Defining *Staphylococcus aureus* Virulence Factors in Bacterial Pneumonia and Influenza Super-infection

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Staphylococcus aureus is an important pulmonary pathogen that can cause severe pneumonia and often complicates influenza infections. The emergence of methicillin-resistant *S. aureus* (MRSA) over the past few decades has made treatment more difficult in staphylococcal pneumonia and super-infection, due to antibiotic resistance and increased virulence compared to methicillin-sensitive strains. Understanding how this pathogen can invade and establish infection in the lung is important for the development of future therapeutics. To understand the bacterial genetic requirements in both infection contexts, I established methods for *S. aureus* transposon sequencing in the lung. Successful establishment of infection by bacterial pathogens requires adhesion to host components. Staphylococcal species express a broad range of cell wall-anchored proteins (CWAs) that are involved in adhesion to host cells, extracellular matrix proteins, and bacterial biofilms as well as nutrient acquisition and immune evasion. CWAs have known roles in upper respiratory tract colonization; however, their role in the lung is unknown. Thus, I screened several CWA family members in the context of *S. aureus* pneumonia and influenza, *S. aureus* super-infection. Lacking individual CWAs during bacterial pneumonia had variable effects on bacterial burden, immune infiltrate, and acute lung injury. However, influenza was the main driver of super-infection susceptibility regardless of CWA mutant. CWAs did influence inflammatory signaling

in both settings, suggesting that they maintain an impact on the immune system in the lung. To understand inflammation in the context of *S. aureus* pneumonia alone, I characterized the role of a cell membrane associated protein, second immunoglobulin-binding protein (Sbi). Sbi induced inflammation in the lung differently than the structurally related protein, staphylococcal protein A. Induction of inflammation was independent of Sbi antibody binding and may be due to its role in complement evasion. I then characterized a novel CWA, *S. aureus* surface protein D (SasD) in the context of pneumonia. Mice infected with a SasD mutant had decreased bacterial burden, inflammatory responses, and mortality compared to wildtype *S. aureus*. These data suggest that cell surface exposed proteins are important factors to investigate further in the context of *S. aureus* pulmonary infections and may be future therapeutic or vaccine targets.

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ACME: arginine catabolic mobile element

ADAM10: a disintegrin and metalloproteinase domain-containing protein 10

Agr: accessory gene regulator

AM: alveolar macrophage

AMP: antimicrobial peptide

C5aR: complement component C5a receptor

C5L2: C5a anaphylatoxin chemotactic receptor or complement component C5a receptor 2

CA-MRSA: community-associated MRSA

CAP: community-acquired pneumonia

CCN1: cellular communication network factor 1

CDC: centers for disease control and prevention

CFU: colony forming unit

CHIPs: chemotaxis inhibiting protein of *S. aureus*

CR: complement receptor

CWA: cell wall-anchored protein

DD1α: D-alanyl-alanine ligase

Eap: extracellular adherence protein

ECM: extracellular matrix

Efb: extracellular fibrinogen-binding protein

EGFR: epidermal growth factor receptor

Ehp: Efb homologous protein

Fab: fragment antigen binding region of immunoglobulin

Fc: fragment crystallizable region of immunoglobulin

FcR: Fc receptor

G-CSF: granulocyte colony-stimulating factor

HA-MRSA: hospital-associated MRSA

HAP: Hospital-acquired pneumonia

Hla: alpha-hemolysin, also known as alpha-toxin or α-toxin

Hlg: gamma-hemolysin or γ-hemolysin

IFN: interferon

IFNAR: IFNα receptor, also known as type I IFN receptor

IFNLR: interferon lambda receptor, also known as IL-28 receptor

IFNα: interferon alpha (type I IFN)

IFNβ: interferon beta (type I IFN)

IFNγ: interferon gamma (type II IFN)

IFNλ: interferon lambda (type III IFN)

Ig: Immunoglobulin

IL-: interleukin-

IRF: interferon-regulatory factor

JAK: Janus kinase

LTA: lipoteichoic acid

Luk: leukocidin

MAC: membrane attack complex

MARCO: macrophage receptor with collagenous structure

MGEs: mobile genetic elements

MPO: myeloperoxidase

MRSA: methicillin-resistant *S. aureus*

MSSA: methicillin-sensitive *S. aureus*

NET: neutrophil extracellular trap

NLRC4: NLR family CARD domain-containing protein 4

NLRP3: NOD-, LRR- and pyrin domain-containing protein 3

PAMPs: Pathogen-associated molecular patterns

PhnD: Phosphonate-ABC transporter periplasmic binding protein

PIA: polysaccharide intercellular adhesin

PRRs: PAMP recognition receptors

PSMs: Phenol-soluble modulins

PVL: Panton-Valentine Leukocidin

RNS: reactive nitrogen species

ROS: reactive oxygen species

Rot: repressor of toxins

Sae: *S. aureus* exoprotein expression or staphylococcal accessory element

SAK: staphylokinase

SCC*mec:* staphylococcal cassette chromosome *mec*

SCIN: staphylococcal complement inhibitor

ScpA: staphopain A

SEIX: staphylococcal enterotoxin-like toxin X

SEO: staphylococcal enterotoxin O

SplA: serine protease-like protein A

SR: scavenger receptor

SSTIs: skin and soft tissue infections

STAT: signal transducer and activator of transcription

STING: stimulator of interferon genes

TACE: tumor necrosis factor-alpha converting enzyme

Th1: type 1 immunity

Th17: type 17 immunity

Th2: type 2 immunity

TLR: toll-like receptor

TNFR1: tumor necrosis factor receptor 1

VAP: ventilator-associated pneumonia

vWbp: vWF binding protein

vWF: von Willebrand factor

WT: wildtype

β-toxin: beta-toxin, also known as beta-hemolysin

γδ T: gamma delta T cell

δ-toxin: delta-toxin

Preface

I would like to take the time to thank those who have helped me along my graduate career and made this work possible. First, I would like to thank my advisor Dr. John Alcorn for his support and trust in pursuing a microbiology focused project. This is a new direction for his laboratory and myself, and there were many stumbling blocks along the way. However, his belief in me (and his extraordinary letter of recommendation writing skills) helped me continue the pursuit of this project even when it appeared to not have any direction. Your grounding energy has been helpful on so many occasions when anxiety and stress got the best of me; reminding me to pursue hobbies outside of the lab has been greatly beneficial for my well-being. Thank you for giving me the room to grow as a scientist, speaker, and mentor throughout my time in your laboratory.

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Thank you all, from the bottom of my heart.

1.0 Introduction

1.1 Staphylococcus aureus

Staphylococcus aureus is a Gram-positive bacterium in the family *Staphylococcaceae*, characterized by its spherical shape and grape-like clusters[1]. *S. aureus* is one of the first pathogens described and continues to be a common pathogen today. It has the ability to cause a wide variety of infections, ranging from asymptomatic carriage to invasive diseases such as endocarditis, pneumonia, and bacteremia[2, 3].

S. aureus was first described in 1881 by Alexander Ogston as the sole microorganism isolated from abscesses capable of producing new abscesses when injected into rodents[4]. In 1882, Ogston coined the term *Staphylococcus* for the genus based on his microscopic observations[5]. In 1884, Friedrich Julius Rosenbach divided the genus into two species based on colony coloring, the yellow *S. aureus* ("aureus" golden) and white *S. albus* (now known as *S. epidermidis*)[6, 7].

Approximately 10-30% of the population is colonized with *S. aureus* on some part of their body[8, 9]. The primary reservoir for *S. aureus* is the anterior nares, with approximately 30% of individuals colonized at any given time, ranging from 10^4-10^5 CFU/ml in persistent colonizers[10, 11]. Nasal carriage is a significant risk for staphylococcal infection, with 50-80% of infecting isolates originating from the patient[12, 13]. While the nares are the most regarded site of colonization, *S. aureus* can also colonize the skin as well as the intestines[9]. Colonization of different body parts

is highly correlated within an individual, likely from frequent touching and nose picking and subsequent redistribution on the body[9].

1.1.1 Emergence of Methicillin-resistant S. aureus (MRSA)

S. aureus' ability to acquire antibiotic resistance has complicated efforts to control staphylococcal infections. Antibiotic resistance to penicillin occurred rapidly after it was introduced into clinical use in the mid 1940s due to penicillinase-containing strains[3, 14]. A penicillinase-resistant penicillin derivative, methicillin, was introduced in 1959 to counteract these strains, however, resistance was found within a year[14]. Known as methicillin-resistant *S. aureus* (MRSA), these strains contain the gene *mecA*, which encodes an alternative penicillin binding protein 2a (PBP2a) that is resistant to nearly all beta-lactam antibiotics[14, 15].

From the 1960s-1990s, MRSA strains were confined to hospitals and other health care centers in the United States[15]. Known collectively as hospital-associated MRSA (HA-MRSA), these strains became endemic in larger urban hospitals where affected patients were more susceptible to infection due to age or comorbid conditions[3, 14]. These strains tended to cause invasive infections such as pneumonia or bacteremia[15].

However, since the mid 1990s, MRSA has become increasingly prevalent in the community, leading to new community associated (CA-MRSA) strains[14, 15]. CA-MRSA strains tend to infect younger healthier patients as well as manifest predominantly as skin and soft tissue infections (SSTIs)[14]. HA- and CA-MRSA strains are typically distinguished molecularly, with the main difference being by staphylococcal cassette chromosome *mec* (SCC*mec*) type. HA-MRSA strains

carry relatively large SCC*mec* types, which may contain resistance to other antibiotics, while CA-MRSA strains contain smaller SCC*mec* types that can carry other virulence factors, such as Panton-Valentine leukocidin (PVL)[15]. Because CA-MRSA strains have the capacity to infect otherwise healthy younger individuals, it is thought that they have enhanced virulence compared to HA-MRSA strains[2, 14]. The CDC put forth a formal definition of CA-MRSA disease, which is contracted within 48 hours of hospital admission by patients not undergoing surgery, prolonged hospitalization, catheterization, or hemodialysis[16]. However, most MRSA infections are now acquired outside of hospital settings, and distinction between HA- and CA-MRSA strains is very blurred. Initially in the United States there were 8 distinct clusters based on pulsed-field gel electrophoresis (PFGE), designated USA100-800 with USA300 and USA400 being CA-MRSA strains[17]. The most prevalent MRSA strain in the United States is now USA300[2, 15, 18]. USA300 has also become increasingly common as an asymptomatic colonizing strain[15] and accounts for both CA-MRSA and HA-MRSA cases[2].

1.1.1.1 Genetic Differences of MRSA

Most of the genetic diversity of MRSA occurs within the accessory genome, where virulence mediators, immune evasion, and antibiotic resistance products are located[19]. The accessory components compromise ~25% of the *S. aureus* genome and consists of mobile genetic elements (MGEs) such as pathogenicity islands, chromosomal cassettes, transposons, plasmids, and bacteriophages, acquired by horizontal gene transfer between strains, which leads to a large variety in virulence expression depending on the strain[19]. This is one of the challenges in analyzing strains, as successful MRSA lineages will often differ from their predecessors at multiple loci[19].

Aside from methicillin-resistance, there are other differences between MRSA and methicillin-sensitive (MSSA) strains. For example, *mecA* is carried within a larger MGE known as SCC*mec,* which may carry other resistance or virulence genes[3, 15]. While the exact differences between MSSA and MRSA will vary depending on what strains are used in comparison, most of the MRSA characterization has occurred in USA300. Additional MGEs found in USA300 include the arginine catabolic mobile element (ACME), which may play a role in pathogenesis by helping MRSA colonize the skin, and PVL, a toxin with controversial function[15, 19]. MGEs can also include factors such as superantigens, proteases, lipoproteins, hemolysins, leukocidins, and phenol-soluble modulins (PSMs)[15, 19].

While MGEs play a predominant role in MRSA pathogenicity, there are also core genome differences that have been characterized. Most of these differences lead to increased expression of key virulence factors such as the alpha-toxin, PSMs, and proteases[2, 15, 19]. Differential expression of global gene regulators *Agr*, *SarA*, and SaeR*S* likely explain many of the differences in expression seen between MSSA and MRSA[2, 15, 19].

The most well studied, *Agr* (accessory gene regulator) is a peptide-based quorum sensing system that up-regulates many toxins and virulence factors when cell density reaches a certain threshold, allowing for the control of establishment versus virulence[20]. The *Agr* system regulates expression of virulence factors such as the alpha-toxin, delta-toxin, PSMs, and protein A[2, 9, 19]. USA300 is known to have a hyperactive *Agr* system, which likely influences the increased level of toxin expression seen in this strain[2]. Indeed, *Δagr* mutants are highly attenuated in several animal models of infection including sepsis, pneumonia and skin abscess models[2]. *S. aureus*

exoprotein expression (s*ae*) drives expression via a two-component system relying on a histidine kinase (SaeS) and response regulator (SaeR)[21]. This system regulates virulence factors such as coagulase, alpha-toxin, beta- and gamma-hemolysins, PVL, and nuclease[21]. SarA is known to regulate the *Agr* system as well as exert post-transcriptional regulation of targets by binding and altering mRNA turnover rates[21]. Additionally, SarA is a repressor of protease production, which can have several effects on phenotype such as biofilm formation and accumulation of virulence proteins.

1.2 Staphylococcal Cell-wall Anchored Proteins

S. aureus expresses surface proteins that are covalently attached to the cell wall, collectively known as the cell-wall anchored proteins (CWAs). These CWAs are crucial to the success of *S. aureus*, both as a pathogen and commensal. *S. aureus* can express up to 24 different CWAs, but the precise combination of CWAs depends on the strain and growth conditions[22]. Because of the limited repertoire of CWA proteins expressed, many of these proteins have evolved to perform several important interactions with the host. Several CWAs are multifunctional due to distinct domains binding different ligands or a single domain having several host ligands[22, 23, 24]. Additionally, many CWAs have evolved to have functional redundancy with other CWAs, making elucidation of mechanisms in these proteins difficult in null mutants[22].

As these proteins are exposed on the cell surface of *S. aureus*, these proteins are in direct contact with the host, and unsurprisingly have evolved to carry out functions related to colonization as well as evasion of the host immune system. Originally, all were defined as MSCRAMM

(Microbial Surface Component Recognizing Adhesive Matrix Molecules) proteins due to early characterization of those CWA proteins that bind host extracellular matrix (ECM), such as fibrinogen and fibronectin. However, not all CWAs bind ECM components and have other functions such as nutrient acquisition and immune evasion.

1.2.1 CWA Classification

All CWAs contain a secretory signal sequence at the N-terminus, directing the proteins to the secretory (Sec) apparatus located in the membrane[22, 25]. The Sec translocon is a complex formed in the membrane by SecY, SecE, and SecG. Protein translocation requires SecA, a cytoplasmic ATPase that pushes unfolded precursors through the translocon. SecDF, a chaperone powered by the proton motive force, assists in folding the translocated proteins. The signal peptide of the translocated precursor protein is cleaved by a signal peptidase (SpsB)[25].

After translocation, CWAs are covalently linked to peptidoglycan. The C-terminal end of CWAs contains a characteristic sorting signal motif, LPXTG (Leu-Pro-X-Thr-Gly; where X is any amino acid), which is recognized by the transpeptidase Sortase A (SrtA). SrtA cleaves between the threonyl (T) and glycyl (G) residues and forms a thioester bond between its active cysteine and the carboxyl group of the threonyl residue. Nucleophilic attack by the peptidoglycan intermediate (lipid II) leads to a surface protein-lipid II intermediate that is subsequently incorporated into peptidoglycan via penicillin binding proteins[22, 25].

CWAs can be classified into four subfamilies based on structural and functional motifs (Figure 1 and Table 1). These are the MSCRAMM, NEAT, three-helical bundle, and G5-E subfamilies and are described below.

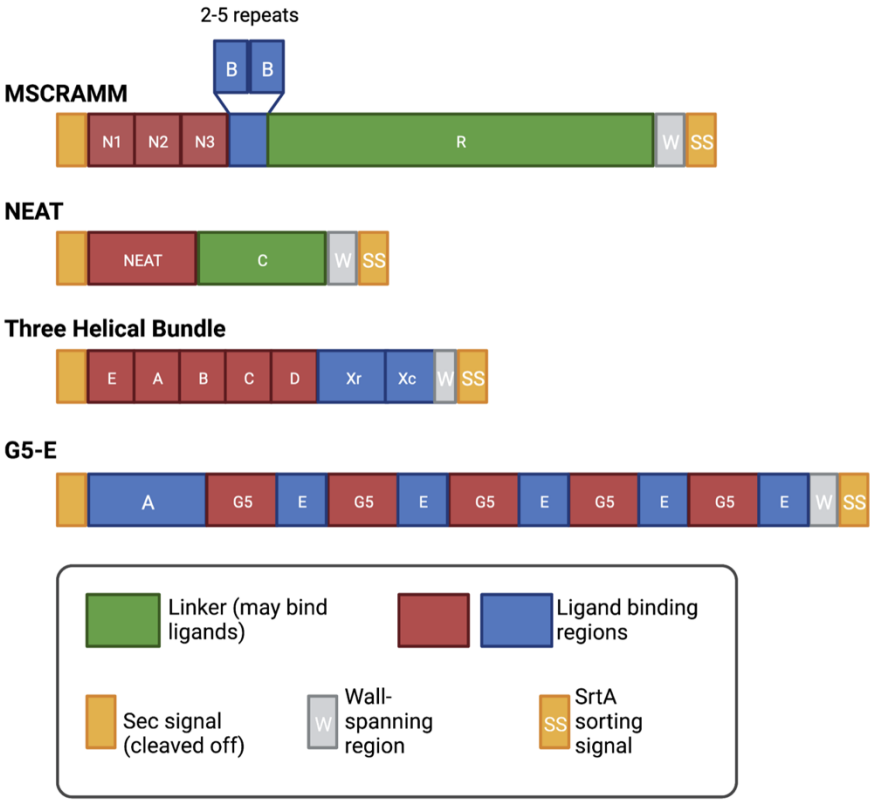

Figure 1: Structure of Cell Wall-anchored Protein Families

Staphylococcal CWAs are divided into four families based on structural motifs. These families follow the same translation product pattern with a Sec signal sequence at the N-terminus and a wall-spanning region (W) and sorting signal (SS) at the C-terminus. The MSCRAMM family is defined by tandemly linked IgG-like folded domains (N) known as the A domain. The R domain in made up of either fibronectin binding domains (FnBPs) or serine-aspartate repeats (Clfs +Sdrs). Sdr proteins also contain 2-5 B repeats located between the A and R domains. Cna contains an A domain and B domains but lacks the unstructured R domain. The NEAT (near-iron transporter) motif family contains the iron-regulated surface determinants (IsdABH). The NEAT domain has a similar structure to the Ig-like beta-sandwich. IsdA, IsdB, and IsdH, contain one, two, or three NEAT domains, respectively. The three-helical bundle motif family contains five tandemly linked triple-helical bundle domains (EABCD) that bind to IgG. This family also contains a variable length region (Xr) and a constant (Xc) region that contains the wall-spanning region (W). The G5-E repeat family contains alternating repeats of G5 and E domains. Member SasG contains an A domain, however, this is cleaved to facilitate biofilm formation. Figure made with Biorender.com.

1.2.1.1 The MSCRAMM family

The most prevalent and well-studied subfamily of CWAs is the MSCRAMM family (Table 1). This term previously applied to all CWAs that mediate binding to host ECM, but now is used to define those proteins that share structural similarities and common mechanisms for binding[22]. MSCRAMMs are also found in many other Gram-positive bacteria such as *S. epidermidis*, coagulase-negative staphylococci, enterococci, and streptococci[22, 23].

All MSCRAMM proteins contain at least two adjacent subdomains containing IgG-like folds in the N-terminal ligand binding A domain. The A region contains three separately folded subdomains: N1, N2, and N3, with N2 and N3 being IgG-like folds. The A domain is separated from the cell wall anchoring region by unstructured repeats that form flexible stalks, known as the R domain. The R domain is composed of either serine-aspartate dipeptide repeats (SD repeats) or ~29 residue fibronectin-binding repeats. These linker regions connect via proline-rich domain that spans from the cell wall to the peptidoglycan-binding sorting signal. Some MSCRAMM proteins (SdrC, SdrD, SdrE and Bbp) contain between two and five 110-113 residue B repeats, located between the A and R domains. These repeats are folded separately and form a rigid rod that projects the A domain away from the cell and are dependent on Ca^{2+} for structural integrity. Some B domains may have ligand-binding abilities or influence ligand binding of the A domain[23]. Another MSCRAMM protein, Cna, has a modified A domain, with N1 and N2 being the IgG-like folded subdomains. Cna also has B repeats, which lack sequence similarity to other family members, and lacks the unstructured R region[22, 23].

These proteins bind through the 'dock, lock and latch' (DLL) mechanism. Ligands can dock to the open apo form of the protein, leading to conformational changes that create a closed form, which locks ligands in place. The N2 and N3 domains are folded in a variant form of the IgG fold (known as DEv-IgG), which is composed to two beta sheets that contain nine individual beta strands. These two subdomains are arranged in a specific manner that creates a trench between N2 and N3, where ligands will dock. First, a ligand will bind ('dock') to the trench between N2 and N3 subdomains. This leads to a disordered portion of N3 binding the ligand and undergoing a conformational change to create two additional beta strands, one to 'lock' the ligand in place in the trench, and another to 'latch' to the N2 domain[22, 23]. This leads to strong ligand binding after DLL completion, where forces to separate the MSCRAMM protein and ligand are equivalent to those needed to break a covalent bond[26, 27]. Additionally, these bonds are potentiated by shear stress, allowing for bacteria to be tethered to surfaces in the presence of moving fluids, such as the blood stream[28]. Within the family, there are slight modifications to this binding mechanism, typically in the orientation of the N1 and N2 beta sheets, changing the shape of the ligand binding trench. Some MSCRAMM members can bind ligands only in their apo form, while others can bind ligands in both the apo and closed form, such as ClfA binding the C-terminus of fibrinogen[29]. Cna uses a modified DLL mechanism known as the 'collagen hug' where N1 and N2 are the IgG-like folds with a longer linker between the domains creating a hole rather than a trench to accommodate a collagen triple-helix rod[22, 23].

1.2.1.2 The NEAT motif family

Near iron transporter (NEAT) motif proteins are involved in heme capture from hemoglobin and help bacteria survive in the host, where iron is limited. NEAT domains are 125 residues and are conserved with neighbor putative iron transporters[30] and have a structure similar to the Ig-like

beta-sandwich fold[31]. Because the NEAT domain is tied to iron capture, these proteins are collectively known as iron-regulated surface (Isd) proteins[22]. However, the Isd family refers more to the genomic area in which these proteins are found, rather than being a CWA specific family; there are Isd proteins that are not attached to the cell wall via sortase A as well as members that are not found in the cell wall[30]. The three Isd family members that are canonical CWAs are IsdA, IsdB, and IsdH, which all contain the LPXTG motif and contain one, two, or three NEAT domains, respectively. IsdC is bound in the cell wall, but through a different transpeptidase, Sortase B, which recognizes NPTQN motif and is embedded in the cell wall and not surface exposed[30]. Each NEAT domain binds one heme molecule within a hydrophobic pocket. In most Isd family members, the binding and transfer of heme is unidirectional, eventually leading to heme binding to the membrane receptor, IsdE.

1.2.1.3 Three-helical Bundle family

This family only contains one CWA, staphylococcal protein A (SpA). SpA is a multifunctional protein that is ubiquitous in *S. aureus* and is often used in strain typing due to its variation in the Xr region[22, 23]. At the N-terminus, SpA contains five separately folded three helical bundles in tandem (EABCD), each of which can bind to several different ligands. Each three-helical bundle can exist in a slightly different conformation, allowing for plasticity needed to bind structurally diverse ligands. Located between the EABCD modules and the cell surface is the region Xr, which is composed of octapeptide repeats that are highly variable number, followed by a conserved region Xc, which contains the wall-spanning region[22].

While SpA is the only CWA in this family, there other staphylococcal secreted proteins that share similar structures. Second immunoglobulin-binding protein (Sbi) is non-covalently

associated with lipoteichoic acid (LTA) in the cell wall, contains four three-helical bundles, two of which are similar to protein A. Additional small, secreted proteins SCIN and Efb also contain single three-helical bundles[22].

1.2.1.4 The G5-E repeat family

This family contains G5 domains, which are characterized by five conserved glycine residues that adopt a β-triple helix-β-like fold. These G5 repeats are in tandem and are separated by 50 residue sequences known as E regions[22]. Both the G5 and E domains are comprised of two triple-stranded beta sheets with a central collagen-like helical region[24]. While a single G5 and E repeat will be intrinsically disordered and unfolded, $G5_1$-E-$G5_2$ moieties will fold into an elongated structure, where $G5_2$ triggers folding of the central E domain, which in turn will promote folding of $G5_1$ [32]. Proteins in this family form highly extended fibrillar structures and can have the ability to mask shorter adhesions, which may help control different outcomes such as adhesion, invasion, or biofilm formation[24].

1.2.2 CWAs as Virulence Factors

It is known that CWAs can have a variety of functions, such as host adhesion, self/biofilm adhesion, nutrient acquisition, and immune evasion. There are also several CWAs that have unknown functions, but likely play a role in at least one of those categories listed.

For testing, isogenic mutants along with complemented controls are tested for virulence in models of infection. However, functional redundancy can make it difficult to detect reductions in virulence based on deletion of one factor. To overcome this problem, single CWAs can be

expressed in surrogate hosts, such as *Lactococcus lactis* or *S. carnosus*[22]. Additionally, the background strain can have an influence on elucidating CWA functions, as differences in CWA repertoire or expression of global regulators such as *Agr* could affect phenotypes seen during infection. Furthermore, other differences between laboratory-adapted and clinical strains can also complicate function elucidation and many staphylococcal virulence factors are specific or preferential to human ligands.

Here the focus will be on the CWAs studied herein, listed in by alphabetical order in each subfamily. For summary of CWA functions see Figure 2. See Table 1 for more details on CWAs not covered here as well as reviews[22, 23, 24, 33, 34, 35].

1.2.2.1 MSCRAMM

ClfA. ClfA was first characterized as a fibrinogen binding protein and was termed 'clumping factor' due to mutants failing to form clumps in soluble fibrinogen or adhere to immobilized fibrinogen[36]. It was later discovered that this binding occurred at the C-terminal end of the fibrinogen γ chain[37, 38] through the DLL mechanism[29].

ClfA is a major virulence factor in bacteremia, sepsis, and infective endocarditis[39, 40, 41, 42, 43]. ClfA has been characterized to bind to human platelets[44] later attributed to ClfA's ability to bind fibrinogen, which can then bind the integrin complex GPIIb/IIIa (also known as $\alpha_{IIb}\beta_3$) on platelets[45]. This can lead to platelet activation and aggregation with additional signals through complement (IgG + FcγRIIa or complement + CR)[46]. Additionally, ClfA mediates adhesion to endothelial cells through a similar mechanism with integrin $\alpha_V\beta_3$, leading to endothelial apoptosis and increased permeability of vessels[47]. Damage to the endothelium will trigger release of von

Willebrand factor (vWF), which can also be exploited as an attachment site. ClfA binds the secreted bacterial factor vWbp (vWF binding protein), which in turn will bind vWF and anchor *S. aureus* to the vascular endothelium under shear stress[48]. As ClfA promotes bacterial survival in the blood, this allows for more opportunity to disseminate to other organs, where ClfA also plays a role such as the kidneys[49], joints[50], bones[51] and skin[52].

Additionally, ClfA has also been shown to inhibit phagocytosis via fibrinogen-dependent and -independent mechanisms[53]. ClfA binds complement factor I in a fibrinogen-dependent manner[54], acting as a cofactor for factor I, leading to increased cleavage of C3b into iC3b and reduced deposition of C3b on the bacterial surface[55]. It was also recently discovered that ClfA can also bind complement factor H independent of fibrinogen, also contributing to preventing opsonization[56].

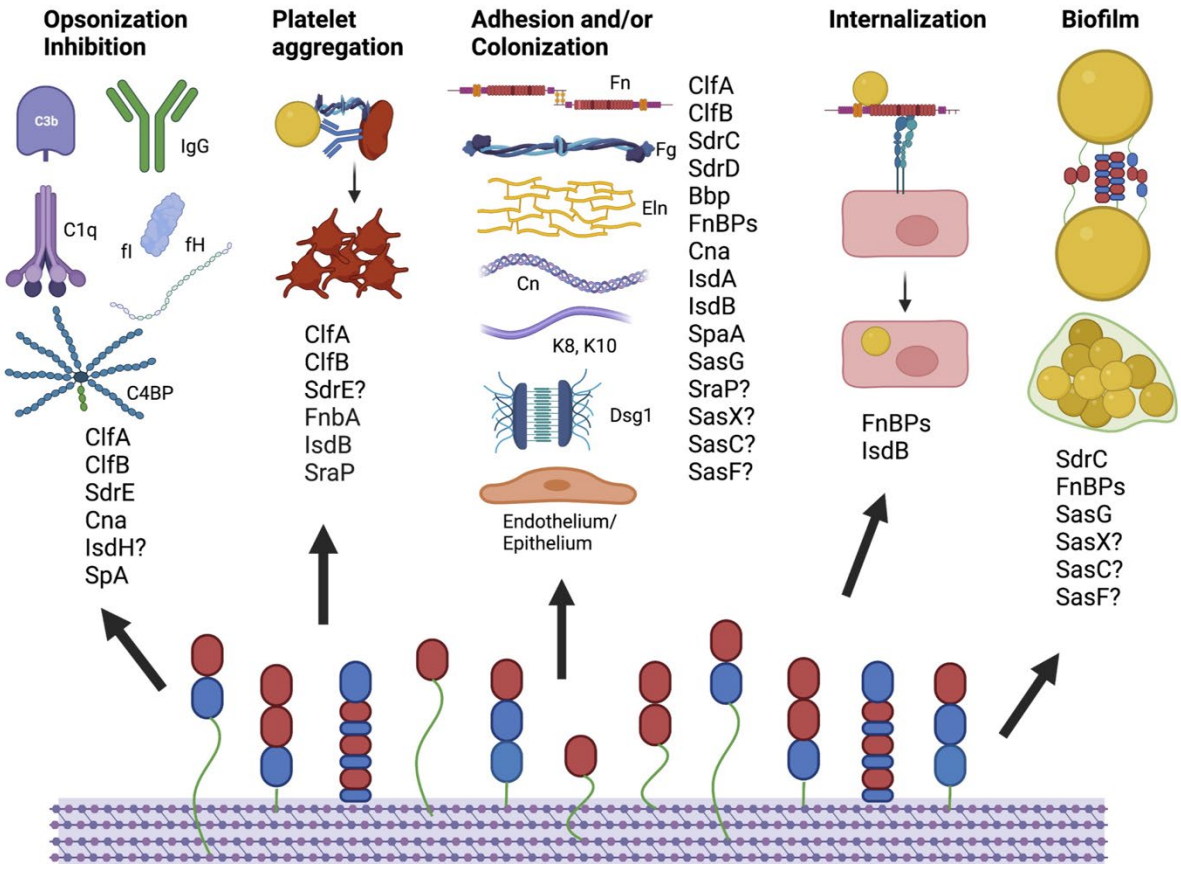

Figure 2: Main Roles of Cell Wall-anchored Proteins

Staphylococcal CWAs can perform many various functions that can be divided into five main categories: opsonization inhibition, platelet aggregation, adhesion and/or colonization, internalization into nonprofessional phagocytes, and biofilm. Opsonization inhibition primarily occurs through binding and inhabiting various components of the complement system or binding the Fc region of IgG. Platelet aggregation requires both binding and a secondary signal (complement or IgG) to induce activation of platelets and the formation of a clot. The main role of CWAs is adhesion to host components or epithelial or endothelial cells. Some CWAs can also facilitate binding and internalization into nonprofessional phagocytes, which is dependent on integrins. CWAs can also promote biofilm formation through homophilic interactions on nearby cells or through unknown mechanisms. ? Denotes a CWA that is presumed to have this function and/or it has not been directly tested. Abbreviations: C3b: complement component C3b, IgG: immunoglobulin G, fI: Factor I, fH: Factor H, C4BP: complement C4b binding protien, Fn: fibronectin, Fg: fibrinogen, Eln: elastin, Cn: collagen, K8: keratin 8, K10: keratin 10, Dsg1: desmoglein 1. Figure made with Biorender.com.

ClfB. ClfB was discovered after ClfA due to its ability to also bind Southern Blot probes specific to the dipeptide repeat R domain and mediates similar clumping in soluble fibrinogen[57, 58, 59]. ClfB can also bind fibrinogen but binds the fifth repeat of the α chain[60]. ClfB can also bind and aggregate platelets similar to ClfA but with a longer lag time[61]. However, the A domain of ClfB is cleaved by the metalloprotease aureolysin, resulting in loss of binding at the end of exponential growth[59]. Similar to ClfA, ClfB is implicated in endocarditis and kidney abscesses[49, 62].

ClfB's major function appears to be related to adhesion and colonization of the nose[63, 64, 65, 66]. Immunization with ClfB leads to reduced nasal colonization in mice[67] and a mutant (Δ*clfB*) strain leads to reduced experimental colonization in humans[64]. ClfB was first characterized to facilitate binding to human desquamated stratified squamous nasal epithelial cells through a type I cytokeratin, keratin 10 (K10)[68]. The binding site was later found to be in the glycine loop region of the C-terminus tail of K10[69]. An additional ligand found was loricrin, an abundant protein that makes up the cornified envelope of squamous epithelial cells. This is presumed to be the major ligand for nasal colonization by ClfB due to the abundance in the nose, and that lacking loricrin leads to an 80% reduction in colonization[63]. ClfB can also bind a type II cytokeratin, keratin 8 (K8), at the tail region similar to K10[70]. However, K8 is not found in the skin and is expressed in simple epithelium[71], but the relevance of this has not been investigated.

ClfB has also been implicated in colonization and infection of the skin. *S. aureus* strains associated with patients of Atopic Dermatitis (AD) bind ClfB ligand loricrin more strongly than those from healthy controls[65]. Binding of ClfB binding in AD is also influenced by the level of

NMF (natural moisturizing factor) found, potentially due to altered cell surface morphology which could mask ClfB ligands[66]. ClfB plays a critical role in initial abscess formation through the ligand loricrin. Loricrin is expressed within the wall of a skin abscess, facilitating the binding of ClfB, and in turn promoting the development of an abscess[72].

FnbBPs. Binding to fibronectin was one of the first adhesion phenotypes described in *S. aureus*[73, 74] and was initially thought to be mediated through one protein, named fibronectin-binding protein[75, 76]. *S. aureus* was found to bind the N-terminal region as well as heparin-binding domain of fibronectin[77]. This protein was renamed fibronectin-binding protein A (FnbA) after the discovery of a second fibronectin-binding protein, FnbB[78]. These two proteins (collectively known as fibronectin binding proteins, FnBPs) share high homology with each other and are both found in 60-70% of *S. aureus* strains[79]. Protein expression on surface is inversely correlated with protease V8 levels (more V8, less FnBPs)[79]. Due to their similarity, these two proteins have many overlapping ligands and are described together below.

One of the more interesting roles of the FnBPs is the invasion into nonphagocytic cells[35, 80, 81]. This is a passive process on the side of the bacterium and requires binding the integrin $\alpha_5\beta_1$ with fibronectin acting as a bridge[80]. On the host side, it requires activation of signal transduction pathways as well as host cell cytoskeleton involvement[82]. This phenomenon can occur with either FnBP, and expression of only one is required for invasion to occur. Internalization into nonphagocytic cells has been seen for human endothelial cells[83], bovine cell lines[84, 85], murine fibroblasts[86], human keratinocytes[87], and lung epithelial cells[88, 89]. This uptake into cells may provide a safe harbor for the bacteria away from antimicrobials and immune cells as well as help

explain recurrent infections[35, 81]. Indeed, small colony variants express FnBPs at high levels, which may help facilitate their uptake into surrounding cells when they are released[90].

In addition to fibronectin binding, FnBPs can also bind fibrinogen and elastin using their A domain[91, 92, 93, 94, 95]. Binding to fibrinogen occurs at the same site as ClfA, the C-terminal region of the γ chain[91, 92]. Elastin binding uses the same region of the A domain as fibrinogen, although competitive binding between fibrinogen and elastin may depend on the specific isoform used[94, 95]. FnBPs can also bind plasminogen in a different region of the A domain and these proteins can bind both fibrinogen and plasminogen at the same time[96]. This was shown to be a combination of strong fibrinogen-plasminogen interactions and weak plasminogen-FnBP interactions[97]. This likely helps to contribute to activation of the zymogen, leading to reduced opsonization, dissemination into tissues, and/or biofilm formation[96, 97]. FnBPs are also implicated in biofilm formation in HA-MRSA strains, potentially due to differences in expression between MSSA and MRSA strains[98, 99]. FnBPs also have the ability to form homophilic interactions between A domains on adjacent cells similar to SdrC[100]. Recently, FnbB was described as the receptor for H3 histones[101]. Histones are a component of neutrophil extracellular traps (NETs) and H3 is known to have anti-staphylococcal activity[102]. This study showed that FnbB binds H3 likely for 2 main reasons: 1) to prevent histone interaction with the bacterial membrane, and 2) to promote the degradation through its ability to bind and activate plasminogen[101]. FnBPs have been implicated in many infections such as endocarditis[41, 103], indwelling medical device/foreign body infections[104, 105, 106, 107], and skin infections[108]. However, FnbB is more associated with invasive infections such as endocarditis or osteomyelitis compared to FnbA[109].

