INVESTIGATING NEUTROPHIL CELL FATE FOLLOWING INTERACTIONS WITH *STAPHYLOCOCCUS AUREUS*

by

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DEDICATION

First and foremost, I dedicate this thesis to my Montana family. You have all stood by me, through thick, thin, and all the craziness that accompanied to make this journey the best part of my life yet. Thank you, MC Rollins, who showed me the meaning of True Grit. Thank you, Will Thompson, who has never given up on my ability to learn a real skier's stance and taught me more about life then I thought possible. Thank you, Kayla Lyons, the most graceful skier I have ever known, for being the most true and steadfast friend. Thank you, Chester Fox, for helping us build a home and always being up for a quiet conversation. Thank you, Nate Berry, for our brilliant dynamic and friendship. Thank you, Dr. James Cwick, for helping me maintain my sanity during the COVID-19 lockdown. We will always have game night. Thank you, Maria Predtechenskaya, for your warmth and humor. Thank you, Kelly and Skylar Sullivan, Claire Wright, and Alasdair Boyler. This is also dedicated to the Lyons Family who have taken us in, and through their guidance and love, have helped us make a home here in the valley. We appreciate you all so much.

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ABSTRACT

Staphylococcus aureus is a ubiquitous pathogen with a growing list of antibiotic resistant capabilities. This gram-positive bacterium is able to cause a range of diseases, from a benign state of nasal colonization to fatal endocarditis. The ability to exist along this spectrum is largely dependent on the molecular dialog that takes place between the pathogen and the host, specifically white blood cells known as neutrophils. Neutrophils are the front line of defense against *S. aureus* infections. By modulating neutrophil behavior and inducing premature cell death, *S. aureus* has an advantage during an infectious state. In this thesis, I provide a method for studying this host and pathogen dynamic, and moreover, I investigate the mechanism by which *S. aureus* inhibits the neutrophil inflammatory response by repressing NF-κB. Here I show that *S. aureus* secretes a protein 30-50kDa in size, which both decreases total amount of NF-κB and activated NF-κB in neutrophils. This potent mystery protein is able to repress IL-8 production and does this all in a lysis independent manner. Additionally, the mystery protein is able to inhibit NF-κB activity in another cell type, the monocyte. It was previously believed that the *S. aureus* protein SSL3 was responsible for deactivating NF-κB, but herein, I show this is not the case. These findings reopen the need to examine the mechanism by which *S. aureus* modulates neutrophil inflammatory responses. Inhibition of the inflammatory response is likely linked the premature cell death seen during *S. aureus* infections. By utilizing these clues, the field is closer to understanding the intricacies of this host and pathogen dynamic, opening avenues to developing novel infection treatment methods.

CHAPTER ONE

NF-KB, FRIEND OR FOE DURING *STAPHYLOCOCCUS AUREUS* INFECTION

Staphylococcus aureus and Neutrophils, the Ambivalent Neighbors

Staphylococcus aureus (*S. aureus*) is a ubiquitous Gram-positive bacterium. Causing over 120,000 cases of blood stream infections per year in the US alone, *S. aureus* is known for being a leading cause of hospital and community acquired infections (Kourtis, 2019). This pathogen can cause disease states ranging from mild soft-tissue infections, to deadly septicemia (Lowy, 1998), and necrotizing pneumonia (Uhlemann, Otto, Lowy, & DeLeo, 2014). In the US a single clone, USA300, has become widespread due to an increased ability to colonize human tissue and increased virulence (Otto, 2010). In addition to dealing with increased virulence of modern *S. aureus* strains, treating these infections is becoming increasingly more complicated due to the rise in antibiotic resistance. *S. aureus* has an aptitude for developing or incorporating antibiotic resistance genes, and has devised a mechanism for skirting antibiotic-induced death from most of the mainstream antibiotics available today (Foster, 2017). For example, methicillin is a β-lactam antibiotic related to penicillin; resistance works by interfering with a protein called PBP2 which is involved in the synthesis of peptidoglycan, an integral part of the cell wall. Through the creation of PBP2', which has a different structure than PBP2, peptidoglycan synthesis cannot be targeted by methicillin or other β-lactams (Foster, 2017). These bacteria are now known as methicillin resistant *Staphylococcus aureus*, or MRSA. Vancomycin, colloquially called the last line of defense antibiotic, also works by interfering with peptidoglycan synthesis by binding to partially constructed peptidoglycan strands. In brief, through the introduction of the *vanA* operon by

horizontal gene transfer, *S. aureus* creates an altered peptidoglycan structure to which vancomycin cannot bind (McGuinness, Malachowa, & DeLeo, 2017). The result is vancomycin resistant *Staphylococcus aureus*, or VRSA. That being said, vancomycin resistance comes at a fitness cost to *S. aureus* and is a possible reason why VRSA is still uncommon today.

Despite its infamy as a clinically relevant pathogen, *S. aureus* is a highly adaptable commensal organism. This bacterium is known to establish persistent nasal colonization in 20% of individuals, intermittent carriage in 30% of individuals, and no life time colonization for the other 50% (Wertheim et al., 2005). *S. aureus* colonization is seen at higher rates in individuals that are older, smoke, and have a co-morbid condition such as obesity and diabetes. However, after adjusting for sociodemographic factors and co-morbidities, one study did not find a significant correlation between MRSA colonization and death (Mendy, Vieira, Albatineh, & Gasana, 2016). The widespread population colonization of such a virulent pathogen is remarkable and speaks to the ability of *S. aureus* to interact with the host immune response.

From the host point of view, the innate immune cell that is most critical for controlling *S. aureus* infections is the neutrophil, otherwise known as a polymorphonuclear leukocyte or PMN. One study demonstrated that in a cutaneous infection model, neutrophil-depleted mice developed severe skin lesions which lead to bacteremia and necrotizing ulcers not seen in control mice (Mölne, Verdrengh, & Tarkowski, 2000). Even when neutrophils are at normal levels, they still must contend with a virulence arsenal. By looking at differences in gene expression of community-associated (CA) MRSA strains and healthcare-associated (HA) strains during neutrophil challenge, it is seen that CA-MRSA strains are more virulent (J. M. Voyich et al., 2005). In

addition, CA-MRSA are not only better at avoiding neutrophil-mediated killing compared to HA-MRSA, but cause more widespread tissue destruction in a mouse model (J. M. Voyich et al., 2005). There are many examples of strategies used by *S. aureus* and neutrophils to kill each other in their quest for dominance. In brief, these are some of the mechanisms used by neutrophils. The first interactions of neutrophils and *S. aureus* are those of recognition. Neutrophils will recognize pathogen associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs). An example of a PRR is Toll Like Receptor (TLR) 1/2, which recognizes the PAMPs of lipopeptides (Pandey, Kawai, & Akira, 2015). This process starts an inflammatory cascade resulting in the production cytokines that attract more neutrophils to a site of infection. This process will be explained in greater detail further in this chapter. Another way neutrophils recognize *S. aureus* is through Fc-mediated phagocytosis. The bacteria of interest, in this case *S. aureus*, becomes coated with opsonins from human serum. These opsonins include immunoglobulins (Igs) and complement, which are recognized respectively by Fc receptors (FcRs) and complement receptors (CRs) (Y. Wang & Jönsson, 2019). *S. aureus*, when coated in IgG, is recognized by an FcγR, which triggers receptor mediated phagocytosis (Guerra, Borgogna, Patel, Sward, & Voyich, 2017).

During the process of phagocytosis, many events happen in short order. First, there is the assembly of the NADPH oxidase complex, which leads to the production of superoxide and subsequent reactive oxygen species (ROS), such as hydrogen peroxide (DeLeo, Lee-Ann, Michael, & Nauseef, 1999). ROS reacts with an enzyme called myeloperoxidase (MPO), which converts it to HOCl, the active component in bleach (Nauseef, 2014). If bombardment with bleach is not enough to kill a pathogen in a phagosome, the neutrophil plies the phagosome with an assortment

of antimicrobial peptides and proteins. Examples of these include serine proteases like elastase, and peptides like α-defensins (Othman, Sekheri, & Filep, 2021). Combined, this creates a hostile environment meant to kill invading pathogens, all while protecting the host tissue.

S. aureus does not go down without a fight and has its own arsenal to combat neutrophil killing. Like how neutrophils use TLR1/2 to sense the presence of *S. aureus*, *S. aureus* uses the SaeR/S two component system to sense outside stimuli and respond with a wide range of produced exoproteins (J. M. M. Voyich et al., 2009). SaeR/S controls many well-known virulence factors. For example, while neutrophils phagocytose microbes opsonized in IgG, *S. aureus* created Protein A will bind to Fcγ of IgG, inhibiting the FcγR from binding and inhibiting Fc-mediated phagocytosis (Guerra et al., 2017). In another example, to prevent the production of hypochlorous acid, a SaeR/S controlled protein called SPIN will bind to MPO, preventing the hydrogen peroxide from reaching the MPO active site (de Jong et al., 2017). To further inhibit the use of ROS, *S. aureus* produces a catalase (not controlled by SaeR/S) that converts hydrogen peroxide back to oxygen and water (Mandell, 1975), which increases *S. aureus* survival in the phagosome. The SaeR/S system does more than just inhibit neutrophil microbicidal activity, as it encodes a number of leukotoxins that attack neutrophils in return. One example is LukGH, a bi-component toxin that binds to the neutrophil cell surface marker CD11b, and thus specialized to kill neutrophils (Janesch et al., 2017).

S. aureus is infamous for its ability to secrete proteins that modulate the innate and adaptive immune responses. Staphylococcal superantigens (SAgs) are a group of proteins that dysregulate T-cell proliferation which result in some of *S. aureus*' most infamous disease manifestations, such as necrotizing pneumonia and toxic shock syndrome (Fraser & Proft, 2008; Tuffs et al., 2017). The

staphylococcal superantigen-like proteins (SSLs), like SAgs, are secreted, but unlike SAgs do not cause Vβ-specific T-cell proliferation, but do have unique and overlapping immunomodulatory abilities (Benson, Lilo, Nygaard, Voyich, & Torres, 2012; Fraser & Proft, 2008; Tuffs et al., 2017). The majority of the SSL genes are synergistically controlled by the SaeR/S TCS, and the transcription factor regulator of toxins (Rot), which is indirectly controlled by the Agr system (Benson et al., 2012). The dual nature of this regulation may be important so the SSL genes are expressed during specific circumstances, such as early infection, and the nature of specific SSLs will be explored in greater depth later.

There are also examples of mechanisms *S. aureus* uses to modulate inflammation to promote infection. The SaeR/S two component system of USA400 has been shown to promote IFN-γ, TNF-α, IL-6, and IL-2 production in mouse models (Watkins, Pallister, & Voyich, 2011). In human monocytes, SaeR/S mediated factors are able to decrease production of TNF-α, and in a co-culture model with neutrophils, this causes a decrease in bactericidal activity (Sward et al., 2018). SaeR/S mediated factors can also decrease the ability of neutrophils to recruit other neutrophils by blocking IL-8 production. The is done through the inhibition of NF-κB signaling, which is also linked to enhanced neutrophil cell death (Zurek et al., 2015). By both inhibiting neutrophils' ability to recruit more immune cells, and by accelerating the death of neutrophils present, the SaeR/S two component system allows *S. aureus* to shape its microenvironment during early stages of infection.

Although neutrophil cell death is necessary to maintain inflammatory homeostasis, neutrophils have many different modes of cell death, not all of which are beneficial to the host. The antimicrobial proteins, peptides, and ROS contained in neutrophils and mentioned above, must

be dealt with in a controlled manner, or else leak into host tissues causing inflammation and tissue damage. Therefore, neutrophils have several modes of cell death, all situationally linked. In brief, as this topic will be explored in depth later, neutrophils apoptose and are subsequently removed by macrophages in a process called efferocytosis. This process is beneficial for maintaining tissue homeostasis (summarized in (Kobayashi, Malachowa, & DeLeo, 2017)). Other modes of neutrophil cell death are much more pro-inflammatory. For instance, while combating a pathogen, neutrophils may create a neutrophil extracellular trap (NET), which coats the pathogen in a web of DNA, ROS, and antimicrobial enzymes like histones. This mode of death is coined NETosis (Brinkmann et al., 2004; Fuchs et al., 2007; Nauseef & Kubes, 2016). Another mode of cell death is necroptosis, which as the name implies, is a messy cell death where neutrophil components spill into the extracellular space (reviewed in (Grootjans, Vanden Berghe, & Vandenabeele, 2017)). Interestingly, virulent strains of *S. aureus* have been shown to induce both of these modes of neutrophil cell death (Bhattacharya et al., 2018; Greenlee-Wacker et al., 2014), modulating immune cells to its own advantage. As two of these death modes, apoptosis and necroptosis, intersect at the transcription factor NF-κB, it is prudent to take a closer look at this complicated protein in more depth.

The Role of NF-κB, the Double-Edged Sword, an Overview

At the cornerstone of inflammation, innate and adaptive immune responses, cellular development, cancer, cell survival and death, lies the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factors. These five transcription factors are p50, p52, p65 (RelA), RelB, and c-Rel. By forming homo or heterodimers (Hayden & Ghosh, 2011; Pahl, 1999), they form a number of viable combinations which result in specific gene expression patterns (A.

Hoffmann, Natoli, & Ghosh, 2006; Mitchell, Vargas, & Hoffmann, 2016; Verstrepen et al., 2008). These homo- and heterodimers bind to κB sites on DNA to promote the transcription of over 150 genes as a mediator of stress responses (Pahl, 1999). The signaling pathway that results in NF-κB transcription is complex, starting at a variety of pattern recognition receptors (PRRs) that initiate signaling when bound to PAMPs (Pandey et al., 2015).

This chapter will focus on the instigation of the NF-κB signaling pathway, and the resulting cell death in the context of a *Staphylococcus aureus* infection. Two important PRRs here are TLR1/2 and IL-1R. There are many ways PMNs recognize *S. aureus* (for example, receptor mediated, complement, etc.), but these are more relevant to phagocytosis. It has been shown through the work of several groups, including Chekabab et al. and Zurek et al., that NF-κB signaling can be mediated by *S. aureus*. Therefore, we will focus on the pattern recognition receptors (PRRs) toll-like receptor 1/2 (TLR1/2) and interleukin-1 receptor (IL-1R), as these activate immediate antimicrobial activities on the cellular level, in both neutrophils and other cell types. TLR signaling can lead to MyD88-dependent pathways that result in NF-κB activation, and TRIF-dependent pathways that result in IRF3 activation with some NF-κB signaling (Hayden & Ghosh, 2011). For the purposes of the research in this dissertation, we will tour canonical MyD88-dependent NF-κB activation.

Upon TLR1/2 activation, signaling will go through the MyD88-like adaptor protein (MAL or TIRAP) and MyD88 complex, to the IRAK1/4/ TRAF6 complex, to the TAK1/TAB complex, and finally to the IκB Kinase (IKK) complex (Verstrepen et al., 2008). Stimulation by IL-1R is similar except IL-1R directly interacts with the MyD88/IRAK1/4/ TRAF6 complex, which directly interacts with the TAK1/TAB complex (Verstrepen et al., 2008). The details of these signaling

complexes are beyond the scope of this review and are summarized in Verstrepen et al., 2008. The inhibitor of κB Kinase (IKK) subsists of two catalytic units, the IKKα and IKKβ, along with the regulatory protein NF-κB essential modulator (NEMO) (Verstrepen et al., 2008). In homeostasis, NF-κB sits in an inactivated state in the cytosol, bound to IκB. Upon signaling pathway activation, IKK phosphorylates IκB, marking it for degradation, and allowing NF-κB to translocate to the nucleus for transcription (Hayden & Ghosh, 2011; Verstrepen et al., 2008). Different varieties of IκB, such as IκBα provide more of a NF-κB override off function, while IκBβ and IκBε prevent NF-κB positive oscillation during drawn out times of stimulation (Alexander Hoffmann, Levchenko, Scott, & Baltimore, 2002).

Upon release of NF-κB from its inhibitors, it will translocate to the nucleus and bind to various κB sites, instigating translation of numerous context dependent genes (A. Hoffmann et al., 2006). In the case of stimulation by *S. aureus*, these will include IL-8 (also known as CXCL1 in humans and KC in mice) and IL-1β, to attract neutrophils to the site of infection, and spur inflammatory activity in surrounding tissues (Coeshott et al., 1999; E. Hoffmann, Dittrich-Breiholz, Holtmann, & Kracht, 2002). The resulting proinflammatory microenvironment predisposes cells for accelerated cell death. Although there are some cases where specific stimuli will cause NF-κB to turn into a repressor of anti-apoptotic genes, leading to cell death (Dutta, Fan, Gupta, Fan, & Gélinas, 2006), overwhelmingly NF-κB is the gateway factor inhibiting cell death, specifically by preventing apoptosis (Kettritz et al., 1998). In the event of NF-κB pathway inhibition some other main players (caspase-8, caspase-1) can direct the mode of cell death (X. Wang, Yousefi, & Simon, 2018).

Resolution of inflammation is critical for maintaining homeostasis, and apoptosis is one way to contain inflammation. In the context of aged neutrophils heading towards apoptosis, gene expression shifts and amongst other things, genes reducing oxidative stress are expressed (Kobayashi et al., 2003). This is hypothesized to reduce local tissue damage. In support of this anti-inflammatory hypothesis, macrophages that undergo efferocytosis of apoptotic neutrophils not only do not produce pro-inflammatory cytokines themselves, but can secrete anti-inflammatory cytokines to keep the surrounding area of neutrophil cell death in homeostasis (Fadok et al., 1998). Acute inflammation is necessary part of wound healing and begins either from sensing PAMPs or damage associated molecular patterns (DAMPs). This inflammation is associated with NF-κB being 'on,' resulting in production of cytokines like IL-8, and the induction of an antiapoptotic state (Kettritz et al., 1998). Initially, neutrophils are needed in the wound site to summon other immune cells and to phagocytose invading pathogens. However, chronic inflammation, or inflammation well beyond the acute infiltration of immune cells, leads to protracted wound healing and an increase in inflammation (discussed in Selders, Fetz, Radic, & Bowlin, 2017)). This can be due to delayed clean up by macrophages, followed by necrotic cell lysis, and leakage of neutrophil components that can directly damage tissues. In order to expedite healing, and aid in the return of homeostasis, there are different therapies to target and inhibit NF-κB activity, which could induce the apoptotic state and allow for macrophage efferocytosis (Kebir & Filep, 2013). Targeting neutrophils to undergo apoptosis could relieve uncontrolled inflammation in more settings than just wound healing. For example, rheumatoid arthritis is marked by chronic NF-κB activity and irresolvable inflammation (Makarov, 2001). Classic anti-inflammatory drugs like aspirin and

glucocorticoids are used to treat rheumatoid arthritis, and they work in-part by inhibiting NF-κB activation (Makarov, 2001).

NF-κB transcription is more than just a 'central switch' of inflammation (Pahl, 1999). Its activity is a double-edged sword as pathogen control is dependent on its ability to function, and excessive or under promotion can be detrimental to the host and clearance. It is also important to keep in mind that the role of NF-κB in neutrophils is cell type specific. NF-κB signaling can play a more inflammatory role in immune cells, while this pathway is more focused on maintaining tissue homeostasis in non-immune cells, such as the epithelium (Pasparakis, 2009). In addition, human neutrophils naturally lack p52 and RelB which target genes in lymphocytes associated with longevity (Hayden & Ghosh, 2011; McDonald, Bald, & Cassatella, 1997). Therefore, there are less NF-κB homo- and heterodimer combinations available to human neutrophils. Neutrophils contain abundant amounts of c-Rel, p50/NF-κB1, and p65/RelA (McDonald et al., 1997), which reflects the genes they express during infection-specific inflammation.

S. aureus Modulation of NF-κB Signaling

S. aureus has a complicated but understudied relationship with NF-κB. When comparing *S. aureus* and its relationships to inflammation, it is relatively understudied compared to other pathogens including *Yersinia pestis* and multidrug-resistant *Mycobacterium tuberculosis*, both of which have various redundant mechanisms to suppress inflammation during early colonization (Deng et al., 2014; Faridgohar & Nikoueinejad, 2017; Huang, Wang, Lin, & Kuo, 2014; Pathak et al., 2007; Sweet, Conlon, Golenbock, Goguen, & Silverman, 2007; Vagima et al., 2015; Zhou et al., 2005). That being said, *S. aureus* does have a number of methods to navigate its relationship with inflammation. It is likely that *S. aureus* has a different relationship with NF-κB depending on

the context of the infection model and the strain of *S. aureus*. For example, the strain USA300, using live pathogen, and in a neutrophil infection model saw NF-kB inhibition and decreased IL-8 expression through a SaeR/S mediated factor (Zurek et al., 2015). A study using supernatants from ATCC29213 also saw that when incubated with airway epithelial cells, there was a decrease in NF-κB activity and IL-8 production (Chekabab et al., 2015). In a model with 16HBE cells, supernatant from the strains MSSA NCTC 6571, MSSA Newman, MSSA209, MRSA41, and MRSA USA400 all repressed IL-8 expression, while MSSA SH1000 and MSSA RN4220 activated IL-8 expression (Ji, Bolhuis, & Watson, 2019). The suppressive factor in question was seen to be heat stable and less than 3kDa in size. Another study using *S. aureus* strain ATCC25923 and RAW264.7 macrophages demonstrated activation of NF-κB and expression of various cytokines (Ma et al., 2019). Suppression of inflammation in the form of IL-8 has shown to be conserved between virulent *S. aureus* strains, while missing from avirulent strains (Tajima, Seki, Shinji, & Masuda, 2007). Another unique suppressive factor has been defined in the strain *S. aureus* MSSA476. This protein is a homologue of human TIR, and therefore called staphylococcal TIR domain protein (TirS), which inhibits signaling through both TLR2 activated NF-κB signaling and MAPK pathways (Askarian et al., 2014). All of these studies showed different outcomes, likely due to the strains used and the specific contexts of the infection models, making it hard to draw cross-study conclusions due to the inconsistencies, and this will be explored later. These differences have also made it difficult to identify the *S. aureus* factors influencing NF-κB.

The protein factors that are most published on in the context of *S. aureus* repression of NF-κB would be SSL3 and SSL4.The SaeR/S two-component system, discussed earlier, is known to regulate the SSLs, including 3 and 4 (Tuffs et al., 2017). SSL3 is able to specifically bind and

inhibit TLR2 activity in both human and murine PMNs and macrophages, which has been shown to inhibit IL-8 secretion in HEK cells (Bardoel et al., 2012). SSL3-mediated IL-8 inhibition is specific to the TLR2 pathway, and not seen when cells are stimulated with IL-1β (Bardoel et al., 2012). This does not mean that inhibiting TLR2 will not later inhibit the IL-1R mediated IL-1β response, just that SSL3 is specific for TLR2, as both TLR1/2 and TLR2/6 heterodimers. Mice deficient in TLR2 have an 80% reduction in IL-1β protein compared to WT, and a 53% decrease in MPO activity compared to WT mice (Cho et al., 2012). We also posit that there may be yet undefined inhibitors specific to the IL-1R signaling pathway. SSL3 mediated inhibition of TLR2 activity works in two stages, where first the protein binds to the staphylococcal lipopeptides, and second, when a lipopeptide has been bound by TLR2, SSL3 will prevent heterodimer formation with either TLR1 or TLR6 (Koymans, Feitsma, Brondijk, et al., 2015). SSL4 shares sequence homology with SSL3, and has been shown to also bind to TLR2, although with less potency as an inhibitor (Koymans, Feitsma, Brondijk, et al., 2015).

