

THE STAPHYLOCOCCUS AUREUS TWO COMPONENT SYSTEM, SAER/S,  
MODULATES MONOCYTE PRODUCTION OF TNF- $\alpha$  TO INFLUENCE  
NEUTROPHIL FUNCTIONS

by

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A dissertation submitted in partial fulfillment  
of the requirements for the degree

of

Doctor of Philosophy

in

Immunology and Infectious Diseases

MONTANA STATE UNIVERSITY  
Bozeman, Montana

April 2018

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## ACKNOWLEDGEMENTS

First and foremost, I would like to thank my mentor, Dr. Jovanka Voyich, for making my graduate studies an invaluable experience. It has been a privilege to study under Dr. Voyich and I have been humbled by her outstanding scientific expertise, professorship, and communication skills. Most importantly, I have appreciated Dr. Voyich's continual support, encouragement, generosity and willingness to talk even amidst her relentless schedule. Dr. Voyich has been a great role model and an inspiration and it has been an honor to study and learn from her.

To my committee members, Dr. Mark Jutila, Dr. Mark Quinn, and Dr. Valerie Copié, thank you for furthering my education and for your flexibility. The input you all provided in our meetings led to the development and completion of my project, and for that I am truly grateful. I would also like to thank all the members of the Voyich lab, past and present. Our experiences in and outside of the lab are memories that I will never forget. Thank you for making every day a great experience.

Last but not least, I would like to thank my family and wife that supported me through the years. Through thick and thin, you were always there for me. Your words of encouragement and wisdom, paired with unfailing love, allowed me to succeed to heights I never thought were possible. Thank you for being a strong foundation and thank you for all that you have done and continue to do.

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## ABSTRACT

*Staphylococcus aureus* (*S. aureus*) is a commensal organism that colonizes the anterior nares of more than half the population. Although most individuals colonized with *S. aureus* remain asymptomatic, showing no signs of complications, colonization is associated with a predisposition to infection. *S. aureus* infections include skin- and soft-tissue infections as well as life-threatening infections, such as necrotizing fasciitis, necrotizing pneumonia, and sepsis. To date, it is not clearly understood how *S. aureus* transitions from a commensal organism to a deadly pathogen but evidence highlights that this capacity is largely dependent on two-component gene-regulatory systems that control expression of cytolytic and immunomodulatory virulence factors. The SaeR/S two-component system (SaeR/S TCS) of *S. aureus* is critical for the regulation of virulence factors that enables immune evasion and attenuates killing of *S. aureus* by human neutrophils. However, the precise SaeR/S-dependent mechanisms used by *S. aureus* to overcome and effective neutrophil response remains incompletely define. To advance our understanding, we studied SaeR/S-dependent immunomodulation of TNF- $\alpha$ . TNF- $\alpha$  is an important inflammatory mediator because it can recruit neutrophils to the site of infection and promote increased neutrophil killing of *S. aureus*. Using primary human cells, we demonstrated that the SaeR/S system reduced early monocyte production of TNF- $\alpha$  and showed that this modulation influenced the neutrophil priming and subsequent staphylocidal activity. These results demonstrated that *S. aureus* could reduce TNF- $\alpha$  early during infection to diminish neutrophil production of reactive oxygen species. As increased TNF- $\alpha$  is associated with morbidity and mortality during systemic infections, we propose that SaeR/S modulation of monocyte-derived TNF- $\alpha$  is important for reducing protective immune responses during localized infections. These data add to our understanding of how *S. aureus* disrupts early inflammatory responses to initiate infection.

## CHAPTER ONE

## INTRODUCTION TO DISSERTATION

Pathogenic *Staphylococcus aureus* (*S. aureus*) and methicillin-resistant *S. aureus* (MRSA) account for diverse diseases ranging in severity from mild skin and soft-tissue infections to life-threatening bacteremia, endocarditis, and necrotizing pneumonia<sup>1</sup>. In 2011, there was an estimated 80,461 invasive MRSA infections in the United States with an associated 11,285 deaths<sup>2</sup>. Although invasive MRSA infections declined by 31% compared to 2005, these estimates largely reflected infections caused by hospital-associated MRSA (HA-MRSA). In contrast, infections caused by community-associated MRSA (CA-MRSA) declined rather minimally. Unlike HA-MRSA, which causes infections in individuals with underlying predispositions, CA-MRSA can cause disease in otherwise healthy people independent of clinical environments<sup>1</sup>. Furthermore, the threat of multi-drug resistant *S. aureus* is a major global health concern and necessitates the development of alternative therapeutic approaches to mitigate infection. Currently, immunomodulation by which *S. aureus* influences host pro-inflammatory responses to promote pathogenesis is an emerging field of study<sup>3</sup>. Characterizing protective or detrimental immune responses elicited during *S. aureus* infection could provide insight into developing immunotherapies or prophylactic vaccines. However, characterizing staphylococcal immunomodulatory mechanisms requires a strong understanding of *S. aureus* virulence, pathogenesis and subsequent immune responses.

Prominent differences between HA-MRSA and CA-MRSA exist due to mobile genetic elements and altered regulation and non-synonymous mutations in core genes<sup>4</sup>.

One mobile genetic element that has been largely investigated is the Staphylococcal cassette chromosome (*SCCmec*), which has a variety of insertion sequence (IS) elements, transposons and integrated plasmids known to confer antibiotic-resistance<sup>1</sup>. Differences in the *SCCmec*, however, cannot explain the heightened virulence of specific CA-MRSA strains, as nearly all strains possess this element<sup>1</sup> and methicillin-susceptible strains can also be very pathogenic<sup>5</sup>. Another factor encoded by a mobile genetic element that is predominantly associated with CA-MRSA strains is the Panton-Valentine leukocidin (PVL)<sup>6,7</sup>. PVL is strongly associated with necrotizing pneumonia and skin and soft-tissue infections, however, strains lacking PVL have been shown to be as virulent in mouse and human models of infection<sup>7-9</sup>. These reports suggest that the heightened pathogenesis of CA-MRSA strains is influenced by other underlying virulence determinants, such as virulence factors under the control of global gene regulatory systems. Nonetheless, investigating individual staphylococcal virulence determinants is valued due to the potential of creating a target candidate that could be selectively inhibited through alternative therapeutic treatments. However, this strategy has proven to be challenging due to the abundance of virulence factors and the redundancy in their mechanism of action.

Currently, a primary focus is elucidating the differential regulation, production, and influence of staphylococcal virulence factors on the immune system. The relevance of this focus is exemplified from studies characterizing similar pathogenesis between HA-MRSA and CA-MRSA strains despite differences in acquired mobile genetic elements<sup>9</sup>. The similarities in virulence correlated to differential expression of core genome-encoded virulence determinants, such as cytolytic *S. aureus* exoproteins<sup>9</sup>. Many

of the *S. aureus* exoproteins are under the control of global gene-regulatory systems, which represent prime targets for selective inhibition to attenuate production of key virulence factors<sup>9,10</sup>. If *S. aureus* virulence factors can be nullified through selective inhibition, it may prevent early host tissue destruction and immune-dysfunction and allow for an enhanced immune response to occur. In particular, the staphylococcal two-component gene regulatory systems control expression of multiple virulence factors and represent major candidates for selective inhibition. *S. aureus* has 16 two-component gene regulatory systems that sense environmental signals and control expression of multiple virulence and immunomodulatory factors<sup>11,12</sup>. If these global regulatory systems are attenuated, it could prevent production of virulence determinants and ultimately lead to an improved immune response and overall host outcome.

To further our understanding of *S. aureus* immunomodulation there must also be efforts focused on studying immune responses during *S. aureus* infection. Focusing on the inflammatory response is a promising approach because cytokines produced early during interaction between *S. aureus* and the immune response can largely influence the subsequent course of disease<sup>13</sup>. Although the production of inflammatory signals in the context of *S. aureus* infections has been extensively studied, significant gaps remain in our understanding as to how early regulation of inflammatory cytokines influence downstream immune responses. To address immunomodulation and the role of specific staphylococcal virulence determinants, we herein characterize a role of the *S. aureus* SaeR/S two-component gene regulatory system and demonstrate that it is essential for diminishing production of a critical pro-inflammatory cytokine, tumor-necrosis factor- $\alpha$  (TNF- $\alpha$ ) from monocytes, which can then influence neutrophil priming. The studies

described in this dissertation will provide insight into the influence of SaeR/S-modulation of monocyte-derived TNF- $\alpha$  and how this modulation regulates neutrophil priming and antimicrobial functions.

### Overview of Dissertation

Herein, we evaluated a role for the staphylococcal two-component gene regulatory system, SaeR/S, on the influence of monocyte-derived TNF- $\alpha$  production and the subsequent effect on neutrophil functions. To provide background for the studies performed in this dissertation, Chapter two will focus on the current and foundational literature regarding the host innate immune response during inflammation and infection with *S. aureus* and will end with the hypothesis of the dissertation. Topics in Chapter 2 will elucidate literature on neutrophil production, migration, priming, antibacterial mechanisms and cell fate as well as how *S. aureus* can influence these functions to promote pathogenesis. In Chapter 3, we demonstrate the ability of SaeR/S to modulate early monocyte production of TNF- $\alpha$ . Modulation of monocyte-derived TNF- $\alpha$  occurred in both whole blood and isolated peripheral blood mononuclear cells (PBMCs) and was independent of cell lysis. We further elucidate the role of TNF- $\alpha$  modulation on neutrophil priming. Specifically, we implement novel co-culture models and conditioned media assays to demonstrate that SaeR/S modulation of TNF- $\alpha$  directly influences neutrophil ROS production and staphylocidal activity. In Chapter 4, co-authored work will be discussed. In Chapter 5, we further explore the role of TNF- $\alpha$  modulation in the context of *saeR/S*-mediated pathogenesis and influence on monocyte subsets. Neutrophil

functions, including ROS and apoptosis, in response to different concentrations TNF- $\alpha$  will be characterized. Also included are preliminary data elucidating the specific immunomodulatory factors responsible for reducing monocyte derived TNF- $\alpha$ .

## CHAPTER TWO

## BACKGROUND

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

*Staphylococcus aureus* (*S. aureus*) is a Gram-positive bacterium that persistently colonizes one out of three individuals<sup>14</sup>. Although most individuals colonized with *S. aureus* remain asymptomatic throughout their lifetime, colonization is associated with a predisposition to disease<sup>14,15</sup>. Disease caused by *S. aureus* predominate as recurrent skin and soft-tissue infections (SSTI) but can also disseminate and cause invasive life-threatening complications<sup>16</sup>. Due to the inherent ability to disseminate and the high prevalence of *S. aureus* in the community, this pathogen remains a leading cause of morbidity and mortality in the United States<sup>2</sup>. This is exemplified by the fact that *S. aureus* infections are estimated to cause 400,000 hospital admissions in the United States annually and 12 million annual outpatient visits worldwide<sup>17</sup>. Severe life-threatening diseases caused by *S. aureus* are also compounded by the rapid emergence of resistance to antibiotics. Currently, staphylococcal strains have acquired resistance to all  $\beta$ -lactam antibiotics, including penicillin and methicillin, which developed shortly after their introduction<sup>1</sup>. Resistance to  $\beta$ -lactam antibiotics is associated with the acquisition of the staphylococcal cassette chromosome (SCC*mec*)<sup>1</sup>, which is a mobile genetic element containing insertion sequence elements, transposons and integrated plasmids<sup>1</sup>. There are currently eight distinct SCC*mec* types among *S. aureus* strains, with SCC*mec*IV being

predominant in strains of community-associated methicillin-resistant *S. aureus* (CA-MRSA)<sup>18</sup>.

CA-MRSA strain, USA300 (LAC), is the leading cause of MRSA infections in the United States<sup>19</sup>. USA300 can cause infection in otherwise healthy individuals and, in comparison to hospital-associated MRSA (HA-MRSA) strains, USA300 is thought to exhibit enhanced virulence, also referred to as hypervirulence<sup>9</sup>. The hypervirulence of USA300 correlates to an augmented ability to cause dermonecrotic lesions as well as invasive diseases such as bacteremia, endocarditis, and necrotizing fasciitis<sup>20–22</sup>. Many studies demonstrate that these complications arise from essential immune evasion mechanisms and altered transcriptional regulation of virulence factors<sup>10,23,24</sup>. These immune evasion mechanisms of USA300 include influencing chemotaxis of innate immune cells, interfering with complement and phagocytosis, inhibiting the detrimental effects of antimicrobial peptides, and altering neutrophil cell fate<sup>25</sup>. Additionally, USA300 has a diverse repertoire of secreted pore-forming toxins that can bind to specific receptors on immune cells and result in host cell lysis<sup>26</sup>. These immune evasion and modulatory strategies combined with the rapid development of antibiotic-resistance in USA300 necessitates the development for alternative forms of treatment.

The necessity for alternative forms of treatment is further exemplified by the immune evasion and immunomodulatory strategies of USA300, which are regulated by two-component gene regulatory systems. In particular, the *S. aureus* exoprotein expression system (SaeR/S) controls transcription of virulence factors that have a conserved consensus binding sequence within the promoter region<sup>22</sup>. In responses to host derived molecules, such as neutrophil  $\alpha$ -defensin or H<sub>2</sub>O<sub>2</sub>, the SaeR/S-system can

activate expression of specific genes within 5-10 minutes that subsequently produce virulence factors to promote survival<sup>27,28</sup>. As production of these antimicrobial molecules and rapid activation of SaeR/S are observed early following phagocytosis, these studies highlight that *S. aureus* and neutrophils are well acquainted very early in infection. If the neutrophil antibacterial mechanisms prevail, *S. aureus* will be eliminated leading to resolution of infection but if *S. aureus* prevails, many of the produced virulence factors can subsequently regulate proximal cell cytokine production, leading to enhanced inflammation and exacerbation of infection<sup>29,30</sup>. Nonetheless, in order for *S. aureus* to be a successful pathogen and exacerbate infection, it must first overcome responses of the neutrophil.

#### The Assassin and Foe, *S. aureus*-Neutrophil Interactions

Polymorphonuclear leukocytes (PMNs or neutrophils) are an abundant innate immune cell that are essential during bacterial and fungal infections<sup>31</sup>. Neutrophils are generated from the bone marrow and upon maturation, are released into the circulatory system where they account for approximately 60-70% of the total leukocyte population<sup>32</sup>. Neutrophils in circulation are equipped with granules and secretory vesicles that have antibacterial proteins<sup>33</sup>. In addition to these vesicles, neutrophils utilize NADPH-oxidase to generate reactive oxygen species (ROS) that can also mediate direct killing of intra- and extracellular pathogens<sup>33</sup>. The importance of neutrophils and their associated mechanisms during *S. aureus* infection is demonstrated in individuals with genetic and acquired immunodeficiency conditions. For example, individuals with chronic granulomatous disease (CGD) are characterized by a defect in the NADPH oxidase complex leading to an

impairment of ROS production<sup>34</sup>. CGD patients and others with defects in neutrophil functions are associated with an increased susceptibility to *S. aureus*; thus, highlighting the importance of neutrophil responses during infection<sup>35</sup>. Despite the abundance of neutrophils in circulation and their efficient arsenal of antibacterial properties, *S. aureus* utilizes mechanisms to disrupt every neutrophil function, ranging from recruitment and activation to ROS and apoptosis.

#### Monocytes in Inflammation and Role in Neutrophil Recruitment

Monocytes, dendritic cells, and macrophages are mononuclear phagocytic cells that comprise the myeloid lineage and are important for neutrophil recruitment. Monocytes mature in the bone-marrow and are derived from macrophage and dendritic cell progenitors (MDPs) that have differentiated into common monocyte progenitors (cMOPs)<sup>36</sup>. Upon maturation, cMOPs decrease expression of CXC-chemokine receptor 4 (CXCR4), which promotes egress from the bone marrow followed by circulation in the blood and spleen<sup>36</sup>. Circulating monocytes can be further distinguished as patrolling or non-classical monocytes that have endothelial cell-supporting functions or transmigrating classical monocytes that enter into tissue in response to appropriate stimuli. In humans, classical monocytes are characterized by high expression of CD14, the major co-receptor with TLR4 that binds to LPS, whereas non-classical monocytes express lower levels of CD14 as well as CD16, which is the Fcγ receptor type III (FcγRIII)<sup>37</sup>. Non-classical monocytes can quickly arise from classical monocytes, which constitute 80-95% of total monocytes in circulation<sup>38</sup>. Under steady state conditions the half-life of classical monocytes is very short and without appropriate signaling these monocytes undergo

spontaneous apoptosis and are removed<sup>39</sup>. Following maturation, classical monocytes increase expression of MHC class II, CD11c and CCR7, which is important for adaptive immunity because they can present antigens to cognate T cells in the draining lymph node<sup>36</sup>. Alternatively, classical monocytes can enter into tissue and lymph nodes, convert into non-classical monocytes, and during inflammation mature into monocyte-derived macrophages and dendritic cells which have much longer lifespans<sup>37</sup>.

Monocytes that have traversed the endothelium exhibit increased expression of IL-1 $\beta$  and TNF- $\alpha$  in response to microbial challenge, which are important for neutrophil recruitment and activation<sup>36</sup>. In addition, these monocytes have increased expression of MHC class II, CD11c and CCR7 and lose expression of CD14 and CD62L<sup>36</sup>. Pro-inflammatory signals within the tissue environment can further induce monocytes to increase in size and can promote changes in cytoplasmic constituents such as lysosomes, mitochondria and endoplasmic reticulum<sup>36</sup>. These changes in monocyte features are thought to give rise to monocyte-derived macrophages, however, resident tissue macrophages are largely derived from embryogenesis and can maintain populations through self-renewal<sup>36</sup>. Although the origin of macrophages can vary considerably between tissues, a conserved feature of these cells is to undergo polarization into M1 or M2 macrophages. Polarization of M1 macrophages occurs in the presence of infectious agents and inflammatory mediators, such as TNF- $\alpha$  and IFN- $\gamma$ , and is characterized by production of inflammatory cytokines (IL-12, IL-23, IL-1 $\beta$ , TNF- $\alpha$ ) and the ability to produce reactive oxygen species and nitric oxide<sup>40</sup>. Conversely, M2 polarization occurs in response to IL-4, IL-13, and IL-10 and is characterized low production of IL-12 and

IL-23 and increased production of IL-10 and TGF- $\beta$ <sup>40</sup>. Together, M1 macrophages are associated with increased microbicidal activity and are important during initial stages of infection, while M2 macrophages dampen the inflammatory response and contribute to tissue remodeling and resolution of infection. In the presence of microbial pathogens, classical monocytes can further differentiate into tumor necrosis factor and nitric oxide synthase-producing dendritic cells (TIP-DCs) or differentiate into inflammatory macrophages that promote inflammation or pro-resolving macrophages involved in tissue repair<sup>36</sup>. Although cells of the myeloid lineage have bactericidal properties, including the production of reactive nitrogen intermediates (RNIs) and reactive oxygen intermediates (ROIs), their primary innate immune function is to recruit neutrophils to the site of infection and prime them for appropriate responses.

### Neutrophil Recruitment

Neutrophil recruitment to the site of infection is a highly regulated process. Upon infection, local resident cells recognize conserved pathogen motifs through pattern recognition receptors (PRRs) and initiate a pro-inflammatory response. The pro-inflammatory response is composed of cytokines and chemokines that directly or indirectly recruit circulating neutrophils. Following recruitment from circulation and migration to the site of infection, neutrophils encounter *S. aureus* and elicit responses to eliminate infection. In turn, *S. aureus* utilizes immunomodulatory and virulence determinants to attenuate the neutrophil response. To elucidate how *S. aureus* attenuates neutrophil antibacterial responses, it is important to delineate neutrophil homeostasis during inflammation and infection. The subsequent sections will review neutrophil recruitment and function in

inflammation as well as responses elicited by neutrophils and *S. aureus* during infection.

Neutrophils are differentiated hematopoietic stem cells (HSC) that originate in the perivascular niche of the bone marrow<sup>41</sup>. The perivascular niche provides retention signals that allow differentiation of a HSC, starting from a myeloblast and ending with a fully differentiated neutrophil<sup>42</sup>. There are several stages of neutrophil maturation which can be distinguished based upon the formation of granules. There are three types of neutrophil granules that commence following differentiation of the myeloblast to a promyelocyte: azurophilic (primary), specific (secondary), and gelatinase (tertiary) granules<sup>43</sup>. Primary granules contain myeloperoxidase (MPO), defensins, and the protease azurocidin<sup>44</sup>. Following differentiation from the promyelocyte to myelocyte stage, the specific granules are synthesized, which contain lactoferrin, flavocytochrome b<sub>558</sub> and collagenase<sup>45</sup>. Maturation of the myelocyte progresses to a metamyelocyte then band cell and finally the mature neutrophil<sup>33</sup>. During the later stages of granulopoiesis, tertiary granules containing gelatinase develop as well as secretory vesicles that contain alkaline phosphatase, formylated peptide receptors (FPRs) and IgG immunoglobulin Fc receptors<sup>43</sup>. The maturation of granules and secretory vesicles equips the neutrophil with antibacterial mechanisms that can then be used to eliminate pathogens.

Granule development is followed by decreased expression of chemokine receptors, such as CXCR4, which allows disassociation from the perivascular niche and egress of neutrophils from the bone marrow into the blood stream<sup>41</sup>. Under steady state conditions the neutrophil will survey the endothelium for inflammatory signals and if a response is not warranted, the neutrophil will undergo apoptosis within 5-10 hours and be cleared from circulation<sup>46</sup>. However, if a microbial challenge is present it will induce the release of

inflammatory cytokines which activates the endothelium and leads to neutrophil recruitment and migration to the infected loci<sup>41</sup>. Inflammation can also result in the release of growth factors, such as G-CSF and GM-CSF, and further stimulate granulopoiesis and the release of neutrophils into the bloodstream<sup>41</sup>. These events are highly regulated and important for neutrophil recruitment to localized infections.