SdrC. Similar to the discovery of ClfB, the Sdr (Serine Aspartate repeat) family members (SdrC, SdrD, SdrE) were discovered by Southern Blot probe binding to the R domain composed of SD (serine aspartate) repeats[110]. These proteins carried additional repeated B motifs, which are not present in ClfA and ClfB. SdrC is found in all *S. aureus* strains and containing only SdrC is correlated with MSSA strains[111, 112]. SdrC has been implicated in nasal colonization, as it promotes adhesion to human desquamated nasal epithelial cells[111] but the mechanism is unknown.

SdrC's role appears to be primarily through mediating adhesion. The first ligand of SdrC was discovered to be β-neurexin 1β through a phage display library[113]. As this protein is expressed in neuronal cells, it is unclear how this ligand interaction facilitates colonization or infection. SdrC has also been shown to promote homophilic interactions with additional SdrC proteins[114]. The N2 domain of SdrC contains a region that promotes self-dimerization with another SdrC protein, promoting biofilm expansion. This SdrC-SdrC interaction is weak and low affinity[115] and authors suggest that this may favor detachment and dissemination. Recently, homophilic interactions were discovered to be mediated by metal chelating residues binding Ca^{2+} between two N2 domains[116]. Additionally, SdrC also engages in hydrophobic interactions with abiotic surfaces, which may be due to the protein's high percentage of hydrophobic residues[115]. These interactions are stronger than the self-dimerization, and likely contribute to initiation of biofilms on abiotic surfaces. Interestingly, using a peptide of β-neurexin 1β blocks adhesion to abiotic surfaces as well as biofilm formation[115].

SdrD. Unlike SdrC, SdrD is associated with MRSA strains[112]. SdrD has also been implicated in nasal colonization by binding to desquamated nasal epithelial cells[117]. SdrD can facilitate

binding to human keratinocytes through its interaction with desmoglein 1 (Dsg1)[118]. Dsg1 is a cadherin found in intercellular junctions known as desmosomes which are abundant in tissues exposed to mechanical stress (i.e. skin or heart)[119]. However, it is unclear if this interaction promotes nasal colonization as well.

SdrD likely contributes to virulence *in vivo,* however, those mechanisms are not clearly defined. Expression of *sdrD* is upregulated during *ex vivo* incubation with blood[120], likely due to interactions with neutrophils[121]. SdrD promotes survival in whole blood independent of C3b deposition or phagocytosis, which may or may not be related to its ability to promote neutrophil lysis[121]. The presence of the *sdrD* gene has been implicated in bone infections[112, 122] and SdrD has been shown to play a role in systemic infections and abscesses[49, 121].

SdrE. SdrE induces platelet aggregation but with a longer lag time than ClfA and ClfB[45]. This interaction was dependent on plasma and the authors suggest that SdrE may be using a plasma protein as a bridge to platelets. It was later discovered that SdrE can bind to complement components Factor H and C4b-binding protein (C4BP)[123, 124], however, it is unclear if either was mediating the aggregation of platelets. Factor H bound to SdrE is still active and can act as a cofactor for Factor I-mediated cleavage of C3b, resulting in less C3b deposition, C5a generation, and decreased opsonization[123]. C4BP is recruited to the cell surface of *S. aureus*, increasing the degradation of C3 classical convertase, cleavage of C4b, and decreased opsonization[124]. SdrE can recruit both Factor H and C4BP at the same time without competitive binding, suggesting that complement evasion may be the main function of this protein. The presence of SdrE is correlated

with invasiveness[111] and has been implicated in abscesses[49]. Use of SdrE as a vaccine candidate had better results than other CWAs IsdA, IsdB, and SdrD[125].

An isoform of SdrE, known as bone sialoprotein-binding protein (Bbp), was discovered before the other Sdr family members through its ability to bind bone sialoprotein, an ECM component of bone[126, 127]. *S. aureus* strains will express neither or one of these isoforms[128]. *S. aureus* isolates from osteomyelitis express Bbp and patients who have suffered a bone or joint infection with *S. aureus* had anti-Bbp antibodies[109, 127, 129]. Despite this, only SdrE has been shown to be correlated with invasiveness of a strain[111]. Bbp is also known to bind human fibrinogen α chain[130] as well as C4BP[124].

1.2.2.2 NEAT

IsdB. After the discovery of IsdA (originally called staphylococcal transferrin-binding protein A, StbA)[131], additional iron-regulated genes were discovered in a locus that is regulated by iron, including IsdB[132, 133]. IsdB was found to bind hemoglobin and is required for *S. aureus* to use hemoglobin as the sole iron source[132, 133]. IsdB preferentially binds human hemoglobin but can bind murine hemoglobin as well[49, 134, 135]. IsdB had been implicated in organ abscesses after systemic infection[49, 135]. Patients who have *S. aureus* endocarditis also contain anti-IsdB antibodies[136].

IsdB can also bind to several other ligands, similarly to IsdA (Table 1). IsdB can bind to the platelet integrin GPIIb/IIIa and promote aggregation[137]. Unlike other CWAs that can bind GPIIb/IIIa, this interaction does not require a fibrinogen bridge nor IgG-FcγRIIa binding, and IsdB binds directly with high affinity. This interaction requires the high-affinity conformation of the

integrin and occurs at a different site than hemoglobin binding[138]. IsdB can also bind to human cells that express other β3 integrins such as 293T, HeLa, and breast cancer epithelial (MCF-7) cells[138]. IsdB can also mediate invasion into 293T and HeLa cells and plays a role even when FnBPs are present. However, since IsdB is iron regulated, this only occurs during iron-limiting conditions[138]. Binding to $\alpha_V\beta_3$ integrin was shown to be increased under mechanical tension[139]. Recently, IsdB was discovered to bind vitronectin, a plasma and ECM protein[140]. This is thought to be an additional mechanism for binding β3 containing integrins, as IsdB can mediate adhesion and internalization with or without vitronectin. IsdB can also bind vWF directly at a different site as vitronectin [136]. This promotes binding to endothelial cells and the interaction is likely stronger under shear stress.

1.2.2.3 Three Helical Bundle

Sbi[1]. Initially, by Western Blot it was difficult to differentiate Sbi from SpA due to their similar size, overlapping IgG specificity, and cross-reactivity with commercial anti-SpA antibodies; due to this, it was originally thought to be a breakdown product of SpA[141]. This explains some of the early reports of *spa*-negative strains only being slightly less virulent[141, 142]. Some groups found Sbi in the supernatant and not the cell-wall fraction[141, 143], but others found it both in the supernatant and associated with the cell membrane potentially through its interaction with lipoteichoic acid[144]. These authors[144] showed that both the soluble and cell-wall associated forms contributed to survival of *S. aureus* but perform different roles: the IgG-binding domains are only protective when anchored to the cell surface while the complement binding domains are only

[1] While Sbi is not a CWA, it was studied herein and will be covered.

protective when secreted. This was later confirmed[145] and that when surface bound, only the IgG binding domains are exposed, explaining this phenomenon.

Sbi was discovered as a second IgG binding protein via shotgun phage display looking for bacterial ligand binding regions to bovine calf serum[146]. This protein differs from SpA in that it only has two functional IgG-binding domains (domains I and II), can only bind IgG, and lacks the cell-wall anchoring sequence, but is still wall-associated [141, 143, 147]. Expression of Sbi in a number of strains is low in bacterial media but increases in the presence of human and mouse serum[142, 148, 149]. Using phage display, it was also discovered that Sbi could bind other components of the serum[146]. This was followed up and it was discovered that Sbi can also bind β_2-glycoprotien I (β_2-GPI; also known as apolipoprotein H)[142]. This binding occurred outside of the IgG binding region of the protein. Similar to SpA, Sbi is also able to induce inflammatory cytokines through its IgG binding domains, which interact with the TNFα receptor (TNFR1) and epidermal growth factor receptor (EGFR)[149]. This likely occurs in the same region (IgG binding domains) of Sbi as SpA, however, in this study, all four domains were used.

It was later discovered that Sbi also has two domains (III and IV) involved in complement binding [143]. Sbi binds to complement component C3 and preincubation of Sbi with human serum leads to inhibition of all three complement pathways[143]. The Sbi fragment containing domains III and IV specifically binds to C3 in the sub-fragment regions C3dg and C3a. The fourth domain can inhibit complement by 2 mechanisms: 1) independently inhibiting the alternative pathway or 2) when linked to the 3rd domain, domain IV induces futile consumption of C3 via fluid phase activation. The IV fragment was later shown to have a similar structure to other staphylococcal

complement inhibitors Efb, Ehp, and SCIN[150]. The third domain is unfolded and may act as an acceptor for the C3d thioester bond. The proposed model[143, 150] is that domain III is the site for assembly of the alternative pathway C3 convertase that is transiently resistant to activation by Factor H and Factor I. The Fc binding regions of Sbi may also be involved by recruiting IgG molecules that are also targets for C3b deposition. Any C3 convertases that form on IgG are more protected from inactivation by Factor H and Factor I, leading to more consumption of C3 and subsequently reduces opsonization of the staphylococcal surface. Sbi can also bind Factor H and FHR (Factor H related protein 1) in human serum[151]. Factor H can bind Sbi, but binding is enhanced when C3, C3b, or C3d is present, and what C3 form is present dictates its stability. The authors propose a model where C3 binding to Sbi leads to a conformational change in C3 which exposes additional binding sites. This forms a tripartite complex where Factor H keeps functionality and may increase the amount of complement inhibition occurring at the site of infection. It could be argued that the original characterization of domains III-IV inhibition of the alternative pathway[143, 150] could be due to this regions ability to bind Factor H. Sbi can also bind C3/C3b simultaneously with plasminogen to enhance cleavage of C3/C3b[152]. Plasminogen is activated to plasmin either by staphylokinase or a host kinase to plasmin, which can degrade C3/C3b bound to Sbi. Plasminogen binding is mediated by domains III-IV, which can also degrade C3a. Sbi virulence has been implicated in skin abscesses and atopic dermatitis and likely occurs through its TNFR1 and complement binding mechanisms, although this has not been directly shown[153, 154, 155].

1.2.2.4 G5-E

SasG. SasG was discovered in 2003 by an *in silico* analysis of *S. aureus* genomes searching for CWAs[156]. The sequence and structure of SasG is very similar to another *S. aureus* protein, Pls, which is only found in MRSA strains[157]. Patients who have recovered from *S. aureus* infections have antibodies reactive to SasG, and the presence of SasG is correlated with invasive strains versus nasal carriage isolates[156]. SasG is also present in most clinical isolates from humans regardless of subtypes of *Agr*, coagulase and capsules[158].

Through its N-terminal A domain, SasG promotes adhesion to desquamated nasal epithelial cells but not buccal or non-differentiated keratinocytes[159]. SasG binding was inhibited by Pls and Aap, a related protein found in *S. epidermidis*, suggesting that these proteins share a binding site. SasG was also shown to reduce adherence to fibrinogen and fibronectin but promote bacterial aggregation. As Pls is thought to be a masking protein to prevent other CWAs from binding their ligands[157], SasG could have a similar role. This was later confirmed[160] and SasG was shown to mask the ability of several other CWAs (SpA, ClfB, and FnBPs) to bind to their ligands. While SasG is able to mask ClfB's ability to bind K10, an important ligand in the nose, adherence is still maintained though SasG's adhesive properties. SasG has a fibrillar structure and is expressed densely across the surface of the cell as peritrichous fibrils[160], which explains its ability to mask adhesion of other CWAs. The number of repeats contained in SasG affects the ability of this protein to mask other CWAs' function.

SasG's G5 domain structure has been implicated in biofilm formation[161] and cleavage of SasG after the A domain does promote protein-based biofilms in *S. aureus* independent of PIA[160]. It was

later discovered that the G5-E domain is responsible for biofilm formation and that it is dependent on Zn^{2+} binding[162, 163]. These domains engage in zinc dependent homophilic bonds across the stretched protein, which have been called a "zinc zipper". Five or more repeats are necessary for biofilm formation[160], likely helping to contribute to the strength of self-association as well as withstanding shear forces. SasG's role in biofilm formation occurs early during the accumulation phase, but not primary attachment. The proposed model is cell-wall attached SasG is cleaved (either spontaneously or through an inhibitor resistant protease) within the repeat region leading to fragments of different lengths being released into the supernatant. Some of these fragments will reattach to newly exposed repeats in a Zn^{2+} dependent manner. Cleaved and exposed SasG B domains on neighboring cells can interact in a Zn^{2+} dependent manner, leading to cell accumulation and biofilm formation[164]. A second mechanism of zinc promoting biofilms through SasG is zinc will induce collapsing of other cell surface components, leading to an even greater projection of SasG on the surface to interact with SasG on other cells[163]. Interestingly, since SasG and Aap of *S. epidermidis* are very similar, they can promote interspecies biofilms[163].

1.2.2.5 Unknown

SasD. SasD was also discovered in 2003 by an *in silico* analysis of *S. aureus* genomes searching for CWAs[156]. A total of 10 LPXTG proteins were identified and were named using the convention *S. aureus* surface (Sas) protein. SasD was found to be present in all six genomes analyzed and does not appear to have any repeat domains like many other CWA proteins. SasD, along with SasF, have a modified LPXTG motif (LPXAG), but this does not appear to alter cell-wall association based on analysis of SasF in cell-wall fractions[156]. Another study looking at the cell wall distribution of SasD found it expressed as discrete puncta versus the canonical ring-like expression of most CWAs (along the cell wall division plane)[165]. The difference in distribution

was attributed to the absence of the YSIRK/GS signal motif and further explored with SasF. SasF (and SasD) do not contain this motif and are found in discrete puncta near the cell pole. However, when the motif is expressed in a complemented SasF, expression is now seen as ring-like. How this difference in distribution affects function and or virulence is unknown.

In a study looking at proteomic and transcriptomic regulation of CWAs, trypsin-cleaved SasD peptides representing 39% coverage did not map to any other CWAs, suggesting that it does not contain repetitive domains with other members, unlike the Sdr family[166]. SasD was shown to have bell-shape *agr*-like expression, with protein expression peaking at OD_{600nm}=1.8 in tryptic soy broth (TSB) and expression was very low in the Newman *agr*- strain. Expression was increased in RPMI media, both in wildtype (WT) and *agr*- strains, which the authors contribute to iron regulation, although this increase in expression is ~15% of IsdB's increase at the same OD. Transcriptomic analysis shows that SasD mRNA peaks at OD=2.2 and is increased ~2-fold in the *agr*- strain, suggesting differences in regulation.

SasD is one of the proteins used in *sas* sequence typing of *S. aureus*[167, 168]. In a study looking at genetic evolution of MRSA strains, SasD was found to have variation levels similar to housekeeping genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL*)[168]. There were 7 alleles found containing 11 polymorphic sites found in 147 MSSA and MRSA isolates. In a study looking at clonal diversity in animal-associated *S. aureus* strains, SasD was found to have 13 alleles containing 16 polymorphic sites in 118 strains[167]. This discrepancy could be due to the difference in clonal types that colonize/infect human and animals. In another study it was found that this protein appears to have little sequence variation based on comparison of 58 *S. aureus* isolates from

various lineages (ST5, CC5, ST8, CC8, ST30, CC30)[169]. It was also found in 55/58 strains tested and was missing in 2 human- and 1 bovine-derived strains.

Table 1: Ligands and Binding Mechanisms of Staphylococcal CWAs

Protein[a] (Abbreviation)	Ligand[b]	Binding mechanism[b]	Function[b]	References
MSCRAMM Family				
Clumping factor A (ClfA)	Fibrinogen γ chain C-terminus	DLL	Adhesion; Immune Evasion	[29, 37, 38]
	Fibrinogen γ-globular domain of the D region	Unknown; Monoclonal antibody tefibazumab (anti-ClfA) blocks binding to this site	Adhesion?; Immune Evasion?	[170]
	Human platelets via GPIIb/IIIa	Through fibrinogen	Platelet binding	[44, 45]
	GPIIb/IIIa AND IgG+ FcγRIIa OR Complement opsonin + CR	Fibrinogen + complement binding	Platelet activation and aggregation	[46]

Table 1 Continued

Clumping factor A (ClfA) cont.	Integrin $\alpha_V\beta_3$	Through fibrinogen	Adhesion to endothelial cells	[47]
	Complement Factor I	Fibrinogen binding leads to conformational change allowing Factor I to bind and be active	Acts as cofactor, increased degradation of C3b to iC3b and less C3b binding	[54, 55]
	Complement Factor H	Unknown (likely DLL?)	C3 convertase degradation, Increased C3b degradation, reduced opsonization	[56]
	Von Willebrand factor binding protein (vWbp)	ClfA binds vWbp through DLL, which in turn will bind vWF	Adhesion	[48, 171]
	Annexin 2	Unknown	Bovine mammary epithelial cell adhesion and invasion.	[172]
Clumping factor B (ClfB)	Fibrinogen α chain repeat 5	DLL	Adhesion	[60, 173]
	Keratin 10 Tail region	DLL	Nasal colonization	[68, 69, 173]

Table 1 Continued

Clumping factor B (ClfB) cont.	Loricrin, multiple locations	DLL	Nasal colonization; Adherence to Atopic Dermatitis corneocytes; Skin abscesses	[63, 65, 72]
	Keratin 8	Presumed DLL?	Adhesion?	[70]
	Platelets (through (GPIIb/IIIa and IgG+ FcγRIIa ?)	Through fibrinogen	Platelet aggregation	[61]
	Annexin 2	Unknown	Bovine mammary epithelial cell adhesion and invasion.	[174]
Serine-aspartate repeat protein C (SdrC)	Desquamated nasal epithelium	Unknown	Nasal colonization	[117]
	β-neurexin 1β N-terminus	DLL	Adhesion?	[113]

Table 1 Continued

Serine-aspartate repeat protein C (SdrC) cont.	SdrC homophilic interactions	Ca^{2+} dependent co-binding of N2 domains	Biofilm formation	[114, 115, 116]
	Abiotic surfaces	Through hydrophobic residues?	Biofilm formation	[115]
Serine-aspartate repeat protein D (SdrD)	Desquamated nasal epithelium	Unknown	Nasal colonization	[117]
	Desmoglein 1	Unknown	Binding to keratinocytes	[118]
Serine-aspartate repeat protein E (SdrE)	Factor H	Modified DLL mechanism	Increased degradation of C3 convertases, reduced opsonization	[56, 123, 175]
	Unknown	Binds in A region of SdrE	Platelet aggregation	[45]
	C4b-binding protein (C4BP)	Unknown	Degradation of classical C3 convertase, C4b degradation, reduced opsonization	[124, 176]

Table 1 Continued

Bone sialoprotein-binding protein (Bbp) *(isoform of SdrE)*	Bone sialoprotein	Unknown	Role in bone infections?	[126]
	C4b-binding protein (C4BP)	Unknown	Degradation of classical C3 convertase, C4b degradation, reduced opsonization	[124]
	Fibrinogen α-chain	DLL	Adhesion	[130, 177]
Fibronectin-binding protein A (FnbA)	Fibronectin	Fibronectin binding repeats, tandem β-zipper	Adhesion	[83, 178, 179]
	Integrin $\alpha_5\beta_1$	Bridge through fibronectin	Invasion of nonphagocytic cells	[80, 82]
	Fibrinogen γ chain C-terminus	DLL	Adhesion	[91, 93]
	Elastin	DLL	Adhesion	[94, 95]

Table 1 Continued

Fibronectin-binding protein A (FnbA) cont.	Plasminogen	N3 domain of FnbA; Binding to Fibrinogen leads to confirmational change, allowing binding to plasminogen	Activation of plasminogen; Immune evasion	[96, 97]
	FnbA homophilic interactions	FnbA A domain, Zn^{2+} dependent	Biofilm	[98, 99, 100]
	Platelets (through (GPIIb/IIIa and IgG+ FcγRIIa ?)	Through fibrinogen	Platelet aggregation	[180]
Fibronectin-binding protein B (FnbB)	Fibronectin	Fibronectin binding repeats, tandem β-zipper	Adhesion	[83, 178, 179]
	Integrin $\alpha_5\beta_1$	Bridge through fibronectin	Invasion of nonphagocytic cells	[80, 82]
	Fibrinogen γ chain C-terminus	DLL	Adhesion	[92]
	Elastin	DLL	Adhesion	[94]

Table 1 Continued

Fibronectin-binding protein B (FnbB) cont.	Histone H3	DLL	Prevent membrane damage; increase degradation; immune evasion	[101]
	Plasminogen	N3 domain of FnbB; Binding to Fibrinogen leads to confirmational change, allowing binding to plasminogen	Activation of plasminogen; Immune evasion	[96, 97]
	FnbB homophilic interactions?	FnbB A domain?; Zn^{2+} dependent	Biofilm	[99]
Collagen adhesion (Cna)	Collagen triple helix	Collagen hug	Adhesion	[181, 182]
	Complement C1q	Modified Collagen hug	Inhibition of Classical complement pathway	[183, 184]
	Laminin	Modified Collagen hug	Adhesion to basement membrane	[184, 185]

Table 1 Continued

NEAT Family				
Iron-regulated surface protein A (IsdA) *(aka StbA or SasE)*	Heme	NEAT domain	Heme uptake, iron acquisition	[133, 186, 187]
	Fibrinogen β and γ chains	NEAT domain	Adhesion; nasal colonization	[188, 189]
	Fibronectin	NEAT domain	Adhesion; nasal colonization	[188, 189]
	Keratin 10	NEAT domain	Adhesion; nasal colonization	[190]
	Loricrin	NEAT domain	Adhesion; nasal colonization	[190]
	Involucrin	NEAT domain	Adhesion; nasal colonization	[190]
	Desquamated epithelial cells	NEAT domain? Though interactions listed above?	Adhesion; nasal colonization	[117]
	Apolactoferrin	NEAT domain	Inhibition of lactoferrin; nasal colonization?	[191]
	Transferrin	NEAT domain?	Iron acquisition	[131]

Table 1 Continued

Iron-regulated surface protein A (IsdA) cont.	Alteration of surface hydrophilicity	C- domain of IsdA	Resistance to bactericidal lipids and AMPs; Skin colonization	[192]
Iron-regulated surface protein B (IsdB) *(aka FrpB, SirH, or SasJ)*	Hemoglobin/ Methemoglobin	$NEAT_1$ domains	Heme extraction through $NEAT_2$; can also bind haptoglobin-hemoglobin complex; iron acquisition	[133, 135, 193, 194]
	β3-containing integrins (GPIIb/IIIa, $\alpha_V\beta_3$)	Outside of NEAT domains	Platelet aggregation; Adhesion and internalization	[137, 138, 139]
	Vitronectin	NEAT domains	Acts as bridge to integrins; adhesion and internalization	[140]
	Von Willebrand factor	NEAT domain	Adhesion to endothelial cells	[136]

Table 1 Continued

Iron-regulated surface protein H (IsdH) (*aka HarA or SasI)*	Haptoglobin-Hemoglobin complex	NEAT domains	Binding of haptoglobin-hemoglobin complex ($NEAT_1$ + $NEAT_2$); inhibits binding of complexes by host scavenger receptor CD163 ($NEAT_1$); Heme extraction; Iron Acquisition	[193, 195, 196, 197]
	Hemoglobin/ Methemoglobin	$NEAT_2$ and $NEAT_3$ domains	Heme extraction; Iron Acquisition.	[196, 198, 199, 200, 201, 202 203]
	Heme	$NEAT_3$ domain	Iron Acquisition	[196, 204]
	Unknown	Unknown	Enhanced degradation of C3b to iC3b and C3d; reduced phagocytosis	[205]

Table 1 Continued

Three Helical Bundle Family				
Staphylococcal Protein A (SpA)	IgG Fc	EABCD domains (IgG binding domains)	Inhibition of opsonophagocytosis via human IgG1, IgG2, and IgG4	206, 207, 208, 209, 210, 211, 212
	Fab heavy chain V_H3 family region of IgM, IgA, IgE, IgG	IgG binding domains	B cell super-antigen; Leads to nonspecific activation of B cells, proliferation, migration to spleen, or clonal deletion via apoptosis	213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224
	Von Willebrand factor	vWF A1, D'/D3 domains; SpA IgG binding domains (in Fc binding region)	Adhesion to endothelial cells; platelet adhesion; Mechanical (shear) stress likely leads to conformational changes that expose binding sites	225, 226, 227, 228, 229, 230, 231

Table 1 Continued

Staphylococcal Protein A (SpA) cont.	TNFR1+ EGFR; Unknown[c]	TNFR1: IgG binding domains EGFR: IgG binding domains Unknown: Xr region	Inflammatory signaling (TNFα, IL-6, IFNβ) in airway epithelium	[232, 233, 234, 235, 236]
Second binding protein for IgG (Sbi)[d]	IgG Fc	Domains I and II (IgG binding domains)	Inhibition of opsonophagocytosis via human IgG1, IgG2, and IgG4	[141, 146, 147]
	Complement C3	Domains III and IV (Complement binding domains)	Complement Evasion; Activation of alternative pathway; Futile consumption of C3; Reduced opsonophagocytosis by neutrophils	[143, 150, 237 144]
	β_2-glycoprotien I (β_2-GPI; aka apolipoprotein H	Complement binding domains?	Unknown	[142]

Table 1 Continued

Second binding protein for IgG (Sbi)[d] cont.	Factor H	Complement binding domains; binds better when C3 is present	Complement Evasion; Inhibits Alternative pathway	[151]
	Plasminogen	Complement binding domains	Complement Evasion; Cleaves C3, C3b, C3a	[152]
	Lipoteichoic Acid (LTA)	C-terminal Y domain of Sbi	Allows for membrane bound Sbi	[145]
	TNFR1 + EGFR	In domains I-IV (IgG binding domains?)	Induction of inflammatory signaling in macrophages	[149]
G5-E Family				
***S. aureus* surface protein G (SasG)**	Desquamated epithelial cells	A region of SasG	Adhesion; nasal colonization	[159, 160]
	SasG homophilic interactions	Zn^{2+} dependent binding of G5-E domains	Biofilm formation	[160, 162, 163, 164, 238]
	No Ligand	Steric hindrance/ masking other CWAs	Inhibition of adhesion binding	[160]

Table 1 Continued

Plasmin-sensitive surface protein (Pls)[e]	No Ligand	Steric hindrance/ masking other CWAs	Inhibition of adhesion binding; reduced internalization	[157, 239, 240, 241]
	Unknown	Glycosylation status?	Biofilm formation	[241]
	Desquamated epithelial cells	A region of Pls	Adhesion; nasal colonization	[159]
Unknown				
Adenosine synthase A (AdsA) (*aka SasH)*	Enzymatic function	Convert AMP/ADP/ATP to adenosine	Bacterial survival; Reduced killing by inhibiting oxidative burst in neutrophils; inhibition of macrophage phagocytosis; inhibition of Th1/Th17 immunity	[242, 243, 244, 245]
	Enzymatic function	Converts nuclease-digested NETs into deoxyadenosine	Degradation of NETs; induced apoptosis of macrophages	[246, 247]

Table 1 Continued

Serine-rich adhesion for platelets (SraP) *(aka SasA)*	N-acetylneuraminic acid (Neu5Ac) containing glycoproteins; Salivary agglutinin gp340	β-grasp fold domain	Platelet binding; Adhesion and invasion; Oral colonization?	[248, 249, 250]
***S. aureus* protein X (SasX)**	Unknown	Unknown	Adhesion to human nasal epithelial cells; bacterial aggregates; biofilm; neutrophil evasion	[251]
***S. aureus* surface protein C (SasC)**	Unknown	N-terminal domain	Bacterial aggregation; biofilm accumulation	[252]
***S. aureus* surface protein F (SasF)**	Unknown	Unknown	Linoleic acid resistance; skin abscess formation	[108, 253]

Table 1 Continued

***S. aureus* surface protein F (SasF) cont.**	Unknown	Unknown	Invasive infections? (Bone implant infection, bovine mastitis, endocarditis); biofilm?	254, 255, 256
SasB, SasD, SasK, SasL	Unknown	Unknown	Unknown	156

a aka: also known as

b ? denotes presumed ligand/binding/function has not been directly tested

c See Staphylococcal Pneumonia section for more details

d Not a CWA but was studied herein and is covered

e Note: Pls is encoded in SCC*mec* type I typically found in HA-MRSA strai

1.3 *S. aureus* Host-Pathogen Interactions

The innate immune system is an important part of host defense against staphylococcal infections. Phagocytes, consisting of neutrophils and macrophages, play a large role in controlling *S. aureus* infections. Because of this, a majority of staphylococcal virulence factors function by avoiding recognition, phagocytosis, and killing by phagocytes. *S. aureus* is a successful pathogen that produces a wide array of virulence factors including toxins, immune evasion factors, proteases, and non-protein factors[9]. However, it is important to note that some of these virulence factors are specific to humans and may not show virulence in some models.

Here, the focus is on the early stages of staphylococcal infection, focusing on the major components of innate immune system and the host-pathogen interactions.

1.3.1 Opsonization

One of the first lines of defense against bacteria is to tag them for phagocytosis via opsonization[9, 257]. The two main types of opsonins are complement and immunoglobulins (Igs), which are deposited on the bacterial surface and are recognized by receptors that recognize the Fc region of Igs (Fc receptors; FcRs) or complement (complement receptors; CRs) and initiate the phagocytic process. *S. aureus* has several virulence factors that can interfere with opsonization with either complement or Igs.

The complement system is part of the innate immune humoral response and is a complex system of proteases leading to soluble and surface-bound factors that contribute to phagocytosis and other immune processes[258]. There are three pathways of complement, all which converge on the cleavage of C3 into surface deposited C3b and a soluble mediator C3a by C3 convertases. Continuation of this cascade eventually leads to the cleavage of C5 to C5a and C5b, and initiation of the membrane attack complex (MAC) composed of C5b, C6, C7, C8, and C9, which can form pores and lyse some pathogens. C3a and C5a can also act as chemoattractants to bring more phagocytes to the area of inflammation[258]. There are several receptors that recognize surface-deposited factors and expression of these receptors depends on the type of phagocyte. For example, neutrophils express CR1 (CD35) and CR3 (CD11b/CD18) while macrophages express CR3 and CR4 (CD11c/CD18)[257]. *S. aureus* protein SCIN is the most central to inhibiting complement, leading to fewer C3 convertases, less C3b deposition, and subsequently less C5a and MAC formation. Sbi can also bind complement Factor H and C3 to prevent complement cascade activation. There are several other proteins that can bind and inhibit different parts of the complement pathway, such as Efb, Cna, and Eap. *S. aureus* protease aureolysin can cleave C3, leading to less functional C3b to be deposited[9, 259]. CHIPs is a protein that can inhibit both neutrophil and macrophage chemotaxis by binding to phagocytic receptors such as the C5a receptor[260].

Opsonization with Igs occurs when antigen specific Igs are produced by the adaptive immune system in response to a pathogen. Igs will bind to pathogens via their antigen-specific binding site in their Fab region and subsequently be recognized via their Fc region and FcRs on immune cells[9, 257]. There are several subtypes of Igs (IgG1, IgG2, IgG3, IgG4, IgM, IgA) and each subtype has

specific functions and FcRs. The subtype of Ig made and what antigen the Ig is specific to is dependent on the infection type and immune response (antigen presentation, memory, Ig maturation, etc.). In *S. aureus* infections, Igs are typically directed against surface molecules such as peptidoglycan, wall teichoic acid, and surface expressed proteins, such as CWAs (see 1.2 above) Since most humans are exposed to staphylococci early in life, people will likely already have some level of staphylococcal specific Igs prior to an active infection. Thus, having naturally occurring Ig types, such as IgG, against several staphylococcal antigens will help facilitate this process. Additionally, IgG can feed into the classical pathway of complement, increasing the level of opsonization[257]. *S. aureus* produces several proteins that can interfere with Ig deposition, with the most characterized being SpA. SpA binds the Fc region to prevent recognition by phagocytic FcRs. Additionally, binding the Fc region of IgG prevents activation of the classical pathway of complement fixation. Sbi exclusively binds the Fc region of IgG while proteases V8 and SAK can cleave Igs[9, 172].

1.3.2 Phagocytosis

Phagocytosis is the process of engulfing other cells, cell fragments, or microorganisms. This process not only sequesters the components phagocytosed, but also leads to their eventual elimination via degradation[261]. There are several phases in the phagocytic process, starting from attachment of the opsonized microbe via receptors (CRs or FcRs), pseudopod extensions around the attached microbe, and completion of the engulfment resulting in the formation of a phagosome[261]. After formation of the phagosome, it will fuse with granules (neutrophils) or the lysosome (macrophages) containing bactericidal components. Concurrently, an oxidative burst is

initiated in the phagosome via NADPH-dependent oxidases leading to the generation of reactive oxygen species (ROS)[257, 262].

In addition to inhibition of opsonization, phagocytosis can be inhibited by *S. aureus,* typically through masking of the pathogen from the immune system. Coating of the surface of the bacteria with sugars or host components decreases the antigenicity of the pathogen, and thus may prevent the opsonization and the phagocytic process all together[9, 19]. The expression of a capsular exopolysaccharide is common in some clinical strains; however, this is not produced by USA300[9]. PIA is another exopolysaccharide that can act as a shield as well as a major component of staphylococcal biofilm matrix.[9] Biofilms themselves are protective against phagocytosis and can form on abiotic surfaces such as indwelling medical devices or on tissue surfaces such as the heart valves in endocarditis[22]. After initial adhesion to a surface, the biofilm matrix is produced consisting of PIA exopolysaccharide, extracellular DNA, teichoic acids, and other biofilm promoting proteins[9]. Bacterial aggregates in the blood are also protective against phagocytosis, with several *S. aureus* proteins known to bind host components to help facilitate aggregation in the blood[9, 22]. Coagulase and vWbp together bind prothrombin and create a complex known as staphylothrombin. Staphylothrombin can then cleave fibrinogen in the absence of vascular damage, and fibrinogen binding proteins can also adhere to fibrin clots or activate aggregation of platelets[9].

1.3.3 Phagocyte-mediated Killing

1.3.3.1 Neutrophils

In the immune system, the predominant phagocytic cell is the neutrophil, accounting for 40-60% of all leukocytes in humans. Neutrophils are part of the first line of defense against a pathogen as they are the first recruited cell type to an area of inflammation, show a fast response, and have several bactericidal mechanisms. These cells can be rapidly mobilized from the bone marrow and can secrete cytokines to recruit in additional immune cells. Neutrophils have two main mechanisms for killing pathogens after phagocytosis: generation of ROS and granules filled with proteases and antimicrobial peptides (AMPs).

Neutrophils contain several types of granules characterized by what they contain; primary (azurophilic) granules contain myeloperoxidase (MPO) and secondary (specific) granules that do not; however, granules are heterogenous and can be subdivided further based on various markers. MPO is one of the most abundant proteins in the primary granules and catalyzes the generation of hypochlorous acid that aids in bacterial killing. Additional ROS molecules are generated within the phagosome such as hydrogen peroxide, hydroxyl radicals, and superoxide. Inducible nitric oxide synthetase (iNOS) generates NO which will react with superoxide to form reactive nitrogen species (RNS) that are also antimicrobial. Additional components found in granules include proteins involved in metal binding/sequestering (lactoferrin, lipocalin), cationic AMPs (defensins, cathelicidin type peptides), and serine proteases (cathepsin G, elastase)[257].

While neutrophils are essential for the clearance of *S. aureus* infections, this bacterium has developed methods to avoid killing after phagocytosis. Staphylococci can survive within the

phagosome and have developed several mechanisms to avoid killing[257, 263]. *S. aureus* has several mechanisms to resist ROS damage. Staphyloxanthin, the orange pigment that gives *S. aureus* its name, scavenges free radicals from ROS activity through its conjugated double bonds. *S. aureus* also expressed several enzymes to help detoxify ROS such as catalase, superoxide dismutase, and lactate dehydrogenase. AMPs are commonly positively charged and function by forming pores in the bacterial membrane. To avoid this, *S. aureus* will reduce the negative charge of the membrane and cell wall to avoid AMP interactions. Lysozyme, an enzyme known to cleave Gram-positive cell walls, is inefficient for *S. aureus* due to modifications of its peptidoglycan[9, 260]. Staphylokinase (SAK) can bind and inhibit the bactericidal activity of neutrophil defensins while Eap can inhibit serine proteases neutrophil elastase, cathepsin G, and proteinase 3[260, 264].