Now that how *S. aureus* may be modulating NF-κB has been explored, it is important to review how the context of neutrophil and *S. aureus* recognition can alter neutrophil responses. Engagement of different receptors impacts the activation of NF-κB. For example, *S. aureus* supernatant is recognized by through TLR1/2 (Chekabab et al., 2015). While live pathogen is recognized by IL-1R (Miller et al., 2006, 2007) and TLR1/2. This is relevant as initially, neutrophils may come into contact with the debris caused by previous neutrophil and *S. aureus* interactions, along with *S. aureus* supernatant, before they interact with a whole pathogen. These interactions may prime the neutrophils to act differently upon initial contact with the live bacterium, as the TLR1/2 response differs from the IL-1R response at the gene level (Cho et al.,

2012; Miller et al., 2006). *S. aureus* can also be recognized by TLR2 ultimately resulting in the production of pro-IL1β. Caspase-1 causes the formation of the mature cytokine as IL-1β, which is recognized by IL-1R, starting a new signaling pathway. This effect was observed in a mouse subcutaneous inoculation model with *S. aureus* strain SH1000 (Cho et al., 2012). At four hours post infection RNA was extracted from skin biopsies and upregulated genes included CXCL1 (KC), IL-1β, and inflammasome related NLRP3 (Cho et al., 2012). Genes involved in the inflammatory response, cell growth and proliferation, cell death, and cell movement, expressed by neutrophils were also shown to be significantly upregulated (Cho et al., 2012). In a different experiment described in the same paper, after 6 hours IL-1β production was increased in neutrophils corresponding with an increase in MOI of MRSA strain USA300 LAC, and was likely dependent on TLR2, NOD2, and FPR1 signaling in an overlapping and redundant manner (Cho et al., 2012). Another study saw that macrophages exposed to USA300 produced significant levels of IL-1β compared to a control, although levels were reduced when the macrophages were exposed to PMNs+USA300 (Greenlee-Wacker et al., 2014) suggesting a predominant role for PMNs in NF-κB signaling following interaction with *S. aureus*. As the neutrophil is the foremost cell for both controlling *S. aureus* and secreting IL-1β, this relationship merits further study.

It is important to note that although NF-κB targets so many genes, the actual gene expression is specific to the stimulatory context (Pahl, 1999).The dependence of TLR2, NOD2, and FPR1 on neutrophil recruitment and IL-1β show that there are multiple stages of neutrophil and *S. aureus* interaction, where the first neutrophils are initially activated by PRRs such as TLR2. This induces NF-κB signaling, and secrete chemokines and cytokines such as IL-8/KC and pro-IL-1β. What could be considered a second wave of activation, denoted by the transformation of

pro-IL-1β into IL-1β, its subsequent recognition by IL-1R, results in the eventual secretion of other factors such as IL-6, IL-8, and GM-CSF (Verstrepen et al., 2008). It has been shown in long term infection mouse models with *S. aureus* strain SH1000, the second wave, or responses controlled by the IL-1R response, are more critical for resolution in a necrotic wound model compared to the early responses by TLR2 (A. Hoffmann et al., 2006). The majority of IL-1β is produced upon inflammasome interaction, and not cleaved by neutrophil serine proteases. These ultimately attract another wave of neutrophils that produce large amounts of IL-1β (Cho et al., 2012), which may, themselves be necrotic as IL-1β secretion is RIPK-3 dependent (Wicki et al., 2016), and will be discussed more in a later section. Even though the TLR2 mediated signaling, or wave one, and the IL-1R mediated signaling, or wave two, both converge on NF-κB, they differ in their transcriptional output. This could be for a variety of reasons (A. Hoffmann et al., 2006; Miller et al., 2006; Verstrepen et al., 2008), such as differences in the IκB proteins that respond, the different protein combinations of the NF-κB unit, and more, but the discussion as to why TLR2 responses differ from IL-1R responses is beyond the scope of this paper.

NF-κB Modulation as a Way to Promote *S. aureus* Colonization

NF-κB modulation by *S. aureus* and by other pathogens has largely been looked at in the framework of pathogenic factors, but as mentioned above, NF-κB activity and subsequent cytokine and chemokine production is largely context dependent, with some groups demonstrating activation, and some inhibition. By inhibiting NF-κB during resolution of inflammation, healing can be prevented and inflammation is maintained (mentioned in (Hayden & Ghosh, 2011)), which is detrimental for the host, and propagates the idea that the ultimate goal of *S. aureus* is to cause immune activation. This is not necessarily true, as despite its formidable arsenal of virulence

factors, and as described earlier, *S. aureus* is known to colonize the anterior nares of the nose in 30% of humans, without necessarily causing overt inflammation(Wertheim et al., 2005). This suggests that large amounts of inflammation may not be ideal for the pathogen. Early subtle inhibition of NF-κB, on the other hand, may prevent inflammation and the subsequent waves of immune cells through the lack of IL-8 then IL-1β, therefore making a more welcoming home for *S. aureus*. TLR2 is critical to early stages of colonization, as TLR2 deficiencies are linked to nasal colonization in mice (González-Zorn et al., 2005). Thus, the modulation of NF-κB by *S. aureus* could be useful in the staged preceding interaction with the innate immune system. Some groups have speculated that modulation of NF-κB is useful in the context of early colonization, and not necessarily an active infection. For example, activities that inhibit IL-8 (Zurek et al., 2015), are in conflict with the ability of the leukotoxins PVL and HLA to induce NF-κB dependent IL-8 expression and release (Dragneva et al., 2001; König, Prévost, Piémont, & König, 1995). It was shown early on that *S. aureus* actively represses certain virulence factors during the colonization stage, while activating those factors during infection (Reviewed in (Novick, 2003)). So although NF-κB modulation has been studied in the context of an active infection, there is a right time and place for these activities, and the relevance of NF-κB modulation has not been thoroughly examined for the function of colonization.

The idea that suppression of the NF-κB pathway is for initial temporary colonization is supported by MyD88 deficient mice showing decreased IL-1β and KC (IL-8) at 6 hours after *S. aureus* inoculation, and this trend was resolving by 24 hours (Miller et al., 2006). Increases in these cytokines later could be due to RIPK-1 mediated cell death and NF-κB activation down the line (Grootjans et al., 2017). Additionally, in the subcutaneous inoculation model with *S. aureus*

strain SH1000, TLR2 deficient mice were able to heal their skin lesions at similar rates to their WT counterparts, while IL-1R deficient mice experienced both larger lesions and decreased neutrophil recruitment (Miller et al., 2006). This is in contrast to a model using various clinically relevant strains, bacterial supernatant, and airway epithelial cells, where the predominant mode of NF-κB recognition came through TLR1/2 signaling (Chekabab et al., 2015). Both IL-1R and TLR1/2 mediated activation of the NF-κB pathway signal through MyD88 (Bernthal et al., 2011), therefore it may be beneficial for *S. aureus* to have redundant methods for inhibiting the NF-κB pathway. TLR2 mediated recognition happens when gram positive bacterial cell walls have degraded, therefore, pre-mediated inhibition of this signaling pathway would be advantageous to a bacteria in the early stages of colonization, before innate immune cells are summoned. The production of IL-1β is dependent on first the production of pro-IL-1β from TLR2 and NOD2 activation, and later a second signal from the activation of the NLRP3 inflammasome which causes caspase-1 to activate, cleave pro-IL-1β, and secrete it (Cho et al., 2012). IL-1R is activated through the ligands IL-1α and IL-1β, which are expressed constitutively by epithelial cells, and by activated innate immune cells, respectively (Miller et al., 2007). In a subcutaneous skin infection, IL-1β has been shown to be the ligand needed to induce effective immune control, and the addition of recombinant IL-1β to IL-1β deficient mice is able to rescue the host's ability to resolve lesions (Miller et al., 2007). The activation of pro-IL1β comes in the form of cleavage by caspase-1, which is dependent on an active inflammasome in an ongoing infection (Bernthal et al., 2011; Opdenbosch & Lamkanfi, 2019). Therefore, the IL-1β response and subsequent activation of NF-κB is also more relevant in a situation where there is active damage and immune cell recruitment, making repression of this pathway helpful for early colonization of the host. The initial NF-κB

response is critical to defining the host's relationship with *S. aureus*, in an either inflammatory or tolerogenic manner. *S. aureus* immunomodulation may very well turn this versatile transcription factor into a double-edged sword that leads to an inappropriate response, or a finely-tuned tool that allows the body to make room for a commensal factor.

The Impact of NF-κB on Cell Fate

Upon inhibition of the NF-κB signaling pathway, either by host or pathogen factors, cells default to caspase-mediated apoptosis (X. Wang et al., 2018), which is generally beneficial for the host. In brief, there are two main modes of apoptosis, the extrinsic (death receptor pathway) and the intrinsic (mitochondrial pathways). The extrinsic pathway begins the apoptosis process by initial recognition of a signal by a 'death domain,' for example, TNF-α is recognized by TNFR1, or FasL is recognized by FasR (Jan & Chaudhry, 2019). This starts a signaling cascade culminating in the activation of procaspase 8. The activation of caspase 8 leads to the execution phase, leading to the physiological hallmarks of apoptosis (Elmore, 2007). While the extrinsic pathway is dependent on explicit recognition of a death signal, the intrinsic pathway is more associated with the loss of factors that prevent cell death programs, which induces apoptosis. For example, inhibition of NF-κB, an anti-apoptotic factor, could induce the intrinsic pathway (X. Wang et al., 2018). In addition, positive triggers for the intrinsic pathway could involve shocking stimuli like hypoxia or radiation. The intrinsic pathway revolves around instability induced in the mitochondria, where membrane potential is lost, and ultimately caspase 9 is activated, leading to the execution phase and the physiological hallmarks of apoptosis (Elmore, 2007). The execution pathway involves many proteins, including caspases 3, 6, 7, and 10 (Jan & Chaudhry, 2019). For

more on the differences between extrinsic and intrinsic apoptosis, and the final execution pathway, refer to the reviews by Susan Elmore, 2007, and Rehmat Jan and Gul-e-Saba Chaudhry, 2019.

Apoptosis of infected cells may lead to pathogen death either upon autophagy (Bai, Feldman, Chmura, Ovrutsky, & Su, 2013), or through macrophage induced killing after efferocytosis (Behar & Briken, 2019). Upon inhibition of Caspase-8, cells will progress to other cell death pathways, including necroptosis, with the role of RIPK-3 being dependent on the original stimuli (X. Wang et al., 2018). RIPK-1 is involved in both the apoptosis and necroptosis pathways (Peterson et al., 2017). Although the physiological relevance of these different cell death pathways are still under investigation, it is widely known that certain pathogens activate or inactivate different cell death pathways to their own advantage. For example, *S. aureus* has been documented in neutrophils inducing accelerated apoptosis (Zurek et al., 2015), vital NETosis (Brinkmann et al., 2004; Pilsczek et al., 2010), and necroptosis (Greenlee-Wacker et al., 2014), depending on the model and time point examined. Changing the amount of NF-κB activation in a cell has consequences for cell fate outcome. In the context of *S. aureus* mediated NF-κB modulation, there is still work to be done to discover the exact mechanism of modulation, which death pathways may be involved, and what is the overall ramification for the host tissue.

As indicated above, the studies of the influence of NF-κB on cell fate in *S. aureus* are incomplete. Zurek et al., 2015, demonstrated that inhibition of NF-κB signaling by *S. aureus* resulted in accelerated apoptosis (Zurek et al., 2015). Other studies demonstrate that *S. aureus* infection causes diversion from the canonical apoptosis pathway in favor of more inflammatory options (Greenlee-Wacker et al., 2014). Under normal conditions, a cell will first opt for activation of the NF-κB signaling pathway and an inflammatory response. Under suppression of NF-κB, the

cell will direct towards caspase-8 mediated apoptosis. When this option too fails, the cell will undergo formation of either the ripoptosome or necrosome complexes, which can lead to necroptosis, or regulated necrosis (reviewed in (Pasparakis & Vandenabeele, 2015; X. Wang et al., 2018)). The relationship between *S. aureus* and necroptosis is understudied, and this relationship has been hotly contested. One study showed that neutrophil necroptosis is beneficial for *S. aureus* clearance, as it inhibits excessive IL-1β release in *mlkl* deficient mice (Kitur et al., 2016). That being said, it has been shown that human PMNs stimulated with *S. aureus* undergo necroptosis in a MLKL independent manner (Greenlee-Wacker, Kremserová, & Nauseef, 2017), therefore the aforementioned study may not have been looking at true necroptosis. On the other hand, it has been shown that 15-50% of ingested *S. aureus* can survive inside of neutrophils, and these neutrophils undergo RIPK-3 mediated necroptosis (Greenlee-Wacker et al., 2017). For this reason, we suggest that the initial inhibition of NF-κB is a mechanism to drive neutrophils towards an inflammatory death pathway, like NETosis or necroptosis, that will increase *S. aureus* chances of survival. For example, this necroptotic state can prevent efferocytosis by macrophages, proliferating pathogenic spread (Behar & Briken, 2019; Greenlee-Wacker et al., 2017). Of note, PMNs were seen to undergo necroptosis at high *S. aureus* MOI (10:1), but necroptosis at a low MOI (1:1) required additional caspase inhibitors (Greenlee-Wacker et al., 2014). It is unclear if this is due to a lack of contribution on the pathogen side, or if at a low MOI, the *S. aureus* respond differently to the PMNs. Additionally, most necroptosis occurs in a RIPK-1, MLKL, and RIPK-3 dependent manner (X. Wang et al., 2018). Necroptosis induced by USA300 is RIPK-3 dependent, while independent of RIPK-1 and MLKL (Greenlee-Wacker et al., 2017; X. Wang et al., 2018), raising questions as to the nature of this necrotic death, and the ability of USA300 to fast-track

neutrophils to this inflammatory death. that IL-1β secretion was linked to pyroptosis instead of necroptosis (Kitur et al., 2016).

It has been shown that deficits in TLR2 correlated with decreased IL-1β production and larger lesions in mice, compared to WT (Cho et al., 2012), demonstrating the importance of TLR2 in initiating an inflammatory response to infection. And so, the question becomes, how does IL-1β relate to cell death in neutrophils, and later, does *S. aureus* prevent NF-κB signaling, and IL-1β production to its own advantage. Secretion of IL-1β involves the NLRP3/ASC/caspase-1 inflammasome, which is activated in response to staphylococcal pore forming toxins or NOD (Holzinger et al., 2012; Mankan, Dau, Jenne, & Hornung, 2012; Melehani, James, DuMont, Torres, & Duncan, 2015). Pro-IL-1β becomes the mature form through cleavage, the majority of which is done by caspase-1, and it has been shown that cleavage is possible by neutrophil proteases and caspase-8 (Coeshott et al., 1999; Guma et al., 2010; Vince et al., 2012). IL-1β lacks a secretion motif, and therefore the two prime mechanisms of IL-1β secretion are through pyroptosis and necroptosis, which also cause the secretion of different DAMPs (reviewed in (Opdenbosch & Lamkanfi, 2019)). One study suggested that NF-κB regulated genes act to negatively regulated caspase-1-dependent IL-1β secretion (Greten et al., 2007), which makes sense as NF-κB genes are pro-survival and anti-apoptotic, gatekeeping further inflammatory death pathways. Upon stimulation by monosodium urate (MSU) crystals or LPS, IL-8 production leads to the production of IL-1β, which in turn is necessary for second wave production of IL-8 for maximum neutrophil recruitment (Martinez et al., 2004; Matsukawa et al., 1998). This is interesting in that MSU crystals are associated with inducing neutrophil necroptosis (X. Wang et al., 2018). Together, this suggests that in the event of NF-κB failure, or pathogen mediated inhibition, the cell is able to proceed to

death pathways that are more immune signaling in nature by secreting DAMPs and IL-1β, and neutrophils are more predisposed to this mode of death due to the large number of staphylococcal pore-forming toxins that activate NLRP3, such as PVL and LukGH (Holzinger et al., 2012; Melehani et al., 2015). Therefore, between the combination of TLR2 mediated NF-κB inhibition, and the bombardment of pore-forming toxins, is the necrotic cell death outcome beneficial to the host for clearance, or an escape mechanism for the *S. aureus*. By encouraging cell death in a necrotic fashion, it could be that the pathogen does not have to contend with potentially more bactericidal ROS mediated killing methods.

Study Caveats

As alluded to in previous sections, the context of these host and pathogen studies can highly influence not just the overall experimental outcome, but the subtleties of which pathways are activated over others. Overall, at later time points during an active *S. aureus* infection, the NF-κB pathway is highly active and the local environment is inflammatory, which is critical for resolution of the infection. The activity of the *S. aureus* stimulated NF-κB pathway is likely dependent on a number of factors, including the time point, the MOI, and the strain used. When NF-κB is promoted, it could be due to MOI or a time point that overwhelms initial attempts to inhibit the pro-inflammatory response. Of note, one study using LAC saw a 15 to 100 fold reduction in IL-1β production compared to studies using other MOIs, culture conditions, and strains (Cho et al., 2012). The timing of inflammatory events is relevant as it has been shown that inhibition of NF-κB signaling during the resolution of infection extends the duration of inflammation, and prevents apoptosis in a mouse model (Lawrence, Gilroy, Colville-Nash, & Willoughby, 2001). As preventing NF-κB signaling, and the subsequent infiltration of neutrophils, is beneficial for *S.*

aureus survival, it makes sense that this selective pressure has resulted in several different NF-κB mechanisms, with others likely still to be discovered.

I present these considerations as there have been many papers published on the subject of *S. aureus* SSL3 and SSL4 proteins repressing the NF-κB pathway in neutrophils. As I move into the hypothesis and chapters of this thesis, keep in mind the caveats I have mentioned as they are likely the source of any discrepancies between the previously published studies, and my work.

Hypothesis

S. aureus is a ubiquitous colonizer and pathogen among the human population. The duality of how this bacterium can both live in harmony with a human host and be the cause of fatal infection is of great scientific interest. The work of this dissertation focuses on host-pathogen interactions. Specifically, experiments on early interactions of *S. aureus* and neutrophils are conducted, in order to better understand how initial recognition of the pathogen influences inflammatory outcomes. Many studies have described *S. aureus* manipulation of inflammation in the host, although these studies describing NF-κB repression often do not identify a mechanism of inhibition. As described earlier, it is possible to see inhibitory effects from supernatant alone, potent enough to mask the presence of *S. aureus* in a polymicrobial infection model (Chekabab et al., 2015). It has also been shown that in the presence of live USA300, the repressive effects are controlled by the SaeR/S two component system (Zurek et al., 2015). Chapter Two will highlight the overarching methodology for how neutrophils are extracted from whole human blood, and describe the use of an assay to quantify neutrophil cytotoxicity. These methodologies set the stage for the experiments described in Chapter Three. The overarching hypothesis for this work is that *S. aureus* modulates NF-κB to impact neutrophil function and promote pathogen survival. Data

indicate that *S. aureus* represses NF-κB expression in neutrophils through a secreted protein, 30kDa to 50kDa in size that is regulated primarily by the SaeR/S system. These studies pave the way for future studies, described in Chapter 4, to identify the exact SaeR/S-regulated proteins responsible for repression of NF-κB and for examining the molecular mechanisms of NF-κB modulation and how this impacts neutrophil cell fate.

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CHAPTER TWO

QUANTIFYING THE CYTOTOXICITY OF *STAPHYLOCOCCUS AUREUS* AGAINST HUMAN POLYMORPHONUCLEAR LEUKOCYTES

Contribution of Authors and Co-Authors

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Co-Author: Kyler B Pallister

Contributions: Prepared reagents and acted.

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Video Article

Quantifying the Cytotoxicity of *Staphyloccus aureus* Against Human Polymorphonuclear Leukocytes

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Abstract

Staphylococcus aureus is capable of secreting a wide range of leukocidins that target and disrupt the membrane integrity of polymorphonuclear leukocytes (PMNs or neutrophils). This protocol describes both the purification of human PMNs and the quantification of *S. aureus* cytotoxicity against PMNs in three different sections. Section 1 details the isolation of PMNs and serum from human blood using density centrifugation. Section 2 tests the cytotoxicity of extracellular proteins produced by *S. aureus* against these purified human PMNs. Section 3 measures the cytotoxicity against human PMNs following the phagocytosis of live *S. aureus*. These procedures measure disruption of PMN plasma membrane integrity by *S. aureus* leukocidins using flow cytometry analysis of PMNs treated with propidium iodide, a DNA binding fluorophore that is cell membrane impermeable. Collectively, these methods have the advantage of rapidly testing *S. aureus* cytotoxicity against primary human PMNs and can be easily adapted to study other aspects of host-pathogen interactions.

Video Link

The video component of this article can be found at https://www.jove.com/video/60681/

Introduction

Staphylococcus aureus is a Gram-positive bacterium that causes a wide spectrum of diseases in humans. This prominent pathogen produces numerous virulence factors that contribute to different aspects of infection. These include surface molecules that allow *S. aureus* to adhere to different types of host tissue[1], extracellular proteins that interfere with the host immune response[2], and an array of secreted toxins that target different types of host cells[3]. In this report, we describe a method that quantifies the cytotoxicity of extracellular proteins produced by *S. aureus* against human polymorphonuclear leukocytes (PMNs or neutrophils), primary effector cells of the host innate immune response.

PMNs are the most abundant leukocytes in mammals. These circulating immune cells are rapidly recruited to the site of host tissue insult in response to danger signals produced by resident cells or by compounds unique to invading microbes. The extracellular input from these molecules and from direct contacts with activated resident host cells during extravasation increase the activation state of PMNs in a process known as priming[4,5]. Primed PMNs that have reached distressed tissue then execute important innate immune responses designed to prevent the establishment of infection. These include the binding and internalization, or phagocytosis, of invading microorganisms that triggers a cascade of intracellular events cumulating in microbe destruction by a battery of potent antimicrobial compounds[5].

PMNs play an essential role protecting humans from invading pathogens and are particularly important for preventing *S. aureus* infection[4]. However, this bacterium produces a wide range of virulence genes that impede different PMN functions. These include extracellular proteins that block recognition of signaling molecules, prevent adhesion to host tissue, inhibit production of antimicrobial compounds, and compromise plasma membrane integrity[4]. *S. aureus* orchestrates the temporal expression of these virulence genes through the collective input from multiple two-component sensory systems that recognize specific environmental cues. The SaeR/S two-component system is a major up-regulator of *S. aureus* virulence gene transcription during infection[6,7,8,9,10,11]. In particular, this two-component system has been shown to be critical for the production of bi-component leukocidins that specifically target human PMNs[12].

This protocol is broken into three different sections. The first section describes the purification of PMNs from human blood using density gradient centrifugation using a protocol that has been adapted from methods established by Bøyum[13] and Nauseef[14]. The second and third sections detail two different techniques to examine *S. aureus* cytotoxicity; one intoxicates PMNs with extracellular proteins produced by *S. aureus* while the other examines the ability of living bacteria to damage PMNs following phagocytosis. These procedures use propidium iodide to measure the loss of PMN plasma membrane integrity caused by *S. aureus* pore-forming toxins. Propidium iodide is a DNA-binding fluorophore that is normally cell membrane impermeable but can cross plasma membranes that have been disrupted by *S. aureus* toxins. Flow cytometry analysis allows the rapid quantification of propidium iodide-positive PMNs to measure the relative cytotoxicity of *S. aureus* strains. Methicillin-resistant *S. aureus*

(MRSA) identified as pulsed-field gel electrophoresis type USA300 and an isogenic deletion mutant of *saeR/S* in this strain (USA300Δ*saeR/S*) have been used as models to demonstrate how these procedures can quantify the cytotoxicity of *S. aureus* against human PMNs.

Protocol

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 this study.

1. Purification of human polymorphonuclear leukocytes and isolation of human serum

NOTE: All reagents should be routinely checked for the presence of endotoxin using a commercially available endotoxin detection kit and should contain <25.0 pg/mL endotoxin to prevent unwanted priming of PMNs.

