DISRUPTION OF NEUTROPHIL REACTIVE OXYGEN SPECIES PRODUCTION BY *STAPHYLOCOCCUS AUREUS*

by

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DEDICATION

This dissertation is dedicated to my parents for the sacrifices they have made so I can fulfill my potential. They always encouraged me to never stop learning and pursue an education, and they still do.

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ABSTRACT

Staphylococcus aureus (*S. aureus*) is a bacterial pathogen that causes a wide range of human disease, from skin infections to invasive endocarditis. Neutrophils are the most abundant white blood cell in the human body, and the first line of defense following *S. aureus* infection. Even though neutrophils are equipped with an arsenal of bactericidal mechanisms, *S. aureus* survives neutrophil encounter. The mechanisms used by *S. aureus* to survive neutrophil killing remain unresolved. Previous studies have shown that the *S. aureus* SaeR/S two-component gene regulatory system is essential to survive neutrophil killing. Herein, we tested the hypothesis that *S. aureus* uses SaeR/S-dependent mechanisms to reduce neutrophil bactericidal mechanisms. First, we determined that *S. aureus* uses genes under the regulation of SaeR/S to inhibit neutrophil reactive oxygen species (ROS) production independent of previously defined mechanisms. Subsequently, we helped characterize a novel *S. aureus* SaeR/S-regulated virulence factor that inhibits human myeloperoxidase (MPO) activity to prevent formation of the highly bactericidal agent hypochlorous acid. Thus, *S. aureus* SaeR/S-regulated factors disrupt the neutrophil bactericidal mechanism with most efficacy against it, which is killing by oxidative mechanisms. We then focused on the role of *S. aureus* SaeR/S-regulated secreted leukocidins on neutrophil ROS production. While *S. aureus* leukocidins show redundancy inducing neutrophil pore formation, we determined that the surface receptors engaged by leukocidins induce distinct signaling pathways leading to ROS production. We showed that specific kinases are required for the differential production of neutrophil ROS induced by the *S. aureus* leukocidins LukGH and Panton-Valentine leukocidin (PVL). Importantly, the signaling pathways induced by *S. aureus* leukocidins through neutrophil surface receptors differ from the signals induced by physiological ligands through the same surface receptors. These results suggest *S. aureus* leukocidins "short-circuit" neutrophil signals to induce aberrant ROS production. In conclusion, *S. aureus* SaeR/S-regulated factors prevent proper bacterial clearance by disrupting neutrophil ROS production. These data provide us with a better understanding of the specific mechanisms used by *S. aureus* to survive neutrophil killing leading to pathogenesis.

CHAPTER ONE

INTRODUCTION TO *STAPHYLOCOCCUS AUREUS* AND NEUTROPHILS

Staphylococcus aureus

Staphylococcus aureus (*S. aureus*) is a Gram-positive bacterium that causes a wide range of disease in humans. The Scottish surgeon Alexander Ogston is credited for coining the term *Staphylococcus* originating from the Greek words *staphyle* and *kokkos*, meaning "bunch of grapes" and "berry", respectively (Licitra, 2013; Ogston, 1882b). Shortly after in 1884, the German physician Friedrich Julius Rosenbach coined the term *aureus* from the Latin word *aurum* meaning "gold" due to the color of the bacterial colonies on nutrient rich agar (Licitra, 2013). Indeed, early drawings by Ogston clearly displayed clusters of grape-shaped bacteria isolated from surgical abscesses and resemble recent scanning electron micrographs of *S. aureus* [Figure 1].

Early on while trying to determine the cause of "blood poisoning" in patients' abscesses, the observation that "micrococci" were also present on the skin of healthy individuals did not escape Ogston (Ogston, 1882a).

> It is therefore, it seems to me, permissible to formulate what we know, in words like those of Wolff, viz., *micro-organisms do not exist in the blood and tissues of healthy men.* But if they do not exist within, they are present in enormous numbers without. They not only rest, but develop on the surfaces of the human body.

Once again, Ogston correctly interpreted his observations since *S. aureus*

is a commensal organism that asymptomatically and persistently colonizes about 30% of the human population (with up to 60% of the human population intermittently colonized) primarily in the anterior nares but also found in the pharynx and external sites like the neck, forearm, hands, genital area, and ankles (Graham et al., 2006; Sollid et al., 2014; Wertheim et al., 2005). Colonization occurs in healthy individuals but a complex interaction of factors appears to influence human susceptibility to *S. aureus* colonization, which include ethnicity, age, gender, and medical conditions such as diabetes, HIV, and liver disease (Wertheim et al., 2005). Asymptomatic colonizers can be a reservoir for infection, as shown by studies that identified *S. aureus* nasal colonizing isolates identical to isolates in patients with furunculosis and bacteremia (Valentine and Hall-Smith, 1952; von Eiff et al., 2001). Yet, studies have questioned whether nasal colonization is a risk factor for infection since decolonization with antibiotics have yielded conflicting results with both reported decreases and insignificant changes in

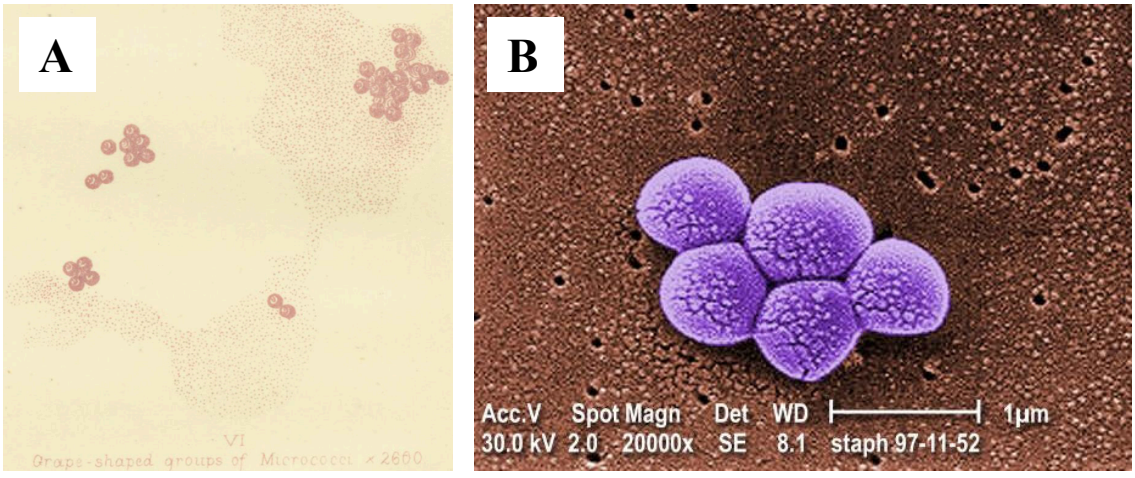


Figure 1. Images of *S. aureus*. A) Drawing by Scottish surgeon Alexander Ogston of grape-shaped bacteria likely *S. aureus* from the surgical site of a patient, 2600x magnification [adapted from (Ogston, 1881)]. B) Falsely colored scanning electron micrograph of *S. aureus* isolated from a patient with a vancomycin-resistant strain (image credit to the Public Health Image Library).

the rates of infection following decolonization (Coates et al., 2009). The effectiveness of *S. aureus* decolonization is also questioned by high rates of nasal recolonization. Additionally, while the anterior nares are the most common site for detection of *S. aureus*, studies have also failed to link nasal colonization with predisposition to skin infection (Miller et al., 2009). The failure to reduce *S. aureus* infection rates as a result of nasal decolonization could be attributed to differences in the strains of *S. aureus* used between studies. For example, only 6% of individuals are colonized with healthcare-associated and methicillin-sensitive *S. aureus* strains at a site other than the anterior nares. This is in contrast to 25% of individuals colonized with community-associated methicillin-resistant strains in non-nasal sites (Yang et al., 2010). Thus, different strains are likely to inhabit different body sites likely influencing subsequent infection. Furthermore, decolonization strategies are hindered by the ability of *S. aureus* to acquire resistance to antibiotics like mupirocin, which is used for elimination of nasal carriage (Antonov et al., 2015; Poovelikunnel et al., 2015). Collectively, colonization plays an important role in *S. aureus* pathogenesis but more studies are needed to identify better methods to eliminate carriage and to determine if indeed decolonization will prevent disease.

Antibiotic Resistance

Staphylococcus aureus is the epitome of antibiotic resistance. In 1928, Dr. Alexander Fleming discovered the antibiotic penicillin in agar plates inoculated with *S. aureus* that were allowed to air dry (Fleming, 1929). These plates were contaminated with a mold that was first misidentified by Fleming as *Penicillium rubrum* but later

corrected by Charles Thom to *Penicillium notatum* (Gaynes, 2017). Remarkably, the *Penicillium* mold inhibited the growth of *S. aureus* and other bacteria by producing penicillin [Figure 2]. However, it was not until 1940 when a group from the University of Oxford published the first *in vivo* experiments showing the efficacy of penicillin in treating *S. aureus* infections in mice (Chain et al., 1940). With the advent of the Second World War, a furious undertaking to mass-produce penicillin began with the help of the United States government. *Penicillium chrysogenum* was chosen as the strain for mass production since it produced six times more penicillin than *P. notatum*. By the end of 1943, enough penicillin was available to treat all of the Allied Forces; in contrast, the United States had enough penicillin in 1942 to treat fewer than 100 patients (Gaynes, 2017).

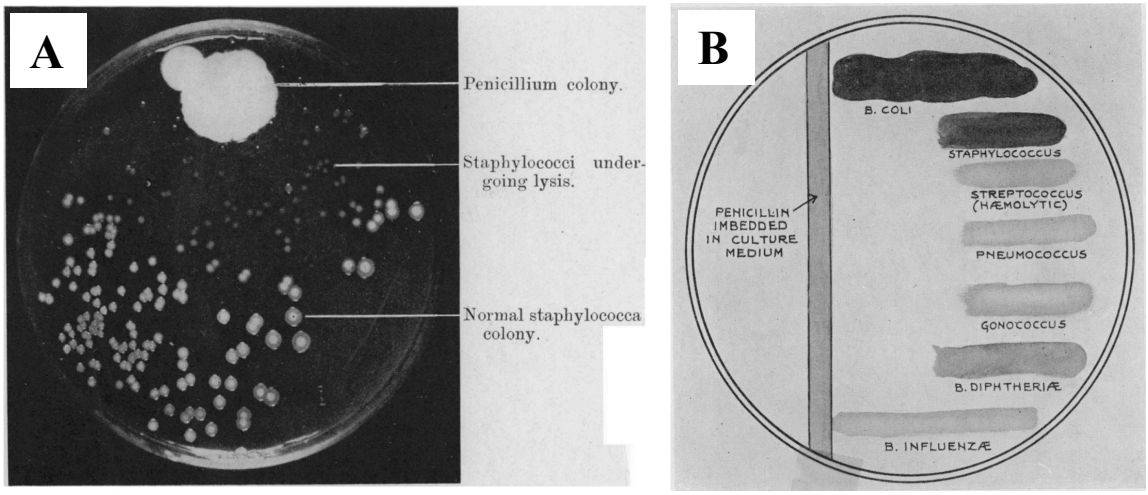


Figure 2. The discovery of penicillin by Dr. Alexander Fleming. A) Original photograph showing a zone of *S. aureus* growth inhibition originating from a *Penicillium* colony observed by Fleming. B) The broth from the cultured *Penicillium* mold showed inhibitory action to *S. aureus* and other bacteria. Images adapted from (Fleming, 1929).

Penicillin was viewed as a "magic bullet" to treat bacterial infections, but this sentiment was short-lived. In his 1945 Nobel Lecture, just three years after the mass production of penicillin, Alexander Fleming warned the world about the potential of *S. aureus* to develop resistance to penicillin (Fleming, 1945).

> But I would like to sound one note of warning. Penicillin is to all intents and purposes non-poisonous so there is no need to worry about giving an overdose and poisoning the patient. There may be a danger, though, in underdosage. It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body. The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant.

By the end of 1944, *S. aureus* resistance to penicillin was described in the literature (Kirby, 1944). By the mid-1950s, penicillin resistant *S. aureus* strains were pandemic (Chambers and Deleo, 2009). To combat the increase in penicillin resistance, new penicillin derivatives were synthesized that were immune to the beta-lactamase which conferred resistance to penicillin. In 1959, the antibiotic "celbenin", later called by its most common name methicillin, was first introduced and showed bactericidal activity towards penicillin-resistant *S. aureus* (Knox, 1960). By the end of 1960, methicillin-resistant *S .aureus* (MRSA) were isolated from England hospitals (Jevons, 1961). To date, *S. aureus* has gained resistance to every antibiotic used against it and the mechanisms of resistance to select antibiotics are summarized in Table 1 (Foster, 2017). The ability to gain antibiotic resistance has severely hindered our ability to treat *S. aureus* infections, enabled the geographical expansion of resistant strains to cause disease, and

represents a major health crisis compounded by a reduction in new antibiotic approvals (Ventola, 2015).

Epidemiology

The most recent epidemiological statistics show *S. aureus* is a leading cause of human infectious disease. Annually, *S. aureus* is responsible for about 600,000 hospitalizations in the United States (Klein et al., 2017). A multi-state survey of healthcare-associated infections, which defined healthcare-associated infections as 1. "infections not present or incubating on admission to the survey hospital" or 2. infection present on admission due to a surgical procedure performed at the hospital within the previous 28 days or 1 year in the case of an implant, identified *S. aureus* as the second leading cause of infection behind *Clostridium difficile* (*C. difficile*) (Magill et al., 2014). However, while *C. difficile* infections were strictly limited to gastrointestinal infections, *S. aureus* was the leading cause of healthcare-associated pneumonia and surgical-site infections and was also a causative agent of gastrointestinal, urinary tract, and bloodstream infections [Figure 3].

Table 1. Select antibiotics used against *S. aureus* and mechanisms used by *S. aureus* to gain antibiotic resistance.

Antibiotic	Mechanism of action against *S. aureus*	Mechanism conferring *S. aureus* antibiotic resistance
Penicillin	Inhibits cell wall synthesis	Acquisition of *blaZ* gene that encodes beta-lactamase to deactivate active site of penicillin
Methicillin	Inhibits PBP2, enzyme involved in cell wall synthesis	Acquisition of *mecA*, a PBP2 homologue with a buried active site inaccessible to methicillin
Vancomycin	Inhibits cell wall synthesis	1. Full resistance: Acquisition of *van* gene, enzyme able to use D-lactate instead of D-alanine in cell wall synthesis 2. Intermediate resistance: Single step mutations leading to increased thickness of cell wall preventing antibiotic from reaching its lethal target
Daptomycin	Binds negatively charged phosphatidylglycerol head groups in cell membrane causing depolarization, permeabilization, and leakage of ions	1. gain-of-function mutation in *mrpF* leading to increased production of positively charged lysyl-phosphatidylglycerol to repel daptomycin and reduce amount of negatively charged phosphatidylglycerol 2. Mutations in the Agr global regulator lead to increased release of phospholipids to intercept daptomycin before reaching its target

Antibiotic	Mechanism of action against *S. aureus*	Mechanism conferring *S. aureus* antibiotic resistance
Mupirocin	Inhibits protein synthesis by preventing charging of the isoleucyl tRNA	1. Mutations leading to decrease binding to Ile-tRNA synthetase 2. Acquisition of insensitive Ile-tRNA synthetase

Infections caused by *S. aureus* are not limited to a hospital setting. Community-associated *S. aureus* infections, roughly defined as an infection acquired outside of a hospital setting or not due to recent medical or surgical procedures, are estimated to be four times less likely than hospital-associated infections (Centers for Disease Control and Prevention, 2015). Community-associated methicillin-susceptible *S. aureus* (CA-MSSA) infections gradually increased from the 1960s to the 1980s, although exact rates of community-associated infections were difficult to determine due to lack of surveillance (Chambers, 2001). MRSA infections were largely limited to healthcare settings until the 1990s when cases of hypervirulent community-associated methicillin-resistant *S. aureus* (CA-MRSA) infecting children and leading to death were first reported in the United States (Centers for Disease Control and Prevention (CDC), 1999; Herold et al., 1998). The pathology of CA-MRSA infections is most commonly presented as skin and soft-tissue infections, but can also cause severe invasive infections including necrotizing fasciitis, necrotizing pneumonia, osteomyelitis, and thrombosis (Chambers and Deleo, 2009; Gonzalez et al., 2005a, 2005b; Miller et al., 2005; Nourse et al., 2007).

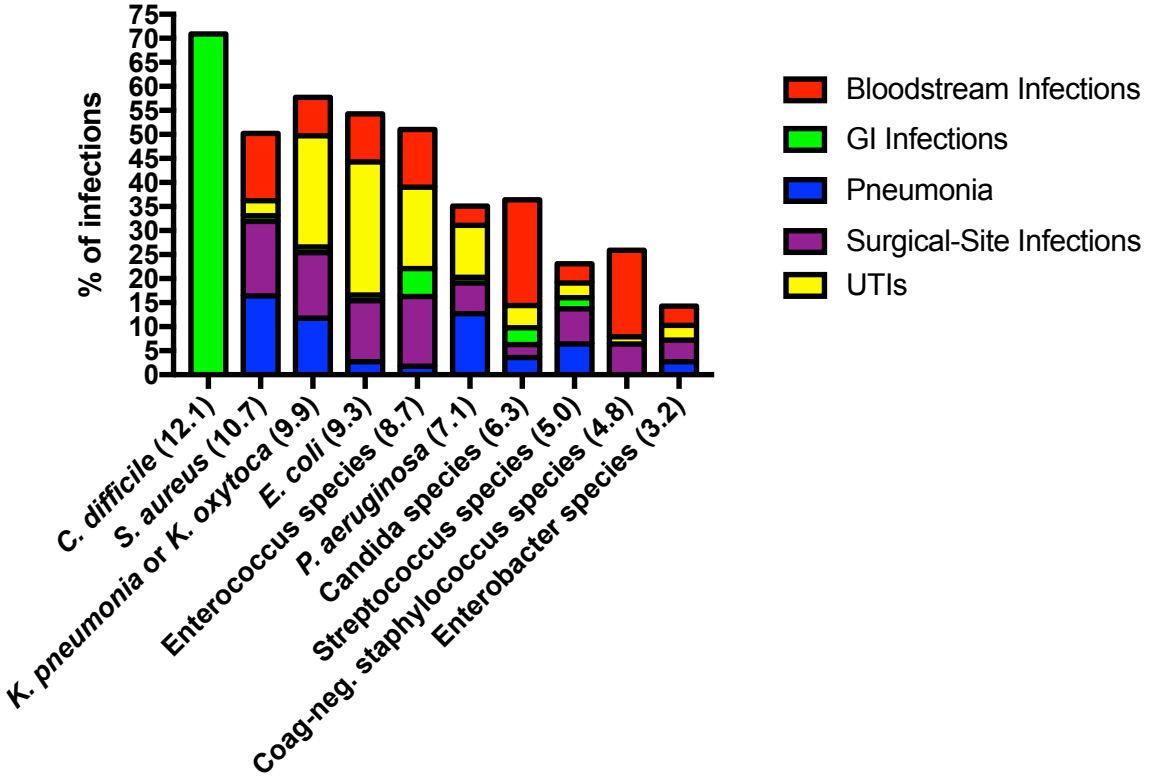


Figure 3. Top causative agents of healthcare-associated infections. *C. difficile* caused 12.1% of all healthcare-associated infections but were all limited to gastrointestinal (GI) infections. *S. aureus* caused 10.7% of all healthcare-associated infections, only behind *C. difficile,* but is able to cause a wide range of diseases (Magill et al., 2014). UTIs, urinary tract infections; coag-neg., coagulase-negative.

Analysis of the *S. aureus* strains causing community-associated infections suggested a separate clonal lineage from hospital-associated strains (Herold et al., 1998). The first wave of CA-MRSA infections in the United States were caused by the USA400 (MW2) clone, which was the strain responsible for the lethal infection of the children mentioned above, and USA300 as determined by pulsed-field gel electrophoresis (PFGE) typing (McDougal et al., 2003). In the United States, USA300 is the predominant cause

of CA-MRSA infections (Centers for Disease Control and Prevention, 2015) [Figure 4]. The factors responsible for increased pathogenicity by CA-MRSA strains have not been fully characterized. In animal models, community-associated *S. aureus* strains show enhanced survival and increased killing of mice compared to hospital-associated strains (Li et al., 2009; Voyich et al., 2005). In addition, community-associated strains show a propensity for increased survival following neutrophil phagocytosis and increased ability to kill neutrophils (Voyich et al., 2005). Several studies have focused on USA300 for its enhanced production of virulence factors that target neutrophils for destruction (Li et al., 2009; Montgomery et al., 2008). Of these secreted virulence factors, the Panton-Valentine Leukocidin (PVL), covered in more detail in Chapter 4, has garnered attention for its positive correlation in severe CA-MRSA infections like necrotizing pneumonia (Gillet et al., 2002, 2007). However, studies have questioned its role in causing severe infection due to a lack of clear phenotype in mutant PVL *S. aureus* strains using animal models (Bubeck Wardenburg et al., 2007, 2008; Voyich et al., 2006). The identification of the C5a receptor as the target for PVL and its specificity for the human receptor in comparison to the mouse ortholog C5a receptor may explain the lack of phenotype of *S. aureus* PVL mutant strains in animal models (Spaan et al., 2013). Nonetheless, clear determinants for the increase in CA-MRSA infections and their enhanced pathogenicity remain elusive.

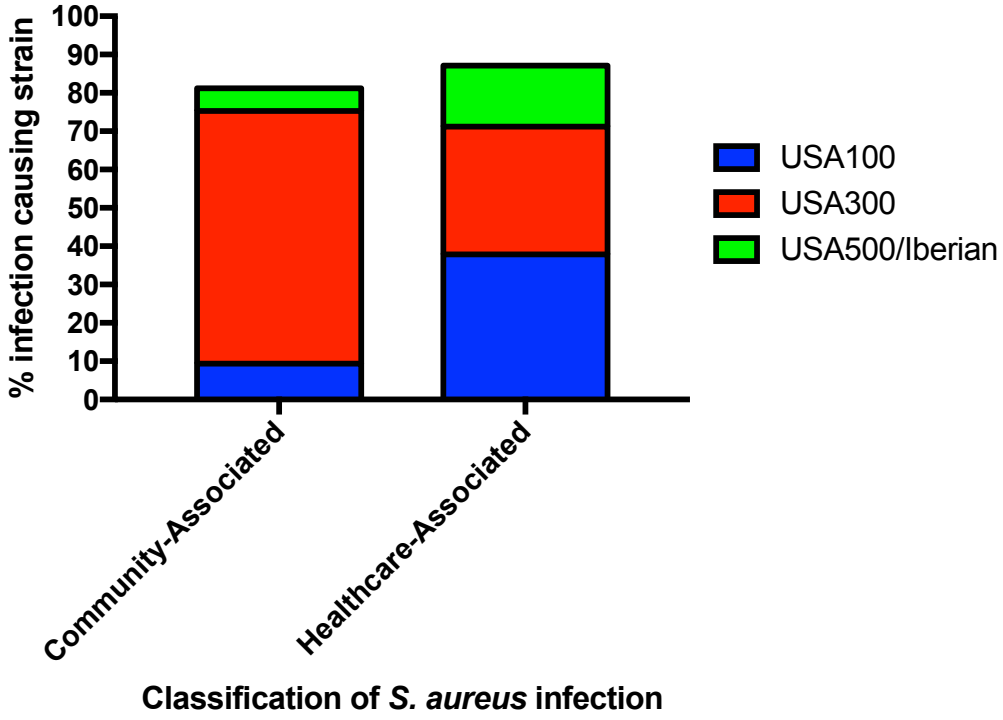


Figure 4. *S. aureus* clones causing infection. The *S. aureus* USA300 clone causes the majority of CA-MRSA infections. In contrast, multiple *S. aureus* clones cause similar nosocomial infection rates.

Economic Impact

S. aureus infections are a tremendous economic burden on the United States healthcare system. MRSA infections are estimated to cost the healthcare system up to $9.7 billion annually (Klein et al., 2007). The excess cost of treating a MRSA infection compared to a MSSA infection is estimated to be up to $35,000 (Klein et al., 2007). A study focused on CA-MRSA infections estimated a $2.2 billion annual burden to the healthcare system and up to $13.8 billion to society (Lee et al., 2013). These dollar amounts do not take into account the human suffering component and the emotional toll

on families. A better understanding of the mechanisms used by *S. aureus* to cause disease will lead to the development of therapeutics and reduce the economic burden.

Neutrophils

One of the overarching hypotheses behind the enhanced virulence of USA300 and other CA-MRSA strains of *S. aureus* is the ability to circumvent killing by polymorphonuclear leukocytes (also known as PMNs or neutrophils). Neutrophils are the most abundant leukocytes in the bloodstream of healthy individuals, constituting between 50-60% of leukocytes (Hsieh et al., 2007). They are characterized by a multi-lobed nucleus, hence the name polymorphonuclear, and an abundancy of granules in their cytoplasm. They mature from myeloblasts in the bone marrow producing up to 2×10^{11} cells per day (Borregaard, 2010). Under steady state conditions, the post-mitotic maturation phase from metamyelocyte to entrance into the bloodstream (as a neutrophil) is estimated to take six days (Dancey et al., 1976). Once exiting the bone marrow, neutrophils are estimated to patrol the bloodstream for 13-19 hours before being ingested by macrophages (Lahoz-Beneytez et al., 2016; McCracken and Allen, 2014). The proper maintenance of neutrophil circulation to health is exemplified by neutropenia, which increases susceptibility to infection, and neutrophilia, which causes dyspnea and hypoxia (McCracken and Allen, 2014).

Microbial Clearance

Recruitment. Neutrophils are the first line of defense following breach of epithelial barriers by pathogens such as *S. aureus*. Studies using mice have shown increased neutrophil recruitment near a site of *S. aureus* infection within 30 minutes (Harding et al., 2014). Endothelial cells or resident tissue cells near a site of infection recognize foreign organisms through pattern recognition receptors and initiate an acute inflammatory cascade that recruits neutrophils. Circulating neutrophils in the bloodstream follow a chemokine gradient established on the surface of endothelial cells in a heparan sulfate dependent mechanism to hone near the site of infection (Massena et al., 2010). Activated endothelial cells express P- and E-selectins to increase neutrophil recruitment and bind to glycosylated ligands (for example, P-selectin glycoprotein ligand 1) on the neutrophil effectively tethering the neutrophil to the endothelial cells resulting in neutrophil rolling. Subsequently, neutrophil movement through the blood vessels is arrested by attachment to integrins (Kolaczkowska and Kubes, 2013). Near the site of arrest, neutrophils extravasate from the blood vessels through the tight junctions between endothelial cells and pericytes arriving closer to the infected tissue (Kolaczkowska and Kubes, 2013). During chemotaxis and extravasation, neutrophil surface receptors are engaged and signals are relayed in both “inside-out” and “outside-in” signaling pathways that initiate and prepare the neutrophil for engagement of a pathogen (Springer and Dustin, 2012).

Phagocytosis. Once neutrophils reach the site of infection, *S. aureus* is engaged through immunoglobulin and complement receptors to initiate bacterial ingestion, a

process known as phagocytosis (van Kesse et al., 2014). The peptidoglycan cell wall of *S. aureus* is a major site of immunoglobulin binding and complement deposition. Neutrophil Fcγ receptors bind to immunoglobulins while complement receptor 1 and 3 bind to deposited C3b and iC3b on the staphylococcal surface. Opsonins on the surface of *S. aureus* enhance neutrophil engagement leading to phagocytosis and initiate specific signaling pathways that mobilize neutrophil granules to the forming phagosome containing *S. aureus*. Immunoglobulin- and complement-independent phagocytic pathways exist but these are less defined in relation to *S. aureus* uptake (Gordon, 2016). *S. aureus* employs mechanisms to inhibit neutrophil phagocytosis and these are covered in detail in Chapter 2.

NADPH Oxidase Activation. Neutrophils are equipped with an arsenal of oxidant dependent and independent antimicrobial mechanisms (Nauseef, 2007; Segal, 2005). The susceptibility of bacteria to oxidant dependent or independent killing mechanisms appears to be species specific. For example, *Escherichia coli* and *Pseudomonas aeruginosa* are highly susceptible to non-oxidative killing mechanisms while *S. aureus* is overwhelmingly susceptible to oxidative killing and resistant to non-oxidative mechanisms (Becker et al., 2017; Edwards et al., 1987; McGovern et al., 2011). These differences in killing susceptibility are exemplified in the detrimental clinical outcomes in patients suffering from chronic granulomatous disease (CGD), which have defective production of neutrophil reactive oxygen species (Assari, 2006; Lekstrom-Himes and Gallin, 2000; Roos and de Boer, 2014). Patients with CGD suffer from recurring bacterial and fungal infections like *S. aureus* and *Aspergillus fumigatus*, both highly susceptible to

oxidant killing, but not from *P. aeruginosa* or *E. coli* (Ben-Ari et al., 2012; Gazendam et al., 2016).

The production of neutrophil reactive oxygen species (ROS) is controlled by complex pathways that involve membrane and cytoplasmic proteins, lipid metabolism, phosphorylation events, ion fluxes, and short-lived radicals; all requiring temporal and spatial accuracy to ensure proper pathogen killing and prevent self-harm from toxic oxygen metabolites. The core protein machinery in neutrophils that initiates the formation of the superoxide anion from molecular oxygen is the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Segal, 2008). In resting neutrophils, NADPH oxidase components are separated in the membrane, cytoplasm, and granules. The membrane bound $gp91^{phox}$ and $p22^{phox}$, collectively termed flavocytochrome b558, are found on the neutrophil membrane and the membranes of specific, secretory, and gelatinase granules (Borregaard et al., 2007). The trimeric complex of $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$, as well as the inactivate GDP-bound Rac2, are found in the cytoplasm (Nunes et al., 2013). Recognition of *S. aureus*, as well as other pathogens, through the engagement of immunoglobulin and complement on the surface of *S. aureus* by Fc receptors and complement receptors, respectively, initiates a signaling cascade that recruits the cytoplasmic NADPH oxidase components to the membrane bound $gp91^{phox}$ and $p22^{phox}$. Notably, pathogens are not the only activators of NADPH oxidase since cytokines like tumor necrosis factor alpha (TNF-α) can also stimulate ROS production (Gao et al., 2007).

The assembled NADPH oxidase components and the active GTP-bound Rac2 are localized to the phagosome containing *S. aureus*. Cytoplasmic NADPH donates a pair of electrons that are shuttled through flavin adenine dinucleotide (FAD) and heme groups in $gp91^{phox}$ to molecular oxygen creating superoxide (Winterbourn and Kettle, 2013). Superoxide can spontaneously dismutate or react with myeloperoxidase (MPO) to form hydrogen peroxide (Kettle et al., 2007). Then, myeloperoxidase catalyzes the conversion of hydrogen peroxide to the highly bactericidal hypochlorous acid (HOCl) in the presence of chloride (Klebanoff et al., 2013). Computational modeling has estimated HOCl to reach the millimolar concentration range in a phagosome devoid of granule proteins (Winterbourn et al., 2006). However, the diffusion of HOCl is limited by the proximity to MPO and the majority of HOCl is expected to react with host protein methionine and cysteine amino acids. Even so, chlorination of *S. aureus* proteins by human neutrophils is detected *in vitro* suggesting that HOCl has phagosomal bactericidal activity (Chapman et al., 2002; Green et al., 2014). The mechanisms by which neutrophil ROS production is terminated remain largely unknown but recent studies have characterized oxidant sensing proteins that modulate neutrophil function after ROS production and present an interesting avenue to investigate termination mechanisms (DeCoursey and Ligeti, 2005; Syed Mortadza et al., 2015; Wang et al., 2016). In addition, neutrophil ROS production is involved in modulating apoptosis but ROS contribution following phagocytosis-induced cell death has yet to be fully characterized (Kebir and Filep, 2013).

Sensing the Host by *S. aureus*

S. aureus is by no means a bystander when neutrophils engage and commence their antimicrobial program. Protein sensors in the staphylococcal membrane recognize host components that allow *S. aureus* to mount a survival response (Ji et al., 2015; Yang et al., 2012, 2013; Zurek et al., 2014). Among these sensory systems, the *S. aureus* exoprotein expression regulator/sensor (SaeR/S) system stands out for its control of virulence factors that are essential to survive neutrophil killing (Voyich et al., 2009). The SaeR/S gene regulatory system operon is composed of four genes, *saeP, saeQ, saeR,* and *saeS,* and was first identified for its regulation of secreted toxins that destroy human immune cells (Giraudo et al., 1994, 1996, 1997). SaeR/S is erroneously referred to as a two-component system (TCS) since SaeR and SaeS are sufficient for activation of downstream gene targets while the roles of SaeP and SaeQ remain unknown (Giraudo et al., 1999; Novick, 2003). The SaeS histidine kinase intramembrane sensor is activated by human neutrophils and individual neutrophil components including α-defensin and hydrogen peroxide (Flack et al., 2014; Geiger et al., 2008; Liu et al., 2015; Zurek et al., 2014). Based on preliminary studies of SaeS and studies of other regulatory systems, it is hypothesized that activation leads to autophosphorylation of SaeS at a conserved histidine residue in the C-terminal cytoplasmic domain. The activation signal is relayed via phosphorylation from SaeS to SaeR at an aspartate residue. Phosphorylated SaeR likely forms a monomer that contains a winged helix-turn-helix module and binds DNA at the 5'-TTAAN$_7$TTAA-3' consensus binding sequence upstream of target genes and initiates transcription (Fan et al., 2015; Ko et al., 2016; Liu et al., 2016; Nygaard et al.,

2010; Sun et al., 2010). Thus, environmental cues signal through the staphylococcal membrane via SaeS and SaeR to initiate a response against neutrophils.

The SaeR/S regulon controls expression of ~8% of the *S. aureus* genome (Voyich et al., 2009). Gene ontology suggests SaeR/S targets are involved in virulence, immunomodulation, metabolism, protein translation and post-translational modifications, amino acid and ion transport, proteolysis, and hypothetical proteins. The function of many SaeR/S targets remain undefined. However, SaeR/S-regulated factors involved in immune modulation and cell lysis have been the most studied and are covered in more detail in Chapter 2 and Chapter 4, respectively.

SaeR/S is vital for *S. aureus* to survive neutrophil killing. Previous studies have shown that *S. aureus* have a survival defect upon neutrophil challenge in an SaeR/S-dependent mechanism (Sward et al., 2018; Voyich et al., 2009). These studies utilized isogenic *saeR/S* mutants in both the USA300 and USA400 CA-MRSA strains. Interestingly, wild-type *S. aureus* shows increased resistance to neutrophil killing within 30 minutes of exposure compared to the *saeR/S* mutant. This temporal factor is important because neutrophils are still intact at this early time point and thus SaeR/S-regulated leukocidins are not expected to play a role in the increased survival. As mentioned earlier, *S. aureus* is especially sensitive to killing by neutrophil reactive oxygen species. Yet, the role of *S. aureus* SaeR/S-regulated factors in modulating neutrophil reactive oxygen species production has not been investigated. *S. aureus* is equipped antioxidant mechanisms that include superoxide dismutase, catalase, staphyloxanthin, and methionine reductases, but these are not regulated by SaeR/S (Ballal and Manna, 2009;

Clauditz et al., 2006; Cosgrove et al., 2007; Hall et al., 2017; Pang et al., 2014). The unique SaeR/S-dependent mechanisms used by *S. aureus* to disrupt neutrophil reactive oxygen species production is explored in this dissertation.

Hypothesis

Neutrophils produce ROS to kill *S. aureus,* but *S. aureus* is resistant to human neutrophil killing in a SaeR/S-dependent mechanism. The SaeR/S-dependent mechanisms used by *S. aureus* to survive neutrophil killing following phagocytosis are unknown. Herein, we tested the hypothesis that SaeR/S-regulated factors disrupt neutrophil reactive oxygen species production to increase *S. aureus* survival. In Chapter 3, we show that *S. aureus* reduces the production of neutrophil hydrogen peroxide and hypochlorous acid in a SaeR/S-dependent mechanism. Through an international collaboration (Appendix A), we helped characterize the Staphylococcal Peroxidase Inhibitor, SPIN, a novel SaeR/S-regulated human specific virulence factor that binds human myeloperoxidase and inhibits the conversion of hydrogen peroxide to hypochlorous acid.

To further explore SaeR/S-dependent mechanisms that may impact reactive oxygen species production we next investigated the role of leukocidins. The production of leukocidins by *S. aureus* to lyse immune cells is a hallmark mechanism of staphylococcal immune evasion and significantly influenced by SaeR/S. The last ten years have greatly advanced our knowledge of leukotoxin-neutrophil interactions by identifying the host receptors targeted to cause neutrophil lysis. However, our

understanding of the effects of *S. aureus* leukotoxins on neutrophils outside of lytic activity is understudied. In Chapter 4, we hypothesized that leukotoxins regulated by SaeR/S induce "outside-in" signaling in neutrophils to disrupt signaling pathways. We focused on the production of neutrophil reactive oxygen species in response to secreted leukocidins. Our studies show that the *S. aureus* SaeR/S-regulated secretome induces neutrophil reactive oxygen species production in leukotoxin GH- and Panton-Valentine Leukocidin-dependent mechanisms. Unexpectedly, leukotoxin GH induced neutrophil reactive oxygen species extracellularly while Panton-Valentine Leukocidin induced intracellular and extracellular reactive oxygen species production. Data in Chapter 4 identifies that the signaling pathways dictating the spatial differences in neutrophil oxidant production are specific to the type of intoxication. These data define novel SaeR/S-dependent mechanisms used by *S. aureus* to disrupt neutrophil reactive oxygen species production. Collectively, studies suggest that SaeR/S-regulated factors are active in disrupting every step of neutrophil interaction with *S. aureus* – initial recognition, internalization, reactive oxygen species mediated killing, neutrophil death, and escape from within the neutrophil.

REFERENCES CITED

Anderson, K. E., Boyle, K. B., Davidson, K., Chessa, T. A. M., Kulkarni, S., Jarvis, G. E., et al. (2008). CD18-dependent activation of the neutrophil NADPH oxidase during phagocytosis of Escherichia coli or Staphylococcus aureus is regulated by class III but not class I or II PI3Ks. *Blood* 112, 5202–5211. doi:10.1182/blood-2008-04-149450.

Antonov, N. K., Garzon, M. C., Morel, K. D., Whittier, S., Planet, P. J., and Lauren, C. T. (2015). High prevalence of mupirocin resistance in Staphylococcus aureus isolates from a pediatric population. *Antimicrob. Agents Chemother.* 59, 3350–3356. doi:10.1128/AAC.00079-15.

Assari, T. (2006). Chronic Granulomatous Disease; fundamental stages in our understanding of CGD. *Med. Immunol.* 5, 4. doi:10.1186/1476-9433-5-4.

Ballal, A., and Manna, A. C. (2009). Regulation of superoxide dismutase (sod) genes by SarA in Staphylococcus aureus. *J. Bacteriol.* 191, 3301–10. doi:10.1128/JB.01496-08.

Becker, K. A., Li, X., Seitz, A., Steinmann, J., Koch, A., Schuchman, E., et al. (2017). Neutrophils Kill Reactive Oxygen Species-Resistant Pseudomonas aeruginosa by Sphingosine. *Cell. Physiol. Biochem.* 43, 1603–1616. doi:10.1159/000482024.

Ben-Ari, J., Wolach, O., Gavrieli, R., and Wolach, B. (2012). Infections associated with chronic granulomatous disease: linking genetics to phenotypic expression. *Expert Rev. Anti. Infect. Ther.* 10, 881–94. doi:10.1586/eri.12.77.

Borregaard, N. (2010). Neutrophils, from Marrow to Microbes. *Immunity* 33, 657–670. doi:10.1016/j.immuni.2010.11.011.

Borregaard, N., Sørensen, O. E., and Theilgaard-Mönch, K. (2007). Neutrophil granules: a library of innate immunity proteins. *Trends Immunol.* 28, 340–345. doi:10.1016/j.it.2007.06.002.

Bubeck Wardenburg, J., Bae, T., Otto, M., Deleo, F. R., and Schneewind, O. (2007). Poring over pores: alpha-hemolysin and Panton-Valentine leukocidin in Staphylococcus aureus pneumonia. *Nat. Med.* 13, 1405–6. doi:10.1038/nm1207-1405.

Bubeck Wardenburg, J., Palazzolo-Ballance, A. M., Otto, M., Schneewind, O., and DeLeo, F. R. (2008). Panton-Valentine leukocidin is not a virulence determinant in murine models of community-associated methicillin-resistant Staphylococcus aureus disease. *J. Infect. Dis.* 198, 1166–70. doi:10.1086/592053.

Centers for Disease Control and Prevention (2015). Active Bacterial Core Surveillance Report, Emerging Infections Program Network, MethicillinResistant Staphylococcus aureus, 2015.

Centers for Disease Control and Prevention (CDC) (1999). Four pediatric deaths from community-acquired methicillin-resistant Staphylococcus aureus — Minnesota and North Dakota, 1997-1999. *MMWR. Morb. Mortal. Wkly. Rep.* 48, 707–10. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21033181.

Chain, E., Florey, H. W., Adelaide, M. B., Gardner, A. D., Oxfd, D. M., Heatley, N. G., et al. (1940). Penicillin As a Chemotherapeutic Agent. *Lancet* 236, 226–228. doi:10.1016/S0140-6736(01)08728-1.

Chambers, H. F. (2001). The changing epidemiology of Staphylococcus aureus? *Emerg. Infect. Dis.* 7, 178–182. doi:10.3201/eid0702.700178.

Chambers, H. F., and Deleo, F. R. (2009). Waves of resistance: Staphylococcus aureus in the antibiotic era. *Nat. Rev. Microbiol.* 7, 629–41. doi:10.1038/nrmicro2200.

Chapman, A. L. P., Hampton, M. B., Senthilmohan, R., Winterbourn, C. C., and Kettle, A. J. (2002). Chlorination of bacterial and neutrophil proteins during phagocytosis and killing of Staphylococcus aureus. *J. Biol. Chem.* 277, 9757–62. doi:10.1074/jbc.M106134200.

Clauditz, A., Resch, A., Wieland, K.-P., Peschel, A., and Gotz, F. (2006). Staphyloxanthin Plays a Role in the Fitness of Staphylococcus aureus and Its Ability To Cope with Oxidative Stress. *Infect. Immun.* 74, 4950–4953. doi:10.1128/IAI.00204-06.

Coates, T., Bax, R., and Coates, A. (2009). Nasal decolonization of Staphylococcus aureus with mupirocin: Strengths, weaknesses and future prospects. *J. Antimicrob. Chemother.* 64, 9–15. doi:10.1093/jac/dkp159.

Cosgrove, K., Coutts, G., Jonsson, I.-M., Tarkowski, A., Kokai-Kun, J. F., Mond, J. J., et al. (2007). Catalase (KatA) and alkyl hydroperoxide reductase (AhpC) have compensatory roles in peroxide stress resistance and are required for survival, persistence, and nasal colonization in Staphylococcus aureus. *J. Bacteriol.* 189, 1025–35. doi:10.1128/JB.01524-06.

Dancey, J. T., Deubelbeiss, K. A., Harker, L. A., and Finch, C. A. (1976). Neutrophil kinetics in man. *J. Clin. Invest.* 58, 705–15. doi:10.1172/JCI108517.

de Jong, N. W. M., Ramyar, K. X., Guerra, F. E., Nijland, R., Fevre, C., Voyich, J. M., et al. (2017). Immune evasion by a staphylococcal inhibitor of myeloperoxidase. *Proc. Natl. Acad. Sci. U. S. A.* 114, 9439–9444. doi:10.1073/pnas.1707032114.

DeCoursey, T. E., and Ligeti, E. (2005). Regulation and termination of NADPH oxidase activity. *Cell. Mol. Life Sci.* 62, 2173–2193. doi:10.1007/s00018-005-5177-1.

DuMont, A. L., Yoong, P., Day, C. J., Alonzo, F., McDonald, W. H., Jennings, M. P., et al. (2013). Staphylococcus aureus LukAB cytotoxin kills human neutrophils by targeting the CD11b subunit of the integrin Mac-1. *Proc. Natl. Acad. Sci. U. S. A.* 110, 10794–9. doi:10.1073/pnas.1305121110.

Edwards, S. W., Say, J. E., and Hart, C. A. (1987). Oxygen-dependent killing of Staphylococcus aureus by human neutrophils. *J. Gen. Microbiol.* 133, 3591–7. doi:10.1099/00221287-133-12-3591.

Fan, X., Zhang, X., Zhu, Y., Niu, L., Teng, M., Sun, B., et al. (2015). Structure of the DNA-binding domain of the response regulator SaeR from Staphylococcus aureus. *Acta Crystallogr. Sect. D Biol. Crystallogr.* 71, 1768–1776. doi:10.1107/S1399004715010287.

Flack, C. E., Zurek, O. W., Meishery, D. D., Pallister, K. B., Malone, C. L., Horswill, A. R., et al. (2014). Differential regulation of staphylococcal virulence by the sensor kinase SaeS in response to neutrophil-derived stimuli. *Proc. Natl. Acad. Sci. U. S. A.* 111, E2037-45. doi:10.1073/pnas.1322125111.

Fleming, A. (1929). On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of B. influenzae. *Br. J. Exp. Pathol.* 10, 226–236. doi:10.1093/clinids/2.1.129.

Fleming, A. (1945). “The Nobel Prize in Physiology or Medicine 1945”. Nobelprize.org. Nobel Media AB 2014. Web. 5 Jul 2018. <http://www.nobelprize.org/nobel_prizes/medicine/laureates/1945/>.

Foster, T. J. (2017). Antibiotic resistance in Staphylococcus aureus. Current status and future prospects. *FEMS Microbiol. Rev.* 41, 430–449. doi:10.1093/femsre/fux007.

Futosi, K., Fodor, S., and M??csai, A. (2013). Neutrophil cell surface receptors and their intracellular signal transduction pathways. *Int. Immunopharmacol.* 17, 638–650. doi:10.1016/j.intimp.2013.06.034

Gao, X. P., Zhu, X., Fu, J., Liu, Q., Frey, R. S., and Malik, A. B. (2007). Blockade of class IA phosphoinositide 3-kinase in neutrophils prevents NADPH oxidase activation- and adhesion-dependent inflammation. *J. Biol. Chem.* 282, 6116–6125. doi:10.1074/jbc.M610248200.

Gaynes, R. (2017). The discovery of penicillin—new insights after more than 75 years of clinical use. *Emerg. Infect. Dis.* 23, 849–853. doi:10.3201/eid2305.161556.

Gazendam, R. P., van Hamme, J. L., Tool, A. T. J., Hoogenboezem, M., van den Berg, J. M., Prins, J. M., et al. (2016). Human Neutrophils Use Different Mechanisms To Kill *Aspergillus fumigatus* Conidia and Hyphae: Evidence from Phagocyte Defects. *J. Immunol.* 196, 1272–1283. doi:10.4049/jimmunol.1501811.

Geiger, T., Goerke, C., Mainiero, M., Kraus, D., and Wolz, C. (2008). The virulence regulator sae of Staphylococcus aureus: Promoter activities and response to phagocytosis-related signals. *J. Bacteriol.* 190, 3419–3428. doi:10.1128/JB.01927-07.

Gillet, Y., Issartel, B., Vanhems, P., Fournet, J. C., Lina, G., Bes, M., et al. (2002). Association between Staphylococcus aureus strains carrying gene for Panton-Valentine leukocidin and highly lethal necrotising pneumonia in young immunocompetent patients. *Lancet* 359, 753–759. doi:10.1016/S0140-6736(02)07877-7.

Gillet, Y., Vanhems, P., Lina, G., Bes, M., Vandenesch, F., Floret, D., et al. (2007). Factors Predicting Mortality in Necrotizing Community-Acquired Pneumonia Caused by Staphylococcus aureus Containing Panton-Valentine Leukocidin. *Clin. Infect. Dis.* 45, 315–321. doi:10.1086/519263.

Giraudo, a T., Raspanti, C. G., Calzolari, a, and Nagel, R. (1994). Characterization of a Tn551-mutant of Staphylococcus aureus defective in the production of several exoproteins. *Can. J. Microbiol.* 40, 677–681. doi:10.1139/m94-107.

Giraudo, A. T., Calzolari, A., Cataldi, A. A., Bogni, C., and Nagel, R. (1999). The sae locus of Staphylococcus aureus encodes a two-component regulatory system. *FEMS Microbiol. Lett.* 177, 15–22. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10436918 [Accessed November 30, 2015].

Giraudo, A. T., Cheung, A. L., and Nagel, R. (1997). The sae locus of Staphylococcus aureus controls exoprotein synthesis at the transcriptional level. *Arch. Microbiol.* 168, 53–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9211714 [Accessed November 30, 2015].

Giraudo, A. T., Rampone, H., Calzolari, A., and Nagel, R. (1996). Phenotypic characterization and virulence of a sae- agr- mutant of Staphylococcus aureus. *Can J Microbiol* 42, 120–123. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8742355.

Gonzalez, B. E., Hulten, K. G., Dishop, M. K., Lamberth, L. B., Hammerman, W. a, Mason, E. O., et al. (2005a). Pulmonary manifestations in children with invasive community-acquired Staphylococcus aureus infection. *Clin. Infect. Dis.* 41, 583–590. doi:10.1086/432475.

Gonzalez, B. E., Martinez-Aguilar, G., Hulten, K. G., Hammerman, W. A., Coss-Bu, J., Avalos-Mishaan, A., et al. (2005b). Severe Staphylococcal sepsis in adolescents in the era of community-acquired methicillin-resistant Staphylococcus aureus. *Pediatrics* 115, 642–8. doi:10.1542/peds.2004-2300.

Gordon, S. (2016). Phagocytosis: An Immunobiologic Process. *Immunity* 44, 463–475. doi:10.1016/j.immuni.2016.02.026.

Graham, P. L., Lin, S. X., and Larson, E. L. (2006). A U.S. population-based survey of Staphylococcus aureus colonization. *Ann. Intern. Med.* 144, 318–25. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16520472.

Green, J. N., Kettle, A. J., and Winterbourn, C. C. (2014). Protein chlorination in neutrophil phagosomes and correlation with bacterial killing. *Free Radic. Biol. Med.* 77, 49–56. doi:10.1016/j.freeradbiomed.2014.08.013.

Hall, J. W., Yang, J., Guo, H., and Ji, Y. (2017). The Staphylococcus aureus AirSR Two-Component System Mediates Reactive Oxygen Species Resistance via Transcriptional Regulation of Staphyloxanthin Production. *Infect. Immun.* 85, 1–12. doi:10.1128/IAI.00838-16.

Harding, M. G., Zhang, K., Conly, J., and Kubes, P. (2014). Neutrophil Crawling in Capillaries; A Novel Immune Response to Staphylococcus aureus. *PLoS Pathog.* 10, e1004379. doi:10.1371/journal.ppat.1004379.

Hawkins, P. T., and Stephens, L. R. (2015). PI3K signalling in inflammation. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* 1851, 882–897. doi:10.1016/j.bbalip.2014.12.006.

Herold, B. C., Immergluck, L. C., Maranan, M. C., Lauderdale, D. S., Gaskin, R. E., Boyle-Vavra, S., et al. (1998). Community-acquired methicillin-resistant Staphylococcus aureus in children with no identified predisposing risk. *JAMA* 279, 593–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9486753.

Hsieh, M. M., Everhart, J. E., Byrd-Holt, D. D., Tisdale, J. F., and Rodgers, G. P. (2007). Prevalence of neutropenia in the U.S. population: age, sex, smoking status, and ethnic differences. *Ann. Intern. Med.* 146, 486–92. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17404350.

Ibrahim, F. B. M., Pang, S. J., and Melendez, A. J. (2004). Anaphylatoxin signaling in human neutrophils. A key role for sphingosine kinase. *J. Biol. Chem.* 279, 44802–44811. doi:10.1074/jbc.M403977200.

Janesch, P., Rouha, H., Weber, S., Malafa, S., Gross, K., Maierhofer, B., et al. (2017). Selective sensitization of human neutrophils to LukGH mediated cytotoxicity by Staphylococcus aureus and IL-8. *J. Infect.* 74, 473–483. doi:10.1016/j.jinf.2017.02.004.

Jevons, M. P. (1961). "Celbenin" -resistant Staphylococci. *Br. Med. J.* 1, 124–125. doi:10.1136/bmj.1.5219.124-a.

Ji, C. J., Kim, J. H., Won, Y. Bin, Lee, Y. E., Choi, T. W., Ju, S. Y., et al. (2015). Staphylococcus aureus PerR is a hypersensitive hydrogen peroxide sensor using iron-mediated histidine oxidation. *J. Biol. Chem.* 290, 20374–20386. doi:10.1074/jbc.M115.664961.

Johansson, M. W., Patarroyo, M., Oberg, F., Siegbahn, A., and Nilsson, K. (1997). Myeloperoxidase mediates cell adhesion via the alpha M beta 2 integrin (Mac-1, CD11b/CD18). *J. Cell Sci.* 110 (Pt 9, 1133–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9175709.

Kanai, F., Liu, H., Field, S. J., Akbary, H., Matsuo, T., Brown, G. E., et al. (2001). The PX domains of p47phox and p40phox bind to lipid products of PI(3)K. *Nat. Cell Biol.* 3, 675–678. doi:10.1038/35083070.

Kebir, D. El, and Filep, J. G. (2013). Modulation of neutrophil apoptosis and the resolution of inflammation through β2 integrins. *Front. Immunol.* 4, 1–15. doi:10.3389/fimmu.2013.00060.

Kettle, A. J., Anderson, R. F., Hampton, M. B., and Winterbourn, C. C. (2007). Reactions of superoxide with myeloperoxidase. *Biochemistry* 46, 4888–4897. doi:10.1021/bi602587k [doi].

Kirby, W. M. (1944). EXTRACTION OF A HIGHLY POTENT PENICILLIN INACTIVATOR FROM PENICILLIN RESISTANT STAPHYLOCOCCI. *Science* 99, 452–3. doi:10.1126/science.99.2579.452.

Klebanoff, S. J., Kettle, A. J., Rosen, H., Winterbourn, C. C., and Nauseef, W. M. (2013). Myeloperoxidase: a front-line defender against phagocytosed microorganisms. *J. Leukoc. Biol.* 93, 185–98. doi:10.1189/jlb.0712349.

Klein, E., Smith, D. L., and Laxminarayan, R. (2007). Hospitalizations and deaths caused by methicillin-resistant Staphylococcus aureus, United States, 1999-2005. *Emerg. Infect. Dis.* 13, 1840–6. doi:10.3201/eid1312.070629.

Klein, E. Y., Mojica, N., Jiang, W., Cosgrove, S. E., Septimus, E., Morgan, D. J., et al. (2017). Trends in Methicillin-Resistant Staphylococcus aureus Hospitalizations in the United States, 2010-2014. *Clin. Infect. Dis.* 65, 1921–1923. doi:10.1093/cid/cix640.

Knox, R. (1960). A New Penicillin (BRL 1241) Active Against Penicillin-Resistant Staphylococci. *Br. Med. J.* 2, 690–693. doi:10.1136/bmj.2.5200.690.

Ko, T. P., Huang, C. Y., Hsieh, T. J., Chen, S. C., Chen, Y. R., Yang, C. S., et al. (2016). Crystal structures of Staphylococcal SaeR reveal possible DNA-binding modes. *Biochem. Biophys. Res. Commun.* 474, 686–690. doi:10.1016/j.bbrc.2016.05.009.

Kolaczkowska, E., and Kubes, P. (2013). Neutrophil recruitment and function in health and inflammation. *Nat. Rev. Immunol.* 13, 159–75. doi:10.1038/nri3399.

Lahoz-Beneytez, J., Elemans, M., Zhang, Y., Ahmed, R., Salam, A., Block, M., et al. (2016). Human neutrophil kinetics: Modeling of stable isotope labeling data supports short blood neutrophil half-lives. *Blood* 127, 3431–3438. doi:10.1182/blood-2016-03-700336.

Lau, D., Mollnau, H., Eiserich, J. P., Freeman, B. A., Daiber, A., Gehling, U. M., et al. (2005). Myeloperoxidase mediates neutrophil activation by association with CD11b/CD18 integrins. *Proc. Natl. Acad. Sci. U. S. A.* 102, 431–6. doi:10.1073/pnas.0405193102.

Lee, B. Y., Singh, A., David, M. Z., Bartsch, S. M., Slayton, R. B., Huang, S. S., et al. (2013). The economic burden of community-associated methicillin-resistant Staphylococcus aureus (CA-MRSA). *Clin. Microbiol. Infect.* 19, 528–536. doi:10.1111/j.1469-0691.2012.03914.x.

Lekstrom-Himes, J. A., and Gallin, J. I. (2000). Immunodeficiency diseases caused by defects in phagocytes. *N. Engl. J. Med.* 343, 1703–14. doi:10.1056/NEJM200012073432307.

Li, M., Diep, B. A., Villaruz, A. E., Braughton, K. R., Jiang, X., DeLeo, F. R., et al. (2009). Evolution of virulence in epidemic community-associated methicillin-resistant Staphylococcus aureus. *Proc. Natl. Acad. Sci. U. S. A.* 106, 5883–8. doi:10.1073/pnas.0900743106.

Licitra, G. (2013). Etymologia: Staphylococcus. *Emerg. Infect. Dis.* 19, 1553. doi:10.3201/eid1909.ET1909.

Lishko, V. K., Podolnikova, N. P., Yakubenko, V. P., Yakovlev, S., Medved, L., Yadav, S. P., et al. (2004). Multiple binding sites in fibrinogen for integrin ??M?? 2 (Mac-1). *J. Biol. Chem.* 279, 44897–44906. doi:10.1074/jbc.M408012200.

Liu, Q., Cho, H., Yeo, W.-S., and Bae, T. (2015). The Extracytoplasmic Linker Peptide of the Sensor Protein SaeS Tunes the Kinase Activity Required for Staphylococcal Virulence in Response to Host Signals. *PLOS Pathog.* 11, e1004799. doi:10.1371/journal.ppat.1004799.

Liu, Q., Yeo, W., and Bae, T. (2016). The SaeRS Two-Component System of Staphylococcus aureus. *Genes (Basel).* 7, 81. doi:10.3390/genes7100081.

Magill, S. S., Edwards, J. R., Bamberg, W., Beldavs, Z. G., Dumyati, G., Kainer, M. A., et al. (2014). Multistate Point-Prevalence Survey of Health Care–Associated Infections. *N. Engl. J. Med.* 370, 1198–1208. doi:10.1056/NEJMoa1306801.

Massena, S., Christoffersson, G., Hjertström, E., Zcharia, E., Vlodavsky, I., Ausmees, N., et al. (2010). A chemotactic gradient sequestered on endothelial heparan sulfate induces directional intraluminal crawling of neutrophils. *Blood* 116, 1924–31. doi:10.1182/blood-2010-01-266072.

McCracken, J. M., and Allen, L.-A. H. (2014). Regulation of human neutrophil apoptosis and lifespan in health and disease. *J. Cell Death* 7, 15–23. doi:10.4137/JCD.S11038.

McDougal, L. K., Steward, C. D., Killgore, G. E., Chaitram, J. M., McAllister, S. K., and Tenover, F. C. (2003). Pulsed-field gel electrophoresis typing of oxacillin-resistant Staphylococcus aureus isolates from the United States: establishing a national database. *J. Clin. Microbiol.* 41, 5113–20. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=262524&tool=pmcentrez&rendertype=abstract [Accessed November 13, 2012].

McGovern, N. N., Cowburn, A. S., Porter, L., Walmsley, S. R., Summers, C., Thompson, A. A. R., et al. (2011). Hypoxia Selectively Inhibits Respiratory Burst Activity and Killing of Staphylococcus aureus in Human Neutrophils. *J. Immunol.* 186, 453–463. doi:10.4049/jimmunol.1002213.

Miller, L. G., Perdreau-Remington, F., Rieg, G., Mehdi, S., Perlroth, J., Bayer, A. S., et al. (2005). Necrotizing fasciitis caused by community-associated methicillin-resistant Staphylococcus aureus in Los Angeles. *N. Engl. J. Med.* 352, 1445–53. doi:10.1056/NEJMoa042683.

Miller, M., Cook, H. A., Furuya, E. Y., Bhat, M., Lee, M. H., Vavagiakis, P., et al. (2009). Staphylococcus aureus in the community: Colonization versus infection. *PLoS One* 4. doi:10.1371/journal.pone.0006708.

Mócsai, A., Zhang, H., Jakus, Z., Kitaura, J., Kawakami, T., and Lowell, C. A. (2003). G-protein-coupled receptor signaling in Syk-deficient neutrophils and mast cells. *Blood* 101, 4155–4163. doi:10.1182/blood-2002-07-2346.

Montgomery, C. P., Boyle-Vavra, S., Adem, P. V., Lee, J. C., Husain, A. N., Clasen, J., et al. (2008). Comparison of Virulence in Community-Associated Methicillin-Resistant Staphylococcus aureus Pulsotypes USA300 and USA400 in a Rat Model of Pneumonia. *J. Infect. Dis.* 198, 561–570. doi:10.1086/590157.

Nauseef, W. M. (2007). How human neutrophils kill and degrade microbes: an integrated view. *Immunol. Rev.* 219, 88–102. doi:10.1111/j.1600-065X.2007.00550.x.

Nourse, C., Starr, M., and Munckhof, W. (2007). Community-acquired methicillin-resistant Staphylococcus aureus causes severe disseminated infection and deep venous thrombosis in children: Literature review and recommendations for management. *J. Paediatr. Child Health* 43, 656–661. doi:10.1111/j.1440-1754.2007.01153.x.

Novick, R. P. (2003). Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol. Microbiol.* 48, 1429–49. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12791129 [Accessed June 16, 2013].

Nunes, P., Demaurex, N., and Dinauer, M. C. (2013). Regulation of the NADPH oxidase and associated ion fluxes during phagocytosis. *Traffic* 14, 1118–31. doi:10.1111/tra.12115.

Nygaard, T. K., Pallister, K. B., Ruzevich, P., Griffith, S., Vuong, C., and Voyich, J. M. (2010). SaeR binds a consensus sequence within virulence gene promoters to advance USA300 pathogenesis. *J. Infect. Dis.* 201, 241–54. doi:10.1086/649570.

Ogston, A. (1881). Report upon micro-organisms in surgical diseases. *Br. Med. J.* 1, 369–375. doi:10.1136/bmj.1.1054.369.

Ogston, A. (1882a). Micrococcus Poisoning. *J. Anat. Physiol.* 17, 24–58. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17231450.

Ogston, A. (1882b). Micrococcus Poisoning. *J. Anat. Physiol.* 17, 24–58. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1310127/pdf/janatphys00147-0031.pdf.

Pang, Y. Y., Schwartz, J., Bloomberg, S., Boyd, J. M., Horswill, A. R., and Nauseef, W. M. (2014). Methionine sulfoxide reductases protect against oxidative stress in staphylococcus aureus encountering exogenous oxidants and human neutrophils. *J. Innate Immun.* 6, 353–364. doi:10.1159/000355915.

Podolnikova, N. P., Podolnikov, A. V., Haas, T. A., Lishko, V. K., and Ugarova, T. P. (2015). Ligand recognition specificity of leukocyte integrin ??m??2 (Mac-1, CD11b/CD18) and its functional consequences. *Biochemistry* 54, 1408–1420. doi:10.1021/bi5013782.

Poovelikunnel, T., Gethin, G., and Humphreys, H. (2015). Mupirocin resistance: Clinical implications and potential alternatives for the eradication of mrsa. *J. Antimicrob. Chemother.* 70, 2681–2692. doi:10.1093/jac/dkv169.

Roos, D., and de Boer, M. (2014). Molecular diagnosis of chronic granulomatous disease. *Clin. Exp. Immunol.* 175, 139–49. doi:10.1111/cei.12202.

Segal, A. W. (2005). How neutrophils kill microbes. *Annu. Rev. Immunol.* 23, 197–223. doi:10.1146/annurev.immunol.23.021704.115653.

Segal, A. W. (2008). The function of the NADPH oxidase of phagocytes and its relationship to other NOXs in plants, invertebrates, and mammals. *Int. J. Biochem. Cell Biol.* 40, 604–618. doi:10.1016/j.biocel.2007.10.003.

Sollid, J. U. E., Furberg, A. S., Hanssen, A. M., and Johannessen, M. (2014). Staphylococcus aureus: Determinants of human carriage. *Infect. Genet. Evol.* 21, 531–541. doi:10.1016/j.meegid.2013.03.020.

Spaan, A. N., Henry, T., Van Rooijen, W. J. M., Perret, M., Badiou, C., Aerts, P. C., et al. (2013). The staphylococcal toxin panton-valentine leukocidin targets human C5a receptors. *Cell Host Microbe* 13, 584–594. doi:10.1016/j.chom.2013.04.006.

Spaan, A. N., Schiepers, A., de Haas, C. J. C., van Hooijdonk, D. D. J. J., Badiou, C., Contamin, H., et al. (2015). Differential Interaction of the Staphylococcal Toxins Panton-Valentine Leukocidin and γ-Hemolysin CB with Human C5a Receptors. *J. Immunol.* 195, 1034–43. doi:10.4049/jimmunol.1500604.

Springer, T. A., and Dustin, M. L. (2012). Integrin inside-out signaling and the immunological synapse. *Curr. Opin. Cell Biol.* 24, 107–115. doi:10.1016/j.ceb.2011.10.004.

Sun, F., Li, C., Jeong, D., Sohn, C., He, C., and Bae, T. (2010). In the Staphylococcus aureus two-component system sae, the response regulator SaeR binds to a direct repeat sequence and DNA binding requires phosphorylation by the sensor kinase SaeS. *J. Bacteriol.* 192, 2111–27. doi:10.1128/JB.01524-09.

Sward, E. W., Fones, E. M., Spaan, R. R., Pallister, K. B., Haller, B. L., Guerra, F. E., et al. (2018). Staphylococcus aureus SaeR/S-Regulated Factors Decrease Monocyte-Derived Tumor Necrosis Factor-α to Reduce Neutrophil Bactericidal Activity. *J. Infect. Dis.* 217, 943–952. doi:10.1093/infdis/jix652.

Syed Mortadza, S. A., Wang, L., Li, D., and Jiang, L.-H. (2015). TRPM2 Channel-Mediated ROS-Sensitive Ca2+ Signaling Mechanisms in Immune Cells. *Front. Immunol.* 6, 1–7. doi:10.3389/fimmu.2015.00407.

Ueda, T., Rieu, P., Brayer, J., and Arnaout, M. A. (1994). Identification of the complement iC3b binding site in the beta 2 integrin CR3 (CD11b/CD18). *Proc. Natl. Acad. Sci. U. S. A.* 91, 10680–4. doi:10.1073/pnas.91.22.10680.

Valentine, F. C. ., and Hall-Smith, S. . (1952). Superficial staphylococcal infection. *Lancet (London, England)* 2, 351–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14955999.

van Kesse, K. P. M., Bestebroer, J., and van Strijp, J. A. G. (2014). Neutrophil-mediated phagocytosis of Staphylococcus aureus. *Front. Immunol.* 5, 1–12. doi:10.3389/fimmu.2014.00467.

Ventola, C. L. (2015). The antibiotic resistance crisis: part 1: causes and threats. *P T A peer-reviewed J. Formul. Manag.* 40, 277–83. doi:Article.

von Eiff, C., Becker, K., Machka, K., Stammer, H., and Peters, G. (2001). Nasal carriage as a source of Staphylococcus aureus bacteremia. Study Group. *N. Engl. J. Med.* 344, 11–6. doi:10.1056/NEJM200101043440102.

Voyich, J. M., Braughton, K. R., Sturdevant, D. E., Whitney, A. R., Saïd-Salim, B., Porcella, S. F., et al. (2005). Insights into mechanisms used by Staphylococcus aureus to avoid destruction by human neutrophils. *J. Immunol.* 175, 3907–19.

Voyich, J. M., Otto, M., Mathema, B., Braughton, K. R., Whitney, A. R., Welty, D., et al. (2006). Is Panton-Valentine leukocidin the major virulence determinant in community-associated methicillin-resistant Staphylococcus aureus disease? *J. Infect. Dis.* 194, 1761–70. doi:10.1086/509506.

Voyich, J. M., Vuong, C., DeWald, M., Nygaard, T. K., Kocianova, S., Griffith, S., et al. (2009). The SaeR/S gene regulatory system is essential for innate immune evasion by Staphylococcus aureus. *J. Infect. Dis.* 199, 1698–706. doi:10.1086/598967.

Wang, G., Cao, L., Liu, X., Sieracki, N. A., Di, A., Wen, X., et al. (2016). Oxidant Sensing by TRPM2 Inhibits Neutrophil Migration and Mitigates Inflammation. *Dev. Cell* 38, 453–462. doi:10.1016/j.devcel.2016.07.014.

Wertheim, H. F., Melles, D. C., Vos, M. C., van Leeuwen, W., van Belkum, A., Verbrugh, H. A., et al. (2005). The role of nasal carriage in Staphylococcus aureus infections. *Lancet Infect. Dis.* 5, 751–762. doi:10.1016/S1473-3099(05)70295-4.

Winterbourn, C. C., Hampton, M. B., Livesey, J. H., and Kettle, A. J. (2006). Modeling the reactions of superoxide and myeloperoxidase in the neutrophil phagosome: Implications for microbial killing. *J. Biol. Chem.* 281, 39860–39869. doi:10.1074/jbc.M605898200.

Winterbourn, C. C., and Kettle, A. J. (2013). Redox reactions and microbial killing in the neutrophil phagosome. *Antioxid. Redox Signal.* 18, 642–60. doi:10.1089/ars.2012.4827.

Wymann, M. P., Bulgarelli-Leva, G., Zvelebil, M. J., Pirola, L., Vanhaesebroeck, B., Waterfield, M. D., et al. (1996). Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. *Mol. Cell. Biol.* 16, 1722–1733. doi:10.1128/MCB.16.4.1722.

Yang, E. S., Tan, J., Eells, S., Rieg, G., Tagudar, G., and Miller, L. G. (2010). Body site colonization in patients with community-associated methicillin-resistant Staphylococcus aureus and other types of S. aureus skin infections. *Clin. Microbiol. Infect.* 16, 425–431. doi:10.1111/j.1469-0691.2009.02836.x.

Yang, S. J., Bayer, A. S., Mishra, N. N., Meehl, M., Ledala, N., Yeaman, M. R., et al. (2012). The Staphylococcus aureus two-component regulatory system, grars, senses and confers resistance to selected cationic antimicrobial peptides. *Infect. Immun.* 80, 74–81. doi:10.1128/IAI.05669-11.

Yang, S. J., Xiong, Y. Q., Yeaman, M. R., Bayles, K. W., Abdelhady, W., and Bayer, A. S. (2013). Role of the LytSR two-component regulatory system in adaptation to cationic antimicrobial peptides in staphylococcus aureus. *Antimicrob. Agents Chemother.* 57, 3875–3882. doi:10.1128/AAC.00412-13.

Zhan, Y., Virbasius, J. V., Song, X., Pomerleau, D. P., and Wayne Zhou, G. (2002). The p40phox and p47phox PX domains of NADPH oxidase target cell membranes via direct and indirect recruitment by phosphoinositides. *J. Biol. Chem.* 277, 4512–4518. doi:10.1074/jbc.M109520200.

Zurek, O. W., Nygaard, T. K., Watkins, R. L., Pallister, K. B., Torres, V. J., Horswill, A. R., et al. (2014). The Role of Innate Immunity in Promoting SaeR/S-Mediated Virulence in Staphylococcus aureus. *J. Innate Immun.* 6, 21–30. doi:10.1159/000351200.

CHAPTER TWO

EPIC IMMUNE BATTLES OF HISTORY: NEUTROPHILS VS. *STAPHYLOCOCCUS AUREUS*

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Epic Immune Battles of History: Neutrophils vs. *Staphylococcus aureus*

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Neutrophils are the most abundant leukocytes in human blood and the first line of defense after bacteria have breached the epithelial barriers. After migration to a site of infection, neutrophils engage and expose invading microorganisms to antimicrobial peptides and proteins, as well as reactive oxygen species, as part of their bactericidal arsenal. Ideally, neutrophils ingest bacteria to prevent damage to surrounding cells and tissues, kill invading microorganisms with antimicrobial mechanisms, undergo programmed cell death to minimize inflammation, and are cleared away by macrophages. *Staphylococcus aureus* (*S. aureus*) is a prevalent Gram-positive bacterium that is a common commensal and causes a wide range of diseases from skin infections to endocarditis. Since its discovery, *S. aureus* has been a formidable neutrophil foe that has challenged the efficacy of this professional assassin. Indeed, proper clearance of *S. aureus* by neutrophils is essential to positive infection outcome, and *S. aureus* has developed mechanisms to evade neutrophil killing. Herein, we will review mechanisms used by *S. aureus* to modulate and evade neutrophil bactericidal mechanisms including priming, activation, chemotaxis, production of reactive oxygen species, and resolution of infection. We will also highlight how *S. aureus* uses sensory/regulatory systems to tailor production of virulence factors specifically to the triggering signal, e.g., neutrophils and defensins. To conclude, we will provide an overview of therapeutic approaches that may potentially enhance neutrophil antimicrobial functions.

Keywords: host-pathogen interactions, innate immunity, immune evasion, host defense, phagocytosis, chemotaxis, *Staphylococcus aureus*

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INTRODUCTION

Polymorphonuclear leukocytes (PMNs or neutrophils) are the first line of defense against bacterial pathogens that have breached epithelial barriers. Within minutes of bacterial invasion, neutrophils respond to soluble factors including chemokines and cytokines and are recruited to the site of infection where they ingest microbes. Subsequently, neutrophils expose microorganisms to antimicrobial proteins, peptides, and reactive oxygen species to kill the invading pathogen. This is a delicate process that must eliminate the pathogen while controlling excessive inflammation. Concurrently, neutrophils secrete cytokines and chemokines to continue to recruit neutrophils

and enhance other host responses to infection. Finally, neutrophil death is essential for proper resolution of infection and must be regulated to minimize bystander damage while continuing to signal if more immune response is needed or if tissue repair should begin. These potent mechanisms are effective at eliminating most fungal and bacterial microorganisms. However, successful pathogens have developed strategies to disrupt various neutrophil functions to cause infection.

S. aureus is a highly-adaptable Gram-positive pathogen estimated to colonize 50–60% of the population (Wertheim et al., 2005; Gorwitz et al., 2008). It is also a leading cause of infections ranging from superficial skin abscesses to life-threatening diseases, including septicemia and necrotizing pneumonia (Klevens et al., 2007; Kobayashi et al., 2015). The ability of *S. aureus* to cause human disease is based in part on its ability to evade the innate immune response, thereby circumventing rapid elimination. Many factors contribute to *S. aureus* pathogenesis. These include production of numerous toxins, such as the barrel forming two-component toxins capable of directly lysing host immune cells (Menestrina et al., 2003), and tissue destroying enzymes including protease, lipase, and hyaluronidase, as well as many surface proteins and adhesins linked to virulence (Lowy, 1998). In this review, we will focus on evasion strategies used by *S. aureus* to disrupt neutrophil functions essential for bacterial clearance. First, we will highlight virulence factors produced by *S. aureus* to alter neutrophil priming, activation, chemotaxis, and adhesion. Then, we will discuss strategies used by *S. aureus* to subvert neutrophil killing by antimicrobial peptides and proteins and reactive oxygen species. Additionally, we will examine recent literature investigating mechanisms used by *S. aureus* to modulate neutrophil cell death programs. Finally, we will highlight the reciprocal communication between *S. aureus* and the neutrophil emphasizing sensing and adaptive responses used by *S. aureus* to recognize and respond to neutrophil challenge. The review will conclude with an overview of potential therapeutic approaches aimed at disrupting bacterial sensing and signaling to decrease production of virulence factors during neutrophil interaction and discuss putative immunotherapies to boost immune responses to *S. aureus* while limiting inflammatory damage caused by neutrophils.

STRATEGIES USED BY *S. AUREUS* TO DISRUPT NEUTROPHIL PRIMING, ACTIVATION, CHEMOTAXIS AND ADHESION

Neutrophils are initially recruited to a site of infection by following chemokine gradients in a process termed chemotaxis. Taking cues from activated endothelium, neutrophils slow their movement through blood vessels by selectin-mediated tethering to the endothelium followed by complete movement arrest through interaction with integrins on the endothelium. Extravasation from the blood vessels through the endothelial barrier is required for neutrophils to access interstitial fluid and migrate via a chemotactic gradient to the site of infection where ingestion of bacteria can take place. For detailed reviews of neutrophil chemotaxis, adhesion to the epithelium, and transmigration, please refer to (Kolaczkowska and Kubes, 2013; de Oliveira et al., 2016). Herein, we will focus on virulence factors produced by *S. aureus* to inhibit specific neutrophil receptors from binding host and bacterial derived ligands, which results in impaired neutrophil priming, activation, chemotaxis, and adhesion to the endothelium.

NEUTROPHIL PRIMING: A POTENTIAL TARGET OF *S. AUREUS*?

Priming refers to the ability of a primary agonist to enhance a neutrophil's response to a secondary stimulus (Swain et al., 2002). There are many known neutrophil priming agents including: complement components C3a and C5a (Skjeflo et al., 2014), interferon-γ (IFN-γ) (Edwards et al., 1988), interleukin-8 (IL-8) (Mitchell et al., 2003), and tumor-necrosis factor-α (TNF-α; Rainard et al., 2000). Bacterial derived products such as N-formyl methionyl peptide, formyl-methionyl-leucyl phenylalanine (fMLF), peptidoglycan, and *S. aureus* cytolytic toxins also demonstrate an ability to prime neutrophils (Elbim et al., 1994; El-Benna et al., 2008; Clarke et al., 2010; Malachowa et al., 2012). The ability of these agents to prime neutrophils is typically not universal in that concentration and neutrophil response can vary drastically (Swain et al., 2002). Primed neutrophil responses influence many neutrophil functions including increases in adhesion, phagocytosis, superoxide production, and degranulation (Ellis and Beaman, 2004). It can also influence neutrophil apoptosis (Wright et al., 2013). Thus, priming can set the stage for subsequent neutrophil-pathogen interactions and influences outcome of this interaction.