Additionally, neutrophils are able to trap and kill extracellular bacteria through the release of their chromatin, known as neutrophil extracellular traps (NETs). The DNA is also associated with antimicrobial peptides, histones, and proteases to help facilitate bacterial killing[257, 265]. NET formation is induced via proinflammatory stimuli such as ROS, cytokines, and bacterial exposure[265]. *S. aureus* primarily evades NETs via the expression of a nuclease, Nuc, which can degrade the chromatin and subsequently allow *S. aureus* to escape being killed by AMPs or proteases[9, 265]. Additionally, the enzyme adenosine synthase is secreted and is able to convert NETs to deoxyadenosine, which can trigger death of immune cells via caspase activation[265]. Recently, it was discovered that NETs can influence macrophage phagocytosis and killing,[266] and so NET avoidance likely plays a larger role than previously realized.

1.3.3.2 Macrophages

Macrophages are another key component of the innate immune system. Unlike neutrophils, macrophages can be tissue resident, with circulating monocytes entering tissue to differentiate into macrophages as needed during infection. They are typically less reactive than neutrophils, likely to control off target inflammation that can occur with neutrophil activation. As well as acting as professional phagocytes, macrophages are also antigen presenting cells (APCs), playing a role in tying the innate and adaptive immune responses[172]. Upon sensing environmental signals, macrophages display a spectrum of functional characteristics that can generally be categorized into microbicidal M1 or tissue repair/inflammation resolution M2 macrophages[262]. Macrophages can recognize *S. aureus* for phagocytosis through complement and Fc receptors (CRs and FcRs, respectively) as well as receptors known as scavenger receptors (SRs)[172]. SRs can recognize a wide variety of pathogenic molecules such as polysaccharides, lipids, CpG, and LTA and can induce phagocytosis in the absence of traditional opsonins. While a majority of *S. aureus* is efficiently phagocytosed and killed by macrophages, some can survive and eventually escape, similar to what occurs in neutrophils [172, 263].

Macrophage phagosomes function similarly to neutrophil phagosomes, however macrophage phagosomes fuse with lysosomes rather than granules (which are not present in macrophages)[172]. This leads to an acidification of the phagolysosome, which can negatively impact bacterial growth and increase efficacy of AMPs[172, 262]. This is primarily mediated through the function of a vacuolar associated ATPase (v-ATPase), which pumps H^+ ions into the lumen of the phagosome[172, 267]. In macrophages, ROS is generated similarly to neutrophils, with a major difference being the amount of MPO present in each cell type (less in macrophages), leading to different concentrations of

various ROS[172]. Additionally, mitochondria ROS (mROS) also plays a role in macrophages, by sending mROS to the phagosome via mitochondria-derived vesicles or by mitochondria associating with the phagosome membrane[172, 268]. Macrophages also use nutritional immunity mechanisms to sequester nutrients away from microbes[172, 262]. Metal ions iron and manganese are sequestered to restrict bacterial growth, while copper and zinc are unrestricted and used to overwhelm bacteria with toxicity[172, 267].

S. aureus can also resist killing in macrophages through similar mechanisms as neutrophils such as resisting ROS/RNS, and AMPs. Additionally, *S. aureus* can survive and multiply in mature phagosomes in both murine and human macrophages[269]. *S. aureus* is able to sense the acidification of the phagolysosome through the GraXRS regulatory system, which triggers transcription of genes involved in surviving the hostile environment, such as AMP resistance[262]. Several studies have shown that *S. aureus* containing phagolysosomes do not acidify appropriately compared to other bacteria such as *Streptococcus pneumoniae*, which may help facilitate later escape from the phagosome[172]. There is evidence that the alpha-toxin can inhibit mitochondrial association with phagolysosomes via induction of caspase 1, which may explain part of this phenomenon[268, 270]. To overcome sequestering of metals within the phagosome, *S. aureus* will express metal transporters as well as metallophores/siderophores to bind metal ions[172]. While not directly tied to phagocytosis, *S. aureus* can also influence the polarization of macrophages, which may allow for inefficient bactericidal behavior[172].

1.3.4 Killing of Phagocytes

While many of the mechanisms to avoid killing by phagocytes occurs within the phagosome, *S. aureus* also has several mechanisms to actively avoid phagocytes by inducing their death. Dead or dying phagocytes are not microbicidal, and thus killing these cells before being phagocytosed is another mechanism to evade killing within the host[271]. *S. aureus* expresses a number of pore-forming toxins, leading to loss of membrane integrity and lysis of affected cells. Collectively called leukotoxins, the function of these toxins is primarily geared towards leukocytes such as neutrophil and macrophages[257, 265, 267]. The most well characterized staphylococcal pore-forming toxin is the alpha-toxin. The alpha-toxin acts as a cytolysin for many cell types including leukocytes by forming a membrane-spanning pore. This is a receptor dependent process, using the host protein ADAM10 (a disintegrin and metalloprotease domain-containing protein 10). In high concentrations, pore formation leads to inflammatory signaling, such as NLRP3 inflammasome signaling, and subsequent cell death (pyroptosis or necroptosis) [267, 272]. *S. aureus* has several other pore-forming toxins known as bicomponent leukocidins such as PVL (encoded by *lukS-PV* and *lukF-PV*), LukDE, LukAB (also called LukGH), and HlgAB or HlgCB combinations of the gamma-hemolysin. These toxins require two subunits, with fast (F) subunit recognizing the host membrane protein receptor (for example, a chemokine receptor) and the second slow (S) subunit is then recruited and leads to dimerization and pore formation. Their receptors are almost exclusively all chemokine receptors located on various immune cells, but mainly on phagocytes[257, 265, 272]. Phenol-soluble modulins (PSMs), including the delta-toxin, are amphipathic alpha-helical peptides that are receptor independent, likely due to their ability to insert into lipid bilayers. PSMs have more characterization in their interactions with neutrophils but have been shown to affect

macrophages as well[267]. Additionally, both PSMs and LukAB have been shown to lyse neutrophils from within[257, 265].

1.4 Staphylococcal Bacterial Pneumonia

1.4.1 Epidemiology

Prior to the emergence of CA-MRSA*, S. aureus* was an uncommon cause of pneumonia in healthy individuals who had not previously been infected with influenza [273, 274]. In the 1950s, sporadic cases were described in very young infants and adults with preexisting diseases (cardiopulmonary disease, alcoholism, diabetes) and acquired their pneumonia in the hospital. Mortality rates at the time in the ranged from 12-15% in children, 20% in young adults, 30-50% post-influenza, and 84% of patients with bacteremic primary pneumonia[274]. In the 1960s-1980s, *S. aureus* accounted for 1-10% of community-acquired pneumonia (CAP) and ≥ 10% of hospital-acquired pneumonia (HAP)[275]. Those who had severe infection requiring hospitalization typically were older and had preexisting diseases and acquired infections from a health care setting[274, 275].

In the 1980s-mid 1990s, *S. aureus* was associated with 20-40% of nosocomial pneumonia cases[276, 277]. In a study of nosocomial pneumonia from 1985 to 1988, *S. aureus* was the second most common bacterial cause after *Pseudomonas aeruginosa*[278, 279]. Between 1983 and 1992, *S. aureus* became the primary cause of HAP in the United States, accounting for 15-30% of the cases[277, 280]. This was consistent with reports from Europe in the same time period (30%)[281]. In a six-year prospective Spanish hospital study of *S. aureus* bacteremic pneumonia, the authors found

94% of MRSA pneumonia cases were nosocomially acquired compared to 70% for MSSA pneumonia and those with MRSA had longer hospitalization times[282]. Ventilated patients were at high risk of developing *S. aureus* pneumonia, with studies reporting 10-60% of ventilator-associated pneumonia (VAP) caused by *S. aureus*[276, 277, 283]. MRSA VAP patients typically had worse morbidity and mortality than MSSA VAP patients[284, 285]. This increase in incidence of *S. aureus* HAP overlaps with the spread of HA-MRSA and this likely contributed to this rise[280, 286, 287]. In a study done on the National Nosocomial Infections Surveillance (NNIS) system database from 1992-2003 found that MRSA accounted for 55% of *S. aureus* infections in the ICU[288]. In a retrospective study of the 2000-2001 Nationwide Inpatient Sample (NIS) database ICD-9-CM discharge diagnosis codes, *S. aureus* was the most frequently isolated pathogen in the intensive care unit (ICU) and patients typically had three times the length of stay and five times the risk of death compared to patients not infected with *S. aureus*[289]. However, this study did not differentiate MSSA from MRSA, so it is unclear what percentage of their findings was due to MRSA.

As CA-MRSA began to spread[18, 290, 291, 292, 293], there was an increase in MRSA CAP cases[274, 294, 295, 296, 297, 298, 299]. While CA-MRSA is more associated with SSTIs, CA-MRSA CAP can still cause severe disease in young healthy adults and children[295, 300, 301]. These cases differed from HA-MRSA cases in that they were primarily necrotizing pneumonia[302], which was thought to be due to the increased rate of PVL-positivity in these strains[295, 296, 303, 304, 305]. Necrotizing pneumonia is characterized by hemoptysis, leukopenia, high fever, and lung necrosis or abscesses[306]. This can progress rapidly and become fatal in healthy adults and children, with a mortality rate as high as 60%[307, 308]. CA-MRSA CAP cases are also commonly associated with recent or concurrent influenza-like illness and clusters of infections are temporally related to influenza A outbreaks[309,

[310, 311], which likely contributes to the level of tissue damage seen in the lungs of these patients (See 1.5 below).

1.4.2 Staphylococcal Virulence

There have been several studies done on staphylococcal virulence factors *in vitro* and *in vivo*. Here, the focus will be on the major virulence factors associated with pneumonia. For more information see reviews[272, 312, 313, 314].

Panton-Valentine Leukocidin. The first toxin associated with CA-MRSA necrotizing pneumonia is Panton-Valentine leukocidin (PVL)[304, 315]. While MSSA can also encode PVL, the association with necrotizing pneumonia is in CA-MRSA strains[303, 305, 316]. PVL is a bicomponent pore that targets neutrophils, monocytes, and macrophages that results in cell death[317]. The receptors for PVL are complement receptors, C5aR and C5L2, which are found on phagocytes and explain its cell specificity[318]. PVL is secreted as its monomers, LukS-PV and LukF-PV, which will associate with their receptor and initiate pore-formation. After pore-formation, neutrophils undergo caspase-3 and caspase-9 mediated cell death[319]. In macrophages, PVL can lead to induction of the NLRP3 inflammasome and activation of cathepsin B, ultimately leading to programmed necrosis and cell death[320, 321].

Both human and rabbit phagocytes are susceptible to PVL induced cytotoxicity, but not murine or nonhuman primate phagocytes[322]. This explains the difficulty to associate PVL with pneumonia in infection models and the conflicting literature[323, 324, 325]. However, a humanized mouse shows increased PVL virulence[326]. PVL may still be able to induce inflammation in mice

without the ability to lyse phagocytes[327, 328]. Clinical meta-analyses also fail to find a clear connection between PVL and necrotizing pneumonia independent of the CA-MRSA background[329, 330, 331]. USA300, the more prevalent CA-MRSA in the United States, expresses higher levels of PVL and is more virulent in mouse models than USA400, which may also help to explain the lack of evidence for PVL[332].

Alpha-toxin. The most well characterized virulence factor in the lungs is the alpha-toxin (also known as alpha-hemolysin; Hla). Hla is a pore forming toxin that assembles into heptamers at the cell membrane by binding to its receptor, ADAM10 [333, 334, 335]. Hla has been shown to alter the lung alveolar epithelium by a variety of mechanisms such as altering focal adhesions, inducing proinflammatory signaling and calcium fluxes, leading to decreases in antimicrobial surfactant secretion and mitochondrial depolarization. This leads to increased epithelial injury and subsequent lung damage and edema[336, 337, 338, 339, 340, 341, 342, 343]. Infection with strains lacking Hla is associated with lower morbidity and mortality compared to wild-type strains in pneumonia[270, 344, 345, 346]. Disruption of ADAM10 expression in the lung is also protective against a lethal pneumonia model[347]. Antibodies against Hla are protective in pneumonia models[348, 349, 350, 351, 352], supporting a role for this virulence factor in the lung. Hla is not only helpful for *S. aureus* but can help Gram-negative bacteria in a co-infection model[270].

Hla induces the NLPR3 inflammasome[353, 354] and this interaction has been heavily studied[268, 346, 355]. Hla mediated NLRP3 induction, through K^+ efflux, in the lung leads to activation of capase-1 and subsequent generation of IL-1β, IL-18 and pyroptosis induction[272, 354]. Hla-mediated NLRP3 inflammasome activation can be used as a way to survive in the macrophage phagosome by

preventing acidification[268]. Interestingly, IL-1β appears to be a biproduct of this phenomenon as blocking IL-1β signaling can increase survival without affecting bacterial burden[268, 346]. More recent work suggests that the NLRP3 inflammasome mechanism may be a biproduct of (or in combination with) necroptosis[356, 357, 358]. This study shows that Hla induces MLKL phosphorylation, which forms a pore as part of the cell death pathway, ultimately leading to K^+ efflux induced activation of the NLRP3 inflammasome. There is also some evidence that the NLRP6 inflammasome may be involved in both pyroptosis and necroptosis in the lung, although it is unclear if Hla is involved in this process[359]. At this time, it is unclear if both pyroptosis and necroptosis are induced in the lung and what roles they play in staphylococcal pneumonia. In the skin, there is some evidence that both cell death pathways are induced and play different roles: 1) pyroptosis helps with bacterial clearance of *S. aureus* while 2) necroptosis helps to control the level of inflammation[360].

Protein A. Protein A (SpA) is one of the most well-studied and multifunctional virulence factors in *S. aureus*. The most well-characterized role of SpA is immune evasion by binding Igs[22]. While this likely has a role in pneumonia, it has not been directly tested and will not be covered here; for more details, on SpA functions see Table 1. SpA was the first virulence factor described in the lung, although early studies did not find it anti-phagocytic[361, 362]. *S. aureus* strains isolated from patients with airway infections have increased expression of protein A[363].

In human airway epithelial cells, SpA induces pro-inflammatory signaling through binding of TNFR1[232]. This was also confirmed in an *in vivo* murine pneumonia model, as TNFR1 null mice and WT mice infected with *Δspa* had attenuated disease. The IgG binding domains of SpA

were later confirmed to be mediating this interaction with TNFR1[233]. SpA induces expression of TNFR1 on the apical surface of airway epithelium and leads to increased shedding of the receptor[232]. Shedding is mediated by another SpA interaction, with EGFR, leading to its phosphorylation[235]. EGFR signaling leads to phosphorylation and activation of a metalloprotease TACE (TNF-α converting enzyme; aka ADAM17), which cleaves TNFR1. Additionally, *S. aureus* induction of TACE (likely through the SpA-EGFR interaction) leads to increased IL-6 signaling and recruitment of macrophages via CCL-2[234]. SpA, through its Xr region, can also induce type I IFN (IFNβ) transcription via signal transducer and activator of transcription 1/3 (STAT1/STAT3) activation[236]. In this study, the Xr region also induced IL-6 in an IFNβ- and STAT3-dependent manner, although it is unclear how and if this relates to the IL-6 signaling through TACE. SpA can also facilitate invasion of polarized airway epithelial cell monolayers via the paracellular junctions[364]. SpA activation of EGFR, along with the protease calpain, leads to cleavage of occludin and E-cadherin, facilitating bacterial transmigration through the cell-cell junctions.

Phenol-soluble modulins. Phenol-soluble modulins (PSMs) are a family of short amphipathic α-helical peptides; PSMα1-α4, PSMβ1, PSMβ2, and the *S. aureus* δ-toxin[365]. PSMs, along with many other toxins, are expressed at higher levels in MRSA strains[366]. PSMs are regulated by the virulence regulator *Agr*, and cytolysis of host cells most likely occurs through nonspecific receptor-independent mechanisms, although the cellular receptor formyl peptide receptor 2 (FPR2) induces inflammatory effects[365, 367]. Few studies have looked at the effect of PSMs specifically in the lung[356, 368, 369]. PSMs can induce inflammatory signaling and cytotoxicity in A549 human lung epithelial cells[369]. PSMα can also facilitate phagosomal escape in non-professional phagocytes[370 371] allowing for intercellular replication, but this has only been shown

in CFTR-complemented cystic fibrosis upper airway epithelial cell line (S9). PSMs can help to induce IL-1β release and necroptosis in macrophages[356, 372], an important immune cell in the lung during staphylococcal infections. Mice treated with an inhibitor of PSM expression (RNAIII-inhibiting peptide) had a dose dependent decrease in weight loss, mortality, and bacterial burden[368]. This phenotype was dependent on the presence of neutrophils and the receptor FRP2, leading to TNF-α mediated necroptosis of these cells.

Beta-toxin. The beta-toxin (β-toxin, also known as sphingomyelinase C) is a Mg^{2+}-dependent neutral sphingomyelinase that hydrolyzes membrane sphingomyelin of host cells to generate phosphorylcholine and ceramide[373, 374]. This toxin can lyse several types of cells, including red blood cells, monocytes, lymphocytes, and neutrophils[374, 375, 376]. There are few studies that have investigated this toxin in pneumonia[377, 378]. Purified β-toxin can inhibit ciliary beat frequency of rabbit ciliated epithelial cells isolated from the maxillary sinus[377]. In a mouse model of pneumonia, β-toxin leads to increased neutrophil efflux and airway permeability[378]. This toxin also induced shedding of syndecan-1, a major heparan sulfate proteoglycan of epithelial cells, which the authors suggest is the mechanism behind reduced neutrophil migration as reported in a bleomycin-induced acute lung injury model[379]. The beta-toxin has also been implicated in helping to induce the NLRP3 inflammasome and necroptosis in macrophages[354, 356].

Iron Acquisition. Iron is essential to bacterial growth and hemoglobin is a preferred source of iron for *S. aureus*[380]. *S. aureus* has two iron acquisition systems, the heme transport system (Hts) [380] and the heme-iron by the iron regulated surface determinant (Isd) system (See 1.0 above). Ferric uptake regulator (Fur) is a transcription repressor that is used to sense iron-limited

environments and regulate expression of proteins involved in iron acquisition as well as proteins involved in infection[30]. In a shotgun proteomics approach to identify airway proteins involved in early *S. aureus* airway infection, the authors found that 7% of host peptides at 6 hours were of hemoglobin[381]. This was further supported as *S. aureus* isolated from the bronchoalveolar lavage at 30 minutes and 6 hours after infection were associated with hemoglobin. Fur is required for full virulence in a murine model of staphylococcal pneumonia[382]. Fur helps to regulate hemolysin and cytotoxin expression and lacking Fur (*Δfur*) leads to reduced bacterial survival in the presence of neutrophils or in the lungs. However, the authors suggest that the lungs are not an iron-limited environment, as *Δfur S. aureus* has reduced survival even with higher expression of iron acquiring proteins. This is supported by other groups' work in mouse models[383, 384]. Thus, the Fur requirement could be due to its ability to regulate other global regulators (*Agr*, *Sae*, *Rot*) in *S. aureus*[385].

Cell wall-anchored proteins. Because of the overlapping structures and functions of CWAs, it is likely that these proteins work together during colonization and infection in the host. Because of this, there is not one CWA (with the exception of SpA described above) that has been described as "THE" CWA required for pneumonia. Several CWAs have been implicated in nasal colonization (ClfB, SdrC, SdrD, IsdA, SasG, SasX), which could influence staphylococcal pneumonia. In a Cotton rat nasal colonization model, upregulation of CWA genes *clfB, sdrD, sdrC, sasG* is seen in USA300[256]. In humans, expression of *clfB* and *fnbA*, and *isdA* are increased[10]. See Table 1 and reviews[272, 386, 387] for more details on colonization.

CWAs are required during pneumonia, as a Sortase A mutant has reduced virulence in a mouse pneumonia model[388]. This is also seen when SrtA inhibitors are used during pneumonia[389, 390, 391, 392]. Indeed, CWA specific antibodies are found in patients with bacteremic pneumonia[393] and vaccination with CWAs is protective in murine pneumonia models[394, 395, 396, 397]. However, little work has been done to investigate these proteins, other than SpA, in pneumonia. Fibronectin binding proteins (FnBPs) have been shown to adhere to human airway epithelium[398] and have a role in internalization *in vitro*[399]. A FnBP deletion mutant shows increased growth in a rat model of pneumonia, which the authors contribute to immune evasion via internalization[399]. Adenosine synthase A (AdsA) has been shown to inhibit alveolar macrophage (AM) phagocytosis and AM production of type IIA secretory phospholipase A2 (sPLA2-IIA) in a guinea pig model[244].

1.4.3 Immune Responses to Staphylococcal Pneumonia

Here the focus is on lung-specific innate immune responses to *S. aureus*. For more details see reviews[272, 313, 314].

Physical Barriers. The first line of defense is the physical barriers found within the airway. Mucus is a protective barrier that traps and eliminates pathogens via mucociliary clearance[400, 401]. Additionally, there are several factors found in the mucus that can help fight bacterial infections. Mucin 5b is required for bacterial defense within the lungs and Muc5b$^{-/-}$ mice have dramatic increases in *S. aureus* (150-389-fold) burden[402]. This mucin is highly involved in mucociliary clearance in the respiratory tract and lacking this mucin leads to chronic infection and death. Pulmonary surfactant proteins found in the mucus can also have immunomodulatory affects[403]. Specifically, SP-A and SP-D are protective in staphylococcal pneumonia and SP-A acts as an

opsonin for AM phagocytosis[404, 405]. However, *S. aureus* does target both mucins and surfactants with serine protease SplA and cysteine protease staphopain A (ScpA), respectively[406, 407]. Additionally, *S. aureus* expresses PhnD, which allows small staphylococcal microaggregates to form in lung alveoli, leading to ciliary inhibition through Hla[343].

Alveolar Macrophages. In the lung, the main resident macrophage is the alveolar macrophage[408, 409]. These cells are found in the airways and alveolar spaces and play a critical role in regulating immune responses and inflammation in the lung. These cells are responsible for phagocytosis of pathogens and particles, secretion of cytokines, and initiating recruitment of other immune cells.

Depletion of AMs through clodronate liposomes or anti-GR-1 antibody treatment can lead to high bacterial burden and inflammatory cytokine levels, as well as mortality rates as high as 90%[410, 411]. Depletion of AMs leads to a dysregulated immune response, with a larger recruitment of $CD4^+$ T cells and more lung inflammation. As described elsewhere in this review, *S. aureus* expresses several virulence factors that can lead to macrophage death, and this is no different in the lung[356, 358, 372] and blocking AM death leads to better outcomes in mice. Toxin-induced inflammasome signaling leads to IL-1β release, leading to a larger recruitment of neutrophils to the lung, which can be detrimental (see section below).

While phagocytosis of pathogens is very important in the lung, the level of phagocytosis and phagocyte recruitment depends on the bacterial pathogen[412]. The main phagocytic receptor on AMs is MARCO (macrophage receptor with collagen structure) a scavenger receptor that can

recognize a broad array of ligands[413, 414]. This receptor can bind both unopsonized particles as well as pathogens such as *S. aureus* in mice and humans. In mice lacking MARCO, mortality is significantly increased in *S. pneumoniae* and *S. aureus* infections[415, 416]. Acidification of the AM phagosome through v-ATPase is required for *S. aureus* killing[417]. Recognition of *S. aureus* through stimulator of interferon genes (STING), an endoplasmic reticulum associated protein which recognizes bacterial dinucleotides, is protective in pneumonia[418]. Mice that lack STING have increased macrophage necroptosis, which ultimately leads to higher bacterial burden, lung damage, and mortality.

Another role of AM phagocytosis is the clearing of dead/dying cells including neutrophils through a process known as efferocytosis. *S. aureus* Hla can lead to reductions in CCN1 and DD1α, leading to reduced efferocytosis of neutrophils[419]. As *S. aureus* can also use neutrophils for a protected reservoir (see below), this also likely contributes to persistence within the lungs. Despite the importance of these cells, *S. aureus* has been shown to survive in alveolar macrophages[268, 420] and there is a report of *S. aureus* being found in rat AMs 30 days after infection[421]. This is likely through their ability to resist killing by macrophages, and in some cases, *S. aureus'* ability to lyse macrophages after phagocytosis, leading to chronic infection[422]. In other bacterial pneumonias, AMs can induce apoptosis when their killing abilities are overwhelmed, however this does not appear to happen with *S. aureus*, potentially through its ability to upregulate inhibitors of apoptosis[423]. A recent report shows that AMs patrolling and phagocytosing bacterial pathogens, such as *S. aureus*, is used to regulate immune responses in the lungs with low inoculum, and when this is inhibited, more neutrophils and subsequent inflammation occurs[424]. Not surprisingly, CA-MRSA stains associated with pneumonia have more resistance to AM mediated killing[425].

Neutrophils. Neutrophils are also an essential component of the innate immune system. These cells help mediate pathogen killing through phagocytosis and subsequent killing, production of NETs, production of ROS, and secretion of inflammatory cytokines[426]. In the lung, depletion of neutrophils leads to 80% mortality in mice during staphylococcal pneumnonia[427], although this was not replicated in another study[411]. This is likely due to the reduced clearance of *S. aureus*, leading to larger inflammatory cytokine release in the lung, and not systemic bacteremia. This has been supported by other work[428]. Influx of neutrophils occurs within the first 6 hours of pulmonary infection due to early induction of inflammatory cytokines[429]. Additionally, neutrophils are required to prevent dissemination of nasal colonizing *S. aureus* into the lower airways[430]. Treatment with G-CSF has been shown to lead to better outcomes in pneumonia models[430, 431], likely through the recruitment of neutrophils to help control bacterial numbers. The presence of lipoteichoic acid and peptidoglycan is enough to induce neutrophil recruitment into the airways[432]. *S. aureus* can also induce neutrophil death through PSMα1 through a TNFα- and FPR2-dependent manner[368], leading to worse outcomes. Recently, it was shown that the NLRC4 inflammasome is detrimental for neutrophil recruitment in the lung[433]. NLRC4-driven necroptosis in the lung, along with IL-18, leads to suppression of gamma delta (γδ) T cell IL-17A-dependent neutrophil recruitment.

Hla-mediated damage has been shown to increase neutrophil recruitment into the lung through CXC chemokines[345]. However, in this model, increased neutrophil recruitment leads to more inflammation and lung injury, which increases the bacterial burden in the lung, potentially through increased staphylococcal attachment. Neutrophils have been implicated in mediating inflammatory damage in response to staphylococcal pneumonia[232, 434, 435, 436, 437], suggesting that

there is a fine balance between bacterial control and immune-mediated damage. *S. aureus* can induce IFNγ expression in neutrophils 24 hours post infection in a NADPH-oxidase dependent manner[434]. Increases in IFNγ were shown to induce NETosis in the lung. In a recent report, it was shown that NETosis can lead to increased AM phagocytosis and killing of *S. aureus*[266], which likely also occurs in the lung. However, *S. aureus* expresses a nuclease, Nuc, which can degrade NETs[438] and AdsA, which can convert NETs to deoxyadenosine[246] and induce macrophage apoptosis. NADPH-oxidase is also required for *S. aureus* clearance, but only in neutrophils[428], which the authors tie to neutrophil's ability to create ROS. *S. aureus* super antigen SEIX has been shown to bind human and mammalian neutrophils through glycoprotein receptors[439]. This prevents bacterial binding to neutrophils and subsequent phagocytic killing, leading to necrotizing pneumonia in rabbits. Another super antigen, SEO, induces inflammatory cytokine release from neutrophils during pneumonia, but does not drive pathogenicity[440]. This is dependent on toll-like receptor (TLR)4 TLR4, but not TLR2, and K^+ efflux induced NLRP3 inflammasome activation. In the context of asthma, *S. aureus* extracellular vesicles (EVs) induce neutrophilic inflammation in the lungs and a more skewed Th17 immune response[441]. Vaccination with staphylococcal EVs can protect mice in a lethal pneumonia model[442] by inducing Th1 immune responses, but this is associated with T cell responses and not neutrophils.

1.5 Influenza Super-infection

1.5.1 Influenza

Influenza is a viral respiratory disease with symptoms associated with an upper respiratory tract infection including fever, sore throat, runny nose, cough, headache, muscle pain, and fatigue. However, influenza can also lead to severe lower respiratory tract infections in some people. In some cases, influenza can also lead to non-respiratory complications in the heart, central nervous system, and other organs[443].

Influenza circulates in seasonal epidemics in the northern and southern hemispheres with those most affected being the very young and very old. In a typical year, 3-5 million cases of severe illness resulting in ~500,000 deaths are caused by seasonal influenza virus infection in the world[444]. In the United States from 2010 to 2017, the CDC estimated that influenza infection resulted in 9.2-35.6 million illnesses and 140,000-710,000 hospitalizations[443, 445]. Influenza-associated death in the United States range from 5,000-52,000 per year depending on the circulating viruses[446]. However, influenza can also cause pandemics due to its ability to genetically reassort (see below). The incidence of influenza increases due to the lack of preexisting immunity, although the numbers are dependent on the pandemic virus itself. There have been five major pandemics since the early 1900s: the infamous 1918 (H1N1) Spanish influenza pandemic, 1957 (H2N2) Asian influenza pandemic, 1968 (H3N2) Hong Kong influenza, and 2009 (H1N1) Swine influenza [447]. The most severe pandemic was the 1918 pandemic, with over 50 million deaths worldwide. However, many of these deaths can be attributed to additional secondary pneumonias (see below).

The influenza viruses, consisting of types A-D, belong to the family *Orthomyxoviridae* and are enveloped negative-sense single-stranded RNA viruses with a segmented genome[443, 447]. Influenza A and influenza B are the most common viruses to cause disease in humans and contain eight RNA segments encoding RNA polymerase subunits, viral glycoproteins hemagglutinin (HA) and neuraminidase (NA), viral nucleoprotein, matrix protein, membrane protein, nonstructural protein, and nuclear export protein. The two glycoproteins are located on the surface of the virus particle are the most antigenically variable due to them being the main targets of protective antibodies[443]. In influenza A, HA and NA are classified into antigenically diverse subtypes: HA1-16 and NA1-9, and individual viruses are classified based on what HA and NA types they contain (i.e., H1N1, H3N2)[443]. Viruses are also named based on their host, place of isolation, isolate number, and year of isolation; for example, Influenza A/Turkey/Ontario/6118/1968 (H8N4) is a influenza A virus isolated from a turkey in Ontario in 1968, numbered isolate 6118, and has HA subtype 8 and NA subtype 4[443].

Due to the segmented genome, genetic reassortment of individual RNA segments can occur when two strains of the same type (2 influenza A or 2 influenza B) infect the same cell. Influenza A can infect humans as well as several other animals such as pigs, poultry, horses, wild migratory birds, and domestic animals. Because of this, there is a large animal reservoir, which not only keeps the virus circulating, but also serves as reassortment sites primarily for human, pig, and bird influenza A strains. Many of these strains can infect the same host and can infect the same cell. For example, the 1957 and 1968 pandemics were caused by a reassortment between avian and human strains while the 2009 pandemic was caused by reassortment of human, avian, and swine strains in pigs[447]. This phenomenon, known as antigenic shift, can lead to novel strains with avian

or swine components which have not been seen in human populations, leading to pandemics[448]. Influenza additionally undergoes antigenic drift due to accumulating mutations, primarily in HA and NA, to avoid the immune response. Because of this phenomenon, influenza vaccines need to be updated frequently to account for the drifted strains as they occur and circulate.

At the cellular level, infection takes place in epithelial cells of the respiratory tract of humans and mammals and in the intestinal epithelium in birds. The virus will bind to the target cell via HA, which binds to sialic acid present on the glycoproteins found on the cellular surface; human strains prefer α2,6 linkage while avian prefer α2,3 linkage to the rest of the oligosaccharide portion of the glycoprotein. After binding, the virus is internalized in an endosome which is then acidified, leading to a conformational change in HA that allows for fusion of the viral envelope with the endosomal membrane. This in turn releases the viral contents, and the genome will be imported into the nucleus for transcription and replication of the viral genome. At later stages of infection, the genome is exported from the nucleus and eventually migrates to the plasma membrane, where membrane proteins have already been assembled and the virion is packaged with genetic material and viral proteins. Budding of new virions occur, using the host membrane as the viral membrane, and NA activity prevents HA binding to sialic acid present on the host cell and virion[443].

The innate immune response recognizes influenza through viral conserved components, known as pathogen associated molecular patterns (PAMPs) through host pathogen recognition receptors (PRRs). Recognition of viral RNAs through retinoic acid-inducible gene I (RIG-I) and TLRs 3, 7, and 8 leads to activation of transcription factors interferon regulatory factor (IRF) 3 and 7, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), leading to

expression of interferons (IFNs) and pro-inflammatory cytokines (TNFα, IL-6, IL-1β). Type I IFNs (IFNα and IFNβ) and Type III IFNs (IFNλ1-4) play important roles in the antiviral response, both in the infected cells as well as those surrounding the infection. Following expression of IFNs, they interact with their receptors IFNAR and IFNLR for type I and III, respectively, in an autocrine or paracrine manner, leading to activation of Janus kinase (JAK)–signal transducer and activator of transcription (STAT) signaling. Phosphorylated STAT1 an STAT2 along with IRF9 lead to expression of IFN-stimulated genes (ISGs). Once expressed, ISGs will then inhibit viral infection through several mechanisms: inhibiting viral entry, transcription of viral genes, genome replication, viral budding and increasing degradation of viral mRNA and proteins[443, 449].

Alveolar macrophages are critical for limiting viral spread in the lungs and regulating the initiation of adaptive immunity. Natural killer (NK) cells kill virally infected cells to prevent viral spread. Dendritic cells (DCs) along with AMs act as antigen presenting cells, with DCs migrating to draining lymph nodes to activate naïve and memory T cells. $CD8^+$ T cells will express cytotoxic activity and kill infected cells and restrict viral replication. $CD4^+$ T cells differentiate mainly into Th1 T cells and help shape immune responses through cytokine secretion (IFNγ, TNFα, IL-2) as well as help $CD8^+$ T cells and B cells produce antibodies. B cells are primarily responsible for producing influenza specific antibodies, leading to viral neutralization and elimination[443, 449].

1.5.2 Influenza Bacterial Super-infection

While influenza on its own can cause morbidity and mortality, this is enhanced when there is a secondary bacterial infection, colloquially known as super-infection. Those most susceptible to

super-infection are also those who are more susceptible to influenza (very young, very old, immunocompromised, pregnant)[450]. However, healthy children and young adults are still susceptible to morbidity and mortality due to super-infection. The most infamous situation of this is during the 1918 pandemic, where of those 50 million deaths, only a small percentage died during peak viral infection (<5%)[451]. Majority of the deaths occurred between 7- and 14-days post infection with secondary bacterial pneumonia estimated in 95% of deaths[451, 452, 453]. In the 1957 pandemic, and estimated 70% of cases were super-infected[454, 455, 456] and in the 1968 pandemic saw an estimated 48% super-infection rate[457, 458]. The large drop in super-infection between the 1918 and 1957 and 1968 pandemics is likely due to introduction of antibiotics, influenza vaccines, and improved public health measures. In the 2009 pandemic, super-infection rates are estimated between 25-50% depending on the study[310, 459, 460, 461, 462, 463]. However, super-infection is not limited to pandemic influenza, and occurs in every influenza season[464]. In seasonal influenza, super-infection is associated with increased hospitalization, morbidity, and mortality[459, 465, 466, 467]. Additionally, viral bacterial super-infections are not limited to influenza viruses, but have been characterized in other viral respiratory illnesses: respiratory syncytial virus, rhinovirus, human coronavirus, parainfluenza virus, and adenovirus[468]. However, the focus here will be on influenza super-infections.

The most prevalent bacteria involved in super-infection has changed over time. In the 1918 pandemic, the predominant bacteria was streptococcal species (primarily *S. pneumoniae* and some *S. pyogenes*), accounting for 40% of super-infection deaths[452]. In the 1957 pandemic, *S. aureus* was the predominant pathogen, accounting for 44% of deaths[469]. The 1968 pandemic had *S. pneumoniae* as the predominant bacteria accounting for 48% of super-infections[457]. Since the 2009

pandemic, *S. aureus*, with MRSA being a major contributor, has become the main super-infecting bacteria[310, 470] (Figure 3). The increase in pneumococcal vaccination has likely contributed to this shift[471].