Neutrophil recruitment to localized infections is influenced by residential cells and subsequent changes on the surface of endothelium. During acute infection, local resident mast cells, macrophages and dendritic cells secrete TNF- $\alpha$ , IL-1 $\beta$  and other inflammatory mediators that activate endothelial cells to induce neutrophil recruitment<sup>47</sup>. Activated endothelial cells rapidly upregulate pre-stored P-selectin from secretory vesicles and synthesize E-selectin shortly thereafter<sup>33</sup>. P-selectin and E-selectin are adhesion molecules that initiate neutrophil tethering and rolling by transiently binding to neutrophil ligands, including P-selectin glycoprotein ligand-1 (PSGL-1), E-selectin ligand-1 (ESL-1), and L-selectin<sup>47</sup>. Firm adhesion then follows by binding of neutrophil  $\beta_2$  integrins, such as CD11a/CD18 (LFA-1) and CD11b/CD18 (MAC-1), to endothelial cell surface molecules, such as ICAM-1 and ICAM-2<sup>48</sup>. Expression of MAC-1 by neutrophils is further enhanced by chemoattractants displayed on the endothelial surface and initiates intraluminal crawling and neutrophil transmigration<sup>33</sup>. Neutrophil transmigration by which the neutrophil leaves the vasculature, requires ICAM-1/2 and VCAM-1<sup>33</sup>. These well-characterized molecules and others promote passage across the endothelial cell layer either paracellularly or transcellularly<sup>47</sup>. Following extravasation, neutrophils encounter chemotactic molecules and proinflammatory cytokines that create a gradient and facilitate

migration to the infection foci. Bacteria-derived *N*-formyl-methionyl-leucyl-phenylalanine (fMLF) or the complement component C5a are chemotactic molecules that facilitate neutrophil migration to the site of infection<sup>47</sup>. Although the cascade of neutrophil recruitment is highly efficient, *S. aureus* implements multiple mechanisms to disrupt this process.

Neutrophil tethering, rolling and extravasation can be attenuated by the staphylococcal enterotoxin-like X (SEIX) and the staphylococcal superantigen-like 5 (SSL5)<sup>25</sup>. These proteins directly bind to PSGL-1 on the surface of neutrophils and thus can inhibit binding to P-selectin and E-selectin on activated endothelium<sup>49</sup>. Additionally, the chemotaxis inhibitory protein (CHIPS) is an efficient secreted *S. aureus* virulence factor that inhibits neutrophil chemotaxis<sup>25</sup>. This exoprotein shows affinity for both the C5a receptor (C5aR) and formyl peptide receptor (FPR) present on neutrophils, which inhibit complement mediated chemotaxis and formylated peptide mediated chemotaxis, respectively<sup>50</sup>. Neutrophil expression of FPR and formyl-peptide receptor-like-1 (FPRL1) can also be bound by *S. aureus* FPRL1 inhibitory protein (FLIPr) and inhibit neutrophil chemotaxis toward chemoattractant agents<sup>51</sup>. Despite these mechanisms, high concentrations and varieties of chemoattractants, chemokines, and priming agents promote neutrophils migration and accumulation at the site of infection.

### Neutrophil Priming

Priming refers to the ability of a primary agonist to enhance a neutrophil's response to a secondary stimulus<sup>52</sup>. Priming agents are produced from residential cells, such as dendritic cells and macrophages, as well as monocytes and mast cells. Production of

priming agents by these cells are produced upon activation of pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) and NOD-like receptors (NLRs), which in turn activate transcription of inflammatory genes<sup>53</sup>. Activation of residential macrophages or monocyte subsets through PRRs can induce expression of over 100 inflammatory genes, including those that encode priming agents such as complement components C3a and C5a, interferon- $\gamma$  (IFN- $\gamma$ ), interleukin-8 (IL-8), interleukin-1 (IL-1) and tumor-necrosis factor- $\alpha$  (TNF- $\alpha$ )<sup>54–58</sup>. These inflammatory mediators, such as IL-1 and TNF- $\alpha$ , subsequently facilitate neutrophil recruitment by binding to and activating endothelial cells to up-regulate E-selectin, ICAM-1 and VCAM-1<sup>59,60</sup>. Furthermore, inflammatory mediators, such as TNF- $\alpha$ , promote cytokine production in other proximal cells by binding to TNF receptors (TNFR) that activates the transcription factor NF- $\kappa$ B, which controls expression of inflammatory genes that can further induce neutrophil priming<sup>61</sup>. Although the ability of these agents to prime neutrophils is typically not universal in that concentration and neutrophil responses can vary drastically, the overall outcome of neutrophil priming can increase adhesion, phagocytosis, superoxide production, degranulation and influence apoptosis<sup>62,63</sup>.

Less is known about the impact of *S. aureus* on neutrophil priming and how modulation of neutrophil priming agents by *S. aureus* influence neutrophil functions. Previous studies have investigated neutrophil priming with conditioned media isolated from peripheral blood mononuclear cells (PBMCs) challenged with killed *S. aureus*<sup>64,65</sup>. These studies identified that TNF- $\alpha$  within the conditioned medium from PBMCs challenged with killed *S. aureus* increased neutrophil staphylocidal activity compared to

medium from unstimulated PBMCs. These studies indicate that inflammatory mediators produced by PBMCs and residential cells in response to *S. aureus* can influence the outcome of neutrophil-*S. aureus* interactions. In congruence with this concept, *S. aureus* has been shown to promote robust production of IFN- $\gamma$ , which correlated to poorer outcomes in mouse models of infection<sup>66,67</sup>. This is consistent with findings in mouse wound and peritonitis *S. aureus* infection studies, which demonstrated that increased bacterial burdens at the infection foci was associated with production of IFN- $\gamma$ <sup>67,68</sup>. As IFN- $\gamma$  can prime neutrophil oxidative burst and granule exocytosis, it is likely that in these studies, priming by IFN- $\gamma$  lead to unregulated neutrophil responses making them less effective at killing *S. aureus*<sup>62</sup>. In contrast, IFN- $\gamma$  has been shown to promote neutrophil staphylocidal activity and is an important treatment therapy for patients with chronic granulomatous disease<sup>55,69</sup>. Thus, location, timing and concentration of inflammatory mediators can largely influence neutrophil priming and subsequent interactions with *S. aureus*.

Bacterial derived components can also prime neutrophils and include agents such as fMLF, peptidoglycan, LPS (Gram-negative bacteria), and *S. aureus* cytolytic toxins<sup>25</sup>. *S. aureus* cytolytic toxins include hemolysins and leukotoxins, which can lyse cells when present at high concentrations by forming pores in the cell membrane. Two characterized *S. aureus* cytolytic toxins are Panton-Valentine leukocidin (PVL) and leukotoxin GH (LukG/H, also known as LukA/B). At high concentrations, these factors maintain pore forming capabilities but at lower sublytic concentrations these factors can induce neutrophil priming functions, including increasing neutrophil binding, uptake and killing

of *S. aureus*<sup>70</sup>. This is exemplified by studies demonstrating that PVL induces an enhanced neutrophil ROS production, increases granule exocytosis, and promotes release of neutrophil priming agents<sup>70-72</sup>. Similarly, LukG/H has been shown to increase surface expression of neutrophil complement receptor 3 (CR3, MAC-1), resulting in enhanced binding and clearance of *S. aureus*<sup>73</sup>. In addition, studies have shown *S. aureus* hemolysin, alpha-toxin, induced production of inflammatory mediators, such as IL-8, which is a well characterized neutrophil priming agent<sup>74</sup>. Together, these studies highlight the importance of priming agents produced by residential cells on neutrophil functions and provide insight into the priming effects of *S. aureus* virulence factors. Production of priming agents is critical for enhancing neutrophil bactericidal mechanisms, which is important following interaction with *S. aureus*.

#### Neutrophil Phagocytosis and Bactericidal Mechanisms

A primary goal after neutrophil extravasation and priming is to promote resolution of inflammation by recognizing and eliminating microbial pathogens. *S. aureus* expresses an array of pathogen associated molecular patterns (PAMPs), such as peptidoglycan, lipoprotein, and lipoteichoic acid which interact with pattern recognition receptors (PRRs) on the surface of neutrophils. Binding of neutrophil receptors to promote phagocytosis of *S. aureus* is further enhanced through opsonization. Opsonization coats *S. aureus* with serum complement components and/or immunoglobulins that can be recognized by neutrophil receptors. Among the antibody receptors, neutrophils express CD16, CD32 and CD64 which can recognize the antibody Fc region of IgG immunoglobulins<sup>75</sup>. On the other hand, well-characterized neutrophil complement receptors include, C1qR, CD35 (CR1),

CD11b/CD18 (CR3) and CD11c/CD18 (CR4)<sup>75</sup>. Together, neutrophil PRR, complement and antibody receptors enable uptake of *S. aureus*, but *S. aureus* can also elicit virulence mechanisms to alter these processes.

*S. aureus* has several virulence factors that can impede neutrophil recognition and phagocytosis as well as neutralize the activity of ROS and antibacterial mechanisms. Complement, which aids in neutrophil phagocytosis of opsonized *S. aureus*, can be inhibited through production of the staphylococcal complement inhibitor (SCIN), staphylokinase, and extracellular fibrinogen binding protein (Efb)<sup>25</sup>. These factors largely influence the ability of C3 convertase to convert and deposit C3b onto the bacterial membrane and, in turn, inhibit recognition by neutrophil complement receptors<sup>76</sup>. *S. aureus* can also produce virulence factors that target opsonization by inhibiting immunoglobulin function, including protein A, second binding protein of immunoglobulin (Sbi), and clumping factor A (ClfA)<sup>76</sup>. Protein A and Sbi bind the Fc region of IgG immunoglobulins while ClfA binds to fibrinogen on the bacterial cell surface, which interferes with bacterial adherence to immobilize ligands and inhibits phagocytosis<sup>76,77</sup>. Protein A is a pluripotent virulence factor of *S. aureus* that is a highly expressed surface component or can be freely secreted into the extracellular environment. This protein can inhibit opsonophagocytosis as well as bind to the Fab region of B-cell receptors leading to programmed cell death<sup>78,79</sup>. Furthermore, protein A can also induce TNFR shedding by inducing the TNF- $\alpha$  converting enzyme (TACE), which can further attenuate pro-inflammatory responses<sup>80</sup>. In addition, the presence of a capsular polysaccharide composed of N-acetyl mannosaminuronic acid, N-acetyl L-fucosamine and N-acetyl D-fucosamine has been shown to prevent recognition of staphylococcal surface associated proteins by neutrophil receptors<sup>81</sup>. The capsule

polysaccharide expressed by *S. aureus* has been demonstrated to block killing of neutrophils by covering C3b deposited on the bacterial surface<sup>82</sup>. It is important to note, however, that at high plasma concentrations capsule expression loses the ability to block phagocytosis, which indicates that capsule expression is not essential for *S. aureus* virulence<sup>81</sup>. This is further supported by the finding that USA300 lacks a capsular polysaccharide but does not show impaired phagocytosis relative to capsule positive strains<sup>82</sup>. This might be due to a mechanism where extracellular fibrinogen binding protein (Efb) and clumping factor A (ClfA) bind C3b and fibrinogen to create a fibrinogen shield, that prevents C3b mediated phagocytosis and neutrophil recognition<sup>81</sup>. Together, these virulence factors are used to inhibit activity of immunoglobulins and complement, which lead to decreased neutrophil recognition and phagocytosis.

#### Neutrophil Antibacterial Mechanisms

Following phagocytosis, *S. aureus* is engulfed into the phagosome and is targeted by an array of oxygen-dependent and independent microbicidal agents. The prominent oxygen dependent killing mechanism of neutrophils occurs through the formation of the NADPH oxidase complex on the phagosomal membrane. Formation of the NADPH oxidase complex requires cytosolic translocation of p40<sup>phox</sup>, p47<sup>phox</sup>, p67<sup>phox</sup> and GTPase Rac2 to the phagosome and the association with flavocytochrome b<sub>558</sub><sup>83</sup>. This complex then transfers electrons from cytosolic NADPH to molecular oxygen to generate superoxide anions and secondary ROS metabolites, including singlet oxygen and hydrogen peroxide<sup>83</sup>. In addition, degranulation by which secretory vesicles and specific and tertiary granules fuse to the phagosome, contribute to oxygen-dependent phagosomal mediated killing<sup>84</sup>.

Primary granules also fuse to the phagosome and provide myeloperoxidase (MPO) and oxygen-independent antimicrobial agents, including  $\alpha$ -defensin, elastase, azurocidin and lysozyme<sup>84</sup>. The superoxide produced by the active NADPH oxidase complex can undergo dismutation into hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) either spontaneously or catalyzed by sodium oxide dismutase<sup>85</sup>. In the presence of MPO and chloride,  $\text{H}_2\text{O}_2$  can then be converted to hypochlorous acid/hypochlorite anion, which is a major effector in the oxidative defense mechanisms and is a highly bactericidal agent<sup>85</sup>. Combined, these well-characterized efforts are used to eliminate invading bacteria; however, there are numerous *S. aureus* factors that disrupt these mechanisms.

In the phagosome, *S. aureus* employs evasion strategies to influence both non-oxidative and oxidative neutrophil killing. One prominent evasion strategy to prevent non-oxidative killing by neutrophils is by modulating the composition of its peptidoglycan. The *dlt* operon functions to substitute negatively charged amino acids present with positively charged D-alanine, which reduces the attraction of cationic peptides<sup>86</sup>. Similarly, *S. aureus* can transfer positively charged L-lysine into the negatively charged lipid phosphatidylglycerol<sup>87</sup>. These slight modifications create electrostatic repulsions that prevent neutrophil antimicrobial peptides, such as  $\alpha$ -defensin, from binding and degrading the *S. aureus* cell membrane. In addition to evasion of non-oxidative killing mechanisms, *S. aureus* can reduce or inhibit neutrophil oxidative killing mechanisms. *S. aureus* superoxide dismutase and catalase inactivate harmful superoxide radicals by converting superoxide into hydrogen peroxide and hydrogen peroxide into molecular oxygen and water<sup>88</sup>. The staphylococcal peroxidase inhibitor (SPIN) of *S. aureus* influences the

oxidative burst of neutrophils by binding and inhibiting MPO<sup>89</sup>. SPIN is predominantly regulated by the SaeR/S regulon and likely contributes to the SaeR/S-dependent reduction of neutrophil ROS<sup>90</sup>. In addition, iron-regulated surface determinants (Isd) which shuttle heme-iron across the *S. aureus* cell envelope from host iron sources, has been shown to be important for resistance to killing by human neutrophils and H<sub>2</sub>O<sub>2</sub><sup>91</sup>. The sequestration of iron by Isd prevents the generation of secondary ROS, such as hydroxyl radical, by influencing Fenton chemistry thereby limiting oxidative damage<sup>91</sup>. Lastly, staphyloxanthin is an antioxidant that increases tolerance to H<sub>2</sub>O<sub>2</sub> and singlet oxygen produced by neutrophils and, uniquely, provides *S. aureus* with its phenotypic golden pigmentation<sup>92</sup>. Collectively, *S. aureus* has many redundant mechanisms to protect against antimicrobial peptides and ROS products once inside the neutrophil phagosome.

### Neutrophil Apoptosis

Neutrophil cell death is the terminal response during homeostasis, inflammation, and infection. Neutrophils can undergo programmed cell death, known as apoptosis, mediated by intrinsic and extrinsic pathways that influence pro- and anti-apoptotic proteins. Under normal homeostasis, neutrophils constitutively express anti-apoptotic protein, Mcl-1, which prevents activation of pro-apoptotic proteins, Bax, Bad, and Bak<sup>93</sup>. The pro-apoptotic proteins promote apoptosis by creating pores in the mitochondrial membrane that in turn releases cytochrome c. Cytochrome c initiates cysteine-aspartic proteases, also known as caspases, that degrade cellular components and lead to program cell death<sup>93</sup>. Pro-apoptotic proteins have a relatively long half-life and are less susceptible to external factors that reduce neutrophil apoptosis, such as GM-CSF, leukotriene B<sub>4</sub>, and

C5a<sup>94</sup>. The anti-apoptotic proteins, however, can be upregulated following activation but in steady-state conditions, are more susceptible to proteasomal degradation and thus have a relatively short half-life<sup>94</sup>.

Spontaneous apoptosis is influenced by extrinsic pathways involving soluble extracellular factors that bind to membrane bound receptors. These pathways include FAS/FAS ligand, TNF- $\alpha$ /TNFR, and TRAIL-mediated apoptosis<sup>93</sup>. Binding of these receptors triggers the formation of the death-inducing signaling complex (DISC), which ultimately leads to caspase activation and neutrophil apoptosis<sup>94</sup>. Alternatively, neutrophil extravasation and migration to the infection foci can prolong neutrophil survival by outside-in signaling through binding of B<sub>2</sub> integrins, such as LFA-1 and MAC-1, which promote activation of MCL-1<sup>94</sup>. Spontaneous apoptosis is also influenced by binding of ligands to G-protein coupled receptors (GPCR) present in the surface of neutrophils. Binding of GPCR CXCR1 and CXCR2 by IL-8 activates phosphatidylinositol lipid kinase (PI3K) promoting translocation and phosphorylation of Akt to the plasma membrane. Activation of Akt subsequently delays spontaneous apoptosis<sup>95</sup>. Apoptosis is a highly regulated process that is caspase-dependent and requires energy in the form of ATP. This process ultimately leads to decreased neutrophil cytokine production, chromatin condensation, cell shrinkage, and increased expression of phosphatidylserine<sup>96</sup>. Apoptotic neutrophils are recognized by bone marrow and spleen derived macrophages and are phagocytosed through a process known as efferocytosis, which removes them from circulation.

Neutrophil apoptosis during infection is critical for resolution of inflammation. Almost immediately after antigen recognition and phagocytosis, program cell death is

initiated through rapid expression of neutrophil pro-apoptotic genes<sup>97</sup>. Following phagocytosis, neutrophil ROS further act as intracellular signaling molecules that promote apoptosis by causing mitochondrial outer-membrane permeability, which release cytochrome c that can activates caspases<sup>98</sup>. This is further supported by the negative influence of ROS on Akt activation: in the presence of an ROS inhibitor, such as glutathione, Akt phosphorylation is four times higher compared to untreated neutrophils<sup>95</sup>. The production of ROS is also associated with an alternative form of cell death through formation of neutrophil extracellular traps (NETs), a process referred to as NETosis<sup>99</sup>. NETs are composed of DNA and antimicrobial proteins and are extruded into the surrounding environment to trap and kill bacteria<sup>99</sup>. The exact contribution of ROS in NETosis is unclear but highly important as exemplified in patients with chronic granulomatous disease (CGD). CGD patients lack a functional NADPH oxidase and have impaired neutrophil apoptosis following ingestion of bacteria and a compromised ability to form NETs<sup>100,101</sup>. Together, neutrophil apoptosis and NETosis are regulated by intrinsic and extrinsic activities that promote resolution of infection, however, both processes can be influenced by *S. aureus* during infection.

Alternative to apoptosis, necrosis is a form of cell death that occurs when ATP is depleted and is generally characterized as a caspase-independent and receptor interacting protein kinase (RIP)-dependent process<sup>102</sup>. *S. aureus* can induce necrosis by production of pore-forming toxins (PFTs), including PVL,  $\gamma$ -hemolysin,  $\alpha$ -toxin,  $\alpha$ -type phenol soluble modulins and LukAB<sup>103,104</sup>. These PFTs target neutrophils and monocytes, as well as natural killer cells, dendritic cells and T lymphocytes in both receptor-dependent and independent mechanisms<sup>104</sup>. Pore formation by these toxins leads to necrosis

characterized by cytoplasmic swelling, ruptured plasma membrane, lytic nucleus, and release of cytotoxic and inflammatory mediators that damage the surrounding tissue and promotes inflammation<sup>102</sup>. In addition to causing cell lysis, *S. aureus* can cause accelerated neutrophil cell death, also referred to as programmed necrosis or necroptosis<sup>105</sup>. The SaeR/S TCS of *S. aureus* has been shown to promote necroptosis by inhibiting IL-8 production and suppressing NF-kB activation, which are important regulators of the anti-apoptotic protein Mcl-1<sup>30</sup>. The ability of *S. aureus* to cause necrosis is consistent with the finding that *S. aureus* promotes activation of receptor-interacting protein-1 (RIP-1), which is independent of the classical apoptotic pathway mediated by effector caspases<sup>106</sup>. *S. aureus* inhibition of ROS production, as mentioned previously, would influence neutrophil apoptosis and NET formation. Additionally, *S. aureus* secretes nuclease, regulated by the SaeR/S-TCS, that is demonstrated to degrade NET products<sup>107</sup>. Lastly, *S. aureus* can inhibit macrophage efferocytosis of neutrophils containing *S. aureus* by inducing upregulating of CD47 on the neutrophil surface, which is a prominent “don’t eat me” signal<sup>106</sup>. Together, these dysfunctions lead to increased inflammation and tissue damage which can abrogate resolution of infection.

