INSIGHTS INTO THE RECIPROCAL COMMUNICATION BETWEEN NEUTROPHILS AND *STAPHYLOCOCCUS AUREUS*

by

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ABSTRACT

Staphylococcus aureus (*S. aureus*) is a highly adaptable pathogen that can cause endocarditis, skin abscesses, tissue necrosis, and sepsis. *S. aureus* success can be partially attributed to its ability to colonize and subsequently infect a wide variety of host tissues. This capacity is dependent on elaborate two-component gene-regulatory systems that control expression of virulence and immunomodulatory factors. The *S. aureus* exoprotein expression (SaeR/S) system is recognized as a major regulator of virulence that significantly contributes to the pathogen's ability to evade killing by the human neutrophil. However, it is unclear how this system becomes activated and how the SaeR/S system modulates neutrophil function. In this study, we elucidated how *S. aureus* evades neutrophil killing by studying the reciprocal communication between the host and pathogen. We demonstrated that only select SaeR/S-regulated genes (as opposed to all targets) were transcriptionally up-regulated in response to stimulation by neutrophils as well as α-defensin and show that the mouse skin environment (that lacks alpha-defensin) promoted transcription of specific *saeR/S*-targets, different from the expression profile elicited following neutrophil interaction or α-defensin. These results were unexpected and demonstrated differential activation of *saeR/S* targets was dependent on specific stimuli. Furthermore, we studied the influence of SaeR/S on neutrophil function and showed that this system promoted accelerated cell death by decreasing NF-kB activity, and in-turn IL-8 production, to promote neutrophil lysis. These findings underscored the importance of neutrophil signaling demonstrating that neutrophil-derived production of IL-8 was necessary for this cell to kill *S. aureus* effectively. It follows that treatment of human neutrophils with recombinant IL-8 significantly increased neutrophil staphylocidal activity. Finally, we propose that both timing and magnitude of inflammation in neutrophils play major roles in dictating the outcome of staphylococcal disease and that alteration in the innate ability of neutrophils to produce IL-8 may increase susceptibility to *S. aureus* infections. Taken together, the findings define novel pathogen- and host-derived factors that play pivotal roles in the course of *S. aureus* infection.

CHAPTER ONE

INTRODUCTION

Background

Staphylococcus aureus (*S. aureus*) is a Gram-positive bacterium that can cause a variety of infections ranging from endocarditis, sepsis, necrotizing fasciitis to skin and soft tissue infections. *S. aureus* is the leading cause of hospital acquired infections including both native valve and prosthetic valve infective endocarditis and is associated with higher mortality rates relative to other microbes (1-4). In the United States, *S. aureus* contributes to 12 million annual outpatient visits for skin and soft-tissue infections (5). Despite its pathogenic capabilities, approximately 30% of the population is colonized with *S. aureus* with the primary anatomical site being the anterior nares, followed by the skin (6). *S. aureus* is such a successful colonizer that it is estimated that nearly every individual is a carrier at some point during one's lifespan (7). These observations underline the importance of the host immune system in determining susceptibility to staphylococcal infections as well as the remarkable ability of *S. aureus* to tightly regulate its virulence.

S. aureus has been recognized as a human pathogen for centuries. The first reported case of *S. aureus* infection, described by Sir Alexander Ogston dates back to 1882; however, the biblical description of "incurable boils" in the sixth Egyptian plague along with Job's boils might be the first documented reference of staphylococcal disease (8,9). The advent of mass production of penicillin in 1941 marked the beginning of the

emergence of strains with resistance to antibiotics. Within one year of its introduction, *S. aureus* strains emerged resistant to penicillin and today, 90% of the strains are resistant to penicillin (10). It follows, within one year of the use of methicillin, methicillin resistant *S. aureus* (MRSA) emerged making strains resistant to the entire class of beta-lactam antibiotics. Currently, ~65% of the strains are MRSA (8). The glycopeptide antibiotic vancomycin is considered a last-line antimicrobial for the treatment of drug-resistant *S. aureus*. As expected, vancomycin resistant *S. aureus* (VRSA) strains emerged in the early 2000s (8), but the strains seem to have an unexplained fitness defect and fortunately the VRSA strains, to date, have not increased at the rate previously observed with MRSA (8,11).

Community-Associated Methicillin Resistant
Staphylococcus aureus (CA-MRSA) USA300

Besides being a paradigm for the development of drug-resistance, *S. aureus* is a major health threat because of its ability to infect immunocompromised as well as immunocompetent patients in hospital and community settings. The emergence of the community-associated (CA) strains in the 1990s redefined the epidemiology of *S. aureus* infections and demonstrated that all individuals were at risk of contracting a *S. aureus* infection (12). USA300 (LAC) is currently the predominant strain responsible for majority of skin and soft tissue infections (12,13). While the prevalence of USA300 over other CA-*S. aureus* strains has not been attributed to single virulence factors, differences in the genetic makeup and transcriptional regulation leading to the so-called hyper-virulence have been proposed to be the key contributors (14-16).

SaeR/S Two Component System

S. aureus success in hospitals and the community can be partially attributed to its ability to colonize and subsequently infect a wide variety of host tissues. This capacity is dependent on elaborate two-component (TC) gene-regulatory systems that tightly control expression of virulence and immuno-modulatory factors in response to different stimuli (1,17-20). The *S. aureus* exoprotein expression (SaeR/S) system, first identified by Dr. Rose Nagel's team in 1994 (21), is among 16 TC systems and has been identified as a major regulator of *S. aureus* virulence factors that are essential for innate immune evasion (22-36). This system directly activates the expression of genes encoding many virulence factors, including α and β-hemolysins (*hla*, *hlb*), two-component leukotoxins (*hlgBC*, *lukAB/GH*) and adhesins such as coagulase, fibronectin-binding protein A, extracellular adherence protein, and extracellular matrix–binding protein (29,37,38). Moreover, the SaeR recognition sequence has been identified within virulence gene promoters that are transcriptionally regulated by *saeR/S* (29,39-42).

The system is under the regulation of two promoters (P1 and P3) and consists of four open reading frames (ORFs): *saeP, saeQ, saeR, saeS*. P3 is constitutively expressed and transcribes *saeS* and *saeR* while P1 is an inducible promoter with two SaeR binding regions that can transcribe the entire *sae* operon (43,44). The genes *saeR* and *saeS* code for a response regulator and a histidine kinase, respectively, while *saeP* is predicted to be a lipoprotein and *saeQ* is a putative membrane protein (45,46). It is hypothesized that upon *S. aureus* encounter with an external stimuli, SaeS a member of the EnvZ family of histidine protein kinases becomes autophosphorylated at the histidine residue

followed by the phosphate group transfer to asparyl group on the response regulator SaeR (43). SaeR, a member of the OmpR family of response regulators (43), resides in the cytoplasm and upon phosphorylation SaeR binds promoter regions containing the SaeR binding site and promotes transcription of its targets (29,40). However, the site of phosphorylation and abundance on SaeR has not been determined and the role of SaeP and Q in regulating virulence is poorly understood. Studies suggest these accessory genes play a role in dephosphorylating SaeS and thus negatively regulating the Sae system. This observation was further supported by an increase in SaeR/S-regulated transcript levels in the absence of P1 promoter (45). Still, many questions remain as to the precise molecular function of each of the genes of the Sae system.

In addition to regulating staphylococcal virulence factors, the SaeR/S system modulates host responses to promote infection. SaeR/S contributes to a robust pro-inflammatory response characterized by production of TNF-α, IFN-γ, IL-1β and IL-6 that correlates with higher bacterial burdens and mortality in a mouse model of invasive disease (27). These results were confirmed in human whole blood assays that showed reduced production of TNF-α, IFN-γ, IL-6 and IL-2 in blood treated with secreted factors from Δ*saeR/S* versus wild-type *S. aureus* (27) and in a mouse model of peritonitis that demonstrated *saeR/S* promoted IFNγ production in neutrophils. This same study correlated IFNγ production by neutrophils with exacerbated disease (47). In addition, the SaeR/S system has been shown to be essential for pathogenesis in multiple mouse and/or rabbit models of skin infection, osteomyelitis, infective endocarditis and pneumonia (26,29,30,35,48). In conclusion, the SaeR/S TC system is an essential

regulatory network that *S. aureus* employs to detect the host environment and activate its virulence factors accordingly. A complete understanding of the system's activation and role *in vivo* will be essential to developing novel drug targets against staphylococcal disease.

Neutrophil Granulopoiesis

The neutrophil (polymorphonuclear leukocyte or PMN) is the most abundant white blood cell and serves as the first line of defense against bacterial and fungal infections. The timing and magnitude of neutrophil influx to the sites of injury are some of the key determining factors of the infection outcome, making granulopoiesis an essential and tightly regulated process. Granulopoiesis takes place in the bone marrow where myeloblasts undergo differentiation into neutrophilic promyelocytes which develop into myelocytes and eventually become mature neutrophils (51). Approximately 10^11 neutrophils are derived from myeloid progenitors daily. While only ~2% of PMN reserves enter blood circulation, this number fluctuates depending on cytokines and growth factors present in the bone marrow and peripheral tissues (52). During steady state granulopoiesis, CXCR4 and CXCR2 ligands, stromal-cell-derived factor-1 (SDF-1) and CXCL2, are the primary determinants of neutrophil retention and release from the bone marrow, respectively (53). SDF-1 is primarily produced by the stromal cells while CXCL2 comes from the endothelial cells (52).

Neutrophil production is strongly linked to granulocyte colony stimulating factor (G-CSF) as it controls CXC4-CXC2 axis and its synthesis is dictated by tissue resident cell-derived IL-17 and IL-23 which are elevated during PMN depletion (54). The

importance of G-CSF in granulopoiesis is further demonstrated in patients with G-CSF receptor mutations who are neutropenic (55). Given its vast influence on PMN production, recombinant G-CSF has been successfully used for treatment of cyclic and chemotherapy-induced neutropenia over the past 30 years (56). Moreover, G-CSF-mediated PMN production is dependent on TLR4 and TRIFF signaling suggesting pathogen pattern recognition (PPR) signaling is critical for granulopoiesis (57). The influence of PPR on steady-state PMN development is further supported by studies with germ-free mice and rats that observed significantly lower baseline PMN levels compared to animals with conventional microbiome (58,59).

Emergency granulopoiesis, defined as *de novo* production of neutrophils during a pathogen invasion, can be induced directly when myeloid progenitors recognize pathogen-derived components and subsequently proliferate. Indirect emergency granulopoiesis is elicited by mature hematopoietic and non- hematopoietic cells that secrete G-CSF and GM-CSF which enhance PMN precursor production in the bone marrow (52). Interestingly, in mice, PMN progenitors have been shown to mature at the site of *S. aureus* skin infection suggesting the bone marrow might not be the only location for PMN development (60). Further studies are necessary to determine if human PMNs can also mature in peripheral tissues during an infection.

While stages of PMN maturation are similar between steady-state and emergency granulopoiesis, the transcription factors involved in neutrophil development are vastly different and thus highlight the role of inflammation during an infection (61). IL-6 and myeloid-derived reactive oxygen species (ROS) are two recently identified

inflammatory agents that regulate emergency granulopoiesis (62,63). These findings support the observation that even in the absence of the three major myeloid growth factors (G-CSF, GM-CSF, M-CSF), emergency granulopoiesis can still occur (64).

In addition to *de novo* synthesis, there are studies that suggest mature PMNs that reside in the lung might be a source of mature neutrophils during an injury. However, the role of the pulmonary marginated PMN pool remains to be determined since there are limited studies investigating the role of this population (65-67). For example, it has been shown that the PMN reserve in the lung is released during epinephrine spikes while other studies suggest that PMN presence in the lung is an artifact of the adjacent cardiac output (68,69).

Neutrophils in Staphylococcal Disease

Neutrophils (polymorphonuclear leukocytes or PMNs) are essential for clearance of staphylococcal infections as patients with neutrophil defects are highly susceptible to *S. aureus* disease (70-74). Furthermore, studies in mice demonstrate that in addition to PMNs being essential to clearance of *S. aureus*, the number of PMNs recruited to the site of *S. aureus* infection is important since complete depletion or too many PMNs can have a detrimental outcome (75,76). Thus, there is a fine balance between PMNs causing damage to the host, frequently linked to elevated pro-inflammatory responses (77,78), and a response causing clearance of the pathogen. What triggers an appropriate PMN response i.e. activation state of cells and communication within the immune system, to resolve a *S. aureus* infection, is not completely defined. To address this, the

host factors that contribute to susceptibility to *S. aureus* as well as the mechanisms used by the pathogen to disrupt PMN responses at different stages of infection need to be taken into consideration and are discussed below.

Neutrophil Recruitment to the Site of Infection

Upon interaction with *S. aureus* in the tissue, resident cells and endothelium become activated and secrete a variety of cytokines and chemokines, such as TNF-alpha, IL-1β, IL-17, Gro-α, Gro-β, IL-8, C3b, and leukoterine B4 (LTB4), which guide neutrophils to the site of infection (51,67). Activation of the endothelium induces, expression of selectins facilitating interaction with the circulating neutrophils. Briefly, P-selectin binds PSGL-1 on the neutrophil while E-selectin associates with three primary ligands on circulating neutrophils, L-selectin, CD44 and ESL-1, causing the cells to slow down and tether to the endothelium for initial leukocyte capture. As neutrophils come in contact with endothelium, their secretory vesicles fuse with the outer cell membrane resulting in immediate expression of chemotactic receptors as well as β2-integrins that bind ICAM on the endothelium for firm adherence (51,79,80). Subsequently, neutrophils extravasate (enter the tissue directly through or between endothelial cells) and infiltrate to the site of infection upon detection of several chemoattractants including C5a, *S. aureus*-derived N-formylated peptides (fMLPs) and phenol soluble molecules (PSMs) that bind neutrophil's C5a, FP1 and FP2 receptors, respectively (67,81). How neutrophils discriminate between the different chemoattractants is quite intricate and dependent on several factors, including the concentration and type of chemoattractants present. For example, C5a and fMLPs are

the dominant (i.e preferred) chemoattractants that signal via p38 MAPK pathway while the intermediate IL-8 and LTB4 chemokines require PI3K signaling (82). In the presence of C5a and fMLPs, IL-8 and LTB4 signaling is masked by PI3K antagonist phosphate and tensin homolog (PTEN) which inhibits PI3K signaling and results in PMNs preferential chemotaxis towards C5a and fMLPs. (82,83). Although receptor desensitization does not play a role in PMNs ability to differentiate between the aforementioned chemotactic factors (82), this process along with cross-desensitization is essential for cells' navigation through chemotactic gradients that have been shown to vary depending on the tissue type (67,84,85).

S. aureus has multiple mechanisms by which it can inhibit PMN recruitment. Superantigen-like protein 5 (SSL5) can hinder PMN rolling by binding PSGL-1 (86) and Sae-regulated extracellular adhesion protein (Eap) interrupts PMN adhesion by engaging ICAM-1 on the endothelium (87,88). Unlike fMLPs that attract PMNs, staphylococcal chemotaxis inhibitory protein (CHiP) blocks neutrophil's FP and C5a receptors while SSL7 hinders C5a synthesis and staphopain A cleaves CXCR2 receptors (89-91). Nonetheless, clinical manifestations of *S. aureus* abscesses associated with PMNs suggest neutrophils overcome the aforementioned inhibitions. The initial hindrance and/or delay of the PMN influx, however, might be a strategy employed by *S. aureus* to aid the establishment of infection.

The phenotype of tissue neutrophils is quite different from that of the circulating PMNs. It is estimated that PMN lifespan is less than 24 hours in the blood, although recent studies suggest ~ 5 days, and in the tissues PMN lifespan can be extended up-to

several days (92,93). The process of diapedesis itself and engagement of neutrophil pathogen recognition receptors (PRRs) such as TLR, NOD and peptidoglycan recognition protein (PGRP) with pathogen-derived factors contribute to neutrophil activation that facilitates degranulation, oxidative burst, cytokine release and prolong neutrophil lifespan (94,95).

Neutrophil Phagocytosis and Antimicrobial Defense Mechanisms

PMNs readily phagocytose opsonized *S. aureus* by engaging complement and Fc receptors (CR, FcR) with complement particles and immunoglobulins deposited on the microbe, respectively. Immunoglobulins not only opsonize the pathogen but can neutralize toxins and activate the classical complement cascade (96). As in the case of neutrophil recruitment, phagocytosis can be inhibited by *S. aureus* virulence factors that have anti-phagocytic properties. Protein A, SSL10, formyl peptide receptor-like 1 inhibitor (FLIPr), FLIPr-like and SaeR/S-regulated IgG-binding protein (Sbi) bind to the Fc portion of the IgG and thus inhibit neutrophils' FcR binding (97-99). Furthermore, Sae-regulated extracellular fibrinogen binding (Efb), Eap, staphylococcal complement inhibitor (SCIN), and aureolysin can prevent C3b synthesis and/or deposition (100-104) while staphylokinase converts plasminogen into plasmin that subsequently cleaves IgG and C3b from the pathogen's surface (105).

Collectively, *S. aureus* is decorated in its ability to disrupt phagocytosis, however, the efficacy of phagocytosis *in vitro* and *in vivo* is incompletely defined. This is in part due to the difficulty of measuring phagocytosis *in vivo* along with the different

methodology, sites of infection studied, and strains employed. For example, synchronized phagocytosis via centrifugation induces rapid ingestion of majority of USA300 by PMNs within 15 minutes (48). Such high efficacy has been also observed at 24 hours post-infection in a model of *S. aureus* bacteremia (75). In contrast, Sae-dependent vWBP and coa virulence factors have been shown to prevent PMN phagocytosis via formation of pseudocapsules by Newman *S. aureus* strain in the presence of fibrinogen (106). In addition, Sae-regulated Efb factor can inhibit PMN phagocytosis *in vitro* and *in vivo* model of bacteremia with heat-killed *S. aureus* (103).

Despite the discrepancies in data pertaining phagocytosis, the ability of *S. aureus* to survive PMN phagocytosis is a recognized virulence mechanism (30,48,75,107). Inside the neutrophil, *S. aureus* is enclosed within a phagosome which fuses with granule-containing vesicles, a process known as degranulation. Granules are subdivided into primary (azurophilic or alpha), secondary (specific or beta) and tertiary (gamma) vesicles and released in the reverse order of formation sequence in the bone marrow (51,108). In addition to storing antimicrobial peptides and enzymes ranging from alpha-defensin, LL-37, elastase, gelatinase, lactoferrin to myeloperoxidase (MPO) and proteases, they also contain receptors (i.e. CD11b/CD18, CD66, CD67) and NADPH-oxidase subunits (Gp91phox/p22phox) known as flavocytochrome b_{568}(51,108,109). The oxidative burst takes place when phagosome- or cell membrane bound flavocytochrome b_{568} assembles with cytosolic p67phox/p40phox/p42phox/Rac subunits to facilitate electron transfer resulting in reduction of oxygen to superoxide (O_2^-) anions. Superoxide anion rapidly dismutates to H_2O_2 which is an essential substrate for OH^- and MPO-

catalyzed hypochlorous acid (HOCl) production (109). HOCl is a potent antimicrobial agent that can also react with amines to form toxic mono- and di-chloramines (110). In addition to direct antimicrobial properties, ROS has been shown to promote killing in an indirect manner. Reeves et al. proposed that the charge generated by O_2^- production within phagosomes is counterbalanced by influx of K^+ which results in optimal conditions for protease activation and release implying ROS contributes to pathogen killing indirectly (111). This observation is further supported by the observation that patients with Chronic Granulomatous Disease (CGD), whose cells cannot produce O_2^- due to having defects in the NADPH complex, are highly susceptible to fungal and bacterial infections. In contrast, patients with MPO deficiency, which still permits O_2^- and H_2O_2 production and thus K^+-mediated protease activation proposed to directly kill bacteria, are susceptible to *Candida albicans* but are healthy otherwise (112).

Given that *S. aureus* survives the neutrophil evasion, it is no surprise that it can counteract the toxic ROS response. *S. aureus* can scavenge oxygen radicals and neutralize H_2O_2 with the aid of catalase, superoxide dismutase, yellow carotenoid pigment and iron-regulating surface proteins (113-115). Staphylokinase and aurolysin reduce the antimicrobial properties of the granule-containing phagosomes by cleaving alpha-defensin and LL-37, respectively (116,117). In addition, a four-component staphylococcal *dltABCD* operon serves to substitute additional D-alanines onto the techoic acids within peptidoglycan in order to reduce the negative charge of the cell surface and to decrease attraction of antimicrobial peptides (118). A similar mechanism is elicited by the multiple peptide resistance factor (mprF) that modifies membrane

lipids with L-lysine to oppose antimicrobial agents (119).

Neutrophil Extracellular Traps

Neutrophil extracellular trap (NET) formation is an additional antimicrobial mechanism employed by neutrophils during infection. The mechanism consists of DNA ejection along with histones and antimicrobial peptides (120). NET induction can be induced by PMN exposure to phorbol myristate acetate (PMA) and has been shown to be dependent on Raf-MEK-ERK pathway and NADPH oxidase suggesting it is a programmed mechanism (121,122). In the case of *S. aureus*-mediated NET formation, Thammavongsa et al., has shown that *S. aureus* nuclease (Nuc), controlled by Sae, and adenosine synthase (AdsA) can utilize human NETs to synthesize 2'-deoxyadenosine which kills macrophages (123). This observation was further supported *in vivo* bacteremia model where wild-type infection caused macrophage depletion in the infected kidney in *nuc* and *adsA* dependent manner (123). Human NETs can be also elicited by LukGH/LukAB toxin regulated by the Sae system, but they do not have bactericidal effects towards *S. aureus*. In the same report, NETs were induced by electroporation suggesting that NET formation can also take place in a nonspecific manner (124). MPO-deficient neutrophils cannot make NETs which supports the observation that NETs are not essential to killing *S. aureus* in a clinical setting since MPO-deficient patients are not highly susceptible to bacterial infections (125). In contrast, murine NET formation was not only beneficial for inhibition of *S*. aureus dispersal, but also required for enhanced bacterial killing in a model of staphylococcal sepsis (126). Given the discrepancies, ranging from beneficial to detrimental (123,126),

the role of NETs in *S. aureus* pathogenesis is most likely to be dependent on multiple factors, including the infection type, animal model employed as well as the host's health status.

S. aureus-induced Neutrophil Cell Death

Neutrophil cell death, although a final process, has a major influence on the progression of a staphylococcal infection. In an ideal scenario, infected neutrophils are cleared from the infection site by resident macrophages that then inhibit PMN influx via IL-17/IL-23/G-CSF axis (50) (discussed under "Neutrophil Granulopoiesis"). If neutrophils lyse abruptly, undergo accelerated or delayed apoptosis or NETosis macrophage efferocytosis can be inhibited. This dysfunction leads to increased inflammation and tissue damage which can delay or prevent resolution of infection (126). Indeed, the clinically relevant strains of *S. aureus* have been shown to have the ability to modulate PMN lifespan and this is a major strategy by which *S. aureus* can evade the microbicidal effects of the neutrophil. (30,104,127-129).

Despite having morphology of apoptotic cells, such as membrane blebbing and condensed chromatin, *S. aureus*-infected neutrophils undergo abrupt lysis without involvement of caspases in SaeR/S-dependent manner suggesting that *S. aureus*-infected PMNs undergo programmed necrosis, or necroptosis (127,129-131). This has been supported by a recent finding that USA300-infected neutrophils undergo cell death in a receptor-interacting-protein 1-dependent manner associated with necroptosis cell death (130). In the same study, macrophages did not engulf infected PMNs preventing proper detoxication of spent PMNs pathway demonstrating a mechanism by which USA300

clearance is inhibited (130). Furthermore, we have shown that SaeR/S system inhibits NF-kB activity necessary for neutrophil survival highlighting a unique mechanism used by this pathogen to evade the neutrophil. Which SaeR/S-regulated factors modulate PMN responses is not completely defined. However, Eap has been shown to hinder NF-kB DNA binding in THP-1 cells (86) and a number of studies have demonstrated that SaeR/S-regulated two-component toxins (LukAB/GH, LukED, HlgABC, LukSF) contribute to PMN damage and/or lysis (132-136) highlighting the importance of the Sae system in PMN evasion.

Hypothesis

Herein, we have tested the hypothesis that differential regulation of the SaeR/S system is induced by specific host-derived stimuli (Chapter 2) resulting in evasion and destruction of the neutrophil (Chapter 3). In Chapter 2, we investigated the specificity of SaeR/S activation by examining the influence of the external SaeS loop on subsequent transcription of SaeR/S-regulated virulence factors (38,128). We demonstrated that the SaeR/S system is essential for transcription of virulence factors in the presence of human polymorphonuclear leukocytes (PMNs or neutrophils) as well as during murine skin infection (38). These observations confirmed previously published data that demonstrated up-regulation of *saeR* and *saeS* during neutrophil phagocytosis (104). These studies suggested that activation of the SaeR/S TC system and subsequent transcription of its virulence factors are dependent on the external environment as

neutrophil-derived alpha-defensin promoted SaeR/S activation while other host peptides, even at lethal doses, did not influence SaeR/S-mediated transcription (38).

In Chapter 3, we studied the host side and defined the pathogen- and host-derived mechanisms that contribute to altered PMN fate following *S. aureus* phagocytosis (129). We showed that the SaeR/S system influences PMN cell-fate by promoting plasma membrane damage, lysis and accelerating programmed cell death in accordance with previous reports (30,129). On the host side, this phenotype was linked to suppression of NF-kB phosphorylation by the SaeR/S system, followed by decreased IL-8 production which were necessary for neutrophils to survive and clear the pathogen successfully (129). Inhibition of NF-kB with parthenolide resulted in neutrophils' inability to clear USA300 or Δ*saeR/S* mutant strain highlighting the importance of the NF-kB to *S. aureus* clearance during neutrophil interaction.

Ongoing studies focus on defining the effect of neutrophil density and function on clearance of *S. aureus* infection. Preliminary studies demonstrate subcutaneous skin infection with USA300 was associated with higher levels of neutrophils in the abscess which was also reflected by higher expression of chemokines, cytokines and growth factors that play indirect or direct roles in neutrophil influx when compared to that of the Δ*saeR/S* mutant-infected mice. Given that neutrophil concentration at the infection site plays an essential role to pathogen clearance, we are currently assessing the influence of the neutrophil density on PMN function and *S. aureus* survival. Herein, we report that neutrophil antimicrobial properties as well as cell death rate are altered at low density during interaction with *S. aureus*. Furthermore, neutrophils at low density are not

capable of killing *S. aureus* underlying the importance of an adequate concentration of neutrophils necessary to clear the infection. These findings not only apply to neutropenic patients who are highly susceptible to fungal and bacterial infections but also provide new insight into susceptibility of healthy individuals to staphylococcal infections implying that inadequate neutrophil density at the site of *S. aureus* insult may result in infection. In conclusion, our studies focus on defining the intricate roles between neutrophils and *S. aureus* that play a significant role in dictating the outcome of infection.

Literature Cited

1. Fowler VG, Miro JM, Hoen B, Cabell CH, Abrutyn E, Rubinstein E, et al. Staphylococcus aureus endocarditis: a consequence of medical progress. JAMA. 2005 Jun 22;293(24):3012–21.

2. Miro JM, Anguera I, Cabell CH, Chen AY, Stafford JA, Corey GR, et al. Staphylococcus aureus native valve infective endocarditis: report of 566 episodes from the International Collaboration on Endocarditis Merged Database. Clin Infect Dis. 2005 Aug 15;41(4):507–14.

3. Wang A, Athan E, Pappas PA, Fowler VG, Olaison L, Paré C, et al. Contemporary clinical profile and outcome of prosthetic valve endocarditis. JAMA. 2007 Mar 28;297(12):1354–61.

4. Otto M. Basis of virulence in Community-Associated Methicillin-Resistant Staphylococcus aureus. Annu Rev Microbiol. 2010 Oct 13;64(1):143–62.

5. McCaig LF, McDonald LC, Mandal S, Jernigan DB. Staphylococcus aureus-associated skin and soft tissue infections in ambulatory care. Emerging Infect Dis. 2006 Nov;12(11):1715–23.

6. Popovich KJ, Aroutcheva A, Hota B, Beavis KG, Hayden MK, Weinstein RA. Anatomic sites of colonization with Community-Associated Methicillin-Resistant Staphylococcus aureus. Infection Control and Hospital Epidemiology. 2014 Sep;35(9):1192–4.

7. Peacock SJ, de Silva I, Lowy FD. What determines nasal carriage of Staphylococcus aureus? Trends in Microbiology. 2001 Dec;9(12):605–10.

8. Stryjewski ME, Corey GR. Methicillin-Resistant Staphylococcus aureus: An evolving pathogen. Clin Infect Dis. 2013 Dec 16;58:S10–9.

9. Davis S, Schaller J, Wedgwood R, Harvard M. Job's syndrome: recurrent,‘ cold’, staphylococcal abscesses. The Lancet. Elsevier; 1966;287(7445):1013–5.

10. Lowy FD. Antimicrobial resistance: the example of Staphylococcus aureus. Journal of Clinical Investigation. 2003 May 1;111(9):1265–73.

11. Perichon B, Courvalin P. VanA-Type Vancomycin-Resistant Staphylococcus aureus. Antimicrobial Agents and Chemotherapy. 2009 Oct 16;53(11):4580–7.

12. Chambers HF, DeLeo FR. Waves of resistance: Staphylococcus aureus in the antibiotic era. Nature Publishing Group; 2009 Sep 1;:1–13.

13. King MD, Humphrey BJ, Wang YF, Kourbatova EV, Ray SM, Blumberg HM. Emergence of community-acquired methicillin-resistant Staphylococcus aureus USA 300 clone as the predominant cause of skin and soft-tissue infections. Ann Intern Med. Am Coll Physicians; 2006;144(5):309–17.