1. Bring 50 mL of 3% dextran-0.9% NaCl (w/v), 35 mL of 0.9% NaCl (w/v), 20 mL of 1.8% NaCl (w/v), 12 mL of 1.077 g/mL density gradient solution, and 20 mL of injection- or irrigation-grade water to room temperature.
2. To isolate human serum, incubate 4 mL of freshly drawn human blood without anti-coagulant at 37 °C in a 15 mL glass tube for 30 min. After incubation, centrifuge sample at 2,000–3,000 × *g* for 10 min at room temperature. Transfer the upper serum layer into a fresh 15 mL conical centrifuge tube and place on ice.
3. Combine 25 mL of freshly drawn heparinized (1000 units/mL) whole human blood with 25 mL of room temperature 3% dextran-0.9% NaCl (1:1 ratio) in two replicate 50 mL conical centrifuge tubes (50 mL total volume per tube). Mix by gently rocking each 50 mL conical tube and then let stand at room temperature for 30 min.
4. After incubation at room temperature, two separate layers will appear. Transfer the top layer of each dextran-blood mixture into new 50 mL conical tubes and centrifuge at 450 x *g* for 10 min at room temperature with low or no brakes.
5. Carefully aspirate both supernatants and discard without disturbing the cell pellets. Gently resuspend each cell pellet in 2 mL of room temperature 0.9% NaCl, combine the resuspended pellets in a single 50 mL conical tube, then add the remaining 0.9% NaCl (final volume of 35 mL).
6. Carefully underlay 10 mL of room temperature of 1.077 g/mL density gradient solution beneath the cell suspension using a hand pipette. Spin at 450 x *g* for 30 min at room temperature with low or no brakes. Gently aspirate the supernatant without disturbing the cell pellet. Supernatant will contain peripheral blood mononuclear cells that can be collected as previously described[14].
7. Lyse the red blood cells by resuspending the cell pellet in 20 mL of room temperature water. Mix gently by rocking the tube for 30 s. The lysis of red blood cells will be accompanied by a distinct decrease in turbidity.
8. Immediately add 20 mL of 1.8% NaCl (at room temperature [RT]) and centrifuge sample at 450 × *g* for 10 min at room temperature.
   NOTE: It is important to minimize the time that PMNs are left in water alone following red blood cell lysis to maximize PMN yield and prevent PMN lysis and/or activation.
9. Carefully aspirate the supernatant without disturbing the cell pellet. Gently resuspend the cell pellet in 2 mL of RT RPMI 1640 medium and place on ice.
10. Count cells using a hemocytometer. Resuspend purified PMNs at a concentration of 1 x 10^7 cells/mL with ice-cold RPMI and keep on ice.
11. Combine 100 µL of purified PMNs (1 x 10^6 cells) with 300 µL of ice-cold Dulbecco's phosphate-buffered saline (DPBS) containing 1 µL of propidium iodide stain in two replicate flow cytometry tubes. For a positive control for plasma membrane damage, add 40 µL of 0.5% Triton X-100 solution into one of the flow cytometry tubes and mix thoroughly.
12. Use flow cytometry to measure the forward scatter, side scatter, and propidium iodide staining (excitation/emission maxima at 535/617 nm) of purified cells (**Figure 1**).
    NOTE: Forward and side scatter analysis will identify unwanted populations of lymphocytes and monocytes. Propidium iodide will only stain cells with a compromised plasma membrane and purified PMNs that have pronounced populations of propidium iodide positive cells should not be used. For these studies, purified PMNs were only used if they comprised >98% of purified cells and <5% stained positive for propidium iodide.
13. Prepare a 96-well plate for PMN cytotoxicity assays by coating individual wells that will be used in this assay with 100 µL of 20% isolated human serum that has been diluted with DPBS.
    NOTE: Plating PMNs directly on plastic or glass will cause activation of the cells. Be sure to include at least one negative control well that will only receive media and at least one positive control well that will receive 0.05% Triton X-100.
14. Incubate the plate at 37 °C for 30 min. Following incubation, wash the coated wells twice with ice-cold DPBS to remove any excess serum. Gently tap the plate upside down to remove any residual DPBS and place on ice.
15. Gently add 100 µL of purified human PMNs at 1 x 10^7 cells/mL to each coated well (1 x 10^6 PMNs/well). Allow PMNs to settle in wells by incubating the plate on ice for at least 5 min. Keep the plate level to allow even distribution of cells in each well and leave on ice to avoid unwanted activation of PMNs.

2. Cytotoxicity assay of *S. aureus* extracellular proteins against human polymorphonuclear leukocytes

1. Culture *S. aureus* overnight in tryptic soy broth (TSB) using a shaking incubator set at 37 °C. For these studies, 20 mL of TSB in separate 150 mL Erlenmeyer flasks were inoculated with frozen cultures of *S. aureus* strains USA300 or USA300Δ*saeR/S* and grown for approximately 14 h with shaking at 250 rpm.
2. Subculture *S. aureus* by performing a 1:100 dilution of overnight bacterial culture with fresh media. Incubate at 37 °C with shaking until the bacteria reach early stationary growth phase.
   NOTE: For these experiments, 20 mL of tryptic soy broth in 150 mL Erlenmeyer flasks were inoculated with 200 µL of overnight cultured USA300 or USA300Δ*saeR/S* and incubated at 37 °C with shaking at 250 rpm for 5 h.

3. When bacteria have reached early stationary growth phase, transfer 1 mL of subcultured *S. aureus* into a 1.5 mL microcentrifuge tube and centrifuge at 5,000 × *g* for 5 min at room temperature.
4. Following centrifugation, transfer supernatant into a 3 mL syringe. Pass supernatants through a 0.22 µm filter and into a new 1.5 mL microcentrifuge tube on ice.
5. Perform serial dilutions of supernatants with ice-cold media used to culture *S. aureus*.
   NOTE: For the experiments shown, supernatants from USA300 and USA300Δ*saeR/S* underwent four consecutive 1/2 log dilutions with ice-cold TSB.
6. Gently add supernatant samples or media alone (for negative and positive controls) to individual wells of 96-well plate containing PMNs on ice from step 1.15. For these experiments, 10 µL of USA300 or USA300Δ*saeR/S* supernatant samples were added to each well. Gently rock plate to distribute supernatants in wells and incubate at 37 °C.
7. At desired times, remove the plate from incubator and place on ice. Add 40 µL of 0.5% Triton X-100 to the positive control well.
8. Gently pipette the samples up and down in each well to completely pull off all PMNs adhered to plate, then transfer the samples to flow cytometry tubes on ice that contain 300 µL of ice-cold DPBS with 1 µL of propidium iodide.
9. Measure the proportion of propidium iodide-positive PMNs using flow cytometry (**Figure 2A**). When bound to DNA, propidium iodide has excitation/emission at 535/617 nm.

3. *S. aureus* cytotoxicity assay against human polymorphonuclear leukocytes following phagocytosis

NOTE: Growth curves defined by the optical density at 600 nm (OD_{600}) and concentration of bacteria must be determined empirically for the *S. aureus* strains to be tested before beginning this assay. Success of these experiments requires the consistent harvest of equal concentrations of each *S. aureus* strain tested at mid-exponential growth phase using the OD_{600} of sub-cultured bacteria.

1. Start overnight cultures of *S. aureus* strains and subculture bacteria as described in steps 2.1.1 and 2.1.2.
2. Harvest subcultured *S. aureus* when it has reached mid-exponential growth by transferring 1 mL of cultured bacteria to a 1.5 mL microcentrifuge tube and centrifuging at 5,000 × *g* for 5 min at room temperature.
   NOTE: Under our growth conditions, USA300 and USA300Δ*saeR/S* reached mid-exponential growth phase after approximately 135 min of incubation[6].
3. Wash *S. aureus* following centrifugation by aspirating the supernatant, resuspending the pelleted bacteria in 1 mL of DPBS, vortexing the sample for 30 s, and centrifuging at 5,000 × *g* for 5 min at room temperature.
4. Opsonize *S. aureus* by resuspending the bacterial pellet in 1 mL of 20% human serum diluted with DPBS and incubating at 37 °C with agitation for 15 min.
5. Centrifuge opsonized bacteria at 5,000 x g for 5 minutes at room temperature. Wash *S. aureus* following centrifugation by aspirating the supernatant, resuspending the pelleted bacteria in 1 mL DPBS, then vortex the sample until the bacterial pellet is completely broken apart plus an additional 30 seconds. Centrifuge bacteria at 5,000 × *g* for 5 min at room temperature.
6. Resuspend opsonized *S. aureus* strains in 1 mL RPMI, vortex the sample until bacterial pellet is completely broken apart, and then for an additional 30 s. Place bacteria on ice.
7. Dilute opsonized *S. aureus* strains to the desired concentration with ice-cold RPMI. Vortex for 30 s and place on ice.
8. Confirm the concentration of opsonized *S. aureus* by plating 1:10 serial dilutions of bacteria on tryptic soy agar.
   NOTE: Because differences in the concentration of bacteria used in this assay can have a major impact on subsequent PMN plasma membrane permeability (**Figure 3A**), it is very important that the concentration of each strain tested is determined for every experiment and is equivalent between strains.
9. Gently add 100 µL/well of each *S. aureus* strain or RPMI (for positive and negative controls) to PMNs in the 96-well plate on ice from step 1.14. Gently rock plate to distribute *S. aureus* in wells.
10. Synchronize phagocytosis by centrifuging the plate at 500 × *g* for 8 min at 4 °C[15]. Incubate plate at 37 °C immediately following centrifugation (T = 0).
11. At desired times, remove plate from incubator and place on ice. Add 40 µL of 0.5% Triton X-100 to the positive control well.
12. Gently pipette the samples up and down in each well to completely pull off all PMNs adhered to plate, then transfer the samples to flow cytometry tubes on ice containing 200 µL of ice-cold DPBS with 1 µL of propidium iodide.
13. Analyze samples for propidium iodide staining using flow cytometry as described in step 2.9.

Representative Results

We have demonstrated how the procedures described above can be used to relatively quantify the cytotoxicity of *S. aureus* against human PMNs using MRSA PFGE-type USA300 and an isogenic deletion mutant of *saeR/S* in this strain (USA300Δ*saeR/S*) generated in previous studies[6]. PMNs isolated using the procedures described in section 1 of this protocol were stained with propidium iodide and examined using flow cytometry. Forward and side scatter plots were used to illustrate contamination of purified PMNs by monocytes or lymphocytes (**Figure 1A,B**) and PMN integrity was determined using propidium iodide staining (**Figure 1C**). The described method of human PMN purification can consistently yield 0.5 x 10^7 to 1 x 10^8 PMNs that are >98% pure and are >95% propidium iodide negative.

The cytotoxicity of extracellular proteins produced by USA300 and USA300Δ*saeR/S* were tested against purified PMNs (**Figure 2**) following the procedures described in section 2 of this protocol. These experiments demonstrate a concentration dependent increase in the propidium iodide staining of purified PMNs following 30 min of intoxication with extracellular proteins produced by USA300 (**Figure 2B**). Previous studies have demonstrated that the SaeR/S two-component system is important for expression of numerous bi-component leukocidins that target human PMNs[8,10,11,16]. Congruent with these previous findings, very few propidium iodide-positive PMNs were detected following exposure to extracellular proteins produced by USA300Δ*saeR/S* (**Figure 2B**). Further experiments demonstrated a steady increase in the proportion of lysed PMNs following intoxication by USA300 extracellular proteins that plateaued after approximately 30 min (**Figure 2C**). Minimal lysis of human

PMNs was noted at all timepoints following exposure to extracellular proteins produced by USA300Δ*saeR/S*. These results illustrate the utility of this assay for the relative quantification of cytotoxicity by extracellular *S. aureus* proteins against human PMNs.

We tested USA300 and USA300Δ*saeR/S* using the *S. aureus* cytotoxicity assay against human PMNs following phagocytosis that is described in section 3 of this protocol (**Figure 3**). A concentration dependent increase in the proportion of propidium iodide positive PMNs was observed 90 min after the phagocytosis of USA300 (**Figure 3A**). A significant decrease was observed in the proportion of PMNs that were propidium iodide positive following the phagocytosis of USA300Δ*saeR/S* (**Figure 3A**), supporting other results that indicate the SaeR/S two-component system is important for the cytotoxicity of *S. aureus* against human PMNs (**Figure 2**)[7,11]. As previously mentioned and demonstrated in **Figure 3A**, differences in *S. aureus* concentration have a pronounced impact on PMN lysis following phagocytosis. Enumeration of the USA300 and USA300Δ*saeR/S* inoculum used in each of these experiments demonstrated that the contrast in cytotoxicity between these strains was not due to differences in the concentration of bacteria used (**Figure 3B**). These findings show how the *S. aureus* cytotoxicity assay against human PMNs following phagocytosis can be used to assess the ability of different *S. aureus* strains to compromise human PMN plasma membrane integrity.

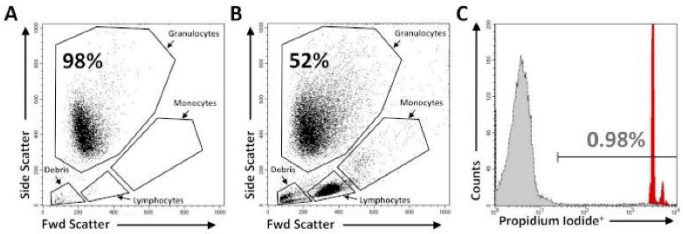

Figure 1: Flow cytometry analysis of purified PMNs. Representative flow cytometry dot plots of (**A**) purified human PMNs and (**B**) PMNs that have been purposely contaminated with peripheral blood mononuclear cells. (**C**) Representative flow cytometry histogram demonstrating minimal propidium iodide staining (<1%) of purified PMNs (shaded grey) as compared to PMNs treated with 0.05% Triton X-100 (shaded red). Please click here to view a larger version of this figure.

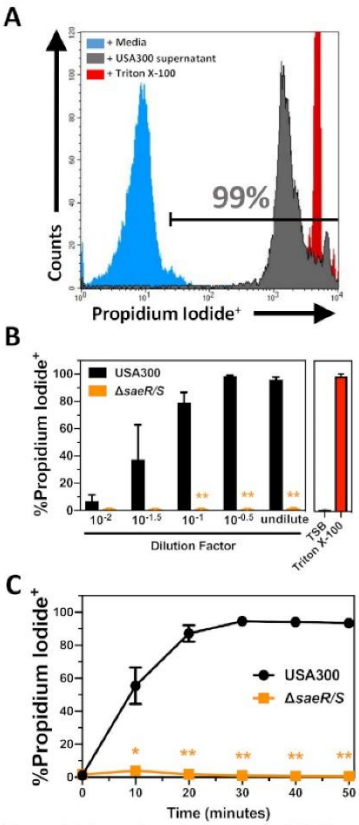

Figure 2: Flow cytometry analysis of PMNs intoxicated with extracellular proteins produced by *S. aureus*. (**A**) Representative flow cytometry histogram of PMNs stained with propidium iodide after 30 min of incubation with media control (shaded blue), filtered USA300 supernatant at a final concentration of 1:110 (shaded grey), or 0.05% Triton X-100 (shaded red). (**B**) The proportion of propidium iodide positive PMNs after 30 min of incubation with different concentrations of USA300 or USA300Δ*saeR/S* supernatants. (**C**) The proportion of propidium iodide positive PMNs over time following incubation with USA300 or USA300Δ*saeR/S* supernatant at a final concentration of 1:110. Data are presented as mean ± SEM of at least 3 separate experiments with * $p \leq 0.05$ and ** $p \leq 0.005$ as determined by two-tailed t-test. Please click here to view a larger version of this figure.

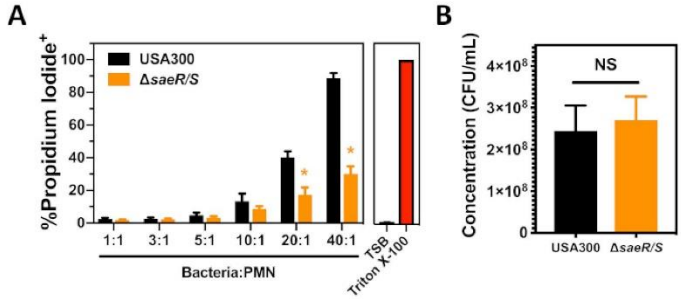

Figure 3: Flow cytometry analysis of PMNs following phagocytosis of *S. aureus*. (**A**) The proportion of propidium iodide positive PMNs 90 min after the phagocytosis of different concentrations of USA300 or USA300Δ*saeR/S*. (**B**) Concentration of opsonized *S. aureus* strains used for the experiments shown in panel A. Data are presented as mean ± SEM of 4 separate experiments with * $p \leq 0.01$ as determined by two-tailed t-test. Please click here to view a larger version of this figure.

Discussion

This protocol describes the purification of PMNs from human blood and two distinct assays that use propidium iodide for quantifying the cytotoxicity of *S. aureus* against these important innate immune cells. The success of these procedures will depend upon the quality of purified PMNs and the appropriate preparation of *S. aureus* and extracellular proteins produced by this pathogen. For the isolation of PMNs, it is important to minimize PMN activation during and after purification by using reagents free of endotoxin contamination, treating cell preparations gently, and keeping cells at the appropriate temperature. Signs that indicate activation of PMNs include clumping of cells during purification and when more than 5% of isolated cells stain positive for propidium iodide. Because of the relatively short life span of PMNs, these cells must be isolated from human blood and tested in the same day. PMNs will begin to exhibit signs of spontaneous apoptosis if left on ice for more than 3 h after purification. As mentioned earlier, it is very important that every PMN preparation is carefully evaluated using flow cytometry analysis of forward and side scatter as well as propidium iodide staining to ensure the purity and integrity of isolated cells.

The expression of bi-component leukocidins by *S. aureus* is responsible for the majority of compromised PMN plasma membrane integrity that is observed using the assays described in this protocol. Variation in the expression of these toxins and other pore-forming peptides, such as phenol-soluble modulins, between strains of *S. aureus* will produce differences in cytotoxicity against human PMNs. Significant deviations during in vitro growth between *S. aureus* strains will also influence expression of pore-forming toxins and subsequent cytotoxicity. In addition, the ratio of *S. aureus* to PMNs in phagocytosis assays has a major impact on subsequent PMN plasma membrane permeability (**Figure 3A**) and these experiments require the consistent harvest of equal concentrations of each *S. aureus* strain tested at mid-exponential growth phase using the OD_{600} of subcultured bacteria. Given these considerations, it is very important to define growth curves for all strains that will be examined before beginning cytotoxicity assays. We do not recommend these methods for analyzing *S. aureus* cytotoxicity with strains that exhibit significant growth differences in vitro.

USA300 is a virulent MRSA isolate that is known to be highly cytotoxic against human PMNs[15] and the loss of SaeR/S in this strain dramatically reduces transcription of numerous bi-component leukocidins that target human PMNs[6,12], making these strains ideal models for comparing cytotoxicity using the assays described. However, there is extensive genetic variation between different *S. aureus* isolates and the parameters detailed in these protocols may not result in substantial changes in cytotoxicity against human PMNs when testing other *S. aureus* strains. Tailoring the growth conditions, volumes of supernatants added, or ratio of bacteria to PMNs may be required for success with these methods using other strains of *S. aureus*.

Disclosures

The authors have nothing to disclose.

Acknowledgments

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CHAPTER THREE

SECRETED PROTEINS FROM *STAPHYLOCOCCUS AUREUS* SUPPRESS NF-KB

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Manuscript Information

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SECRETED PROTEINS FROM *STAPHYLOCOCCUS AUREUS* SUPPRESS NF-KB

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Abstract

Staphylococcus aureus (*S. aureus*) is a ubiquitous bacterium that induces a spectrum of disease, from existing as a commensal microbe to being the causative agent of necrotic lung infections. The ability of *S. aureus* to exist on this spectrum can be largely attributed to the exquisite regulation of numerous adhesins, immunomodulatory proteins, and toxins. In this study, we examined the ability of *S. aureus* to inhibit NF-κB signaling, and subsequent IL-8 production, in human neutrophils. Through a combination of techniques, we show that NF-κB repression is caused by secreted proteins regulated by both the SaeR/S and Agr systems. The factor(s) that decrease NF-κB-p65 is between 30kDa and 50kDa in size. The ability of secreted proteins to decrease NF-κB was maintained in the presence of the canonical NF-κB trigger, lipopolysaccharide (LPS). Surprisingly, this observation was only observed in *S. aureus* PFGE-type USA300 strain LAC but was not conserved in PFGE-type USA400 strain MW2. Collectively, these data identify another mechanism used by *S. aureus* to modulate inflammation by reducing NF-κB-p65.

Importance

The relationship between initial *Staphylococcus aureus* (*S. aureus*) infections and inflammation triggered by the innate immune system is an understudied area with conflicting publications. Here we show that the *S. aureus* PFGE type USA300 strain LAC secretes proteins that reduces NF-κB-p65. Although the mechanism behind this repression needs to be further defined, our study adds to previous observations and highlight that *S. aureus* targets NF-κB to impact the host inflammatory response to infection.

Introduction

Staphylococcus aureus (*S. aureus*) colonizes the anterior nares of approximately 30-50% of the population (Gorwitz et al., 2008; Wertheim et al., 2005). Although *S. aureus* generally exists as a commensal organism, this bacterium can cause disease ranging from benign soft-tissue abscesses to lethal infections such as necrotizing fasciitis. Through the acquisition of antibiotic resistance, *S. aureus* is increasingly more difficult to treat in community and hospital settings. The front line of defense against *S. aureus* infections is the human neutrophil, a highly inflammatory leukocyte that produces different antimicrobial proteins, peptides, and reactive oxygen species (ROS) when activated (Guerra et al., 2017; Rigby & Deleo, 2011). Neutrophils are also capable of producing cytokines, such as IL-8, that prime and attract more neutrophils as well as prevent premature apoptosis (Kettritz et al., 1998; Wozniak, Betts, Murphy, & Rokicinski, 1993). This ability is critical for controlling early bacterial infections (Yoshio Hirao, Tsugiyasu Kanda, Yoshimasa Aso, Masato Mitsuhashi, & Isao Kobayashi, 2000). In turn, *S. aureus* produces cytolytic toxins, superantigens, and immunomodulatory proteins that manipulate the host immune response (Guerra et al., 2017). Manipulation of the inflammatory environment likely gives *S. aureus* an edge when establishing infection in a new host.

The cornerstone of neutrophil inflammation is the pro-inflammatory transcription factor, nuclear factor kappa-light chain enhancer of activated B cells (NF-κB). NF-κB is activated through the recognition of pathogen-associated molecular patterns (PAMPs), often through toll-like receptors (TLRs). This recognition starts a signaling cascade that results in the activation of NF-κB, translocation into the nucleus, and the transcription of anti-apoptosis and pro-inflammatory products including IL-8 (Hayden & Ghosh, 2011). These inflammatory responses, although

potentially damaging to the host if uncontrolled, are necessary to summon other immunological factors and control infections. We have previously demonstrated that *S. aureus*, through the action of the *S. aureus* exoprotein secretion system SaeR/S, represses NF-κB expression and subsequent cytokine production (Zurek et al., 2015). In this study, we advanced our previous observations and those made by others (Zurek, Ji, Bolhuis, and Watson 2019a; Koymans et al. 2017; Chekabab et al. 2015) to demonstrate that the prominent *S. aureus* isolate identified as PFGE type USA300 strain LAC can repress NF-κB-p65 expression by a SaeR/S-regulated secreted protein between 30kDa and 50kDa. To our surprise, this mechanism was not observed in the clinically relevant isolate PFGE-type USA400 strain MW2, suggesting that this mechanism of NF-κB repression is not conserved across clinically relevant strains.