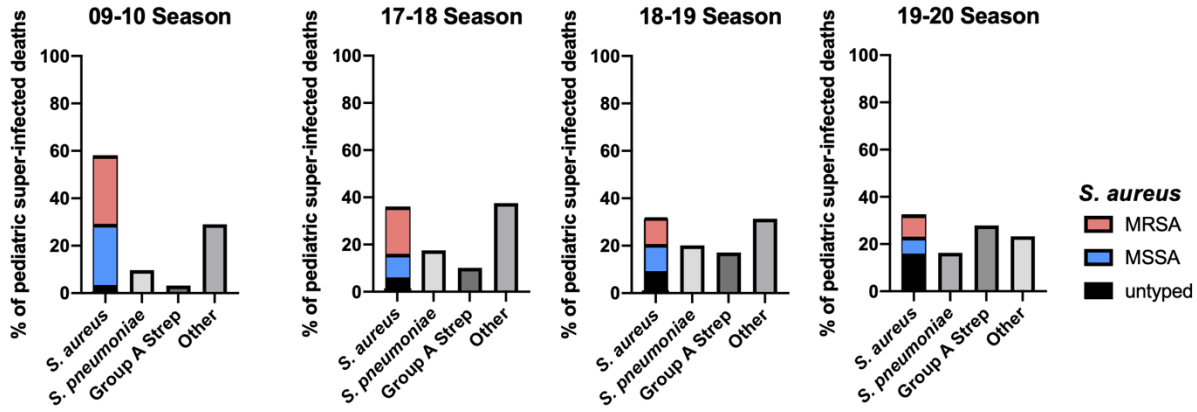

Figure 3: *S. aureus* is the Main Super-infecting Bacteria

The CDC tracks pediatric deaths from influenza each season and track data such as super-infections and the causal bacteria. Since the 2009 pandemic (most left), *S. aureus* has been the main super-infecting bacteria. This holds for the last three seasons with available data (17-18, 18-19, 19-20). The 20-21 season did not have sufficient data due to COVID19 pandemic and the 21-22 season is ongoing and data has not been compiled at time of writing. *S. aureus* data can be further broken down into methicillin-resistant *S. aureus* (MRSA), methicillin-sensitive *S. aureus* (MSSA) or untyped *S. aurues*. Data taken from FluView (https://gis.cdc.gov/grasp/fluview/pedfludeath.html).

Fatal super-infection cases generally result from a combination of severe pneumonia and acute respiratory distress syndrome (ARDS) due to high levels of inflammation. The decline from pneumonia to ARDS can occur rapidly in some cases. Descriptions from the 1918 pandemic describe healthy young individuals becoming acutely ill and dying within hours, even if they were not aware of their impending death:

> "Two hours after admission they have the Mahogany spots over the cheek bones, and a few hours later you can begin to see the Cyanosis extending from their ears and spreading all over the face, until it is hard to distinguish the coloured men from the white. It is only a matter of a few hours then until death comes, and it is simply a struggle for air until they suffocate."[472]

Viral invasion of the lower respiratory tract, in particular the alveolar epithelial cells, can drive the development of severe disease[473, 474]. Once breached, viral PAMPs and cytokines amplify inflammation through the endothelium[475] and this inflammation is only increased during bacterial super-infection. Involvement of significant portions of the lung, a combination of viral infection, bacterial infection, and immune mediated damage and inflammation, can lead to physical and physiological changes in the lung: obstruction of airways, loss of alveolar structure, loss of epithelial integrity, degradation of lung ECM, and fluid accumulation. This ultimately can lead to the inability to perform efficient gas exchange in the lung, leading to ARDS and potentially death[450, 476].

1.5.3 Lung and Immune Changes During Super-infection

Susceptibility to super-infection typically occurs five to seven days post influenza infection. On average, patients will present to the hospital 5.2 days after symptoms begin[310]. This susceptibility is multifactorial and involves changes in several different pathways ultimately creating an environment where bacteria can take a foothold, typically to higher levels than in a naïve lung[477, 478]. While it has been known for some time that viral infections, in particular influenza A, can lead to bacterial pneumonias, determining how susceptibility occurs can be difficult in humans. Because of this, most of the understanding of viral predisposition to super-

infection has been done primarily in mouse models studying viral-bacterial-immune dynamics. A majority of the research done on super-infection has focused on how influenza alters antibacterial responses in the lung. Many of the changes that occur in the immune system during the window of susceptibility can be tied back to changes in interferon signaling[479, 480, 481] . For more in-depth reviews see[482, 483, 484, 485, 486, 487, 488, 489]. Here is a brief review of the known mechanisms of susceptibility.

Physiologic changes. Many of the bacteria known to cause super-infection are nasal commensals, and inflammation from influenza has been shown to increase dissemination from the nasopharynx to the lung[490, 491]. Viral- and immune-mediated damage can lead to epithelial and endothelial disruption, which could lead to several outcomes: increase in bacterial nutrients, exposure or increased expression of cryptic adhesion receptors for bacteria, and increased exposure of ECM, which contains several components bacteria can bind[484, 490, 492, 493]. Influenza-bacterial binding has been shown to increase both internalization and adhesion of bacteria within the lung[494, 495] and has been documented for other pulmonary viruses[485, 496, 497]. This suggests that even the physiologic changes can have profound effects on bacterial invasion into the lungs. This is supported by a study that found super-infecting *S. aureus* isolates to be less virulent as well as more closely related to nasal colonizing strains than those found in bacterial pneumonia alone[498].

Disruption of innate immunity. Influenza causes dysregulation of AMs and neutrophils, limiting their ability to eliminate bacteria from the lung. Influenza is known to cause depletion of AMs from the lung, and inflammatory monocytes are recruited to fill the niche[499]. As described elsewhere in this dissertation, loss of AMs can lead to dysregulation of immune responses in the

lung, which likely compounds the loss of these cells. Macrophages also have lower expression of MARCO and other scavenger receptors[480, 486]. Super-infection typically has a higher recruitment of neutrophils, which is correlated with mortality likely due to inflammation and damage[477, 500]. This is likely increased further due to bacterial toxins known to lyse phagocytes. There is also an impairment of bacterial killing in both macrophages and neutrophils due to inhibition of NADPH oxidase[501, 502]. TLR differences have been reported, with TLR4 desensitized in *S. pneumoniae* super-infection[503] and TLR9 increasing susceptibility in *S. aureus* superinfection[504]. In *S. aureus* super-infection, NETosis has been shown to be increased in super-infection, however it does not help to control bacterial numbers and likely adds to lung damage[505].

Interferons also play a large role in super-infection susceptibility. Influenza induces type I (IFNα and IFNβ) and type III IFN (IFNλ) early on during infection[506, 507]. Type I IFNs lead to increases in bacterial burden during super-infection, which is abrogated in mice lacking the type I IFN receptor (IFNαR1)[477, 479]. This also appears to happen with type III IFN[481]. This promotes pro-inflammatory cytokines IL-6, TNFα and immunosuppressive IL-10, altering the overall immune environment[485]. Type II IFN, IFNγ, also plays a role in super-infection and can inhibit phagocytosis responses in AMs[480, 508]. While IFNγ can increase macrophage phagocytosis and killing of bacteria[508, 509], the large amounts of IFNγ appear to be inhibitory[480, 486]. IFNs are known to inhibit type 17 immunity, an important arm of the immune system for controlling extracellular bacterial infections[477, 510] (see below).

Disruption of adaptive immunity. The antiviral response is Th1 dominant, with major cytokines being IFNγ, IL-12, and TNFα. Natural killer (NK) cells, $CD4^+$ T and $CD8^+$ T cells,

heavily involved in Th1 immunity, are critical to clearance of viral infections. However, as described above, increased levels of IFNγ can increase super-infection susceptibility. Additionally, IFNγ can also inhibit TNFα made by NK cells, which can reduce macrophage uptake and killing even further[511]. Type 2 (Th2) responses are predominantly associated with allergies and tissue homeostasis, but also play a role in super-infection susceptibility. Type 2 cytokine IL-13 can be protective due to its ability to attenuate IFNγ early on during influenza, but later on is downregulated and likely influences the high levels of IFNγ[512]. Immunosuppressive cytokine IL-10 can also increase susceptibility to super-infection and is regulated through IL-27, which can inhibit Th17 responses. Additionally, it has been shown that type 2 innate lymphoid cells (ILC2s) have a protective role against super-infection[445].

Type 3 responses (also known as type 17; Th17) are critical for clearance of extracellular pathogens including bacteria and is characterized by the cytokines IL-17, IL-23, and IL-22[513]. Both IL-17 and IL-22 promote clearance of bacteria through the recruitment of phagocytes and induction of AMPs. Th1 immunity is known to suppress Th17 immunity, and this is a major contributing factor to super-infection susceptibility. Influenza infection attenuates the production of IL-17 by both $CD4^+$ and γδ T cells and exogenous treatment with IL-23 or IL-1β rescues bacterial clearance[477, 510, 514]. IL-22 is also protective during super-infection and can be produced by type 3 ILCs[515, 516].

1.6 Gap of Knowledge

While there has been much work done elucidating the immune responses in the lung to both bacterial pneumonia and influenza bacterial super-infection, little is known about bacterial behavior within the lung. In the context of *S. aureus*, there is epidemiological data supporting the study of this pathogen in the lung as described above. *S. aureus* can cause severe pneumonia and often complicates influenza infections, leading to morbidity and mortality, as well as a large financial burden on the healthcare system[517]. The emergence of MRSA over the past few decades has only complicated treatment of staphylococcal pneumonia and super-infection, due to antibiotic resistance and increased virulence compared to MSSA. Thus, understanding how this pathogen can invade and establish infection in the lung is important not only for the pursuit of knowledge, but also for development of future therapeutics that will benefit society. Additionally, discovery of virulence factors in the lung may extend beyond this organ, as virulence factors are often implicated in many infection sites (see 1.2.2 above).

Based on previous work in super-infection, it is known that antiviral immunity will inhibit antibacterial immunity within the lung. There have been several mechanisms described as underlying causes of increased susceptibility to secondary bacterial infection, such as the inhibition of Th17 immunity via Th1 responses, depletion of AMs, and reduction of bactericidal mechanisms[482, 483, 486]. There also has been work done in looking at physiological differences in the lung during bacterial pneumonia and influenza super-infection. Influenza induces damage to epithelial and endothelial cells within the lung, likely making it easier for bacterial pathogens to adhere and obtain nutrients[484]. Additionally, there is known synergism that can occur between viruses and bacteria in the lung. Specifically for influenza, viral-bacterial binding can increase

bacterial internalization and viral and bacterial virulence factors can synergize[343, 484, 490]. However, the largely unexplored aspect of super-infection is the bacteria itself. Does the bacterium itself play a role in super-infection susceptibility or is this only dependent on the immunological and physiological differences seen in the lung? Additionally, how does the presence of a viral infection alter the behavior of bacteria to ensure survival?

Due to the complicated nature of the super-infection model, understanding how virulence works in bacterial pneumonia can help contextualize bacterial responses during super-infection. Additionally, it is important to study on its own outside of the context of super-infection as bacterial pneumonia can lead to negative outcomes. How does *S. aureus* establish infection in the lung? Is persistence solely dependent on bacterial adhesion and immune evasion, or is there another unknown mechanism that plays a role? How can *S. aureus* induce enough damage within the lung to lead to necrotizing pneumonia? Is the damage more dependent on the immune response to *S. aureus* or *S. aureus* itself? Are there *S. aureus* pathways that could be targeted to prevent or treat pneumonia as well as super-infection?

Most of the work presented here looks specifically at the role of CWAs in the context of lung infections. CWAs have well described functions in both colonization and infection and can contribute through several different mechanisms (see Table 1 and Figure 2). As many of these proteins have described roles in nasal colonization[22, 63, 518], it is reasonable to hypothesize that these proteins may play a role in the lower respiratory tract. Staphylococcal protein A, a CWA, is one of the major virulence factors described in the lung[232, 233, 234, 235, 236]. While there are no other known CWAs that have similar structure or function to SpA, there are 23 other CWAs that *S. aureus* can

express and these likely influence pulmonary infections to some degree. In the context of bacterial pneumonia, do CWAs play a role in establishing infection, and if so, what are the mechanisms by which these proteins work? In the context of super-infection, does a preceding viral infection alter the requirement for these proteins in the lung? Can there be therapeutics developed against these proteins to help treat staphylococcal pneumonia or super-infection?

Herein, I begin to explore staphylococcal virulence in the lung in both *S. aureus* pneumonia and influenza *S. aureus* super-infection.

2.0 Developing Methods for *S. aureus* Transposon Sequencing (Tn-seq) in the Lung

2.1 Introduction

Community-acquired and healthcare-associated pneumonia, caused by both viral and bacterial pathogens, is the leading cause of death in children worldwide[519]. Historically, staphylococcal pneumonia was considered an uncommon cause of community-acquired pneumonia (CAP), accounting for 1-10% of CAP cases[275]. However, the increase in prevalence of methicillin-resistant *S. aureus* (MRSA), and especially community-acquired MRSA (CA-MRSA), has led to an increase in morbidity and mortality[314, 520]. Although MRSA more commonly causes hospital-acquired or healthcare-associated pneumonia, CAP now accounts for 30% of all MRSA pneumonia cases and can have 30-day mortality rates as high as 30%[520, 521]. These outcomes are due in part to the lack of treatment options for clinicians, which consist only of antibiotics and occasionally corticosteroids[522]. Although several papers have looked at major virulence factors and regulators expressed by *S. aureus*/MRSA during pulmonary infection, the mechanisms controlling establishment and persistence of *S. aureus* in the lung are still unknown.

While *S. aureus* has always been highly associated with influenza infections in the young and the elderly, it has emerged as the major cause of pneumonia associated with influenza since the 2009 H1N1 pandemic[523]. Although influenza can be fatal alone, severe influenza pneumonia is often exacerbated by bacterial infection resulting in poor patient outcomes even in previously healthy individuals. In the 1918 pandemic, which killed an estimated 50 million people, 95% of those deaths were caused by secondary bacterial infections[452, 524]. In the 2009 H1N1 pandemic,

25-50% of hospitalized patients were super-infected with bacterial pneumonia, with *S. aureus* causing 74.4% of super-infections in critically ill patients and 38.7% of super-infected pediatric deaths during the pandemic (CDC). Several studies have been published investigating *S. aureus* influenza super-infections but have focused on the effects of antiviral immunity on bacterial clearance, with few papers focusing on *S. aureus'* role in super-infection[18]. However, there is literature suggesting that *S. aureus,* as well as other lung pathogens, can have differential expression of genes based on the lung environment, although this has not been actively explored[525, 526, 527, 528].

One way to elucidate virulence mechanisms *in vivo* is through transposon sequencing (Tn-seq). Transposon sequencing is a technique that combines transposon mutagenesis with sequencing to track individual strains in a large pool of bacterial mutants[529]. High density transposon mutant libraries have been used to characterize essential and conditionally essential genes in a variety of bacterial species and infection models[530, 531, 532, 533]. Additionally, this technique can also identify host genes involved in pathogenesis by changing the environment and keeping the pathogen constant and comparing gene fitness of the pathogen in both conditions[529, 534]. This has been done for other bacterial species in bacterial pneumonia and influenza super-infection to help understand how the lung environment differs in each infection type[526, 535, 536]. Thus, this is a method that may help to elucidate virulence mechanisms that differ in staphylococcal pneumonia and influenza *S. aureus* super-infection. Here we develop a model for using *S. aureus* Tn-seq in the lung.

2.2 Materials and Methods

2.2.1 Mice

Six- to eight-week-old male WT C57BL/6NTac mice were purchased from Taconic Farms. Mice were maintained under pathogen-free conditions within the animal facilities at the UPMC Children's Hospital of Pittsburgh. All studies were performed on sex- and age-matched mice. Animal studies were conducted with approval from the University of Pittsburgh Institutional Animal Care and Use Committee.

2.2.2 Infections

Influenza A/PR/8/34 (H1N1) was grown in chicken eggs as previously described[537]. Methicillin-resistant *S. aureus* (MRSA) strain USA300 was used. TnMRSA[530] was a gift from our collaborator Anthony Richardson at the University of Pittsburgh. For infection, one aliquot corresponding to $1x10^{10}$ CFU was grown overnight in 100 ml of Tryptic Soy Broth (TSB) (BD Bacto™) overnight at 37° C at 250 rpm. MRSA and TnMRSA dose were calculated using OD_{660} measurement of the culture and application of a calculated extinction coefficient. For pneumonia studies, mice were inoculated with varying CFU (see figures) of MRSA or TnMRSA in 50 µl of sterile PBS. For super-infection, mice were inoculated with 100 plaque forming units (PFU) of influenza in 50 µl of sterile PBS and were infected six days later with MRSA or TnMRSA as described above. All infections were performed via oropharyngeal aspiration. Lungs were harvested 24 hours after MRSA challenge using pentobarbital injection (300 mg/kg) and cervical

dislocation. Whole lungs were separated into lobes, placed in 2 ml of sterile PBS, and homogenized. Epithelial cells were removed by centrifugation at 2000 rpm for 5 minutes and the bacteria were subsequently pelleted at 10,000 xg for 5 minutes. The bacteria pellet was resuspended in TSB and incubated at 37 C at 250 rpm for 5 hours. Bacteria were pelleted at 10,000 xg for 10 minutes, resuspended in 1 ml of 8% DMSO TSB, and frozen until DNA extraction.

2.2.3 Tn-seq Library Preparation and Sequencing

Methods were derived from[530, 538]. DNA extraction was performed using DNeasy Blood and Tissue kit (Qiagen) using the bacteria protocol and 0.5 mg/ml of lysostaphin (Sigma) in combination with lysozyme. After extraction, DNA was sonicated for 20 seconds at 25% intensity and placed on ice and repeated for a total of eight times. DNA was purified using QIAquick PCR purification kit (Qiagen) following manufacturer's protocol. A 3' poly-C tail was added using dCTP and ddCTP (Roche), enzyme Terminal Deoxynucleotidyl Transferase (Promega) and incubated for 1 hour at 37 C followed by heat inactivation for 20 minutes at 75 C. Excess nucleotides were removed using DTR Gel filtration cartridges (EdgeBio) following manufacturer's directions. Primer sequences are listed in Table 2. PCR1 was performed with primers to the poly-C tail and to the transposon sequence. PCR2 was performed using olj511 and index barcoding primers. Final PCR products were purified using AMPure XP PCR purification magnetic beads (Beckman Coulter) to selectively remove small and large fragments and subsequently concentrated using QIAquick PCR purification kit (Qiagen). Samples were pooled and sequenced using the NextSeq 500/550 High Output kit (75 cycles) (Illumina). Tn-seq library preparation confirmation was conducted using TOPO™ TA Cloning™ kit for sequencing (Invitrogen) following manufacturer's directions. Sanger sequencing was performed by Genewiz.

2.2.4 Statistics

Data were analyzed using Prism 8 (GraphPad). Analyses comparing two groups were performed using an unpaired t test. For analyses assessing more than two groups, Kruskal-Wallis with Dunn's multiple comparisons correction was used. Analyses comparing two variables were tested via Two-way analysis of variance (ANOVA) with Sidak's multiple comparisons correction. All figures show combined data from multiple replicate studies and are graphed as mean ± standard error of the mean (SEM). N values are numbers of animals per independent experiment. Statistical significance ($p \leq 0.05$) is indicated in figure legends, with p values between 0.05 and 0.1 displayed numerically.

2.3 Results

2.3.1 Transposon Sequencing (Tn-seq) Methodology Background

Tn-seq is done by constructing a transposon insertion library in which each clone contains a transposon insertion at a different site so that most or all nonessential genes contain insertions (and therefore gene disruptions) in the library population[529]. The library is then grown under an experimental condition and the relative frequency of each mutant in the population is determined at the end of the experiment by next-generation sequencing of the transposon junctions (Figure 4 A). The number of sequence reads for a particular insertion corresponds to the frequency of the insertion mutant in the population and allows the fitness contribution of each gene to be quantified

by comparing the frequency of transposon junction reads before (input) and after the experiment (output) (Figure 4 B)[529, 539].

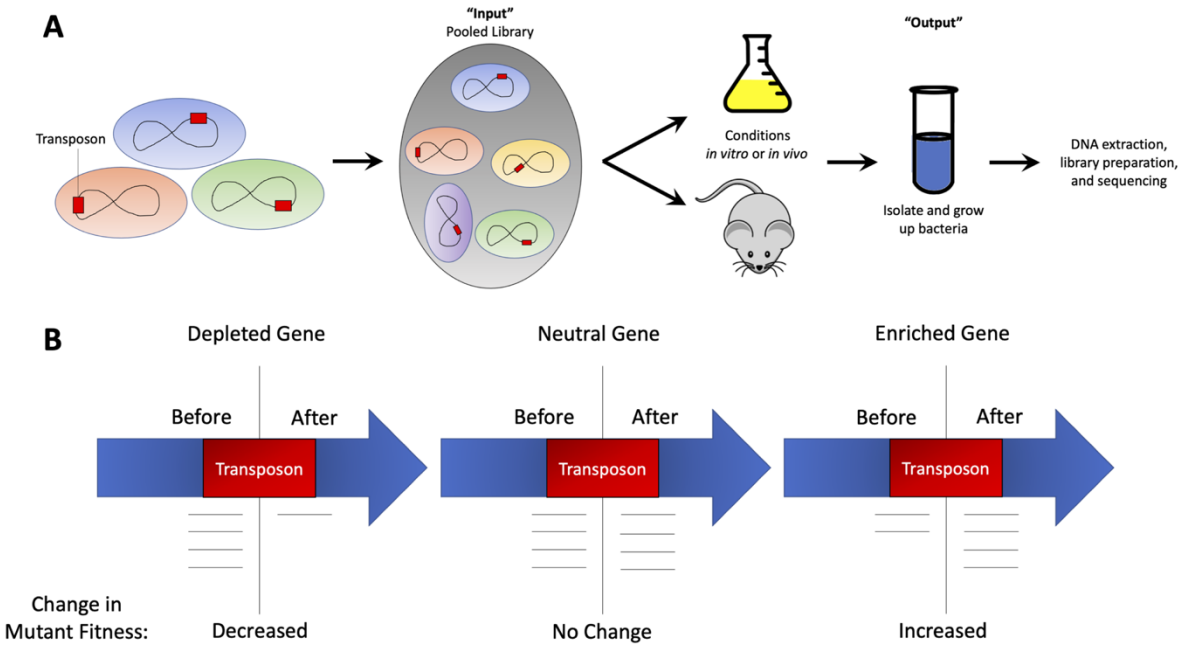

Figure 4: Transposon Sequencing (Tn-seq) Methodology

A. A high-density transposon insertion library contains multiple insertions in nonessential genes where each individual mutant contains an insertion in a non-essential gene. The library is pooled and grown under conditions of interest. Viable mutants are recovered and grown up over a short time period to increase bacterial numbers for downstream library preparations. The library serves as the "input" which is compared to the "output" post-condition. B. After sequencing, the relative frequency of transposons within a gene are compared between input and output pools. An unaffected gene would have similar relative frequency between the input and output pools since there is no selective pressure at that loci. In a depleted gene, the relative frequency of reads is decreased in the output (the gene is important in the condition), with the opposite for an enriched gene.

We used a previously constructed MRSA USA300 strain (LAC) Tn-seq library (TnMRSA)[530]. This library consists of approximately 77,000 mutants containing the transposon

bursa aurealis and is selectable by erythromycin resistance. After infection, we modified already existing Tn-seq processing protocols[530, 538] (Figure 5; see methods). Briefly, after purification of genomic DNA from pooled TnMRSA mutants, the DNA was fragmented via sonication. After several rounds of amplification and addition of PCR primer encoded Illumina adapters, the transposons were sequenced.

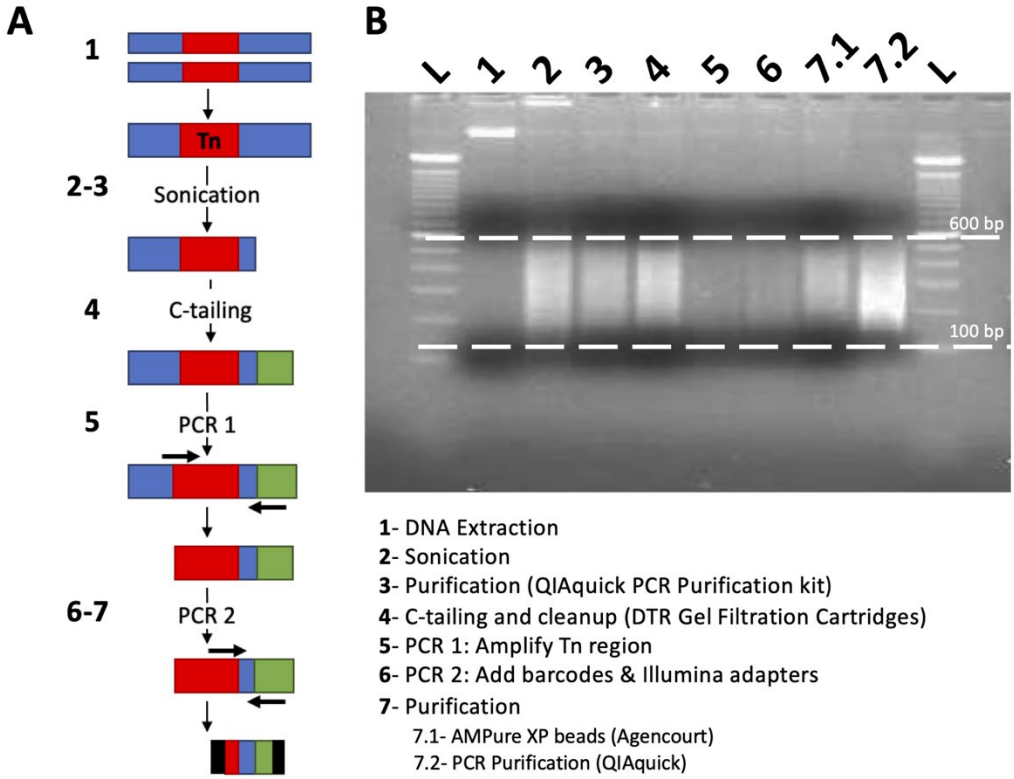

Figure 5: Tn-seq Library Preparation

Pictorial representation (A) or agarose gel image (B) of Tn-seq library preparation. Purification of genomic DNA from a pooled population of mutants is fragmented via sonication. The addition of a poly-C tail adds a site for primer annealing in PCR 1. Amplification of transposon junction sites using a poly-G and Tn-specific primers is performed in PCR 1. PCR 2 adds a barcode index for multiplexing as well as Illumina adapters for sequencing[7,8].

2.3.2 Determining Dosage of TnMRSA in Staphylococcal Pneumonia and Influenza Super-infection.

One of the limitations of Tn-seq is determining if transposon mutants are deleted due to bacterial killing or stochastic removal during the establishment of infection[529]. To prevent bottlenecks in the host, a large enough infectious dose must be given so that there are 10-100 cells

of each transposon insertion in the inoculum[534, 539]. Thus, for a ~77,000 mutant library, the minimum dose for proper recovery of Tn mutants should be ~$7.7x10^5$ CFU. As *S. aureus* is rapidly cleared from the murine lung compared to other bacterial species such as *S. pneumoniae* (Figure 6 A; personal observation), we wanted to use an inoculum that was a few logs higher to ensure enough bacterial recovery for sequencing. Preceding influenza is known to increase bacterial burden in the lung[477], and we wanted similar recovery rates from pneumonia and super-infected animals to ensure that differences seen in sequencing were due to actual physiological differences in the host and not due to differences in bacterial recovery numbers. After comparing infection of mice with USA300 MRSA and TnMRSA, we determined dosages for both staphylococcal pneumonia and influenza super-infection (Figure 6 B and C). At the dosages tested, we had similar whole lung bacterial burden as well as morbidity measured by weight loss. We decided to continue with a TnMRSA dose $1x10^8$ CFU and Flu-TnMRSA doses $5x10^7$ and $1x10^8$. We continued with two Flu-TnMRSA doses as our n was small as well as to ensure we had enough mice survive super-infection for sequencing.

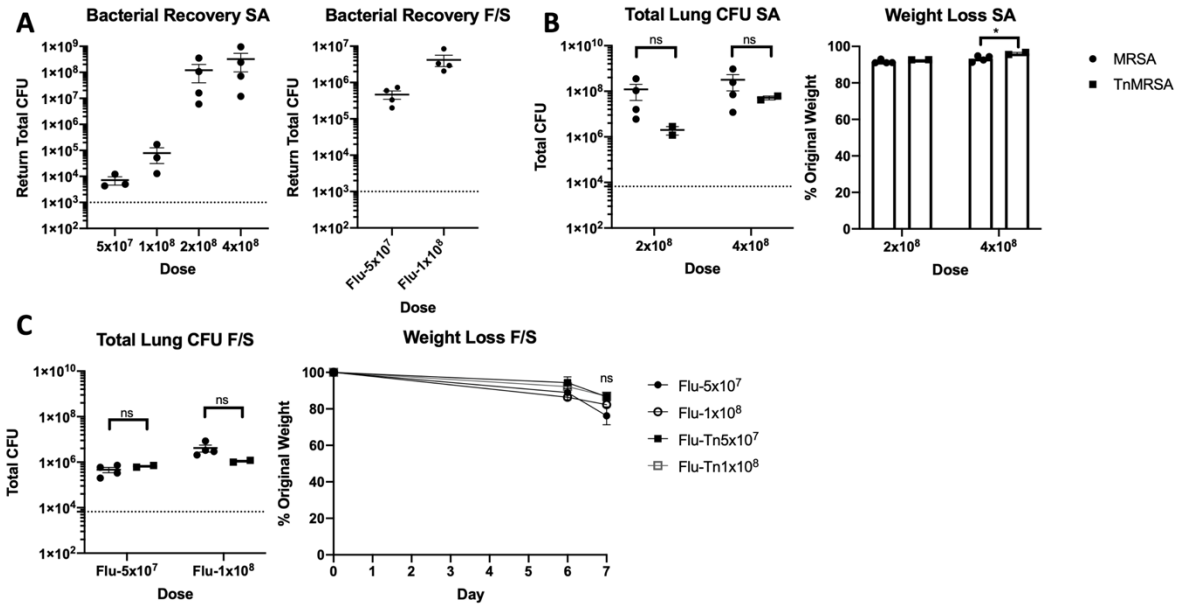

Figure 6: Determining TnMRSA Dosage for Lung Tn-seq

A. Bacterial Recovery in MRSA pneumonia (SA) or MRSA influenza super-infection (F/S) after 24 hours of infection. B. Total lung CFU and weight loss of mice infected with MRSA or TnMRSA. C. Total lung CFU and weight loss of mice infected with influenza for 6 days followed by infection with MRSA or TnRMSA for 24 hours. Statistics tested by Two-way ANOVA with Sidak's multiple comparison correction. * $p<0.05$. N=2-4, combination of 1-2 experiments, data graphed as mean ± SEM (standard error of the mean).

2.3.3 TnMRSA Infection During Pneumonia and Super-infection

After determining our dosages for both pneumonia and super-infection, we performed Tn-seq experiments with more animals including PBS controls. Compared to PBS controls, mice infected with TnMRSA had high bacterial burdens and lost weight (Figure 7 A). This held for Flu-TnMRSA infected animals as well, although weight loss was not significantly different in the Flu-PBS group (Figure 7 B). This was to be expected as a majority of the weight loss during super-

infection occurs due to influenza. After comparison of the one TnMRSA and two Flu-TnMRSA dosages, we determined that moving forward a Flu-TnMRSA dosage of $5x10^7$ was sufficient for similar bacterial recovery and weight loss after accounting for influenza-mediated weight loss (Figure 7 C). After recovery from the lungs, Tn-seq bacteria were grown *in vitro* for five hours to ensure enough DNA for sequencing. After this time, we saw high bacterial numbers that should be sufficient for sequencing (Figure 7 D).

Unfortunately, after Tn-seq processing, we were unable to obtain our sequence data for analysis. This was due to our processed DNA not binding to the Illumina flow cell (Figure 8 A) despite presence of DNA. This was later discovered to be due to our adapter-containing primers not being full length (Figure 8 B). This was due to ordering the incorrect purification method for our primers (standard desalting) which does not guarantee that all primers are full length versus more stringent purification methods used for sequencing primers (high performance liquid chromatography (HPLC) or polyacrylamide gel electrophoresis (PAGE)). Additionally, another group published *S. aureus* Tn-seq within the lung[540], although they focused solely on *S. aureus* pneumonia. Due the technological issues, graduate student career timing, and an already existing publication, this project was not pursued further.

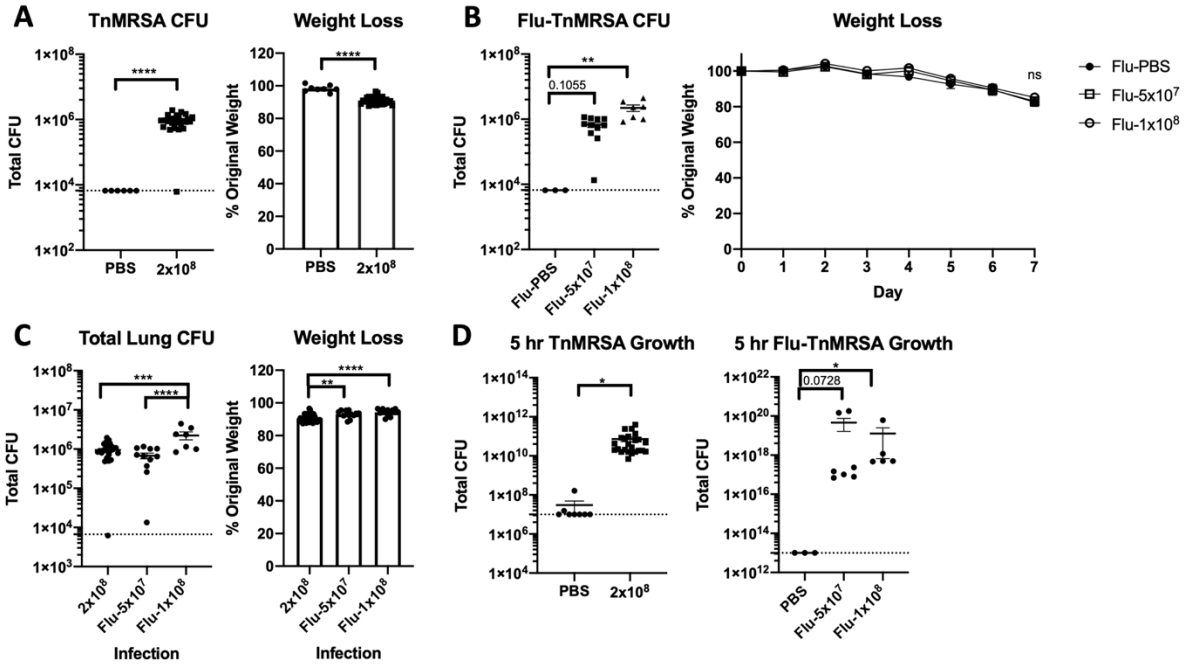

Figure 7: Tn-seq Studies in the Lung

A. Total lung CFU and weight loss in mice infected with PBS or TnMRSA for 24 hours. B. Total lung CFU and wight loss in mice infected with influenza for 6 days followed by PBS or TnMRSA infection for 24 hours. C. Comparison of Total CFU of TnMRSA and weight loss due to TnMRSA independent of infleunza weight loss of TnMRSA infections (same data as A and B). D. Total CFU of TnMRSA after 5 hours of growth. Statistics tested by unpaired T test (A,D left panel), Kruskall Wallis with Dunn's multiple comparisons correction (B left panel, C, D right panel), or Two-way ANOVA with Sidak's correction (B right panel). $p<0.05$, ** $p<0.01$, ***$p<0.001$, ****$p<0.0001$. N=6-8 (2 for PBS groups), combination of 3 experiments, data graphed as mean ± SEM (standard error of the mean).

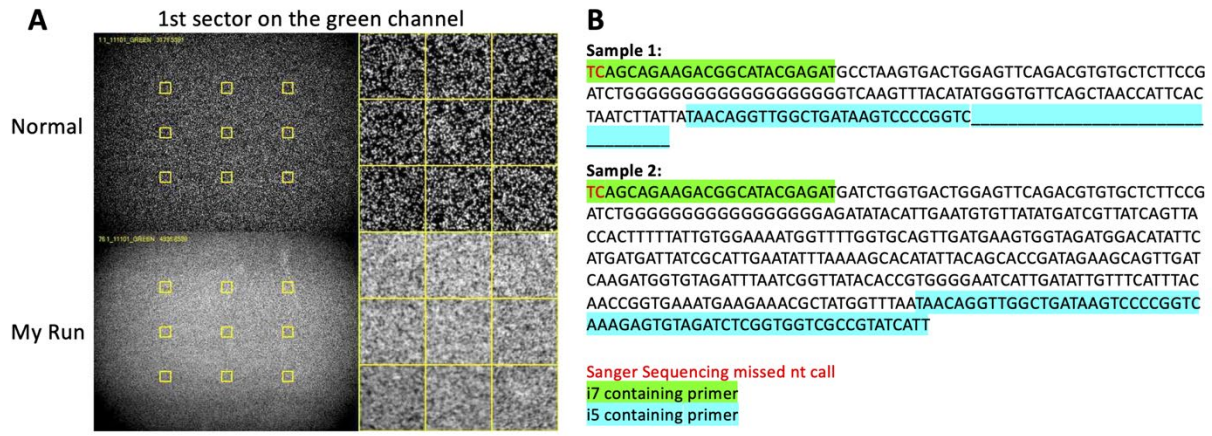

Figure 8: Tn-seq Sequencing Failed Due to Lack of DNA Binding

A. Image of the flow cell in the green channel during sequencing of my run versus a typical run. B. Analysis of DNA sequences revealed that a majority of DNA fragments did not have full length primers (Sample 1) that contain the Illumina adapter sequences need for binding the flow cell (Sample 2).