Not much is known about the impact of *S. aureus* on neutrophil priming. Earlier studies investigated priming of neutrophils with conditioned medium from peripheral blood mononuclear cells (PBMC) challenged with killed *S. aureus* (Ferrante et al., 1989; Bates et al., 1991). These studies demonstrated increased neutrophil staphylocidal activity following priming with conditioned media from PBMCs challenged with killed *S. aureus* vs. priming with medium from unstimulated PBMCs. This enhanced bactericidal activity was shown to be dependent on TNF-α produced by PBMCs in response to killed *S. aureus* (Ferrante et al., 1989, 1993; Bates et al., 1991). These studies suggest that the response of PBMCs and resident cells to *S. aureus* may strongly impact the outcome of neutrophil-*S. aureus* interactions. Supporting this idea are studies demonstrating that *S. aureus* promotes production of IFN-γ and this production has been linked to poorer outcome of infection in mouse models (Watkins et al., 2011, 2013). IFN-γ impacts neutrophil function in many ways, including priming of oxidative burst and degranulation mechanisms (Ellis and Beaman, 2004). Using a murine wound model of *S. aureus* infection, IFN-γ-mediated CXC chemokine production by T cells promoted a robust recruitment of neutrophils that resulted in elevated *S. aureus* burdens at the infectious foci (McLoughlin et al., 2008). Subsequent studies using a mouse peritonitis model

also demonstrated IFN-γ was associated with higher bacterial burdens but that the source of IFN-γ was neutrophils (Watkins et al., 2013). Potentially, priming by IFN-γ causes overactivation and inflammation in neutrophils making them less effective at clearing *S. aureus*. The role of IFN-γ and neutrophil bactericidal activity against *S. aureus* warrants further investigation especially considering that IFN-γ is used to treat patients with chronic granulomatous disease (Gallin et al., 1991). CGD patients receiving recombinant IFN-γ show a decrease in *S. aureus* infections (Gallin et al., 1991). Furthermore, *in vitro* studies have demonstrated that priming of human neutrophils with IFN-γ increased bactericidal activity against *S. aureus* (Edwards et al., 1988). Thus, more studies are needed to determine under what conditions IFN-γ promotes an effective neutrophil response against clinically relevant strains of *S. aureus*. Clearly, source, timing and amount of IFN-γ play an important role in outcome of *S. aureus*-neutrophil interactions.

S. aureus produces hemolysins, and bi-component leukocidins that directly impact immune cell function (Seilie and Bubeck Wardenburg, 2017). Many of these toxins act by forming pores in the membrane of immune cells causing lysis. Among these, the bi-component toxins leukotoxin GH (LukGH, also known as LukAB) and Panton-Valentine leukocidin (PVL) have the ability to cause neutrophil pore-formation when present at high concentrations. However, at sublytic concentrations, these factors can promote enhanced binding, uptake and killing of *S. aureus* through priming of neutrophils (Graves et al., 2012). This is also consistent with reports that have demonstrated the ability of PVL to enhance neutrophil superoxide, granule exocytosis, and release of leukotriene B_4 and IL-8 (König et al., 1995; Colin and Monteil, 2003). In addition, low doses of alpha-toxin, a single component toxin encoded by *hla*, has been shown to promote transcriptional activity leading to production of inflammatory mediators that can prime neutrophils including IL-8 (Dragneva et al., 2001).

As mentioned above, most of the studies thus far have not directly investigated how modulation of known neutrophil priming agents (such as cytokines and chemokines) by *S. aureus* actually influences neutrophil function. Also, the impact of *S. aureus* toxins and immunomodulatory proteins on neutrophil priming has not been thoroughly investigated. Taken together, targeting neutrophil priming may be very important for *S. aureus* to gain an edge on evading the neutrophil. However, additional studies are needed to understand the impact of *S. aureus* on neutrophil priming.

KEY FACTORS USED BY *S. AUREUS* TO DISRUPT NEUTROPHIL ACTIVATION, CHEMOTAXIS AND ADHESION

In contrast to our understanding of how *S. aureus* may impact priming, there is an abundance of data that highlight how specific *S. aureus* factors impact neutrophil activation, chemotaxis and adhesion. In this section, we highlight individual virulence factors that have been studied for their ability to disrupt these key mechanisms (**Figure 1**).

CHIPS

The chemotaxis inhibitory protein of *S. aureus* (CHIPS) is a 14.1-kDa exoprotein that inhibits neutrophil migration and activation (De Haas et al., 2004). CHIPS prevents neutrophils from responding to both host- and bacteria-derived chemoattractants. Neutrophil activation by host-derived C5a is inhibited by CHIPS binding to the C5a receptor (C5aR). Similarly, neutrophil detection of bacteria-derived formylated peptides is inhibited by CHIPS binding to the formyl peptide receptor (FPR). CHIPS shows high affinity to both C5aR and FPR with K_D-values of 1.1 ± 0.2 and 35.4 ± 7.7 nM, respectively; the affinity of C5aR and FPR for their natural ligands is within the CHIPS binding affinity to C5aR and FPR (Falk et al., 1982; Huey and Hugli, 1985; Postma et al., 2004). While C5aR and FPR are G-protein coupled receptors (GPCRs), the active blocking domain in CHIPS is distinct for inhibiting C5aR and FPR (Haas et al., 2005). By using FITC-labeled CHIPS, Postma et al. demonstrated that CHIPS is not internalized following binding to neutrophil C5aR and FPR and its activity is ATP and cell-signaling independent (Postma et al., 2004). It has also been demonstrated that CHIPS binds to C5aR and FPR extracellularly and does not act as an agonist since receptor binding does not induce calcium mobilization (De Haas et al., 2004). It was further demonstrated that intravenously administered CHIPS was able to inhibit mouse neutrophil influx following intraperitoneal administration of C5a despite a 30-fold lower affinity of CHIPS for mouse C5aR (De Haas et al., 2004). Finally, neutralizing antibodies to CHIPS have been observed in human sera isolated from both normal donor controls and *S. aureus* infected samples, implying CHIPS plays an active role during staphylococcal infection (Wright et al., 2007).

FLIPR AND FLIPR-LIKE

Neutrophils express formyl peptide receptor-like-1 (FPRL1). This receptor is activated by diverse peptides and proteins including: the synthetic peptides Trp-Lys-Tyr-Met-Val-D-Met-NH2 (WKYMVm), and L-conformer WKYMVM, lipoxin A_4, the T21/DP107 leucine zipper-like domain of the HIV-1 envelope protein gp41, serum amyloid A, the mitochondrial peptide fragment MYFINILTL, the antimicrobial peptide LL-37, and prion peptide fragments (Fiore et al., 1994; Su et al., 1999a,b; Chiang et al., 2000; De Yang et al., 2000; Christophe et al., 2001; Le et al., 2001). Activation of FPRL1 on neutrophils leads to intracellular calcium mobilization and chemotaxis, while superoxide production through FPRL1 is ligand dependent (Bae et al., 2003). *S. aureus* produces two proteins that bind FPRL1 and inhibit its activation. FPRL1 inhibitory protein (FLIPr) is a secreted protein that binds and antagonizes both FPRL1 and FPR, but FPRL1 antagonism by FLIPr is much stronger than on FPR (Prat et al., 2006). Similarly, a second FPRL1 antagonist termed FLIPr-like shares 73% overall homology with FLIPr (Prat et al., 2009). FLIPr and FLIPr-like show similar inhibitory activity toward FPRL1 following stimulation with the FPRL1 agonist MMK-1; however, FLIPr-like antagonism toward FPR is 100-fold stronger than that of FLIPr following stimulation with fMLF (Prat et al., 2009). As expected, neutrophil exposure to purified

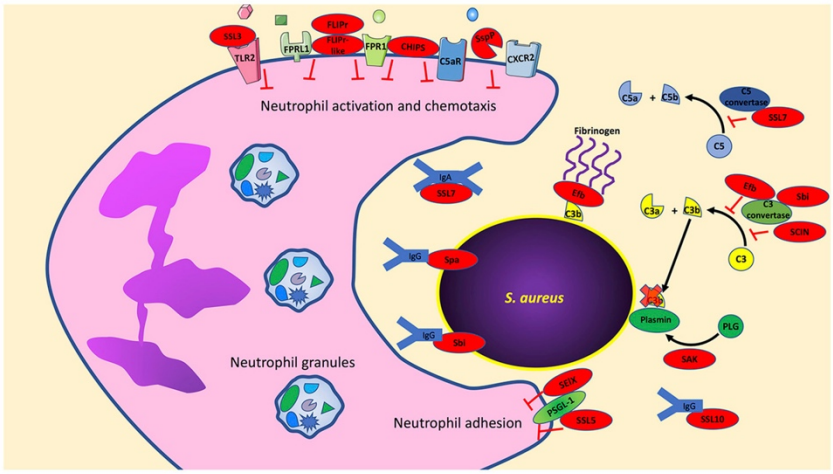


FIGURE 1 | *S. aureus* has an arsenal of virulence factors to inhibit neutrophil activation, chemotaxis, and phagocytosis. Multiple virulence factors produced by *S. aureus* target key host effector proteins, for example, Efb, Sbi, and SCIN target the complement protein C3 convertase to prevent formation of C3a and C3b. Further studies are needed to determine if the production of these virulence factors are truly redundant or if they have multiple host targets as is the case with Sbi, which also targets immunoglobulins. Bacterial components indicated in red. PLG, plasminogen; SAK, staphylokinase; SspP, staphopain A.

FLIPr and FLIPr-like completely inhibits neutrophil chemotaxis toward the FPRL1 agonist MMK-1 (Prat et al., 2006, 2009).

FLIPr and FLIPr-like have also been shown to bind different FcγR isoforms to block IgG binding (Stemerding et al., 2013). Stemerding et al. showed that FLIPr preferentially binds to FcγR class II receptors while FLIPr-like can bind to FcγR cla ss I, II, and III receptors. As expected, neutrophil pretreatment with FLIPr or FLIPr-like significantly reduced phagocytosis of *S. aureus* opsonized with purified IgG (Stemerding et al., 2013). However, it should be noted that phagocytic inhibition of neutrophils toward *S. aureus* opsonized with human serum by FLIPr and FLIPr-like was only observed at low serum concentrations (<1% serum). At higher serum concentrations (1–10% serum), FLIPr and FLIPr-like do not inhibit neutrophil-mediated phagocytosis of opsonized *S. aureus*. Under these higher serum conditions, neutrophil phagocytosis of opsonized *S. aureus* is likely mediated by complement receptors, which are not antagonized by FLIPr and FLIPr-like proteins. It follows that experiments using neutrophils treated with FLIPr and FLIPr-like had reduced uptake of *S. aureus* only when opsonized with complement inactivated serum thus showing the specific inhibition of FLIPr and FLIPr-like toward serum immunoglobulins (Stemerding et al., 2013).

STAPHOPAIN A

The CXCR2 chemokine receptor (also known as IL8RB) is highly expressed on human neutrophils and has high specificity for the potent neutrophil chemoattractants CXCL1, CXCL2, and CXCL7, as well as CXCL8 (IL-8, which can also bind to CXCR1) (Yoshimura et al., 1987; Baggiolini et al., 1989; Ben-Baruch et al., 1997; Ritzman et al., 2010). *S. aureus* infection induces CXCL1, CXCL2, CXCL8 production resulting in neutrophil recruitment (Mempel et al., 2003; Sasaki et al., 2003; Olaru and Jensen, 2010). To prevent neutrophil recruitment induced by CXCR2 ligands, *S. aureus* secretes the cysteine protease staphopain A which cleaves the N-terminus of CXCR2 required for ligand binding (Laarman et al., 2012). Neutrophils pretreated with staphopain A blocked calcium mobilization following stimulation with CXCL1 and CXCL7. Importantly, these chemokines only bind CXCR2 (and not CXCR1). In contrast, staphopain A did not inhibit neutrophil calcium mobilization following stimulation with CXCL8 since it can also signal through CXCR1. The impacts of staphopain A *in vivo* are lacking since staphopain A is specific for human CXCR2 and does not cleave murine CXCR2 (Laarman et al., 2012).

SEIX AND SSL5

Neutrophils express P-selectin glycoprotein ligand 1 (PSGL-1) on the cellular surface to bind P- and E-selectin on activated endothelial cells or platelets and L-selectin expressed on leukocytes (Moore et al., 1995; Guyer et al., 1996; Hidalgo et al., 2007; Huo and Xia, 2009; Stadtmann et al., 2013). PSGL-1 binding to selectins tethers neutrophils to activated endothelial cells and is a first step in the process of transmigration. *S. aureus* secretes two proteins that inhibit PSGL-1 binding to

selectins. The staphylococcal enterotoxin-like toxin X (SElX) is a highly conserved superantigen that binds glycosylated PSGL-1 on neutrophils and inhibits binding to P-selectin (Wilson et al., 2011; Fevre et al., 2014). Similarly, the staphylococcal superantigen-like 5 (SSL5) protein directly binds PSGL-1 on neutrophils inhibiting rolling on endothelial cells (Bestebroer et al., 2007). In addition to binding PSGL-1, SSL5 also binds to other GPCRs but only inhibits the ligands that require the N-terminal domain of their respective receptors for activation (Bestebroer et al., 2009). Thus, SSL5 pretreatment of neutrophils inhibited activation induced by C3a, C5a, CXCL1, and CXCL8 (Bestebroer et al., 2009).

SSL3

Toll-like receptor 2 (TLR2) recognizes staphylococcal peptidoglycan-associated lipoproteins (Fournier and Philpott, 2005; Kurokawa et al., 2009). TLR2 can also discriminate between diacylated and triacylated lipoproteins by associating with TLR6 or TLR1, respectively (Takeuchi et al., 2001, 2002). The recognition of staphylococcal lipoproteins such as the staphylococcal iron transporter C (SitC) are exclusively dependent on TLR2 to induce immune activation (Stoll et al., 2005; Kurokawa et al., 2009; Müller et al., 2010). The importance of TLR2 during *S. aureus* infection is highlighted by $TLR2^{-/-}$ mice that show increased susceptibility and nasal colonization to *S. aureus* compared to wild-type mice (Takeuchi et al., 2000; González-Zorn et al., 2005; Hoebe et al., 2005). In addition, $TLR2^{-/-}$ mice infected with *S. aureus* show alterations in cytokines that modulate neutrophil function including TNF-α and IL-1β (Knuefermann et al., 2004). Engagement of TLR2 influences many neutrophil functions including: adhesion molecule expression, reactive oxygen species production (following stimulation with fMLF), and modulates CXCL8, and chemokine receptor expression (Sabroe et al., 2003). *S. aureus* produces the staphylococcal superantigen-like 3 protein that binds TLR2 to inhibit activation in neutrophils and other cell types expressing TLR2 (Bardoel et al., 2012; Yokoyama et al., 2012). Crystal structures showed that SSL3 binding to TLR2 reduced the lipopeptide binding pocket by ∼50%, which inhibited binding of the TLR2 agonist Pam_2CSK_4 (Koymans et al., 2015). The same study demonstrated SSL3 can bind a preformed TLR2- Pam_2CSK_4 complex and this blocked TLR2-TLR1 and TLR2-TLR6 heterodimerization thereby inhibiting downstream signaling.

STRATEGIES USED BY *S. AUREUS* TO INHIBIT NEUTROPHIL PHAGOCYTOSIS

Phagocytosis is a process by which neutrophils, and other phagocytes, ingest particles from their extracellular environment including bacteria and host cells. In neutrophils, this sequestration results in the formation of an intracellular compartment termed the phagosome following invagination of the cellular membrane. Neutrophil granules fuse with the phagosome to release antimicrobial peptides and proteins, as well as produce reactive oxygen species to kill invading microorganisms. Neutrophils bind to pathogen-associated molecular patterns on the *S. aureus* surface to initiate the phagocytic process. Furthermore, binding of immunoglobulins to *S. aureus* and complement activation enhances phagocytosis, as well as engages different neutrophil receptors resulting in branching downstream signaling. *S. aureus* employs strategies and produces a wide range of virulence factors to disrupt neutrophil phagocytosis. Herein, we will highlight select strategies used by *S. aureus* to disrupt neutrophil phagocytosis. Since a complete review on the strategies used by *S. aureus* to avoid phagocytosis is outside the scope of this publication, we recommend previous detailed reviews focusing on immunoglobulin and complement evasion by *S. aureus* to inhibit phagocytosis (Lambris et al., 2008; Serruto et al., 2010; van Kesse et al., 2014).

CAPSULE SYNTHESIS

The production of capsular polysaccharide by *S. aureus* has been proposed as an antiphagocytic evasion strategy but its actual role in inhibiting neutrophil phagocytosis remains controversial. Indeed, previous studies have shown that *S. aureus* strains producing high levels of capsular polysaccharide withstand neutrophil killing better than microencapsulated strains (Xu et al., 1992; Thakker et al., 1998). However, it should be noted that under these experimental conditions neutrophil killing of *S. aureus* was correlated to the ability of neutrophils to associate with bacteria without clearly showing ingestion. Furthermore, the experimental conditions used suspended instead of adherent neutrophils which can greatly influence neutrophil ingestion of *S. aureus* (Lu et al., 2014). Also, an intraperitoneal *S. aureus* infection model showed no differences in neutrophil intracellular staphylococcal survival between a highly and microencapsulated strain (Gresham et al., 2000). Notably, the predominant clinical isolate USA300 is unencapsulated, further questioning the role of capsule synthesis in *S. aureus* pathogenicity (Montgomery et al., 2008; Carrel et al., 2015).

COMPLEMENT INHIBITION

Human pathogens have developed strategies to evade complement, thus inhibiting immune recognition, cytokine production, and neutrophil uptake (Lambris et al., 2008; Serruto et al., 2010). *S. aureus* produces several virulence factors that target different machinery in the complement system. The staphylococcal complement inhibitor (SCIN), is a 9.8-kDa exoprotein that specifically binds and inhibits human C3 convertases (Rooijakkers et al., 2005a, 2006). SCIN binding to C3 convertases does not prevent C3 binding but completely inhibits generation of C3b and thus opsonization of *S. aureus* by C3b (Rooijakkers et al., 2005a; Ricklin et al., 2009; Garcia et al., 2010). In addition, SCIN stabilizes C3 convertases by competing with factor H which accelerates decay of C3 convertases (Ricklin et al., 2009). *In vitro*, recombinant SCIN can significantly inhibit neutrophil phagocytosis of human serum opsonized *S. aureus* resulting in increased bacterial survival (Rooijakkers et al.,

2005a). *In vivo* studies with SCIN are lacking since it is human specific, but SCIN is immunogenic since a limited study with 80 healthy people and 20 individuals with recurring staphylococcal infections showed all produced antibodies to SCIN (Rooijakkers et al., 2005a).

S. aureus is also equipped with virulence factors that target complement without direct binding to C3 convertase. Staphylokinase is a secreted protein that binds human plasminogen converting it into its active form plasmin (Parry et al., 2000; Mölkänen et al., 2002; Rooijakkers et al., 2005b). Plasmin, a serine protease, is bound to the *S. aureus* surface and degrades C3 convertase-dependent C3b to prevent deposition on the bacterial surface. Human neutrophils show decreased phagocytic activity toward human serum opsonized *S. aureus* (depleted of IgG and IgM to rule out immunoglobulin mediated phagocytosis) and pretreated with recombinant staphylokinase (Rooijakkers et al., 2005b). In addition, *S. aureus* secretes the extracellular fibrinogen-binding (Efb) protein that binds to C3 to prevent cleavage to C3b and can also directly bind C3b deposited on the extracellular bacterial membrane (Lee et al., 2004; Ko et al., 2013). As its name implies, the extracellular fibrinogen-binding protein also contains a fibrinogen binding domain that recruits fibrinogen and inhibits neutrophil phagocytosis (Ko et al., 2013). Complement receptor recognition of C3b is blocked by a thick layer of fibrinogen linked by Efb on the bacterial surface (Ko et al., 2013). *Ex vivo* studies with human blood exposed to Efb and *S. aureus* showed a significant reduction in neutrophil phagocytosis compared to *S. aureus* alone. Similarly, supernatants from wild-type *S. aureus* reduced neutrophil phagocytosis of opsonized *S. aureus* compared to supernatants from Efb-deficient *S. aureus* in the presence of fibrinogen. Finally, neutrophils from mice intraperitoneally infected with GFP-expressing wild-type *S. aureus* or an Efb-deficient mutant showed significantly higher ingestion of the Efb mutant compared to the wild-type (Ko et al., 2013). While this observation suggests that sufficient levels of Efb are produced by *S. aureus* to have an *in vivo* effect, it should be noted that intraperitoneal injection was performed with *S. aureus* grown to late exponential phase without washing culture toxins. Thus, increased and unknown levels of Efb were likely present from the beginning giving an advantage to *S. aureus* to prevent neutrophil phagocytosis.

INHIBITION OF IMMUNOGLOBULIN RECOGNITION

Complement and immunoglobulins function as opsonins to enhance *S. aureus* recognition and ingestion by neutrophils. While *S. aureus* produces virulence factors that inhibit complement activation and deposition on the bacterial surface, the efficacy of these virulence factors *in vivo* is controversial since immunoglobulin opsonization plays a redundant role in opsonophagocytosis. In fact, opsonophagocytosis assays investigating the role of *S. aureus* virulence factors that disrupt complement must be done in the absence of immunoglobulins to observe an inhibitory effect on phagocytosis because *S. aureus* produces virulence factors that inhibit opsonization by immunoglobulins. Protein A, encoded by the *spa* gene, is one of the better characterized virulence factors produced by *S. aureus* that inhibits neutrophil phagocytosis. Protein A is a 42-kDa secreted and membrane bound protein that binds the constant Fcγ region of IgG thus preventing proper engagement of Fcγ receptors on neutrophils and antigen recognition (Forsgren and Sjöquist, 1966; Kronvall et al., 1970; Sjödahl, 1977). Indeed, early studies clearly showed that purified protein A inhibited neutrophil phagocytosis of *S. aureus* independent of capsule polysaccharide on the bacterial surface (Dossett et al., 1969; King and Wilkinson, 1981). More recent studies have demonstrated protein A induces B cell proliferation and production of V_H3 serum IgG and IgM (Pauli et al., 2014; Kim et al., 2016). Although abundant, these antibodies do not provide protection against *S. aureus*. Generation of protein A variants that were unable to bind immunoglobulin induce a protective antibody response presumably via allowing Fc mediated uptake of opsonized *S. aureus* (Falugi et al., 2013). These data suggest that mechanisms of neutrophil recognition and uptake of *S. aureus* are likely critical to resolution of infection.

S. aureus produces a second binding protein of immunoglobulin (termed Sbi) that also binds IgG to inhibit neutrophil phagocytosis (Jacobsson and Frykberg, 1995; Zhang et al., 1998, 1999; Smith et al., 2011). Human neutrophils can phagocytose significantly more opsonized Sbi-deficient *S. aureus* compared to wild-type, and Sbi plays a protective role in *ex vivo* whole human blood killing assays (Smith et al., 2011). Interestingly, Sbi binds to lipoteichoic acid in the bacterial cell surface to remain anchored, while it can also exist in a secreted form that binds complement protein C3 to induce its degradation away from the bacterial surface (Burman et al., 2008; Upadhyay et al., 2008). Sbi also plays another immunomodulatory role that was demonstrated to impact neutrophil function. In a mouse model of peritonitis Sbi induced production of IL-6 and CXCL-1, which resulted in neutrophil recruitment and subsequently an exacerbated inflammatory response (Gonzalez et al., 2015).

In addition to Sbi, *S. aureus* secretes other proteins that bind both immunoglobulins and complement proteins to disrupt neutrophil phagocytosis. The 23-kDa staphylococcal superantigen-like 7 (SSL7) exoprotein binds to human IgA with high affinity (K_D of 1.1 nM) and inhibits FcαR binding on the neutrophil surface (Langley et al., 2005). Using an *ex vivo* whole blood infection model it was demonstrated that binding of IgA by SSL7 reduced neutrophil phagocytosis of *S. aureus* (Bestebroer et al., 2010). SSL7 also binds to the C5 complement protein (K_D of 18 nM) and inhibits C5a generation in an IgA binding-dependent manner, as well as formation of the C5b-9 membrane attack complex (Langley et al., 2005; Bestebroer et al., 2010; Laursen et al., 2010; Lorenz et al., 2013). *In vivo* mouse studies showed that administration of purified SSL7 inhibited neutrophil recruitment following exposure to heat-killed *S. aureus*. Thus, SSL7 serves diverse functions that include inhibiting neutrophil phagocytosis, recruitment, and complement activation (Bestebroer et al., 2010; Lorenz et al., 2013). Another staphylococcal superantigen-like protein termed SSL10 also binds to IgG (K_D of 220 nM) and to the complement protein C1q (Itoh et al., 2010). SSL10 significantly

inhibited neutrophil ingestion of IgG-opsonized *S. aureus* by blocking binding of FcγR (Patel et al., 2010).

Taken together, it is clear that *S. aureus* produces virulence factors that decrease neutrophil phagocytosis via production of physical barriers (capsule polysaccharide), inhibition of complement activation leading to decreased deposition of opsonins, and disruption of immunoglobulin binding to bacterial antigens. However, the efficacy of these virulence factors has been difficult to determine. Neutrophil-mediated phagocytosis of *S. aureus* is efficient in the absence of opsonins. *In vitro* studies have shown that adherent neutrophils rapidly ingest both opsonized and unopsonized *S. aureus* (Lu et al., 2014). Thus, neutrophils contain pattern-recognition receptors that are sufficient for phagocytosis of *S. aureus* independent of opsonins. Clearly, the redundancy in neutrophil receptor-mediated phagocytosis mechanisms compounds studies and strongly suggests the combination of *S. aureus* virulence factors are needed to thwart the innate ability of neutrophils to recognize this pathogen.

EVASION STRATEGIES USED BY *S. AUREUS* TO SURVIVE NEUTROPHIL KILLING

S. aureus is clearly decorated to avoid ingestion by neutrophils. Additionally, *S. aureus* is loaded with mechanisms to disarm potent neutrophil bactericidal mechanisms. Soon after initiation of the phagocytic process, and possibly before complete sealing of the phagosome, neutrophil granules mobilize to fuse and release antimicrobial agents into the phagosome containing *S. aureus* (Flannagan et al., 2009). Following phagocytosis, neutrophils expose *S. aureus* to antimicrobial peptides, proteins, and reactive oxygen species to degrade essential bacterial proteins and disrupt homeostasis resulting in bacterial death (**Figure 2**). In this section, we will highlight strategies used by *S. aureus* to avoid oxidative and non-oxidative mechanisms of neutrophil killing. Although we separate *S. aureus* evasion of oxidative and non-oxidative killing, it is important to remember that these mechanisms likely have synergistic antimicrobial activity in a physiological setting.

S. AUREUS EVASION OF NON-OXIDATIVE KILLING

The *dlt* operon in *S. aureus*, containing genes *dltABCD* is involved in activation and transfer of D-alanine, and increases tolerance to cationic antimicrobial peptides (AMPs), including human neutrophil peptide-1 (HNP-1, also known as alpha-defensin), by incorporation of positively charged D-alanine into teichoic acids (Peschel et al., 1999; Collins et al., 2002). Teichoic acids are highly negatively charged by deprotonized phosphate groups that electrostatically interact with cationic peptides. Thus, a *S. aureus dlt* mutant strain retains a negatively charged teichoic acid backbone and is significantly more susceptible to killing by cationic peptides (Peschel et al., 1999). *In vitro*, *S. aureus*

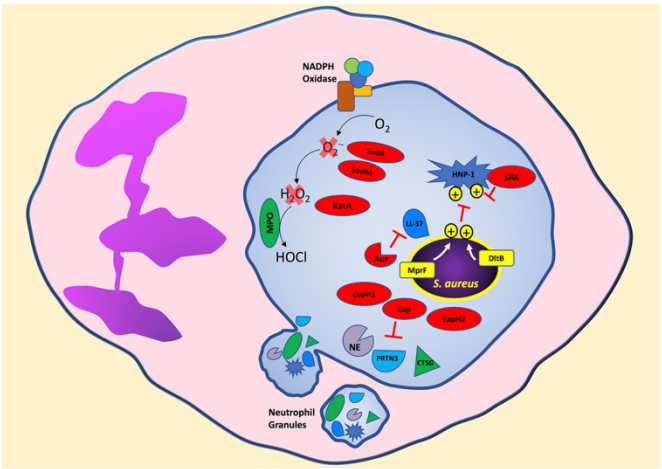


FIGURE 2 | *S. aureus* produces virulence factors that target different neutrophil bactericidal mechanisms following phagocytosis. Cationic antimicrobial peptides are ineffective toward *S. aureus* due to the presence of positive charges on the bacterial surface transferred by MprF and DltB. *S. aureus* secretes virulence factors that degrade antimicrobial proteins and enzymes released into the neutrophil phagosome. In addition, neutrophil reactive oxygen species production is decreased by *S. aureus* virulence factors that degrade intermediate reactive oxygen species like superoxide and hydrogen peroxide to reduce the formation of the highly bactericidal chemical agent hypochlorous acid. Bacterial components indicated in red. Aur, aureolysin; PRTN3, proteinase 3; CTSG, cathepsin G; SAK, staphylokinase.

dlt mutants are highly susceptible to neutrophil non-oxidative killing mechanisms whereas wild-type *S. aureus* is resistant and mainly susceptible to neutrophil oxidative killing (Collins et al., 2002). The importance of the *dlt* operon to increase *S. aureus* survival was observed in an *in vivo* mouse model of sepsis, which showed increased host mortality infected with wild-type *S. aureus* compared to the non-lethal *S. aureus dlt* mutant (Collins et al., 2002). Similarly, the *mprF* gene in *S. aureus* encoding lysylphosphatidylglycerol (LPG) synthetase confers resistance to cationic AMPs by transferring the positively charged L-lysine to the negatively charged lipid phosphatidylglycerol creating LPG (Peschel et al., 2001; Oku et al., 2004; Ernst et al., 2009). Electrostatic repulsion between cationic AMPs and the attached lysine prevents binding of cationic AMPs and disruption of the bacterial membrane. Wild-type *S. aureus* is more resistant to killing by human neutrophils compared to a *S. aureus mprF* mutant (Peschel et al., 2001). Thus, *S. aureus* modifies its cell surface as an evasion strategy by increasing electrostatic repulsion of neutrophil antimicrobial peptides leading to decreased bacterial killing.

In addition to cell membrane modifications to decrease efficacy of antimicrobial peptides by electrostatic repulsion, *S. aureus* produces proteins that directly bind, inhibit, and degrade antimicrobial peptides. Staphylokinase, which activates plasminogen to the active serine protease plasmin leading to degradation of IgG and C3b (Rooijakkers et al., 2005b), also binds HNP-1 and inhibits its bactericidal activity (Jin et al., 2004). The cathelicidin LL-37, which has potent staphylococcal bactericidal activity, is degraded by the *S. aureus* secreted metalloprotenaise aureolysin (Sieprawska-Lupa et al., 2004). Thus, *S. aureus* strains producing aureolysin are significantly more resistant to LL-37 than aureolysin-negative strains. Furthermore, the extracellular adherence protein (Eap) and the Eap-homologs 1 and 2 (EapH1 and EapH2) are neutrophil serine protease inhibitors (Harraghy et al., 2005). Purified Eap, EapH1, or EapH2 inhibit the activity of neutrophil elastase, proteinase 3, and cathepsin G which are found in neutrophil azurophilic granules and are bactericidal to *S. aureus* (Papayannopoulos et al., 2010; Stapels et al., 2014).

S. AUREUS EVASION OF OXIDATIVE KILLING

Neutrophil activation leads to assembly of the NADPH oxidase resulting in production of reactive oxygen species (ROS; DeLeo and Quinn, 1996; DeLeo et al., 1999; Bréchard and Tschirhart, 2008; Nunes et al., 2013). Assembly of the membrane and cytoplasmic NADPH oxidase components results in electrons shuttled from NADPH to oxygen producing superoxide. Spontaneous and myeloperoxidase (MPO) catalyzed dismutation of superoxide produces hydrogen peroxide, which in the presence of MPO and chloride results in the highly bactericidal agent hypochlorous acid/hypochlorite anion (Kettle et al., 2007; Klebanoff et al., 2013). Neutrophil ingestion exposes *S. aureus* to concentrated ROS in the phagosome resulting in oxidation and chlorination of bacterial and host proteins (Green et al., 2014). Inhibition of neutrophil ROS production by diphenyleneiodonium significantly increases *S. aureus* survival following neutrophil phagocytosis even with functioning non-oxidative killing mechanisms (Hampton and Winterbourn, 1995; Hampton et al., 1996). Individuals with chronic granulomatous disease carry mutations in components of NADPH oxidase preventing formation of superoxide and hydrogen peroxide and suffer from recurring bacterial infections including *S. aureus* (Lekstrom-Himes and Gallin, 2000; Assari, 2006). Thus, uninhibited neutrophil ROS production is essential for *S. aureus* clearance.

Bacterial pathogens have developed strategies to inhibit ROS killing (Flannagan et al., 2009). *S. aureus* produces several enzymes in response to neutrophils to degrade and detoxify ROS. The ROS chain of production begins with the formation of superoxide from oxygen. *S. aureus* produces two superoxide dismutases, encoded by *sodA* and *sodM*, that convert superoxide into hydrogen peroxide and molecular oxygen (Karavolos et al., 2003). Superoxide dismutase activity assessed on nondenaturing polyacrylamide gel electrophoresis from the *S. aureus* cytoplasm shows three distinct bands consisting of SodA and SodM homodimers, and a SodA-SodM hybrid (Clements et al., 1999; Valderas and Hart, 2001). Wild-type *S. aureus* is more resistant to superoxide stress compared to isogenic *sodA*, or *sodM*, or *sodAsodM*-deficient strains (Karavolos et al., 2003). In a mouse subcutaneous model of infection, significantly higher wild-type *S. aureus* colony forming units were recovered from the site of infection compared to single or double *sod* mutant strains suggesting a role for superoxide dismutase in virulence (Karavolos et al., 2003). The expression of *sodA* and *sodM* is increased under oxidative stress and regulated by the transcriptional regulator SarA (Ballal and Manna, 2009). Interestingly, *S. aureus* uses multiple regulatory systems to respond to oxidative stress. KatA, the only catalase encoded by *S. aureus* that degrades hydrogen peroxide to water and oxygen, is co-regulated by the ferric uptake repressor (Fur) and the peroxide response regulator (Horsburgh et al., 2001b). As expected, *katA* deficient *S. aureus* is more sensitive to killing by hydrogen peroxide compared to wild-type *S. aureus* (Horsburgh et al., 2001a). Furthermore, the alkyl hydroperoxide reductase (AhpC) increases *S. aureus* resistance to the organic-hydroperoxide cumene hydroperoxide and its absence increases *S. aureus* resistance to hydrogen peroxide by a compensatory increase in *katA* expression (Cosgrove et al., 2007). However, the significance of KatA in surviving neutrophil ROS production is unclear since studies have shown no significant differences in neutrophil killing of wild-type *S. aureus* compared to an isogenic *katA* deficient strain (Cosgrove et al., 2007).

Superoxide dismutase and catalase protect *S. aureus* from ROS through enzymatic degradation. *S. aureus* also produces virulence factors that inhibit ROS killing by different mechanisms. The iconic yellow-golden pigment of *S. aureus* is a result of the antioxidant carotenoid named staphyloxanthin and synthesized by genes encoding *crtM* and *crtN* (Wieland et al., 1994; Pelz et al., 2005). The production of staphyloxanthin increases *S. aureus* resistance to killing by hydrogen peroxide and singlet oxygen (Liu et al., 2005). In

addition, staphyloxanthin increases *S. aureus* survival during exposure to neutrophil ROS; in contrast, staphyloxanthin does not confer protection during exposure to neutrophils from patients with chronic granulomatous disease or neutrophils pretreated with diphenyleneiodonium which have deficiencies in ROS production (Liu et al., 2005). Following exposure to neutrophil ROS, surviving bacteria undergo repair of oxidized proteins to maintain homeostasis. *S. aureus* encodes four methionine sulfoxide reductases that play a role in reducing oxidized methionine residues (Singh, 2003; Schöneich, 2005; Singh et al., 2015). Deletion of the methionine sulfoxide reductases *msrA1* and *msrB* increases *S. aureus* susceptibility to exogenous hydrogen peroxide and hypochlorous acid. Furthermore, a *msrA1* and *msrB* double mutant strain of *S. aureus* is more susceptible to neutrophil killing compared to wild-type (Pang et al., 2014). Finally, the SaeR/S TCS also plays a role in regulating virulence factors that decrease neutrophil hydrogen peroxide and hypochlorous acid production following *S. aureus* phagocytosis (Guerra et al., 2016). The SaeR/S-regulated virulence factors that decrease neutrophil ROS remain unknown and are an active area of research, but are independent of superoxide dismutase and catalase activity since their expression is not regulated by SaeR/S (Rogasch et al., 2006; Voyich et al., 2009; Nygaard et al., 2010; Sun et al., 2010).

Human neutrophils extrude DNA decorated with antimicrobial proteins termed neutrophil extracellular traps (NETs) to ensnare and kill bacteria as a terminal cell fate pathway that has been named NETosis and is dependent on ROS (Brinkmann et al., 2004; Fuchs et al., 2007; Galluzzi et al., 2012). Neutrophil exposure to *S. aureus* is a potent inducer of NETs (Pilsczek et al., 2010). However, *S. aureus* escapes NETs by secreting nuclease (*nuc*), an SaeR/S-regulated factor (Berends et al., 2010; Olson et al., 2013). Furthermore, the nuclease products from DNA degradation 2′-deoxyadenosine-3′-monophosphate and 2′-deoxyadenosine-5′-monophosphate are converted by the *S. aureus* exoprotein adenosine synthase (*adsA*) into 2′-deoxyadenosine (dAdo), which induces caspase-3-mediated apoptosis in macrophages (Thammavongsa et al., 2013). A murine intravenous infection model showed macrophages are unable to diffuse into kidney abscesses containing neutrophils and wild-type *S. aureus*; however, infection with a *nuc* or *adsA* deficient *S. aureus* strain allowed macrophages to efficiently infiltrate the neutrophil abscess (Thammavongsa et al., 2013).

S. AUREUS MODULATION OF NEUTROPHIL FATE

Neutrophils undergo an apoptotic differentiation program in response to bacterial pathogens to limit host damage caused by a prolonged inflammatory response (Kobayashi et al., 2003). For excellent reviews on the mechanisms of neutrophil apoptosis and how bacterial pathogens modulate neutrophil fate, we recommend (Kennedy and Deleo, 2009; Rigby and DeLeo, 2012; Kobayashi et al., 2017). *S. aureus* produces pore-forming toxins that lyse neutrophils, other leukocytes, and red blood cells and this lytic activity promotes an intense inflammatory response. While these toxins clearly play a role in lysing neutrophils and other immune cells, their role if any beyond neutrophil cytolysis is unknown. Notable exceptions include Panton-Valentine Leukocidin (PVL) and LukGH which have been shown to prime neutrophils at sublytic concentrations and promote neutrophil extracellular trap formation, respectively (Graves et al., 2012; Malachowa et al., 2013). In addition, neutrophils exposed to *S. aureus* have been shown to undergo cell death with intact phagosomes undermining the role of cytolytic toxins in *S. aureus* escape from neutrophils (Kobayashi et al., 2010). For reviews on the lytic properties of these toxins and their targets, please refer to (DuMont and Torres, 2014; Reyes-Robles and Torres, 2016). Herein, we will highlight recent studies suggesting *S. aureus* modulates neutrophil fate to disrupt proper neutrophil clearance following phagocytosis of *S. aureus* independent of lytic activity.

Following phagocytosis, neutrophils are ingested by macrophages in a process called efferocytosis to remove spent neutrophils and limit inflammation (Martin et al., 2014). *S. aureus* inhibits macrophage efferocytosis of *S. aureus* laden neutrophils by upregulating the "don't eat me" signal CD47 (Greenlee-Wacker et al., 2014). Interestingly, macrophage production of cytokines that modulate neutrophil fate was altered and inflammasome activation was reduced in response to neutrophils harboring *S. aureus* compared to *S. aureus* alone. *In vitro*, human neutrophils containing *S. aureus* do not undergo the classical apoptotic death pathway as they fail to activate caspase-3, as well as caspase-2, -8, and -9. Instead, neutrophil death in this *in vitro* model in response to *S. aureus* is dependent on receptor-interacting protein 1 (RIP-1) which is a hallmark of programmed cell death or necroptosis. Studies by Zurek et al. showed that the SaeR/S TCS plays a role in modulating neutrophil fate by inhibiting IL-8 production and NF-κB activation (Greenlee-Wacker et al., 2014; Zurek et al., 2015). Programmed neutrophil death in response to wild-type *S. aureus* was accelerated compared to exposure to a *saeR/S* deletion mutant.

In addition, ROS production is implicated in programmed neutrophil death leading to NET formation (NETosis) and as discussed above *S. aureus* inhibits neutrophil ROS production (Fuchs et al., 2007; Akong-Moore et al., 2012). Interestingly, Pilsczek et al. described a NET forming mechanism that did not require neutrophil lysis and is ROS independent. Instead, DNA-containing vesicles budding from the nuclear membrane are released while maintaining neutrophil plasma membrane integrity (Pilsczek et al., 2010). Thus, these anuclear neutrophils may still retain phagocytic and chemotactic activity, as previously observed (Malawista et al., 1989). Further studies are needed to determine if inhibition of neutrophil ROS production by *S. aureus* modulates NETosis and to better elucidate ROS independent and dependent NET formation and NETosis. However, it is clear that *S. aureus* alters the neutrophil death program through a variety of mechanisms that impact not only cellular signaling by the neutrophil but clearance via efferocytosis.

THE OTHER SIDE OF THE EQUATION: THE ABILITY OF *S. AUREUS* TO SENSE NEUTROPHILS

As reviewed above, the neutrophil has many mechanisms to sense and respond to *S. aureus* and likewise individual *S. aureus* factors have evolved to disrupt every step of the neutrophil response to the invading pathogen. However, the ability of *S. aureus* to sense the host has been underestimated. The *S. aureus* genome consists of 16 two-component systems (TCS) that sense environmental stimuli and regulate gene expression accordingly (Kawada-Matsuo et al., 2011). Of these 16 TCSs, SaeR/S is recognized as a major contributor to *S. aureus* pathogenesis and neutrophil evasion (Voyich et al., 2009; **Figure 3**). Geiger et al. was the first to demonstrate that the TCS SaeR/S contained an upstream promoter element that could be activated by human neutrophil products H_2O_2 and alpha-defensin (Geiger et al., 2008). SaeR/S target genes are differentially regulated in response to whole blood, neutrophils and neutrophil components (H_2O_2, alpha-defensin, and calprotectin; Voyich et al., 2005; Palazzolo-Ballance et al., 2007; Malachowa et al., 2011; Flack et al., 2014; Zurek et al., 2014; Cho et al., 2015). The nine amino acid extracellular loop of SaeS plays an important role in sensing different neutrophil-derived stimuli (Flack et al., 2014; Liu et al., 2015). Highly conserved residues M31, W32, and F33 on SaeS are essential to the appropriate activation of *sae*-target genes. Residue M31 is essential to the activation of *sae* regulon and plays an important role in sensing neutrophil-derived stimuli including HNP-1. Strains with mutations in aromatic residues W32 and F33 have disrupted normal basal signaling of SaeS in the absence of inducing signals, yet both mutant kinases have appropriate activation of effector genes following exposure to neutrophil-derived stimuli (Flack et al., 2014). This posits that response regulators turn-on only subsets of genes based on the host stimulus. Such observations support a unique hypothesis that emerging strains of bacteria are not more virulent because they harbor new virulence factors but are better able to sense and respond to their human hosts.

SaeR/S is the best studied for its role in sensing the neutrophil but additional studies are needed to understand just how *S. aureus* senses the neutrophil *in vivo*. Certainly other regulatory systems play a role either directly or potentially by responding to the activation of SaeR/S, however, these studies are currently limited. Considering HNP-1 is recognized early following phagocytosis, the recognition of HNP-1 is likely very important for *S. aureus* adaptation to the neutrophil environment. Interestingly, the recognition of HNP-1 by SaeS has not been demonstrated to influence the genes in *S. aureus* directly correlated with combating the antimicrobial capacity of antimicrobial peptides [e.g., *dlt* and *mprF* are regulated by the GraRS two-component system (Yang et al., 2012)] but instead

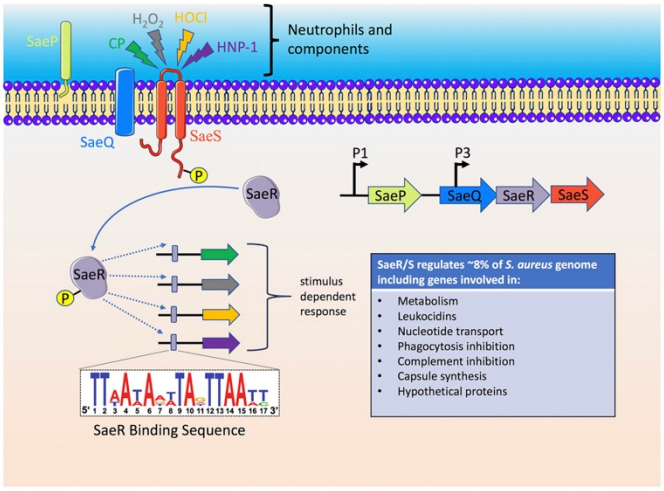


FIGURE 3 | The *S. aureus* SaeR/S two-component system senses neutrophils and neutrophil components to activate an anti-host immune response. Activation of the SaeS histidine kinase results in autophosphorylation which is subsequently transferred to the SaeR response regulator. A promoter binding region recognized by phosphorylated SaeR (SaeR binding sequence) initiates gene transcription of SaeR/S-regulated virulence factors that target neutrophil antimicrobial mechanisms, cell fate and cellular signaling. The immediate genes transcribed by SaeR/S are dependent on the stimulus, i.e., the SaeR/S transcriptional profile is dynamic and specific to the stimulus. Since bacterial sensory-regulatory systems activate multiple virulence factors, therapeutic approaches to inhibit bacterial sensing, and activation is an active area of research. CP, calprotectin.

appears to ready *S. aureus* for combating neutrophil reactive oxygen species and for producing cytolytic toxins. Additionally, the GraRS system recognizes cationic peptides including LL-37, and polymyxin B but does not recognize HNP-1 implying a complex interplay of sensory systems to combat the neutrophil (Yang et al., 2012). Another interesting two-component gene regulatory system is WalK/R. Studies have demonstrated this system is essential for *S. aureus* viability (Dubrac and Msadek, 2004; Dubrac et al., 2007). However, studies using a strain of *S. aureus* with a constitutively active WalR response regulator demonstrated constitutive expression caused up-regulation of virulence genes that are known to be SaeR-regulated including *coa*, *hla hlgA*, *hlgB*, *hlgC*, and *sbi* (Delauné et al., 2012). Although the exact mechanism of communication between WalR and SaeR/S is unknown data suggest WalR influences the SaeR/S system indirectly. This is inferred since deletion of *saeR/S* in a strain that constitutively expressed WalR showed the impact of WalR on the virulence factors during growth *in vitro* was through SaeR/S (Delauné et al., 2012). However, in mouse models of infection constitutive expression of WalR in *S. aureus* caused increased neutrophil recruitment and enhanced bacterial clearance compared to wild-type *S. aureus*. Constitutive expression of WalR decreasing virulence *in vivo* was independent of SaeR/S and linked to WalR dependent peptidoglycan release promoting an inflammatory environment that recruited neutrophils (Delaune et al). Currently the stimulus recognized by WalK is unknown. Understanding what stimulus(i) activates this system may reveal how WalK/R and SaeR/S regulation of virulence are linked. Additionally, the fatty acid kinase VfrB, that lacks any traditional DNA-binding domain, has been shown to influence gene expression of well-defined SaeR/S-dependent genes (Bose et al., 2014; Krute et al., 2017). However, when a *vfrB* mutant strain was exposed to the known SaeS-activation stimulus HNP-1, this kinase no longer impacted virulence genes regulated by SaeR/S (Krute et al., 2017). These data further emphasize the importance of defining virulence regulation in physiologically relevant environments and highlight the complexity of virulence gene regulation in *S. aureus*.

THE CHALLENGE OF INVESTIGATING NEUTROPHIL—*S. AUREUS* INTERACTIONS *IN VIVO*

Investigating the neutrophil-*S. aureus* interactions *in vivo* has been challenging. The relevance of using the murine immune system to mimic interactions between *S. aureus* and the human immune system is heavily debated (Buer and Balling, 2003; Kim et al., 2014; Montgomery et al., 2014; Reizner et al., 2014; Tseng et al., 2015). Interestingly, though HNP-1 is recognized as the most prominent trigger of *saeR/S*, murine neutrophils are deficient in its production (Inaba et al., 2010). Not only do they differ in the production of HNP-1 but notable differences are observed in chemokine production, cell trafficking, and susceptibility to secreted toxins (Johnston et al., 1990; Singer and Sansonetti, 2004; Parker and Prince, 2012; Tseng et al., 2015). Often the importance of single *S. aureus* virulence genes is highlighted in models of infection; however, canonical *S. aureus* toxins such as HlgA, HlgC, and LukF-PVL have extremely limited lytic activity on murine cells (Liu, 2009; Baba-Moussa et al., 2011; Parker and Prince, 2012; Vandenesch et al., 2012). While deficiencies in the murine model are apparent it remains the most prominent and cost effective method to explore relationships *in vivo* between *S. aureus* and the host immune system. To that end, advances are being made toward creating more effective murine models that retain benefits such as availability of working with immune-deficient lines while displaying similar disease characteristics as observed in human hosts (Montgomery et al., 2014; Tseng et al., 2015). Tseng et al. developed a "humanized" murine model wherein NOD/SCID/IL2rγ^{null} mice are engrafted with human $CD34^{+}$ umbilical cord blood cells leading to the production of human immune cells within mice (Tseng et al., 2015). These mice demonstrated susceptibility to *S. aureus* infections at concentrations similar to what is observed in humans as well as increased susceptibility to LukF-PVL. In future efforts, humanized murine models such as this may be key to understanding how *S. aureus* regulates its pathogenesis in the context of a more relevant host environment and may be critical to advance research into effective therapeutics.

POTENTIAL OF THERAPEUTICS TO AUGMENT NEUTROPHIL FUNCTION AGAINST *S. AUREUS*

Vaccine development to prevent or reduce *S. aureus* infections has not been successful (Fowler and Proctor, 2014). Antibody based vaccines targeting individual virulence factors have not conferred significant protection against *S. aureus*, and in some cases have even increased mortality (Fowler et al., 2013). As discussed above, *S. aureus* produces virulence factors that target specific neutrophil receptors to inhibit neutrophil functions. However, *S. aureus* virulence factors show redundancy in the neutrophil functions they inhibit. For example, multiple virulence factors target the complement pathway and immunoglobulin recognition to inhibit neutrophil phagocytosis and neutralizing one of these virulence factors is not likely to have a significant effect *in vivo*. Thus, vaccine approaches that simultaneously target multiple *S. aureus* virulence factors are needed and encouragingly some are in the pipeline (Torre et al., 2015; Frenck et al., 2016).

Targeting *S. aureus* sensory/regulatory systems has great potential for therapeutic and vaccine development. Theoretically, chemical inhibitors and neutralizing antibodies of sensory/regulatory systems could inhibit *S. aureus* from sensing its environment and producing virulence factors that disrupt host antimicrobial functions. The SaeR/S TCS is an attractive candidate for vaccine development since SaeR/S-regulated factors disrupt key neutrophil functions that include production of ROS, cytokine expression, and cell fate (Watkins et al., 2011, 2013; Nygaard et al., 2013; Zurek et al., 2014; Guerra et al., 2016). Many of the best studied immunomodulatory factors and cytolytic proteins that target neutrophil function are regulated by SaeR/S including Sbi, Efb, HlgABC, Hla, LukG/H (LukA/B),

and PVL (Nygaard et al., 2010). Thus, inhibiting SaeS from sensing its environment has the potential to prevent expression of multiple virulence factors in response to host signals. In this line of thinking, targeting *S. aureus* quorum sensing via inhibition of Agr has been proposed as a therapeutic approach to inhibit production of virulence of factors (Clatworthy et al., 2007; Park et al., 2007; Cegelski et al., 2008; Kaufmann et al., 2008; Njoroge and Sperandio, 2009; Rutherford and Bassler, 2012). For a review on the *S. aureus* Agr regulatory system please refer to (Painter et al., 2014). In brief, findings have identified synthetic peptides, small molecules, and antibodies that disrupt *S. aureus* Agr-dependent quorum sensing either by blocking the sensor histidine kinase AgrC from recognizing its cognate signal autoinducing peptide (AIP), by inhibiting the AgrA response regulator from binding DNA, or by antibody interference of AIP (Park et al., 2007; Kaufmann et al., 2008; Kirchdoerfer et al., 2011; Tal-Gan et al., 2013; Broderick et al., 2014; Murray et al., 2014; Sully et al., 2014). Using this approach, reductions in mouse dermonecrosis following infection with *S. aureus*, increased bacterial clearance, and reduced hemolytic activity have been reported (Tal-Gan et al., 2013; Sully et al., 2014). In addition, the development of materials with quorum sensing inhibitors to apply during a skin infection is an active area of research and showing promising results (Broderick et al., 2014; Kratochvil et al., 2017). However, studies are needed to fully elucidate the effects of inhibiting sensory/regulatory systems since this can also increase the production of negatively regulated virulence factors and the impact on cross talk between regulatory systems that might result in compensation by one system at the loss of another, is not known.

Modulation of the inflammatory response may be another attractive target for therapeutics that might increase the likelihood of an effective neutrophil response. However, additional research is needed to identify key inflammatory mediators that resolve *S. aureus* infection and to understand those that exacerbate disease. Studies have identified IL-17 as a key determinant of proper host defense during *S. aureus* cutaneous infection (Cho et al., 2010). Murphy et al. identified γδ T cells as the major source of IL-17 during peritonitis caused by *S. aureus* and identified a subset of memory γδ T cells that enhanced IL-17 production to protect the host during subsequent *S. aureus* infection (Murphy et al., 2014). Thus, immunotherapies that enhance host bactericidal functions by neutralizing detrimental inflammatory responses, increasing beneficial cytokines at the site of infection, or stimulating the expansion of protective T cell subpopulations in response to *S. aureus* show therapeutic potential. Further research is also needed to understand site-specific cytokine requirements to mount an effective host response to *S. aureus* infection.

Lastly, as discussed above, the use of mouse models has undeniably increased our understanding of *S. aureus*-host interactions. However, due to the specificity of many of the *S. aureus* virulence factors toward human cells, improved models of disease are needed like the "humanized" mouse as discussed above. With improved models and advances in understanding neutrophil-*S. aureus* interactions, perhaps future reviews will be able to document neutrophils as the clear winner in this epic immune battle.

In conclusion, the ability of *S. aureus* to survive a neutrophil encounter is thought to contribute significantly to the virulence of this pathogen. This is exemplified by the observed increase in susceptibility to *S. aureus* infections in individuals suffering from defects that alter normal neutrophil function, such as chronic granulomatous disease, leukocyte adhesion deficiency, and neutropenia (Bodey et al., 1966; Pincus et al., 1976; Dale et al., 1979; Lekstrom-Himes and Gallin, 2000). It is clear that neutrophil antimicrobial activity is essential to eliminate *S. aureus*. However, studies have also demonstrated that increased neutrophil numbers at the site of infection can exacerbate disease (Gresham et al., 2000; McLoughlin et al., 2008) and that modulation of the neutrophil inflammatory response can significantly impact outcome of infection (Watkins et al., 2013; Zurek et al., 2015). Clearly more studies are needed to define the neutrophil responses that resolve a *S. aureus* infection. Finding the right balance of controlling inflammation while maintaining an effective neutrophil antimicrobial response will be key to the design of an effective therapeutic.

AUTHOR CONTRIBUTIONS

FG and JV wrote, edited, prepared manuscript. TB, DP, and ES wrote sections and contributed equally.

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REFERENCES

Akong-Moore, K., Chow, O. A., Von Kö Ckritz-Blickwede, M., Nizet, V., Wehkamp, J., and Fischer-Bosch, M. (2012). Influences of chloride and hypochlorite on neutrophil extracellular trap formation. *PLoS ONE* 7:e42984. doi: 10.1371/journal.pone.0042984

Assari, T. (2006). Chronic granulomatous disease; fundamental stages in our understanding of CGD. *Med. Immunol.* 5:4. doi: 10.1186/1476-9433-5-4

Baba-Moussa, L., Sina, H., Scheftel, J. M., Moreau, B., Sainte-Marie, D., Kotchoni, S. O., et al. (2011). Staphylococcal Panton-Valentine leucocidin as a major virulence factor associated to furuncles. *PLoS ONE* 6:e25716. doi: 10.1371/journal.pone.0025716

Bae, Y.-S., Park, J. C., He, R., Ye, R. D., Kwak, J.-Y., Suh, P.-G., et al. (2003). Differential signaling of formyl peptide receptor-like 1 by Trp-Lys-Tyr-Met-Val-Met-CONH2 or lipoxin A4 in human neutrophils. *Mol. Pharmacol.* 64, 721–730. doi: 10.1124/mol.64.3.721

Baggiolini, M., Walz, A., and Kunkel, S. L. (1989). Neutrophil-activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils. *J. Clin. Invest.* 84, 1045–1049. doi: 10.1172/JCI114265

Ballal, A., and Manna, A. C. (2009). Regulation of superoxide dismutase (sod) genes by SarA in *Staphylococcus aureus*. *J. Bacteriol.* 191, 3301–3310. doi: 10.1128/JB.01496-08

Bardoel, B. W., Vos, R., Bouman, T., Aerts, P. C., Bestebroer, J., Huizinga, E. G., et al. (2012). Evasion of Toll-like receptor 2 activation by staphylococcal superantigen-like protein 3. *J. Mol. Med.* 90, 1109–1120. doi: 10.1007/s00109-012-0926-8

Bates, E. J., Ferrante, A, and Beard, L. J. (1991). Characterization of the major neutrophil-stimulating activity present in culture medium conditioned by *Staphylococcus aureus*-stimulated mononuclear leucocytes. *Immunology* 72, 448–450.

Ben-Baruch, A., Grimm, M., Bengali, K., Evans, G. A., Chertov, O., Wang, J. M., et al. (1997). The differential ability of IL-8 and neutrophil-activating peptide-2 to induce attenuation of chemotaxis is mediated by their divergent capabilities to phosphorylate CXCR2 (IL-8 receptor B). *J. Immunol.* 158, 5927–5933.

Berends, E. T., Horswill, A. R., Haste, N. M., Monestier, M., Nizet, V., and Von Köckritz-Blickwede, M. (2010). Nuclease expression by *Staphylococcus aureus* facilitates escape from neutrophil extracellular traps. *J. Innate Immun.* 2, 576–586. doi: 10.1159/000319909

Bestebroer, J., Aerts, P. C., Rooijakkers, S. H., Pandey, M. K., Köhl, J., Van Strijp, J. A. G., et al. (2010). Functional basis for complement evasion by staphylococcal superantigen-like 7. *Cell. Microbiol.* 12, 1506–1516. doi: 10.1111/j.1462-5822.2010.01486.x

Bestebroer, J., Poppelier, M. J., Ulfman, L. H., Lenting, P. J., Denis, C. V., Van Kessel, K. P. M., et al. (2007). Staphylococcal superantigen-like 5 binds PSGL-1 and inhibits P-selectin-mediated neutrophil rolling. *Blood* 109, 2936–2943. doi: 10.1182/blood-2006-06-015461

Bestebroer, J., Van Kessel, K. P., Azouagh, H., Walenkamp, A. M., Boer, I. G. J., Romijn, R. A., et al. (2009). Staphylococcal SSL5 inhibits leukocyte activation by chemokines and anaphylatoxins. *Blood* 113, 328–337. doi: 10.1182/blood-2008-04-153882

Bodey, G. P., Buckley, M., Sathe, Y. S., and Freireich, E. J. (1966). Quantitative relationships between circulating leukocytes and infection in patients with acute leukemia. *Ann. Intern. Med.* 64, 328–340. doi: 10.7326/0003-4819-64-2-328

Bose, J. L., Daly, S. M., Hall, P. R., and Bayles, K. W. (2014). Identification of the *Staphylococcus aureus* vfrAB Operon, a novel virulence factor regulatory locus. *Infect. Immun.* 82, 1813–1822. doi: 10.1128/IAI.01655-13

Bréchard, S., and Tschirhart, E. J. (2008). Regulation of superoxide production in neutrophils: role of calcium influx. *J. Leukoc. Biol.* 84, 1223–1237. doi: 10.1189/jlb.0807553

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., et al. (2004). Neutrophil extracellular traps kill bacteria. *Science* 303, 1532–1535. doi: 10.1126/science.1092385

Broderick, A. H., Stacy, D. M., Tal-Gan, Y., Kratochvil, M. J., Blackwell, H. E., and Lynn, D. M. (2014). Surface coatings that promote rapid release of peptide-based AgrC inhibitors for attenuation of quorum sensing in *Staphylococcus aureus*. *Adv. Healthc. Mater.* 3, 97–105. doi: 10.1002/adhm.201300119

Buer, J., and Balling, R. (2003). Mice, microbes and models of infection. *Nat. Rev. Genet.* 4, 195–205. doi: 10.1038/nrg1019

Burman, J. D., Leung, E., Atkins, K. L., O'Seaghdha, M. N., Lango, L., Bernado, P., et al. (2008). Interaction of human complement with Sbi, a staphylococcal immunoglobulin-binding protein: Indications of a novel mechanism of complement evasion by *Staphylococcus aureus*. *J. Biol. Chem.* 283, 17579–17593. doi: 10.1074/jbc.M800265200

Carrel, M., Perencevich, E. N., and David, M. Z. (2015). USA300 Methicillin-Resistant *Staphylococcus aureus*, United States, 2000-2013. *Emerging Infect. Dis.* 21, 1973–1980. doi: 10.3201/eid2111.150452

Cegelski, L., Marshall, G. R., Eldridge, G. R., and Hultgren, S. J. (2008). The biology and future prospects of antivirulence therapies. *Nat. Rev. Microbiol.* 6, 17–27. doi: 10.1038/nrmicro1818

Chiang, N., Fierro, I. M., Gronert, K., and Serhan, C. N. (2000). Activation of lipoxin A(4) receptors by aspirin-triggered lipoxins and select peptides evokes ligand-specific responses in inflammation. *J. Exp. Med.* 191, 1197–1208. doi: 10.1084/jem.191.7.1197

Cho, H., Jeong, D.-W., Liu, Q., Yeo, W. S., Vogl, T., Skaar, E. P., et al. (2015). Calprotectin increases the activity of the SaeRS two component system and murine mortality during *Staphylococcus aureus* infections. *PLoS Pathog.* 11:e1005026. doi: 10.1371/journal.ppat.1005026

Cho, J. S., Pietras, E. M., Garcia, N. C., Ramos, R. I., Farzam, D. M., Monroe, H. R., et al. (2010). IL-17 is essential for host defense against cutaneous *Staphylococcus aureus* infection in mice. *J. Clin. Invest.* 120, 1762–1773. doi: 10.1172/JCI 40891

Christophe, T., Karlsson, A., Dugave, C., Rabiet, M. J., Boulay, F., and Dahlgren, C. (2001). The synthetic peptide Trp-Lys-Tyr-Met-Val-Met-NH2 specifically activates neutrophils through FPRL1/Lipoxin A4 receptors and is an agonist for the orphan monocyte-expressed chemoattractant receptor FPRL2. *J. Biol. Chem.* 276, 21585–21593. doi: 10.1074/jbc.M007769200

Clarke, T. B., Davis, K. M., Lysenko, E. S., Zhou, A. Y., Yu, Y., and Weiser, J. N. (2010). Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. *Nat. Med.* 16, 228–231. doi: 10.1038/nm.2087

Clatworthy, A. E., Pierson, E., and Hung, D. T. (2007). Targeting virulence: a new paradigm for antimicrobial therapy. *Nat. Chem. Biol.* 3, 541–548. doi: 10.1038/nchembio.2007.24

Clements, M. O., Watson, S. P., and Foster, S. J. (1999). Characterization of the major superoxide dismutase of *Staphylococcus aureus* and its role in starvation survival, stress resistance, and pathogenicity. *J. Bacteriol.* 181, 3898–3903.

Colin, D. A., and Monteil, H. (2003). Control of the oxidative burst of human neutrophils by Staphylococcal Leukotoxins control of the oxidative burst of human neutrophils by Staphylococcal Leukotoxins. *Infect. Immun.* 71, 3724–3729. doi: 10.1128/IAI.71.7.3724-3729.2003

Collins, L. V., Kristian, S. A., Weidenmaier, C., Faigle, M., Van Kessel, K. P., Van Strijp, J. A. G., et al. (2002). *Staphylococcus aureus* strains lacking D-alanine modifications of teichoic acids are highly susceptible to human neutrophil killing and are virulence attenuated in mice. *J. Infect. Dis.* 186, 214–219. doi: 10.1086/341454

Cosgrove, K., Coutts, G., Jonsson, I.-M., Tarkowski, A., Kokai-Kun, J. F., Mond, J. J., et al. (2007). Catalase (KatA) and alkyl hydroperoxide reductase (AhpC) have compensatory roles in peroxide stress resistance and are required for survival, persistence, and nasal colonization in *Staphylococcus aureus*. *J. Bacteriol.* 189, 1025–1035. doi: 10.1128/JB.01524-06

Dale, D. C., Guerry, D., Wewerka, J. R., Bull, J. M., and Chusid, M. J. (1979). Chronic neutropenia. *Medicine* 58, 128–144. doi: 10.1097/00005792-197903000-00002

De Haas, C. J. C., Veldkamp, K. E., Peschel, A., Weerkamp, F., Van Wamel, W. J., Heezius, E. C., et al. (2004). Chemotaxis Inhibitory Protein of *Staphylococcus aureus*, a Bacterial Antiinflammatory Agent. *J. Exp. Med.* 3687900, 687–695. doi: 10.1084/jem.20031636

de Oliveira, S., Rosowski, E. E., and Huttenlocher, A. (2016). Neutrophil migration in infection and wound repair: going forward in reverse. *Nat. Rev. Immunol.* 16, 378–391. doi: 10.1038/nri.2016.49

De Yang, Chen, Q., Schmidt, A. P., Anderson, G. M., Wang, J. M., Wooters, J., et al. (2000). LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. *J. Exp. Med.* 192, 1069–1074. doi: 10.1084/jem.192.7.1069

Delauné, A., Dubrac, S., Blanchet, C., Poupel, O., Mäder, U., Hiron, A., et al. (2012). The WalKR system controls major staphylococcal virulence genes and is involved in triggering the host inflammatory response. *Infect. Immun.* 80, 3438–3453. doi: 10.1128/IAI.00195-12

DeLeo, F. R., Allen, L. A., Apicella, M., and Nauseef, W. M. (1999). NADPH oxidase activation and assembly during phagocytosis. *J. Immunol.* 163, 6732–6740.

DeLeo, F. R., and Quinn, M. T. (1996). Assembly of the phagocyte NADPH oxidase: molecular interaction of oxidase proteins. *J. Leukoc. Biol.* 60, 677–691.

Dossett, J. H., Kronvall, G., Williams, R. C., and Quie, P. G. (1969). Antiphagocytic effects of staphylococcal protein A. *J. Immunol.* 103, 1405–1410.

Dragneva, Y., Anuradha, C. D., Valeva, A., Hoffmann, A., Bhakdi, S., and Husmann, M. (2001). Subcytocidal attack by staphylococcal alpha-toxin

activates NF-kappaB and induces interleukin-8 production. *Infect. Immun.* 69, 2630–2635. doi: 10.1128/IAI.69.4.2630-2635.2001

Dubrac, S., and Msadek, T. (2004). Identification of genes controlled by the essential YycG / YycF two-component system of *Staphylococcus aureus*. *J. Bacteriol.* 186, 1175–1181. doi: 10.1128/JB.186.4.1175-1181.2004

Dubrac, S., Boneca, I. G., Poupel, O., and Msadek, T. (2007). New insights into the WalK/WalR (YycG/YycF) essential signal transduction pathway reveal a major role in controlling cell wall metabolism and biofilm formation in *Staphylococcus aureus*. *J. Bacteriol.* 189, 8257–8269. doi: 10.1128/JB.00645-07

DuMont, A. L., and Torres, V. J. (2014). Cell targeting by the *Staphylococcus aureus* pore-forming toxins: It's not just about lipids. *Trends Microbiol.* 22, 21–27. doi: 10.1016/j.tim.2013.10.004

Edwards, S. W., Say, J. E., and Hughes, V. (1988). Gamma interferon enhances the killing of *Staphylococcus aureus* by human neutrophils. *J. Gen. Microbiol.* 134, 37–42. doi: 10.1099/00221287-134-1-37

El-Benna, J., Dang, P. M., and Gougerot-Pocidalo, M. A. (2008). Priming of the neutrophil NADPH oxidase activation: role of p47phox phosphorylation and NOX2 mobilization to the plasma membrane. *Semin. Immunopathol.* 30, 279–289. doi: 10.1007/s00281-008-0118-3

Elbim, C., Bailly, S., Chollet-Martin, S., Hakim, J., and Gougerot-Pocidalo, M. A. (1994). Differential priming effects of proinflammatory cytokines on human neutrophil oxidative burst in response to bacterial N-formyl peptides. *Infect. Immun.* 62, 2195–201.

Ellis, T. N., and Beaman, B. L. (2004). Interferon-gamma activation of polymorphonuclear neutrophil function. *Immunology* 112, 2–12. doi: 10.1111/j.1365-2567.2004.01849.x

Ernst, C. M., Staubitz, P., Mishra, N. N., Yang, S. J., Hornig, G., Kalbacher, H., et al. (2009). The bacterial defensin resistance protein MprF consists of separable domains for lipid lysinylation and antimicrobial peptide repulsion. *PLoS Pathog.* 5, 1–9. doi: 10.1371/journal.ppat.1000660

Falk, W., Harvath, L., and Leonard, E. J. (1982). Only the chemotactic subpopulation of human blood monocytes expresses receptors for the chemotactic peptide N-formylmethionyl-leucyl-phenylalanine. *Infect. Immun.* 36, 450–454.

Falugi, F., Kim, H. K., and Missiakas, D. M. (2013). Role of Protein A in the evasion of host adaptive immune responses. *MBio* 4, 1–9. doi: 10.1128/mBio.00575-13

Ferrante, A., Harvey, D. P., and Bates, E. J. (1989). *Staphylococcus aureus*-stimulated mononuclear leucocyte-conditioned medium increases the neutrophil bactericidal activity, and augments oxygen radical production and degranulation in response to the bacteria. *Clin. Exp. Immunol.* 78, 366–371.

Ferrante, A., Martin, A. J., Bates, E. J., Goh, D. H., Harvey, D. P., Parsons, D., et al. (1993). Killing of *Staphylococcus aureus* by tumor necrosis factor-alpha-activated neutrophils. The role of serum opsonins, integrin receptors, respiratory burst, and degranulation. *J. Immunol.* 151, 4821–4828.

Fevre, C., Bestebroer, J., Mebius, M. M., de Haas, C. J., van Strijp, J. A. G., Fitzgerald, J. R., et al. (2014). *Staphylococcus aureus* proteins SSL6 and SElX interact with neutrophil receptors as identified using secretome phage display. *Cell. Microbiol.* 16, 1646–1665. doi: 10.1111/cmi.12313

Fiore, S., Maddox, J. F., Perez, H. D., and Serhan, C. N. (1994). Identification of a human cDNA encoding a functional high affinity lipoxin A4 receptor. *J. Exp. Med.* 180, 253–260. doi: 10.1084/jem.180.1.253

Flack, C. E., Zurek, O. W., Meishery, D. D., Pallister, K. B., Malone, C. L., Horswill, A. R., et al. (2014). Differential regulation of staphylococcal virulence by the sensor kinase SaeS in response to neutrophil-derived stimuli. *Proc. Natl. Acad. Sci. U.S.A.* 111, E2037–E2045. doi: 10.1073/pnas.1322125111

Flannagan, R. S., Cosío, G., and Grinstein, S. (2009). Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. *Nat. Rev. Microbiol.* 7, 355–366. doi: 10.1038/nrmicro2128

Forsgren, A., and Sjöquist, J. (1966). "Protein A" from *S. aureus*. I. Pseudo-immune reaction with human gamma-globulin. *J. Immunol.* 97, 822–827.

Fournier, B., and Philpott, D. J. (2005). Recognition of *Staphylococcus aureus* by the innate immune system. *Clin. Microbiol. Rev.* 18, 521–540. doi: 10.1128/CMR.18.3.521-540.2005

Fowler, V. G., Allen, K. B., Moreira, E. D., Moustafa, M., Isgro, F., Boucher, H. W., et al. (2013). Effect of an investigational vaccine for preventing *Staphylococcus aureus* infections after cardiothoracic surgery: a randomized trial. *JAMA* 309, 1368–1378. doi: 10.1001/jama.2013.3010

Fowler, V. G., and Proctor, R. A. (2014). Where does a *Staphylococcus aureus* vaccine stand? *Clin. Microbiol. Infect.* 20, 66–75. doi: 10.1111/1469-0691.12570

Frenck, R. W., Buddy Creech, C., Sheldon, E. A., Seiden, D. J., Kankam, M. K., Baber, J., et al. (2016). Safety, tolerability, and immunogenicity of a 4-antigen *Staphylococcus aureus* vaccine (SA4Ag): results from a first-in-human randomised, placebo-controlled phase 1/2 study. *Vaccine* 35, 375–384. doi: 10.1016/j.vaccine.2016.11.010

Fuchs, T. A., Abed, U., Goosmann, C., Hurwitz, R., Schulze, I., Wahn, V., et al. (2007). Novel cell death program leads to neutrophil extracellular traps. *J. Cell Biol.* 176, 231–241. doi: 10.1083/jcb.200606027

Gallin, J. I., Malech, H. L., Melnick, D. A., Weening, R. S., and Roos, D. (1991). A controlled trial of interferon gamma to prevent infection in chronic granulomatous disease. The International Chronic Granulomatous Disease Cooperative Study Group. *N. Engl. J. Med.* 324, 509–516. doi: 10.1056/NEJM199102213240801

Galluzzi, L., Vitale, I., Abrams, J. M., Alnemri, E. S., Baehrecke, E. H., Blagosklonny, M. V., et al. (2012). Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. *Cell Death Differ.* 19, 107–120. doi: 10.1038/cdd.2011.96

Garcia, B. L., Ramyar, K. X., Tzekou, A., Ricklin, D., McWhorter, W. J., Lambris, J. D., et al. (2010). Molecular basis for complement recognition and inhibition determined by crystallographic studies of the Staphylococcal Complement Inhibitor (SCIN) Bound to C3c and C3b. *J. Mol. Biol.* 402, 17–29. doi: 10.1016/j.jmb.2010.07.029

Geiger, T., Goerke, C., Mainiero, M., Kraus, D., and Wolz, C. (2008). The virulence regulator sae of *Staphylococcus aureus*: promoter activities and response to phagocytosis-related signals. *J. Bacteriol.* 190, 3419–3428. doi: 10.1128/JB.01927-07

Gonzalez, C. D., Ledo, C., Giai, C., Garófalo, A., and Gómez, M. I. (2015). The Sbi protein contributes to *Staphylococcus aureus* inflammatory response during systemic infection. *PLoS ONE* 10:e0131879. doi: 10.1371/journal.pone.0131879

González-Zorn, B., Senna, J. P., Fiette, L., Shorte, S., Testard, A., Chignard, M., et al. (2005). Bacterial and host factors implicated in nasal carriage of methicillin-resistant *Staphylococcus aureus* in mice. *Infect. Immun.* 73, 1847–1851. doi: 10.1128/IAI.73.3.1847-1851.2005

Gorwitz, R. J., Kruszon-Moran, D., McAllister, S. K., McQuillan, G., McDougal, L. K., Fosheim, G. E., et al. (2008). Changes in the prevalence of nasal colonization with *Staphylococcus aureus* in the United States, 2001-2004. *J. Infect. Dis.* 197, 1226–1234. doi: 10.1086/533494

Graves, S. F., Kobayashi, S. D., Braughton, K. R., Whitney, A. R., and Sturdevant, D. E., Rasmussen, D. L., et al. (2012). Sublytic concentrations of *Staphylococcus aureus* Panton-Valentine leukocidin alter human PMN gene expression and enhance bactericidal capacity. *J. Leukoc. Biol.* 92, 361–374. doi: 10.1189/jlb.1111575

Green, J. N., Kettle, A. J., and Winterbourn, C. C. (2014). Protein chlorination in neutrophil phagosomes and correlation with bacterial killing. *Free Radic. Biol. Med.* 77, 49–56. doi: 10.1016/j.freeradbiomed.2014.08.013

Greenlee-Wacker, M. C., Rigby, K. M., Kobayashi, S. D., Porter, A. R., DeLeo, F. R., and Nauseef, W. M. (2014). Phagocytosis of *Staphylococcus aureus* by human neutrophils prevents macrophage efferocytosis and induces programmed necrosis. *J. Immunol.* 192, 4709–4717. doi: 10.4049/jimmunol.1302692

Gresham, H. D., Lowrance, J. H., Caver, T. E., Wilson, B. S., Cheung, A. L., and Lindberg, F. P. (2000). Survival of *Staphylococcus aureus* inside neutrophils contributes to infection. *J. Immunol.* 164, 3713–3722. doi: 10.4049/jimmunol.164.7.3713

Guerra, F. E., Addison, C. B., de Jong, N. W., Azzolino, J., Pallister, K. B., van Strijp, J. A. G., et al. (2016). *Staphylococcus aureus* SaeR/S-regulated factors reduce human neutrophil reactive oxygen species production. *J. Leukoc. Biol.* 100, 1–6. doi: 10.1189/jlb.4VMAB0316-100RR

Guyer, D. A., Moore, K. L., Lynam, E. B., Schammel, C. M., Rogelj, S., McEver, R. P., et al. (1996). P-selectin glycoprotein ligand-1 (PSGL-1) is a ligand for L-selectin in neutrophil aggregation. *Blood* 88, 2415–2421.

Haas, P. J., De Haas, C. J., Poppelier, M. J., Van Kessel, K. P., Van Strijp, J. A., Dijkstra, K., et al. (2005). The structure of the C5a receptor-blocking domain of chemotaxis inhibitory protein of *Staphylococcus aureus* is related

to a group of immune evasive molecules. *J. Mol. Biol.* 353, 859–872. doi: 10.1016/j.jmb.2005.09.014

Hampton, M. B., and Winterbourn, C. C. (1995). Modification of neutrophil oxidant production with diphenyleneiodonium and its effect on bacterial killing. *Free Radic. Biol. Med.* 18, 633–639. doi: 10.1016/0891-5849(94)00181-I

Hampton, M., Kettle, A., and Winterbourn, C. (1996). Involvement of superoxide and myeloperoxidase in oxygen-dependent killing of *Staphylococcus aureus* by neutrophils. *Infect. Immun.* 64, 3512–3517.