Results

Secretion of a *S. aureus* Factor Represses NF-κB and Cytokine Expression

It is advantageous to *S. aureus* to repress an inflammatory response while establishing its microenvironment. It has been previously shown that *S. aureus* can suppress NF-κB and subsequent IL-8 production in both neutrophils and airway epithelial cells (Chekabab et al., 2015; Zurek et al., 2015). We previously demonstrated that type USA300 could repress NF-κB (Zurek et al., 2015) and that this repression was regulated by the SaeR/S two-component gene regulatory system in *S. aureus*. In the current study we investigated if secreted factors from overnight cultures reduced NF-κB compared to unstimulated neutrophils (Figure 3.1). By stimulating neutrophils with sterile filtered supernatants from the wild type USA300, USA300Δ*saeR/S*, and USA300Δ*agr* mutant strains, the overall amount of p65 protein was seen to be reduced by wild type supernatant, but not the USA300Δ*saeR/S*, and USA300Δ*agr* mutant strain supernatant (supplemental Figures

3.1A-B). Next, an ELISA was used to compare the amount of NF-κB phosphorylated, and therefore active, S536 of the P65 subunit (Figure 3.1A) produced in neutrophils exposed to either the USA300 supernatant, or the USA300Δ*saeR/S* and USA300Δ*agr* supernatant. Neutrophils exposed to USA300Δ*saeR/S* and USA300Δ*agr* produced NF-κB similar to unstimulated neutrophils while wildtype USA300 significantly reduced activated NF-κB below the resting state (Figure 3.1A). We previously demonstrated that the SaeR/S two component system mediated a reduction of NF-κB phosphorylation which corresponded to reduced IL-8 production (Zurek et al., 2015). To determine if USA300 supernatant could reduce IL-8 transcription, neutrophils were exposed to *S. aureus* supernatant and IL-8 transcript abundance was assessed at two-hours post-exposure (Figure 3.1B). Neutrophils exposed to Δ*saeR/S* and Δ*agr* supernatants had increases in IL-8 expression relative to resting and wild-type exposed cells. Exposure to USA300Δ*saeR/S* supernatant significantly activated NF-κB relative to neutrophils treated with wild type USA300 supernatant and above a base line of NF-κB activation for neutrophils in a resting state. The secreted factor that influences NF-κB expression is also regulated by *agr*, however, the Δ*agr* supernatant exposed neutrophils did not demonstrate statistically significant differences when compared to USA300. To investigate if the secreted factor was conserved in another clinically relevant strain, we exposed human neutrophils to USA400 supernatant and corresponding isogenic mutants of SaeR/S or Agr in this strain. Surprisingly, USA400 supernatant did not significantly alter total amounts of NF-κB between the different mutant supernatant groups (Figure 3.1C).

Reduction of NF-κB Expression is Caused by a Secreted Protein and Occurs in both Neutrophils and Monocytes

Proteinase K digestion was used to demonstrate that the factor of interest is a protein. Following treatment with proteinase K no significant differences in NF-κB phosphorylation were observed between WT, Δ*saeR/S*, and Δ*agr* supernatant stimulation (Figure 3.2A). To further characterize our protein of interest, size exclusion filters of 0.2μM, 50kDa, or 30kDa were used. Reduced NF-κB phosphorylation was seen in WT supernatants following filtration with 0.2uM, and 50kDa. Interestingly, the differences in NF-κB phosphorylation was absent in neutrophils exposed to supernatants following filtration at 30kDa (Figure 3.2B). This indicates that the protein controlled by SaeR/S (and to some degree, Agr), is likely between 30kDa and 50kDa in size. Reduced NF-κB phosphorylation was independent of cell death since lysis was significantly reduced in neutrophils incubated with supernatants from the 30kDa and 50kDa filters (Supplemental Figure 3.2A).

We next utilized THP1-Blue™ NF-κB monocyte to confirm our observations from Figure 3.1 and to determine if the *S. aureus* factor could reduce NF-κB expression following stimulation with the canonical NF-κB trigger LPS. We used supernatant that was filtered with the 50kDa filter to retain viability at this long time point. When THP1-Blue™ NF-κB monocytes were incubated with a combination of WT supernatants and LPS, the WT supernatants were seen to suppress the LPS induced activation in a dose dependent manner (Figure 3.2C).

Reduced Expression of NF-κB is not Caused by SSL3 or SSL4

Previous studies have shown that SSL3 and SSL4 are able to bind to TLR2, which could inhibit NF-κB signaling (Koymans, Feitsma, Brondijk, et al., 2015; Koymans et al., 2017). As shown previously, USA300 can repress NF-κB signaling, both through live pathogen interaction (Zurek et al., 2015), and as shown here through a secreted protein(s) (Figure 3.1). This is in contrast to USA400, that did not produce a secreted factor that reduces total amount of NF-κB protein (Figure 3.1E). These observations led us to believe there may be significant differences between the SSLs of the strains, but when aligned by BLAST, SSL3 in USA300 and USA400 are shown to be 98% identical, with 12 gaps between the two strains (Supplemental Figure 3.2A). Structural analysis demonstrates that a DVID insertion in USA400 is located on the other side of the protein from the TLR1/2 binding site and likely does not interfere with binding (data not shown). When comparing SSL4 by BLAST, these two strains are 93% identical, with 31 gaps (data not shown). To investigate if *ssl3* and *ssl4* contributed to the reduced NF-κB phosphorylation in neutrophils, we first measured *ssl3* and *ssl4* transcript abundance during *S. aureus* growth in culture and following phagocytosis. *ssl3* and *ssl4* transcripts were expressed in USA300, USA400 and corresponding isogenic Δ*saeR/S* strains from overnight (stationary phase) and exponential cultures (Figure 3.3A). There was increased expression in *ssl3* and *ssl4* transcripts during stationary growth compared to expression during exponential growth. These data imply that SSL3 and SSL4 are likely present in supernatants from both USA300 and USA400 strains and are not responsible for the reduced NF-κB phosphorylation observed. When examining USA300 *ssl3* and *ssl4* following phagocytosis by neutrophils, we did observe an increase in transcript abundance of *ssl3* and *ssl4* in USA300 that had been phagocytosed by neutrophils that was not observed for USA300Δ*saeR/S*

(Figure 3.3B), suggesting these genes are under the regulatory control of this two-component system. When examining *ssl3* and *ssl4* expression during exponential growth versus stationary growth, it was seen that both of these transcripts were made in abundance in both the wild type and Δ*saeR/S* strains, indicating that they would be present in the supernatant used for the experiment in Figure 3.1C. For phagocytosed USA400, we observed a similar increase *ssl4* transcript abundance but not for *ssl3* transcript abundance when USA400 was stimulated by PMNs (Figure 3.3D).

As a first attempt to identify the secreted factors responsible for the reduction in NF-κB expression we screened *S. aureus* supernatants from isogenic mutant strains of genes coding proteins within the 30-50kDa range. This included knockouts in the genes *ssl3*, *ssl4*, *hla*, *LukGH*, and *pvl*, with a *ssl11* knockout included which was slightly below 30kDa at 24.98kDa (Table 2). These isogenic deletion mutants were selected based on published data demonstrating the expression of these genes is regulated by both SaeR/S and Agr (Alonzo, III, & Torres, 2014; Benson et al., 2012; Li & Cheung, 2008). The *hla* mutant strain has been characterized in previous studies as indicated in Materials and Methods. The *ssl3/4* and *ssl11* strains were generated for this study and had no significant defects in growth compared to the parental USA300 strain (Supplemental Figure 3.4). The ssL11 mutant has a predicted protein size of 24.98kDa and was used as both a size control, and to explore a possible link to PMN migration which is explained in the discussion section. None of the mutant strains investigated demonstrated significant differences in phosphorylation state from WT USA300 (Figure 3.4), implying that the proteins knocked-out were not responsible for the repression of NF-κB activation.

Discussion

NF-κB and cytokine repression by *S. aureus* has been described before, but results in this study indicate a mechanism that is distinct from these reports (Chekabab et al., 2015; Ji et al., 2019; Koymans et al., 2017). We previously showed that *S. aureus* can repress IL-8 production through inhibition of NF-κB activity when neutrophils are exposed to live USA300 (Zurek et al., 2015). Here we show that this effect is mediated by both the SaeR/S and the Agr two-component systems and can be induced by proteins in diluted overnight supernatant (Figure 3.1A). These potent secreted factors also repressed IL-8 transcription (Figure 3.1B). In addition, we demonstrated that this repression is caused by one or more proteins that are between 30kDa to 50kDa in size (Figure 3.2B). Furthermore, we show that this repressive ability is not conserved in the clinically relevant strain, USA400 (Figure 3.1C). This concurs with a previous study showing non-significant differences in IL-8 production in 16HBE cells when stimulated by USA400 versus resting, although the IL-8 repressive agent they examined was non-proteinaceous and determined to be less than 3kDa in size (Ji et al., 2019).

Based on previous observations by Bardoel et al, and Koymans et al., we originally anticipated that SSL3 and/or SSL4 proteins would be responsible for the inhibited phosphorylation of the transcription factor NF-κB. However, we found that SSL3 and SSL4 were not responsible for the measured decrease in NF-κB- p65 in our studies. Both the Δ*saeR/S* and Δ*agr* mutants produced *ssl3* and *ssl4* in overnight culture (Figure 3.3A) (Benson et al., 2012), but supernatants from these cultures where not able to repress NF-κB activation. In addition, we demonstrate that the deletion of *ssl3* and *ssl4* in USA300 had no influence on NF-κB activation under the conditions tested. Previous studies have demonstrated that SSL11 from USA300 disrupts motility in the

neutrophil-like cells, dHL60 by locking the cell into an adhesive state (Chen, Yang, & Barbieri, 2019). When p65 is lacking in mice, neutrophil migration into infected tissues is attenuated (Alcamo et al., 2001), suggesting a potential link between the observed NF-κB inhibition, neutrophil migration, and SSL11. However, supernatant produced by USA300 lacking *ssl11* did not influence NF-κB activation relative to supernatant produced by wild-type USA300, suggesting SSL11 is not responsible for the repression of NF-κB phosphorylation observed in these studies. None of the individual toxin knockouts screened, i.e., Δ*hla*, Δl*ukGH*, nor Δ*pvl*, showed a difference from the WT suppression (Figure 3.4), which agrees with a previous study showing that Hla from USA300 does not influence IL-8 production in whole blood (T. K. Nygaard, Pallister, Zurek, & Voyich, 2013). We decided to screen leukotoxins, based on their sizes, regulation by SaeR/S and Agr and also because it is not unheard of for *Staphylococcal* toxins to have secondary effects, as shown by several studies demonstrating that leukotoxins induce neutrophil cell death via NETosis (Bhattacharya et al., 2018; Mazzoleni, Zimmermann-Meisse, Smirnova, Tarassov, & Prévost, 2021).

The identity of the repressive proteins and the mechanism of this repression is beyond the scope of this paper, although this method may be linked to the overall reduction in total p65 protein, as seen in Figure 3.1C. Future studies will investigate additional virulence factors between 30-50 kD and also generate double and triple knockout strains since we anticipate more than one protein is responsible for the significant reduction in NF-κB-p65. Although the physiologic relevance of NF- κB-p65 reduction is currently unknown, it is possible *S. aureus* uses the ability to stay hidden during early colonization or to reduce inflammation while the pathogen establishes a biofilm. Regardless, our findings add to previous studies by us and others that indicate NF- κB

modulation is a virulence mechanism used by *S. aureus*. Given the major axis point of NF-κB in both cell survival and the entry point for various modes of neutrophil cell death, defining the mechanism behind this *S. aureus* modulation will further illuminate the intricacies of this host and pathogen interaction.

Materials and Methods

Bacterial Strains and Culture

CA-MRSA strains USA300 or USA400 and corresponding isogenic mutants were used in this study. Unless otherwise indicated, overnight media consisted of tryptic soy broth (TSB) (EMD Millipore; Darmstadt, Germany), with the subculture supplemented with 0.5% (w/v) glucose (as described Voyich 2005). When needed, spectinomycin was included in the medium at a 0.5 mg/mL concentration. Subcultures were created by inoculating a 1:100 dilution of the overnight culture. Overnight cultures had CFUs/mL between 4-15x10^9 CFUs/mL. Bacteria were harvested by centrifugation for 5 minutes at 8,000xg. Supernatants were sterile-filtered and diluted with 1:30 DPBS. This dilution was empirically determined to maintain the phenotype of reduced NF-κB without complete lysis of cells (data not shown).

Construction of the Δ*ssl3* and Δ*ssl4* double mutant, and Δ*ssl11* deletion mutant was preformed using allelic exchange with the plasmid pKOR1 as described previously (Bae & Schneewind, 2006; T. K. Nygaard et al., 2018). The primers used to generate these mutants are listed in Table 1. Primers containing *attb* sites were used to amplify proteins of interest from the USA300 genome for BP Clonase II (ThermoFisher Scientific) mediated insertion into the pKOR1 plasmid. For growth curves OD_{600} reading were measured every half hour by a Nanodrop 2000C UV-Vis Spectrophotometer (ThermoFischer Scientific; Wilmington, DE, United States). Colony forming units (CFUs) of serially diluted samples were enumerated on TSA after incubation overnight at 37°C with 5% CO_2 as described (Collins et al., 2020; Dankoff et al., 2020; J. M. Voyich et al., 2005). Growth curves for Δ*lukGH* not shown. The Δ*pvl* mutant was kindly provided

by Michael Otto and all other mutants used in this study were generated in previous studies(T. K. K. Nygaard et al., 2010; T. K. Nygaard et al., 2012).

Neutrophil Isolation

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. Human neutrophils (polymorphonuclear leukocytes, PMNs) were isolated under endotoxin-free conditions (<25 pg ml^{-1}) as previously described (Dankoff et al., 2020; J. M. Voyich et al., 2005; J. M. M. Voyich et al., 2009). Purity (<1% PBMC contamination) and viability (<2% propidium iodide positivity) of neutrophil preparations were assessed by flow cytometry on a FACS Calibur instrument and BD Biosciences Cell Quest Pro software (version 0.3.3flb).

Plasma Membrane Damage

Propidium iodide (PI) uptake was used as a measure of plasma membrane permeability to assess damage of neutrophils by secreted *S. aureus* proteins as described (Dankoff et al., 2020; T. K. Nygaard et al., 2012). Briefly, bacterial strains were cultured overnight at 37°C with shaking (250 RPM) in TSB. After, 1mL of bacteria were collected, and centrifuged for 5 minutes at 8,000xg. Supernatants were sterile-filtered as described above and diluted with DPBS. Neutrophils were exposed to supernatants for 1 hour at 37°C with 5% CO_2. After incubation, cells were stained with 0.5 µL PI (1 mg mL^{-1} Life Technologies) and analyzed by flow cytometry on a FACS Calibur flow cytometer (BD Biosciences; Franklin Lakes, New Jersey).

Phosphorylated and Total Protein P65 NF-κB ELISA

Plates (96-well,Cellstar®, Greiner Bio-one) were prepared by coating the wells with 20% normal human serum, and incubated for at least 30 minutes at 37°C. These wells were then flushed twice with DPBS. Neutrophils, at a concentration of 10^6 cells per well, were incubated with supernatants (as described above), or *E. coli* derived lipopolysaccharide (LPS, used as a positive control), at indicated concentrations, for two hours. The cells were then processed and analyzed in accordance to manufacturer recommendations for the NFκB-p65 (Total/Phospho) Human InstantOne™ ELISA Kit (ThermoFisher Scientific; Waltham, MA).

Western Blot Analysis

Supernatants from overnight cultures in TSB without glucose were harvested as described above. Plates (96-well,Cellstar®, Greiner Bio-one) were prepared by coating the wells with 20% normal human serum, and incubated for at least 30 minutes at 37°C. These wells were then flushed twice with DPBS. Neutrophils, 1 million cells per well, were stimulated by supernatants for 2 hours, at 37°C, before being collected and lysed with RIPA Buffer. Samples (20 μL) were resolved using 10% SDS-PAGE gels, (150 V for 50 minutes), and transferred onto nitrocellulose (at 10mAmps overnight). Membranes were washed and blocked in DPBS containing 5% (w/v) milk solution for 10 minutes followed by incubation with either rabbit anti-GAPDH primary antibody (Cellular Technologies, D16H11), or mouse anti-p65-NF-κB (Santa Cruz Antibodies, sc-8008) at the recommended dilution of 1:1,000. The membranes were incubated overnight, with rocking at 4°C. GAPDH and p65 NF-κB were detected after a 2 hour incubation with goat anti-mouse IgG coupled to horseradish peroxidase (at 1:10,000 dilution) (Jackson ImmunoResearch; West Grove, PA, United States), or goat anti-rabbit IgG coupled to horseradish peroxidase (at 10,000 dilution)

(Jackson ImmunoResearch; West Grove, PA, United States), and then developed using 5 mL 3,3',5,5'-tetramethylbenzidine (TMB) substrate. Images were taken with a Gel Doc Imager (ProteinSimple; San Jose, CA, United States) and analyzed by ImageJ densitometry software (Schindelin et al., 2012), with amounts of p65 NF-κB normalized to amounts of GAPDH per well.

THP1-Blue™ NF-κB *Assay*

THP1-Blue™ NF-κB Monocyte Cells (InvivoGen; San Diego, CA), were cultured in accordance to manufacturer recommendations. Supernatants from *S. aureus* strains were collected as described above. THP1-Blue™ cells were suspended in manufacturer provided growth media at a concentration of 100,000 cells/well, and were incubated with supernatants for 24 hours at 37°C. After incubation, samples were incubated with QUANTI-Blue™ Solution, in accordance to manufacturer recommendations, and quantified by a 450nm well scan with an Epoch2 Microplate Spectrophotometer (BioTek; Winooski, VT). Individual experiments were done in duplicate, with averages taken between technical repeats. LPS from *E. coli*, was used at a concentration of 10ng or 1ng per well.

qRT-PCR

TaqMan® gene expression experiments were performed as previously described (T. K. K. Nygaard et al., 2010; J. M. Voyich et al., 2005). For experiments measuring *S. aureus* transcription, *S. aureus* strains were cultured as above and grown to an OD600 of 1.5 (mid-exponential) and exposed to media only or neutrophils at an MOI of 10:1. After 30 minutes RNA was extracted as described (T. K. K. Nygaard et al., 2010). Relative quantification of *S. aureus* target genes was determined using the $2^{-\Delta\Delta Ct}$ method and change in expression of target transcripts were normalized to that of the housekeeping gene gyrase B (*gryB*) and relative to either USA300 (strain LAC)

transcript levels or USA400 (strain MW2) grown in TSB only. The *gyrB* forward sequence is CAAATGATCACAGCTTTGGTACAG and the reverse sequence is CGGCATCAGTCATAATGACGAT with the probe sequence of 5' 6FAM AATCGGTGGCGACTTTGATCTAGCGAAAG. For experiments measuring human gene transcripts, neutrophils were isolated, prepared, and exposed to *S. aureus* supernatants as described above. At a 2 hour timepoint, they underwent an RNA extraction (RNeasy® Mini Kit, QIAGEN), as done previously (Kobayashi et al., 2002). Relative quantification of neutrophil gene targets were determined using the $2^{-\Delta\Delta Ct}$ method and change in expression of target transcripts were normalized to that of the housekeeping gene GAPDH, and relative to neutrophil only transcript levels. Primer/probe sequences generated for this study are described in Table 1, and on demand primer and probe set included the TaqMan® Gene Expression Assays for GAPDH (Hs02758991_g1) (T. K. K. Nygaard et al., 2010; J. M. Voyich et al., 2005).

Statistics

Statistical analyses were performed using GraphPad Prism version 8.0 (GraphPad Software, La Jolla, CA, United States) with *t*-tests and ANOVA as indicated. Error bars represent the standard error of the mean (SEM).

Acknowledgements

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Figures and Tables

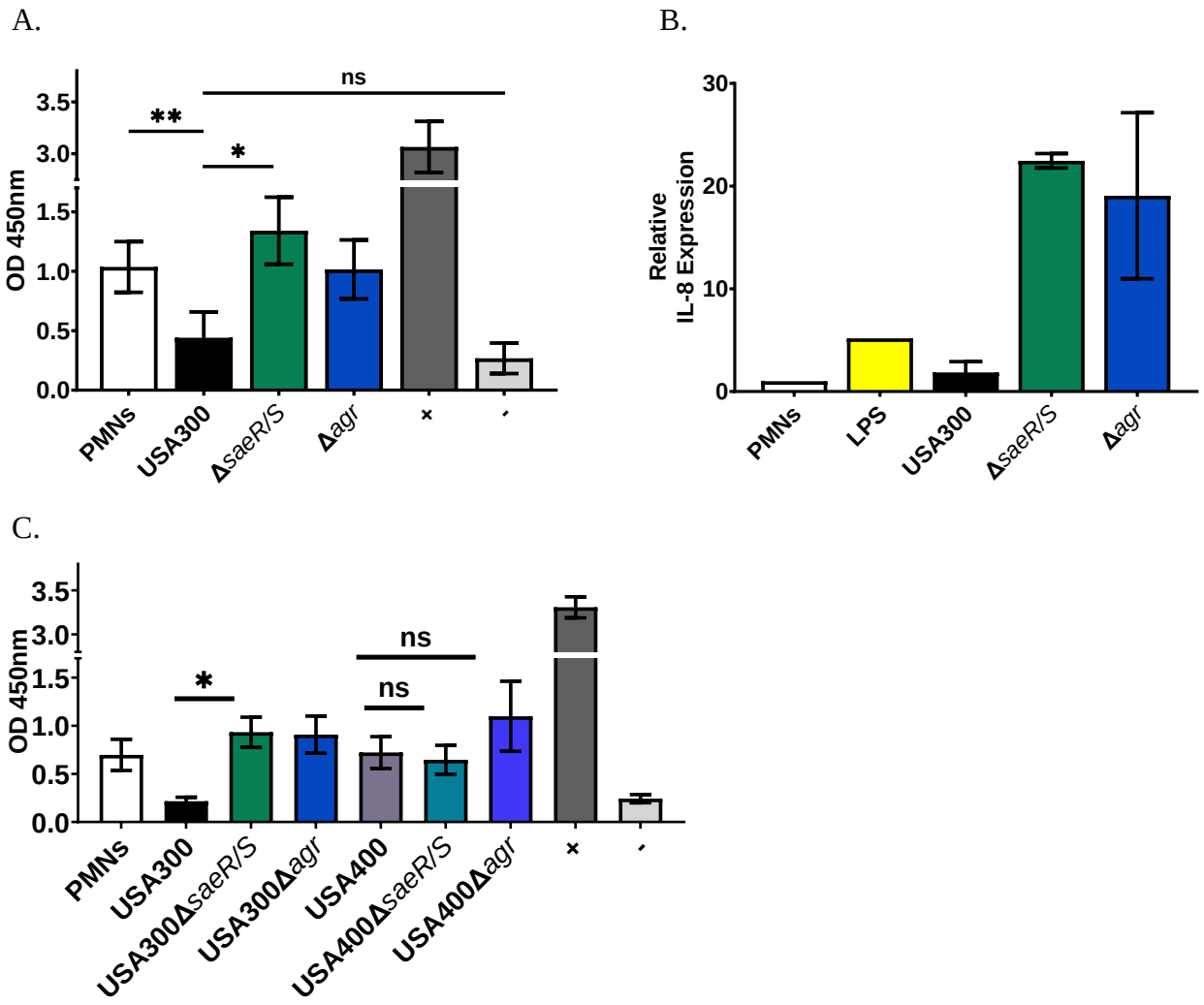


Figure 3.1. A USA300, secreted factor, is a potent NF-κB inhibitor. A) Concentrations of phosphorylated p65 (P+ S536) as determined by ELISA for $1x10^6$ PMNs incubated with supernatant for two hours, and analyzed at OD 450nm. Data are mean ± SEM of four independent experiments. B) TaqMan® RT-PCR data of relative IL-8 expression normalized to GAPDH of resting human PMNs after a two-hour incubation with supernatant. Data are mean ± SEM of two independent experiments. C) Relative concentrations of total p65 in USA300 and USA400 strains as measured by ELISA for 1x106 PMNs incubated with supernatant for two hours and analyzed at 450nm. Data are mean ± SEM of five independent experiments. –, negative control and +, positive control. *p≤0.05 and **p≤0.001 as determined by one-way repeated-measures ANOVA with Tukey's post-test.