2.4 Discussion

Transposon sequencing is a powerful technique to elucidate virulence mechanisms *in vivo* that may be difficult to discover otherwise. To date, this technique has been used for several lung pathogens[525, 533, 540, 541, 542, 543, 544, 545]. Many of these studies have found similar requirements for pathways involved in persistence within the lung. The bacterial pathways that appear to be most impacted by the lung environment are amino acid and nucleic acid metabolism and biosynthesis[525, 540, 542, 543, 544, 545]. This is not surprising, as amino acids and nucleic acids are not only part of macromolecules needed by the bacteria, but also that these compounds feed into several different biochemical pathways. In *Klebsiella pneumoniae* infection, it was found that branched-chain

amino acid metabolism is not only important for nutrient purposes, but also plays a role in copper toxicity[544]. In *P. aeruginosa*, pyrimidine biosynthesis is tied to biomass formation in the presence of fluoroquinolone treatment[545]. This is supported by other studies that have found similar requirements in other pneumonia-causing bacteria[546, 547, 548]. Other pathways include cell wall/membrane synthesis, serum resistance, oxidative stress, and classical virulence factors[525, 540, 542]. This suggests that bacterial persistence within the host requires adaptation to a nutrient-poor, high stress environment while still replicating and producing virulence factors.

The *S. aureus* pneumonia Tn-seq found similar findings[540]. The major required clusters of orthologous groups (COGs) included energy production and conversion, nucleotide transport and metabolism, posttranslational modifications, and translation. Mutants in central metabolism also had defects in biofilm formation, arachidonic acid survival, and macrophage extracellular and intercellular survival. Interestingly, extracellular acidification rate of mutants correlated with intercellular survival in macrophages. This further suggests that metabolism is central to many mechanisms of *in vivo* virulence, especially for those related to growth *in vivo* and tolerance to host immune defenses.

There have also been a few super-infection Tn-seq studies performed[526, 535]. When comparing *Haemophilus influenzae*[536] and influenza, *H. influenzae*[526] Tn-seq studies, this group found that there were many genes that had overlapping requirement in both infections. In the influenza, *S. pneumoniae* paper[535], the authors found that of the 24 genes identified, no genes were essential for *S. pneumoniae* or super-infection alone. This supports the idea that core metabolic functions are required in both settings. However, the *H. influenzae* super-infection paper still found differential requirements for some metabolic cofactors/coenzymes as well as genes involved in environmental

stresses[526]. The differences in these studies may be due to the depth of transposon mutagenesis; *S. pneumoniae* library had 2000 mutants while the *H. influenzae* library had ~75,000. Despite this, both groups suggest that the influenza-induced changes in the lung did affect how the bacteria respond to their environment. It is thought that the influenza-infected lung has more available nutrients for bacteria due to the cellular damage[484]. Indeed, this is seen with a putative *S. pneumoniae* aminotransferase mutant (*ΔPA*) that grew better in the presence of influenza-infected lung homogenate compared to naïve lung homogenate[535]. Similar changes are likely to occur in super-infection with *S. aureus*. Core metabolism pathways are likely still required for survival in the lung, but there may be reduced requirements for particular pathways that have abundant nutrients in an influenza-infected lung. For example, *H. influenzae* does not require histidine biosynthesis during super-infection, likely due the increased availability from influenza infection[526]. Other amino acids may also be more available to bacteria in the influenza-infected lung such as proline and serine as these are released from cultured epithelial cells infected with influenza[549].

There are also likely differences in the host response to bacteria, which has been described extensively in the super-infection literature[482, 486]. During super-infection, *H. influenzae* does not require a glycosylation pathway needed to avoid complement, whereas it is required during mono-infection[526]. *S. aureus* can express a capsular polysaccharide, although USA300 does not express this[9, 19] and so it would not be detected using the *S. aureus* Tn-seq library used here or in the published study[540]. Additionally, many of the known virulence proteins would not likely show up in a Tn-seq screen based on the nature of the technique. *S. aureus* produces several secreted factors that have known roles in immune evasion[9, 19, 260], however these would not be detected using this

method as Tn-seq is unable to detect genetic requirements for secreted proteins[530]. However, there may be unknown connections to metabolism pathways that influence immune avoidance, as seen in the *S. aureus* pneumonia study[540].

While we were unable to obtain sequencing results from these studies, we now have the methodology to continue this project in super-infection. Infection dosages and Tn-seq processing has been optimized in our laboratory. A caveat to our data is the high limit of detection of bacterial counts due to inefficient plating that was done. This is a combination of limited graduate student experience at the time these studies were performed as well as using 10 µl "dot plating" versus conventional 100 µl spread plates for bacterial enumeration. This likely explains the high CFU reading of the PBS groups post-infection and post-5-hour growth. If repeated, bacterial enumeration should be done on spread plates for more accurate numbers. Additionally, mice in our facility can carry *S. epidermidis* and this bacterium was present on many of the plates counted (personal observation). In the future, mice should likely be dipped in ethanol to prevent contamination from their commensal flora for more accurate Tn-seq results. An additional control that should be added to these studies is a 24-hour *in vitro* growth control in TSB, the media the library was constructed in. While this was performed in some of the Tn-seq studies, analysis of growth via plating was not conducted.

3.0 Screening *S. aureus* Mutants in Bacterial Pneumonia and Influenza Super-infection

3.1 Summary

In the respiratory tract, *Staphylococcus aureus* is associated with infections ranging from asymptomatic colonization to severe necrotizing pneumonia. While staphylococcal pneumonia has always been highly associated with influenza infections, it has emerged as the major cause of pneumonia associated with influenza since the 2009 H1N1 pandemic. This is partially due to the increase in prevalence of community-associated methicillin-resistant *S. aureus* (CA-MRSA). Despite the clinical relevance of *S. aureus*, little is known about which *S. aureus*/MRSA factors are important in pneumonia and influenza super-infection. Successful establishment of infection by bacterial pathogens requires adhesion to host cells. Staphylococcal species express a broad range of surface proteins that are involved in adhesion to host cells, known as cell wall-anchored proteins (CWAs). CWA proteins have known roles in upper respiratory tract colonization, however their role in the lung is unknown. Therefore, we seek to understand the role of these proteins in the context of lung infection in both MRSA pneumonia and influenza MRSA super-infection. We screened several CWA mutants in the context of MRSA pneumonia and influenza MRSA super-infection to determine their roles in both contexts. Here, we find that CWAs have variable effects in bacterial pneumonia, with changes seen in bacterial burden, immune infiltrate, and lung injury. However, influenza increases these readouts regardless of CWA mutant, to similar levels as influenza alone. Only the inflammatory signature of each CWA mutant stays consistent in both infections. This supports that influenza is the main driver of super-infection and that CWAs likely play a minor role.

Part of the data presented here is found in "Novel Requirements for Staphylococcal Cell Wall-Anchored Protein SasD in Pulmonary Infection" by Jennifer A. Grousd, Abigail M. Riesmeyer, Vaughn S. Cooper, Jennifer M. Bomberger, Anthony R. Richardson, and John F. Alcorn. Currently found on BioRxiv (https://www.biorxiv.org/content/10.1101/2022.04.01.486802v1).

3.2 Introduction

Influenza is a seasonal respiratory virus that causes an estimated 294,000-518,000 deaths worldwide each year[550]. In the 2018-2019 season, there was an estimated 29 million influenza cases in the United States alone[551]. A frequent complication of influenza is secondary bacterial pneumonia (colloquially referred to as super-infection), which has higher morbidity and mortality than influenza alone[448, 552, 553]. Samples from the infamous 1918 influenza pandemic showed that a majority of deaths were caused by secondary bacterial pneumonia[452]. This was again seen in the 2009 H1N1 pandemic, with about a third of influenza fatalities associated with some bacterial infection[554, 555]. Since 2009, *Staphylococcus aureus* is a major contributor to influenza bacterial super-infections[310, 461, 556, 557]. *S. aureus* increases the risk of morbidity and mortality, with community circulating methicillin-resistant *S. aureus* (MRSA) likely playing a role[558, 559, 560].

Much of the literature of super-infection focuses on the immunological differences in antibacterial immunity that occur with a preceding influenza infection. In general, the antiviral response to influenza will inhibit the clearance of a bacterial infection through mechanisms such as increased interferon production, increased alveolar macrophage death, and downregulation of bacterial pattern recognition receptors[482, 484, 490, 561]. There are also physiological differences that

occur due to influenza infection that may increase susceptibility to secondary bacterial infections. Many of the bacteria known to cause super-infection are nasal commensals, and inflammation from influenza has been shown to increase dissemination from the nasopharynx to the lung[490, 491]. The virus itself, and the immune response to the virus, can lead to epithelial and endothelial damage, which could lead to increased nutrient resources as well as potentially expose cryptic receptors for bacterial adherence to cells or basement membrane components[484, 490, 492, 493]. Once the bacteria are attached, bacterial toxins could synergize with influenza to cause even further damage and inflammation in the lung, potentially leading to the increased morbidity and death seen in super-infection[343, 484, 490].

Although there has been much work elucidating the mechanisms of super-infection in terms of immunological and physiological dysregulation, not much work has been done focusing on the bacterial side of this equation. For *S. aureus* specifically, there are few major virulence factors described within the lung, even within the context of *S. aureus* pneumonia. Because the super-infection literature suggests that influenza can influence bacterial adherence, we decided to explore surface proteins of *S. aureus*, collectively known as the cell wall-anchored proteins (CWAs), since many of these proteins have known roles in adhesion[22, 518]. *S. aureus* can express up to 24 CWAs, with the most prevalent subfamily known as microbial surface component recognizing adhesive matrix molecule (MSCRAMM) proteins[22, 518]. These CWAs are secreted via the Sec apparatus and are covalently attached to the cell wall via the Sortase A enzyme that recognizes the cell wall sorting motif LPXTG[22, 23, 25, 562]. Because these are surface exposed proteins, they are in direct contact with the host and have a variety of known functions such as host adhesion, biofilm formation, immune evasion, and nutrient acquisition for both colonization and invasive infection[22,

[518]. Some of the CWAs have been described to play a part in nasal colonization[22, 63, 518]. These proteins have also been targets of vaccination and monoclonal antibody efforts against *S. aureus*[22, 563, 564]. Because these CWA proteins play an important part in *S. aureus* colonization and infection, we decided to screen several CWA members in the lung in both bacterial pneumonia and influenza super-infection. Our results support the literature findings that the increase in bacterial burden and immune responses during super-infection is primarily driven by influenza infection.

3.3 Materials and Methods

3.3.1 Mice.

Six- to eight-week-old male WT C57BL/6NTac mice were purchased from Taconic Farms. Mice were maintained under pathogen-free conditions within the animal facilities at the UPMC Children's Hospital of Pittsburgh. All studies were performed on sex- and age-matched mice. Animal studies were conducted with approval from the University of Pittsburgh Institutional Animal Care and Use Committee.

3.3.2 *S. aureus* strains.

USA300 MRSA strain JE2[565] was the WT strain for all studies. All strains used in study are listed in Table 3 and are derived from the Nebraska Transposon Mutant Library[565] (BEI Resources), with strains *srtA::Tn, sdrE::Tn, sdrC::Tn, fnbB::Tn, isdB::Tn, sasG::Tn,* and

sasD::Tn gifted from Dr. Ken Urish, University of Pittsburgh. All mutants were confirmed by PCR using gene-and transposon-specific primers in Table 4. *S. aureus* strains were grown in Tryptic Soy Broth (BD Bacto^{TM}) overnight at 37^{o} C at 250 rpm. Overnight cultures were diluted 1:100 and grown until OD_{660}~1, approximating logarithmic growth phase. MRSA dose was calculated using OD_{660} measurement of the culture and application of a calculated extinction coefficient. For growth curves, overnight cultures were diluted 1:200 in a 96-well plate in sexaplicate. Plates were grown at 37^{o}C at 282 rpm in a Synergy H1 Hybrid Multi-Mode Reader (BioTek). Optical density measurements at 660 nm were taken every 30 minutes. Growth rate (μ) was calculated from at least two independent experiments using the equation $A_t=A_{t-1}*e^{\mu t}$. The μ_{max} was calculated as the average of the three highest μ rates.

3.3.3 Murine Models.

Influenza A/PR/8/34 (H1N1) was grown in chicken eggs as previously described[537]. Mice were inoculated with PBS vehicle or 100 plaque forming units (PFU) of influenza in 50 μl of sterile PBS. Six days later, mice were infected with $1x10^8$ colony forming units (CFU) of MRSA in 50 μl of sterile PBS. All infections were performed via oropharyngeal aspiration. Mice were harvested 24 hours after MRSA challenge using pentobarbital injection (300 mg/kg) and cervical dislocation. During harvest, the lung was lavaged with 1 ml sterile PBS. BAL cells were pelleted and red blood cells were lysed (ACK lysis buffer, Gibco). Cells were resuspended, placed on slides via cytospin, stained with Hema 3 (Thermo Fisher), and quantified. The right upper lung lobe was homogenized in 1 ml PBS and plated on tryptic soy agar for determination of bacterial burden. The remaining right lung was frozen in liquid nitrogen and stored at -80^{o}C for gene expression analysis.

3.3.4 Biofilm Assay

Overnight cultures were diluted 1:100, plated in quadruplicate in 96 well plate and incubated at 37 C for 24 hours. The following day, the plate was washed with sterile PBS, dried for 30 minutes, and fixed with 95% ethanol for ten minutes. Wells were stained with 0.1% wt/vol filtered crystal violet for 15 minutes and washed by submerging the plate in sterile water three times. The plate was air dried for one hour, decolorized with 33% glacial acetic acid for 10 minutes, and read at 570 nm.

3.3.5 Macrophage experiments.

For experiments, $7x10^5$ RAW264.7 cells were plated in 6-well plates, infected at an MOI 10, and spun at 250 xg for 5 minutes at 4^o C to synchronize infection. Cells were infected for 1 hour in the absence of antibiotics, media was replaced with antibiotic- and serum-free media with and without gentamicin (100 ug/ml) for 1 hour, then replaced with antibiotic-free media for an additional hour. At collection, cells were lysed with 1% Triton X-100 at room temperature for 10 minutes and 50 µl was collected for CFU determination. Phagocytosis was calculated by the equation ((CFU+gentamicin)/(CFU-gentamicin))*100. RLT (Qiagen) was added to the wells and collected and ran through a Qiashredder and frozen at -80^{o}C until RNA extraction.

3.3.6 RNA extraction and qPCR.

RNA was extracted from mouse lungs using the Absolutely Total RNA Purification Kit (Agilent). RNA extraction from cell culture experiments were performed using the Qiagen RNeasy

kit (Qiagen). RNA was quantified and converted to cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad). Quantitative PCR was performed using SsoAdvanced Universal Probes Supermix (Bio-Rad) and TaqMan primer-probe sets (ThermoFisher Scientific) listed in Table 5 on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Gene expression was calculated using the ΔΔct method using *hprt* as a housekeeping gene and normalized to the average WT *S. aureus* values unless otherwise stated.

3.3.7 Multiplex and Heatmap analysis.

Lung homogenate cytokines were assessed using the Bio-Plex Pro Mouse Cytokine 23-plex Assay (Bio-Rad). For clustering analysis, all data was combined and samples with missing data and MIP-1α, due to poor detection, were excluded. The average for each cytokine per mutant was used. Using R (version 4.1.0) in RStudio (version 1.4.1717), data was log-transformed and scaled to Z score and clustered by cytokine using the hclust function and Pearson correlation. The resulting heatmap was visualized using Heatmap.2 function in the gplots package.

3.3.8 Statistical Analysis.

Data were analyzed using Prism 8 (GraphPad). Analyses comparing two groups were performed using Mann-Whitney test or an unpaired t test. For analyses assessing more than two groups, Kruskal-Wallis with Dunn's multiple comparisons correction was used. Analyses comparing two variables were tested via Two-way analysis of variance (ANOVA) with Sidak's

multiple comparisons correction. All figures show combined data from multiple replicate studies and are graphed as mean ± standard error of the mean (SEM). N values are numbers of animals per independent experiment. Statistical significance ($p \leq 0.05$) is indicated in figure legends, with p values between 0.05 and 0.1 displayed numerically.

3.4 Results

Since cell wall-anchored proteins (CWAs) are exposed on the cell surface of *S. aureus*, we hypothesized that these proteins may be playing a role in colonization and/or infection in the lung. We screened nine CWA mutants (*fnbB::Tn, clfA::Tn, clfB::Tn, sdrC::Tn, sdrD::Tn, sdrE::Tn, isdB::Tn, sasG::Tn*, and *sasD::Tn*) and the corresponding wildtype (WT) strain JE2 in the context of bacterial pneumonia and influenza super-infection. We also included a Sortase A (*srtA::Tn*) mutant, the enzyme responsible for attaching these CWAs to the cell wall of *S. aureus.* In terms of bacterial burden within the lung (Figure 9 A), lacking individual CWA proteins during bacterial pneumonia led to varying decreases in burden when compared to the WT strain. The differences in bacterial burden did not appear to be due to differences in *in vitro* growth rates of the various mutants (Figure 24 A and B). Interestingly, the Sortase A mutant did not have a significant decrease in bacterial burden. The mutant lacking SasG (*S. aureus* surface protein G; *sasG::Tn*) had the largest decrease in burden during bacterial pneumonia. During super-infection, mutants had increased burden versus single infection, although only the WT and ClfA mutant (clumping factor A; *clfA::Tn*) were significantly increased. The mutant lacking IsdB (iron-regulated surface determinant protein B; *isdB::Tn*) was the only mutant that had significantly decreased burden in

both bacterial pneumonia and super-infection. Lacking *S. aureus* CWAs did not impact viral burden in the lung (Figure 24 C).

To look at lung inflammation with CWA mutants, we examined the number of cells in the airway via bronchoalveolar lavage (BAL) (Figure 9 B). Cellular immune infiltrates in the lung varied based on the mutant. During super-infection, almost all mutants had significantly higher numbers of BAL cells in the airways. The number of BAL cells during super-infection are at influenza alone levels, suggesting this is the main driver of immune infiltrate during super-infection. To look at acute lung injury and leak, we measured total protein in the BAL (Figure 9 C). Although not as variable, the level of protein in the BAL during bacterial pneumonia varied based on the mutant. Interestingly, the mutant lacking FnbB (fibronectin binding protein B; *fnbB::Tn*) had the highest level of protein in the BAL in bacterial pneumonia. Lung leak also significantly increased during super-infection compared to bacterial pneumonia. Only the WT had significantly increased BAL protein during super-infection compared to influenza alone.

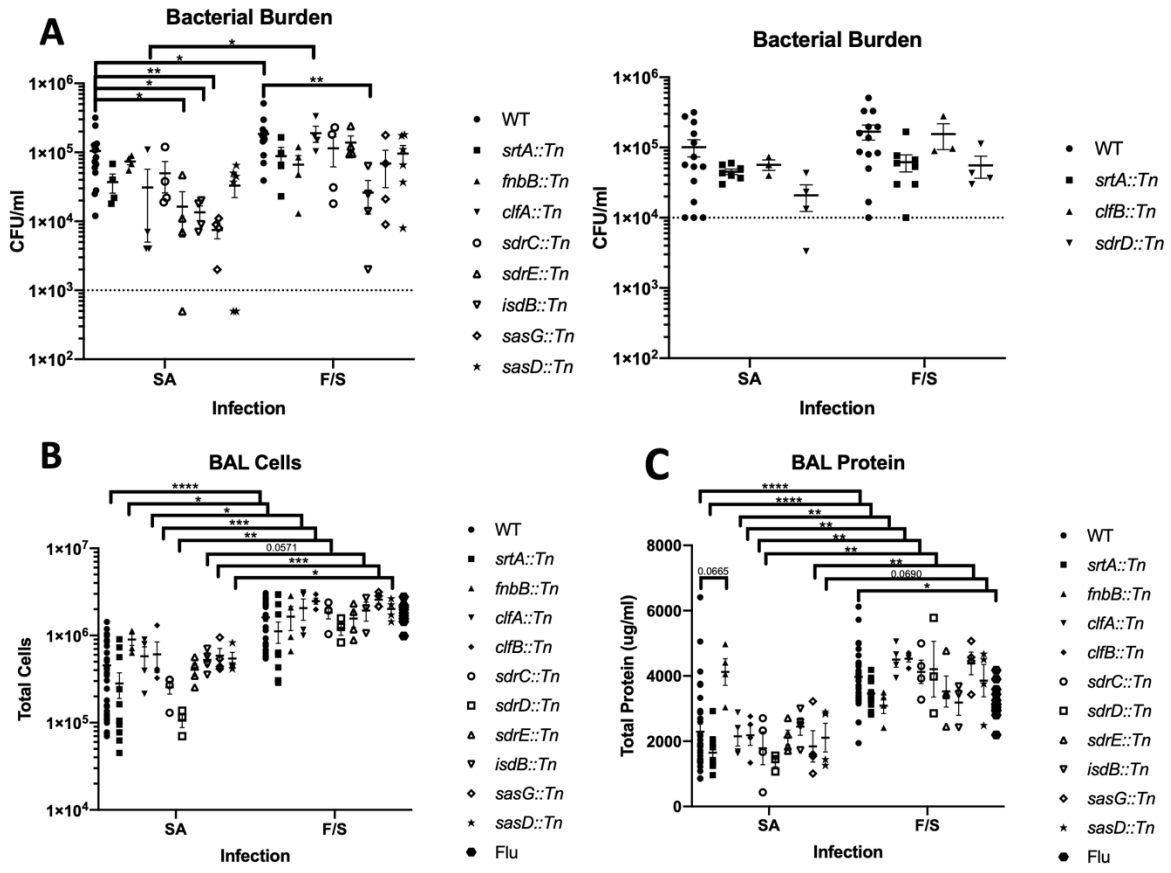

Figure 9: Differential Impact of *S. aureus* CWA Mutants in Bacterial Pneumonia and Influenza Super-Infection

Mice were inoculated with PBS or 100 PFU of influenza on day 0 and six days later were infected with PBS or $1x10^8$ CFU of WT MRSA or a strain lacking individual CWA (see graphs) and harvested 24 hours later. A. Bacterial burden in bacterial pneumonia (SA) or influenza super-infection (FS) 24 hours post MRSA infection. Mice with undetectable CFU were graphed as half of the limit of detection. B-C. Total cells (B) or total protein (C) in the bronchoalveolar lavage (BAL) in bacterial pneumonia (SA) or influenza super-infection (FS). Statistics tested by Two-way ANOVA with Sidak's multiple comparison correction. * $p<0.05$, ** $p<0.01$, ***$p<0.001$, ****$p<0.0001$. N=2-4, combination of several experiments, data graphed as mean ± SEM (standard error of the mean). srtA: Sortase A, fnbB: fibronectin binding protein B, clfA/B: clumping factor A/B, sdrC/D/E: serine-aspartate repeat containing protein C/D/E, isdB: iron-regulated surface determinant B, sasD/G: *S. aureus* surface protein D/G.

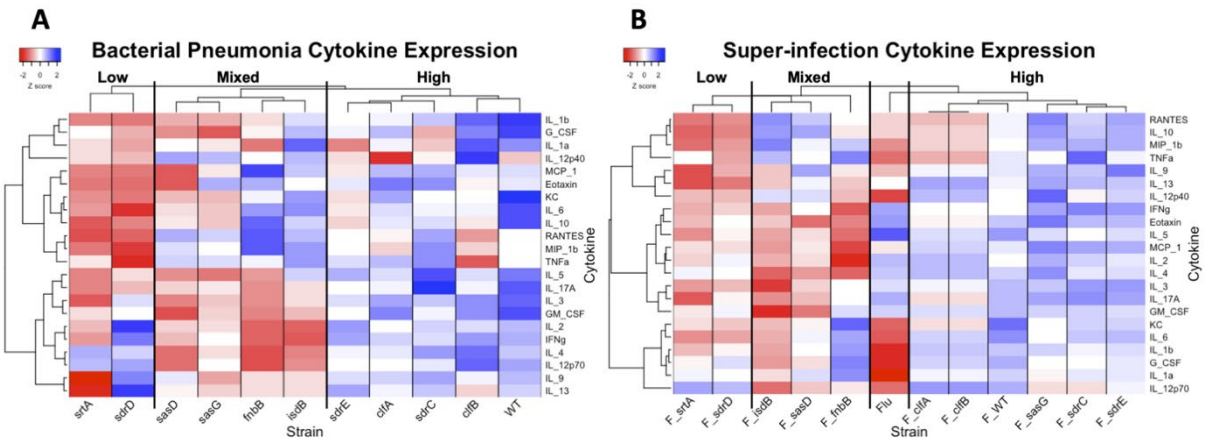

Figure 10: CWA Mutant Induced Cytokine Expression in Bacterial Pneumonia and Influenza Super-Infection

A. Cytokine expression in bacterial pneumonia. There are three clusters (from left to right): low inflammation, mixed inflammation, and high inflammation. B. Cytokine expression in super-infection. There are four clusters (from left to right): low inflammation, mixed inflammation, influenza alone (Flu), and high inflammation. Cytokines were measured via multiplex analyses of lung homogenate. For each cytokine, average values for each MRSA strain were log transformed and converted to Z scores. Heatmap clustering was performed by cytokine using the Pearson correlation and graphed using the heatmap.2 function of the gplots package in R. srtA: Sortase A, fnbB: fibronectin binding protein B, clfA/B: clumping factor A/B, sdrC/D/E: serine-aspartate repeat containing protein C/D/E, isdB: iron-regulated surface determinant B, sasD/G: *S. aureus* surface protein D/G.

Next, we examined the inflammatory response to CWA mutants via cytokines in lung homogenate. To determine if immune signatures were similar in CWA subfamilies, we visualized the cytokine data by clustering analyses (Figure 10 A and B). In bacterial pneumonia, there were three clusters of inflammatory responses which we termed: low inflammation, mixed inflammation, and high inflammation (Figure 10 A). Unsurprisingly, the Sortase A mutant, which lacks all CWAs on the cell surface, had the lowest level of cytokine induction. *srtA::Tn* had significant decreases in type 2 cytokines IL-9 ($p<0.0001$) and IL-13 ($p=0.0025$), but not IL-4 and IL-5. This was not driven by IL-33 expression, as *srtA::Tn* trended towards increased IL-33

expression via qPCR (p=0.0710) (data not shown). The other mutant in the low inflammation cluster was *sdrD::Tn* (serine aspartate repeat containing protein D), which had higher levels of expression of type 1 and type 2 cytokines compared to *srtA::Tn.* The mutants found in the mixed inflammation cluster typically had higher levels of innate immunity cytokines and chemokines, but lower levels of type 1, 2, and 17 cytokines. The high inflammation cluster, which contained the WT strain as well as most Clf and Sdr members, had the highest levels of cytokines. During super-infection, the clustering of CWAs by cytokine expression was very similar to bacterial pneumonia (Figure 10 B). Again, the strains cluster into three groups distinct from influenza alone and the only mutant that switched clusters was *sasG::Tn*.

Due to the inflammatory changes seen, we wanted to look at early CWA interactions with the immune system. As alveolar macrophages (AMs) are likely the first immune cell to encounter *S. aureus* in the lung, we decided to determine if there were inflammatory differences in RAW264.7 macrophages infected with WT or CWA mutant MRSA (Figure 11). Macrophages were infected without (-G) and with (+G) gentamicin to kill external *S. aureus* to determine if there were differences in external and internal *S. aureus* signals (Figure 11 A). Lacking all CWAs or individual CWAs led to mostly significant decreases in bacterial burden regardless of gentamicin treatment. This is also reflected in their survival rates, and for some mutants, may be tied to their reduced ability to form biofilm (Figure 25 A and B). Mutants lacking ClfA and ClfB had the highest burden after the wildtype, although this is not significant. Macrophages infected with *isdB::Tn* had significantly less burden as well as reduced expression of cytokines (Figure 11 B), similar to the phenotype seen *in vivo*. The mutant lacking SasG also had significantly less burden, similar to the Sortase A mutant (Figure 11 A left panel), however *sasG::Tn* survival is much lower

than *srtA::Tn* (Figure 25 A). Macrophages also expressed lower levels of cytokines when infected with *sasG::Tn* (Figure 11 C). Unsurprisingly, lacking all CWAs led to reduced bacterial burden, but not overall survival (Figure 11 A and Figure 25 A). Compared to individual CWA mutants, the *srtA::Tn* strain was phagocytosed the least, although not significant (Figure 25 C). As this mutant is lacking several surface proteins, it is unsurprising that the bacterial responses in macrophages were reduced (Figure 11 D).

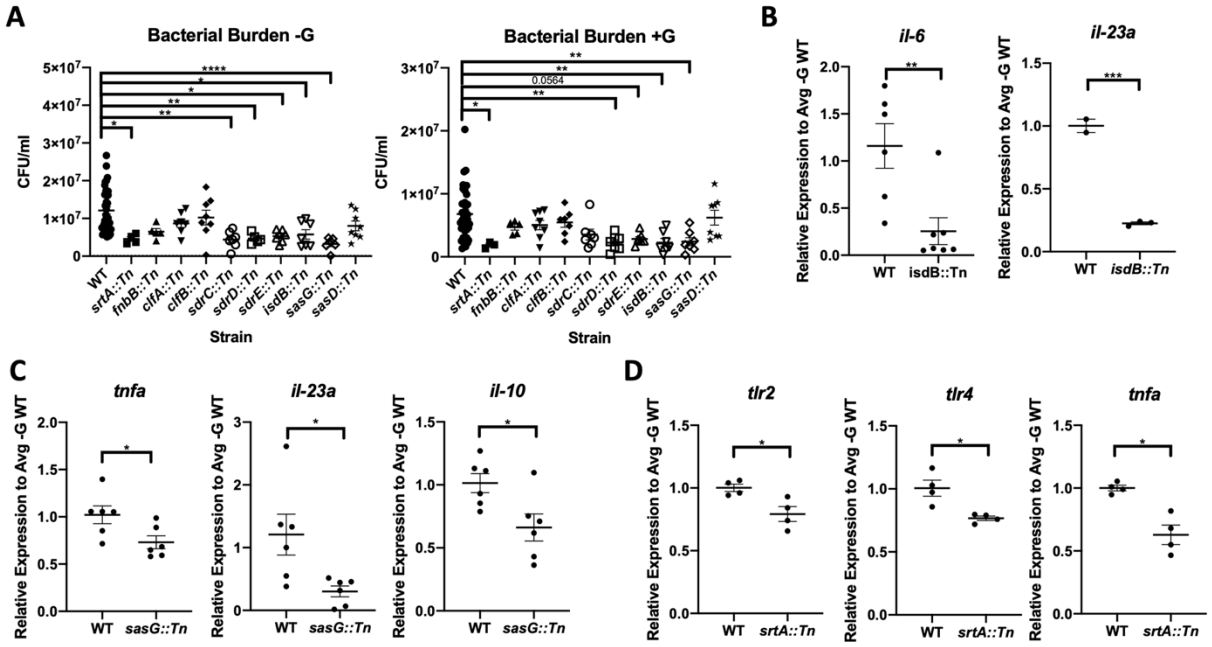

Figure 11: CWA Mutant Interactions with Macrophage Cell Line RAW264.7.

A-D. RAW264.7 macrophages infected with WT or CWA mutant (see graph) MRSA for 3 hours at an MOI of 10. Macrophages were infected for one hour in the absence of antibiotics, media was then replaced with antibiotic- and serum-free media with (+G) or without (-G) gentamicin for 1 hour and changed to antibiotic free media. A. Bacterial burden of macrophages after 3 hours of infection without (-G) and with (+G) 1 hour 100 ug/ml gentamycin treatment. B. Gene expression in RAW264.7 macrophages infected with either WT or *isdB::Tn* MRSA without gentamicin treatment. C. Gene expession of macrophages infeced with either WT or *sasG::Tn* MRSA without gentamicin treatment.D. Gene expression in RAW264.7 macrophages infected with either WT or *srtA::Tn* MRSA without gentamicin treatment. Statistics tested by Kruskal-Wallis with Dunn's multiple comparisons correction (A) or Mann-Whitney (B-D).* $p<0.05$, ** $p<0.01$, *** $p<0.001$, ****$p<0.0001$. N=3-4, combination of several experiments, except for right panel of B where n=2 in one experiment. Data graphed as mean ± SEM (standard error of the mean). srtA: Sortase A, fnbB: fibronectin binding protein B, clfA/B: clumping factor A/B, sdrC/D/E: serine-aspartate repeat containing protein C/D/E, isdB: iron-regulated surface determinant B, sasD/G: *S. aureus* surface protein D/G**.**

3.5 Discussion

The majority of the viral super-infection literature focuses on the differences in immune responses between bacterial pneumonia and influenza bacterial super-infection. It is well documented that preceding influenza greatly impairs the antibacterial response within the lung[482, 486]. Few studies have examined bacterial factors in single or super-infection in the lung. The literature suggests that increased inflammation and tissue damage lead to increased adhesion within the lung, contributing to increases in bacterial burden[484, 490]. However, to our knowledge, there has been no specific testing of bacterial adhesion components *in vivo* during single or viral super-infection in the lung. Most studies that have investigated *S. aureus* virulence factors in the lung have focused on secreted toxins, such as the alpha-toxin[270, 272, 343, 356, 566]. While toxin-mediated damage contributes to lung pathology, the alpha-toxin has been shown to decrease adhesion to lung epithelial cells[567]. Thus, we wanted to determine if proteins with known adhesion properties influenced the outcomes of single or super-infection.

Our data supports the finding that changes due to influenza infection are the primary driver of super-infection (Figure 12), with influenza increasing bacterial burden, immune recruitment, and acute lung injury seen in the model. Interestingly, regardless of what CWA was removed, influenza appeared to "level the playing field" for the mutants, with endpoints being much higher and tighter grouped in super-infection than in bacterial pneumonia alone. *S. aureus* strains seen in super-infected individuals are less virulent and more closely related to nasal colonizing strains than those strains found in bacterial pneumonia patients[498]. This is likely due to the increased inflammation and damage within lung as well as a more dysregulated immune response during super-infection leading to less aggressive colonizing strains taking hold in the lung. However,

viral-bacterial synergism is likely adding to this phenomenon, as influenza can increase both internalization and adhesion of bacteria within the lung[494, 495]. This is not specific to influenza, as the same phenomenon is seen in rhinovirus-*S. aureus* super-infections[496, 497].

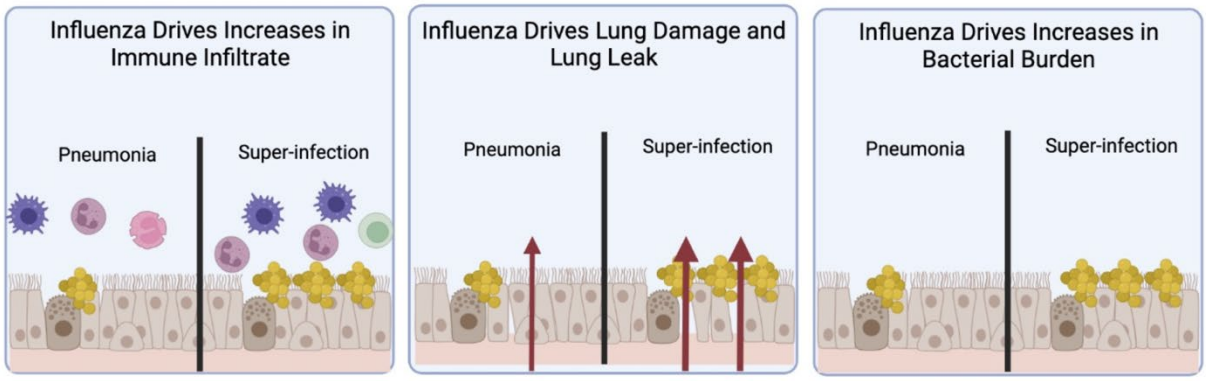

Figure 12: Influenza Drives Super-infection Regardless of CWA Mutant.