Harraghy, N., Kormanec, J., Wolz, C., Homerova, D., Goerke, C., Ohlsen, K., et al. (2005). sae is essential for expression of the staphylococcal adhesins Eap and Emp. *Microbiology* 151, 1789–1800. doi: 10.1099/mic.0.27902-0

Hidalgo, A., Peired, A. J., Wild, M. K., Vestweber, D., and Frenette, P. S. (2007). Complete identification of E-Selectin Ligands on neutrophils reveals distinct functions of PSGL-1, ESL-1, and CD44. *Immunity* 26, 477–489. doi: 10.1016/j.immuni.2007.03.011

Hoebe, K., Georgel, P., Rutschmann, S., Du, X., Mudd, S., Crozat, K., et al. (2005). CD36 is a sensor of diacylglycerides. *Nature* 433, 523–527. doi: 10.1038/nature03253

Horsburgh, M. J., Clements, M. O., Crossley, H., Ingham, E., and Foster, S. J. (2001a). PerR controls oxidative stress resistance and iron storage proteins and is required for virulence in *Staphylococcus aureus*. *Infect. Immun.* 69, 3744–3754. doi: 10.1128/IAI.69.6.3744-3754.2001

Horsburgh, M. J., Ingham, E., and Foster, S. J. (2001b). In *Staphylococcus aureus*, fur is an interactive regulator with PerR, contributes to virulence, and Is necessary for oxidative stress resistance through positive regulation of catalase and iron homeostasis. *J. Bacteriol.* 183, 468–475. doi: 10.1128/JB.183.2.468-475.2001

Huey, R., and Hugli, T. E. (1985). Characterization of a C5a receptor on human polymorphonuclear leukocytes (PMN). *J. Immunol.* 135, 2063–2068.

Huo, Y., and Xia, L. (2009). P-selectin Glycoprotein Ligand-1 Plays a crucial role in the selective recruitment of Leukocytes into the Atherosclerotic arterial wall. *Trends Cardiovasc. Med.* 19, 140–145. doi: 10.1016/j.tcm.2009.07.006

Inaba, Y., Ashida, T., Ito, T., Ishikawa, C., Tanabe, H., Maemoto, A., et al. (2010). Expression of the antimicrobial peptide alpha-defensin/cryptdins in intestinal crypts decreases at the initial phase of intestinal inflammation in a model of inflammatory bowel disease, IL-10-deficient mice. *Inflamm. Bowel Dis.* 16, 1488–1495. doi: 10.1002/ibd.21253

Itoh, S., Hamada, E., Kamoshida, G., Yokoyama, R., Takii, T., Onozaki, K., et al. (2010). Staphylococcal superantigen-like protein 10 (SSL10) binds to human immunoglobulin G (IgG) and inhibits complement activation via the classical pathway. *Mol. Immunol.* 47, 932–938. doi: 10.1016/j.molimm.2009.09.027

Jacobsson, K., and Frykberg, L. (1995). Cloning of ligand-binding domains of bacterial receptors by phage display. *Biotechniques* 18, 878–885.

Jin, T., Bokarewa, M., Foster, T., Mitchell, J., Higgins, J., and Tarkowski, A. (2004). *Staphylococcus aureus* resists human defensins by production of staphylokinase, a novel bacterial evasion mechanism. *J. Immunol.* 172, 1169–1176. doi: 10.4049/jimmunol.172.2.1169

Johnston, S. C., Dustin, M. L., Hibbs, M. L., and Springer, T. A. (1990). On the species specificity of the interaction of LFA-1 with intercellular adhesion molecules. *J. Immunol.* 145, 1181–1187.

Karavolos, M. H., Horsburgh, M. J., Ingham, E., and Foster, S. J. (2003). Role and regulation of the superoxide dismutases of *Staphylococcus aureus*. *Microbiology* 149, 2749–2758. doi: 10.1099/mic.0.26353-0

Kaufmann, G. F., Park, J., and Janda, K. D. (2008). Bacterial quorum sensing: a new target for anti-infective immunotherapy. *Expert Opin. Biol. Ther.* 8, 719–724. doi: 10.1517/14712598.8.6.719

Kawada-Matsuo, M., Yoshida, Y., Nakamura, N., and Komatsuzawa, H. (2011). Role of two-component systems in the resistance of *Staphylococcus aureus* to antibacterial agents. *Virulence* 2, 427–430. doi: 10.4161/viru.2.5.17711

Kennedy, A. D., and Deleo, F. R. (2009). Neutrophil apoptosis and the resolution of infection. *Immunol. Res.* 43, 25–61. doi: 10.1007/s12026-008-8049-6

Kettle, A. J., Anderson, R. F., Hampton, M. B., and Winterbourn, C. C. (2007). Reactions of superoxide with myeloperoxidase. *Biochemistry* 46, 4888–4897. doi: 10.1021/bi602587k

Kim, H. K., Falugi, F., Missiakas, D. M., and Schneewind, O. (2016). Peptidoglycan-linked protein A promotes T cell-dependent antibody expansion during *Staphylococcus aureus* infection. *Proc. Natl. Acad. Sci. U.S.A.* 113, 5718–5723. doi: 10.1073/pnas.1524267113

Kim, H. K., Missiakas, D., and Schneewind, O. (2014). Mouse models for infectious diseases caused by *Staphylococcus aureus*. *J. Immunol. Methods* 410, 88–99. doi: 10.1016/j.jim.2014.04.007

King, B. F., and Wilkinson, B. J. (1981). Binding of human immunoglobulin G to protein A in encapsulated *Staphylococcus aureus*. *Infect. Immun.* 33, 666–672.

Kirchdoerfer, R. N., Garner, A. L., Flack, C. E., Mee, J. M., Horswill, A. R., Janda, K. D., et al. (2011). Structural basis for ligand recognition and discrimination of a quorum-quenching antibody. *J. Biol. Chem.* 286, 17351–17358. doi: 10.1074/jbc.M111.231258

Klebanoff, S. J., Kettle, A. J., Rosen, H., Winterbourn, C. C., and Nauseef, W. M. (2013). Myeloperoxidase: a front-line defender against phagocytosed microorganisms. *J. Leukoc. Biol.* 93, 185–198. doi: 10.1189/jlb.0712349

Klevens, R. M., Morrison, M. A., Nadle, J., Petit, S., Gershman, K., Ray, S., et al. (2007). Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *JAMA* 298, 1763–1771. doi: 10.1001/jama.298.15.1763

Knuefermann, P., Sakata, Y., Baker, J. S., Huang, C. H., Sekiguchi, K., Hardarson, H. S., et al. (2004). Toll-like receptor 2 mediates *Staphylococcus aureus*-induced myocardial dysfunction and cytokine production in the heart. *Circulation* 110, 3693–3698. doi: 10.1161/01.CIR.0000143081.13042.04

Ko, Y. P., Kuipers, A., Freitag, C. M., Jongerius, I., Medina, E., van Rooijen, W. J., et al. (2013). Phagocytosis Escape by a *Staphylococcus aureus* protein that connects complement and coagulation proteins at the bacterial surface. *PLoS Pathog.* 9:e1003816. doi: 10.1371/journal.ppat.1003816

Kobayashi, S. D., Braughton, K. R., Palazzolo-Ballance, A. M., Kennedy, A. D., Sampaio, E., Kristosturyan, E., et al. (2010). Rapid neutrophil destruction following phagocytosis of *Staphylococcus aureus*. *J. Innate Immun.* 2, 560–575. doi: 10.1159/000317134

Kobayashi, S. D., Braughton, K. R., Whitney, A. R., Voyich, J. M., Schwan, T. G., Musser, J. M., et al. (2003). Bacterial pathogens modulate an apoptosis differentiation program in human neutrophils. *Proc. Natl. Acad. Sci. U.S.A.* 100, 10948–10953. doi: 10.1073/pnas.1833375100

Kobayashi, S. D., Malachowa, N., and DeLeo, F. R. (2015). Pathogenesis of *Staphylococcus aureus* abscesses. *Am. J. Pathol.* 185, 1518–1527. doi: 10.1016/j.ajpath.2014.11.030

Kobayashi, S. D., Malachowa, N., and DeLeo, F. R. (2017). Influence of microbes on neutrophil life and death. *Front. Cell. Infect.* Microbiol. 7:159. doi: 10.3389/fcimb.2017.00159

Kolaczkowska, E., and Kubes, P. (2013). Neutrophil recruitment and function in health and inflammation. *Nat. Rev. Immunol.* 13, 159–175. doi: 10.1038/nri3399

König, B., Prévost, G., Piémont, Y., and König, W. (1995). Effects of *Staphylococcus aureus* leukocidins on inflammatory mediator release from human granulocytes. *J. Infect. Dis.* 171, 607–613. doi: 10.1093/infdis/171.3.607

Koymans, K. J., Feitsma, L. J., Brondijk, T. H., Aerts, P. C., Lukkien, E., Lossl, P., et al. (2015). Structural basis for inhibition of TLR2 by staphylococcal superantigen-like protein 3 (SSL3). *Proc. Natl. Acad. Sci. U.S.A.* 112, 11018–11023. doi: 10.1073/pnas.1502026112

Kratochvil, M. J., Yang, T., Blackwell, H. E., and Lynn, D. M. (2017). Nonwoven polymer nanofiber coatings that inhibit quorum sensing in *Staphylococcus aureus*: toward new nonbactericidal approaches to infection control. *ACS Infect. Dis.* 3, 271–280. doi: 10.1021/acsinfecdis.6b00173

Kronvall, G., Grey, H. M., and Williams, R. C. (1970). Protein A reactivity with mouse immunoglobulins. Structural relationship between some mouse and human immunoglobulins. *J. Immunol.* 105, 1116–1123.

Krute, C. N., Rice, K. C., and Bose, J. L. (2017). VfrB is a key activator of the *Staphylococcus aureus* SaeRS two-component system. *J. Bacteriol.* 199, e00828–16. doi: 10.1128/JB.00828-16

Kurokawa, K., Lee, H., Roh, K. B., Asanuma, M., Kim, Y. S., Nakayama, H., et al. (2009). The triacylated ATP binding cluster transporter substrate-binding lipoprotein of *Staphylococcus aureus* functions as a native ligand for toll-like receptor 2. *J. Biol. Chem.* 284, 8406–8411. doi: 10.1074/jbc.M809618200

Laarman, A. J., Mijnheer, G., Mootz, J. M., van Rooijen, W. J., Ruyken, M., Malone, C. L., et al. (2012). *Staphylococcus aureus* Staphopain A inhibits CXCR2-dependent neutrophil activation and chemotaxis. *EMBO J.* 31, 3607–3619. doi: 10.1038/emboj.2012.212

Lambris, J. D., Ricklin, D., and Geisbrecht, B. V. (2008). Complement evasion by human pathogens. *Nat. Rev. Microbiol.* 6, 132–142. doi: 10.1038/nrmicro1824

Langley, R., Wines, B., Willoughby, N., Basu, I., Proft, T., and Fraser, J. D. (2005). The Staphylococcal Superantigen-Like Protein 7 Binds IgA and Complement C5 and Inhibits IgA-Fc RI binding and serum killing of bacteria. *J. Immunol.* 174, 2926–2933. doi: 10.4049/jimmunol.174.5.2926

Laursen, N. S., Gordon, N., Hermans, S., Lorenz, N., Jackson, N., Wines, B., et al. (2010). Structural basis for inhibition of complement C5 by the SSL7 protein from *Staphylococcus aureus*. *Proc. Natl. Acad. Sci. U.S.A.* 107, 3681–3686. doi: 10.1073/pnas.0910565107

Le, Y., Yazawa, H., Gong, W., Yu, Z., Ferrans, V. J., Murphy, P. M., et al. (2001). The neurotoxic prion peptide fragment PrP(106-126) is a chemotactic agonist for the G protein-coupled receptor formyl peptide receptor-like 1. *J. Immunol.* 166, 1448–1451. doi: 10.4049/jimmunol.166.3.1448

Lee, L. Y., Höök, M., Haviland, D., Wetsel, R. A., Yonter, E. O., Syribeys, P., et al. (2004). Inhibition of complement activation by a secreted *Staphylococcus aureus* Protein. *J. Infect. Dis.* 190, 571–579. doi: 10.1086/422259

Lekstrom-Himes, J. A., and Gallin, J. I. (2000). Immunodeficiency diseases caused by defects in phagocytes. *N. Engl. J. Med.* 343, 1703–1714. doi: 10.1056/NEJM200012073432307

Liu, G. Y. (2009). Molecular pathogenesis of *Staphylococcus aureus* infection. *Pediatr. Res.* 65, 71–77. doi: 10.1203/PDR.0b013e31819dc44d

Liu, G. Y., Essex, A., Buchanan, J. T., Datta, V., Hoffman, H. M., Bastian, J. F., et al. (2005). *Staphylococcus aureus* golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. *J. Exp. Med.* 202, 209–215. doi: 10.1084/jem.20050846

Liu, Q., Cho, H., Yeo, W.-S., and Bae, T. (2015). The extracytoplasmic linker peptide of the sensor protein SaeS tunes the kinase activity required for Staphylococcal Virulence in response to host signals. *PLoS Pathog.* 11:e1004799. doi: 10.1371/journal.ppat.1004799

Lorenz, N., Clow, F., Radcliff, F. J., and Fraser, J. D. (2013). Full functional activity of SSL7 requires binding of both complement C5 and IgA. *Immunol. Cell Biol.* 91, 469–476. doi: 10.1038/icb.2013.28

Lowy, F. D. (1998). *Staphylococcus aureus* infections. *N. Engl. J. Med.* 339, 520–532. doi: 10.1056/NEJM199808203390806

Lu, T., Porter, A. R., Kennedy, A. D., Kobayashi, S. D., and DeLeo, F. R. (2014). Phagocytosis and killing of *Staphylococcus aureus* by human neutrophils. *J. Innate Immun.* 6, 639–649. doi: 10.1159/000360478

Malachowa, N., Kobayashi, S. D., Braughton, K. R., Whitney, A. R., Parnell, M. J., Gardner, D. J., et al. (2012). *Staphylococcus aureus* leukotoxin GH promotes inflammation. *J. Infect. Dis.* 206, 1185–1193. doi: 10.1093/infdis/jis495

Malachowa, N., Kobayashi, S. D., Freedman, B., Dorward, D. W., and DeLeo, F. R. (2013). *Staphylococcus aureus* leukotoxin GH promotes formation of neutrophil extracellular traps. *J. Immunol.* 191, 6022–6029. doi: 10.4049/jimmunol.1301821

Malachowa, N., Whitney, A. R., Kobayashi, S. D., Sturdevant, D. E., Kennedy, A. D., Braughton, K. R., et al. (2011). Global changes in *Staphylococcus aureus* gene expression in human blood. *PLoS ONE* 6:e18617. doi: 10.1371/journal.pone.0018617

Malawista, S. E., Van Blaricom, G., and Breitenstein, M. G. (1989). Cryopreservable neutrophil surrogates. Stored cytoplasts from human polymorphonuclear leukocytes retain chemotactic, phagocytic, and microbicidal function. *J. Clin. Invest.* 83, 728–732. doi: 10.1172/JCI113939

Martin, C. J., Peters, K. N., and Behar, S. M. (2014). Macrophages clean up: efferocytosis and microbial control. *Curr. Opin. Microbiol.* 17, 17–23. doi: 10.1016/j.mib.2013.10.007

McLoughlin, R. M., Lee, J. C., Kasper, D. L., and Tzianabos, A. O. (2008). IFN-gamma regulated chemokine production determines the outcome of *Staphylococcus aureus* infection. *J. Immunol.* 181, 1323–1332. doi: 10.4049/jimmunol.181.2.1323

Mempel, M., Voelcker, V., Köllisch, G., Plank, C., Rad, R., Gerhard, M., et al. (2003). Toll-Like Receptor expression in human keratinocytes: Nuclear Factor κB controlled gene activation by *Staphylococcus aureus* is Toll-Like Receptor 2 but Not Toll-Like Receptor 4 or platelet activating factor receptor dependent. *J. Invest. Dermatol.* 121, 1389–1396. doi: 10.1111/j.1523-1747.2003.12630.x

Menestrina, G., Dalla Serra, M., Comai, M., Coraiola, M., Viero, G., Werner, S., et al. (2003). Ion channels and bacterial infection: the case of beta-barrel pore-forming protein toxins of *Staphylococcus aureus*. *FEBS Lett.* 552, 54–60. doi: 10.1016/S0014-5793(03)00850-0

Mitchell, G. B., Albright, B. N., and Caswell, J. L. (2003). Effect of interleukin-8 and granulocyte colony-stimulating factor on priming and activation of bovine neutrophils. *Infect. Immun.* 71, 1643–1649. doi: 10.1128/IAI.71.4.1643-1649.2003

Mölkänen, T., Tyynelä, J., Helin, J., Kalkkinen, N., and Kuusela, P. (2002). Enhanced activation of bound plasminogen on *Staphylococcus aureus* by staphylokinase. *FEBS Lett.* 517, 72–78. doi: 10.1016/S0014-5793(02)02580-2

Montgomery, C. P., Boyle-Vavra, S., Adem, P. V., Lee, J. C., Husain, A. N., Clasen, J., et al. (2008). Comparison of virulence in community-associated methicillin-resistant *Staphylococcus aureus* Pulsotypes USA300 and USA400 in a rat model of Pneumonia. *J. Infect. Dis.* 198, 561–570. doi: 10.1086/590157

Montgomery, C. P., Daniels, M., Zhao, F., Alegre, M. L., Chong, A. S., and Daum, R. S. (2014). Protective immunity against recurrent *Staphylococcus aureus* skin infection requires antibody and interleukin-17A. *Infect. Immun.* 82, 2125–2134. doi: 10.1128/IAI.01491-14

Moore, K. L., Patel, K. D., Bruehl, R. E., Fugang, L., Johnson, D. A., Lichenstein, H. S., et al. (1995). P-selectin glycoprotein ligand-1 mediates rolling of human neutrophils on P-selectin. *J. Cell Biol.* 128, 661–671. doi: 10.1083/jcb.128.4.661

Müller, P., Müller-Anstett, M., Wagener, J., Gao, Q., Kaesler, S., Schaller, M., et al. (2010). The *Staphylococcus aureus* lipoprotein SitC colocalizes with Toll-like receptor 2 (TLR2) in murine keratinocytes and elicits intracellular TLR2 accumulation. *Infect. Immun.* 78, 4243–4250. doi: 10.1128/IAI.00538-10

Murphy, A. G., O'Keeffe, K. M., Lalor, S. J., Maher, B. M., Mills, K. H., and McLoughlin, R. M. (2014). *Staphylococcus aureus* infection of mice expands a population of memory γδ T cells that are protective against subsequent infection. *J. Immunol.* 192, 3697–3708. doi: 10.4049/jimmunol.1303420

Murray, E. J., Crowley, R. C., Truman, A., Clarke, S. R., Cottam, J. A., Jadhav, G. P., et al. (2014). Targeting *Staphylococcus aureus* quorum sensing with nonpeptidic small molecule inhibitors. *J. Med. Chem.* 57, 2813–2819. doi: 10.1021/jm500215s

Njoroge, J., and Sperandio, V. (2009). Jamming bacterial communication: new approaches for the treatment of infectious diseases. *EMBO Mol. Med.* 1, 201–210. doi: 10.1002/emmm.200900032

Nunes, P., Demaurex, N., and Dinauer, M. C. (2013). Regulation of the NADPH oxidase and associated ion fluxes during phagocytosis. *Traffic* 14, 1118–1131. doi: 10.1111/tra.12115

Nygaard, T. K., Pallister, K. B., Ruzevich, P., Griffith, S., Vuong, C., and Voyich, J. M. (2010). SaeR binds a consensus sequence within virulence gene promoters to advance USA300 pathogenesis. *J. Infect. Dis.* 201, 241–254. doi: 10.1086/649570

Nygaard, T. K., Pallister, K. B., Zurek, O. W., and Voyich, J. M. (2013). The impact of α-toxin on host cell plasma membrane permeability and cytokine expression during human blood infection by CA-MRSA USA300. *J. Leukoc. Biol.* 94, 971–979. doi: 10.1189/jlb.0213080

Oku, Y., Kurokawa, K., Ichihashi, N., and Sekimizu, K. (2004). Characterization of the *Staphylococcus aureus* mprF gene, involved in lysinylation of phosphatidylglycerol. *Microbiology* 150, 45–51. doi: 10.1099/mic.0.26706-0

Olaru, F., and Jensen, L. E. (2010). *Staphylococcus aureus* stimulates neutrophil targeting chemokine expression in keratinocytes through an autocrine IL-1alpha signaling loop. *J. Invest. Dermatol.* 130, 1866–1876. doi: 10.1038/jid.2010.37

Olson, M. E., Nygaard, T. K., Ackermann, L., Watkins, R. L., Zurek, O. W., Pallister, K. B., et al. (2013). *Staphylococcus aureus* nuclease is a SaeR/S-dependent virulence factor. *Infect. Immun.* doi: 10.1128/iai.01242-12

Painter, K. L., Krishna, A., Wigneshweraraj, S., and Edwards, A. M. (2014). What role does the quorum-sensing accessory gene regulator system play during *Staphylococcus aureus* bacteremia? *Trends Microbiol.* 22, 676–685. doi: 10.1016/j.tim.2014.09.002

Palazzolo-Ballance, A. M., Reniere, M. L., Braughton, K. R., Sturdevant, D. E., Otto, M., Kreiswirth, B. N., et al. (2007). Neutrophil microbicides induce a pathogen survival response in community-associated methicillin-resistant

Staphylococcus aureus. *J. Immunol.* 180, 500–509. doi: 10.4049/jimmunol.180.1.500

Pang, Y. Y., Schwartz, J., Bloomberg, S., Boyd, J. M., Horswill, A. R., and Nauseef, W. M. (2014). Methionine sulfoxide reductases protect against oxidative stress in *Staphylococcus aureus* encountering exogenous oxidants and human neutrophils. *J. Innate Immun.* 6, 353–364. doi: 10.1159/000355915

Papayannopoulos, V., Metzler, K. D., Hakkim, A., and Zychlinsky, A. (2010). Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. *J. Cell Biol.* 191, 677–691. doi: 10.1083/jcb.201006052

Park, J., Jagasia, R., Kaufmann, G. F., Mathison, J. C., Ruiz, D. I., Moss, J. A., et al. (2007). Infection control by antibody disruption of bacterial quorum sensing signaling. *Chem. Biol.* 14, 1119–1127. doi: 10.1016/j.chembiol.2007.08.013

Parker, D., and Prince, A. (2012). Immunopathogenesis of *Staphylococcus aureus* pulmonary infection. *Semin. Immunopathol.* 34, 281–297. doi: 10.1007/s00281-011-0291-7

Parry, M. A., Zhang, X. C., and Bode, W. (2000). Molecular mechanisms of plasminogen activation: bacterial cofactors provide clues. *Trends Biochem. Sci.* 25, 53–59. doi: 10.1016/S0968-0004(99)01521-2

Patel, D., Wines, B. D., Langley, R. J., and Fraser, J. D. (2010). Specificity of staphylococcal superantigen-like protein 10 toward the human IgG1 Fc domain. *J. Immunol.* 184, 6283–6292. doi: 10.4049/jimmunol.0903311

Pauli, N. T., Kim, H. K., Falugi, F., Huang, M., Dulac, J., Henry Dunand, C., et al. (2014). *Staphylococcus aureus* infection induces protein A-mediated immune evasion in humans. *J. Exp. Med.* 211, 2331–2339. doi: 10.1084/jem.20141404

Pelz, A., Wieland, K. P., Putzbach, K., Hentschel, P., Albert, K., and Götz, F. (2005). Structure and biosynthesis of staphyloxanthin from *Staphylococcus aureus*. *J. Biol. Chem.* 280, 32493–32498. doi: 10.1074/jbc.M505070200

Peschel, A., Jack, R. W., Otto, M., Collins, L. V., Staubitz, P., Nicholson, G., et al. (2001). *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with l-lysine. *J. Exp. Med.* 193, 1067–1076. doi: 10.1084/jem.193.9.1067

Peschel, A., Otto, M., Jack, R. W., Kalbacher, H., Jung, G., and Götz, F. (1999). Inactivation of the dlt operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides. *J. Biol. Chem.* 274, 8405–8410. doi: 10.1074/jbc.274.13.8405

Pilsczek, F. H., Salina, D., Poon, K. K., Fahey, C., Yipp, B. G., Sibley, C. D., et al. (2010). A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to *Staphylococcus aureus*. *J. Immunol.* 185, 7413–7425. doi: 10.4049/jimmunol.1000675

Pincus, S. H., Boxer, L. A., and Stossel, T. P. (1976). Chronic neutropenia in childhood. Analysis of 16 cases and a review of the literature. *Am. J. Med.* 61, 849–861. doi: 10.1016/0002-9343(76)90409-5

Postma, B., Poppelier, M. J., van Galen, J. C., Prossnitz, E. R., van Strijp, J. A. G., de Haas, C. J., et al. (2004). Chemotaxis inhibitory protein of *Staphylococcus aureus* binds specifically to the C5a and formylated peptide receptor. *J. Immunol.* 172, 6994–7001. doi: 10.4049/jimmunol.172.11.6994

Prat, C., Bestebroer, J., de Haas, C. J., van Strijp, J. A., and van Kessel, K. P. (2006). A new staphylococcal anti-inflammatory protein that antagonizes the formyl peptide receptor-like 1. *J. Immunol.* 177, 8017–8026. doi: 10.4049/jimmunol.177.11.8017

Prat, C., Haas, P.-J., Bestebroer, J., de Haas, C. J. C., van Strijp, J. A. G., and van Kessel, K. P. M. (2009). A homolog of formyl peptide receptor-like 1 (FPRL1) inhibitor from *Staphylococcus aureus* (FPRL1 inhibitory protein) that inhibits FPRL1 and FPR. *J. Immunol.* 183, 6569–6578. doi: 10.4049/jimmunol.0801523

Rainard, P., Riollet, C., Poutrel, B., and Paape, M. J. (2000). Phagocytosis and killing of *Staphylococcus aureus* by bovine neutrophils after priming by tumor necrosis factor-alpha and the des-arginine derivative of C5a. *Am. J. Vet. Res.* 61, 951–959. doi: 10.2460/ajvr.2000.61.951

Reizner, W., Hunter, J. G., O'Malley, N. T., Southgate, R. D., Schwarz, E. M., and Kates, S. L. (2014). A systematic review of animal models for *Staphylococcus aureus* osteomyelitis. *Eur. Cell. Mater.* 27, 196–212. doi: 10.22203/eCM.v027a15

Reyes-Robles, T., and Torres, V. J. (2016). *Staphylococcus aureus* Pore-Forming Toxins. *Curr. Top. Microbiol. Immunol.* 6, 23–27. doi: 10.1007/82_2016_16

Ricklin, D., Tzekou, A., Garcia, B. L., Hammel, M., McWhorter, W. J., Sfyroera, G., et al. (2009). A molecular insight into complement evasion by the staphylococcal complement inhibitor protein family. *J. Immunol.* 183, 2565–2574. doi: 10.4049/jimmunol.0901443

Rigby, K. M., and DeLeo, F. R. (2012). Neutrophils in innate host defense against *Staphylococcus aureus* infections. *Semin. Immunopathol.* 34, 237–259. doi: 10.1007/s00281-011-0295-3

Ritzman, A. M., Hughes-Hanks, J. M., Blaho, V. A., Wax, L. E., Mitchell, W. J., and Brown, C. R. (2010). The chemokine receptor CXCR2 ligand KC (CXCL1) mediates neutrophil recruitment and is critical for development of experimental lyme arthritis and carditis. *Infect. Immun.* 78, 4593–4600. doi: 10.1128/IAI.00798-10

Rogasch, K., Rühmling, V., Pané-Farré, J., Höper, D., Weinberg, C., Fuchs, S., et al. (2006). Influence of the two-component system SaeRS on global gene expression in two different *Staphylococcus aureus* strains. *J. Bacteriol.* 188, 7742–7758. doi: 10.1128/JB.00555-06

Rooijakkers, S. H. M., Ruyken, M., Roos, A., Daha, M. R., Presanis, J. S., Sim, R. B., et al. (2005a). Immune evasion by a staphylococcal complement inhibitor that acts on C3 convertases. *Nat. Immunol.* 6, 920–927. doi: 10.1038/ni1235

Rooijakkers, S. H., Ruyken, M., van Roon, J., van Kessel, K. P., van Strijp, J. A., and van Wamel, W. J. (2006). Early expression of SCIN and CHIPS drives instant immune evasion by *Staphylococcus aureus*. *Cell. Microbiol.* 8, 1282–1293. doi: 10.1111/j.1462-5822.2006.00709.x

Rooijakkers, S. H., van Wamel, W. J., Ruyken, M., van Kessel, K. P., and van Strijp, J. A. (2005b). Anti-opsonic properties of staphylokinase. *Microbes Infect.* 7, 476–484. doi: 10.1016/j.micinf.2004.12.014

Rutherford, S. T., and Bassler, B. L. (2012). Bacterial quorum sensing: its role in virulence and possibilities for its control. *Cold Spring Harb. Perspect. Med.* 2, 1–26. doi: 10.1101/cshperspect.a012427

Sabroe, I., Prince, L. R., Jones, E. C., Horsburgh, M. J., Foster, S. J., Vogel, S. N., et al. (2003). Selective roles for Toll-like receptor (TLR)2 and TLR4 in the regulation of neutrophil activation and life span. *J. Immunol.* 170, 5268–5275. doi: 10.4049/jimmunol.170.10.5268

Sasaki, T., Kano, R., Sato, H., Nakamura, Y., Watanabe, S., and Hasegawa, A. (2003). Effects of staphylococci on cytokine production from human keratinocytes. *Br. J. Dermatol.* 148, 46–50. doi: 10.1046/j.1365-2133.2003.05017.x

Schöneich, C. (2005). Methionine oxidation by reactive oxygen species: reaction mechanisms and relevance to Alzheimer's disease. *Biochim. Biophys. Acta* 1703, 111–119. doi: 10.1016/j.bbapap.2004.09.009

Seilie, E. S., and Bubeck Wardenburg, J. (2017). *Staphylococcus aureus* pore-forming toxins: the interface of pathogen and host complexity. *Semin. Cell Dev. Biol.* doi: 10.1016/j.semcdb.2017.04.003. [Epub ahead of print].

Serruto, D., Rappuoli, R., Scarselli, M., Gros, P., and van Strijp, J. A. (2010). Molecular mechanisms of complement evasion: learning from staphylococci and meningococci. *Nat. Rev. Microbiol.* 8, 393–399. doi: 10.1038/nrmicro2366

Sieprawska-Lupa, M., Mydel, P., Krawczyk, K., Wójcik, K., Puklo, M., Lupa, B., et al. (2004). Degradation of human antimicrobial peptide LL-37 by *Staphylococcus aureus* -derived proteinases. *Antimicrob. Agents Chemother.* 48, 4673–4679. doi: 10.1128/AAC.48.12.4673-4679.2004

Singer, M., and Sansonetti, P. J. (2004). IL-8 is a key chemokine regulating neutrophil recruitment in a new mouse model of *Shigella*-induced colitis. *J. Immunol.* 173, 4197–4206. doi: 10.4049/jimmunol.173.6.4197

Singh, V. K. (2003). Multiple methionine sulfoxide reductase genes in *Staphylococcus aureus*: expression of activity and roles in tolerance of oxidative stress. *Microbiology* 149, 2739–2747. doi: 10.1099/mic.0.26442-0

Singh, V. K., Vaish, M., Johansson, T. R., Baum, K. R., Ring, R. P., Singh, S., et al. (2015). Significance of four methionine sulfoxide reductases in *Staphylococcus aureus*. *PLoS ONE* 10:e0117594. doi: 10.1371/journal.pone.0117594

Sjödahl, J. (1977). Structural studies on the four repetitive Fc-binding regions in protein A from *Staphylococcus aureus*. *Eur. J. Biochem.* 78, 471–490. doi: 10.1111/j.1432-1033.1977.tb11760.x

Skjeflo, E. W., Christiansen, D., Espevik, T., Nielsen, E. W., and Mollnes, T. E. (2014). Combined inhibition of complement and CD14 efficiently attenuated the inflammatory response induced by *Staphylococcus aureus* in a human whole blood model. *J. Immunol.* 192, 2857–2864. doi: 10.4049/jimmunol.1300755

Smith, E. J., Visai, L., Kerrigan, S. W., Speziale, P., and Foster, T. J. (2011). The Sbi protein is a multifunctional immune evasion factor of *Staphylococcus aureus*. *Infect. Immun.* 79, 3801–3809. doi: 10.1128/IAI.05075-11

Stadtmann, A., Germena, G., Block, H., Boras, M., Rossaint, J., Sundd, P., et al. (2013). The PSGL-1-L-selectin signaling complex regulates neutrophil adhesion under flow. *J. Exp. Med.* 210, 2171–2180. doi: 10.1084/jem.20130664

Stapels, D. A., Ramyar, K. X., Bischoff, M., von Kockritz-Blickwede, M., Milder, F. J., Ruyken, M., et al. (2014). *Staphylococcus aureus* secretes a unique class of neutrophil serine protease inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* 111, 13187–13192. doi: 10.1073/pnas.1407616111

Stemerding, A. M., Köhl, J., Pandey, M. K., Kuipers, A., Leusen, J. H., Boross, P., et al. (2013). *Staphylococcus aureus* formyl peptide receptor-like 1 inhibitor (FLIPr) and its homologue FLIPr-like are potent FcγR antagonists that inhibit IgG-mediated effector functions. *J. Immunol.* 191, 353–362. doi: 10.4049/jimmunol.1203243

Stoll, H., Dengjel, J., Nerz, C., and Götz, F. (2005). *Staphylococcus aureus* deficient in lipidation of prelipoproteins is attenuated in growth and immune activation. *Infect. Immun.* 73, 2411–2423. doi: 10.1128/IAI.73.4.2411-2423.2005

Su, S. B., Gao, J., Gong, W., Dunlop, N. M., Murphy, P. M., Oppenheim, J. J., et al. (1999a). T21/DP107, A synthetic leucine zipper-like domain of the HIV-1 envelope gp41, attracts and activates human phagocytes by using G-protein-coupled formyl peptide receptors. *J. Immunol.* 162, 5924–5930.

Su, S. B., Gong, W., Gao, J. L., Shen, W., Murphy, P. M., Oppenheim, J. J., et al. (1999b). A seven-transmembrane, G protein-coupled receptor, FPRL1, mediates the chemotactic activity of serum amyloid A for human phagocytic cells. *J. Exp. Med.* 189, 395–402. doi: 10.1084/jem.189.2.395

Sully, E. K., Malachowa, N., Elmore, B. O., Alexander, S. M., Femling, J. K., Gray, B. M., et al. (2014). Selective chemical inhibition of agr quorum sensing in *Staphylococcus aureus* promotes host defense with minimal impact on resistance. *PLoS Pathog.* 10:e1004174. doi: 10.1371/journal.ppat.1004174

Sun, F., Li, C., Jeong, D., Sohn, C., He, C., and Bae, T. (2010). In the *Staphylococcus aureus* two-component system sae, the response regulator SaeR binds to a direct repeat sequence and DNA binding requires phosphorylation by the sensor kinase SaeS. *J. Bacteriol.* 192, 2111–2127. doi: 10.1128/JB.01524-09

Swain, S. D., Rohn, T. T., and Quinn, M. T. (2002). Neutrophil priming in host defense: role of oxidants as priming agents. *Antioxid. Redox Signal.* 4, 69–83. doi: 10.1089/152308602753625870

Takeuchi, O., Hoshino, K., and Akira, S. (2000). Cutting Edge: TLR2-Deficient and MyD88-deficient mice are highly susceptible to *Staphylococcus aureus* infection. *J. Immunol.* 165, 5392–5396. doi: 10.4049/jimmunol.165.10.5392

Takeuchi, O., Kawai, T., Mühlradt, P. F., Morr, M., Radolf, J. D., Zychlinsky, A., et al. (2001). Discrimination of bacterial lipoproteins by Toll-like receptor 6. *Int. Immunol.* 13, 933–940. doi: 10.1093/intimm/13.7.933

Takeuchi, O., Sato, S., Horiuchi, T., Hoshino, K., Takeda, K., Dong, Z., et al. (2002). Cutting edge: role of Toll-Like Receptor 1 in mediating immune response to microbial lipoproteins. *J. Immunol.* 169, 10–14. doi: 10.4049/jimmunol.169.1.10

Tal-Gan, Y., Stacy, D. M., Foegen, M. K., Koenig, D. W., and Blackwell, H. E. (2013). Highly potent inhibitors of quorum sensing in *Staphylococcus aureus* revealed through a systematic synthetic study of the group-III autoinducing peptide. *J. Am. Chem. Soc.* 135, 7869–7882. doi: 10.1021/ja3112115

Thakker, M., Park, J. S., Carey, V., and Lee, J. C. (1998). *Staphylococcus aureus* serotype 5 capsular polysaccharide is antiphagocytic and enhances bacterial virulence in a murine bacteremia model. *Infect. Immun.* 66, 5183–5189.

Thammavongsa, V., Missiakas, D. M., and Schneewind, O. (2013). *Staphylococcus aureus* degrades neutrophil extracellular traps to promote immune cell death. *Science* 342, 863–866. doi: 10.1126/science.1242255

Torre, A., Bacconi, M., Sammicheli, C., Galletti, B., Laera, D., Fontana, M. R., et al. (2015). Four-component *Staphylococcus aureus* vaccine 4C-staph enhances fc?? receptor expression in neutrophils and monocytes and mitigates *S. aureus* infection in neutropenic mice. *Infect. Immun.* 83, 3157–3163. doi: 10.1128/IAI.00258-15

Tseng, C. W., Biancotti, J. C., Berg, B. L., Gate, D., Kolar, S. L., Müller, S., et al. (2015). Increased susceptibility of humanized NSG mice to panton-valentine leukocidin and *Staphylococcus aureus* skin infection. *PLoS Pathog.* 11, 1–17. doi: 10.1371/journal.ppat.1005292

Upadhyay, A., Burman, J. D., Clark, E. A., Leung, E., Isenman, D. E., Van Den Elsen, J. M., et al. (2008). Structure-function analysis of the C3 binding region of *Staphylococcus aureus* immune subversion protein Sbi. *J. Biol. Chem.* 283, 22113–22120. doi: 10.1074/jbc.M802636200

Valderas, M. W., and Hart, M. E. (2001). Identification and characterization of a second superoxide dismutase gene (sodM) from *Staphylococcus aureus*. *J. Bacteriol.* 183, 3399–3407. doi: 10.1128/JB.183.11.3399-3407.2001

van Kesse, K. P., Bestebroer, J., and van Strijp, J. A. (2014). Neutrophil-mediated phagocytosis of *Staphylococcus aureus*. *Front. Immunol.* 5:467. doi: 10.3389/fimmu.2014.00467

Vandenesch, F., Lina, G., and Henry, T. (2012). *Staphylococcus aureus* Hemolysins, bi-component Leukocidins, and Cytolytic Peptides: a redundant arsenal of membrane-damaging virulence factors? *Front. Cell. Infect. Microbiol.* 2:12. doi: 10.3389/fcimb.2012.00012

Voyich, J. M., Braughton, K. R., Sturdevant, D. E., Whitney, A. R., Saïd-Salim, B., Porcella, S. F., et al. (2005). Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. *J. Immunol.* 175, 3907–3919. doi: 10.4049/jimmunol.175.6.3907

Voyich, J. M., Vuong, C., DeWald, M., Nygaard, T. K., Kocianova, S., Griffith, S., et al. (2009). The SaeR/S gene regulatory system is essential for innate immune evasion by *Staphylococcus aureus*. *J. Infect. Dis.* 199, 1698–1706. doi: 10.1086/598967

Watkins, R. L., Pallister, K. B., and Voyich, J. M. (2011). The SaeR/S gene regulatory system induces a pro-inflammatory cytokine response during *Staphylococcus aureus* infection. *PLoS ONE* 6:e19939. doi: 10.1371/journal.pone.0019939

Watkins, R. L., Zurek, O. W., Pallister, K. B., and Voyich, J. M. (2013). The SaeR/S two-component system induces interferon-gamma production in neutrophils during invasive *Staphylococcus aureus* infection. *Microbes Infect.* 15, 749–754. doi: 10.1016/j.micinf.2013.05.004

Wertheim, H. F., Melles, D. C., Vos, M. C., van Leeuwen, W., van Belkum, A., Verbrugh, H. A., et al. (2005). The role of nasal carriage in *Staphylococcus aureus* infections. *Lancet Infect. Dis.* 5, 751–762. doi: 10.1016/S1473-3099(05)70295-4

Wieland, B., Feil, C., Gloria-Maercker, E., Thumm, G., Lechner, M., Bravo, J. M., et al. (1994). Genetic and biochemical analyses of the biosynthesis of the yellow carotenoid 4,4'-diaponeurosporene of *Staphylococcus aureus*. *J. Bacteriol.* 176, 7719–7726. doi: 10.1128/jb.176.24.7719-7726.1994

Wilson, G. J., Seo, K. S., Cartwright, R. A., Connelley, T., Chuang-Smith, O. N., Merriman, J. A., et al. (2011). A novel core genome-encoded superantigen contributes to lethality of community-associated MRSA necrotizing pneumonia. *PLoS Pathog.* 7:e1002271. doi: 10.1371/journal.ppat.1002271

Wright, A. J., Higginbottom, A., Philippe, D., Upadhyay, A., Bagby, S., Read, R. C., et al. (2007). Characterisation of receptor binding by the chemotaxis inhibitory protein of *Staphylococcus aureus* and the effects of the host immune response. *Mol. Immunol.* 44, 2507–2517. doi: 10.1016/j.molimm.2006.12.022

Wright, H. L., Thomas, H. B., Moots, R. J., and Edwards, S. W. (2013). RNA-Seq reveals activation of both common and cytokine-specific pathways following neutrophil priming. *PLoS ONE* 8:e58598. doi: 10.1371/journal.pone.0058598

Xu, S., Arbeit, R. D., and Lee, J. C. (1992). Phagocytic killing of encapsulated and microencapsulated *Staphylococcus aureus* by human polymorphonuclear leukocytes. *Infect. Immun.* 60, 1358–1362.

Yang, S. J., Bayer, A. S., Mishra, N. N., Meehl, M., Ledala, N., Yeaman, M. R., et al. (2012). The *Staphylococcus aureus* two-component regulatory system, grars, senses and confers resistance to selected cationic antimicrobial peptides. *Infect. Immun.* 80, 74–81. doi: 10.1128/IAI.05669-11

Yokoyama, R., Itoh, S., Kamoshida, G., Takii, T., Fujii, S., Tsuji, T., et al. (2012). Staphylococcal superantigen-like protein 3 binds to the toll-like receptor 2 extracellular domain and inhibits cytokine production induced by *Staphylococcus aureus*, cell wall component, or lipopeptides in murine macrophages. *Infect. Immun.* 80, 2816–2825. doi: 10.1128/IAI.00399-12

Yoshimura, T., Matsushima, K., Tanaka, S., Robinson, E. A., Appella, E., Oppenheim, J. J., et al. (1987). Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines. *Proc. Natl. Acad. Sci. U.S.A.* 84, 9233–9237. doi: 10.1073/pnas.84.24.9233

Zhang, L., Jacobsson, K., Strom, K., Lindberg, M., and Frykberg, L. (1999). *Staphylococcus aureus* expresses a cell surface protein that binds both IgG and beta2-glycoprotein I. *Microbiology* 145(Pt 1), 177–183. doi: 10.1099/13500872-145-1-177

Zhang, L., Jacobsson, K., Vasi, J., Lindberg, M., and Frykberg, L. (1998). A second IgG-binding protein in *Staphylococcus aureus*. *Microbiology* 144(Pt 4), 985–991. doi: 10.1099/00221287-144-4-985

Zurek, O. W., Nygaard, T. K., Watkins, R. L., Pallister, K. B., Torres, V. J., Horswill, A. R., et al. (2014). The role of innate immunity in promoting SaeR/S-Mediated Virulence in *Staphylococcus aureus*. *J. Innate Immun.* 6, 21–30. doi: 10.1159/000351200

Zurek, O. W., Pallister, K. B., and Voyich, J. M. (2015). *Staphylococcus aureus* Inhibits Neutrophil-derived IL-8 to promote cell death. *J. Infect. Dis.* 212, 1–5. doi: 10.1093/infdis/jiv124

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CHAPTER THREE

STAPHYLOCOCCUS AUREUS SaeR/S-REGULATED FACTORS REDUCE HUMAN NEUTROPHIL REACTIVE OXYGEN SPECIES PRODUCTION

Contribution of Authors and Co-Authors

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Contributions: oversight, project design, data analysis, figure presentation, and manuscript writing

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Brief Conclusive Report

Staphylococcus aureus SaeR/S-regulated factors reduce human neutrophil reactive oxygen species production

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ABSTRACT

Neutrophils are the first line of defense after a pathogen has breached the epithelial barriers, and unimpaired neutrophil functions are essential to clear infections. *Staphylococcus aureus* is a prevalent human pathogen that is able to withstand neutrophil killing, yet the mechanisms used by *S. aureus* to inhibit neutrophil clearance remain incompletely defined. The production of reactive oxygen species (ROS) is a vital neutrophil antimicrobial mechanism. Herein, we test the hypothesis that *S. aureus* uses the SaeR/S two-component gene regulatory system to produce virulence factors that reduce neutrophil ROS production. With the use of ROS probes, the temporal and overall production of neutrophil ROS was assessed during exposure to the clinically relevant *S. aureus* USA300 (strain LAC) and its isogenic mutant LAC*ΔsaeR/S*. Our results demonstrated that SaeR/S-regulated factors do not inhibit neutrophil superoxide (O_2^-) production. However, subsequent neutrophil ROS production was significantly reduced during exposure to LAC compared with LAC*ΔsaeR/S*. In addition, neutrophil H_2O_2 production was reduced significantly by SaeR/S-regulated factors by a mechanism independent of catalase. Consequently, the reduction in neutrophil H_2O_2 resulted in decreased production of the highly antimicrobial agent hypochlorous acid/hypochlorite anion (HOCl/$^-$OCl). These findings suggest a new evasion strategy used by *S. aureus* to diminish a vital neutrophil antimicrobial mechanism.

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Introduction

PMNs (leukocytes or neutrophils) are the most abundant WBC in the human body and the first line of defense during bacterial infection [1]. Following migration to the site of infection and phagocytosis, neutrophils expose pathogens to an abundance of microbicidal components, including cationic peptides, proteases, and potent ROS [2, 3]. Assembly and activation of the NADPH oxidase system result in the production of O_2^- from molecular O_2, followed by dismutation to H_2O_2 and to the formation of the highly bactericidal agent HOCl/$^-$OCl, catalyzed by the enzyme MPO [4–6].The production of neutrophil ROS is highly effective at killing many pathogens, including the Gram-positive pathogen *S. aureus* [7, 8]. Microbicidal capacity of ROS against *S. aureus* is exemplified further by the observed increase in susceptibility to infections in individuals with genetic defects in any of the 5 structural components of the NADPH oxidase complex, resulting in chronic granulomatous disease [9, 10]. Collectively, neutrophil microbicidal systems are very efficient at killing ingested bacteria and limiting inflammation.

Despite the neutrophil's capacity to contain most bacterial pathogens, its antimicrobial mechanisms are not fully effective in killing *S. aureus*, and bacterial survival following neutrophil phagocytosis has been proposed as a virulence strategy used by this bacterium [11, 12]. This is best exemplified by the epidemic of CA-MRSA that began in the late 1990s [13]. CA-MRSA can cause uncomplicated skin and soft-tissue infections, as well as invasive, life-threatening illnesses in otherwise healthy individuals [13–15], and its emergence has fostered research investigating the ability of *S. aureus* to survive after neutrophil phagocytosis.

Survival of *S. aureus* following neutrophil phagocytosis is dependent on the concerted effort of multiple virulence factors [11, 12, 16–18]. The *S. aureus* SaeR/S two-component system regulates virulence genes essential for evasion of neutrophil killing [12, 19, 20]. In brief, the *sae* locus consists of 4 open-reading frames (*saePQRS*). *SaeS* and *saeR* code for the two-component module of the SaeR/S system, where SaeS is the histidine kinase, and SaeR is the cognate response regulator. SaeR/S-regulated genes are up-regulated in response to

Abbreviations: CA-MRSA = community-associated methicillin-resistant *Staphylococcus aureus*, DPBS = Dulbecco's PBS, HOCl/$^-$OCl = hypochlorous acid/hypochlorite anion, LAC HK = heat-killed LAC, MPO = myeloperoxidase, O_2^- = superoxide, PMN = polymorphonuclear leukocyte, ROS = reactive oxygen species, SOD = superoxide dismutase, WT = wild-type

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

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neutrophil-derived components, including human α-defensin-1 and H_2O_2 [19, 21, 22]. Studies using isogenic *saeR/S* deletion mutants have shown that neutrophil lysis is reduced, and *S. aureus* has significantly reduced survival following neutrophil phagocytosis in the absence of SaeR/S [11, 12]. SaeR also controls the expression of secreted virulence factors, including those that contribute to neutrophil lysis, such as leukocidin G/H (*lukG/H* also known as *lukA/B*), γ hemolysins (*hlgA, B, C*), and Panton-Valentine leukocidin (*lukF/S-PV*) [12, 23]. Additionally, SaeR-regulated factors influence neutrophil cell fate contributing to pathogen survival [22].

In this study, we investigate further the role of *S. aureus* SaeR/S in modulating neutrophil function by examining its influence on ROS production. Our results demonstrate that SaeR/S-regulated factors decrease neutrophil-derived H_2O_2 and HOCl production by a mechanism independent of catalase activity.

MATERIALS AND METHODS

Bacterial strains and culture

WT *S. aureus* pulsed-field gel electrophoresis-type USA300 (strain LAC) [24] and its previously generated isogenic *saeR/S* mutant strain (LACΔ*saeR/S*) [23] were grown in tryptic soy broth containing 0.5% glucose and harvested at midexponential growth, as described previously [11, 22, 23].

Neutrophil and ROS assays

Human neutrophils were isolated from heparinized venous blood of healthy volunteer donors in accordance with a protocol approved by the Institutional Review Board for Human Subjects at Montana State University. Human neutrophils were isolated as described previously [11, 12]. For all ROS assays, 1×10^6 neutrophils (loaded with various probes, described below) were exposed to 1×10^7 bacteria (10:1 bacteria:PMN ratio) in 96-well serum-coated plates in duplicate. Neutrophils were also exposed to LAC HK (5 min at 99°C), 500 ng/ml PMA, or RPMI, and phagocytosis was synchronized [11]. All measurements were done using a SpectraMax Paradigm Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Luminol. Neutrophil-derived ROS detection by the oxidation of cell-permeable luminol, resulting in chemiluminescence, was measured as described previously [25]. Neutrophils were stained with 100 μM luminol for 15 min in the dark at 4°C. Chemiluminescence was measured in 1 min intervals. The final concentrations of SOD and catalase were 50 and 2000 U/ml, respectively.

Isoluminol. Neutrophil-derived O_2^- production by the oxidation of cell-impermeable isoluminol, resulting in chemiluminescence, was measured as described previously [25]. Neutrophils were stained with 100 μM isoluminol for 15 min in the dark at 4°C. Chemiluminescence was measured in 1 min intervals.

Amplex Red. Neutrophil-derived extracellular H_2O_2 production was measured using the Amplex Red Hydrogen Peroxide/Peroxidase Kit (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions. Amplex Red oxidation was measured in 1 min intervals with 535/595 nm excitation/emission wavelengths.

Secreted catalase. The Amplex Red Catalase Assay Kit (Thermo Fisher Scientific) was used following the manufacturer's suggested protocol. Supernatants from PMN and/or bacterial incubations were collected following 90 min exposure at 37°C and sterile filtered with 0.22 μm syringe filters.

R19-S. Neutrophil-derived HOCl production was measured using R19-S (FutureChem, Seoul, South Korea) [26, 27]. Oxidation of R19-S was measured in 1 min intervals with 485/535 nm excitation/emission wavelengths. Alternatively, neutrophil intracellular HOCl production at 90 min was measured by flow cytometry with a BD FACSCalibur (BD Biosciences, San Jose, CA, USA) using 488/530 nm emission/excitation wavelengths.

Neutrophil phagocytosis assay

Phagocytosis of *S. aureus* by human neutrophils was determined with fluorescence microscopy, as described previously [11]. FITC-labeled bacteria were added (10:1 bacteria:neutrophil ratio), and phagocytosis was synchronized as above. To counterstain uningested bacteria, samples were stained with anti-FITC conjugated to Alexa Fluor 594 (Thermo Fisher Scientific), and mounted coverslips were evaluated using fluorescence microscopy. The number of *S. aureus* bound and/or ingested was evaluated in 25 or 50 neutrophils per experiment from separate fields of view, and percent phagocytosis was calculated as (number of ingested bacteria per cell/total number of PMN-associated bacteria per cell, bound or ingested) × 100.

HOCl killing

A mixture of $HOCl/^-OCl$ was generated by mixing 10 ml commercially available Clorox, 5 ml DPBS, and 160 μl 36.5–38.0% hydrochloric acid. After overnight incubation in the dark, the concentration of $HOCl/^-OCl$ was calculated using Beer's Law [28]. The average pH of the solution was 7.51 ± 0.09, similar to the acid dissociation constant for $HOCl/^-OCl$ (pKa = 7.44) [28, 29]. Bacteria (1×10^7), resuspended in DPBS, were mixed with $HOCl/^-OCl$, diluted in DPBS to desired working concentrations and incubated at 37°C for 30 min. Bacteria were enumerated following overnight incubation at 37°C. Bacterial survival was calculated relative to bacterial concentration following exposure to DPBS only.

Statistical procedures

Statistical analyses were performed using GraphPad Prism version 6.0a (GraphPad Software, La Jolla, CA, USA) with *t* tests and ANOVA as indicated, and error bars represent the SEM.

RESULTS AND DISCUSSION

SaeR/S-regulated factors decrease human neutrophil ROS production

Previous studies have shown that human neutrophils fail to kill *S. aureus* completely after phagocytosis, and SaeR/S-regulated factors are at least partially responsible for reduced bactericidal activity [11, 12]. However, the mechanisms affected by SaeR/S-regulated factors leading to reduced neutrophil staphylococcal killing have yet to be elucidated fully. To this end, neutrophil ROS production in response to WT *S. aureus* strain LAC and its isogenic Δ*saeR/S* mutant strain (LACΔ*saeR/S*) was analyzed using probes to measure different ROS. First, intracellular neutrophil ROS production was measured with luminol. Whereas there were no significant differences in the time to maximum neutrophil ROS production, total neutrophil ROS production was reduced significantly in response to the *S. aureus* LAC compared with LACΔ*saeR/S* (**Fig. 1A** and **B**). To determine if extracellular ROS contributed to abundance of intracellular ROS, neutrophils were exposed to LAC or LACΔ*saeR/S*, including SOD or catalase. As expected and in congruence with published findings, the addition of exogenous SOD reduced total neutrophil ROS production in response to LAC and LACΔ*saeR/S* (Fig. 1C) [30, 31]. In the presence of SOD, significant differences remained in total neutrophil intracellular ROS production during exposure to LAC and LACΔ*saeR/S* (Fig. 1D). The addition of exogenous catalase also reduced total neutrophil intracellular ROS production in response to LAC and LACΔ*saeR/S* compared with untreated neutrophils exposed to bacteria (Fig. 1E). However, neutrophils produced similar amounts of intracellular ROS in

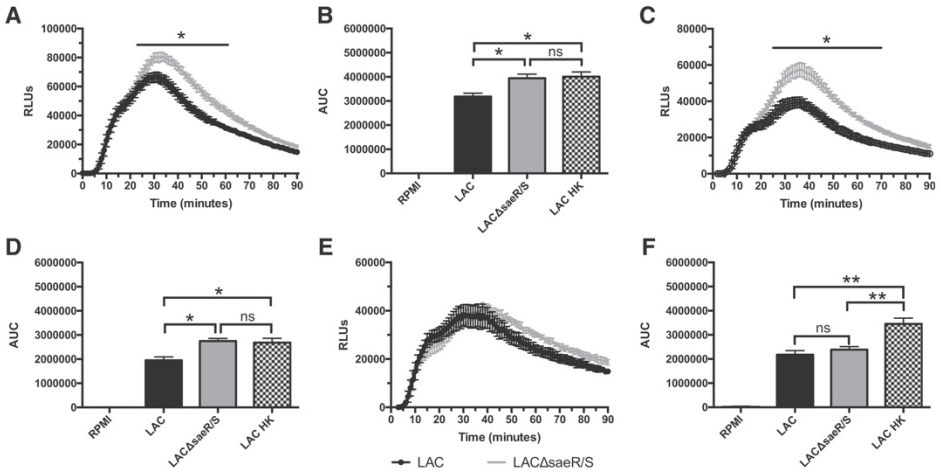


Figure 1. SaeR/S-regulated factors decrease intracellular ROS. Human PMNs were preloaded with luminol, as described in Materials and Methods, and exposed to LAC, LAC*Δ saeR/S*, or RPMI, and chemiluminescence was measured. (A) Time-dependent neutrophil intracellular ROS production following exposure to *S. aureus* LAC or LAC*Δ saeR/S*. (B) Total relative neutrophil ROS production determined by calculating the area under the curve (AUC) from A. (C) Time-dependent neutrophil intracellular ROS production following exposure to *S. aureus* LAC or LAC*Δ saeR/S* in the presence of exogenous SOD. (D) Total relative neutrophil ROS production determined by calculating the area under the curve from C. (E) Time-dependent neutrophil intracellular ROS production following exposure to *S. aureus* LAC or LAC*Δ saeR/S* in the presence of exogenous catalase. (F) Total relative neutrophil ROS production determined by calculating the area under the curve from E. Data represent 5 separate experiments, using 5 different neutrophil donors; $*P \leq 0.05$, $**P \leq 0.01$, as determined by two-way ANOVA (A, C, and E) and one-way ANOVA (B, D, and F). RLUs, Relative luminescence units.

response to LAC and LAC*Δ saeR/S* in the presence of exogenous catalase (Fig. 1F). Collectively, these results suggest that *S. aureus* SaeR/S-regulated factors reduce intracellular ROS production. Additionally, neutrophils exposed to LAC HK produced similar amounts of intracellular ROS as those exposed to LAC*Δ saeR/S* (Fig. 1B and D), confirming that reduction of neutrophil ROS by *S. aureus* is an active process that requires a viable organism and SaeR/S. Importantly, the neutralization of extracellular H_2O_2 with exogenous catalase eliminated differences in intracellular neutrophil ROS production in response to LAC and LAC*Δ saeR/S*. These results suggest that *S. aureus* SaeR/S-regulated factors reduce the production of neutrophil-derived H_2O_2, leading to a reduction in overall ROS production. Of note, neutrophil propidium iodide uptake was not significantly different between neutrophils exposed to LAC or LAC*Δ saeR/S* after 90 min, confirming that differences in ROS production were not a result of differences in neutrophil membrane damage (data not shown). As differences in uptake of LAC and LAC*Δ saeR/S* could influence ROS abundance, we assessed neutrophil phagocytosis using fluorescence microscopy. There were no differences in neutrophil ingestion of WT LAC compared with the LAC*Δ saeR/S* mutant (Supplemental Fig. 1), which is in agreement with previous findings [12].

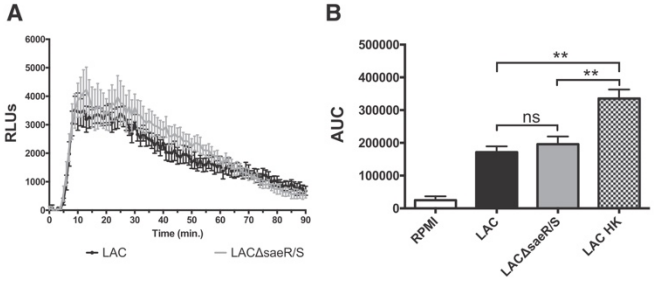


Figure 2. Human neutrophil extracellular O_2^- production following exposure to WT *S. aureus* LAC and LAC*Δ saeR/S* using isoluminol. (A) Time-dependent neutrophil extracellular O_2^- production following exposure to *S. aureus* LAC or LAC*Δ saeR/S*. (B) Total relative neutrophil O_2^- production determined by calculating the area under the curve from A. Data represent 4 separate experiments, using 4 different neutrophil donors; $**P \leq 0.01$, as determined by two-way ANOVA (A) and one-way ANOVA (B).

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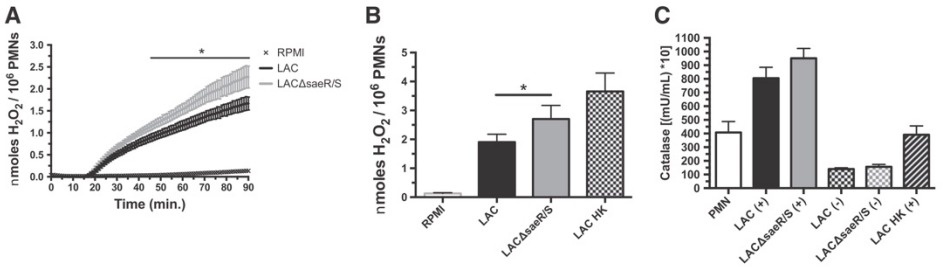


Figure 3. Human neutrophil extracellular H_2O_2 production is significantly reduced by SaeR/S-regulated *S. aureus* factors. (A) Time-dependent neutrophil extracellular H_2O_2 production was measured using Amplex Red, following exposure *S. aureus* LAC or LAC*Δ*saeR/S* or RPMI as a control. (B) Neutrophil-derived extracellular H_2O_2 production calculated from an H_2O_2 standard curve. (C) Secreted catalase following *S. aureus* exposure to human neutrophils. Bacteria exposed (+) or not exposed (−) to neutrophils. Data represent 4 separate experiments (A and C) and 5 separate experiments (B), with $^*P \leq 0.05$, as determined by two-way ANOVA (A) and paired *t* test (B).

SaeR/S-regulated factors reduce the production of neutrophil-derived H_2O_2

Luminol measures overall intracellular ROS production. As our results showed that extracellular ROS played a significant role in enhancing intracellular neutrophil ROS production (Fig. 1A–F) and suggested that SaeR/S-regulated factors reduce the production of neutrophil-derived H_2O_2, we used isoluminol and Amplex Red probes to measure specifically O_2^- and H_2O_2, respectively. Cell-impermeable isoluminol was used to measure neutrophil extracellular O_2^- production in response to LAC and LAC*Δsaer/S*. We hypothesized that neutrophil O_2^- production would not be reduced by SaeR/S-regulated factors, as significant differences remained in overall neutrophil intracellular ROS production during exposure to LAC and LAC*Δsaer/S* in the presence of exogenous SOD (Fig. 1C and D). The neutrophil O_2^- burst occurred within 10 min after recording luminescence (**Fig. 2A**). Consistent with results shown in Fig. 1C and D, there were no significant differences in neutrophil O_2^- production during exposure to LAC or LAC*Δsaer/S* (Fig. 2B). This suggests that activation and assembly of the NADPH oxidase complex are not affected by SaeR/S-regulated factors. Exposure to LAC HK did result in significantly increased O_2^- production compared with LAC and LAC*Δsaer/S*. This is expected, as *S. aureus* produces SOD to neutralize O_2^- via *sodA* and *sodM*, but these genes are not regulated by SaeR/S [12, 23, 32, 33].

As the measurement of neutrophil intracellular ROS with luminol in the presence of catalase suggested that SaeR/S-regulated factors reduce production of extracellular H_2O_2 (Fig. 1C and D), we used the H_2O_2-specific probe Amplex Red to measure neutrophil extracellular H_2O_2 production in response to *S. aureus* LAC and LAC*Δsaer/S*. Detectable neutrophil-derived H_2O_2 was observed within 20 min of exposure to LAC and LAC*Δsaer/S* (**Fig. 3A**). Significant differences in extracellular H_2O_2 production between neutrophils exposed to LAC and LAC*Δsaer/S* were observed, starting at 43 min. In addition, there were significant increases in molar amounts of H_2O_2 produced by neutrophils in response to LAC*Δsaer/S* versus LAC at the end of the 90 min assay (Fig. 3B). Differences in extracellular H_2O_2 production by neutrophils confirmed results showing overall reduction in ROS—and no significant differences between neutrophils exposed to LAC and LAC*Δsaer/S*—when the cell-impermeable catalase was present (Fig. 1E and F). Importantly, there were no differences in the secreted catalase that could degrade extracellular H_2O_2 (Fig. 3C), confirming that SaeR/S does not regulate catalase following neutrophil phagocytosis.

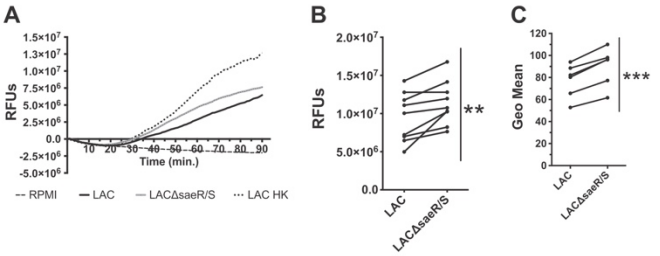


Figure 4. Human neutrophil intracellular hypochlorite production is reduced significantly by SaeR/S-regulated *S. aureus* factors. HOCl production was measured in human neutrophils using R19-S, following exposure to WT *S. aureus* LAC or LAC*Δsaer/S*. (A) Representative plot of time-dependent neutrophil intracellular HOCl production following exposure to *S. aureus* LAC or LAC*Δsaer/S* or LAC HK or RPMI as a control. (B) Relative neutrophil-derived HOCl production at the end of the 90 min assay from A. (C) Relative neutrophil-derived intracellular HOCl measured by flow cytometry. Data represent 9 separate experiments (B) and 6 separate experiments (C); $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, as determined by paired *t* test (B and C). RFUs, Relative fluorescence units.

SaeR/S-regulated factors reduce the production of neutrophil-derived HOCl

HOCl is present in neutrophil phagosomes and is highly bactericidal. H_2O_2 is a precursor to the production of MPO-catalyzed HOCl. Therefore, we measured intracellular HOCl production using the cell-permeable R19-S probe to assess how SaeR/S-regulated factors affect HOCl production (**Fig. 4A**). Consistent with our results showing that SaeR/S reduced H_2O_2 production, SaeR/S-regulated factors significantly decreased neutrophil HOCl production (Fig. 4B and C). Production of neutrophil H_2O_2 and HOCl was detected at ~20 min, with the maximum H_2O_2 burst occurring earlier than the HOCl burst (Supplemental Fig. 2).

The formation of H_2O_2 from O_2^- can occur spontaneously or is catalyzed by SOD or MPO [34, 35]. Based on our observations, we propose that unidentified SaeR/S-regulated factor(s) interfere with the enzymatic reactions catalyzed by MPO to produce H_2O_2 and HOCl. Inasmuch as there are no differences in secreted catalase between neutrophils exposed to *S. aureus* LAC and LACΔ*saeR/S* (Fig. 3C), we propose that the significant decrease in H_2O_2 produced by neutrophils exposed to LAC compared with LACΔ*saeR/S* is a result of SaeR/S-regulated factors inhibiting the SOD activity of MPO to produce H_2O_2. In addition, SaeR/S-regulated factors may interfere with the chlorination activity of MPO, resulting in decreased production of HOCl. Ongoing studies are determining if decreased HOCl production is a direct result of lower H_2O_2 production or if SaeR/S-regulated factors directly interfere with both the dismutase and chlorination activity of MPO.

WT *S. aureus* LAC and LACΔ*saeR/S* are equally susceptible to reagent HOCl

S. aureus SaeR/S-regulated factors significantly decreased neutrophil-derived HOCl production in response to WT LAC versus LACΔ*saeR/S* (Fig. 4). To determine if the WT and mutant were differentially susceptible to HOCl, we exposed LAC and LACΔ*saeR/S* to varied concentrations of HOCl. As shown in **Fig. 5**, LAC and LACΔ*saeR/S* were equally susceptible to HOCl exposure. HOCl concentrations below 5 μM did not kill *S. aureus*. However, there was a precipitous decrease in *S. aureus* survival following exposure between 5 and 15 μM HOCl. These data are in agreement with previously published findings [36]. Future studies will investigate the physiologically relevant question as to whether reduction in ROS makes WT *S. aureus* more resistant to neutrophil killing reflective of clinical syndromes, demonstrating the importance of ROS in controlling *S. aureus* [9, 10]. It is possible that a SaeR/S-mediated reduction in ROS makes the ROS amount insufficient to kill *S. aureus*, as in vitro studies have demonstrated a fine line between ROS amounts that are effective versus amounts that the pathogen can tolerate (**Fig. 5** and ref. [21]).

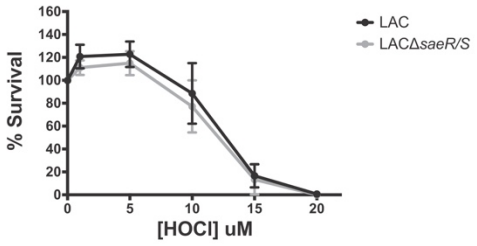


Figure 5. *S. aureus* LAC and LACΔ*saeR/S* are equally susceptible to killing by HOCl. Bacteria (1×10^7) were exposed to different concentrations of HOCl for 30 min at 37°C. Subsequently, bacteria were serially diluted and plated on trypticase soy agar plates, and CFUs were enumerated following overnight incubation at 37°C. Data represent 5 separate experiments.

The importance of detoxifying ROS is demonstrated by the many mechanisms used by *S. aureus* that include scavenging and neutralizing ROS, such as O_2^- and H_2O_2, with SODs (SodA and SodM) and catalase (KatA), respectively [37, 38]. In addition, the iron-regulated surface-determinant proteins IsdA and IsdB have been implicated in increasing *S. aureus* resistance to killing by H_2O_2 [21], as well as methionine sulfoxide reductases to reduce oxidized methionine residues following oxidative stress that increase *S. aureus* resistance to ROS [39]. Unlike previously described mechanisms to neutralize ROS, our data suggest that *S. aureus* can directly inhibit H_2O_2 and HOCl production, resulting in increased *S. aureus* survival. Future studies will focus on identifying specific SaeR/S-regulated factors that inhibit neutrophil ROS production.

AUTHORSHIP

F.E.G. contributed to project design and experimental procedures, analyzed data, provided the figure presentation, and wrote the manuscript. C.B.A., J.A., N.W.M.d.J., K.B.P., and J.v.S. contributed to project design and experimental procedures. J.M.V. provided oversight and contributed to project design, data analysis, figure presentation, and manuscript writing.

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DISCLOSURES

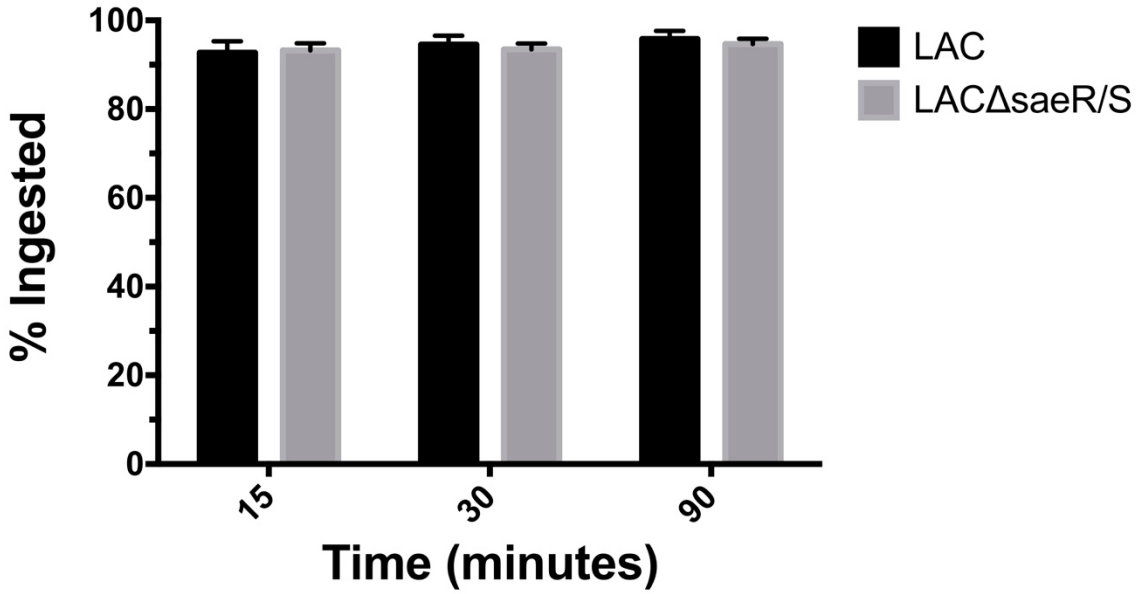
The authors have declared that there are no conflicts of interest.

REFERENCES

1. Amulic, B., Cazalet, C., Hayes, G. L., Metzler, K. D., Zychlinsky, A. (2012) Neutrophil function: from mechanisms to disease. *Annu. Rev. Immunol.* **30,** 459–489.
2. Nordenfelt, P., Tapper, H. (2011) Phagosome dynamics during phagocytosis by neutrophils. *J. Leukoc. Biol.* **90,** 271–284.
3. Hurst, J. K. (2012) What really happens in the neutrophil phagosome? *Free Radic. Biol. Med.* **53,** 508–520.

