (Figure A2). Of potential consequence to pathogenesis were genes in the arc operon, which controls the expression of arginine deiminase in S. aureus$^{347}$. While the role of arginine in biofilm formation and pathogenesis is still debated, studies show that expression of proteins encoded by the arc operon is required to produce polysaccharide intracellular adhesin, the main polysaccharide found in S. aureus biofilm matrices$^{348}$. However, the
USA300LAC strain of *S. aureus* can form biofilms independent of PIA\textsuperscript{115}. Additionally, our studies show that the neutrophil-killing activity generated from biofilm spent media does not require PIA production (Figure 2.A). Thus, the role of Arc proteins in assisting biofilm mediated neutrophil killing is likely independent of PIA. Recent studies show that the *arc* operon drives the synthesis of host polyamines. Most *S. aureus* strains are sensitive to polyamines. USA300LAC however expresses a polyamine acetyl transferase, SpeG, that detoxifies these products. A series of enzymatic conversions performed by the *arc* genes allows for the secretion of SpeG. Thus, these mutants might be showing a lack of neutrophil-killing activity due to polyamine toxicity, owing to the lack of SpeG production\textsuperscript{349}. This might explain the observations made in Figure A2 and requires further investigation.
Figure A.2. Neutrophil killing from biofilm spent media is influenced by arginine metabolism.

Neutrophil killing activity for biofilm spent media from transposon mutants in genes associated with arginine metabolism. Percentage cell death for all neutrophil killing assays was calculated in comparison to neutrophils treated with 0.1% sodium dodecyl sulfate as a positive control, whereas neutrophils in Hank’s Balanced Salt Solution (HBSS) were used as a negative control (+HBSS). Results represent an average of 6 independent experiments ±s.e.m. **P<0.01, ***P<0.001 in comparison to wild type, using one-way analysis of variance and a Tukey’s post hoc analysis.
4. DNA repair/transcription/translation. As described in Chapter 1, the regulatory mechanisms involved in the expression of *S. aureus* virulence factors is a complex network, about which much remains to be known. While multiple global regulators have been identified as influencing virulence, very little is understood about the direct regulation of virulence genes such as those encoding the leukocidin proteins. It was therefore not surprising to find that the mutants that showed a loss of neutrophil killing in this group were mostly associated with previously uncharacterized proteins. Nevertheless, an interesting observation in this group of mutants was the identification of proteins in the LysR family of transcription factors. The LysR family of transcriptional regulators is well characterized as being a group of catabolite-responsive regulators that respond to changes in sugar and amino acid levels to affect the expression of genes in *Bacillus subtilis*\(^{350,351}\). Recently, studies have identified homologues of these proteins in the Newman strain of *S. aureus*, a strain that is genetically diverse from the USA300LAC strain\(^ {352}\). Mutations in these regulators were found to downregulate pigment production and expression of capsular polysaccharides, causing decreased bacterial burdens in an acute pulmonary model of infection\(^ {353}\). These proteins could therefore similarly play important roles in USA300LAC, including the regulation of leukocidins. These results therefore warrant further studies. Furthermore, measurement of the expression levels for global regulators such as Agr, SaeRS and Rot (known to be required for the expression of biofilm derived killing activity) in mutants with a loss of biofilm killing could provide a better understanding of whether the identified genes act downstream of these regulators, to control the expression of
neutrophil killing proteins (including PVL, HlgAB). Lastly, an analysis of expression levels for PVL and HlgAB in these mutants would provide information on whether the mechanisms for regulation are directly associated with leukocidin expression and secretion.

5. **Others.** Here we identify a loss of neutrophil killing with transposon mutants in genes encoding for hypothetical proteins, with no known function.

   Collectively, the data from this screen identifies some exciting targets for genes that might play a role in the neutrophil killing activity of *S. aureus* biofilms. This screen represents 1, 532 of 1952 non-essential genes with a transposon insertion. Assessing the neutrophil-killing activity of the 420 remaining mutants, as well as performing a secondary screen to confirm these preliminary results, will provide a clearer image of genes that potentially play key roles in the synthesis, regulation, secretion and folding of leukocidins as well as other neutrophil killing factors released by biofilms.