14. Montgomery CP, Boyle-Vavra S, Adem PV, Lee JC, Husain AN, Clasen J, et al. Comparison of Virulence in Community‐Associated Methicillin‐Resistant Staphylococcus aureus pulsotypes USA300 and USA400 in a rat model of pneumonia. J Infect Dis. 2008 Aug 15;198(4):561–70.

15. Jones MB, Montgomery CP, Boyle-Vavra S, Shatzkes K, Maybank R, Frank BC, et al. Genomic and transcriptomic differences in community acquired methicillin resistant Staphylococcus aureus USA300 and USA400 strains. BMC Genomics. 2014 Dec 19;15(1):1145.

16. Li M, Diep BA, Villaruz AE, Braughton KR, Jiang X, DeLeo FR, et al. Evolution of virulence in epidemic community-associated methicillin-resistant Staphylococcus aureus. Proc Natl Acad Sci USA. 2009 Apr 7;106(14):5883–8.

17. Miller JF, Mekalanos JJ, Falkow S. Coordinate regulation and sensory transduction in the control of bacterial virulence. Science. 1989 Feb 17;243(4893):916–22.

18. Diep BA, Gill SR, Chang RF, Phan TH, Chen JH, Davidson MG, et al. Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant Staphylococcus aureus. The Lancet. 2006 Mar;367(9512):731–9.

19. Gordon RJ, Lowy FD. Pathogenesis of Methicillin‐Resistant Staphylococcus aureus Infection. Clin Infect Dis. 2008 Jun;46(S5):S350–9.

20. Nygaard TK, DeLeo FR, Voyich JM. Community-associated methicillin-resistant Staphylococcus aureus skin infections: advances toward identifying the key virulence factors. Curr Opin Infect Dis. 2008 Apr;21(2):147–52.

21. Giraudo AT, Raspanti CG, Calzolari A, Nagel R. Characterization of a Tn551-mutant of Staphylococcus aureus defective in the production of several exoproteins. NRC Research Press Ottawa, Canada; 1994 Aug;40(8):677–81.

22. Giraudo AT, Cheung AL, Nagel R. The sae locus of Staphylococcus aureus controls exoprotein synthesis at the transcriptional level. Arch Microbiol. Springer; 1997;168(1):53–8.

23. Giraudo AT, Calzolari A, Cataldi AA, Bogni C. The sae locus of Staphylococcus aureus encodes a two-component. 1999 Jul 15;:1–8.

24. Giraudo AT, Mansilla C, Chan A, Raspanti C, Nagel R. Studies on the Expression of regulatory locus sae in Staphylococcus aureus. Current Microbiology. 2003 Apr 1;46(4):246–50.

25. Cabell CH, Jollis JG, Peterson GE, Corey GR, Anderson DJ, Sexton DJ, et al. Changing patient characteristics and the effect on mortality in endocarditis. Arch Intern Med. 2002 Jan 14;162(1):90–4.

26. Xiong YQ, Willard J, Yeaman MR, Cheung AL, Bayer AS. Regulation of Staphylococcus aureus alpha-toxin gene (hla) expression by agr, sarA, and sae in vitro and in experimental infective endocarditis. J Infect Dis. 2006 Nov 1;194(9):1267–75.

27. Watkins RL, Pallister KB, Voyich JM. The SaeR/S Gene regulatory system induces a pro-inflammatory cytokine response during Staphylococcus aureus infection. Diep BA, editor. PLoS ONE. 2011 May 13;6(5):e19939.

28. Hoen B, Alla F, Selton-Suty C, Béguinot I, Bouvet A, Briançon S, et al. Changing profile of infective endocarditis: results of a 1-year survey in France. JAMA. 2002 Jul 3;288(1):75–81.

29. 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 Jan 15;201(2):241–54.

30. Voyich JM, Vuong C, DeWald M, Nygaard TK, Kocianova S, Griffith S, et al. The SaeR/S gene regulatory system is essential for innate immune evasion by Staphylococcus aureus. J Infect Dis. 2009 Jun;199(11):1698–706.

31. Steinhuber A, Goerke C, Bayer MG, Döring G, Wolz C. Molecular architecture of the regulatory locus sae of Staphylococcus aureus and its impact on expression of virulence factors. Journal of Bacteriology. 2003 Nov;185(21):6278–86.

32. Benton BM, Zhang JP, Bond S, Pope C, Christian T, Lee L, et al. Large-scale identification of genes required for full virulence of Staphylococcus aureus. Journal of Bacteriology. 2004 Dec;186(24):8478–89.

33. Bae T, Banger AK, Wallace A, Glass EM, Aslund F, Schneewind O, et al. Staphylococcus aureus virulence genes identified by bursa aurealis mutagenesis and nematode killing. Proc Natl Acad Sci USA. 2004 Aug 17;101(33):12312–7.

34. Beenken KE, Mrak LN, Zielinska AK, Atwood DN, Loughran AJ, Griffin LM, et al. Impact of the functional status of saeRSon in vivophenotypes of Staphylococcus aureus sarA mutants. Molecular Microbiology. 2014 May 12;92(6):1299–312.

35. Cassat JE, Hammer ND, Campbell JP, Benson MA, Perrien DS, Mrak LN, et al. A secreted bacterial protease tailors the Staphylococcus aureus virulence repertoire to modulate bone remodeling during osteomyelitis. Cell Host and Microbe. 2013 Jun;13(6):759–72.

36. Olson ME, Nygaard TK, Ackermann L, Watkins RL, Zurek OW, Pallister KB, et al. Staphylococcus aureus nuclease is a SaeR/S-dependent virulence factor. Infection and Immunity. 2013 Feb 4.

37. Klevens RM, Morrison MA, Nadle J, Petit S, Gershman K, Ray S, et al. Invasive methicillin-resistant Staphylococcus aureus infections in the United States. JAMA. 2007 Oct 17;298(15):1763–71.

38. Zurek OW, Nygaard TK, Watkins RL, Pallister KB, Torres VJ, Horswill AR, et al. The role of innate immunity in promoting SaeR/S-mediated virulence in Staphylococcus aureus. J Innate Immun. 2014;6(1):21–30.

39. Lautenschlager S, Herzog C, Zimmerli W. Course and outcome of bacteremia due to Staphylococcus aureus: evaluation of different clinical case definitions. Clin Infect Dis. 1993 Apr;16(4):567–73.

40. Sun F, Li C, Jeong D, Sohn C, He C, Bae T. 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. Journal of Bacteriology. 2010 Mar 29;192(8):2111–27.

41. Ringberg H, Thorén A, Lilja B. Metastatic complications of Staphylococcus aureus septicemia. To seek is to find. Infection. 2000 Apr;28(3):132–6.

42. Fowler VG, Olsen MK, Corey GR, Woods CW, Cabell CH, Reller LB, et al. Clinical identifiers of complicated Staphylococcus aureus bacteremia. Arch Intern Med. 2003 Sep 22;163(17):2066–72.

43. Adhikari RP, Novick RP. Regulatory organization of the staphylococcal sae locus. Microbiology. 2008 Mar 1;154(3):949–59.

44. Geiger T, Goerke C, Mainiero M, Kraus D, Wolz C. The virulence regulator Sae of Staphylococcus aureus: promoter activities and response to phagocytosis-related signals. Journal of Bacteriology. 2008 Apr 30;190(10):3419–28.

45. Jeong D-W, Cho H, Jones MB, Shatzkes K, Sun F, Ji Q, et al. The auxiliary protein complex SaePQ activates the phosphatase activity of sensor kinase SaeS in the SaeRS two-component system of Staphylococcus aureus. Molecular Microbiology. 2012 Oct; 86(2); 331-48.

46. Jeong DW, Cho H, Lee H, Li C, Garza J, Fried M, et al. Identification of the P3

promoter and distinct roles of the two promoters of the SaeRS two-component system in Staphylococcus aureus. Journal of Bacteriology. 2011 Aug 25;193(18):4672–84.

47. 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. Elsevier Masson SAS; 2013 Sep 10;15(10-11):749–54.

48. Montgomery CP, Boyle-Vavra S, Daum RS. Importance of the global regulators Agr and SaeRS in the pathogenesis of CA-MRSA USA300 infection. DeLeo FR, editor. PLoS ONE. 2010 Dec 2;5(12):e15177.

49. Borregaard N. Neutrophils, from marrow to microbes. Immunity. Elsevier Inc; 2010 Nov 24;33(5):657–70.

50. Manz MG, Boettcher S. Emergency granulopoiesis. Nat Rev Immunol. Nature Publishing Group; 2014 Apr 22;14(5):302–14.

51. Eash KJ, Greenbaum AM, Gopalan PK, Link DC. CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow. Journal of Clinical Investigation. 2010 Jul 1;120(7):2423–31.

52. Stark MA, Huo Y, Burcin TL, Morris MA, Olson TS, Ley K. Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17. immunity. 2005 Mar;22(3):285–94.

53. Dong F, Hoefsloot LH, Schelen AM, Broeders CA, Meijer Y, Veerman AJ, et al. Identification of a nonsense mutation in the granulocyte-colony-stimulating factor receptor in severe congenital neutropenia. Proc Natl Acad Sci USA. 1994 May 10;91(10):4480–4.

54. Dale DC, Mackey MC. Understanding, treating and avoiding hematological disease: better medicine through mathematics? Bull Math Biol. 2014 Sep 12.

55. Bugl S, Wirths S, Radsak MP, Schild H, Stein P, Andre MC, et al. Steady-state neutrophil homeostasis is dependent on TLR4/TRIF signaling. Blood. 2013 Jan 31;121(5):723–33.

56. Balmer ML, Schurch CM, Saito Y, Geuking MB, Li H, Cuenca M, et al. Microbiota-derived compounds drive steady-state granulopoiesis via MyD88/TICAM signaling. The Journal of Immunology. 2014 Nov 7;193(10):5273–83.

57. Ohkubo T, Tsuda M, Suzuki S, Borai El N, Yamamura M. Peripheral blood neutrophils of germ-free rats modified by in vivo granulocyte-colony-

stimulating factor and exposure to natural environment. Scand J Immunol. 1999 Jan;49(1):73–7.

58. Kim MH, Granick JL, Kwok C, Walker NJ, Borjesson DL, Curry FRE, et al. Neutrophil survival and c-kit+-progenitor proliferation in Staphylococcus aureus-infected skin wounds promote resolution. Blood. 2011 Mar 24;117(12):3343–52.

59. Bardoel BW, Kenny EF, Sollberger G, Zychlinsky A. The balancing act of neutrophils. Cell Host and Microbe. Elsevier Inc; 2014 May 14;15(5):526–36.

60. Walker F, Zhang H-H, Matthews V, Weinstock J, Nice EC, Ernst M, et al. IL6/sIL6R complex contributes to emergency granulopoietic responses in G-CSF- and GM-CSF-deficient mice. Blood. 2008 Apr 15;111(8):3978–85.

61. Kwak H-J, Liu P, Bajrami B, Xu Y, Park S-Y, Nombela-Arrieta C, et al. Myeloid cell-derived reactive oxygen species externally regulate the proliferation of myeloid progenitors in emergency granulopoiesis. Immunity. Elsevier Inc; 2015 Jan 20;42(1):159–71.

62. Hibbs ML, Quilici C, Kountouri N, Seymour JF, Armes JE, Burgess AW, et al. Mice lacking three myeloid colony-stimulating factors (G-CSF, GM-CSF, and M-CSF) still produce macrophages and granulocytes and mount an inflammatory response in a sterile model of peritonitis. The Journal of Immunology. 2007 May 2;178(10):6435–43.

63. Bierman HR, Kelly KH, Cordes FL, Byron RL, Polhemus JA, Rappoport S. The release of leukocytes and platelets from the pulmonary circulation by epinephrine. Blood. 1952 Jul;7(7):683–92.

64. Summers C, Rankin SM, Condliffe AM, Singh N, Peters AM, Chilvers ER. Neutrophil kinetics in health and disease. Trends in Immunology. Elsevier Ltd; 2010 Aug 1;31(8):318–24.

65. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. Nat Rev Immunol. Nature Publishing Group; 2013 Mar 1;13(3):159–75.

66. Athens JW, Haab OP, Raab SO, Mauer AM, Ashenbrucker H, Cartwright GE, et al. Leukokinetic studies. IV. The total blood, circulating and marginal granulocyte pools and the granulocyte turnover rate in normal subjects. Journal of Clinical Investigation. 1961 Jun;40:989–95.

67. Tak T, Tesselaar K, Pillay J, Borghans JAM, Koenderman L. What's your age again? Determination of human neutrophil half-lives revisited. Journal of Leukocyte Biology. 2013 Sep 30;94(4):595–601.

68. Bodey GP, Buckley M, Sathe YS, Freireich EJ. Quantitative relationships between circulating leukocytes and infection in patients with acute leukemia. Ann Intern Med. 1966 Feb;64(2):328–40.

69. Pincus SH, Boxer LA, Stossel TP. Chronic neutropenia in childhood. Analysis of 16 cases and a review of the literature. Am J Med. 1976 Dec;61(6):849–61.

70. Dale DC, Guerry D, Wewerka JR, Bull JM, Chusid MJ. Chronic neutropenia. Medicine (Baltimore). 1979 Mar;58(2):128–44.

71. Lekstrom-Himes JA, Gallin JI. Immunodeficiency diseases caused by defects in phagocytes. N Engl J Med. 2000 Dec 7;343(23):1703–14.

72. Dinauer MC. Methods in Molecular Biology. Quinn MT, DeLeo FR, editors. Totowa, NJ: Humana Press; 2014. 15 p.

73. Gresham HD, Lowrance JH, Caver TE, Wilson BS, Cheung AL, Lindberg FP. Survival of Staphylococcus aureus inside neutrophils contributes to infection. J Immunol. 2000 Apr 1;164(7):3713–22.

74. Mölne L, Verdrengh M, Tarkowski A. Role of neutrophil leukocytes in cutaneous infection caused by Staphylococcus aureus. Infection and Immunity. 2000 Nov;68(11):6162–7.

75. Brown K, Brain S, Pearson J, Edgeworth J, Lewis S, Treacher D. Neutrophils in development of multiple organ failure in sepsis. The Lancet. 2006 Jul;368(9530):157–69.

76. Rittirsch D, Flierl MA, Ward PA. Harmful molecular mechanisms in sepsis. Nat Rev Immunol. 2008 Oct;8(10):776–87.

77. Borregaard N, Miller LJ, Springer TA. Chemoattractant-regulated mobilization of a novel intracellular compartment in human neutrophils. Science. 1987 Sep 4;237(4819):1204–6.

78. Borregaard N, Sørensen OE, Theilgaard-Mönch K. Neutrophil granules: a library of innate immunity proteins. Trends in Immunology. 2007 Aug;28(8):340–5.

79. Kretschmer D, Gleske A-K, Rautenberg M, Wang R, Köberle M, Bohn E, et al. Human Formyl Peptide Receptor 2 senses highly pathogenic Staphylococcus aureus. Cell Host and Microbe. Elsevier Ltd; 2010 Jun 17;7(6):463–73.

80. Heit B. An intracellular signaling hierarchy determines direction of migration in opposing chemotactic gradients. J Cell Biol. 2002 Oct 7;159(1):91–102.

81. Heit B, Robbins SM, Downey CM, Guan Z, Colarusso P, Miller BJ, et al. PTEN functions to 'prioritize' chemotactic cues and prevent “distraction” in migrating neutrophils. Nat Immunol. 2008 Jun 8;9(7):743–52.

82. Bednar F, Song C, Bardi G, Cornwell W, Rogers TJ. Cross-desensitization of CCR1, but not CCR2, following activation of the Formyl Peptide Receptor FPR1. The Journal of Immunology. 2014 May 16;192(11):5305–13.

83. Olson TS, Ley K. Chemokines and chemokine receptors in leukocyte trafficking. Am J Physiol Regul Integr Comp Physiol. 2002 Jul;283(1):R7–28.

84. Bestebroer J, Poppelier MJJG, Ulfman LH, Lenting PJ, Denis CV, Van Kessel KPM, et al. Staphylococcal superantigen-like 5 binds PSGL-1 and inhibits P-selectin-mediated neutrophil rolling. Blood. 2007 Apr 1;109(7):2936–43.

85. Haggar A, Ehrnfelt C, Holgersson J, Flock J-I. The extracellular adherence protein from Staphylococcus aureus inhibits neutrophil binding to endothelial cells. Infection and Immunity. 2004 Oct;72(10):6164–7.

86. Athanasopoulos AN, Economopoulou M, Orlova VV, Sobke A, Schneider D, Weber H, et al. The extracellular adherence protein (Eap) of Staphylococcus aureus inhibits wound healing by interfering with host defense and repair mechanisms. Blood. 2006 Apr 1;107(7):2720–7.

87. de Haas CJC. Chemotaxis Inhibitory Protein of Staphylococcus aureus, a bacterial antiinflammatory agent. Journal of Experimental Medicine. 2004 Feb 23;199(5):687–95.

88. Langley R, Wines B, Willoughby N, Basu I, Proft T, Fraser JD. The Staphylococcal Superantigen-Like Protein 7 Binds IgA and Complement C5 inhibits IgA-Fc RI binding and serum killing of bacteria. The Journal of Immunology. 2005 Feb 22;174(5):2926–33.

89. Laarman AJ, Mijnheer G, Mootz JM, van Rooijen WJM, Ruyken M, Malone CL, et al. Staphylococcus aureus Staphopain A inhibits CXCR2-dependent neutrophil activation and chemotaxis. The EMBO Journal. Nature Publishing Group; 2012 Jul 31;31(17):3607–19.

90. Pillay J, Braber den I, Vrisekoop N, Kwast LM, de Boer RJ, Borghans JAM, et al. In vivo labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days. Blood. 2010 Jul 29;116(4):625–7.

91. Cheretakis C, Leung R, Sun CX, Dror Y, Glogauer M. Timing of neutrophil tissue repopulation predicts restoration of innate immune protection in a murine bone marrow transplantation model. Blood. 2006 Oct 15;108(8):2821–6.

92. Sabroe I, Prince LR, Jones EC, Horsburgh MJ, Foster SJ, Vogel SN, et al. Selective roles for Toll-like receptor (TLR)2 and TLR4 in the regulation of neutrophil activation and life span. J Immunol. 2003 May 15;170(10):5268–75.

93. Kobayashi SD, Voyich JM, DeLeo FR. Regulation of the neutrophil-mediated inflammatory response to infection. Microbes and Infection. 2003 Nov;5(14):1337–44.

94. Kobayashi SD, Voyich JM, Buhl CL, Stahl RM, DeLeo FR. Global changes in gene expression by human polymorphonuclear leukocytes during receptor-mediated phagocytosis: cell fate is regulated at the level of gene expression. Proc Natl Acad Sci USA. 2002 May 14;99(10):6901–6.

95. Patel D, Wines BD, Langley RJ, Fraser JD. Specificity of Staphylococcal Superantigen-Like Protein 10 toward the Human IgG1 Fc Domain. The Journal of Immunology. 2010 May 18;184(11):6283–92.

96. Atkins KL, Burman JD, Chamberlain ES, Cooper JE, Poutrel B, Bagby S, et al. S. aureus IgG-binding proteins SpA and Sbi: Host specificity and mechanisms of immune complex formation. Molecular Immunology. 2008 Mar;45(6):1600–11.

97. Stemerding AM, Kohl J, Pandey MK, Kuipers A, Leusen JH, Boross P, et al. Staphylococcus aureus Formyl Peptide Receptor-like 1 Inhibitor (FLIPr) and its homologue FLIPr-like are potent Fc R antagonists that inhibit IgG-mediated effector functions. The Journal of Immunology. 2013 Jun 21;191(1):353–62.

98. Rooijakkers SHM, Ruyken M, Roos A, Daha MR, Presanis JS, Sim RB, et al. Immune evasion by a staphylococcal complement inhibitor that acts on C3 convertases. Nat Immunol. 2005 Aug 7;6(9):920–7.

99. Lee LYL, Liang X, Hook M, Brown EL. Identification and characterization of the C3 binding domain of the Staphylococcus aureus extracellular fibrinogen-binding protein (Efb). Journal of Biological Chemistry. 2004 Nov 24;279(49):50710–6.

100. Laarman AJ, Ruyken M, Malone CL, van Strijp JAG, Horswill AR, Rooijakkers SHM. Staphylococcus aureus metalloprotease aureolysin cleaves complement C3 to mediate immune evasion. The Journal of Immunology. 2011 May 19;186(11):6445–53.

101. Ko Y-P, Kuipers A, Freitag CM, Jongerius I, Medina E, van Rooijen WJ, et al. Phagocytosis escape by a Staphylococcus aureus protein that connects complement and coagulation proteins at the bacterial surface. Skaar EP, editor. PLoS Pathog. 2013 Dec 12;9(12):e1003816.

102. Woehl JL, Stapels DAC, Garcia BL, Ramyar KX, Keightley A, Ruyken M, et al. The extracellular adherence protein from Staphylococcus aureus inhibits the classical and lectin pathways of complement by blocking formation of the C3 proconvertase. The Journal of Immunology. 2014 Dec 5;193(12):6161–71.

103. Rooijakkers SHM, van Wamel WJB, Ruyken M, van Kessel KPM, van Strijp JAG. Anti-opsonic properties of staphylokinase. Microbes and Infection. 2005 Mar;7(3):476–84.

104. Voyich JM, Braughton KR, Sturdevant DE, Whitney AR, Saïd-Salim B, Porcella SF, et al. Insights into mechanisms used by Staphylococcus aureus to avoid destruction by human neutrophils. J Immunol. 2005 Sep 15;175(6):3907–19.

105. Guggenberger C, Wolz C, Morrissey JA, Heesemann J. Two Distinct Coagulase-Dependent Barriers Protect Staphylococcus aureus from neutrophils in a three dimensional in vitro infection model. Sullam PM, editor. PLoS Pathog. 2012 Jan 12;8(1):e1002434.

106. Spaan AN, Surewaard BGJ, Nijland R, Van Strijp JAG. Neutrophils versus Staphylococcus aureus: A biological tug of war. Annu Rev Microbiol. 2013 Sep 8;67(1):629–50.

107. Sengeløv H, Follin P, Kjeldsen L, Lollike K, Dahlgren C, Borregaard N. Mobilization of granules and secretory vesicles during in vivo exudation of human neutrophils. J Immunol. 1995 Apr 15;154(8):4157–65.

108. Quinn MT, Ammons MCB, DeLeo FR. The expanding role of NADPH oxidases in health and disease: no longer just agents of death and destruction. Clinical Science. 2006 Jul 1;111(1):1.

109. Klebanoff SJ. Myeloperoxidase: friend and foe. Journal of Leukocyte Biology. 2005 Jan 26;77(5):598–625.

110. Reeves EP, Lu H, Jacobs HL, Messina CGM, Bolsover S, Gabella G, et al. Killing activity of neutrophils is mediated through activation of proteases by K+ flux. Nature. 2002 Mar 21;416(6878):291–7.

111. Lehrer RI, Cline MJ. Leukocyte myeloperoxidase deficiency and disseminated candidiasis: the role of myeloperoxidase in resistance to Candida infection. Journal of Clinical Investigation. 1969 Aug;48(8):1478–88.

112. DeLeo FR, Diep BA, Otto M. Host Defense and pathogenesis in Staphylococcus aureus infections. Infectious Disease Clinics of North America. 2009 Mar;23(1):17–34.

113. Liu GY. Staphylococcus aureus golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. Journal of Experimental Medicine. 2005 Jul 11;202(2):209–15.

114. Palazzolo-Ballance AM, Reniere ML, Braughton KR, Sturdevant DE, Otto M, Kreiswirth BN, et al. Neutrophil microbicides induce a pathogen survival response in community-associated methicillin-resistant Staphylococcus aureus. J Immunol. 2008 Jan 1;180(1):500–9.

115. Jin T, Bokarewa M, Foster T, Mitchell J, Higgins J, Tarkowski A. Staphylococcus aureus resists human defensins by production of staphylokinase, a novel bacterial evasion mechanism. J Immunol. 2004 Jan 15;172(2):1169–76.

116. Sieprawska-Lupa M, Mydel P, Krawczyk K, Wójcik K, Puklo M, Lupa B, et al. Degradation of human antimicrobial peptide LL-37 by Staphylococcus aureus-derived proteinases. Antimicrobial Agents and Chemotherapy. 2004 Dec;48(12):4673–9.

117. Peschel A, Otto M, Jack RW, Kalbacher H, Jung G, Gotz F. Inactivation of the dlt operon in Staphylococcus aureus confers sensitivity to defensins, protegrins, and other antimicrobial peptides. J Biol Chem. 1999 Mar 26;274(13):8405–10.

118. Peschel A, Jack RW, Otto M, Collins LV, Staubitz P, Nicholson G, et al. 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. 2001 May 7;193(9):1067–76.

119. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. Science. 2004 Mar 5;303(5663):1532–5.

120. Hakkim A, Fuchs TA, Martinez NE, Hess S, Prinz H, Zychlinsky A, et al. Activation of the Raf-MEK-ERK pathway is required for neutrophil extracellular trap formation. Nat Chem Biol. 2010 Dec 19;7(2):75–7.

121. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, et al. Novel cell death program leads to neutrophil extracellular traps. J Cell Biol. 2007 Jan 8;176(2):231–41.

122. Thammavongsa V, Missiakas DM, Schneewind O. Staphylococcus aureus degrades neutrophil extracellular traps to promote immune cell death. Science. American Association for the Advancement of Science; 2013;342(6160):863–6.

123. Malachowa N, Kobayashi SD, Freedman B, Dorward DW, DeLeo FR. Staphylococcus aureus Leukotoxin GH Promotes Formation of Neutrophil Extracellular Traps. The Journal of Immunology. 2013 Dec 6;191(12):6022–9.

124. Metzler KD, Fuchs TA, Nauseef WM, Reumaux D, Roesler J, Schulze I, et al. Myeloperoxidase is required for neutrophil extracellular trap formation: implications for innate immunity. Blood. 2011 Jan 20;117(3):953–9.

125. McDonald B, Urrutia R, Yipp BG, Jenne CN, Kubes P. Intravascular neutrophil extracellular traps capture bacteria from the bloodstream during sepsis. Cell Host and Microbe. Elsevier Inc; 2012 Sep 13;12(3):324–33.

126. Bratton DL, Henson PM. Neutrophil clearance: when the party is over, clean-up begins. Trends in Immunology. Elsevier Ltd; 2011 Aug 1;32(8):350–7.

127. Kobayashi SD, Braughton KR, Palazzolo-Ballance AM, Kennedy AD, Sampaio E, Kristosturyan E, et al. Rapid neutrophil destruction following phagocytosis of Staphylococcus aureus. J Innate Immun. 2010;2(6):560–75.

128. Flack CE, Zurek OW, Meishery DD, Pallister KB, Malone CL, Horswill AR, et al. Differential regulation of staphylococcal virulence by the sensor kinase SaeS in response to neutrophil-derived stimuli. Proc Natl Acad Sci USA. 2014 May 13;111(19):E2037–45.

129. Zurek OW, Pallister KB, Voyich JM. Staphylococcus aureus inhibits neutrophil-derived IL-8 to promote cell death. J Infect Dis. 2015 Feb 26.

130. Greenlee-Wacker MC, Rigby KM, Kobayashi SD, Porter AR, DeLeo FR, Nauseef WM. Phagocytosis of Staphylococcus aureus by human neutrophils prevents macrophage efferocytosis and induces programmed necrosis. The Journal of Immunology. 2014 May 15;192(10):4709–17.

131. Erttmann SF, Gekara NO, Fällman M. Bacteria Induce Prolonged PMN Survival via a phosphatidylcholine-specific phospholipase C- and protein kinase C-dependent mechanism. Srinivasula SM, editor. PLoS ONE. 2014 Jan 31;9(1):e87859.

132. DuMont AL, Nygaard TK, Watkins RL, Smith A, Kozhaya L, Kreiswirth BN, et al. Characterization of a new cytotoxin that contributes to Staphylococcus aureus pathogenesis. Molecular Microbiology. 2010 Dec 13;79(3):814–25.

133. Ventura CL, Malachowa N, Hammer CH, Nardone GA, Robinson MA, Kobayashi SD, et al. Identification of a novel Staphylococcus aureus two-component leukotoxin using cell surface proteomics. Horsburgh MJ, editor. PLoS ONE. 2010 Jul 16;5(7):e11634.

134. Alonzo F III, Benson MA, Chen J, Novick RP, Shopsin B, Torres VJ. Staphylococcus aureus leucocidin ED contributes to systemic infection by targeting neutrophils and promoting bacterial growth in vivo. Molecular Microbiology. 2011 Dec 20;:no–no.

135. Malachowa N, Whitney AR, Kobayashi SD, Sturdevant DE, Kennedy AD, Braughton KR, et al. Global changes in Staphylococcus aureus gene expression in human blood. Chakravortty D, editor. PLoS ONE. 2011 Apr 15;6(4):e18617.

136. Graves SF, Kobayashi SD, Braughton KR, Whitney AR, Sturdevant DE, Rasmussen DL, et al. Sublytic concentrations of Staphylococcus aureus Panton-Valentine leukocidin alter human PMN gene expression and enhance bactericidal capacity. Journal of Leukocyte Biology. 2012 May 11.

CHAPTER TWO

THE ROLE OF INNATE IMMUNITY IN PROMOTING SAER/S-MEDIATED VIRULENCE IN *STAPHYLOCOCCUS AUREUS*

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The Role of Innate Immunity in Promoting SaeR/S-Mediated Virulence in *Staphylococcus aureus*

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Key Words
Staphylococcus aureus · USA300 · SaeR/S · Two-component systems · Virulence · Gene expression · Neutrophils · α-Defensin

Abstract
The ability of *Staphylococcus aureus* to infect tissues is dependent on precise control of virulence through gene-regulatory systems. While the SaeR/S two-component system has been shown to be a major regulator of *S. aureus* virulence, the influence of the host environment on SaeR/S-regulated genes (*saeR/S* targets) remains incompletely defined. Using QuantiGene 2.0 transcriptional assays, we examined expression of genes with the SaeR binding site in USA300 exposed to human and mouse neutrophils and host-derived peptides and during subcutaneous skin infection. We found that only some of the *saeR/S* targets, as opposed to the entire SaeR/S virulon, were activated within 5 and 10 min of interacting with human neutrophils as well as α-defensin. Furthermore, mouse neutrophils promoted transcription of *saeR/S* targets despite lacking α-defensin, and the murine skin environment elicited a distinctive expression profile of *saeR/S* targets. These findings indicate that *saeR/S*-mediated transcription is unique to and dependent on specific host stimuli. By using isogenic USA300Δ*saeR/S* and USA300Δ*agr* knockout strains, we also determined that SaeR/S is the major regulator of virulence factors, while Agr, a quorum-sensing two-component system, has moderate influence on transcription of the *saeR/S* targets under the tested physiological conditions.