#### *S. aureus* SaeR/S Two-Component System

The ability of *S. aureus* to disrupt every step of the neutrophil response is largely attributed to virulence genes under the regulation of two-component gene-regulatory systems. Two-component gene-regulatory systems sense environmental stimuli and trigger responses at the transcriptional level. Most two-component systems are composed of a transmembrane histidine kinase and an intracellular response regulator<sup>108</sup>. Activation

of the transmembrane region leads to histidine kinase autophosphorylation that can then transfer phosphate groups to amino acids on the response regulator<sup>108</sup>. Phosphorylation of the response regulator promotes binding to specific genes and activates their transcription<sup>109</sup>. Among the 16 identified two-component systems, the *S. aureus* exoprotein expression (SaeR/S) system is the predominant systems for sensing neutrophils and neutrophil products<sup>10,27,110</sup>.

The SaeR/S TCS is composed of a histidine kinase sensor, SaeS, and a response regulator, SaeR. Both *saeS* and *saeR* are transcribed from the *sae* operon under the control of two promoters, P1 and P3<sup>111</sup>. P3 is constantly active and transcribes *saeS* and *saeR*, while the P1 promoter is inducible and transcribes the entire *sae* operon of *saeP*, *saeQ*, *saeR*, and *saeS*<sup>111</sup>. The role of *saeP* and *saeQ* in regulating virulence is incompletely understood but it is predicted that *saeP* is a lipoprotein and *saeQ* is a putative transmembrane protein that together negatively regulate SaeR/S<sup>112</sup>. The SaeS sensor contains a nine amino acid extracellular loop that can rapidly activate SaeR upon sensing neutrophil-derived stimuli<sup>27,110</sup>. Three highly conserved residues important for SaeS signalling include, M31, W32, and F33, which if mutated inhibit activation of the *sae* regulon and disrupt normal basal signalling of SaeS<sup>110</sup>. The subsequent phosphorylation and activation of SaeR leads to transcription of virulence genes that have a conserved SaeR recognition binding sequence<sup>22</sup>. Virulence factors transcribed by SaeR binding include:  $\alpha$ -  $\beta$ - and  $\gamma$ -hemolysins ( encoded by *hla*, *hly*, *hlgABC*), leukotoxins (*hlgBC*, *lukAB/GH*, *PVL*), nucleases, staphylococcal binding immunoglobulin protein (*sbi*), and adhesins, such as coagulase, fibronectin binding protein A (FnBPA), extracellular adherence protein (Eap) and extracellular matrix binding protein (Emp)<sup>22,27</sup>.

The observations that the SaeR/S TCS is rapidly activated and controls expression of key virulence determinants highlights the involvement of *saeR/S* in *S. aureus* evasion and modulation of innate immunity.

### SaeR/S Cytolytic Factors

SaeR/S regulates expression of membrane damaging toxins, leukotoxins, and superantigens that have diverse effects on immune responses.  $\beta$ -hemolysin and  $\alpha$ -hemolysin are regulated by SaeR/S and are cytolytic membrane-damaging toxins<sup>5,22,113</sup>.  $\beta$ -hemolysin targets sphingomyelin present in the plasma membrane and is shown to target both erythrocytes and monocytes resulting in lysis and can promote killing of proliferating lymphocytes<sup>114,115</sup>. Alpha-hemolysin has been shown to induce programmed cell death and plasma membrane permeability in human T cells, B cells and monocytes and can exacerbate infection by inducing inflammatory cytokines and chemokines, including IL-8, IL- $\beta$ , TNF- $\alpha$ , KC and MIP-2<sup>28,74,116</sup>. PVL and LukAB are bi-component leukotoxins regulated by SaeR/S that assemble to form  $\beta$ -barrel pores in leukocytes<sup>104</sup>. Although the role of PVL is not critical for inducing mortality in murine models of sepsis, purified PVL has been identified to induce necrotic lesions in the skin and lungs of rabbits and mice, respectively<sup>8,117,118</sup>. PVL also demonstrates the ability to associate with neutrophils through binding complement receptor C5aR and induces pro-inflammatory responses that recruit neutrophils and induce secretion of leukotriene B4 and oxygen metabolites<sup>71,119</sup>. LukAB (LukG/H) is an SaeR/S-regulated factor that binds to human receptor CD11b can promote lysis of human neutrophils, macrophages, monocytes and dendritic cells<sup>118,120</sup>. SaeR/S regulation of enterotoxins, such as *seb*, *sec*,

and superantigen-like proteins *ssl(s)*, significantly contribute to conditions such as sepsis and toxic shock syndromes (TSS) associated with *S. aureus*<sup>24</sup>. Staphylococcal enterotoxins (SE) B (SEB) and C (SEC) promote TSS by inducing robust pro-inflammatory cytokine responses mediated by binding to MHC class II molecules of antigen presenting cells, which subsequently can activate T-cell receptors and signalling<sup>121,122</sup>. SaeR/S-regulated superantigen-like proteins can further influence neutrophil tethering to endothelial cells by binding to P-selectin and can bind complement and immunoglobulin proteins preventing recognition by innate immune cells and reducing opsonophagocytosis<sup>123,124</sup>.

#### SaeR/S Immunomodulatory Factors

In addition to regulating cytolytic toxins, the SaeR/S-TCS is involved in immune evasion and immunomodulation. Invasive murine infections have demonstrated that SaeR/S-regulated factors induce robust production of pro-inflammatory cytokines, such as TNF- $\alpha$ , IFN- $\gamma$ , IL-6 and IL-2<sup>66</sup>. The robust production of these cytokines correlated to enhanced bacterial survival and host mortality<sup>66</sup>. Findings were also replicated in human whole blood challenged with SaeR/S-secreted factors<sup>66</sup>. These results are consistent with the ‘cytokine storm’ response that is characterized by multiple organ failure and mortality due to an uncontrolled pro-inflammatory response<sup>66</sup>. In congruence with these findings, mouse models of peritonitis and subcutaneous infections have also demonstrated the relevance of SaeR/S in inducing elevated levels of neutrophil IFN- $\gamma$ <sup>67</sup>. Consistent with these observations, invasive challenge with *S. aureus* in IFN- $\gamma$  deficient mice demonstrated increased bacterial clearance and reduced cellular toxicity compared to

wild-type mice, indicating that immunomodulation of IFN- $\gamma$  contributes to *S. aureus* pathogenesis<sup>67</sup>. *In vitro* models similarly show immunomodulation through the ability of SaeR/S to inhibit production of neutrophil derived IL-8, which subsequently affected neutrophil cell death and promoted bacterial survival<sup>30</sup>. Additionally, the SaeR/S-regulated factor, Sbi, has been shown to induce production of pro-inflammatory cytokines and chemokines through binding and inducing shedding of TNF- $\alpha$  receptor (TNFR), which can bind soluble TNF- $\alpha$  and reduce its functional activity<sup>22,29</sup>. These findings further coincide with the SaeR/S TCS in promoting dermonecrotic lesions in mouse and rabbit skin infection models, which is largely influenced by local inflammatory responses<sup>22,24,125</sup>. Together these studies characterize a role for the SaeR/S TCS in modulating inflammatory responses and provide insight into the pleiotropic functions of inflammatory mediators during infection.

#### Pleiotropic functions of TNF- $\alpha$ during *S. aureus* infection

Many of the inflammatory mediators influenced by the SaeR/S TCS of *S. aureus* are involved in neutrophil extravasation, priming, and antibacterial defenses. However, these inflammatory mediators are also involved in exacerbating *S. aureus* infections, highlighting their various functions during localized or systemic infections. One primary pleiotropic cytokine that appears to be regulated by the SaeR/S-system and influences multiple responses during infections with *S. aureus* is TNF- $\alpha$ . Elevated TNF- $\alpha$  during murine models of systemic *S. aureus* infection have been shown to directly correlate to overall host morbidity and mortality<sup>66,126,127</sup>. Although this is well demonstrated, the concentration of *S. aureus* during systemic infection may also be a contributing factor. This

is exemplified in a study, which found that inhibition of TNF- $\alpha$  prior to systemic challenge with low dose *S. aureus*, resulted in over 60% mortality compared to no mortality in mice that didn't receive treatment<sup>128</sup>. Studies have also demonstrated that TNF- $\alpha$ /lymphotoxin-alpha double mutant mice display increased mortality during *S. aureus* sepsis infections<sup>129</sup>. Additionally, increases in postoperative *S. aureus* infections in humans with rheumatoid arthritis receiving TNF- $\alpha$  inhibitors for treatment have been observed<sup>130</sup>. Collectively, these data support the hypothesis that TNF- $\alpha$  production is import for control of *S. aureus* infection.

In contrast to systemic infections, local production of TNF- $\alpha$  by residential cells is needed for neutrophil recruitment and correlate to protective immune responses during *S. aureus* infection<sup>132,133</sup>. The importance of cytokine production by localized residential cells is further exemplified in patients deficient in intracellular signaling molecules MYD88 and IRAK4. Individuals with these deficiencies have a reduced ability to activate transcription and expression of pro-inflammatory cytokines, including TNF- $\alpha$ , IL-6 and IL-8<sup>34</sup>. These deficiencies contribute to a predisposition to recurrent pyogenic infections, such as severe *S. aureus* skin infections<sup>34</sup>. Together, these studies highlight that the inflammatory response and the concentration of bacteria are critical to determining host outcome and more studies are necessary to determine overall responses of TNF- $\alpha$  and the role of SaeR/S during infection with *S. aureus*.

### Hypothesis

Work described in this dissertation, tests the **hypothesis that the *S. aureus* SaeR/S two-component gene regulatory system influences neutrophil bactericidal activity by modulating early production of tumor-necrosis factor- $\alpha$  (TNF- $\alpha$ )** (Chapter 3). A previous finding demonstrated that the SaeR/S-TCS induced a robust TNF- $\alpha$  response in invasive mouse models of infection that coincided with an onset of significant mortality<sup>66</sup>. The robust cytokine response was measured 10 hours post-infection and was thought to contribute to mortality through inducing a cytokine storm, which results in multiple organ failure and death. Similar studies have shown that elevated levels of TNF- $\alpha$  during systemic challenge of *S. aureus* correlate to enhance morbidity and mortality, which highlight the detrimental response of elevated levels of TNF- $\alpha$  in the bloodstream<sup>126,127</sup>. On the other hand, early production of TNF- $\alpha$  in response to *S. aureus* is critical for neutrophil recruitment, priming, and antibacterial mechanisms, which highlights the importance of TNF- $\alpha$  during localized infections<sup>133,134</sup>. These studies indicated that early production of TNF- $\alpha$  during localized *S. aureus* infection promotes a protective immune response, while long-term elevated systemic levels of TNF- $\alpha$  correlated to disease progression. To further these findings, we assessed the ability of clinically relevant strains of *S. aureus* to induce early levels of TNF- $\alpha$ , as well as the subsequent influence on neutrophil functions. Using a co-culture model of human neutrophils and peripheral blood mononuclear cells we characterize a prominent role for the SaeR/S-TCS of *S. aureus* in modulating early monocyte-derived TNF- $\alpha$ . We further demonstrate that this modulation impedes neutrophil priming, ROS production

and bactericidal activity. Collectively, these data identify diminished monocyte-derived TNF- $\alpha$  as another evasion mechanism used by *S. aureus* to diminish neutrophil microbicidal activity.

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CHAPTER THREE

STAPHYLOCOCCUS AUREUS SAER/S-REGULATED FACTORS DECREASE  
MONOCYTE-DERIVED TUMOR NECROSIS FACTOR- $\alpha$  TO REDUCE  
NEUTROPHIL BACTERICIDAL ACTIVITY

Contribution of Authors and Co-Authors

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Manuscript Information Page

Eli W. Sward, Elizabeth M. Fones, Russel R. Spaan, Kyler B. Pallister, Brandon L. Haller, Fermin E. Guerra, Oliwia W. Zurek, Tyler K. Nygaard and Jovanka M. Voyich

Journal of Infectious Diseases

State of Manuscript:

☐ Prepared for submission to a peer-reviewed journal  
☐ Officially submitted to a peer-review journal  
☐ Accepted by a peer-reviewed journal  
☒ Published in a peer-reviewed journal

Publisher: Oxford University Press for the Infectious Disease Society of America

Issue in which manuscript appears: March 2018, Volume 217, Issue 6: 854-856.

## Major Article

### ***Staphylococcus aureus* SaeR/S-regulated Factors Decrease Monocyte-derived TNF- $\alpha$ to Reduce Neutrophil Bactericidal Activity**

Eli W. Sward<sup>1</sup>, Elizabeth M. Fones<sup>1</sup>, Russel R. Spaan<sup>1</sup>, Kyler B. Pallister<sup>1</sup>, Brandon L. Haller<sup>1</sup>, Fermin E. Guerra<sup>1</sup>, Oliwia W. Zurek<sup>2</sup>, Tyler K. Nygaard<sup>1</sup>, and Jovanka M. Voyich<sup>1\*</sup>.

Running Title: *S. aureus* Disrupts Neutrophil Priming

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Word Count: Abstract (140); Text (3455)

## Footnotes

Potential Conflicts of Interest. All authors: No reported conflicts.

Financial Support. This work was supported by the U.S. National Institutes of Health (Grants NIH-

R01A1090046, NIH-PAR98-072, and NIH-RR020185 for a fellowship award to F.E.G. and O.W.Z.), as well

as funds from the Montana University System Research Initiative (51040-MUSRI2015-03) and Montana

State University Agriculture Experiment Station and an equipment grant from Murdock Charitable Trust.

Presented in part. 48th Annual Meeting of the Society of Leukocyte Biology, Salt Lake City, Utah 23-25,

October 2014; International Conference on Gram positive Pathogens, Omaha NE, 12-15,

October 2014; 18th International Conference on Bacilli and Gram positive Bacteria,

Florence, Italy 19-21, June 2015

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## Abstract

The ability of *Staphylococcus aureus* to evade killing by human neutrophils significantly contributes to disease progression. In this study, we characterize an influential role for the *S. aureus* SaeR/S-two component gene regulatory system in suppressing monocyte production of TNF- $\alpha$  to subsequently influence human neutrophil priming. TNF- $\alpha$  production from monocytes was significantly reduced following challenge with wild type *S. aureus* (USA300) compared to an isogenic *saeR/S* deletion mutant (USA300 $\Delta$ *saeR/S*). We observed that priming of neutrophils using conditioned medium (CM) from PBMCs stimulated with USA300 $\Delta$ *saeR/S* significantly increased neutrophil bactericidal activity against USA300 relative to unprimed neutrophils and neutrophils primed with USA300 CM. The increased neutrophil bactericidal activity was associated with enhanced ROS production that was significantly influenced by elevated TNF- $\alpha$  concentrations. Taken together, our findings identify an immune evasion strategy used by *S. aureus* to impede neutrophil priming and subsequent bactericidal activity.

**Key words:** *Staphylococcus aureus*; neutrophil; human polymorphonuclear leukocytes; SaeR/S-two component gene regulatory system; peripheral blood mononuclear cells; monocytes; TNF- $\alpha$ ; priming,

## Introduction

*Staphylococcus aureus* (*S. aureus*) is a ubiquitous Gram-positive bacterium estimated to permanently colonize one out of three individuals [1]. *S. aureus* typically causes treatable cutaneous abscesses but infections can progress into dermonecrotic lesions and life-

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

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

these immunomodulatory roles, we further demonstrated that SaeR/S-regulated factors can suppress phosphorylation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) and reduce production of IL-8 in human neutrophils to promote cell death and enhance bacterial survival [7]. Collectively, these findings highlight immune evasion strategies by which SaeR/S-regulated factors influence inflammatory signaling to exacerbate infection. However, the specific immune cells that are targeted for immunomodulation by SaeR/S-regulated factors and the subsequent influence on downstream immune responses remains incompletely defined.

Peripheral blood mononuclear cells (PBMCs) have been shown to regulate inflammatory signals that promote neutrophil recruitment and enhance their antimicrobial mechanisms in response to *S. aureus* infection [20–22]. Many PBMC products such as monocyte-derived IL-1 and TNF- $\alpha$  also act as neutrophil priming agents [21,23]. Neutrophil priming is a mechanism that regulates the level of neutrophil activation to provide an appropriate response to a secondary stimulus[23]. Despite the importance of inflammatory mediators produced by PBMCs that are involved in neutrophil priming, there is a lack of studies examining the impact of *S. aureus* on this process. Previous studies have investigated the effects of conditioned media containing inflammatory signals produced by human PBMCs following exposure to heat killed (HK) *S. aureus* on neutrophil functions [24,25]. These studies observed that neutrophil staphylocidal activity was enhanced following priming with conditioned media and that this effect was dependent on PBMC production of TNF- $\alpha$  [25]. While these effects were observed using conditioned media from HK *S. aureus*, it remains to be elucidated if conditioned media from live bacteria elicit a similar response on neutrophil priming.

In the current study, we developed a co-culture model using human PBMCs and neutrophils, as well as conditioned media assays, to demonstrate the importance of TNF- $\alpha$  during monocyte-neutrophil interactions following challenge with live *S. aureus*. We observed that SaeR/S-regulated factors significantly decreased monocyte production of TNF- $\alpha$ . Diminished TNF- $\alpha$  concentrations significantly influenced neutrophil staphylocidal activity by limiting neutrophil priming and subsequent production of reactive oxygen species. Collectively, this study demonstrates that the response of PBMCs to *S. aureus* significantly impacts the outcome of neutrophil-*S. aureus* interactions.

## **Methods**

### Bacterial strains and cultures

Community-associated methicillin-resistant *S. aureus* (CA-MRSA) pulsed-field gel electrophoresis-type USA300 (strain LAC), USA400 (strain MW2), and isogenic *saeR/S* and *hla* deletion mutants (USA300 $\Delta$ *saeR/S*, USA400 $\Delta$ *saeR/S*, and USA300 $\Delta$ *hla*), generated in previous studies [6,10,15], were grown in tryptic soy broth containing 0.5% glucose and harvested at mid-exponential growth, as described previously [6,10,12,15].

### Assessment of TNF- $\alpha$ production from human cells

Heparinized venous blood from healthy donors was collected in accordance with protocol approved by the Institutional Review Board for Human Subjects at Montana State University. All donors provided written consent to participate in the study. For whole blood experiments, 20 $\mu$ l of RPMI only or RPMI with 2x10<sup>6</sup> colony-forming units (CFU) of bacteria was added to 980 $\mu$ l of freshly drawn human whole blood in 1.5ml eppendorf

tubes. Samples were placed on an end-over-end mixer (20 RPM, 37°C, 5% CO<sub>2</sub>) and at designated time points, samples were stained with FITC mouse anti-human CD14, PE mouse anti-human CD3, and APC mouse anti-human TNF or IgG-1 isotype control (BD Biosciences). Intracellular staining was performed using a BD Pharmigen intracellular stain kit following the manufactures protocol and all samples were analyzed using a FACS Calibur Flow Cytometer (BD Bioscience). Alternatively, serum was collected by centrifugation ( 524 x g, 10 minutes), filtered using a 0.2µm filter (PALL Life Science), and stored at -80°C until quantitation of TNF-α was performed using a TNF-α specific enzyme-linked immunosorbant assay (ELISA, BD Bioscience).

Human neutrophils (polymorphonuclear leukocytes or PMNs) and peripheral blood mononuclear cells (PBMCs) were isolated under endotoxin-free conditions (<25pg/ml) as previously described [6,12]. Purity (<1% PBMC contamination) and viability (<2% propidium iodide positive) of neutrophil preparations were assessed by flow cytometry (FACS Calibur; BD Biosciences). For co-culture assays, 7x10<sup>5</sup> neutrophils were combined with 3x10<sup>5</sup> PBMCs at a physiological ratio and exposed to 2x10<sup>6</sup> colony forming units (CFUs) of *S. aureus* opsonized with 50% normal human serum (NHS) in a 96-well NHS coated plate. After bacteria were added, phagocytosis was synchronized by centrifugation (524 x g, 7 minutes, 10°C) and then incubated at 37°C [12]. At designated time points, samples were stained as above and analyzed using flow cytometry.

For PBMC conditioned media (CM) experiments, 5x10<sup>6</sup> PBMCs were challenged with 1x10<sup>7</sup> opsonized bacteria in a 24-well serum coated plate that was then synchronized and incubated at 37°C[12]. At designated time points, samples were filtered using 0.2µm

filter (PALL Life Science), plated to ensure they did not contain live bacteria, and stored at 4°C for further use as CM or at -80°C for assessment of intracellular TNF- $\alpha$  production using ELISA as described above.

#### TNF- $\alpha$ inhibition Assays

Calbiochem TNF- $\alpha$  inhibitor C<sub>3</sub> H<sub>3</sub> F<sub>3</sub> N<sub>3</sub> O<sub>2</sub> • 2HCl • 1/2H<sub>2</sub> O (CAS 1049741-03-8; Millipore Sigma) was re-suspended according to manufacturer protocol and used at the final concentration of 4.6  $\mu$ M. TNF- $\alpha$  inhibitor was added to PMNs (10<sup>6</sup> cells) only, or PMNs (7x10<sup>5</sup> cells) + PBMCs (3x10<sup>5</sup> cells) for 10 minutes on ice. After treatment, cells were exposed to 2x10<sup>6</sup> opsonized bacteria, phagocytosis was synchronized using centrifugation (524 x g, 7 minutes, 10°C) then incubated at 37°C with 5% CO<sub>2</sub>. [12].