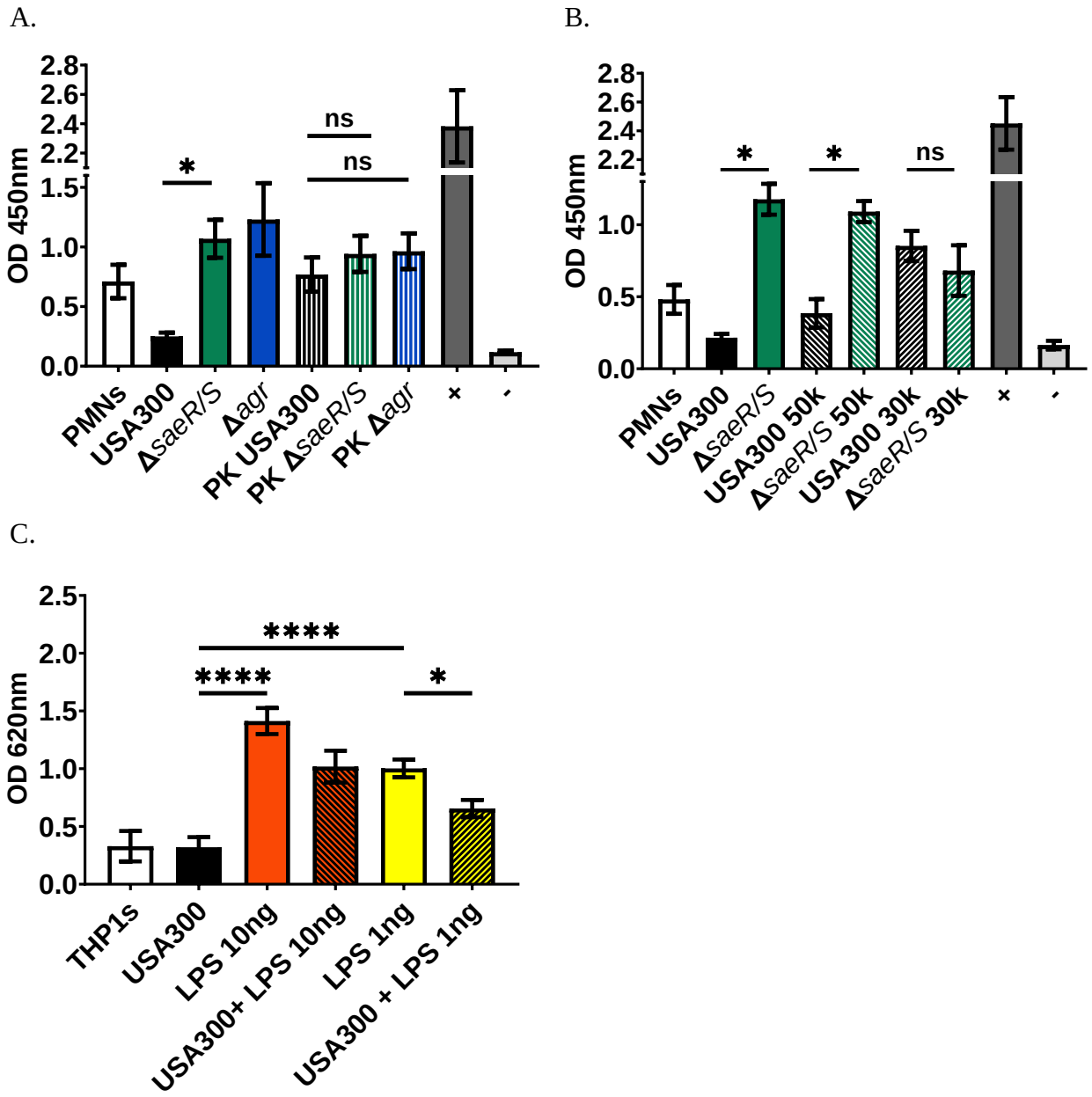


Figure 3.2. NF-κB expression in PMNs is reduced by a SaeR-regulated protein ~30k to 50kDa in size. A) Relative concentrations of phosphorylated p65 (P^+ S536) as determined by ELISA for $1x10^6$ PMNs incubated with supernatant for two hours (A and B). A) following proteinase K treatment (PK). Data is a mean ± SEM of five independent experiments. B) Relative concentrations of phosphorylated p65 (P+ S536) as determined by ELISA WT and Δ*saeR/S* supernatant was filtered with either the standard 0.2μM filter, a 50kDa filter, or a 30kDa filter, as described in Materials and Methods. Data is a mean ± SEM of six independent experiments. C) THP1-Blue™ NF-κB cells were incubated with 50kDa filtered1:30 WT supernatant, LPS 10ng, LPS 1ng, or a combination thereof, for 24 hours, then developed at OD 620nm. Data is a mean ± SEM of six independent experiments. -, negative control, +, positive control, *p≤0.05, **p≤0.01, *** p≤0.001, ****p≤0.0001, ns, not significant, as determined by one-way repeated-measures ANOVA with Tukey's post-test.

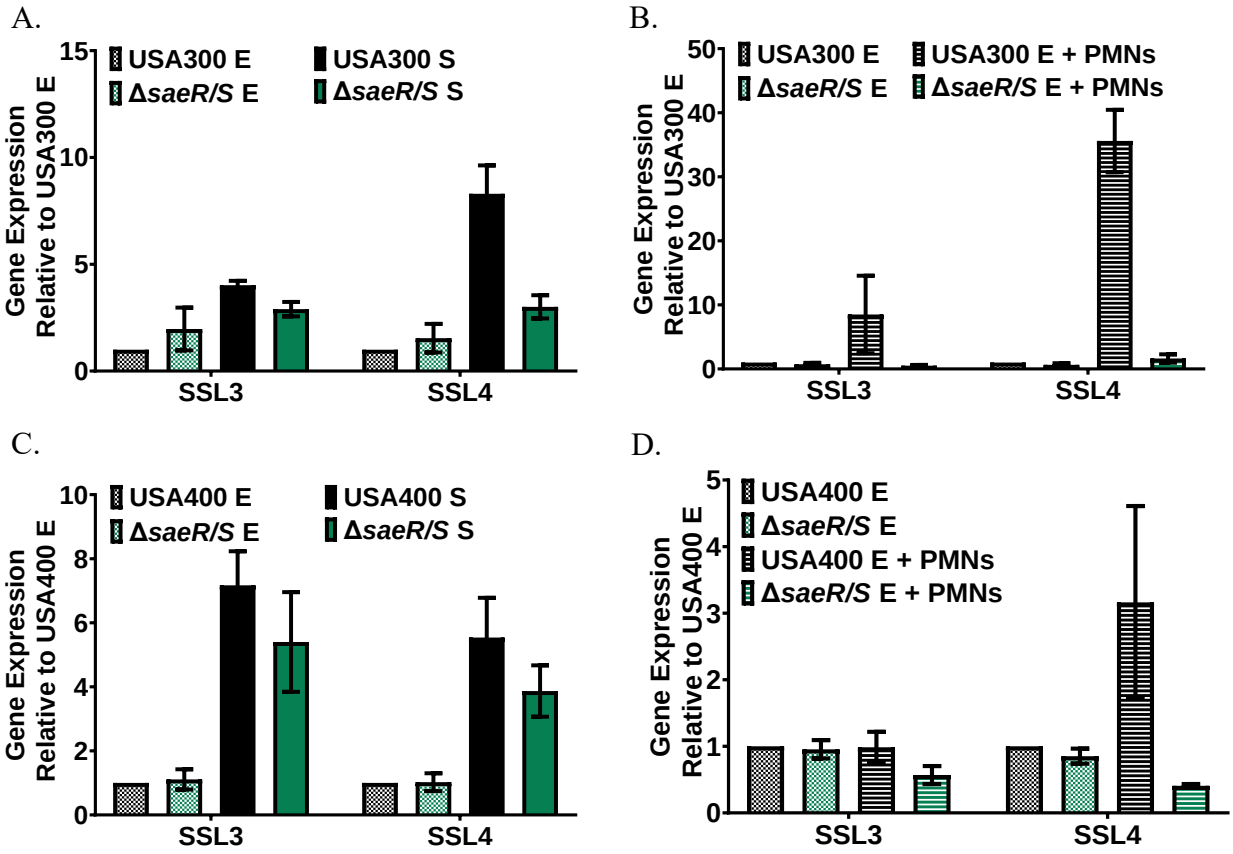


Figure 3.3. *ssl3* and *ssl4* relative transcript abundance in USA300 and USA400 strains. TaqMan® RT-PCR data shown is normalized to *gyrB* and relative to USA300 WT (A, B) or USA400 WT (C, D) at exponential growth (E). A) *ssl3* and *ssl4* transcript abundance in USA300 during exponential growth (E) and stationary growth (S). B) USA300 *ssl3* and *ssl4* expression during exponential growth (E) only or following challenge with 1 x 10^6 PMNs. C) *ssl3* and *ssl4* transcript abundance in USA400 during exponential growth (E) and stationary growth (S). D) Transcript abundance of USA400 *ssl3* and *ssl4* during exponential growth (E) only or following challenge with 1 x 10^6 PMNs. Samples were analyzed in triplicate and results are from two biological replicates, error bars represent mean ± SEM.

A.

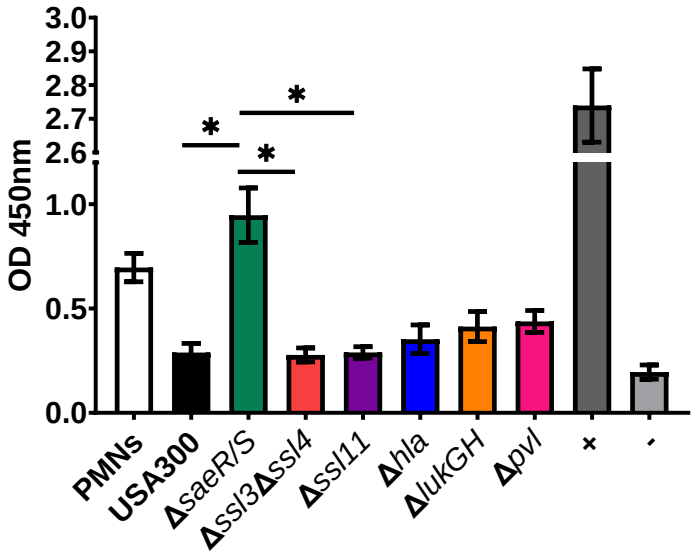
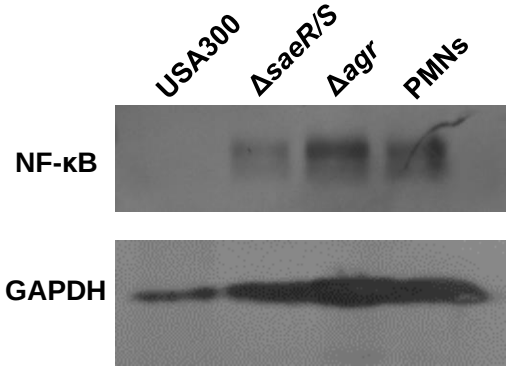


Figure 3.4. Comparison of USA300 NF-κB phosphorylation in USA300 isogenic mutant strains. Total amounts of phosphorylated p65 (P^+ S536) as determined by ELISA for $1x10^6$ PMNs incubated with supernatant for two hours. Data are mean ± SEM of nine independent experiments. -, negative control, and +, positive control. *$p \leq 0.05$ as determined by one-way repeated-measures ANOVA with Tukey's post-test.

A.



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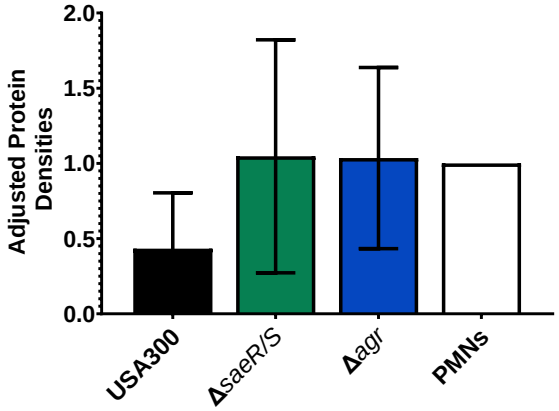
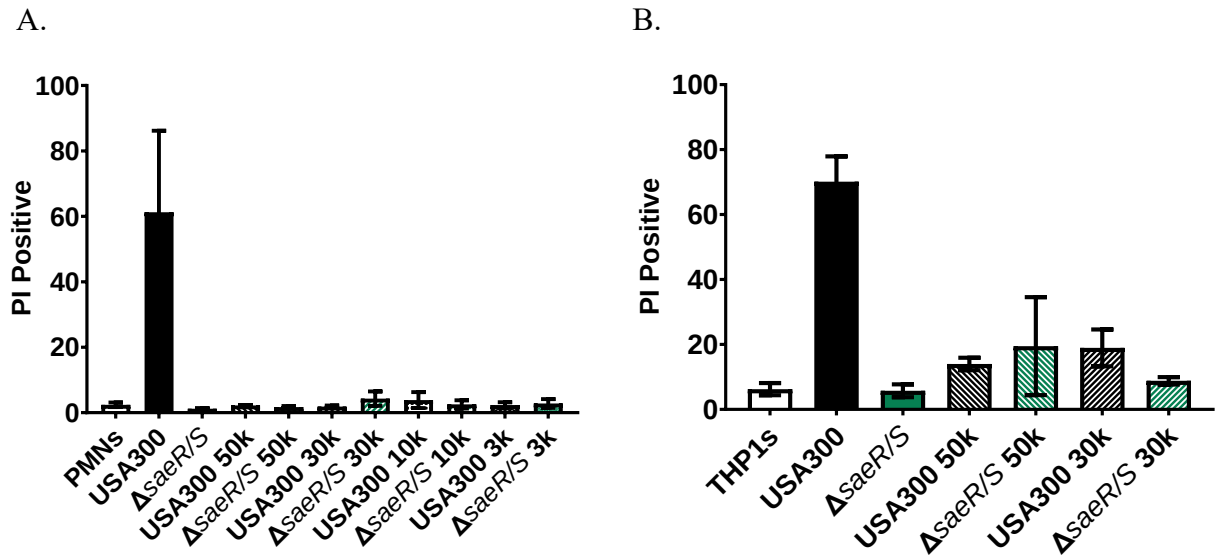


Supplemental Figure 3.1. Wild type supernatants deduce the amount of NF-κB protein. A) Representative Western blot of p65 protein from data collected in B. B) Representative Western blot of p65 protein from data collected in A. Diluted supernatant (1:30) from late exponential cultures from WT USA300 significantly reduces the p65 protein compared to USA300Δ*saeR/S*. Quantification of p65 using densitometry analysis after $1x10^6$ PMNs were stimulated with diluted supernatant for two hours. Data shown are amounts relative to resting PMNs, normalized to GAPDH density, and is presented as mean ± SEM of three independent experiments.



Supplemental Figure 3.2. Plasma membrane damage in either PMNs or THP1-Blue™ NF-κB cells after exposure to *S. aureus* supernatants. A) PMNs were incubated for two hours with supernatants following filtration as described previously. PMNs were then incubated with propidium iodide (PI), and examined for plasma membrane permeability via flow cytometry. Data is a mean ± SEM of two independent experiments. B) THP1-Blue™ NF-κB cells were incubated for 24 hours with supernatants following filtration with, 0.2μ M or 50kDa, or 30kDa then incubated with PI, and examined for cell death via flow cytometry. Data is a mean ± SEM of two independent experiments.

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USA300 and USA400 SSL3 Protein Alignments
97.8% identity in 356 residues overlap; Score: 1744.0; Gap frequency: 1.1%

USA300          1 MKMRTIAKTSLALGLLTTGAITVTTQSVKAEKIQSTKVDKVPTLKAERLAMINITAGANS
USA400          1 MKMRTIAKTSLALGLLTTGAITVTTQSVKAEKIQSTKVDKVPTLKAERLAMINITAGANS
                  ************************************************************

USA300         61 ATTQAANTRQERTPKLEKAPNTNEEKTSASKIEKISQPKQEEQKTLNISATPAPKQEQSQ
USA400         61 ATTQAANTRQERTPKLEKAPNTNEEKTSASKIEKISQPKQEEQKTLNISATPAPKQEQSQ
                  ************************************************************

USA300        121 TTTESTTPKTKVTTPPSTNTPQPMQSTKSDTPQSPTIKQAQTDMTPKYEDLRAYYTKPSF
USA400        121 TTTESTTQQTKMTTPPSTNTPQPMQSTKSDTPQSPTIKQAQTDMTPKYEDLRAYYTKPSF
                  *******  ** ********************************************

USA300        181 EFEKQFGFMLKPWTTVRFMNVIPNRFIYKIALVGKDEKKYKDGPYDNIDVFIVLEDNKYQ
USA400        181 EFEKQFGFLLKPWTTVRFMNVIPNRFIYKIALVGKDEKKYKDGPYDNIDVFIVLEDNKYQ
                  ******** ************************************************

USA300        241 LKKYSVGGITKTNSKKVNHKVELSITKKDNQGMISRDVSEYMITKEEISLKELDFKLRKQ
USA400        241 LKKYSVGGITKTNSKKVNHKVELSITKKDNQGMISRDVSEYMITKEEISLKELDFKLRKQ
                  ************************************************************

USA300        301 LIEKHNLYGNMGSGTIVIKMKNGGKYTFELHKKLQEHRMA----GTNIDNIEVNIK
USA400        301 LIEKHNLYGNMGSGTIVIKMKNGGKYTFELHKKLQEHRMADVIDGTNIDNIEVNIK
                  ***************************************    ************
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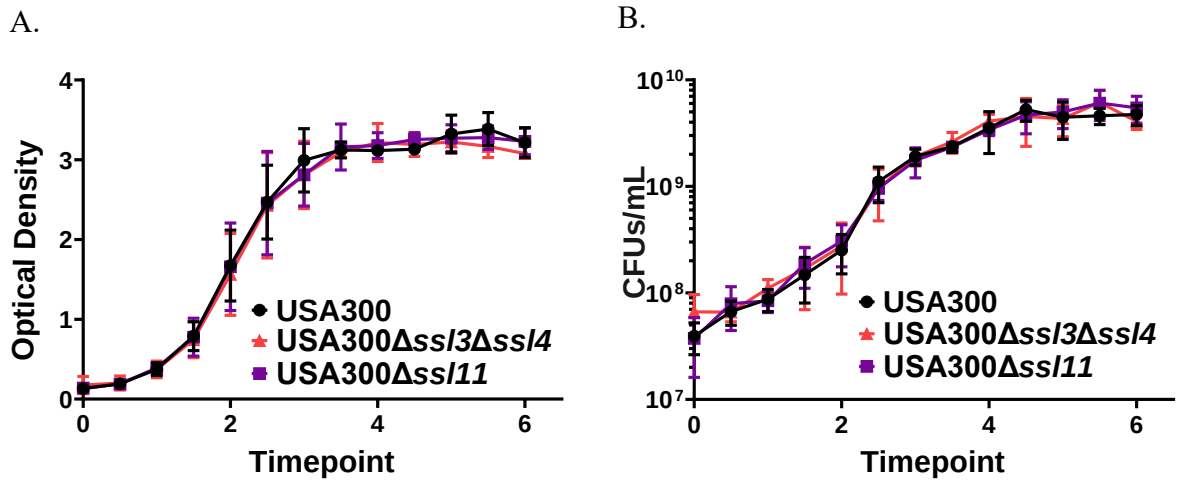
Supplemental Figure 3.3. SSL3 in USA300 and USA400, when aligned by BLAST, are 98% identical. Yellow indicates the 12 differences between the two stains.



Supplemental Figure 3.4. USA300, USA300Δ*ssl3*Δ*ssl4*, and USA300Δ*ssl11* demonstrate no difference in growth. A) The three aforementioned strains showed no significant difference in growth as shown by OD600 measurements taken every half hour for six hours. B) The three aforementioned strains showed no significant difference in growth as calculated by CFUs/mL every half hour for six hours. Data is a mean ± SEM of three independent experiments.

Table 1. Primers and probes used in this study.

Primer	Sequence	Description
Construction of USA300Δ*ssl3*Δ*ssl4*, USA300Δ*ssl11*, and USA300Δ*lukGH*.		
Forward, Top	5'c-GGGGACAAGTTTGTACAAAAAAGCAGGCcagttcatgccgaaaagaaacc -3'	USA300, *ssl3*
Reverse, Top	5'- GGTGGTGCATGCgttaaaagccctagtgctaaactg -3'	USA300, *ssl3*
Forward, Bottom	5'- GGTGGTGCATGCgcagatgtcatagatggcac -3'	USA300, *ssl4*
Reverse, Bottom	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTctaccgtcaatgacgtcac -3'	USA300, *ssl4*
Forward, Top	5'- GGG GAC AAG TTT GTA CAA AAA AGC AGG C ctc acaggaactg cgattc -3'	USA300, *ssl11*
Reverse, Top	5'- GGTGGTGCATGCccctagtgctaaacttgctttag -3'	USA300, *ssl11*
Forward, Bottom	5'- GGT GGT GCA TGC gggtga tgttattgat ggcag -3'	USA300, *ssl11*
Reverse, Bottom	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTcttcaatgcctcatcttcgtc -3'	USA300, *ssl11*
Forward, Top	5'- AAA CTG AAG GgA GCT cCT CAT CAA C -3'	USA300, *lukGH*
Reverse, Top	5'- AGT GTA TGg ATC cTG TTT GAG TGT AGA G -3'	USA300, *lukGH*
Forward, Bottom	5'- TGA TAT GTg TCG ACA TGT GAA TAA TAT CAC -3'	USA300, *lukGH*
Reverse, Bottom	5'- TAA TCT AAG CTT TCC ATG AGG CAA TTC -3'	USA300, *lukGH*
Taqman® primer/probe sequences		
Forward	CTTGGCAGCCTTCCTGATTT	Human, *il-8*
Reverse	GGGTGGAAAGGTTTGGAGTATG	Human, *il-8*
Probe	6FAMCAGCTCTGTGTGAAGGTGCAGTTTTAMRA	Human, *il-8*
Forward	CGCTTAAAGCAGAGCGATTAG	USA300, *ssl3*
Reverse	GTGCGTTCTTGTCTTGTGTTAG	USA300, *ssl3*
Probe	AGCAGGTGCAAATTCAGCGACAAC	USA300, *ssl3*
Forward	CAACACCGCCCTCAACTAAA	USA300, *ssl4*
Reverse	TGTTGTCGCATTTGCTGTTTG	USA300, *ssl4*
Probe	CGACAACACCGCCCTCAACTAAAGT	USA300, *ssl4*
Forward	CGCTTAAAGCAGAGCGATTAG	USA400, *ssl3*
Reverse	GTGCGTTCTTGTCTTGTGTTAG	USA400, *ssl3*
Probe	AGCAGGTGCAAATTCAGCGACAAC	USA400, *ssl3*
Forward	GCAACAACACCATCTTCAACTAA	USA400, *ssl4*
Reverse	TGATTGCGGTGCTTCTACTT	USA400, *ssl4*
Probe	CAGCAAACGCGACAACACCATCTT	USA400, *ssl4*

Table 2. Approximate sizes of proteins knocked out in the strains seen in Figure 3.4.

Strain	Protein Size	Source
ssl3	39kDa	WP_000784024.1 size converter AA to kDa
ssl4	34kDa	WP_000705644.1 size converter AA to kDa
ssl11	24.98kDa	WP_000769163.1 size converter AA to kDa
hla	Each monomer is 33.2 kDa	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6110284/ Du et al., 2018
lukGH	LukG 37kDa, LukH 35kDa	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3958006/ DuMont et al., 2014
pvl	LukS 33kDa, LukF 34kDa	https://pubmed.ncbi.nlm.nih.gov/15170101/ Kaneko et al., 2004

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CHAPTER FOUR

CONCLUSIONS AND FUTURE STUDIES

S. aureus is a ubiquitous bacteria known for its role as a pervasive pathogen, and as a member of the normal microbiota of the anterior nares and skin. Its ability to incorporate new antibiotic resistance mechanisms, and its ability to kill immune cells make it a formidable foe, one which, despite ongoing efforts still have not resulted in a vaccine. It is critical to understand how *S. aureus* interacts with its human host, both in colonization and pathogenic contexts in order to better understand what is needed to give the human immune system an 'edge' and avoid pathogenicity.

Chapter Two of this dissertation described a method for isolating neutrophils from whole human blood (Dankoff et al., 2020). This methods manuscript included the description of two neutrophil and *S. aureus* interaction assays. One of the methods described how to evaluate the cytotoxicity of *S. aureus* after phagocytosis by neutrophils. Cytotoxicity is measured by flow cytometry examining cell death through propidium iodide uptake. The other method, which is the basis for most of the experiments in Chapter Three, also uses neutrophils, but examines *S. aureus* cytotoxicity of extracellular proteins found in *S. aureus* supernatant. Once again, cell death is evaluated by flow cytometry and propidium iodide uptake.