   Additionally, while this screen focuses on the neutrophil killing activity of spent media, performing a similar screen with direct neutrophil- biofilm contact (as opposed to spent media) would provide insight into the role of proteins that are activated by biofilms upon exposure to neutrophils. Previous studies show that the expression of LukAB is increased when planktonic *S. aureus* comes into contact with neutrophils. In Chapter 3, we show that biofilm bacteria also respond to direct neutrophil contact by expressing LukAB. The mechanisms that control this expression are yet to be understood. In conjunction with results obtained from this screen, performing a screen with the transposon library to identify mutants that show a decrease in neutrophil killing
activity when placed in contact with biofilms would provide additional insights into the mechanisms by which \textit{S. aureus} biofilms kill neutrophils.
### Table A.1 Transposon mutants showing a loss of neutrophil killing by biofilm spent media

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<tr>
<th>Category</th>
<th>Gene</th>
<th>Protein description</th>
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<td>SAUSA300_2035</td>
<td>kdpD  sensor histidine kinase, KdpD</td>
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<td></td>
<td>SAUSA300_0101</td>
<td>clfB  staphylococcal tandem lipoprotein clumping factor B</td>
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<td>SAUSA300_2565</td>
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<td><strong>2. Membrane associated</strong></td>
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<tr>
<td></td>
<td>SAUSA300_1334</td>
<td>cap5H  putative membrane protein</td>
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<td><strong>3. Metabolism</strong></td>
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<td>recF  DNA replication and repair protein</td>
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<td>scrB  sucrose-6-phosphate hydrolase</td>
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<td></td>
<td>SAUSA300_0008</td>
<td>hutH  histidine ammonia-lyase</td>
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<td>lysP  lysine specific permease</td>
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<td>SAUSA300_1714</td>
<td>ribE  riboflavin synthase alpha subunit</td>
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<td>SAUSA300_2277</td>
<td>hutL  imidazolepropionase</td>
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<td>SAUSA300_2631</td>
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<td>SAUSA300_1096</td>
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<td>deoC  transcriptional regulator, LysR family domain protein</td>
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<td></td>
<td>SAUSA300_1345</td>
<td>transcriptional regulator, DeoR family asparaginyl-tRNA synthetase</td>
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</table>
Materials and Methods

_Bacterial strains and growth conditions._ Transposon mutants used in these studies were obtained from the Network for Antimicrobial Research in *Staphylococcus aureus* at the University of Nebraska, Medical Center\(^{303}\). The presence of transposon insertions for these mutants was verified by plating onto TSA + 5 \(\mu\)g/mL erythromycin before use and performing PCR reactions with primers specific for the transposon\(^{303}\) as well as the respective genes (Table 2.2).

_Spent media collection._ To generate spent media for neutrophil killing assays, isolated colonies were used to inoculate 5 mL TSB and grown for 16-18 hours at 37°C under shaking (200 RPM) conditions. These were then allowed to grow to an optical density (OD at 600 nm) of 0.5-0.7, equivalent to an exponentially growing *S. aureus* culture. These cultures were then diluted to an OD\(_{600}\) of 0.1 and used to inoculate TSB either as planktonic (5 mL/15 mL test tube) or biofilm cultures (tissue culture treated 96-well plates, 200 \(\mu\)L/well)\(^{354}\). Planktonic (shaking/200 RPM) and biofilm (static) cultures were then allowed to grow for 24 hours, centrifuged and filter sterilized using a 0.22 \(\mu\)m filter.

_Neutrophil isolation._ Neutrophils were isolated from whole blood using previously published methods\(^{305}\).
**LIVE-DEAD killing assays.** Neutrophils were incubated with bacterial spent media for 30 minutes and stained with a 1:1 mixture (by volume) of Syto-9 (3.34 mM) and propidium iodide (20 mM) for 15 minutes (Thermo Fisher), after which the ratio of fluorescence generated by Syto-9 (Ex 485nm/ Em 525nm) and propidium iodide (Ex 525nm/ Em 630nm) was measured using a standard fluorometer. Numbers of neutrophils were calculated as a percentage of the positive (0.1% SDS treated) control (dead).