Compared to bacterial pneumonia caused by *S. aureus,* influenza infection drives many of the changes seen in super-infection regardless of the CWA tested in this study. Influenza drives morbidity to super-infection in three main ways (from left to right panels): 1) influenza drives the increase in immune cells in the lung and the inflammation they cause, 2) influenza drives epithelial and endothelial damage leading to increased lung leak, and these mechanisms (likely in combination with others) ultimately leads to increases in burden due to increased survival of *S. aureus* in the lung regardless of CWA status.

We saw more variability in the endpoints studied during bacterial pneumonia, likely because adhesion in the lung is more difficult in a homeostatic state. SasG has a known role in biofilm formation[160, 164], which may explain the decrease in burden seen in bacterial pneumonia. SasG has also been shown to adhere to human desquamated nasal epithelial cells via an unknown ligand[159], so it unclear if lacking SasG would have a pronounced impact on murine lung cell adhesion. IsdB is the receptor for hemoglobin and part of the heme acquisition system to attain iron, an important

bacterial nutrient[135]. While this protein has higher affinity for human hemoglobin, it still plays a role in murine models[49, 135]. Thus, it is unsurprising that lacking this CWA had an impact on bacterial survival in both bacterial pneumonia and influenza super-infection. In bacterial pneumonia, *fnbB::Tn* had elevated protein in the BAL, which may be caused by the high bacterial burden and immune cell infiltrate. FnbB, along with FnbA (not tested in this study), have been shown to play a role in invasion into nonprofessional phagocytes via fibronectin-integrin $\alpha_5\beta_1$ interactions[81, 82, 568]. This phenomenon has been shown *in vitro* for alveolar epithelium and a FnbB deletion mutant was found to have increased protein leak in a rat model of pneumonia[399]. This suggests that internalization of *S. aureus,* and subsequent immune evasion, may reduce inflammation in the lung. SdrD is known to play a role during nasal colonization as well as help promote survival of *S. aureus* in the blood[117, 121, 256]. Lacking this CWA may allow for reduced number of immune cells during bacterial pneumonia as well as lower cytokine expression.

CWAs are known to bind to several proteins within the host such as fibrinogen and fibronectin[24]. In this study we did not explore bacterial adhesion to specific ligands, but it is likely a combination of several ligands, as described at other host sites such as the nose[387]. CWAs also have overlapping ligands, such as ClfA, ClfB, FnbA, and FnbB all binding fibrinogen[24]. Because we only looked at single CWA mutants, some of the functions of these proteins in bacterial pneumonia and super-infection could be masked.

Even though the CWA mutants had more clear phenotypes in bacterial pneumonia compared to super-infection, the cytokine signature in both settings appears to be driven by the expression of these CWAs. The mutants found in each cluster were consistent in both bacterial pneumonia

and super-infection, with the exception of *sasG::Tn*. This suggests that while a majority of the inflammation in the lung is driven by influenza, at least some part of the immune response is shaped by the presence of these CWAs on the cell surface of the bacteria. As SasG has a known role in biofilm formation and influenza is known to induce dissemination of *S. aureus* biofilms[160, 164, 491], this effect could influence how the immune system reacts to this mutant. A majority of the MSCRAMM proteins (*clfA::Tn, clfB::Tn, sdrC::Tn, sdrE::Tn*) cluster together in the high inflammation cluster. This is what we expected to find, as these proteins have similar domains used for ligand binding and this may influence the immune response[23]. ClfA has been shown to be a T cell activator driving Th1 and Th17 activation[569]. While we did not see any significant changes in IL-2 or IFNγ, we did see a nearly significant decrease in IL-17A (p=0.0571) with the *clfA::Tn* mutant. Unsurprisingly, the Sortase A mutant, which lacks all CWAs on the cell surface, induced the lowest expression of cytokines. It is important to note that the Sortase A mutant still makes all the CWAs, but they are secreted into the environment instead of covalently attached to the cell wall. However, it does suggest that the influence on immune signaling is greatest when the CWAs are still attached to the bacteria. However, more testing would be needed in defining the portions of each CWA responsible for altering immune signaling.

Alveolar macrophages patrol the airways looking for pathogens and help coordinate immune responses in the lung[408]. Thus, early interactions with AMs likely shape the overall immune response to bacteria. As CWAs are expressed on the cell surface, they likely influence how *S. aureus* is either recognized or what inflammatory mediators are made by macrophages. Many of these CWAs can bind host proteins and mask bacterial recognition and lacking them likely leads to better macrophage recognition and inflammatory responses. Unsurprisingly, lacking all CWAs

had the largest decrease burden, although not significant, and had decreased expression of bacterial recognition proteins TLR2 and TLR4. While this reduction is less than 2-fold, this does suggest that CWAs may play a minor role in either recognition of *S. aureus* or in early macrophage signaling leading to increased TLR expression. Interestingly, *srtA::Tn* infection led to decreases in TNFα but not IL-6 expression, which could be explained by timing, differences in gene and protein regulation, or that a secreted protein is has a redundant effect on the immune system response. For example, both protein A, a CWA, and Sbi, a secreted and cell-wall associated protein, can both induce inflammatory signaling through the TNFα receptor[149, 232].

The bacterial survival and inflammatory response in macrophages reflected what was seen *in vivo*. The increased inflammation seen in Clf mutants in the lung could partially be explained by their increased survival when interacting with macrophages. However, Sdr mutants, which had similar inflammation *in vivo* to Clf mutants, did not survive similarly in macrophages. As neutrophils are the first blood immune cell to enter the tissue[570], it could be that these cells are playing a larger role in the overall inflammatory response to those CWAs. SdrD is known to increase survival of *S. aureus* in the presence of neutrophils[121] and other Sdr proteins could have similar interactions. Infection with *isdB::Tn* led to lower expression levels of inflammatory cytokines, which was also seen in the lungs. While there was a significant reduction in *il-23a* expression in macrophages, clearance of *isdB::Tn* from the lung was not affected. This could be due to poor survival of the mutant itself, timing, or a different cell type *in vivo* responsible for the production of IL-23, such as dendritic cells. Biofilm formation also likely plays a role in how these bacteria are sensed. Those CWA mutants that produced lower biofilm mass tended to not survive as well when in the presence of macrophages. Biofilms can be protective against phagocytosis[571],

and while we did not see any obvious trends other than with *srtA::Tn,* this could be due to the particular experimental setup that was used (several washes could have removed more bacteria leading to inaccurate phagocytosis rates). Bacterial microaggregates in the lung have been shown to help mediate damage through the alpha-toxin[343] and CWAs could be involved in forming microaggregates, as FnbA, FnbB, SdrC, and SasG can form homophilic interactions with themselves on nearby cells[99, 114, 160].

In conclusion, our data supports the idea that influenza is the major driver of susceptibility to super-infection. CWAs do not appear to have a major virulence role in super-infection, likely due to the influenza-induced changes in the lung environment and the immune response. However, they do significantly impact the inflammatory milieu and this may affect lung tissue injury. In pneumonia, CWAs have varying levels of influence on bacterial burden, although much of this could be due to overlapping ligands between CWAs. CWAs do, however, have an influence on inflammatory signaling in the lung, and this could be due to early interactions with immune cells such as macrophages.

4.0 Investigating the Role of Sbi in the Lung

4.1 Summary

Severe community-acquired pneumonia (CAP) can lead to high levels of morbidity and mortality in patients. This is partly due to the increase in inflammation that can affect lung function. Understanding the basis of inflammation during CAP could lead to better clinical treatment. *Staphylococcus aureus* is a major contributor to infections including pneumonia. One of the characterized virulence factors in the lung is staphylococcal protein A (SpA), which can induce inflammatory signaling in the lung in addition to its ability to bind immunoglobulins. A related protein, second binding protein for IgG (Sbi), shares homology with SpA, however it is unclear if Sbi induces inflammation in the lung similar to SpA. Here we investigate the role of Sbi during staphylococcal pneumonia. We find that Sbi induces inflammatory changes to the lung and lacking Sbi leads to improved survival. These inflammatory changes differ from those induced by SpA and are not dependent on immunoglobin binding. This suggests that Sbi induces inflammation through a different mechanism, such as complement evasion.

4.2 Introduction

Pneumonia is a significant cause of morbidity and mortality worldwide. In 2016, the Global Burden of Diseases, Injuries, and Risk Factors (GBD) Study on lower respiratory infections estimated that 336.5 million lower respiratory tract infections occurred, leading to 652,572

deaths[572]. In the United States, approximately 1.5 million cases of adult community-acquired pneumonia (CAP) occur annually, with a 1-year mortality rate of 30.6%[573]. While thought to be less severe than hospital-acquired or ventilator-associated pneumonia, CAP can still lead to high morbidity and mortality[574, 575]. Severe CAP cases can lead to sepsis, acute respiratory distress syndrome (ARDS), and ultimately death[574, 576]. Even patients who recover clinically with CAP can still have subclinical levels of inflammation, which is associated with an increased risk of death[577]. Understanding how inflammation is induced during pneumonia may lead to better treatment options for patients.

Staphylococcus aureus is an important respiratory pathogen that can lead to severe cases of CAP[272]. In a recent study looking at patients with severe staphylococcal pneumonia, 89% required hospitalization, over 70% required mechanical ventilation, and 33% died as result of their infection[578]. *S. aureus*, and in particular methicillin-resistant *S. aureus* (MRSA) can have high levels of expression of various virulence factors that can lead to inflammation[9, 15]. *S. aureus* toxins and other factors can directly kill immune cells or induce high levels of inflammation[172, 260]. However, in the lung, many of these inflammatory mechanisms are still not understood. One of the well characterized virulence factors in the lung is staphylococcal protein A (SpA). In addition to its role in avoiding opsonization by binding Fc regions of immunoglobulin, SpA can activate clotting by binding von Willebrand factor, act as a superantigen for B cells, as well as induce inflammation during staphylococcal pneumonia[22, 232, 518]. The latter is mediated through two distinct mechanisms on the lung epithelium: 1) inducing TNFR1 and EGFR signaling through its IgG binding domains[232, 233, 234, 235] and 2) by inducing IFNβ signaling through its Xr domain[236].

S. aureus expresses a second binding protein for IgG (Sbi) that also mediates escape from opsonization of both IgG and complement[150, 151]. Sbi contains four globular domains, the first two are homologous to the immunoglobulin binding domains of SpA , while the other two domains are responsible for interfering with the complement system[144]. Because Sbi and SpA have homology in both IgG binding domains as well as the Xr domain, it is possible that Sbi may play a similar role in the lung. Unlike SpA, Sbi is associated with the cell envelope and secreted rather than being anchored to the cell wall[144]. As such, Sbi may have farther reaching affects than SpA. Thus, this study was aimed at investigating the role of Sbi in the induction of inflammatory responses in staphylococcal pneumonia.

4.3 Materials and Methods

4.3.1 Mice.

Six- to eight-week-old male WT C57BL/6NTac mice were purchased from Taconic Farms. B6.129S2-*Ighm*^{tm1Cgn}/J (μMT) and corresponding wildtype mice were purchased from Jackson Laboratory. Mice were maintained under pathogen-free conditions within the animal facilities at the UPMC Children's Hospital of Pittsburgh. All studies were performed on sex- and age-matched mice. Animal studies were conducted with approval from the University of Pittsburgh Institutional Animal Care and Use Committee.

4.3.2 *S. aureus* strains.

Strains used in this study are listed in Table 3. USA300 MRSA strain $JE2^{565}$ was the WT strain for all studies. Strain sbi A100.2 was generated via phage 11 transduction of *sbi::Tn* lysate into the wildtype JE2 strain, selected with 5 μg/ml erythromycin and confirmed by PCR (Table 4). *S. aureus* strains were grown in Tryptic Soy Broth (BD Bacto™) overnight at 37° C at 250 rpm. Overnight cultures were diluted 1:100 and grown until OD_{660}~1, approximating logarithmic growth phase. MRSA dose was calculated using OD_{660} measurement of the culture and application of a calculated extinction coefficient. For growth curves, overnight cultures were diluted 1:200 in a 96-well plate in sexaplicate. Plates were grown at 37°C at 282 rpm in a Synergy H1 Hybrid Multi-Mode Reader (BioTek). Optical density measurements at 660 nm were taken every 30 minutes. Growth rate (μ) was calculated from at least two independent experiments using the equation $A_t=A_{t-1}*e^{\mu t}$. The μ_{max} was calculated as the average of the three highest μ rates.

4.3.3 Murine Models.

Mice were inoculated with $1x10^8$ colony forming units (CFU) of MRSA in 50 μl of sterile PBS. All infections were performed via oropharyngeal aspiration. Mice were harvested 6 or 24 hours after MRSA challenge using pentobarbital injection (300 mg/kg) and cervical dislocation. In mortality studies, a dose of $2x10^8$ CFU was used. During harvest, the lung was lavaged with 1 ml sterile PBS. BAL cells were pelleted and red blood cells were lysed (ACK lysis buffer, Gibco). Cells were resuspended, placed on slides via cytospin, stained with Hema 3 (Thermo Fisher), and quantified. The right upper lung lobe was homogenized in 1 ml PBS and plated on tryptic soy agar

for determination of bacterial burden. The remaining right lung was frozen in liquid nitrogen and stored at -80ºC for gene expression analysis. The left lobe was perfused with 10% formalin and embedded in paraffin. Lung sections were stained with hematoxylin and eosin and inflammatory features were evaluated via microscopy after sample blinding. Lung homogenate cytokines were assessed using the Bio-Plex Pro Mouse Cytokine 23-plex Assay (Bio-Rad).

4.3.4 Macrophage experiments.

For experiments, $7x10^5$ RAW264.7 cells were plated in 6-well plates, infected at an MOI 10, and spun at 250 xg for 5 minutes at 4^o C to synchronize infection. Cells were infected for 1 hour in the absence of antibiotics, media was replaced with antibiotic- and serum-free media with and without gentamicin (100 ug/ml) for 1 hour, then replaced with antibiotic-free media for an additional hour. At collection, cells were lysed with 1% Triton X-100 at room temperature for 10 minutes and 50 µl was collected for CFU determination. Phagocytosis was calculated by the equation ((CFU+gentamicin)/(CFU-gentamicin))*100. RLT (Qiagen) was added to the wells and collected and ran through a Qiashredder and frozen at -80ºC until RNA extraction.

4.3.5 RNA extraction and qPCR.

RNA was extracted from mouse lungs using the Absolutely Total RNA Purification Kit (Agilent). RNA extraction from cell culture experiments were performed using the Qiagen RNeasy kit (Qiagen). RNA was quantified and converted to cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad). Quantitative PCR was performed using SsoAdvanced Universal Probes Supermix

(Bio-Rad) and TaqMan primer-probe sets (ThermoFisher Scientific) listed in Table 5 on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Gene expression was calculated using the ΔΔct method using *hprt* as a housekeeping gene and normalized to the average WT *S. aureus* values unless otherwise stated.

4.3.6 Statistical Analysis.

Data were analyzed using Prism 8 (GraphPad). Analyses comparing two groups were performed using an unpaired t test. For mortality studies, log-ranked Mantel Cox test was used. All figures show combined data from multiple replicate studies and are graphed as mean ± standard error of the mean (SEM). N values are numbers of animals per independent experiment. Statistical significance ($p \leq 0.05$) is indicated in figure legends, with p values between 0.05 and 0.15 displayed numerically.

4.4 Results

To determine if Sbi is playing a role during staphylococcal pneumonia, mice were infected with wildtype (WT) MRSA or MRSA lacking Sbi (*sbi::Tn*). Infection with *sbi::Tn* led to reduced bacterial burden in the lung (Figure 13 A). Despite the reduction in bacterial burden in the lung, the total immune infiltrate was not different in mice infected with the mutant or WT MRSA (Figure 13 B). However, the ratio of neutrophils:macrophages trended lower in the mutant infected mice, suggesting that there may be immune changes occurring. Indeed, there were decreases in

expression of *il-17a*, an important cytokine in staphylococcal clearance, as well as an IL-17-regulated antimicrobial peptide lipocalin 2 (*lcn2*) (Figure 13 C). Additionally, a mucin involved in bacterial clearance in the lung, mucin 5b (*muc5b*) had a trend towards increased expression. There were also decreases in inflammatory cytokines IL-1α and IL-1β as well as chemoattractants MCP-1 (CCL2) and MIP-1α (CCL3) (Figure 13 D).

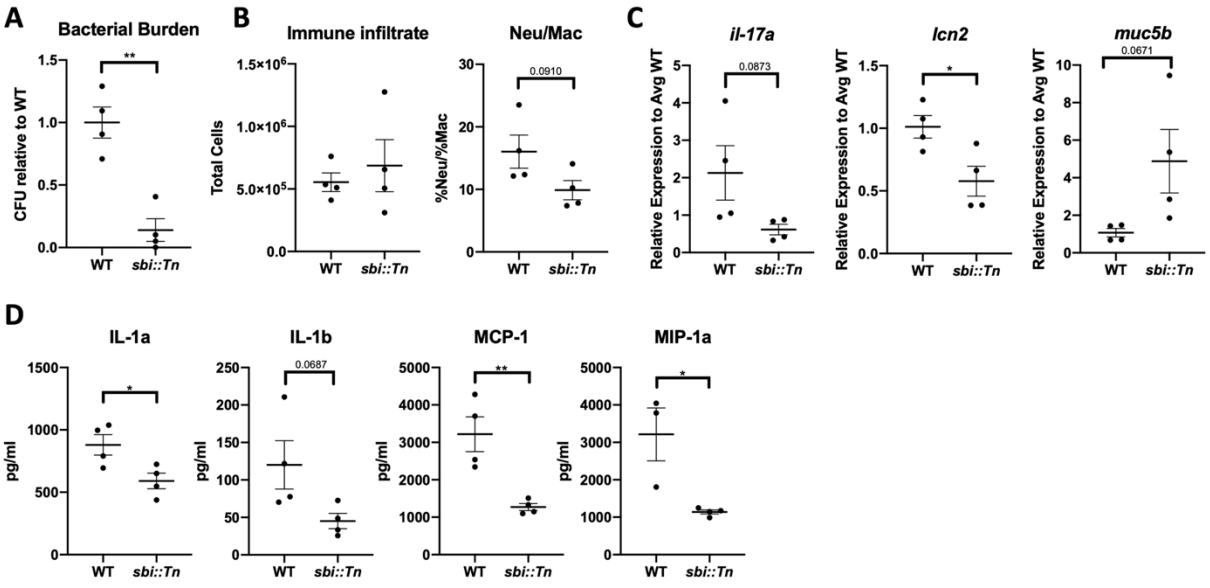

Figure 13: Sbi Induces Inflammatory Changes in the Lung.

A-D. Mice were infected with 1x10^8 CFU WT MRSA or MRSA lacking Sbi (*sbi::Tn*) for 24 hours. A. Bacterial burden in mice infected with MRSA for 24 hours. B. Immune infiltrate and ratio of neutrophils:macrophages in the bronchoalveolar lavage (BAL). C. Cytokine protein levels in the lung homogenate. D. Gene expression levels in the lung. Statistics done by unpaired T test, * $p<0.05$, ** $p<0.01$. N=2, combination of two experiments, data graphed as mean ± SEM.

At 6 hours post infection, there was no difference in bacterial burden in mice infected with either WT or *sbi::Tn* MRSA (Figure 14 A). However, there was a significant decrease in the

immune infiltrate in the lung, due to reduced numbers of neutrophils (Figure 14 B). At this time point, transcripts for *il-10* and murine cathelicidin antimicrobial peptide *(camp*) trended towards decrease (Figure 14 C). There was also a decrease in G-CSF and MIP-1β (CCL4) (Figure 14 D).

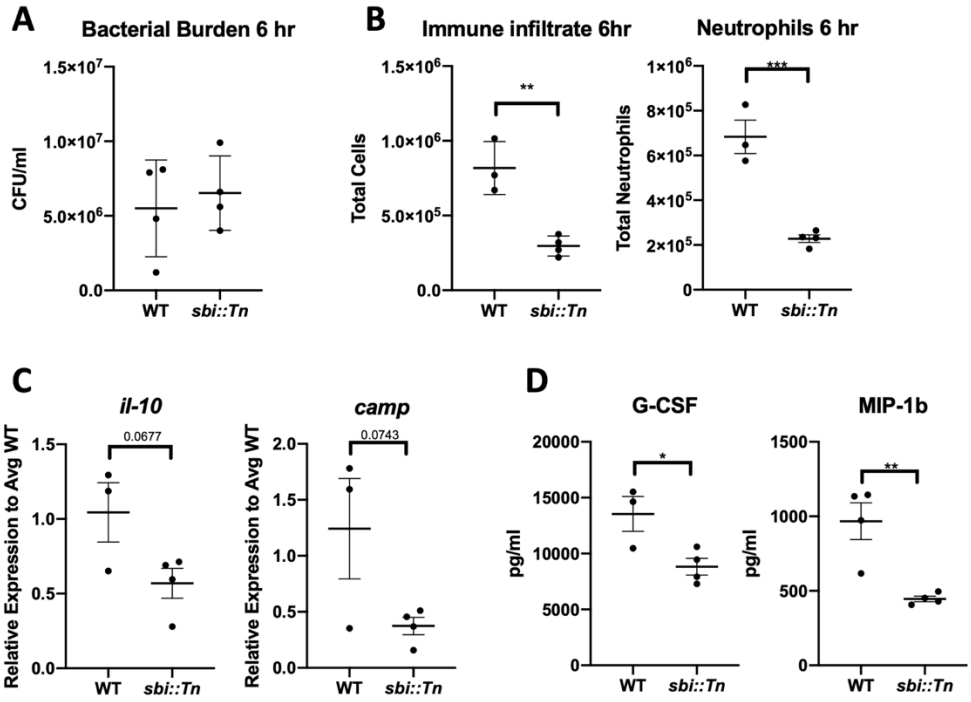

Figure 14: Sbi Induces Changes at 6 hours post Infection

A-D. Mice were infected with 1x108 CFU WT MRSA or MRSA lacking Sbi (sbi::Tn) for 6 hours. A. Bacterial burden in mice infected with MRSA for 6 hours. B. Immune infiltrate and total neutrophil number in the bronchoalveolar lavage (BAL). C. Gene expression levels in the lung. D. Cytokine protein levels in the lung homogenate. Statistics done by unpaired T test, * $p<0.05$, ** $p<0.01$, ***$p<0.001$. N=2, combination of two experiments, data graphed as mean ± SEM.

Due to the differences seen in neutrophil recruitment, which is coordinated by alveolar macrophages[579], and that Sbi is known to induce inflammation in macrophages[149], we decided to

look at infection of RAW264.7 macrophages infected with either WT or *sbi::Tn* MRSA. Macrophages infected with *sbi:Tn* had significantly reduced bacterial burden compared to macrophages infected with WT MRSA (Figure 15 A). This may be due to increased rates of phagocytosis and reduced survival of the mutant (Figure 15 B). Infection with *sbi::Tn* led to an increase in *il-23a* transcript, an important cytokine in the induction of the Th17 response (Figure 15 C). This may explain the increase in *il-17a* transcript seen at 6 hours post infection in the lung (Figure 26 A). There was also a trend towards increased arginase 1 (*arg1*) expression, a marker of M2 macrophages (Figure 15 C). This suggests that Sbi is contributing to inflammation caused by macrophages.

Due to the changes seen during *in vivo* and *in vitro* infection with *sbi::Tn* compared to WT MRSA, we created a transduced mutant, sbi A100.2, to eliminate the potential for mutations in the transposon mutant. Mice infected with sbi A100.2 had no difference in bacterial burden compared to mice infected with WT MRSA (Figure 16 A). However, there was a significant increase in the immune infiltrate in the lung, leading to an almost significant increase in the neutrophil:macrophage ratio, likely due to the trend towards increased neutrophils in the lung (Figure 16 B). Infection with sbi A100.2 also led to trending decreases in cytokines IL-10, IL4 and IL-12p70 (Figure 16 C). Additionally, we saw a significant reduction in mortality in mice infected with sbi A100.2 compared to WT MRSA (Figure 16 D), likely due to changes in inflammation in the lung. While there were statistically significant differences in growth *in vitro* (Figure 26 B and C), these changes are functionally small and likely do not influence the inflammatory changes seen.

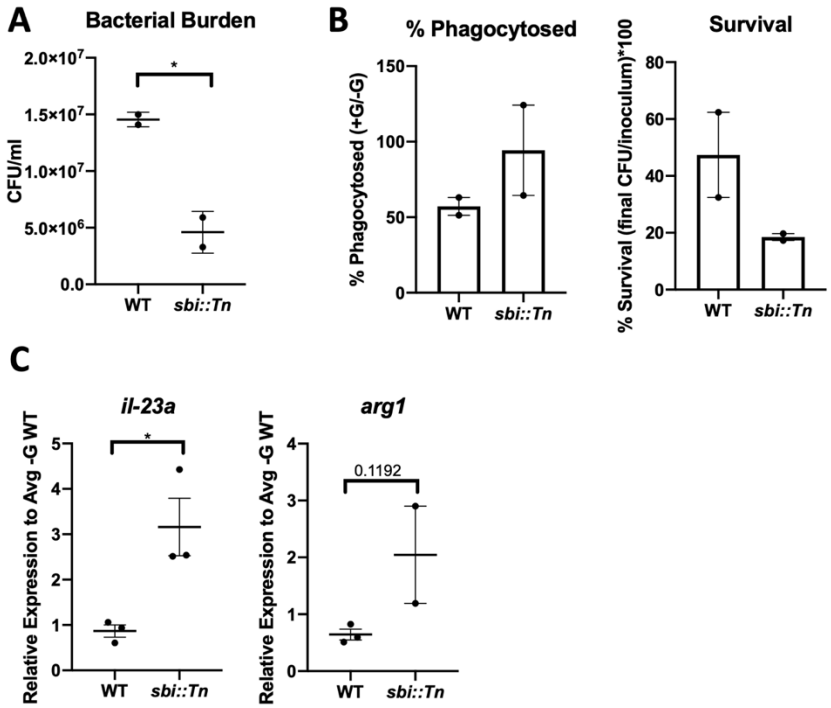

Figure 15: Sbi Induces Inflammatory Changes in RAW264.7 Macrophages

A-C. RAW264.7 macrophages infected with WT or sbi::Tn MRSA for 3 hours at an MOI of 10. Macrophages were infected for one hour in the absence of antibiotics, media was then replaced with antibiotic- and serum-free media with or without gentamicin for 1 hour, and changed to antibiotic free media. CFU and transcript graphs show without gentamicin conditions. A. Bacterial burden RAW264.7 macrophages. B. % Phagocytosed bacteria and % Survival in RAW264.7 cells. % Phagocytosed is calculated by the following equation: ((average CFU with gentamicin)/(average CFU without gentamicin))*100. And % Survival is calculated as (Final CFU/inoculum CFU)*100. C. Gene expression in RAW264.7 macrophages infected with WT or sbi::Tn MRSA for 3 hours. Statistics done by unpaired T test, * $p<0.05$, ** $p<0.01$, ***$p<0.001$. N=2, combination of two experiments, data graphed as mean ± SEM.

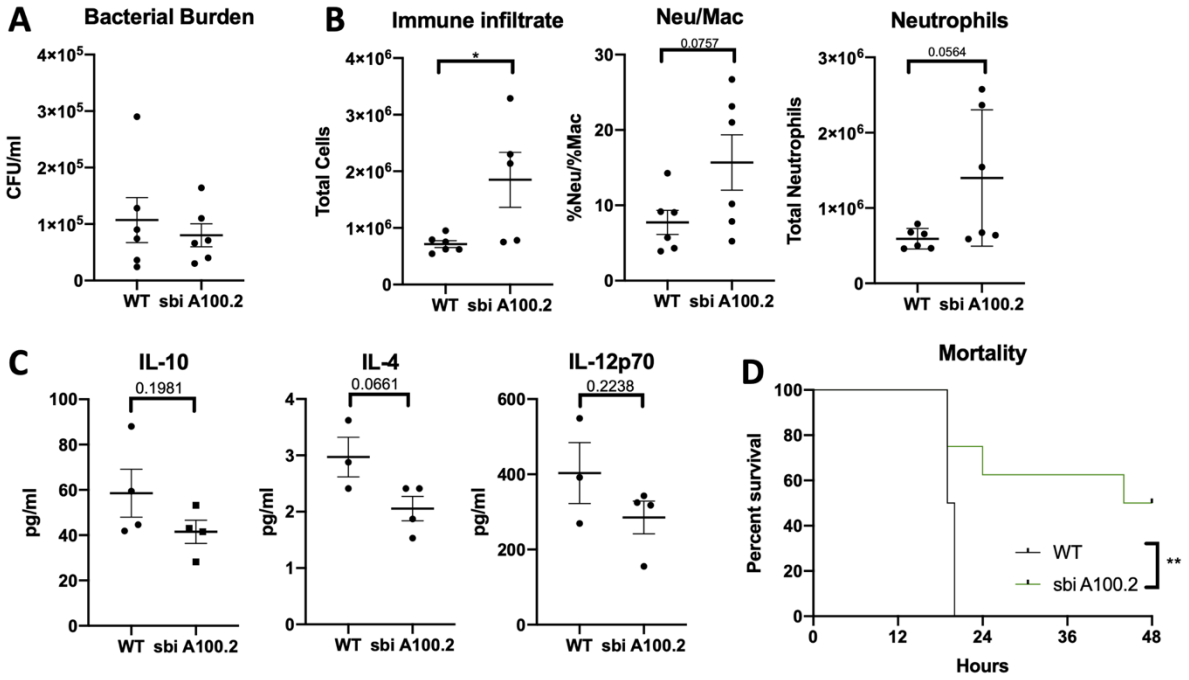

Figure 16: Mice Infected with sbi A100.2 have Decreased Inflammation

A-C. Mice were infected with 1x108 CFU WT MRSA or MRSA lacking Sbi (sbi A100.2) for 24 hours. A. Bacterial burden in mice infected with MRSA for 24 hours. B. Immune infiltrate, ratio of neutrophils:macrophages, and total neutrophil number in the bronchoalveolar lavage (BAL). C. Cytokine protein levels in the lung homogenate. D. Mice were infected with a lethal dose (2x108 CFU) of WT or MRSA lacking Sbi (sbi A100.2). Statistics done by unpaired test (A-C) or log-ranked Mantel Cox test (D), * $p<0.05$, ** $p<0.01$. N=3-4, combination of two experiments (A,B,D) or one experimetn (C), data graphed as mean ± SEM.

Macrophages infected with sbi A100.2 had a trend towards decreased bacterial burden (Figure 17 A). Unlike the *sbi::Tn* mutant, there does not appear to be a difference in phagocytosis between the bacterial strains, and survival mimics the difference in bacterial burden (Figure 17 B). Interestingly, we saw a significant reduction in *il-1β* transcript in macrophages infected with sbi A100.2 but see no differences in *il-6* or *tnfα* (Figure 17 C).

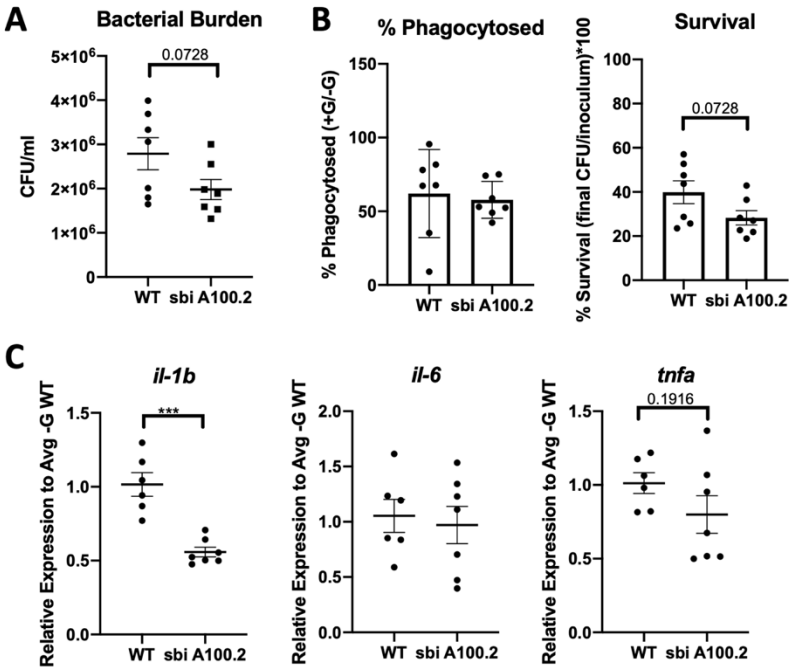

Figure 17: RAW264.7 Infection with sbi A100.2 Induces Some Changes in Inflammation

A-C. RAW264.7 macrophages infected with WT or sbi A100.2 MRSA for 3 hours at an MOI of 10. Macrophages were infected for one hour in the absence of antibiotics, media was then replaced with antibiotic- and serum-free media with or without gentamicin for 1 hour, and changed to antibiotic free media. CFU and transcript graphs show without gentamicin conditions. A. Bacterial burden RAW264.7 macrophages. B. % Phagocytosed bacteria and % Survival in RAW264.7 cells. % Phagocytosed is calculated by the following equation: ((average CFU with gentamicin)/(average CFU without gentamicin))*100. % Survival is calculated as (Final CFU/inoculum CFU)*100. C. Gene expression in RAW264.7 macrophages infected with WT or sbi A100.2 for 3 hours. Statistics done by unpaired T test, ***$p<0.001$. N=3-4, combination of two experiments, data graphed as mean ± SEM.

Since Sbi can prevent opsonization by binding the Fc regions of IgG, we wanted to rule out that this was mediating the phenotype seen. We performed a mortality study using WT B6 mice as well as μMT mice that lack B cells. Regardless of mouse phenotype, sbi A100.2 causes significantly less death in mice (Figure 18). This suggests that antibody binding is not mediating this effect *in vivo*.

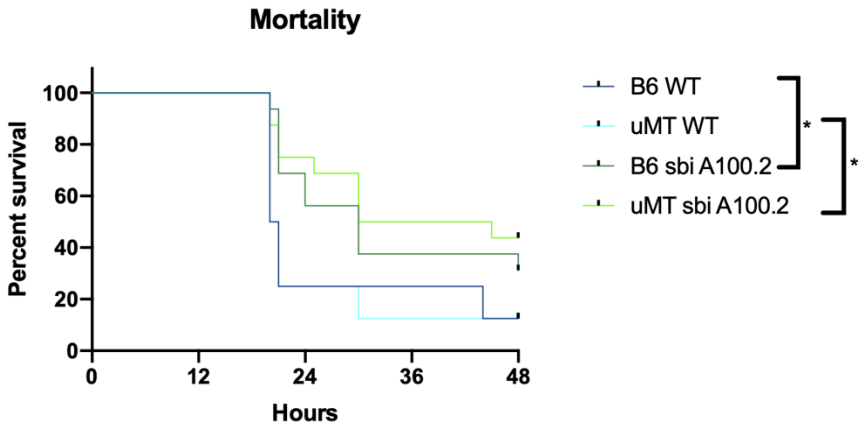

Figure 18: Mortality Difference in sbi A100.2 is Not Dependent on the Presence of Antibodies

Wildtype (B6) or mice without B cells (uMT) were infected with a lethal dose ($2x10^8$ CFU) of WT or MRSA lacking Sbi (sbi A100.2). Statistics done log-ranked Mantel Cox test, * $p<0.05$, N=4, combination of four experiments, data graphed as mean ± SEM.

4.5 Discussion

Our data suggests that Sbi is playing a role in the induction of inflammation in the lung. While there is not a difference in bacterial burden in mice infected with WT or sbi A100.2 MRSA at 24 hours post infection, there are other inflammatory changes seen. Inflammatory differences observed are likely associated with an increase in survival in these mice. This mortality difference is not dependent on antibodies, suggesting that the mechanism for this increase in survival is likely due to Sbi's role in complement evasion and/or mediating inflammatory signaling in the lung.

While we do see inflammatory changes, there is no clear answer to what is mediating these changes. Some of this is due to limited data; many endpoints such as cytokine levels and gene

expression have not been fully tested in these studies. For example, no assays were performed to determine if IFNβ levels change in the lung. Additionally, some experiments were only performed once and may not have sufficient statistical power to make any conclusions. In the future, it will be important to complete examination of these endpoints to determine what pathways may be affected. Additionally, there are some large differences between the *sbi::Tn* and sbi A100.2 strains (i.e., bacterial burden, immune infiltrate, cytokine differences), suggesting that there may be additional mutation(s) in the transposon mutant. To avoid this possibility, all future work should be focused on the sbi A100.2 mutant.

Other than having similar mortality data in sbi A100.2 and CCL2 (MCP-1) induction in *sbi::Tn*, the phenotype seen with Sbi mutants does not mimic what has been seen with SpA in the lung[232, 234]. We see the opposite trend on neutrophil recruitment and do not see changes in IL-6 or TNFα as reported for SpA[232, 234, 236]. This could be due to SpA masking the effect of Sbi in the lung. To rule out this possibility, future studies should include a *spa*-null strain and/or create a new MRSA strain lacking both SpA and Sbi. Additionally, a complemented Sbi strains should also be made.