#### Bactericidal Assays

PMNs or PMNs supplemented with PBMCs (co-cultures) were exposed to opsonized bacteria at a 2:1 multiplicity of infection (MOI, bacteria:cell ratio) in a 96-well serum coated plate and phagocytosis was synchronized as described above [12]. At designated time points, 100ul of sample was placed in 900ul reverse osmosis water, vortexed, and placed on ice for 15 minutes. Serial dilutions were performed and CFUs were enumerated by plating on tryptic soy agar (TSA) as previously described [6,10,12]. For PBMC bactericidal assays, 5x10<sup>6</sup> PBMCs in a 24-well serum coated plate were challenged with 1x10<sup>7</sup> opsonized bacteria, synchronized using centrifugation (524 x g, 7 minutes, 10°C)

and incubated at 37°C [12]. At designated time points, bacterial survival was assessed using serial dilutions in water and CFUs were enumerated on TSA.

#### Neutrophil priming with TNF- $\alpha$ or PBMC conditioned media

For reactive oxygen species (ROS) assays, neutrophils were loaded at room temperature with 25  $\mu$ M 2',7'-dihydrochlorofluorescein diacetate (DCF; Molecular Probes) for 30 min in RPMI/H [12]. DCF-containing PMNs ( $10^6$ ) were then added to a 96-well serum-coated plate containing 50 $\mu$ l of PBMC CM, RPMI only, or PBMC CM containing TNF- $\alpha$  inhibitor, for 30 minutes at 37°C. TNF- $\alpha$  inhibitor was added to CM for a final concentration of 4.5 ng/ml for 10-20 minutes at room temperature prior to addition to PMNs. After treatment, PMNs were exposed to opsonized bacteria at a ratio of 1:2 (PMN:bacteria), and phagocytosis was synchronized using centrifugation (524 x g, 7 minutes, 10°C) [12]. Measurements were done using a SpectraMax Paradigm Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) or by flow cytometry using 488/530 nm emission/excitation wavelengths.

## **Results**

### **SaeR/S-regulated factors decrease TNF- $\alpha$ production in human whole blood**

To determine if SaeR/S-regulated factors influenced TNF- $\alpha$  production, viable wild-type *S. aureus* (USA300) or isogenic *saeR/S*-deletion mutant (USA300 $\Delta$ *saeR/S*) were exposed to human whole blood and TNF- $\alpha$  secretion was quantitated at 1, 3, and 5-hours using ELISA (Figure 1). Production of TNF- $\alpha$  occurred as early as 1 hour post-exposure to *S.*

*aureus* and, as expected, challenge with USA300 had significantly increased production compared to USA300 $\Delta$ *saeR/S* (Fig. 1A). However, by 3 hours, whole blood exposed to USA300 $\Delta$ *saeR/S* resulted in significant increase in TNF- $\alpha$  concentrations compared to USA300 and this trend continued at 5 hours (Fig. 1A). We then used flow cytometry to identify the source of TNF- $\alpha$  in blood following exposure to *S. aureus*. CD14<sup>+</sup> monocytes were identified as the primary source of TNF- $\alpha$  and challenge with USA300 $\Delta$ *saeR/S* resulted in a significant increase in TNF- $\alpha$  production from CD14<sup>+</sup> cells at 3 hours post-challenge compared to challenge with USA300 (Fig. 1 B, C). Experiments using an isotype control antibody verified specificity of intracellular TNF- $\alpha$  production (data not shown). Additionally, the proportions of CD14<sup>+</sup> cells between challenge with USA300 and USA300 $\Delta$ *saeR/S* were similar (5-10% of total cells, Supplemental Fig. 1) indicating these results did not arise from differences in total monocyte populations. The USA300 $\Delta$ *saeR/S* strain also demonstrated increased bacterial survival compared to USA300 at 1 hour but by 3 hours there were no significant differences between the strains (Fig. 1D). These results reflect previous observations showing that the USA300 $\Delta$ *saeR/S* strain does not have a growth defect in blood at early time points [15].

### **SaeR/S decreases TNF- $\alpha$ production in human monocytes**

Since CD14<sup>+</sup> monocytes were the predominant source of TNF- $\alpha$  production in human blood exposed to *S. aureus*, we next investigated TNF- $\alpha$  production in purified peripheral blood mononuclear cells (PBMCs). To reduce the potential influence of bacterial cytolytic activity between USA300 and USA300 $\Delta$ *saeR/S*, we used a low multiplicity of infection

(MOI) of 2:1 (bacteria:PBMC). Similar to observations using whole blood, CD14<sup>+</sup> monocytes were identified as the primary producers of TNF- $\alpha$  and significant increases in intracellular monocyte TNF- $\alpha$  expression was observed at 3 hours following challenge with USA300 $\Delta$ *saeR/S* compared to USA300 (Fig. 2A). To demonstrate that the observed effects of the *SaeR/S* TCS on TNF- $\alpha$  production were not specific to strain USA300, we also challenged PBMCs with USA400 (strain MW2) and an isogenic *saeR/S*-deletion mutant (USA400 $\Delta$ *saeR/S*). In congruence with our findings, PBMCs challenged with USA400 showed a significant decrease in TNF- $\alpha$  production from CD14<sup>+</sup> monocytes compared to challenge with USA400 $\Delta$ *saeR/S* (Supplemental Fig. 2A).

We have recently demonstrated that at high MOIs (10 bacteria:1 monocyte) the *SaeR/S*-regulated  $\alpha$ -toxin (Hla) significantly increases monocyte programmed cell death and plasma membrane permeability [15]. To determine if observed increases in TNF- $\alpha$  at a lower MOI were due to the impact of *SaeR/S*-regulated factors on monocyte viability, we investigated TNF- $\alpha$  production and viability of PBMCs following exposure to USA300, USA300 $\Delta$ *saeR/S* and USA300 $\Delta$ *hla*. ELISA results demonstrated that PBMCs exposed to USA300 $\Delta$ *hla* elicited a similar TNF- $\alpha$  response to PBMCs challenged with USA300, but a significantly lower TNF- $\alpha$  response than PBMCs challenged with USA300 $\Delta$ *saeR/S* at 3 and 5 hours post exposure (Fig. 2B). Additionally, PBMCs challenged with USA300 caused significant increases in monocyte cell membrane permeability compared to USA300 $\Delta$ *saeR/S* and USA300 $\Delta$ *hla*. However, there were no significant differences in membrane permeability of CD14<sup>+</sup> cells following exposure to USA300 $\Delta$ *saeR/S* and USA300 $\Delta$ *hla* (Fig. 2C). These results confirm previous findings demonstrating that Hla significantly contributes to monocyte cell

membrane damage but indicate that there are other SaeR/S-regulated factors and mechanisms responsible for reducing monocyte production of TNF- $\alpha$  [15]. Lastly, we also assessed bacterial viability following challenge with PBMCs (Fig. 2D). PBMCs were unable to control challenge with USA300 and USA300 $\Delta hla$  but bacterial survival of USA300 $\Delta saeR/S$  was significantly reduced at 3 and 5 hours (Fig. 2D). Collectively, these results demonstrate that the SaeR/S TCS is required for bacterial survival following exposure to PBMCs and suggest that reduced monocyte production of TNF- $\alpha$  by SaeR/S-regulated factors is independent of cell lysis.

#### **TNF- $\alpha$ production by PBMCs increases PMN bactericidal activity**

TNF- $\alpha$  has been shown to influence neutrophil bactericidal activity and ROS production [26–28]. To determine if the influence of SaeR/S-regulated factors on monocyte-derived TNF- $\alpha$  could directly influence neutrophil staphylocidal activity, we developed a co-culture model that combined isolated human neutrophils and PBMCs at a ratio of 7:3 (PMN:PBMC). The neutrophil bactericidal activity following challenge with USA300 $\Delta saeR/S$  was not significantly increased by the addition of PBMCs (Fig. 3A). However, we did demonstrate the importance TNF- $\alpha$  in these co-cultures by treating them with a TNF- $\alpha$  specific inhibitor (iTNF), which lead to significant increases in USA300 $\Delta saeR/S$  survival (Fig. 3A). Inhibition of TNF- $\alpha$  had no significant influence on neutrophil bactericidal activity in USA300 challenged co-cultures. These data suggest that the levels of TNF- $\alpha$  following challenge with USA300 are significantly reduced by SaeR/S-regulated factors and are insufficient to augment neutrophil bactericidal activity. ELISA results further demonstrated that co-cultures challenged with USA300 $\Delta saeR/S$

resulted in significantly increased TNF- $\alpha$  cytokine production at 3 hours post-challenge compared to USA300, and also confirmed the specificity of the TNF- $\alpha$  inhibitor (Fig. 3B). Taken together, these results demonstrate that SaeR/S-regulated factors influence early monocyte production of TNF- $\alpha$ , which significantly influences neutrophil bactericidal activity.

### **Conditioned media from PBMCs Challenged with USA300 $\Delta$ saeR/S Primes Neutrophils**

To address if PBMCs exposed to *S. aureus* produce secreted factors that could prime neutrophils, neutrophils were treated with filtered (bacteria-free) conditioned media (CM) from PBMCs challenged with USA300 or USA300 $\Delta$ saeR/S for 3 hours. CM-primed neutrophils were then challenged with a secondary stimulus of USA300 and subsequent ROS production was measured. Around 60 minutes post exposure, USA300-challenged neutrophils that were primed with USA300 $\Delta$ saeR/S CM had significantly increased ROS production compared to infected neutrophils primed with USA300 CM or unprimed neutrophils (Fig. 4A and B). Furthermore, treatment of USA300 $\Delta$ saeR/S CM with the TNF- $\alpha$  inhibitor, prior to neutrophil priming, significantly attenuated the enhanced ROS response (Fig. 4A and B). In congruence with these findings, neutrophils primed with USA400 $\Delta$ saeR/S CM also demonstrated significant increases in ROS production around 60 minutes post exposure compared to neutrophils primed with USA400 CM. Additionally, the increased ROS response in neutrophils primed with USA400 $\Delta$ saeR/S CM was significantly attenuated by addition of the TNF- $\alpha$  inhibitor (Supplemental Fig. 2 B, C).

These results indicate that the disruption of neutrophil priming by the SaeR/S-modulation of TNF- $\alpha$  is conserved between different strains of *S. aureus*.

To confirm if CM from USA300 $\Delta$ saeR/S exposed PBMCs acted as a priming agent, we exposed DCF-labeled neutrophils to USA300 CM or USA300 $\Delta$ saeR/S CM and subsequently challenged with N-formyl-methionine-leucyl-phenylalanine (fMLF). fMLF is a bacterial-derived chemotactic peptide that induces a respiratory burst by binding to formyl peptide receptors (FPRs) that are present on neutrophils and upregulated following neutrophil priming[29]. In congruence with our previous findings, USA300 $\Delta$ saeR/S CM significantly enhanced the neutrophil ROS response compared to USA300 CM following fMLF stimulation and the influence of TNF- $\alpha$  was confirmed using the TNF- $\alpha$  inhibitor (Fig. 4C). Additionally, no significant differences were observed in plasma membrane damage following priming with CM from USA300 or USA300 $\Delta$ saeR/S (data not shown). Lastly, the influence of priming with USA300 $\Delta$ saeR/S CM on neutrophil bactericidal activity was investigated. Priming PMNs with USA300 $\Delta$ saeR/S CM significantly enhanced killing of *S. aureus* at 30 and 60 minutes and this effect was not observed following priming with USA300 CM (Fig. 4D). The enhanced bactericidal activity of PMNs primed with USA300 $\Delta$ saeR/S CM was in-part attributed to TNF- $\alpha$ , as treatment of USA300 $\Delta$ saeR/S CM with the TNF- $\alpha$  inhibitor significantly reduced the increased neutrophil staphylocidal activity (Fig. 4D). These results demonstrate that factors regulated by the SaeR/S system reduce PBMC cytokine production of TNF- $\alpha$  to subsequently impact the neutrophil response to a secondary stimulus, including *S. aureus* and fMLF. Taken together, these data identify that

disruption of neutrophil priming is a virulence mechanism used by *S. aureus* to impede an effective neutrophil response.

## Discussion

The success of *S. aureus* as a pathogen is largely attributed to evasion mechanisms that circumvent killing by neutrophils. These mechanisms can directly target specific neutrophil functions, including neutrophil recruitment, phagocytosis, and the generation of reactive oxygen species (ROS) [2,3,8,30]. In addition to these strategies, we observed that SaeR/S-regulated virulence factors impeded neutrophil priming and ROS production by decreasing monocyte production of TNF- $\alpha$ .

Elevated TNF- $\alpha$  is historically considered to be a biomarker for predicting severe *S. aureus* bacteremia with potentially fatal outcomes [31,32]. In agreement, we have demonstrated in invasive models that induction of TNF- $\alpha$  expression by SaeR/S-regulated factors was associated with increased bacterial burden and mortality [11]. In contrast, findings have also shown that local production of TNF- $\alpha$  by residential cells can correlate to a protective immune response [33,34]. For example, TNF- $\alpha$  production by residential cells has been shown to significantly influence neutrophil recruitment from the microvasculature and that inhibition of TNF- $\alpha$  can reduce neutrophil recruitment by as much as 40-70% [33,34]. These findings highlight the pleiotropic influence of TNF- $\alpha$  during different immune responses. Herein, our data indicate that elevated TNF- $\alpha$  concentrations correlate to enhanced neutrophil responses that we propose is important during the initial stages of infection.

TNF- $\alpha$  is a robust priming agent that can enhance the production of neutrophil ROS [23] and has been observed to increase staphylocidal activity in a dose dependent manner [28,35]. The importance of ROS in controlling *S. aureus* infections is illustrated by the observed increase in susceptibility to infections in individuals with mutations in components of NADPH oxidase, as seen in patients suffering from chronic granulomatous disease[36]. *S. aureus* uses many mechanisms to detoxify ROS including superoxide dismutase and catalase[37,38]. In previous studies we demonstrated that SaeR/S-regulated factors also reduced neutrophil ROS products by decreasing hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and hypochlorous acid (HOCl) production, likely inhibiting myeloperoxidase [8,39]. In the current study, we observed that factors regulated by SaeR/S significantly impeded TNF- $\alpha$  production by PBMCs to diminish neutrophil ROS response to secondary challenge with either *S. aureus* or fMLF. Collectively, these observations underscore the importance of ROS production in controlling *S. aureus* and demonstrate inhibition of ROS is a major mechanism used by this pathogen to survive neutrophil phagocytosis.

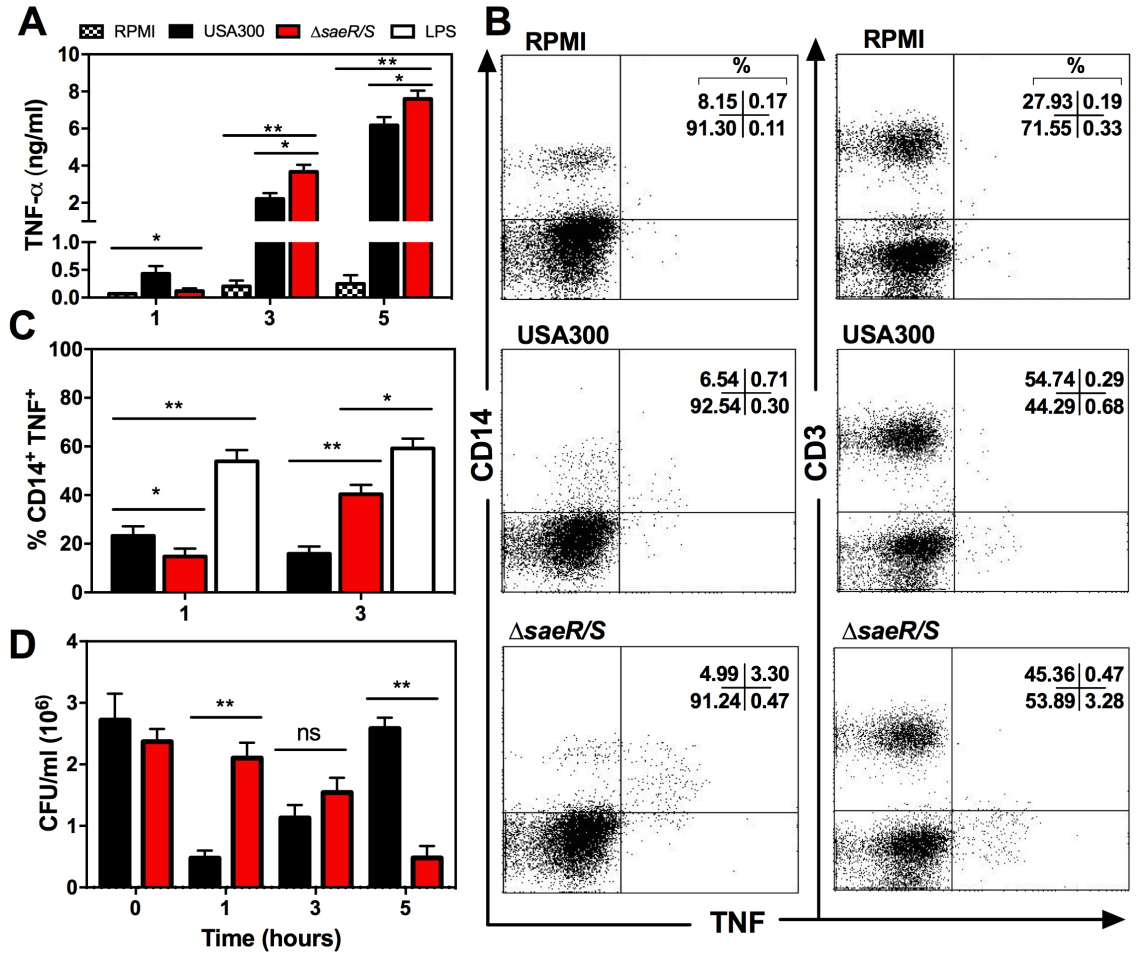
TNF- $\alpha$  also impacts neutrophil cell fate by activating NF- $\kappa$ B [40]. NF- $\kappa$ B is a transcription factor that regulates anti-apoptotic signals, including production of IL-8 and TNF- $\alpha$  [41,42]. We have previously demonstrated that SaeR/S-regulated factors diminished both NF- $\kappa$ B phosphorylation and IL-8 production which accelerated neutrophil cell death and improved survival of *S. aureus* [7]. TNF- $\alpha$  has been shown to increase NF- $\kappa$ B activation and IL-8 production in neutrophils [40]. Therefore, it is possible that reduced production of TNF- $\alpha$  in PBMCs by SaeR/S-regulated factors suppresses NF- $\kappa$ B activation and IL-8 production in neutrophils. Alternatively, NF- $\kappa$ B regulates TNF- $\alpha$  production and suppression of NF- $\kappa$ B activation by SaeR/S-regulated factors could impede proper TNF- $\alpha$

production and subsequent IL-8 production[42]. Future studies will investigate if these *S. aureus* immunomodulation mechanisms are linked and contribute to accelerated neutrophil death.

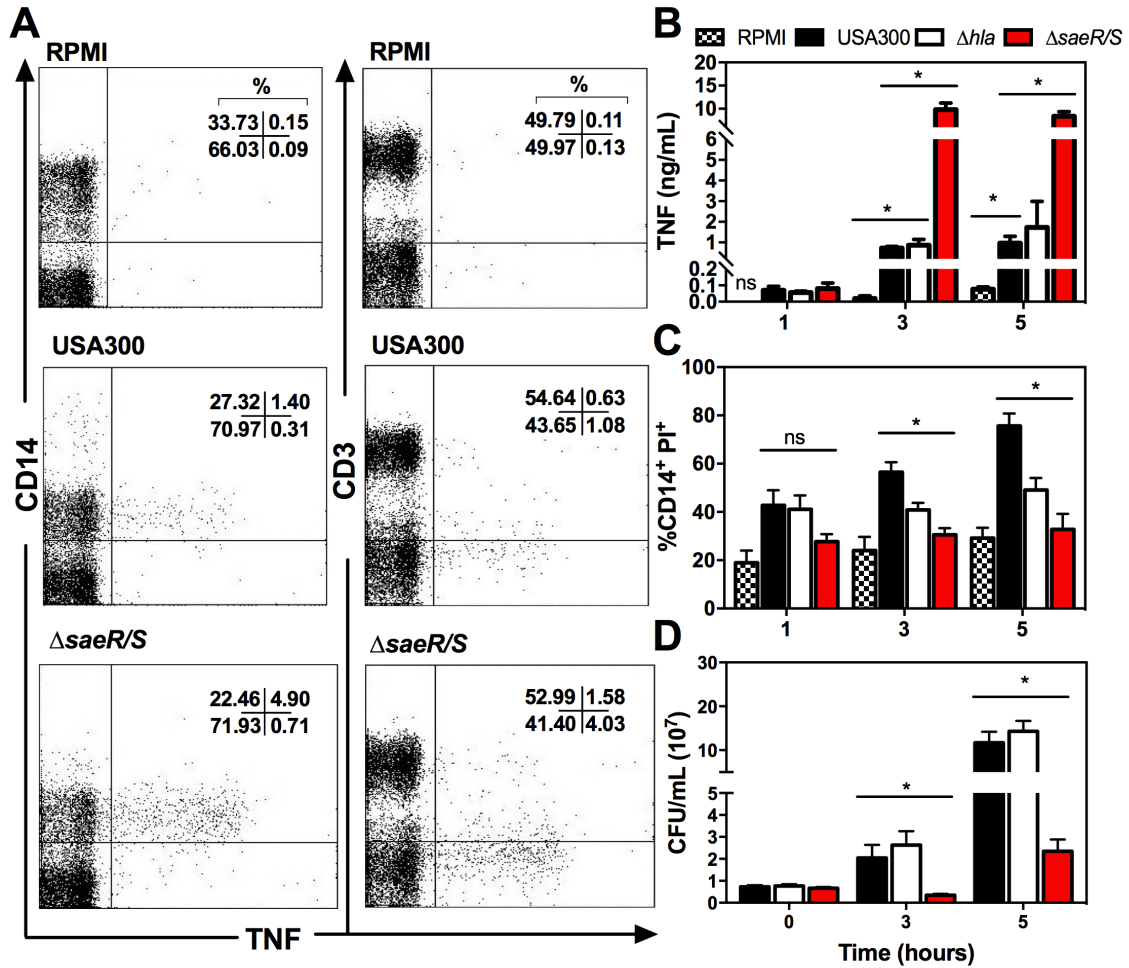
Although we identified that SaeR/S-regulated factors decreased TNF- $\alpha$  production from human monocytes, the mechanism responsible for this decrease remains undefined. As previous studies have shown that Hla is cytolytic towards human monocytes [43,44], we used a USA300 $\Delta$ *hla* strain of *S. aureus* to determine if monocyte production of TNF- $\alpha$  was associated with cell viability. Our data demonstrate that Hla was not responsible for the observed decrease in TNF- $\alpha$  and suggest that cell lysis is not the predominant mechanism. The SaeR/S-regulated factor LukGH (LukAB) has been observed to target monocytes and contribute to mediating phagosomal escape from macrophages [10,45,46]. Although macrophages and monocytes are different, it is possible that LukGH is influencing monocyte phagosomal functions to induce variations in TNF- $\alpha$  cytokine production [47,48]. Notably, LukGH and Panton-Valentine Leukocidin (PVL) have also been observed to influence neutrophil priming [49,50]. The SaeR/S-regulated immunomodulatory protein Sbi has been shown to increase TNF- $\alpha$  production in murine peritoneal macrophages in vivo during peritonitis [10,19] and might also account for the differences in TNF- $\alpha$  production observed in this study. Focusing on specific SaeR/S-regulated cytolytic and immunomodulatory factors responsible for reducing TNF- $\alpha$  will be the topic of future research.