Introduction

Staphylococcus aureus is a pathogen of major medical concern that can cause infections ranging from mild skin abscesses to necrotizing pneumonia and sepsis in immunocompromised and healthy individuals. It contributes to 12 million annual outpatient visits for skin and soft tissue infections and is the leading cause of infective endocarditis worldwide [1, 2]. The pathogen's success in hospital and community settings can be partially attributed to elaborate two-component gene-regulatory systems that tightly control expression of virulence and immunomodulatory factors in response to environmental stimuli. The SaeR/S system is recognized as a major regulator of *S. aureus* virulence and is required for successful establishment of skin and invasive infections as well as survival following human neutrophil phagocytosis [3–6].

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Upon activation of the histidine kinase SaeS, the cytosolic response regulator SaeR promotes expression of genes with the SaeR binding site which include leukotoxins, hemolysins, adhesins and proteases that are responsible for *saeR/S*-mediated virulence [3–5, 7]. While expression of genes with the consensus SaeR binding site was shown to be dependent on the SaeR/S system in vitro, *rot* and *arlR* transcription was unaffected by the absence of *saeR/S,* suggesting that expression of genes with the SaeR binding site might be influenced by other regulatory networks and/or environmental conditions [3]. Furthermore, the SaeR/S system has 2 membrane proteins, SaeP and SaeQ, that most likely serve to negatively control the system's activity [8]. Despite the essential role of the SaeR/S system in evading innate immunity, the conditions under which SaeR/S becomes activated as well as the subsequent transcription of genes with SaeR binding sites have not been completely defined.

To this end, we designed QuantiGene 2.0 assays with *S. aureus* genes that contain the SaeR recognition sequence in the promoter region (referred to as *saeR/S* targets) and control targets that do not have the SaeR binding site *(agrA, mecA* and *dltA)* [3, 7]. Using this technique, we were able to measure gene expression changes in *S. aureus* during initial interactions with human and mouse neutrophils and host-derived peptides as well as during subcutaneous skin infection. We also began to elucidate the influence of the quorum-sensing gene regulator Agr on SaeR/S regulon expression, as *agr* controls the expression of a number of virulence factors, including those with SaeR binding sites, depending on the growth conditions [9]. By measuring transcript levels of *saeR/S* targets in *saeR/S* and *agr* isogenic mutants, we found that *saeR/S* is the predominant regulator of genes containing the SaeR binding domain while *agr* has a moderate impact on *saeR/S* target transcription during initial interactions with human neutrophils and in vivo during murine skin infection. Furthermore, we demonstrate that while *saeR/S* regulates expression of a number of virulence factors under physiologically relevant conditions, only a subset of these effectors is transcriptionally upregulated in the presence of neutrophils as well as α-defensin. Supporting this finding, we show that the mouse skin environment promotes transcription of specific *saeR/S* targets, differing from the expression profile elicited following neutrophil interaction or α-defensin, further emphasizing that activation of this two-component system and the subsequent downstream regulation are dependent on the stimuli sensed by *S. aureus*.

Materials and Methods

Bacterial Strains and Culture

Wild-type USA300 (LAC) was used to generate USA300Δ*saeR/S,* USA300Δ*saeR/S*+Comp (complemented with a plasmid encoding *saeR* and *saeS*) and USA300Δ*agr* (isogenic knockout of *agrBDCA*), as previously described [3, 4, 10]. Bacteria used in the studies were cultured in tryptic soy broth with 0.5% glucose and harvested at midexponential growth phase (optical density at 600 nm = 1.5) [11].

Neutrophil and Purified Peptide Assays

Human neutrophils (polymorphonuclear leukocytes, PMNs) were isolated from heparinized venous blood of healthy donors 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. Erythrocytes were removed by dextran sedimentation, and PMNs were separated from peripheral blood mononuclear cells using Histopaque-1077® (Sigma Aldrich) followed by water lysis of remaining erythrocytes under endotoxin-free conditions (<25.0 pg/ml) as described elsewhere [11]. Bone marrow-derived mouse neutrophils were isolated from BALB/c mice using a Percoll gradient and resuspended in RPMI plus 5 mM HEPES media [12]. PMN phagocytosis was synchronized by centrifuging PMNs and bacteria at 380 *g* for 8 min at 4°C [11]. For activation assays with host-derived components, USA300 [5×10^7 colony-forming units (CFUs)] was treated with water or a sublethal dose (confirmed by CFU assays) of either human α-defensin (HNP-1, Millipore), β-defensin (hBD-3, AnaSpec), LL-37 (AnaSpec) or dermcidin (DCD, AnaSpec), all resuspended in water and tested at 0.48 μM, apart from H_2O_2, which was tested at 0.55 mM (AMRESCO). Strains were incubated at 37°C with shaking (250 rpm) for 10 and 30 min (as indicated). The control experiment with lethal doses (determined using CFU assays) was performed by exposing USA300 to α-defensin (4.8 μM) or LL-37 (9.6 μM) for 30 min. At designated time points, samples were lysed with RLT-β-mercaptoethanol buffer and homogenized using FastPrep, and *S. aureus* RNA was isolated using the RNeasy method (Qiagen) [11, 13].

Mouse Studies

Mouse studies performed for this investigation conformed to the NIH guidelines and were approved by the Institutional Animal Care and Use Committee at Montana State University. Crl:SKH1-hrBR mice (Charles River) were inoculated subcutaneously with 2×10^7 CFUs of USA300, USA300Δ*saeR/S* or USA300Δ*agr* in 50 μl of PBS [3, 14]. Eight hours after infection, mice were sacrificed, and skin biopsies were harvested with a 9-mm punch, homogenized and centrifuged. The supernatants were removed, the remaining bacteria pellets were lysed with RLT-β-mercaptoethanol buffer (Qiagen), and *S. aureus* RNA was harvested as described above [11, 13].

QuantiGene 2.0 Assays

QuantiGene 2.0 assays (Affymetrix) were performed on 2 biological replicates of the aforementioned experiments [15]. In brief, purified RNA samples were captured by microsphere beads unique to each gene of interest and hybridized overnight to target-specific DNA probes with biotinylated sites, which were recognized by addition of streptavidin-conjugated R-phycoerythrin. The amplified signal was detected together with the capture bead specific to each gene of interest using a Luminex flow cytometer (BioRad).

Quality control of QuantiGene 2.0 was assessed in preliminary experiments using varied doses of RNA to determine the optimum amount of RNA for subsequent assays. Each *S. aureus* RNA sample (50–100 ng) was assayed in duplicate, and transcript abundance of 15 genes with the SaeR recognition sequence and 3 genes *(agrA*, *mecA* and *dltA)* without the SaeR binding site was detected as the mean fluorescent signal normalized to *gyrB* expression levels. This endogenous control was constitutively expressed in *S. aureus* under the tested conditions. Changes in *S. aureus* gene expression in response to host stimuli were determined by comparing transcript levels in *S. aureus* strain(s) treated with the described stimulus to those in the same strain grown in media (time-matched) (fig. 1, 2). To define the influence of *saeR/S* and *agr* on gene expression, transcript levels in the wild-type strain treated with the relevant host component were compared to gene expression in mutant strains exposed to the same stimulus (fig. 3, 5). Samples that consisted of host RNA alone demonstrated that the probes were *S. aureus* RNA specific (data not shown). QuantiGene 2.0 results were confirmed using TaqMan quantitative RT-PCR (qRT-PCR) as previously described [3, 11], demonstrating that this assay is a suitable alternative to global gene expression assessment methods.

Results

A Subset of saeR/S-*Regulated Genes Is Activated during Early Human Neutrophil Interactions*

While promoter activity and transcript levels of *saeR/S* have been shown to be affected by PMN phagocytosis, individual PMN components and pH changes [9, 11, 16, 17], the effect of host-derived components on *saeR/S* targets has not been completely defined. To this end, we employed QuantiGene 2.0 assays to measure expression changes of 15 genes with the SaeR binding site [3, 7] in a USA300 wild-type strain (LAC) during initial interactions with human neutrophils (fig. 1a). By comparing transcript levels in neutrophil-treated USA300 to those in USA300 grown in media, we identified targets that were immediately activated and thus may be essential to successful initiation of infection by *S. aureus*. Five and 10 min after interaction, transcription of a specific subset of genes, namely *hlgA*, *hlgB*, *hlgC* (γ-hemolysins), *lukA* (leukotoxin A/B or G/H) and *sbi* (immunoglobulin-binding protein), was strongly upregulated in USA300 (fig. 1a; online suppl. table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000351200). In contrast, *lukD* (leukotoxin E/D), *lukF-PVL* (Panton-Valentine leukocidin), *hla* (α-hemolysin), *ssl7* (superantigen-like protein) and *splA* (serine protease) expression did not increase despite the presence of the SaeR binding region (fig. 1a; online suppl. table 1). Although *saeS* transcript levels were unaffected by the treatment, the transcriptional activity of *saeP* and *saeR* increased slightly after interaction with neutrophils while *agrA* and *arlR* expression did not increase (fig. 1a; online suppl. table 1). To confirm the validity of the QuantiGene 2.0 assay, we used TaqMan qRT-PCR to measure changes in the gene expression of USA300 in the presence of neutrophils. In congruence with the QuantiGene 2.0 data, after 10 min, *lukA* transcript was upregulated while *lukF-PVL* and *hla* expression did not change significantly (fig. 1b). Together, these findings suggest that only a subset of *saeR/S*-regulated genes is transcriptionally activated during initial interactions with human neutrophils.

Specific Host Stimuli Promote Transcription of saeR/S-*Regulated Genes*

Unlike LL-37, which does not promote activation of the *sae* promoter, α-defensin and H_2O_2 have been shown to increase *saeR/S* transcript levels [16]. However, the effect of neutrophil-derived components on *saeR/S*-regulated genes has not been completely defined. To address this, we treated USA300 with α-defensin, LL-37 and H_2O_2 for 10 min and investigated changes in transcript levels of *saeR/S* targets. Human α-defensin had the most robust effect on *saeR/S* targets, relatively similar to that of USA300 treated with neutrophils (fig. 1a; online suppl. table 1). Expression of γ-hemolysins *(hlgA, hlgB, hlgC),* leukotoxin AB/GH *(lukA)* and genes of the SaeR/S system was increased, while changes in expression of the remaining target genes were minimal. Despite their antimicrobial properties, LL-37 and H_2O_2 had no effect on transcript abundance of *saeR/S* targets (fig. 1a; online suppl. table 1).

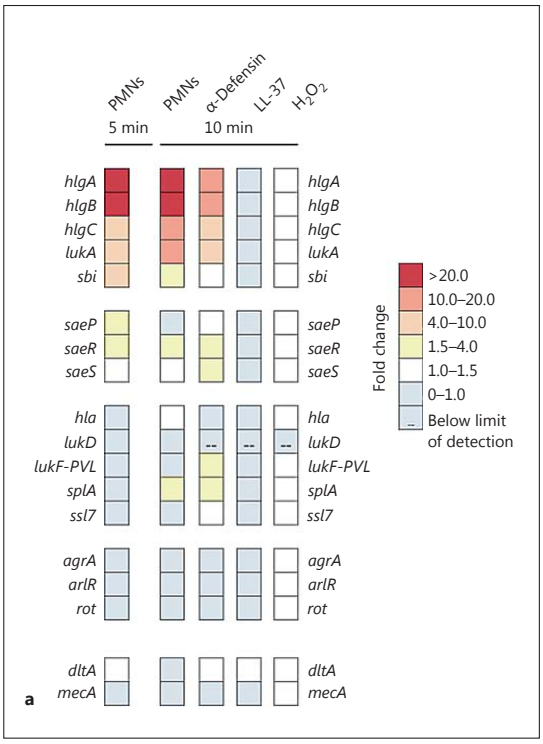
We next wanted to determine whether peptide-mediated killing could promote transcription of *saeR/S* targets to test if cell death is a prominent activator of the SaeR/S system. We measured expression of *saeR/S* targets in *S. aureus* subjected to a lethal dose of α-defensin or LL-37 for 30 min. Even at a higher dosage, LL-37 did not elicit a transcriptional change in *saeR*/S targets, while α-defensin triggered a similar response to that of the sublethal dose (data not shown), suggesting that the transcriptional activation of *saeR/S* targets noted in our studies is due to specific host-derived components as opposed to any antimicrobial insult.

While the expression profiles of *saeR/S* targets were similar between neutrophil- and α-defensin-treated groups (fig. 1a), the difference in the magnitude of expression suggested that α-defensin might contribute only partially to activating *saeR/S* targets in USA300 following neutrophil interaction. To address this, we examined gene expression in *saeR/S* targets after 30-min exposure

to purified human α-defensin, human neutrophils and bone marrow-derived mouse neutrophils that do not produce α-defensins [18] (fig. 2a, b; online suppl. table 2). Despite the absence of α-defensins, *saeR/S*-regulated gene expression increased substantially in response to mouse PMNs (fig. 2a, b; online suppl. table 2). Transcript levels of *hlgA, hlgB, hlgC, lukA, sbi, hla, splA, saeP, saeR* and *saeS* were upregulated, suggesting that, in addition to α-defensin, there are other neutrophil-specific signals that promote activation of *saeR/S* targets (fig 2a). Also, *lukF-PVL* was only upregulated in the presence of α-defensin and human neutrophils, while α-hemolysin *(hla)* and immunoglobulin-binding protein *(sbi)* were increased due to α-defensin and mouse PMNs, further highlighting the dependence of *saeR/S* target expression on the external environment (fig. 2a). We next compared the magnitude of responses among the 3 treatment groups and found that *hlgABC* transcript levels were the highest in the mouse neutrophil-treated group, while α-defensin promoted strong transcription of *lukA* and *sbi* when compared to transcript levels in USA300 treated with human or mouse neutrophils (fig. 2b). In addition, expression of *saeR, saeS* and *saeP* was above the limit of detection in USA300 treated with human neutrophils. To confirm that the regulatory system becomes activated by human neutrophils at 30 min, as reported previously [11], we employed TaqMan qRT-PCR to measure *saeR* levels under the same conditions (data not shown). Transcript levels of *saeR* were increased 10-fold, demonstrating that human neutrophils promote SaeR/S activation 30 min after interaction. These findings suggest that the downstream activation of *saeR/S* targets is independent of *saeR* transcript levels and specific to the host-derived signal.

The SaeR/S Two-Component System Is the Major Regulator of Virulence Factors during Neutrophil Interaction

Although extensive in vitro studies have identified genes regulated by the SaeR/S system [3, 4, 7], we wanted to determine if expression of the relevant targets was also



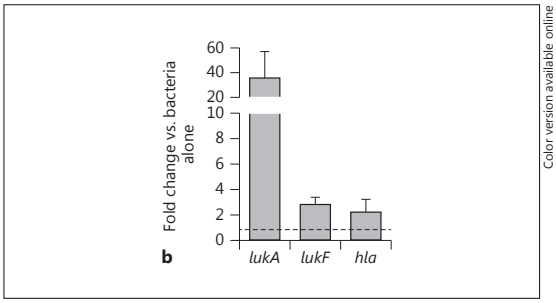
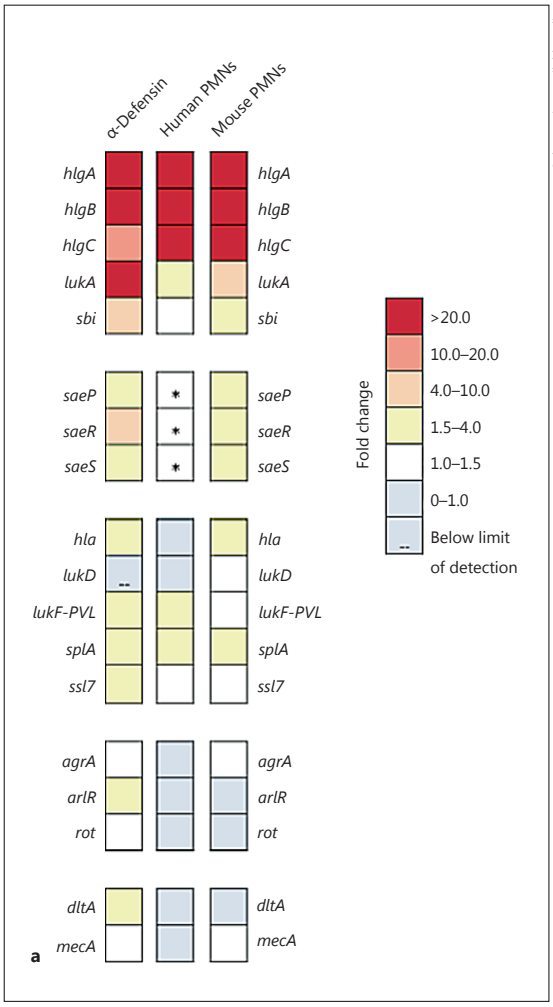


Fig. 1. Differential transcription of *saeR/S* targets is dependent on the host stimulus. **a** QuantiGene 2.0 assays were used to assess transcript levels of the listed *saeR/S* targets in USA300 exposed to human neutrophils (10:1, bacteria to PMN), α-defensin (0.48 μM), LL-37 (0.48 μM) or H_2O_2 (0.55 mM). Data are reported as the mean fold change of 2 separate experiments as shown in online supplementary table 1. Transcript levels in *S. aureus* exposed to the stimulus were normalized to *gyrB* and calibrated to those from *S. aureus* strains grown in media only (time-matched). **b** TaqMan qRT-PCR confirmation of QuantiGene 2.0 data. The fold change is the relative gene expression of *lukA, lukF-PVL* and *hla* measured in USA300 calibrated to *gyrB* levels after 10-min interaction with human neutrophils (10:1, bacteria to PMN) and normalized to baseline gene expression of USA300 grown in media (indicated by the dotted line).

saeR/S-dependent under physiologically relevant conditions. We addressed this by investigating gene expression in USA300Δ*saeR/S* and USA300Δ*agr,* following early interaction with human neutrophils. USA300Δ*agr* was included because previous studies have shown *agr* to have an influence on expression of *saeR/S* targets [9]. By comparing transcript levels of USA300 to those in the mutant strains in the presence of neutrophils, we identified that genes triggered in USA300 *(hlgA*, *hlgB*, *hlgC*, *lukA* and *sbi)* were abolished in USA300Δ*saeR/S* (fig. 3). Although expression of *saeP, hla, lukF-PVL* and *splA* was not upregulated in USA300 treated with neutrophils (fig. 1a), their transcript levels were substantially downregulated in USA300Δ*saeR/S* (fig. 3). In contrast, expression of *rot* and *arlR* was similar in USA300 and USA300Δ*saeR/S,* suggesting that these targets are not solely regulated by *saeR/S* under the conditions tested, an observation supported by in vitro studies (fig. 3) [3]. By assessing the fold change between USA300 and USA300Δ*agr* gene expression in response to neutrophils, we found that transcription of *hlgA, hlgB, hlgC, lukF-PVL* and *splA* was slightly reduced in the absence of *agr* (fig. 3). Furthermore, to confirm that the noted changes were specific to *saeR/S,* we also assessed gene expression during neutrophil interaction using a USA300Δ*saeR/S* strain complemented with a plasmid containing *saeR/S* (online suppl. fig. 1). Complementation restored the wild-type gene expression levels, confirming that transcriptional changes in response to neutrophils are *saeR/S*-mediated (online suppl. fig. 1). These findings suggest that while the SaeR/S system is the major regulator of the relevant genes, Agr has moderate influence over *saeR/S*-regulated gene expression in the presence of human neutrophils.



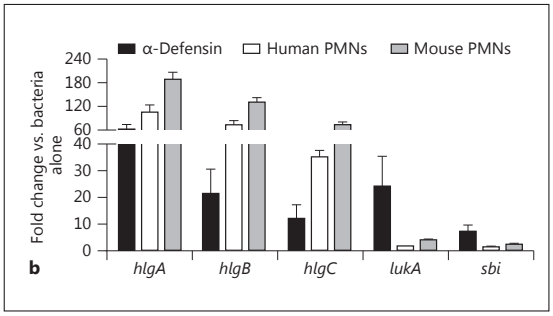


Fig. 2. α-Defensin is not required for neutrophil-mediated activation of the SaeR/S system. USA300 was exposed to α-defensin (0.48 μM), human neutrophils and mouse neutrophils (10:1, bacteria to PMN) for 30 min, and changes in expression were measured using the QuantiGene 2.0 platform. **a** Fold change of *saeR/S* targets. **b** Quantitative comparison of *saeR/S* targets (from **a**) substantially affected by α-defensin, human neutrophils and mouse neutrophils. Data are shown as the mean fold change of 2 separate experiments calculated as stated in figure 1 and listed in online supplementary table 2. Asterisks indicate gene targets with expression above the limit of detection.

saeR/S *Targets Are Activated following Murine Skin Infection*

Next, we expanded on the aforementioned findings by investigating expression of *saeR/S*-regulated genes in vivo. Since *saeR/S* and *agr* are essential to successful initiation of *S. aureus* skin infection [3, 19, 20], we hypothesized that the skin environment will activate *saeR/S* targets. To this end, we isolated *S. aureus* RNA from skin abscesses of mice 8 h following infection with USA300, USA300Δ*saeR/S* or USA300Δ*agr* and measured gene expression using the QuantiGene 2.0 assay. To assess changes in *saeR/S* target expression elicited by the skin environment, we compared gene transcription in *S. aureus* strains from the abscess to transcript levels in corresponding strains at midexponential growth, representing the initial inoculum. With the exception of *sbi, saeR/S* targets *hlgA, hlgB, hlgC* and *lukA,* previously shown to be activated by mouse neutrophils (fig. 2a, b), were also highly expressed in USA300 and USA300Δ*agr* but abolished in the USA300Δ*saeR/S* strain (fig. 4a; online suppl. table 3). On the other hand, only the skin environment triggered robust transcription (fold change in parentheses) of *hla* (6.4), *splA* (7.65) and *ssl7* (3.95) in USA300 (fig. 4b; online suppl. table 3) when compared to mouse neutrophil-induced expression of *hla* (1.52), *splA* (2.43) and *ssl7* (1.16) (fig. 2a, b; online suppl. table 2), further emphasizing that the specificity of the transcriptional response in *S. aureus* is dependent on the external environment. Our results also confirm previous studies that showed the *saeR/S* mutant to have increased levels of *mecA* expression, which are likely due to an indirect effect since *mecA* does not have the SaeR binding site [3].

In order to determine the influence of *saeR/S* and *agr* on expression of the relevant targets, we compared transcript levels of the wild type to those in the mutant strains 8 h after skin infection. Genes that were downregulated in USA300Δ*saeR/S* during neutrophil interaction (fig. 3) were also substantially repressed in USA300Δ*saeR/S* in the skin, and *lukF-PVL* and *splA* were downregulated in USA300Δ*agr* (fig. 5). Surprisingly, *hlgA, hlgB, hlgC* and *ssl7,* which were repressed in the presence of neutrophils in USA300Δ*agr* (fig. 3), were upregulated during skin infection (fig. 5). While higher transcript levels of the virulence factors in USA300Δ*agr* were unexpected, they correlate with increased expression of *saeR/S* in the USA300Δ*agr* strain during skin infection (fig. 5). Additionally, increased transcription of *agrA* in the *saeR/S* mutant during skin infection implies that *saeR/S* and *agr* might compensate for each other's absence to promote transcription of essential virulence factors (fig. 5).

Locus	USA300 vs. USA300Δ*saeR/S*	USA300 vs. USA300Δ*agr*
agrA	2.1	–2,259.5
arlR	1.8	–2.0
dltA	1.7	1.6
hla	–141.2	2.6
hlgA	–149.0	–5.3
hlgB	–221.0	–4.4
hlgC	–49.7	–4.6
lukA	–56.5	–2.2
lukD	–2.5	–2.0
lukF-PVL	–3.1	–4.1
mecA	0.7	1.7
rot	0.9	1.4
saeP	–153.1	1.5
saeR	–16,547.6	1.6
saeS	–2,283.5	1.5
sbi	–361.8	1.5
splA	–13.1	–2.6
ssl	–2.0	–2.0

Genes regulated by:
SaeR/S
Agr
SaeR/S and Agr
Not regulated

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Fig. 3. USA300 genes regulated by *saeR/S* and *agr* following interaction with human neutrophils. QuantiGene 2.0 assays were used to assess transcript levels of the listed *saeR/S*-regulated genes in USA300, USA300Δ*saeR/S* or USA300Δ*agr* exposed to human neutrophils (10:1, bacteria to PMN). The mean fold change of 2 separate experiments was calculated by comparing *gyrB*-calibrated transcript levels in wild-type USA300 to those in USA300Δ*saeR/S* and USA300Δ*agr* isogenic knockout strains 10 min after neutrophil interaction. Blue and yellow depict genes regulated by *saeR/S* and *agr,* respectively, and genes influenced by both systems are shaded green.

Discussion

The ability of *S. aureus* to evade neutrophils as well as establish invasive and skin infections has been linked to the two-component system SaeR/S that regulates expression of virulence factors and immunomodulatory proteins [3–5, 7, 20]. In this investigation, we studied physiological triggers of SaeR/S and how host stimuli affect *saeR/S*-regulated gene expression. We found that a robust transcriptional response of genes with the SaeR binding site in USA300 takes place as early as 5 min after human neutrophil interaction. The immediate activation of the specific *saeR/S* targets *hlgA, hlgB, hlgC, lukA* and *sbi* in response to human PMNs suggests that these effectors might be required for initiation of infection in humans. Our findings correspond with previous reports that showed *hlgABC, lukAB/lukGH* and *sbi* promote neutrophil cell death and/or *S. aureus* survival during neutrophil phagocytosis [21–24]. Lack of pronounced transcription of *hla* during early neutrophil interaction also coincides with studies that demonstrated Hla does not promote cell death of human neutrophils; however, this toxin plays an important role in targeting lymphocyte populations [25]. Given that the

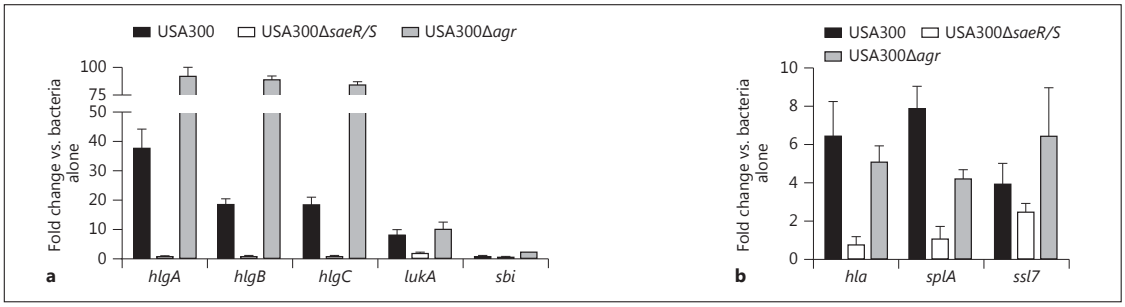

Fig. 4. The skin environment promotes *saeR/S* target transcription. QuantiGene 2.0 assays were used to assess transcript levels of the genes shown in skin abscesses 8 h after subcutaneous inoculation with USA300, USA300Δ*saeR/S* or USA300Δ*agr*. **a** Genes previously shown to be activated by other stimuli (fig. 1, 2). **b** Additional targets activated by the skin environment. Transcript levels were normalized to *gyrB* and calibrated to those from corresponding *S. aureus* strains at midexponential growth phase. Data are reported as the mean fold change of 2 separate experiments with 2 mice per group (values shown in online suppl. table 3).

SaeR/S system promotes PMN damage and is required for *S. aureus* survival following phagocytosis [4], our findings imply that *saeR/S* activation triggers a PMN-specific transcriptional response facilitating upregulation of virulence factors essential to evade neutrophil killing. This idea is supported by the observation that not all genes containing the SaeR binding site were activated in response to neutrophils even though their transcription is dependent on the SaeR/S system [3, 7]. While the quorum system Agr had influence over several *saeR/S* targets, its impact on expression of the virulence factors during early interaction with the neutrophils was not as robust. This finding was expected as transcription of *agr* is not stimulated by the neutrophil environment at the multiplicities of infection tested [11; this study] and is not essential to USA300 survival during PMN interaction [10]. Collectively, these findings highlight the specificity of the *saeR/S*-mediated transcriptional response to neutrophils.

Another goal of our study was to define expression of *saeR/S* targets during the initial interactions with neutrophil-derived components. We found that human α-defensin elicited transcriptional changes of *saeR/S* targets 10 and 30 min after treatment, in accordance with previous reports [8, 16, 17]. While we cannot rule out that α-defensin-mediated cell membrane disruption promotes activation of SaeS, especially since antimicrobial peptides elicit cell damage through various mechanisms [26], LL-37 did not activate *saeR/S* at sublethal nor at lethal concentrations. This further emphasizes that SaeR/S activation is dependent on particular stimuli as opposed to any type of microbial damage.