Chapter Three included data that indicated a SaeR/S-regulated protein inhibits NF-κB activation, the findings are summarized in Figure 4.1. The basis of Chapter Three was that of a follow up study to Zurek et al., a previous publication from this lab (Zurek et al., 2015). This previous study documented a USA300 SaeR/S mediated mechanism of NF-κB repression, which led to accelerated apoptosis, along with reduced IL-8 production (Zurek et al., 2015). The main

objective of Chapter Three was to further characterize this repressive ability, with the goal of identifying the *S. aureus* factors responsible and to examine the mechanisms NF-κB mediated cell death. We hypothesized that the NF-κB mediated repression was caused by a secreted protein, and that this repressive activity was independent of the *S. aureus* mediated cytotoxicity described in Chapter Two. To test this, I did a simple experiment incubating neutrophils with diluted *S. aureus* supernatant, then did a Western blot to assess levels of overall NF-κB protein. This showed a reduction in overall NF-κB protein in the conditions incubated with wild type supernatant compared to the Δ*saeR/S* or Δ*agr* supernatant. These data demonstrated that NF-κB repression is mediated by SaeR/S, and to some degree Agr. To investigate if a reduction in NF-κB protein reduces the overall amount of activated NF-κB, we used the same setup as before, but assessed phosphorylated NF-κB with an ELISA, which confirmed that neutrophils incubated with wild type supernatant compared to resting, Δ*saeR/S*, or Δ*agr* supernatant had less activated NF-κB. To add to the findings of Zurek et al., we looked at the ability of wild type supernatant to impede IL-8 production via qRT-PCR, and saw that the neutrophils incubated with wild type *S. aureus* had IL-8 transcript levels at two hours similar to resting, while the Δ*saeR/S* and Δ*agr* groups had a twenty fold increase in IL-8 transcript abundance over resting neutrophils. The observed phenotype was not conserved in the USA400 *S. aureus*. Additional experiments identified the *S. aureus* factor to be a secreted protein, approximately 30-50kDa in size. In addition, we saw that the repressive ablitity of this factor is very potent and a 1:30 dilution of supernatant could inhibit LPS induced NF-κB activation in a human monocyte line.

As discussed in Chapter 1 it is difficult to compare our results with previously published observations due to the differences in experimental designs. For example, time points analyzed

strongly influence NF-kB expression. Shorter stimulatory time periods, here which would be reflective of initial host pathogen interactions, typically result in activation and then suppression of NF-κB behaviors, while longer time points reflect robust inflammatory response, increases in NF-κB, and extended life spans of neutrophils (Hayden & Ghosh, 2011; Mitchell et al., 2016). Other differences in outcomes of experiments reflect cell lines, human donor variation (Janesch et al., 2017), or mice strains used to measure an outcome. Many papers, for example, utilize C57BL/6J mice, and according to Opdenbosch et al., 2019, they have a 'differential inflammasome response' due to a mutated *Nlrp1b* allele (Opdenbosch et al., 2019), therefore potentially skewing inflammation results. Additionally, mouse and rat species do not have caspase-10, which plays a part in apoptosis (Opdenbosch & Lamkanfi, 2019) and also respond differently to *S. aureus* toxins due to differences in receptors between human and mouse cells.

Much like how the differences between lab strain K12 *E. coli* and enterohemorrhagic O157:H7 *E. coli* make them not interchangeable for experiments, *S. aureus* strains cover such a breadth of differences that results from one strain do not necessarily apply to another. The history of evolution for hospital-associated (HA) MRSA and community-associated (CA) MRSA is covered extensively in a review by Uhlemann et al., but in brief, HA-MRSA infections historically were obtained in the hospital by immuno-compromised individuals. Over the last 20 years, there have been the emergence of more virulent CA-MRSA strains that infect otherwise healthy individuals (Uhlemanne et al., 2014). These CA-MRSA strains are commonly classified by pulsed field gel electrophoresis types, into groups such as and not limited to USA400, EMRSA-15, MRSA252, and the most common, USA300 (Uhlemann et al., 2014). Common laboratory strains include SH1000, and RN4220. Strain Newman is often utilized in assays and it has a mutation in

SaeS making the SaeR/S TCS perpetually turned ‘on’. Differences between these strains are vast, from phenotypes seen in infections, to prophage encoded virulence factors, preference for host species, eccentricities concerning individual regulation of virulence factors, and truncation of prominent virulence factors (reviewed in greater depth in (Alonzo et al., 2014; Guerra et al., 2017; Turner et al., 2019; Uhlemann et al., 2014)). In addition to strain variability, the media the *S. aureus* is grown in can affect the production of virulence factors. Additionally, growth phase and MOI, are factors that can influence how host cells respond to this multifaceted pathogen. It is important to keep these considerations in mind when reviewing conflicting studies.

Identifying the Protein(s) Involved in NF-κB Suppression

Many previous studies had described the *S. aureus* proteins of SSL3 and SSL4 as inhibitors of the NF-κB pathway by binding to TLR1/2 (Koymans, Feitsma, Harma Brondijk, et al., 2015; Koymans et al., 2017; Yokoyama et al., 2012). When analyzing SSL3 and SSL4 production under a variety of conditions, from overnight growth to *S. aureus* activation by neutrophils, we did show SaeR/S mediated control of *ssl3* and *ssl4* transcript when exposed to neutrophils. What we did not see, was SaeR/S mediated production of *ssl3* and *ssl4* transcript in overnight growth, which was the source of the supernatant used in our studies. Additionally, we had not seen the suppressive activity conserved in USA400 a strain that also produces *ssl3* and *ssl4* transcript. Together, this evidence indicates that SSL3 and SSL4 are not the primary factors repressing NF-κB in the context of our experiments. Additionally, in ELISA experiments using a Δ*ssl3*Δ*ssl4* isogenic mutant strain NF-κB was not restored to the levels observed in the Δ*saeR/S* mutant strain. Other isogenic knockout strains tested included Δ*ssl11*, Δ*hla*, Δ*lukGH*, and Δ*pvl*. SSL11 has a history of repression neutrophil migration (Chen et al., 2019), which is known to be partially controlled by

NF-κB. Additionally, the individual toxins tested have sizes between 30-50kDa. Unfortunately, none of the strains tested caused an activation of NF-κB activity, and therefore I did not find the mystery SaeR/S mediated repressive protein. Therefore, although we did not identify the protein responsible for repressing NF-κB activation and subsequent IL-8 production we demonstrated that the NF-κB repression is caused by a secreted protein, 30-50kDa in size, and that it is not conserved in the clinically relevant strain USA400. Together, these hints will pave the way for future studies to define the mechanism of SaeR/S mediated NF-κB repression.

Future Studies: Cell Death, Imagestream Work, NETosis:

The overarching theme of my research was always about how the context of neutrophil interactions with *S. aureus* leads to different modes of cell death. As briefly discussed in the introduction, neutrophils are well-known for their ability to undergo cell death in a variety of dramatic ways. There are a number of great reviews available on cell death, and neutrophil cell death in specific (Fox, Leitch, Duffin, Haslett, & Rossi, 2010; Grootjans et al., 2017; Kobayashi et al., 2017; X. Wang et al., 2018; Yipp & Kubes, 2013), but here I wanted to highlight three mode of cell death. These are apoptosis, necroptosis, and NETosis. Apoptosis, or spontaneous cell death, can occur after either phagocytosis and killing of a pathogen (PICD), or as a result of the neutrophil reaching the end of its short life (Kobayashi et al., 2017). This process is critical to maintaining homeostasis in the host as it prevents leakage of inflammatory products into the host tissue. Early studies defined apoptosis by several hallmarks, including condensation of chromatin, membrane blebbing, phosphatidylserine (PS) on the outer plasma membrane, and gradual disruption in membrane integrity (Krysko, Vanden Berghe, D'Herde, & Vandenabeele, 2008; Savill et al., 1989). Membrane integrity can readily be examined with propidium iodide uptake via flow

cytometry (Dankoff et al., 2020), and nuclear condensation can be observed through microscopy (Fox et al., 2010; Savill et al., 1989). Another infamous mode of cell death is necroptosis, or regulated necrosis, which is dependent on MLKL, RIPK1, and many other mediators (reviewed in (Pasparakis & Vandenabeele, 2015)). Hallmarks include rapid swelling of the cytoplasm, intact nuclei, and after membrane rupture, secretion of intracellular components {reviewed in (Krysko et al., 2008)}. The differences between apoptosis and necroptosis can be leveraged, and examined to some degree by traditional flow cytometry, but the differences are even more clear cut when using imaging flow cytometry (Krysko et al., 2008; Pietkiewicz, Schmidt, & Lavrik, 2015).

The majority of this dissertation examined neutrophil cell death in the context of apoptosis, and these studies are setting the stage for an investigation into necrosis. The pathways of apoptosis and necroptosis intersect at NF-κB. In a closed conformation, RIPK1 activates NF-κB and is therefore anti-apoptotic, but in an open conformation, promotes apoptosis and necroptosis {reviewed in (Grootjans et al., 2017)}. This relationship is summarized in Figure 4.2, and future studies will be geared towards better understanding how host-pathogen interactions influence cell death. My research described a mechanism by which *S. aureus* USA300 uses a secreted SaeR/S mediated protein to suppress NF-κB activity in human neutrophils and monocytes, thereby inhibiting the production of IL-8. To understand which protein is responsible for the phenotype described and how repression of NF-κB affects ultimate cell death, several follow up experiments are necessary.

The original goal of the study was to determine how NF-κB repression is linked to altered cell death in neutrophils. Upon confirmation of the identity of the protein and creation of an isogenic mutant strain, experiments could then be done to confirm how the factor influences cell

death. While a previous study in our lab demonstrated the link between NF-κB repression and accelerated apoptosis (Zurek et al., 2015), other papers have demonstrated a link between USA300 activity and neutrophil necroptosis (Greenlee-Wacker et al., 2017, 2014). There are observations by others that support NF-κB repression as a starting point for a necrotic outcome (X. Wang et al., 2018). Cell death could be assessed using an assay described by Pietkiewicz et al., where apoptosis and necroptosis can be distinguished from each other on the single cell level by use of annexin V and propidium iodide staining with an Imaging Flow Cytometer (Pietkiewicz et al., 2015). By leveraging the differences between the apoptotic and necroptotic cell death pathways, gates distinguishing between different nuclear morphologies and key features of the cell membrane can differentiate between these different kinds of cell death. Different isogenic *S. aureus* mutants could be screened, to better understand the protein players in inducing necrosis.

The way in which *S. aureus* modulates cell death is incompletely understood. By further exploring how *S. aureus* inhibits NF-κB activity, and how this is related to subsequent cell death, we will have a better understanding of how the host and this iconic pathogen coexist.

Role of NETosis in *S. aureus* Neutrophil Interactions

As part of my dissertation, I performed preliminary studies on how *S. aureus* induces NETosis in neutrophils. There are several different types of NETosis, all stimuli dependent. These include canonical or suicidal NETosis, vital NETosis, and a form of NETosis where only mitochondrial DNA is extruded from the cell. These different types of NETosis and their hallmarks are reviewed in detail (Delgado-Rizo et al., 2017). *S. aureus* is able to induce the rapid and ROS-independent version of NETosis called vital NETosis (Pilsczek et al., 2010), although the mechanism of how this is done is still unknown. Additionally, bicomponent leukotoxins, have

been linked to NET formation (Bhattacharya et al., 2018; Malachowa, Kobayashi, Freedman, Dorward, & DeLeo, 2013). The SaeR/S mediated protein EAP has been implicated in NET formation, although the mechanism behind this is unclear (Eisenbeis et al., 2018). To examine NETosis I developed a method to differentiate between normal neutrophils and pre-vital neutrophils by examining cell morphology through imaging flow cytometry. Much like the cell death types above could be differentiated by key differences, the differences between NETosis types can be leveraged to investigate this understudied cell death pathway (Figure 4.3). By utilizing this novel screening method, and through the use of various NET-centric quantification assays, I began to examine how *S. aureus* reprograms neutrophils to induce vital NETosis over suicidal NETosis. Specifically, I investigated the hypothesis that the SaeR/S mediated virulence factors induced vital NETosis through leukotoxin mediation of ROS and prevent suicidal NETosis through SPIN and EAP which may be inhibiting the translocation of MPO and NE, respectively (de Jong et al., 2017; Eisenbeis et al., 2018; Nauseef & Kubes, 2016; Pilsczek et al., 2010; Yipp et al., 2012). My preliminary studies were inconclusive and additional studies are needed to resolve the key players in *S. aureus* induced NETosis. However, these experiments did identify a technique that might be utilized in future experiments to measure translocation and colocalization of neutrophil elastase and MPO in response to specific *S. aureus* virulence factors (Figure 4.4). Additional experiments could be designed to examine Vital NETosis phenotypes, both through imaging flow cytometry and NET Quantification Assays (PicoGreen reagent Quant-iT™ PicoGreen® dsDNA Detection kit, Invitrogen) and histone citrullination (Citrullinated Histone H3 ELISA Kit™, Cayman Chemical).

Figures

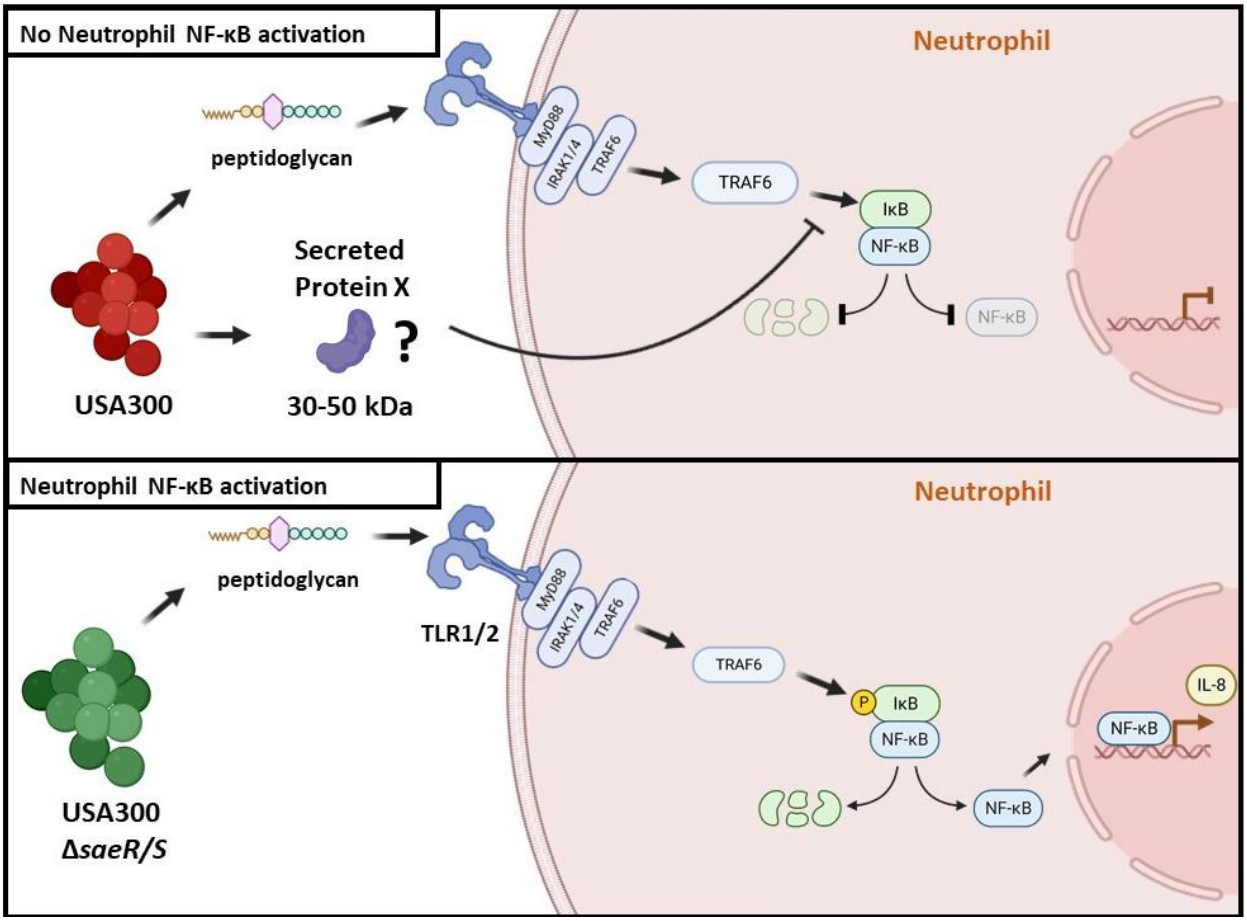

Figure 4.1. *S. aureus* USA300 inhibits NF-κB activation through an unknown secreted protein. In this model, TLR1/2 is activated by various *S. aureus* membrane proteins, such as peptidoglycan. This activation of TLR1/2 results in the activation of NF-κB and inflammatory responses like IL-8 production. Top Panel: When neutrophils are exposed to USA300 supernatants, NF-κB activation is inhibited by an unknown protein (Protein X). This protein was determined to be 30-50kDa in mass, and is secreted by USA300 *S. aureus*. Bottom Panel: USA300Δ*saeR/S* does not exhibit NF-κB inhibitory properties, and neutrophil exposure to USA300Δ*saeR/S* supernatant results in robust IL-8 production through NF-κB activation, indicating that Protein X is regulated

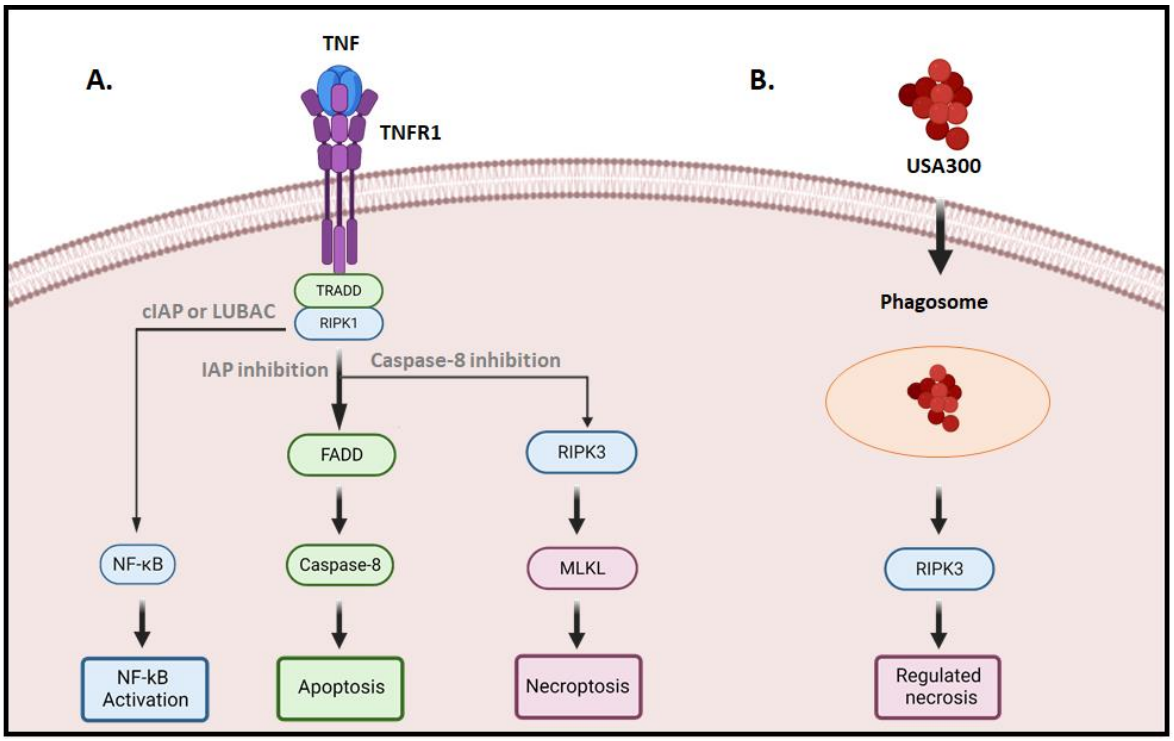

Figure 4.2. Models of cell death through apoptosis or necroptosis dependent on inhibition of the NF-κB pathway. A. As a well studied pathway, TNF-α recognition starts off a signaling pathway triggering NF-κB activation, resulting in an anti-apoptotic state. Through the inhibition of this pathway, the apoptotic pathway begins, involving caspases such as Caspase-8. Upon inhibition of Caspase-8, the necroptotic pathway is activated, involving proteins like RIPK3. B. USA300 has been shown to induce regulated necrosis, along with accelerated apoptosis, although the mechanisms behind these altered neutrophil cell death pathways remain unknown. We have shown a mechanism by which USA300 can inhibit NF-κB activation, which may be the starting point for inducing accelerated apoptosis or regulated necrosis described by others (Zurek and Greenlee-Wacker).

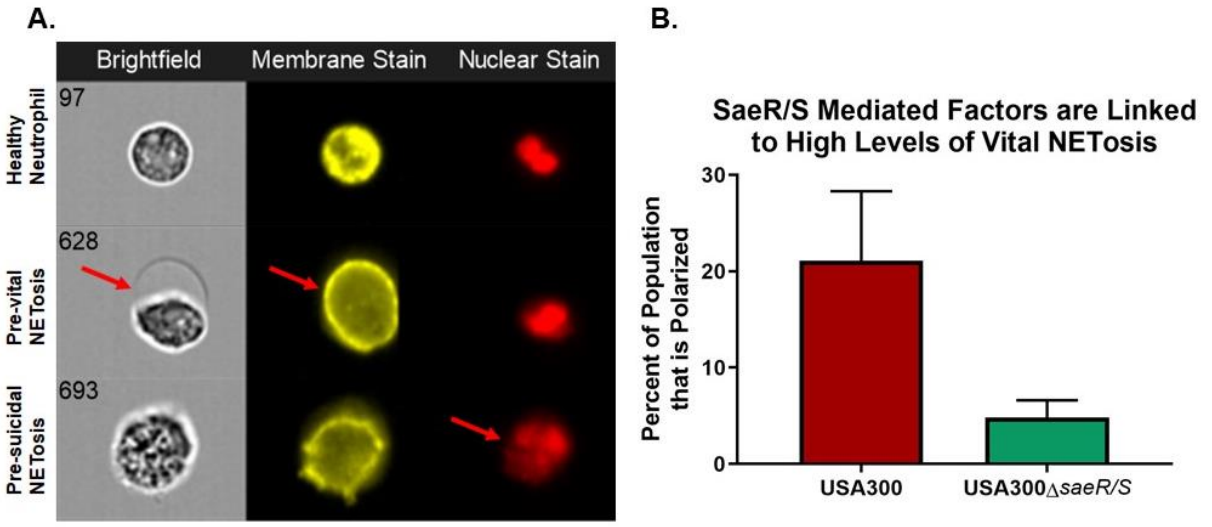

Figure 4.3. Imaging Flow Cytometry can leverage differences in human neutrophil cell morphology to quantify pre-NETosis behavior. A) Representative images demonstrating a healthy neutrophil, a pre-vital neutrophil, and a pre-suicidal neutrophil (as indicated). Red arrows indicate the main morphological differences between pre-vital and pre-suicidal NETosis. B) Morphological differences from A quantified via image analysis indicate neutrophils exposed to USA300 S. *aureus* induce high levels of polarization, indicative of vital NETosis, while neutrophils exposed to the USA300ΔsaeR/S *S. aureus* strain do not.