In the current study, we developed a co-culture model of human neutrophils and PBMCs to demonstrate that SaeR/S-regulated factors significantly reduce early monocyte production of TNF- $\alpha$  and that this reduction influenced neutrophil responses to *S. aureus*,

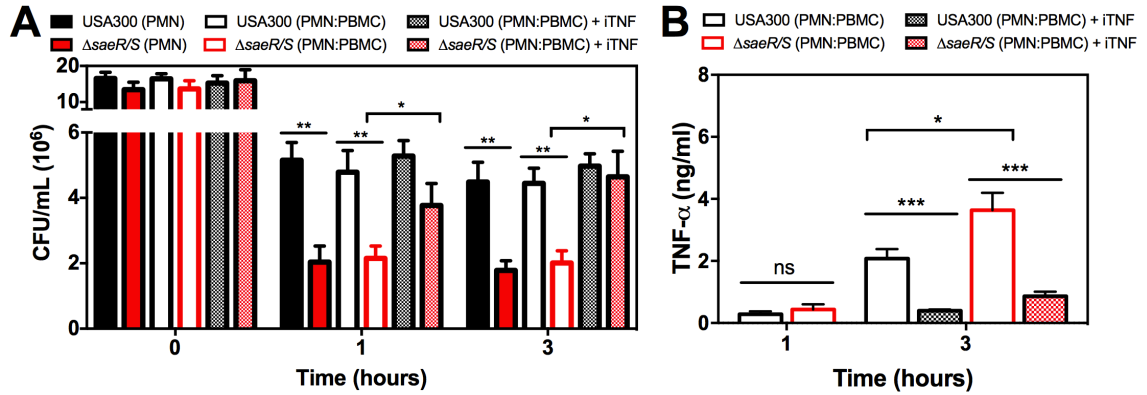
including ROS production and bactericidal activity. These data identify an immune evasion strategy used by *S. aureus* that targets neutrophil priming through SaeR/S-mediated reduction of monocyte derived TNF- $\alpha$  to limit the initial inflammatory response and survive neutrophil phagocytosis.



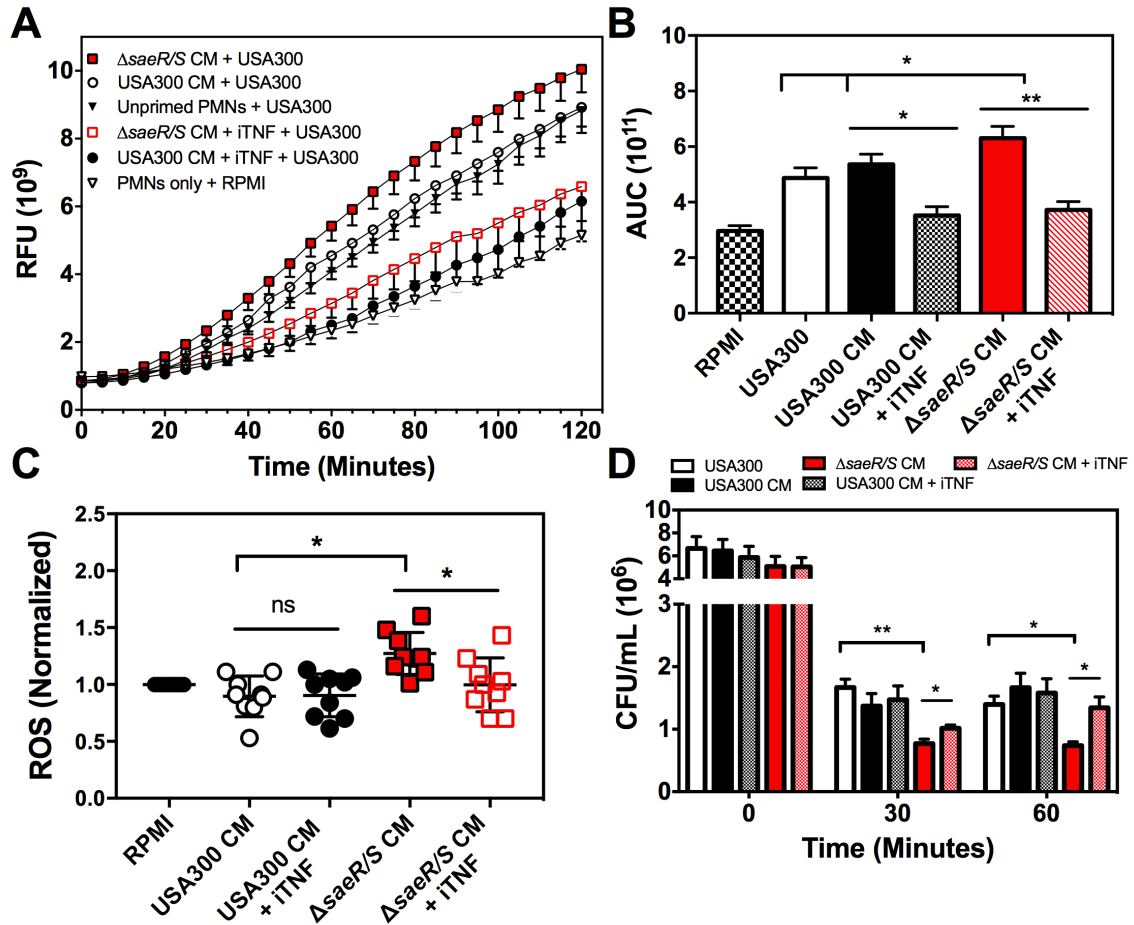
**Figure 1: SaeR/S suppresses TNF- $\alpha$  production in human blood from CD14<sup>+</sup> monocytes.** Human whole blood was exposed to USA300, USA300 $\Delta$ saeR/S, LPS, or RPMI for 1, 3, and 5 hours and TNF- $\alpha$  production was measured. **A.** Serum TNF- $\alpha$  cytokine concentrations were measured at designated time points using ELISA. **B.** Representative flow cytometry dot plots of human blood stained for monocyte (CD14<sup>+</sup>), T-cell (CD3<sup>+</sup>) and production of TNF- $\alpha$ , at 3 hours post-challenge with USA300 and USA300 $\Delta$ saeR/S. **C.** Compiled flow cytometry results of TNF- $\alpha$  and CD14<sup>+</sup> positive monocytes of total CD14<sup>+</sup> monocyte population. **D.** Bacterial survival of USA300 and USA300 $\Delta$ saeR/S in human whole blood. Data shown are the means  $\pm$  SEM of 10 experiments and statistics were determined by one-way ANOVA followed by Tukey's post-test. \*P<0.05, \*\*P<0.01.



**Figure 2: SaeR/S decreases TNF-α production in isolated human PBMCs.** Isolated human PBMCs were exposed to RPMI, USA300, USA300Δ*saeR/S*, or USA300Δ*hla* at a MOI of 2:1 (bacteria:PBMC) for 1, 3, and 5 hours. **A.** Representative flow cytometry dot plots of CD14<sup>+</sup> and CD3<sup>+</sup> cells producing TNF-α at 3 hours. **B.** At designated time points TNF-α from PBMCs exposed to bacteria was quantified using ELISA. **C.** CD14<sup>+</sup> PBMC plasma membrane permeability was assessed by propidium iodide uptake and measured using flow cytometry. **D.** Bacterial survival following exposure to PBMCs was assessed at designated time points. Data shown are the means ± SEM for 10 experiments. Statistics were determined by one-way ANOVA followed by Tukey's post-test. \*P<0.05.

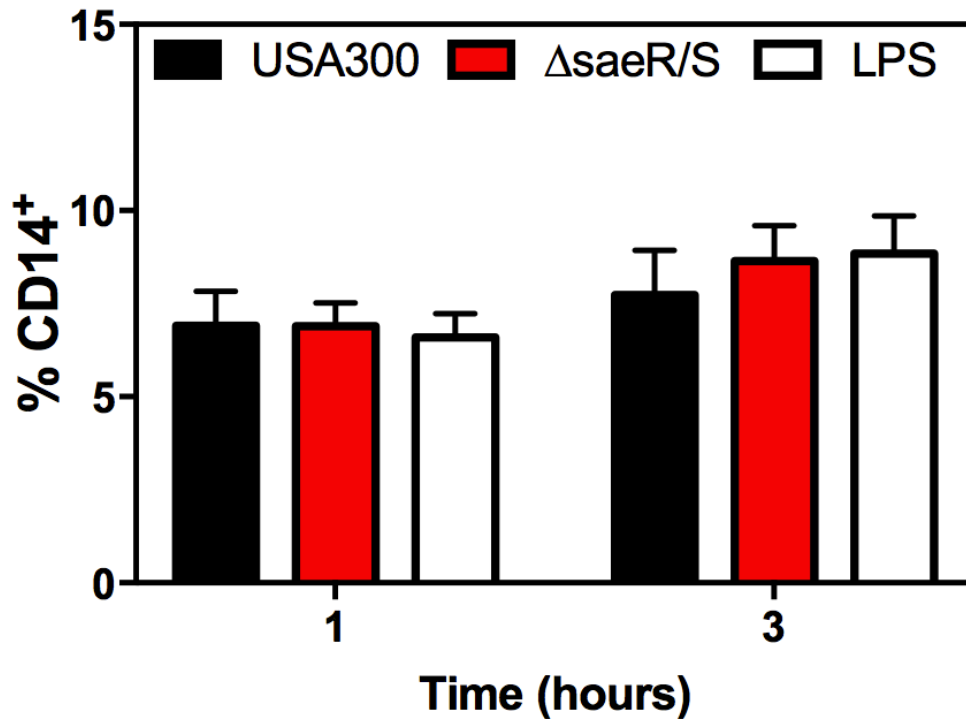


**Figure 3: TNF- $\alpha$  production from PBMCs increases PMN bactericidal activity.** PMNs supplemented with PBMCs only, or pretreated with 4.6 $\mu$ M TNF- $\alpha$  inhibitor (iTNF), were challenged with USA300 or USA300 $\Delta$ saeR/S at an MOI of 2. **A.** Bactericidal activity of untreated or iTNF-treated PMNs supplemented with PBMCs was compared to bactericidal activity of PMNs alone at 0, 1 and 3 hours. **B.** TNF- $\alpha$  cytokine concentrations were quantitated using ELISA. Data shown are the means  $\pm$  SEM of 10 experiments and statistics were determined by one-way ANOVA followed by Tukey's post-test. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

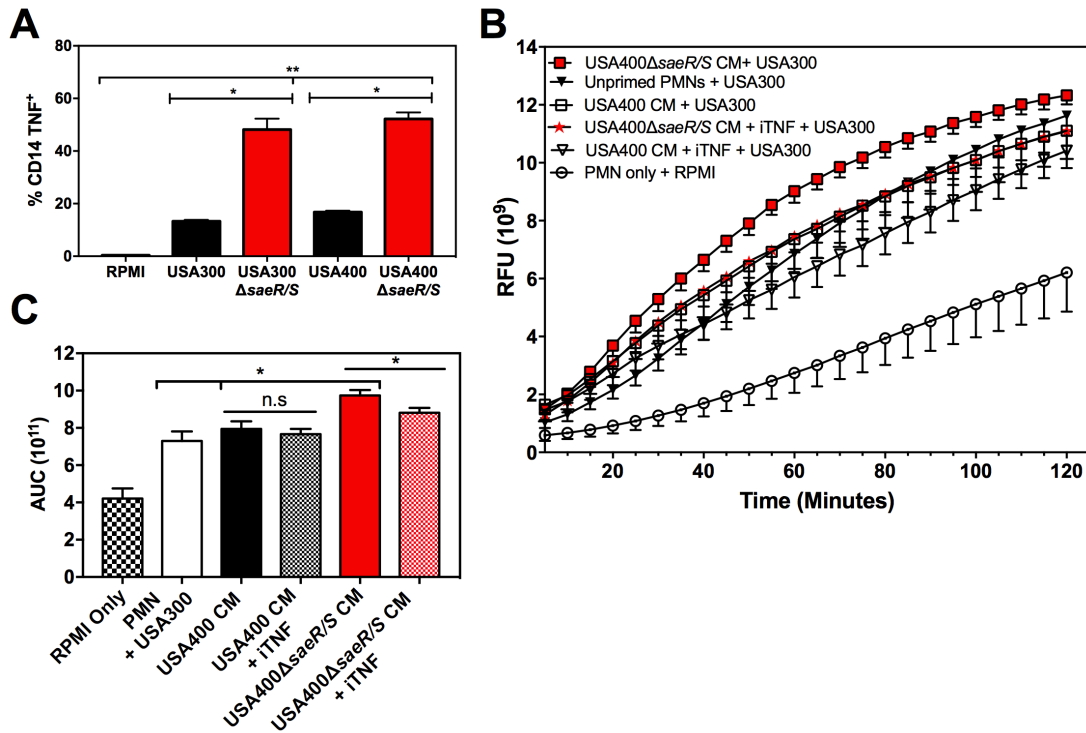


**Figure 4: Conditioned media from USA300 $\Delta$ saeR/S exposed PBMCs increases neutrophil ROS response to secondary challenge with USA300 and fMLF.** Conditioned media (CM) from PBMCs exposed to USA300 or USA300 $\Delta$ saeR/S (MOI of 2:1) for 3 hours was used to prime neutrophils for 30 minutes prior to challenge with live USA300 or secondary stimulus, fMLF. **A.** DCF-labeled neutrophils unprimed or CM-primed were challenged with USA300 or RPMI only and reactive oxygen species (ROS) production was measured over 120 minutes via spectrometry. **B.** Data from (A) showing total RFUs calculated using area under the curve (AUC). **C.** Intracellular ROS production from primed DCF-labeled neutrophils following challenge with 1 $\mu$ M fMLF was measured by flow cytometry at 40 minutes post-secondary challenge. **D.** Bacterial viability at 0, 30, and 60 minutes following interaction with neutrophils primed with USA300 CM, or USA300 $\Delta$ saeR/S CM, or CM treated with iTNF, or RPMI and challenged with USA300.

Data shown are the means  $\pm$  SEM from 7 experiments for (A, B, and D), and 9 experiments for (C). Statistics were determined by one-way ANOVA followed by Tukey's post-test. \* $P < 0.05$ , \*\* $P < 0.01$ . RFUs=Relative Fluorescent Units.



**Supplemental Figure 1: Percent CD14<sup>+</sup> monocytes in human blood.** Human whole blood was exposed to USA300, USA300 $\Delta$ saeR/S, or LPS for 1 and 3 hours and total CD14<sup>+</sup> monocytes were measured. Compiled flow cytometry results of total CD14<sup>+</sup> monocyte cell populations measured at 1 and 3 hours. Data shown are the means  $\pm$  SEM of 10 experiments.



**Supplemental Figure 2: USA400 SaeR/S-dependent decrease in TNF- $\alpha$  production influences neutrophil ROS response to secondary challenge with USA300.** Isolated human PBMCs were exposed to RPMI, USA300, USA300 $\Delta$ saeR/S, USA400, or USA400 $\Delta$ saeR/S at a MOI of 2:1 (bacteria:PBMC) for 3 hours. **A.** Compiled flow cytometry results of TNF- $\alpha$  and CD14<sup>+</sup> positive monocytes of total CD14<sup>+</sup> monocyte population. **B.** DCF-labeled neutrophils unprimed or CM-primed were challenged with USA300 or RPMI only and reactive oxygen species (ROS) production was measured over 120 minutes via spectrometry. **C.** Data from (B) showing total RFUs calculated using area under the curve (AUC). Data shown are the means  $\pm$  SEM of 3 experiments (A) and 5 experiments (B, C). Statistics were determined by one-way ANOVA followed by Tukey's post-test. \*P<0.05, \*\*P<0.01. RFUs=Relative Fluorescent Units. Data using USA300 in A are from separate experiments than those shown in Figure 1C.

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CHAPTER FOUR

STAPHYLOCOCCUS AUREUS INHIBITS TUMOR NECROSIS FACTOR- $\alpha$  IN  
MONOCYTE SUBSETS TO INFLUENCE NEUTROPHIL FUNCTIONS

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

Manuscript Information Page

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Journal of Infectious Diseases

State of Manuscript:

- ☒ Prepared for submission to a peer-reviewed journal
- ☐ Officially submitted to a peer-review journal
- ☐ Accepted by a peer-reviewed journal
- ☐ Published in a peer-reviewed journal

## Introduction

*Staphylococcus aureus* (*S. aureus*) is a Gram-positive pathogen that persistently colonizes one out of three individuals<sup>1</sup>. Although individuals colonized with *S. aureus* remain asymptomatic throughout their lifetime, colonization is a primary risk factor for infection<sup>2</sup>. Infections caused by *S. aureus* include self-limiting skin- and soft-tissue infections, which can progress into severe life-threatening complications, such as necrotizing pneumonia, necrotizing fasciitis, sepsis and death<sup>3</sup>. Treatment of *S. aureus* is further complicated by the rapid development of antibiotic resistance and is a global health concern due to the ability to cause infections in healthy individuals within community settings. Currently, community-associated methicillin-resistant *S. aureus* (CA-MRSA), strain USA300, is the leading cause of MRSA infections in the United States and is associated with 400,000 hospital admissions annually and 12 million annual outpatient visits worldwide<sup>4</sup>. Due to the potential of a post-antibiotic era and high mortality rates associated with *S. aureus* infections, studies focusing on developing alternative forms of treatment are necessary. Immunotherapies and selective inhibition of staphylococcal virulence factors are likely to be successful alternative therapies, but require a further understanding of *S. aureus* pathogenesis and protective host immune responses.

The pathogenesis of USA300 and associated mortality is largely associated with the production of cytolytic and immunomodulatory virulence factors under the control of two-component gene-regulatory systems. Currently, there are 16 putative staphylococcal two-component gene-regulatory systems (TCS) that have diverse functions in pathogenesis<sup>5</sup>. One TCS that is important for *S. aureus* virulence is the *S. aureus*

exoprotein expression system (SaeR/S). SaeR/S is rapidly expressed following neutrophil phagocytosis and regulates specific virulence factors with conserved binding sequences within 5 to 10 minutes following interaction with neutrophils or host associated stimuli<sup>6-9</sup>. The SaeR/S-TCS also demonstrates the ability to modulate cytokine production. Following *S. aureus*-neutrophil interaction, SaeR/S-regulated factors were shown to decrease phosphorylation and activation of NF- $\kappa$ B and reduce production of IL-8<sup>10</sup>. In turn, this promoted accelerated neutrophil apoptosis, or programmed necrosis, and lead to increased bacterial survival. In congruence with these findings, we have demonstrated that the SaeR/S-TCS influenced monocyte cytokine production of TNF- $\alpha$  during early time points of challenge, which subsequently altered neutrophil priming, ROS production, and ability to kill *S. aureus*. Previous studies have demonstrated that secreted factors could influence cytokine production in dendritic cells<sup>11,12</sup>, however, it remains unclear if secreted SaeR/S-regulated factors are responsible for modulating TNF- $\alpha$  in our models of infection.