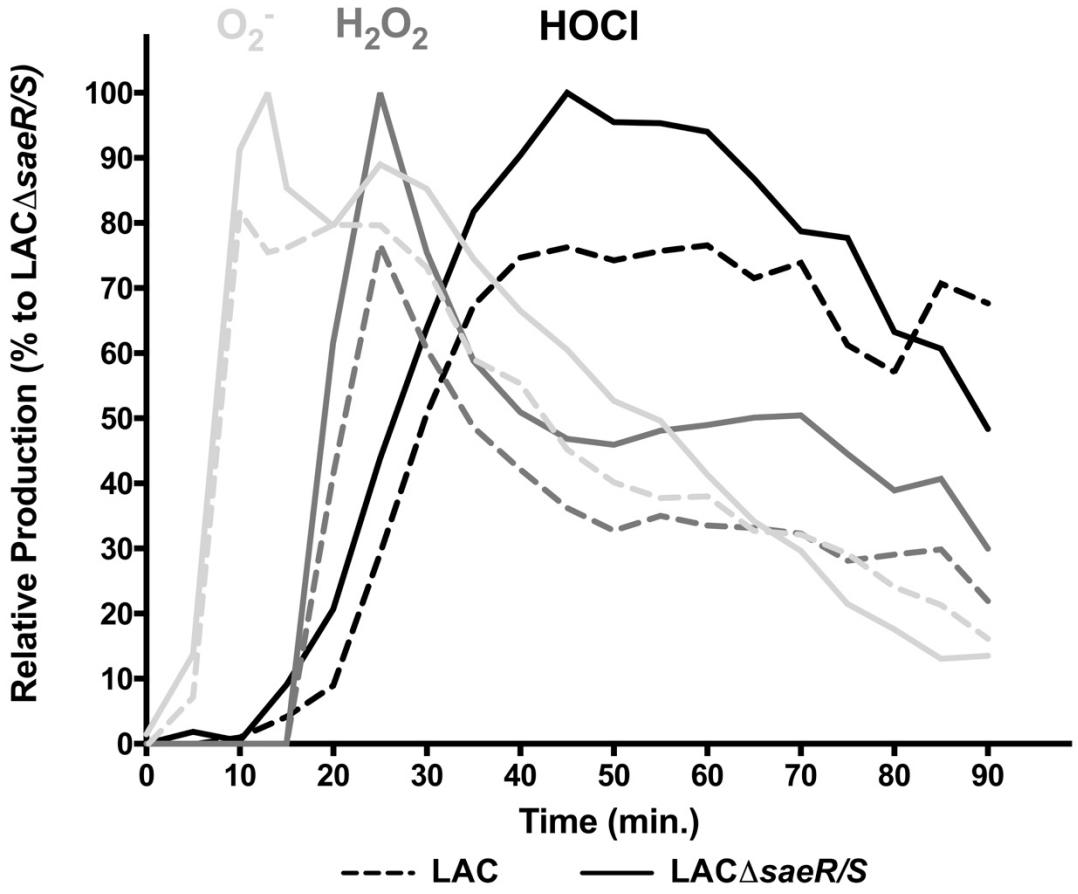
4. DeLeo, F. R., Quinn, M. T. (1996) Assembly of the phagocyte NADPH oxidase: molecular interaction of oxidase proteins. *J. Leukoc. Biol.* **60,** 677–691.
5. DeLeo, F. R., Allen, L. A., Apicella, M., Nauseef, W. M. (1999) NADPH oxidase activation and assembly during phagocytosis. *J. Immunol.* **163,** 6732–6740.
6. Winterbourn, C. C., Kettle, A. J. (2013) Redox reactions and microbial killing in the neutrophil phagosome. *Antioxid. Redox Signal.* **18,** 642–660.
7. Hampton, M. B., Winterbourn, C. C. (1995) Modification of neutrophil oxidant production with diphenyleneiodonium and its effect on bacterial killing. *Free Radic. Biol. Med.* **18,** 633–639.
8. Hampton, M. B., Kettle, A. J., Winterbourn, C. C. (1996) Involvement of superoxide and myeloperoxidase in oxygen-dependent killing of *Staphylococcus aureus* by neutrophils. *Infect. Immun.* **64,** 3512–3517.
9. Lekstrom-Himes, J. A., Gallin, J. I. (2000) Immunodeficiency diseases caused by defects in phagocytes. *N. Engl. J. Med.* **343,** 1703–1714.
10. Roos, D., de Boer, M. (2014) Molecular diagnosis of chronic granulomatous disease. *Clin. Exp. Immunol.* **175,** 139–149.
11. Voyich, J. M., Braughton, K. R., Sturdevant, D. E., Whitney, A. R., Saïd-Salim, B., Porcella, S. F., Long, R. D., Dorward, D. W., Gardner, D. J., Kreiswirth, B. N., Musser, J. M., DeLeo, F. R. (2005) Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. *J. Immunol.* **175,** 3907–3919.
12. Voyich, J. M., Vuong, C., DeWald, M., Nygaard, T. K., Kocianova, S., Griffith, S., Jones, J., Iverson, C., Sturdevant, D. E., Braughton, K. R., Whitney, A. R., Otto, M., DeLeo, F. R. (2009) The SaeR/S gene regulatory system is essential for innate immune evasion by *Staphylococcus aureus*. *J. Infect. Dis.* **199,** 1698–1706.
13. DeLeo, F. R., Chambers, H. F. (2009) Reemergence of antibiotic-resistant *Staphylococcus aureus* in the genomics era. *J. Clin. Invest.* **119,** 2464–2474.
14. Miller, L. G., Perdreau-Remington, F., Rieg, G., Mehdi, S., Perlroth, J., Bayer, A. S., Tang, A. W., Phung, T. O., Spellberg, B. (2005) Necrotizing fasciitis caused by community-associated methicillin-resistant *Staphylococcus aureus* in Los Angeles. *N. Engl. J. Med.* **352,** 1445–1453.
15. Kreisel, K. M., Stine, O. C., Johnson, J. K., Perencevich, E. N., Shardell, M. D., Lesse, A. J., Gordin, F. M., Climo, M. W., Roghmann, M.-C. (2011) USA300 methicillin-resistant *Staphylococcus aureus* bacteremia and the risk of severe sepsis: is USA300 methicillin-resistant *Staphylococcus aureus* associated with more severe infections? *Diagn. Microbiol. Infect. Dis.* **70,** 285–290.
16. Genestier, A. L., Michallet, M. C., Prévost, G., Bellot, G., Chalabreysse, L., Peyrol, S., Thivolet, F., Etienne, J., Lina, G., Vallette, F. M., Vandenesch, F., Genestier, L. (2005) *Staphylococcus aureus* Panton-Valentine leukocidin directly targets mitochondria and induces Bax-independent apoptosis of human neutrophils. *J. Clin. Invest.* **115,** 3117–3127.
17. Bubeck Wardenburg, J., Bae, T., Otto, M., Deleo, F. R., Schneewind, O. (2007) Poring over pores: alpha-hemolysin and Panton-Valentine leukocidin in *Staphylococcus aureus* pneumonia. *Nat. Med.* **13,** 1405–1406.
18. Wang, R., Braughton, K. R., Kretschmer, D., Bach, T.-H. L., Queck, S. Y., Li, M., Kennedy, A. D., Dorward, D. W., Klebanoff, S. J., Peschel, A., DeLeo, F. R., Otto, M. (2007) Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nat. Med.* **13,** 1510–1514.
19. Flack, C. E., Zurek, O. W., Meishery, D. D., Pallister, K. B., Malone, C. L., Horswill, A. R., Voyich, J. M. (2014) Differential regulation of staphylococcal virulence by the sensor kinase SaeS in response to neutrophil-derived stimuli. *Proc. Natl. Acad. Sci. USA* **111,** E2037–E2045.
20. Zurek, O. W., Pallister, K. B., Voyich, J. M. (2015) *Staphylococcus aureus* inhibits neutrophil-derived IL-8 to promote cell death. *J. Infect. Dis.* **212,** 934–938.
21. Palazzolo-Ballance, A. M., Reniere, M. L., Braughton, K. R., Sturdevant, D. E., Otto, M., Kreiswirth, B. N., Skaar, E. P., DeLeo, F. R. (2008) Neutrophil microbicides induce a pathogen survival response in community-associated methicillin-resistant *Staphylococcus aureus*. *J. Immunol.* **180,** 500–509.
22. Zurek, O. W., Nygaard, T. K., Watkins, R. L., Pallister, K. B., Torres, V. J., Horswill, A. R., Voyich, J. M. (2014) The role of innate immunity in promoting SaeR/S-mediated virulence in *Staphylococcus aureus*. *J. Innate Immun.* **6,** 21–30.
23. Nygaard, T. K., Pallister, K. B., Ruzevich, P., Griffith, S., Vuong, C., Voyich, J. M. (2010) SaeR binds a consensus sequence within virulence gene promoters to advance USA300 pathogenesis. *J. Infect. Dis.* **201,** 241–254.
24. Diep, B. A., Gill, S. R., Chang, R. F., Phan, T. H., Chen, J. H., Davidson, M. G., Lin, F., Lin, J., Carleton, H. A., Mongodin, E. F., Sensabaugh, G. F., Perdreau-Remington, F. (2006) Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant *Staphylococcus aureus*. *Lancet* **367,** 731–739.
25. Bylund, J., Björnsdottir, H., Sundqvist, M., Karlsson, A., Dahlgren, C. (2014) Measurement of respiratory burst products, released or retained, during activation of professional phagocytes. *Methods Mol. Biol.* **1124,** 321–338.
26. Chen, X., Lee, K.-A., Ha, E.-M., Lee, K. M., Seo, Y. Y., Choi, H. K., Kim, H. N., Kim, M. J., Cho, C.-S., Lee, S. Y., Lee, W.-J., Yoon, J. (2011) A specific and sensitive method for detection of hypochlorous acid for the imaging of microbe-induced HOCl production. *Chem. Commun. (Camb.)* **47,** 4373–4375.
27. Ng, H. P., Zhou, Y., Song, K., Hodges, C. A., Drumm, M. L., Wang, G. (2014) Neutrophil-mediated phagocytic host defense defect in myeloid Cftr-inactivated mice. *PLoS One* **9,** e106813.
28. Morris, J. C. (1966) The acid ionization constant of HOCl from 5 to 35°. *J. Phys. Chem.* **70,** 3798–3805.
29. Kettle, A. J., Albrett, A. M., Chapman, A. L., Dickerhof, N., Forbes, L. V., Khalilova, I., Turner, R. (2014) Measuring chlorine bleach in biology and medicine. *Biochim. Biophys. Acta* **1840,** 781–793.
30. Ohno, Y., Gallin, J. I. (1985) Diffusion of extracellular hydrogen peroxide into intracellular compartments of human neutrophils. Studies utilizing the inactivation of myeloperoxidase by hydrogen peroxide and azide. *J. Biol. Chem.* **260,** 8438–8446.
31. Gerber, C. E., Bruchelt, G., Falk, U. B., Kimpfler, A., Hauschild, O., Kuçi, S., Bächi, T., Niethammer, D., Schubert, R. (2001) Reconstitution of bactericidal activity in chronic granulomatous disease cells by glucose-oxidase-containing liposomes. *Blood* **98,** 3097–3105.
32. Rogasch, K., Rühmling, V., Pané-Farré, J., Höper, D., Weinberg, C., Fuchs, S., Schmudde, M., Bröker, B. M., Wolz, C., Hecker, M., Engelmann, S. (2006) Influence of the two-component system SaeRS on global gene expression in two different *Staphylococcus aureus* strains. *J. Bacteriol.* **188,** 7742–7758.
33. Sun, F., Li, C., Jeong, D., Sohn, C., He, C., Bae, T. (2010) In the *Staphylococcus aureus* two-component system sae, the response regulator SaeR binds to a direct repeat sequence and DNA binding requires phosphorylation by the sensor kinase SaeS. *J. Bacteriol.* **192,** 2111–2127.
34. McCord, J. M., Fridovich, I. (1969) Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* **244,** 6049–6055.
35. Kettle, A. J., Anderson, R. F., Hampton, M. B., Winterbourn, C. C. (2007) Reactions of superoxide with myeloperoxidase. *Biochemistry* **46,** 4888–4897.
36. Chapman, A. L. P., Hampton, M. B., Senthilmohan, R., Winterbourn, C. C., Kettle, A. J. (2002) Chlorination of bacterial and neutrophil proteins during phagocytosis and killing of *Staphylococcus aureus*. *J. Biol. Chem.* **277,** 9757–9762.
37. Horsburgh, M. J., Clements, M. O., Crossley, H., Ingham, E., Foster, S. J. (2001) PerR controls oxidative stress resistance and iron storage proteins and is required for virulence in *Staphylococcus aureus*. *Infect. Immun.* **69,** 3744–3754.
38. Karavolos, M. H., Horsburgh, M. J., Ingham, E., Foster, S. J. (2003) Role and regulation of the superoxide dismutases of *Staphylococcus aureus*. *Microbiology* **149,** 2749–2758.
39. Singh, V. K., Vaish, M., Johansson, T. R., Baum, K. R., Ring, R. P., Singh, S., Shukla, S. K., Moskovitz, J. (2015) Significance of four methionine sulfoxide reductases in *Staphylococcus aureus*. *PLoS One* **10,** e0117594.

KEY WORDS:
innate immunity · bacteria · host-pathogen interactions · two-component systems



Supplemental Figure 1. Phagocytosis by human neutrophils is not influenced by SaeR/S-regulated factors. Percentage of LAC and LAC*saeR/S* strains ingested by human PMNs was calculated using the equation: [(number of ingested bacteria per cell / total number of PMN-associated bacteria, bound or ingested)*100]. Results are from 3 separate donors.

su



Supplemental Figure 2. ROS production by *S. aureus* LAC or LAC∆*saeR/S* during exposure to human neutrophils. Superoxide (O_2-), hydrogen peroxide (H_2O_2) and hypohclorous acid (HOCl) production by human neutrophils was measured isoluminol, Amplex® Red, and R19-S, respectively. Results are shown as the average change over time in 5-minute intervals for each ROS measured and normalized to neutrophils exposed to LAC∆*saeR/S*.

CHAPTER FOUR

STAPHYLOCOCCUS AUREUS LEUKOCIDINS MODULATE HUMAN NEUTROPHIL REACTIVE OXYGEN SPECIES PRODUCTION

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Contributions: oversight, project design, data analysis, figure presentation, and manuscript writing

Manuscript Information

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INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a Gram-positive commensal pathogen that causes a wide range of infections in humans, from superficial skin and soft-tissue infections to invasive endocarditis, necrotizing fasciitis, and necrotizing pneumonia (Miller et al., 2005; Tong et al., 2015). Over the last twenty years, an epidemiological transition in staphylococcal disease occurred, characterized by the emergence of community-associated methicillin-resistant *S. aureus* (CA-MRSA) strains causing infections in healthy individuals without predisposing conditions (Centers for Disease Control and Prevention (CDC), 1999; Chambers, 2001; Herold et al., 1998). *S. aureus* pulsed-field gel electrophoresis type USA300 emerged as the predominant cause of CA-MRSA infections in the United States and has also become a leading cause of healthcare-associated MRSA infections (Centers for Disease Control and Prevention, 2015; Nimmo, 2012). The factors contributing to the increased incidence of *S. aureus* USA300 infections remain unknown. Vaccine development against *S. aureus* has yet to prove successful, and our ability to treat infections is alarmingly limited by the ability of *S. aureus* to rapidly acquire antibiotic resistance (Foster, 2017; Fowler and Proctor, 2014).

S. aureus is equipped with an arsenal of virulence factors to evade host immune responses and destroy immune cells (Guerra et al., 2017; Yoong and Torres, 2013). Polymorphonuclear leukocytes, also known as PMNs or neutrophils, are the first line of host defense once *S. aureus* breaches the epithelial barriers and are quickly recruited to the site infection (Harding et al., 2014; Kolaczkowska and Kubes, 2013). Neutrophil surface receptors engage *S. aureus* and initiate the process of internalizing the bacterial

pathogen in a process termed phagocytosis (van Kesse et al., 2014). Phagocytosis leads to the enclosure of *S. aureus* within a membrane bound structure called the phagosome, wherein *S. aureus* is exposed to an array of antimicrobial peptides, proteins, and reactive oxygen species (ROS). For most pathogens, production of these antimicrobial factors results in disruption of the bacterial membrane and oxidation and chlorination of essential proteins (Green et al., 2014; Winterbourn and Kettle, 2013). However, *S. aureus* mounts a rapid counterattack that initiates with transcriptional upregulation and secretion of leukocidins that form pores through the neutrophil plasma membrane, as well as secretion of proteins that inhibit neutrophil ROS production (Guerra et al., 2016; Voyich et al., 2005; Zurek et al., 2014).

Staphylococcal bi-component leukocidins are essential to neutrophil evasion and upregulated by the SaeR/S and Agr regulatory systems within minutes in response to human neutrophils (Flack et al., 2014; Gordon et al., 2013; Voyich et al., 2009; Zurek et al., 2014). Each bi-component leukocidin contains an S subunit that recognizes the host receptor and subsequently binds the F subunit, which induces pore formation on the host cell membrane. Clinically relevant *S. aureus* strains produce up to five bi-component leukocidins: *hlgAB, hlgCB, lukSF-PV, lukED,* and *lukGH* (also known as *lukAB*). The mechanism of LukGH binding to its host receptor differs from the other bi-component leukotoxins since LukG and LukH heterodimers form in solution before binding to its host receptor (Badarau et al., 2015). Each host receptor binding S component shows affinity for multiple chemokine or complement receptors (with the exception of LukH) on the surface of neutrophils, albeit each S subunit shows preferential binding to one

receptor based on binding affinity. On the neutrophil surface, HlgA preferentially binds the chemokine receptor CXCR1 (Spaan et al., 2014); HlgC binds the complement receptor C5aR (Spaan et al., 2014); LukS-PV preferentially binds C5aR (Spaan et al., 2013); LukE binds CXCR1 and CXCR2 (Reyes-Robles et al., 2013); LukGH binds to the CD11b subunit of complement receptor 3 (DuMont et al., 2013). The bi-component leukocidins show high specificity for their human receptor counterparts, with the exception of LukED, and has hindered *in vivo* animal studies to determine the contribution of each leukotoxin to staphylococcal pathogenesis.

While the identification of *S. aureus* bi-component leukocidin host receptors and the mechanisms inducing pore formation have garnered the most attention, less is known about the effects leukocidin binding has on host cell signaling. *S. aureus* bi-component leukocidins bind G protein-coupled receptors (GPCRs; CXCR1, CXCR2, and C5aR) and the β2 integrin family through binding complement receptor 3, which is a heterodimer composed of CD11b/CD18 (Podolnikova et al., 2015; Raghuwanshi et al., 2012; Sarma and Ward, 2012). Host-derived ligand binding to these receptors induces signaling pathways involved in neutrophil recruitment, priming, bactericidal mechanisms through recognition of pathogens and ROS production, and cell death (Fu et al., 2004; Ibrahim et al., 2004; Mitchell et al., 2003; Raghuwanshi et al., 2012; Rajagopalan and Rajarathnam, 2004; Rubel et al., 2001; Zurek et al., 2015). Previous studies have shown NADPH oxidase activation downstream of GPCRs and integrin signaling resulting in the production of human neutrophil ROS. For example, the CXCR1/CXCR2 ligand interleukin-8 (IL-8) induces superoxide production, as well as C5a binding to its cognate

C5a receptor (Fu et al., 2004; Ibrahim et al., 2004). CD11b/CD18 is a promiscuous receptor with many known ligands binding to the I-domain of CD11b (Balsam et al., 1998; Mesri et al., 1998; Podolnikova et al., 2015). CD11b ligands have been shown to modulate neutrophil ROS production in cooperation with cytokine receptors, and the Src-family kinases (SFKs) and Syk tyrosine kinases are involved in integrin-mediated signaling (Berton et al., 2005; Futosi et al., 2013; Lau et al., 2005; Zhao et al., 2003; Zhou et al., 2013).

Since multiple *S. aureus* leukocidins bind human neutrophil surface receptors to induce pore formation, targeting multiple receptors on the neutrophil surface to induce lysis seems like a redundant virulence mechanism. Since GPCR signaling leads to activation of phosphoinositide 3-kinase (PI3K) and phospholipases (Futosi et al., 2013), leukocidins upregulated in response to neutrophils and neutrophil components may play a critical role in neutrophil signaling that leads to ROS production. In neutrophils, PI3K is critical to nicotinamide adenine dinucleotide phosphate (NAPDH) oxidase activation by regulating the attachment of the cytosolic oxidase components to the plasma or phagosome membrane. In resting neutrophils, NADPH oxidase components are separated in the membrane, cytoplasm, and granules. The membrane bound $gp91^{phox}$ and $p22^{phox}$, collectively termed flavocytochrome b558, are found on the neutrophil membrane and the membranes of specific, secretory, and gelatinase granules (Borregaard et al., 2007). The trimeric complex of $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$, as well as the inactivate GDP-bound Rac2, are found in the cytoplasm (Nunes et al., 2013). Recognition of *S. aureus*, as well as other pathogens, through the engagement of immunoglobulin and complement on the

surface of *S. aureus* by Fc receptors and complement receptors, respectively, initiates a signaling cascade that recruits the cytoplasmic NADPH oxidase components to the membrane bound $gp91^{phox}$ and $p22^{phox}$. Notably, pathogens are not the only activators of NADPH oxidase since cytokines like tumor necrosis factor alpha (TNF-α) can also stimulate ROS production (Gao et al., 2007).

Several lines of evidence suggest neutrophil ROS production is crucial for clearance of *S. aureus*. In the clinical setting, the dependence on oxidative killing mechanisms to protect against *S. aureus* is exemplified in patients with chronic granulomatous disease (CGD) who have a mutation in one of the NADPH oxidase components required for ROS production. Neutrophils from individuals with CGD have significant decreases in ROS production and suffer from recurring life-threatening infections with *S. aureus* the leading causative agent (Ben-Ari et al., 2012; Roos and de Boer, 2014).

In the current study, we investigate the impact of leukocidins on human neutrophil ROS production. We identify that *S. aureus* spatially modulates neutrophil ROS production and uses LukGH and PVL to disrupt signaling pathways that dysregulate extracellular and intracellular ROS production. Surprisingly, we demonstrate that PI3K is required for LukGH stimulation of extracellular neutrophil superoxide production, but that extracellular ROS induced by PVL primarily utilized a PI3K-independent mechanism. Unlike LukGH, PVL also induced robust neutrophil intracellular ROS production that was PI3K and Src-family kinases dependent. The implications of PVL, a soluble agonist, inducing neutrophil intracellular ROS production are discussed. These

data identify another mechanism used by predominant *S. aureus* leukocidins to disrupt neutrophil function.

RESULTS

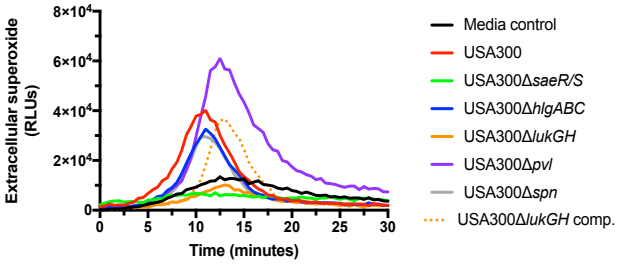
S. aureus bi-component leukocidins modulate human neutrophil ROS production

To determine if *S. aureus* SaeR/S-regulated secreted factors modulate human neutrophil ROS production, we exposed neutrophils to *S. aureus* culture supernatants and measured extracellular superoxide and intracellular ROS. Neutrophils exposed to supernatants from wildtype *S. aureus* USA300 produced extracellular superoxide and intracellular ROS, while neutrophils exposed to supernatants from a *saeR/S* isogenic knockout mutant (USA300Δ*saeR/S*) lacked a ROS response above the baseline ROS [Figure 1A-D]. These results suggested SaeR/S-regulated secreted factors induced both an extracellular and intracellular ROS response. To determine the role of secreted *S. aureus* bi-component leukocidins in inducing neutrophil ROS production, we also exposed neutrophils to supernatants from three different leukocidin isogenic mutants, which have previously been shown to be at least partially regulated by SaeR/S (Zurek et al., 2014). Neutrophil extracellular superoxide production was significantly reduced by supernatants from USA300Δ*lukGH* to a similar extent as neutrophils exposed to the supernatants from USA300Δ*saeR/S*, suggesting that neutrophil extracellular superoxide was primarily induced by LukGH. In contrast, neutrophils exposed to supernatants from USA300Δ*pvl* showed increased extracellular superoxide, suggesting that the presence of PVL decreases extracellular superoxide. Interestingly, supernatants lacking Hlg

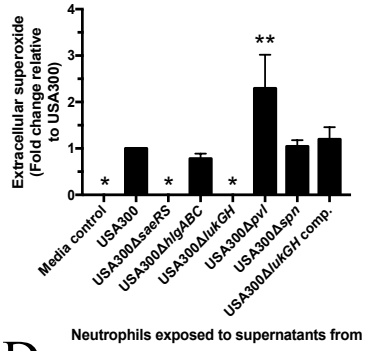
(USA300Δ*hlgABC*), which like PVL targets C5aR (Spaan et al., 2015), did not differentially influence neutrophil ROS production compared to USA300. Supernatants from the recently identified human myeloperoxidase inhibitor, the staphylococcal peroxidase inhibitor (SPIN; *spn*), also did not impact neutrophil ROS production compared to USA300 (de Jong et al., 2017).

These results were inversed when examining neutrophil intracellular ROS production. Neutrophils exposed to supernatants from USA300Δ*lukGH* showed increased intracellular ROS production, whereas they showed decreased extracellular superoxide production [Figure 1A-D]. Thus, the presence of LukGH appeared to stimulate neutrophil extracellular superoxide while decreasing intracellular ROS. Likewise, supernatants from USA300Δ*pvl* decreased neutrophil intracellular ROS while increasing extracellular superoxide compared to the wildtype USA300 supernatant. Thus, PVL appeared to increase neutrophil intracellular ROS and decrease extracellular superoxide. These results were not due to differences in the ability of supernatants to cause neutrophil pore formation since they caused equal membrane permeability compared to the wildtype [Figure 1E]. However, the role of SaeR/S in modulating leukocidins was clear since it did not cause membrane permeability above the baseline.

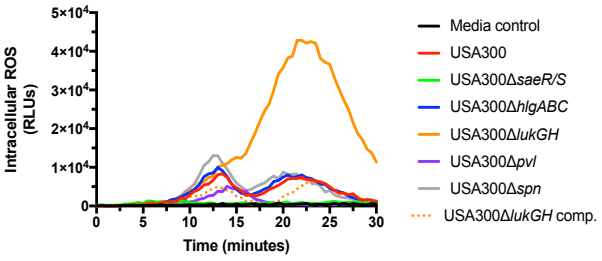
A


Extracellular superoxide (RLUs)
8×10^4
6×10^4
4×10^4
2×10^4
0
0 5 10 15 20 25 30
Time (minutes)
Media control
USA300
USA300ΔsaeR/S
USA300ΔhlgABC
USA300ΔlukGH
USA300Δpvl
USA300Δspn
USA300ΔlukGH comp.

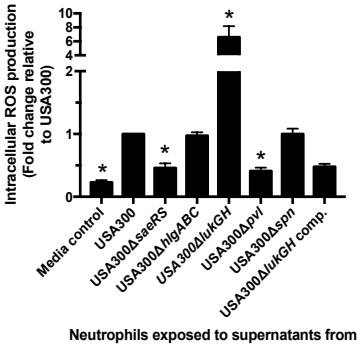
B


Extracellular superoxide (Fold change relative to USA300)
4
3
2
1
0
*
**
Media control
USA300
USA300ΔsaeRS
USA300ΔhlgABC
USA300ΔlukGH
USA300Δpvl
USA300Δspn
USA300ΔlukGH comp.
Neutrophils exposed to supernatants from

C


Intracellular ROS (RLUs)
5×10^4
4×10^4
3×10^4
2×10^4
1×10^4
0
0 5 10 15 20 25 30
Time (minutes)
Media control
USA300
USA300ΔsaeR/S
USA300ΔhlgABC
USA300ΔlukGH
USA300Δpvl
USA300Δspn
USA300ΔlukGH comp.

D


Intracellular ROS production (Fold change relative to USA300)
10
8
6
4
2
1
0
*
Media control
USA300
USA300ΔsaeRS
USA300ΔhlgABC
USA300ΔlukGH
USA300Δpvl
USA300Δspn
USA300ΔlukGH comp.
Neutrophils exposed to supernatants from

E

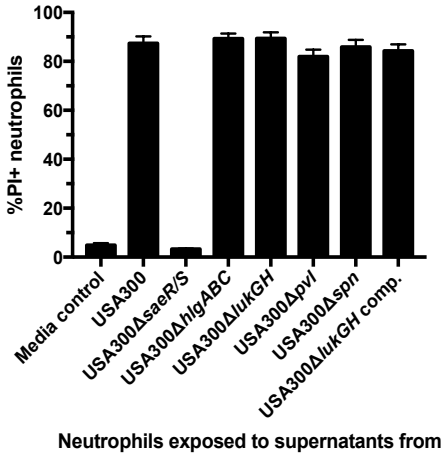

%PI+ neutrophils
100
80
60
40
20
0
Media control
USA300
USA300ΔsaeR/S
USA300ΔhlgABC
USA300ΔlukGH
USA300Δpvl
USA300Δspn
USA300ΔlukGH comp.
Neutrophils exposed to supernatants from

Figure 1. *S. aureus* SaeR/S-regulated secreted factors differentially modulate human neutrophil ROS production. A) Representative plot of human neutrophil extracellular superoxide production in response to supernatants from USA300 and isogenic mutants. B) Area under the neutrophil superoxide peak from 7 neutrophil donors normalized to the area from the superoxide response to supernatant from USA300. C) Representative plot of human neutrophil intracellular ROS production in response to supernatants from USA300 and isogenic mutants. D) Area under the curve from neutrophil intracellular ROS production from 8 neutrophil donors and normalized to the area under the curve to supernatant from USA300. E) Propidium iodide uptake by neutrophils exposed to supernatants from *S. aureus* from 7 donors. * $p < 0.05$, ** $p < 0.01$, as determined by one-way ANOVA with Dunnett's multiple comparisons test. Column bars display average with standard error of the mean. RLUs, relative light units; %PI+, % propidium iodide positive.

Neutrophil ROS production is distinctly modulated by LukGH and PVL

Exposure to *S. aureus* supernatants suggested that LukGH and PVL preferentially induced neutrophil extracellular and intracellular ROS, respectively. To test these findings, we exposed neutrophils to purified recombinant LukGH (rLukGH) or PVL (rPVL) and measured neutrophil extracellular superoxide and intracellular ROS. Neutrophils exposed to purified rLukGH showed pronounced extracellular superoxide production [Figure 2A,E]. In contrast, neutrophil intracellular ROS production in response to LukGH was minimal [Figure 2C,E]. Similarly, neutrophils exposed to PVL produced extracellular superoxide [Figure 2B,F], but also produced significant intracellular ROS [Figure 2D,F]. By comparing the relative amount of neutrophil extracellular superoxide and intracellular ROS during exposure to rLukGH or rPVL from the same blood donors, it is evident that LukGH induced more neutrophil extracellular superoxide than PVL; however, only PVL induced neutrophil intracellular ROS production [Figure 2E-F]. We confirmed these findings by using an intracellular ROS fluorescent probe, dihydroethidium, and analyzing neutrophils by flow cytometry [Figure

S1]. Again, only neutrophils exposed to rPVL showed increased intracellular ROS, and the concentration of rLukGH that induced the highest neutrophil extracellular superoxide production did not induce intracellular ROS. The exposure to rLukGH or rPVL also induced neutrophil pore formation, as shown by uptake in propidium iodide and with similar EC50 as previously reported [Figure 2G-H] (Janesch et al., 2017). To our knowledge, this is the first evidence for a preferential induction of neutrophil intracellular ROS by PVL or any other *S. aureus* bi-component leukocidin.

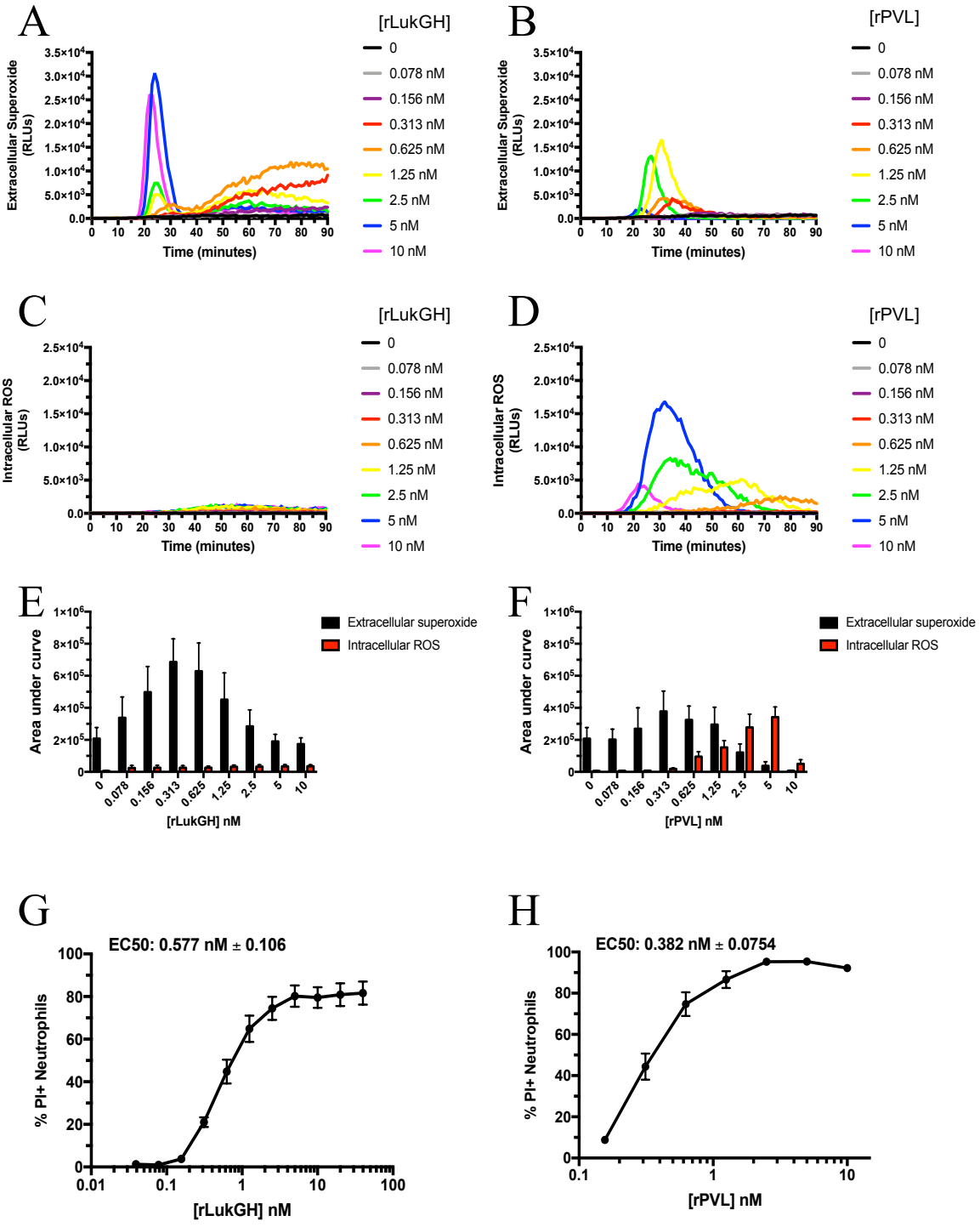

A
Extracellular Superoxide (RLUs)
Time (minutes)
[rLukGH]
0
0.078 nM
0.156 nM
0.313 nM
0.625 nM
1.25 nM
2.5 nM
5 nM
10 nM
B
Extracellular Superoxide (RLUs)
Time (minutes)
[rPVL]
0
0.078 nM
0.156 nM
0.313 nM
0.625 nM
1.25 nM
2.5 nM
5 nM
10 nM
C
Intracellular ROS (RLUs)
Time (minutes)
[rLukGH]
0
0.078 nM
0.156 nM
0.313 nM
0.625 nM
1.25 nM
2.5 nM
5 nM
10 nM
D
Intracellular ROS (RLUs)
Time (minutes)
[rPVL]
0
0.078 nM
0.156 nM
0.313 nM
0.625 nM
1.25 nM
2.5 nM
5 nM
10 nM
E
Area under curve
Extracellular superoxide
Intracellular ROS
[rLukGH] nM
F
Area under curve
Extracellular superoxide
Intracellular ROS
[rPVL] nM
G
EC50: 0.577 nM ± 0.106
% PI+ Neutrophils
[rLukGH] nM
H
EC50: 0.382 nM ± 0.0754
% PI+ Neutrophils
[rPVL] nM

Figure 2. *S. aureus* leukocidins LukGH and PVL induce distinct neutrophil ROS responses. (A) Representative plot of rLukGH-induced neutrophil extracellular superoxide production. (B) Representative plot of rPVL-induced neutrophil extracellular superoxide production. (C) rLukGH does not induce neutrophil intracellular ROS production while rPVL does, as shown in (D). Equal y-axis scale between (C) and (D) shows the clear difference in neutrophil intracellular ROS induced by rPVL but not rLukGH. (E) and (F) display the relative neutrophil extracellular superoxide and intracellular ROS, respectively, in response to rLukGH or rPVL from 5 donors. (G) and (H) show the concentration dependent neutrophil plasma membrane damage induced by rLukGH and rPVL from 4 or 5 donors, respectively.

Physiological ligands do not induce the same neutrophil ROS response through the receptors targeted by LukGH and PVL

The complement C5 fragment has previously been reported to induce neutrophil ROS production through interaction with the C5aR (Ibrahim et al., 2004). Thus, we wanted to determine whether the PVL intracellular ROS response was a general mechanism through the C5a receptor, which is also the target receptor for PVL (Spaan et al., 2013). Human neutrophils exposed to purified recombinant C5a showed only prolonged extracellular superoxide production and lacked intracellular ROS production, as opposed to neutrophils exposed to PVL that displayed extracellular superoxide and robust intracellular ROS production [Figure 3]. It should be noted that neutrophils exposed to rPVL show initial superoxide production that then is quickly halted [Figure S2]. Similarly, we decided to determine if physiological ligands of CD11b induced a similar neutrophil ROS response as rLukGH. Neutrophils exposed to iC3b or fibrinogen, known CD11b ligands that also interact with the I-domain of CD11b required for binding of LukGH, did not induce neutrophil extracellular or intracellular ROS [Figure 3] (DuMont et al., 2013; Lishko et al., 2004; Ueda et al., 1994). In contrast, rLukGH

induced robust neutrophil extracellular superoxide production but not intracellular ROS [Figure 3]. Even though iC3b, fibrinogen, and LukGH require the I-domain for binding to CD11b, iC3b or fibrinogen did not inhibit rLukGH pore formation [Figure S3]. Human MPO, which is suggested to be a CD11b ligand, also did not prevent rLukGH plasma membrane damage (Johansson et al., 1997; Lau et al., 2005; Podolnikova et al., 2015). Prevention of LukGH pore formation by anti-CD11b monoclonal antibodies was epitope specific and only partial, as previously shown (DuMont et al., 2013) [Figure S3].

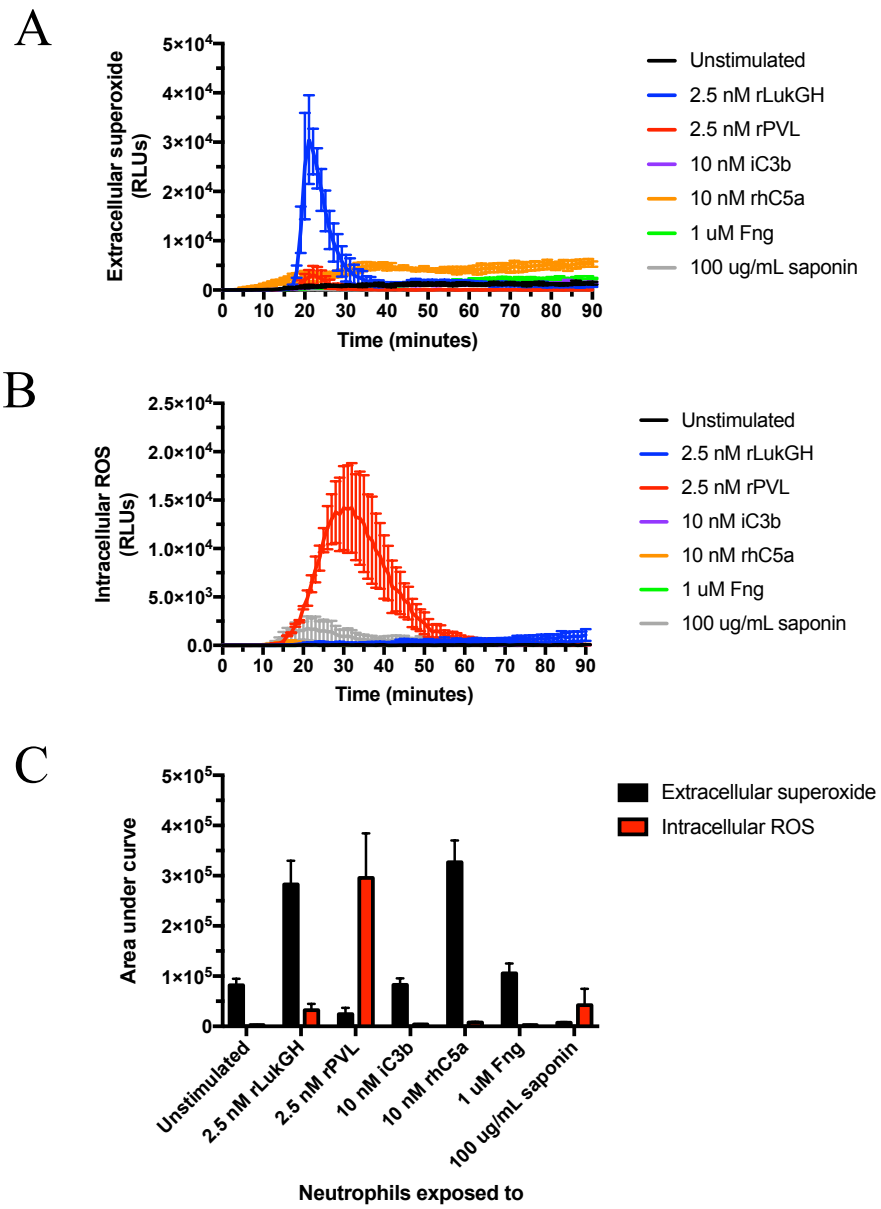


Figure 3. Physiological ligands to receptors targeted by LukGH and PVL fail to elicit similar neutrophil ROS responses. A) The CD11b ligands iC3b or fibrinogen (Fng) do not induce neutrophil extracellular superoxide, whereas rLukGH induces extracellular superoxide. The C5a receptor ligand rhC5a induces prolonged neutrophil superoxide production, whereas rPVL induces a short extracellular superoxide burst. (B) rPVL, and to a much lesser extent the membrane permeabilizing agent saponin, induce intracellular ROS. (C) Displays the relative neutrophil extracellular superoxide and intracellular ROS in response to rLukGH, rPVL, and physiological ligands targeting the same receptors as LukGH and PVL. Plots show average responses from 3 separate donors with the standard error of the mean.

PI3K is required for LukGH and C5a stimulation of neutrophil superoxide production but only partially required for PVL-induced neutrophil ROS production

Since the PI3K phosphorylated lipid products have an important role in regulating neutrophil ROS production partially by recruiting $p47^{phox}$ and $p40^{phox}$ to membranes through their PX domains, we tested the effect of the general PI3K inhibitor wortmannin on neutrophil ROS production in response to rLukGH, rPVL, and rhC5a (Hawkins and Stephens, 2015; Kanai et al., 2001; Nunes et al., 2013; Wymann et al., 1996; Zhan et al., 2002). The neutrophil extracellular superoxide production induced by rLukGH and rhC5a was >80% percent inhibited by wortmannin [Figure 4A-B]. However, the neutrophil extracellular superoxide production induced by rPVL was only partially inhibited by wortmannin, suggesting a significant role for PI3K-independent mechanisms in modulating neutrophil superoxide production specifically to PVL. The neutrophil intracellular ROS production induced by rPVL was largely dependent on PI3K with ~90% inhibition in the presence of wortmannin [Figure 4C-D].

The dichotomous findings in the role of PI3K regulating neutrophil extracellular and intracellular ROS production in response to rPVL led us to investigate the role of specific class I PI3K isoforms and class III PI3K in neutrophil ROS production. We treated neutrophils with specific PI3K isoform inhibitors prior to stimulation with rLukGH, rPVL, and rhC5a. As shown in Figure 4E, neutrophil extracellular superoxide induced by rLukGH was largely dependent on the class I isoforms PI3Kβ and PI3Kγ. Comparatively, PI3Kδ showed a reduced impact and PI3Kα appears to only minimally impact extracellular superoxide production induced by rLukGH. The neutrophil

extracellular superoxide production induced by rhC5a was also largely dependent on PI3Kβ and PI3Kγ, but PI3Kδ was equally important in response to rhC5a. Additionally, PI3Kα was partially required for the rhC5a-induced neutrophil extracellular superoxide. In contrast, neutrophil superoxide oxide production in response to rPVL was only partially dependent on any of the class I PI3K isoforms, which is in congruence with the partial effect of wortmannin on PVL-induced neutrophil superoxide production [Figure 4A-B]. Neutrophil intracellular ROS production induced by rPVL was significantly more dependent on class I PI3Kβ than the α, δ, or γ isoforms [Figure 4F].

Since previous studies have shown the lone class III PI3K, Vps34, is important for intracellular phagosome ROS production in response to *S. aureus* (Anderson et al., 2008), we used a Vps34 inhibitor to determine its role inducing neutrophil extracellular superoxide and rPVL-induced neutrophil intracellular ROS. As shown in Figure 4G, Vps34 did not have a significant role in regulating neutrophil extracellular ROS production in response to rLukGH, rPVL, or rhC5a. Likewise, the Vps34 inhibitor reduced neutrophil intracellular ROS by only ~20% in response to rPVL [Figure 4H].

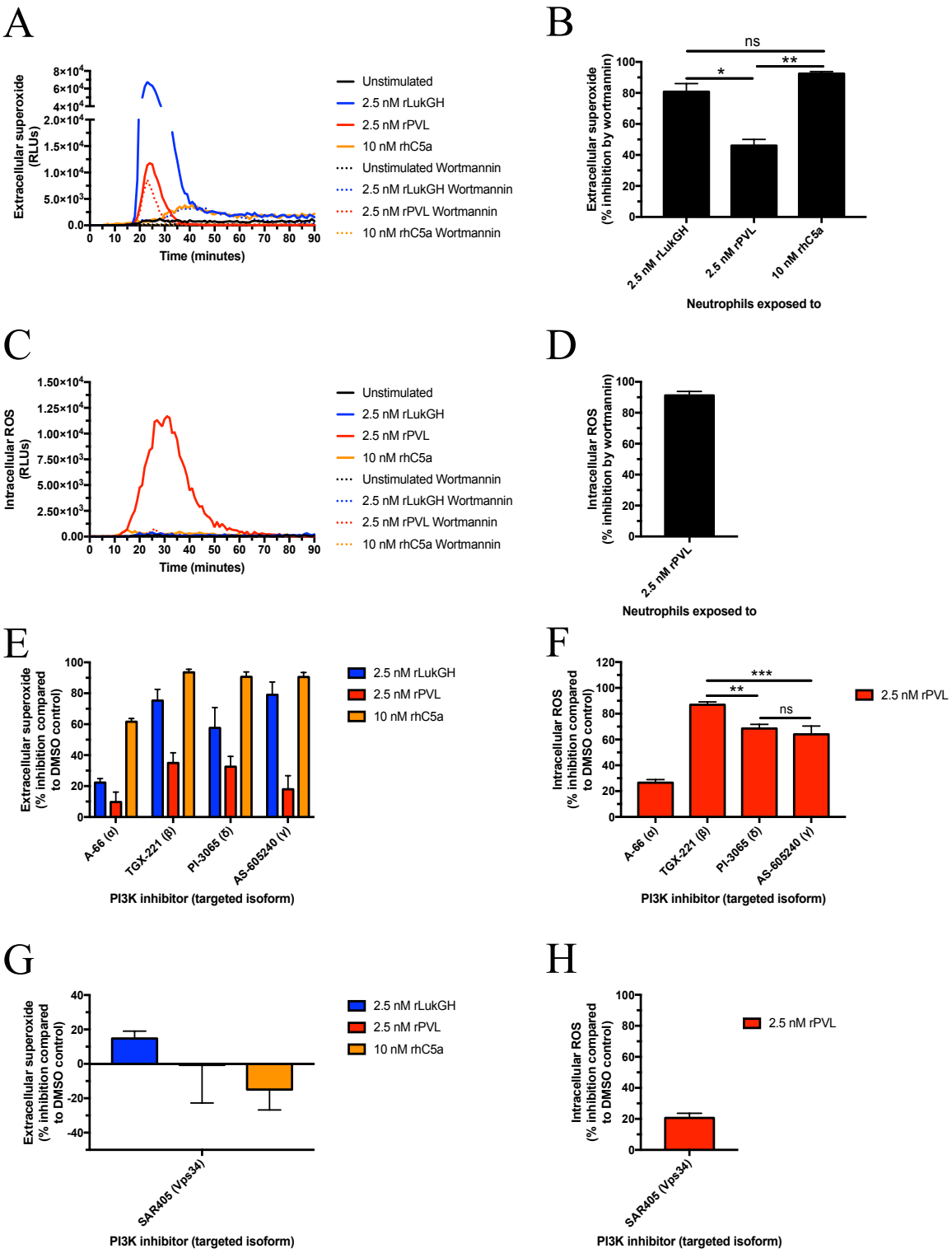

A
Extracellular superoxide (RLUs)
Time (minutes)
Unstimulated
2.5 nM rLukGH
2.5 nM rPVL
10 nM rhC5a
Unstimulated Wortmannin
2.5 nM rLukGH Wortmannin
2.5 nM rPVL Wortmannin
10 nM rhC5a Wortmannin
B
Extracellular superoxide (% inhibition by wortmannin)
ns
*
**
2.5 nM rLukGH
2.5 nM rPVL
10 nM rhC5a
Neutrophils exposed to
C
Intracellular ROS (RLUs)
Time (minutes)
Unstimulated
2.5 nM rLukGH
2.5 nM rPVL
10 nM rhC5a
Unstimulated Wortmannin
2.5 nM rLukGH Wortmannin
2.5 nM rPVL Wortmannin
10 nM rhC5a Wortmannin
D
Intracellular ROS (% inhibition by wortmannin)
2.5 nM rPVL
Neutrophils exposed to
E
Extracellular superoxide (% inhibition compared to DMSO control)
2.5 nM rLukGH
2.5 nM rPVL
10 nM rhC5a
A-66 (α)
TGX-221 (β)
PI-3065 (δ)
AS-605240 (γ)
PI3K inhibitor (targeted isoform)
F
Intracellular ROS (% inhibition compared to DMSO control)
***
**
ns
2.5 nM rPVL
A-66 (α)
TGX-221 (β)
PI-3065 (δ)
AS-605240 (γ)
PI3K inhibitor (targeted isoform)
G
Extracellular superoxide (% inhibition compared to DMSO control)
2.5 nM rLukGH
2.5 nM rPVL
10 nM rhC5a
SAR405 (Vps34)
PI3K inhibitor (targeted isoform)
H
Intracellular ROS (% inhibition compared to DMSO control)
2.5 nM rPVL
SAR405 (Vps34)
PI3K inhibitor (targeted isoform)

Figure 4. Involvement of PI3K in human neutrophil ROS production to *S. aureus* LukGH, PVL, and C5a. A) Representative plot of neutrophil extracellular ROS production and the effect of the PI3K general inhibitor wortmannin. B) The inhibitory effect of wortmannin on neutrophil extracellular superoxide in response to rLukGH, rPVL, and rhC5a from 4 donors. C) Representative plot of neutrophil intracellular ROS production and the effect of wortmannin. D) Percent inhibition of wortmannin on neutrophil intracellular ROS in response to rPVL from 4 donors. E) Percent inhibition of specific PI3K class I isoform inhibitors on neutrophil extracellular superoxide in response to rLukGH, rPVL, and rhC5a from 5 donors. F) Percent inhibition of specific PI3K class I isoform inhibitors on neutrophil intracellular ROS in response to rPVL from 6 donors. G) Percent inhibition of the PI3K class III isoform Vps34 on neutrophil extracellular superoxide production in response to rLukGH, rPVL, and rhC5a. H) Percent inhibition of intracellular ROS production in response to PVL from 4 donors. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as determined by one-way ANOVA with Tukey's multiple comparisons test. Column bars display average with standard error of the mean.

Modulation of neutrophil ROS by Src family kinases and Syk in response to LukGH, PVL, and C5a

Neutrophil integrin signaling is dependent on Src family kinases (SFKs) and Syk while the reliance of GPCRs on SFKs and Syk is less defined (Futosi et al., 2013; Mócsai et al., 2003). To further elucidate the signaling pathways required for neutrophil ROS production in response to LukGH, PVL, and C5a, we used chemical inhibitors of SFKs and Syk. The neutrophil extracellular superoxide response induced by rLukGH and rhC5a was highly dependent on SFKs, while the rPVL-induced neutrophil extracellular superoxide was unaffected by inhibition of SFKs [Figure 5A]. In contrast, rPVL-induced neutrophil extracellular superoxide production was completely dependent on Syk with >90% inhibition in the presence of the specific Syk inhibitor PRT-060-318. Syk inhibition partially inhibited rLukGH and rhC5a induced neutrophil extracellular superoxide. While SFKs were not required for neutrophil extracellular superoxide

production in response to rPVL, SFKs were essential for rPVL-induced neutrophil intracellular ROS [Figure 5B]. On the other hand, Syk was only partially required for intracellular ROS induced by rPVL.

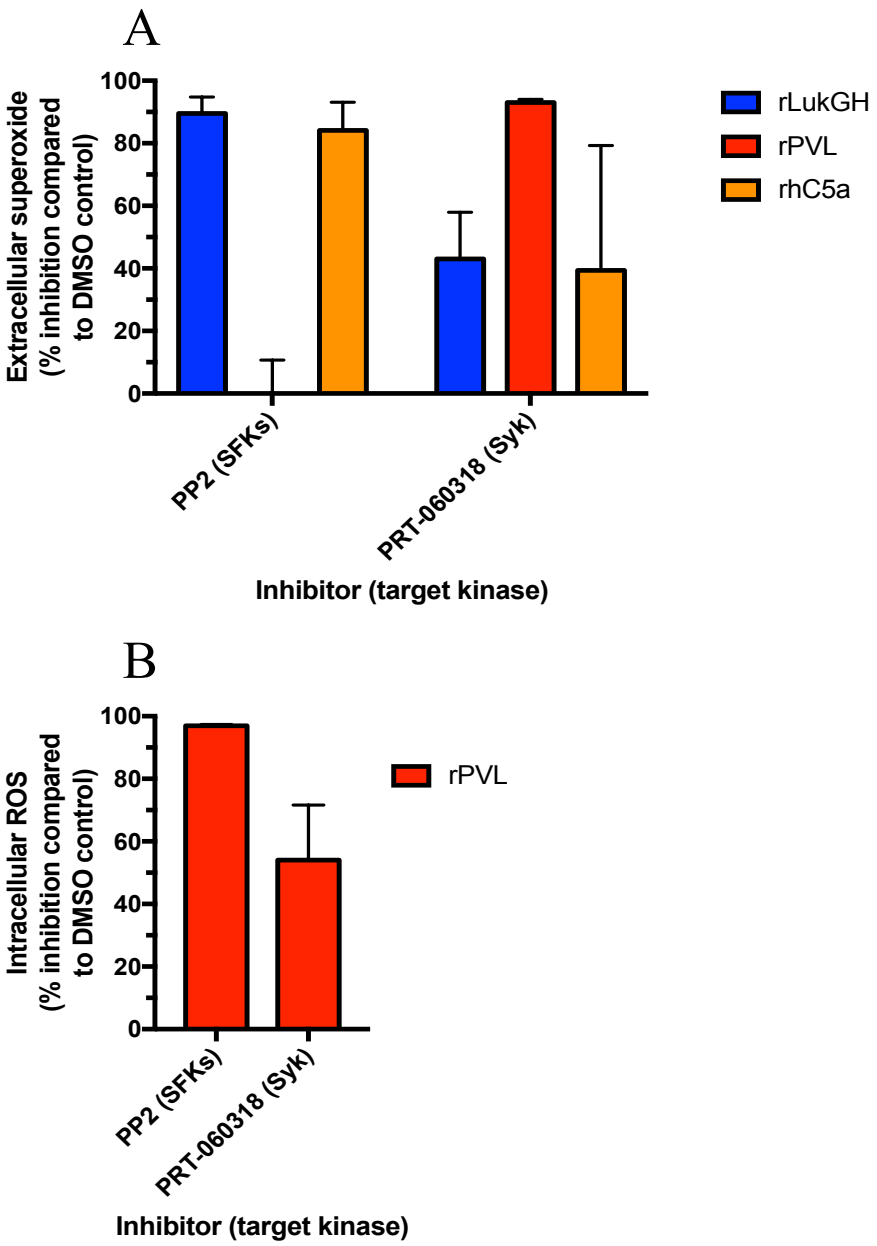


Figure 5. Src family kinases and Syk are involved in neutrophil ROS production in response to LukGH, PVL, and C5a. A) Src family kinases (SFKs) are required for neutrophil extracellular superoxide induced by rLukGH and rhC5a but not rPVL. In contrast, Syk is required for rPVL-induced neutrophil extracellular superoxide but only partially required in response to rLukGH and rhC5a. B) SFKs are required for neutrophil intracellular ROS induced by rPVL but Syk only partially. Data is from 2 donors and column bars show the average with the standard error of the mean.

DISCUSSION

Recent studies have identified the inhibition of neutrophil ROS production as a virulence mechanism used by *S. aureus* to survive neutrophil killing (de Jong et al., 2017; Guerra et al., 2016; Sward et al., 2018). *S. aureus* killing by neutrophils is highly dependent on oxidant production, as opposed to other organisms like *Pseudomonas aeruginosa* and *Escherichia coli* that are susceptible to non-oxidative killing (Becker et al., 2017; McGovern et al., 2011). In addition, *S. aureus* employs strategies to limit damage by neutrophil ROS including catalase and superoxide dismutase, as well as the antioxidant staphyloxanthin that gives *S. aureus* its iconic golden pigment (Ballal and Manna, 2009; Clauditz et al., 2006; Cosgrove et al., 2007; Hall et al., 2017; Pelz et al., 2005). Thus, the inhibition and dysregulation of neutrophil ROS production by *S. aureus* is a virulence strategy to circumvent the neutrophil killing mechanisms with most efficacy against *S. aureus*.

The production of neutrophil reactive oxygen species (ROS) is controlled by complex pathways that involve membrane and cytoplasmic proteins, lipid metabolism, phosphorylation events, ion fluxes, short-lived radicals, requiring temporal and spatial accuracy to ensure proper pathogen killing and prevent self-harm from toxic oxygen metabolites. Class I PI3Ks phosphorylate the membrane lipid phosphatidylinositol-4,5-disphosphate (PtdIns(4,5)P2)) to phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3)). Phosphatase activity converts PtdIns(3,4,5)P3 into PtdIns(3,4)P2 (Luo and Mondal, 2015). The PX domain of the $p47^{phox}$ subunit of NADPH oxidase contains two binding pockets involved in attachment to the membrane; one pocket preferentially

binds PtdIns(3,4)P2 and the second pocket can bind phosphatidylserine or phosphatidic acid (Karathanassis et al., 2002; Nunes et al., 2013). In contrast, the PX domain of the $p40^{phox}$ subunit preferentially binds to phosphatidylinositol-3-phosphate (PtdIns(3)P) through the class III PI3K Vps34 on phagosomes and this binding is essential for intracellular superoxide production in response to phagocytosed *S. aureus* (Ellson et al., 2006; Karathanassis et al., 2002; Suh et al., 2006). Activation through GPCRs also activates phospholipase C to hydrolyze PtdIns(4,5)P2 into diacylglycerol and inositol-1,4,5-trisphosphate (Ins(1,4,5)P3). Diacylglycerol is the physiological activator of protein kinase C and its phosphorylation on $p47^{phox}$ activates NADPH oxidase activity (El Benna et al., 1996; Madani et al., 2001; Nguyen et al., 2017; Suh and Hille, 2005). Ins(1,4,5)P3 induces the release of intracellular calcium stores and intake of extracellular calcium, which is required protein kinase C activation, assembly of NADPH oxidase and sustained superoxide production (El Chemaly et al., 2010; Lipp and Reither, 2011; Nunes et al., 2013).

Few studies have shown that *S. aureus* secreted products have activating properties towards neutrophils including upregulation of CD11b, priming, neutrophil extracellular trap formation, ROS production, and calcium mobilization (Colin and Monteil, 2003; König et al., 1995; Malachowa et al., 2013; Schmitz et al., 1997; Spaan et al., 2013; Veldkamp et al., 1997). Since *S. aureus* is sensitive to neutrophil ROS production, we hypothesized leukocidins secreted by *S. aureus* could directly activate neutrophil ROS production as a virulence strategy. Indeed, we demonstrated that exposure of neutrophils to *S. aureus* secreted products stimulated neutrophil ROS

production in a SaeR/S-dependent mechanism. Exposure of neutrophils to supernatants from specific isogenic mutants suggested the neutrophil ROS response was differentially modulated by LukGH and PVL. Neutrophils exposed to purified LukGH produced extracellular superoxide but no detectable intracellular ROS. The inhibition of PI3Ks by wortmannin showed that phosphatidylinositol lipid signaling was required for neutrophil extracellular superoxide in response to LukGH. Since "outside-in" signaling through integrins requires Src-family kinases (SFKs) and Syk tyrosine kinase, we used chemical inhibitors of these kinases to further elucidate the signaling pathways downstream of LukGH binding to the CD11b/CD18 integrin (Futosi et al., 2013; Mócsai et al., 2006). The neutrophil extracellular superoxide induced by LukGH required SFKs and to a lesser extent Syk (Figure 6). Importantly, the stimulation through CD11b was specific to LukGH since the physiological ligands fibrinogen and iC3b did not induce extracellular superoxide.

Surprisingly, purified PVL induced both neutrophil extracellular superoxide and intracellular ROS (Figure 3). In contrast to LukGH, neutrophil extracellular superoxide production induced by PVL was largely PI3K-independent since PI3K inhibition with wortmannin did not abolish extracellular superoxide. Furthermore, neutrophil extracellular superoxide did not require SFKs but fully required Syk activity. The magnitude of extracellular superoxide induced by LukGH is consistently greater than that induced by PVL. Our data suggest LukGH uses two diverging signaling pathways, an SFK-Syk-dependent and SFK-dependent/Syk-independent, to induce extracellular superoxide. The combinatorial effect of these two pathways could explain the higher

extracellular superoxide induced by LukGH compared to PVL, which only depends on a Syk-dependent pathway. Unlike LukGH, PVL induced robust neutrophil intracellular ROS production, which was unexpected since PVL is a soluble protein and intracellular ROS is associated with particulate phagosome formation. Interestingly, the signaling pathway required for PVL-induced neutrophil intracellular ROS production mimicked that of LukGH-induced extracellular superoxide formation; that is, SFKs and PI3K (primarily through the PI3Kβ isoform) were required while Syk was partially required. This latter finding is interesting since GPCR activation through Syk is a controversial area of study (Mócsai et al., 2003). Even though C5a and PVL signal through C5aR, they elicit spatially different ROS production in neutrophils. Neutrophil stimulation with C5a only induced extracellular superoxide production, which similar to LukGH required SFKs and to a lesser extent Syk. This further supports a novel role for Syk in human neutrophil GPCR signaling through C5aR.

Our results begin to unravel additional effects of *S. aureus* leukocidins on human neutrophils. *S. aureus* leukocidins display redundancy in neutrophil pore-formation, but they do not show redundancy inducing neutrophil signaling pathways. LukGH induction of neutrophil extracellular ROS production directly through the αMβ2 integrin (CD11b/CD18) opposes the activity of physiological CD11b/CD18 ligands, which are shown to inhibit NADPH oxidase activation in the absence of a co-stimulatory signal (Zhao et al., 2003). We were able to confirm that the physiological CD11b/CD18 ligand fibrinogen inhibits spontaneous neutrophil superoxide production (data not shown). To our knowledge, only myeloperoxidase (MPO) has previously been shown to induce

neutrophil extracellular superoxide production directly through CD11b (Lau et al., 2005). In our studies, MPO did not inhibit LukGH pore formation in neutrophils suggesting that MPO and LukGH bind different sites on CD11b or that LukGH can displace MPO from CD11b (Figure S3). Future studies will determine if MPO induces neutrophil extracellular superoxide production under our assay conditions. Additionally, our data does not rule out the possibility of LukGH using other receptors to induce signaling or pore formation since anti-CD11b antibodies and CD11b physiological ligands showed poor ability to inhibit LukGH pore formation (Figure S3).

The neutrophil non-phagosomal intracellular ROS induced by PVL is of particular interest since the signaling pathway differs from that of C5a, even though PVL and C5a share the same binding receptor. This suggests that other receptors are involved in PVL-neutrophil interaction. A recent study using a CRISPR-Cas9 library screen in U937 cells confirmed C5aR is needed for PVL-induced cytotoxicity (Tromp et al., 2018). Surprisingly, PTPRC (CD45) was a higher cytotoxic hit than C5aR. CD45 is a leukocyte specific receptor-like protein phosphatase better known for its role in T and B cell activation. Interestingly, CD45 can activate SFKs that subsequently activate Syk. Thus, the signal crosstalk by PVL-C5aR(GPCR)-CD45(SFKs/Syk) will be further investigated since SFK activity is essential for PVL-induced intracellular ROS.

Collectively, non-phagosomal intracellular ROS production induced in neutrophils by a *S. aureus* secreted toxin may be an ideal mechanism to prevent proper staphylococcal clearance. By secreting PVL, *S. aureus* potentially “short-circuits” neutrophil signaling to prematurely activate the NADPH oxidase components

intracellularly before phagocytosis of *S. aureus*. The extracellular superoxide production induced by PVL is potentially a general mechanism induced through C5aR signaling since C5a also induces neutrophil extracellular superoxide production. But, why preferential induction of intracellular and not extracellular ROS? Under our assay conditions, neutrophil intracellular ROS production is luminol-based, which is dependent on MPO. Azurophilic (primary) granules contain MPO and are spatially separated from secondary granules that contain $gp91^{phox}/p22^{phox}$. Our results suggest PVL induces primary and secondary granule fusion expending cellular antimicrobial resources prior to *S. aureus* phagocytosis, thus potentially reprogramming neutrophil fate to favor pathogen survival. Current studies using immunofluorescence are underway to analyze neutrophil granule localization in response to PVL. The robust neutrophil intracellular ROS induced by PVL is also likely to activate antioxidant mechanisms. This is supported by published data showing upregulation of the nuclear factor erythroid 2-related factor (Nrf2) related genes in response to PVL (Graves et al., 2012). Nrf2 is a cytoplasmic redox sensing transcription factor activated by oxidative stress, and ongoing studies are investigating its role in altering neutrophil cell death during PVL-induced intracellular ROS production (Ma, 2013).

In conclusion, *S. aureus* secreted leukocidins not only bind neutrophil surface receptors to induce pore formation but also to induce distinct signaling pathways that differ from signaling pathways induced by physiological ligands (Figure 6). The signaling pathways induced by *S. aureus* leukocidins are likely a virulence mechanism to prematurely activate neutrophil antimicrobial mechanisms prior to phagocytosis. Thus,

neutrophil reactive oxygen species production, which is the primary bactericidal mechanism used by neutrophils to kill *S. aureus*, is disrupted and likely contributes to altered neutrophil fate ultimately leading to *S. aureus* immune evasion and survival.

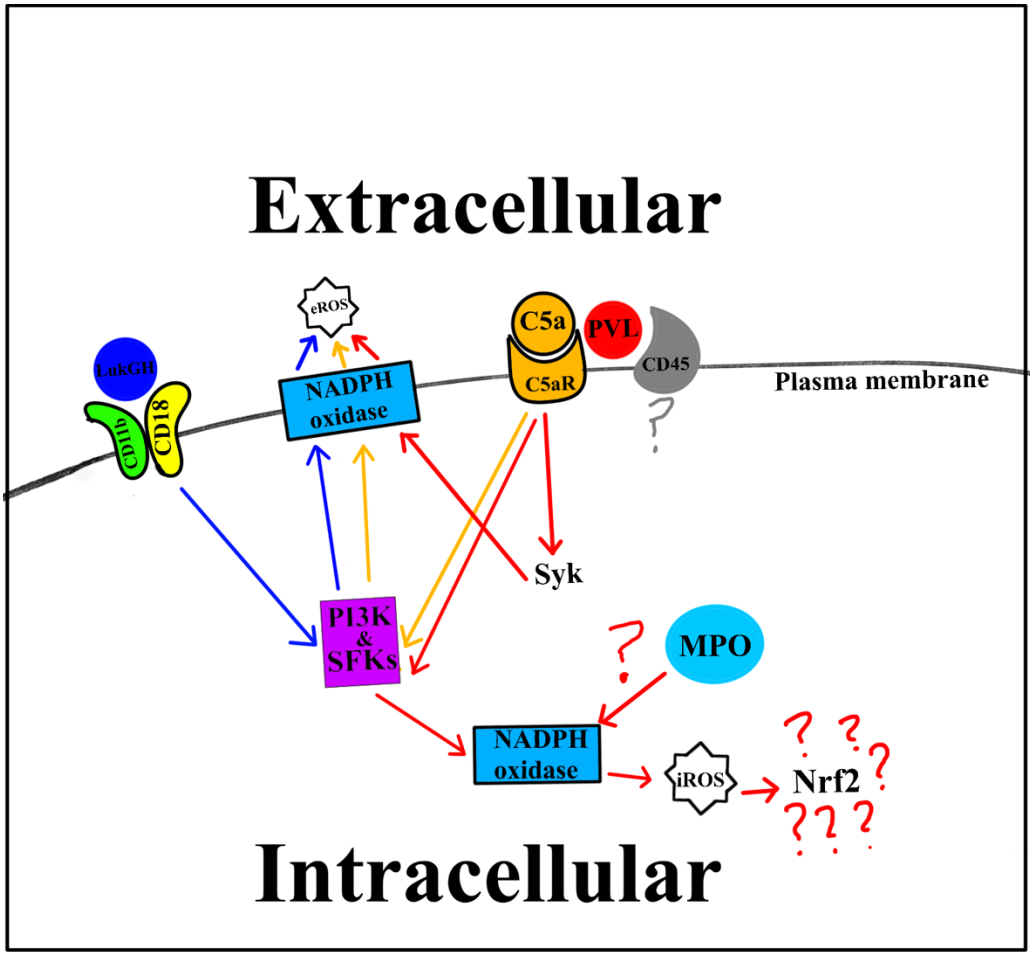


Figure 6. LukGH, PVL, and C5a neutrophil signaling leading to ROS production. The color of the arrows corresponds to the ligand. For example, LukGH (colored blue) requires PI3K and Src-family kinases (SFKs) to induce extracellular ROS (eROS). Question marks signify unknown mechanisms and future studies. iROS, intracellular ROS.

MATERIALS AND METHODS

Bacterial strains and supernatants

Wildtype *S. aureus* pulsed-field gel electrophoresis-type USA300 (Diep et al., 2006), and isogenic mutant USA300Δ*saeR/S*, USA300Δ*pvl,* were generated previously (Voyich et al., 2006, 2009). USA300Δ*lukGH,* USA300Δ*hlgABC,* USA300Δ*spn*, and the USA300Δ*lukGH* with a *lukGH* complemented plasmid (USA300Δ*lukGH* comp.) were generated for this study using previously published methods (Nygaard et al., 2012). All strains were grown in tryptic soy broth supplemented with 0.5% glucose and harvested at mid-exponential growth, as described previously (Nygaard et al., 2010; Voyich et al., 2005; Zurek et al., 2014). Bacteria were opsonized in Dulbecco's phosphate buffered saline (DPBS) containing 50% human serum for 30 minutes, 37°C, with intermittent shaking. Human serum was removed after centrifugation, bacteria were washed once with DPBS by centrifugation, and the remaining opsonized bacterial pellets were resuspended in RPMI 1640. Opsonized bacteria were diluted to $1x10^7$ CFUs/mL in 600 μL RPMI final volume in 24-well serum-coated plates. Bacterial cultures were collected after incubating for 5 hours, 37°C, 5% CO_2, and supernatants were harvested by sterile-filtration (0.2 μm). Sterile-filtered supernatants were diluted 1:3 and were used immediately or stored undiluted at -80°C until use.

Human neutrophil isolation and ROS assays

Heparinized venous blood from healthy volunteers was collected in accordance with a protocol approved by the Institutional Review Board for Human Subjects at Montana State University. All donors provided written consent to participate in the study. Neutrophils were isolated under endotoxin-free conditions (< 25 pg/mL) by erythrocyte sedimentation with dextran and neutrophil sedimentation with Ficoll-Paque PLUS, as previously described, and resuspended in RPMI at $1x10^7$ neutrophils/mL (Voyich et al., 2005, 2009). For all neutrophil ROS assays, the same ROS probe staining procedures were followed as described below. Neutrophil extracellular superoxide production was measured with cell-impermeable, superoxide dismutase-inhibitable Diogenes (National Diagnostics, Atlanta, GA, USA) following the manufacturer's protocol. Briefly, purified neutrophils were diluted 1:2 with Diogenes for 15 minutes, 4°C, in the dark. For intracellular ROS production, neutrophils were diluted 1:2 with 400 μM luminol (Sigma-Aldrich, St. Louis, MO, USA) for 15 minutes, 4°C, in the dark. For intracellular ROS detection with dihydroethidium (DHE; Thermo Fisher Scientific, Waltham, MA, USA), purified neutrophils were diluted 1:2 with 40 μM DHE for 15 minutes, 4°C, in the dark. After staining, 100 μL ($5x10^5$ neutrophils) of stained neutrophils were loaded into plasma-coated 96-well plates. For intracellular ROS detection with luminol and DHE, neutrophils were supplemented with 500 U/mL each of superoxide dismutase (Sigma-Aldrich) and catalase (MilliporeSigma, Burlington, MA, USA) to neutralize extracellular superoxide and hydrogen peroxide, respectively. After adding inhibitors and stimulants, as described below, Diogenes or luminol stained neutrophils were placed in a 37°C

heated SpectraMax Paradigm Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) and chemiluminescence was measured in 30 second or minute intervals for specified times. For DHE intracellular ROS detection, neutrophils were analyzed by flow cytometry in a BD FACSCalibur (BD Biosciences, San Jose, CA, USA) at the specified time.

Neutrophil intoxication

Neutrophils stained with Diogenes and luminol as described above were stimulated with sterile-filtered bacterial supernatants (diluted 1:3). Neutrophil ROS production by chemiluminescence was measured every 30 seconds for 30 minutes. Purified recombinant LukGH and the PVL subunits LukF-PV and LukS-PV were acquired from IBT Bioservices (Rockville, MD, USA). Equimolar concentrations of LukF-PV and LukS-PV were mixed to form functional LukSF-PV leukocidin (PVL). LukGH, PVL, fibrinogen (MilliporeSigma, Burlington, MA, USA), iC3b (Quidel, San Diego, CA, USA), recombinant human C5a (R&D Systems), or saponin (MilliporeSigma, Burlington, MA, USA) were added to probe stained neutrophils and chemiluminescence was measured in 1-minute intervals for 90 minutes. The area under the curve for each condition was integrated to calculate total ROS production. Neutrophils were exposed to propidium iodide and analyzed by flow cytometry to determine cell membrane permeability, as previously described (Flack et al., 2014).

CD11b neutralization

Neutrophils were incubated with monoclonal anti-CD11b clone LM2/1 (Thermo Fisher Scientific), clone ICRF44 (BioLegend, San Diego, CA, USA), isotype control (BioLegend), iC3b, fibrinogen, or human myeloperoxidase (R&D Systems, Minneapolis, MN, USA) at specified concentrations for 30 minutes at 4°C. Then, neutrophils were exposed to 5 nM LukGH for 1 hour at 37°C, 5% CO_2. Following incubation, neutrophil cell permeability was analyzed by propidium iodide uptake with flow cytometry (Flack et al., 2014).

Neutrophil inhibition of PI3K, Src family kinases, and Syk

Following loading with Diogenes or luminol, neutrophils were incubated for 15 minutes at 4°C, in the dark with 100 nM wortmannin, or 500 nM A-66, or 500 nM TGX-221, or 100 nM PI-3065, or 500 nM AS-605240, or 1 μM SAR405 (all from Cayman Chemical Company, Ann Arbor, MI, USA). Amount and exposure time was based on concentrations used in published studies that demonstrated inhibition of PI3K and specific isoforms (Anderson et al., 2008; Essig et al., 2017; Jamieson et al., 2011; Kobayashi et al., 2011; Pittini et al., 2016). To inhibit Src family kinases or Syk activity, neutrophils were preincubated with 10 μM PP2 or 1 μM PRT-060318, respectively (both from Selleckchem, Houston, TX, USA) for 15 minutes at 4°C, in the dark (Fumagalli et al., 2013; Reilly et al., 2011). Subsequently, neutrophils were stimulated as described

above and the area under the curve for each condition was integrated to calculate total ROS production.

Statistical procedures

Statistical analyses were performed using GraphPad Prism version 7.0a (GraphPad Software, La Jolla, CA, USA) with *t* tests and ANOVA as indicated. Error bars represent the standard error of the mean.

SUPPLEMENTAL FIGURES

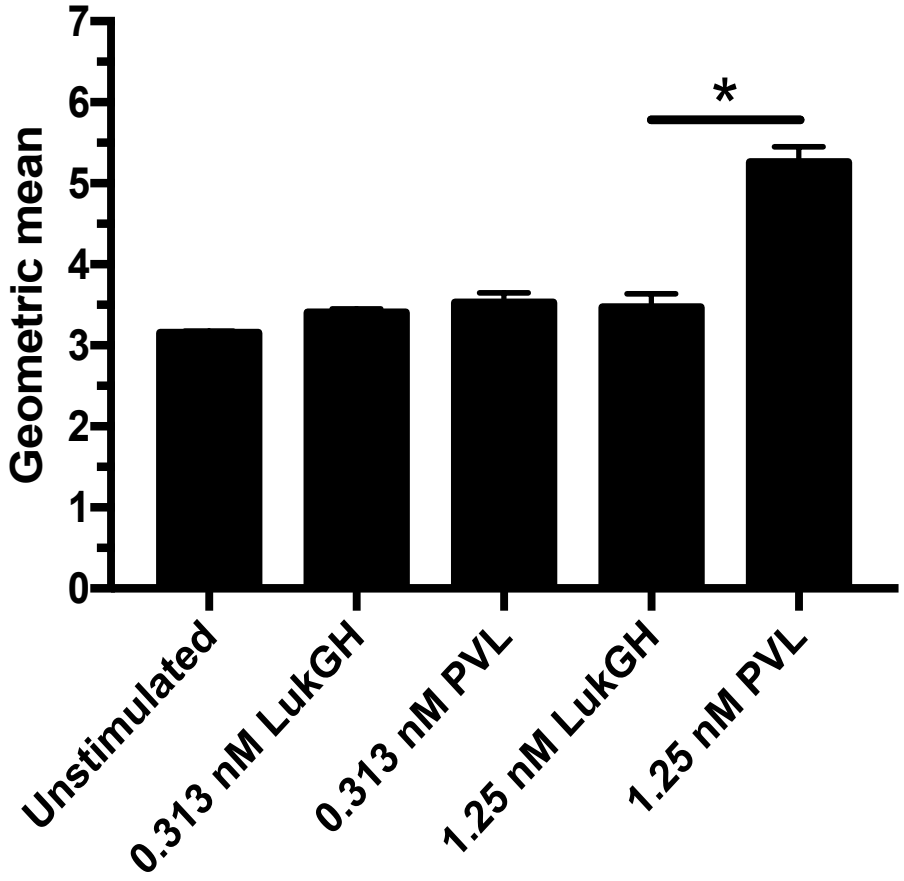


Figure S1. Neutrophil intracellular ROS production detected with dihydroethidium. Only neutrophils exposed to rPVL produce intracellular ROS. Lower concentrations of rPVL (0.313 nM) induce extracellular superoxide production, which under our conditions does not leak into the neutrophil to react intracellularly. Data is from 4 donors. * $p < 0.05$, as

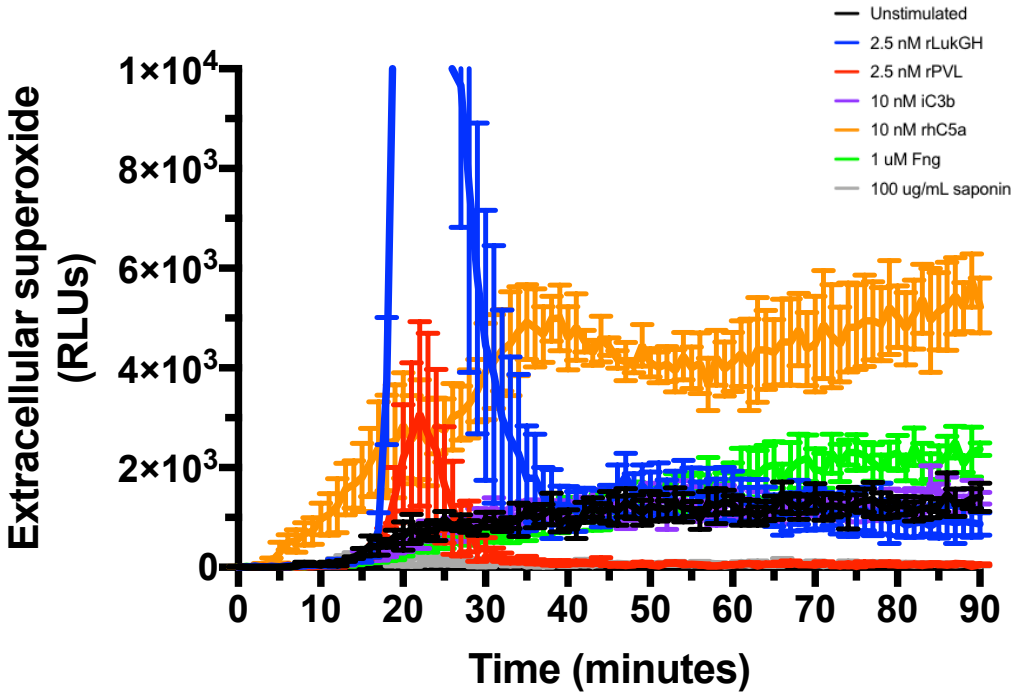


Figure S2. Magnification of Figure 3A to show extracellular superoxide induced by rPVL. Neutrophil superoxide production induced by PVL is consistently lower than the superoxide production induced by LukGH. RLUs, relative light units.