Another explanation for the differences seen could be due to Sbi's role in complement evasion, which SpA plays no known role in. Complement activation is known to recruit neutrophils[580, 581] and lacking a complement evasion protein may lead to higher levels of C3a- and C5a-mediated chemotaxis. However, in the *sbi::Tn* mutant, we see fewer neutrophils in the lung at 6 hours post-infection, but this has not been tested in the sbi A100.2 mutant and cannot be ruled out. Additionally, early complement activation could lead to better controlled overall inflammatory

responses in the lung. Inflammation in the lung is a double-edged sword; inflammation is needed to mediate bacterial clearance, but too much inflammation can lead to lung damage and decreased lung function, which can lead to death. Indeed, we saw lower levels of inflammatory cytokines and reduced mortality in mice infected with sbi A100.2 but no difference in burden. In the future, it will be important to look at the complement system in mice infected with sbi A100.2 to determine if this could explain the differences seen.

In our macrophage infections, *sbi::Tn* and sbi A100.2 have similar trends in terms of bacterial burden, however, the inflammatory changes seen are different. This suggests that Sbi may be playing a role in terms of bacterial survival when interacting with macrophages, but this requires further testing. Heat-inactivated serum or serum free media was used in these experiments, so complement would not be playing a role. Sbi has been shown to play a role in inducing inflammation in macrophages also through TNFR1 and EGFR[149]. Recombinant Sbi led to increases in IL-1β, TNFα, and IL-6 protein, and we do see a significant decrease in *il-1β* transcript in RAW264.7 cells infected with sbi A100.2. We also see a trend towards decreased *tnfα,* but this is far from significance. This could be explained by differences seen between peritoneal macrophages and RAW264.7 cells, differences in bacterial strains (Newman versus JE2), or differences in bacterial-macrophage cultures. In the future, it will be important to perform infections with BMDMs or *ex vivo* macrophages as well as inhibit TNFR1 and EGFR signaling to determine if this mechanism is mediating the inflammation.

5.0 Characterization of a Novel CWA, SasD

5.1 Summary

Staphylococcus aureus cell wall-anchored proteins (CWAs) are involved in many aspects of infection such as adhesion, immune evasion, biofilm formation, and nutrient acquisition. Despite their important role in colonization and infection, not all CWAs have been characterized. One such CWA is *S. aureus* surface protein D (SasD). In a screen of CWA mutants in the context of bacterial pneumonia and influenza super-infection, we found that the uncharacterized SasD induced changes in both inflammatory and homeostatic lung markers. Here, we further characterized a SasD mutant (sasD A50.1) in the context of pneumonia. Mice infected with sasD A50.1 have decreased bacterial burden, inflammatory responses, and mortality compared to wildtype *S. aureus*. Mice also have reduced levels of IL-1β, likely derived from macrophages. Reductions in IL-1β transcript levels as well as increased macrophage viability point at differences in cell death pathways. These data identify a novel virulence factor for *S. aureus* that influences inflammatory signaling within the lung.

5.2 Introduction

Staphylococcus aureus is a common pathogen that has evolved both as a commensal flora as well as a major cause of invasive infections. Infection with *S. aureus* can range from mild skin infections to severe pneumonia, sepsis, and death[9, 314]. As a successful pathogen, *S. aureus*

expresses several mechanisms to establish infection and evade the host immune system such as resistance to phagosomal bactericidal mechanisms, pore forming toxins, complement evasion proteins, and super-antigens[2, 9]. Furthermore, methicillin-resistant *S. aureus* (MRSA) antibiotic resistance and additional virulence determinants can lead to more difficulty in clinical treatment[15].

One family of proteins implicated in establishing colonization and infection are the cell wall-anchored proteins (CWAs). CWAs are covalently attached to the peptidoglycan cell wall by the enzyme Sortase A, which recognizes the c-terminal cell-wall sorting signal LPXTG[25]. CWAs facilitate binding to host extracellular matrix and plasma proteins, evasion of several host defense mechanisms, invasion of endothelial and epithelial cells, biofilm formation, and iron acquisition from hemoglobin[22, 33]. Many of these family members contain common domain structures that facilitate ligand binding or project ligand binding regions away from the cell wall. However, there are some CWAs that do not have sequence or predicted structure similarities and their function is unknown[159]. One of these proteins is *S. aureus* surface protein D (SasD). While this protein was discovered in 2003, little work has been done to characterize this protein[156, 165, 166].

In a screen of CWAs in the context of staphylococcal pneumonia and *S. aureus* influenza super-infections (see 3.0 above), we discovered that mice infected with MRSA lacking SasD (*sasD::Tn*) had lower inflammation than mice infected with wildtype MRSA. Thus, we decided to characterize a transduced version of this mutant, sasD A50.1, in the context of MRSA pneumonia.

Data presented here are found in "Novel Requirements for Staphylococcal Cell Wall-Anchored Protein SasD in Pulmonary Infection" by Jennifer A. Grousd, Abigail M. Riesmeyer,

Vaughn S. Cooper, Jennifer M. Bomberger, Anthony R. Richardson, and John F. Alcorn. Currently found on BioRxiv (https://www.biorxiv.org/content/10.1101/2022.04.01.486802v1).

5.3 Materials and Methods

5.3.1 Mice.

Six- to eight-week-old male and female WT C57BL/6NTac mice were purchased from Taconic Farms. Mice were maintained under pathogen-free conditions within the animal facilities at the UPMC Children's Hospital of Pittsburgh. All studies were performed on sex- and age-matched mice. Animal studies were conducted with approval from the University of Pittsburgh Institutional Animal Care and Use Committee.

5.3.2 *S. aureus* strains.

USA300 MRSA strain JE2[565] was the WT strain for all studies. Strain sasD A50.1 was generated via phage 11 transduction of *sasD::Tn* lysate into the wildtype JE2 strain, selected with 5 µg/ml erythromycin and confirmed by PCR (Table 4). *S. aureus* strains were grown in Tryptic Soy Broth (BD Bacto™) overnight at 37° C at 250 rpm. Overnight cultures were diluted 1:100 and grown until OD_{660}~1, approximating logarithmic growth phase. MRSA dose was calculated using OD_{660} measurement of the culture and application of a calculated extinction coefficient. For growth curves, overnight cultures were diluted 1:200 in a 96-well plate in sexaplicate. Plates were

grown at 37ºC at 282 rpm in a Synergy H1 Hybrid Multi-Mode Reader (BioTek). Optical density measurements at 660 nm were taken every 30 minutes. Growth rate (μ) was calculated from at least two independent experiments using the equation $A_t=A_{t-1}*e^{\mu t}$. The μ_{max} was calculated as the average of the three highest μ rates.

5.3.3 Murine Models.

Mice were inoculated with $1x10^8$ colony forming units (CFU) of MRSA in 50 μl of sterile PBS. All infections were performed via oropharyngeal aspiration. Mice were harvested 6 or 24 hours after MRSA challenge using pentobarbital injection (300 mg/kg) and cervical dislocation. In mortality studies, a dose of $2x10^8$ CFU was used. During harvest, the lung was lavaged with 1 ml sterile PBS. BAL cells were pelleted and red blood cells were lysed (ACK lysis buffer, Gibco). Cells were resuspended, placed on slides via cytospin, stained with Hema 3 (Thermo Fisher), and quantified. The right upper lung lobe was homogenized in 1 ml PBS and plated on tryptic soy agar for determination of bacterial burden. The remaining right lung was frozen in liquid nitrogen and stored at -80ºC for gene expression analysis. The left lobe was perfused with 10% formalin and embedded in paraffin. Lung sections were stained with hematoxylin and eosin and inflammatory features were evaluated via microscopy after sample blinding. Lung homogenate cytokines were assessed using the Bio-Plex Pro Mouse Cytokine 23-plex Assay (Bio-Rad). For competitive index studies, mice were inoculated with a 1:1 ratio of JE2 and sasD A50.1 strains at a total of $1x10^8$ CFU. Whole lungs were homogenized in 2 ml of sterile PBS and plated on tryptic soy agar with and without erythromycin (5 μg/ml). Competitive index was calculated as the ratio of sasD A50.1:JE2 CFU at sacrifice divided by the ratio at the time of inoculation.

5.3.4 Macrophage experiments.

RAW264.7 cells were used and BMDMs were isolated as previously described[582]. For experiments, $7x10^5$ cells were plated in 6-well plates, infected at an MOI 10 (RAW264.7) or 50 (BMDMs) and spun at 250 xg for 5 minutes at 4^o C to synchronize infection. For RAW264.7 experiments, cells were infected for 1 hour in the absence of antibiotics, media was replaced with antibiotic- and serum-free media with and without gentamicin (100 ug/ml) for 1 hour, then replaced with antibiotic-free media for an additional hour. At collection, cells were lysed with 1% Triton X-100 at room temperature for 10 minutes and 50 µl was collected for CFU determination. Phagocytosis was calculated by the equation ((CFU+gentamicin)/(CFU-gentamicin))*100. RLT (Qiagen) was added to the wells and collected and ran through a Qiashredder and frozen at -80ºC until RNA extraction. For BMDM experiments, cells were rested overnight, treated with 10 ng/ml IFNγ (R&D Systems) for 24 hours. BMDMs were infected for 3 hours, washed, and resuspended in antibiotic free RPMI media. BMDM viability was determined by trypan blue (Gibco) staining and the Countess 3 automatic cell counter (Invitrogen). BMDMs wells were combined and incubated in RIPA buffer (25mM Tris, 150 mM NaCl, 1% NP-40, 0.1% SDS, 5 mM EDTA, 0.5% sodium deoxycholate) for 30 minutes at 4°C with agitation, centrifuged at 10,000 rpm for 10 minutes at 4°C, and frozen at -80°C until Western Blot. Primary antibodies were rabbit anti-IL-1β (Abcam 254360), rabbit anti-caspase 1 (Abcam 138483), rabbit anti-caspase p20 (Invitrogen PA5-99390), and mouse anti-β-actin (Cell Signaling 8H10D10). Samples were thawed, proteins were quantified using BCA protein assay (Pierce), boiled in Laemmli buffer (Bio-Rad), and loaded on a 4-20% gel (Bio-Rad). Proteins were transferred to a PVDF membrane using the Trans-Blot Turbo transfer system (Bio-Rad). Blots were probed with primary antibodies and donkey anti-mouse or

goat anti-rabbit secondary antibodies conjugated to IRDye® 800CW or 680RD fluorophores (LI-COR). Blots were imaged using the Odyssey CLx and analyzed using Image Studio (LI-COR). Relative protein expression is normalized to beta-actin levels in each sample.

5.3.5 RNA extraction and qPCR.

RNA was extracted from mouse lungs using the Absolutely Total RNA Purification Kit (Agilent). RNA extraction from cell culture experiments were performed using the Qiagen RNeasy kit (Qiagen). RNA was quantified and converted to cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad). Quantitative PCR was performed using SsoAdvanced Universal Probes Supermix (Bio-Rad) and TaqMan primer-probe sets (ThermoFisher Scientific) listed in Table 5 on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Gene expression was calculated using the ΔΔct method using *hprt* as a housekeeping gene and normalized to the average WT *S. aureus* values unless otherwise stated.

5.3.6 Statistical Analysis.

Data were analyzed using Prism 8 (GraphPad). Analyses comparing two groups were performed using Mann-Whitney test or an unpaired t test. For analyses assessing more than two groups, Kruskal-Wallis with Dunn's multiple comparisons correction was used. Analyses comparing two variables were tested via Two-way analysis of variance (ANOVA) with Sidak's multiple comparisons correction. Mortality data were analyzed by a log rank (Mantel-Cox) test. All figures show combined data from multiple replicate studies and are graphed as mean ± standard error of the mean (SEM). N values are numbers of animals per independent experiment. Statistical

significance ($p \leq 0.05$) is indicated in figure legends, with p values between 0.05 and 0.1 displayed numerically.

5.4 Results

We identified that the mutant lacking SasD (*S. aureus* surface protein D; *sasD::Tn*) induced reduced levels of cytokines G-CSF, CXCL1, MCP-1, and IL-1β compared to the WT strain during bacterial pneumonia (Figure 27 A). Additionally, we also saw increased levels of epithelial and lung homeostasis marker gene expression including tight junction protein 1 (*tjp1*) and mucin 5b (*muc5b*) (Figure 27 B). This suggested to us that this mutant may be causing less inflammation in the lung leading to improved epithelial and lung function. There is very little known about SasD and to our knowledge, there has been no *in vivo* characterization of this CWA. Therefore, we decided to characterize this mutant in the context of bacterial pneumonia. To eliminate the potential for unknown mutations in the transposon mutant, we transduced the mutant into the JE2 strain, creating the strain sasD A50.1. This strain had no difference in *in vitro* growth compared to the WT strain (Figure 28 A and B). At 24 hours post infection, mice infected with sasD A50.1 had reduced bacterial burden and immune infiltrate in the BAL (Figure 19 A and B). Infection with sasD A50.1 led to a decrease and increase in percentage of neutrophils and eosinophils, respectively. While there were no changes in genes related to neutrophil function (Figure 28 C), we did see a 50% reduction in the neutrophil:eosinophil ratio in the lung (Figure 19 C and D). The change in bacterial burden and immune cell infiltrate did not lead to differences in acute lung injury by BAL protein (Figure 19 E) or histological score (Figure 28 D-E). However, we did see significant changes to transcripts related to lung homeostasis (Figure 28 F). Additionally, we saw

a significant delay in mortality in mice infected with sasD A50.1 compared to WT *S. aureus* (Figure 19 F). The changes seen 24 hours post infection in mice infected with sasD A50.1 may be because of a decrease in survival of the mutant in the lung, as this mutant had a reduced competitive index when mice were infected with a 1:1 ratio of mutant:WT bacteria (Figure 19 G).

To evaluate the inflammatory state within the lung, we looked at cytokine expression in the lung 24 hours post infection. Similar to the original screen, we saw decreases in protein levels of IL-17A, CXCL1, GM-CSF, and IL-1β in mice infected with sasD A50.1 (Figure 20 A). We also saw a decrease in transcript levels for *il-17a, cxcl1, and il-1β* (Figure 20 B). Because we saw a decrease in IL-1β and *S. aureus* is known to induce the NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3) inflammasome[354], we also looked at inflammasome components. We saw a significant decrease in NLRP3 transcript, but not the general inflammasome adaptor ASC (apoptosis-associated speck-like protein containing C-terminal caspase recruitment domain [CARD]; *pycard*) (Figure 20 B). We did not see differences in alpha-toxin activity, the known inducer of the NLRP3 inflammasome[354], via hemolysis of blood agar in either strain (data not shown).

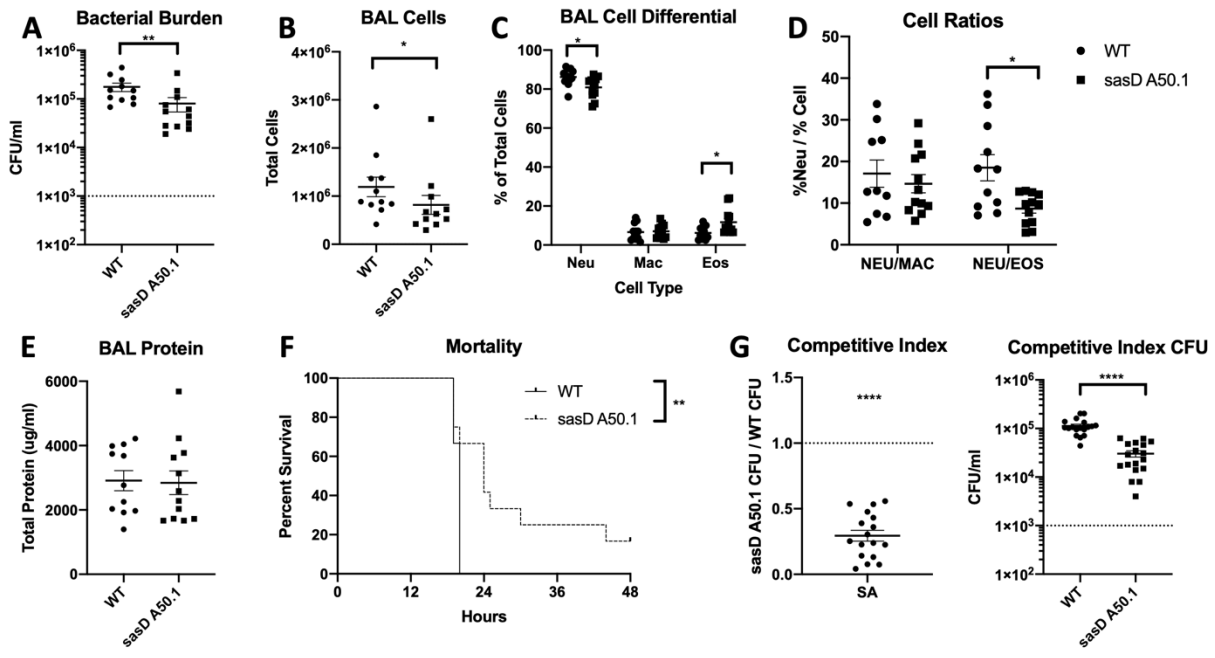

Figure 19: SasD Impacts *S. aureus* Bacterial Pneumonia Outcomes

A-E. Mice were infected with $1x10^8$ CFU WT MRSA or MRSA lacking SasD (sasD A50.1) for 24 hours. A. Bacterial burden in mice infected with MRSA for 24 hours. B. Total cells in the bronchoalveolar lavage (BAL). C-D. Cell differentials (C) and neutrophil cell ratios (D) of BAL cells. E. Total protein in the BAL. F. Mice were infected with a lethal dose ($2x10^8$ CFU) of WT or MRSA lacking SasD (sasD A50.1). G. Competitive index of WT and mutant sasD A50.1 MRSA in the lung. Mice were infected with a 1:1 ratio of WT:sasD A50.1 for a total dose of $1x10^8$ CFU for 24 hours. Whole lungs were collected in 2 ml PBS and homogenized and plated for CFU with and without antibiotic selection. Competitive index is calculated as the ratio of Mutant CFU:WT CFU at 24 hpi. Statistics tested by student Mann-Whitney (A,B, D, G), Two-way ANOVA with Sidak's multiple comparisons (C), log-ranked Mantel Cox test (F), one sample T-test with H_0 set to 1 (1:1 ratio of mutant:WT) (G). * $p<0.05$, ** $p<0.01$,**** $p<0.0001$. N=4-8, combination of several experiments, data graphed as mean ± SEM

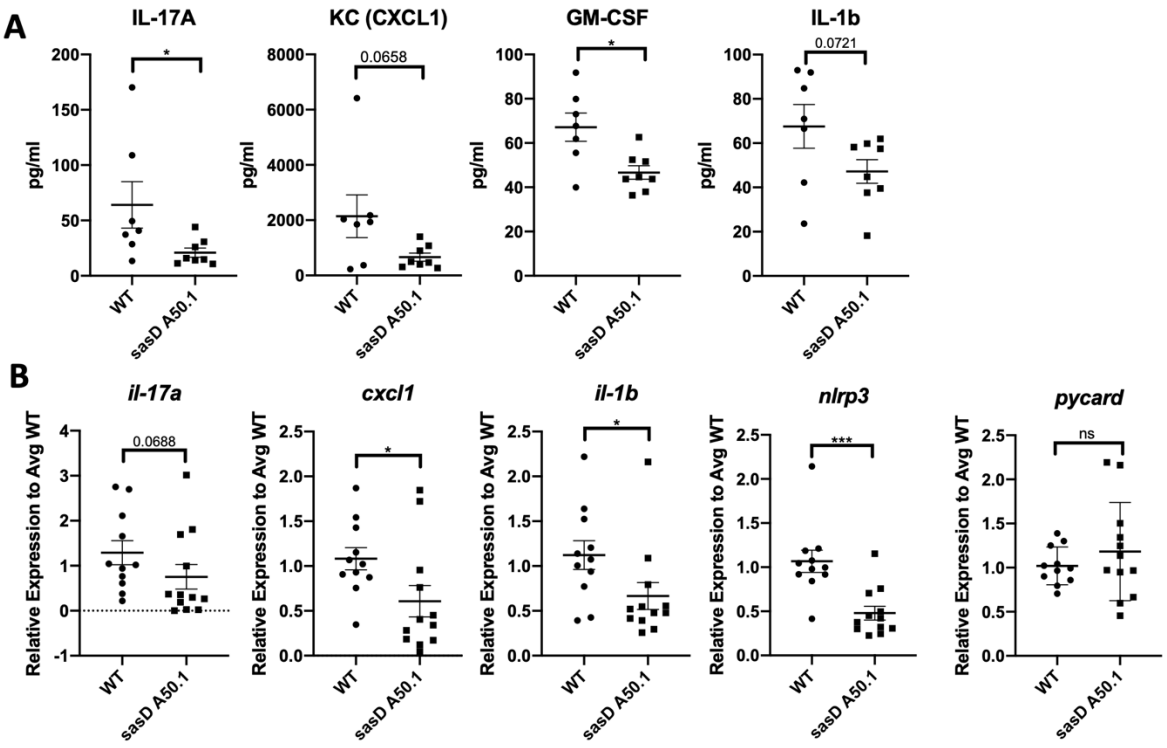

Figure 20: SasD Increases Inflammation in Mice Infected with MRSA

Mice were infected with $1x10^8$ CFU WT MRSA or MRSA lacking SasD (sasD A50.1) for 24 hours. A. Cytokine protein levels in lung homogenate. B. Gene expression levels of cytokines and inflammasome components relative to average WT levels in the lung. Statistics done by Mann-Whitney test, * $p<0.05$, *** $p<0.001$. N=4, combination of several experiments, data graphed as mean ± SEM.

Mice infected with sasD A50.1 for 6 hours also had a reduction in bacterial burden compared with WT (Figure 21 A). While the total number of immune cells recruited into the airway was not different between WT and sasD A50.1 infected animals (Figure 21 B), we did see significant decreases in the percentages and total numbers of macrophages in the BAL (Figure 21 C-D). This increase in the neutrophil:macrophage ratio in the lung during this early timepoint (Figure 21 E) may be associated with less neutrophil recruitment later during infection. Unlike 24 hours post infection, we did see a significant difference in acute lung injury via protein in the BAL (Figure

21 F) and there was a significant difference in peribronchial inflammation via histology (Figure 29 A-B). However, we did not see changes in lung homeostasis transcripts (Figure 29 C). We also saw a survival defect of this mutant early on in infection via competitive index (Figure 21 G), which does not appear to be due to differences in early recruited neutrophil function (Figure 29 D).

The inflammatory response to sasD A50.1 at 6 hours post infection was very similar to 24 hours post infection (Figure 22). Again, we saw reductions in IL-17A, and IL-1β with the addition of a decrease in G-CSF and IL-6 (Figure 22 A). By transcript levels, we saw reductions in both *il-17a and il-23a* (Figure 22 B), suggesting that the antibacterial immunity response is not as robust to sasD A50.1 compared to the WT at this time point, potentially due to the difference in survival between the two strains (Figure 21 G). We also saw a reduction in inflammasome components NLRP3 but not ASC (Figure 22 B).

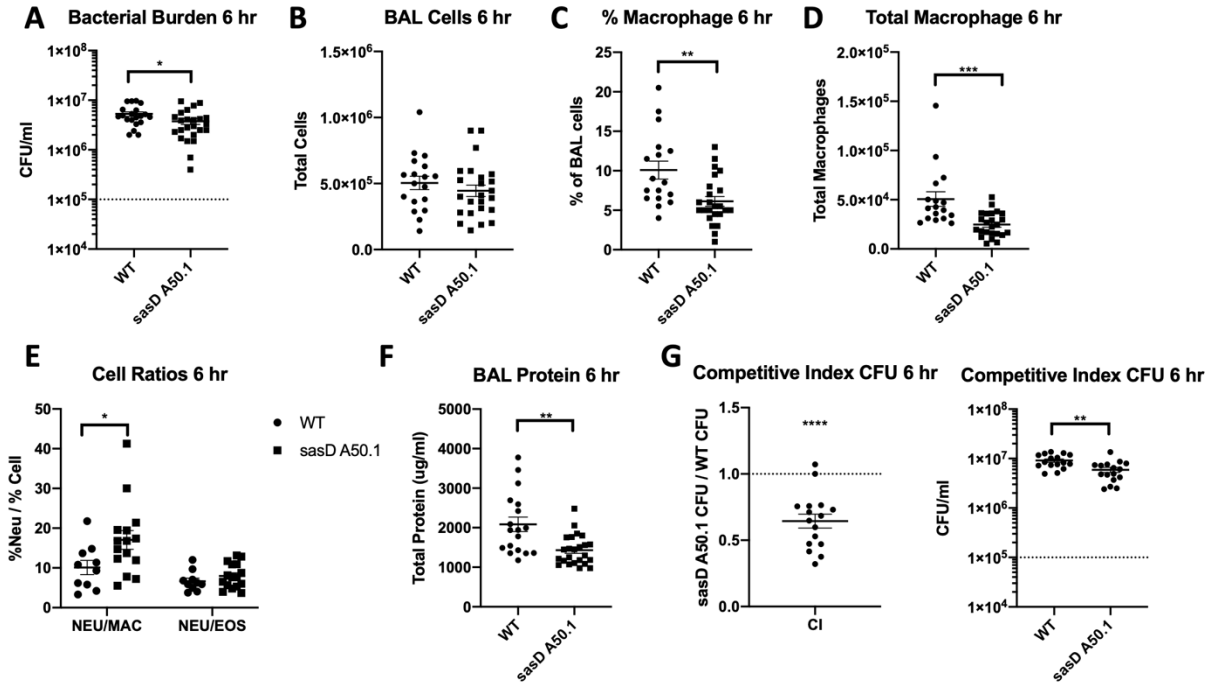

Figure 21: SasD Impacts Initiation of Host Defense against MRSA in Bacterial Pneumonia

A-F. Mice were infected with $1x10^8$ CFU WT MRSA or MRSA lacking SasD (sasD A50.1) for 6 hours. A. Bacterial burden in mice infected with MRSA for 6 hours. B. Total cells in the bronchoalveolar lavage. C-D. Percentage (C) and total number (D) of macrophages in the BAL. E. Neutrophil cell ratios in the BAL. F. Total protein in the BAL. G. Competitive index of WT and mutant sasD MRSA in the lung. Male and female mice were infected with a 1:1 ratio of WT:sasD A50.1 for a total dose of $1x10^8$ CFU for 6 hours. Whole lungs were collected in 2 ml PBS and homogenized and plated for CFU with and without antibiotic selection. Competitive index is calculated as the ratio of Mutant CFU:WT CFU at 6 hpi. Statistics tested by student Mann-Whitney (A, C, D, F, G), Two-way ANOVA with Sidak's multiple comparisons (E), one sample T-test with H_0 set to 1 (1:1 ratio of mutant:WT) (G). * $p<0.05$, ** $p<0.01$, ***$p<0.001$, **** $p<0.0001$. N=8, combination of several experiments, data graphed as mean ± SEM.

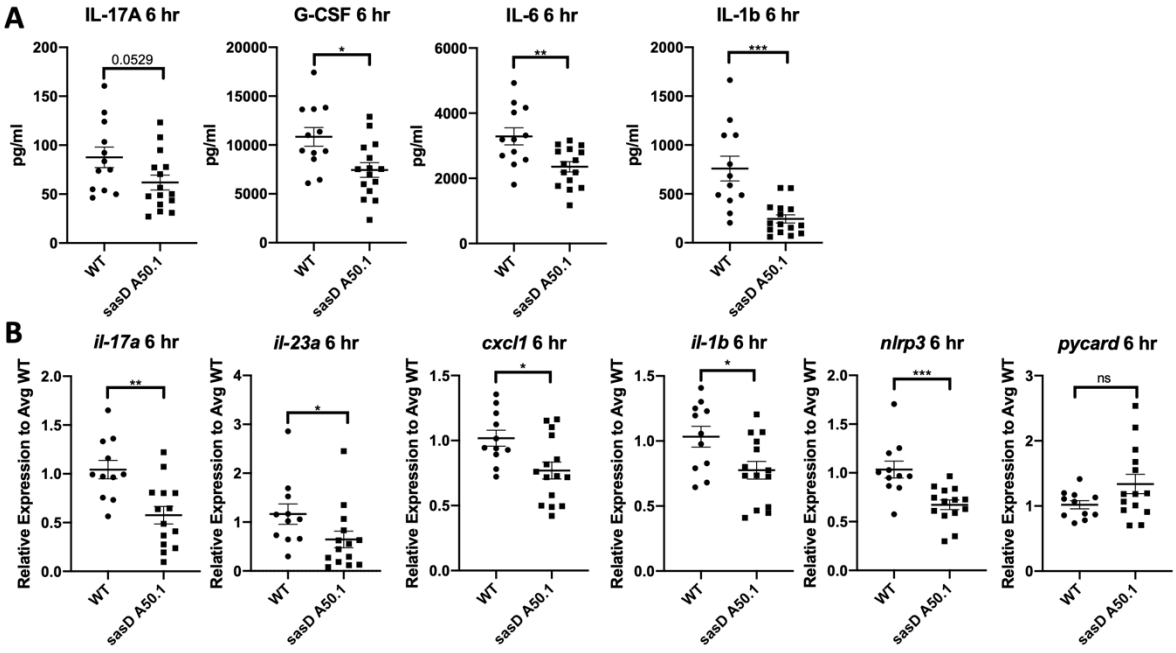

Figure 22: SasD is Required for Early Inflammation During Infection with MRSA

Mice were infected with $1x10^8$ CFU WT MRSA or MRSA lacking SasD (sasD A50.1) for 6 hours. A. Cytokine protein levels in lung homogenate. B. Gene expression levels of cytokines and inflammasome components relative to average WT levels. Statistics done by student Mann-Whitney, * $p<0.05$,** $p<0.01$, *** $p<0.001$. N=8, combination of several experiments, data graphed as mean ± SEM.

Because of the differences seen in macrophages early on during infection, we looked at early macrophage interactions with sasD A50.1. Using the macrophage cell line RAW264.7, we saw a significant reduction in transcript expression of IL-1β and TNFα despite no difference in bacterial burden or amount of phagocytosed bacteria (Figure 23 A and B). In bone marrow-derived macrophages (BMDMs), infection with sasD A50.1 led to an increase in % viability compared to WT infected BMDMs at 3 hours post infection (Figure 23 C). At this timepoint we did not see any differences in pro-IL-1β or caspase 1 activity (Figure 23 D and E). However, we do see similar changes in BMDMs via qPCR compared to RAW264.7 cells (Figure 23 F).

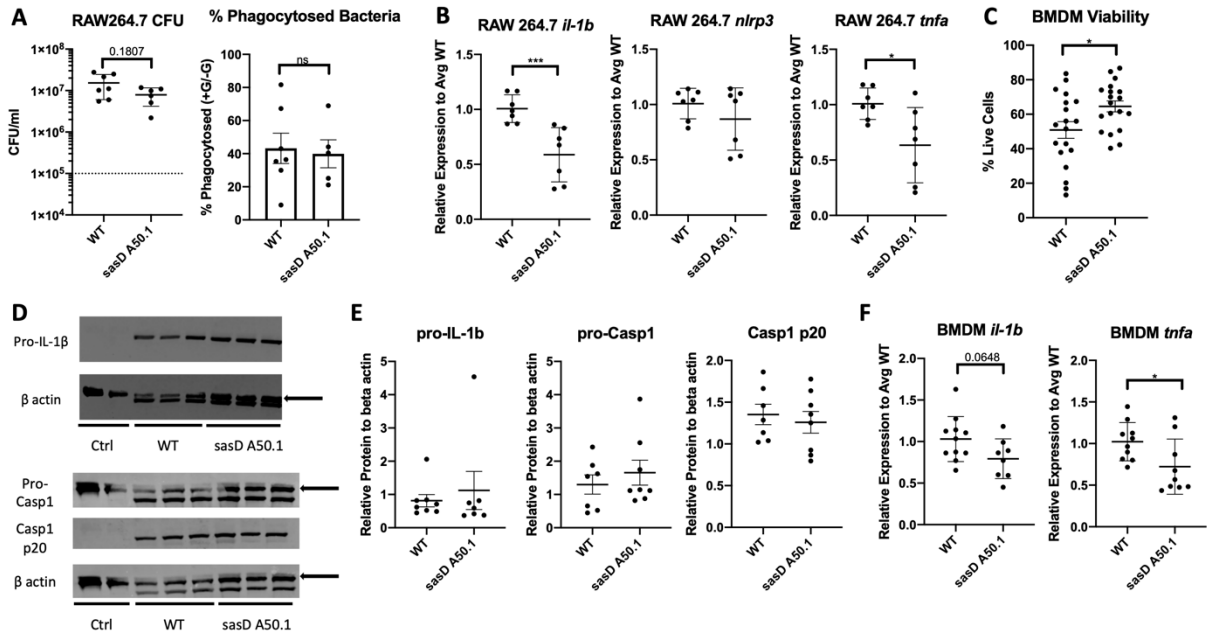

Figure 23: SasD Increases Macrophage Inflammation and Decreases Survival.

A-B. RAW264.7 macrophages infected with WT or sasD A50.1 MRSA for 3 hours at an MOI of 10. Macrophages were infected for one hour in the absence of antibiotics, media was then replaced with antibiotic- and serum-free media with or without gentamicin for 1 hour, and changed to antibiotic free media. CFU and transcript graphs show without gentamicin conditions. A. Bacterial burden and % phagocytosed bacteria in RAW264.7 macrophages. % Phagocytosed bacteria is calculated by the following equation: ((average CFU with gentamicin)/(average CFU without gentamicin))*100. B. Gene expression in RAW264.7 macrophages infected with WT or sasD A50.1 MRSA for 3 hours. C-F. Bone marrow-derived macrophages (BMDMs) were infected with WT or sasD A50.1 MRSA for 3 hours at an MOI of 50 in the absence of antibiotics. C. Viability measured by trypan blue staining of BMDMs 3 hours post infection. D-E. Representative images (D) and quantification of western blot analyses (E) of BMDM levels of IL-1β and caspase 1. One to three wells were combined per sample and protein levels are normalized to beta actin in each sample. Arrows denote which band was used for quantification. F. Gene expression in BMDM macrophages infected with WT or sasD A50.1 MRSA for 3 hours. Statistics tested by Mann-Whitney, * $p<0.05$, *** $p<0.001$. N=4-7, combination of several experiments, data graphed as mean ± SEM.

5.5 Discussion

During our screen (see 3.0 above) we found that the *sasD::Tn* mutant had decreased levels of myeloid cytokines and increased gene expression of lung homeostatic markers in bacterial pneumonia. We found similar findings with a transduced mutant. Most striking was the delay in mortality in mice infected with sasD A50.1, which may be explained by the decrease in bacterial survival seen with sasD A50.1 infection compared to the WT strain. What could be causing this decrease in bacterial survival is unknown, as this protein is uncharacterized. While it is known that SasD has a punctate surface expression versus a ring-like distribution of most CWAs[156, 165], it is unclear if this is contributing to the differences seen in our model. This decrease in bacterial survival of sasD A50.1 could explain the inflammatory differences seen early and late during bacterial pneumonia.

At 6 hours post infection, there is a significant reduction in the macrophages in the lung, as well as IL-1β. It is known that macrophage derived IL-1β can induce excessive inflammation and pathology in the lung[583, 584]. The reduction in IL-1β could be explained by the decrease in macrophages early during infection. This decrease in inflammation continued at 24 hours post infection, where there was a reduction in the levels of neutrophils, which can cause excessive damage themselves[585]. While we did not see functional changes in neutrophils via qPCR, we did see a decrease in the neutrophil:eosinophil ratio within the lung at 24 hours post infection with sasD A50.1. Eosinophils have been implicated in antibacterial immunity[586], and the increase ratio of eosinophils could help control the bacterial burden in the lung. It has been shown that IL-33 induction of type 2 responses is protective in lethal models of *S. aureus* sepsis and pneumonia by counterbalancing pro-inflammatory responses[587, 588]. While we did not see any differences in IL-

33 (data not shown) or gross pathology at 24 hours post infection, we did see a reduction in type 17 cytokines and neutrophils, which has been shown to be protective in patients with *S. aureus* infection[588, 589]. Thus, the reduction in inflammation or alteration of inflammatory cell ratios could help explain the delayed mortality seen in mice.