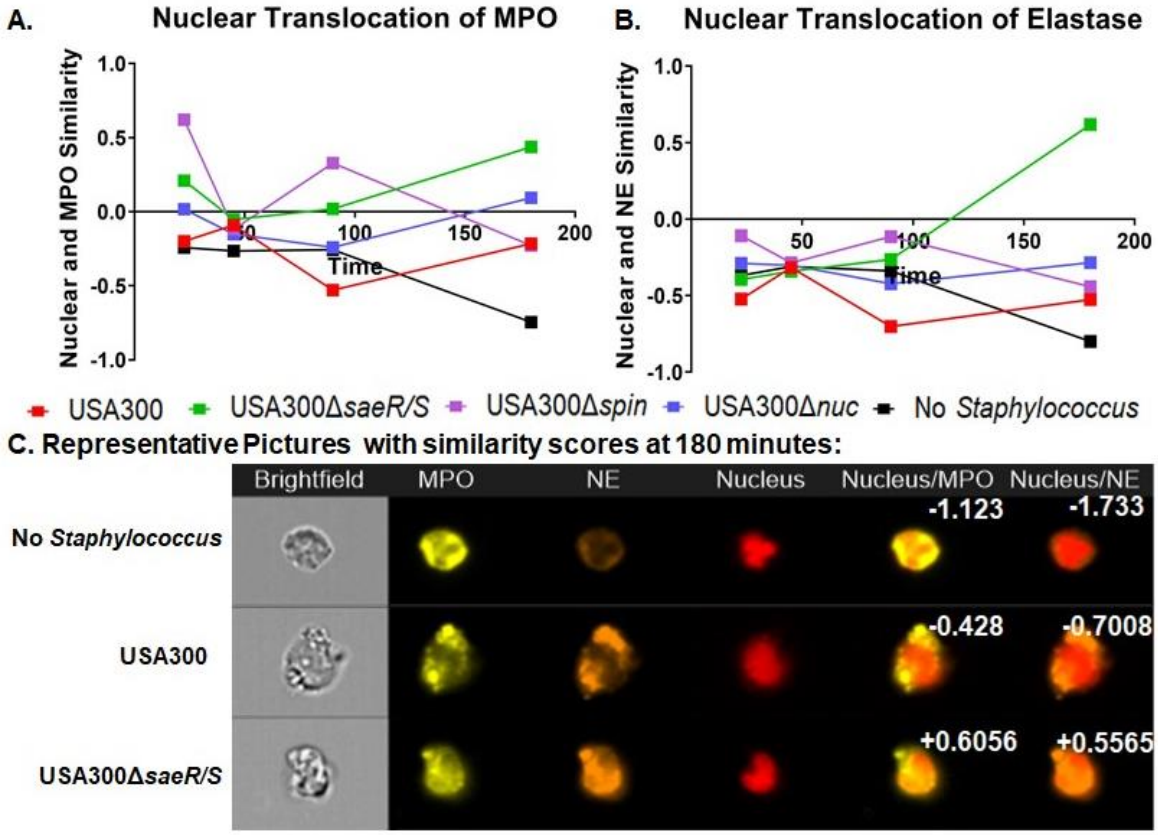

Figure 4.4. SaeR/S mediated factors inhibit pre-suicidal NETosis behavior in human neutrophils. A and B) By 180 minutes, neutrophils exposed to USA300Δ*saeR/S* has higher translocation scores for both MPO and elastase migration into the nucleus compared to USA300. C) Representative images used to generate the translocation scores shown in A and B.

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APPENDICES

APPENDIX A

CO-AUTHORED PUBLICATIONS

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ORIGINAL RESEARCH
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CD103 (αE Integrin) Undergoes Endosomal Trafficking in Human Dendritic Cells, but Does Not Mediate Epithelial Adhesion

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Dendritic cell (DC) expression of CD103, the α subunit of αEβ7 integrin, is thought to enable DC interactions with E-cadherin-expressing gastrointestinal epithelia for improved mucosal immunosurveillance. In the stomach, efficient DC surveillance of the epithelial barrier is crucial for the induction of immune responses to *H. pylori*, the causative agent of peptic ulcers and gastric cancer. However, gastric DCs express only low levels of surface CD103, as we previously showed. We here tested the hypothesis that intracellular pools of CD103 in human gastric DCs can be redistributed to the cell surface for engagement of epithelial cell-expressed E-cadherin to promote DC-epithelial cell adhesion. In support of our hypothesis, immunofluorescence analysis of tissue sections showed that $CD103^+$ gastric DCs were preferentially localized within the gastric epithelial layer. Flow cytometry and imaging cytometry revealed that human gastric DCs expressed intracellular CD103, corroborating our previous findings in monocyte-derived DCs (MoDCs). Using confocal microscopy, we show that CD103 was present in endosomal compartments, where CD103 partially co-localized with clathrin, early endosome antigen-1 and Rab11, suggesting that CD103 undergoes endosomal trafficking similar to β1 integrins. Dynamic expression of CD103 on human MoDCs was confirmed by internalization assay. To analyze whether DC-expressed CD103 promotes adhesion to E-cadherin, we performed adhesion and spreading assays on E-cadherin-coated glass slides. In MoDCs generated in the presence of retinoic acid, which express increased CD103, intracellular CD103 significantly redistributed toward the E-cadherin-coated glass surface. However, DCs spreading and adhesion did not differ between E-cadherin-coated slides and slides coated with serum alone. In adhesion assays using E-cadherin-positive HT-29 cells, DC binding was significantly improved by addition of Mn^{2+} and decreased in the presence of EGTA, consistent with the dependence of integrin-based interactions on divalent cations. However, retinoic acid failed to increase DC adhesion, and a CD103 neutralizing

antibody was unable to inhibit DC binding to the E-cadherin positive cells. In contrast, a blocking antibody to DC-expressed E-cadherin significantly reduced DC binding to the epithelium. Overall, these data indicate that CD103 engages in DC-epithelial cell interactions upon contact with epithelial E-cadherin, but is not a major driver of DC adhesion to gastrointestinal epithelia.

Keywords: gastrointestinal epithelium, integrin alpha E, antigen-presenting cell, cell adhesion, endosome

INTRODUCTION

Dendritic cells (DCs) frequently interact with the epithelial layer of the gastric mucosa, as shown in previous studies (1–3). As professional antigen-presenting cells, DCs control the immune response to *Helicobacter pylori* (*H. pylori*) (4), a bacterial pathogen that causes chronic gastritis, peptic ulcer disease, and gastric cancer (5–7). Specifically, the type of T cell response induced by the DCs largely determines whether *H. pylori* infection causes only mild inflammation or leads to severe inflammatory pathologies including ulcers or cancer (8–10). For those DCs that are located immediately beneath or within the gastric epithelium, their spatial interactions with the epithelial cells have important functional implications for the immune response to *H. pylori*. First, DCs that reside within the epithelial layer or extend transepithelial dendrites have direct access to the gastric lumen for *H. pylori* antigen sampling (1–3, 11). Second, positioning of gastric DCs immediately below the epithelium increases the probability for pathogen capture upon epithelial barrier breach, and third, the close proximity of DCs to epithelial cells likely enhances the paracrine effects of epithelial-derived mediators that regulate DC function (12–14). In spite of the importance of DC-epithelial interactions for gastrointestinal immune responses, the molecular mechanisms of these interactions are not well-defined.

Binding of DC-expressed CD103 (αEß7 integrin) to epithelial E-cadherin was proposed as a potential mechanism for DC adhesion to epithelial cells (15–17). CD103, the α subunit of αEß7 integrin, is widely recognized as an important DC subset and lineage marker in humans and mice (18–20). Specifically, CD103 identifies a DC subset termed conventional DC1 that is able to cross-present exogenous antigens to CD8 T cells and that induces mucosal tolerance to commensals and dietary antigens (18, 21). The functional role of CD103 has been extensively studied in transfected cells lines, where the A-domain of the αE (CD103) integrin subunit was shown to interact with the top surface of E-cadherin domain 1, and in intestinal intraepithelial lymphocytes (IELs), where CD103 anchors the IELs within the epithelial layer (22–24). Inspite of its frequent use as a DC marker, the function of CD103 in primary human DCs has received little investigative attention. Therefore, the goal of our study was to determine whether CD103 enables DCs in the human stomach to interact with the epithelium through E-cadherin engagement.

Notably, previous studies from our laboratory and others have shown that surface CD103 expression of gastric DCs is low compared to CD103 expression on DCs in other tissue compartments, such as the small intestine (14, 25–27). This low surface CD103 expression was unexpected, since gastric DCs have a tolerogenic capacity similar to that of human intestinal DCs (14, 28) and also are efficient producers of retinoic acid (RA), properties generally associated with intestinal $CD103^+$ DC subsets (14, 29, 30). However, we also showed that human monocyte-derived DCs express considerable amounts of CD103 in intracellular compartments (26). Other integrins including α5β1, α6β4, and αMβ2 are expressed in endosomal compartments and recirculate through the membrane to enable dynamic and tightly regulated interactions with their respective ligands (31–33). Therefore, we hypothesized that intracellular pools of αE integrin/CD103 present in human gastric DCs can be redistributed to the cell surface for engagement of epithelial cell-expressed E-cadherin in the stomach to promote DC-epithelial cell adhesion. Interestingly, our experiments revealed that CD103 undergoes endosomal trafficking in human DCs and is engaged upon DC contact with epithelial E-cadherin, but is not the major adhesion factor that mediates epithelial cell binding.

MATERIALS AND METHODS

Human Blood and Tissue Samples

Heparinized blood samples were obtained with local IRB approval from healthy adult volunteers in Birmingham, AL (IRB# X120806005), or Bozeman, MT (IRB #DB082817 and #DB092614). Gastric tissue specimens were obtained with Institutional Review Board (IRB) approval and informed consent from non-*H. pylori*-infected adult subjects undergoing elective gastric bypass surgery or sleeve gastrectomy for treatment of obesity at the University of Alabama at Birmingham (IRB# F120815005) or were provided as exempt specimens by the National Disease Research Interchange (Philadelphia, PA; IRB# DB062615-EX).

Antibodies

The following mouse anti-human monoclonal antibodies were used for flow cytometry, imaging cytometry and confocal analysis of MoDCs: HLA-DR (clone L243), CD11c (B-ly6), CD103/αE (B-Ly7,) CD3 (HIT3a), CD19 (SJ25C1), CD45 (2D1), CD56 (MY31), E-cadherin (67A4), CD49d (9F10) purchased from eBioscience, Biolegend, or Tonbo, all San Diego, CA. Endosomal compartments were labeled with rabbit anti-human clathrin (D3C6, Cell Signaling, Danvers, MA), rabbit anti-human EEA-1 (polyclonal), mouse anti-human Rab7a (Rab7-117), and rabbit anti-human Rab11 (polyclonal), all from Abcam, Cambridge, MA. The following monoclonal antibodies were

used for staining of paraffin-embedded tissue sections: anti-human HLA-DR (LN-3) and anti-human CD103 [EPR4166(2)], both Abcam, Cambridge, MA. The following antibodies were used in neutralization assays: anti-human CD103 (2G5) (Beckman Coulter, France) and anti-human E-cadherin (SHE78-7) (Thermo Fisher Scientific, Waltham, MA). Appropriate isotype-matched control antibodies were used in all experiments.

Dendritic Cells

To obtain human gastric DCs, mucosal tissue was subjected to three rounds of EDTA treatment and then digested with collagenase solution to obtain lamina propria mononuclear cells, as described previously (14, 26). Gastric DCs were then enriched using MACS sorting for HLA-DR$^+$ cells (Miltenyi Biotec, Auburn, CA).

To generate monocyte-derived DCs (MoDCs), PBMCs were isolated using Ficoll density gradient centrifugation, and MoDCs were differentiated from MACS-isolated CD14$^+$ blood monocytes by culturing 2×10^6 monocytes per well in 24-well plates in complete medium (DMEM, 10% heat-inactivated human AB serum and antibiotics) or serum-free medium (X-Vivo 10, with HEPES and L-Glutamine) supplemented with recombinant human GM-CSF (25 ng/mL), and IL-4 (17 ng/mL), both from R&D Systems, Minneapolis, MN (26, 28). To enhance DC CD103 expression, 100 nM retinoic acid (RA, Sigma, St. Louis, MO) was added to some MoDC cultures, as described previously (26). Cytokines and RA were replenished after 3 days, and after 5–6 days, non-adherent cells were harvested as MoDCs by vigorous pipetting.

Immunofluorescent Labeling of Tissues and Cells for Microscopy

We used 4 μm paraffin-embedded sections to analyze CD103 expression by human gastric DCs *in situ*. Sections were de-paraffinized and then incubated in a vegetable steamer for 30 min in pre-heated Unmasking Solution (Vector Laboratories, Burlingame, CA) for antigen retrieval. Sections were then blocked in normal goat serum and incubated in the presence of primary antibodies overnight. Species specific secondary antibodies labeled with Alexa 488 or Alexa 555 (SouthernBiotech, Birmingham, AL) were added for 30 min. Finally, nuclei were stained with DAPI, and sections were mounted in Fluoroshield (Sigma-Aldrich) and sealed with nail varnish. For microscopic analysis of MoDCs, cells were stained either directly on glass-bottom plates or chamber slides (CD103 distribution and spreading assays) or were stained in suspension and then spotted onto glass slides (endosomal markers). For intracellular labeling, DCs were permeabilized with Cytofix/Cytoperm solution (Becton Dickinson) for 20 min at 4°C, washed with PermWash buffer (Becton Dickinson) and then were incubated with antibodies for 30 min at 4°C. Nuclei were labeled with DRAQ5 or DAPI.

Microscopy and Image Analysis

Immunofluorescence analysis of slides was performed on an Olympus BX60 upright fluorescence microscope equipped with a DS-Ri1 digital camera and with NIS Elements software (Nikon, Melville, NY) or on an EVOS FL Cell Imaging system (Thermo Fisher Scientific). Confocal microscopy images were acquired using a Zeiss LSM 510 META system, with a 63× objective and a step size of 0.5 μm, or an inverted Leica SP5 Confocal Scanning Laser Microscope (Leica, Wetzlar, Germany) with a 20× objective or a 63× water immersion objective with Immersol (W 2010, Zeiss, Oberkochen, Germany). Digital image analysis was performed using ImageJ 1.48v software. The distribution of CD103$^+$ DCs in relation to the epithelium in gastric tissue sections was determined by manual counting on digital images, using NIS Elements software. Three-dimensional co-localization of red (endosomal markers) and green (CD103) voxels in confocal stacks was determined using the JaCOP plugin to calculate Mander's co-localization coefficient (34). Part of this procedure involved screening 16 algorithms for optimal exclusion of background staining; the Bernsen method of auto-thresholding was chosen and applied objectively to all images (35). In tissue sections, regions of interest were set to exclude surface and glandular epithelial cells.

FACS Analysis

For flow cytometry, cells were labeled with pre-determined optimum concentrations of antibodies at 4°C for 15 min, followed by washing in FACS Stain Buffer (BD Biosciences). For intracellular staining, cells were fixed and permeabilized with Cytofix/Cytoperm (BD Bioscience), and antibodies we added in the presence of BD PermWash buffer. Dead cells were labeled with LIVE/DEAD® yellow dye (Life Technologies, Carlsbad, CA). A BD LSR or LSRII was used for flow cytometry; and data were analyzed using FlowJo V10 software (Treestar, Ashland, OR). Gastric DCs were gated as live CD45^{pos}/lineage^{neg}/HLA-DR^{high} cells. The lineage cocktail contained antibodies to CD3, CD19, and CD56.

Imaging Cytometry

Imaging cytometry was performed using an ImageStreamX Mark II (Amnis Corp., Seattle, WA). Cells were prepared as described for FACS analysis, with 7-AAD or DAPI used to label nuclei in fixed cells. Data were analyzed with IDEAS software v6.1 (Amnis Corp.). The following channels were recorded: Ch 1–brightfield, Ch 2–CD103 FITC, channel 3–CD11c PE, and channel 5–7-AAD or channel 7–DAPI. DCs were gated as focused cells based on Gradient RMS Ch 1, single cells based on Aspect Ratio and Area Ch1, and Intensity of CD11c PE in Ch 3.

Internalization Assay

Relative rates of internalization of CD103 were determined in MoDCs using a variation of the method from Chen et al. (36). Briefly, aliquots of MoDCs generated in the presence of retinoic acid (100 nM) were chilled to 4°C, then incubated with FITC labeled mouse anti-human CD103 for 30 min. Unbound antibody was then removed by washing in ice cold media. Time zero samples were left on ice, and internalization was initiated in the remaining samples by resuspending the samples in 37°C media and incubating them at that temperature for the indicated times. At each time point, samples were quickly washed in ice cold FACS buffer and left on ice to inhibit further

internalization. After washing, cells were incubated for 30 min with anti-mouse IgG eFluor660 to label remaining cell surface anti-CD103 antibodies. Cells were then washed again in cold FACS buffer and analyzed with a BD LSR flow cytometer. Mean fluorescent intensities were normalized to the highest value of that fluorophore in each experiment.

Adhesion and Spreading Assays

For adhesion and spreading assays with recombinant E-cadherin, glass bottom 24-well or 96-well plates were coated with goat anti-human IgG at 5 μg/mL and incubated overnight at 4°C. Non-adherent IgG was washed off with PBS + Ca^{2+}, and Fc-tagged recombinant human E-cadherin (Acro Biosystems, Newark, DE) at 2 μg/mL or an equal volume human serum was added to wells and incubated at room temperature for 1 h. Alternatively, wells were coated directly with 0.2 μg/mL recombinant human E-cadherin (R&D systems, Minneapolis, MN) for 1 h. After coating, wells were washed, blocked with human serum, and washed again. MoDCs were incubated for 30 min with an anti-human CD103 neutralizing antibody (20 μg/mL), an appropriate isotype control antibody or were left untreated and then were added to the plates at 5×10^5/mL. After incubation for 40 min at 37°C, non-adherent cells were gently washed off and cells were fixed with Cytofix/Cytoperm (BD biosciences, San Jose, CA). MoDCs were blocked with 10% goat serum and stained with HLA-DR FITC, a secondary IgG2a FITC to enhance signal, and DAPI to label cell nuclei. Wells were then imaged on an EVOS FL Cell imaging system and analyzed using ImageJ.

To quantify DC adhesion to gastrointestinal epithelial cells, HT-29 cells were cultured on 48 well plates for 3 days at a starting concentration of 3×10^5 per well to obtain a 100% confluency for co-culture. MoDCs generated in medium alone or in the presence of RA were harvested and pre-treated with DC culture medium containing one of the following for 30 min at 37°C: 2 mM Mn^{2+}; 1 mM EGTA; 2 mM Mn^{2+} and 1 mM EGTA; anti-human CD103 (2–20 μg/mL); or anti-human E-cadherin (5 μg/mL). MoDCs were next plated with the HT-29 monolayers at 2×10^5 cells per well and incubated for 2 h at 37°C, with antibodies or other additives remaining in the culture media. Following incubation, non-adherent cells were gently washed off with media, and adherent MoDCs and HT-29 cells were harvested using 0.25% trypsin/1 mM EDTA (Millipore, Darmstadt, Germany). Cells were then stained with HLA-DR FITC antibody, counting beads were added, and recovered MoDCs were quantified by flow cytometry. Experiments with <5% DC adhesion were excluded from the analyses, because it was difficult to interpret minor changes in adhesion when overall adhesion levels were extremely low. Experiments with low overall adhesion correlated neither with low DC CD103 or E-cadherin expression levels nor with a specific passage number or reduced E-cadherin expression of the HT-29 cells.

To quantify CD103 expression following MoDC HT-29 co-culture, a subset of MoDCs were incubated without HT-29 cells and subjected to the same 0.25% trypsin/1 mM EDTA treatment. MoDCs incubated alone and those co-cultured with HT-29 cells were then stained for CD103 expression and analyzed by flow cytometry.

Statistical Analysis

Data were analyzed using GraphPad Prism 6.05. Results are presented as mean ± SEM. Differences between values were analyzed for statistical significance by the two-tailed Student's *t*-test or one- or two-way ANOVA with appropriate *post-hoc* analysis as indicated. Differences were considered significant at $P < 0.05$.

RESULTS

Gastric Intraepithelial DCs Contain a Significant CD103-Expressing DC Subset

Flow cytometric analyses of gastric DCs have shown that $CD103^+$ DCs are rare in both human and murine stomach (14, 25–27). Here, we used immunofluorescence analysis of human gastric tissue sections to analyze CD103 expression by gastric mononuclear phagocytes in more detail. Mucosal DCs, and possibly some macrophages, were identified based on high expression of HLA-DR in conjunction with an irregular cell morphology. Notably, our previous studies showed that gastric HLA-DR^{high} cells are $CD45^+$ leukocytes that express the DC-specific transcription factor zDC, but not the intestinal macrophage marker CD13 and that do not include B cells, T cells, mast cells, or NK cells (2, 14). Our immunofluorescence analyses confirmed that CD103 expression by HLA-DR^{high} cells in the gastric mucosa relatively rare (**Figure 1a**). Likewise, the majority of $CD103^+$ cells, likely T lymphocytes, were negative for HLA-DR expression (**Figure 1a**). However, individual $CD103^+$ HLA-DR^+ DCs were detected in close association with the gastric glandular epithelium, either at intraepithelial sites, or directly below the epithelium (**Figures 1b,c**). We next performed a quantitative analysis of the distribution of $CD103^-$ and $CD103^+$ DC subsets in relationship to the gastric epithelium by counting DCs at intraepithelial, subepithelial (with epithelial contact), and lamina propria sites (no epithelial contact) (**Figures 1d,e**). Although intraepithelial HLA-DR^+ DCs represented <2% of all gastric mucosal DCs, a proportion ($P \leq 0.05$) of these cells expressed CD103. Specifically, 46.1% of intraepithelial gastric HLA-DR^+ cells were positive for CD103 compared to only 10.7 and 6.9% of $CD103^+$ DCs at subepithelial and lamina propria sites, respectively (**Figure 1e**). These observations suggest that, in spite of an overall low expression of CD103 by human gastric DCs, CD103 might still contribute to DC interactions with the gastric epithelium in those DCs that are integrated into the epithelial cell layer.

Intracellular Expression of CD103 (αE Integrin) in Human Monocyte-Derived and Gastric DCs

Human monocyte-derived DCs (MoDCs) contain intracellular as well as surface-expressed CD103 (26). Having shown that gastric DCs express low levels of surface CD103 overall (14, 26), but that surface CD103 expression is more frequent on gastric intraepithelial DCs (**Figures 1b,e**), we hypothesized that intracellular CD103 pools may be recruited to the cell membrane to mediate binding to epithelial E-cadherin. Therefore, we next

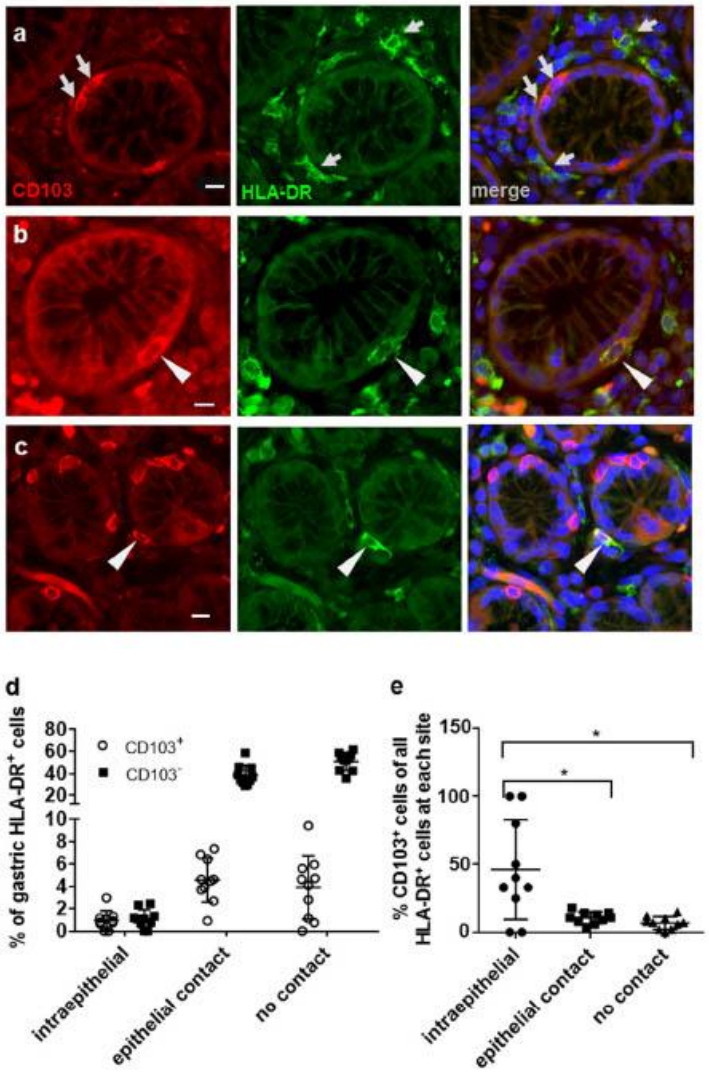

FIGURE 1 | Distribution of CD103+ DCs in human gastric mucosa. Paraffin-embedded tissue sections from the gastric mucosa of non-*H. pylori*-infected human subjects were immunofluorescently labeled for HLA-DR (Alexa 488, green) and CD103 (Alexa 555, red). Nuclei were stained with DAPI. **(a)** High magnification single color and merged images of gastric mucosa with multiple cells positive for either CD103 or HLA-DR (arrows). **(b)** Occasional intraepithelial HLA-DR$^+$ DCs and **(c)** subepithelial DCs with epithelial contact show staining for CD103. Arrowheads point out HLA-DR$^+$ DCs that co-express CD103. Bar = 20 µm. **(d,e)** Quantitative analysis of HLA-DR$^+$ DCs with and without CD103 expression at intraepithelial, subepithelial (with epithelial contact), and lamina propria sites (no epithelial contact). Tissue sections from 10 human subjects were analyzed. *$P \leq 0.05$, ANOVA with Tukey's *post-hoc* test.

analyzed whether primary human gastric DCs also express intracellular CD103. As shown in **Figures 2A,C**, significant levels of CD103 were detected in both gastric DCs and MoDCs when cells were permeabilized prior to immunolabeling, consistent with intracellular expression. In addition, intracellular CD103 expression was confirmed by imaging flow cytometry

(**Figures 2D,E**). In contrast, we did not observe significant intracellular expression of α4 integrin, in spite of high surface expression (**Figure 2B**).