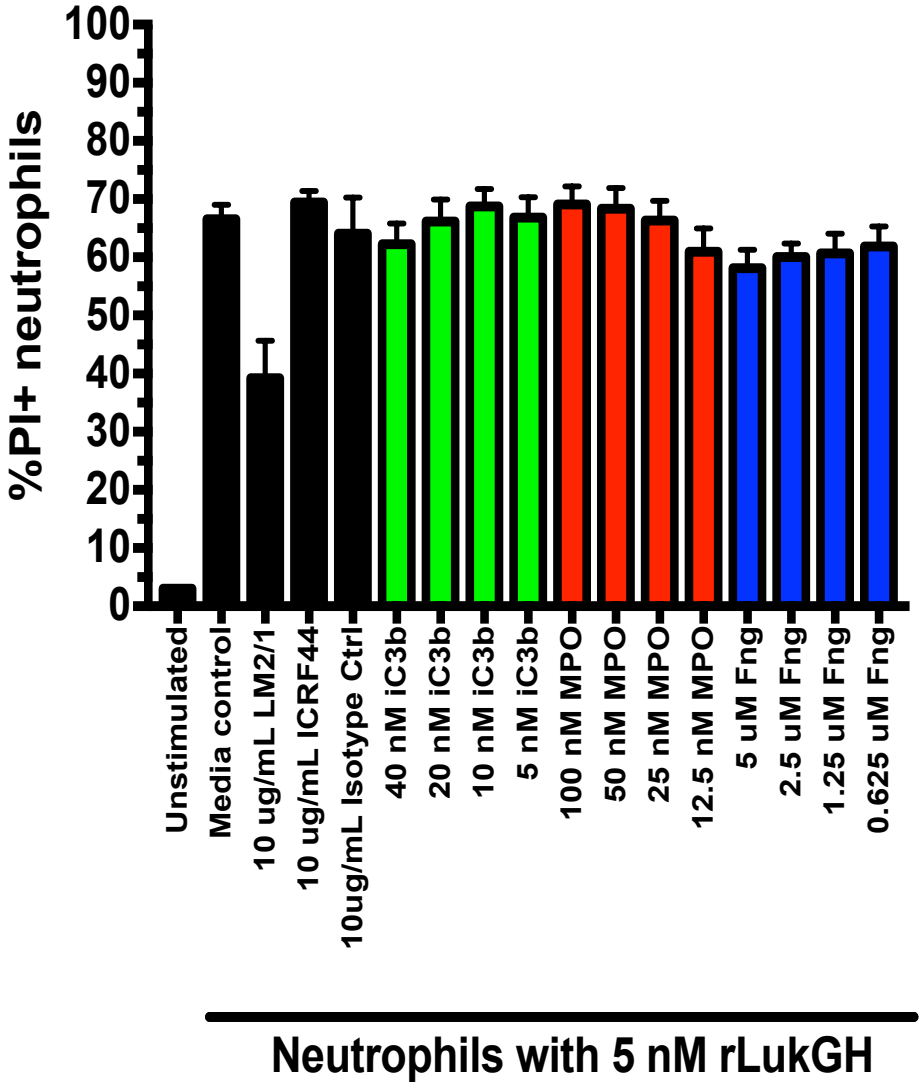


Figure S3. CD11b ligands do not inhibit LukGH-induced neutrophil cell membrane permeability. Anti-CD11b clones LM2/1 and ICRF44 target different CD11b epitopes.

REFERENCES CITED

Anderson, K. E., Boyle, K. B., Davidson, K., Chessa, T. A. M., Kulkarni, S., Jarvis, G. E., et al. (2008). CD18-dependent activation of the neutrophil NADPH oxidase during phagocytosis of Escherichia coli or Staphylococcus aureus is regulated by class III but not class I or II PI3Ks. *Blood* 112, 5202–5211. doi:10.1182/blood-2008-04-149450.

Badarau, A., Rouha, H., Malafa, S., Logan, D. T., Ha??kansson, M., Stulik, L., et al. (2015). Structure-function analysis of Heterodimer Formation, oligomerization, and receptor binding of the staphylococcus aureus bi-component toxin LukGH. *J. Biol. Chem.* 290, 142–156. doi:10.1074/jbc.M114.598110.

Ballal, A., and Manna, A. C. (2009). Regulation of superoxide dismutase (sod) genes by SarA in Staphylococcus aureus. *J. Bacteriol.* 191, 3301–10. doi:10.1128/JB.01496-08.

Balsam, L. B., Liang, T. W., and Parkos, C. a (1998). Functional mapping of CD11b/CD18 epitopes important in neutrophil-epithelial interactions: a central role of the I domain. *J. Immunol.* 160, 5058–5065.

Becker, K. A., Li, X., Seitz, A., Steinmann, J., Koch, A., Schuchman, E., et al. (2017). Neutrophils Kill Reactive Oxygen Species-Resistant Pseudomonas aeruginosa by Sphingosine. *Cell. Physiol. Biochem.* 43, 1603–1616. doi:10.1159/000482024.

Ben-Ari, J., Wolach, O., Gavrieli, R., and Wolach, B. (2012). Infections associated with chronic granulomatous disease: linking genetics to phenotypic expression. *Expert Rev. Anti. Infect. Ther.* 10, 881–94. doi:10.1586/eri.12.77.

Berton, G., Mócsai, A., and Lowell, C. A. (2005). Src and Syk kinases: Key regulators of phagocytic cell activation. *Trends Immunol.* 26, 208–214. doi:10.1016/j.it.2005.02.002.

Borregaard, N., Sørensen, O. E., and Theilgaard-Mönch, K. (2007). Neutrophil granules: a library of innate immunity proteins. *Trends Immunol.* 28, 340–345. doi:10.1016/j.it.2007.06.002.

Centers for Disease Control and Prevention (2015). Active Bacterial Core Surveillance Report, Emerging Infections Program Network, MethicillinResistant Staphylococcus aureus, 2015.

Centers for Disease Control and Prevention (CDC) (1999). Four pediatric deaths from community-acquired methicillin-resistant Staphylococcus aureus — Minnesota and North Dakota, 1997-1999. *MMWR. Morb. Mortal. Wkly. Rep.* 48, 707–10.

Chambers, H. F. (2001). The changing epidemiology of Staphylococcus aureus? *Emerg. Infect. Dis.* 7, 178–182. doi:10.3201/eid0702.700178.

Clauditz, A., Resch, A., Wieland, K.-P., Peschel, A., and Gotz, F. (2006). Staphyloxanthin Plays a Role in the Fitness of Staphylococcus aureus and Its Ability To Cope with Oxidative Stress. *Infect. Immun.* 74, 4950–4953. doi:10.1128/IAI.00204-06.

Colin, D. A., and Monteil, H. (2003). Control of the Oxidative Burst of Human Neutrophils by Staphylococcal Leukotoxins Control of the Oxidative Burst of Human Neutrophils by Staphylococcal Leukotoxins. *Infect. Immun.* 71, 3724–3729. doi:10.1128/IAI.71.7.3724.

Cosgrove, K., Coutts, G., Jonsson, I.-M., Tarkowski, A., Kokai-Kun, J. F., Mond, J. J., et al. (2007). Catalase (KatA) and alkyl hydroperoxide reductase (AhpC) have compensatory roles in peroxide stress resistance and are required for survival, persistence, and nasal colonization in Staphylococcus aureus. *J. Bacteriol.* 189, 1025–35. doi:10.1128/JB.01524-06.

de Jong, N. W. M., Ramyar, K. X., Guerra, F. E., Nijland, R., Fevre, C., Voyich, J. M., et al. (2017). Immune evasion by a staphylococcal inhibitor of myeloperoxidase. *Proc. Natl. Acad. Sci. U. S. A.* 114, 9439–9444. doi:10.1073/pnas.1707032114.

Diep, B. A., Gill, S. R., Chang, R. F., Phan, T. H., Chen, J. H., Davidson, M. G., et al. (2006). Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant Staphylococcus aureus. *Lancet* 367, 731–9. doi:10.1016/S0140-6736(06)68231-7.

DuMont, A. L., Yoong, P., Day, C. J., Alonzo, F., McDonald, W. H., Jennings, M. P., et al. (2013). Staphylococcus aureus LukAB cytotoxin kills human neutrophils by targeting the CD11b subunit of the integrin Mac-1. *Proc. Natl. Acad. Sci. U. S. A.* 110, 10794–9. doi:10.1073/pnas.1305121110.

El Benna, J., Faust, R. P., Johnson, J. L., and Babior, B. M. (1996). Phosphorylation of the respiratory burst oxidase subunit p47phox as determined by two-dimensional phosphopeptide mapping. Phosphorylation by protein kinase C, protein kinase A, and a mitogen-activated protein kinase. *J. Biol. Chem.* 271, 6374–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8626435 [Accessed October 6, 2014].

El Chemaly, A., Okochi, Y., Sasaki, M., Arnaudeau, S., Okamura, Y., and Demaurex, N. (2010). VSOP/Hv1 proton channels sustain calcium entry, neutrophil migration, and superoxide production by limiting cell depolarization and acidification. *J. Exp. Med.* 207, 129–39. doi:10.1084/jem.20091837.

Ellson, C. D., Davidson, K., Ferguson, G. J., O'Connor, R., Stephens, L. R., and Hawkins, P. T. (2006). Neutrophils from p40phox-/- mice exhibit severe defects in NADPH oxidase regulation and oxidant-dependent bacterial killing. *J. Exp. Med.* 203, 1927–37. doi:10.1084/jem.20052069.

Essig, K., Hu, D., Guimaraes, J. C., Alterauge, D., Edelmann, S., Raj, T., et al. (2017). Roquin Suppresses the PI3K-mTOR Signaling Pathway to Inhibit T Helper Cell Differentiation and Conversion of Treg to Tfr Cells. *Immunity* 47, 1067–1082.e12. doi:10.1016/j.immuni.2017.11.008.

Flack, C. E., Zurek, O. W., Meishery, D. D., Pallister, K. B., Malone, C. L., Horswill, A. R., et al. (2014). Differential regulation of staphylococcal virulence by the sensor kinase SaeS in response to neutrophil-derived stimuli. *Proc. Natl. Acad. Sci. U. S. A.* 111, E2037-45. doi:10.1073/pnas.1322125111.

Foster, T. J. (2017). Antibiotic resistance in Staphylococcus aureus. Current status and future prospects. *FEMS Microbiol. Rev.* 41, 430–449. doi:10.1093/femsre/fux007.

Fowler, V. G., and Proctor, R. A. (2014). Where does a Staphylococcus aureus vaccine stand? *Clin. Microbiol. Infect.* 20, 66–75. doi:10.1111/1469-0691.12570.

Fu, H., Bylund, J., Karlsson, A., Pellmé, S., and Dahlgren, C. (2004). The mechanism for activation of the neutrophil NADPH-oxidase by the peptides formyl-Met-Leu-Phe and Trp-Lys-Tyr-Met-Val-Met differs from that for interleukin-8. *Immunology* 112, 201–10. doi:10.1111/j.1365-2567.2004.01884.x.

Fumagalli, L., Campa, C. C., Germena, G., Lowell, C. A., Hirsch, E., and Berton, G. (2013). Class I Phosphoinositide-3-Kinases and Src Kinases Play a Nonredundant Role in Regulation of Adhesion-Independent and -Dependent Neutrophil Reactive Oxygen Species Generation. *J. Immunol.* 190, 3648–3660. doi:10.4049/jimmunol.1201951.

Futosi, K., Fodor, S., and M??csai, A. (2013). Neutrophil cell surface receptors and their intracellular signal transduction pathways. *Int. Immunopharmacol.* 17, 638–650. doi:10.1016/j.intimp.2013.06.034.

Gao, X. P., Zhu, X., Fu, J., Liu, Q., Frey, R. S., and Malik, A. B. (2007). Blockade of class IA phosphoinositide 3-kinase in neutrophils prevents NADPH oxidase activation- and adhesion-dependent inflammation. *J. Biol. Chem.* 282, 6116–6125. doi:10.1074/jbc.M610248200.

Gordon, C. P., Williams, P., and Chan, W. C. (2013). Attenuating Staphylococcus aureus virulence gene regulation: A medicinal chemistry perspective. *J. Med. Chem.* 56, 1389–1404. doi:10.1021/jm3014635.

Graves, S. F., Kobayashi, S. D., Braughton, K. R., Whitney, a. R., Sturdevant, D. E., Rasmussen, D. L., et al. (2012). Sublytic concentrations of Staphylococcus aureus Panton-Valentine leukocidin alter human PMN gene expression and enhance bactericidal capacity. *J. Leukoc. Biol.* 92, 361–374. doi:10.1189/jlb.1111575.

Green, J. N., Kettle, A. J., and Winterbourn, C. C. (2014). Protein chlorination in neutrophil phagosomes and correlation with bacterial killing. *Free Radic. Biol. Med.* 77, 49–56. doi:10.1016/j.freeradbiomed.2014.08.013.

Guerra, F. E., Addison, C. B., de Jong, N. W. M., Azzolino, J., Pallister, K. B., van Strijp, J. A. G., et al. (2016). Staphylococcus aureus SaeR/S-regulated factors reduce human neutrophil reactive oxygen species production. *J. Leukoc. Biol.* 100, 1–6. doi:10.1189/jlb.4VMAB0316-100RR.

Guerra, F. E., Borgogna, T. R., Patel, D. M., Sward, E. W., and Voyich, J. M. (2017). Epic Immune Battles of History: Neutrophils vs. Staphylococcus aureus. *Front. Cell. Infect. Microbiol.* 7, 1–19. doi:10.3389/fcimb.2017.00286.

Hall, J. W., Yang, J., Guo, H., and Ji, Y. (2017). The Staphylococcus aureus AirSR Two-Component System Mediates Reactive Oxygen Species Resistance via Transcriptional Regulation of Staphyloxanthin Production. *Infect. Immun.* 85, 1–12. doi:10.1128/IAI.00838-16.

Harding, M. G., Zhang, K., Conly, J., and Kubes, P. (2014). Neutrophil Crawling in Capillaries; A Novel Immune Response to Staphylococcus aureus. *PLoS Pathog.* 10, e1004379. doi:10.1371/journal.ppat.1004379.

Hawkins, P. T., and Stephens, L. R. (2015). PI3K signalling in inflammation. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* 1851, 882–897. doi:10.1016/j.bbalip.2014.12.006.

Herold, B. C., Immergluck, L. C., Maranan, M. C., Lauderdale, D. S., Gaskin, R. E., Boyle-Vavra, S., et al. (1998). Community-acquired methicillin-resistant Staphylococcus aureus in children with no identified predisposing risk. *JAMA* 279, 593–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9486753.

Ibrahim, F. B. M., Pang, S. J., and Melendez, A. J. (2004). Anaphylatoxin signaling in human neutrophils. A key role for sphingosine kinase. *J. Biol. Chem.* 279, 44802–44811. doi:10.1074/jbc.M403977200.

Jamieson, S., Flanagan, J. U., Kolekar, S., Buchanan, C., Kendall, J. D., Lee, W.-J., et al. (2011). A drug targeting only p110α can block phosphoinositide 3-kinase signalling and tumour growth in certain cell types. *Biochem. J.* 438, 53–62. doi:10.1042/BJ20110502.

Janesch, P., Rouha, H., Weber, S., Malafa, S., Gross, K., Maierhofer, B., et al. (2017). Selective sensitization of human neutrophils to LukGH mediated cytotoxicity by Staphylococcus aureus and IL-8. *J. Infect.* 74, 473–483. doi:10.1016/j.jinf.2017.02.004.

Johansson, M. W., Patarroyo, M., Oberg, F., Siegbahn, A., and Nilsson, K. (1997). Myeloperoxidase mediates cell adhesion via the alpha M beta 2 integrin (Mac-1, CD11b/CD18). *J. Cell Sci.* 110 (Pt 9, 1133–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9175709.

Kanai, F., Liu, H., Field, S. J., Akbary, H., Matsuo, T., Brown, G. E., et al. (2001). The PX domains of p47phox and p40phox bind to lipid products of PI(3)K. *Nat. Cell Biol.* 3, 675–678. doi:10.1038/35083070.

Karathanassis, D., Stahelin, R. V., Bravo, J., Perisic, O., Pacold, C. M., Cho, W., et al. (2002). Binding of the PX domain of p47phoxto phosphatidylinositol 3, 4-bisphosphate and phosphatidic acid is masked by an intramolecular interaction. *EMBO J.* 21, 5057–5068. doi:10.1093/emboj/cdf519.

Kobayashi, N., Ueki, K., Okazaki, Y., Iwane, A., Kubota, N., Ohsugi, M., et al. (2011). Blockade of class IB phosphoinositide-3 kinase ameliorates obesity-induced inflammation and insulin resistance. *Proc. Natl. Acad. Sci.* 108, 5753–5758. doi:10.1073/pnas.1016430108.

Kolaczkowska, E., and Kubes, P. (2013). Neutrophil recruitment and function in health and inflammation. *Nat. Rev. Immunol.* 13, 159–75. doi:10.1038/nri3399.

König, B., Prévost, G., Piémont, Y., and König, W. (1995). Effects of Staphylococcus aureus leukocidins on inflammatory mediator release from human granulocytes. *J. Infect. Dis.* 171, 607–13. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7533198.

Lau, D., Mollnau, H., Eiserich, J. P., Freeman, B. A., Daiber, A., Gehling, U. M., et al. (2005). Myeloperoxidase mediates neutrophil activation by association with CD11b/CD18 integrins. *Proc. Natl. Acad. Sci. U. S. A.* 102, 431–6. doi:10.1073/pnas.0405193102.

Lipp, P., and Reither, G. (2011). Protein kinase C: the “masters” of calcium and lipid. *Cold Spring Harb. Perspect. Biol.* 3, 1–17. doi:10.1101/cshperspect.a004556.

Lishko, V. K., Podolnikova, N. P., Yakubenko, V. P., Yakovlev, S., Medved, L., Yadav, S. P., et al. (2004). Multiple binding sites in fibrinogen for integrin ??M?? 2 (Mac-1). *J. Biol. Chem.* 279, 44897–44906. doi:10.1074/jbc.M408012200.

Luo, H. R., and Mondal, S. (2015). Molecular control of PtdIns(3,4,5)P3 signaling in neutrophils. *EMBO Rep.* 16, 149–163. doi:10.15252/embr.201439466.

Ma, Q. (2013). Role of Nrf2 in Oxidative Stress and Toxicity. *Annu. Rev. Pharmacol. Toxicol.* 53, 401–426. doi:10.1146/annurev-pharmtox-011112-140320.

Madani, S., Hichami, a, Legrand, a, Belleville, J., and Khan, N. a (2001). Implication of acyl chain of diacylglycerols in activation of different isoforms of protein kinase C. *FASEB J.* 15, 2595–2601. doi:10.1096/fj.01-0753int.

Malachowa, N., Kobayashi, S. D., Freedman, B., Dorward, D. W., and DeLeo, F. R. (2013). Staphylococcus aureus leukotoxin GH promotes formation of neutrophil extracellular traps. *J Immunol* 191, 6022–6029. doi:10.4049/jimmunol.1301821.

McGovern, N. N., Cowburn, A. S., Porter, L., Walmsley, S. R., Summers, C., Thompson, A. A. R., et al. (2011). Hypoxia Selectively Inhibits Respiratory Burst Activity and Killing of Staphylococcus aureus in Human Neutrophils. *J. Immunol.* 186, 453–463. doi:10.4049/jimmunol.1002213.

Mesri, M., Plescia, J., and Altieri, D. C. (1998). Dual regulation of ligand binding by CD11b I domain. *J. Biol. Chem.* 273, 744–748. doi:10.1074/jbc.273.2.744.

Miller, L. G., Perdreau-Remington, F., Rieg, G., Mehdi, S., Perlroth, J., Bayer, A. S., et al. (2005). Necrotizing fasciitis caused by community-associated methicillin-resistant Staphylococcus aureus in Los Angeles. *N. Engl. J. Med.* 352, 1445–53. doi:10.1056/NEJMoa042683.

Mitchell, G. B., Albright, B. N., and Caswell, J. L. (2003). Effect of interleukin-8 and granulocyte colony-stimulating factor on priming and activation of bovine neutrophils. *Infect. Immun.* 71, 1643–1649. doi:10.1128/IAI.71.4.1643-1649.2003.

Mócsai, A., Abram, C. L., Jakus, Z., Hu, Y., Lanier, L. L., and Lowell, C. A. (2006). Integrin signaling in neutrophils and macrophages uses adaptors containing immunoreceptor tyrosine-based activation motifs. *Nat. Immunol.* 7, 1326–1333. doi:10.1038/ni1407.

Mócsai, A., Zhang, H., Jakus, Z., Kitaura, J., Kawakami, T., and Lowell, C. A. (2003). G-protein-coupled receptor signaling in Syk-deficient neutrophils and mast cells. *Blood* 101, 4155–4163. doi:10.1182/blood-2002-07-2346.

Nguyen, G. T., Green, E. R., and Mecsas, J. (2017). Neutrophils to the ROScue: Mechanisms of NADPH Oxidase Activation and Bacterial Resistance. *Front. Cell. Infect. Microbiol.* 7. doi:10.3389/fcimb.2017.00373.

Nimmo, G. R. (2012). USA300 abroad: Global spread of a virulent strain of community-associated methicillin-resistant Staphylococcus aureus. *Clin. Microbiol. Infect.* 18, 725–734. doi:10.1111/j.1469-0691.2012.03822.x.

Nunes, P., Demaurex, N., and Dinauer, M. C. (2013). Regulation of the NADPH oxidase and associated ion fluxes during phagocytosis. *Traffic* 14, 1118–31. doi:10.1111/tra.12115.

Nygaard, T. K., Pallister, K. B., DuMont, A. L., DeWald, M., Watkins, R. L., Pallister, E. Q., et al. (2012). Alpha-toxin induces programmed cell death of human T cells, B cells, and monocytes during USA300 infection. *PLoS One* 7, e36532. doi:10.1371/journal.pone.0036532.

Nygaard, T. K., Pallister, K. B., Ruzevich, P., Griffith, S., Vuong, C., and Voyich, J. M. (2010). SaeR binds a consensus sequence within virulence gene promoters to advance USA300 pathogenesis. *J Infect Dis* 201, 241–254. doi:10.1086/649570.

Pelz, A., Wieland, K. P., Putzbach, K., Hentschel, P., Albert, K., and Götz, F. (2005). Structure and biosynthesis of staphyloxanthin from Staphylococcus aureus. *J. Biol. Chem.* 280, 32493–32498. doi:10.1074/jbc.M505070200.

Pittini, Á., Casaravilla, C., Allen, J. E., and Díaz, Á. (2016). Pharmacological inhibition of PI3K class III enhances the production of pro- and anti-inflammatory cytokines in dendritic cells stimulated by TLR agonists. *Int. Immunopharmacol.* 36, 213–217. doi:10.1016/j.intimp.2016.04.028.

Podolnikova, N. P., Podolnikov, A. V., Haas, T. A., Lishko, V. K., and Ugarova, T. P. (2015). Ligand recognition specificity of leukocyte integrin ??m??2 (Mac-1, CD11b/CD18) and its functional consequences. *Biochemistry* 54, 1408–1420. doi:10.1021/bi5013782.

Raghuwanshi, S. K., Su, Y., Singh, V., Haynes, K., Richmond, A., and Richardson, R. M. (2012). The Chemokine Receptors CXCR1 and CXCR2 Couple to Distinct G Protein-Coupled Receptor Kinases To Mediate and Regulate Leukocyte Functions. *J. Immunol.* 189, 2824–2832. doi:10.4049/jimmunol.1201114.

Rajagopalan, L., and Rajarathnam, K. (2004). Ligand selectivity and affinity of chemokine receptor CXCR1: Role of N-terminal domain. *J. Biol. Chem.* 279, 30000–30008. doi:10.1074/jbc.M313883200.

Reilly, M. P., Sinha, U., André, P., Taylor, S. M., Pak, Y., DeGuzman, F. R., et al. (2011). PRT-060318, a novel Syk inhibitor, prevents heparin-induced thrombocytopenia and thrombosis in a transgenic mouse model. *Blood* 117, 2241–2246. doi:10.1182/blood-2010-03-274969.

Reyes-Robles, T., Alonzo, F., Kozhaya, L., Lacy, D. B., Unutmaz, D., and Torres, V. J. (2013). Staphylococcus aureus Leukotoxin ED targets the chemokine receptors CXCR1 and CXCR2 to Kill leukocytes and promote infection. *Cell Host Microbe* 14, 453–459. doi:10.1016/j.chom.2013.09.005.

Roos, D., and de Boer, M. (2014). Molecular diagnosis of chronic granulomatous disease. *Clin. Exp. Immunol.* 175, 139–49. doi:10.1111/cei.12202.

Rubel, C., Fernandez, G. C., Dran, G., Bompadre, M. B., Isturiz, M. A., and Palermo, M. S. (2001). Fibrinogen Promotes Neutrophil Activation and Delays Apoptosis. *J. Immunol.* 166, 2002–2010. doi:10.4049/jimmunol.166.3.2002.

Sarma, J. V., and Ward, P. A. (2012). New developments in C5a receptor signaling. *Cell Heal. Cytoskelet.* 4, 73–82. doi:10.2147/CHC.S27233.New.

Schmitz, F. J., Veldkamp, K. E., Van Kessel, K. P., Verhoef, J., and Van Strijp, J. a (1997). Delta-toxin from Staphylococcus aureus as a costimulator of human neutrophil oxidative burst. *J. Infect. Dis.* 176, 1531–1537. doi:10.1086/514152.

Spaan, A. N., Henry, T., Van Rooijen, W. J. M., Perret, M., Badiou, C., Aerts, P. C., et al. (2013). The staphylococcal toxin panton-valentine leukocidin targets human C5a receptors. *Cell Host Microbe* 13, 584–594. doi:10.1016/j.chom.2013.04.006.

Spaan, A. N., Schiepers, A., de Haas, C. J. C., van Hooijdonk, D. D. J. J., Badiou, C., Contamin, H., et al. (2015). Differential Interaction of the Staphylococcal Toxins Panton-Valentine Leukocidin and γ-Hemolysin CB with Human C5a Receptors. *J. Immunol.* 195, 1034–43. doi:10.4049/jimmunol.1500604.

Spaan, A. N., Vrieling, M., Wallet, P., Badiou, C., Reyes-Robles, T., Ohneck, E. A., et al. (2014). The staphylococcal toxins γ-haemolysin AB and CB differentially target phagocytes by employing specific chemokine receptors. *Nat. Commun.* 5. doi:10.1038/ncomms6438.

Suh, B. C., and Hille, B. (2005). Regulation of ion channels by phosphatidylinositol 4,5-bisphosphate. *Curr. Opin. Neurobiol.* 15, 370–378. doi:10.1016/j.conb.2005.05.005.

Suh, C.-I., Stull, N. D., Li, X. J., Tian, W., Price, M. O., Grinstein, S., et al. (2006). The phosphoinositide-binding protein p40phox activates the NADPH oxidase during FcgammaIIA receptor-induced phagocytosis. *J. Exp. Med.* 203, 1915–25. doi:10.1084/jem.20052085.

Sward, E. W., Fones, E. M., Spaan, R. R., Pallister, K. B., Haller, B. L., Guerra, F. E., et al. (2018). Staphylococcus aureus SaeR/S-Regulated Factors Decrease Monocyte-Derived Tumor Necrosis Factor-α to Reduce Neutrophil Bactericidal Activity. *J. Infect. Dis.* 217, 943–952. doi:10.1093/infdis/jix652.

Tong, S. Y. C., Davis, J. S., Eichenberger, E., Holland, T. L., and Fowler, V. G. (2015). Staphylococcus aureus infections: Epidemiology, pathophysiology, clinical manifestations, and management. *Clin. Microbiol. Rev.* 28, 603–661. doi:10.1128/CMR.00134-14.

Tromp, A. T., Van Gent, M., Abrial, P., Martin, A., Jansen, J. P., De Haas, C. J. C., et al. (2018). Human CD45 is an f-component-specific receptor for the staphylococcal toxin Panton-Valentine leukocidin. *Nat. Microbiol.* 3, 708–717. doi:10.1038/s41564-018-0159-x.

Ueda, T., Rieu, P., Brayer, J., and Arnaout, M. A. (1994). Identification of the complement iC3b binding site in the beta 2 integrin CR3 (CD11b/CD18). *Proc. Natl. Acad. Sci. U. S. A.* 91, 10680–4. doi:10.1073/pnas.91.22.10680.

van Kesse, K. P. M., Bestebroer, J., and van Strijp, J. A. G. (2014). Neutrophil-mediated phagocytosis of Staphylococcus aureus. *Front. Immunol.* 5, 1–12. doi:10.3389/fimmu.2014.00467.

Veldkamp, K. E., Van Kessel, K. P. M., Verhoef, J., and Van Strijp, J. A. G. (1997). Staphylococcal culture supernates stimulate human phagocytes. *Inflammation* 21, 541–551. doi:10.1023/A:1027315814817.

Voyich, J. M., Braughton, K. R., Sturdevant, D. E., Whitney, A. R., Saïd-Salim, B., Porcella, S. F., et al. (2005). Insights into mechanisms used by Staphylococcus aureus to avoid destruction by human neutrophils. *J. Immunol.* 175, 3907–19. Available at: http://www.jimmunol.org/content/175/6/3907.full [Accessed January 22, 2014].

Voyich, J. M., Otto, M., Mathema, B., Braughton, K. R., Whitney, A. R., Welty, D., et al. (2006). Is Panton-Valentine leukocidin the major virulence determinant in community-associated methicillin-resistant Staphylococcus aureus disease? *J Infect Dis* 194, 1761–1770. doi:10.1086/509506.

Voyich, J. M., Vuong, C., DeWald, M., Nygaard, T. K., Kocianova, S., Griffith, S., et al. (2009). The SaeR/S gene regulatory system is essential for innate immune evasion by Staphylococcus aureus. *J. Infect. Dis.* 199, 1698–706. doi:10.1086/598967.

Winterbourn, C. C., and Kettle, A. J. (2013). Redox reactions and microbial killing in the neutrophil phagosome. *Antioxid. Redox Signal.* 18, 642–60. doi:10.1089/ars.2012.4827.

Wymann, M. P., Bulgarelli-Leva, G., Zvelebil, M. J., Pirola, L., Vanhaesebroeck, B., Waterfield, M. D., et al. (1996). Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. *Mol. Cell. Biol.* 16, 1722–1733. doi:10.1128/MCB.16.4.1722.

Yoong, P., and Torres, V. J. (2013). The effects of Staphylococcus aureus leukotoxins on the host: Cell lysis and beyond. *Curr. Opin. Microbiol.* 16, 63–69. doi:10.1016/j.mib.2013.01.012.

Zhan, Y., Virbasius, J. V., Song, X., Pomerleau, D. P., and Wayne Zhou, G. (2002). The p40phox and p47phox PX domains of NADPH oxidase target cell membranes via direct and indirect recruitment by phosphoinositides. *J. Biol. Chem.* 277, 4512–4518. doi:10.1074/jbc.M109520200.

Zhao, T., Benard, V., Bohl, B. P., and Bokoch, G. M. (2003). The molecular basis for adhesion-mediated suppression of reactive oxygen species generation by human neutrophils. 112. doi:10.1172/JCI200319108.Introduction.

Zhou, H., Liao, J., Aloor, J., Nie, H., Wilson, B. C., Fessler, M. B., et al. (2013). CD11b/CD18 (Mac-1) is a novel surface receptor for extracellular double-stranded RNA to mediate cellular inflammatory responses. *J. Immunol.* 190, 115–25. doi:10.4049/jimmunol.1202136.

Zurek, O. W., Nygaard, T. K., Watkins, R. L., Pallister, K. B., Torres, V. J., Horswill, A. R., et al. (2014). The Role of Innate Immunity in Promoting SaeR/S-Mediated Virulence in Staphylococcus aureus. *J. Innate Immun.* 6, 21–30. doi:10.1159/000351200.

Zurek, O. W., Pallister, K. B., and Voyich, J. M. (2015). Staphylococcus aureus Inhibits Neutrophil-derived IL-8 to Promote Cell Death. *J. Infect. Dis.* 212, 1–5. doi:10.1093/infdis/jiv124.

CHAPTER FIVE

CONCLUSIONS AND FUTURE STUDIES

S. aureus continues to be a major cause of human infectious disease. Neutrophils are quickly recruited to a site of *S. aureus* infection and equipped with an arsenal of antimicrobial mechanisms. Yet, *S. aureus* survives neutrophil killing. The mechanisms used by *S. aureus* to circumvent neutrophil killing remain unknown, but our work has defined specific strategies used by *S. aureus* to disrupt neutrophil bactericidal mechanisms.

S. aureus SaeR/S-regulated factors are rapidly upregulated in response to neutrophils. We showed SaeR/S-regulated factors reduce neutrophil reactive oxygen species production to decrease neutrophil killing (Guerra et al., 2016). Furthermore, a collaborative study identified the staphylococcal peroxidase inhibitor, SPIN, as a SaeR/S-regulated factor that binds human myeloperoxidase to inhibit formation of the highly bactericidal agent hypochlorous acid (de Jong et al., 2017). In Chapter 4, we began to unravel the signaling pathways induced by *S. aureus* secreted leukocidins to induce neutrophil ROS production. These latter studies challenge the assumption that neutrophil pore formation by leukocidins is a dead-end pathway through cell lysis. Instead, we show that leukocidins manipulate neutrophil signaling pathways to induce ROS production. The ability of soluble agonists like the *S. aureus* Panton-Valentine leukocidin (PVL) to induce neutrophil extracellular and intracellular ROS production is likely a virulence mechanism to disrupt proper neutrophil bactericidal mechanisms. Thus, *S. aureus* inhibits

and disrupts neutrophil ROS production, which is the most effective bactericidal mechanism against *S. aureus*.

The model in Figure 1 summarizes the work in this dissertation supporting the hypothesis that *S. aureus* uses SaeR/S-dependent mechanisms to reduce neutrophil bactericidal mechanisms. Once the initial wave of neutrophils reaches the site of infection, *S. aureus* senses the host through SaeR/S and mounts a quick response to survive the initial engagement. In this first round, *S. aureus* relies on SaeR/S-regulated factors like SPIN to survive the neutrophil phagosome. During this initial engagement, *S. aureus* also starts to secrete leukocidins and other factors to disrupt phagosome integrity and escape the neutrophil. *S. aureus* survival of the initial neutrophil encounter is critical since it prevents proper clearance and induces *S. aureus* to secrete leukocidins. Thus, incoming waves of neutrophils will encounter leukocidins secreted by *S. aureus* and induce premature neutrophil ROS production. Essentially, leukocidins may act as a *S. aureus* soluble shield to disrupt neutrophil signaling involved in antimicrobial mechanisms.

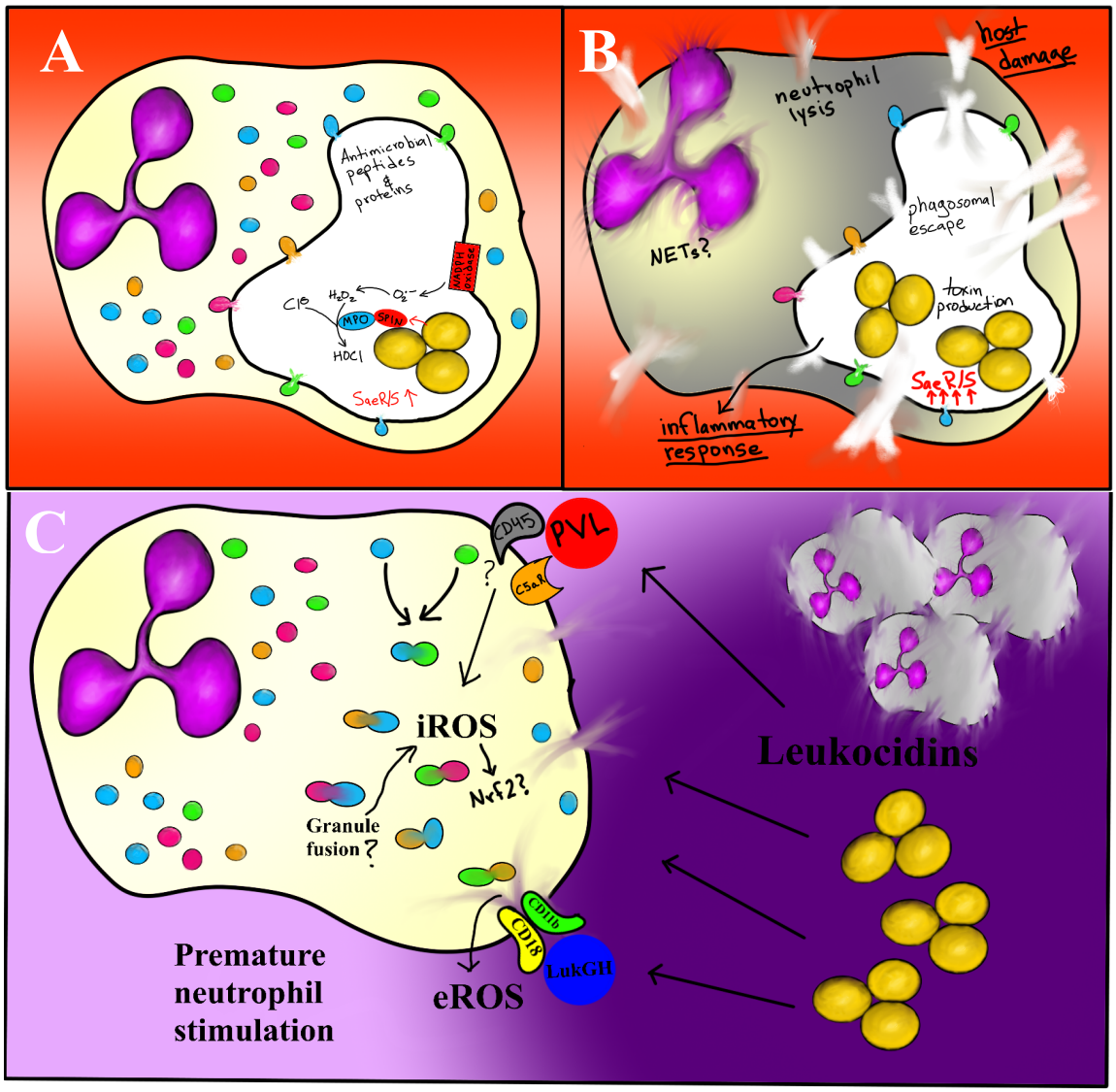


Figure 1. Model schematic of *S. aureus* survival during neutrophil encounter. A) *S. aureus* senses neutrophils using SaeR/S to amount a quick response to survive phagosome bactericidal activity. B) Inhibiting neutrophil ROS production allows *S. aureus* to rupture the phagosome using leukocidins. C) The production of leukocidins by *S. aureus* creates a soluble shield to induce neutrophil ROS production before reaching bacteria.

Future studies will continue to focus on the aberrant neutrophil signaling pathways induced by *S. aureus* leukocidins. In regard to PVL induced neutrophil ROS production, the following will be investigated:

1. Determine the contribution of canonical GPCR signaling in neutrophil extracellular and intracellular ROS production. We will use specific inhibitors of GPCR signaling, like pertussis toxin and gallein, to identify the contribution of PVL-C5aR signaling in neutrophil ROS production.
2. Determine the contribution of CD45 in PVL signaling. The PVL-CD45 interaction is likely to activate Src-family kinases (SFKs) and downstream Syk. Our studies suggest SFKs and Syk play essential roles in modulating neutrophil ROS production in response to PVL. A small chemical inhibitor of CD45 phosphatase activity has been described recently (Perron et al., 2014). Anti-CD45 antibodies are also available to determine the specific contribution of CD45-dependent neutrophil ROS production induced by PVL.
3. Elucidate neutrophil granule localization in response PVL. Our results suggest azurophilic granules containing MPO and secondary and tertiary granules containing the membrane bound $gp91^{phox}$ and $p22^{phox}$ NADPH oxidase components are co-localizing to induce luminol-based chemiluminescence. We will utilize confocal microscopy with anti-MPO and anti-$gp91^{phox}$ antibodies to determine granule localization following exposure to PVL.
4. Determine the consequences of neutrophil intracellular ROS production in response to PVL. To begin, we will focus on the activation of the Nrf2

transcription factor since previous studies suggest Nrf2-activated genes are upregulated in response to PVL (Graves et al., 2012). We will use immunoblotting to determine Nrf2 activation. A particular question of interest will be whether Nrf2 is activated to rescue neutrophils from oxidative stress or as a dead-end signaling pathway to attenuate neutrophil inflammatory responses during cell death.

As for neutrophil extracellular ROS induced by LukGH, our future experiments will focus to:

1. Determine the SFK-dependent but Syk-independent signaling pathway contributing to neutrophil extracellular ROS production. Current studies suggest SFKs are essential for integrin signaling through adapter proteins (DAP12, FcRγ, and uncharacterized proteins), which then activate Syk (Futosi et al., 2013). While SFKs are required for neutrophil extracellular ROS production induced by LukGH through the CD11b/CD18 integrin, Syk inhibition only partially attenuates the ROS response. This suggests a diverging signaling pathway between SFKs and Syk that affects neutrophil extracellular ROS production. SFKs have previously been shown to directly activate $p47^{phox}$ through phosphorylation of tyrosine residues (Chowdhury et al., 2005). Thus, it is possible that $p47^{phox}$ phosphorylation by SFKs bypasses Syk to directly activate NADPH oxidase. We will begin to tease apart this puzzle by determining the extent of $p47^{phox}$ phosphorylation during neutrophil stimulation by LukGH and how SFKs inhibition changes this phosphorylation.

Finally, our most frequently used *in vitro* neutrophil-*S. aureus* interaction model gives the host an advantage since neutrophils can phagocytose *S. aureus* within 10 minutes. This is likely too rapid for *S. aureus* to mount a quick response to disrupt the initial neutrophil engagement, especially as it relates to the secretion of leukocidins. Thus, future experimental conditions will allow *S. aureus* to incubate and secrete leukocidins before exposure to neutrophils. We will monitor neutrophil ROS production to determine if leukocidins induce neutrophil ROS production before phagocytosis. Clearly, much remains to be determined regarding how *S. aureus* disrupts neutrophil function.

"Carry on."

REFERENCES CITED

Chowdhury, A. K., Watkins, T., Parinandi, N. L., Saatian, B., Kleinberg, M. E., Usatyuk, P. V, et al. (2005). Src-mediated tyrosine phosphorylation of p47phox in hyperoxia-induced activation of NADPH oxidase and generation of reactive oxygen species in lung endothelial cells. *J. Biol. Chem.* 280, 20700–11. doi:10.1074/jbc.M411722200.

de Jong, N. W. M., Ramyar, K. X., Guerra, F. E., Nijland, R., Fevre, C., Voyich, J. M., et al. (2017). Immune evasion by a staphylococcal inhibitor of myeloperoxidase. *Proc. Natl. Acad. Sci. U. S. A.* 114, 9439–9444. doi:10.1073/pnas.1707032114.

Futosi, K., Fodor, S., and M??csai, A. (2013). Neutrophil cell surface receptors and their intracellular signal transduction pathways. *Int. Immunopharmacol.* 17, 638–650. doi:10.1016/j.intimp.2013.06.034.

Graves, S. F., Kobayashi, S. D., Braughton, K. R., Whitney, a. R., Sturdevant, D. E., Rasmussen, D. L., et al. (2012). Sublytic concentrations of Staphylococcus aureus Panton-Valentine leukocidin alter human PMN gene expression and enhance bactericidal capacity. *J. Leukoc. Biol.* 92, 361–374. doi:10.1189/jlb.1111575.

Guerra, F. E., Addison, C. B., de Jong, N. W. M., Azzolino, J., Pallister, K. B., van Strijp, J. A. G., et al. (2016). Staphylococcus aureus SaeR/S-regulated factors reduce human neutrophil reactive oxygen species production. *J. Leukoc. Biol.* 100, 1–6. doi:10.1189/jlb.4VMAB0316-100RR.

Perron, M. D., Chowdhury, S., Aubry, I., Purisima, E., Tremblay, M. L., and Saragovi, H. U. (2014). Allosteric Noncompetitive Small Molecule Selective Inhibitors of CD45 Tyrosine Phosphatase Suppress T-Cell Receptor Signals and Inflammation In Vivo. *Mol. Pharmacol.* 85, 553–563. doi:10.1124/mol.113.089847.

APPENDIX

CO-AUTHORED PUBLICATIONS

PNAS PNAS PNAS

Check for updates

Immune evasion by a staphylococcal inhibitor of myeloperoxidase

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Staphylococcus aureus **is highly adapted to its host and has evolved many strategies to resist opsonization and phagocytosis. Even after uptake by neutrophils, *S. aureus* shows resistance to killing, which suggests the presence of phagosomal immune evasion molecules. With the aid of secretome phage display, we identified a highly conserved protein that specifically binds and inhibits human myeloperoxidase (MPO), a major player in the oxidative defense of neutrophils. We have named this protein "staphylococcal peroxidase inhibitor" (SPIN). To gain insight into inhibition of MPO by SPIN, we solved the cocrystal structure of SPIN bound to a recombinant form of human MPO at 2.4-Å resolution. This structure reveals that SPIN acts as a molecular plug that prevents H_2O_2 substrate access to the MPO active site. In subsequent experiments, we observed that SPIN expression increases inside the neutrophil phagosome, where MPO is located, compared with outside the neutrophil. Moreover, bacteria with a deleted gene encoding SPIN showed decreased survival compared with WT bacteria after phagocytosis by neutrophils. Taken together, our results demonstrate that *S. aureus* secretes a unique proteinaceous MPO inhibitor to enhance survival by interfering with MPO-mediated killing.**

immune evasion | myeloperoxidase | neutrophil | phagocytosis | *Staphylococcus aureus*

The bacterium *Staphylococcus aureus* is a rising threat to human health. Thirty percent of healthy adults are colonized with this bacterium, resulting in an increased risk for infections ranging from abscesses to endocarditis (1). Neutrophils play a prominent role in fighting staphylococcal infections (2), as their intracellular granules contain numerous antimicrobial proteins and components for generating bactericidal reactive oxygen species (ROS). After *S. aureus* is phagocytosed, neutrophils' azurophilic granules fuse with the phagosome and release their contents (3). The five essential components of NADPH oxidase then assemble in the phagosomal membrane and become active (4). Active NADPH oxidase produces superoxide from O_2, which converts to hydrogen peroxide (H_2O_2) either spontaneously or by the action of superoxide dismutase. Myeloperoxidase (MPO) catalyses the reaction of H_2O_2 with chloride to generate hypochlorous acid (HOCl), which is a major effector in the oxidative defense of neutrophils (5). MPO also forms radicals by oxidizing a wide range of substrates, such as tyrosine, nitrite, nitric oxide, and phenols (6).

While the pathogen is taken up rapidly by phagocytes, mainly neutrophils and macrophages, not all bacteria are killed and these phagocytes can therefore act as so called "Trojan Horses" and distribute a pathogen away from the initial site of infection (7). To counteract the manifold antimicrobial defenses of neutrophils, *S. aureus* has evolved specific evasion molecules to inhibit intracellular killing (8). For example, the golden pigment staphyloxanthin serves as an antioxidant and is known to protect *S. aureus* against ROS (9). Catalase is yet another enzyme important for resistance against oxidative stress. This enzyme converts H_2O_2 into H_2O and O_2 and is considered to be a virulence factor. *S. aureus* also expresses an alkyl hydroperoxide reductase (*ahpC*) that contributes catalase-like activity. Whereas AhpC is believed to detoxify endogenously produced hydrogen peroxide, catalase appears more important for protection against external oxidative stress (10). Finally, *S. aureus* produces specific evasion proteins that disrupt phagosomal membranes, such as phenol-soluble modulins, hemolysin-α, and leukocidin AB (8). Together, these evasion molecules are believed to contribute to bacterial survival following phagocytosis.

Proteomic studies have shown that between 100 and 200 proteins are secreted from *S. aureus*, many with unknown functions (11). Consequently, known evasion molecules are likely to represent only a small fraction of the total repertoire. Therefore, we recently developed a phage display approach (12) as a nonbiased high-throughput method to screen for new potential staphylococcal immune evasion molecules. Since *S. aureus* can survive within the phagosome, but also because recent work suggests that SaeR/S regulated factors exist that inhibit neutrophil ROS production (13), we screened this staphylococcal phage library against several intracellular proteins of neutrophils.

Through this approach, we identified the hypothetical protein NWMN_0402 as an evasion factor. We have named this protein "staphylococcal peroxidase inhibitor" (SPIN), as it is able to bind and inhibit MPO. Here, we characterize SPIN and detail the structural basis for MPO inhibition by SPIN. We further show that the production of SPIN is up-regulated after phagocytosis of

Significance

Staphylococcus aureus **secretes numerous proteins to evade our innate immune system, for example to evade opsonization and phagocytosis by neutrophils. Here we describe the discovery that *S. aureus* has evolved a protein, called SPIN, that specifically binds and inhibits the human myeloperoxidase enzyme (MPO). MPO is located inside the granules of neutrophils and is important in the oxidative burst against pathogens. We identify the molecular mode of action of SPIN inhibiting MPO, illustrate this with the cocrystal structure, and show that SPIN is important for bacterial survival by MPO-dependent killing. Our study shows that *S. aureus* fights back after it is engulfed by neutrophils, which will help our understanding of the complex nature of *S. aureus* infections.**

Author contributions: R.N., C.F., J.M.V., B.L.G., K.P.M.v.K., J.A.G.v.S., B.V.G., and P.J.A.H. designed research; N.W.M.d.J., K.X.R., F.E.G., A.J.M., B.L.G., and B.V.G. performed research; C.F., K.P.M.v.K., and P.J.A.H. were involved in the discovery of the protein and its function; and N.W.M.d.J., K.X.R., K.P.M.v.K., J.A.G.v.S., B.V.G., and P.J.A.H. wrote the paper.

The authors declare no conflict of interest.

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.wwpdb.org (PDB ID code 5UZU).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1707032114/-/DCSupplemental.

S. aureus by human neutrophils and that SPIN protects *S. aureus* from MPO-mediated killing.

Results

Identification of SPIN. We designed a secretome phage display strategy to screen for unidentified evasion molecules that target neutrophil granule proteins, as described previously (12, 14). Isolated DNA of *S. aureus* strain Newman was randomly sheared and the resulting fragments were cloned in the pDJ01 phagemid vector for specific display of secretome proteins. Phages were produced upon addition of the VCSM13 helper phage. Screening was performed against total degranulate from TNF-primed, formylmethionine-leucyl-phenylalanine–stimulated neutrophils. After four rounds of selection, 48 clones were sequenced. The major hit was enriched in 36 clones and corresponded to the hypothetical ORF NWMN_0402 (accession no. BAF66674 strain Newman) (Fig. S1*A*). We named this protein SPIN due to its ability to inhibit MPO, as described in *SPIN Binds and Inhibits MPO*.

The gene encoding SPIN, denoted as *spn*, is located on genomic island α, also known as νSaα. This island is found in all *S. aureus* genomes and contains a cluster of genes encoding known evasion molecules, most notably the staphylococcal superantigen-like (*ssl*) proteins (8). *spn* is located downstream of the *ssl* cluster with the coding sequence oriented in the reverse direction (Fig. S1*B*). The genes in νSaα are often variable and each *S. aureus* lineage has a unique combination of gene variants (15). However, *spn* is located in the conserved region of νSaα (Fig. S1*C*). It was present in 83 of 84 clinical strains isolated from intensive care unit patients as well as in all completed *S. aureus* genomes included in our analysis (15).

The amino acid sequence of SPIN is highly conserved among almost all *S. aureus* clonal lineages, both human- and animal-associated strains, with 92.4% of residues conserved (Fig. S1*D*). Only two strains of clonal complex (CC)5 lineage, Mu3 and Mu50, encoded a truncated version of SPIN, lacking 35 amino acids at the C terminus. To determine if SPIN is produced in vivo during colonization or infection of the human host, sera from 20 healthy individuals were tested for the presence of antibodies against SPIN. These sera showed substantial amounts of antibody, and elicited values comparable to CHIPS (chemotaxis inhibitory protein of *S. aureus*) (Fig. S1*E*). CHIPS is a known *S. aureus* immune evasion molecule (16) that yielded a mean fluorescence intensity value in a previous multiplex assay indicative of high IgG levels, whereas the superantigens (e.g., SEI) did not (17). Because SPIN is highly conserved in *S. aureus* strains, has a high prevalence, and is expressed in vivo, we further investigated this protein as a potential evasion molecule acting on one of the granular proteins of neutrophils.

SPIN Binds and Inhibits MPO. Since we identified SPIN as a potential evasion protein, recombinant SPIN was coupled to CNBr-activated Sepharose beads and used for affinity chromatography to probe neutrophil degranulate for its binding partner. Eluted proteins were visualized by silver-stained PAGE and identified by mass spectrometry. Although elastase, proteinase 3, cathepsin G, azurocidin, lactoferrin, and MPO were all identified as potential binding partners, a subsequent SPIN capture ELISA against these potential targets only showed evidence for SPIN binding to MPO (Fig. 1*A*). Next, we used conventional surface plasmon resonance (SPR) (Fig. 1*B*) as well as AlphaScreen equilibrium competitive binding assays (Fig. S2) to validate our ELISA result. Both SPR and AlphaScreen were consistent with low nanomolar affinity binding between SPIN and MPO, and yielded comparable K_D values of 10 ± 0.6 nM and 2.0 ± 0.07 nM, respectively.

Since SPIN forms a high-affinity complex with MPO, we hypothesized that SPIN disrupts MPO function. Indeed, a colorimetric MPO activity assay demonstrated that SPIN inhibits MPO in a dose-dependent manner (Fig. 1*C*) with an apparent IC_{50} of ∼7 nM. Subsequently, we investigated whether SPIN was secreted as a functional protein in staphylococcal culture supernatant. Both

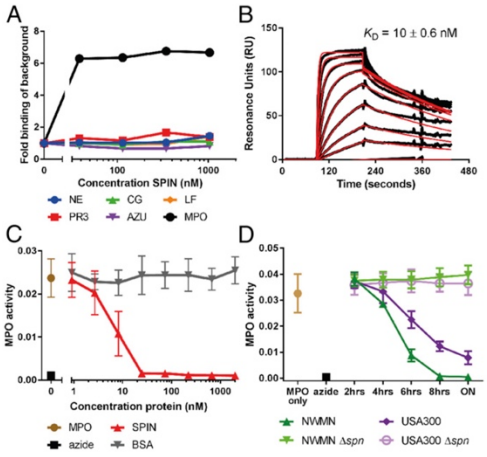


Fig. 1. SPIN binds and inhibits MPO. (*A*) An ELISA-type binding assay of C-His SPIN to all candidate proteins. AZU, azurocidin; CG, cathepsin G; LF, lactoferrin; MPO, myeloperoxidase; NE, neutrophil elastase; PR3, proteinase 3. (*B*) Characterization of SPIN binding to MPO by SPR, where SPIN was injected over a surface of native MPO. (*C* and *D*) Recombinant SPIN and *S. aureus* supernatant inhibit MPO in a dose-dependent manner. ON is overnight culture. Bars express SD with $n = 3$ for *B*–*D*.

WT strains Newman and USA300 accumulated increasing levels of MPO inhibitory activity in conditioned culture medium over time. Moreover, we also found that strain Newman supernatant showed a higher degree of inhibition compared with USA300. To determine whether this MPO inhibitory activity was caused by the SPIN protein, we generated isogenic KOs of the SPIN gene in both Newman and USA300 backgrounds. Conditioned culture medium from both KOs was completely devoid of MPO inhibitory activity (Fig. 1*D*).

We also performed a titration analysis on samples of conditioned culture supernatant from WT *S. aureus* strains Newman and USA300 (Fig. S3 *A* and *B*). The IC_{50} of these samples was 0.12% and 0.30% supernatant for Newman and USA300, respectively. By comparing this to the IC_{50} from recombinant SPIN (Fig. 1*C*), the amount of SPIN present in the overnight supernatant could be estimated. This appeared to be 5.9 μM for Newman and 2.4 μM for USA300. Interestingly, the supernatant from the Mu50 strain, which contains a truncated version of *spn* (Fig. S1*D*), showed no detectable MPO inhibitory activity in its supernatant (Fig. S3*C*), indicating a nonfunctional SPIN-variant. The slight inhibitory effect observed at low dilutions of the conditioned media is due to the inhibitory color of Todd Hewitt Broth (THB) itself (Fig. S3*C*).

Structural Basis for MPO Inhibition by SPIN. SPIN shares no significant sequence homology to other characterized proteins. Thus, we sought structural information to better understand the physical basis for SPIN binding to and inhibition of MPO. We succeeded in crystallizing a complex of SPIN bound to a recombinant form of MPO, collected X-ray diffraction data to 2.4-Å Bragg-limiting resolution, and solved the structure by molecular replacement (Table S1). Electron density maps calculated after the initial placement of a model for human MPO revealed a strong, contiguous feature that could not be explained by the presence of MPO (Fig. 2*A*). A model of the SPIN polypeptide was completed through a combination of automated chain tracing and manual building, and the structure of the complex was refined to final R_{work}/R_{free} values of 18.4% and 24.1%, respectively (Table S1). The SPIN

protein consists of the common α-helix bundle fold found in other staphylococcal evasion proteins, with a unique addition of a prominent β-hairpin at its N terminus (Fig. 2*B*). As there are no biochemical features that would promote stability of this β-hairpin in the absence of ligand (e.g., a disulfide bond), we suspect that this region of SPIN is disordered in the unbound state.

The interaction between SPIN and MPO buries 1,633 Å^2 from the bacterial inhibitor and is comprised of two conceptually distinct interfaces (Fig. 2*C*). The first interface lies at the side of the peroxidase active site entrance, and accounts for 726 Å^2 (~45%) buried in the complex. Sixteen of the 20 SPIN residues associated with this site are found on either the first or second helix of the inhibitor. The sidechains of five such residues form either hydrogen bonds or salt-bridges with groups donated by MPO, and likely help impart specificity for this enzyme. The second interface accounts for 907 Å^2 (~55%) of the area buried in the complex, and is derived exclusively from residues within the SPIN β-hairpin. All positions in this region of SPIN form extensive contacts with residues that line the MPO active site channel. We find the Gly residue in the β-hairpin turn particularly noteworthy, since it not only allows the second strand to complete the hairpin but also dictates the extent to which this hairpin can penetrate the MPO active site. The overall effect of this structural feature allows SPIN to act as a molecular plug and thereby prevent access of the H_2O_2 substrate to the reactive heme located deep within the MPO active site channel (Fig. 2*D*).

Production of SPIN by *S. aureus* Is Up-Regulated After Phagocytosis by Neutrophils. To better understand the biological context of SPIN production by *S. aureus*, we compared SPIN gene expression and promoter activity in bacteria that had been phagocytosed and not phagocytosed by neutrophils. We used strain USA300, since SPIN is constitutively expressed at low levels by strain Newman. SPIN gene expression over time was monitored using qPCR, either with or without phagocytosis. We observed a highly significant, eightfold increase in *spn* expression following phagocytosis compared with samples without neutrophils (Fig. 3*A*). This outcome is comparable to our earlier microarray study, where enhanced *spn* expression was detected after exposure to azurophilic granule proteins (18). Importantly, we did not detect a similar change in *spn* expression when we used bacteria lacking SaeR/S, which regulates production of multiple evasion factors (19). As a control, we found no significant change in *agrA* expression under any of these conditions (20) (Fig. S4).

Next, we used GFP promoter-reporter USA300 bacteria in a fibrin-thrombin matrix gel to immobilize cells. Time-lapse imaging of the bacteria revealed up-regulation of the *spn* promoter-driven GFP signal following phagocytosis by neutrophils (arrows, Fig. 3*B*). In contrast, the *spn* promoter appeared inactive in those bacteria that had not been phagocytosed (circles, Fig. 3*B*); this observation correlates with *spn* promoter activity for THB-grown bacteria, which showed activity only after 2 h of growth (Fig. S5). We further quantified the total GFP

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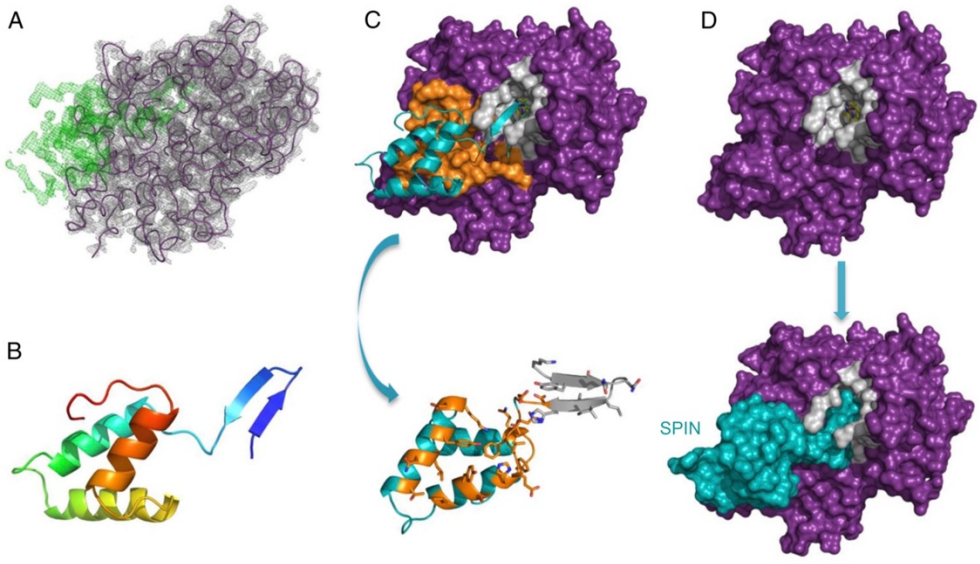


Fig. 2. Structural basis for inhibition of MPO by SPIN. (*A*) Electron density maps (2.4-Å resolution) calculated after initial placement of an MPO model (R_{free} = 28%). $2F_o$–F_c density contoured at 1.5 σ (gray cage) is shown for the MPO model (purple wire), and F_o–F_c density contoured at 3.0 σ (green cage) is attributable to SPIN. (*B*) Structure of the SPIN polypeptide depicted as a ribbon diagram. N terminus of the protein is indicated in indigo, the C terminus in red. The orientation of SPIN has been maintained across *A* and *B* for clarity. (*C*) Representation of the final model for the SPIN/MPO complex. (*Upper*) SPIN is shown as a cyan ribbon while MPO is depicted as a molecular surface. Residues comprising the first SPIN binding interface are colored orange, and residues lining the MPO active site channel are colored gray. (*Lower*) SPIN is drawn with the residues found at the first interface colored orange, and residues interacting with the MPO active site channel are colored gray. The sidechains of interfacing residues are depicted in ball-and-stick convention. Note the orientation of SPIN in the *Lower* panel is flipped 180° in the viewing plane relative to the *Upper* panel. (*D*) Surface representations provide insight into the physical basis for MPO inhibition by SPIN. (*Upper*) MPO is shown as a molecular surface with the residues lining the active site channel in gray. (*Lower*) SPIN is drawn as a cyan molecular surface according to its position in the final model of the SPIN/MPO complex. The location of the reactive site heme from native human MPO (33) is shown as a colored ball-and-stick. Note that the SPIN β-hairpin appears to completely occlude access of small molecules to the reactive site heme.

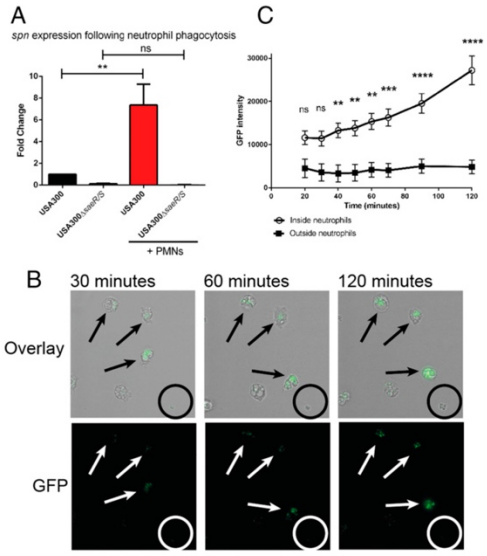


Fig. 3. Production of SPIN is up-regulated after phagocytosis inside neutrophils. (*A*) Analysis of *spn* expression following exposure to human neutrophils. Bars express SD with $n = 3$. Statistical significance was determined using one-way ANOVA. (*B*) Time-lapse analysis of SPIN expression shown as GFP fluorescence promoter-reporter USA300 in a fibrin-thrombin matrix gel. An overlay of bright-field and GFP (*Upper*) and GFP alone (*Lower*) is shown. (Magnification: 40×.) The time indicates the duration after start of phagocytosis. Arrows indicate bacteria inside neutrophils and the circle indicates a colony of bacteria growing outside neutrophils. One representative experiment is shown from three independent experiments. (*C*) Quantification of total GFP signal from neutrophil-resident or free bacteria. Three different experiments with 44 neutrophils and 26 colonies growing outside neutrophils were analyzed. Bars express SEM. Significance was determined by two-way ANOVA with Bonferroni posttest correction for multiple comparison. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$; ns, not significant.

signal for either free or neutrophil-resident bacteria for 2 h, beginning at 20 min. We found that GFP intensity increased more than twofold over time for phagocytosed, but not free-living bacteria (Fig. 3*C*).

Production of SPIN Promotes Survival Against Neutrophil-Derived ROS. Since SPIN inhibits MPO and is up-regulated following phagocytosis, we next investigated the relevance of SPIN to *S. aureus* evasion of neutrophil killing mechanisms. First we used an artificial system that mimics phagosomal ROS production using glucose oxidase (GO), which forms H_2O_2 in the presence of glucose. We evaluated bacterial survival after the addition of MPO and NaCl, which generates the bactericidal reagent HOCl (Fig. 4 *A* and *B*). Excessive quantities of GO result in bactericidal concentrations of H_2O_2, which makes it impossible to evaluate MPO-mediated killing. In limiting concentrations of GO, increasing MPO concentrations led to killing of *S. aureus* Newman (Fig. 4*A*, *Left*) and USA300 (Fig. 4*A*, *Right*). Addition of SPIN restored the survival of bacteria (Fig. 4*A*). Moreover, adding increasing concentrations of SPIN (Fig. 4*B*) resulted in a dose-dependent rescue from HOCl killing for both Newman and USA300. This demonstrates clearly that the presence of SPIN is important for protection from MPO-generated HOCl.

SPIN is neither able to bind nor inhibit mouse, horse, cow, or rabbit MPOs, hampering the development of an in vivo animal model system (Fig. S6). We therefore set out to investigate how the presence or absence of the SPIN gene influences *S. aureus* survival following phagocytosis by human neutrophils. First, the contribution of MPO to staphylococcal killing by neutrophils was investigated using the membrane-permeable MPO inhibitor AZM198 (21). Incubation of intact neutrophils with this inhibitor before washing and lysis was equally as effective as direct treatment of lysates, indicating that AZM198 is membrane-permeable and completely inhibits MPO (Fig. S7). We quantified bacterial killing by neutrophils with and without AZM198 pretreatment, and observed a difference of 26% between neutrophils with active and inactive MPO. This illustrates the modest extent of MPO-dependent staphylococcal killing by neutrophils in our experiments (Fig. 4*C*).

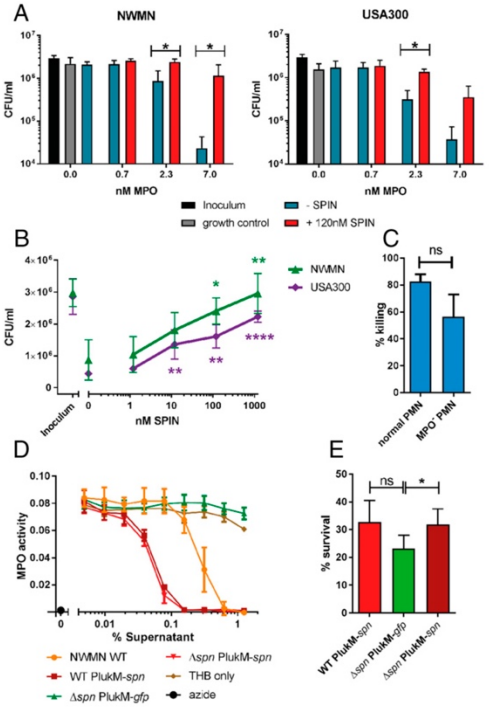


Fig. 4. SPIN is important for evasion of MPO-dependent neutrophil killing. (*A*) *S. aureus* strains Newman (*Left*) and USA300 (*Right*) were killed in a dose-dependent manner by adding 0.7–7.0 nM MPO in a coupled glucose oxidase-MPO system. Addition of 120 nM SPIN prevented ROS killing. Statistical significance was determined by two-way ANOVA with Bonferroni posttest correction for multiple comparison. (*B*) SPIN provides dose-dependent protection from HOCl-mediated killing for strains Newman and USA300 relative to 0 nM SPIN. Statistical significance was determined by one-way ANOVA with Bonferroni posttest correction for multiple comparison. (*C*) Differential bacterial survival of Newman WT from neutrophils with active or membrane-permeable AZM198-inactivated MPO. (*D*) Supernatants of overnight culture of strains WT PlukM-*spn*, Δ*spn* PlukM-*spn*, Δ*spn* PlukM-*gfp* were diluted, tested for MPO activity, and compared with Newman WT. Bars express SD with $n = 3$ (*A*–*D*). (*E*) Effect of *S. aureus* survival from isolated neutrophils after 60 min of phagocytosis. Statistical analysis determined by paired Student *t* test for *C* and *E*. Bars express SEM with $n = 6$. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{****}P \leq 0.0001$; ns, not significant.

Given the contribution of MPO in killing of phagocytosed *S. aureus*, we used the strong *lukM* promoter (22) and used the Newman strain to increase intraphagosomal SPIN production (WT PlukM-*spn*), thereby ensuring maximal levels of MPO inhibition in our bactericidal assays (Fig. 4*D*). We used the same plasmid to complement the isogenic *spn* KO (Δ*spn* PlukM-*spn*) and prepared a strain of the *spn* KO expressing GFP from the same plasmid (PlukM-*gfp*) to serve as a negative control. Supernatants from overnight cultures of these strains were compared with Newman WT for their ability to inhibit MPO (Fig. 4*D*). As predicted, the amount of SPIN produced in the WT PlukM-*spn* and Δ*spn* PlukM-*spn* was increased approximately fivefold relative to Newman WT, as judged by IC_{50} values. Importantly, these strains grew with similar kinetics in THB (Fig. S8*A*) and showed comparable phagocytosis by neutrophils at both 15- and 60-min time points (Fig. S8 *B* and *C*). Therefore, we used these three strains to detect differences in bacterial survival at 60 min following phagocytosis (Fig. 4*E*). Indeed, we observed a *spn*-dependent, significant difference in killing between the negative control strain Δ*spn* and the complemented strain that overexpressed *spn*. Although the difference between the Δ*spn* strain and the overexpressing WT strain was not statistically significant, it nevertheless showed a trend towards increased survival similar to the complemented strain. Taken together, these data show that SPIN is an important component to *S. aureus* survival of the oxidative killing mechanisms deployed within the neutrophil phagosome.

Discussion

Here we describe SPIN as a component of the *S. aureus* immune evasion arsenal that impairs bacterial killing by neutrophils. The phagocytosed bacteria must confront numerous antibacterial systems within the phagosome. Considering this as a potentially important site for execution of an immune evasion strategy, we set out to identify any previously unknown *S. aureus* proteins that inhibit components within neutrophil granules. By using an innovative phage-display approach that is limited to *S. aureus* secreted proteins (Fig. S1), we discovered that SPIN binds MPO with low nanomolar affinity and inhibits its activity (Fig. 1). Thus, together with the recently characterized neutrophil serine protease inhibitors, Eap, EapH1, and EapH2 (23), SPIN constitutes part of an elaborate staphylococcal innate immune evasion program that specifically targets the antibacterial enzymes stored within the azurophilic granules of neutrophils.

From experiments using promoter-GFP reporter bacteria, we observed that the promoter of *spn* is stimulated following uptake of *S. aureus* by neutrophils compared with bacteria outside neutrophils or in the first 2 h of growth in THB (Fig. 3 *B* and *C* and Fig. S5). Thus, SPIN is specifically up-regulated intracellularly, where MPO is located. A similar result was also observed when looking at mRNA levels (Fig. 3*A*), where the production of SPIN was increased over eightfold 30 min after phagocytosis. Moreover, there was no up-regulation observed when bacteria lacking the SaeR/S two-component regulatory system were used. The SaeR/S system plays an important role in neutrophil evasion (19) and regulates factors that reduce neutrophil ROS (13). SaeR/S is up-regulated comparably to *spn* (≈3- to 10-fold 30 min after phagocytosis) (20) and the promoter of SPIN contains the binding consensus sequence of SaeR (24). At least two of the 11 *ssl* genes (*ssl5* and *ssl8*) are regulated via the Sae system (25) and are located directly upstream of the SPIN gene. Thus, we propose that SPIN is largely regulated via the SaeR/S regulon and likely contributes to the SaeR/S-dependent reduction of neutrophil ROS (13).

By using a simplified system consisting of an enzymatic H_2O_2-generating system and Cl^- as a substrate for MPO, we found that SPIN had a clear dose-dependent and statistically significant effect on *S. aureus* survival (Fig. 4 *A* and *B*). However, in the complex and necessarily redundant process of bacterial killing within neutrophils, a multitude of oxygen radicals, proteases, and other enzymes must work in concert to ensure effective elimination of *S. aureus* cells. Although the contribution of MPO and its main product, HOCl, to *S. aureus* killing has been studied since the early 1970s (5), we and others believe this process remains incompletely understood (26). For example, it was previously believed that MPO is the main component for killing *S. aureus*, because neutrophils from MPO-deficient patients showed a log difference in bacterial survival after phagocytosis (5). Using the potent and cell-permeable MPO inhibitor, AZM198, we found that while untreated neutrophils kill 82 ± 5% of bacteria, MPO-depleted neutrophils are still able to kill 56 ± 16% (Fig. 4*C*). Thus, under the specific test conditions in our assays, roughly one-fourth of the staphylococcal killing is due to MPO. Nevertheless, when a strain of *S. aureus* overexpressing *spn* was compared with a *spn*-deleted counterpart (22) (Fig. 4*D*), we observed a statistically significant difference in bacterial survival dependent on SPIN (Fig. 4*E*). This not only confirms the relevance of SPIN-mediated inhibition of MPO in the physiological context, it also provides a starting point for reanalysis of previous studies where the presence of SPIN may have adversely affected data interpretation.

SPIN consists of a three α-helix bundle fold, which is commonly found among *S. aureus* innate immune evasion proteins, including Efb, Ecb (also called Ehp), Sbi, and SCINs (27). The triple-helix motif is a thermodynamically stable structure which can accommodate high sequence diversity and adapt to binding a wide variety of targets (28). However, this simple structural motif is typically modified by an N-terminal extension that imparts specific function to the protein in question (27). Indeed, this appears to be the case for SPIN as well, as is suggested by the cocrystal structure of SPIN/MPO (Fig. 2). To our knowledge, SPIN is unique as an example of a pathogen-derived protein whose role is to specifically bind and inhibit MPO. SPIN is not the only known inhibitor of MPO, however, as it has previously been shown that the host serum protein ceruloplasmin (CP) binds MPO with a K_D of ∼30 nM (29) and also blocks MPO activity (30). Examination of the crystal structure of CP bound to MPO (31) indicates that the mechanism through which CP inhibits MPO is conceptually distinct from that of SPIN. Indeed, even though the MPO active site appears to remain partly solvent accessible in the CP/MPO complex, CP attenuates MPO activity by disturbing essential transitions between the redox states of the peroxidase catalytic heme (32). In contrast, our structure of SPIN/MPO shows that SPIN forms a molecular plug that is likely to completely occlude access of substrates to the MPO active site (Fig. 2). Separately, while CP and SPIN share partial overlap in their binding sites on MPO and recognize their target with similar affinities, they display no obvious homology to one another at the sequence or structural levels. This suggests that the MPO-inhibitory capacity of SPIN has arisen through independent evolutionary processes from that of CP.

In conclusion, we identified an evasion protein called SPIN which is able to bind and inhibit human MPO. By solving the cocrystal structure of SPIN/MPO, we demonstrate the molecular mechanism of inhibition, via insertion of an N-terminal hairpin into the active site of MPO to block substrate access. We further show the expression of SPIN is up-regulated inside the neutrophil, where MPO is located, and that its presence helps in evading MPO-mediated killing. Various proteins secreted by *S. aureus* are known to be human-specific, which leads to restrictions in investigating this pathogen in animal models (8, 16). This seems to account for SPIN as well since it inhibits only human MPO and not MPO of other animals we have tested (Fig. S6). By adding this evasion molecule to the arsenal of previously identified evasion molecules, we can better understand the elaborate mechanism by which *S. aureus* evades killing through a multifaceted strategy that acts within the phagosomal compartment.

Materials and Methods

Bacterial Strains and Constructs. The bacterial strains and constructs used in this study are described in detail in *SI Materials and Methods*.

Identification, Expression, and Purification of SPIN. SPIN was subsequently produced in *Escherichia coli* and purified according to procedures described in *SI Materials and Methods*. Primers used in this study are listed in Table S2.

Binding Studies. Detailed procedures about ELISAs, SPR, and the AlphaScreen assay are described in *SI Materials and Methods*.

MPO Activity Assay. SPIN activity was determined by measuring the MPO activity via the redox indicator *o*-Dianisidine. For detailed procedures, see *SI Materials and Methods*.

Crystallization, Structure Determination, Refinement, and Analysis. A description of crystal cell constants, diffraction data quality, and properties of the final model for the SPIN/MPO complex can be found in Table S1. See *SI Materials and Methods* for detailed information about the SPIN/MPO crystal structure.

Analysis of *spn* and *agrA* Expression Following Exposure to Human Neutrophils. Gene expression was assessed using TaqMan RNA-to-C_T 1-*Step* Kit (Life Technologies). Informed consent for blood draw was obtained from all subjects, in accordance with the Declaration of Helsinki. Approval from the medical ethics committee of the University Medical Center Utrecht was attained (METC-protocol 07–125/C approved on 1 March 2010). For detailed procedures, see *SI Materials and Methods*.

Time-Lapse Microscopy and Phagocytosis Assay. Detailed information about time-lapse microscopy of promoter reporter USA300 bacteria and phagocytosis is described in *SI Materials and Methods*.

Bactericidal Assays. Bactericidal assays with MPO and GO or neutrophils is described in detail in *SI Materials and Methods*.

Graphical and Statistical Analyses. MPO activity analyses and statistical analyses were performed with Graphpad Prism v6. Statistical significance was calculated using ANOVA and Student's *t* test.