Another unresolved question is if SaeR/S factors influence monocyte subsets. Macrophages can arise through self-renewal or by differentiated monocytes from peripheral blood circulation<sup>13</sup>. Circulating monocytes are broadly characterized as classical or non-classical monocytes that have different expression of CD14, the major co-receptor with TLR4 that bind to LPS, and CD16, the Fc $\gamma$  receptor type III<sup>14</sup>. Classical monocytes constitute 80-90% of circulating monocytes and are characterized by high expression of CD14 and are devoid of CD16 (CD14<sup>++</sup>CD16<sup>-</sup>)<sup>14</sup>. Classical monocytes can further differentiate into an intermediate monocyte subset (CD14<sup>++</sup>CD16<sup>+</sup>), which can

develop into non-classical monocytes characterized by expression of CD16 and lower levels of CD14 (CD14<sup>+</sup>CD16<sup>+</sup>)<sup>14</sup>. Increased populations of classical and non-classical monocyte subsets have been observed in humans during invasive *S. aureus* infections, which indicate that intermediate monocytes may play an important role during infection<sup>15</sup>. In addition, these three monocyte subsets have unique cytokine production profiles. In response to LPS or Pam3cys, a synthetic TLR2 agonist, non-classical monocytes demonstrated increased TNF- $\alpha$  production relative to classical monocytes and intermediate monocytes, with intermediate monocytes producing significantly less than the others<sup>16,17</sup>. As intermediate monocytes may constitute a large population of differentiating classical monocytes that have reduced cytokine production, this subset may play a significant role in the inflammatory response to *S. aureus* infection. However, to date, studies that focus on differences in monocyte subsets and associated cytokine production during *S. aureus* are lacking and remain incompletely defined.

In the current study, we assessed for differences in monocyte subsets and TNF- $\alpha$  production during challenge with *S. aureus* and examined the ability of SaeR/S-secreted factors to influence monocyte-derived TNF- $\alpha$  production. Our findings demonstrate that SaeR/S-regulated factors may promote an intermediate monocyte subset that is associated with reduced TNF- $\alpha$  production and that secreted factors negatively regulated by SaeR/S can induce TNF- $\alpha$  production in monocytes. We further elucidated a direct role of TNF- $\alpha$  on neutrophil bacterial killing, ROS, MPO, and apoptosis following secondary challenge with USA300. Using these models, we suggest that reduced monocyte-production of TNF- $\alpha$  by SaeR/S-regulated factors results in accelerated neutrophil cell

death, or necrosis, whereas higher TNF- $\alpha$  concentrations promote programmed cell death, or apoptosis. Collectively, this study demonstrates that the SaeR/S system can influence TNF- $\alpha$  in multiple ways, which influences the outcome of neutrophil-*S. aureus* interactions.

## **Materials and Methods**

### Bacterial strains and cultures

Community-associated methicillin-resistant *S. aureus* (CA-MRSA) pulsed-field gel electrophoresis-type USA300 (strain LAC), and isogenic *saeR/S*, *lukF-PVL*, *lukA*, and *agr* deletion mutants (USA300 $\Delta$ *saeR/S*, USA300 $\Delta$ *pvl*, USA300 $\Delta$ *lukA*, USA300 $\Delta$ *agr*), were generated in previous studies<sup>8,9,18–21</sup>. For assays using *S. aureus* supernatants, 20 mL of TSB without glucose was inoculated with 200 $\mu$ L USA300 or USA300 $\Delta$ *saeR/S* overnight culture, grown for 5 hours with shaking (250 rpm), centrifuged (5,000xg, 5 minutes, 4°C), and filtered (0.2 $\mu$ M), as described previously<sup>22</sup>. For infection assays, 1mL of *S. aureus* grown to mid exponential was harvested by centrifugation (5,000xg, 5 minutes, 4°C), washed twice with 1mL DPBS, and re-suspended in 1mL RPMI without hepes<sup>22</sup>.

### Infection of human blood and isolated neutrophils and peripheral blood mononuclear cells (PBMCs)

Heparinized venous blood from healthy donors was collected in accordance with a protocol approved by the Institutional Review Board for Human Subjects at Montana State University. All donors provided written consent to participate in the study. For

human whole blood live infection assays, RPMI or RPMI containing  $2 \times 10^6$  colony forming units (CFU) of bacteria was added to 1mL of freshly drawn human whole blood in 1.5mL Eppendorf tubes and placed on an end-over-end mixer (20 RPM, 37°C, 5% CO<sub>2</sub>). For whole blood supernatant assays, 5-hour sterile-filtered USA300 or USA300 $\Delta$ *saeR/S* supernatants were added to 1mL human whole blood at designated final dilutions in 1.5mL eppendorf tubes and placed on an end-over-end mixer (20 RPM, 37°C, 5% CO<sub>2</sub>).

Human neutrophils (polymorphonuclear leukocytes or neutrophils) and peripheral blood mononuclear cells (PBMCs), cells were isolated under endotoxin-free conditions (<25pg/ml) as previously described<sup>9,23</sup>. Purity (<1% PBMC contamination) and viability (<2% propidium iodide positive) of neutrophil preparations were assessed by flow cytometry (FACS Calibur; BD Biosciences). For neutrophil and PBMC live infection assays,  $1 \times 10^6$  isolated PBMCs or neutrophils were challenged with  $2 \times 10^6$  CFUs of *S. aureus* in a 96-well normal human serum (NHS) coated plate and phagocytosis was synchronized by centrifugation (524 x g, 7 minutes, 10°C) and then incubated at 37°C<sup>23</sup>. For bacterial supernatant assays,  $1 \times 10^6$  isolated PBMCs were challenged with 5-hour sterile-filtered supernatants at designated final dilutions in a 96-well normal human serum (NHS) coated plate and incubated at 37°C<sup>23</sup>.

#### Assessment of cell surface expression and TNF- $\alpha$ production from human cells

At designated time points following challenge of whole blood with live *S. aureus* or bacterial supernatants, samples were stained with FITC mouse anti-human CD14 and APC mouse anti-human TNF- $\alpha$  or IgG-1 isotype control (BD Biosciences). Intracellular

staining was performed using a BD Pharmingen intracellular stain kit following the manufactures protocol and all samples were analyzed using a FACS Calibur Flow Cytometer (BD Bioscience).

At designated time points following challenge of PBMCs with live *S. aureus* or bacterial supernatants, samples were stained with FITC or PercP mouse anti-human CD14, FITC mouse anti-human CD16, and APC mouse anti-human TNF- $\alpha$  or IgG-1 isotype control (BD Biosciences). After staining, 1mL of FACS Buffer (BD Biosciences) was added and samples were centrifuged (400 x g, 8 minutes, 4°C). Supernatant was aspirated and 250  $\mu$ L of IC Fixation Buffer (BD Biosciences) was added for 20 minutes. Samples were washed twice with 1X Perm Wash (BD Biosciences) and centrifuged between wash steps (400 x g, 8 minutes, 4°C). For intracellular staining of TNF- $\alpha$ , APC mouse anti-human TNF- $\alpha$  was diluted 1:100 in 1X Perm Wash and 100 $\mu$ L was added to samples for 30 minutes on ice. Samples were washed and centrifuged using Perm Wash (BD Bioscience) and 250 $\mu$ L of 1% paraformaldehyde diluted in FACS Buffer was added for the final step. Samples were analyzed using a FACS Calibur Flow Cytometer (BD Bioscience).

#### Neutrophil priming with TNF- $\alpha$

For reactive oxygen species (ROS) assays, neutrophils were loaded at room temperature with 25  $\mu$ M 2',7'-dihydrochlorofluorescein diacetate (DCF; Molecular Probes) for 30 min in RPMI/H<sup>23</sup>. DCF-containing PMNs ( $1 \times 10^6$ ) were then added to a 96-well serum-coated plate containing 0.1ng/ml, 1ng/ml, or 10ng/ml recombinant TNF- $\alpha$  (BioLegend) for 30 minutes at 37°C. After treatment, PMNs were exposed to opsonized bacteria at a

ratio of 1:2 (PMN:bacteria), and phagocytosis was synchronized using centrifugation (524 x g, 7 minutes, 10°C)<sup>23</sup>. Measurements were done using a SpectraMax Paradigm Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) using 488/530nm emission/excitation wavelengths.

At designated time points, bacterial survival following challenge with *S. aureus* at a 1:2 MOI (PMN:bacteria) was assessed using serial dilutions in water and CFUs were enumerated on tryptic soy agar (TSA). PMN lysis was quantitated using lactate dehydrogenase (LDH) assays (Promega) and myeloperoxidase (MPO) enzyme activity was quantitated as described by the manufacturer<sup>24</sup>. At designated time points, neutrophil cell death was measured by chromatin condensation using flow cytometry as described previously<sup>25</sup>.

## **Results and Discussion**

### **Defining Monocyte Subsets Responsible for TNF- $\alpha$ Production**

Although the response of monocytes to *S. aureus* has been studied, it remains to be elucidated which monocyte subset(s) is/are responsible for TNF- $\alpha$  production and if the SaeR/S TCS of *S. aureus* can influence this process. As mentioned previously, the majority of monocytes in circulation are CD14<sup>++</sup>CD16<sup>-</sup>, referred to as classical monocytes, and a smaller percentage are non-classical monocytes characterized by CD14<sup>+</sup> CD16<sup>+</sup> expression<sup>14</sup>. In accordance with the literature, classical monocytes can rapidly differentiate into non-classical monocytes through an intermediate monocyte subset<sup>14</sup>. These monocyte subsets have altered production of pro-inflammatory cytokines,

including production of TNF- $\alpha$ <sup>16</sup>. As the SaeR/S-TCS was shown to decrease monocyte production of TNF- $\alpha$ , we conducted experiments exploring differences in monocyte subsets during challenge with wild-type *S. aureus*, USA300, or an isogenic *saeR/S* deletion mutant (USA300 $\Delta$ *saeR/S*). In agreement with previous findings, isolated human PBMCs challenged with USA300 exhibited a significant decrease in total monocyte production of TNF- $\alpha$  at 3-hours post-challenge compared to PBMCs challenged with USA300 $\Delta$ *saeR/S* (Fig. 1A). As TNF- $\alpha$  production can vary between different monocyte subsets<sup>17</sup>, flow cytometry was used to assess CD14 and CD16 surface expression profiles following challenge with USA300 or USA300 $\Delta$ *saeR/S*. In congruence with the literature, the majority of monocytes in unchallenged (RPMI only) PBMCs were classical monocytes, however, there were significant reductions in classical monocytes following challenge with USA300 and USA300 $\Delta$ *saeR/S* (Fig. 1B). These reductions correlated to differences in intermediate and non-classical monocytes. Specifically, when PBMCs were challenged with USA300 there was a significant increase in intermediate monocytes relative to challenge with USA300 $\Delta$ *saeR/S* and control medium (Fig. 1C). Furthermore, PBMCs challenged with USA300 $\Delta$ *saeR/S* demonstrated increased levels of non-classical monocytes compared to PBMCs challenged with USA300 but no significant differences were observed when compared to control medium (Fig. 1D). These results indicate that the SaeR/S-TCS influences monocyte TNF- $\alpha$  production through potentially promoting an intermediate monocyte subset that does not fully differentiate into a pro-inflammatory non-classical monocyte. Future studies are necessary to confirm these results and will include assays measuring for differences in phagocytosis and surface expression of HLA-

DR, CD11b and TLRs<sup>26</sup>. Together, these studies indicate that the SaeR/S TCS influences CD14, CD16, and TNF- $\alpha$  expression and provide direction for future studies exploring *in vivo* modulation of monocyte subsets during *S. aureus* infection.

### **SaeR/S Secreted Factors Influence Monocyte TNF- $\alpha$ Production**

Though the SaeR/S TCS reduces monocyte production of TNF- $\alpha$  and possibly monocyte maturation, it is unclear what SaeR/S-regulated factor(s) is responsible for these effects. Previous studies reported herein have demonstrated that the SaeR/S-TCS reduced TNF- $\alpha$  production in monocytes following challenge with live USA300. These results indicate that SaeR/S regulated factors can influence signaling pathways following phagocytosis, which is supported by other studies showing SaeR/S-regulated factors LukAB and PVL, as well as the Agr sensory system, to influence monocyte phagosomal functions<sup>27–29</sup>. To identify if these virulence factors influenced TNF- $\alpha$  production, PBMCs were challenged with USA300 and isogenic *saeR/S*, *lukA*, *lukF-PVL*, and *agr* deletion mutants (USA300 $\Delta$ *saeR/S*, USA300 $\Delta$ *lukA*, USA300 $\Delta$ *pvl*, and USA300 $\Delta$ *agr*) and intracellular CD14 TNF- $\alpha$  production was assessed. At 3-hours post-challenge, CD14<sup>+</sup> monocytes demonstrated significantly reduced TNF- $\alpha$  production when challenged with USA300 compared to both USA300 $\Delta$ *saeR/S* and USA300 $\Delta$ *pvl* (Fig. 2). In contrast, neither USA300 $\Delta$ *lukA* or USA300 $\Delta$ *agr* appeared to influence TNF- $\alpha$  production from CD14<sup>+</sup> monocytes. In addition, TNF- $\alpha$  modulation by PVL was independent of cell membrane permeability as challenge with USA300 $\Delta$ *pvl* showed similar propidium iodide uptake as PBMCs challenged with USA300 (Fig 2B). These preliminary data indicate that PVL is

an important SaeR/S-regulated factor for influencing monocyte-derived TNF- $\alpha$  production during challenge with live *S. aureus*.

To explore if additional secreted factors influenced TNF- $\alpha$ , human whole blood was exposed to 5-hour sterile-filtered USA300 and USA300 $\Delta$ saeR/S supernatants and monocyte-derived production of TNF- $\alpha$  was assessed. At 3-hours post-challenge, high concentrations of USA300 $\Delta$ saeR/S supernatants induced significant increases in intracellular TNF- $\alpha$  production compared to USA300 supernatants (Fig. 2C). There was also a noticeable concentration dependent effect on the ability of USA300 $\Delta$ saeR/S supernatants to induce TNF- $\alpha$  in whole blood, suggesting that TNF- $\alpha$  modulation is due to a virulence factor(s) negatively regulated by SaeR/S. Similar experiments using bacterial supernatants were performed on isolated human PBMCs. In congruence with findings from whole blood, PBMCs challenged with high concentrations of USA300 $\Delta$ saeR/S supernatants demonstrated increased CD14<sup>+</sup> TNF- $\alpha$  production compared to challenge with USA300 supernatants (Fig. 2D). Lastly, cell membrane permeability was measured by propidium iodide uptake to determine if differences in TNF- $\alpha$  production were associated with monocyte lysis. In contrast to PBMCs challenged with live bacteria, significant differences in cell membrane permeability between challenge with USA300 and USA300 $\Delta$ saeR/S supernatants were observed (Fig. 2E). These preliminary results indicate that SaeR/S-regulated PVL is important for regulating monocyte-derived TNF- $\alpha$  and that if at high enough concentrations in the extracellular environment, SaeR/S-regulated factors can induce monocyte lysis and subsequently influence TNF- $\alpha$  production. Future studies are necessary to identify

SaeR/S-negatively regulated factors that may increase monocyte-derived TNF- $\alpha$  production. A recent study by Cruciana et al. identified that two small acidic dimeric proteins, EsxA and EsxB, increased dendritic cell production of TNF- $\alpha$ . Both EsxA and EsxB are encoded in the *ess* gene cluster, which is negatively regulated by SaeR/S, indicating that these proteins are potential candidates for the observed SaeR/S-dependent modulation of TNF- $\alpha$ <sup>12</sup>. However, additional studies are required to test if EsxA and EsxB influence monocyte production of TNF- $\alpha$ .

### **Assessing the Direct Influence of TNF- $\alpha$ on Neutrophil Functions**

Previous studies, including those reported in Chapter 3, demonstrated that TNF- $\alpha$  from conditioned media produced by PBMCs enhances neutrophil killing of *S. aureus*<sup>30,31</sup>. These studies highlighted a primary role for TNF- $\alpha$  in neutrophil functions, however, other inflammatory mediators within conditioned media may also contribute to the observed effects. To minimize the influence of other factors within conditioned media, neutrophil priming assays were performed using different concentrations of recombinant TNF- $\alpha$  (rTNF- $\alpha$ ) and neutrophil bacterial killing, ROS production, and apoptosis were measured. Priming of neutrophils with rTNF- $\alpha$  enhanced bacterial killing of USA300 at 1- and 3-hours post-challenge, which likely correlates to increased neutrophil ROS and degranulation. To assess these possibilities, ROS production and degranulation were measured. Both 1.0 and 10ng/ml rTNF- $\alpha$  demonstrated increased ROS production over 3-hours and MPO activity (used as a marker for degranulation) at 1- and 3-hours post-challenge (Fig. 1C).

The SaeR/S TCS of USA300 has recently been shown to induce rapid neutrophil death by inhibiting Nf-kB activation and reducing IL-8 production<sup>10</sup>. The observed accelerated neutrophil cell death was associated with increased bacterial survival, which could be prevented through administration of recombinant IL-8. These studies highlight that inflammatory mediators can significantly influence neutrophil cell death and improve overall neutrophil-*S. aureus* outcomes. Based on this study and the results that rTNF- $\alpha$  improved neutrophil staphylocidal activity (Fig. 3A), experiments were performed to test if neutrophil priming with TNF- $\alpha$  could influence apoptosis. Preliminary data demonstrated that at 3-hours post-challenge, neutrophils primed with 0.1ng/ml and 1.0ng/ml rTNF- $\alpha$  showed significantly less chromatin condensation compared to untreated neutrophils challenged with USA300 (Fig. 3D). In contrast, neutrophils primed with 10ng/ml rTNF- $\alpha$  exhibited increased chromatin condensation compared to the other treatments. Preliminary data also indicate that at all rTNF- $\alpha$  concentrations, lactate dehydrogenase (LDH) release was prominently reduced at 5-hours post-challenge compared to untreated neutrophils challenged with USA300, although these results did not reach significance (Fig. 3E). These findings are consistent with studies demonstrating that neutrophils treated with low TNF- $\alpha$  concentrations are anti-apoptotic whereas high TNF- $\alpha$  concentrations are pro-apoptotic<sup>32,33</sup> and indicate that TNF- $\alpha$  induced neutrophil apoptosis is important for staphylocidal activity. Together, these studies demonstrate that increased TNF- $\alpha$  enhances neutrophil *S. aureus* killing that is associated with increased ROS and MPO activity and suggest that TNF- $\alpha$  is important for inducing programmed cell death or apoptosis, rather than programmed necrosis<sup>10</sup>.

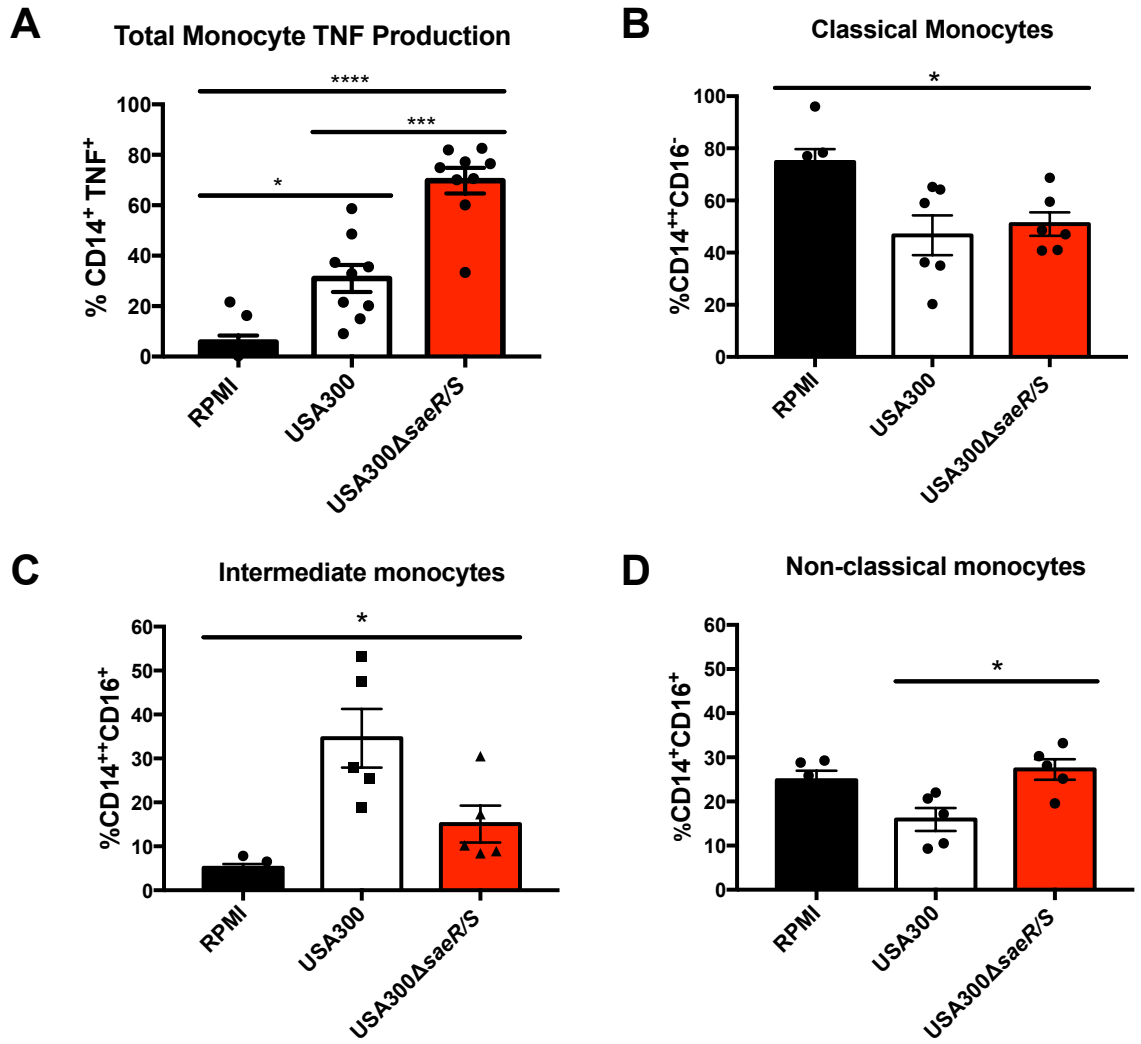


Figure 1. *SaeR/S* modulates monocyte expression of CD14 and CD16 to influence TNF- $\alpha$  production. PBMCs were challenged with USA300 or USA300 $\Delta$ *saeR/S* at a 2:1 MOI (bacteria:PBMC) and assessed for extracellular CD14 and CD16 expression, and intracellular production of TNF- $\alpha$  at 3-hours. **A)** Total PBMC CD14 production of TNF- $\alpha$ . Total populations of **B)** classical monocytes (CD14<sup>++</sup>CD16<sup>-</sup>), **C)** intermediate monocytes (CD14<sup>++</sup>CD16<sup>-</sup>) and **D)** non-classical monocytes (CD14<sup>+</sup>CD16<sup>+</sup>) were assessed using flow cytometry. Compiled flow cytometry results of PBMC CD16 expression at 3-hours post-challenge. Data shown are the means  $\pm$  SEM of 9 experiments for (A) and 5-6 experiments for (B,C and D). Statistics were determined by one-way ANOVA followed by Tukey's post-test. \*P<0.05, \*\*\*P<0.01, \*\*\*\*P<0.001.