Locus	USA300 vs. USA300Δ*saeR/S*	USA300 vs. USA300Δ*agr*
agrA	2.5	–280.0
arlR	1.1	1.9
dltA	–2.1	1.8
hla	–129.0	1.3
hlgA	–63.6	3.3
hlgB	–39.4	2.8
hlgC	–27.5	2.6
lukA	–15.7	1.0
lukD	ULD	ULD
lukF-PVL	–4.0	–3.1
mecA	10.0	0.7
rot	0.9	1.5
saeP	–5.5	2.6
saeR	–3,289.4	2.3
saeS	–3,089.1	2.0
sbi	–108.3	3.7
splA	–6.5	–2.9
ssl7	1.2	3.1

Genes regulated by:
SaeR/S
Agr
SaeR/S and Agr
Not regulated

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Fig. 5. USA300 genes regulated by *saeR/S* and *agr* during subcutaneous skin infection. The mean fold change of 2 separate experiments, described in figure 4, was calculated by comparing *gyrB*-calibrated transcript levels in wild-type USA300 to those in USA300Δ*saeR/S* and USA300Δ*agr* 8 h after inoculation. ULD = Under the limit of detection. Blue and yellow depict genes regulated by *saeR/S* and *agr,* respectively, and genes influenced by both systems are shaded green.

α-Defensin, contained within primary azurophilic granules of the human PMN, has been suggested to be the molecular trigger of SaeR/S [8, 16, 17]; however, the expression profile elicited by α-defensin was relatively different in magnitude from that of the human neutrophil.

This observation prompted us to determine whether neutrophils had to contain α-defensin to promote *saeR/S* target activation by exposing *S. aureus* to mouse neutrophils. We chose mouse PMNs because they are similar in function to the human neutrophil but do not contain α-defensin [18]. Surprisingly, mouse PMNs upregulated transcription of *saeR/S* targets even more robustly than human neutrophils. Based on these observations, we propose that while α-defensin is an essential activator of the SaeR/S system, there are other neutrophil-derived components and processes such as phagocytosis, reactive oxygen species production and/or degranulation that might promote transcription of *saeR/S* targets.

Next, we wanted to define transcript changes of *saeR/S* targets in vivo. As expected, the skin environment promoted a robust transcription of *saeR/S* targets with γ-hemolysins and leukotoxin AB/GH transcript levels being substantially high 8 h after inoculation. Interestingly, *lukAB* and *hlgABC* have been shown to be dispensable for abscess formation during skin infection in rabbits and mice, respectively [27, 28]. This could be due to the redundancy of various toxins produced by *S. aureus* that might compensate for the lack of HlgABC or LukAB/GH. It remains to be determined whether these toxins play a role in modulating immune responses that could be important for the progression of infection.

The specificity of the *saeR/S*-mediated response during skin infection was further highlighted by increased expression of α-hemolysin transcript, which is essential to dermonecrosis formation [19, 27]. Given that skin abscesses are composed of various cell types, including lymphocytes that are susceptible to Hla, our results suggest there are additional skin-derived triggers of the SaeR/S system. To begin to address this, we measured mRNA levels of *saeR/S* targets following exposure to skin-derived peptides β-defensin and dermicidin; however, neither of the stimuli promoted SaeR/S activation (data not shown). While the infiltrated neutrophils at the site of infection and the long exposure to the skin environment likely contribute to the observed expression profile, future investigations are needed to identify triggers of the SaeR/S system in the skin.

To confirm the impact of SaeR/S on gene expression in vivo, we examined *saeR/S* target expression in USA300Δ*saeR/S* and USA300Δ*agr*. As in the case of human neutrophils, expression of the majority of the genes with the SaeR binding site was dependent on the SaeR/S system during skin infection. Although deletion of *agr* results in reduced pathogenesis of *S. aureus* in the skin [20, 29], the transcript levels of several virulence factors, including α-hemolysin, which contributes to dermonecrosis, were either equal or substantially higher in the *agr* mutant when compared to the wild-type strain. This discrepancy can be explained by the role of Agr in posttranscriptional regulation of toxins such as α-hemolysin which translation is dependent on *agr* [30]. Furthermore, genes that were upregulated in the *agr* mutant in the skin were downregulated in the same mutant during neutrophil interaction. The biphasic regulation could be due to a variety of factors including the difference in external stimuli, bacteria growth phase and/or timing (10 min with neutrophils vs. 8 h in the skin). These findings further underscore that regulation of transcription mediated by two-component systems is complex and highly specific to the environment sensed by *S. aureus*.

The finding that only select *saeR/S*-regulated genes, as opposed to all targets with the SaeR recognition sequence, were transcriptionally upregulated in response to *saeR/S*-activating stimuli was unexpected. We suggest that differential activation of *saeR/S* targets is dependent on specific stimuli, most likely in concert with other regulatory systems, RNA decay mechanisms [31] and/or transcription factors such as negative regulator of toxins, which has been shown to repress *saeR/S*-regulated genes [32]. In addition, the temporal expression of targets such as *lukA* and *sbi,* which declined over the 30-min exposure to human neutrophils, as opposed to *hlgABC* mRNA levels that increased over time, suggests that the transcription kinetics of *saeR/S* targets is important and should be investigated in respect to *S. aureus* pathogenesis. Finally, previous studies proposed that the availability of phosphorylated SaeR (SaeR-P) and arrangement of the SaeR binding sites influence the differential regulation of the *saeR/S* targets [33, 34]. While some genes might require low levels of SaeR-P to be transcriptionally active, others will be activated in the presence of high SaeR-P concentrations [33]. Since transcript levels of *saeR/S* do not correlate with the robustness of the downstream gene expression [33; this study], it is likely that SaeR-P levels might dictate the magnitude of the gene transcription downstream of the SaeR/S system. Thus, future studies are needed to determine the effect of external stimuli on SaeR-P levels along with the influence of differences within the SaeR binding site sequence on differential transcription of *saeR/S* targets.

To our knowledge, this is the first study to identify genes that are regulated by *saeR/S* and *agr* under physiologically relevant conditions and determine the effects of host-derived stimuli including skin on *saeR/S* target gene expression. Our findings suggest that the SaeR/S system is

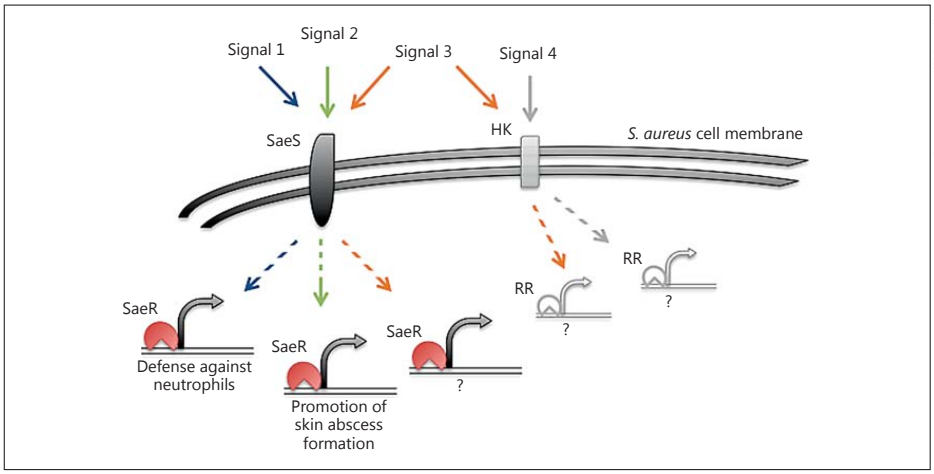

Fig. 6. A model representing the dependence of *saeR/S*-mediated transcription on host-derived signals. Depending on the source of SaeR/S system activation, only specific subsets of genes with the SaeR binding site are activated during early interactions with host components and subcutaneous skin infection. In the presence of neutrophils and α-defensin (signal 1), activation of the SaeR/S system leads to promotion of gene transcription necessary to evade neutrophils. When stimulated by the skin environment (signal 2), specific *saeR/S* targets, including genes that promote skin abscess formation, are transcriptionally activated and vary in type and robustness of response from those stimulated by signal 1. Given that mouse neutrophils promote SaeR/S activation despite lacking α-defensin, there are additional host-derived components (signal 3) that can activate the SaeR/S system and potentially stimulate other gene-regulatory networks promoting pathogen survival. Additionally, activation of the SaeR/S system is highly specific to the external environment since not all host-derived factors (signal 4) promote transcription of SaeR/S targets. HK = Histidine kinase; RR = response regulator.

a major regulator of virulence factors in the initial stages of *S. aureus* interaction with the innate immune system, while the Agr system has a partial influence on *saeR/S* target expression. Given that SaeR/S is required for successful establishment of skin and invasive *S. aureus* infections, subsequent studies are necessary to determine which of the *saeR/S* targets are responsible for *saeR/S*-mediated pathogenesis. Moreover, with the recent findings that the WalKR system potentially influences SaeR/S activity [35], future analyses will include the contribution of additional regulatory networks to gene expression of *saeR/S* targets.

Collectively, our findings provide insight into gene regulation in *S. aureus* and highlight the importance of examining downstream events of gene-regulatory systems upon their stimulation. To that end, we hypothesize that activation of SaeR targets depends on the stimulus and that only specific subsets of genes respond on a transcriptional level, depending on the environment (fig. 6). This viable strategy allows the pathogen to tailor its response by expending energy only on factors necessary to survive the pending host challenge.

Acknowledgments

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Disclosure Statement

The authors declare that there are no competing financial interests.

References

1 Fowler VG, Miro JM, Hoen B, Cabell CH, Abrutyn E, Rubinstein E, et al: *Staphylococcus aureus* endocarditis: a consequence of medical progress. JAMA 2005;293:3012–3021.

2 McCaig LF, McDonald LC, Mandal S, Jernigan DB: *Staphylococcus aureus*-associated skin and soft tissue infections in ambulatory care. Emerg Infect Dis 2006;12:1715–1723.

3 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;201:241–254.

4 Voyich JM, Vuong C, DeWald M, Nygaard TK, Kocianova S, Griffith S, et al: The SaeR/S gene regulatory system is essential for innate immune evasion by *Staphylococcus aureus.* J Infect Dis 2009;199:1698–1706.

5 Watkins RL, Pallister KB, Voyich JM: The SaeR/S gene regulatory system induces a proinflammatory cytokine response during *Staphylococcus aureus* infection. PLoS One 2011;6:e19939.

6 Montgomery CP, Daum RS: Transcription of inflammatory genes in the lung after infection with community-associated methicillin-resistant *Staphylococcus aureus:* a role for panton-valentine leukocidin? Infect Immun 2009;77:2159–2167.

7 Sun F, Li C, Jeong D, Sohn C, He C, Bae T: 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 2010;192:2111–2127.

8 Jeong D-W, Cho H, Jones MB, Shatzkes K, Sun F, Ji Q, et al: The auxiliary protein complex SaePQ activates the phosphatase activity of sensor kinase SaeS in the SaeRS two-component system of *Staphylococcus aureus.* Mol Microbiol 2012;86:331–348.

9 Novick RP: The staphylococcal saeRS system coordinates environmental signals with agr quorum sensing. Microbiology 2003;149:2709–2717.

10 Pang YY, Schwartz J, Thoendel M, Ackermann LW, Horswill AR, Nauseef WM: agr-dependent interactions of *Staphylococcus aureus* USA300 with human polymorphonuclear neutrophils. J Innate Immun 2010;2:546–559.

11 Voyich JM, Braughton KR, Sturdevant DE, Whitney AR, Saïd-Salim B, Porcella SF, et al: Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. J Immunol 2005;175:3907–3919.

12 Siemsen DW, Schepetkin IA, Kirpotina LN, Lei B, Quinn MT: Neutrophil isolation from nonhuman species. Methods Mol Biol 2007;412:21–34.

13 Voyich JM, Sturdevant DE, DeLeo FR: Analysis of *Staphylococcus aureus* gene expression during PMN phagocytosis. Methods Mol Biol 2008;431:109–122.

14 Voyich JM, Otto M, Mathema B, Braughton KR, Whitney AR, Welty D, et al: Is Panton-Valentine leukocidin the major virulence determinant in community-associated methicillin-resistant *Staphylococcus aureus* disease? J Infect Dis 2006;194:1761–1770.

15 Affymetrix: QuantiGene 2.0 Assay User Manual. https://www.panomics.com/downloads/UM13074_QG2Manual_RevB_080102.pdf (accessed January 20, 2013).

16 Geiger T, Goerke C, Mainiero M, Kraus D, Wolz C: The virulence regulator Sae of *Staphylococcus aureus:* promoter activities and response to phagocytosis-related signals. J Bacteriol 2008;190:3419–3428.

17 Palazzolo-Ballance AM, Reniere ML, Braughton KR, Sturdevant DE, Otto M, Kreiswirth BN, et al: Neutrophil microbicides induce a pathogen survival response in community-associated methicillin-resistant *Staphylococcus aureus.* J Immunol 2008;180:500–509.

18 Eisenhauer PB, Lehrer RI: Mouse neutrophils lack defensins. Infect Immun 1992;60:3446–3447.

19 Kennedy AD, Wardenburg JB, Gardner DJ, Long D, Whitney AR, Braughton KR, et al: Targeting of alpha-hemolysin by active or passive immunization decreases severity of USA300 skin infection in a mouse model. J Infect Dis 2010;202:1050–1058.

20 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.

21 DuMont AL, Nygaard TK, Watkins RL, Smith A, Kozhaya L, Kreiswirth BN, et al: Characterization of a new cytotoxin that contributes to *Staphylococcus aureus* pathogenesis. Mol Microbiol 2010;79:814–825.

22 Ventura CL, Malachowa N, Hammer CH, Nardone GA, Robinson MA, Kobayashi SD, et al: Identification of a novel *Staphylococcus aureus* two-component leukotoxin using cell surface proteomics. PLoS One 2010;5:e11634.

23 Malachowa N, Whitney AR, Kobayashi SD, Sturdevant DE, Kennedy AD, Braughton KR, et al: Global changes in *Staphylococcus aureus* gene expression in human blood. PLoS One 2011;6:e18617.

24 Smith EJ, Visai L, Kerrigan SW, Speziale P, Foster TJ: The Sbi protein is a multifunctional immune evasion factor of *Staphylococcus aureus.* Infect Immun 2011;79:3801–3809.

25 Nygaard TK, Pallister KB, DuMont AL, DeWald M, Watkins RL, Pallister EQ, et al: Alpha-toxin induces programmed cell death of human T cells, B cells, and monocytes during USA300 Infection. PLoS One 2012;7:e36532.

26 Midorikawa K, Ouhara K, Komatsuzawa H, Kawai T, Yamada S, Fujiwara T, et al: *Staphylococcus aureus* susceptibility to innate antimicrobial peptides, beta-defensins and CAP18, expressed by human keratinocytes. Infect Immun 2003;71:3730–3739.

27 Kobayashi SD, Malachowa N, Whitney AR, Braughton KR, Gardner DJ, Long D, et al: Comparative analysis of USA300 virulence determinants in a rabbit model of skin and soft tissue infection. J Infect Dis 201;204:937–941.

28 Malachowa N, Kobayashi SD, Braughton KR, Whitney AR, Parnell MJ, Gardner DJ, et al: *Staphylococcus aureus* leukotoxin GH promotes inflammation. J Infect Dis 2012;206:1185–1193.

29 Wright JS, Jin R, Novick RP: Transient interference with staphylococcal quorum sensing blocks abscess formation. Proc Natl Acad Sci USA 2005;102:1691–1696.

30 Novick RP, Ross HF, Projan SJ, Kornblum J, Kreiswirth B, Moghazeh S: Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. EMBO J 1993;12:3967–3975.

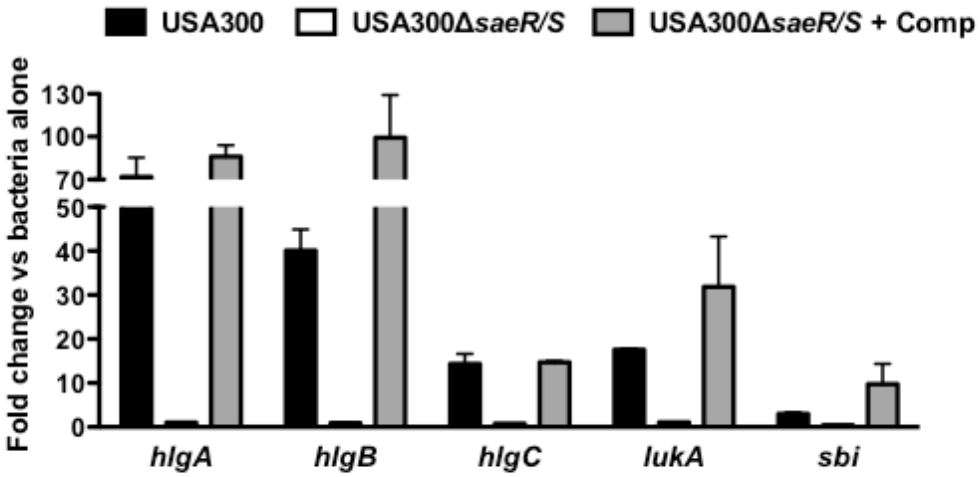
31 Marincola G, Schäfer T, Behler J, Bernhardt J, Ohlsen K, Goerke C, et al: RNase Y of *Staphylococcus aureus* and its role in the activation of virulence genes. Mol Microbiol 2012;85:817–832.

32 Said-Salim B, Dunman PM, McAleese FM, Macapagal D, Murphy E, McNamara PJ, et al: Global regulation of *Staphylococcus aureus* genes by Rot. J Bacteriol 2003;185:610–619.

33 Mainiero M, Goerke C, Geiger T, Gonser C, Herbert S, Wolz C: Differential target gene activation by the *Staphylococcus aureus* two-component system saeRS. J Bacteriol 2010;192:613–623.

34 Cho H, Jeong DW, Li C, Bae T: Organizational requirements of the SaeR binding sites for a functional P1 promoter of the sae operon in *Staphylococcus aureus.* J Bacteriol 2012;194:2865–2876.

35 Delaune A, Dubrac S, Blanchet C, Poupel O, Mader U, Hiron A, et al: The WalKR system controls major staphylococcal virulence genes and is involved in triggering the host inflammatory response. Infect Immun 2012;80:3438–3453.



Online Suppl. Fig. 1. Transcription of genes stimulated by neutrophils in USA300 is dependent on *saeR/S* expression. QuantiGene 2.0 assays were used to measure transcript levels of *hlgA, hlgB, hlgC, lukA* and *sbi* in USA300, USA300Δ*saeR/S* or USA300Δ*saeR/S* + Comp subjected to human neutrophils for 10 minutes (10:1 bacteria to PMN). Gene expression in PMN-treated *S. aureus* strains was normalized to *gyrB* and calibrated to transcript levels in corresponding strains grown in media. Besides USA300Δ*saeR/S* that was assessed once, data are shown as mean fold change of two separate experiments.

Online Suppl. Table 1. Host-factors differentially regulate *saeR/S* targets

		Human PMNs	**Human PMNs**	**α-Defensin**	**LL-37**	**H_2O_2**
		5 min	10 min	10 min	10 min	10 min
ID	Locus	USA300	USA300	USA300	USA300	USA300
SAUSA300_1992	***agrA***	0.61 +/- 0.4	0.86 +/- 0.16	0.92 +/- 0.12	0.93 +/- 0.04	1.21 +/- 0.06
SAUSA300_1308	***arlR***	0.76 +/- 0.23	0.59 +/- 0.01	0.97 +/- 0.01	0.79 +/- 0.04	1.09 +/- 0.2
SAUSA300_0835	***dltA***	1.3 +/- 0.79	0.56 +/- 0.13	1.12 +/- 0.08	1.28 +/- 0.2	1.08 +/- 0.31
SAUSA300_1058	***hla***	0.77 +/- 0.37	1.15 +/- 0.13	0.94 +/- 0.02	0.71 +/- 0.11	1.1 +/- 0.41
SAUSA300_2365	***hlgA***	24.65 +/- 12.98	71.86 +/- 19.15	17.32 +/- 5.23	0.69 +/- 0.09	1.05 +/- 0.17
SAUSA300_2366	***hlgB***	23.33 +/- 9.2	40.1 +/- 6.8	10.08 +/- 3.9	0.83 +/- 0.22	1.31 +/- 0.4
SAUSA300_2367	***hlgC***	7.08 +/- 1.11	14.39 +/- 3.19	7.51 +/- 2.5	0.7 +/- 0.14	1.03 +/- 0.16
SAUSA300_1974	***lukA***	4.35 +/- 2.96	17.59 +/- 0.28	4.46 +/- 0.66	0.78 +/- 0.55	1.03 +/- 0.59
SAUSA300_1768	***lukD***	0.58 +/- 0.34	0.75 +/- 0.14	0 +/- 0	0 +/- 0	0 +/- 0
SAUSA300_1381	***lukF-PVL***	0.7 +/- 0.13	0.94 +/- 0.23	1.53 +/- 0.11	0.88 +/- 0.07	1.12 +/- 0.23
SAUSA300_0032	***mecA***	0.73 +/- 0.25	0.68 +/- 0.03	0.91 +/- 0.08	0.97 +/- 0.1	1.16 +/- 0.11
SAUSA300_1708	***rot***	0.66 +/- 0.19	0.49 +/- 0.16	0.85 +/- 0.04	0.87 +/- 0.05	1 +/- 0.04
SAUSA300_0693	***saeP***	2.65 +/- 1.22	0.8 +/- 0.13	1.27 +/- 0.38	0.6 +/- 0.06	1.17 +/- 0.23
SAUSA300_0691	***saeR***	2 +/- 0.21	1.78 +/- 0.35	2.17 +/- 1.05	0.63 +/- 0.03	1.1 +/- 0.3
SAUSA300_0690	***saeS***	1.02 +/- 0.35	1.09 +/- 0.25	1.74 +/- 0.76	0.67 +/- 0.01	1.18 +/- 0.3
SAUSA300_2364	***sbi***	7.73 +/- 2.43	3.02 +/- 0.45	1.31 +/- 0.34	0.68 +/- 0.04	1.26 +/- 0.27
SAUSA300_1758	***splA***	0.86 +/- 0.12	1.59 +/- 0.75	1.96 +/- 0.31	0.85 +/- 0.03	1.13 +/- 0.28
SAUSA300_0401	***ssl7***	0.4 +/- 0.06	0.56 +/- 0.08	1.27 +/- 0.01	0.91 +/- 0.07	1.18 +/- 0.09

Gene expression in USA300 treated with the depicted stimulus was measured by QuantiGene 2.0 assays and normalized to *gyrB* transcript levels. Ratio of gene expression in stimulus-treated vs untreated *S. aureus* (time-matched) is shown as mean fold change +/- standard deviation of two separate experiments. 0 = Gene expression under limit of detection.

Online Suppl. Table 2. Host-factors differentially regulate *saeR/S* targets

		α-Defensin	**Human PMNs**	**Mouse PMNs**
		30 min	30 min	30 min
ID	Locus	USA300	USA300	USA300
SAUSA300_1992	***agrA***	1.23 +/- 0.28	0.38 +/- 0.01	1.03 +/- 0.47
SAUSA300_1308	***arlR***	1.58 +/- 0.46	0.4 +/- 0.08	0.92 +/- 0.2
SAUSA300_0835	***dltA***	2.16 +/- 0.64	0.36 +/- 0.1	0.6 +/- 0.12
SAUSA300_1058	***hla***	1.98 +/- 1.34	0.74 +/- 0.17	1.52 +/- 0.49
SAUSA300_2365	***hlgA***	61.41 +/- 19.07	103.94 +/- 28.11	187.88 +/- 25.04
SAUSA300_2366	***hlgB***	21.53 +/- 12.88	73.01 +/- 15.32	130.23 +/- 17.07
SAUSA300_2367	***hlgC***	12.04 +/- 7.41	35.06 +/- 3.57	72.6 +/- 10.73
SAUSA300_1974	***lukA***	24.03 +/- 16.01	1.7 +/- 0.01	4.06 +/- 0.55
SAUSA300_1768	***lukD***	0 +/- 0	0.71 +/- 0.02	1.16 +/- 0.13
SAUSA300_1381	***lukF-PVL***	3.74 +/- 0.21	1.82 +/- 0.46	1.37 +/- 0.14
SAUSA300_0032	***mecA***	1.33 +/- 0.33	0.63 +/- 0.04	1.03 +/- 0.19
SAUSA300_1708	***rot***	1.37 +/- 0.57	0.89 +/- 0.46	0.97 +/- 0.04
SAUSA300_0693	***saeP***	3.04 +/- 2.11	*	2.09 +/- 0.46
SAUSA300_0691	***saeR***	5.85 +/- 3.85	*	2.33 +/- 0.6
SAUSA300_0690	***saeS***	3.6 +/- 1.97	*	1.67 +/- 0.12
SAUSA300_2364	***sbi***	7.15 +/- 3.66	1.39 +/- 0.45	2.42 +/- 0.63
SAUSA300_1758	***splA***	3.82 +/- 2.38	1.52 +/- 0.95	2.43 +/- 0.1
SAUSA300_0401	***ssl7***	2.65 +/- 0.58	1.1 +/- 0.93	1.16 +/- 0.09

Gene expression in USA300 treated with the depicted stimulus was measured by QuantiGene 2.0 assays and normalized to *gyrB* transcript levels. Ratio of gene expression in stimulus-treated vs untreated *S. aureus* (time-matched) is shown as mean fold change +/- standard deviation of two separate experiments. 0 = Gene expression under the limit of detection; *Gene expression above limit of detection.

Online Suppl. Table 3. Skin environment promotes transcription of *saeR/S* targets

ID	Locus	USA300	USA300Δ*saeR/S*	USA300Δ*agr*
SAUSA300_1992	***agrA***	1.25 +/- 0.37	1.84 +/- 0.14	0 +/- 0
SAUSA300_1308	***arlR***	1.67 +/- 0.8	1.46 +/- 0.56	0.95 +/- 0.04
SAUSA300_0835	***dltA***	1.21 +/- 0.36	0.78 +/- 0.3	0.51 +/- 0.25
SAUSA300_1058	***hla***	6.4 +/- 4.19	0.79 +/- 1.11	5.08 +/- 1.44
SAUSA300_2365	***hlgA***	37.32 +/- 13.37	0.54 +/- 0.77	124.68 +/- 45.88
SAUSA300_2366	***hlgB***	18.39 +/- 2.14	0 +/- 0	115.78 +/- 6.72
SAUSA300_2367	***hlgC***	18.21 +/- 3.14	0.67 +/- 0.94	101.37 +/- 9.21
SAUSA300_1974	***lukA***	7.86 +/- 4.68	1.81 +/- 0.17	9.96 +/- 5.44
SAUSA300_1768	***lukD***	0 +/- 0	0 +/- 0	0 +/- 0
SAUSA300_1381	***lukF-PVL***	2.84 +/- 0.28	2.11 +/- 0.13	3.01 +/- 0.52
SAUSA300_0032	***mecA***	0.63 +/- 0.05	2.26 +/- 0.23	1.08 +/- 0.04
SAUSA300_1708	***rot***	3.45 +/- 0.46	2.82 +/- 1.38	0.47 +/- 0.17
SAUSA300_0693	***saeP***	1.52 +/- 0.18	3.12 +/- 1.85	4.13 +/- 1.1
SAUSA300_0691	***saeR***	1.35 +/- 0.63	0 +/- 0	2.36 +/- 0.52
SAUSA300_0690	***saeS***	1.28 +/- 0.26	0 +/- 0	1.99 +/- 0.23
SAUSA300_2364	***sbi***	0.82 +/- 0.37	0.4 +/- 0.57	2.14 +/- 0.23
SAUSA300_1758	***splA***	7.65 +/- 0.98	1.09 +/- 1.53	3.2 +/- 0.58
SAUSA300_0401	***ssl7***	3.95 +/- 1.67	2.5 +/- 0.93	6.41 +/- 4.24

Gene expression in USA300, USA300Δ*saeR/S* and USA300Δ*agr* was measured by QuantiGene 2.0 assays and normalized to *gyrB* transcript levels. Ratio of gene expression in strains from the skin abscess 8 hours post-infection vs bacteria grown at mid-exponential phase (strain-matched) is shown as mean fold change +/- standard deviation of two separate experiments. 0 = Gene expression under the limit of detection.

CHAPTER THREE

STAPHYLOCOCCUS AUREUS INHIBITS NEUTROPHIL-DERIVED IL-8 TO PROMOTE CELL DEATH

Contribution of Authors and Co-Authors

Author: Oliwia W Zurek

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

Co-Authors: Kyler B. Pallister

Contributions: Assisted in experiments and critiqued the manuscript.

Co-Author: Jovanka M. Voyich

Contributions: Conceived the study; provided funding and edited/critiqued manuscript in every stage of preparation.

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BRIEF REPORT

Staphylococcus aureus Inhibits Neutrophil-derived IL-8 to Promote Cell Death

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While *Staphylococcus aureus* accelerates human neutrophil cell death, the underlying host- and pathogen-derived mechanisms remain incompletely defined. Previous studies demonstrated that the *S. aureus* SaeR/S sensory system is essential for pathogen survival following neutrophil phagocytosis. Herein, we demonstrate that the SaeR/S system promoted accelerated cell death, suppressed phosphorylation of nuclear factor-κB, and reduced interleukin-8 (IL-8) production in human neutrophils. Treatment of neutrophils with recombinant IL-8 significantly reduced bacterial burden and apoptosis. Our findings demonstrate a mechanism by which *S. aureus* suppresses the early neutrophil-derived IL-8 response to disrupt cell fate and promote disease.

Keywords. cell fate; IL-8; neutrophils; NF-κB; pathogenesis; SaeR/S; *Staphylococcus aureus*.

Staphylococcus aureus is a common pathogen that can infect immunocompromised as well as healthy individuals. Human polymorphonuclear leukocytes (PMNs or neutrophils) are the first line of defense against *S. aureus*. It follows that the ability of *S. aureus* to initiate infection includes survival after PMN phagocytosis and alteration of PMN cell fate [1–3]. Although it has been shown that *S. aureus* alters neutrophil lifespan by inducing rapid neutrophil death (referred to as programmed necrosis or necroptosis), the host and pathogen mechanisms associated with this phenotype have not been fully elucidated [2, 3]. To advance our understanding of how *S. aureus* influences PMN cell fate to promote disease, we investigated the role of the SaeR/S 2-component system (TCS) in PMN cell death.