Since we saw a change in IL-1β production both early and late during infection, we decided to examine the inflammasome. *S. aureus* is known to prime and activate the NLRP3 inflammasome via pore-forming toxins, such as the alpha-toxin[354]. The NLRP3 inflammasome activates caspase 1, which cleaves pro-IL-1β[272]. We did see a significant downregulation of *il-1β* and *nlrp3* transcripts but not the more common ASC (*pycard*) component, suggesting that potentially the priming step of the NLRP3 inflammasome expression may be reduced. Priming of the NLRP3 inflammasome is thought to be due to sensing of *S. aureus* lipoproteins and toll-like receptor (TLR) 2 and 4 signaling[354, 590]. While we did not see changes in expression in TLR-2 or -4 in macrophages (data not shown), we cannot rule out the possibility that SasD may be involved in the sensing of *S. aureus*. When infected with sasD A50.1, RAW264.7 cells had a reduction in *il-1β* and *tnfα* without a significant change in bacterial burden or bacterial phagocytosis. In BMDMs, we saw a similar trend in gene expression as well as an increase in viability when infected with sasD A50.1 compared to WT *S. aureus*. While we did not see any differences in pro-IL-1β or caspase 1 activity at 3 hours post infection, there may be other cell death pathways involved such as necroptosis. Blocking necroptosis has been shown to reduce bacterial burden and damage during *S. aureus* pneumonia[356], similar to the sasD A50.1 *in vivo* phenotype, and can feed into NLRP3 inflammasome and pyroptosis induction[360]. Thus, the decrease in IL-1β could be due to changes in cell death pathways that funnel into the NLRP3 inflammasome in macrophages.

In conclusion, we identified a critical role for SasD in bacterial pneumonia associated with increased bacterial burden, inflammation, and mortality. SasD may contribute to survival of *S. aureus* in the lung as there is decreased bacterial survival in the mutant at both 6 and 24 hours post infection. SasD promotes induction of early IL-1β production in macrophages, which consequentially recruits neutrophils into the lung at later timepoints, leading to increased inflammation. These data suggest that early targeting of SasD in the lung may reduce future inflammation signaling during staphylococcal pneumonia.

6.0 Conclusions and Future Directions

Determining *in vivo* virulence factors for bacterial infections can be difficult due to the inherent complexity of host-pathogen interactions and individual physiologies. The process of infection is multifactorial even when only the bacterium or host is considered. However, to fully understand infectious processes it is important to look at both the host and pathogen, as many of these responses have likely coevolved over evolutionary time due to the ever-continuing arms race between pathogens and their hosts. In the bacterium, there is a balance between evading immune responses and adapting to the host environment while the host immune system balances pathogen clearance with host inflammation and damage. Thus, understanding host-pathogen interactions together may illuminate aspects of infection otherwise unnoticed. Influenza bacterial super-infections are even more complex as it involves more than one type of pathogen in the host. Due to this, many of the studies looking at super-infection susceptibility have been focused on the immune system, which can be studied and manipulated in mouse models quite well. However, just as the immune system is not rigid, bacteria also can have varying responses to environmental stimuli. Thus, understanding how bacteria adapt to host environments can be an important step in elucidating mechanisms within the host.

One of the main techniques available to understand bacterial adaptations to host environments is Tn-seq. By looking at the genetic requirements needed to establish infection in a particular host environment, there can be understanding of the stressors bacteria face and how they adapt to their new environment. Additionally, understanding adaptations to the host environment can give information as to how the host is responding to the pathogen. For example, Tn-seq has been used

to find genetic requirements for bacterial survival with phagocytes[540, 541, 591, 592, 593], which confirms the importance of ROS/RNS and complement-mediated opsonophagocytosis by these immune cells. In the lung, there have been many metabolic pathways implicated in bacterial survival, which gives insight into how bacteria adapt to the lung environment. Additionally, it has given insight into how core metabolic pathways can lead to changes in factors already known for bacterial survival such as macrophage survival, complement evasion, and biofilm formation[525, 536, 540, 542, 543, 544, 545]. Tn-seq studies performed in the context of super-infection have found that core metabolic pathways are required in both contexts, with some differences based on more available nutrients as well as a dysregulated immune system in the lung[526, 535]. While Tn-seq has been performed with *S. aureus* in the context of pneumonia[540], it is still unexplored in the context of super-infection. However, a major caveat of Tn-seq is that it cannot identify all requirements in a particular environment due to its inability to detect secreted protein requirements, bacterial cheating and survival through other mutants, and limited detection of small open reading frames[530, 534, 539, 594]. Thus, it cannot be the only technique used to discover virulence factors.

To fully understand the role of bacteria during super-infection, understanding how bacteria behave in a previously uninfected lung is important. The addition of another factor, viral infection, is likely to impact both the host and bacteria response and complicate mechanism determination. As the Alcorn Laboratory primarily focuses on *S. aureus* influenza super-infections, elucidating mechanisms in *S. aureus* pneumonia is essential to understanding the full context of super-infection. Additionally, understanding *S. aureus* virulence in pneumonia is equally important on its own, as it can be a cause of necrotizing pneumonia[302, 306].

Adherence is one of the first steps in establishing infection. Without the ability to bind to host components, or to neighboring bacteria for biofilm formation, bacteria have a higher chance of being cleared by the immune system. CWAs play a large role in staphylococcal adherence to host components as well as cell-cell adhesion in biofilms. Additionally, several of these proteins are implicated in nasal colonization and immune evasion[22, 23, 518]. As these proteins are located in the cell wall, they are exposed to the exterior environment, which increases the chance that these proteins are sensed by the immune system and/or *S. aureus* has developed mechanisms to shield these proteins from detection. Indeed, many of these proteins have been implicated in shielding *S. aureus* from host recognition by covering the bacteria in host factors as well as alter immunological signaling[22, 23, 518] (see Table 1). Thus, these proteins are a good place to start to elucidate the mechanisms of bacterial persistence in the lung.

In the context of bacterial pneumonia*, S. aureus* CWAs have varying degrees of influence on bacterial persistence (see 3.0 above). Many of the CWA mutants tested here had decreased survival in the lung compared to WT MRSA. This is not surprising as losing immune evasion or adhesion proteins will affect the ability of bacteria to survive in a stressful environment. Reduced survival in the lung likely leads to less severe activation of the immune response and consequently less immune mediated inflammation and damage within the lung. This may be a positive or negative consequence, depending on how much inflammation is actually required to clear the infection. Much of *S. aureus* clearance in the lung is mediated by neutrophils[427] and neutrophils can be beneficial or detrimental depending on the context. There needs to be enough recruitment and bactericidal activity to clear the infection, but excess recruitment and activity of neutrophils can lead to host damage. While immune mediated damage may not be life threatening in other organs,

in the lung, excess inflammation can lead to organ dysfunction and death due to its important role in respiration[576]. Additionally, *S. aureus* is known to form microaggregates in the lung to help facilitate damage mediated through the alpha-toxin[343]. This was attributed to PhnD, however, there may be a role for CWA(s), especially those known to form homophilic bonds between neighboring bacteria. Here, the focus was not on toxin-mediated damage, however in the future, it may be helpful to explore CWAs and the alpha-toxin together to determine if they play a role in that context.

In the context of influenza super-infection, the CWAs tested did not have a major role in controlling bacterial persistence, immune recruitment into the airways, or lung damage. This supports the paradigm that influenza and the immune response to influenza are the major drivers of susceptibility to super-infection[482, 483, 484, 486]. As most of the super-infecting bacteria are commensals of the upper respiratory tract, secondary bacterial infection may be a consequence of opportunity for the bacteria rather than any particular bacterial behavior changes to promote super-infection. There is support for *S. aureus* in this context, as super-infecting strains are more closely related to nasal colonization strains than those that cause pneumonia[498]. However, this does not rule out the possibility that there are non-CWA virulence factor(s) that play a major role in super-infection susceptibility. For example, dissemination of nasal biofilms to the lung occurs during influenza infection[491594,595] and so proteins involved in sensing external stimuli and/or promoting dissemination of biofilms may be an important avenue to explore.

The one context tested that CWAs have a role in both pneumonia and super-infection is inflammatory signaling. Many of these proteins have similar structures and are grouped into

subfamilies based on this[22, 23, 518]. Thus, it is unsurprising that subfamily members, such as MSCRAMMs, would elicit similar immune signatures. Additionally, lacking all CWAs on the cell surface leading to reduced inflammatory signaling is not surprising. While these proteins are still secreted outside of the bacterium, many of these proteins cannot perform their functions of immune evasion because they are no longer attached to the bacteria. Thus, losing several immune evasion mechanisms, on top of their adhesion functions, likely leads to less inflammation and immune activity needed to clear these bacteria, which is ultimately better for the host. This can be seen in pneumonia mortality differences in MRSA mutants lacking Sbi or SasD (see 4.0 and 5.0 above). What was surprising is that the clustering of CWA mutants did not change much in super-infection. While the expression level and clustering of cytokines are different between these two contexts, it does suggest that CWAs do influence inflammatory signaling and/or that these CWAs are recognized similarly in both contexts. Some of this could be due to timing as cytokines were measured 24 hours post *S. aureus* infection in both contexts. The addition of bacterial PAMPs would induce immunological signaling in the lung, even if abrogated compared to pneumonia alone. However, it is unclear if this is a transient change just seen at this time point, or if this is a shift in the overall immune response in the lung. Looking at the cytokine signature at earlier and later time points to determine if it resembles influenza alone would be helpful. Many *S. aureus* proteins have been implicated in altering inflammatory signaling[9, 149, 232] and this potentially could be a bacterial 'behavior' that increases susceptibility to super-infection due to the overwhelming inflammation caused. Inflammation is known to alter cell surface expression of various molecules, and this influences adhesion and nutrient availability for the bacteria[484, 490, 492, 493, 578]. Thus, studying staphylococcal CWAs in the context of inflammation inducing proteins may be a viable avenue to pursue. Additionally, the one CWA that did move inflammation clusters is SasG, which

further supports research into biofilm mechanisms in the context of super-infection as mentioned above.

One reason as to why most CWAs did not have a strong phenotype in either pneumonia or influenza super-infection could be due to the nature of the mutants. These mutants come from the Nebraska Transposon Mutant Library (NTML)[565], which has been shown to have additional mutations due to the nature of the library creation[595, 596]. Thus, creating new mutants in a clean background is essential to understand these proteins' role in the lung. This was done for the two mutants studied in more detail, Sbi and SasD (See 4.0 and 5.0 above), but should be performed for the others if they are explored in more detail. Additionally, many of these CWAs have overlapping ligands and mechanisms in the host and so missing one CWA may not have a clear phenotype due to compensation of other CWAs. Many of the seminal work on CWAs has included exogenous expression in a bacterial host that does not express CWAs such as *Lactococcus lactis* or *S. carnosus*[22]. This allows for clear delineation of individual CWA function without the worry of compensation by other proteins. Due to the lack of previous experience in the Alcorn Laboratory with genetic manipulation of bacterial species as well as graduate student career timing, this was not performed for SasD. However, for future publications, it will be necessary to include this as a control.

While Sbi is not a CWA, it is a cell membrane associated protein that has known roles in immune evasion and induction of inflammatory signaling. Additionally, it has a similar structure to SpA, one of the major virulence factors in the lung. Based on experiments here, Sbi's role is not through IgG binding as there is no difference in survival in B6 and μMT mice (see 3.0 above).

Based on current data, it does not appear that Sbi is inducing inflammation through its Xr domain like SpA. However, this cannot be ruled out at this time. SpA is still present in the sbi A100.2 strain, and so this could be masking the phenotype of Sbi. Additionally, signaling through TNFR1 or EGFR was not investigated, and so we cannot rule out that this is a possible mechanism. If Sbi does not alter inflammation through signaling changes, then the changes seen are likely due to complement evasion activity of this protein. To explore this further, Sbi domain I and II and Sbi domain III and IV fragments should be created and used to determine which aspect of the protein is responsible for the induction of inflammation.

We also characterize a CWA with no known function, SasD. While we did not find a direct function for this protein, this is the first instance of *in vitro* or *in vivo* infection models with this protein and it will continue to be explored in the Alcorn Laboratory. SasD does influence inflammatory signaling in the lung, including IL-1β likely derived from alveolar macrophages. SasD also appears to alter macrophage survival, however, this does not appear to be mediated through caspase 1. At this time, it is unclear if the NLRP3 inflammasome is responsible for the IL-1β release, however this is the most well characterized inflammasome for *S. aureus* and mechanism for IL-1β release[346, 353, 354, 356, 597]. Few studies have implicated NLRC4 and NLRP6 inflammasomes with *S. aureus*, but these pathways are largely unexplored[359, 433]. Additionally, it is unclear if IL-1β is directly tied to cell death via pyroptosis, or a biproduct of inflammasome activation or induction of another cell death pathway such as necroptosis. Both pyroptosis and necroptosis have been implicated in *S. aureus* infections and play different roles in bacterial clearance and inflammation[360]. MRSA lacking SasD had a survival defect *in vivo*, suggesting that this protein is playing some role in bacterial persistence in the lung. This CWA is also interesting

due to its modified cell wall sorting motif, LPXAG, and the absence of the YSIRK/GS signal motif [156 165], however it is unclear at this time if these aspects influence the role of this protein.

In conclusion, understanding staphylococcal virulence in the context of both bacterial pneumonia and influenza super-infection is important for understanding host-pathogen interactions in the lung. Elucidation of unknown mechanisms of bacterial survival within the lung in both contexts can be at least partially done through the technique Tn-seq. Additionally, screening several CWAs in the context of both infections shows support for influenza and influenza induced immunity being the main drivers of super-infection. Exploring CWAs in the context of other bacterial virulence factors (biofilm, inflammation, etc.) may be helpful in fully elucidating their functions in the lung in both infections. Two surface exposed proteins explored here, Sbi and SasD, influence inflammatory signaling within the lung during pneumonia and this likely leads to increased mortality. Further exploration into these proteins, and additional CWAs, may lead to novel discoveries in terms of host-pathogen interactions, lung virulence factors, *S. aureus* virulence factors, as well as potential therapeutics to treat or prevent *S. aureus* pneumonia and influenza *S. aureus* super-infection.

Appendix A Supplemental Figures

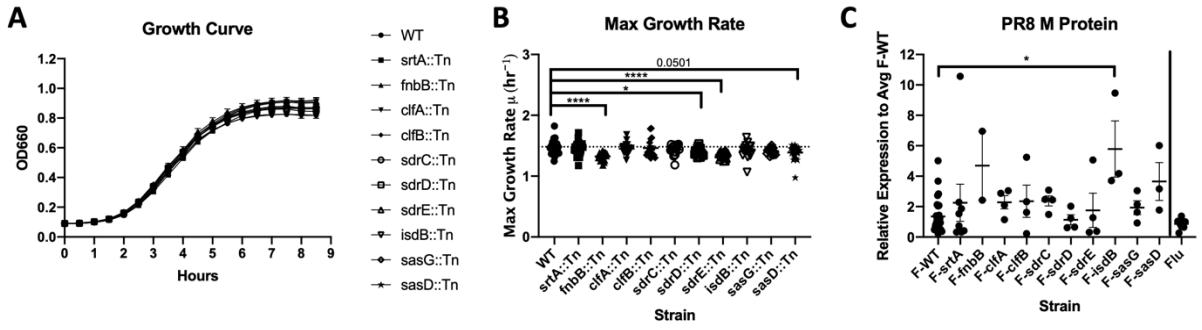

Figure 24: Bacterial Growth and Viral Burden

A. WT or mutant MRSA (see graphs) were grown overnight in tryptic soy broth and diluted 1:200 in a 96-well microtiter plate in sexaplicate. Plates were grown at 37°C at 282 rpm continuously. Measurements at 660 nm were taken every 30 minutes. Combination of at least 2 experiments per strain. B. Max growth rate of WT or mutant MRSA strains (see graphs). Growth rate (μ) was calculated off at least two independent experiments using the equation $A_t=A_{t-1}*e^{\mu t}$ (see methods). The μ_{max} was calculated as the average of the three highest μ rates. C. Relative expression of influenza PR8 M protein via qPCR normalized to the average F-WT values. Statistics tested by Kruskal-Wallis with Dunn's multiple comparisons correction (B-C). * $p<0.05$, ****$p<0.0001$. N=2-6, combination of several experiments, data graphed as mean ± SEM. srtA: Sortase A, fnbB: fibronectin binding protein B, clfA/B: clumping factor A/B, sdrC/D/E: serine-aspartate repeat containing protein C/D/E, isdB: iron-regulated surface determinant B, sasD/G: *S. aureus* surface protein D/G.

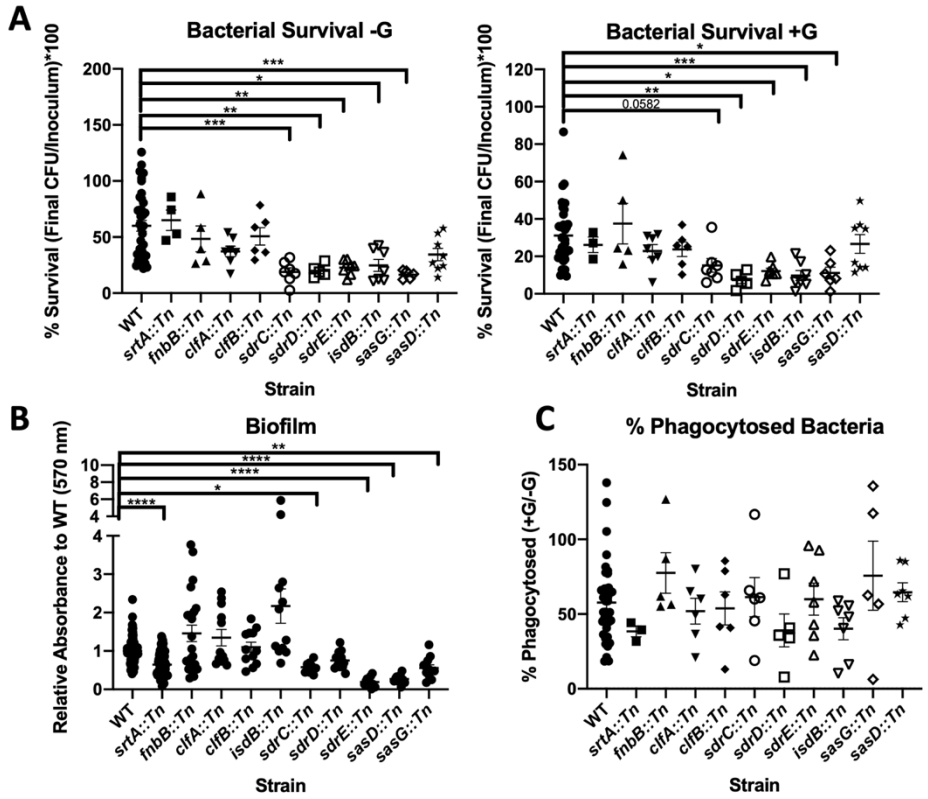

Figure 25: Survival of CWA Mutants with RAW264.7 Macrophages

A,C. RAW264.7 macrophages infected with WT or CWA mutant (see graph) MRSA for 3 hours at an MOI of 10. Macrophages were infected for one hour in the absence of antibiotics, media was then replaced with antibiotic- and serum-free media with (+G) or without (-G) gentamicin for 1 hour and changed to antibiotic free media. A. Bacterial survival of WT and CWA mutant MRSA (see graph) without (-G) and with (+G) 100 ug/ml gentamicin in the presence of RAW264.7 macrophages. B. Biofilm mass as measured by crystal violet staining and read at 570 nm (see methods). C. % phagocytosed bacteria in RAW264.7 macrophages infected with WT or CWA mutant MRSA (see graph). % Phagocytosed bacteria is calculated by the following equation: ((CFU with gentamicin)/(CFU without gentamicin))*100. Statistics tested by Kruskal-Wallis with Dunn's multiple comparisons. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ****$p<0.0001$. N=3-4 combination of several experiments (A,C) or N=10-12 of a single experiment (B), data graphed as mean ± SEM (standard error of the mean). srtA: Sortase A, fnbB: fibronectin binding protein B, clfA/B: clumping factor A/B, sdrC/D/E: serine-aspartate repeat containing protein C/D/E, isdB: iron-regulated surface determinant B, sasD/G: *S. aureus* surface protein D/G.

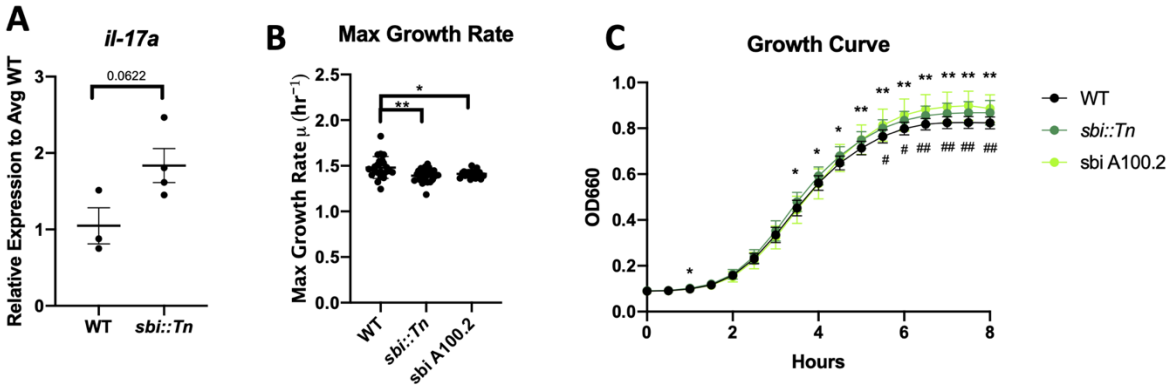

Figure 26: Supplemental Data for Sbi

A. Gene expression of *il-17a* in mice were infected with WT MRSA or MRSA lacking Sbi (sbi::Tn) for 6 hours. B. WT or mutant MRSA (see graphs) were grown overnight in tryptic soy broth and diluted 1:200 in a 96-well microtiter plate in sexaplicate. Plates were grown at 37°C at 282 rpm continuously. Measurements at 660 nm were taken every 30 minutes. Combination of at least 2 experiments per strain. Statistical significance is between WT and *sbi::Tn* MRSA strains (*) or between WT and sbi A100.2 (#). C. Max growth rate of WT or mutant MRSA strains (see graphs). Growth rate (μ) was calculated off at least two independent experiments using the equation $At=At-1*e^{\mu t}$ (see methods). The μmax was calculated as the average of the three highest μ rates. Statistics done by unpaired t test (A) Kruskal-Wallis Test with Dunn's multiple comparisons (B), Mixed-effects model with Dunnett's multiple comparisons correction (C). * $p<0.05$, * $p<0.01$, $^{\#}$ $p<0.05$, $^{\#\#}$ $p<0.01$. N=3-4 (A), 6 (B-C), combination of several experiments (B-C), data graphed as mean ± SEM.

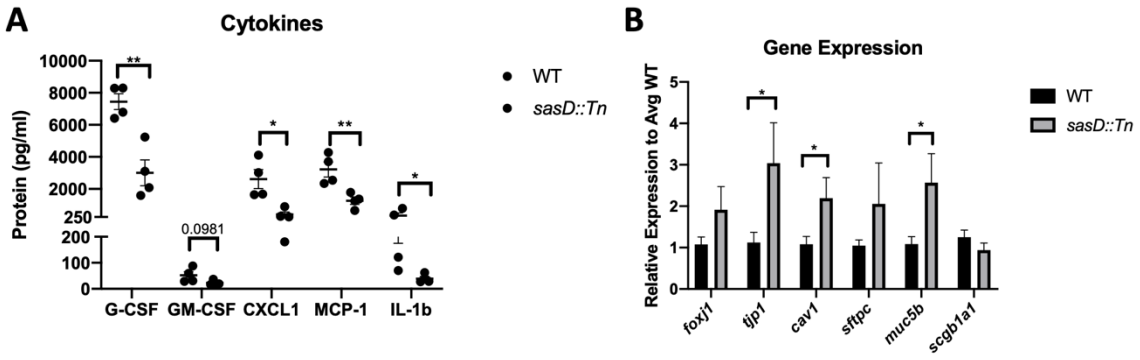

Figure 27: SasD Increases Inflammatory Cytokines and Decreases Lung Homeostatic Gene Expression

A. Lung homogenate protein levels of cytokines in mice infected with WT or sasD::Tn MRSA during bacterial pneumonia. B. Gene expression of lung epithelial markers in mice infected with WT or sasD::Tn MRSA during bacterial pneumonia. Statistics tested by unpaired t test. * $p<0.05$, ** $p<0.01$. N=2, combination of several experiments, data graphed as mean ± SEM.

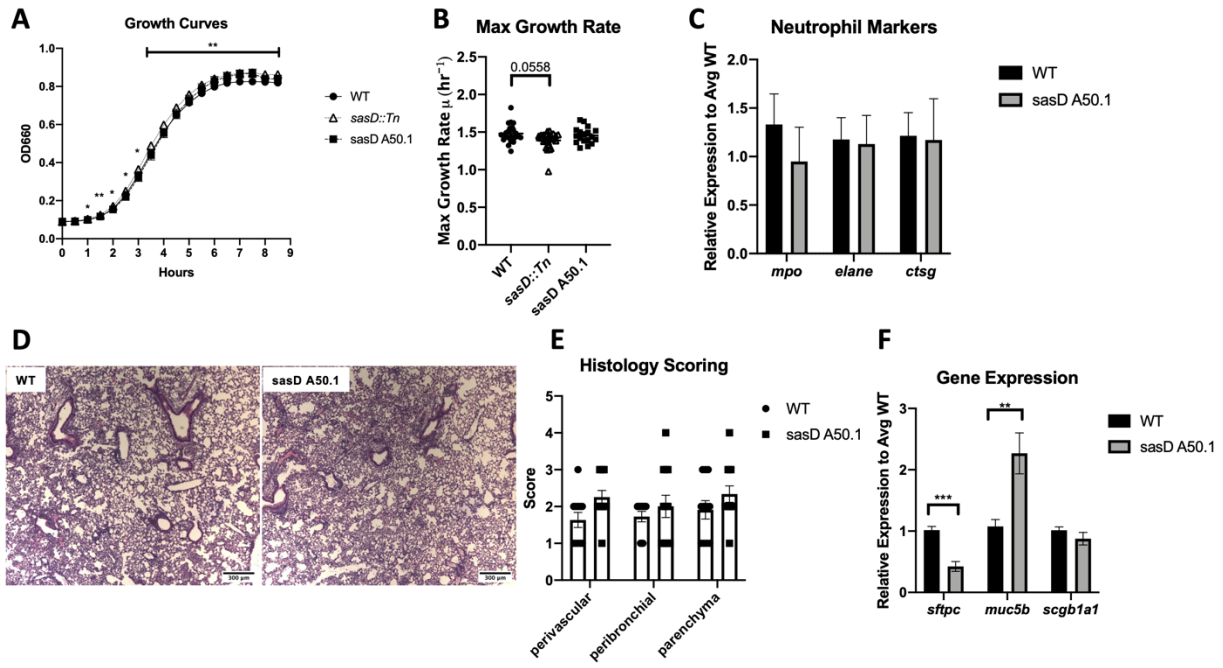

Figure 28: Characterization of SasD During Pneumonia

A. WT or mutant MRSA (see graphs) were grown overnight in tryptic soy broth and diluted 1:200 in a 96-well microtiter plate in sexaplicate. Plates were grown at 37°C at 282 rpm continuously. Measurements at 660 nm were taken every 30 minutes. Combination of at least 2 experiments per strain. Statistical significance is between WT and sasD::Tn MRSA strains. B. Max growth rate of WT or mutant MRSA strains (see graphs). Growth rate (μ) was calculated off at least two independent experiments using the equation $At=At\text{-}1*e^{\mu t}$ (see methods). The μmax was calculated as the average of the three highest μ rates. C. Gene expression of neutrophil markers relative to the average WT values. D. Histology scoring of H&E-stained lung sections. E. Gene expression of lung epithelial markers relative to the average WT values. Statistics done by Mixed-effects model with Dunnett's multiple comparisons correction (A), Kruskal-Wallis Test with Dunn's multiple comparisons correction (B), unpaired T test (C-E). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, N=2-6, combination of multiple experiments, data graphed as mean ± SEM.

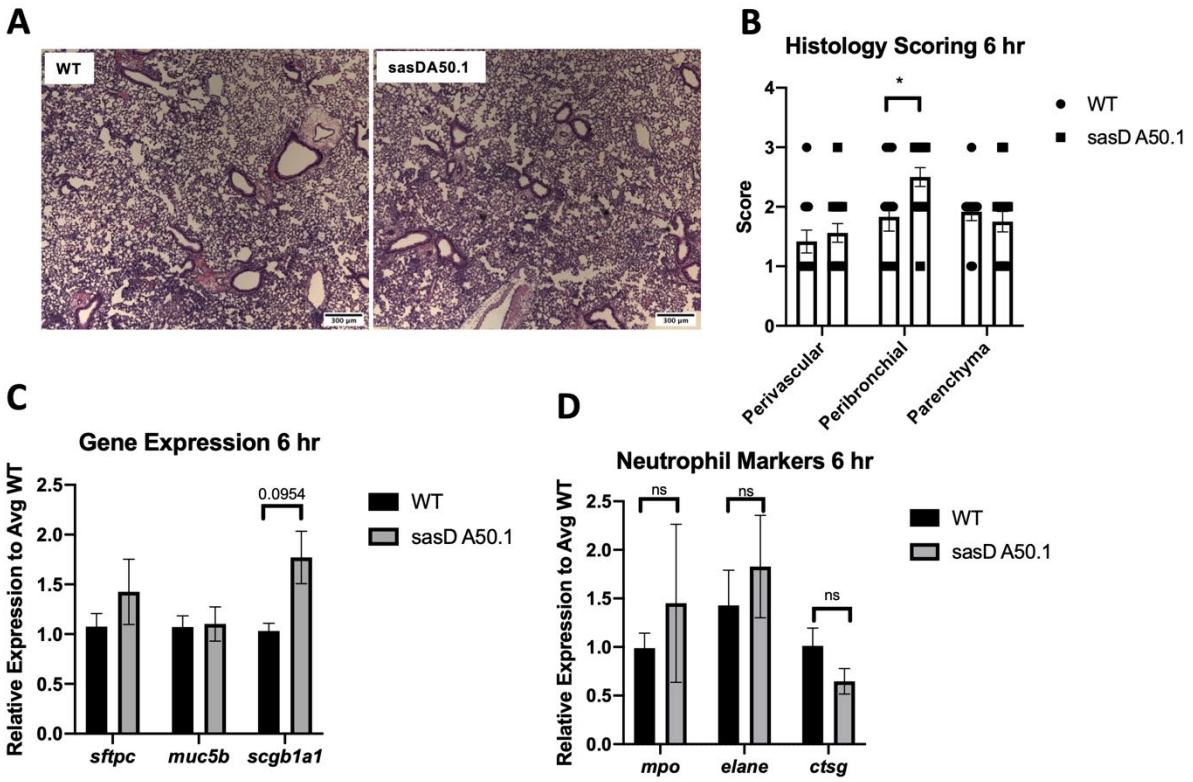

Figure 29: Characterization of Early SasD Infection

A. Histology scoring of H&E-stained lung sections. B-C. Gene expression of lung epithelial (B) and neutrophil (C) markers relative to the average WT values. Statistics done by Two-way ANOVA with Sidak's multiple comparisons correction (A), unpaired T test (B-C). * $p<0.05$, n=4-6, combination of multiple experiments, data graphed as mean ± SEM.

Appendix B Supplemental Materials and Methods

Table 2: Tn-seq Primers

Primer	Sequence[a]	Purpose	References
olj376	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGG GGGGGGGGGGGGGG	PCR 1	530, 538
olj510	CCAAAATCCGTTCCTTTTTCATAGTTCCTATATAGT TATACGC	PCR 1	530, 538
Olj511	*AATGATACGGCGACCACC*GAGATCTACACTCTTTGA CCGGGGACTTATCAGCCAACCTGTTA	PCR 2	530, 538
INDEX 1	*CAAGCAGAAGACGGCATACGA*GAT**CGTGAT**GTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT	PCR 2	530, 538
INDEX 2	*CAAGCAGAAGACGGCATACGA*GAT**ACATCG**GTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT	PCR 2	530, 538
INDEX 3	*CAAGCAGAAGACGGCATACGA*GAT**GCCTAA**GTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT	PCR 2	530, 538
INDEX 4	*CAAGCAGAAGACGGCATACGA*GAT**TGGTCA**GTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT	PCR 2	530, 538
INDEX 5	*CAAGCAGAAGACGGCATACGA*GAT**CACTGT**GTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT	PCR 2	530, 538
INDEX 6	*CAAGCAGAAGACGGCATACGA*GAT**ATTGGC**GTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT	PCR 2	530, 538
INDEX 7	*CAAGCAGAAGACGGCATACGA*GAT**GATCTG**GTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT	PCR 2	530, 538

Table 2 Continued

INDEX 8	*CAAGCAGAAGACGGCATACGA*GAT**TCAAGT**GTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT	PCR 2	530, 538
INDEX 9	*CAAGCAGAAGACGGCATACGA*GAT**CTGATC**GTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT	PCR 2	530, 538
INDEX 10	*CAAGCAGAAGACGGCATACGA*GAT**AAGCTA**GTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT	PCR 2	530, 538
INDEX 11	*CAAGCAGAAGACGGCATACGA*GAT**GTAGCC**GTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT	PCR 2	530, 538
INDEX 12	*CAAGCAGAAGACGGCATACGA*GAT**TACAAG**GTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT	PCR 2	530, 538
olj512	ACACTCTTTGACCGGGGACTTATCAGCCAACCTGTT A	Sequencing	530, 538

a Underlined=transposon-specific sequence; Italics=Illumina adapter sequence, Bold=barcode region

Table 3: *S. aureus* Strains Used

Strain	Description	NTML #	Reference
JE2	NTML Wildtype strain		[565]
JE2 *srtA::Tn*	NTML mutant	NE1787	[565]
JE2 *clfA::Tn*	NTML mutant	NE543	[565]
JE2 *clfB::Tn*	NTML mutant	NE391	[565]
JE2 *fnbB::Tn*	NTML mutant	NE728	[565]
JE2 *sdrC::Tn*	NTML mutant	NE432	[565]
JE2 *sdrD::Tn*	NTML mutant	NE1289	[565]
JE2 *sdrE::Tn*	NTML mutant	NE98	[565]
JE2 *isdB::Tn*	NTML mutant	NE1102	[565]
JE2 *sasG::Tn*	NTML mutant	NE825	[565]
JE2 *sasD::Tn*	NTML mutant	NE1032	[565]
sasD A50.1	Transduced strain from JE2 *sasD::Tn*		This study
JE2 *sbi::Tn*	NTML mutant	NE453	[565]
sbi A100.2	Transduced from strain JE2 *sbi::Tn*		This study

Table 4: *S. aureus* Primers Used

Strain	Primer (5'-3')	Source	Transposon Primer Used
JE2 *srtA::Tn*	CCAAACGCCTGTCTTTTCAT	This study	Buster
JE2 *clfA::Tn*	AAACACGCAATTCGGAAAAA	This study	Buster
JE2 *clfB::Tn*	TTCGCACTGTTTGTGTTTGC	This study	Upstream
JE2 *fnbB::Tn*	CTCCCGCCTTAATTCCTTCT	This study	Upstream
JE2 *sdrC::Tn*	CAATATTTCCGTCCAGGATCA	This study	Buster
JE2 *sdrD::Tn*	CAAAAAGGTAGATGCCAAAACTG	This study	Upstream
JE2 *sdrE::Tn*	CCATCAGGAGAGGTCATTGC	This study	Buster
JE2 *isdB::Tn*	CAAACCAACACCATCACCTG	This study	Buster
JE2 *sasG::Tn*	TGGAAAGTTTCATGGGCAAC	This study	Buster
JE2 *sasD::Tn*	CATGCCGACACAACTTCAAT	This study	Upstream
sasD A50.1	CATGCCGACACAACTTCAAT	This study	Upstream
JE2 *sbi::Tn*	GTTGCTAGTTGGGGCAGCA	This study	Upstream
sbi A100.2	GTTGCTAGTTGGGGCAGCA	This study	Upstream

Table 5: TaqMan Primer-Probes Used

Gene	Catalog Number
IL-17a	Mm00439618_m1
IL-23a	Mm00518984_m1
IL-6	Mm00446190_m1
Tnfα	Mm00443258_m1
IL-10	Mm00439614_m1
TLR2	Mm00442346_m1
TLR4	Mm00445273_m1
Cxcl1	Mm04207460_m1
IL-1β	Mm00434228_m1
NLRP3	Mm00840904_m1
Pycard	Mm00445747_g1
Foxj1	Mm01267279_m1
TJP1	Mm00493699_m1
Cav1	Mm00483057_m1
Sftpc	Mm00488144_m1
Scgb1a1	Mm00442046_m1
Muc5b	Mm00466376_m1
MPO	Mm01298424_m1
ELANE	Mm00469310_m1
CTSG	Mm00456011_m1
Lcn2	Mm01324470_m1
Camp	Mm00438285_m1
Arg1	Mm00475988_m1

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