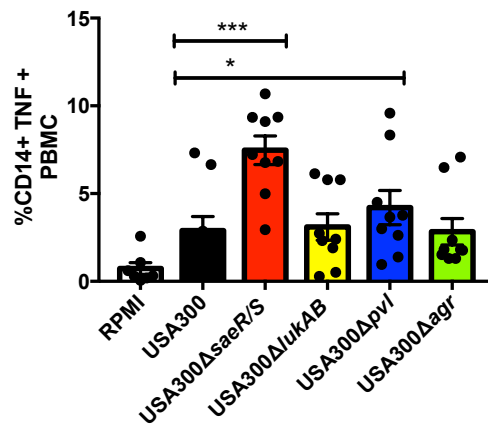
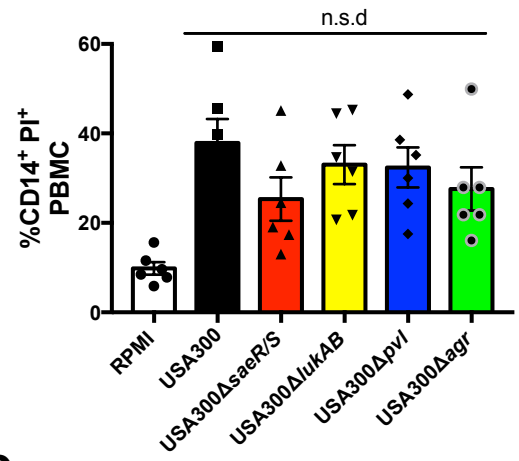
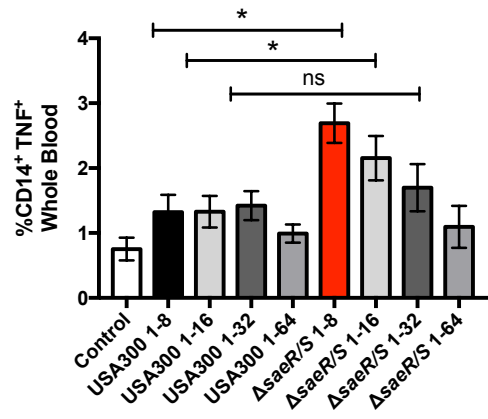
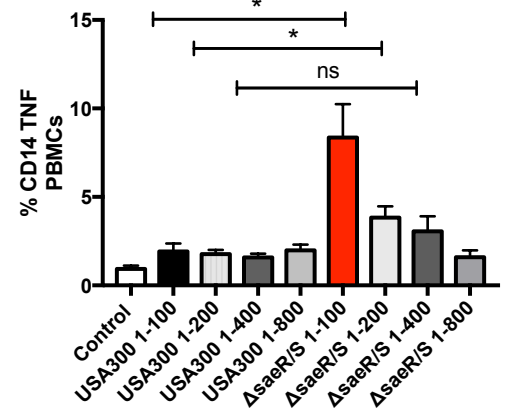
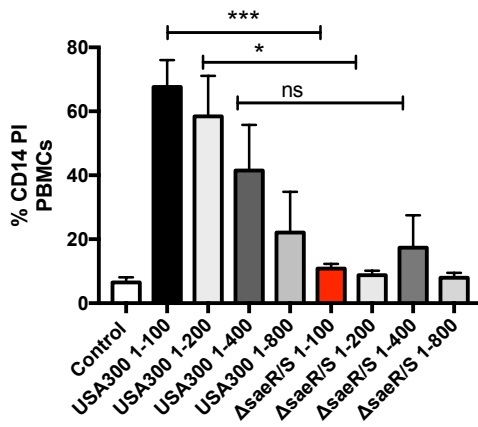
**A****B****C****D****E**

Figure 2. Identifying the SaeR/S-regulated factor that decreases monocyte-derived TNF- $\alpha$ . Isolated human PBMCs were challenged with USA300 or isogenic deletion mutants ( $\Delta$ *saeR/S*,  $\Delta$ *lukA*,  $\Delta$ *pvl*,  $\Delta$ *agr*) at a 2:1 MOI (bacteria:PBMC) for 3-hours and assessed for **A)** CD14<sup>+</sup> TNF- $\alpha$  production and **B)** CD14<sup>+</sup> cell membrane permeability measured using propidium iodide uptake. Bacterial strains were grown for 5-hours in TSB-no glucose and were sterile filtered to obtain bacteria free supernatants. **C)** 5-hour bacterial supernatants were added to 1ml human whole blood at the designated final dilution and at 3-hour post-challenge, CD14<sup>+</sup> TNF- $\alpha$  production was measured. 5-hour bacterial supernatants were added to isolated human PBMCs at the designated final dilution and at 3-hour post-challenge **D)** CD14<sup>+</sup> TNF- $\alpha$  and **E)** cell membrane permeability (propidium iodide uptake) were measured. All samples were evaluated using flow cytometry. Data shown are the means  $\pm$  SEM of 9 experiments for (A), 6 experiments for (B), and 6 experiments for (C, D and E). Statistics were determined by one-way ANOVA followed by Tukey's post-test. \*P<0.05, \*\*\*P<0.001. n.s.d. and ns = no significant differences.

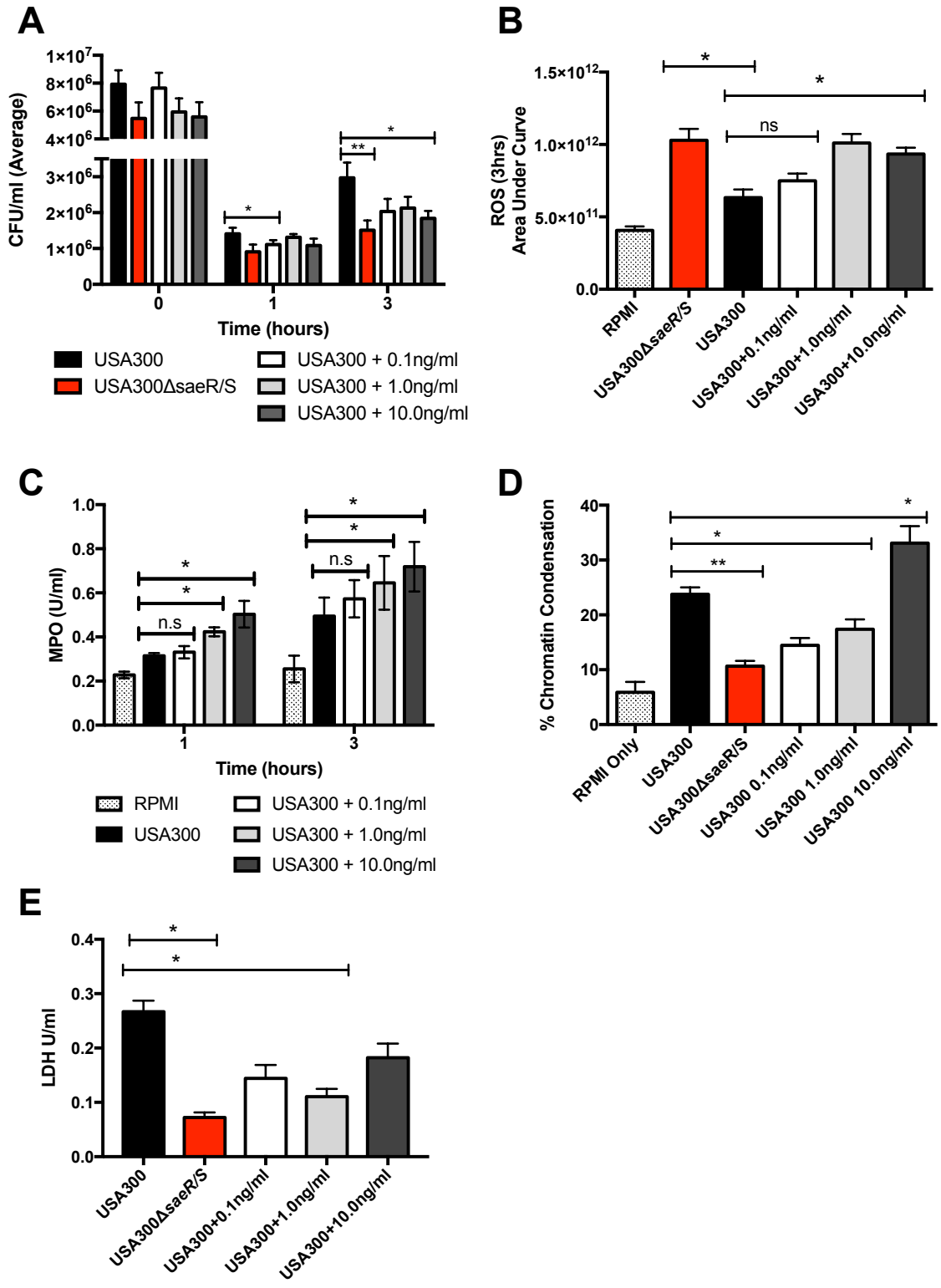


Figure 3. Recombinant TNF- $\alpha$  influences neutrophil functions in response to *S. aureus*. Neutrophils were primed for 30 minutes at 37°C with 0.1, 1.0, and 10.0ng/ml rTNF- $\alpha$  then challenged with USA300 or USA300 $\Delta$ saeR/S at an MOI of 2:1 (bacteria:PMN). **A)** Bacterial survival of USA300 or USA300 $\Delta$ saeR/S was assessed between neutrophils primed with rTNF- $\alpha$  or untreated. **B)** ROS production in response to USA300 or USA300 $\Delta$ saeR/S was measured in TNF- $\alpha$  primed or untreated neutrophils labeled with 25  $\mu$ M 2',7'-dihydrochlorofluorescein diacetate for 3 hours post-challenge. Overall ROS was determined using area under the curve (AUC). **C)** Myeloperoxidase (MPO) activity was measured between rTNF- $\alpha$  primed or untreated neutrophils challenged with USA300 at 3 hours post challenge. **D)** Neutrophil chromatin condensation was measured between primed and untreated neutrophils challenged with USA300 or USA300 $\Delta$ saeR/S at 3-hours post-challenge. **E)** Lactate dehydrogenase (LDH) release was compared between primed and untreated neutrophils at 5-hours post challenge with UAS300 or USA300 $\Delta$ saeR/S. Data shown are the means  $\pm$  SEM of 10 experiments for (A,B), 3 and 7 experiments (1- and 3-hour, respectively) for (C), 7 experiments for (D) and 4 experiments for (E). Statistics were determined by one-way ANOVA followed by Tukey's post-test. \*P<0.05, \*\*P<0.01. n.s = not significant.

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## CHAPTER FIVE

## CONCLUSION

The work described explores the role of *S. aureus* modulation of monocyte-derived TNF- $\alpha$  on neutrophil functions. Previous findings demonstrated that the SaeR/S system influenced neutrophil production of IFN- $\gamma$ , which has been shown to be a priming agent that enhances neutrophil ROS and staphylocidal activity<sup>1-3</sup>. Although these studies indicated that IFN- $\gamma$  induced over activation of neutrophil functions resulting in mortality, they highlighted that the SaeR/S system could influence pro-inflammatory cytokine production. To explore this concept, the current studies examined the influence of SaeR/S on early production of TNF- $\alpha$ , as this cytokine is produced quickly during infection and can influence many neutrophil responses<sup>4-7</sup>. Using human whole blood and isolated peripheral blood mononuclear cells, we have found that the SaeR/S-TCS of *S. aureus* reduced early monocyte production of TNF- $\alpha$  and that this modulation was independent of cell lysis. Preliminary data indicate that this modulation of TNF- $\alpha$  was not mediated by SaeR/S-regulated factor alpha-hemolysin ( $\alpha$ -hemolysin) and suggest that Pantone Valentine leucocidin (PVL) but not LukAB (LukG/H) or factors regulated by the Agr sensory system influenced this process. Using co-culture models of PMNs supplemented with PBMCs, we demonstrated that TNF- $\alpha$  production was important for bacterial killing, which was exemplified by inhibiting TNF- $\alpha$  in the co-culture setup. These results are consistent with findings of increased susceptibility to *S. aureus*

infections in individuals receiving TNF- $\alpha$  inhibitors to treat complications such as rheumatoid arthritis<sup>8</sup>.

We also used filtered conditioned media assays where neutrophils were exposed to supernatants produced by PBMCs challenged with USA300 or USA300 $\Delta$ saeR/S to determine if secreted factors produced by these cells could prime neutrophils. USA300 $\Delta$ saeR/S conditioned media promoted increased neutrophil ROS production and enhanced killing of *S. aureus* at 30 and 60 minutes post-challenge. Treatment of USA300 $\Delta$ saeR/S CM with a TNF- $\alpha$  inhibitor, prior to neutrophil priming, significantly attenuated the enhanced ROS response. These results correlated to previous findings demonstrating that TNF- $\alpha$  produced from PBMCs challenged with killed *S. aureus* enhanced neutrophil staphylocidal activity, which further supports the significance of SaeR/S-mediated modulation of TNF- $\alpha$  on neutrophil functions<sup>9,10</sup>. These findings were also replicated using another clinically relevant strain of *S. aureus*, MW2, which indicates that early modulation of monocyte-derived TNF- $\alpha$  is a conserved *S. aureus* virulence mechanism to influence neutrophil functions. Taken together, these data identify that disruption of neutrophil priming is a virulence mechanism associated with the SaeR/S-system and is used by *S. aureus* to impede a proper neutrophil ROS response and subsequent staphylocidal activity.

Based on these observations, we propose a model of how *S. aureus* influences neutrophil functions through influencing monocyte-derived TNF- $\alpha$ . During a localized infection, activation of the SaeR/S system by reactive oxygen species or alpha-defensin initiates transcription of specific virulence factors<sup>11</sup>. In turn SaeR/S-regulated factors

reduce Nf-kB activation<sup>12</sup>. Nf-kB is important for activating transcription and production of pro-inflammatory cytokines, such as TNF- $\alpha$ <sup>13</sup>. Reduction of TNF- $\alpha$  by monocytes impedes neutrophil priming. Absence of priming influences neutrophil phagocytosis, ROS, degranulation, cytokine production, and apoptosis<sup>4-7</sup>. We propose that SaeR/S-mediated modulation of monocyte-derived TNF- $\alpha$  is an evasion strategy that allows *S. aureus* to reduce the inflammatory response, promote immune evasion, and subsequently lead to dissemination to other tissues.

In conclusion, the research in this dissertation advances our understanding and provides insight into how *S. aureus* influences cytokine production of TNF- $\alpha$  to reduce neutrophil functions. We identified that early monocyte-derived TNF- $\alpha$  is important for neutrophil-*S. aureus* interactions and that the SaeR/S system suppressed this process. Ongoing studies will assess the influence of *S. aureus* and secreted factors on monocyte maturation and define the mechanisms for TNF- $\alpha$  modulation. Given that *S. aureus* can cause infections in both hospital and community settings and is associated with increasing antibiotic resistance, it is important that we continue to define both *S. aureus* virulence factors and key host immune responses that will lead to alternative therapeutics to treat or prevent *S. aureus* infections.

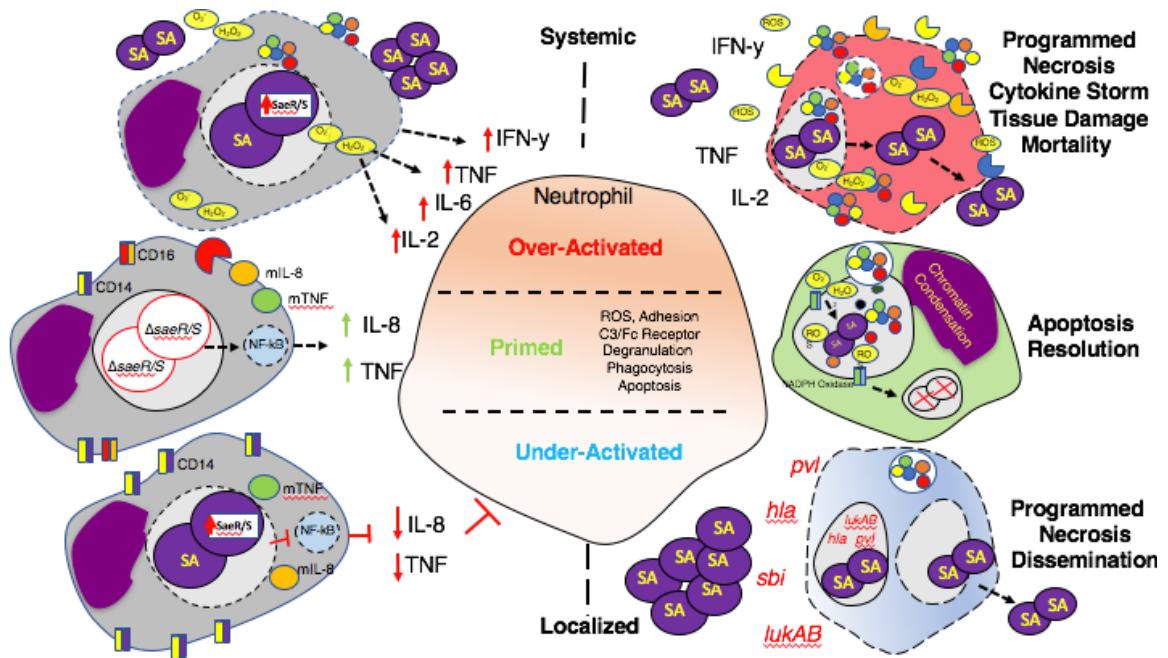


Figure 1. Model of *S. aureus saeR/S*-mediated modulation of monocyte-derived TNF- $\alpha$  and the influence on neutrophil functions. Over-activated: During systemic infection, innate immune cells phagocytose *S. aureus* and activate the *SaeR/S*-TCS<sup>6,35</sup>. The *SaeR/S*-TCS induces a robust inflammatory response leading to over-activation of surrounding cells, including neutrophils. These neutrophils fail to contain inflammatory mediators, ROS, and antimicrobial peptides and, in turn, induce programmed necrosis, cytokine storm, tissue damage and death. Under Activated: In contrast, during a localized infection, residential cells phagocytose *S. aureus* and activate the *SaeR/S* TCS, which decreases phosphorylation and activation of NF- $\kappa$ B, leading to reduced production of inflammatory mediators<sup>6,10</sup>. *SaeR/S*-regulated factors also prolong the intermediate monocyte subset, which further decreases the inflammatory response. Decreased inflammatory mediators suppress neutrophil activation, including reduced ROS<sup>24</sup>, leading

to an under-activated neutrophil. Under-activation of neutrophils leads to *S. aureus* replication and virulence factor production, which results in increased programmed necrosis and dissemination to other tissues<sup>10</sup>. Primed: When the SaeR/S TCS is inhibited, the non-classical monocyte subset is promoted and induces activation of NF- $\kappa$ B, which contributes to production of inflammatory mediators. These inflammatory mediators prime the neutrophil for an enhanced response and results in bacterial clearance, neutrophil apoptosis, and resolution of infection.

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## APPENDICES

APENDIX A

EPIC IMMUNE BATTLES OF HISTORY: NEUTROPHILS VS.  
STAPHYLOCOCCUS AUREUS

APPENDIX A

CO-AUTHORED WORK

EPIC IMMUNE BATTLES OF HISTORY: NEUTROPHILS VS.  
STAPHYLOCOCCUS AUREUS

Contribution of Authors and Co-Authors

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Contributions: Wrote edited and prepared manuscript.

Co-Authors: Timothy R. Borgogna, Delisha M. Patel, Eli W. Sward

Contributions: Wrote sections and contributed equally.

Co-Author: Jovanka M. Voyich

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

Manuscript Information Page

Fermin E. Guerra, Timothy R. Borgogna, Delisha M. Patel, Eli W. Sward and Jovanka M. Voyich

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Publisher: Frontiers Media SA

Issue in which manuscript appears: June 2017, Volume 7, Article 286.