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The SaeR/S TCS regulates production of virulence factors that promote evasion and destruction of neutrophils [1, 4, 5]. Moreover, neutrophil components, including α-defensin, trigger activation of the SaeR/S TCS and induce a transcriptional response tailored for its environment [6]. In this study, we demonstrate that the SaeR/S TCS contributes to accelerated neutrophil cell death via decreased nuclear factor-κB (NF-κB) phosphorylation and reduced interleukin-8 (IL-8) production by human neutrophils. Furthermore, we identified IL-8 to be an essential neutrophil-derived cytokine that can prolong PMN survival and promote bacterial clearance during staphylococcal disease.

MATERIALS AND METHODS

Bacteria Cultures

USA300, USA400, and isogenic USA300Δ*saeR/S* (Δ*saeR/S*) and USA400Δ*saeR/S* mutant strains were previously generated [4, 7] and prepared for use in human PMN assays as described in [7].

Human PMN Isolation

Human neutrophils were isolated from heparinized venous blood obtained from healthy donors [7]. Purity (less than 1% peripheral blood mononuclear cell [PBMC] contamination) and viability were assessed via flow cytometry (fluorescence-activated cell sorter [FACS], FACSCalibur, BD Biosciences). PMNs (1×10^6) were plated onto serum-coated 96- or 24-well plates and exposed to 2×10^6 (low multiplicity of infection [MOI], 2:1) or 1×10^7 (high MOI, 10:1) colony forming units (CFUs) of USA300 or Δ*saeR/S* and phagocytosis was synchronized [7].

Cell Death Assays

At designated time points, PMNs exposed to USA300 or Δ*saeR/S* were subjected to Annexin V (Trevigen) and propidium iodide (PI) or nuclear condensation or terminal deoxynucleotidyltransferase dUTP nick-end labeling (TUNEL) assays as described [8]. PMN lysis was quantified using lactate dehydrogenase (LDH) assays and calculated as percent lysis compared to maximum LDH release (USA300 + PMNs at 10:1 ratio) (Promega, [1]). CaspGLOW assays (eBioscience) were used to analyze PMN caspase activity via FACS per manufacturer's suggestion.

NF-κB Inactivation and IL-8 Assays

Neutrophils (1×10^6) were plated in duplicate and incubated with 0.2% dimethyl sulfoxide (DMSO) or 20 μM parthenolide (Sigma) in DMSO for 30 minutes at 37°C and 5% CO_2. USA300 or Δ*saeR/S* were added (low MOI) and phagocytosis was synchronized [7]. At designated time points, samples were either

subjected to nuclear condensation or TUNEL assays described above or used to determine PMN bactericidal activity (CFUs enumerated as described in [7]). To determine the role of IL-8, neutrophils were treated with recombinant IL-8 (25 ng/mL, R&D) or with anti-IL-8 (1 μg/mL, R&D) and isotype (1 μg/mL, R&D) 30 minutes prior to bacterial treatment. At 6 hours postinfection, nuclear condensation of PMNs was assessed along with bacterial survival.

Intracellular Detection of Phosphorylated NF-κB p65 Subunit and IL-8

Heparinized whole blood was treated with Roswell Park Memorial Institute (RPMI) medium or RPMI medium containing 1×10^7 CFUs USA300 or Δ*saeR/S* and incubated with end-over-end mixing (20 rpm, 37°C with 5% CO_2) for 5, 60, and 120 minutes. Using BD Biosciences whole-blood intracellular stain kit, samples were stained with anti-CD11b (BD Biosciences) or isotype control for 15 minutes at room temperature (RT). The optimal intracellular stain with anti-NF-κB-p65(P) (eBioscience) or corresponding isotype control was achieved after a 30 minute-incubation at RT. PMNs were determined by forward scatter/side scatter profile and high CD11b expression. For IL-8 detection, at 3 and 6 hours postexposure, infected PMNs or PMNs in media were stained with anti-CD11b or isotype control, fixed, and permeabilized per manufacture's recommendation (eBioscience). Additionally, cells were incubated with anti-IL-8 or corresponding isotype control (BD biosciences) for 30 minutes and analyzed by FACS.

Cytokine Assays

Supernatants from PMNs (1×10^6) or PBMCs (1×10^4) subjected to USA300 or Δ*saeR/S* (low MOI) were collected and sterile filtered at 6 and 18 hours. Procarta assays (Affymetrix) were used to detect interferon-γ (IFN-γ), IL-6, macrophage inflammatory protein-1α (MIP-1α), IL-1α, IL-1β, IL-8, vascular endothelial growth factor (VEGF), and tumor necrosis factor-α (TNF-α). The samples were read in duplicate using a Luminex cytometer (Bio-Rad). Additionally, supernatants from PMNs (1×10^6) or PBMCs (1×10^4) subjected to USA300 or Δ*saeR/S* (low MOI) were collected and sterile filtered at 6 and 18 hours and assayed in duplicate using R&D Quantikine enzyme-linked immunosorbent assay (ELISA) for IL-8 and VEGF.

Study Approval

Healthy blood donors were informed and gave written consent prior to participation. All studies were conducted in accordance with a protocol approved by the Institutional Review Board for Human Subjects at Montana State University.

RESULTS AND DISCUSSION

To test the hypothesis that the SaeR/S TCS influences PMN cell fate, we subjected human PMNs to USA300 or USA300Δ*saeR/S* (Δ*saeR/S*) strains at low MOI and measured cellular features associated with programmed cell death (Figure 1*A*, Supplementary Figure 1*A*–*D*). Consistent with published observations [3], USA300 promoted significant exposure of phosphatidylserine and plasma membrane damage within 6 hours of interaction (Supplementary Figure 1*A* and *B*). By 9 hours, the majority of USA300-treated cells were undergoing late apoptosis and/or necrosis, depicted by condensed nuclei (Figure 1*A*), fragmented DNA (Supplementary Figure 1*C*), and nearly complete PMN lysis (Supplementary Figure 1*D*). The aforementioned apoptotic features were significantly reduced in neutrophils infected with the Δ*saeR/S* mutant (Figure 1*A* and Supplementary Figure 1*A*–*D*), demonstrating that SaeR/S contributes to accelerated neutrophil death following *S. aureus* phagocytosis.

To determine if SaeR/S interferes with caspase activation, reported to be inhibited by USA300 [2], we measured active caspases in PMNs following interaction with USA300 and Δ*saeR/S* at high MOI (Supplementary Figure 1*E*). The substantial decrease in caspase activity in PMNs exposed to USA300 as opposed to Δ*saeR/S* demonstrated that caspase inhibition is dependent on SaeR/S-regulated factors. At low MOI, however, caspases were neither activated by USA300 nor Δ*saeR/S* mutant (Supplementary Figure 1*F*). Given these observations, we aimed to identify additional host-derived factors that contribute to SaeR/S-mediated cell death.

Because cytokines play an essential role in modulating neutrophil lifespan [9] and because the SaeR/S system has been shown to influence cytokine levels in whole blood and murine models of infection [5], we examined the influence of SaeR/S on secreted cytokine levels in PMNs during infection with USA300 and Δ*saeR/S* at 6 and 18 hours. We identified cytokines predominantly made by neutrophils by comparing cytokine profiles in purified PMNs to PBMCs representative of the contamination in the cell preparation (1×10^4 PBMCs was equivalent to <1.0% contamination in PMN preparations). Based on the cytokine survey, IFN-γ, IL-6, MIP-1α, IL-1α, IL-1β, and TNF-α were mainly produced by PBMCs (data not shown). However, IL-8 and VEGF were neutrophil-derived and, therefore, examined in subsequent experiments. At 6 and 18 hours, USA300-treated PMNs produced significantly less IL-8 than the Δ*saeR/S* group (Figure 1*B*). VEGF levels were higher in USA300-infected PMNs than the Δ*saeR/S* group at 6 hours and similar between the 2 groups at 18 hours (Supplementary Figure 2*A*); thus, reduced IL-8 during USA300 infection was not a result of a general protein synthesis decline.

To further demonstrate that PMNs were the source of IL-8, we measured intracellular IL-8 in PMNs by FACS. Infection with the Δ*saeR/S* mutant promoted a significant increase of IL-8 expressing PMNs in contrast to PMNs only at 3 hours (Supplementary Figure 2*B*) and when compared to both PMNs only and USA300-treated PMNs at 6 hours postexposure (Figure 1*C*). This trend was also observed during USA400 and USA400Δ*saeR/S* infection

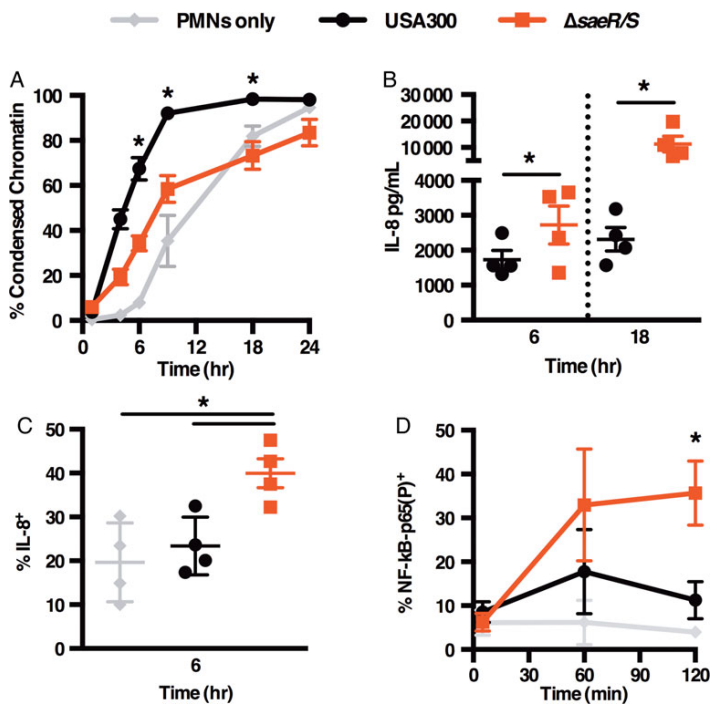

Figure 1. SaeR/S alters cell fate and suppresses IL-8 production in neutrophils. PMNs were subjected to USA300 or Δ*saeR/S* at low MOI. Percent PMNs with condensed chromatin assessed via FACS at designated times (*A*). IL-8 release from PMNs assessed by ELISA (*B*) and intracellular IL-8 measured in PMNs at 6 hours postinfection via FACS (*C*). Whole blood was treated with USA300 or Δ*saeR/S* and percent NF-κB-p65(P)$^{+}$ cells from CD11b^{Hi+} population was quantified by FACS at designated time points (*D*). Data shown are the means ± SEM of 4–10 experiments (*A*) or 3–4 experiments (*B–D*). **P* < .05 determined by 1-way ANOVA with Tukey's posttest (*A*, *C*, and *D*) comparing USA300 to Δ*saeR/S* groups or paired *t* test (*B*). Abbreviations: ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter; IL-8, interleukin-8; MOI, multiplicity of infection; nuclear factor-κB, NF-κB; NF-κB-p65(P), phosphorylated NF-κB p65 subunit; PMNs, polymorphonuclear leukocytes; SEM, standard error of the mean.

(Supplementary Figure 2*C*), confirming the SaeR/S-dependent inhibition of PMN-derived IL-8. Last, we assessed SaeR/S system's global impact on IL-8 release in whole blood and observed a significant decrease in IL-8 released in serum samples from the USA300-treated group when compared to the Δ*saeR/S*-treated blood at 3 hours postinfection (Supplementary Figure 2*D*). VEGF levels were similar between both groups, further highlighting the specificity of SaeR/S-dependent IL-8 suppression (Supplementary Figure 2*E*).

IL-8 is an essential chemokine that promotes neutrophil recruitment, antimicrobial functions, and prolonged survival in vitro [9] and during infection with pathogenic microorganisms, including *Anaplasma phagocytophilum*, *Chlamydophila pneumoniae*, and *Paracoccidioides brasiliensis* [10, 11]. Given that USA300 does not promote de novo IL-8 transcription [3], we wanted to determine if IL-8 reduction during USA300 infection is associated with SaeR/S-dependent modulation of NF-κB activity. To test this, we employed FACS to examine phosphorylation of the phosphorylated NF-κB p65 subunit (NFκB-p65[P]) in PMNs from human blood infected with USA300 or Δ*saeR/S*. At 2 hours, the percentage of PMNs with NF-κB-p65(P) was significantly reduced following interaction with USA300 when compared to Δ*saeR/S*-infected cells (Figure 1*D*).

Our observation that *S. aureus* suppresses NF-κB is supported by published data linking NF-κB inhibition to accelerated cell death following infection with *Escherichia coli*, *Yersinia pestis*, and *Salmonella typhimurium* [12]. Given NF-κB's regulation of antiapoptotic factors such as myeloid cell leukemia-1 (Mcl-1) and caspase inhibitors that can extend cell lifespan [11], we wanted to determine whether functional NF-κB is necessary for neutrophil survival during infection with *S. aureus*. To test this, we measured nuclear condensation and DNA fragmentation in PMNs pretreated with the NF-κB inhibitor parthenolide. PMN lifespan was shortened significantly in Δ*saeR/S*-infected PMNs following parthenolide pretreatment compared to untreated neutrophils infected with Δ*saeR/S* (Figure 2*A*, confirmed

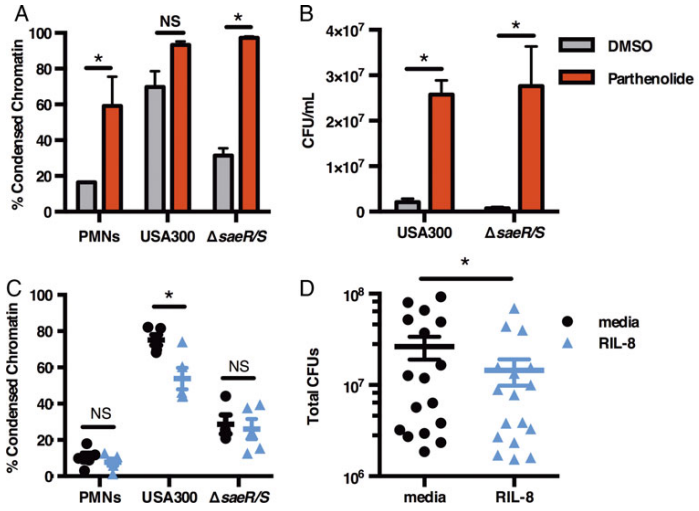

Figure 2. IL-8 promotes neutrophil survival and bacterial clearance. PMNs treated with 20 μM parthenolide or DMSO control were infected with USA300 or Δ*saeR/S* at low MOI for 6 hours followed by analysis of PMNs with condensed chromatin (*A*) and bacterial burden (*B*). In C–D, PMNs were exposed to RIL-8 (25 ng/mL) and infected with USA300 or Δ*saeR/S* for 6 hours followed by FACS analysis of condensed chromatin (*C*) or assessment of USA300 survival. *D*, Data shown are the means ± SEM of 3–5 separate experiments. **P* < .05 determined by 1-way ANOVA with Tukey's posttest (*A–C*) or by paired *t* test (*D*) comparing USA300 to Δ*saeR/S* groups. Abbreviations: ANOVA, analysis of variance; CFU, colony-forming unit; DMSO, dimethyl sulfoxide; FACS, fluorescence-activated cell sorter; IL-8, interleukin-8; MOI, multiplicity of infection; NS, not significant; PMNs, polymorphonuclear leukocytes; RIL-8, recombinant IL-8; SEM, standard error of the mean.

by TUNEL shown in Supplementary Figure 3*A*). Notably, cell death of parthenolide-treated PMNs infected with USA300 did not increase significantly when compared to the USA300 group without treatment underpinning the intrinsic ability of USA300 to inhibit NF-κB in PMNs (Figure 2*A*). NF-κB involvement in spontaneous PMN apoptosis [13] was confirmed by PMNs that underwent accelerated death in the presence of parthenolide (Figure 2*A*). Using LDH assays, we showed that the observed effect of parthenolide was not due to cell poisoning (Supplementary Figure 3*B*). Parthenolide treatment also significantly ($P < .05$ determined by t test, n = 3) abolished IL-8 release by 95% in USA300-infected PMNs (DMSO 1426 ± 94 pg/mL vs parthenolide 75 ± 39 pg/mL), but did not alter VEGF production (DMSO 214 ± 52 pg/mL vs parthenolide 215 ± 21 pg/mL), further suggesting that NF-κB inhibition was specific.

Finally, bacterial survival of both USA300 and Δ*saeR/S* was significantly increased in PMNs exposed to parthenolide (Figure 2*B*), while parthenolide alone did not promote bacterial growth (data not shown). The observation that inhibition of NF-κB in Δ*saeR/S*-treated neutrophils resulted in accelerated cell death and bacterial survival similar to USA300-treated neutrophils emphasizes the importance of the PMN response to resolution of *S. aureus* infection. Herein, we demonstrate that the SaeR/S TCS reduced phosphorylation of NF-κB to promote evasion of PMN killing. Our results are supported by skin infection models where lack of MyD88 protein, essential to NF-κB activation, led to larger abscess formation and *S. aureus* survival [14]. Also, patients with NF-κB signaling deficiencies are highly susceptible to staphylococcal infections [15], further emphasizing the importance of NF-κB-dependent inflammation during staphylococcal disease.

To determine the role of IL-8 during *S. aureus*–neutrophil interaction, PMNs were replenished with recombinant IL-8 (RIL-8). The percentage of USA300-treated PMNs undergoing cell death was significantly reduced when compared to PMNs exposed to USA300 without treatment (Figure 2*C*). IL-8 can inhibit PMN apoptosis via the PI3K/Akt pathway, leading to subsequent activation of NF-κB and antiapoptotic Mcl-1 production, needed for neutrophil survival [10]. Therefore, future studies are necessary to define the precise mechanism of how SaeR/S-derived IL-8 alters PMN cell fate. In correlation with improved neutrophil survival, RIL-8 significantly enhanced PMN bactericidal activity against USA300, demonstrated by a significant decrease in USA300 survival (Figure 2*D*). Finally, the importance of IL-8 in bacterial clearance was underscored by increased USA300 burden in PMNs treated with neutralizing IL-8 antibody (Supplementary Figure 3*C*).

Taken together, our findings identify a mechanism employed by *S. aureus* to evade human neutrophils by which the SaeR/S

TCS disrupts PMN cell fate via suppression of NF-κB and IL-8 production to promote bacterial survival. We propose that both timing and magnitude of inflammation in PMNs play major roles in dictating the outcome of staphylococcal disease and that alteration in the innate ability of PMNs to produce IL-8 may increase susceptibility to *S. aureus* infections.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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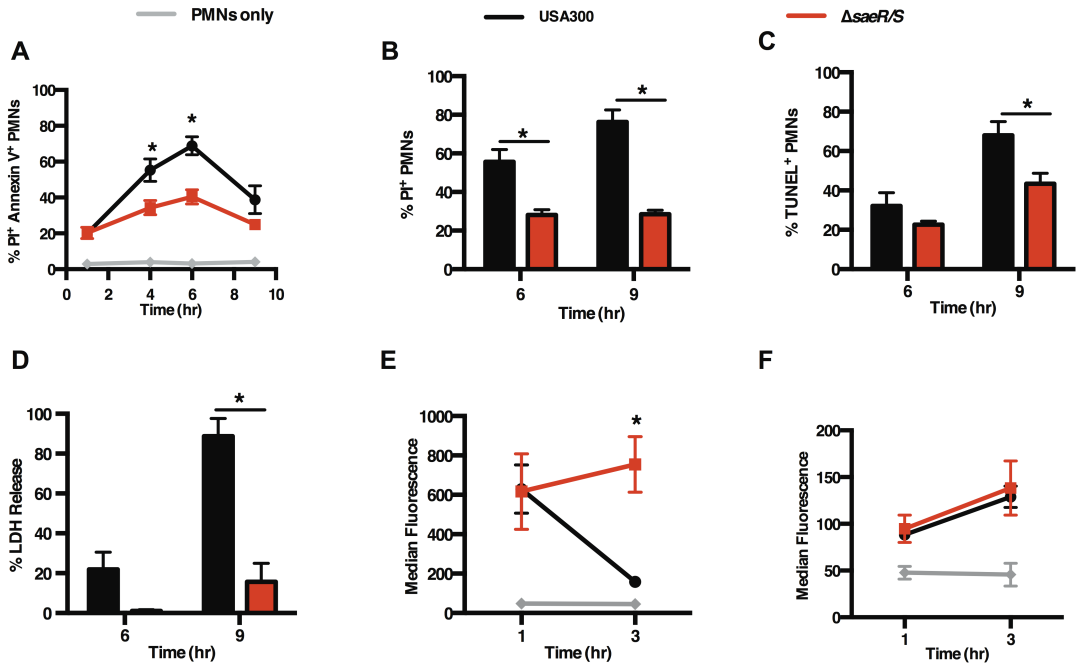
Potential conflicts of interest. All authors: No reported conflicts.

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

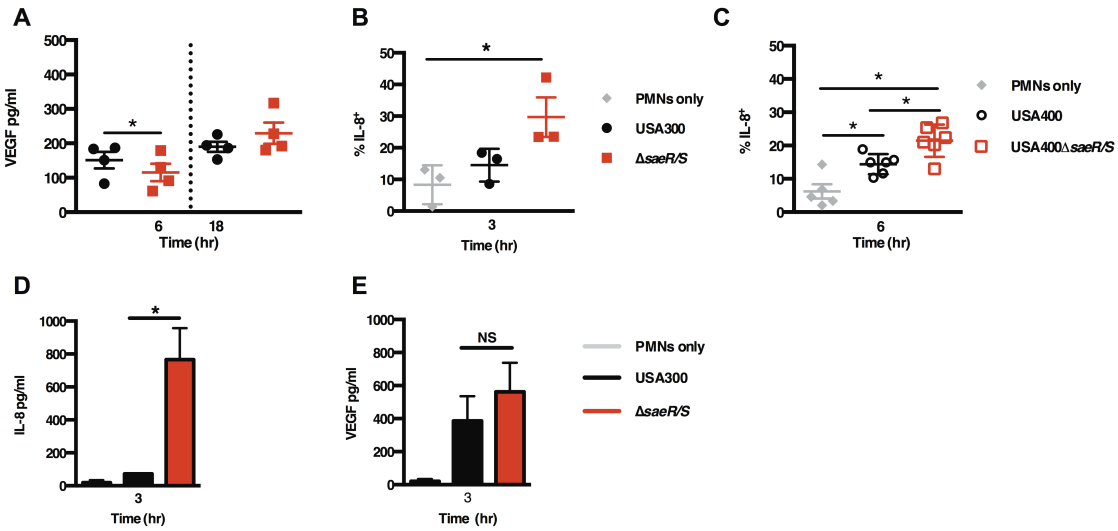
1. Voyich JM, Vuong C, DeWald M, et al. The SaeR/S gene regulatory system is essential for innate immune evasion by *Staphylococcus aureus*. J Infect Dis **2009**; 199:1698–706.
2. Greenlee-Wacker MC, Rigby KM, Kobayashi SD, Porter AR, DeLeo FR, Nauseef WM. Phagocytosis of *Staphylococcus aureus* by human neutrophils prevents macrophage efferocytosis and induces programmed necrosis. J Immunol **2014**; 192:4709–17.
3. Kobayashi SD, Braughton KR, Palazzolo-Ballance AM, et al. Rapid neutrophil destruction following phagocytosis of *Staphylococcus aureus*. J Innate Immun **2010**; 2:560–75.
4. Nygaard TK, Pallister KB, Ruzevich P, Griffith S, Vuong C, Voyich JM. SaeR binds a consensus sequence within virulence gene Ppomoters to advance USA300 pathogenesis. J Infect Dis **2010**; 201:241–54.
5. Watkins RL, Pallister KB, Voyich JM. The SaeR/S gene regulatory system Iiduces a pro-inflammatory cytokine response during *Staphylococcus aureus* infection. PLOS One **2011**; 6:e19939.
6. 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.
7. 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.
8. Voyich JM, DeLeo FR. Host-pathogen interactions: leukocyte phagocytosis and associated sequelae. Methods Cell Sci **2002**; 24: 79–90.
9. Kettritz R, Gaido ML, Haller H, Luft FC, Jennette CJ, Falk RJ. Interleukin-8 delays spontaneous and tumor necrosis factor-alpha-mediated apoptosis of human neutrophils. Kidney Int **1998**; 53:84–91.
10. Sarkar A, Hellberg L, Bhattacharyya A, et al. Infection with *Anaplasma phagocytophilum* activates the phosphatidylinositol 3-Kinase/Akt and NF-kB survival pathways in neutrophil granulocytes. Infect Immun **2012**; 80:1615–23.
11. McCracken JM, Allen LA. Regulation of human neutrophil apoptosis and lifespan in health and disease. JCD **2014**; 7:15–23.
12. Neish AS, Naumann M. Microbial-induced immunomodulation by targeting the NF-κB system. Trends Microbiol. Elsevier Ltd **2011**; 19: 596–605.
13. Ward C. NF-kappa B activation is a critical regulator of human granulocyte apoptosis in vitro. J Biol Chem **1999**; 274:4309–18.
14. Miller LS, O'Connell RM, Gutierrez MA, et al. MyD88 mediates neutrophil recruitment initiated by IL-1R but not TLR2 activation in immunity against *Staphylococcus aureus*. Immunity **2006**; 24:79–91.
15. Picard C, Casanova JL, Puel A. Infectious diseases in patients with IRAK-4, MyD88, NEMO, or IkB deficiency. Clin Microbiol Rev **2011**; 24:490–7.

Supplementary Figure 1



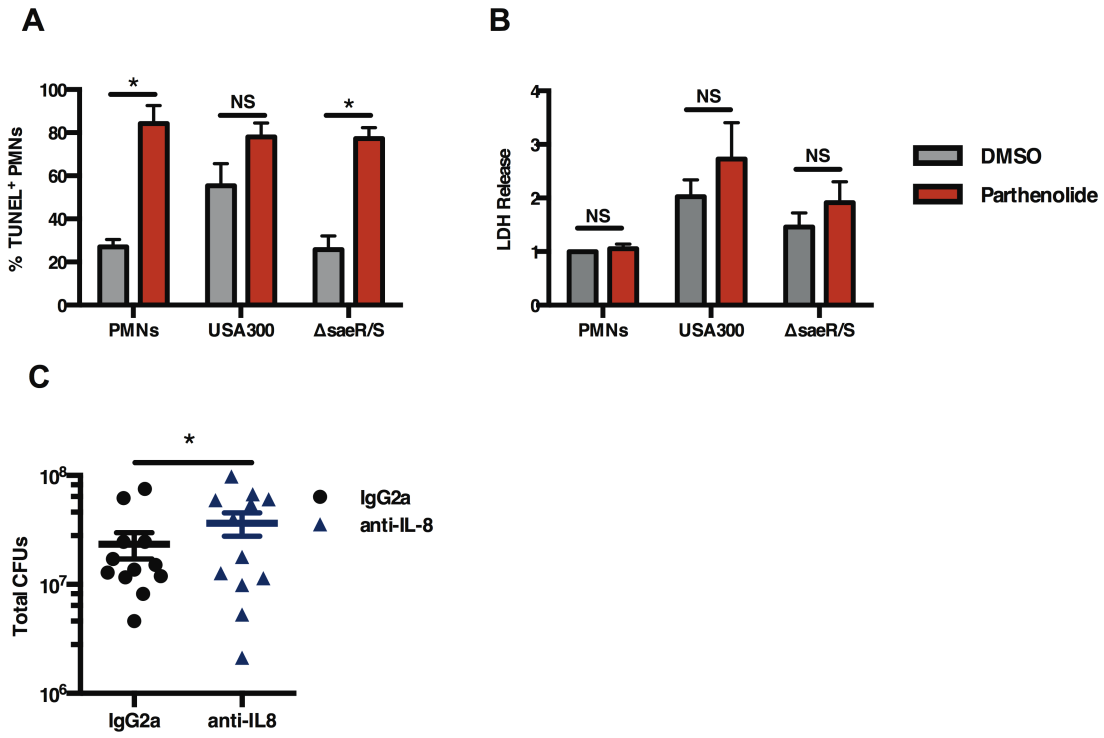
Supplementary Figure 1. SaeR/S contributes significantly to accelerated PMN cell death induced by USA300. Assessment of cell death in human PMNs (grey) subjected to USA300 (black) and Δ*saeR/S* (red) at low MOI. Results are shown as percent PMNs positive for PI and Annexin V (A) and plasma membrane damage (B) assessed via FACs at designated times. Percent PMNs with fragmented DNA was assayed by TUNEL (C). Cell lysis measured by LDH release at 6 and 9 hours compared to maximum LDH release as described in methods (D). Median fluorescence of active caspases in PMNs measured by FACs at high (E) and low MOI (F). Data shown are the means ± SEM of 3-6 experiments. *$p<0.05$ determined by One-Way ANOVA with Tukey's posttest comparing USA300 to Δ*saeR/S* groups.

Supplementary Figure 2



Supplementary Figure 2. SaeR/S suppresses IL-8 production. VEGF release from PMNs interacting with USA300 or Δ*saeR/S* at low MOI was measured by ELISA (A). In B-C, FACs was employed to analyze IL-8 expression in PMNs ($CD11b^{Hi+}$) 3 hour post-interaction with USA300 or Δ*saeR/S* (B) and following 6 hour interaction with USA400 and USA400Δ*saeR/S* at low MOI (C). IL-8 (D) and VEGF (E) were quantified by ELISA from filtered supernatants of human blood alone (grey bar) or infected for 3 hours with $1x10^7$ CFUs of USA300 (black bar) or Δ*saeR/S* (red bar). Results are the mean ± SEM of 3-6 (A-C) or 2-3 experiments (D-E) with different blood donors. *$p<0.05$ determined by One-Way ANOVA with Tukey's posttest comparing USA300 to Δ*saeR/S* groups or paired t-test in (A).