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1. Lowy FD (1998) *Staphylococcus aureus* infections. *N Engl J Med* 339:520–532.
2. Rigby KM, DeLeo FR (2012) Neutrophils in innate host defense against *Staphylococcus aureus* infections. *Semin Immunopathol* 34:237–259.
3. Faurschou M, Borregaard N (2003) Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect* 5:1317–1327.
4. Babior BM, Lambeth JD, Nauseef W (2002) The neutrophil NADPH oxidase. *Arch Biochem Biophys* 397:342–344.
5. Klebanoff SJ, Hamon CB (1972) Role of myeloperoxidase-mediated antimicrobial systems in intact leukocytes. *J Reticuloendothel Soc* 12:170–196.
6. Kettle AJ, Winterbourn CC (1997) Myeloperoxidase: A key regulator of neutrophil oxidant production. *Redox Rep* 3:3–15.
7. Thwaites GE, Gant V (2011) Are bloodstream leukocytes Trojan Horses for the metastasis of *Staphylococcus aureus*? *Nat Rev Microbiol* 9:215–222.
8. Spaan AN, Surewaard BGJ, Nijland R, van Strijp JA (2013) Neutrophils versus *Staphylococcus aureus*: A biological tug of war. *Annu Rev Microbiol* 67:629–650.
9. Liu GY, et al. (2005) *Staphylococcus aureus* golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. *J Exp Med* 202:209–215.
10. Cosgrove K, et al. (2007) Catalase (KatA) and alkyl hydroperoxide reductase (AhpC) have compensatory roles in peroxide stress resistance and are required for survival, persistence, and nasal colonization in *Staphylococcus aureus*. *J Bacteriol* 189:1025–1035.
11. Kusch H, Engelmann S (2014) Secrets of the secretome in *Staphylococcus aureus*. *Int J Med Microbiol* 304:133–141.
12. Fevre C, et al. (2014) *Staphylococcus aureus* proteins SSL6 and SElX interact with neutrophil receptors as identified using secretome phage display. *Cell Microbiol* 16:1646–1665.
13. Guerra FE, et al. (2016) *Staphylococcus aureus* SaeR/S-regulated factors reduce human neutrophil reactive oxygen species production. *J Leukoc Biol* 100:1005–1010.
14. Fevre C, Scheepmaker L, Haas PJ (2017) Identifying bacterial immune evasion proteins using phage display. *Methods Mol Biol* 1535:43–61.
15. McCarthy AJ, Lindsay JA (2013) *Staphylococcus aureus* innate immune evasion is lineage-specific: A bioinfomatics study. *Infect Genet Evol* 19:7–14.
16. de Haas CJC, et al. (2004) Chemotaxis inhibitory protein of *Staphylococcus aureus*, a bacterial antiinflammatory agent. *J Exp Med* 199:687–695.
17. Verkaik NJ, et al. (2009) Anti-staphylococcal humoral immune response in persistent nasal carriers and noncarriers of *Staphylococcus aureus*. *J Infect Dis* 199:625–632.
18. Palazzolo-Ballance AM, et al. (2008) Neutrophil microbicides induce a pathogen survival response in community-associated methicillin-resistant *Staphylococcus aureus*. *J Immunol* 180:500–509.
19. Voyich JM, et al. (2009) The SaeR/S gene regulatory system is essential for innate immune evasion by *Staphylococcus aureus*. *J Infect Dis* 199:1698–1706.
20. Voyich JM, et al. (2005) Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. *J Immunol* 175:3907–3919.
21. Björnsdottir H, et al. (2015) Neutrophil NET formation is regulated from the inside by myeloperoxidase-processed reactive oxygen species. *Free Radic Biol Med* 89:1024–1035.
22. Vrieling M, et al. (2015) Bovine *Staphylococcus aureus* secretes the leukocidin LukMF' to kill migrating neutrophils through CCR1. *MBio* 6:e00335.
23. Stapels DA, et al. (2014) *Staphylococcus aureus* secretes a unique class of neutrophil serine protease inhibitors. *Proc Natl Acad Sci USA* 111:13187–13192.
24. Nygaard TK, et al. (2010) SaeR binds a consensus sequence within virulence gene promoters to advance USA300 pathogenesis. *J Infect Dis* 201:241–254.
25. Pantrangi M, Singh VK, Wolz C, Shukla SK (2010) Staphylococcal superantigen-like genes, ssl5 and ssl8, are positively regulated by Sae and negatively by Agr in the Newman strain. *FEMS Microbiol Lett* 308:175–184.
26. Klebanoff SJ, Kettle AJ, Rosen H, Winterbourn CC, Nauseef WM (2013) Myeloperoxidase: A front-line defender against phagocytosed microorganisms. *J Leukoc Biol* 93:185–198.
27. Serruto D, Rappuoli R, Scarselli M, Gros P, van Strijp JA (2010) Molecular mechanisms of complement evasion: Learning from staphylococci and meningococci. *Nat Rev Microbiol* 8:393–399.
28. Koymans KJ, Vrieling M, Gorham RD, Jr, van Strijp JAG (2016) Staphylococcal immune evasion proteins: Structure, function, and host adaptation. *Curr Top Microbiol Immunol* 6:23–27.
29. Bakhautdin B, Goksoy Bakhautdin E, Fox PL (2014) Ceruloplasmin has two nearly identical sites that bind myeloperoxidase. *Biochem Biophys Res Commun* 453:722–727.
30. Segelmark M, Persson B, Hellmark T, Wieslander J (1997) Binding and inhibition of myeloperoxidase (MPO): A major function of ceruloplasmin? *Clin Exp Immunol* 108:167–174.
31. Samygina VR, et al. (2013) Ceruloplasmin: Macromolecular assemblies with iron-containing acute phase proteins. *PLoS One* 8:e67145.
32. Chapman ALP, et al. (2013) Ceruloplasmin is an endogenous inhibitor of myeloperoxidase. *J Biol Chem* 288:6465–6477.
33. Fiedler TJ, Davey CA, Fenna RE (2000) X-ray crystal structure and characterization of halide-binding sites of human myeloperoxidase at 1.8 A resolution. *J Biol Chem* 275:11964–11971.
34. van den Berg S, et al. (2015) A human monoclonal antibody targeting the conserved staphylococcal antigen IsaA protects mice against *Staphylococcus aureus* bacteremia. *Int J Med Microbiol* 305:55–64.
35. Waterhouse AM, Procter JB, Martin DM, Clamp M, Barton GJ (2009) Jalview version 2—A multiple sequence alignment editor and analysis workbench. *Bioinformatics* 25:1189–1191.
36. Carver TJ, et al. (2005) ACT: The Artemis comparison tool. *Bioinformatics* 21:3422–3423.
37. Bose JL, Fey PD, Bayles KW (2013) Genetic tools to enhance the study of gene function and regulation in *Staphylococcus aureus*. *Appl Environ Microbiol* 79:2218–2224.
38. Pang YY, et al. (2010) agr-Dependent interactions of *Staphylococcus aureus* USA300 with human polymorphonuclear neutrophils. *J Innate Immun* 2:546–559.
39. van Kessel KPM, van Strijp JAG, Verhoef J (1991) Inactivation of recombinant human tumor necrosis factor-alpha by proteolytic enzymes released from stimulated human neutrophils. *J Immunol* 147:3862–3868.
40. Rooijakkers SHM, van Wamel WJB, Ruyken M, van Kessel KPM, van Strijp JA (2005) Anti-opsonic properties of staphylokinase. *Microbes Infect* 7:476–484.
41. Bosse R, Illy C, Chelsky D (2002) *Application Note. Principles of AlphaScreen* (PerkinElmer Life Sciences, Boston).
42. Otwinowski Z, Minor W (1997) Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol* 276:307–326.
43. Zwart PH, et al. (2008) Automated structure solution with the PHENIX suite. *Structural Proteomics High-Throughput Methods*, eds Kobe B, Guss M, Huber T (Humana Press, Totowa, NJ), pp 419–435.
44. Afonine PV, et al. (2010) Joint X-ray and neutron refinement with phenix.refine. *Acta Crystallogr D Biol Crystallogr* 66:1153–1163.
45. Emsley P, Lohkamp B, Scott WG, Cowtan K (2010) Features and development of Coot. *Acta Crystallogr D Biol Crystallogr* 66:486–501.
46. Krissinel E, Henrick K (2007) Inference of macromolecular assemblies from crystalline state. *J Mol Biol* 372:774–797.
47. Voyich JM, Sturdevant DE, DeLeo FR (2008) Analysis of *Staphylococcus aureus* gene expression during PMN phagocytosis. *Methods Mol Biol* 431:109–122.
48. Denys GA, Grover P, O'Hanley P, Stephens JT, Jr (2011) In vitro antibacterial activity of E-101 Solution, a novel myeloperoxidase-mediated antimicrobial, against Gram-positive and Gram-negative pathogens. *J Antimicrob Chemother* 66:335–342.
49. Bestebroer J, et al. (2007) Staphylococcal superantigen-like 5 binds PSGL-1 and inhibits P-selectin-mediated neutrophil rolling. *Blood* 109:2936–2943.

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BACTERIAL INFECTIONS

Increased Pilus Production Conferred by a Naturally Occurring Mutation Alters Host-Pathogen Interaction in Favor of Carriage in *Streptococcus pyogenes*

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ABSTRACT Studies of the human pathogen group A *Streptococcus* (GAS) define the carrier phenotype to be an increased ability to adhere to and persist on epithelial surfaces and a decreased ability to cause disease. We tested the hypothesis that a single amino acid change (Arg135Gly) in a highly conserved sensor kinase (LiaS) of a poorly defined GAS regulatory system contributes to a carrier phenotype through increased pilus production. When introduced into an *emm* serotype-matched invasive strain, the carrier allele (the gene encoding the LiaS protein with an arginine-to-glycine change at position 135 [$liaS^{R135G}$]) recapitulated a carrier phenotype defined by an increased ability to adhere to mucosal surfaces and a decreased ability to cause disease. Gene transcript analyses revealed that the *liaS* mutation significantly altered transcription of the genes encoding pilus in the presence of bacitracin. Elimination of pilus production in the isogenic carrier mutant decreased its ability to colonize the mouse nasopharynx and to adhere to and be internalized by cultured human epithelial cells and restored the virulence phenotype in a mouse model of necrotizing fasciitis. We also observed significantly reduced survival of the isogenic carrier mutant compared to that of the parental invasive strain after exposure to human neutrophils. Elimination of pilus in the isogenic carrier mutant increased the level of survival after exposure to human neutrophils to that for the parental invasive strain. Together, our data demonstrate that the carrier mutation ($liaS^{R135G}$) affects pilus expression. Our data suggest new mechanisms of pilus gene regulation in GAS and that the invasiveness associated with pilus gene regulation in GAS differs from the enhanced invasiveness associated with increased pilus production in other bacterial pathogens.

KEYWORDS group A *Streptococcus*, carrier, sensor kinase, pilus, colonization, asymptomatic carriage

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Colonization of a susceptible host is a critical first step in the infection cycle of all bacterial pathogens. Upon contact with a host, a complex interplay between host immune and clearance mechanisms and the ability of a pathogen to subvert host defenses ensues. The result is a continuum ranging from asymptomatic carriage (henceforth referred to simply as carriage) to the breach of host barriers, dissemination, and a multitude of disease manifestations (1). Currently, the vast majority of research and, hence, our understanding of infectious disease pathogenesis are based on the ability of a bacterial pathogen to cause disease. However, the rates of carriage of many bacterial pathogens, such as *Neisseria meningitidis* (2–4), *Streptococcus pneumoniae* (5),

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Staphylococcus aureus (6), *Streptococcus agalactiae* (group B *Streptococcus* [GBS]) (7–9), and *Streptococcus pyogenes* (group A *Streptococcus* [GAS]) (10), far exceed the rates of disease caused by these pathogens. Thus, study of the bacterial molecular mechanisms that contribute to carriage is critical to identifying enhanced strategies to prevent disease.

GAS is an ideal model organism for the study of bacterial carriage. The diseases caused by GAS range from relatively benign superficial diseases (e.g., impetigo, pharyngitis) to severe and life-threatening conditions (e.g., toxic shock syndrome, necrotizing fasciitis). In the United States, the annual rate of occurrence of serious invasive disease caused by GAS is very low at approximately 3 cases/100,000 population (11). GAS is also carried in the throats of healthy individuals in the absence of symptoms. The rate of GAS carriage among children ranges from 5 to 15% (10), a rate that far exceeds that of any disease caused by GAS. The mechanisms by which GAS causes disease have been intensely investigated and have been the subject of several reviews (12). On the other hand, the GAS carrier state has been described as an enigma for decades, with few efforts being made to explore the underlying bacterial or host mechanisms of GAS carriage (13, 14). Improving our understanding of the bacterial molecular mechanisms contributing to GAS carriage may enhance our understanding of the basic biology of this important human pathogen and facilitate the development of an effective vaccine.

Control of gene expression by regulatory systems along with mutational adaptation may favor bacterial persistence after disease resolution. Comparison of the whole genomes of carriage and disease-causing strains of GAS has identified mutations unique to carrier strains that contribute to GAS carriage (15–18). A recurring theme that has emerged from GAS comparative genomic studies is that mutations affecting bacterial cell surface molecules alter the host-pathogen interaction and are critical to the development of carriage and a carrier phenotype. Gene regulation in GAS, similar to that in other bacteria, uses several two-component systems (TCS) that consist of a membrane-anchored histidine kinase (HK) for sensing environmental stimuli that, upon activation, phosphorylates a cytoplasmic response regulator (RR), leading to altered gene transcription (19). GAS genomes have up to 13 TCS, and the functions of many remain poorly understood (20). Recently, a GAS carrier strain with a mutation leading to a single amino acid change in a sensor kinase (LiaS) of the three-component system (3CS) LiaFSR was identified (17). Importantly, this was the first mutation in a human GAS carrier strain found to alter global gene regulation. The carrier mutation in *liaS* (causing an arginine-to-glycine change at position 135 of the LiaS protein [*liaS*^{R135G}]) alters the transcription of genes encoding several virulence factors (17). The relative contribution of individual differential gene expression to the carrier phenotype has not been further explored.

Pili are long filamentous surface structures made by GAS and other bacterial pathogens that have received increased attention, especially in Gram-positive organisms. The GAS pilus was first identified in 2005 (21), and it is now known to be a heteropolymeric structure consisting of a backbone protein (BP) and ancillary proteins (AP) (22) and is the antigenic target for serologic T typing first described by Lancefield (21). Studies suggest that the presence of pilus on the GAS cell surface may simultaneously promote epithelial cell colonization and reduce virulence (23, 24), phenotypes consistent with a carrier state. Regulation of GAS pilus gene expression occurs through stand-alone regulators, including RofA/Nra (25–27) and MsmR (28), and varies by serotype but may also involve GAS TCS. Studies in *S. pneumoniae* (29) and GBS (30) suggest that the LiaFSR three-component system may directly or indirectly regulate pilus gene expression. However, to date, no studies provide direct evidence of LiaFSR pilus gene regulation in GAS.

In the study described here, we tested the hypothesis that the *liaS*^{R135G} carrier mutation increases pilus gene transcription and that increased pilus production contributes to the carrier phenotype. In contrast to previous studies, we show that activation of the LiaFSR 3CS by bacitracin results in a significant increase in pilus gene transcription in the isogenic carrier mutant. Elimination of pilus production in the isogenic carrier mutant decreases its ability to adhere to and persist on epithelial surfaces and restores

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virulence, essentially reversing the carrier phenotype. Our studies are the first to provide direct evidence that the LiaFSR regulatory system alters pilus gene regulation and that increased pilus production contributes to carriage.

RESULTS

Activation of the LiaFSR 3CS significantly increases pilus gene transcription and protein expression in the carrier mutant. Previous RNA sequencing (RNA-seq) analyses assessing the global gene transcriptional impact of the *liaS*^{R135G} mutation failed to reveal any significant differences in pilus gene transcription (17). In contrast, deletion of *liaS* (*hk03*) in *Streptococcus pneumoniae* significantly increased pilus gene transcription (29), whereas deletion of *liaR* in *Streptococcus agalactiae* significantly decreased pilus gene transcription (30). We hypothesized that the absence of significant changes in pilus gene transcripts in the isogenic carrier mutant was due to the relative inactivity of the LiaFSR three-component system under the previous conditions tested. Activation of LiaFSR- and LiaR-dependent gene activation in response to bacitracin is well established in *Bacillus subtilis* (31), *S. aureus* (32), and *S. pneumoniae* (33). We tested our hypothesis by measuring the bacitracin-induced activation of LiaFSR through gene transcription. Prior to assaying transcript levels, we first assayed the ability of the parental invasive strain MGAS10870 to grow in the presence of bacitracin. MGAS10870 was grown to mid-exponential phase and, upon reaching mid-exponential-phase growth, was treated with bacitracin at concentrations ranging from the MIC (0.025 μg/ml for MGAS10870 and 0.0125 μg/ml for the isogenic mutant MGAS10870 *liaS*^{R135G}) to over 100-fold the MIC (5.0 μg/ml). The highest concentration of bacitracin resulted in a significant reduction in growth beginning at the 60-min time point (Fig. 1A). No significant differences in growth were observed for the other concentrations used. Additionally, no significant differences in the numbers of CFU between treated and untreated cultures were observed up to bacitracin concentrations of 1.0 μg/ml (Fig. 1A, dashed lines).

We next assayed activation in GAS bacteria harvested at 30-min intervals following the addition of bacitracin using quantitative real-time PCR to measure the transcript levels of *liaFSR* and compared those levels to the transcript levels from untreated cells. LiaFSR is known to be autoregulated in *B. subtilis* (31); however, whether autoregulation occurs in GAS is unknown. Significant increases in *liaFSR* transcript levels following the addition of bacitracin occurred at concentrations of $\geq$1 μg/ml and $\geq$60 min posttreatment (Fig. 1B). To provide further evidence of LiaFSR activation, we also assayed the transcript levels of *spxA2* (*spyM3_1799*) following the addition of bacitracin. This gene was assayed because deletion of LiaR in *S. agalactiae* was found to eliminate *spxA* transcription (30), and more recently, LiaR has been shown to bind to a 25-bp consensus motif in the promoter of the homologous *spxA* in *Streptococcus mutans* (34). Examination of the *spxA2* promoter sequence in a serotype M3 GAS strain revealed a similar motif with approximately 85% identity (data not shown). Further, *spxA2* was the most highly differentially gene expressed in RNA-seq studies comparing the wild type and its isogenic carrier (*liaS*^{R135G}) mutant (17). Consistent with *spxA2* gene regulation by LiaFSR, we observed significantly increased *spxA2* transcript levels following the addition of bacitracin (Fig. 1B, *spxA2*). The transcription of *spxA2* was also concentration and time dependent. However, at the highest concentration used (5.0 μg/ml), transcript levels continued to increase at the 90-min time point, coinciding with a significantly reduced growth rate (Fig. 1B, *spxA2*). Overall, examination of *liaFSR* and *spxA2* transcript levels revealed a peak at 60 min following treatment with bacitracin at 1.0 μg/ml, suggestive of LiaFSR activation.

Having established the *in vitro* conditions under which the LiaFSR 3CS was activated, we next tested the hypothesis that the carrier mutation *liaS*^{R135G} significantly alters pilus gene transcription. The genes necessary for pilus biosynthesis in GAS are contiguously located in a chromosomal region with genes encoding fibronectin binding proteins, collagen binding proteins, and T antigens (FCT) (35). The T antigen is composed of the pilus subunits, including a backbone protein (*spyM3_100*, *tee3*) and two

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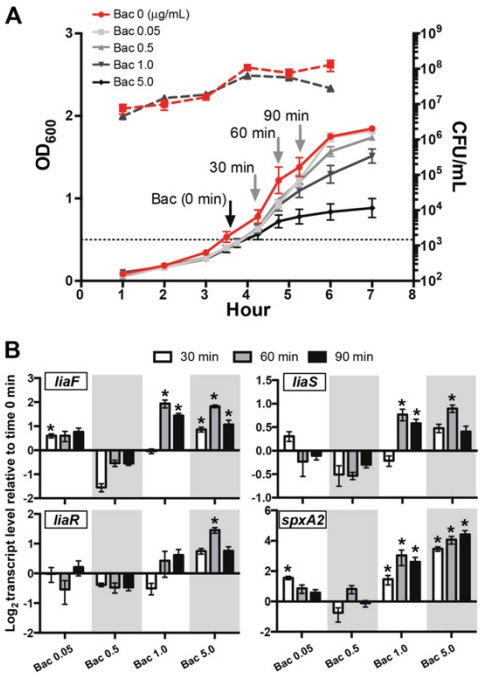


FIG 1 Activation of LiaFSR by bacitracin (Bac) and effect on gene transcription in the serotype M3 invasive strain MGAS10870. (A) MGAS10870 was grown in rich medium (THY), and bacitracin was added when the OD_{600} was 0.5 (dotted line). Cells were harvested at 30-min intervals (gray arrows) for RNA extraction and quantitative real-time PCR. Dashed lines, number of CFU during growth in the presence of bacitracin (5.0 μg/ml; black dashed line) or without bacitracin (red dashed line). (B) Quantitative real-time PCR of *liaF*, *liaS*, *liaR*, and *spxA2* at 30 min (white bars), 60 min (gray bars), and 90 min (black bars) following the addition of bacitracin. *, $P < 0.05$ (*t* test, unpaired, Welch's correction) compared to the transcript level at time zero.

ancillary proteins (*spyM3_0098* and *spyM3_102*) (22). Pilus subunits and the associated sortases necessary for assembly are oriented in a predicted operon (Fig. 2A) under the control of a single promoter (Database of Prokaryotic Operons, version 2.0; http://csbl.bmb.uga.edu/DOOR/ [36]). We measured the transcript levels of the genes encoding an ancillary protein (*spyM3_0098*, *cbp*) and the backbone protein (*spyM3_0100*, *tee3*) from cells of MGAS10870 and the isogenic carrier mutant (MGAS10870 *liaS*^{R135G}) following treatment with bacitracin (1.0 μg/ml, 60 min) (Fig. 2B). Consistent with our hypothesis, we observed significantly increased pilus gene transcription in the isogenic carrier mutant compared to the parental strain following treatment with bacitracin (Fig. 2C).

It is possible that modest but significant increases in transcription do not result in increased protein expression in the isogenic carrier mutant compared to the invasive parental strain. We generated an affinity-purified polyclonal antibody against the major pilus backbone protein, Tee3 (SpyM3_0100), to test the hypothesis that the isogenic carrier mutant produced more pilus protein than the parental invasive strain did. Cell wall and secreted proteins were purified after treatment with bacitracin (1.0 μg/ml), as described above for transcript analyses (Fig. 2B). As predicted following Western immunoblotting using the anti-Tee3 antibody, the isogenic carrier mutant showed increased amounts of the Tee3 protein compared to the parental strain in both purified

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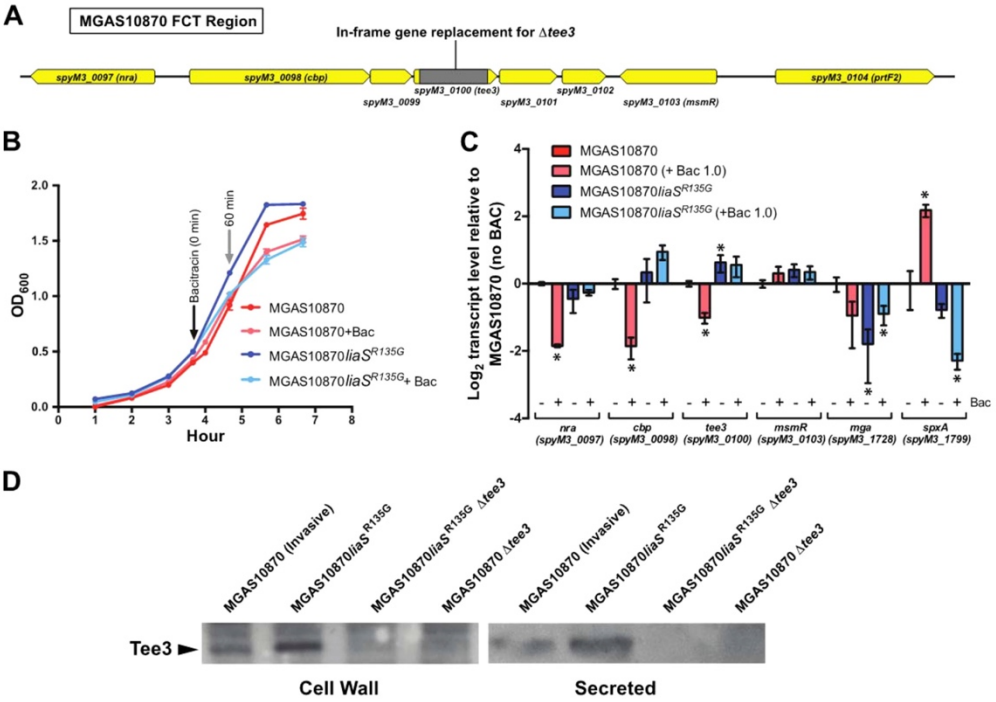


FIG 2 Effect of bacitracin treatment on pilus gene transcript levels. (A) FCT region in serotype M3 GAS. The spectinomycin resistance gene, *aad9* (gray block), was used to construct in-frame deletions of the pilus backbone protein (*tee3*, *spyM3_0100*). (B) Growth curves for MGAS10870 and MGAS10870 *liaS*^{R135G} in rich medium with and without bacitracin at 1.0 μg/ml. Bacitracin was added when the OD_{600} was 0.5, and cells were harvested 60 min after that for RNA extraction and quantitative real-time PCR. (C) Quantitative real-time PCR for determination of the transcript levels of pilus genes (*cbp* and *tee3*), pilus gene regulators (*nra* and *msmR*), and *spxA2*. *, $P < 0.05$ (*t* test, unpaired, Welch's correction) compared to those in parental strain MGAS10870. (D) Western immunoblotting for Tee3 in the parental invasive strain (MGAS10870), an isogenic carrier mutant, and derived pilus-negative mutants. Cells were treated with bacitracin as described in the legend to panel B, proteins were extracted, and equal amounts of purified cell wall and secreted proteins were probed with an affinity-purified antibody to Tee3 as described in Materials and Methods.

cell wall and secreted protein extracts (2-fold and 1.7-fold increases, respectively; Fig. 2D).

Increased pilus gene transcription in the isogenic carrier mutant is not due to altered gene transcription of known pilus gene regulators. Pilus gene regulation may occur through a number of transcriptional regulators and appears to be M protein serotype and FCT type dependent (25–28). Serotype M3 GAS (FCT type 3) strains may regulate pilus gene expression through regulators that reside within the FCT chromosomal region, including Nra (SpyM3_0097) (26) and MsmR (SpyM3_0103) (28). Additionally, transcriptome analysis following the deletion of the stand-alone regulator Mga suggests that Mga is an activator of pilus in serotype M1 GAS strains (37). One potential explanation for the significant increase in pilus gene transcription in the isogenic carrier mutant relative to the parental strain is alteration of gene transcription in one or more of the known pilus gene regulators. A previous transcriptome analysis of the isogenic carrier strain at two time points failed to identify significant differences in either *nra* or *msmR* transcript levels. However, *mga* transcript levels in the isogenic carrier mutant decreased approximately 2-fold in early exponential phase (17). To explore the potential mechanism of altered pilus gene transcription, we assayed transcripts of *nra*, *msmR*, and *mga* in the isogenic carrier and wild-type parental strains after bacitracin treatment

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(1.0 μg/ml, 60 min). Consistent with previous RNA-seq data (17), we observed an approximately 2-fold decrease in *mga* transcription in the isogenic carrier mutant in the absence or presence of bacitracin (Fig. 2C). Interestingly, we observed a significant decrease in the transcript levels of both pilus genes and the pilus gene regulator *nra* in the parental strain (MGAS10870) after bacitracin exposure (Fig. 2C). No significant differences in the transcript levels of either *nra* or *msmR* were observed between the isogenic carrier mutant and the parental strain (Fig. 2C). Nra is a positive regulator of pilus gene transcription in serotype M53 but a negative regulator in serotype M49 (26). Thus, Nra may act as a positive regulator of pilus gene transcription in serotype M3 GAS strains, and the decreased transcription observed in the wild-type MGAS10870 strain may be responsible for the differences in pilus gene expression compared to that in the isogenic carrier strain. Our data indicate that the increased pilus gene transcription conferred by the *liaS*^{R135G} carrier mutation is not mediated by other known pilus gene regulators.

Deletion of the major pilus subunit gene in the *liaS*^{R135G} carrier mutant decreases the ability to colonize the mouse nasopharynx and adhere to and be internalized by cultured human epithelial cells. We have defined the carrier phenotype to be one characterized by an increased ability to adhere to epithelial surfaces and a decreased ability to cause disease, a definition supported by investigation of independent carrier-specific mutations and their contribution to the carrier phenotype (15–18). The *liaS*^{R135G} carrier mutation causes an increased frequency of mouse colonization in a model of nasopharyngeal infection (17). Further, in serotype M1 GAS strains, elimination of pilus decreases adherence to cultured human epithelial cells (23). Inasmuch as induction of the LiaFSR 3CS by bacitracin *in vitro* leads to alteration of pilus gene transcription, we hypothesized that the *liaS*^{R135G} mutation results in increased pilus gene transcription *in vivo* and contributes to the carrier phenotype, including an increased ability to colonize the mouse nasopharynx. If this is true, elimination of the GAS pilus would lead to decreased recovery following mouse nasopharyngeal infection. To test this hypothesis, we created an in-frame replacement of the major pilus subunit gene (*spyM3_0100*, *tee3*) to generate a pilus-negative carrier mutant (MGAS10870 *liaS*^{R135G} Δ*tee3*) and a wild-type invasive strain (MGAS10870 Δ*tee3*) (Fig. 2A). Both pilus-negative strains lacked bacterial cell surface pilus expression, as determined by Western immunoblotting using an affinity-purified anti-Tee3 polyclonal antibody (Fig. 2D). Mice infected in the nasopharynx with the parental invasive strain (MGAS10870), isogenic carrier mutant (MGAS10870 *liaS*^{R135G}), pilus-negative invasive strain (MGAS10870 Δ*tee3*), or pilus-negative carrier mutant (MGAS10870 *liaS*^{R135G} Δ*tee3*) were swabbed daily for 14 days to determine the number of bacterial CFU. Elimination of pilus production significantly reduced the ability of the isogenic carrier strain to colonize the mouse nasopharynx but had only a modest effect on that of the invasive strain (Fig. 3A). Consistent with the findings of previous studies, beginning at day 5 bacteria were significantly more frequently recovered from mice infected with the *liaS*^{R135G} carrier mutant than from mice infected with the parental strain (Fig. 3A). The pilus-negative *liaS*^{R135G} mutant was recovered from mice significantly less frequently than the *liaS*^{R135G} mutant but significantly more frequently than the pilus-negative wild-type invasive strain (Fig. 3A).

In addition to decreased recovery following mouse nasopharyngeal infection, we hypothesized that elimination of pilus would also significantly reduce adherence of the carrier mutant to cultured human epithelial cells. Thus, we performed *in vitro* adherence assays comparing the pilus-negative mutants to their parental counterparts. We observed significantly increased adherence of MGAS10870 *liaS*^{R135G} to HaCaT cells compared to the parental strain, MGAS10870 (Fig. 3B, HaCaT). We also assayed adherence to HEp-2 cells and observed a phenotype similar to that for adherence to HaCaT cells (Fig. 3B, HEp-2). Elimination of the pilus backbone protein had no significant effect on the adherence of the parental invasive strain to either HaCaT or HEp-2 cells (Fig. 3B). The pilus-negative carrier mutant had significantly reduced adherence to HEp-2 cells and a trend toward reduced adherence to HaCaT cells (Fig. 3B). Interestingly, the

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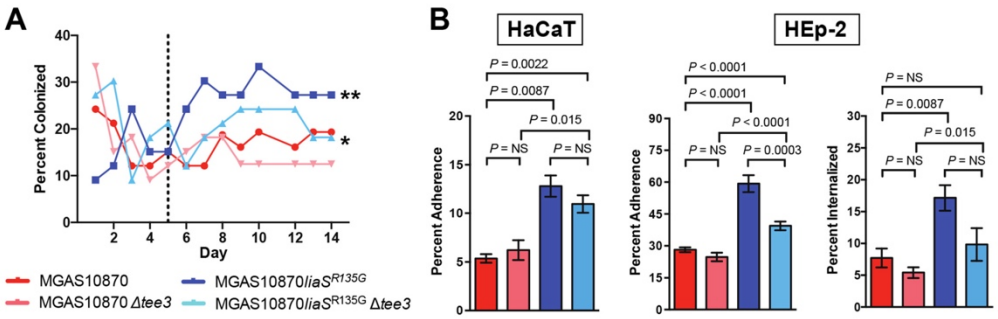


FIG 3 Elimination of pilus in MGAS10870 *liaS*^{R135G} decreases recovery following mouse nasopharyngeal infection and *in vitro* adherence to and internalization by human epithelial cells. (A) Percentage of mice from which bacteria were recovered following nasopharyngeal infection with the respective GAS strains. **, $P < 0.05$ (Kruskal-Wallis repeated-measures ANOVA) relative to mice infected with all other strains after day 5 (dotted line); *, $P < 0.05$ compared to both the parental and pilus-negative parental strains after day 5. (B) Adherence to cultured human epithelial cells. Assays of adherence to HaCaT and HEp-2 cells were performed in replicates of 12 on at least two occasions. Internalization assays were performed using HEp-2 cells. Percent adherence or percent internalization was calculated by dividing the colony counts following the assay by the input number of bacterial cells and multiplying by 100. *P* values were determined by the Mann-Whitney U test. NS, not significant.

pilus-negative carrier mutant maintained significantly increased adherence compared to that of either the parental or the pilus-negative parental strain (Fig. 3B), suggesting that additional molecules are involved in adherence in the carrier mutant.

We also assessed the contribution of the GAS pilus to the ability of the parental and isogenic carrier mutants to be internalized by human epithelial cells. Previous studies suggest that pilus contributes to the ability of GAS to be internalized by epithelial cells (24). Thus, we hypothesized that the increased pilus gene transcription conferred by the carrier mutation would also lead to increased internalization by HEp-2 cells. We performed gentamicin protection assays to determine the level of internalization of the parental, isogenic carrier, and pilus-negative strains. The isogenic carrier mutant had an almost 3-fold increased ability to be internalized by HEp-2 cells compared to the wild-type, parental strain (Fig. 3B). Elimination of pilus did not significantly alter the ability of either the parental or the isogenic carrier mutant to be internalized by HEp-2 cells. However, there was a trend toward significance for the pilus-negative isogenic carrier mutant ($P = 0.065$). Overall, the data suggest that pilus is a contributor to epithelial cell adherence and internalization in the isogenic carrier mutant but that additional factors also contribute to this phenotype.

Deletion of the pilus major subunit gene partially restores virulence in the *liaS*^{R135G} carrier mutant in a mouse model of necrotizing fasciitis. It has been shown that deletion of pilus in serotype M1 GAS strains results in increased virulence in mouse models of invasive GAS infection (23, 24). Thus, we next hypothesized that if increased pilus expression is a contributor to the carrier phenotype (i.e., decreased virulence) conferred by the *liaS*^{R135G} mutation, elimination of pilus would restore virulence in isogenic carrier mutant MGAS10870 *liaS*^{R135G}. Using strains identical to those used for mouse nasopharyngeal infection, mice were infected intramuscularly and followed for survival. No significant difference in survival was observed between mice infected with the isogenic *liaS*^{R135G} carrier mutant strain and mice infected with the pilus-negative *liaS*^{R135G} mutant (MGAS10870 *liaS*^{R135G} Δ*tee3*) (Fig. 4A). However, consistent with an inverse relationship between pilus expression and virulence, we observed significantly greater mortality in mice infected with the MGAS10870 Δ*tee3* mutant strain than mice infected with the carrier *liaS*^{R135G} mutant strain (Fig. 4A). Examination of mouse limbs at 72 h postinfection revealed that the pilus-negative *liaS*^{R135G} mutant caused an infection that appeared similar to that caused by the wild type, with larger areas of devitalized tissue and necrosis that were indistinguishable from those caused by the parental invasive strain (Fig. 4B). In contrast, the MGAS10870

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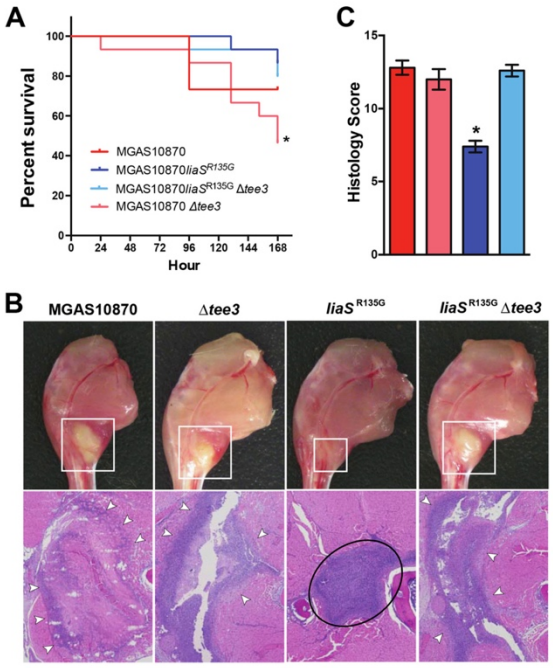


FIG 4 Deletion of the pilus backbone gene in MGAS10870 *liaS*^{R135G} partially restores virulence and reverses the carrier phenotype. (A) Kaplan-Meier survival curve of mice following intramuscular infection with MGAS10870 (parental strain), MGAS10870 Δ*tee3* (parental strain, pilus negative), MGAS10870 *liaS*^{R135G} (carrier mutant), or MGAS10870 *liaS*^{R135G} Δ*tee3* (carrier mutant, pilus negative). *, $P < 0.05$ (log-rank test) relative to both the pilus-positive and pilus-negative carrier mutant. (B) Visual inspection and histopathology of infected mouse limbs at 72 and 24 h postinfection, respectively. (Top row) White boxes, areas of abscess-like formation centered at the inoculation site of the limbs; (bottom row) white arrowheads, areas of marked neutrophil infiltration and necrotic fascial and muscle tissue that extend beyond the border of the field of view; black oval, a well-circumscribed abscess that is limited to the fascial plane and surrounded by healthy muscle tissue (magnification, ×4). (C) Pathology score for wild-type, isogenic carrier, and pilus-negative derivatives based on microscopic examination at 24 h postinfection. *, $P < 0.05$ (Wilcoxon rank sum) compared to all other strains.

liaS^{R135G} strain caused a small isolated area of infection surrounded by healthy tissue (Fig. 4B), similar to previous observations (17). Using a validated pathology score (38), the isogenic carrier mutant (MGAS10870 *liaS*^{R135G}) produced significantly less tissue damage than the wild-type or pilus-negative strain (Fig. 4C). Thus, consistent with our hypothesis, elimination of pilus partially restored virulence in the *liaS*^{R135G} mutant strain.

The isogenic carrier mutant had decreased survival after exposure to human neutrophils, a phenotype reversed upon elimination of pilus production. Previously, an inverse relationship between pilus expression and survival in human blood was reported for serotype M1 GAS strains (23). The *liaS*^{R135G} carrier mutation is known to confer a decreased ability to grow in human blood *ex vivo* (17). We hypothesized that increased pilus transcription and increased pilus expression in the isogenic carrier mutant contribute to this phenotype. Further, we hypothesized that, as reported for serotype M1 GAS strains (23), decreased survival of the isogenic carrier mutant in human blood would be due to increased pilus expression. To test this hypothesis, we performed GAS survival assays following exposure to purified human neutrophils.

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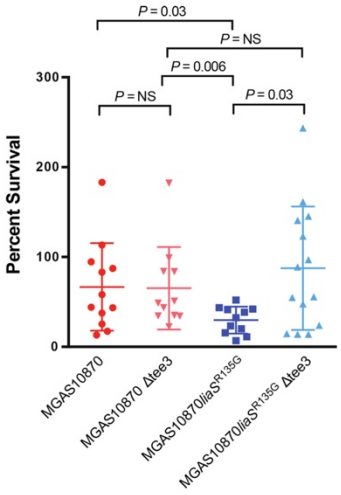


FIG 5 Elimination of pilus in MGAS10870 *liaS*^{R135G} increases survival in purified human neutrophils. The survival of MGAS10870 (parental strain), MGAS10870 Δ*tee3* (parental strain, pilus negative), MGAS10870 *liaS*^{R135G} (carrier mutant), or MGAS10870 *liaS*^{R135G} Δ*tee3* (carrier mutant, pilus negative) was assessed 3 h following interaction with human neutrophils. Percent survival was calculated with the equation (CFU_{+PMN} at 3 h/CFU_{+PMN} at 0 h) × 100. *P* values were determined by the Mann-Whitney U test.

Consistent with our previous observations in whole blood *ex vivo* (17), the isogenic carrier mutant had significantly reduced survival compared to the parental invasive strain (Fig. 5). The pilus-negative carrier mutant strain (MGAS10870 *liaS*^{R135G} Δ*tee3*) had significantly increased survival compared to the pilus-positive carrier mutant (Fig. 5). In contrast, elimination of pilus in the invasive strain did not result in significantly increased survival (Fig. 5).

DISCUSSION

Neither the bacterial nor the host molecular mechanisms that contribute to the persistence of bacterial pathogens in the upper respiratory tract of humans are well understood. Few studies have directly examined the bacterial molecular genetic factors that lead to the persistence of GAS in humans (15, 16, 18). Our findings substantially enhance understanding of the molecular mechanism underlying the carrier mutation (*liaS*^{R135G}) and its contribution to the asymptomatic carrier phenotype in GAS. We demonstrated that the carrier mutation altered pilus gene regulation and that increased pilus gene expression contributed to increased adherence and internalization by epithelial cells and decreased virulence (i.e., the carrier phenotype). This is the first report of pilus as a major contributor to a carrier phenotype in GAS and differs strikingly from the enhanced invasiveness reported for piliated pneumococci (39, 40) and GBS (41, 42).

Our discovery that a GAS carrier mutation alters expression of bacterial surface structures and contributes to the GAS carrier phenotype is in concert with previous findings. We reported a mutation unique to carrier strains in the promoter of the stand-alone regulator Mga that led to decreased transcription of *mga* and Mga-regulated genes, including *emm*, encoding M protein (18). We also identified a carrier mutation that restored the function of the GAS surface protein SclA (streptococcal collagen-like protein A) (16). In addition, we discovered that mutations eliminating GAS capsule production were the most frequently identified mutations in GAS carrier strains (15). In the aggregate, our studies have revealed multiple independent pathways to

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a carrier phenotype defined by increased persistence, decreased virulence, and decreased inflammation, a phenotype that stands in stark contrast to an invasive disease phenotype. A recurring theme has emerged, in that independent genetic changes altering cell surface molecules and, potentially, the host-microbe interaction are critical to the carrier phenotype.

Little is known regarding GAS gene regulation by the three-component system LiaFSR. We previously showed that the *liaS*^{R135G} carrier mutation significantly altered the transcript levels of approximately 7% of the genes in serotype M3 GAS strains (17). Here, we show that gene regulation by LiaFSR and LiaS^{R135G} is affected following activation by bacitracin. Specifically, we show that, consistent with the findings of studies with GBS (30) and *S. pneumoniae* (29), the GAS LiaFSR exerts some level of control over pilus gene transcription. Targeted gene transcription of known pilus gene regulators identified *nra* to be one potential mechanism for the increased pilus gene transcription in the isogenic carrier mutant. However, the association was indirect, as the only significant differences in *nra* transcription following treatment with bacitracin were in the parental invasive strain (in which *nra* transcription was decreased 3.6-fold), suggesting that *nra* acts as a positive regulator of pilus gene transcription, similar to the findings for serotype M53 GAS (26). We also observed a 1.9-fold decrease in *mga* transcription in the isogenic carrier mutant following treatment with bacitracin. Mga-deficient mutants of serotype M1 GAS have decreased pilus gene expression (37), suggesting that Mga is also an activator of pilus gene expression.

It is unknown if the LiaFSR three-component system directly interacts with the pilus operon or known pilus gene regulators. Serotype M1 GAS strains lacking LiaS have a reduced ability to grow under acidic conditions (43). Recently, site-directed mutagenesis suggested that LiaS may contribute to extracellular acidification and may be activated under acidic conditions, and this directly affected biofilm formation (44). Further, GAS pili contribute to biofilm formation, and, at least for a subset of FCT types, a lower environmental pH enhances pilus gene transcription (45). More studies are needed to further define the precise mechanism of pilus gene regulation by LiaFSR and the mechanism by which the *liaS*^{R135G} carrier mutation alters pilus gene transcription.

Our data strongly support the possibility that pilus expression confers a distinct advantage to the development of a carrier phenotype. In studies of serotype M1 GAS strains, mutants lacking pili had a reduced ability to adhere to pharyngeal cells (46), human tonsillar epithelium, and primary human keratinocytes (47). Pilus-defective mutants of serotype M1 GAS were also shown to have a decreased ability to adhere to cultured human epithelial cells and reduced virulence in murine models of invasive disease (23, 24). Consistent with the findings of previous studies, our data demonstrate that increased pilus expression contributes to the increased adherence and decreased virulence of the isogenic carrier mutant. Moreover, our findings echo those of others showing an inverse relationship between expression of bacterial adhesins and virulence (48–50). The pili of several Gram-positive pathogens, including GAS, are under investigation as potential vaccine targets (21, 51). Our findings that pili influence virulence but also carriage and persistence have direct implications in vaccine development.

The current study shows that the LiaFSR 3CS is directly involved in GAS pathogenesis and the host-pathogen interaction. No previous studies have directly linked the LiaFSR regulatory system to immune evasion. The *liaS*^{R135G} single amino acid change results in a significantly decreased ability to survive exposure to human neutrophils. This finding begins to define a mechanism for the observation that the carrier mutation decreases survival in whole human blood *ex vivo* (17). Previous studies of GAS-neutrophil interactions identified the two-component regulatory system Ihk/Irr to be critical to GAS survival (52), and *irr*-negative mutants were more easily killed by neutrophils (53). RNA-seq analysis of the *liaS*^{R135G} mutant at two separate time points *in vitro* did not show any significant changes in *irr* and *ihk* transcription (17). Further investigation is

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needed to determine the precise mechanism by which the LiaFSR 3CS contributes to immune evasion.

Our study has several limitations. We performed targeted gene transcript analysis. It is possible that additional genes are affected by the LiaS^{R135G} carrier amino acid substitution upon activation by bacitracin or other cell wall-active agents. It is also possible that activation of the LiaFSR three-component system by bacitracin remains incomplete and other LiaFSR-activating factors may further contribute to gene regulatory differences. Further, elimination of pilus in the isogenic carrier strain did not fully restore the invasive phenotype. Previous transcriptome studies comparing the parental and isogenic carrier strains showed that several virulence factors are affected (17). Alteration of pilus gene expression contributes to the overall carrier phenotype, but it is likely that the complete phenotype imparted by the *liaS*^{R135G} carrier mutation is multifactorial.

In summary, our study adds substantially to the existing framework of the bacterial molecular mechanisms that contribute to carriage of GAS and other bacterial pathogens. The single amino acid change in LiaS alters the GAS phenotype in favor of asymptomatic carriage. Rather than alter expression of a single factor, the *liaS*^{R135G} mutation likely alters expression of multiple surface/secreted factors, such as pilus, that contribute to the carrier phenotype (Fig. 6). It is reasonable to hypothesize that further study of the gene regulatory changes associated with the *liaS*^{R135G} carrier mutation will reveal new mechanisms contributing to the carrier phenotype.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains used in this study are listed in Table S1 in the supplemental material. MGAS10870 is a serotype M3 GAS invasive strain isolated in 2002 in Ontario, Canada, and its genome has been sequenced to high quality (54). Strain MGAS10870 *liaS*^{R135G} is a previously described isogenic mutant differing from the parental strain, MGAS10870, only by the carrier *liaS* allele (17). All GAS strains were grown in Todd-Hewitt broth containing 0.2% (wt/vol) yeast extract (THY; Difco Laboratories), on THY agar, or on Trypticase soy agar containing 5% sheep blood agar (SBA; Becton Dickinson).

Generation of pilus-negative mutants in strains MGAS10870 and MGAS10870 *liaS*^{R135G}. The plasmids and primers used in this study are listed in Table S2. We used a previously described procedure (55) for in-frame replacement of the gene encoding the pilus backbone protein *tee3* (*spyM3_0100*) with a spectinomycin resistance gene (*aad9*) in strains MGAS10870 and MGAS10870 *liaS*^{R135G}. Experimental details are provided in the supplemental material. Mutants were confirmed and shown, using whole-genome sequencing, to lack spurious mutations.

Anti-Tee3 antibody production and Western immunoblot analysis. Recombinant, purified Tee3 protein was produced using an Impact system (New England BioLabs). Briefly, *tee3* (*spyM3_0100*) was synthesized, optimized for expression in *Escherichia coli*, and cloned into pTXB1 (GenScript). Protein overexpression and purification were carried out as described in the manufacturer's instructions (New England BioLabs). Purified Tee3 protein was subjected to mass spectrometry for identification prior to use in antibody production. Affinity-purified rabbit polyclonal anti-Tee3 antibody was generated by Covance (Denver, PA). Isolation of bacterial proteins and Western immunoblot analysis were carried out as previously described (18).

Mouse virulence studies. All mouse experiments were conducted under a protocol approved by the Houston Medical Research Institute Institutional Animal Care and Use Committee. Mouse intranasal and intramuscular infections were carried out as previously described (17). For nasopharyngeal infections, a total of 30, 3- to 4-week-old female CD1 mice (Harlan Laboratories) were intranasally inoculated with 5×10^7 CFU of the appropriate GAS strain in 50 μl phosphate-buffered saline (PBS), after which the mice were swabbed daily for 14 days. For intramuscular infections, mice ($n = 15$) were infected in the right hind limb with 5×10^6 CFU of the appropriate GAS strain in 100 μl PBS. Mice were evaluated at 24 h postinfection for histopathology. All mice were observed and sacrificed when they reached near mortality, determined using predefined criteria. For histopathology scoring, a pathologist blind to the strain treatment groups reviewed each slide ($n = 5$ per strain) as previously described (38).

Human neutrophil survival assays. Human neutrophils (polymorphonuclear leukocytes [PMNs]) were isolated from heparinized venous blood of healthy volunteer donors ($n = 14$) in accordance with a protocol approved by the Institutional Review Board for Human Subjects at Montana State University. All subjects provided written informed consent. Neutrophils were isolated as previously described (56). Complete procedural details are provided in the supplemental material. Percent survival was determined by dividing the number of bacterial CFU after 3 h exposure by the number of bacterial CFU at time zero.

Cultured human epithelial cell adherence and internalization assays. Adherence to cultured human epithelial cells was carried out as previously described (15). Complete experimental details can be found in the supplemental material. Percent adherence was calculated by dividing the recovered number of CFU by the original inoculum. GAS internalization by cultured epithelial cells was carried out

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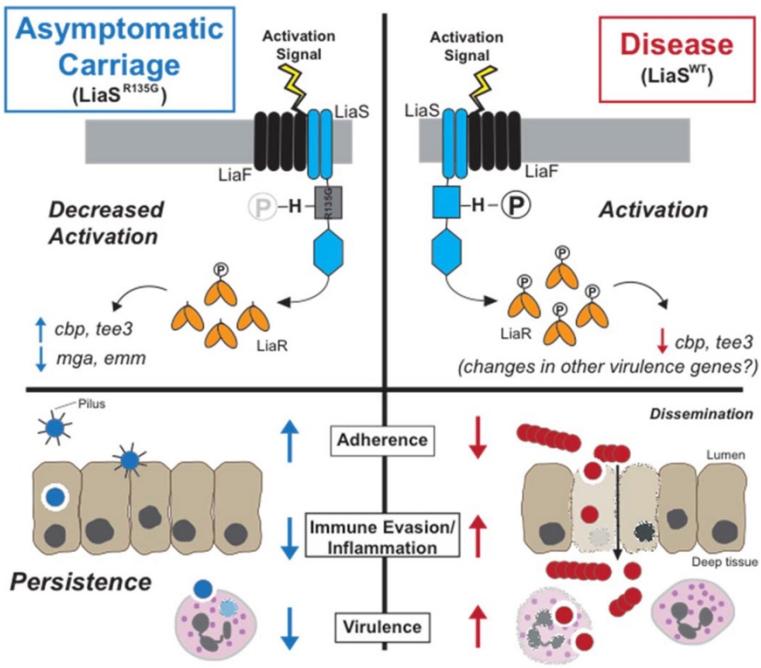


FIG 6 Schematic hypothetical model of the mechanism by which the carrier single amino acid replacement (LiaS^{R135G}) leads to altered pilus expression and the carrier phenotype. LiaS (blue) is a histidine kinase with two transmembrane-spanning domains. Activation signals for LiaS are unknown, but sensing is believed to involve both LiaS and the accessory membrane protein LiaF (black). Upon activation, LiaS undergoes autophosphorylation at the single conserved histidine (H). Following phosphorylation (circled P), the cognate response regulator, LiaR (yellow), is phosphorylated by phosphotransfer from LiaS. However, one hypothesis is that the carrier mutation decreases the autophosphorylation of LiaS, thereby decreasing phosphotransfer to LiaR and altering gene transcription. Decreased activation of LiaS and LiaR leads to alterations in global gene transcription, including the transcription of genes encoding pilus proteins. Ultimately, increased expression of pilus favors increased adherence, decreased immune evasion/inflammation, and decreased virulence, key characteristics of the carrier phenotype and persistence. WT, wild type.

as previously described (15). Percentage internalization was calculated by dividing the recovered number of CFU by the original inoculum. All assays were performed in replicates of 12.

RNA isolation and qRT-PCR analysis. GAS strains were grown overnight in THY, diluted 1:50 in fresh, prewarmed THY, and incubated at 37°C. Growth was monitored hourly using spectrophotometry (determination of the optical density at 600 nm [OD_{600}]), and when the strains reached an OD_{600} of 0.5, bacitracin (Sigma-Aldrich) was added to achieve the final concentration indicated above. Cells were harvested at 30, 60, and 90 min following the addition of bacitracin. RNA was isolated and purified with an RNeasy minikit (Qiagen) as previously described (18). The quantity and quality of RNA were determined using an Agilent 2100 bioanalyzer and an RNA 6000 Nano kit (Agilent Technologies).

TaqMan (Life Technologies) quantitative real-time PCR (qRT-PCR) of cDNA produced using SuperScript III reverse transcriptase (Invitrogen) was performed with an ABI 7500 Fast real-time system. The TaqMan primers and probes used in the analyses are listed in Table S2. The endogenous control gene *tufA* was used for all TaqMan analyses. Transcript levels were compared between strains using the $\Delta\Delta C_T$ threshold cycle (C_T) method (User Bulletin No. 2, ABI Prism 7700 sequence detection system; Life Technologies). All reactions were performed in triplicate using RNA purified from at least three biological replicates.

Statistics. All statistical analyses were performed with Prism (version 6) software (GraphPad Software, Inc.). A Mann-Whitney U test was used to compare survival following exposure to purified human neutrophils and adherence to or internalization by cultured human epithelial cells for the strains. A two-tailed *t* test (unequal variance) was used to compare gene transcript levels. A log rank test was used to compare survival, and Kruskal-Wallis analysis of variance (ANOVA) was used to compare the rates of nasopharyngeal GAS recovery from mice. The Wilcoxon rank sum test was used to compare histopathology scores. A *P* value of <0.05 was considered significant for all tests unless otherwise indicated.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00949-16.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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We have no conflicts of interest to declare.

REFERENCES

1. Casadevall A, Pirofski LA. 2003. The damage-response framework of microbial pathogenesis. Nat Rev Microbiol 1:17–24. https://doi.org/10.1038/nrmicro732.
2. Cartwright KA, Stuart JM, Jones DM, Noah ND. 1987. The Stonehouse survey: nasopharyngeal carriage of meningococci and *Neisseria lactamica*. Epidemiol Infect 99:591–601. https://doi.org/10.1017/S0950268800066449.
3. Caugant DA, Hoiby EA, Magnus P, Scheel O, Hoel T, Bjune G, Wedege E, Eng J, Froholm LO. 1994. Asymptomatic carriage of *Neisseria meningitidis* in a randomly sampled population. J Clin Microbiol 32:323–330.
4. Claus H, Maiden MC, Wilson DJ, McCarthy ND, Jolley KA, Urwin R, Hessler F, Frosch M, Vogel U. 2005. Genetic analysis of meningococci carried by children and young adults. J Infect Dis 191:1263–1271. https://doi.org/10.1086/428590.
5. Bogaert D, De Groot R, Hermans PW. 2004. *Streptococcus pneumoniae* colonisation: the key to pneumococcal disease. Lancet Infect Dis 4:144–154. https://doi.org/10.1016/S1473-3099(04)00938-7.
6. Wertheim HF, Melles DC, Vos MC, van Leeuwen W, van Belkum A, Verbrugh HA, Nouwen JL. 2005. The role of nasal carriage in *Staphylococcus aureus* infections. Lancet Infect Dis 5:751–762. https://doi.org/10.1016/S1473-3099(05)70295-4.
7. Campbell JR, Hillier SL, Krohn MA, Ferrieri P, Zaleznik DF, Baker CJ. 2000. Group B streptococcal colonization and serotype-specific immunity in pregnant women at delivery. Obstet Gynecol 96:498–503.
8. Regan JA, Klebanoff MA, Nugent RP. 1991. The epidemiology of group B streptococcal colonization in pregnancy. Vaginal Infections and Prematurity Study Group. Obstet Gynecol 77:604–610.
9. Yancey MK, Schuchat A, Brown LK, Ventura VL, Markenson GR. 1996. The accuracy of late antenatal screening cultures in predicting genital group B streptococcal colonization at delivery. Obstet Gynecol 88:811–815. https://doi.org/10.1016/0029-7844(96)00320-1.
10. Shaikh N, Leonard E, Martin JM. 2010. Prevalence of streptococcal pharyngitis and streptococcal carriage in children: a meta-analysis. Pediatrics 126:e557–e564. https://doi.org/10.1542/peds.2009-2648.
11. Nelson GE, Pondo T, Toews KA, Farley MM, Lindegren ML, Lynfield R, Aragon D, Zansky SM, Watt JP, Cieslak PR, Angeles K, Harrison LH, Petit S, Beall B, Van Beneden CA. 2016. Epidemiology of invasive group A streptococcal infections in the United States, 2005-2012. Clin Infect Dis 63:478–486. https://doi.org/10.1093/cid/ciw248.
12. Walker MJ, Barnett TC, McArthur JD, Cole JN, Gillen CM, Henningham A, Sriprakash KS, Sanderson-Smith ML, Nizet V. 2014. Disease manifestations and pathogenic mechanisms of group A *Streptococcus*. Clin Microbiol Rev 27:264–301. https://doi.org/10.1128/CMR.00101-13.
13. DeMuri GP, Wald ER. 2014. The group A streptococcal carrier state reviewed: still an enigma. J Pediatr Infect Dis Soc 3:336–342. https://doi.org/10.1093/jpids/piu030.
14. Kaplan EL. 1980. The group A streptococcal upper respiratory tract carrier state: an enigma. J Pediatr 97:337–345. https://doi.org/10.1016/S0022-3476(80)80178-8.
15. Flores AR, Jewell BE, Olsen RJ, Shelburne SA, III, Fittipaldi N, Beres SB, Musser JM. 2014. Asymptomatic carriage of group A *Streptococcus* is associated with elimination of capsule production. Infect Immun 82:3958–3967. https://doi.org/10.1128/IAI.01788-14.
16. Flores AR, Jewell BE, Versalovic EM, Olsen RJ, Bachert BA, Lukomski S, Musser JM. 2015. Natural variant of collagen-like protein A in serotype M3 group A *Streptococcus* increases adherence and decreases invasive potential. Infect Immun 83:1122–1129. https://doi.org/10.1128/IAI.02860-14.
17. Flores AR, Jewell BE, Yelamanchili D, Olsen RJ, Musser JM. 2015. A single amino acid replacement in the sensor kinase LiaS contributes to a carrier phenotype in group A *Streptococcus*. Infect Immun 83:4237–4246. https://doi.org/10.1128/IAI.00656-15.
18. Flores AR, Olsen RJ, Wunsche A, Kumaraswami M, Shelburne SA, III, Carroll RK, Musser JM. 2013. Natural variation in the promoter of the gene encoding the Mga regulator alters host-pathogen interactions in group A *Streptococcus* carrier strains. Infect Immun 81:4128–4138. https://doi.org/10.1128/IAI.00405-13.
19. Gao R, Stock AM. 2009. Biological insights from structures of two-component proteins. Annu Rev Microbiol 63:133–154. https://doi.org/10.1146/annurev.micro.091208.073214.
20. Sitkiewicz I, Musser JM. 2006. Expression microarray and mouse virulence analysis of four conserved two-component gene regulatory systems in group A *Streptococcus*. Infect Immun 74:1339–1351. https://doi.org/10.1128/IAI.74.2.1339-1351.2006.
21. Mora M, Bensi G, Capo S, Falugi F, Zingaretti C, Manetti AG, Maggi T, Taddei AR, Grandi G, Telford JL. 2005. Group A *Streptococcus* produce pilus-like structures containing protective antigens and Lancefield T antigens. Proc Natl Acad Sci U S A 102:15641–15646. https://doi.org/10.1073/pnas.0507808102.
22. Falugi F, Zingaretti C, Pinto V, Mariani M, Amodeo L, Manetti AG, Capo S, Musser JM, Orefici G, Margarit I, Telford JL, Grandi G, Mora M. 2008. Sequence variation in group A *Streptococcus* pili and association of pilus backbone types with Lancefield T serotypes. J Infect Dis 198:1834–1841. https://doi.org/10.1086/593176.
23. Crotty Alexander LE, Maisey HC, Timmer AM, Rooijakkers SH, Gallo RL, von Kockritz-Blickwede M, Nizet V. 2010. M1T1 group A streptococcal pili promote epithelial colonization but diminish systemic virulence through neutrophil extracellular entrapment. J Mol Med 88:371–381. https://doi.org/10.1007/s00109-009-0566-9.
24. Nakata M, Koller T, Moritz K, Ribardo D, Jonas L, McIver KS, Sumitomo T, Terao Y, Kawabata S, Podbielski A, Kreikemeyer B. 2009. Mode of expression and functional characterization of FCT-3 pilus region-encoded proteins in *Streptococcus pyogenes* serotype M49. Infect Immun 77:32–44. https://doi.org/10.1128/IAI.00772-08.
25. Kreikemeyer B, Nakata M, Koller T, Hildisch H, Kourakos V, Standar K, Kawabata S, Glocker MO, Podbielski A. 2007. The *Streptococcus pyogenes* serotype M49 Nra-Ralp3 transcriptional regulatory network and its control of virulence factor expression from the novel eno ralp3 epf sagA pathogenicity region. Infect Immun 75:5698–5710. https://doi.org/10.1128/IAI.00175-07.
26. Luo F, Lizano S, Bessen DE. 2008. Heterogeneity in the polarity of Nra regulatory effects on streptococcal pilus gene transcription and virulence. Infect Immun 76:2490–2497. https://doi.org/10.1128/IAI.01567-07.
27. Molinari G, Rohde M, Talay SR, Chhatwal GS, Beckert S, Podbielski A. 2001. The role played by the group A streptococcal negative regulator Nra on bacterial interactions with epithelial cells. Mol Microbiol 40:99–114. https://doi.org/10.1046/j.1365-2958.2001.02373.x.
28. Nakata M, Podbielski A, Kreikemeyer B. 2005. MsmR, a specific positive regulator of the *Streptococcus pyogenes* FCT pathogenicity region and cytolysin-mediated translocation system genes. Mol Microbiol 57:786–803. https://doi.org/10.1111/j.1365-2958.2005.04730.x.
29. Rosch JW, Mann B, Thornton J, Sublett J, Tuomanen E. 2008. Convergence of regulatory networks on the pilus locus of *Streptococcus*

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pneumoniae. Infect Immun 76:3187–3196. https://doi.org/10.1128/IAI.00054-08.
30. Klinzing DC, Ishmael N, Dunning Hotopp JC, Tettelin H, Shields KR, Madoff LC, Puopolo KM. 2013. The two-component response regulator LiaR regulates cell wall stress responses, pili expression and virulence in group B *Streptococcus*. Microbiology 159:1521–1534. https://doi.org/10.1099/mic.0.064444-0.
31. Kesel S, Mader A, Hofler C, Mascher T, Leisner M. 2013. Immediate and heterogeneous response of the LiaFSR two-component system of *Bacillus subtilis* to the peptide antibiotic bacitracin. PLoS One 8:e53457. https://doi.org/10.1371/journal.pone.0053457.
32. Kuroda M, Kuroda H, Oshima T, Takeuchi F, Mori H, Hiramatsu K. 2003. Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in *Staphylococcus aureus*. Mol Microbiol 49:807–821.
33. Majchrzykiewicz JA, Kuipers OP, Bijlsma JJ. 2010. Generic and specific adaptive responses of *Streptococcus pneumoniae* to challenge with three distinct antimicrobial peptides, bacitracin, LL-37, and nisin. Antimicrob Agents Chemother 54:440–451. https://doi.org/10.1128/AAC.00769-09.
34. Shankar M, Mohapatra SS, Biswas S, Biswas I. 2015. Gene regulation by the LiaSR two-component system in *Streptococcus mutans*. PLoS One 10:e0128083. https://doi.org/10.1371/journal.pone.0128083.
35. Bessen DE, Kalia A. 2002. Genomic localization of a T serotype locus to a recombinatorial zone encoding extracellular matrix-binding proteins in *Streptococcus pyogenes*. Infect Immun 70:1159–1167. https://doi.org/10.1128/IAI.70.3.1159-1167.2002.
36. Mao X, Ma Q, Zhou C, Chen X, Zhang H, Yang J, Mao F, Lai W, Xu Y. 2014. DOOR 2.0: presenting operons and their functions through dynamic and integrated views. Nucleic Acids Res 42:D654–D659. https://doi.org/10.1093/nar/gkt1048.
37. Ribardo DA, McIver KS. 2006. Defining the Mga regulon: comparative transcriptome analysis reveals both direct and indirect regulation by Mga in the group A *Streptococcus*. Mol Microbiol 62:491–508. https://doi.org/10.1111/j.1365-2958.2006.05381.x.
38. Olsen RJ, Sitkiewicz I, Ayeras AA, Gonulal VE, Cantu C, Beres SB, Green NM, Lei B, Humbird T, Greaver J, Chang E, Ragasa WP, Montgomery CA, Cartwright J, Jr, McGeer A, Low DE, Whitney AR, Cagle PT, Blasdel TL, DeLeo FR, Musser JM. 2010. Decreased necrotizing fasciitis capacity caused by a single nucleotide mutation that alters a multiple gene virulence axis. Proc Natl Acad Sci U S A 107:888–893. https://doi.org/10.1073/pnas.0911811107.
39. Barocchi MA, Ries J, Zogaj X, Hemsley C, Albiger B, Kanth A, Dahlberg S, Fernebro J, Moschioni M, Masignani V, Hultenby K, Taddei AR, Beiter K, Wartha F, von Euler A, Covacci A, Holden DW, Normark S, Rappuoli R, Henriques-Normark B. 2006. A pneumococcal pilus influences virulence and host inflammatory responses. Proc Natl Acad Sci U S A 103:2857–2862. https://doi.org/10.1073/pnas.0511017103.
40. Iovino F, Hammarlof DL, Garriss G, Brovall S, Nannapaneni P, Henriques-Normark B. 2016. Pneumococcal meningitis is promoted by single cocci expressing pilus adhesin RrgA. J Clin Invest 126:2821–2826. https://doi.org/10.1172/JCI84705.
41. Banerjee A, Kim BJ, Carmona EM, Cutting AS, Gurney MA, Carlos C, Feuer R, Prasadarao NV, Doran KS. 2011. Bacterial pili exploit integrin machinery to promote immune activation and efficient blood-brain barrier penetration. Nat Commun 2:462. https://doi.org/10.1038/ncomms1474.
42. Maisey HC, Quach D, Hensler ME, Liu GY, Gallo RL, Nizet V, Doran KS. 2008. A group B streptococcal pilus protein promotes phagocyte resistance and systemic virulence. FASEB J 22:1715–1724. https://doi.org/10.1096/fj.07-093963.
43. Ichikawa M, Minami M, Isaka M, Tatsuno I, Hasegawa T. 2011. Analysis of two-component sensor proteins involved in the response to acid stimuli in *Streptococcus pyogenes*. Microbiology 157:3187–3194. https://doi.org/10.1099/mic.0.050534-0.
44. Isaka M, Tatsuno I, Maeyama J, Matsui H, Zhang Y, Hasegawa T. 2016. The YvqE two-component system controls biofilm formation and acid production in *Streptococcus pyogenes*. APMIS 124:574–585. https://doi.org/10.1111/apm.12538.
45. Manetti AG, Koller T, Becherelli M, Buccato S, Kreikemeyer B, Podbielski A, Grandi G, Margarit I. 2010. Environmental acidification drives *S. pyogenes* pilus expression and microcolony formation on epithelial cells in a FCT-dependent manner. PLoS One 5:e13864. https://doi.org/10.1371/journal.pone.0013864.
46. Manetti AG, Zingaretti C, Falugi F, Capo S, Bombaci M, Bagnoli F, Gambellini G, Bensi G, Mora M, Edwards AM, Musser JM, Graviss EA, Telford JL, Grandi G, Margarit I. 2007. *Streptococcus pyogenes* pili promote pharyngeal cell adhesion and biofilm formation. Mol Microbiol 64:968–983. https://doi.org/10.1111/j.1365-2958.2007.05704.x.
47. Abbot EL, Smith WD, Siou GP, Chiriboga C, Smith RJ, Wilson JA, Hirst BH, Kehoe MA. 2007. Pili mediate specific adhesion of *Streptococcus pyogenes* to human tonsil and skin. Cell Microbiol 9:1822–1833. https://doi.org/10.1111/j.1462-5822.2007.00918.x.
48. McElroy MC, Cain DJ, Tyrrell C, Foster TJ, Haslett C. 2002. Increased virulence of a fibronectin-binding protein mutant of *Staphylococcus aureus* in a rat model of pneumonia. Infect Immun 70:3865–3873. https://doi.org/10.1128/IAI.70.7.3865-3873.2002.
49. Natanson S, Sela S, Moses AE, Musser JM, Caparon MG, Hanski E. 1995. Distribution of fibronectin-binding proteins among group A streptococci of different M types. J Infect Dis 171:871–878. https://doi.org/10.1093/infdis/171.4.871.
50. Nyberg P, Sakai T, Cho KH, Caparon MG, Fassler R, Bjorck L. 2004. Interactions with fibronectin attenuate the virulence of *Streptococcus pyogenes*. EMBO J 23:2166–2174. https://doi.org/10.1038/sj.emboj.7600214.
51. Nuccitelli A, Cozzi R, Gourlay LJ, Donnarumma D, Necchi F, Norais N, Telford JL, Rappuoli R, Bolognesi M, Maione D, Grandi G, Rinaudo CD. 2011. Structure-based approach to rationally design a chimeric protein for an effective vaccine against group B Streptococcus infections. Proc Natl Acad Sci U S A 108:10278–10283. https://doi.org/10.1073/pnas.1106590108.
52. Voyich JM, Sturdevant DE, Braughton KR, Kobayashi SD, Lei B, Virtaneva K, Dorward DW, Musser JM, DeLeo FR. 2003. Genome-wide protective response used by group A *Streptococcus* to evade destruction by human polymorphonuclear leukocytes. Proc Natl Acad Sci U S A 100:1996–2001. https://doi.org/10.1073/pnas.0337370100.
53. Voyich JM, Braughton KR, Sturdevant DE, Vuong C, Kobayashi SD, Porcella SF, Otto M, Musser JM, DeLeo FR. 2004. Engagement of the pathogen survival response used by group A *Streptococcus* to avert destruction by innate host defense. J Immunol 173:1194–1201. https://doi.org/10.4049/jimmunol.173.2.1194.
54. Beres SB, Carroll RK, Shea PR, Sitkiewicz I, Martinez-Gutierrez JC, Low DE, McGeer A, Willey BM, Green K, Tyrrell GJ, Goldman TD, Feldgarden M, Birren BW, Fofanov Y, Boos J, Wheaton WD, Honisch C, Musser JM. 2010. Molecular complexity of successive bacterial epidemics deconvoluted by comparative pathogenomics. Proc Natl Acad Sci U S A 107:4371–4376. https://doi.org/10.1073/pnas.0911295107.
55. Lukomski S, Hoe NP, Abdi I, Rurangirwa J, Kordari P, Liu M, Dou SJ, Adams GG, Musser JM. 2000. Nonpolar inactivation of the hypervariable streptococcal inhibitor of complement gene (*sic*) in serotype M1 *Streptococcus pyogenes* significantly decreases mouse mucosal colonization. Infect Immun 68:535–542. https://doi.org/10.1128/IAI.68.2.535-542.2000.
56. Voyich JM, Braughton KR, Sturdevant DE, Whitney AR, Said-Salim B, Porcella SF, Long RD, Dorward DW, Gardner DJ, Kreiswirth BN, Musser JM, DeLeo FR. 2005. Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. J Immunol 175:3907–3919. https://doi.org/10.4049/jimmunol.175.6.3907.

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The Journal of Infectious Diseases

MAJOR ARTICLE

Staphylococcus aureus SaeR/S-Regulated Factors Decrease Monocyte-Derived Tumor Necrosis Factor–α to Reduce Neutrophil Bactericidal Activity

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Background. The ability of *Staphylococcus aureus* to evade killing by human neutrophils significantly contributes to disease progression. In this study, we characterize an influential role for the *S. aureus* SaeR/S 2-component gene regulatory system in suppressing monocyte production of tumor necrosis factor alpha (TNF-α) to subsequently influence human neutrophil priming.

Methods. Using flow cytometry and TNF-α specific enzyme-linked immunosorbent assays we identify the primary cellular source of TNF-α in human blood and in purified peripheral blood mononuclear cells (PBMCs) during interaction with USA300 and an isogenic saeR/S deletion mutant (USA300ΔsaeR/S). Assays with conditioned media from USA300 and USA300ΔsaeR/S exposed PBMCs were used to investigate priming on neutrophil bactericidal activity.

Results. TNF-α production from monocytes was significantly reduced following challenge with USA300 compared to USA300ΔsaeR/S. We observed that priming of neutrophils using conditioned medium from peripheral blood mononuclear cells stimulated with USA300ΔsaeR/S significantly increased neutrophil bactericidal activity against USA300 relative to unprimed neutrophils and neutrophils primed with USA300 conditioned medium. The increased neutrophil bactericidal activity was associated with enhanced reactive oxygen species production that was significantly influenced by elevated TNF-α concentrations.

Conclusions. Our findings identify an immune evasion strategy used by S. aureus to impede neutrophil priming and subsequent bactericidal activity.

Keywords. *Staphylococcus aureus*; neutrophil; SaeR/S; monocyte; TNF-α.

Staphylococcus aureus is a ubiquitous Gram-positive bacterium estimated to permanently colonize 1 of 3 individuals [1]. *Staphylococcus aureus* typically causes treatable cutaneous abscesses, but infections can progress into dermonecrotic lesions and life-threatening infections, including septicemia and necrotizing pneumonia [2, 3]. Methicillin-resistant *S. aureus* (MRSA) further represents a major public health concern as it is one of the most common antimicrobial-resistant pathogens to cause infections in both hospital and community settings [4]. This pervasiveness is in part attributed to the ability of *S. aureus* to circumvent protective immune responses, including evasion of human neutrophil killing (reviewed in [5]). We and others have demonstrated that factors under the regulation of the *S. aureus* SaeR/S 2-component gene regulatory system (SaeR/S TCS) contribute significantly to this ability [6–9]. However, the molecular mechanisms dependent on SaeR/S that promote *S. aureus* survival following neutrophil interaction are incompletely defined.

The SaeR/S TCS regulates genes that control cytolytic and immunomodulatory factors that have been shown to significantly contribute to exacerbated infections in murine models of infection and ex vivo human models of infection [6, 10–16]. The contribution of immunomodulation to these negative outcomes has been exemplified through the ability of SaeR/S-regulated factors to induce specific inflammatory signals that promote a detrimental immune response [7, 11, 17, 18]. For example, SaeR/S-regulated staphylococcal binding immunoglobulin protein (Sbi) was shown to induce robust production of interleukin 6 (IL-6) and modulate levels of CXCL-1 during invasive infection [19]. This alteration in cytokine production during the early stages of infection subsequently influenced neutrophil recruitment and contributed to an overall deleterious inflammatory response. Similarly, we have demonstrated the ability of SaeR/S-regulated virulence factors to induce robust production of inflammatory cytokines, including interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), IL-6, and interleukin 2 (IL-2), that are associated with increased bacterial survival and host mortality [11, 18]. In line with these immunomodulatory roles, we further demonstrated that SaeR/S-regulated factors can suppress phosphorylation of nuclear factor-κB (NF-κB) and reduce production of interleukin 8 (IL-8) in

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human neutrophils to promote cell death and enhance bacterial survival [7]. Collectively, these findings highlight immune evasion strategies by which SaeR/S-regulated factors influence inflammatory signaling to exacerbate infection. However, the specific immune cells that are targeted for immunomodulation by SaeR/S-regulated factors and the subsequent influence on downstream immune responses remain incompletely defined.

Peripheral blood mononuclear cells (PBMCs) have been shown to regulate inflammatory signals that promote neutrophil recruitment and enhance their antimicrobial mechanisms in response to *S. aureus* infection [20–22]. Many PBMC products such as monocyte-derived interleukin 1 (IL-1) and TNF-α also act as neutrophil priming agents [21, 23]. Neutrophil priming is a mechanism that regulates the level of neutrophil activation to provide an appropriate response to a secondary stimulus [23]. Despite the importance of inflammatory mediators produced by PBMCs that are involved in neutrophil priming, there is a lack of studies examining the impact of *S. aureus* on this process. Previous studies have investigated the effects of conditioned media (CM) containing inflammatory signals produced by human PBMCs following exposure to heat-killed *S. aureus* on neutrophil functions [24, 25]. These studies observed that neutrophil staphylocidal activity was enhanced following priming with CM and that this effect was dependent on PBMC production of TNF-α [25]. While these effects were observed using CM from heat-killed *S. aureus*, it remains to be elucidated if CM from live bacteria elicit a similar response on neutrophil priming.