Section Contribution of Co-Author

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



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

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**Received:** 30 March 2017

**Accepted:** 12 June 2017

**Published:** 30 June 2017

### Citation:

Guerra FE, Borgogna TR, Patel DM, Sward EW and Voyich JM (2017) Epic Immune Battles of History: Neutrophils vs. *Staphylococcus aureus*. *Front. Cell. Infect. Microbiol.* 7:286. doi: 10.3389/fcimb.2017.00286

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

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

## INTRODUCTION

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

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

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

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

Neutrophils are initially recruited to a site of infection by following chemokine gradients in a process termed chemotaxis. Taking cues from activated endothelium, neutrophils slow their movement through blood vessels by selectin-mediated tethering to the endothelium followed by complete movement arrest through interaction with integrins on the endothelium. Extravasation from the blood vessels through the endothelial barrier is required for neutrophils to access interstitial fluid and migrate via a chemotactic gradient to the site of infection

where ingestion of bacteria can take place. For detailed reviews of neutrophil chemotaxis, adhesion to the epithelium, and transmigration, please refer to (Kolaczowska and Kubes, 2013; de Oliveira et al., 2016). Herein, we will focus on virulence factors produced by *S. aureus* to inhibit specific neutrophil receptors from binding host and bacterial derived ligands, which results in impaired neutrophil priming, activation, chemotaxis, and adhesion to the endothelium.

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

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

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

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

*S. aureus* produces hemolysins, and bi-component leukocidins that directly impact immune cell function (Seilie and Bubeck-Wardenburg, 2017). Many of these toxins act by forming pores in the membrane of immune cells causing lysis. Among these, the bi-component toxins leukotoxin GH (LukGH, also known as LukAB) and Panton-Valentine leukocidin (PVL) have the ability to cause neutrophil pore-formation when present at high concentrations. However, at sublytic concentrations, these factors can promote enhanced binding, uptake and killing of *S. aureus* through priming of neutrophils (Graves et al., 2012). This is also consistent with reports that have demonstrated the ability of PVL to enhance neutrophil superoxide, granule exocytosis, and release of leukotriene B<sub>4</sub> and IL-8 (König et al., 1995; Colin and Monteil, 2003). In addition, low doses of  $\alpha$ -toxin, a single component toxin encoded by *hla*, has been shown to promote transcriptional activity leading to production of inflammatory mediators that can prime neutrophils including IL-8 (Dragneva et al., 2001).

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

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

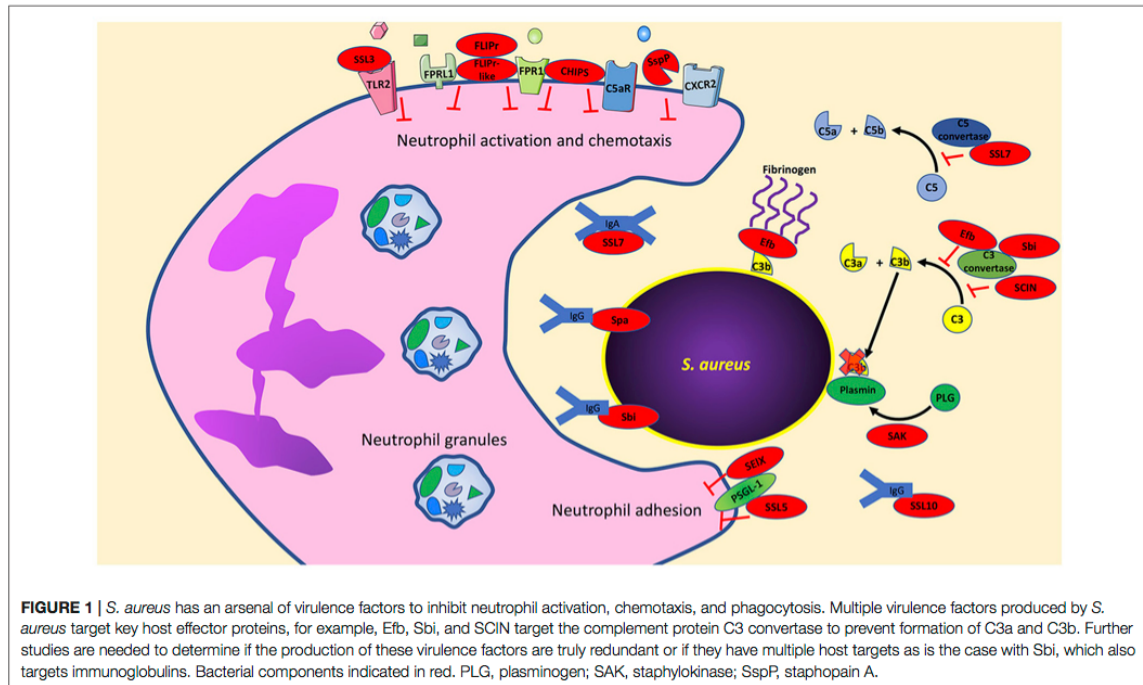
In contrast to our understanding of how *S. aureus* may impact priming, there is an abundance of data that highlight how specific *S. aureus* factors impact neutrophil activation, chemotaxis and adhesion. In this section, we highlight individual virulence factors that have been studied for their ability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

## CHIPS

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

## FLIPR AND FLIPR-LIKE

Neutrophils express formyl peptide receptor-like-1 (FPRL1). This receptor is activated by diverse peptides and proteins including: the synthetic peptides Trp-Lys-Tyr-Met-Val-D-Met-NH<sub>2</sub> (WKYMVM), and L-conformer WKYMVM, lipoxin A<sub>4</sub>, the T21/DP107 leucine zipper-like domain of the HIV-1 envelope protein gp41, serum amyloid A, the mitochondrial peptide fragment MYFINLTL, the antimicrobial peptide LL-37, and prion peptide fragments (Fiore et al., 1994; Su et al., 1999a,b; Chiang et al., 2000; De Yang et al., 2000; Christophe et al., 2001; Le et al., 2001). Activation of FPRL1 on neutrophils leads to intracellular calcium mobilization and chemotaxis, while superoxide production through FPRL1 is ligand dependent (Bae et al., 2003). *S. aureus* produces two proteins that bind FPRL1 and inhibit its activation. FPRL1 inhibitory protein (FLIPr) is a secreted protein that binds and antagonizes both FPRL1 and FPR, but FPRL1 antagonism by FLIPr is much stronger than on FPR (Prat et al., 2006). Similarly, a second FPRL1 antagonist termed FLIPr-like shares 73% overall homology with FLIPr (Prat et al., 2009). FLIPr and FLIPr-like show similar inhibitory activity toward FPRL1 following stimulation with the FPRL1 agonist MMK-1; however, FLIPr-like antagonism toward FPR is 100-fold stronger than that of FLIPr following stimulation with fMLF (Prat et al., 2009). As expected, neutrophil exposure to purified



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

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

## STAPHOPAIN A

The CXCR2 chemokine receptor (also known as IL8RB) is highly expressed on human neutrophils and has high specificity for

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

## SEIX AND SSL5

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

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

### SSL3

Toll-like receptor 2 (TLR2) recognizes staphylococcal peptidoglycan-associated lipoproteins (Fournier and Philpott, 2005; Kurokawa et al., 2009). TLR2 can also discriminate between diacylated and triacylated lipoproteins by associating with TLR6 or TLR1, respectively (Takeuchi et al., 2001, 2002). The recognition of staphylococcal lipoproteins such as the staphylococcal iron transporter C (SitC) are exclusively dependent on TLR2 to induce immune activation (Stoll et al., 2005; Kurokawa et al., 2009; Müller et al., 2010). The importance of TLR2 during *S. aureus* infection is highlighted by TLR2<sup>-/-</sup> mice that show increased susceptibility and nasal colonization to *S. aureus* compared to wild-type mice (Takeuchi et al., 2000; González-Zorn et al., 2005; Hoebe et al., 2005). In addition, TLR2<sup>-/-</sup> mice infected with *S. aureus* show alterations in cytokines that modulate neutrophil function including TNF- $\alpha$  and IL-1 $\beta$  (Knuefermann et al., 2004). Engagement of TLR2 influences many neutrophil functions including: adhesion molecule expression, reactive oxygen species production (following stimulation with fMLF), and modulates CXCL8, and chemokine receptor expression (Sabroe et al., 2003). *S. aureus* produces the staphylococcal superantigen-like 3 protein that binds TLR2 to inhibit activation in neutrophils and other cell types expressing TLR2 (Bardoel et al., 2012; Yokoyama et al., 2012). Crystal structures showed that SSL3 binding to TLR2 reduced the lipopeptide binding pocket by ~50%, which inhibited binding of the TLR2 agonist Pam<sub>2</sub>CSK<sub>4</sub> (Koymans et al., 2015). The same study demonstrated SSL3 can bind a preformed TLR2- Pam<sub>2</sub>CSK<sub>4</sub> complex and this blocked TLR2-TLR1 and TLR2-TLR6 heterodimerization thereby inhibiting downstream signaling.

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

Phagocytosis is a process by which neutrophils, and other phagocytes, ingest particles from their extracellular environment including bacteria and host cells. In neutrophils, this sequestration results in the formation of an intracellular compartment termed the phagosome following invagination of the cellular membrane. Neutrophil granules fuse with the phagosome to release antimicrobial peptides and proteins, as well as produce reactive oxygen species to kill invading

microorganisms. Neutrophils bind to pathogen-associated molecular patterns on the *S. aureus* surface to initiate the phagocytic process. Furthermore, binding of immunoglobulins to *S. aureus* and complement activation enhances phagocytosis, as well as engages different neutrophil receptors resulting in branching downstream signaling. *S. aureus* employs strategies and produces a wide range of virulence factors to disrupt neutrophil phagocytosis. Herein, we will highlight select strategies used by *S. aureus* to disrupt neutrophil phagocytosis. Since a complete review on the strategies used by *S. aureus* to avoid phagocytosis is outside the scope of this publication, we recommend previous detailed reviews focusing on immunoglobulin and complement evasion by *S. aureus* to inhibit phagocytosis (Lambris et al., 2008; Serruto et al., 2010; van Kesse et al., 2014).

### CAPSULE SYNTHESIS

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

### COMPLEMENT INHIBITION

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

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

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

## INHIBITION OF IMMUNOGLOBULIN RECOGNITION

Complement and immunoglobulins function as opsonins to enhance *S. aureus* recognition and ingestion by neutrophils. While *S. aureus* produces virulence factors that inhibit complement activation and deposition on the bacterial surface, the efficacy of these virulence factors *in vivo* is controversial since immunoglobulin opsonization plays a redundant role in opsonophagocytosis. In fact, opsonophagocytosis assays investigating the role of *S. aureus* virulence factors that disrupt complement must be done in the absence of immunoglobulins to observe an inhibitory effect on phagocytosis because *S.*

*aureus* produces virulence factors that inhibit opsonization by immunoglobulins. Protein A, encoded by the *spa* gene, is one of the better characterized virulence factors produced by *S. aureus* that inhibits neutrophil phagocytosis. Protein A is a 42-kDa secreted and membrane bound protein that binds the constant Fc $\gamma$  region of IgG thus preventing proper engagement of Fc $\gamma$  receptors on neutrophils and antigen recognition (Forsgren and Sjöquist, 1966; Kronvall et al., 1970; Sjödahl, 1977). Indeed, early studies clearly showed that purified protein A inhibited neutrophil phagocytosis of *S. aureus* independent of capsule polysaccharide on the bacterial surface (Dossset et al., 1969; King and Wilkinson, 1981). More recent studies have demonstrated protein A induces B cell proliferation and production of V<sub>H</sub>3 serum IgG and IgM (Pauli et al., 2014; Kim et al., 2016). Although abundant, these antibodies do not provide protection against *S. aureus*. Generation of protein A variants that were unable to bind immunoglobulin induce a protective antibody response presumably via allowing Fc mediated uptake of opsonized *S. aureus* (Falugi et al., 2013). These data suggest that mechanisms of neutrophil recognition and uptake of *S. aureus* are likely critical to resolution of infection.

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

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

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

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

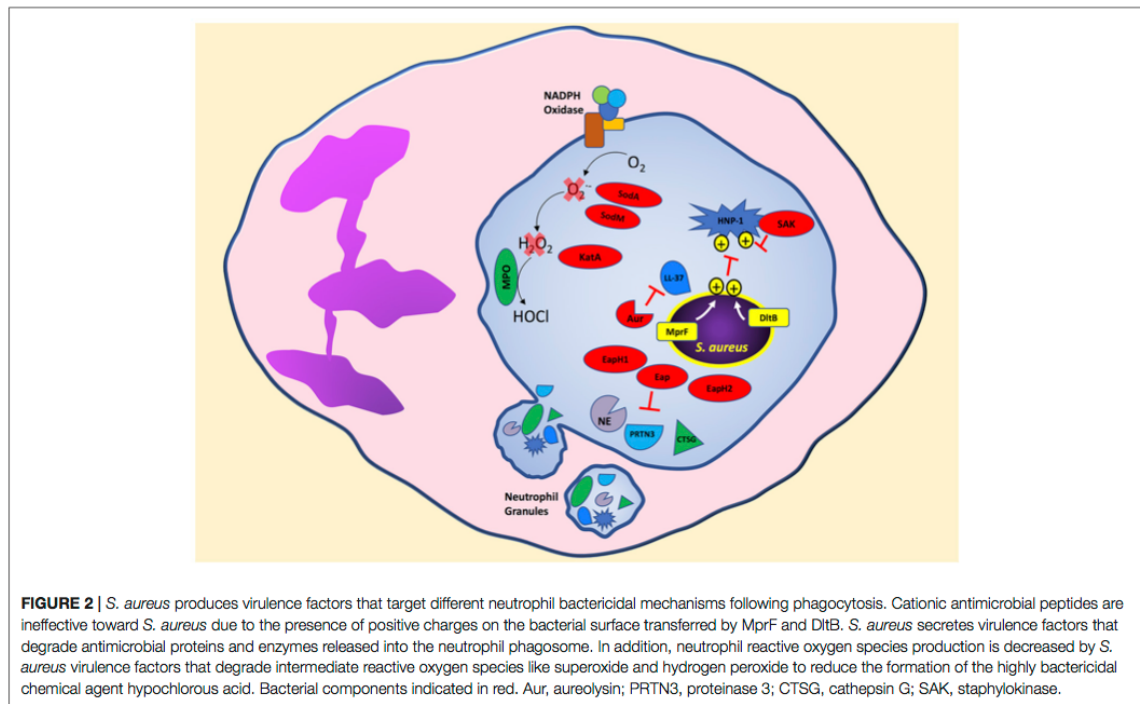
### EVASION STRATEGIES USED BY *S. AUREUS* TO SURVIVE NEUTROPHIL KILLING

*S. aureus* is clearly decorated to avoid ingestion by neutrophils. Additionally, *S. aureus* is loaded with mechanisms to disarm potent neutrophil bactericidal mechanisms. Soon after initiation of the phagocytic process, and possibly before complete sealing

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

### *S. AUREUS* EVASION OF NON-OXIDATIVE KILLING

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



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

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

## S. AUREUS EVASION OF OXIDATIVE KILLING

Neutrophil activation leads to assembly of the NADPH oxidase resulting in production of reactive oxygen species (ROS; DeLeo and Quinn, 1996; DeLeo et al., 1999; Bréchart and Tschirhart, 2008; Nunes et al., 2013). Assembly of the membrane and cytoplasmic NADPH oxidase components results in electrons shuttled from NADPH to oxygen producing superoxide. Spontaneous and myeloperoxidase (MPO) catalyzed dismutation of superoxide produces hydrogen peroxide, which in the presence of MPO and chloride results in the highly bactericidal agent hypochlorous acid/hypochlorite anion (Kettle et al., 2007; Klebanoff et al., 2013). Neutrophil ingestion exposes *S. aureus* to concentrated ROS in the phagosome resulting in oxidation and chlorination of bacterial and host proteins (Green et al., 2014). Inhibition of neutrophil ROS production by

diphenyleneiodonium significantly increases *S. aureus* survival following neutrophil phagocytosis even with functioning non-oxidative killing mechanisms (Hampton and Winterbourn, 1995; Hampton et al., 1996). Individuals with chronic granulomatous disease carry mutations in components of NADPH oxidase preventing formation of superoxide and hydrogen peroxide and suffer from recurring bacterial infections including *S. aureus* (Lekstrom-Himes and Gallin, 2000; Assari, 2006). Thus, uninhibited neutrophil ROS production is essential for *S. aureus* clearance.

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

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

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

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

## S. AUREUS MODULATION OF NEUTROPHIL FATE

Neutrophils undergo an apoptotic differentiation program in response to bacterial pathogens to limit host damage caused by a prolonged inflammatory response (Kobayashi et al., 2003). For excellent reviews on the mechanisms of neutrophil apoptosis and how bacterial pathogens modulate neutrophil fate, we recommend (Kennedy and DeLeo, 2009; Rigby and DeLeo, 2012; Kobayashi et al., 2017). *S. aureus* produces pore-forming

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

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

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



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

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

Investigating the neutrophil-*S. aureus* interactions *in vivo* has been challenging. The relevance of using the murine immune system to mimic interactions between *S. aureus* and the human immune system is heavily debated (Buer and Balling, 2003; Kim et al., 2014; Montgomery et al., 2014; Reizner et al., 2014; Tseng et al., 2015). Interestingly, though HNP-1 is recognized as the most prominent trigger of *saeR/S*, murine neutrophils are deficient in its production (Inaba et al., 2010). Not only do they differ in the production of HNP-1 but notable differences are observed in chemokine production, cell trafficking, and susceptibility to secreted toxins (Johnston et al., 1990; Singer and Sansonetti, 2004; Parker and Prince, 2012; Tseng et al., 2015). Often the importance of single *S. aureus* virulence genes is

highlighted in models of infection; however, canonical *S. aureus* toxins such as HlgA, HlgC, and LukF-PVL have extremely limited lytic activity on murine cells (Liu, 2009; Baba-Moussa et al., 2011; Parker and Prince, 2012; Vandenesch et al., 2012). While deficiencies in the murine model are apparent it remains the most prominent and cost effective method to explore relationships *in vivo* between *S. aureus* and the host immune system. To that end, advances are being made toward creating more effective murine models that retain benefits such as availability of working with immune-deficient lines while displaying similar disease characteristics as observed in human hosts (Montgomery et al., 2014; Tseng et al., 2015). Tseng et al. developed a “humanized” murine model wherein NOD/SCID/IL2r<sup>null</sup> mice are engrafted with human CD34<sup>+</sup> umbilical cord blood cells leading to the production of human immune cells within mice (Tseng et al., 2015). These mice demonstrated susceptibility to *S. aureus* infections at concentrations similar to what is observed in humans as well as increased susceptibility to LukF-PVL. In future efforts, humanized murine models such as this may be key to understanding how *S. aureus* regulates its pathogenesis in the context of a more relevant host environment and may be critical to advance research into effective therapeutics.

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

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

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

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

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

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

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

## AUTHOR CONTRIBUTIONS

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

## FUNDING

This work was supported by the U.S. National Institutes of Health (Grants NIH-R01A1090046, NIH-PAR98-072, and NIH-RR020185 for a fellowship award to FG and DP), as well as funds from the Montana State University Agriculture Experiment Station.

## ACKNOWLEDGMENTS

We would like to acknowledge the Servier Medical Art Powerpoint image bank by Servier (<http://www.servier.com/Powerpoint-image-bank>), which is licensed under a Creative Commons Attribution 3.0 Unported Licence, that was modified to create figures.

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

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APENDIX B

FUTURE STUDIES

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## FUTURE STUDIES

Identifying Cell Subsets in Lungs of Mice Challenged  
with Influenza A Virus and Secondary Infection of *S. aureus*

Current studies are exploring the role of *S. aureus* pathogenesis following antecedent influenza A virus (IAV) infection. It is hypothesized that IAV modulates host immune responses in the lung that lead to activation of SaeR/S-regulated virulence factors, which subsequently promotes *S. aureus* induced murine fatalities. This is supported by studies indicating that IAV dysregulates lung physiology and modulates host immune responses leading to a predisposition to secondary *S. aureus* infection<sup>1</sup>. As the surrounding lung environment during IAV infection includes a variety of different innate and adaptive immune cells, focusing on these recruited subsets will provide insight into the environmental changes that increase *S. aureus* virulence. Preliminary studies indicate that IAV promotes increased recruitment of CD11b monocytes and macrophages compared to murine models challenge with USA300 or USA300 $\Delta$ saeR/S alone. Recruited monocytes due to IAV infection may produce factors recognized by *S. aureus* to survive and evade the immune response and subsequently increase production of SaeR/S causing upregulation of virulence genes. Together, these studies suggest that timing and induction of SaeR/S-regulated virulence factors are critical to the outcome of infection and future studies exploring the immune responses will be important for understanding *S. aureus* induced mortality following antecedent IAV infection.

SaeR/S- System Activation is Dependent on  
Single Amino Acids in SaeR/S Response Regulator

Recent reports have elucidated that the SaeR/S system of *S. aureus* is activated by specific host factors that influence single amino acids in the external loop of SaeS<sup>2,3</sup>. SaeS is the sensory kinase system that phosphorylates the SaeR response regulator upon activation, which in turn initiates transcription and production of specific virulence factors<sup>2,4</sup>. Although the role of specific amino acids in the SaeS extracellular loop in response to host derived stimuli has been well characterized, less is known about the critical amino acids in the SaeR response regulator. Phosphorylation of SaeR is known to occur on conserved aspartic acid residues and preliminary data introducing single point mutations in these residues of SaeR show significantly reduced activity when challenged with neutrophils. Specifically, SaeR residues D51 and D41 when mutated to alanine (D51A and D41A) show significantly reduced levels of neutrophil lysis in both live infection and supernatant cytotoxicity assays. USA300D51A showed significantly reduced neutrophil lysis similar to USA300 $\Delta$ saeR/S indicating that this aspartic acid residue is critical for regulating transcription and production of *S. aureus* virulence factors. Together, these data suggest regulation of SaeR is at the single amino acid level.

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