Supplementary Figure 3



Supplementary Figure 3. Confirmation of the influence of NF-kB and IL-8 on PMN cell fate. PMNs treated with 20μM parthenolide or DMSO control were infected with USA300 or Δ*saeR/S* at low MOI for 6 hours (A-B). Percent PMNs with fragmented DNA (A). In B, LDH release from PMNs normalized to PMNs in DMSO. Viable USA300 or Δ*saeR/S* were recovered from anti-IL-8 or isotype control (1ug/ml) treated PMNs after 6 hours and are shown as CFU/ml (C). Results are the mean ± SEM of three (A-B) and 12 separate experiments (C). *$p<0.05$ determined by One-Way ANOVA with Tukey's posttest comparing DMSO groups to their corresponding parthenolide-treated groups (A-B) or paired t-test (C).

CHAPTER FOUR

CO-AUTHORED WORK

SaeR/S System Activation is Depended on Single Amino Acids in SaeS Histidine Kinase

This original observation demonstrating the specificity of the SaeR/S activation by external stimuli such as alpha-defensin (Chapter 2) was traced, in a follow-up study, to a single amino acid in the external loop of SaeS (1). By investigating the putative extracellular loop of SaeS we demonstrated that recognition of human neutrophil alpha-defensin was dependent on a methionine residue. Introduction of a point mutation in the methionine residue of the putative extracellular domain of SaeS resulted in the loss of virulence factor transcription in the presence of alpha-defensin and PMNs indicating that this particular amino acid is essential for detecting neutrophil-derived stimulus(i) responsible for the system's activation. Additionally, aromatic amino acids in the extracellular domain appear to be capable of refining the response to host stimuli (1). Given that mouse neutrophils induce activation of the SaeR/S system despite their azurophilic granules lacking alpha-defensins (2), these findings strongly suggest additional neutrophil-derived factors can promote activation of the SaeR/S system through refinement of the signal via specific amino acids in the sensory system. Collectively, these data demonstrate virulence regulation in *S. aureus* by the Sae TC system is far more sophisticated than the originally hypothesized 'on-off' paradigm of transcriptional regulation.

SaeR/S System and SaeR/S-regulated Factors Influence the Immune Response during Staphylococcal Infection

Given the essential role of the SaeR/S system in promoting *S. aureus* disease, we began to identify SaeR/S-regulated factors that are contributing to SaeR/S-mediated pathogenesis. In a study by Olson et al, we have shown that SaeR/S-regulated nuclease (Nuc) was directly regulated by the SaeR/S system in vitro and ex vivo independent of the Agr quorum sensing system. Nuc was demonstrated to significantly contribute to pathogenesis during invasive infection in mice highlighting the essential role of the SaeR/S system to *S. aureus* pathogenesis (3).

In another study by Nygaard et al., we showed that alpha-toxin, under regulation of the SaeR/S and Agr systems, promotes cell death of monocytes and modulates an inflammatory response during whole human blood infection (4). Furthermore, *S. aureus* association with monocytes during whole blood human infection correlated with high expression of zinc-dependent metalloprotease ADAM10 which is targeted by Hla. In contrast, Hla did not affect PMN integrity which underscored the ability of virulence factors in targeting specific cell populations for damage thereby promoting disease based on the particular host challenge.

We also investigated the SaeR/S system's influence on the host response during invasive infection (5). While it is well accepted that the SaeR/S system elicits a robust inflammation during an infection, the contribution of neutrophils to this response has not been widely investigated. To this end, we identified murine neutrophils to be the major source of IFN-g during *S. aureus* infection. IFN-g production by the neutrophils was

dependent on the SaeR/S system and its role in promoting staphylococcal disease was highlighted by improved bacterial clearance and survival in mice that are deficient in IFN-g production. Together, these findings underline intricate roles of the SaeR/S system and its virulence factors in modulating the host response to promote *S. aureus* pathogenesis in human and murine models of infection.

Literature Cited

1. Flack CE, Zurek OW, Meishery DD, Pallister KB, Malone CL, Horswill AR, et al. Differential regulation of staphylococcal virulence by the sensor kinase SaeS in response to neutrophil-derived stimuli. Proc Natl Acad Sci USA. 2014 May 13;111(19):E2037–45.

2. Zurek OW, Nygaard TK, Watkins RL, Pallister KB, Torres VJ, Horswill AR, et al. The role of innate immunity in promoting SaeR/S-mediated virulence in Staphylococcus aureus. J Innate Immun. 2014;6(1):21–30.

3. Olson ME, Nygaard TK, Ackermann L, Watkins RL, Zurek OW, Pallister KB, et al. Staphylococcus aureus nuclease is a SaeR/S-dependent virulence factor. Infection and Immunity. 2013 Feb 4.

4. Nygaard TK, Pallister KB, Zurek OW, Voyich JM. The impact of -toxin on host cell plasma membrane permeability and cytokine expression during human blood infection by CA-MRSA USA300. Journal of Leukocyte Biology. 2013 Oct 31;94(5):971–9.

5. 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. Elsevier Masson SAS; 2013 Sep 10;15(10-11):749–54.

CHAPTER FIVE

FUTURE STUDIES AND CONCLUSIONS

Defining the Role of Neutrophil Density on the Outcome of *S. aureus* Infection

Neutropenia can be caused by a number of factors ranging from cancer, HIV, chemotherapy, non-chemotherapy drugs and genetics (1,2). Regardless of the cause, neutropenic patients with PMN blood concentration below $0.5x10^9$ PMNs/L are susceptible to fungal and bacterial infections (3-5). It has been well accepted that the low number of neutrophils is the predisposing factor; however, not all neutropenic patients develop infections and studies suggest neutrophil recruitment and/or density at the tissue sites, rather than PMN numbers in blood, might better predict susceptibility to infections (6,7). Furthermore, studies by Li et al. and Malka et al. demonstrate that neutrophils' ability to kill is severely inhibited at low PMN concentrations even when multiplicity of infection (MOI) is low (8,9). These findings suggest that when PMNs are depleted from the system, establishment of an infection might stem not only from the low numbers of cells, but also from their dysfunctional activity. The underlying reasons for PMNs decreased antimicrobial properties at low concentrations are undefined and are the focus of ongoing and future work.

In our preliminary studies, we aimed to assess the effect of PMN concentration on the cells' ability to kill *S. aureus*. The experimental set up consisted of three different neutrophil quantities (10^5, 10^6, and $4x10^6$ cells) that were treated with USA300 ($2x10^5$,

$2x10^6$ and $8x10^6$ cells) at a constant 2:1 bacteria to neutrophil ratio. We chose the numbers based on clinical data representative of neutropenic PMN concentration (low neutrophil number, LN, 10^6 PMNs/ml), average concentration used in our studies (medium neutrophil number, MN, 10^7 PMNs/ml), as well as a high PMN number (HN, $4x10^7$ PMNs/ml,) to represent a condition of increased neutrophil concentration at the site of infection (10,11). At 3 hours post-interaction, PMNs from the neutropenic concentration (LN) group were ineffective at killing USA300 (Figure 1A). Average (MN) and high neutrophil (HN) groups, in contrast, reduced USA300 burden by 60% and 87% respectively, suggesting that numbers of neutrophils interacting with the pathogen can dictate the efficacy of bacterial clearance (Figure 1A). Membrane damage was also assessed in the same experiments, but there was no difference between LN and MN groups suggesting neutrophil damage was not significantly increased during USA300 interaction at low PMN concentration (Figure 1B).

Currently, we are investigating neutrophil functions that are responsible for the poor killing of *S. aureus* under low density conditions. Following synchronized phagocytosis 95% of USA300 associated with $3x10^5$ PMNs (at MOI of 10) is ingested within 15 minutes (12). To determine whether decreased phagocytosis efficacy might be contributing to improved *S. aureus* survival in LN group, we compared USA300 uptake at MOI of 2 by 10^5 PMNs (LN) and 10^6 PMNs (MN) via fluorescent microscopy. Both groups phagocytosed the majority of PMN-associated USA300 at 15 min post-infection (data not shown) suggesting that, rather than phagocytosis, antimicrobial strategies

employed by PMNs after *S. aureus* uptake are likely to be responsible for the inability of neutrophils to clear the pathogen at low concentration.

Neutrophil ROS production is essential to controlling *S. aureus* infection (13). To determine the effect of PMN concentration on ROS, we labeled LN and MN neutrophils with 2'7'-dihydrodiclorofluorescein diacetate (DCF), infected them with USA300 (MOI of 2) and measured ROS production every-minute with a fluorometer (Figure 2). As expected, 10^6 PMNs (12,14) exposed to PMA or USA300 induced a robust oxidative burst within the first 20 minutes of interaction (12,14) (Figure 2A). However, kinetics of LN ROS production were different and consisted of two prominent bursts, one of which took place around 100 minutes post-infection (Figure 2B). When these data were normalized to PMN quantity, surprisingly, LN PMNs produced more ROS per cell than MN neutrophils in media only (Figure 2C) and during USA300 infection (Figure 2D). These preliminary findings suggest that although low concentration of neutrophils delayed the oxidative burst, the overall yield was higher than that of the MN neutrophils.

The high rate of ROS production in PMNs from the LN group was surprising given their inability to clear *S. aureus* infection (Figure 1A). One potential explanation is that a delayed oxidative burst is advantageous to *S. aureus*. Since *in vivo* antimicrobial properties of ROS are still debated (15,16), the high ROS concentration might not be representative of bactericidal efficacy under these circumstances. Follow-up studies will serve to confirm these results by measuring ROS production in infected PMNs on a single-cell level via flow cytometry (described in (17)).

Since ROS production is linked to cell death in PMNs, (18), we wanted to determine the effect of PMN concentration on cell death to determine if increased ROS in the LN group correlated with cell fate. To this end, we measured chromatin condensation in the three PMN groups at 6 hours post-USA300 infection. In correlation with increased bacterial burden (Fig 1), 94% of LN PMNs had condensed chromatin, indicative of accelerated apoptosis, while only 51% and 30% of PMNs had condensed chromatin in MN and HN groups, respectively (Figure 3). While bacterial burden observed at 3 hours is likely to contribute to accelerated death of PMNs from the LN group, high ROS production might be also responsible for the shortened lifespan (18). To address this we measured USA300 survival in LN neutrophils treated with ROS inhibitor diphenyleneiodonium (DPI) (Figure 4). Reduction of ROS in the LN group resulted in enhanced PMN killing of USA300 suggesting that in the case of neutropenic PMN levels, high ROS production inhibits their bactericidal efficacy (Figure 4) potentially due to alteration of cell fate. Given this finding, we predict that ROS reduction will also aid PMN survival in the LN group which will be addressed in future studies.

Finally, *ex vivo* experiments will be performed using BALB/c mice to determine whether bactericidal properties of endothelium-activated PMNs are also dependent on neutrophil concentration. In brief, we will recruit neutrophils to the peritoneal cavity using thioglycollate (TG) and extract them from the site at 4 hours post-TG injection. Low and medium PMN concentration will be infected *ex vivo* with USA300 at 2:1 ratio followed by assessment of *S. aureus* survival, ROS production and cell death kinetics.

For an *in vivo* model of infection, TG will be used to recruit low and high concentrations of PMNs (described in (19)) followed by USA300 infection (i.p, MOI of 2) and effect of PMN concentration on *S. aureus* survival will be assessed. Based on our *in vitro* studies, we predict that the low concentration of PMNs will not be as effective at clearing *S. aureus* when compared to the high cell recruitment group. Together, these findings will not only contribute to identifying factors that promote establishment of infection under neutropenic conditions but will also provide insight into potential mechanisms by which healthy individuals might acquire staphylococcal infections.

Defining the SaeR/S-mediated Immune Response during Subcutaneous Skin Infection

Studies have shown that the SaeR/S system is essential to USA300 pathogenesis during subcutaneous skin infection (20). While the immune response responsible for SaeR/S-mediated infection persistence and dermonecrosis has not been defined, alpha-hemolysin (Hla), regulated by the SaeR/S and Agr systems, has been shown to contribute to dermonecrosis formation (21,22). On the host side, MyD88, NF-kB, IL-1β, IL-17 and TLR2 signaling contribute to limiting staphylococcal lesion formations via impaired PMN recruitment underscoring the influence of the immune response as well as neutrophils on the outcome of infection (23,24). Other studies suggest that complete depletion of PMNs causes bacteremia during dermatitis and PMN-derived IL-1β is essential to limiting *S. aureus* skin infection (25,26). These findings demonstrate additional roles of neutrophils that include containing the pathogen at the site of injury and being a major source of inflammatory agents. Although the aforementioned findings

significantly contribute to our knowledge of host responses that keep *S. aureus* infection in check, host-derived mechanisms that aid *S. aureus* clearance are not well defined. To this end, we began to characterize immune responses elicited by USA300 and Δ*saeR/S* during subcutaneous skin infection to define key factors that facilitate resolution of staphylococcal disease.

Our published (22) and preliminary data suggest that the SaeR/S system promotes an altered neutrophil response that contributes to the progression of skin infection. This observation is based in part on increased expression of the SaeR/S targets (*hlgABC, lukAB, sbi, hla, splA* and *ssl7*), during subcutaneous skin infection, which have been shown to alter PMN recruitment and integrity in previous studies (27-30). In addition, when compared to Δ*saeR/S*-treated group, USA300 elicits increased production of neutrophil chemoattractants (KC, MIP1-α and CXCL5), proteins contributing to PMN chemotaxis and granulopoiesis (G-CSF, GM-CSF, IL-17, and IL6) as well as pro-inflammatory cytokine (TNF-α, IFN-γ) at 8 and/or 24 hours post infection (Figure 5A-I). These findings are in agreement with a peritonitis model of *S. aureus* infection that associated up-regulated cytokine production with the SaeR/S system. (31). Moreover, USA300 infected mice had higher levels of myeloperoxidase (MPO), indicative of PMN presence, versus the Δ*saeR/S*-treated group at 24 hours post-infection (Figure 6A). Of note, this was independent of bacterial burden since Δ*saeR/S* CFUs were higher or equal to USA300 burden at both time points (Figure 6C-D). The strain lacking the Agr system had a similar effect on the immune response to that of USA300 (Figure 5), even though Δ*agr* has significantly reduced Hla expression required for

formation of dermonecrosis (21). This observation along with similar MPO levels between USA300 and Δ*agr* (Figure 6A) suggest that SaeR/S-regulated factors other than Hla are likely to influence the immune response and PMN influx at the site of skin infection.

These preliminary findings demonstrate that dermonecrosis and persistent skin inflammation are associated with increased neutrophil numbers and pronounced pro-inflammatory responses mediated by SaeR/S-regulated effectors. Future studies will assess abscess morphology from USA300, Δ*saeR/S* and Δ*agr* infected mice by tissue histology to assess the proximity of neutrophils to the bacterial burden. This will serve to determine whether neutrophils are able to reach the site of infection since previous studies have demonstrated that *S. aureus* hinders neutrophil migration (32,33). Such inhibition could potentially explain increased influx of PMNs in the USA300 group since PMNs may recruit to tissue damage but are prohibited from coming in direct contact with the pathogen due to formation of fibrin capsules (33). Additional studies will focus on measuring neutrophil influx and pro-inflammatory cytokines 4 hours post-infection as PMNs have been shown to arrive at the site of injury early and the first response may be imperative to the outcome of infection (34). In addition, we are interested in assessing the contribution of highly expressed virulence factors during subcutaneous skin infection that we have identified previously (22). While *hlgABC* and *lukAB* have been shown to have minimal contribution to abscess formation (29,35), the roles of Sbi, Ssl7 and SplA in skin disease remain to be defined.

Conclusions

The work described here explores the intricate roles of both *S. aureus* and neutrophils that influence the outcome of staphylococcal infection. We have found that specific antimicrobial and/or stress-inducing host components promote SaeR/S-mediated virulence that is essential to *S. aureus* pathogenesis (22). We examined transcription of wild type, Δ*saeR/S* and Δ*agr* mutants in the presence of human neutrophil and mouse skin stimuli since, to date, only *in vitro* studies were performed to compare expression differences between these strains (20,36-38). We found that while SaeR/S is essential for transcription of virulence factors, Agr contributes to their transcription depending on the external stimuli. In addition, we identified *S. aureus* virulence factors, LukAB/GH, HlgABC and Sbi, that are immediately expressed in the presence of neutrophils suggesting they might be essential to initiation of infection (22). This work also led to a follow-up study by Flack et al. where we identified which of the putative extracellular residues on the SaeS histidine kinase were responsible for activating SaeR/S-regulated genes in the presence of alpha-defensin and human neutrophils (39). Together, our research suggests that *S. aureus* has a unique ability to sense the environment by integrating and coordinating specific host-stimuli and refining its virulence accordingly.

In addition to controlling virulence factors during infection, the SaeR/S system enhances rapid cell death of human neutrophils in a caspase-independent manner. We have identified host factors such as NF-kB transcription factor and PMN-derived IL-8 to be essential to withstand USA300 infection (40). Furthermore, depletion of IL-8 resulted in higher bacterial burden while rIL-8 promoted bacterial clearance in USA300-infected

neutrophils underscoring the importance of IL-8 to the outcome of *S. aureus* infection (40). Which of the SaeR/S-regulated factors are responsible for inhibition of NF-kB transcription is not known. However, SaeR/S-regulated Eap factor has been shown to inhibit DNA binding of NF-kB in THP-1 cells in the presence of ICAM integrins suggesting that Eap might be a potential candidate for the observed suppression of neutrophil NF-kB and IL-8 by the SaeR/S system (41).

Based on our observations, we propose a model of how *S. aureus* evades the human neutrophil (described in Figure 7). Upon neutrophil phagocytosis of USA300, neutrophil derived alpha-defensin and additional PMN-derived signal(s) activate the SaeR/S system. This activation is regulated on a single amino acid level of the histidine kinase sensor (SaeS) which dictates transcription of specific virulence factors such as *lukA*, *hlgA* and *sb*i as opposed to the entire SaeR/S-regulated transcriptome (22,39). Subsequently, the SaeR/S system promotes accelerated cell death of the neutrophils which is attributed to SaeR/S-mediated suppression of NF-kB activity and diminished IL-8 production. Based on previously published studies, IL-8 production and its ability to prolong neutrophil lifespan during an infection is likely a result of PI3K/Akt signaling pathway activation resulting in increased NF-kB-mediated Mcl-1 expression which is a major anti-apoptotic protein in the neutrophils (42,43). However, exact mechanisms of how NFkB is being suppressed will be the topic of future studies. Regardless, the importance of IL-8 and NF-kB activation is demonstrated in this study by the improved survival of neutrophils and increased USA300 clearance in the presence of exogenous IL-8.

In conclusion, the research described herein advances our understanding and provides mechanistic insight into how *S. aureus* senses the host and regulates its virulence to facilitate an infection. We identified neutrophil-derived factors that are essential to resisting *S. aureus* and regulation of PMN cell death. Our ongoing studies will assess the importance of neutrophil interactions representative of clinical conditions that render patients susceptible to *S. aureus* disease and define host responses that initiate or aid clearance of USA300 skin infections. Given that immunocompromised and otherwise healthy individuals can acquire *S. aureus* infections, it is essential to continue defining the key pathogenic and host-derived factors that influence the course of staphylococcal disease in order to identify novel preventative measures and therapeutics.

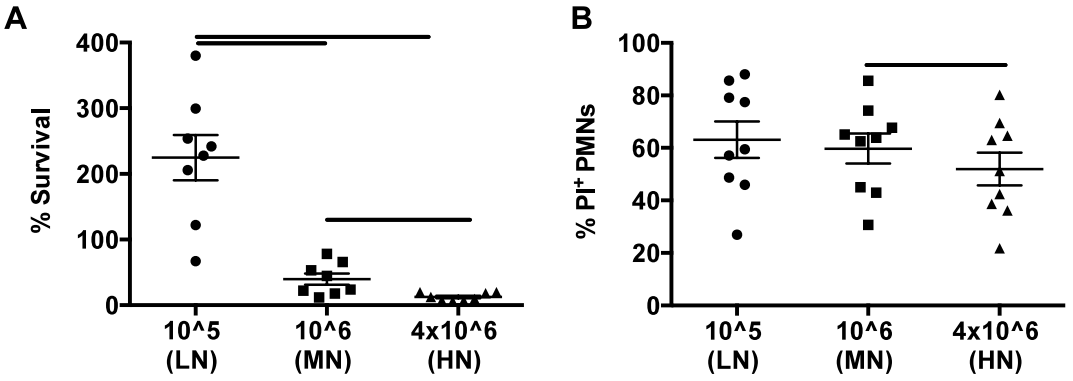


Figure 1. USA300 clearance is dependent on neutrophil concentration. Three different quantities of neutrophils, 10^5, 10^6, and $4x10^6$ cells were treated with $2x10^5$, $2x10^6$ and $8x10^6$ USA300, respectively (2:1 bacteria to PMN ratio). At 3 hours post-interaction, samples were serially diluted and enumerated on TSA plates. Results are shown as percent of USA300 survival compared to the initial dose (A). In the same experiment, another set of infected neutrophils were stained with propidium iodide (PI) and assessed on FACs for membrane damage (B). Data shown as mean ± SEM from 8-9 separate experiments. Line depicts statistical significance between groups ($p<0.05$) determined by One-Way ANOVA with Tukey's posttest.

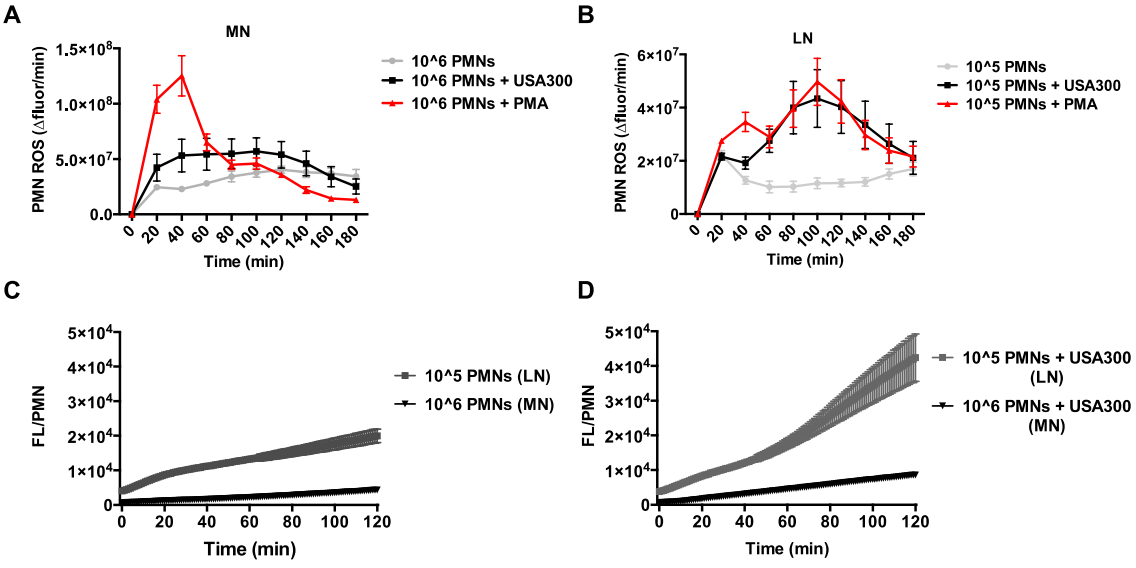


Figure 2. Neutrophils at low concentration undergo a delayed oxidative burst. 10^6 PMNs (A) or 10^5 PMNs (B) were treated with 25 µM DCF for 20 minutes and infected with USA300 at 2:1 ratio (black line), 0.2 µg PMA (red line) or media only (gray line). ROS

was measured every minute up to 180 minutes at 37°C using a fluorometer (ex 485 nm, em 538 nm). In A-B, ROS production is shown as V_{max} over 20 minute intervals. ROS (expressed as fluorescence intensity, FL) normalized to PMN quantity at each time point is shown in C for PMNs in media only and in D for PMNs infected with USA300. Data shown as mean ± SEM from four separate experiments.

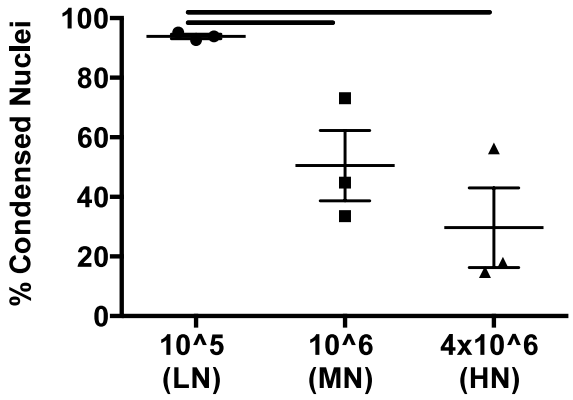


Figure 3. Neutrophil cell death during infection with USA300 is dependent on PMN concentration. Three different PMN quantities (10^5, 10^6, and $4x10^6$) were treated with USA300 at a 2:1 ratio (bacteria:PMN). At 6 hours post-infection, samples were collected and incubated in hypotonic solution with PI overnight and analyzed by FACs. Data are shown as percent of neutrophils with condensed chromatin expressed as mean ± SEM of three separate experiments. Line depicts statistical significance between groups ($p<0.05$) determined by One-Way ANOVA with Tukey's posttest.

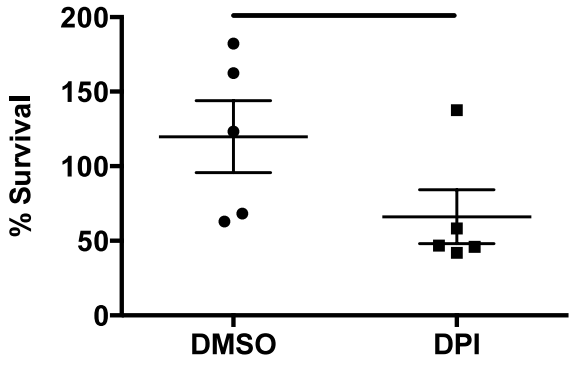


Figure 4. Inhibition of ROS in PMNs at low concentration enhances USA300 killing. PMNs (10^5) were treated with 10 μM DPI or DMSO for 10 minutes and infected with

USA300 (2:1bacteria:PMN). At 3 hours post-infection, samples were serially diluted and plated in duplicate on TSA plates. Data shown as mean ± SEM of percent USA300 survival compared to the initial dose, from five separate experiments. Line depicts statistical significance ($p<0.05$) determined by paired t-test.

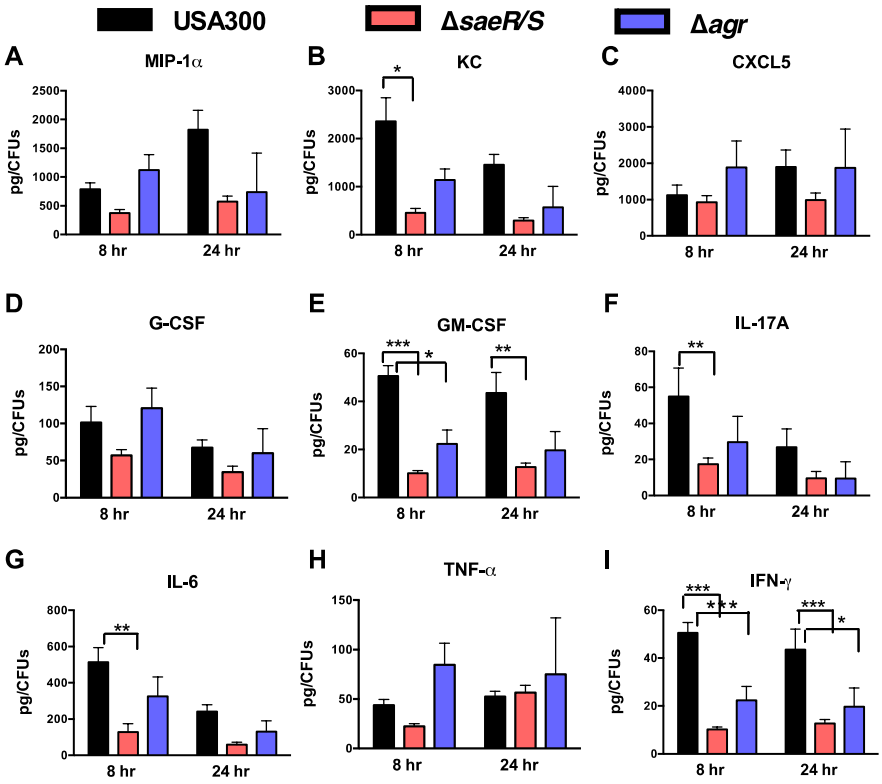


Figure 5. SaeR/S modulates inflammation at the site of subcutaneous skin infection. Mice (hairless Crl:SKH1-hrBR) were injected subcutaneously with 1-4 x10^7 CFUs of USA300, USA300Δ*saeR/S,* USA300Δ*agr* or PBS. At 8 and 24 hours post-infection, 9mm skin biopsies were harvested from the abscess and homogenized for cytokine assessment via Luminex. Data shown as mean ± SEM of two separate experiments (3 mice per treatment group per experiment) with the exception of Δ*agr* (n=3). Line depicts statistical significance ($p<0.05$) between groups determined by One-Way ANOVA with Tukey's posttest.