In the current study, we developed a co-culture model using human PBMCs and neutrophils, as well as CM assays, to demonstrate the importance of TNF-α during monocyte–neutrophil interactions following challenge with live *S. aureus*. We observed that SaeR/S-regulated factors significantly decreased monocyte production of TNF-α. Diminished TNF-α concentrations significantly influenced neutrophil staphylocidal activity by limiting neutrophil priming and subsequent production of reactive oxygen species (ROS). Collectively, this study demonstrates that the response of PBMCs to *S. aureus* significantly impacts the outcome of neutrophil–*S. aureus* interactions.

METHODS

Bacterial Strains and Cultures

Community-associated MRSA pulsed-field gel electrophoresis type USA300 (strain LAC), USA400 (strain MW2), and isogenic *saeR/S* and *hla* deletion mutants (USA300Δ*saeR/S*, USA400Δ*saeR/S*, and USA300Δ*hla*), generated in previous studies [6, 10, 15], were grown in tryptic soy broth containing 0.5% glucose and harvested at midexponential growth, as described previously [6, 10, 12, 15].

Assessment of TNF-α Production From Human Cells

Heparinized venous blood from healthy donors was collected in accordance with the protocol approved by the Institutional Review Board for Human Subjects at Montana State University. All donors provided written consent to participate in the study. For whole-blood experiments, 20 μL of Roswell Park Memorial Institute medium (RPMI) only or RPMI with 2×10^6 colony-forming units (CFU) of bacteria was added to 980 μL of freshly drawn human whole blood in 1.5-mL Eppendorf tubes. Samples were placed on an end-over-end mixer (20 RPM, 37°C, 5% carbon dioxide) and, at designated time points, samples were stained with fluorescein isothiocyanate (FITC) mouse antihuman CD14, phycoerythrin mouse anti-human CD3, and allophycocyanin mouse antihuman TNF or immunoglobulin G1 (IgG1) isotype control (BD Biosciences). Intracellular staining was performed using a BD Pharmigen intracellular stain kit following the manufacturer's protocol, and all samples were analyzed using a FACSCalibur Flow Cytometer (BD Bioscience). Alternatively, serum was collected by centrifugation (524*g* for 10 minutes), filtered using a 0.2-μm filter (PALL Life Science), and stored at –80°C until quantitation of TNF-α was performed using a TNF-α–specific enzyme-linked immunosorbent assay (ELISA, BD Biosciences).

Human neutrophils (polymorphonuclear leukocytes [PMNs]) and peripheral blood mononuclear cells (PBMCs) were isolated under endotoxin-free conditions (<25 pg/mL) as previously described [6, 12]. Purity (<1% PBMC contamination) and viability (<2% propidium iodide positive) of neutrophil preparations were assessed by flow cytometry (FACSCalibur). For co-culture assays, 7×10^5 neutrophils were combined with 3×10^5 PBMCs at a physiological ratio and exposed to 2×10^6 CFUs of *S. aureus* opsonized with 50% normal human serum (NHS) in a 96-well NHS-coated plate. After bacteria were added, phagocytosis was synchronized by centrifugation (524*g* for 7 minutes at 10°C) and then incubated at 37°C [12]. At designated time points, samples were stained as above and analyzed using flow cytometry.

For PBMC CM experiments, 5×10^6 PBMCs were challenged with 1×10^7 opsonized bacteria in a 24-well serum-coated plate that was then synchronized and incubated at 37°C [12]. At designated time points, samples were filtered using a 0.2-μm filter (PALL Life Science), plated to ensure they did not contain live bacteria, and stored at 4°C for further use as CM or at –80°C for assessment of intracellular TNF-α production using ELISA as described above.

TNF-α Inhibition Assays

Calbiochem TNF-α inhibitor $C_{32}H_{32}F_3N_3O_2 \bullet 2\ HCl \bullet 1/2\ H_2O$ (CAS 1049741-03-8; Millipore Sigma) was resuspended according to manufacturer protocol and used at the final concentration of 4.6 μM. TNF-α inhibitor was added to PMNs (10^6 cells) only, or PMNs (7×10^5 cells) plus PBMCs (3×10^5 cells) for 10 minutes on ice. After treatment, cells were exposed to 2×10^6 opsonized bacteria, and phagocytosis was synchronized using centrifugation (524*g* for 7 minutes at 10°C), then incubated at 37°C with 5% carbon dioxide [12].

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Bactericidal Assays

PMNs or PMNs supplemented with PBMCs (co-cultures) were exposed to opsonized bacteria at a 2:1 multiplicity of infection (MOI, bacteria:cell ratio) in a 96-well serum-coated plate and phagocytosis was synchronized as described above [12]. At designated time points, 100 µL of sample was placed in 900 µL reverse osmosis water, vortexed, and placed on ice for 15 minutes. Serial dilutions were performed and CFUs were enumerated by plating on tryptic soy agar as previously described [6, 10, 12]. For PBMC bactericidal assays, 5×10^6 PBMCs in a 24-well serum-coated plate were challenged with 1×10^7 opsonized bacteria, synchronized using centrifugation (524*g* for 7 minutes at 10°C) and incubated at 37°C [12]. At designated time points, bacterial survival was assessed using serial dilutions in water and CFUs were enumerated on tryptic soy agar.

Neutrophil Priming With TNF-α or PBMC Conditioned Media

For ROS assays, neutrophils were loaded at room temperature with 25 µM 2′,7′-dihydrodicloroﬂuorescein diacetate (DCF; Molecular Probes) for 30 minutes in RPMI/4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [12]. DCF-containing PMNs (10^6) were then added to a 96-well serum-coated plate containing 50 µL of PBMC CM, RPMI only, or PBMC CM containing TNF-α inhibitor, for 30 minutes at 37°C. TNF-α inhibitor was added to CM for a final concentration of 4.5 ng/mL for 10–20 minutes at room temperature prior to addition to PMNs. After treatment, PMNs were exposed to opsonized bacteria at a ratio of 1:2 (PMN:bacteria), and phagocytosis was synchronized using centrifugation (524*g* for 7 minutes at 10°C) [12]. Measurements were done using a SpectraMax Paradigm Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, California) or by flow cytometry using 488/530 nm emission/excitation wavelengths.

RESULTS

SaeR/S-Regulated Factors Decrease TNF-α Production in Human Whole Blood

To determine if SaeR/S-regulated factors influenced TNF-α production, viable wild-type *S. aureus* (USA300) or isogenic *saeR*/S-deletion mutant (USA300Δ*saeR/S*) was exposed to human whole blood and TNF-α secretion was quantitated at 1, 3, and 5 hours using ELISA (Figure 1). Production of TNF-α occurred as early as 1 hour postexposure to *S. aureus* and, as expected, challenge with USA300 had significantly increased production compared with USA300Δ*saeR/S* (Figure 1A). However, by 3 hours, whole blood exposed to USA300Δ*saeR/S* resulted in significant increase in TNF-α concentrations compared with USA300, and this trend continued at 5 hours (Figure 1A). We then used flow cytometry to identify the source of TNF-α in blood following exposure to *S. aureus*. $CD14^+$ monocytes were identified as the primary source of TNF-α, and challenge with USA300Δ*saeR/S* resulted in a significant increase in TNF-α production from $CD14^+$ cells at 3 hours postchallenge compared to challenge with USA300 (Figure 1B and 1C). Experiments using an isotype control antibody verified specificity of intracellular TNF-α production (data not shown). Additionally, the proportions of $CD14^+$ cells between challenge with USA300 and USA300Δ*saeR/S* were similar (5%–10% of total cells, Supplementary Figure 1), indicating that these results did not arise from differences in total monocyte populations. The USA300Δ*saeR/S* strain also demonstrated increased bacterial survival compared with USA300 at 1 hour, but by 3 hours there were no significant differences between the strains (Figure 1D). These results reflect previous observations showing that the USA300Δ*saeR/S* strain does not have a growth defect in blood at early time points [15].

SaeR/S Decreases TNF-α Production in Human Monocytes

As $CD14^+$ monocytes were the predominant source of TNF-α production in human blood exposed to *S. aureus*, we next investigated TNF-α production in purified PBMCs. To reduce the potential influence of bacterial cytolytic activity between USA300 and USA300Δ*saeR/S*, we used a low MOI of 2:1 (bacteria:PBMC). Similar to observations using whole blood, $CD14^+$ monocytes were identified as the primary producers of TNF-α, and significant increases in intracellular monocyte TNF-α expression were observed at 3 hours following challenge with USA300Δ*saeR/S* compared with USA300 (Figure 2A). To demonstrate that the observed effects of the SaeR/S TCS on TNF-α production were not specific to strain USA300, we also challenged PBMCs with USA400 (strain MW2) and an isogenic *saeR/S*-deletion mutant (USA400Δ*saeR/S*). In congruence with our findings, PBMCs challenged with USA400 showed a significant decrease in TNF-α production from $CD14^+$ monocytes compared to challenge with USA400Δ*saeR/S* (Supplementary Figure 2*A*).

We have previously demonstrated that at high MOIs (10 bacteria:1 monocyte), the SaeR/S-regulated α-toxin (Hla) significantly increases monocyte programmed cell death and plasma membrane permeability [15]. To determine if observed increases in TNF-α at a lower MOI were due to the impact of SaeR/S-regulated factors on monocyte viability, we investigated TNF-α production and viability of PBMCs following exposure to USA300, USA300Δ*saeR/S*, and USA300Δ*hla*. ELISA results demonstrated that PBMCs exposed to USA300Δ*hla* elicited a similar TNF-α response to PBMCs challenged with USA300, but a significantly lower TNF-α response than PBMCs challenged with USA300Δ*saeR/S* at 3 and 5 hours postexposure (Figure 2B). Additionally, PBMCs challenged with USA300 caused significant increases in monocyte cell membrane permeability compared with USA300Δ*saeR/S* and USA300Δ*hla*. However, there were no significant differences in membrane permeability of $CD14^+$ cells following exposure to USA300Δ*saeR/S* and USA300Δ*hla* (Figure 2C). These results confirm previous findings demonstrating that Hla significantly contributes

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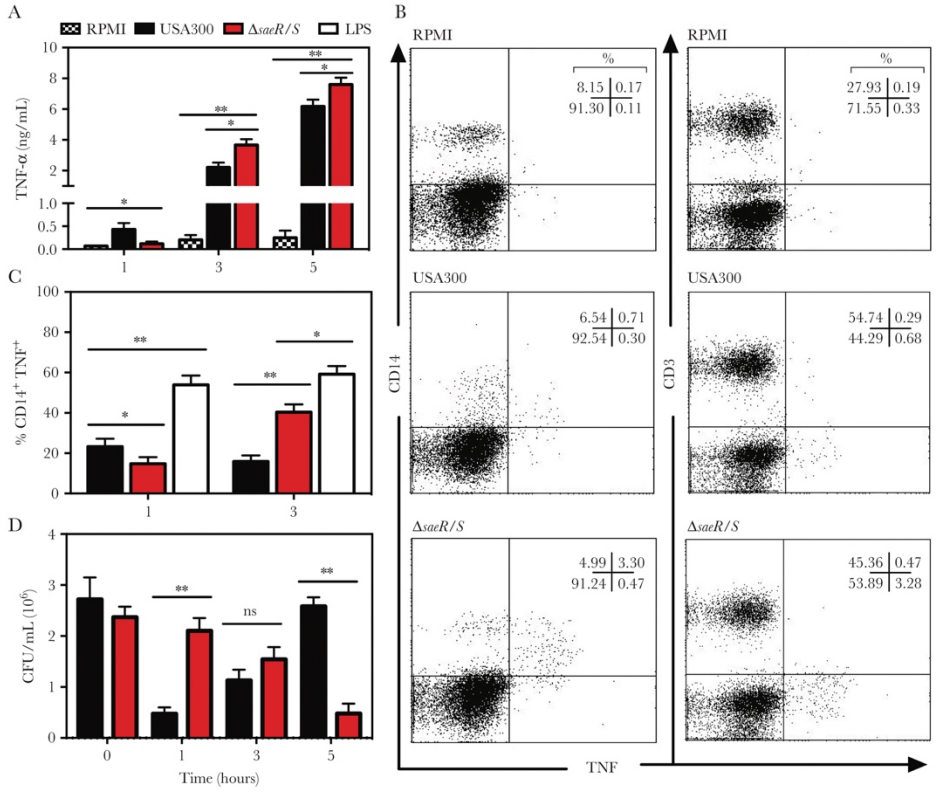


Figure 1. SaeR/S suppresses tumor necrosis factor alpha (TNF-α) production in human blood from CD14⁺ monocytes. Human whole blood was exposed to USA300, USA300Δ*saeR/S*, lipopolysaccharide, or RPMI medium for 1, 3, and 5 hours and TNF-α production was measured. *A*, Serum TNF-α cytokine concentrations were measured at designated time points using enzyme-linked immunosorbent assay. *B*, Representative flow cytometry dot plots of human blood stained for monocyte (CD14⁺), T-cell (CD3⁺), and production of TNF-α, at 3 hours postchallenge with USA300 and USA300Δ*saeR/S*. *C*, Compiled flow cytometry results of TNF-α and CD14⁺ positive monocytes of total CD14⁺ monocyte population. *D*, Bacterial survival of USA300 and USA300Δ*saeR/S* in human whole blood. Data shown are the mean ± standard error of the mean of 10 experiments, and statistics were determined by 1-way analysis of variance followed by Tukey posttest. $*P < .05$, $**P < .01$. Abbreviations: CFU, colony-forming units; LPS, lipopolysaccharide; ns, not significant; TNF-α, tumor necrosis factor alpha.

to monocyte cell membrane damage, but indicate that there are other SaeR/S-regulated factors and mechanisms responsible for reducing monocyte production of TNF-α [15]. Last, we also assessed bacterial viability following challenge with PBMCs (Figure 2D). PBMCs were unable to control challenge with USA300 and USA300Δ*hla*, but bacterial survival of USA300Δ*saeR/S* was significantly reduced at 3 and 5 hours (Figure 2D). Collectively, these results demonstrate that the SaeR/S TCS is required for bacterial survival following exposure to PBMCs and suggest that reduced monocyte production of TNF-α by SaeR/S-regulated factors is independent of cell lysis.

TNF-α Production by PBMCs Increases PMN Bactericidal Activity

TNF-α has been shown to influence neutrophil bactericidal activity and ROS production [26–28]. To determine if the influence of SaeR/S-regulated factors on monocyte-derived TNF-α could directly influence neutrophil staphylocidal activity, we developed a co-culture model that combined isolated human neutrophils and PBMCs at a ratio of 7:3 (PMN:PBMC). The neutrophil bactericidal activity following challenge with USA300Δ*saeR/S* was not significantly increased by the addition of PBMCs (Figure 3A). However, we did demonstrate the importance of TNF-α in these co-cultures by treating them with a TNF-α–specific inhibitor, which led to

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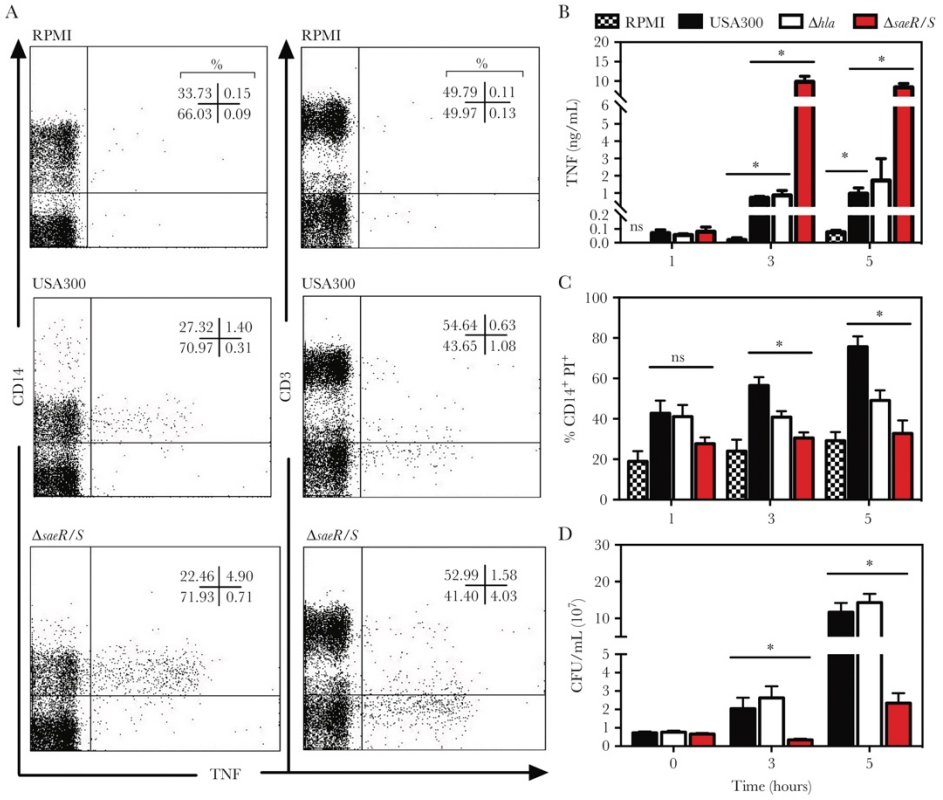


Figure 2. SaeR/S decreases tumor necrosis factor alpha (TNF-α) production in isolated human peripheral blood mononuclear cells (PBMCs). Isolated human PBMCs were exposed to RPMI medium, USA300, USA300Δ*saeR/S*, or USA300Δ*hla* at a multiplicity of infection of 2:1 (bacteria:PBMC) for 1, 3, and 5 hours. *A*, Representative flow cytometry dot plots of $CD14^+$ and $CD3^+$ cells producing TNF-α at 3 hours. *B*, At designated time points, TNF-α from PBMCs exposed to bacteria was quantified using enzyme-linked immunosorbent assay. *C*, $CD14^+$ PBMC plasma membrane permeability was assessed by propidium iodide uptake and measured using flow cytometry. *D*, Bacterial survival following exposure to PBMCs was assessed at designated time points. Data shown are the mean ± standard error of the mean for 10 experiments. Statistics were determined by 1-way analysis of variance followed by Tukey posttest. $^*P < .05$. Abbreviations: CFU, colony-forming units; ns, not significant; PI, propidium iodide; TNF-α, tumor necrosis factor alpha.

significant increases in USA300Δ*saeR/S* survival (Figure 3A). Inhibition of TNF-α had no significant influence on neutrophil bactericidal activity in USA300-challenged co-cultures. These data suggest that the levels of TNF-α following challenge with USA300 are significantly reduced by SaeR/S-regulated factors and are insufficient to augment neutrophil bactericidal activity. ELISA results further demonstrated that co-cultures challenged with USA300Δ*saeR/S* resulted in significantly increased TNF-α cytokine production at 3 hours postchallenge compared with USA300, and also confirmed the specificity of the TNF-α inhibitor (Figure 3B). Taken together, these results demonstrate that SaeR/S-regulated factors influence early monocyte production of TNF-α, which significantly influences neutrophil bactericidal activity.

Conditioned Media From PBMCs Challenged With USA300ΔsaeR/S Primes Neutrophils

To address if PBMCs exposed to *S. aureus* produce secreted factors that could prime neutrophils, neutrophils were treated with filtered (bacteria-free) CM from PBMCs challenged with USA300 or USA300Δ*saeR/S* for 3 hours. CM-primed neutrophils were then challenged with a secondary stimulus of USA300 and subsequent ROS production was measured. Approximately 60 minutes after exposure, USA300-challenged neutrophils

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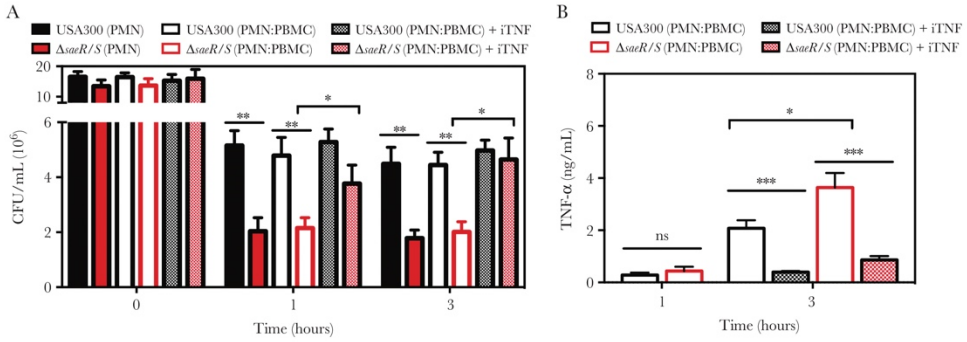


Figure 3. Tumor necrosis factor alpha (TNF-α) production from peripheral blood mononuclear cells (PBMCs) increases polymorphonuclear leukocyte (PMN) bactericidal activity. PMNs supplemented with PBMCs only, or pretreated with 4.6 μM TNF-α inhibitor (iTNF), were challenged with USA300 or USA300Δ*saeR/S* at a multiplicity of infection of 2:1(bacteria to PMN). *A*, Bactericidal activity of untreated or iTNF-treated PMNs supplemented with PBMCs was compared to bactericidal activity of PMNs alone at 0, 1, and 3 hours. *B*, TNF-α cytokine concentrations were quantitated using enzyme-linked immunosorbent assay. Data shown are the mean ± standard error of the mean of 10 experiments and statistics were determined by 1-way analysis of variance followed by Tukey posttest. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$. Abbreviations: CFU, colony-forming units; iTNF, tumor necrosis factor alpha inhibitor; ns, not significant; PBMC, peripheral blood mononuclear cell; PMN, polymorphonuclear leukocyte; TNF-α, tumor necrosis factor alpha.

that were primed with USA300Δ*saeR/S* CM had significantly increased ROS production compared with infected neutrophils primed with USA300 CM or unprimed neutrophils (Figure 4A and 4B). Furthermore, treatment of USA300Δ*saeR/S* CM with the TNF-α inhibitor, prior to neutrophil priming, significantly attenuated the enhanced ROS response (Figure 4A and 4B). In congruence with these findings, neutrophils primed with USA400Δ*saeR/S* CM also demonstrated significant increases in ROS production approximately 60 minutes after exposure compared to neutrophils primed with USA400 CM. Additionally, the increased ROS response in neutrophils primed with USA400Δ*saeR/S* CM was significantly attenuated by addition of the TNF-α inhibitor (Supplementary Figure 2*B* and 2*C*). These results indicate that the disruption of neutrophil priming by the SaeR/S-modulation of TNF-α is conserved between different strains of *S. aureus*.

To confirm if CM from USA300Δ*saeR/S*-exposed PBMCs acted as a priming agent, we exposed DCF-labeled neutrophils to USA300 CM or USA300Δ*saeR/S* CM and subsequently challenged with *N*-formyl-methionine-leucyl-phenylalanine (fMLF). fMLF is a bacterial-derived chemotactic peptide that induces a respiratory burst by binding to formyl peptide receptors that are present on neutrophils and upregulated following neutrophil priming [29]. In congruence with our previous findings, USA300Δ*saeR/S* CM significantly enhanced the neutrophil ROS response compared to USA300 CM following fMLF stimulation and the influence of TNF-α was confirmed using the TNF-α inhibitor (Figure 4C). Additionally, no significant differences were observed in plasma membrane damage following priming with CM from USA300 or USA300Δ*saeR/S* (data not shown). Last, the influence of priming with USA300Δ*saeR/S* CM on neutrophil bactericidal activity was investigated. Priming PMNs with USA300Δ*saeR/S* CM significantly enhanced killing of *S. aureus* at 30 and 60 minutes and this effect was not observed following priming with USA300 CM (Figure 4D). The enhanced bactericidal activity of PMNs primed with USA300Δ*saeR/S* CM was in part attributed to TNF-α, as treatment of USA300Δ*saeR/S* CM with the TNF-α inhibitor significantly reduced the increased neutrophil staphylocidal activity (Figure 4D). These results demonstrate that factors regulated by the SaeR/S system reduce PBMC cytokine production of TNF-α to subsequently impact the neutrophil response to a secondary stimulus, including *S. aureus* and fMLF. Taken together, these data identify that disruption of neutrophil priming is a virulence mechanism used by *S. aureus* to impede an effective neutrophil response.

DISCUSSION

The success of *S. aureus* as a pathogen is largely attributed to evasion mechanisms that circumvent killing by neutrophils. These mechanisms can directly target specific neutrophil functions, including neutrophil recruitment, phagocytosis, and the generation of ROS [2, 3, 8, 30]. In addition to these strategies, we observed that SaeR/S-regulated virulence factors impeded neutrophil priming and ROS production by decreasing monocyte production of TNF-α.

Elevated TNF-α is historically considered to be a biomarker for predicting severe *S. aureus* bacteremia, with potentially fatal outcomes [31, 32]. In agreement, we have demonstrated

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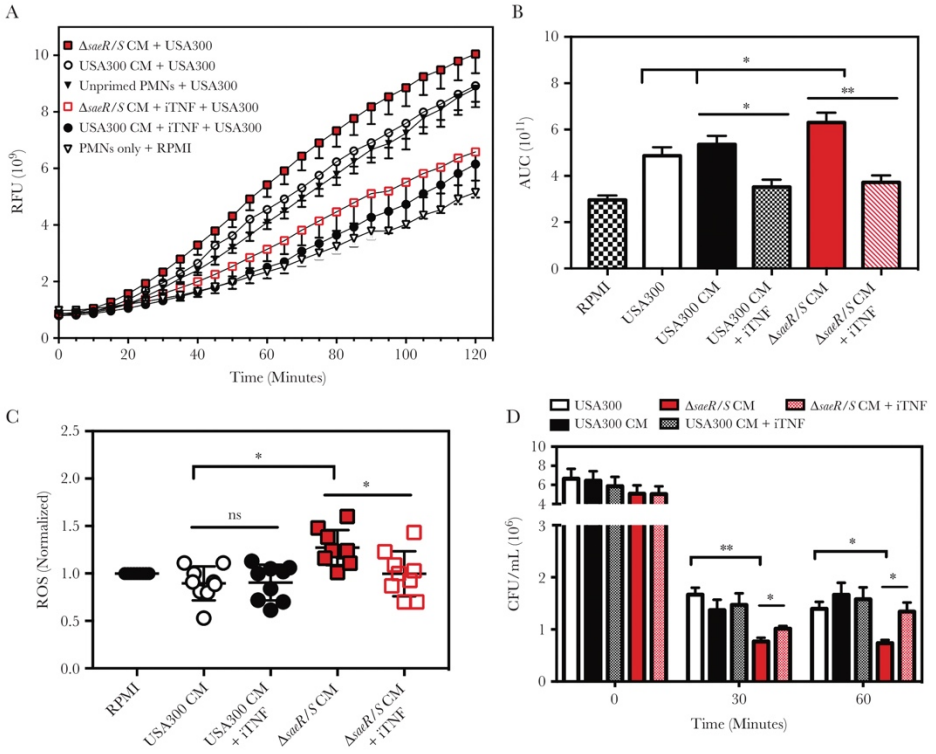


Figure 4. Conditioned media from USA300Δ*saeR/S*-exposed peripheral blood mononuclear cells (PBMCs) increases neutrophil reactive oxygen species (ROS) response to secondary challenge with USA300 and *N*-formyl-methionine-leucyl-phenylalanine (fMLF). Conditioned media (CM) from PBMCs exposed to USA300 or USA300Δ*saeR/S* (multiplicity of infection of 2:1) for 3 hours was used to prime neutrophils for 30 minutes prior to challenge with live USA300 or secondary stimulus, fMLF. *A*, 2′,7′-dihydrodiclorofluorescein diacetate (DCF)–labeled neutrophils unprimed or CM-primed were challenged with USA300 or RPMI medium only and ROS production was measured over 120 minutes via spectrometry. *B*, Data from (*A*) showing total relative fluorescent units calculated using area under the curve. *C*, Intracellular ROS production from primed DCF-labeled neutrophils following challenge with 1 μM fMLF was measured by flow cytometry at 40 minutes post–secondary challenge. *D*, Bacterial viability at 0, 30, and 60 minutes following interaction with neutrophils primed with USA300 CM, USA300Δ*saeR/S* CM, CM treated with tumor necrosis factor alpha inhibitor, or RPMI and challenged with USA300. Data shown are the mean ± standard error of the mean from 7 experiments for *A*, *B*, and *D*, and 9 experiments for *C*. Statistics were determined by 1-way analysis of variance followed by Tukey posttest. **P* < .05, ***P* < .01. Abbreviations: AUC, area under the curve; CFU, colony-forming units; CM, conditioned media; iTNF, tumor necrosis factor alpha inhibitor; ns, not significant; PMN, polymorphonuclear leukocyte; RFU, relative fluorescent unit; ROS, reactive oxygen species.

in invasive models that induction of TNF-α expression by SaeR/S-regulated factors was associated with increased bacterial burden and mortality [11]. In contrast, findings have also shown that local production of TNF-α by residential cells can correlate to a protective immune response [33, 34]. For example, TNF-α production by residential cells has been shown to significantly influence neutrophil recruitment from the microvasculature and that inhibition of TNF-α can reduce neutrophil recruitment by as much as 40%–70% [33, 34]. These findings highlight the pleiotropic influence of TNF-α during different immune responses. Herein, our data indicate that elevated TNF-α concentrations correlate to enhanced neutrophil responses that we propose are important during the initial stages of infection.

TNF-α is a robust priming agent that can enhance the production of neutrophil ROS [23] and has been observed to increase staphylocidal activity in a dose-dependent manner [28, 35]. The importance of ROS in controlling *S. aureus* infections is illustrated by the observed increase in susceptibility to infections in individuals with mutations in components of NADPH

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oxidase, as seen in patients suffering from chronic granulomatous disease [36]. *Staphylococcus aureus* uses many mechanisms to detoxify ROS, including superoxide dismutase and catalase [37, 38]. In previous studies, we demonstrated that SaeR/S-regulated factors also reduced neutrophil ROS products by decreasing hydrogen peroxide and hypochlorous acid production, likely inhibiting myeloperoxidase [8, 39]. In the current study, we observed that factors regulated by SaeR/S significantly impeded TNF-α production by PBMCs to diminish neutrophil ROS response to secondary challenge with either *S. aureus* or fMLF. Collectively, these observations underscore the importance of ROS production in controlling *S. aureus* and demonstrate that inhibition of ROS is a major mechanism used by this pathogen to survive neutrophil phagocytosis.

TNF-α also impacts neutrophil cell fate by activating NF-κB [40], a transcription factor that regulates anti-apoptotic signals, including production of IL-8 and TNF-α [41, 42]. We have previously demonstrated that SaeR/S-regulated factors diminished both NF-κB phosphorylation and IL-8 production, which accelerated neutrophil cell death and improved survival of *S. aureus* [7]. TNF-α has been shown to increase NF-κB activation and IL-8 production in neutrophils [40]. Therefore, it is possible that reduced production of TNF-α in PBMCs by SaeR/S-regulated factors suppresses NF-κB activation and IL-8 production in neutrophils. Alternatively, NF-κB regulates TNF-α production, and suppression of NF-κB activation by SaeR/S-regulated factors could impede proper TNF-α production and subsequent IL-8 production [42]. Future studies will investigate if these *S. aureus* immunomodulation mechanisms are linked and contribute to accelerated neutrophil death.

Although we identified that SaeR/S-regulated factors decreased TNF-α production from human monocytes, the mechanism responsible for this decrease remains undefined. As previous studies have shown that Hla is cytolytic toward human monocytes [43, 44], we used a USA300Δ*hla* strain of *S. aureus* to determine if monocyte production of TNF-α was associated with cell viability. Our data demonstrate that Hla was not responsible for the observed decrease in TNF-α and suggest that cell lysis is not the predominant mechanism. The SaeR/S-regulated factor LukGH (LukAB) has been observed to target monocytes and contribute to mediating phagosomal escape from macrophages [10, 45, 46]. Although macrophages and monocytes are different, it is possible that LukGH is influencing monocyte phagosomal functions to induce variations in TNF-α cytokine production [47, 48]. Notably, LukGH and Panton-Valentine leukocidin have also been observed to influence neutrophil priming [49, 50]. The SaeR/S-regulated immunomodulatory protein Sbi has been shown to increase TNF-α production in murine peritoneal macrophages in vivo during peritonitis [10, 19] and might also account for the differences in TNF-α production observed in this study. Focusing on specific SaeR/S-regulated cytolytic and immunomodulatory factors responsible for reducing TNF-α will be the topic of future research.

In the current study, we developed a co-culture model of human neutrophils and PBMCs to demonstrate that SaeR/S-regulated factors significantly reduce early monocyte production of TNF-α and that this reduction influenced neutrophil responses to *S. aureus*, including ROS production and bactericidal activity. These data identify an immune evasion strategy used by *S. aureus* that targets neutrophil priming through SaeR/S-mediated reduction of monocyte-derived TNF-α to limit the initial inflammatory response and survive neutrophil phagocytosis.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. von Eiff C, Becker K, Machka K, Stammer H, Peters G. Nasal carriage as a source of *Staphylococcus aureus* bacteremia. N Engl J Med **2001**; 344:11–6.
2. Kobayashi SD, Malachowa N, DeLeo FR. Pathogenesis of *Staphylococcus aureus* abscesses. Am J Pathol **2015**; 185:1518–27.
3. McGuinness WA, Kobayashi SD, DeLeo FR. Evasion of neutrophil killing by *Staphylococcus aureus*. Pathogens **2016**; 5:32.
4. Dantes R, Mu Y, Belflower R, et al. National burden of invasive methicillin-resistant *Staphylococcus aureus* infections, United States, 2011. JAMA Intern Med **2013**; 173:1970–8.
5. Guerra FE, Borgogna TR, Patel DM, Sward EW, Voyich JM. Epic immune battles of history: neutrophils vs. *Staphylococcus aureus*. Front Cell Infect Microbiol **2017**; 7:286.

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6. Voyich JM, Vuong C, DeWald M, et al. The SaeR/S gene regulatory system is essential for innate immune evasion by *Staphylococcus aureus*. J Infect Dis **2009**; 199:1698–706.
7. Zurek OW, Pallister KB, Voyich JM. *Staphylococcus aureus* inhibits neutrophil-derived IL-8 to promote cell death. J Infect Dis **2015**; 212:934–8.
8. Guerra FE, Addison CB, de Jong NW, et al. *Staphylococcus aureus* SaeR/S-regulated factors reduce human neutrophil reactive oxygen species production. J Leukoc Biol **2016**; 100:1005–10.
9. Montgomery CP, Boyle-Vavra S, Daum RS. Importance of the global regulators agr and SaeRS in the pathogenesis of CA-MRSA USA300 infection. PLoS One **2010**; 5:e15177.
10. Nygaard TK, Pallister KB, Ruzevich P, Griffith S, Vuong C, Voyich JM. SaeR binds a consensus sequence within virulence gene promoters to advance USA300 pathogenesis. J Infect Dis **2010**; 201:241–54.
11. Watkins RL, Pallister KB, Voyich JM. The SaeR/S gene regulatory system induces a pro-inflammatory cytokine response during *Staphylococcus aureus* infection. PLoS One **2011**; 6:e19939.
12. Voyich JM, Braughton KR, Sturdevant DE, et al. Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. J Immunol **2005**; 175:3907–19.
13. Zurek OW, Nygaard TK, Watkins RL, et al. The role of innate immunity in promoting SaeR/S-mediated virulence in *Staphylococcus aureus*. J Innate Immun **2014**; 6:21–30.
14. Flack CE, Zurek OW, Meishery DD, et al. Differential regulation of staphylococcal virulence by the sensor kinase SaeS in response to neutrophil-derived stimuli. Proc Natl Acad Sci U S A **2014**; 111:E2037–45.
15. Nygaard TK, Pallister KB, DuMont AL, et al. Alpha-toxin induces programmed cell death of human T cells, B cells, and monocytes during USA300 infection. PLoS One **2012**; 7:e36532.
16. Tkaczyk C, Hamilton MM, Datta V, et al. *Staphylococcus aureus* alpha toxin suppresses effective innate and adaptive immune responses in a murine dermonecrosis model. PLoS One **2013**; 8:e75103.
17. Gómez MI, Lee A, Reddy B, et al. *Staphylococcus aureus* protein A induces airway epithelial inflammatory responses by activating TNFR1. Nat Med **2004**; 10:842–8.
18. Watkins RL, Zurek OW, Pallister KB, Voyich JM. The SaeR/S two-component system induces interferon-gamma production in neutrophils during invasive *Staphylococcus aureus* infection. Microbes Infect **2013**; 15:749–54.
19. Gonzalez CD, Ledo C, Giai C, Garófalo A, Gómez MI. The Sbi protein contributes to *Staphylococcus aureus* inflammatory response during systemic infection. PLoS One **2015**; 10:e0131879.
20. Abtin A, Jain R, Mitchell AJ, et al. Perivascular macrophages mediate neutrophil recruitment during bacterial skin infection. Nat Immunol **2014**; 15:45–53.
21. Volk AP, Barber BM, Goss KL, et al. Priming of neutrophils and differentiated PLB-985 cells by pathophysiological concentrations of TNF-α is partially oxygen dependent. J Innate Immun **2011**; 3:298–314.
22. Eberl M, Roberts GW, Meuter S, Williams JD, Topley N, Moser B. A rapid crosstalk of human gammadelta T cells and monocytes drives the acute inflammation in bacterial infections. PLoS Pathog **2009**; 5:e1000308.
23. Swain SD, Rohn TT, Quinn MT. Neutrophil priming in host defense: role of oxidants as priming agents. Antioxid Redox Signal **2002**; 4:69–83.
24. Ferrante A, Harvey DP, Bates EJ. *Staphylococcus aureus*-stimulated mononuclear leucocyte-conditioned medium increases the neutrophil bactericidal activity, and augments oxygen radical production and degranulation in response to the bacteria. Clin Exp Immunol **1989**; 78:366–71.
25. Bates EJ, Ferrante A, Beard LJ. Characterization of the major neutrophil-stimulating activity present in culture medium conditioned by *Staphylococcus aureus*-stimulated mononuclear leucocytes. Immunology **1991**; 72:448–50.
26. Kowanko IC, Ferrante A, Clemente G, Kumaratilake LM. Tumor necrosis factor primes neutrophils to kill *Staphylococcus aureus* by an oxygen-dependent mechanism and *Plasmodium falciparum* by an oxygen-independent mechanism. Infect Immun **1996**; 64:3435–7.
27. Forsberg M, Löfgren R, Zheng L, Stendahl O. Tumour necrosis factor-alpha potentiates CR3-induced respiratory burst by activating p38 MAP kinase in human neutrophils. Immunology **2001**; 103:465–72.
28. Ferrante A, Martin AJ, Bates EJ, et al. Killing of *Staphylococcus aureus* by tumor necrosis factor-alpha-activated neutrophils. The role of serum opsonins, integrin receptors, respiratory burst, and degranulation. J Immunol **1993**; 151:4821–8.
29. Zimmerli W, Seligmann B, Gallin JI. Exudation primes human and guinea pig neutrophils for subsequent responsiveness to the chemotactic peptide N-formylmethionylleucylphenylalanine and increases complement component C3bi receptor expression. J Clin Invest **1986**; 77:925–33.
30. Voyich JM, Braughton KR, Sturdevant DE, et al. Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. J Immunol **2005**; 175:3907–19.
31. van den Berg S, Laman JD, Boon L, et al. Distinctive cytokines as biomarkers predicting fatal outcome of severe *Staphylococcus aureus* bacteremia in mice. PLoS One **2013**; 8:e59107.
32. Gogos CA, Drosou E, Bassaris HP, Skoutelis A. Pro- versus anti-inflammatory cytokine profile in patients with

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severe sepsis: a marker for prognosis and future therapeutic options. J Infect Dis **2000**; 181:176–80.
33. Zhang Y, Ramos BF, Jakschik BA. Neutrophil recruitment by tumor necrosis factor from mast cells in immune complex peritonitis. Science **1992**; 258:1957–9.
34. Malaviya R, Ikeda T, Ross E, Abraham SN. Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-alpha. Nature **1996**; 381:77–80.
35. Bajaj MS, Kew RR, Webster RO, Hyers TM. Priming of human neutrophil functions by tumor necrosis factor: enhancement of superoxide anion generation, degranulation, and chemotaxis to chemoattractants C5a and F-Met-Leu-Phe. Inflammation **1992**; 16:241–50.
36. Lekstrom-Himes JA, Gallin JI. Immunodeficiency diseases caused by defects in phagocytes. N Engl J Med **2000**; 343:1703–14.
37. Karavolos MH, Horsburgh MJ, Ingham E, Foster SJ. Role and regulation of the superoxide dismutases of *Staphylococcus aureus*. Microbiology **2003**; 149:2749–58.
38. Horsburgh MJ, Clements MO, Crossley H, Ingham E, Foster SJ. PerR controls oxidative stress resistance and iron storage proteins and is required for virulence in *Staphylococcus aureus*. Infect Immun **2001**; 69:3744–54.
39. de Jong NWM, Ramyar KX, Guerra FE, et al. Immune evasion by a staphylococcal inhibitor of myeloperoxidase. Proc Natl Acad Sci **2017**; 114:9439–44.
40. Cowburn AS, Deighton J, Walmsley SR, Chilvers ER. The survival effect of TNF-alpha in human neutrophils is mediated via NF-kappa B-dependent IL-8 release. Eur J Immunol **2004**; 34:1733–43.
41. McCracken JM, Allen LA. Regulation of human neutrophil apoptosis and lifespan in health and disease. J Cell Death. **2014**; 7:15–23.
42. Lawrence T. The nuclear factor NF-kappaB pathway in inflammation. Cold Spring Harb Perspect Biol **2009**; 1:a001651.
43. Nygaard TK, Pallister KB, Zurek OW, Voyich JM. The impact of α-toxin on host cell plasma membrane permeability and cytokine expression during human blood infection by CA-MRSA USA300. J Leukoc Biol **2013**; 94:971–9.
44. Wilke GA, Bubeck Wardenburg J. Role of a disintegrin and metalloprotease 10 in *Staphylococcus aureus* alpha-hemolysin-mediated cellular injury. Proc Natl Acad Sci U S A **2010**; 107:13473–8.
45. Münzenmayer L, Geiger T, Daiber E, et al. Influence of Sae-regulated and Agr-regulated factors on the escape of *Staphylococcus aureus* from human macrophages. Cell Microbiol **2016**; 18:1172–83.
46. Dumont AL, Nygaard TK, Watkins RL, et al. Characterization of a new cytotoxin that contributes to *Staphylococcus aureus* pathogenesis. Mol Microbiol **2011**; 79:814–25.
47. Wolf AJ, Arruda A, Reyes CN, et al. Phagosomal degradation increases TLR access to bacterial ligands and enhances macrophage sensitivity to bacteria. J Immunol **2011**; 187:6002–10.
48. Jubrail J, Morris P, Bewley MA, et al. Inability to sustain intraphagolysosomal killing of *Staphylococcus aureus* predisposes to bacterial persistence in macrophages. Cell Microbiol **2016**; 18:80–96.
49. Malachowa N, Kobayashi SD, Braughton KR, et al. *Staphylococcus aureus* leukotoxin GH promotes inflammation. J Infect Dis **2012**; 206:1185–93.
50. Graves SF, Kobayashi SD, Braughton KR, et al. Sublytic concentrations of *Staphylococcus aureus* Panton-Valentine leukocidin alter human PMN gene expression and enhance bactericidal capacity. J Leukoc Biol **2012**; 92:361–74.

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REFERENCES CITED

Akong-Moore, K., Chow, O. A., Von Kö Ckritz-Blickwede, M., Nizet, V., Wehkamp, J., and Fischer-Bosch, M. (2012). Influences of Chloride and Hypochlorite on Neutrophil Extracellular Trap Formation. *PLoS One* 7. doi:10.1371/journal.pone.0042984.

Amulic, B., Cazalet, C., Hayes, G. L., Metzler, K. D., and Zychlinsky, A. (2012). Neutrophil Function: From Mechanisms to Disease. *Annu. Rev. Immunol.* 30, 459–489. Available at: http://www.annualreviews.org/doi/abs/10.1146/annurev-immunol-020711-074942 [Accessed September 23, 2013].

Anderson, K. E., Boyle, K. B., Davidson, K., Chessa, T. A. M., Kulkarni, S., Jarvis, G. E., et al. (2008). CD18-dependent activation of the neutrophil NADPH oxidase during phagocytosis of Escherichia coli or Staphylococcus aureus is regulated by class III but not class I or II PI3Ks. *Blood* 112, 5202–5211. doi:10.1182/blood-2008-04-149450.

Antonov, N. K., Garzon, M. C., Morel, K. D., Whittier, S., Planet, P. J., and Lauren, C. T. (2015). High prevalence of mupirocin resistance in Staphylococcus aureus isolates from a pediatric population. *Antimicrob. Agents Chemother.* 59, 3350–3356. doi:10.1128/AAC.00079-15.

Assari, T. (2006). Chronic Granulomatous Disease; fundamental stages in our understanding of CGD. *Med. Immunol.* 5, 4. doi:10.1186/1476-9433-5-4.

Baba-Moussa, L., Sina, H., Scheftel, J. M., Moreau, B., Sainte-Marie, D., Kotchoni, S. O., et al. (2011). Staphylococcal Panton-Valentine leucocidin as a major virulence factor associated to furuncles. *PLoS One* 6, e25716. doi:10.1371/journal.pone.0025716.

Badarau, A., Rouha, H., Malafa, S., Logan, D. T., Ha??kansson, M., Stulik, L., et al. (2015). Structure-function analysis of Heterodimer Formation, oligomerization, and receptor binding of the staphylococcus aureus bi-component toxin LukGH. *J. Biol. Chem.* 290, 142–156. doi:10.1074/jbc.M114.598110.

Bae, Y.-S., Park, J. C., He, R., Ye, R. D., Kwak, J.-Y., Suh, P.-G., et al. (2003). Differential signaling of formyl peptide receptor-like 1 by Trp-Lys-Tyr-Met-Val-Met-CONH2 or lipoxin A4 in human neutrophils. *Mol. Pharmacol.* 64, 721–30. doi:10.1124/mol.64.3.721.

Baggiolini, M., Walz, A., and Kunkel, S. L. (1989). Neutrophil-activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils. *J. Clin. Invest.* 84, 1045–1049. doi:10.1172/JCI114265.

Ballal, A., and Manna, A. C. (2009). Regulation of superoxide dismutase (sod) genes by SarA in Staphylococcus aureus. *J. Bacteriol.* 191, 3301–10. doi:10.1128/JB.01496-08.

Balsam, L. B., Liang, T. W., and Parkos, C. a (1998). Functional mapping of CD11b/CD18 epitopes important in neutrophil-epithelial interactions: a central role of the I domain. *J. Immunol.* 160, 5058–5065.

Bardoel, B. W., Vos, R., Bouman, T., Aerts, P. C., Bestebroer, J., Huizinga, E. G., et al. (2012). Evasion of Toll-like receptor 2 activation by staphylococcal superantigen-like protein 3. *J. Mol. Med.* 90, 1109–1120. doi:10.1007/s00109-012-0926-8.

Bates, E. J., Ferrante, a, and Beard, L. J. (1991). Characterization of the major neutrophil-stimulating activity present in culture medium conditioned by Staphylococcus aureus-stimulated mononuclear leucocytes. *Immunology* 72, 448–50.

Becker, K. A., Li, X., Seitz, A., Steinmann, J., Koch, A., Schuchman, E., et al. (2017). Neutrophils Kill Reactive Oxygen Species-Resistant Pseudomonas aeruginosa by Sphingosine. *Cell. Physiol. Biochem.* 43, 1603–1616. doi:10.1159/000482024.

Ben-Ari, J., Wolach, O., Gavrieli, R., and Wolach, B. (2012). Infections associated with chronic granulomatous disease: linking genetics to phenotypic expression. *Expert Rev. Anti. Infect. Ther.* 10, 881–94. doi:10.1586/eri.12.77.

Ben-Baruch, A., Grimm, M., Bengali, K., Evans, G. A., Chertov, O., Wang, J. M., et al. (1997). The differential ability of IL-8 and neutrophil-activating peptide-2 to induce attenuation of chemotaxis is mediated by their divergent capabilities to phosphorylate CXCR2 (IL-8 receptor B). *J. Immunol.* 158, 5927–33. doi:10.4049/jimmunol.1002006.

Berends, E. T. M., Horswill, A. R., Haste, N. M., Monestier, M., Nizet, V., and Von Köckritz-Blickwede, M. (2010). Nuclease expression by Staphylococcus aureus facilitates escape from neutrophil extracellular traps. *J. Innate Immun.* 2, 576–586. doi:10.1159/000319909.

Berton, G., Mócsai, A., and Lowell, C. A. (2005). Src and Syk kinases: Key regulators of phagocytic cell activation. *Trends Immunol.* 26, 208–214. doi:10.1016/j.it.2005.02.002.

Bestebroer, J., Aerts, P. C., Rooijakkers, S. H. M., Pandey, M. K., Köhl, J., Van Strijp, J. A. G., et al. (2010). Functional basis for complement evasion by staphylococcal superantigen-like 7. *Cell. Microbiol.* 12, 1506–1516. doi:10.1111/j.1462-5822.2010.01486.x.

Bestebroer, J., Poppelier, M. J. J. G., Ulfman, L. H., Lenting, P. J., Denis, C. V., Van Kessel, K. P. M., et al. (2007). Staphylococcal superantigen-like 5 binds PSGL-1 and inhibits P-selectin-mediated neutrophil rolling. *Blood* 109, 2936–2943. doi:10.1182/blood-2006-06-015461.

Bestebroer, J., Van Kessel, K. P. M., Azouagh, H., Walenkamp, A. M., Boer, I. G. J., Romijn, R. A., et al. (2009). Staphylococcal SSL5 inhibits leukocyte activation by chemokines and anaphylatoxins. *Blood* 113, 328–337. doi:10.1182/blood-2008-04-153882.

Bodey, G. P., Buckley, M., Sathe, Y. S., and Freireich, E. J. (1966). Quantitative relationships between circulating leukocytes and infection in patients with acute leukemia. *Ann. Intern. Med.* 64, 328–40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/5216294 [Accessed May 30, 2017].

Borregaard, N. (2010). Neutrophils, from Marrow to Microbes. *Immunity* 33, 657–670. doi:10.1016/j.immuni.2010.11.011.

Borregaard, N., Sørensen, O. E., and Theilgaard-Mönch, K. (2007). Neutrophil granules: a library of innate immunity proteins. *Trends Immunol.* 28, 340–345. doi:10.1016/j.it.2007.06.002.

Bose, J. L., Daly, S. M., Hall, P. R., and Bayles, K. W. (2014). Identification of the Staphylococcus aureus vfrAB Operon, a Novel Virulence Factor Regulatory Locus. *Infect. Immun.* 82, 1813–1822. doi:10.1128/IAI.01655-13.

Bréchard, S., and Tschirhart, E. J. (2008). Regulation of superoxide production in neutrophils: role of calcium influx. *J. Leukoc. Biol.* 84, 1223–37. doi:10.1189/jlb.0807553.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., et al. (2004). Neutrophil extracellular traps kill bacteria. *Science (80-.).* 303, 1532–1535. doi:10.1126/science.1092385.

Broderick, A. H., Stacy, D. M., Tal-Gan, Y., Kratochvil, M. J., Blackwell, H. E., and Lynn, D. M. (2014). Surface coatings that promote rapid release of peptide-based AgrC inhibitors for attenuation of quorum sensing in Staphylococcus aureus. *Adv. Healthc. Mater.* 3, 97–105. doi:10.1002/adhm.201300119.

Bubeck Wardenburg, J., Bae, T., Otto, M., Deleo, F. R., and Schneewind, O. (2007). Poring over pores: alpha-hemolysin and Panton-Valentine leukocidin in Staphylococcus aureus pneumonia. *Nat. Med.* 13, 1405–6. doi:10.1038/nm1207-1405.

Bubeck Wardenburg, J., Palazzolo-Ballance, A. M., Otto, M., Schneewind, O., and DeLeo, F. R. (2008). Panton-Valentine leukocidin is not a virulence determinant in murine models of community-associated methicillin-resistant Staphylococcus aureus disease. *J. Infect. Dis.* 198, 1166–70. doi:10.1086/592053.

Buer, J., and Balling, R. (2003). Mice, microbes and models of infection. *Nat. Rev. Genet.* 4, 195–205. doi:10.1038/nrg1019.

Burman, J. D., Leung, E., Atkins, K. L., O'Seaghdha, M. N., Lango, L., Bernado, P., et al. (2008). Interaction of human complement with Sbi, a staphylococcal immunoglobulin-binding protein: Indications of a novel mechanism of complement evasion by Staphylococcus aureus. *J. Biol. Chem.* 283, 17579–17593. doi:10.1074/jbc.M800265200.

Bylund, J., Björnsdottir, H., Sundqvist, M., Karlsson, A., and Dahlgren, C. (2014). Measurement of respiratory burst products, released or retained, during activation of professional phagocytes. *Methods Mol. Biol.* 1124, 321–38. doi:10.1007/978-1-62703-845-4_21.

Carrel, M., Perencevich, E. N., and David, M. Z. (2015). USA300 Methicillin-Resistant Staphylococcus aureus, United States, 2000-2013. *Emerg. Infect. Dis.* 21, 1973–80. doi:10.3201/eid2111.150452.

Cegelski, L., Marshall, G. R., Eldridge, G. R., and Hultgren, S. J. (2008). The biology and future prospects of antivirulence therapies. *Nat. Rev. Microbiol.* 6, 17–27. doi:10.1038/nrmicro1818.

Centers for Disease Control and Prevention (2015). Active Bacterial Core Surveillance Report, Emerging Infections Program Network, MethicillinResistant Staphylococcus aureus, 2015.

Centers for Disease Control and Prevention (CDC) (1999). Four pediatric deaths from community-acquired methicillin-resistant Staphylococcus aureus — Minnesota and North Dakota, 1997-1999. *MMWR. Morb. Mortal. Wkly. Rep.* 48, 707–10. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21033181.

Chain, E., Florey, H. W., Adelaide, M. B., Gardner, A. D., Oxfd, D. M., Heatley, N. G., et al. (1940). Penicillin As a Chemotherapeutic Agent. *Lancet* 236, 226–228. doi:10.1016/S0140-6736(01)08728-1.

Chambers, H. F. (2001). The changing epidemiology of Staphylococcus aureus? *Emerg. Infect. Dis.* 7, 178–182. doi:10.3201/eid0702.700178.

Chambers, H. F., and Deleo, F. R. (2009). Waves of resistance: Staphylococcus aureus in the antibiotic era. *Nat. Rev. Microbiol.* 7, 629–41. doi:10.1038/nrmicro2200.

Chapman, A. L. P., Hampton, M. B., Senthilmohan, R., Winterbourn, C. C., and Kettle, A. J. (2002). Chlorination of bacterial and neutrophil proteins during phagocytosis and killing of Staphylococcus aureus. *J. Biol. Chem.* 277, 9757–62. doi:10.1074/jbc.M106134200.

Chen, X., Lee, K.-A., Ha, E.-M., Lee, K. M., Seo, Y. Y., Choi, H. K., et al. (2011). A specific and sensitive method for detection of hypochlorous acid for the imaging of microbe-induced HOCl production. *Chem. Commun. (Camb).* 47, 4373–5. doi:10.1039/c1cc10589b.

Chiang, N., Fierro, I. M., Gronert, K., and Serhan, C. N. (2000). Activation of lipoxin A(4) receptors by aspirin-triggered lipoxins and select peptides evokes ligand-specific responses in inflammation. *J Exp Med* 191, 1197–1208. doi:10.1084/jem.191.7.1197.

Cho, H., Jeong, D.-W., Liu, Q., Yeo, W.-S., Vogl, T., Skaar, E. P., et al. (2015). Calprotectin Increases the Activity of the SaeRS Two Component System and Murine Mortality during Staphylococcus aureus Infections. *PLoS Pathog.* 11, e1005026. doi:10.1371/journal.ppat.1005026.

Cho, J. S., Pietras, E. M., Garcia, N. C., Ramos, R. I., Farzam, D. M., Monroe, H. R., et al. (2010). IL-17 is essential for host defense against cutaneous Staphylococcus aureus infection in mice. *J. Clin. Invest.* 120, 1762–73. doi:10.1172/JCI40891.

Chowdhury, A. K., Watkins, T., Parinandi, N. L., Saatian, B., Kleinberg, M. E., Usatyuk, P. V, et al. (2005). Src-mediated tyrosine phosphorylation of p47phox in hyperoxia-induced activation of NADPH oxidase and generation of reactive oxygen species in lung endothelial cells. *J. Biol. Chem.* 280, 20700–11. doi:10.1074/jbc.M411722200.

Christophe, T., Karlsson, A., Dugave, C., Rabiet, M. J., Boulay, F., and Dahlgren, C. (2001). The Synthetic Peptide Trp-Lys-Tyr-Met-Val-Met-NH2 Specifically Activates Neutrophils through FPRL1/Lipoxin A4 Receptors and is an Agonist for the Orphan Monocyte-expressed Chemoattractant Receptor FPRL2. *J. Biol. Chem.* 276, 21585–21593. doi:10.1074/jbc.M007769200.

Clarke, T. B., Davis, K. M., Lysenko, E. S., Zhou, A. Y., Yu, Y., and Weiser, J. N. (2010). Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. *Nat. Med.* 16, 228–31. doi:10.1038/nm.2087.

Clatworthy, A. E., Pierson, E., and Hung, D. T. (2007). Targeting virulence: a new paradigm for antimicrobial therapy. *Nat. Chem. Biol.* 3, 541–548. doi:10.1038/nchembio.2007.24.

Clauditz, A., Resch, A., Wieland, K.-P., Peschel, A., and Gotz, F. (2006). Staphyloxanthin Plays a Role in the Fitness of Staphylococcus aureus and Its Ability To Cope with Oxidative Stress. *Infect. Immun.* 74, 4950–4953. doi:10.1128/IAI.00204-06.

Clements, M. O., Watson, S. P., and Foster, S. J. (1999). Characterization of the major superoxide dismutase of Staphylococcus aureus and its role in starvation survival, stress resistance, and pathogenicity. *J. Bacteriol.* 181, 3898–3903.

Coates, T., Bax, R., and Coates, A. (2009). Nasal decolonization of Staphylococcus aureus with mupirocin: Strengths, weaknesses and future prospects. *J. Antimicrob. Chemother.* 64, 9–15. doi:10.1093/jac/dkp159.

Colin, D. A., and Monteil, H. (2003). Control of the Oxidative Burst of Human Neutrophils by Staphylococcal Leukotoxins Control of the Oxidative Burst of Human Neutrophils by Staphylococcal Leukotoxins. *Infect. Immun.* 71, 3724–3729. doi:10.1128/IAI.71.7.3724.

Collins, L. V., Kristian, S. A., Weidenmaier, C., Faigle, M., Van Kessel, K. P. M., Van Strijp, J. A. G., et al. (2002). Staphylococcus aureus strains lacking D-alanine modifications of teichoic acids are highly susceptible to human neutrophil killing and are virulence attenuated in mice. *J. Infect. Dis.* 186, 214–9. doi:10.1086/341454.

Cosgrove, K., Coutts, G., Jonsson, I.-M., Tarkowski, A., Kokai-Kun, J. F., Mond, J. J., et al. (2007). Catalase (KatA) and alkyl hydroperoxide reductase (AhpC) have compensatory roles in peroxide stress resistance and are required for survival, persistence, and nasal colonization in Staphylococcus aureus. *J. Bacteriol.* 189, 1025–35. doi:10.1128/JB.01524-06.

Dale, D. C., Guerry, D., Wewerka, J. R., Bull, J. M., and Chusid, M. J. (1979). Chronic neutropenia. *Medicine (Baltimore)*. 58, 128–44. Available at: http://www.ncbi.nlm.nih.gov/pubmed/431399 [Accessed May 30, 2017].

Dancey, J. T., Deubelbeiss, K. A., Harker, L. A., and Finch, C. A. (1976). Neutrophil kinetics in man. *J. Clin. Invest.* 58, 705–15. doi:10.1172/JCI108517.

De Haas, C. J. C., Veldkamp, K. E., Peschel, A., Weerkamp, F., Wamel, W. J. B. Van, Heezius, E. C. J. M., et al. (2004). Chemotaxis Inhibitory Protein of Staphylococcus aureus , a Bacterial Antiinflammatory Agent. *J. Exp. Med. J. Exp. Med* 03687900, 687–695. doi:10.1084/jem.20031636.

de Jong, N. W. M., Ramyar, K. X., Guerra, F. E., Nijland, R., Fevre, C., Voyich, J. M., et al. (2017). Immune evasion by a staphylococcal inhibitor of myeloperoxidase. *Proc. Natl. Acad. Sci. U. S. A.* 114, 9439–9444. doi:10.1073/pnas.1707032114.

de Oliveira, S., Rosowski, E. E., and Huttenlocher, A. (2016). Neutrophil migration in infection and wound repair: going forward in reverse. *Nat. Rev. Immunol.* 16, 378–91. doi:10.1038/nri.2016.49.

De Yang, Chen, Q., Schmidt, A. P., Anderson, G. M., Wang, J. M., Wooters, J., et al. (2000). LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. *J. Exp. Med.* 192, 1069–74. doi:10.1084/jem.192.7.1069.

DeCoursey, T. E., and Ligeti, E. (2005). Regulation and termination of NADPH oxidase activity. *Cell. Mol. Life Sci.* 62, 2173–2193. doi:10.1007/s00018-005-5177-1.

Delauné, A., Dubrac, S., Blanchet, C., Poupel, O., Mäder, U., Hiron, A., et al. (2012). The WalKR system controls major staphylococcal virulence genes and is involved in triggering the host inflammatory response. *Infect. Immun.* 80, 3438–3453. doi:10.1128/IAI.00195-12.

DeLeo, F. R., Allen, L. A., Apicella, M., and Nauseef, W. M. (1999). NADPH oxidase activation and assembly during phagocytosis. *J. Immunol.* 163, 6732–40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10586071 [Accessed December 2, 2013].

DeLeo, F. R., and Chambers, H. F. (2009). Reemergence of antibiotic-resistant Staphylococcus aureus in the genomics era. *J. Clin. Invest.* 119, 2464–74. doi:10.1172/JCI38226.

DeLeo, F. R., and Quinn, M. T. (1996). Assembly of the phagocyte NADPH oxidase: molecular interaction of oxidase proteins. *J. Leukoc. Biol.* 60, 677–91. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8975869 [Accessed December 2, 2013].

Diep, B. A., Gill, S. R., Chang, R. F., Phan, T. H., Chen, J. H., Davidson, M. G., et al. (2006). Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant Staphylococcus aureus. *Lancet* 367, 731–9. doi:10.1016/S0140-6736(06)68231-7.

Dossett, J. H., Kronvall, G., Williams, R. C., and Quie, P. G. (1969). Antiphagocytic effects of staphylococcal protein A. *J. Immunol.* 103, 1405–1410. Available at: http://www.jimmunol.org/content/103/6/1405%5Cnhttp://www.jimmunol.org/content/103/6/1405.abstract?ijkey=ce3459544328c76b1899c9b5e8a083f6f4fa7d31&keytype2=tf_ipsecsha%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/4188886.

Dragneva, Y., Anuradha, C. D., Valeva, A., Hoffmann, A., Bhakdi, S., and Husmann, M. (2001). Subcytocidal attack by staphylococcal alpha-toxin activates NF-kappaB and induces interleukin-8 production. *Infect. Immun.* 69, 2630–5. doi:10.1128/IAI.69.4.2630-2635.2001.

Dubrac, S., Boneca, I. G., Poupel, O., and Msadek, T. (2007). New insights into the WalK/WalR (YycG/YycF) essential signal transduction pathway reveal a major role in controlling cell wall metabolism and biofilm formation in Staphylococcus aureus. *J. Bacteriol.* 189, 8257–8269. doi:10.1128/JB.00645-07.

Dubrac, S., and Msadek, T. (2004). Identification of genes controlled by the essential YycG / YycF two-component system of Staphylococcus aureus. *J. Bacteriol.* 186, 1175–1181. doi:10.1128/JB.186.4.1175.

DuMont, A. L., and Torres, V. J. (2014). Cell targeting by the staphylococcus aureus pore-forming toxins: It's not just about lipids. *Trends Microbiol.* 22, 21–27. doi:10.1016/j.tim.2013.10.004.

DuMont, A. L., Yoong, P., Day, C. J., Alonzo, F., McDonald, W. H., Jennings, M. P., et al. (2013). Staphylococcus aureus LukAB cytotoxin kills human neutrophils by targeting the CD11b subunit of the integrin Mac-1. *Proc. Natl. Acad. Sci. U. S. A.* 110, 10794–9. doi:10.1073/pnas.1305121110.

Edwards, S. W., Say, J. E., and Hart, C. A. (1987). Oxygen-dependent killing of Staphylococcus aureus by human neutrophils. *J. Gen. Microbiol.* 133, 3591–7. doi:10.1099/00221287-133-12-3591.

Edwards, S. W., Say, J. E., and Hughes, V. (1988). Gamma interferon enhances the killing of Staphylococcus aureus by human neutrophils. *J. Gen. Microbiol.* 134, 37–42. doi:10.1099/00221287-134-1-37.

El-Benna, J., Dang, P. M. C., and Gougerot-Pocidalo, M. A. (2008). Priming of the neutrophil NADPH oxidase activation: Role of p47phox phosphorylation and NOX2 mobilization to the plasma membrane. *Semin. Immunopathol.* 30, 279–289. doi:10.1007/s00281-008-0118-3.

El Benna, J., Faust, R. P., Johnson, J. L., and Babior, B. M. (1996). Phosphorylation of the respiratory burst oxidase subunit p47phox as determined by two-dimensional phosphopeptide mapping. Phosphorylation by protein kinase C, protein kinase A, and a mitogen-activated protein kinase. *J. Biol. Chem.* 271, 6374–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8626435 [Accessed October 6, 2014].

El Chemaly, A., Okochi, Y., Sasaki, M., Arnaudeau, S., Okamura, Y., and Demaurex, N. (2010). VSOP/Hv1 proton channels sustain calcium entry, neutrophil migration, and superoxide production by limiting cell depolarization and acidification. *J. Exp. Med.* 207, 129–39. doi:10.1084/jem.20091837.

Elbim, C., Bailly, S., Chollet-Martin, S., Hakim, J., and Gougerot-Pocidalo, M. A. (1994). Differential priming effects of proinflammatory cytokines on human neutrophil oxidative burst in response to bacterial N-formyl peptides. *Infect. Immun.* 62, 2195–201. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8188340.

Ellis, T. N., and Beaman, B. L. (2004). Interferon-gamma activation of polymorphonuclear neutrophil function. *Immunology* 112, 2–12. doi:10.1111/j.1365-2567.2004.01849.x.

Ellson, C. D., Davidson, K., Ferguson, G. J., O'Connor, R., Stephens, L. R., and Hawkins, P. T. (2006). Neutrophils from p40phox-/- mice exhibit severe defects in NADPH oxidase regulation and oxidant-dependent bacterial killing. *J. Exp. Med.* 203, 1927–37. doi:10.1084/jem.20052069.

Ernst, C. M., Staubitz, P., Mishra, N. N., Yang, S. J., Hornig, G., Kalbacher, H., et al. (2009). The bacterial defensin resistance protein MprF consists of separable domains for lipid lysinylation and antimicrobial peptide repulsion. *PLoS Pathog.* 5, 1–9. doi:10.1371/journal.ppat.1000660.

Essig, K., Hu, D., Guimaraes, J. C., Alterauge, D., Edelmann, S., Raj, T., et al. (2017). Roquin Suppresses the PI3K-mTOR Signaling Pathway to Inhibit T Helper Cell Differentiation and Conversion of Treg to Tfr Cells. *Immunity* 47, 1067–1082.e12. doi:10.1016/j.immuni.2017.11.008.

Falk, W., Harvath, L., and Leonard, E. J. (1982). Only the chemotactic subpopulation of human blood monocytes expresses receptors for the chemotactic peptide N-formylmethionyl-leucyl-phenylalanine. *Infect. Immun.* 36, 450–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6282748 [Accessed February 16, 2017].

Falugi, F., Kim, H. K., and Missiakas, D. M. (2013). Role of Protein A in the Evasion of Host Adaptive Immune Responses. *MBio* 4, 1–9. doi:10.1128/mBio.00575-13.Editor.

Fan, X., Zhang, X., Zhu, Y., Niu, L., Teng, M., Sun, B., et al. (2015). Structure of the DNA-binding domain of the response regulator SaeR from Staphylococcus aureus. *Acta Crystallogr. Sect. D Biol. Crystallogr.* 71, 1768–1776. doi:10.1107/S1399004715010287.

Ferrante, A., Harvey, D. P., and Bates, E. J. (1989). Staphylococcus aureus-stimulated mononuclear leucocyte-conditioned medium increases the neutrophil bactericidal activity, and augments oxygen radical production and degranulation in response to the bacteria. *Clin Exp Immunol* 78, 366–371.

Ferrante, A., Martin, A. J., Bates, E. J., Goh, D. H., Harvey, D. P., Parsons, D., et al. (1993). Killing of Staphylococcus aureus by tumor necrosis factor-alpha-activated neutrophils. The role of serum opsonins, integrin receptors, respiratory burst, and degranulation. *J Immunol* 151, 4821–4828.

Fevre, C., Bestebroer, J., Mebius, M. M., de Haas, C. J. C., van Strijp, J. A. G., Fitzgerald, J. R., et al. (2014). Staphylococcus aureus proteins SSL6 and SElX interact with neutrophil receptors as identified using secretome phage display. *Cell. Microbiol.* 16, 1646–1665. doi:10.1111/cmi.12313.

Fiore, S., Maddox, J. F., Perez, H. D., and Serhan, C. N. (1994). Identification of a human cDNA encoding a functional high affinity lipoxin A4 receptor. *J. Exp. Med.* 180, 253–60. doi:10.1084/jem.180.1.253.

Flack, C. E., Zurek, O. W., Meishery, D. D., Pallister, K. B., Malone, C. L., Horswill, A. R., et al. (2014). Differential regulation of staphylococcal virulence by the sensor kinase SaeS in response to neutrophil-derived stimuli. *Proc. Natl. Acad. Sci. U. S. A.* 111, E2037-45. doi:10.1073/pnas.1322125111.

Flannagan, R. S., Cosío, G., and Grinstein, S. (2009). Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. *Nat. Rev. Microbiol.* 7, 355–66. doi:10.1038/nrmicro2128.

Fleming, A. (1929). On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of B. influenzae. *Br. J. Exp. Pathol.* 10, 226–236. doi:10.1093/clinids/2.1.129.

Fleming, A. (1945). “The Nobel Prize in Physiology or Medicine 1945”. Nobelprize.org. Nobel Media AB 2014. Web. 5 Jul 2018. <http://www.nobelprize.org/nobel_prizes/medicine/laureates/1945/>.

Forsgren, A., and Sjöquist, J. (1966). “Protein A” from S. aureus. I. Pseudo-immune reaction with human gamma-globulin. *J. Immunol.* 97, 822–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/4163007.

Foster, T. J. (2017). Antibiotic resistance in Staphylococcus aureus. Current status and future prospects. *FEMS Microbiol. Rev.* 41, 430–449. doi:10.1093/femsre/fux007.

Fournier, B., and Philpott, D. J. (2005). Recognition of Staphylococcus aureus by the innate immune system. *Clin. Microbiol. Rev.* 18, 521–40. doi:10.1128/CMR.18.3.521-540.2005.

Fowler, V. G., Allen, K. B., Moreira, E. D., Moustafa, M., Isgro, F., Boucher, H. W., et al. (2013). Effect of an investigational vaccine for preventing Staphylococcus aureus infections after cardiothoracic surgery: a randomized trial. *JAMA* 309, 1368–78. doi:10.1001/jama.2013.3010.

Fowler, V. G., and Proctor, R. A. (2014). Where does a Staphylococcus aureus vaccine stand? *Clin. Microbiol. Infect.* 20, 66–75. doi:10.1111/1469-0691.12570.

Frenck, R. W., Buddy Creech, C., Sheldon, E. A., Seiden, D. J., Kankam, M. K., Baber, J., et al. (2016). Safety, tolerability, and immunogenicity of a 4-antigen Staphylococcus aureus vaccine (SA4Ag): Results from a first-in-human randomised, placebo-controlled phase 1/2 study. *Vaccine* 35, 375–384. doi:10.1016/j.vaccine.2016.11.010.

Fu, H., Bylund, J., Karlsson, A., Pellmé, S., and Dahlgren, C. (2004). The mechanism for activation of the neutrophil NADPH-oxidase by the peptides formyl-Met-Leu-Phe and Trp-Lys-Tyr-Met-Val-Met differs from that for interleukin-8. *Immunology* 112, 201–10. doi:10.1111/j.1365-2567.2004.01884.x.

Fuchs, T. A., Abed, U., Goosmann, C., Hurwitz, R., Schulze, I., Wahn, V., et al. (2007). Novel cell death program leads to neutrophil extracellular traps. *J. Cell Biol.* 176, 231–241. doi:10.1083/jcb.200606027.

Fumagalli, L., Campa, C. C., Germena, G., Lowell, C. A., Hirsch, E., and Berton, G. (2013). Class I Phosphoinositide-3-Kinases and Src Kinases Play a Nonredundant Role in Regulation of Adhesion-Independent and -Dependent Neutrophil Reactive Oxygen Species Generation. *J. Immunol.* 190, 3648–3660. doi:10.4049/jimmunol.1201951.

Futosi, K., Fodor, S., and M??csai, A. (2013). Neutrophil cell surface receptors and their intracellular signal transduction pathways. *Int. Immunopharmacol.* 17, 638–650. doi:10.1016/j.intimp.2013.06.034.

Gallin, J. I., Malech, H. L., Melnick, D. A., Weening, R. S., and Roos, D. (1991). A controlled trial of interferon gamma to prevent infection in chronic granulomatous disease. The International Chronic Granulomatous Disease Cooperative Study Group. *N. Engl. J. Med.* 324, 509–16. doi:10.1056/NEJM199102213240801.

Galluzzi, L., Vitale, I., Abrams, J. M., Alnemri, E. S., Baehrecke, E. H., Blagosklonny, M. V, et al. (2012). Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. *Cell Death Differ.* 19, 107–20. doi:10.1038/cdd.2011.96.

Gao, X. P., Zhu, X., Fu, J., Liu, Q., Frey, R. S., and Malik, A. B. (2007). Blockade of class IA phosphoinositide 3-kinase in neutrophils prevents NADPH oxidase activation- and adhesion-dependent inflammation. *J. Biol. Chem.* 282, 6116–6125. doi:10.1074/jbc.M610248200.

Garcia, B. L., Ramyar, K. X., Tzekou, A., Ricklin, D., McWhorter, W. J., Lambris, J. D., et al. (2010). Molecular Basis for Complement Recognition and Inhibition Determined by Crystallographic Studies of the Staphylococcal Complement Inhibitor (SCIN) Bound to C3c and C3b. *J. Mol. Biol.* 402, 17–29. doi:10.1016/j.jmb.2010.07.029.

Gaynes, R. (2017). The discovery of penicillin—new insights after more than 75 years of clinical use. *Emerg. Infect. Dis.* 23, 849–853. doi:10.3201/eid2305.161556.

Gazendam, R. P., van Hamme, J. L., Tool, A. T. J., Hoogenboezem, M., van den Berg, J. M., Prins, J. M., et al. (2016). Human Neutrophils Use Different Mechanisms To Kill *Aspergillus fumigatus* Conidia and Hyphae: Evidence from Phagocyte Defects. *J. Immunol.* 196, 1272–1283. doi:10.4049/jimmunol.1501811.

Geiger, T., Goerke, C., Mainiero, M., Kraus, D., and Wolz, C. (2008). The virulence regulator sae of Staphylococcus aureus: Promoter activities and response to phagocytosis-related signals. *J. Bacteriol.* 190, 3419–3428. doi:10.1128/JB.01927-07.

Genestier, A. L., Michallet, M. C., Prévost, G., Bellot, G., Chalabreysse, L., Peyrol, S., et al. (2005). Staphylococcus aureus Panton-Valentine leukocidin directly targets mitochondria and induces Bax-independent apoptosis of human neutrophils. *J. Clin. Invest.* 115, 3117–3127. doi:10.1172/JCI22684.

Gerber, C. E., Bruchelt, G., Falk, U. B., Kimpfler, A., Hauschild, O., Kuçi, S., et al. (2001). Reconstitution of bactericidal activity in chronic granulomatous disease cells by glucose-oxidase–containing liposomes. *Blood* 98, 3097–3105. doi:10.1182/blood.V98.10.3097.

Gillet, Y., Issartel, B., Vanhems, P., Fournet, J. C., Lina, G., Bes, M., et al. (2002). Association between Staphylococcus aureus strains carrying gene for Panton-Valentine leukocidin and highly lethal necrotising pneumonia in young immunocompetent patients. *Lancet* 359, 753–759. doi:10.1016/S0140-6736(02)07877-7.

Gillet, Y., Vanhems, P., Lina, G., Bes, M., Vandenesch, F., Floret, D., et al. (2007). Factors Predicting Mortality in Necrotizing Community-Acquired Pneumonia Caused by Staphylococcus aureus Containing Panton-Valentine Leukocidin. *Clin. Infect. Dis.* 45, 315–321. doi:10.1086/519263.

Giraudo, a T., Raspanti, C. G., Calzolari, a, and Nagel, R. (1994). Characterization of a Tn551-mutant of Staphylococcus aureus defective in the production of several exoproteins. *Can. J. Microbiol.* 40, 677–681. doi:10.1139/m94-107.

Giraudo, A. T., Calzolari, A., Cataldi, A. A., Bogni, C., and Nagel, R. (1999). The sae locus of Staphylococcus aureus encodes a two-component regulatory system. *FEMS Microbiol. Lett.* 177, 15–22. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10436918 [Accessed November 30, 2015].

Giraudo, A. T., Cheung, A. L., and Nagel, R. (1997). The sae locus of Staphylococcus aureus controls exoprotein synthesis at the transcriptional level. *Arch. Microbiol.* 168, 53–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9211714 [Accessed November 30, 2015].

Giraudo, A. T., Rampone, H., Calzolari, A., and Nagel, R. (1996). Phenotypic characterization and virulence of a sae- agr- mutant of Staphylococcus aureus. *Can J Microbiol* 42, 120–123. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8742355.

González-Zorn, B., Senna, J. P. M., Fiette, L., Shorte, S., Testard, A., Chignard, M., et al. (2005). Bacterial and host factors implicated in nasal carriage of methicillin-resistant Staphylococcus aureus in mice. *Infect. Immun.* 73, 1847–1851. doi:10.1128/IAI.73.3.1847-1851.2005.