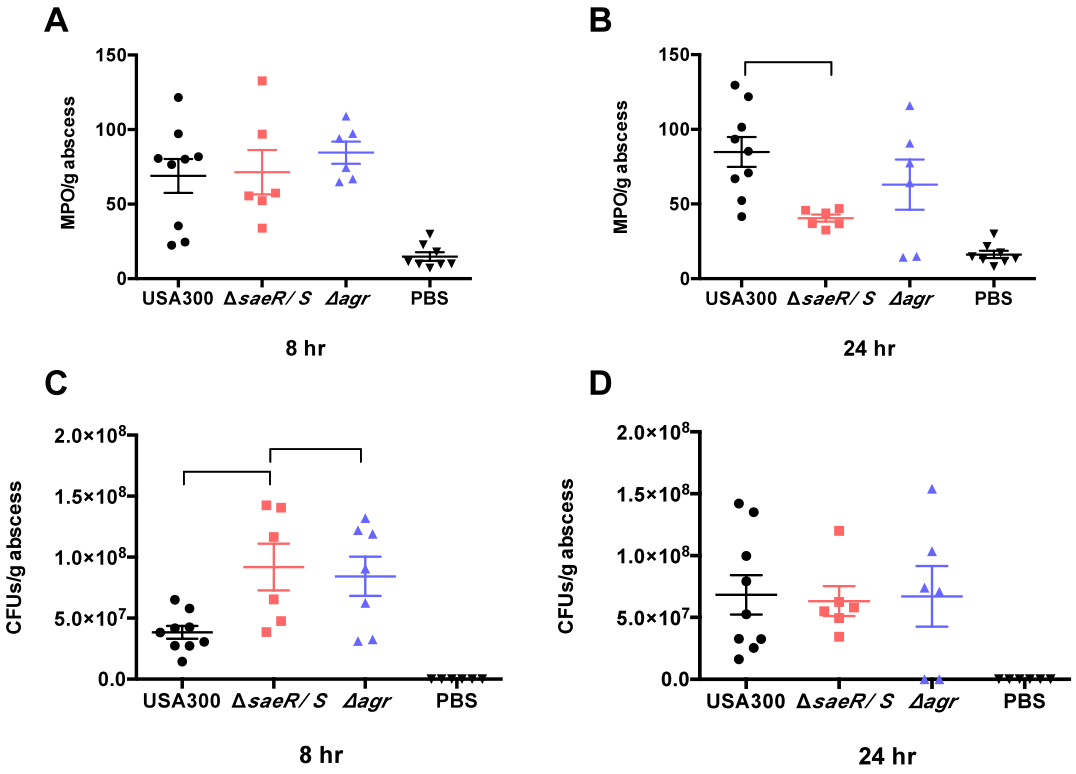


Figure 6. SaeR/S-mediated neutrophil influx is not dependent on bacterial burden. Mice (hairless Crl:SKH1-hrBR) were injected subcutaneously with 1-4 x10^7 CFUs of USA300, USA300Δ*saeR/S,* USA300Δ*agr* or PBS. At 8 and 24 hours post infection, 9mm skin biopsies were harvested from the abscess and homogenized for MPO (A-B) and bacterial burden assessment (C-D). Data shown as mean ± SEM of two-three separate experiments with 3 mice per group/experiment. Line depicts statistical significance ($p<0.05$) between groups determined by One-Way ANOVA with Tukey's posttest.

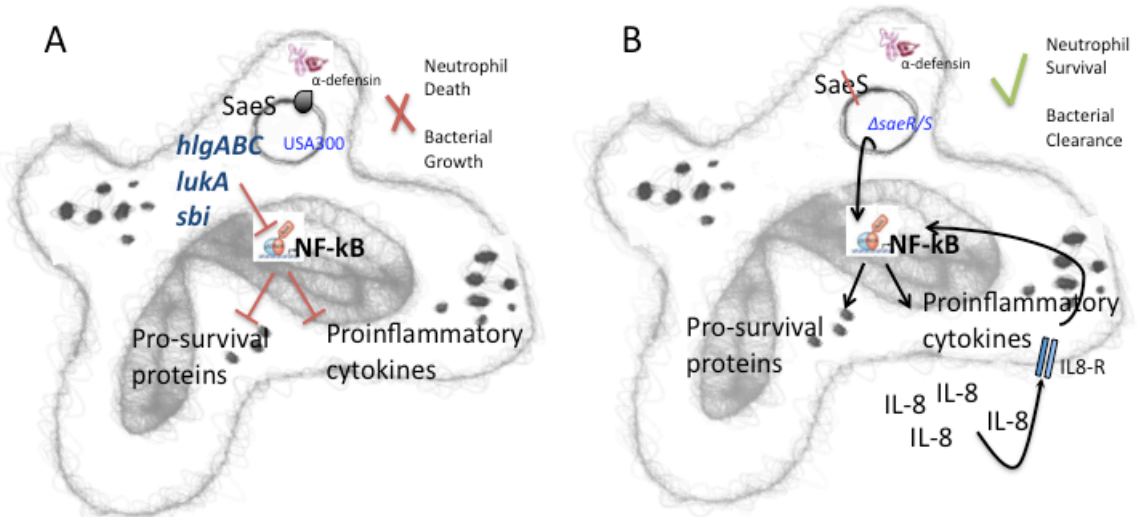


Figure 7. Neutrophil-mediated activation of the SaeR/S system results in accelerated cell death. Following phagocytosis of *S. aureus*, release of alpha-defensins contained within azurophilic granules activate the staphylococcal SaeR/S system resulting in increased transcription of specific virulence factors (A). Simultaneously, SaeR/S inhibits phosphorylation of NF-kB p-65 subunit which results in decreased production of IL-8. Increased expression of the SaeR/S-regulated virulence factors and modulation of the neutrophil immune response via suppression of IL-8 contribute to accelerated cell death and increased bacterial burden (A). In the absence of the SaeR/S system (B), USA300 does not sense alpha-defensin and does not express SaeR/S-regulated virulence factors. It follows that Δ*saeR/S*-infected neutrophils have increased levels of phosphorylated NF-kB resulting in IL-8 production that can elicit NF-kB activation in an autocrine manner leading to improved survival and enhanced antimicrobial functions of neutrophils.

Literature Cited

1. Dale DC. Advances in the treatment of neutropenia. Current Opinion in Supportive and Palliative Care. 2009 Sep;3(3):207–12.

2. Andersohn F, Konzen C, Garbe E. Systematic review: agranulocytosis induced by nonchemotherapy drugs. Ann Intern Med. 2007 May 1;146(9):657–65.

3. Pincus SH, Boxer LA, Stossel TP. Chronic neutropenia in childhood. Analysis of 16 cases and a review of the literature. Am J Med. 1976 Dec;61(6):849–61.

4. Bodey GP, Buckley M, Sathe YS, Freireich EJ. Quantitative relationships between circulating leukocytes and infection in patients with acute leukemia. Ann Intern Med. 1966 Feb;64(2):328–40.

5. Lekstrom-Himes JA, Gallin JI. Immunodeficiency diseases caused by defects in phagocytes. N Engl J Med. 2000 Dec 7;343(23):1703–14.

6. Wright DG, Meierovics AI, Foxley JM. Assessing the delivery of neutrophils to tissues in neutropenia. Blood. 1986 Apr;67(4):1023–30.

7. Silverstein SC, Rabadan R. How many neutrophils are enough (redux, redux)? Journal of Clinical Investigation. 2012 Aug 1;122(8):2776–9.

8. Li Y, Karlin A, Loike JD, Silverstein SC. A critical concentration of neutrophils is required for effective bacterial killing in suspension. Proc Natl Acad Sci USA. 2002 Jun 11;99(12):8289–94.

9. Malka R, Wolach B, Gavrieli R, Shochat E, Rom-Kedar V. Evidence for bistable bacteria-neutrophil interaction and its clinical implications. Journal of Clinical Investigation. 2012 Aug 1;122(8):3002–11.

10. Crawford J, Dale DC, Lyman GH. Chemotherapy-induced neutropenia. Cancer. 2004;100(2):228–37.

11. Hsieh MM, Everhart JE, Byrd-Holt DD, Tisdale JF, Rodgers GP. Prevalence of neutropenia in the U.S. population: age, sex, smoking status, and ethnic differences. Ann Intern Med. 2007 Apr 3;146(7):486–92.

12. Voyich JM, Braughton KR, Sturdevant DE, Whitney AR, Saïd-Salim B, Porcella SF, et al. Insights into mechanisms used by Staphylococcus aureus to avoid destruction by human neutrophils. J Immunol. 2005 Sep 15;175(6):3907–19.

13. Dinauer MC. Methods in Molecular Biology. Quinn MT, DeLeo FR, editors. Totowa, NJ: Humana Press; 2014. 15 p.

14. Kobayashi SD, Braughton KR, Palazzolo-Ballance AM, Kennedy AD, Sampaio E, Kristosturyan E, et al. Rapid neutrophil destruction following phagocytosis of Staphylococcus aureus. J Innate Immun. 2010;2(6):560–75.

15. Segal AW. How neutrophils kill microbes. Annu Rev Immunol. 2005 Apr;23(1):197–223.

16. Segal AW, Harper AM, Garcia RC, Merzbach D. The action of cells from patients with chronic granulomatous disease on Staphylococcus aureus. J Med Microbiol. 1982 Nov;15(4):441–9.

17. Voyich JM, DeLeo FR. Host-pathogen interactions: leukocyte phagocytosis and associated sequelae. Methods Cell Sci. 2002;24(1-3):79–90.

18. Dupre-Crochet S, Erard M, Nusse O. ROS production in phagocytes: why, when, and where? Journal of Leukocyte Biology. 2013 Sep 30;94(4):657–70.

19. Wengner AM, Pitchford SC, Furze RC, Rankin SM. The coordinated action of G-CSF and ELR + CXC chemokines in neutrophil mobilization during acute inflammation. Blood. 2007 Dec 21;111(1):42–9.

20. 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 Jan 15;201(2):241–54.

21. Kennedy AD, Wardenburg JB, Gardner DJ, Long D, Whitney AR, Braughton KR, et al. Targeting of alpha‐hemolysin by active or passive immunization decreases severity of USA300 skin infection in a mouse model. J Infect Dis. 2010 Oct;202(7):1050–8.

22. Zurek OW, Nygaard TK, Watkins RL, Pallister KB, Torres VJ, Horswill AR, et al. The role of innate immunity in promoting SaeR/S-Mediated virulence in Staphylococcus aureus. J Innate Immun. 2014;6(1):21–30.

23. Miller LS, O'Connell RM, Gutierrez MA, Pietras EM, Shahangian A, Gross CE, et al. MyD88 mediates neutrophil recruitment initiated by IL-1R but not TLR2 activation in immunity against Staphylococcus aureus. Immunity. 2006 Jan;24(1):79–91.

24. Cho JS, Pietras EM, Garcia NC, Ramos RI, Farzam DM, Monroe HR, et al. IL-17 is essential for host defense against cutaneous Staphylococcus aureus infection in mice. Journal of Clinical Investigation. 2010 May 3;120(5):1762–73.

25. Mölne L, Verdrengh M, Tarkowski A. Role of neutrophil leukocytes in cutaneous infection caused by Staphylococcus aureus. Infection and Immunity. 2000 Nov;68(11):6162–7.

26. Cho JS, Guo Y, Ramos RI, Hebroni F, Plaisier SB, Xuan C, et al. Neutrophil-derived IL-1β is sufficient for abscess formation in immunity against Staphylococcus aureus in mice. DeLeo FR, editor. PLoS Pathog. 2012 Nov 29;8(11):e1003047.

27. DuMont AL, Nygaard TK, Watkins RL, Smith A, Kozhaya L, Kreiswirth BN, et al. Characterization of a new cytotoxin that contributes to Staphylococcus aureus pathogenesis. Molecular Microbiology. 2010 Dec 13;79(3):814–25.

28. Ventura CL, Malachowa N, Hammer CH, Nardone GA, Robinson MA, Kobayashi SD, et al. Identification of a novel staphylococcus aureus two-component leukotoxin using cell surface proteomics. Horsburgh MJ, editor. PLoS ONE. 2010 Jul 16;5(7):e11634.

29. Malachowa N, Whitney AR, Kobayashi SD, Sturdevant DE, Kennedy AD, Braughton KR, et al. Global Changes in Staphylococcus aureus gene expression in human blood. Chakravortty D, editor. PLoS ONE. 2011 Apr 15;6(4):e18617.

30. Atkins KL, Burman JD, Chamberlain ES, Cooper JE, Poutrel B, Bagby S, et al. S. aureus IgG-binding proteins SpA and Sbi: Host specificity and mechanisms of immune complex formation. Molecular Immunology. 2008 Mar;45(6):1600–11.

31. 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. Elsevier Masson SAS; 2013 Sep 10;15(10-11):749–54.

32. Guggenberger C, Wolz C, Morrissey JA, Heesemann J. Two distinct coagulase-dependent barriers protect Staphylococcus aureus from neutrophils in a three dimensional in vitro infection model. Sullam PM, editor. PLoS Pathog. 2012 Jan 12;8(1):e1002434.

33. Cheng AG, DeDent AC, Schneewind O, Missiakas D. A play in four acts: Staphylococcus aureus abscess formation. Trends in Microbiology. Elsevier Ltd; 2011 May 1;19(5):225–32.

34. Gresham HD, Lowrance JH, Caver TE, Wilson BS, Cheung AL, Lindberg FP. Survival of Staphylococcus aureus inside neutrophils contributes to infection. J Immunol. 2000 Apr 1;164(7):3713–22.

35. Malachowa N, Kobayashi SD, Braughton KR, Whitney AR, Parnell MJ, Gardner DJ, et al. Staphylococcus aureus leukotoxin GH promotes inflammation. J Infect Dis. 2012 Aug 7.

36. Sun F, Li C, Jeong D, Sohn C, He C, Bae T. 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. Journal of Bacteriology. 2010 Mar 29;192(8):2111–27.

37. Novick RP. The staphylococcal saeRS system coordinates environmental signals with agr quorum sensing. Microbiology. 2003 Oct 1;149(10):2709–17.

38. Queck SY, Jameson-Lee M, Villaruz AE, Bach T-HL, Khan BA, Sturdevant DE, et al. RNAIII-independent target gene control by the agr quorum-sensing system: Insight into the evolution of virulence regulation in Staphylococcus aureus. Molecular Cell. 2008 Oct;32(1):150–8.

39. Flack CE, Zurek OW, Meishery DD, Pallister KB, Malone CL, Horswill AR, et al. Differential regulation of staphylococcal virulence by the sensor kinase SaeS in response to neutrophil-derived stimuli. Proc Natl Acad Sci USA. 2014 May 13;111(19):E2037–45.

40. Zurek OW, Pallister KB, Voyich JM. Staphylococcus aureus inhibits neutrophil-derived IL-8 to promote cell death. J Infect Dis. 2015 Feb 26.

41. Athanasopoulos AN, Economopoulou M, Orlova VV, Sobke A, Schneider D, Weber H, et al. The extracellular adherence protein (Eap) of Staphylococcus aureus inhibits wound healing by interfering with host defense and repair mechanisms. Blood. 2006 Apr 1;107(7):2720–7.

42. Allen, McCracken J. Regulation of human neutrophil apoptosis and lifespan in health and disease. JCD. 2014 May;:15.

43. Sarkar A, Hellberg L, Bhattacharyya A, Behnen M, Wang K, Lord JM, et al. Infection with Anaplasma phagocytophilum activates the phosphatidylinositol 3-kinase/akt and NF- B survival pathways in neutrophil granulocytes. Infection and Immunity. 2012 Mar 16;80(4):1615–23.

REFERENCES CITED

Adhikari, R. P., & Novick, R. P. (2008). Regulatory organization of the staphylococcal sae locus. *Microbiology*, *154*(3), 949–959. doi:10.1099/mic.0.2007/012245-0

Allen, & McCracken, J. (2014). Regulation of human neutrophil apoptosis and lifespan in health and disease. *Journal of Cell Death*, 15. doi:10.4137/JCD.S11038

Alonzo, F., III, Benson, M. A., Chen, J., Novick, R. P., Shopsin, B., & Torres, V. J. (2011). Staphylococcus aureus leucocidin ED contributes to systemic infection by targeting neutrophils and promoting bacterial growth in vivo. *Molecular Microbiology*, no–no. doi:10.1111/j.1365-2958.2011.07942

Andersohn, F., Konzen, C., & Garbe, E. (2007). Systematic review: agranulocytosis induced by nonchemotherapy drugs. *Annals of Internal Medicine*, *146*(9), 657–665

Athanasopoulos, A. N., Economopoulou, M., Orlova, V. V., Sobke, A., Schneider, D., Weber, H., et al. (2006). The extracellular adherence protein (Eap) of Staphylococcus aureus inhibits wound healing by interfering with host defense and repair mechanisms. *Blood*, *107*(7), 2720–2727. doi:10.1182/blood-2005-08-3140

Athens, J. W., Haab, O. P., Raab, S. O., Mauer, A. M., Ashenbrucker, H., Cartwright, G. E., & Wintrobe, M. M. (1961). Leukokinetic studies. IV. The total blood, circulating and marginal granulocyte pools and the granulocyte turnover rate in normal subjects. *Journal of Clinical Investigation*, *40*, 989–995. doi:10.1172/JCI104338

Atkins, K. L., Burman, J. D., Chamberlain, E. S., Cooper, J. E., Poutrel, B., Bagby, S., et al. (2008). S. aureus IgG-binding proteins SpA and Sbi: Host specificity and mechanisms of immune complex formation. *Molecular Immunology*, *45*(6), 1600–1611. doi:10.1016/j.molimm.2007.10.021

Bae, T., Banger, A. K., Wallace, A., Glass, E. M., Aslund, F., Schneewind, O., & Missiakas, D. M. (2004). Staphylococcus aureus virulence genes identified by bursa aurealis mutagenesis and nematode killing. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(33), 12312–12317. doi:10.1073/pnas.0404728101

Balmer, M. L., Schurch, C. M., Saito, Y., Geuking, M. B., Li, H., Cuenca, M., et al. (2014). Microbiota-derived compounds drive steady-state granulopoiesis via MyD88/TICAM signaling. *The Journal of Immunology*, *193*(10), 5273–5283. doi:10.4049/jimmunol.1400762

Bardoel, B. W., Kenny, E. F., Sollberger, G., & Zychlinsky, A. (2014). The balancing act of neutrophils. *Cell Host and Microbe*, *15*(5), 526–536. doi:10.1016/j.chom.2014.04.011

Bednar, F., Song, C., Bardi, G., Cornwell, W., & Rogers, T. J. (2014). Cross-Desensitization of CCR1, but not CCR2, following activation of the Formyl Peptide Receptor FPR1. *The Journal of Immunology*, *192*(11), 5305–5313. doi:10.4049/jimmunol.1302983

Beenken, K. E., Mrak, L. N., Zielinska, A. K., Atwood, D. N., Loughran, A. J., Griffin, L. M., et al. (2014). Impact of the functional status of saeRS on in vivo phenotypes of Staphylococcus aureus sarA mutants. *Molecular Microbiology*, *92*(6), 1299–1312. doi:10.1111/mmi.12629

Benton, B. M., Zhang, J. P., Bond, S., Pope, C., Christian, T., Lee, L., et al. (2004). Large-scale identification of genes required for full virulence of Staphylococcus aureus. *Journal of Bacteriology*, *186*(24), 8478–8489. doi:10.1128/JB.186.24.8478-8489.2004

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*(7), 2936–2943. doi:10.1182/blood-2006-06-015461

Bierman, H. R., Kelly, K. H., Cordes, F. L., Byron, R. L., Polhemus, J. A., & Rappoport, S. (1952). The release of leukocytes and platelets from the pulmonary circulation by epinephrine. *Blood*, *7*(7), 683–692

Bodey, G. P., Buckley, M., Sathe, Y. S., & Freireich, E. J. (1966). Quantitative relationships between circulating leukocytes and infection in patients with acute leukemia. *Annals of Internal Medicine*, *64*(2), 328–340

Borregaard, N. (2010). Neutrophils, from marrow to microbes. *Immunity*, *33*(5), 657–670. doi:10.1016/j.immuni.2010.11.011

Borregaard, N., Miller, L. J., & Springer, T. A. (1987). Chemoattractant-regulated mobilization of a novel intracellular compartment in human neutrophils. *Science*, *237*(4819), 1204–1206

Borregaard, N., Sørensen, O. E., & Theilgaard-Mönch, K. (2007). Neutrophil granules: a library of innate immunity proteins. *Trends in Immunology*, *28*(8), 340–345. doi:10.1016/j.it.2007.06.002

Bratton, D. L., & Henson, P. M. (2011). Neutrophil clearance: when the party is over, clean-up begins. *Trends in Immunology*, *32*(8), 350–357. doi:10.1016/j.it.2011.04.009

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., et

al. (2004). Neutrophil extracellular traps kill bacteria. *Science*, *303*(5663), 1532–1535. doi:10.1126/science.1092385

Brown, K., Brain, S., Pearson, J., Edgeworth, J., Lewis, S., & Treacher, D. (2006). Neutrophils in development of multiple organ failure in sepsis. *The Lancet*, *368*(9530), 157–169. doi:10.1016/S0140-6736(06)69005-3

Bugl, S., Wirths, S., Radsak, M. P., Schild, H., Stein, P., Andre, M. C., et al. (2013). Steady-state neutrophil homeostasis is dependent on TLR4/TRIF signaling. *Blood*, *121*(5), 723–733. doi:10.1182/blood-2012-05-429589

Cabell, C. H., Jollis, J. G., Peterson, G. E., Corey, G. R., Anderson, D. J., Sexton, D. J., et al. (2002). Changing patient characteristics and the effect on mortality in endocarditis. *Archives of Internal Medicine*, *162*(1), 90–94

Cassat, J. E., Hammer, N. D., Campbell, J. P., Benson, M. A., Perrien, D. S., Mrak, L. N., et al. (2013). A Secreted bacterial protease tailors the Staphylococcus aureus virulence repertoire to modulate bone remodeling during osteomyelitis. *Cell Host and Microbe*, *13*(6), 759–772. doi:10.1016/j.chom.2013.05.003

Chambers, H. F., & DeLeo, F. R. (2009). Waves of resistance: Staphylococcus aureus in the antibiotic era, 1–13. doi:10.1038/nrmicro2200

Cheng, A. G., DeDent, A. C., Schneewind, O., & Missiakas, D. (2011). A play in four acts: Staphylococcus aureus abscess formation. *Trends in Microbiology*, *19*(5), 225–232. doi:10.1016/j.tim.2011.01.007

Cheretakis, C., Leung, R., Sun, C. X., Dror, Y., & Glogauer, M. (2006). Timing of neutrophil tissue repopulation predicts restoration of innate immune protection in a murine bone marrow transplantation model. *Blood*, *108*(8), 2821–2826. doi:10.1182/blood-2006-04-018184

Cho, J. S., Guo, Y., Ramos, R. I., Hebroni, F., Plaisier, S. B., Xuan, C., et al. (2012). Neutrophil-derived IL-1β is sufficient for abscess formation in immunity against Staphylococcus aureus in mice. *PLoS Pathogens*, *8*(11), e1003047. doi:10.1371/journal.ppat.1003047.g006

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. *Journal of Clinical Investigation*, *120*(5), 1762–1773. doi:10.1172/JCI40891DS1

Crawford, J., Dale, D. C., & Lyman, G. H. (2004). Chemotherapy-induced neutropenia. *Cancer*, *100*(2), 228–237. doi:10.1002/cncr.11882

Dale, D. C. (2009). Advances in the treatment of neutropenia. *Current Opinion in Supportive and Palliative Care*. doi:10.1097/SPC.0b013e32832ea6ae

Dale, D. C., & Mackey, M. C. (2014). Understanding, treating and avoiding hematological disease: better medicine through mathematics? *Bulletin of Mathematical Biology*. doi:10.1007/s11538-014-9995-x

Dale, D. C., Guerry, D., Wewerka, J. R., Bull, J. M., & Chusid, M. J. (1979). Chronic neutropenia. *Medicine*, *58*(2), 128–144

Davis, S., Schaller, J., Wedgwood, R., & Harvard, M. (1966). Job's syndrome: recurrent,‘ cold’, staphylococcal abscesses. *The Lancet*, *287*(7445), 1013–1015

de Haas, C. J. C. (2004). Chemotaxis inhibitory protein of Staphylococcus aureus, a bacterial antiinflammatory agent. *Journal of Experimental Medicine*, *199*(5), 687–695. doi:10.1084/jem.20031636

Delaune, A., Dubrac, S., Blanchet, C., Poupel, O., Mader, U., Hiron, A., et al. (2012). The WalKR system controls major Staphylococcal virulence genes and is involved in triggering the host inflammatory response. *Infection and Immunity*, *80*(10), 3438–3453. doi:10.1128/IAI.00195-12

DeLeo, F. R., Diep, B. A., & Otto, M. (2009). Host defense and pathogenesis in Staphylococcus aureus infections. *Infectious Disease Clinics of North America*, *23*(1), 17–34. doi:10.1016/j.idc.2008.10.003

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. *The Lancet*, *367*(9512), 731–739. doi:10.1016/S0140-6736(06)68231-7

Dinauer, M. C. (2014). *Methods in Molecular Biology*. Vol. 1124, pp. 501–515. Totowa, NJ: Humana Press. doi:10.1007/978-1-62703-845-4_30

Dong, F., Hoefsloot, L. H., Schelen, A. M., Broeders, C. A., Meijer, Y., Veerman, A. J., et al. (1994). Identification of a nonsense mutation in the granulocyte-colony-stimulating factor receptor in severe congenital neutropenia. *Proceedings of the National Academy of Sciences*, *91*(10), 4480–4484

DuMont, A. L., Nygaard, T. K., Watkins, R. L., Smith, A., Kozhaya, L., Kreiswirth, B. N., et al. (2010). Characterization of a new cytotoxin that contributes to Staphylococcus aureus pathogenesis. *Molecular Microbiology*, *79*(3), 814–825. doi:10.1111/j.1365-2958.2010.07490

Dupre-Crochet, S., Erard, M., & Nusse, O. (2013). ROS production in phagocytes: why, when, and where? *Journal of Leukocyte Biology*, *94*(4), 657–670. doi:10.1189/jlb.1012544

Eash, K. J., Greenbaum, A. M., Gopalan, P. K., & Link, D. C. (2010). CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow. *Journal of Clinical Investigation*, *120*(7), 2423–2431. doi:10.1172/JCI41649DS1

Eisenhauer, P. B., & Lehrer, R. I. (1992). Mouse neutrophils lack defensins. *Infection and Immunity*, *60*(8), 3446–3447

Erttmann, S. F., Gekara, N. O., & Fällman, M. (2014). Bacteria induce prolonged PMN survival via a phosphatidylcholine-specific phospholipase C- and protein kinase C-dependent mechanism. *PLoS ONE*, *9*(1), e87859. doi:10.1371/journal.pone.0087859.s005

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. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(19), E2037–E2045. doi:10.1073/pnas.1322125111

Fowler, V. G., Miro, J. M., Hoen, B., Cabell, C. H., Abrutyn, E., Rubinstein, E., et al. (2005). Staphylococcus aureus endocarditis: a consequence of medical progress. *JAMA : the Journal of the American Medical Association*, *293*(24), 3012–3021. doi:10.1001/jama.293.24.3012

Fowler, V. G., Olsen, M. K., Corey, G. R., Woods, C. W., Cabell, C. H., Reller, L. B., et al. (2003). Clinical identifiers of complicated Staphylococcus aureus bacteremia. *Archives of Internal Medicine*, *163*(17), 2066–2072. doi:10.1001/archinte.163.17.2066

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. *The Journal of Cell Biology*, *176*(2), 231–241. doi:10.1083/jcb.200606027

Geiger, T., Goerke, C., Mainiero, M., Kraus, D., & Wolz, C. (2008). The virulence regulator Sae of Staphylococcus aureus: promoter activities and response to phagocytosis-related signals. *Journal of Bacteriology*, *190*(10), 3419–3428. doi:10.1128/JB.01927-07

Giraudo, A. T., Calzolari, A., Cataldi, A. A., & Bogni, C. (1999). The sae locus of Staphylococcus aureus encodes a two-component regulatory system. *FEMS Microbiology Letters 177*(1). 15-22.

Giraudo, A. T., Cheung, A. L., & Nagel, R. (1997). The sae locus of Staphylococcus aureus controls exoprotein synthesis at the transcriptional level. *Archives of Microbiology*, *168*(1), 53–58

Giraudo, A. T., Mansilla, C., Chan, A., Raspanti, C., & Nagel, R. (2003). Studies on the Expression of Regulatory Locus sae in Staphylococcus aureus. *Current Microbiology*, *46*(4), 246–250. doi:10.1007/s00284-002-3853-z

Giraudo, A. T., Raspanti, C. G., Calzolari, A., & Nagel, R. (1994). Characterization of a Tn551-mutant of Staphylococcus aureus defective in the production of several exoproteins. *Http://Dx.Doi.org/10.1139/M94-107*, *40*(8), 677–681. doi:10.1139/m94-107

Gordon, R. J., & Lowy, F. D. (2008). Pathogenesis of Methicillin-Resistant Staphylococcus aureusInfection. *Clinical Infectious Diseases*, *46*(S5), S350–S359. doi:10.1086/533591

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. *Journal of Leukocyte Biology*. doi:10.1189/jlb.1111575

Greenlee-Wacker, M. C., Rigby, K. M., Kobayashi, S. D., Porter, A. R., DeLeo, F. R., & Nauseef, W. M. (2014). Phagocytosis of Staphylococcus aureus by human neutrophils prevents macrophage efferocytosis and induces programmed necrosis. *The Journal of Immunology*, *192*(10), 4709–4717. doi:10.4049/jimmunol.1302692

Gresham, H. D., Lowrance, J. H., Caver, T. E., Wilson, B. S., Cheung, A. L., & Lindberg, F. P. (2000). Survival of Staphylococcus aureus inside neutrophils contributes to infection. *Journal of Immunology (Baltimore, Md. : 1950)*, *164*(7), 3713–3722

Guggenberger, C., Wolz, C., Morrissey, J. A., & Heesemann, J. (2012). Two Distinct Coagulase-dependent barriers protect Staphylococcus aureus from neutrophils in a three dimensional in vitro infection model. *PLoS Pathogens*, *8*(1), e1002434. doi:10.1371/journal.ppat.1002434.t002

Haggar, A., Ehrnfelt, C., Holgersson, J., & Flock, J.-I. (2004). The extracellular adherence protein from Staphylococcus aureus inhibits neutrophil binding to endothelial cells. *Infection and Immunity*, *72*(10), 6164–6167. doi:10.1128/IAI.72.10.6164-6167.2004

Hakkim, A., Fuchs, T. A., Martinez, N. E., Hess, S., Prinz, H., Zychlinsky, A., & Waldmann, H. (2010). Activation of the Raf-MEK-ERK pathway is required for

neutrophil extracellular trap formation. *Nature Chemical Biology*, *7*(2), 75–77. doi:10.1038/nchembio.496

Heit, B. (2002). An intracellular signaling hierarchy determines direction of migration in opposing chemotactic gradients. *The Journal of Cell Biology*, *159*(1), 91–102. doi:10.1083/jcb.200202114

Heit, B., Robbins, S. M., Downey, C. M., Guan, Z., Colarusso, P., Miller, B. J., et al. (2008). PTEN functions to 'prioritize' chemotactic cues and prevent “distraction” in migrating neutrophils. *Nature Immunology*, *9*(7), 743–752. doi:10.1038/ni.1623

Hibbs, M. L., Quilici, C., Kountouri, N., Seymour, J. F., Armes, J. E., Burgess, A. W., & Dunn, A. R. (2007). Mice lacking three myeloid colony-stimulating factors (G-CSF, GM-CSF, and M-CSF) still produce macrophages and granulocytes and mount an inflammatory response in a sterile model of peritonitis. *The Journal of Immunology*, *178*(10), 6435–6443. doi:10.4049/jimmunol.178.10.6435

Hoen, B., Alla, F., Selton-Suty, C., Béguinot, I., Bouvet, A., Briançon, S., et al. (2002). Changing profile of infective endocarditis: results of a 1-year survey in France. *JAMA : the Journal of the American Medical Association*, *288*(1), 75–81

Hsieh, M. M., Everhart, J. E., Byrd-Holt, D. D., Tisdale, J. F., & Rodgers, G. P. (2007). Prevalence of neutropenia in the U.S. population: age, sex, smoking status, and ethnic differences. *Annals of Internal Medicine*, *146*(7), 486–492

Jeong, D. W., Cho, H., Lee, H., Li, C., Garza, J., Fried, M., & Bae, T. (2011). Identification of the P3 promoter and distinct roles of the two promoters of the SaeRS two-component system in Staphylococcus aureus. *Journal of Bacteriology*, *193*(18), 4672–4684. doi:10.1128/JB.00353-11

Jeong, D.-W., Cho, H., Jones, M. B., Shatzkes, K., Sun, F., Ji, Q., et al. (2012). The auxiliary protein complex SaePQ activates the phosphatase activity of sensor kinase SaeS in the SaeRS two-component system of Staphylococcus aureus. *Molecular Microbiology*. 2012 Oct; 86(2); 331-48

Jin, T., Bokarewa, M., Foster, T., Mitchell, J., Higgins, J., & Tarkowski, A. (2004). Staphylococcus aureus resists human defensins by production of staphylokinase, a novel bacterial evasion mechanism. *Journal of Immunology (Baltimore, Md. : 1950)*, *172*(2), 1169–1176

Jones, M. B., Montgomery, C. P., Boyle-Vavra, S., Shatzkes, K., Maybank, R., Frank, B. C., et al. (2014). Genomic and transcriptomic differences in community acquired methicillin resistant Staphylococcus aureus USA300 and USA400 strains. *BMC Genomics*, *15*(1), 1145. doi:10.1186/1471-2164-15-1145

Kennedy, A. D., Wardenburg, J. B., Gardner, D. J., Long, D., Whitney, A. R., Braughton, K. R., et al. (2010). Targeting of alpha-hemolysin by active or passive immunization decreases severity of USA300 skin infection in a mouse model. *The Journal of Infectious Diseases*, *202*(7), 1050–1058. doi:10.1086/656043

Kettritz, R., Gaido, M. L., Haller, H., Luft, F. C., Jennette, C. J., & Falk, R. J. (1998). Interleukin-8 delays spontaneous and tumor necrosis factor-alpha-mediated apoptosis of human neutrophils. *Kidney International*, *53*(1), 84–91. doi:10.1046/j.1523-1755.1998.00741.x

Kim, M. H., Granick, J. L., Kwok, C., Walker, N. J., Borjesson, D. L., Curry, F. R. E., et al. (2011). Neutrophil survival and c-kit+-progenitor proliferation in Staphylococcus aureus-infected skin wounds promote resolution. *Blood*, *117*(12), 3343–3352. doi:10.1182/blood-2010-07-296970

King, M. D., Humphrey, B. J., Wang, Y. F., Kourbatova, E. V., Ray, S. M., & Blumberg, H. M. (2006). Emergence of community-acquired methicillin-resistant Staphylococcus aureus USA 300 clone as the predominant cause of skin and soft-tissue infections. *Annals of Internal Medicine*, *144*(5), 309–317

Klebanoff, S. J. (2005). Myeloperoxidase: friend and foe. *Journal of Leukocyte Biology*, *77*(5), 598–625. doi:10.1189/jlb.1204697

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 : the Journal of the American Medical Association*, *298*(15), 1763–1771. doi:10.1001/jama.298.15.1763

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 Pathogens*, *9*(12), e1003816. doi:10.1371/journal.ppat.1003816.s003

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. *Journal of Innate Immunity*, *2*(6), 560–575. doi:10.1159/000317134

Kobayashi, S. D., Malachowa, N., Whitney, A. R., Braughton, K. R., Gardner, D. J., Long, D., et al. (2011). Comparative analysis of USA300 virulence determinants in a rabbit model of skin and soft tissue infection. *The Journal of Infectious Diseases*, *204*(6), 937–941. doi:10.1093/infdis/jir441

Kobayashi, S. D., Voyich, J. M., & DeLeo, F. R. (2003). Regulation of the neutrophil-

mediated inflammatory response to infection. *Microbes and Infection*, *5*(14), 1337–1344. doi:10.1016/j.micinf.2003.09.013

Kobayashi, S. D., Voyich, J. M., Buhl, C. L., Stahl, R. M., & DeLeo, F. R. (2002). Global changes in gene expression by human polymorphonuclear leukocytes during receptor-mediated phagocytosis: cell fate is regulated at the level of gene expression. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(10), 6901–6906. doi:10.1073/pnas.092148299

Kolaczkowska, E., & Kubes, P. (2013). Neutrophil recruitment and function in health and inflammation. *Nature Reviews Immunology*, *13*(3), 159–175. doi:10.1038/nri3399

Kretschmer, D., Gleske, A.-K., Rautenberg, M., Wang, R., Köberle, M., Bohn, E., et al. (2010). Human Formyl Peptide Receptor 2 senses highly pathogenic Staphylococcus aureus. *Cell Host and Microbe*, *7*(6), 463–473. doi:10.1016/j.chom.2010.05.012

Kwak, H.-J., Liu, P., Bajrami, B., Xu, Y., Park, S.-Y., Nombela-Arrieta, C., et al. (2015). Myeloid cell-derived reactive oxygen species externally regulate the proliferation of myeloid progenitors in emergency granulopoiesis. *Immunity*, *42*(1), 159–171. doi:10.1016/j.immuni.2014.12.017

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. *The EMBO Journal*, *31*(17), 3607–3619. doi:10.1038/emboj.2012.212

Laarman, A. J., Ruyken, M., Malone, C. L., van Strijp, J. A. G., Horswill, A. R., & Rooijakkers, S. H. M. (2011). Staphylococcus aureus metalloprotease aureolysin cleaves complement c3 to mediate immune evasion. *The Journal of Immunology*, *186*(11), 6445–6453. doi:10.4049/jimmunol.1002948

Langley, R., Wines, B., Willoughby, N., Basu, I., Proft, T., & 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. *The Journal of Immunology*, *174*(5), 2926–2933. doi:10.4049/jimmunol.174.5.2926

Lautenschlager, S., Herzog, C., & Zimmerli, W. (1993). Course and outcome of bacteremia due to Staphylococcus aureus: evaluation of different clinical case definitions. *Clinical Infectious Diseases*, *16*(4), 567–573

Lee, L. Y. L., Liang, X., Hook, M., & Brown, E. L. (2004). Identification and characterization of the C3 binding domain of the Staphylococcus aureus extracellular fibrinogen-binding protein (Efb). *Journal of Biological Chemistry*,

279(49), 50710–50716. doi:10.1074/jbc.M408570200

Lehrer, R. I., & Cline, M. J. (1969). Leukocyte myeloperoxidase deficiency and disseminated candidiasis: the role of myeloperoxidase in resistance to Candida infection. *Journal of Clinical Investigation*, *48*(8), 1478–1488. doi:10.1172/JCI106114

Lekstrom-Himes, J. A., & Gallin, J. I. (2000). Immunodeficiency diseases caused by defects in phagocytes. *The New England Journal of Medicine*, *343*(23), 1703–1714. 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. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(14), 5883–5888. doi:10.1073/pnas.0900743106

Li, Y., Karlin, A., Loike, J. D., & Silverstein, S. C. (2002). A critical concentration of neutrophils is required for effective bacterial killing in suspension. *Proceedings of the National Academy of Sciences*, *99*(12), 8289–8294. doi:10.1073/pnas.122244799

Liu, G. Y. (2005). Staphylococcus aureus golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. *Journal of Experimental Medicine*, *202*(2), 209–215. doi:10.1084/jem.20050846

Lowy, F. D. (2003). Antimicrobial resistance: the example of Staphylococcus aureus. *Journal of Clinical Investigation*, *111*(9), 1265–1273. doi:10.1172/JCI18535

Mainiero, M., Goerke, C., Geiger, T., Gonser, C., Herbert, S., & Wolz, C. (2010). Differential target gene activation by the Staphylococcus aureus two-component system saeRS. *Journal of Bacteriology*, *192*(3), 613–623. doi:10.1128/JB.01242-09

Malachowa, N., Kobayashi, S. D., Braughton, K. R., Whitney, A. R., Parnell, M. J., Gardner, D. J., & DeLeo, F. R. (2012). Staphylococcus aureus leukotoxin GH promotes inflammation. *The Journal of Infectious Diseases*. doi:10.1093/infdis/jis495

Malachowa, N., Kobayashi, S. D., Freedman, B., Dorward, D. W., & DeLeo, F. R. (2013). Staphylococcus aureus leukotoxin GH promotes formation of neutrophil extracellular traps. *The Journal of Immunology*, *191*(12), 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*(4), e18617.

doi:10.1371/journal.pone.0018617.g008

Malka, R., Wolach, B., Gavrieli, R., Shochat, E., & Rom-Kedar, V. (2012). Evidence for bistable bacteria-neutrophil interaction and its clinical implications. *Journal of Clinical Investigation*, *122*(8), 3002–3011. doi:10.1172/JCI59832DS1

Manz, M. G., & Boettcher, S. (2014). Emergency granulopoiesis. *Nature Reviews Immunology*, *14*(5), 302–314. doi:10.1038/nri3660

Marincola, G., Schäfer, T., Behler, J., Bernhardt, J., Ohlsen, K., Goerke, C., & Wolz, C. (2012). RNase Y of Staphylococcus aureusand its role in the activation of virulence genes. *Molecular Microbiology*, *85*(5), 817–832. doi:10.1111/j.1365-2958.2012.08144

McCaig, L. F., McDonald, L. C., Mandal, S., & Jernigan, D. B. (2006). Staphylococcus aureus-associated skin and soft tissue infections in ambulatory care. *Emerging Infectious Diseases*, *12*(11), 1715–1723

McDonald, B., Urrutia, R., Yipp, B. G., Jenne, C. N., & Kubes, P. (2012). Intravascular neutrophil extracellular traps capture bacteria from the bloodstream during sepsis. *Cell Host and Microbe*, *12*(3), 324–333. doi:10.1016/j.chom.2012.06.011

Metzler, K. D., Fuchs, T. A., Nauseef, W. M., Reumaux, D., Roesler, J., Schulze, I., et al. (2011). Myeloperoxidase is required for neutrophil extracellular trap formation: implications for innate immunity. *Blood*, *117*(3), 953–959. doi:10.1182/blood-2010-06-290171

Midorikawa, K., Ouhara, K., Komatsuzawa, H., Kawai, T., Yamada, S., Fujiwara, T., et al. (2003). Staphylococcus aureus susceptibility to innate antimicrobial peptides, -defensins and CAP18, expressed by human keratinocytes. *Infection and Immunity*, *71*(7), 3730–3739. doi:10.1128/IAI.71.7.3730-3739.2003

Miller, J. F., Mekalanos, J. J., & Falkow, S. (1989). Coordinate regulation and sensory transduction in the control of bacterial virulence. *Science*, *243*(4893), 916–922

Miller, L. S., O'Connell, R. M., Gutierrez, M. A., Pietras, E. M., Shahangian, A., Gross, C. E., et al. (2006). MyD88 mediates neutrophil recruitment initiated by IL-1R but not TLR2 activation in immunity against Staphylococcus aureus. *Immunity*, *24*(1), 79–91. doi:10.1016/j.immuni.2005.11.011

Miro, J. M., Anguera, I., Cabell, C. H., Chen, A. Y., Stafford, J. A., Corey, G. R., et al. (2005). Staphylococcus aureus native valve infective endocarditis: report of 566 episodes from the International Collaboration on Endocarditis Merged Database. *Clinical Infectious Diseases*, *41*(4), 507–514. doi:10.1086/431979

Montgomery, C. P., & Daum, R. S. (2009). Transcription of inflammatory genes in the lung after infection with Community-Associated Methicillin-Resistant Staphylococcus aureus: a role for panton-valentine leukocidin? *Infection and Immunity*, *77*(5), 2159–2167. doi:10.1128/IAI.00021-09

Montgomery, C. P., Boyle-Vavra, S., & Daum, R. S. (2010). Importance of the global regulators Agr and SaeRS in the pathogenesis of CA-MRSA USA300 infection. *PLoS ONE*, *5*(12), e15177. doi:10.1371/journal.pone.0015177.t002

Montgomery, C. P., Boyle-Vavra, S., Adem, P. V., Lee, J. C., Husain, A. N., Clasen, J., & Daum, R. S. (2008). Comparison of Virulence in Community‐Associated Methicillin‐Resistant Staphylococcus aureus Pulsotypes USA300 and USA400 in a Rat Model of Pneumonia. *The Journal of Infectious Diseases*, *198*(4), 561–570. doi:10.1086/590157

Mölne, L., Verdrengh, M., & Tarkowski, A. (2000). Role of neutrophil leukocytes in cutaneous infection caused by Staphylococcus aureus. *Infection and Immunity*, *68*(11), 6162–6167

Neish, A. S., & Naumann, M. (2011). Microbial-induced immunomodulation by targeting the NF-κB system. *Trends in Microbiology*, *19*(12), 596–605. doi:10.1016/j.tim.2011.08.004

Novick, R. P. (2003). The staphylococcal saeRS system coordinates environmental signals with agr quorum sensing. *Microbiology*, *149*(10), 2709–2717. doi:10.1099/mic.0.26575-0

Novick, R. P., Ross, H. F., Projan, S. J., Kornblum, J., Kreiswirth, B., & Moghazeh, S. (1993). Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. *The EMBO Journal*, *12*(10), 3967–3975

Nygaard, T. K., DeLeo, F. R., & Voyich, J. M. (2008). Community-associated methicillin-resistant Staphylococcus aureus skin infections: advances toward identifying the key virulence factors. *Current Opinion in Infectious Diseases*, *21*(2), 147–152. doi:10.1097/QCO.0b013e3282f64819

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*(5), e36532. doi:10.1371/journal.pone.0036532.g007

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. *The Journal of Infectious Diseases*, *201*(2), 241–

254. doi:10.1086/649570

Nygaard, T. K., Pallister, K. B., Zurek, O. W., & Voyich, J. M. (2013). The impact of -toxin on host cell plasma membrane permeability and cytokine expression during human blood infection by CA-MRSA USA300. *Journal of Leukocyte Biology*, *94*(5), 971–979. doi:10.1189/jlb.0213080

Ohkubo, T., Tsuda, M., Suzuki, S., Borai, El, N., & Yamamura, M. (1999). Peripheral blood neutrophils of germ-free rats modified by in vivo granulocyte-colony-stimulating factor and exposure to natural environment. *Scandinavian Journal of Immunology*, *49*(1), 73–77

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. *Infection and Immunity*. doi:10.1128/IAI.01242-12

Olson, T. S., & Ley, K. (2002). Chemokines and chemokine receptors in leukocyte trafficking. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, *283*(1), R7–28. doi:10.1152/ajpregu.00738.2001

Otto, M. (2010). Basis of Virulence in Community-Associated Methicillin-Resistant Staphylococcus aureus. *Annual Review of Microbiology*, *64*(1), 143–162. doi:10.1146/annurev.micro.112408.134309

Palazzolo-Ballance, A. M., Reniere, M. L., Braughton, K. R., Sturdevant, D. E., Otto, M., Kreiswirth, B. N., et al. (2008). Neutrophil microbicides induce a pathogen survival response in community-associated methicillin-resistant Staphylococcus aureus. *Journal of Immunology (Baltimore, Md. : 1950)*, *180*(1), 500–509

Pang, Y. Y., Schwartz, J., Thoendel, M., Ackermann, L. W., Horswill, A. R., & Nauseef, W. M. (2010). agr-dependent interactions of Staphylococcus aureus USA300 with human polymorphonuclear neutrophils. *Journal of Innate Immunity*, *2*(6), 546–559. doi:10.1159/000319855

Patel, D., Wines, B. D., Langley, R. J., & Fraser, J. D. (2010). Specificity of Staphylococcal Superantigen-Like protein 10 toward the human IgG1 Fc domain. *The Journal of Immunology*, *184*(11), 6283–6292. doi:10.4049/jimmunol.0903311

Peacock, S. J., de Silva, I., & Lowy, F. D. (2001). What determines nasal carriage of Staphylococcus aureus? *Trends in Microbiology*, *9*(12), 605–610

Perichon, B., & Courvalin, P. (2009). VanA-Type Vancomycin-Resistant Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy*, *53*(11), 4580–4587. doi:10.1128/AAC.00346-09

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. *The Journal of Experimental Medicine*, *193*(9), 1067–1076

Peschel, A., Otto, M., Jack, R. W., Kalbacher, H., Jung, G., & Gotz, F. (1999). Inactivation of the dlt operon in Staphylococcus aureus confers sensitivity to defensins, protegrins, and other antimicrobial peptides. *The Journal of Biological Chemistry*, *274*(13), 8405–8410

Picard, C., Casanova, J. L., & Puel, A. (2011). Infectious diseases in patients with IRAK-4, MyD88, NEMO, or IkB deficiency. *Clinical Microbiology Reviews*, *24*(3), 490–497. doi:10.1128/CMR.00001-11

Pillay, J., Braber, den, I., Vrisekoop, N., Kwast, L. M., de Boer, R. J., Borghans, J. A. M., et al. (2010). In vivo labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days. *Blood*, *116*(4), 625–627. doi:10.1182/blood-2010-01-259028

Pincus, S. H., Boxer, L. A., & Stossel, T. P. (1976). Chronic neutropenia in childhood. Analysis of 16 cases and a review of the literature. *The American Journal of Medicine*, *61*(6), 849–861

Popovich, K. J., Aroutcheva, A., Hota, B., Beavis, K. G., Hayden, M. K., & Weinstein, R. A. (2014). Anatomic sites of colonization with Community-Associated Methicillin-Resistant Staphylococcus aureus. *Infection Control and Hospital Epidemiology*, *35*(9), 1192–1194. doi:10.1086/677627

Queck, S. Y., Jameson-Lee, M., Villaruz, A. E., Bach, T.-H. L., Khan, B. A., Sturdevant, D. E., et al. (2008). RNAIII-independent target gene control by the agr quorum-sensing system: insight into the evolution of virulence regulation in Staphylococcus aureus. *Molecular Cell*, *32*(1), 150–158. doi:10.1016/j.molcel.2008.08.005

Quinn, M. T., Ammons, M. C. B., & DeLeo, F. R. (2006). The expanding role of NADPH oxidases in health and disease: no longer just agents of death and destruction. *Clinical Science*, *111*(1), 1. doi:10.1042/CS20060059

Reeves, E. P., Lu, H., Jacobs, H. L., Messina, C. G. M., Bolsover, S., Gabella, G., et al. (2002). Killing activity of neutrophils is mediated through activation of proteases by K+ flux. *Nature*, *416*(6878), 291–297. doi:10.1038/416291a

Ringberg, H., Thorén, A., & Lilja, B. (2000). Metastatic complications of Staphylococcus aureus septicemia. To seek is to find. *Infection*, *28*(3), 132–136

Rittirsch, D., Flierl, M. A., & Ward, P. A. (2008). Harmful molecular mechanisms in sepsis. *Nature Reviews Immunology*, *8*(10), 776–787. doi:10.1038/nri2402

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. *Nature Immunology*, *6*(9), 920–927. doi:10.1038/ni1235

Rooijakkers, S. H. M., van Wamel, W. J. B., Ruyken, M., van Kessel, K. P. M., & van Strijp, J. A. G. (2005b). Anti-opsonic properties of staphylokinase. *Microbes and Infection*, *7*(3), 476–484. doi:10.1016/j.micinf.2004.12.014

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. *Journal of Immunology (Baltimore, Md. : 1950)*, *170*(10), 5268–5275

Said-Salim, B., Dunman, P. M., McAleese, F. M., Macapagal, D., Murphy, E., McNamara, P. J., et al. (2003). Global Regulation of Staphylococcus aureus Genes by Rot. *Journal of Bacteriology*, *185*(2), 610–619. doi:10.1128/JB.185.2.610-619.2003

Sarkar, A., Hellberg, L., Bhattacharyya, A., Behnen, M., Wang, K., Lord, J. M., et al. (2012). Infection with Anaplasma phagocytophilum activates the Phosphatidylinositol 3-Kinase/Akt and NF-kB survival pathways in neutrophil granulocytes. *Infection and Immunity*, *80*(4), 1615–1623. doi:10.1128/IAI.05219-11

Segal, A. W. (2005). How neutrophils kill microbes. *Annual Review of Immunology*, *23*(1), 197–223. doi:10.1146/annurev.immunol.23.021704.115653

Segal, A. W., Harper, A. M., Garcia, R. C., & Merzbach, D. (1982). The action of cells from patients with chronic granulomatous disease on Staphylococcus aureus. *Journal of Medical Microbiology*, *15*(4), 441–449

Sengeløv, H., Follin, P., Kjeldsen, L., Lollike, K., Dahlgren, C., & Borregaard, N. (1995). Mobilization of granules and secretory vesicles during in vivo exudation of human neutrophils. *Journal of Immunology (Baltimore, Md.:1950)*, *154*(8), 4157–4165

Siemsen, D. W., Schepetkin, I. A., Kirpotina, L. N., Lei, B., & Quinn, M. T. (2007). Neutrophil isolation from nonhuman species. *Methods in Molecular Biology-Clifton Then Totowa-*, *412*, 21–34

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. *Antimicrobial Agents and Chemotherapy*, *48*(12), 4673–4679. doi:10.1128/AAC.48.12.4673-4679.2004

Silverstein, S. C., & Rabadan, R. (2012). How many neutrophils are enough (redux, redux)? *Journal of Clinical Investigation*, *122*(8), 2776–2779. doi:10.1172/JCI63939

Smith, E. J., Visai, L., Kerrigan, S. W., Speziale, P., & Foster, T. J. (2011). The Sbi protein is a multifunctional immune evasion factor of Staphylococcus aureus. *Infection and Immunity*, *79*(9), 3801–3809. doi:10.1128/IAI.05075-11

Spaan, A. N., Surewaard, B. G. J., Nijland, R., & Van Strijp, J. A. G. (2013). Neutrophils versus Staphylococcus aureus: A biological tug of war. *Annual Review of Microbiology*, *67*(1), 629–650. doi:10.1146/annurev-micro-092412-155746

Stark, M. A., Huo, Y., Burcin, T. L., Morris, M. A., Olson, T. S., & Ley, K. (2005). Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17. *Immunity*, *22*(3), 285–294. doi:10.1016/j.immuni.2005.01.011

Steinhuber, A., Goerke, C., Bayer, M. G., Döring, G., & Wolz, C. (2003). Molecular architecture of the regulatory Locus sae of Staphylococcus aureus and its impact on expression of virulence factors. *Journal of Bacteriology*, *185*(21), 6278–6286

Stemerding, A. M., Kohl, 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 Fc R antagonists that inhibit IgG-mediated effector functions. *The Journal of Immunology*, *191*(1), 353–362. doi:10.4049/jimmunol.1203243

Stryjewski, M. E., & Corey, G. R. (2013). Methicillin-Resistant Staphylococcus aureus: an evolving pathogen. *Clinical Infectious Diseases*, *58*(suppl 1), S10–S19. doi:10.1093/cid/cit613

Summers, C., Rankin, S. M., Condliffe, A. M., Singh, N., Peters, A. M., & Chilvers, E. R. (2010). Neutrophil kinetics in health and disease. *Trends in Immunology*, *31*(8), 318–324. doi:10.1016/j.it.2010.05.006

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. *Journal of Bacteriology*, *192*(8), 2111–2127. doi:10.1128/JB.01524-09

Tak, T., Tesselaar, K., Pillay, J., Borghans, J. A. M., & Koenderman, L. (2013). What's your age again? Determination of human neutrophil half-lives revisited. *Journal of*

Leukocyte Biology, *94*(4), 595–601. doi:10.1189/jlb.1112571

Thammavongsa, V., Missiakas, D. M., & Schneewind, O. (2013). Staphylococcus aureus degrades neutrophil extracellular traps to promote immune cell death. *Science*, *342*(6160), 863–866

Ventura, C. L., Malachowa, N., Hammer, C. H., Nardone, G. A., Robinson, M. A., Kobayashi, S. D., & DeLeo, F. R. (2010). Identification of a novel Staphylococcus aureus two-component leukotoxin using cell surface proteomics. *PLoS ONE*, *5*(7), e11634. doi:10.1371/journal.pone.0011634.g005

Voyich, J. M., & DeLeo, F. R. (2002). Host-pathogen interactions: leukocyte phagocytosis and associated sequelae. *Methods in Cell Science: an Official Journal of the Society for in Vitro Biology*, *24*(1-3), 79–90

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. *Journal of Immunology (Baltimore, Md. : 1950)*, *175*(6), 3907–3919

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? *Journal of Infectious Diseases*, *194*(12), 1761

Voyich, J. M., Sturdevant, D. E., & DeLeo, F. R. (2008). Analysis of Staphylococcus aureus gene expression during PMN phagocytosis. *Methods in Molecular Biology-Clifton Then Totowa-*, *431*, 109

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. *The Journal of Infectious Diseases*, *199*(11), 1698–1706. doi:10.1086/598967

Walker, F., Zhang, H.-H., Matthews, V., Weinstock, J., Nice, E. C., Ernst, M., et al. (2008). IL6/sIL6R complex contributes to emergency granulopoietic responses in G-CSF- and GM-CSF-deficient mice. *Blood*, *111*(8), 3978–3985. doi:10.1182/blood-2007-10-119636

Wang, A., Athan, E., Pappas, P. A., Fowler, V. G., Olaison, L., Paré, C., et al. (2007). Contemporary clinical profile and outcome of prosthetic valve endocarditis. *JAMA : the Journal of the American Medical Association*, *297*(12), 1354–1361. doi:10.1001/jama.297.12.1354

Ward, C. (1999). NF-kappa B activation is a critical regulator of human granulocyte apoptosis in vitro. *Journal of Biological Chemistry*, *274*(7), 4309–4318

Watkins, R. L., Pallister, K. B., & Voyich, J. M. (2011). The SaeR/S gene regulatory system induces a pro-inflammatory cytokine response during Staphylococcus aureus Infection. *PLoS ONE*, *6*(5), e19939. doi:10.1371/journal.pone.0019939.t001

Watkins, R. L., Zurek, O. W., Pallister, K. B., & Voyich, J. M. (2013). The SaeR/S two-component system induces interferon-gamma production in neutrophils during invasive Staphylococcus aureus infection. *Microbes and Infection / Institut Pasteur*, *15*(10-11), 749–754. doi:10.1016/j.micinf.2013.05.004

Wengner, A. M., Pitchford, S. C., Furze, R. C., & Rankin, S. M. (2007). The coordinated action of G-CSF and ELR + CXC chemokines in neutrophil mobilization during acute inflammation. *Blood*, *111*(1), 42–49. doi:10.1182/blood-2007-07-099648

Woehl, J. L., Stapels, D. A. C., Garcia, B. L., Ramyar, K. X., Keightley, A., Ruyken, M., et al. (2014). The extracellular adherence protein from Staphylococcus aureus inhibits the classical and lectin pathways of complement by blocking formation of the C3 proconvertase. *The Journal of Immunology*, *193*(12), 6161–6171. doi:10.4049/jimmunol.1401600

Wright, D. G., Meierovics, A. I., & Foxley, J. M. (1986). Assessing the delivery of neutrophils to tissues in neutropenia. *Blood*, *67*(4), 1023–1030

Wright, J. S., Jin, R., & Novick, R. P. (2005). Transient interference with staphylococcal quorum sensing blocks abscess formation. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(5), 1691–1696. doi:10.1073/pnas.0407661102

Xiong, Y. Q., Willard, J., Yeaman, M. R., Cheung, A. L., & Bayer, A. S. (2006). Regulation of Staphylococcus aureus alpha-toxin gene (hla) expression by agr, sarA, and sae in vitro and in experimental infective endocarditis. *Journal of Infectious Diseases*, *194*(9), 1267–1275. doi:10.1086/508210

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. *Journal of Innate Immunity*, *6*(1), 21–30. doi:10.1159/000351200

Zurek, O. W., Pallister, K. B., & Voyich, J. M. (2015). Staphylococcus aureus inhibits neutrophil-derived IL-8 to promote cell death. *The Journal of Infectious Diseases*. doi:10.1093/infdis/jiv124