Gonzalez, B. E., Hulten, K. G., Dishop, M. K., Lamberth, L. B., Hammerman, W. a, Mason, E. O., et al. (2005a). Pulmonary manifestations in children with invasive community-acquired Staphylococcus aureus infection. *Clin. Infect. Dis.* 41, 583–590. doi:10.1086/432475.

Gonzalez, B. E., Martinez-Aguilar, G., Hulten, K. G., Hammerman, W. A., Coss-Bu, J., Avalos-Mishaan, A., et al. (2005b). Severe Staphylococcal sepsis in adolescents in the era of community-acquired methicillin-resistant Staphylococcus aureus. *Pediatrics* 115, 642–8. doi:10.1542/peds.2004-2300.

Gonzalez, C. D., Ledo, C., Giai, C., Gar??falo, A., and G??mez, M. I. (2015). The Sbi protein contributes to Staphylococcus aureus inflammatory response during systemic infection. *PLoS One* 10, 1–14. doi:10.1371/journal.pone.0131879.

Gordon, C. P., Williams, P., and Chan, W. C. (2013). Attenuating Staphylococcus aureus virulence gene regulation: A medicinal chemistry perspective. *J. Med. Chem.* 56, 1389–1404. doi:10.1021/jm3014635.

Gordon, S. (2016). Phagocytosis: An Immunobiologic Process. *Immunity* 44, 463–475. doi:10.1016/j.immuni.2016.02.026.

Gorwitz, R. J., Kruszon-Moran, D., McAllister, S. K., McQuillan, G., McDougal, L. K., Fosheim, G. E., et al. (2008). Changes in the prevalence of nasal colonization with Staphylococcus aureus in the United States, 2001-2004. *J. Infect. Dis.* 197, 1226–34. doi:10.1086/533494.

Graham, P. L., Lin, S. X., and Larson, E. L. (2006). A U.S. population-based survey of Staphylococcus aureus colonization. *Ann. Intern. Med.* 144, 318–25. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16520472.

Graves, S. F., Kobayashi, S. D., Braughton, K. R., Whitney, a. R., Sturdevant, D. E., Rasmussen, D. L., et al. (2012). Sublytic concentrations of Staphylococcus aureus Panton-Valentine leukocidin alter human PMN gene expression and enhance bactericidal capacity. *J. Leukoc. Biol.* 92, 361–374. doi:10.1189/jlb.1111575.

Green, J. N., Kettle, A. J., and Winterbourn, C. C. (2014). Protein chlorination in neutrophil phagosomes and correlation with bacterial killing. *Free Radic. Biol. Med.* 77, 49–56. doi:10.1016/j.freeradbiomed.2014.08.013.

Greenlee-Wacker, M. C., Rigby, K. M., Kobayashi, S. D., Porter, A. R., DeLeo, F. R., and Nauseef, W. M. (2014). Phagocytosis of Staphylococcus aureus by human neutrophils prevents macrophage efferocytosis and induces programmed necrosis. *J. Immunol.* 192, 4709–17. doi:10.4049/jimmunol.1302692.

Gresham, H. D., Lowrance, J. H., Caver, T. E., Wilson, B. S., Cheung, a L., and Lindberg, F. P. (2000). Survival of Staphylococcus aureus inside neutrophils contributes to infection. *J. Immunol.* 164, 3713–22. doi:10.4049/jimmunol.164.7.3713.

Guerra, F. E., Addison, C. B., de Jong, N. W. M., Azzolino, J., Pallister, K. B., van Strijp, J. A. G., et al. (2016). Staphylococcus aureus SaeR/S-regulated factors reduce human neutrophil reactive oxygen species production. *J. Leukoc. Biol.* 100, 1–6. doi:10.1189/jlb.4VMAB0316-100RR.

Guerra, F. E., Borgogna, T. R., Patel, D. M., Sward, E. W., and Voyich, J. M. (2017). Epic Immune Battles of History: Neutrophils vs. Staphylococcus aureus. *Front. Cell. Infect. Microbiol.* 7, 1–19. doi:10.3389/fcimb.2017.00286.

Guyer, D. A., Moore, K. L., Lynam, E. B., Schammel, C. M., Rogelj, S., McEver, R. P., et al. (1996). P-selectin glycoprotein ligand-1 (PSGL-1) is a ligand for L-selectin in neutrophil aggregation. *Blood* 88, 2415–21. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8839831.

Haas, P. J., De Haas, C. J. C., Poppelier, M. J. J. C., Van Kessel, K. P. M., Van Strijp, J. A. G., Dijkstra, K., et al. (2005). The structure of the C5a receptor-blocking domain of chemotaxis inhibitory protein of Staphylococcus aureus is related to a group of immune evasive molecules. *J. Mol. Biol.* 353, 859–872. doi:10.1016/j.jmb.2005.09.014.

Hall, J. W., Yang, J., Guo, H., and Ji, Y. (2017). The Staphylococcus aureus AirSR Two-Component System Mediates Reactive Oxygen Species Resistance via Transcriptional Regulation of Staphyloxanthin Production. *Infect. Immun.* 85, 1–12. doi:10.1128/IAI.00838-16.

Hampton, M. B., and Winterbourn, C. C. (1995). Modification of neutrophil oxidant production with diphenyleneiodonium and its effect on bacterial killing. *Free Radic. Biol. Med.* 18, 633–639. doi:10.1016/0891-5849(94)00181-I.

Hampton, M., Kettle, A., and Winterbourn, C. (1996). Involvement of superoxide and myeloperoxidase in oxygen-dependent killing of Staphylococcus aureus by neutrophils. *Infect. Immun.* 64, 3512–3517. Available at: http://iai.asm.org/content/64/9/3512.short [Accessed November 30, 2015].

Harding, M. G., Zhang, K., Conly, J., and Kubes, P. (2014). Neutrophil Crawling in Capillaries; A Novel Immune Response to Staphylococcus aureus. *PLoS Pathog.* 10, e1004379. doi:10.1371/journal.ppat.1004379.

Harraghy, N., Kormanec, J., Wolz, C., Homerova, D., Goerke, C., Ohlsen, K., et al. (2005). sae is essential for expression of the staphylococcal adhesins Eap and Emp. *Microbiology* 151, 1789–1800. doi:10.1099/mic.0.27902-0.

Hawkins, P. T., and Stephens, L. R. (2015). PI3K signalling in inflammation. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* 1851, 882–897. doi:10.1016/j.bbalip.2014.12.006.

Herold, B. C., Immergluck, L. C., Maranan, M. C., Lauderdale, D. S., Gaskin, R. E., Boyle-Vavra, S., et al. (1998). Community-acquired methicillin-resistant Staphylococcus aureus in children with no identified predisposing risk. *JAMA* 279, 593–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9486753.

Hidalgo, A., Peired, A. J., Wild, M. K., Vestweber, D., and Frenette, P. S. (2007). Complete Identification of E-Selectin Ligands on Neutrophils Reveals Distinct Functions of PSGL-1, ESL-1, and CD44. *Immunity* 26, 477–489. doi:10.1016/j.immuni.2007.03.011.

Hoebe, K., Georgel, P., Rutschmann, S., Du, X., Mudd, S., Crozat, K., et al. (2005). CD36 is a sensor of diacylglycerides. *Nature* 433, 523–527. doi:10.1038/nature03253.

Horsburgh, M. J., Clements, M. O., Crossley, H., Ingham, E., and Foster, S. J. (2001a). PerR controls oxidative stress resistance and iron storage proteins and is required for virulence in Staphylococcus aureus. *Infect. Immun.* 69, 3744–54. doi:10.1128/IAI.69.6.3744-3754.2001.

Horsburgh, M. J., Ingham, E., and Foster, S. J. (2001b). In Staphylococcus aureus, fur is an interactive regulator with PerR, contributes to virulence, and Is necessary for oxidative stress resistance through positive regulation of catalase and iron homeostasis. *J. Bacteriol.* 183, 468–75. doi:10.1128/JB.183.2.468-475.2001.

Hsieh, M. M., Everhart, J. E., Byrd-Holt, D. D., Tisdale, J. F., and Rodgers, G. P. (2007). Prevalence of neutropenia in the U.S. population: age, sex, smoking status, and ethnic differences. *Ann. Intern. Med.* 146, 486–92. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17404350.

Huey, R., and Hugli, T. E. (1985). Characterization of a C5a receptor on human polymorphonuclear leukocytes (PMN). *J. Immunol.* 135, 2063–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/4020139 [Accessed February 16, 2017].

Huo, Y., and Xia, L. (2009). P-selectin Glycoprotein Ligand-1 Plays a Crucial Role in the Selective Recruitment of Leukocytes Into the Atherosclerotic Arterial Wall. *Trends Cardiovasc. Med.* 19, 140–145. doi:10.1016/j.tcm.2009.07.006.

Hurst, J. K. (2012). What really happens in the neutrophil phagosome? *Free Radic. Biol. Med.* 53, 508–20. doi:10.1016/j.freeradbiomed.2012.05.008.

Ibrahim, F. B. M., Pang, S. J., and Melendez, A. J. (2004). Anaphylatoxin signaling in human neutrophils. A key role for sphingosine kinase. *J. Biol. Chem.* 279, 44802–44811. doi:10.1074/jbc.M403977200.

Inaba, Y., Ashida, T., Ito, T., Ishikawa, C., Tanabe, H., Maemoto, A., et al. (2010). Expression of the antimicrobial peptide alpha-defensin/cryptdins in intestinal crypts decreases at the initial phase of intestinal inflammation in a model of inflammatory bowel disease, IL-10-deficient mice. *Inflamm. Bowel Dis.* 16, 1488–1495. doi:10.1002/ibd.21253.

Itoh, S., Hamada, E., Kamoshida, G., Yokoyama, R., Takii, T., Onozaki, K., et al. (2010). Staphylococcal superantigen-like protein 10 (SSL10) binds to human immunoglobulin G (IgG) and inhibits complement activation via the classical pathway. *Mol. Immunol.* 47, 932–938. doi:10.1016/j.molimm.2009.09.027.

Jacobsson, K., and Frykberg, L. (1995). Cloning of ligand-binding domains of bacterial receptors by phage display. *Biotechniques* 18, 878–85. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7619494 [Accessed February 20, 2017].

Jamieson, S., Flanagan, J. U., Kolekar, S., Buchanan, C., Kendall, J. D., Lee, W.-J., et al. (2011). A drug targeting only p110α can block phosphoinositide 3-kinase signalling and tumour growth in certain cell types. *Biochem. J.* 438, 53–62. doi:10.1042/BJ20110502.

Janesch, P., Rouha, H., Weber, S., Malafa, S., Gross, K., Maierhofer, B., et al. (2017). Selective sensitization of human neutrophils to LukGH mediated cytotoxicity by Staphylococcus aureus and IL-8. *J. Infect.* 74, 473–483. doi:10.1016/j.jinf.2017.02.004.

Jevons, M. P. (1961). "Celbenin" -resistant Staphylococci. *Br. Med. J.* 1, 124–125. doi:10.1136/bmj.1.5219.124-a.

Ji, C. J., Kim, J. H., Won, Y. Bin, Lee, Y. E., Choi, T. W., Ju, S. Y., et al. (2015). Staphylococcus aureus PerR is a hypersensitive hydrogen peroxide sensor using iron-mediated histidine oxidation. *J. Biol. Chem.* 290, 20374–20386. doi:10.1074/jbc.M115.664961.

Jin, T., Bokarewa, M., Foster, T., Mitchell, J., Higgins, J., and Tarkowski, A. (2004). Staphylococcus aureus resists human defensins by production of staphylokinase, a novel bacterial evasion mechanism. *J. Immunol.* 172, 1169–1176. doi:10.4049/jimmunol.172.2.1169.

Johansson, M. W., Patarroyo, M., Oberg, F., Siegbahn, A., and Nilsson, K. (1997). Myeloperoxidase mediates cell adhesion via the alpha M beta 2 integrin (Mac-1, CD11b/CD18). *J. Cell Sci.* 110 (Pt 9, 1133–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9175709.

Johnston, S. C., Dustin, M. L., Hibbs, M. L., and Springer, T. a (1990). On the species specificity of the interaction of LFA-1 with intercellular adhesion molecules. *J. Immunol.* 145, 1181–7.

Kanai, F., Liu, H., Field, S. J., Akbary, H., Matsuo, T., Brown, G. E., et al. (2001). The PX domains of p47phox and p40phox bind to lipid products of PI(3)K. *Nat. Cell Biol.* 3, 675–678. doi:10.1038/35083070.

Karathanassis, D., Stahelin, R. V., Bravo, J., Perisic, O., Pacold, C. M., Cho, W., et al. (2002). Binding of the PX domain of p47phoxto phosphatidylinositol 3, 4-bisphosphate and phosphatidic acid is masked by an intramolecular interaction. *EMBO J.* 21, 5057–5068. doi:10.1093/emboj/cdf519.

Karavolos, M. H. (2003). Role and regulation of the superoxide dismutases of Staphylococcus aureus. *Microbiology* 149, 2749–2758. doi:10.1099/mic.0.26353-0.

Karavolos, M. H., Horsburgh, M. J., Ingham, E., and Foster, S. J. (2003). Role and regulation of the superoxide dismutases of Staphylococcus aureus. *Microbiology* 149, 2749–2758. doi:10.1099/mic.0.26353-0.

Kaufmann, G. F., Park, J., and Janda, K. D. (2008). Bacterial quorum sensing: a new target for anti-infective immunotherapy. *Expert Opin. Biol. Ther.* 8, 719–724. doi:10.1517/14712598.8.6.719.

Kawada-Matsuo, M., Yoshida, Y., Nakamura, N., and Komatsuzawa, H. (2011). Role of two-component systems in the resistance of Staphylococcus aureus to antibacterial agents. *Virulence* 2, 427–430. doi:10.4161/viru.2.5.17711.

Kebir, D. El, and Filep, J. G. (2013). Modulation of neutrophil apoptosis and the resolution of inflammation through β2 integrins. *Front. Immunol.* 4, 1–15. doi:10.3389/fimmu.2013.00060.

Kennedy, A. D., and Deleo, F. R. (2009). Neutrophil apoptosis and the resolution of infection. *Immunol. Res.* 43, 25–61. doi:10.1007/s12026-008-8049-6.

Kettle, A. J., Albrett, A. M., Chapman, A. L., Dickerhof, N., Forbes, L. V, Khalilova, I., et al. (2014). Measuring chlorine bleach in biology and medicine. *Biochim. Biophys. Acta* 1840, 781–93. doi:10.1016/j.bbagen.2013.07.004.

Kettle, A. J., Anderson, R. F., Hampton, M. B., and Winterbourn, C. C. (2007). Reactions of superoxide with myeloperoxidase. *Biochemistry* 46, 4888–4897. doi:10.1021/bi602587k [doi].

Kim, H. K., Falugi, F., Missiakas, D. M., and Schneewind, O. (2016). Peptidoglycan-linked protein A promotes T cell-dependent antibody expansion during Staphylococcus aureus infection. *Proc. Natl. Acad. Sci.*, 201524267. doi:10.1073/pnas.1524267113.

Kim, H. K., Missiakas, D., and Schneewind, O. (2014). Mouse models for infectious diseases caused by Staphylococcus aureus. *J. Immunol. Methods* 410, 88–99. doi:10.1016/j.jim.2014.04.007.

King, B. F., and Wilkinson, B. J. (1981). Binding of human immunoglobulin G to protein A in encapsulated Staphylococcus aureus. *Infect. Immun.* 33, 666–672.

Kirby, W. M. (1944). EXTRACTION OF A HIGHLY POTENT PENICILLIN INACTIVATOR FROM PENICILLIN RESISTANT STAPHYLOCOCCI. *Science* 99, 452–3. doi:10.1126/science.99.2579.452.

Kirchdoerfer, R. N., Garner, A. L., Flack, C. E., Mee, J. M., Horswill, A. R., Janda, K. D., et al. (2011). Structural basis for ligand recognition and discrimination of a quorum-quenching antibody. *J. Biol. Chem.* 286, 17351–17358. doi:10.1074/jbc.M111.231258.

Klebanoff, S. J., Kettle, A. J., Rosen, H., Winterbourn, C. C., and Nauseef, W. M. (2013). Myeloperoxidase: a front-line defender against phagocytosed microorganisms. *J. Leukoc. Biol.* 93, 185–98. doi:10.1189/jlb.0712349.

Klein, E., Smith, D. L., and Laxminarayan, R. (2007). Hospitalizations and deaths caused by methicillin-resistant Staphylococcus aureus, United States, 1999-2005. *Emerg. Infect. Dis.* 13, 1840–6. doi:10.3201/eid1312.070629.

Klein, E. Y., Mojica, N., Jiang, W., Cosgrove, S. E., Septimus, E., Morgan, D. J., et al. (2017). Trends in Methicillin-Resistant Staphylococcus aureus Hospitalizations in the United States, 2010-2014. *Clin. Infect. Dis.* 65, 1921–1923. doi:10.1093/cid/cix640.

Klevens, R. M., Morrison, M. A., Nadle, J., Petit, S., Gershman, K., Ray, S., et al. (2007). Invasive methicillin-resistant Staphylococcus aureus infections in the United States. *JAMA* 298, 1763–71. doi:10.1001/jama.298.15.1763.

Knox, R. (1960). A New Penicillin (BRL 1241) Active Against Penicillin-Resistant Staphylococci. *Br. Med. J.* 2, 690–693. doi:10.1136/bmj.2.5200.690.

Knuefermann, P., Sakata, Y., Baker, J. S., Huang, C. H., Sekiguchi, K., Hardarson, H. S., et al. (2004). Toll-like receptor 2 mediates Staphylococcus aureus-induced myocardial dysfunction and cytokine production in the heart. *Circulation* 110, 3693–3698. doi:10.1161/01.CIR.0000143081.13042.04.

Ko, T. P., Huang, C. Y., Hsieh, T. J., Chen, S. C., Chen, Y. R., Yang, C. S., et al. (2016). Crystal structures of Staphylococcal SaeR reveal possible DNA-binding modes. *Biochem. Biophys. Res. Commun.* 474, 686–690. doi:10.1016/j.bbrc.2016.05.009.

Ko, Y. P., Kuipers, A., Freitag, C. M., Jongerius, I., Medina, E., van Rooijen, W. J., et al. (2013). Phagocytosis Escape by a Staphylococcus aureus Protein That Connects Complement and Coagulation Proteins at the Bacterial Surface. *PLoS Pathog.* 9, 1–13. doi:10.1371/journal.ppat.1003816.

Kobayashi, N., Ueki, K., Okazaki, Y., Iwane, A., Kubota, N., Ohsugi, M., et al. (2011). Blockade of class IB phosphoinositide-3 kinase ameliorates obesity-induced inflammation and insulin resistance. *Proc. Natl. Acad. Sci.* 108, 5753–5758. doi:10.1073/pnas.1016430108.

Kobayashi, S. D., Braughton, K. R., Palazzolo-Ballance, A. M., Kennedy, A. D., Sampaio, E., Kristosturyan, E., et al. (2010). Rapid neutrophil destruction following phagocytosis of Staphylococcus aureus. *J. Innate Immun.* 2, 560–75. doi:10.1159/000317134.

Kobayashi, S. D., Braughton, K. R., Whitney, A. R., Voyich, J. M., Schwan, T. G., Musser, J. M., et al. (2003). Bacterial pathogens modulate an apoptosis differentiation program in human neutrophils. *Proc. Natl. Acad. Sci.* 100, 10948–10953. doi:10.1073/pnas.1833375100.

Kobayashi, S. D., Malachowa, N., and DeLeo, F. R. (2015). Pathogenesis of Staphylococcus aureus abscesses. *Am. J. Pathol.* 185, 1518–27. doi:10.1016/j.ajpath.2014.11.030.

Kolaczkowska, E., and Kubes, P. (2013). Neutrophil recruitment and function in health and inflammation. *Nat. Rev. Immunol.* 13, 159–75. doi:10.1038/nri3399.

König, B., Prévost, G., Piémont, Y., and König, W. (1995). Effects of Staphylococcus aureus leukocidins on inflammatory mediator release from human granulocytes. *J. Infect. Dis.* 171, 607–13. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7533198.

Koymans, K. J., Feitsma, L. J., Brondijk, T. H. C., Aerts, P. C., Lukkien, E., Lossl, P., et al. (2015). Structural basis for inhibition of TLR2 by staphylococcal superantigen-like protein 3 (SSL3). *Proc. Natl. Acad. Sci. U. S. A.* 112, 11018–11023. doi:10.1073/pnas.1502026112.

Kratochvil, M. J., Yang, T., Blackwell, H. E., and Lynn, D. M. (2017). Nonwoven Polymer Nanofiber Coatings That Inhibit Quorum Sensing in Staphylococcus aureus: Toward New Nonbactericidal Approaches to Infection Control. *ACS Infect. Dis.* doi:10.1021/acsinfecdis.6b00173.

Kreisel, K. M., Stine, O. C., Johnson, J. K., Perencevich, E. N., Shardell, M. D., Lesse, A. J., et al. (2011). USA300 methicillin-resistant Staphylococcus aureus bacteremia and the risk of severe sepsis: is USA300 methicillin-resistant Staphylococcus aureus associated with more severe infections? *Diagn. Microbiol. Infect. Dis.* 70, 285–90. doi:10.1016/j.diagmicrobio.2011.03.010.

Kronvall, G., Grey, H. M., and Williams, R. C. (1970). Protein A reactivity with mouse immunoglobulins. Structural relationship between some mouse and human immunoglobulins. *J. Immunol.* 105, 1116–23. doi:10.1097/MD.0b013e3181d5453d.

Krute, C. N., Rice, K. C., and Bose, J. L. (2016). VfrB is a key activator of the *Staphylococcus aureus* SaeRS two-component system. *J. Bacteriol.*, JB.00828-16. doi:10.1128/JB.00828-16.

Kurokawa, K., Lee, H., Roh, K. B., Asanuma, M., Kim, Y. S., Nakayama, H., et al. (2009). The triacylated ATP binding cluster transporter substrate-binding lipoprotein of Staphylococcus aureus functions as a native ligand for toll-like receptor 2. *J. Biol. Chem.* 284, 8406–8411. doi:10.1074/jbc.M809618200.

Laarman, a. J., Mijnheer, G., Mootz, J. M., van Rooijen, W. J. M., Ruyken, M., Malone, C. L., et al. (2012). Staphylococcus aureus Staphopain A inhibits CXCR2-dependent neutrophil activation and chemotaxis. *EMBO J.* 31, 3607–3619. doi:10.1038/emboj.2012.212.

Lahoz-Beneytez, J., Elemans, M., Zhang, Y., Ahmed, R., Salam, A., Block, M., et al. (2016). Human neutrophil kinetics: Modeling of stable isotope labeling data supports short blood neutrophil half-lives. *Blood* 127, 3431–3438. doi:10.1182/blood-2016-03-700336.

Lambris, J. D., Ricklin, D., and Geisbrecht, B. V. (2008). Complement evasion by human pathogens. *Nat. Rev. Microbiol.* 6, 132–42. doi:10.1038/nrmicro1824.

Langley, R., Wines, B., Willoughby, N., Basu, I., Proft, T., and Fraser, J. D. (2005). The Staphylococcal Superantigen-Like Protein 7 Binds IgA and Complement C5 and Inhibits IgA-Fc RI Binding and Serum Killing of Bacteria. *J. Immunol.* 174, 2926–2933. doi:10.4049/jimmunol.174.5.2926.

Lau, D., Mollnau, H., Eiserich, J. P., Freeman, B. A., Daiber, A., Gehling, U. M., et al. (2005). Myeloperoxidase mediates neutrophil activation by association with CD11b/CD18 integrins. *Proc. Natl. Acad. Sci. U. S. A.* 102, 431–6. doi:10.1073/pnas.0405193102.

Laursen, N. S., Gordon, N., Hermans, S., Lorenz, N., Jackson, N., Wines, B., et al. (2010). Structural basis for inhibition of complement C5 by the SSL7 protein from Staphylococcus aureus. *Proc. Natl. Acad. Sci. U. S. A.* 107, 3681–6. doi:10.1073/pnas.0910565107.

Le, Y., Yazawa, H., Gong, W., Yu, Z., Ferrans, V. J., Murphy, P. M., et al. (2001). The neurotoxic prion peptide fragment PrP(106-126) is a chemotactic agonist for the G protein-coupled receptor formyl peptide receptor-like 1. *J Immunol* 166, 1448–1451. doi:10.4049/jimmunol.166.3.1448.

Lee, B. Y., Singh, A., David, M. Z., Bartsch, S. M., Slayton, R. B., Huang, S. S., et al. (2013). The economic burden of community-associated methicillin-resistant Staphylococcus aureus (CA-MRSA). *Clin. Microbiol. Infect.* 19, 528–536. doi:10.1111/j.1469-0691.2012.03914.x.

Lee, L. Y. L., Höök, M., Haviland, D., Wetsel, R. A., Yonter, E. O., Syribeys, P., et al. (2004). Inhibition of Complement Activation by a Secreted Staphylococcus aureus Protein. *J. Infect. Dis.* 190, 571–579. doi:10.1086/422259.

Lekstrom-Himes, J. A., and Gallin, J. I. (2000). Immunodeficiency diseases caused by defects in phagocytes. *N. Engl. J. Med.* 343, 1703–14. doi:10.1056/NEJM200012073432307.

Li, M., Diep, B. A., Villaruz, A. E., Braughton, K. R., Jiang, X., DeLeo, F. R., et al. (2009). Evolution of virulence in epidemic community-associated methicillin-resistant Staphylococcus aureus. *Proc. Natl. Acad. Sci. U. S. A.* 106, 5883–8. doi:10.1073/pnas.0900743106.

Licitra, G. (2013). Etymologia: Staphylococcus. *Emerg. Infect. Dis.* 19, 1553. doi:10.3201/eid1909.ET1909.

Lipp, P., and Reither, G. (2011). Protein kinase C: the “masters” of calcium and lipid. *Cold Spring Harb. Perspect. Biol.* 3, 1–17. doi:10.1101/cshperspect.a004556.

Lishko, V. K., Podolnikova, N. P., Yakubenko, V. P., Yakovlev, S., Medved, L., Yadav, S. P., et al. (2004). Multiple binding sites in fibrinogen for integrin ??M?? 2 (Mac-1). *J. Biol. Chem.* 279, 44897–44906. doi:10.1074/jbc.M408012200.

Liu, G. Y. (2009). Molecular Pathogenesis of Staphylococcus aureus Infection. *Pediatr Res* 65, 71–77. doi:10.1203/PDR.0b013e31819dc44d.Molecular.

Liu, G. Y., Essex, A., Buchanan, J. T., Datta, V., Hoffman, H. M., Bastian, J. F., et al. (2005). Staphylococcus aureus golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. *J. Exp. Med.* 202, 209–215. doi:10.1084/jem.20050846.

Liu, Q., Cho, H., Yeo, W.-S., and Bae, T. (2015). The Extracytoplasmic Linker Peptide of the Sensor Protein SaeS Tunes the Kinase Activity Required for Staphylococcal Virulence in Response to Host Signals. *PLOS Pathog.* 11, e1004799. doi:10.1371/journal.ppat.1004799.

Liu, Q., Yeo, W., and Bae, T. (2016). The SaeRS Two-Component System of Staphylococcus aureus. *Genes (Basel)*. 7, 81. doi:10.3390/genes7100081.

Lorenz, N., Clow, F., Radcliff, F. J., and Fraser, J. D. (2013). Full functional activity of SSL7 requires binding of both complement C5 and IgA. *Immunol. Cell Biol.* 91, 469–76. doi:10.1038/icb.2013.28.

Lowy, F. D. (1998). Staphylococcus aureus infections. *N. Engl. J. Med.* 339, 520–32. doi:10.1056/NEJM199808203390806.

Lu, T., Porter, A. R., Kennedy, A. D., Kobayashi, S. D., and DeLeo, F. R. (2014). Phagocytosis and Killing of Staphylococcus aureus by Human Neutrophils. *J. Innate Immun.* 6, 639–649. doi:10.1159/000360478.

Luo, H. R., and Mondal, S. (2015). Molecular control of PtdIns(3,4,5)P3 signaling in neutrophils. *EMBO Rep.* 16, 149–163. doi:10.15252/embr.201439466.

Ma, Q. (2013). Role of Nrf2 in Oxidative Stress and Toxicity. *Annu. Rev. Pharmacol. Toxicol.* 53, 401–426. doi:10.1146/annurev-pharmtox-011112-140320.

Madani, S., Hichami, a, Legrand, a, Belleville, J., and Khan, N. a (2001). Implication of acyl chain of diacylglycerols in activation of different isoforms of protein kinase C. *FASEB J.* 15, 2595–2601. doi:10.1096/fj.01-0753int.

Magill, S. S., Edwards, J. R., Bamberg, W., Beldavs, Z. G., Dumyati, G., Kainer, M. A., et al. (2014). Multistate Point-Prevalence Survey of Health Care–Associated Infections. *N. Engl. J. Med.* 370, 1198–1208. doi:10.1056/NEJMoa1306801.

Malachowa, N., Kobayashi, S. D., Braughton, K. R., Whitney, A. R., Parnell, M. J., Gardner, D. J., et al. (2012). Staphylococcus aureus leukotoxin GH promotes inflammation. *J. Infect. Dis.* 206, 1185–1193. doi:10.1093/infdis/jis495.

Malachowa, N., Kobayashi, S. D., Freedman, B., Dorward, D. W., and DeLeo, F. R. (2013). Staphylococcus aureus leukotoxin GH promotes formation of neutrophil extracellular traps. *J Immunol* 191, 6022–6029. doi:10.4049/jimmunol.1301821.

Malachowa, N., Whitney, A. R., Kobayashi, S. D., Sturdevant, D. E., Kennedy, A. D., Braughton, K. R., et al. (2011). Global changes in staphylococcus aureus gene expression in human blood. *PLoS One* 6. doi:10.1371/journal.pone.0018617.

Malawista, S. E., Van Blaricom, G., and Breitenstein, M. G. (1989). Cryopreservable neutrophil surrogates. Stored cytoplasts from human polymorphonuclear leukocytes retain chemotactic, phagocytic, and microbicidal function. *J. Clin. Invest.* 83, 728–732. doi:10.1172/JCI113939.

Martin, C. J., Peters, K. N., and Behar, S. M. (2014). Macrophages clean up: Efferocytosis and microbial control. *Curr. Opin. Microbiol.* 17, 17–23. doi:10.1016/j.mib.2013.10.007.

Massena, S., Christoffersson, G., Hjertström, E., Zcharia, E., Vlodavsky, I., Ausmees, N., et al. (2010). A chemotactic gradient sequestered on endothelial heparan sulfate induces directional intraluminal crawling of neutrophils. *Blood* 116, 1924–31. doi:10.1182/blood-2010-01-266072.

McCord, J. M., and Fridovich, I. (1969). Superoxide Dismutase. AN ENZYMIC FUNCTION FOR ERYTHROCUPREIN (HEMOCUPREIN). *J. Biol. Chem.* 244, 6049–6055. Available at: http://www.jbc.org/content/244/22/6049.short [Accessed February 23, 2016].

McCracken, J. M., and Allen, L.-A. H. (2014). Regulation of human neutrophil apoptosis and lifespan in health and disease. *J. Cell Death* 7, 15–23. doi:10.4137/JCD.S11038.

McDougal, L. K., Steward, C. D., Killgore, G. E., Chaitram, J. M., McAllister, S. K., and Tenover, F. C. (2003). Pulsed-field gel electrophoresis typing of oxacillin-resistant Staphylococcus aureus isolates from the United States: establishing a national database. *J. Clin. Microbiol.* 41, 5113–20. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=262524&tool=pmcentrez&rendertype=abstract [Accessed November 13, 2012].

McGovern, N. N., Cowburn, A. S., Porter, L., Walmsley, S. R., Summers, C., Thompson, A. A. R., et al. (2011). Hypoxia Selectively Inhibits Respiratory Burst Activity and Killing of Staphylococcus aureus in Human Neutrophils. *J. Immunol.* 186, 453–463. doi:10.4049/jimmunol.1002213.

McLoughlin, R. M., Lee, J. C., Kasper, D. L., and Tzianabos, A. O. (2008). IFN-gamma regulated chemokine production determines the outcome of Staphylococcus aureus infection. *J. Immunol.* 181, 1323–32. Available at: http://www.jimmunol.org/content/181/2/1323.full [Accessed August 7, 2013].

Mempel, M., Voelcker, V., Köllisch, G., Plank, C., Rad, R., Gerhard, M., et al. (2003). Toll-Like Receptor Expression in Human Keratinocytes: Nuclear Factor κB Controlled Gene Activation by Staphylococcus aureus is Toll-Like Receptor 2 but Not Toll-Like Receptor 4 or Platelet Activating Factor Receptor Dependent. *J. Invest. Dermatol.* 121, 1389–1396. doi:10.1111/j.1523-1747.2003.12630.x.

Menestrina, G., Dalla Serra, M., Comai, M., Coraiola, M., Viero, G., Werner, S., et al. (2003). Ion channels and bacterial infection: the case of beta-barrel pore-forming protein toxins of Staphylococcus aureus. *FEBS Lett.* 552, 54–60. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12972152 [Accessed May 30, 2017].

Mesri, M., Plescia, J., and Altieri, D. C. (1998). Dual regulation of ligand binding by CD11b I domain. *J. Biol. Chem.* 273, 744–748. doi:10.1074/jbc.273.2.744.

Miller, L. G., Perdreau-Remington, F., Rieg, G., Mehdi, S., Perlroth, J., Bayer, A. S., et al. (2005). Necrotizing fasciitis caused by community-associated methicillin-resistant Staphylococcus aureus in Los Angeles. *N. Engl. J. Med.* 352, 1445–53. doi:10.1056/NEJMoa042683.

Miller, M., Cook, H. A., Furuya, E. Y., Bhat, M., Lee, M. H., Vavagiakis, P., et al. (2009). Staphylococcus aureus in the community: Colonization versus infection. *PLoS One* 4. doi:10.1371/journal.pone.0006708.

Mitchell, G. B., Albright, B. N., and Caswell, J. L. (2003). Effect of interleukin-8 and granulocyte colony-stimulating factor on priming and activation of bovine neutrophils. *Infect. Immun.* 71, 1643–1649. doi:10.1128/IAI.71.4.1643-1649.2003.

Mócsai, A., Abram, C. L., Jakus, Z., Hu, Y., Lanier, L. L., and Lowell, C. A. (2006). Integrin signaling in neutrophils and macrophages uses adaptors containing immunoreceptor tyrosine-based activation motifs. *Nat. Immunol.* 7, 1326–1333. doi:10.1038/ni1407.

Mócsai, A., Zhang, H., Jakus, Z., Kitaura, J., Kawakami, T., and Lowell, C. A. (2003). G-protein-coupled receptor signaling in Syk-deficient neutrophils and mast cells. *Blood* 101, 4155–4163. doi:10.1182/blood-2002-07-2346.

Mölkänen, T., Tyynelä, J., Helin, J., Kalkkinen, N., and Kuusela, P. (2002). Enhanced activation of bound plasminogen on Staphylococcus aureus by staphylokinase. *FEBS Lett.* 517, 72–78. doi:10.1016/S0014-5793(02)02580-2.

Montgomery, C. P., Boyle-Vavra, S., Adem, P. V., Lee, J. C., Husain, A. N., Clasen, J., et al. (2008). Comparison of Virulence in Community-Associated Methicillin-Resistant Staphylococcus aureus Pulsotypes USA300 and USA400 in a Rat Model of Pneumonia. *J. Infect. Dis.* 198, 561–570. doi:10.1086/590157.

Montgomery, C. P., Daniels, M., Zhao, F., Alegre, M. L., Chong, A. S., and Daum, R. S. (2014). Protective immunity against recurrent Staphylococcus aureus skin infection requires antibody and interleukin-17A. *Infect. Immun.* 82, 2125–2134. doi:10.1128/IAI.01491-14.

Moore, K. L., Patel, K. D., Bruehl, R. E., Fugang, L., Johnson, D. A., Lichenstein, H. S., et al. (1995). P-selectin glycoprotein ligand-1 mediates rolling of human neutrophils on P-selectin. *J. Cell Biol.* 128, 661–671. doi:10.1083/jcb.128.4.661.

Morris, J. C. (1966). The Acid Ionization Constant of HOCl from 5 to 35°. *J. Phys. Chem.* 70, 3798–3805. doi:10.1021/j100884a007.

Müller, P., Müller-Anstett, M., Wagener, J., Gao, Q., Kaesler, S., Schaller, M., et al. (2010). The Staphylococcus aureus lipoprotein SitC colocalizes with Toll-like receptor 2 (TLR2) in murine keratinocytes and elicits intracellular TLR2 accumulation. *Infect. Immun.* 78, 4243–50. doi:10.1128/IAI.00538-10.

Murphy, A. G., O'Keeffe, K. M., Lalor, S. J., Maher, B. M., Mills, K. H. G., and McLoughlin, R. M. (2014). Staphylococcus aureus infection of mice expands a population of memory γδ T cells that are protective against subsequent infection. *J. Immunol.* 192, 3697–708. doi:10.4049/jimmunol.1303420.

Murray, E. J., Crowley, R. C., Truman, A., Clarke, S. R., Cottam, J. A., Jadhav, G. P., et al. (2014). Targeting Staphylococcus aureus quorum sensing with nonpeptidic small molecule inhibitors. *J. Med. Chem.* 57, 2813–2819. doi:10.1021/jm500215s.

Nauseef, W. M. (2007). How human neutrophils kill and degrade microbes: an integrated view. *Immunol. Rev.* 219, 88–102. doi:10.1111/j.1600-065X.2007.00550.x.

Ng, H. P., Zhou, Y., Song, K., Hodges, C. A., Drumm, M. L., and Wang, G. (2014). Neutrophil-Mediated Phagocytic Host Defense Defect in Myeloid Cftr-Inactivated Mice. *PLoS One* 9, e106813. doi:10.1371/journal.pone.0106813.

Nguyen, G. T., Green, E. R., and Mecsas, J. (2017). Neutrophils to the ROScue: Mechanisms of NADPH Oxidase Activation and Bacterial Resistance. *Front. Cell. Infect. Microbiol.* 7. doi:10.3389/fcimb.2017.00373.

Nimmo, G. R. (2012). USA300 abroad: Global spread of a virulent strain of community-associated methicillin-resistant Staphylococcus aureus. *Clin. Microbiol. Infect.* 18, 725–734. doi:10.1111/j.1469-0691.2012.03822.x.

Njoroge, J., and Sperandio, V. (2009). Jamming bacterial communication: New approaches for the treatment of infectious diseases. *EMBO Mol. Med.* 1, 201–210. doi:10.1002/emmm.200900032.

Nordenfelt, P., and Tapper, H. (2011). Phagosome dynamics during phagocytosis by neutrophils. *J. Leukoc. Biol.* 90, 271–84. doi:10.1189/jlb.0810457.

Nourse, C., Starr, M., and Munckhof, W. (2007). Community-acquired methicillin-resistant Staphylococcus aureus causes severe disseminated infection and deep venous thrombosis in children: Literature review and recommendations for management. *J. Paediatr. Child Health* 43, 656–661. doi:10.1111/j.1440-1754.2007.01153.x.

Novick, R. P. (2003). Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol. Microbiol.* 48, 1429–49. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12791129 [Accessed June 16, 2013].

Nunes, P., Demaurex, N., and Dinauer, M. C. (2013). Regulation of the NADPH oxidase and associated ion fluxes during phagocytosis. *Traffic* 14, 1118–31. doi:10.1111/tra.12115.

Nygaard, T. K., Pallister, K. B., DuMont, A. L., DeWald, M., Watkins, R. L., Pallister, E. Q., et al. (2012). Alpha-toxin induces programmed cell death of human T cells, B cells, and monocytes during USA300 infection. *PLoS One* 7, e36532. doi:10.1371/journal.pone.0036532.

Nygaard, T. K., Pallister, K. B., Ruzevich, P., Griffith, S., Vuong, C., and Voyich, J. M. (2010a). SaeR binds a consensus sequence within virulence gene promoters to advance USA300 pathogenesis. *J. Infect. Dis.* 201, 241–54. doi:10.1086/649570.

Nygaard, T. K., Pallister, K. B., Ruzevich, P., Griffith, S., Vuong, C., and Voyich, J. M. (2010b). SaeR binds a consensus sequence within virulence gene promoters to advance USA300 pathogenesis. *J Infect Dis* 201, 241–254. doi:10.1086/649570.

Nygaard, T. K., Pallister, K. B., Zurek, O. W., and Voyich, J. M. (2013). The impact of α-toxin on host cell plasma membrane permeability and cytokine expression during human blood infection by CA-MRSA USA300. *J. Leukoc. Biol.* 94, 971–9. doi:10.1189/jlb.0213080.

Ogston, A. (1881). Report upon micro-organisms in surgical diseases. *Br. Med. J.* 1, 369–375. doi:10.1136/bmj.1.1054.369.

Ogston, A. (1882a). Micrococcus Poisoning. *J. Anat. Physiol.* 17, 24–58. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17231450.

Ogston, A. (1882b). Micrococcus Poisoning. *J. Anat. Physiol.* 17, 24–58. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1310127/pdf/janatphys00147-0031.pdf.

Ohno, Y., and Gallin, J. I. (1985). Diffusion of extracellular hydrogen peroxide into intracellular compartments of human neutrophils. Studies utilizing the inactivation of myeloperoxidase by hydrogen peroxide and azide. *J. Biol. Chem.* 260, 8438–46.

Oku, Y., Kurokawa, K., Ichihashi, N., and Sekimizu, K. (2004). Characterization of the Staphylococcus aureus mprF gene, involved in lysinylation of phosphatidylglycerol. *Microbiology* 150, 45–51. doi:10.1099/mic.0.26706-0.

Olaru, F., and Jensen, L. E. (2010). Staphylococcus aureus stimulates neutrophil targeting chemokine expression in keratinocytes through an autocrine IL-1alpha signaling loop. *J. Invest. Dermatol.* 130, 1866–76. doi:10.1038/jid.2010.37.

Olson, M. E., Nygaard, T. K., Ackermann, L., Watkins, R. L., Zurek, O. W., Pallister, K. B., et al. (2013). Staphylococcus aureus nuclease is a SaeR/S-dependent virulence factor. *Infect. Immun.* doi:10.1128/IAI.01242-12.

Painter, K. L., Krishna, A., Wigneshweraraj, S., and Edwards, A. M. (2014). What role does the quorum-sensing accessory gene regulator system play during Staphylococcus aureus bacteremia? *Trends Microbiol.* 22, 676–685. doi:10.1016/j.tim.2014.09.002.

Palazzolo-Ballance, a. M., Reniere, M. L., Braughton, K. R., Sturdevant, D. E., Otto, M., Kreiswirth, B. N., et al. (2007). Neutrophil Microbicides Induce a Pathogen Survival Response in Community-Associated Methicillin-Resistant Staphylococcus aureus. *J. Immunol.* 180, 500–509. doi:10.4049/jimmunol.180.1.500.

Pang, Y. Y., Schwartz, J., Bloomberg, S., Boyd, J. M., Horswill, A. R., and Nauseef, W. M. (2014). Methionine sulfoxide reductases protect against oxidative stress in staphylococcus aureus encountering exogenous oxidants and human neutrophils. *J. Innate Immun.* 6, 353–364. doi:10.1159/000355915.

Papayannopoulos, V., Metzler, K. D., Hakkim, A., and Zychlinsky, A. (2010). Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. *J. Cell Biol.* 191, 677–691. doi:10.1083/jcb.201006052.

Park, J., Jagasia, R., Kaufmann, G. F., Mathison, J. C., Ruiz, D. I., Moss, J. A., et al. (2007). Infection Control by Antibody Disruption of Bacterial Quorum Sensing Signaling. *Chem. Biol.* 14, 1119–1127. doi:10.1016/j.chembiol.2007.08.013.

Parker, D., and Prince, A. (2012). Immunopathogenesis of Staphylococcus aureus pulmonary infection. *Semin. Immunopathol.* 34, 281–297. doi:10.1007/s00281-011-0291-7.

Parry, M. A. A., Zhang, X. C., and Bode, W. (2000). Molecular mechanisms of plasminogen activation: Bacterial cofactors provide clues. *Trends Biochem. Sci.* 25, 53–59. doi:10.1016/S0968-0004(99)01521-2.

Patel, D., Wines, B. D., Langley, R. J., and Fraser, J. D. (2010). Specificity of staphylococcal superantigen-like protein 10 toward the human IgG1 Fc domain. *J. Immunol.* 184, 6283–6292. doi:10.4049/jimmunol.0903311.

Pauli, N. T., Kim, H. K., Falugi, F., Huang, M., Dulac, J., Henry Dunand, C., et al. (2014). Staphylococcus aureus infection induces protein A-mediated immune evasion in humans. *J. Exp. Med.* 211, 2331–9. doi:10.1084/jem.20141404.

Pelz, A., Wieland, K. P., Putzbach, K., Hentschel, P., Albert, K., and Götz, F. (2005). Structure and biosynthesis of staphyloxanthin from Staphylococcus aureus. *J. Biol. Chem.* 280, 32493–32498. doi:10.1074/jbc.M505070200.

Perron, M. D., Chowdhury, S., Aubry, I., Purisima, E., Tremblay, M. L., and Saragovi, H. U. (2014). Allosteric Noncompetitive Small Molecule Selective Inhibitors of CD45 Tyrosine Phosphatase Suppress T-Cell Receptor Signals and Inflammation In Vivo. *Mol. Pharmacol.* 85, 553–563. doi:10.1124/mol.113.089847.

Peschel, A., Jack, R. W., Otto, M., Collins, L. V, Staubitz, P., Nicholson, G., et al. (2001). Staphylococcus aureus resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with l-lysine. *J. Exp. Med.* 193, 1067–1076. doi:10.1084/jem.193.9.1067.

Peschel, A., Otto, M., Jack, R. W., Kalbacher, H., Jung, G., and Götz, F. (1999). Inactivation of the dlt operon in Staphylococcus aureus confers sensitivity to defensins, protegrins, and other antimicrobial peptides. *J. Biol. Chem.* 274, 8405–10. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10085071.

Pilsczek, F. H., Salina, D., Poon, K. K., Fahey, C., Yipp, B. G., Sibley, C. D., et al. (2010). A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to Staphylococcus aureus. *J Immunol* 185, 7413–7425. doi:10.4049/jimmunol.1000675.

Pincus, S. H., Boxer, L. A., and Stossel, T. P. (1976). Chronic neutropenia in childhood. Analysis of 16 cases and a review of the literature. *Am. J. Med.* 61, 849–61. Available at: http://www.ncbi.nlm.nih.gov/pubmed/795298 [Accessed May 30, 2017].

Pittini, Á., Casaravilla, C., Allen, J. E., and Díaz, Á. (2016). Pharmacological inhibition of PI3K class III enhances the production of pro- and anti-inflammatory cytokines in dendritic cells stimulated by TLR agonists. *Int. Immunopharmacol.* 36, 213–217. doi:10.1016/j.intimp.2016.04.028.

Podolnikova, N. P., Podolnikov, A. V., Haas, T. A., Lishko, V. K., and Ugarova, T. P. (2015). Ligand recognition specificity of leukocyte integrin ??m??2 (Mac-1, CD11b/CD18) and its functional consequences. *Biochemistry* 54, 1408–1420. doi:10.1021/bi5013782.

Poovelikunnel, T., Gethin, G., and Humphreys, H. (2015). Mupirocin resistance: Clinical implications and potential alternatives for the eradication of mrsa. *J. Antimicrob. Chemother.* 70, 2681–2692. doi:10.1093/jac/dkv169.

Postma, B., Poppelier, M. J., van Galen, J. C., Prossnitz, E. R., van Strijp, J. A. G., de Haas, C. J. C., et al. (2004). Chemotaxis Inhibitory Protein of Staphylococcus aureus Binds Specifically to the C5a and Formylated Peptide Receptor. *J. Immunol.* 172, 6994–7001. doi:10.4049/jimmunol.172.11.6994.

Prat, C., Bestebroer, J., de Haas, C. J. C., van Strijp, J. A. G., and van Kessel, K. P. M. (2006). A New Staphylococcal Anti-Inflammatory Protein That Antagonizes the Formyl Peptide Receptor-Like 1. *J. Immunol.* 177, 8017–8026. doi:10.4049/jimmunol.177.11.8017.

Prat, C., Haas, P.-J., Bestebroer, J., de Haas, C. J. C., van Strijp, J. a G., and van Kessel, K. P. M. (2009). A homolog of formyl peptide receptor-like 1 (FPRL1) inhibitor from Staphylococcus aureus (FPRL1 inhibitory protein) that inhibits FPRL1 and FPR. *J. Immunol.* 183, 6569–6578. doi:10.4049/jimmunol.0801523.

Raghuwanshi, S. K., Su, Y., Singh, V., Haynes, K., Richmond, A., and Richardson, R. M. (2012). The Chemokine Receptors CXCR1 and CXCR2 Couple to Distinct G Protein-Coupled Receptor Kinases To Mediate and Regulate Leukocyte Functions. *J. Immunol.* 189, 2824–2832. doi:10.4049/jimmunol.1201114.

Rainard, P., Riollet, C., Poutrel, B., and Paape, M. J. (2000). Phagocytosis and killing of Staphylococcus aureus by bovine neutrophils after priming by tumor necrosis factor-alpha and the des-arginine derivative of C5a. *Am. J. Vet. Res.* 61, 951–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10951989 [Accessed March 27, 2017].

Rajagopalan, L., and Rajarathnam, K. (2004). Ligand selectivity and affinity of chemokine receptor CXCR1: Role of N-terminal domain. *J. Biol. Chem.* 279, 30000–30008. doi:10.1074/jbc.M313883200.

Reilly, M. P., Sinha, U., André, P., Taylor, S. M., Pak, Y., DeGuzman, F. R., et al. (2011). PRT-060318, a novel Syk inhibitor, prevents heparin-induced thrombocytopenia and thrombosis in a transgenic mouse model. *Blood* 117, 2241–2246. doi:10.1182/blood-2010-03-274969.

Reizner, W., Hunter, J. G., O'Malley, N. T., Southgate, R. D., Schwarz, E. M., and Kates, S. L. (2014). A systematic review of animal models for Staphylococcus aureus osteomyelitis. *Eur. Cell. Mater.* 27, 196–212. doi:10.1016/j.biotechadv.2011.08.021.Secreted.

Reyes-Robles, T., Alonzo, F., Kozhaya, L., Lacy, D. B., Unutmaz, D., and Torres, V. J. (2013). Staphylococcus aureus Leukotoxin ED targets the chemokine receptors CXCR1 and CXCR2 to Kill leukocytes and promote infection. *Cell Host Microbe* 14, 453–459. doi:10.1016/j.chom.2013.09.005.

Reyes-Robles, T., and Torres, V. J. (2016). Staphylococcus aureus Pore-Forming Toxins. *Curr. Top. Microbiol. Immunol.* 6, 23–27. doi:10.1007/82_2016_16.

Ricklin, D., Tzekou, A., Garcia, B. L., Hammel, M., McWhorter, W. J., Sfyroera, G., et al. (2009). A molecular insight into complement evasion by the staphylococcal complement inhibitor protein family. *J. Immunol.* 183, 2565–2574. doi:10.4049/jimmunol.0901443.

Rigby, K. M., and DeLeo, F. R. (2012). Neutrophils in innate host defense against Staphylococcus aureus infections. *Semin. Immunopathol.* 34, 237–59. doi:10.1007/s00281-011-0295-3.

Ritzman, A. M., Hughes-Hanks, J. M., Blaho, V. A., Wax, L. E., Mitchell, W. J., and Brown, C. R. (2010). The chemokine receptor CXCR2 ligand KC (CXCL1) mediates neutrophil recruitment and is critical for development of experimental lyme arthritis and carditis. *Infect. Immun.* 78, 4593–4600. doi:10.1128/IAI.00798-10.

Rogasch, K., Rühmling, V., Pané-Farré, J., Höper, D., Weinberg, C., Fuchs, S., et al. (2006a). Influence of the two-component system SaeRS on global gene expression in two different Staphylococcus aureus strains. *J. Bacteriol.* 188, 7742–58. doi:10.1128/JB.00555-06.

Rogasch, K., Rühmling, V., Pané-Farré, J., Höper, D., Weinberg, C., Fuchs, S., et al. (2006b). Influence of the two-component system SaeRS on global gene expression in two different Staphylococcus aureus strains. *J. Bacteriol.* 188, 7742–58. doi:10.1128/JB.00555-06.

Rooijakkers, S. H. M., Ruyken, M., Roos, A., Daha, M. R., Presanis, J. S., Sim, R. B., et al. (2005a). Immune evasion by a staphylococcal complement inhibitor that acts on C3 convertases. *Nat. Immunol.* 6, 920–7. doi:10.1038/ni1235.

Rooijakkers, S. H. M., Ruyken, M., van Roon, J., van Kessel, K. P. M., van Strijp, J. A. G., and van Wamel, W. J. B. (2006). Early expression of SCIN and CHIPS drives instant immune evasion by Staphylococcus aureus. *Cell. Microbiol.* 8, 1282–93. doi:10.1111/j.1462-5822.2006.00709.x.

Rooijakkers, S. H. M., van Wamel, W. J. B., Ruyken, M., van Kessel, K. P. M., and van Strijp, J. A. G. (2005b). Anti-opsonic properties of staphylokinase. *Microbes Infect.* 7, 476–84. doi:10.1016/j.micinf.2004.12.014.

Roos, D., and de Boer, M. (2014). Molecular diagnosis of chronic granulomatous disease. *Clin. Exp. Immunol.* 175, 139–49. doi:10.1111/cei.12202.

Rubel, C., Fernandez, G. C., Dran, G., Bompadre, M. B., Isturiz, M. A., and Palermo, M. S. (2001). Fibrinogen Promotes Neutrophil Activation and Delays Apoptosis. *J. Immunol.* 166, 2002–2010. doi:10.4049/jimmunol.166.3.2002.

Rutherford, S. T., and Bassler, B. L. (2012). Bacterial quorum sensing: its role in virulence and possibilities for its control. *Cold Spring Harb. Perspect. Med.* 2, 1–26. doi:10.1101/cshperspect.a012427.

Sabroe, I., Prince, L. R., Jones, E. C., Horsburgh, M. J., Foster, S. J., Vogel, S. N., et al. (2003). Selective roles for Toll-like receptor (TLR)2 and TLR4 in the regulation of neutrophil activation and life span. *J. Immunol.* 170, 5268–5275. doi:10.4049/jimmunol.170.10.5268.

Sarma, J. V., and Ward, P. A. (2012). New developments in C5a receptor signaling. *Cell Heal. Cytoskelet.* 4, 73–82. doi:10.2147/CHC.S27233.New.

Sasaki, T., Kano, R., Sato, H., Nakamura, Y., Watanabe, S., and Hasegawa, A. (2003). Effects of staphylococci on cytokine production from human keratinocytes. *Br. J. Dermatol.* 148, 46–50. doi:10.1046/j.1365-2133.2003.05017.x.

Schmitz, F. J., Veldkamp, K. E., Van Kessel, K. P., Verhoef, J., and Van Strijp, J. a (1997). Delta-toxin from Staphylococcus aureus as a costimulator of human neutrophil oxidative burst. *J. Infect. Dis.* 176, 1531–1537. doi:10.1086/514152.

Schöneich, C. (2005). Methionine oxidation by reactive oxygen species: reaction mechanisms and relevance to Alzheimer's disease. *Biochim. Biophys. Acta* 1703, 111–9. doi:10.1016/j.bbapap.2004.09.009.

Segal, A. W. (2005). How neutrophils kill microbes. *Annu. Rev. Immunol.* 23, 197–223. doi:10.1146/annurev.immunol.23.021704.115653.

Segal, A. W. (2008). The function of the NADPH oxidase of phagocytes and its relationship to other NOXs in plants, invertebrates, and mammals. *Int. J. Biochem. Cell Biol.* 40, 604–618. doi:10.1016/j.biocel.2007.10.003.

Seilie, E. S., and Bubeck Wardenburg, J. (2017). Staphylococcus aureus pore-forming toxins: The interface of pathogen and host complexity. *Semin. Cell Dev. Biol.* doi:10.1016/j.semcdb.2017.04.003.

Serruto, D., Rappuoli, R., Scarselli, M., Gros, P., and van Strijp, J. A. G. (2010). Molecular mechanisms of complement evasion: learning from staphylococci and meningococci. *Nat. Rev. Microbiol.* 8, 393–9. doi:10.1038/nrmicro2366.

Sieprawska-Lupa, M., Mydel, P., Krawczyk, K., Wójcik, K., Puklo, M., Lupa, B., et al. (2004). Degradation of Human Antimicrobial Peptide LL-37 by Staphylococcus aureus -Derived Proteinases. *Antimicrob. Agents Chemother.* 48, 4673–4679. doi:10.1128/AAC.48.12.4673.

Singer, M., and Sansonetti, P. J. (2004). IL-8 Is a Key Chemokine Regulating Neutrophil Recruitment in a New Mouse Model of Shigella-Induced Colitis. *J. Immunol.* 173, 4197 LP-4206.

Singh, V. K. (2003). Multiple methionine sulfoxide reductase genes in Staphylococcus aureus: expression of activity and roles in tolerance of oxidative stress. *Microbiology* 149, 2739–2747. doi:10.1099/mic.0.26442-0.

Singh, V. K., Vaish, M., Johansson, T. R., Baum, K. R., Ring, R. P., Singh, S., et al. (2015). Significance of four methionine sulfoxide reductases in Staphylococcus aureus. *PLoS One* 10, e0117594. doi:10.1371/journal.pone.0117594.

Sjödahl, J. (1977). Structural studies on the four repetitive Fc-binding regions in protein A from Staphylococcus aureus. *Eur. J. Biochem.* 78, 471–90. doi:10.1111/j.1432-1033.1977.tb11760.x.

Skjeflo, E. W., Christiansen, D., Espevik, T., Nielsen, E. W., and Mollnes, T. E. (2014). Combined inhibition of complement and {CD14} efficiently attenuated the inflammatory response induced by Staphylococcus aureus in a human whole blood model. *J. Immunol. {(Baltimore,} Md. 1950)* 192, 2857–2864. doi:10.4049/jimmunol.1300755.

Smith, E. J., Visai, L., Kerrigan, S. W., Speziale, P., and Foster, T. J. (2011). The Sbi protein is a multifunctional immune evasion factor of Staphylococcus aureus. *Infect. Immun.* 79, 3801–3809. doi:10.1128/IAI.05075-11.

Sollid, J. U. E., Furberg, A. S., Hanssen, A. M., and Johannessen, M. (2014). Staphylococcus aureus: Determinants of human carriage. *Infect. Genet. Evol.* 21, 531–541. doi:10.1016/j.meegid.2013.03.020.

Spaan, A. N., Henry, T., Van Rooijen, W. J. M., Perret, M., Badiou, C., Aerts, P. C., et al. (2013). The staphylococcal toxin panton-valentine leukocidin targets human C5a receptors. *Cell Host Microbe* 13, 584–594. doi:10.1016/j.chom.2013.04.006.

Spaan, A. N., Schiepers, A., de Haas, C. J. C., van Hooijdonk, D. D. J. J., Badiou, C., Contamin, H., et al. (2015). Differential Interaction of the Staphylococcal Toxins Panton-Valentine Leukocidin and γ-Hemolysin CB with Human C5a Receptors. *J. Immunol.* 195, 1034–1043. doi:10.4049/jimmunol.1500604.

Spaan, A. N., Vrieling, M., Wallet, P., Badiou, C., Reyes-Robles, T., Ohneck, E. A., et al. (2014). The staphylococcal toxins γ-haemolysin AB and CB differentially target phagocytes by employing specific chemokine receptors. *Nat. Commun.* 5. doi:10.1038/ncomms6438.

Springer, T. A., and Dustin, M. L. (2012). Integrin inside-out signaling and the immunological synapse. *Curr. Opin. Cell Biol.* 24, 107–115. doi:10.1016/j.ceb.2011.10.004.

Stadtmann, A., Germena, G., Block, H., Boras, M., Rossaint, J., Sundd, P., et al. (2013). The PSGL-1-L-selectin signaling complex regulates neutrophil adhesion under flow. *J. Exp. Med.* 210, 2171–80. doi:10.1084/jem.20130664.

Stapels, D. A., Ramyar, K. X., Bischoff, M., von Kockritz-Blickwede, M., Milder, F. J., Ruyken, M., et al. (2014). Staphylococcus aureus secretes a unique class of neutrophil serine protease inhibitors. *Proc Natl Acad Sci U S A* 111, 13187–13192. doi:10.1073/pnas.1407616111.

Stemerding, A. M., Köhl, J., Pandey, M. K., Kuipers, A., Leusen, J. H., Boross, P., et al. (2013). Staphylococcus aureus formyl peptide receptor-like 1 inhibitor (FLIPr) and its homologue FLIPr-like are potent FcγR antagonists that inhibit IgG-mediated effector functions. *J. Immunol.* 191, 353–62. doi:10.4049/jimmunol.1203243.

Stoll, H., Dengjel, J., Nerz, C., and Götz, F. (2005). Staphylococcus aureus deficient in lipidation of prelipoproteins is attenuated in growth and immune activation. *Infect. Immun.* 73, 2411–2423. doi:10.1128/IAI.73.4.2411-2423.2005.

Su, S. B., Gao, J., Gong, W., Dunlop, N. M., Murphy, P. M., Oppenheim, J. J., et al. (1999a). T21/DP107, A synthetic leucine zipper-like domain of the HIV-1 envelope gp41, attracts and activates human phagocytes by using G-protein-coupled formyl peptide receptors. *J Immunol* 162, 5924–5930. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10229829.

Su, S. B., Gong, W., Gao, J. L., Shen, W., Murphy, P. M., Oppenheim, J. J., et al. (1999b). A seven-transmembrane, G protein-coupled receptor, FPRL1, mediates the chemotactic activity of serum amyloid A for human phagocytic cells. *J. Exp. Med.* 189, 395–402. doi:10.1084/jem.189.2.395.

Suh, B. C., and Hille, B. (2005). Regulation of ion channels by phosphatidylinositol 4,5-bisphosphate. *Curr. Opin. Neurobiol.* 15, 370–378. doi:10.1016/j.conb.2005.05.005.

Suh, C.-I., Stull, N. D., Li, X. J., Tian, W., Price, M. O., Grinstein, S., et al. (2006). The phosphoinositide-binding protein p40phox activates the NADPH oxidase during FcgammaIIA receptor-induced phagocytosis. *J. Exp. Med.* 203, 1915–25. doi:10.1084/jem.20052085.

Sully, E. K., Malachowa, N., Elmore, B. O., Alexander, S. M., Femling, J. K., Gray, B. M., et al. (2014). Selective Chemical Inhibition of agr Quorum Sensing in Staphylococcus aureus Promotes Host Defense with Minimal Impact on Resistance. *PLoS Pathog.* 10. doi:10.1371/journal.ppat.1004174.

Sun, F., Li, C., Jeong, D., Sohn, C., He, C., and Bae, T. (2010). In the Staphylococcus aureus two-component system sae, the response regulator SaeR binds to a direct repeat sequence and DNA binding requires phosphorylation by the sensor kinase SaeS. *J. Bacteriol.* 192, 2111–27. doi:10.1128/JB.01524-09.

Swain, S. D., Rohn, T. T., and Quinn, M. T. (2002). Neutrophil priming in host defense: role of oxidants as priming agents. *Antioxid. Redox Signal.* 4, 69–83. doi:10.1089/152308602753625870.

Sward, E. W., Fones, E. M., Spaan, R. R., Pallister, K. B., Haller, B. L., Guerra, F. E., et al. (2018). Staphylococcus aureus SaeR/S-Regulated Factors Decrease Monocyte-Derived Tumor Necrosis Factor-α to Reduce Neutrophil Bactericidal Activity. *J. Infect. Dis.* 217, 943–952. doi:10.1093/infdis/jix652.

Syed Mortadza, S. A., Wang, L., Li, D., and Jiang, L.-H. (2015). TRPM2 Channel-Mediated ROS-Sensitive Ca2+ Signaling Mechanisms in Immune Cells. *Front. Immunol.* 6, 1–7. doi:10.3389/fimmu.2015.00407.

Takeuchi, O., Hoshino, K., and Akira, S. (2000). Cutting Edge: TLR2-Deficient and MyD88-Deficient Mice Are Highly Susceptible to Staphylococcus aureus Infection. *J. Immunol.* 165, 5392–5396. doi:10.4049/jimmunol.165.10.5392.

Takeuchi, O., Kawai, T., Mühlradt, P. F., Morr, M., Radolf, J. D., Zychlinsky, A., et al. (2001). Discrimination of bacterial lipoproteins by Toll-like receptor 6. *Int. Immunol.* 13, 933–40. doi:10.1093/intimm/13.7.933.

Takeuchi, O., Sato, S., Horiuchi, T., Hoshino, K., Takeda, K., Dong, Z., et al. (2002). Cutting Edge: Role of Toll-Like Receptor 1 in Mediating Immune Response to Microbial Lipoproteins. *J. Immunol.* 169, 10–14. doi:10.4049/jimmunol.169.1.10.

Tal-Gan, Y., Stacy, D. M., Foegen, M. K., Koenig, D. W., and Blackwell, H. E. (2013). Highly potent inhibitors of quorum sensing in staphylococcus aureus revealed through a systematic synthetic study of the group-III autoinducing peptide. *J. Am. Chem. Soc.* 135, 7869–7882. doi:10.1021/ja3112115.

Thakker, M., Park, J. S., Carey, V., and Lee, J. C. (1998). Staphylococcus aureus serotype 5 capsular polysaccharide is antiphagocytic and enhances bacterial virulence in a murine bacteremia model. *Infect. Immun.* 66, 5183–5189.

Thammavongsa, V., Missiakas, D. M., and Schneewind, O. (2013). Staphylococcus aureus degrades neutrophil extracellular traps to promote immune cell death. *Science* 342, 863–6. doi:10.1126/science.1242255.

Tong, S. Y. C., Davis, J. S., Eichenberger, E., Holland, T. L., and Fowler, V. G. (2015). Staphylococcus aureus infections: Epidemiology, pathophysiology, clinical manifestations, and management. *Clin. Microbiol. Rev.* 28, 603–661. doi:10.1128/CMR.00134-14.

Torre, A., Bacconi, M., Sammicheli, C., Galletti, B., Laera, D., Fontana, M. R., et al. (2015). Four-component Staphylococcus aureus vaccine 4C-staph enhances fc?? receptor expression in neutrophils and monocytes and mitigates S. aureus infection in neutropenic mice. *Infect. Immun.* 83, 3157–3163. doi:10.1128/IAI.00258-15.

Tromp, A. T., Van Gent, M., Abrial, P., Martin, A., Jansen, J. P., De Haas, C. J. C., et al. (2018). Human CD45 is an f-component-specific receptor for the staphylococcal toxin Panton-Valentine leukocidin. *Nat. Microbiol.* 3, 708–717. doi:10.1038/s41564-018-0159-x.

Tseng, C. W., Biancotti, J. C., Berg, B. L., Gate, D., Kolar, S. L., Müller, S., et al. (2015). Increased Susceptibility of Humanized NSG Mice to Panton-Valentine Leukocidin and Staphylococcus aureus Skin Infection. *PLoS Pathog.* 11, 1–17. doi:10.1371/journal.ppat.1005292.

Ueda, T., Rieu, P., Brayer, J., and Arnaout, M. A. (1994). Identification of the complement iC3b binding site in the beta 2 integrin CR3 (CD11b/CD18). *Proc. Natl. Acad. Sci. U. S. A.* 91, 10680–4. doi:10.1073/pnas.91.22.10680.

Upadhyay, A., Burman, J. D., Clark, E. A., Leung, E., Isenman, D. E., Van Den Elsen, J. M. H., et al. (2008). Structure-function analysis of the C3 binding region of Staphylococcus aureus immune subversion protein Sbi. *J. Biol. Chem.* 283, 22113–22120. doi:10.1074/jbc.M802636200.

Valderas, M. W., and Hart, M. E. (2001). Identification and Characterization of a Second Superoxide Dismutase Gene (sodM) from *Staphylococcus aureus*. *J. Bacteriol.* 183, 3399–3407. doi:10.1128/JB.183.11.3399.

Valentine, F. C. ., and Hall-Smith, S. . (1952). Superficial staphylococcal infection. *Lancet (London, England)* 2, 351–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14955999.

van Kesse, K. P. M., Bestebroer, J., and van Strijp, J. A. G. (2014). Neutrophil-mediated phagocytosis of Staphylococcus aureus. *Front. Immunol.* 5, 1–12. doi:10.3389/fimmu.2014.00467.

Vandenesch, F., Lina, G., and Henry, T. (2012). Staphylococcus aureus Hemolysins, bi-component Leukocidins, and Cytolytic Peptides: A Redundant Arsenal of Membrane-Damaging Virulence Factors? *Front. Cell. Infect. Microbiol.* 2, 12. doi:10.3389/fcimb.2012.00012.

Veldkamp, K. E., Van Kessel, K. P. M., Verhoef, J., and Van Strijp, J. A. G. (1997). Staphylococcal culture supernates stimulate human phagocytes. *Inflammation* 21, 541–551. doi:10.1023/A:1027315814817.

Ventola, C. L. (2015). The antibiotic resistance crisis: part 1: causes and threats. *P T A peer-reviewed J. Formul. Manag.* 40, 277–83.

von Eiff, C., Becker, K., Machka, K., Stammer, H., and Peters, G. (2001). Nasal carriage as a source of Staphylococcus aureus bacteremia. Study Group. *N. Engl. J. Med.* 344, 11–6. doi:10.1056/NEJM200101043440102.

Voyich, J. M., Braughton, K. R., Sturdevant, D. E., Whitney, A. R., Saïd-Salim, B., Porcella, S. F., et al. (2005). Insights into mechanisms used by Staphylococcus aureus to avoid destruction by human neutrophils. *J. Immunol.* 175, 3907–19. Available at: http://www.jimmunol.org/content/175/6/3907.full [Accessed January 22, 2014].

Voyich, J. M., Otto, M., Mathema, B., Braughton, K. R., Whitney, A. R., Welty, D., et al. (2006a). Is Panton-Valentine leukocidin the major virulence determinant in community-associated methicillin-resistant Staphylococcus aureus disease? *J. Infect. Dis.* 194, 1761–70. doi:10.1086/509506.

Voyich, J. M., Otto, M., Mathema, B., Braughton, K. R., Whitney, A. R., Welty, D., et al. (2006b). Is Panton-Valentine leukocidin the major virulence determinant in community-associated methicillin-resistant Staphylococcus aureus disease? *J Infect Dis* 194, 1761–1770. doi:10.1086/509506.

Voyich, J. M., Vuong, C., DeWald, M., Nygaard, T. K., Kocianova, S., Griffith, S., et al. (2009). The SaeR/S gene regulatory system is essential for innate immune evasion by Staphylococcus aureus. *J. Infect. Dis.* 199, 1698–706. doi:10.1086/598967.

Wang, G., Cao, L., Liu, X., Sieracki, N. A., Di, A., Wen, X., et al. (2016). Oxidant Sensing by TRPM2 Inhibits Neutrophil Migration and Mitigates Inflammation. *Dev. Cell* 38, 453–462. doi:10.1016/j.devcel.2016.07.014.

Wang, R., Braughton, K. R., Kretschmer, D., Bach, T.-H. L., Queck, S. Y., Li, M., et al. (2007). Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nat. Med.* 13, 1510–4. doi:10.1038/nm1656.

Watkins, R. L., Pallister, K. B., and Voyich, J. M. (2011). The SaeR/S gene regulatory system induces a pro-inflammatory cytokine response during Staphylococcus aureus infection. *PLoS One* 6, e19939. doi:10.1371/journal.pone.0019939.

Watkins, R. L., Zurek, O. W., Pallister, K. B., and Voyich, J. M. (2013). The SaeR/S two-component system induces interferon-gamma production in neutrophils during invasive Staphylococcus aureus infection. *Microbes Infect.* 15, 749–54. doi:10.1016/j.micinf.2013.05.004.

Wertheim, H. F., Melles, D. C., Vos, M. C., van Leeuwen, W., van Belkum, A., Verbrugh, H. A., et al. (2005). The role of nasal carriage in Staphylococcus aureus infections. *Lancet Infect. Dis.* 5, 751–762. doi:10.1016/S1473-3099(05)70295-4.

Wieland, B., Feil, C., Gloria-Maercker, E., Thumm, G., Lechner, M., Bravo -, J. M., et al. (1994). Genetic and biochemical analyses of the biosynthesis of the yellow carotenoid 4,4'-diaponeurosporene of Staphylococcus aureus. *J. Bacteriol.* 176, 7719–7726.

Wilson, G. J., Seo, K. S., Cartwright, R. A., Connelley, T., Chuang-Smith, O. N., Merriman, J. A., et al. (2011). A novel core genome-encoded superantigen contributes to lethality of community-associated MRSA necrotizing pneumonia. *PLoS Pathog.* 7, e1002271. doi:10.1371/journal.ppat.1002271.

Winterbourn, C. C., Hampton, M. B., Livesey, J. H., and Kettle, A. J. (2006). Modeling the reactions of superoxide and myeloperoxidase in the neutrophil phagosome: Implications for microbial killing. *J. Biol. Chem.* 281, 39860–39869. doi:10.1074/jbc.M605898200.

Winterbourn, C. C., and Kettle, A. J. (2013). Redox reactions and microbial killing in the neutrophil phagosome. *Antioxid. Redox Signal.* 18, 642–60. doi:10.1089/ars.2012.4827.

Wright, A. J., Higginbottom, A., Philippe, D., Upadhyay, A., Bagby, S., Read, R. C., et al. (2007). Characterisation of receptor binding by the chemotaxis inhibitory protein of Staphylococcus aureus and the effects of the host immune response. *Mol. Immunol.* 44, 2507–2517. doi:10.1016/j.molimm.2006.12.022.

Wright, H. L., Thomas, H. B., Moots, R. J., and Edwards, S. W. (2013). RNA-Seq Reveals Activation of Both Common and Cytokine-Specific Pathways following Neutrophil Priming. *PLoS One* 8. doi:10.1371/journal.pone.0058598.

Wymann, M. P., Bulgarelli-Leva, G., Zvelebil, M. J., Pirola, L., Vanhaesebroeck, B., Waterfield, M. D., et al. (1996). Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. *Mol. Cell. Biol.* 16, 1722–1733. doi:10.1128/MCB.16.4.1722.

Xu, S., Arbeit, R. D., and Lee, J. C. (1992). Phagocytic killing of encapsulated and microencapsulated Staphylococcus aureus by human polymorphonuclear leukocytes. *Infect. Immun.* 60, 1358–1362.

Yang, E. S., Tan, J., Eells, S., Rieg, G., Tagudar, G., and Miller, L. G. (2010). Body site colonization in patients with community-associated methicillin-resistant Staphylococcus aureus and other types of S. aureus skin infections. *Clin. Microbiol. Infect.* 16, 425–431. doi:10.1111/j.1469-0691.2009.02836.x.

Yang, S. J., Bayer, A. S., Mishra, N. N., Meehl, M., Ledala, N., Yeaman, M. R., et al. (2012). The Staphylococcus aureus two-component regulatory system, grars, senses and confers resistance to selected cationic antimicrobial peptides. *Infect. Immun.* 80, 74–81. doi:10.1128/IAI.05669-11.

Yang, S. J., Xiong, Y. Q., Yeaman, M. R., Bayles, K. W., Abdelhady, W., and Bayer, A. S. (2013). Role of the LytSR two-component regulatory system in adaptation to cationic antimicrobial peptides in staphylococcus aureus. *Antimicrob. Agents Chemother.* 57, 3875–3882. doi:10.1128/AAC.00412-13.

Yokoyama, R., Itoh, S., Kamoshida, G., Takii, T., Fujii, S., Tsuji, T., et al. (2012). Staphylococcal superantigen-like protein 3 binds to the toll-like receptor 2 extracellular domain and inhibits cytokine production induced by Staphylococcus aureus, cell wall component, or lipopeptides in murine macrophages. *Infect. Immun.* 80, 2816–2825. doi:10.1128/IAI.00399-12.

Yoong, P., and Torres, V. J. (2013). The effects of Staphylococcus aureus leukotoxins on the host: Cell lysis and beyond. *Curr. Opin. Microbiol.* 16, 63–69. doi:10.1016/j.mib.2013.01.012.

Yoshimura, T., Matsushima, K., Tanaka, S., Robinson, E. a, Appella, E., Oppenheim, J. J., et al. (1987). Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines. *Proc. Natl. Acad. Sci. U. S. A.* 84, 9233–9237. doi:10.1073/pnas.84.24.9233.

Zhan, Y., Virbasius, J. V., Song, X., Pomerleau, D. P., and Wayne Zhou, G. (2002). The p40phox and p47phox PX domains of NADPH oxidase target cell membranes via direct and indirect recruitment by phosphoinositides. *J. Biol. Chem.* 277, 4512–4518. doi:10.1074/jbc.M109520200.

Zhang, L., Jacobsson, K., Strom, K., Lindberg, M., and Frykberg, L. (1999). Staphylococcus aureus expresses a cell surface protein that binds both IgG and beta2-glycoprotein I. *Microbiology* 145 (Pt 1, 177–183.

Zhang, L., Jacobsson, K., Vasi, J., Lindberg, M., and Frykberg, L. (1998). A second IgG-binding protein in Staphylococcus aureus. *Microbiology* 144 (Pt 4, 985–91. doi:10.1099/00221287-144-4-985.

Zhao, T., Benard, V., Bohl, B. P., and Bokoch, G. M. (2003). The molecular basis for adhesion-mediated suppression of reactive oxygen species generation by human neutrophils. 112. doi:10.1172/JCI200319108.Introduction.

Zhou, H., Liao, J., Aloor, J., Nie, H., Wilson, B. C., Fessler, M. B., et al. (2013). CD11b/CD18 (Mac-1) is a novel surface receptor for extracellular double-stranded RNA to mediate cellular inflammatory responses. *J. Immunol.* 190, 115–25. doi:10.4049/jimmunol.1202136.

Zurek, O. W., Nygaard, T. K., Watkins, R. L., Pallister, K. B., Torres, V. J., Horswill, A. R., et al. (2014). The Role of Innate Immunity in Promoting SaeR/S-Mediated Virulence in Staphylococcus aureus. *J. Innate Immun.* 6, 21–30. doi:10.1159/000351200.

Zurek, O. W., Pallister, K. B., and Voyich, J. M. (2015). Staphylococcus aureus Inhibits Neutrophil-derived IL-8 to Promote Cell Death. *J. Infect. Dis.* 212, 1–5. doi:10.1093/infdis/jiv124.