Using confocal microscopy, we detected CD103 in vesicular inclusions with a typical endosomal morphology in immature MoDCs with and without concurrent surface CD103 expression (**Figure 3A**). A similar vesicular staining pattern for CD103 was also seen in DCs isolated from human gastric lamina propria (**Figure 3B**). Thus, CD103 expression in endosomal compartments appears to be a common feature of human DCs.

CD103 Partially Co-localizes With Clathrin and Early, Recycling, and Late Endosomal Markers

Previous studies have shown that integrins may undergo endosomal trafficking to allow dynamic interactions with their ligands and facilitate cell migration (32, 37, 38). To characterize the endosomal expression of CD103 in human DCs in more detail, we analyzed co-localization of CD103 with markers for endocytic uptake (clathrin), early endosomes (early endosomal antigen-1, EEA-1), recycling endosomes (Rab11), and late endosomes (Rab7a) in MoDCs (**Figure 4**). In untreated MoDCs, 23.2 ± 1.2% of CD103 was co-localized with clathrin, corresponding to a Manders' co-localization coefficient of 0.232. Lower coefficients were detected for CD103 co-localization with Rab11 (16.7 ± 1.6%) and the late endosomal marker Rab7a (16.2 ± 1.2%), which targets endosomal cargo for lysosomal degradation (39). Only 6.6 ± 0.7% of CD103 co-localized with the early endosomal marker EEA-1. In control slides without primary antibodies, a co-localization co-efficient of 0.71 ± 0.69% was measured (data not shown). Interestingly, MoDCs treated with retinoic acid (RA) showed increased co-localization with clathrin (29.6 ± 1.3%; $P \leq 0.01$), but lower co-localization with Rab11

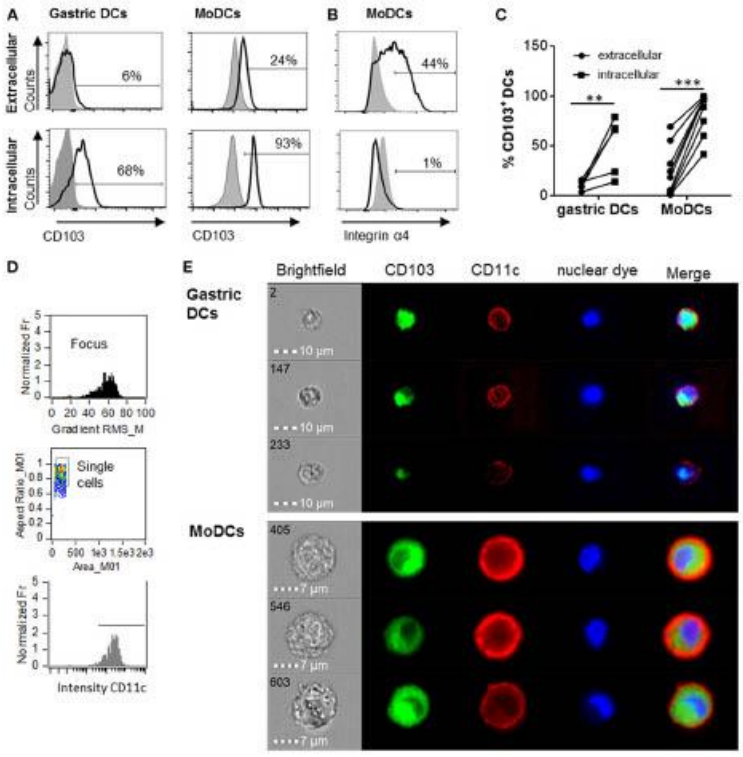

FIGURE 2 | Human gastric and MoDCs contain intracellular CD103 pools. Human gastric DCs gated as live/HLA-DR^{high}/CD45^{pos}/lineage^{neg} cells or MoDCs were labeled with **(A)** an anti-CD103 antibody or **(B)** an anti-α4 integrin antibody using extracellular or intracellular staining protocols. **(A,B)** representative histograms and **(C)** individual values from 4 (gastric DCs) or 9 (MoDCs) independent experiments. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, ANOVA with Tukey's *post-hoc* test. **(D)** Gating strategy for ImageStream imaging cytometry to select for focused, single cells with high CD11c expression. **(E)** Representative images from three independent imaging cytometry experiments show intracellular CD103 in CD11c^{+} gastric DCs and MoDCs, 40× objective. BF, bright field; nuclear dye, 7-AAD.

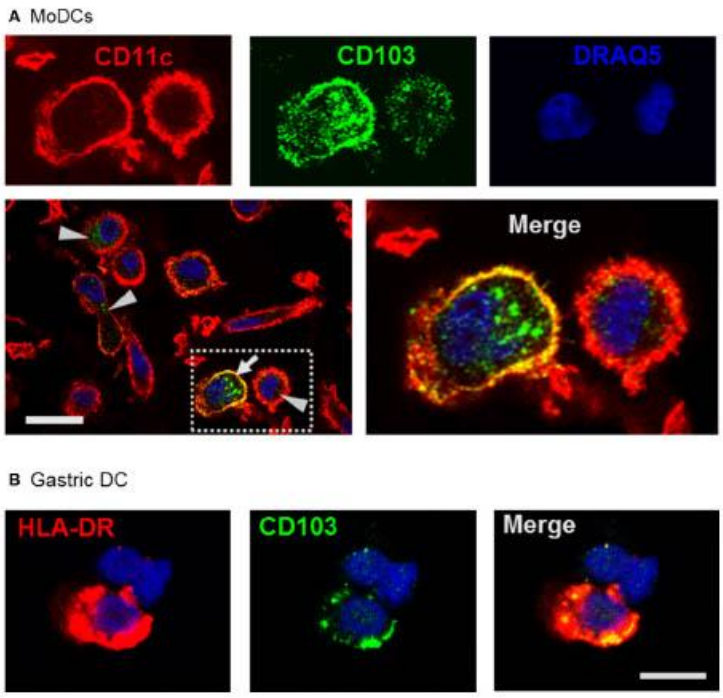

FIGURE 3 | Confocal microscopy analysis shows endosomal expression pattern of CD103 in human monocyte-derived and gastric DCs. **(A)** Confocal images of human MoDCs that were permeabilized and labeled with antibodies to CD103 (Alexa488) and CD11c (Alexa555) Nuclei were labeled with DRAQ5. Arrowheads point to intracellular CD103+ vesicles in DCs without surface CD103 expression, arrow indicates a DC with both surface and intracellular CD103 expression. Representative images from one of three similar experiments are shown. Bar: 20 μm. **(B)** Gastric lamina propria cells were permeabilized and labeled with antibodies to CD103 (Alexa488) and HLA-DR (Alexa555). Image shows a gastric HLA-DR+ DC. Bar: 10 μm.

(12.6 ± 1.1%; $P \leq 0.05$). The partial co-localization of CD103 with endosomal markers suggests that CD103 undergoes some endosomal trafficking in human DCs.

CD103 in Human MoDCs Undergoes Continuous Trafficking Through the Cell Membrane

To functionally analyze whether DC CD103 undergoes endosomal recycling, we performed an internalization assay, as previously described by Chen et al. (36). RA-treated MoDCs were used to achieve a high initial expression of CD103 on the DCs. DCs were then incubated for up to 40 min at 37°C and were kept on ice at all other times to inhibit endosomal trafficking until all cells were collected, with the 0 min sample incubated on ice for the entire 40 min of the assay. As shown in **Figure 5**, the level of CD103 on the cell surface as detected with the secondary reagent decreased significantly with prolonged incubation at 37°C ($P \leq 0.01$), whereas total CD103 expression detected with the primary antibody remained constant. These observations are consistent with internalization of surface expressed CD103 and indicate that CD103 undergoes endosomal recycling, as previously shown for α5β1 and α2β1 integrins (36, 37). Thus, CD103 may be involved in dynamic binding of DCs to the epithelial layer in spite of low surface expression.

Intracellular CD103 Engages in E-cadherin Binding, but Does Not Mediate DC Adhesion to Epithelial Cells

Having shown that human DCs contain dynamic pools of intracellular CD103, we next asked whether intracellular CD103 may be recruited to the DC surface for E-cadherin binding. RA-treated or untreated DCs were incubated on glass-bottom plates coated with recombinant E-cadherin (**Supplemental Figure 1**). The RA-treated DCs expressed increased levels of surface CD103 (**Supplemental Figure 2**). We hypothesized that engagement of E-cadherin by DC CD103 would lead to an accumulation of CD103 staining close to the E-cadherin-coated glass surface (**Figure 6A**). Our analysis of CD103 distribution across the

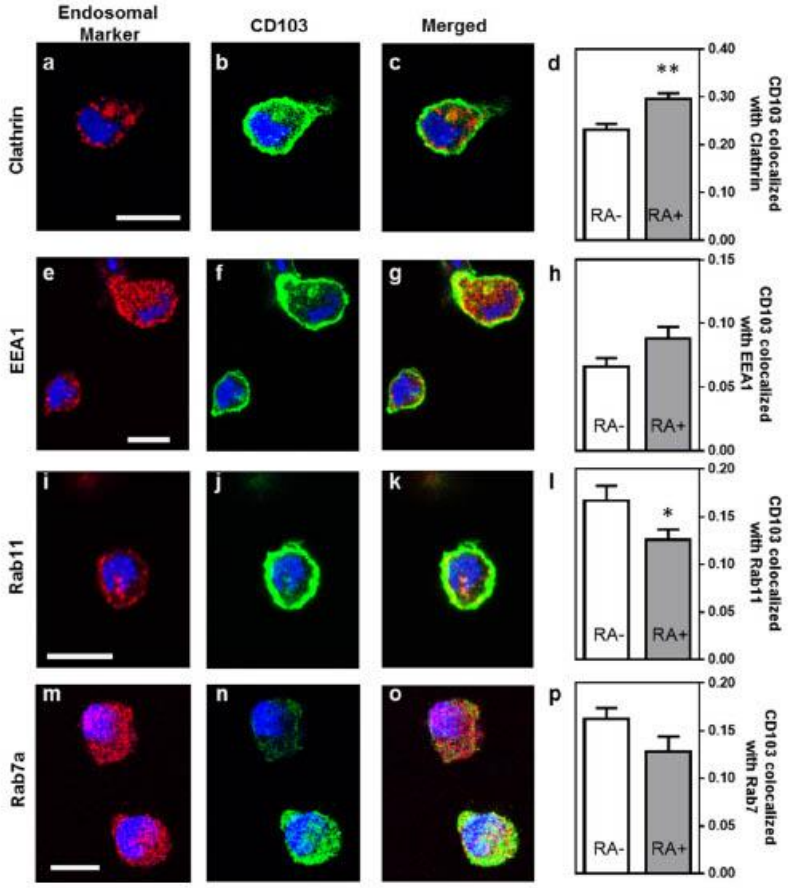

FIGURE 4 | Partial co-localization of CD103 with endosomal markers in human MoDCs. MoDCs generated in medium alone or in the presence of retinoic acid (RA, 100 nM) were permeabilized and stained with antibodies to CD103 (green) and the following endosomal markers (red): **(a–c)** clathrin, **(e–g)** EEA-1, **(i–k)** Rab11, and **(m–o)** Rab7a. Images were obtained by confocal microscopy. Co-localization of CD103 with **(d)** clathrin, **(h)** EEA-1, **(l)** Rab11, and **(p)** Rab7a was calculated as the Manders' colocalization coefficient (M2) using ImageJ. Mean ± SEM of 10 – 17 confocal images obtained from two independent experiments. Bar = 20 µm, $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; unpaired Student's *T*-test.

vertical axis of the DCs showed a small, but significant shift in the proportion of CD103 close to the glass surface in RA-treated MoDCs in wells that were coated with E-cadherin compared to serum alone (**Figure 6B**, $P \leq 0.05$). However, this trend was not observed in MoDCs generated in the absence of RA.

On hard surfaces, such as glass slides, adhesion complexes including integrin-dependent interactions influence cell spreading by enabling cells to extend actin-based lamellipodia (40). A previous study had shown that K562 cells transfected with αE(CD103)β7 formed epithelial protrusions and migrated on E-cadherin-coated surfaces (41). To determine whether CD103 promotes spreading and adhesion of human DCs on E-cadherin-positive surfaces, we used RA-treated and untreated MoDCs that were blocked with a CD103 neutralizing antibody or an isotype control antibody. As shown in **Figure 6C**, RA-treated DCs showed a trend ($P = 0.05$) for increased spreading on E-cadherin. However, DC spreading was not influenced by blocking CD103 on the DCs with a neutralizing antibody. The total number of adhered DCs also did not differ between treatments (data not shown). These results indicate that CD103 may relocate to the cell surface to engage in E-cadherin binding, but that overall DC adhesion to E-cadherin is largely independent of CD103.

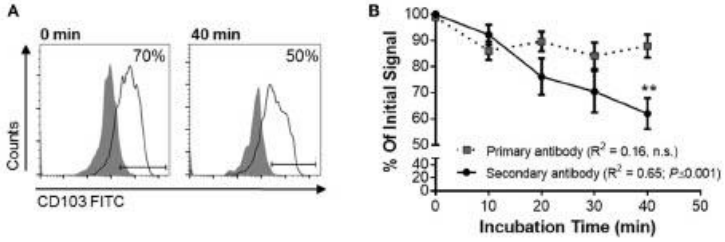

FIGURE 5 | Internalization of surface-expressed CD103 in human MoDCs. Human MoDCs were labeled with an anti-CD103 FITC (mouse IgG1), washed, and chilled with ice-cold buffer. Time zero samples were left on ice, and internalization was initiated in the remaining samples by resuspending the samples in 37°C media and incubating them at that temperature for the indicated times. Following incubation, cells were harvested with cold buffer and then left on ice until all samples were collected. Anti-CD103 antibody remaining on the surface of the DCs was detected with a secondary anti-mouse IgG1 antibody. **(A)** Representative FACS plots and **(B)** pooled data from four independent experiments show a significant decrease in surface CD103 after 40 min. 2-way ANOVA with Sidak's multiple comparisons.

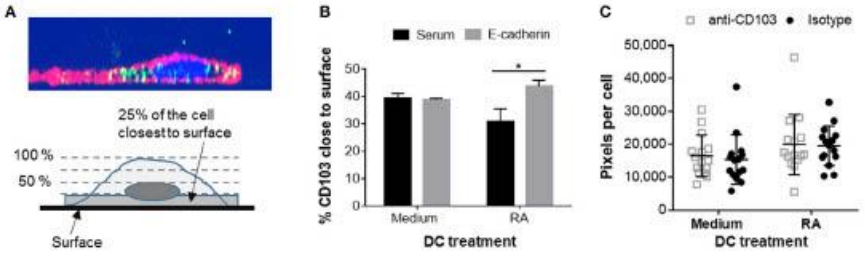

FIGURE 6 | Adhesion of RA-treated MoDCs to E-cadherin-coated surfaces alters distribution of CD103. DC interactions with E-cadherin were analyzed by performing adhesion and spreading assays on glass slides coated with recombinant human E-cadherin. **(A,B)** RA-treated and untreated MoDCs were added to E-cadherin-coated slides and were incubated at 37°C for 40 min. Cells were then fixed, permeabilized and stained for CD103 expression. Z-stack images (0.5 µm step size) of adherent MoDCs were collected by confocal microscopy and analyzed for the distribution of CD103 in relation to the glass surface. **(A)** Top panel: orthogonal representation of an immunofluorescently labeled MoDC adhered to a glass surface and analyzed by z-stack confocal imaging. CD11c: red, CD103: green, DAPI nuclear stain: blue. Bottom panel: graphical representation of the image analysis approach. **(B)** Summarized data from three independent experiments were analyzed. *$P \leq 0.05$, 1-way ANOVA with Tukey's *post-hoc* test. **(C)** Spreading analysis of DCs on E-cadherin-coated glass slides. Glass slides were first coated with anti-human IgG antibodies and then with recombinant human E-cadherin-Fc. MoDCs generated in the presence of medium alone or 100 nM RA were pre-treated with a CD103 neutralizing antibody or an isotype control antibody for 30 min at 4°C. Cells were then added to the slides in the presence of 2 mM Mn^{2+} and incubated at 37°C for 40 min. Cell spreading was analyzed by fluorescence microscopy based on HLA-DR+ pixels per nucleus. Individual data points ($n = 15$ areas), mean and SEM from one representative out of 3 independent experiments are shown.

Bivalent Cations Promote DC Adhesion to E-cadherin-Expressing Gastrointestinal Epithelial Cells

To analyze the interactions between MoDCs and cell-expressed E-cadherin, we performed MoDC adhesion assays with HT-29 cells, a colonic epithelial cell line strongly positive for E-cadherin. Importantly, HT-29 cells have an atypical E-cadherin distribution that involves E-cadherin expression on the apical cell surface (**Figures 7A,B**), but do not have any mutations in the E-cadherin gene *CDH1* (42, 43). DCs were incubated on top of HT-29 monolayers, and adherent cells were recovered by trypsinization after 2 h (**Figure 7C**). On average, 14.4 ± 3.7% of MoDCs were recovered from the cultures as adherent cells (**Figure 7D**). To determine whether integrins including αE integrin (CD103) are involved in DC adhesion to the HT-29 cells, we added 1 mM manganese (Mn^{2+}), a strong activator of integrins that promotes ligand binding (44, 45). Both in RA-treated and untreated MoDCs, addition of Mn^{2+} significantly increased adhesion to HT-29 cells ($P \leq 0.001$ and $P \leq 0.05$, respectively). We also added EGTA, which inactivates bivalent cations, such as Ca^{2+}, Mg^{2+}, and Mn^{2+} that are involved in integrin- and E-cadherin-dependent interactions. EGTA significantly decreased the number of both and RA-treated and untreated DCs that were recovered from the co-cultures ($P \leq 0.05$ and $P \leq 0.01$, respectively). Although there was a trend for increased adhesion in RA-treated MoDCs, RA had no significant effect on DC binding to HT-29 cells, similar to our observations from the spreading analysis (**Figure 6**). Interestingly, co-culture with the HT-29 slightly increased surface expression of CD103 on the MoDCs (**Figure 7E**, $P = 0.056$).

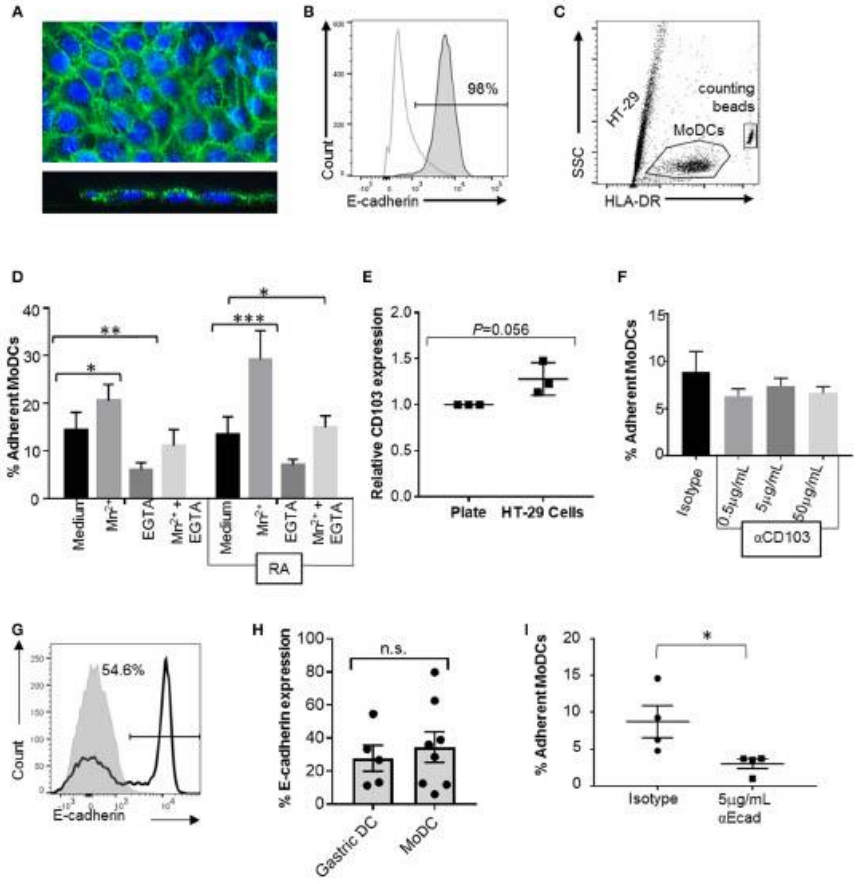

FIGURE 7 | CD103 is not a major mediator of DC adhesion to E-cadherin-expressing epithelial cells. To analyze MoDC binding to E-cadherin expressed by epithelial cells, MoDCs were added to confluent monolayers of HT-29 cells for 2 h. Non-adherent cells were then removed by gentle washing, the remaining cells were collected by trypsinization, and the number of adherent DCs was determined using counting beads and HLA-DR-labeling of the DCs. **(A)** Confocal analysis of an HT-29 monolayer shows E-cadherin expression on the luminal surface. Top panel: maximum Z-projection; bottom panel: orthogonal view. Nuclei are labeled with DRAQ5 (blue). **(B)** Representative FACS histogram of E-cadherin expression on HT-29 cells. Gray line: isotype control; dark gray filled: anti-E-cadherin. **(C)** Gating strategy for counting adherent MoDCs following co-culture with HT-29 cells. **(D)** Percentage of MoDCs adherent to HT-29 cells in the presence of Mn^{2+}, EGTA, or Mn^{2+} + EGTA ($n = 6$). RA indicates that cells were derived in the presence of 100 nM RA. $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$; 2-way ANOVA with Dunnett's multiple comparisons. **(E)** Normalized CD103 expression on MoDCs after recovery from HT-29 co-cultures (with Mn^{2+}). Data from three independent experiments, paired, one-tailed Student's *t*-test. **(F)** MoDCs were incubated with an isotype control antibody or the indicated concentrations of anti-CD103 antibody before and during co-culture with HT-29 cells, and the number of adherent DCs was determined. $N = 3$, $^*P \leq 0.05$; unpaired, two-tailed *T*-test. **(G,H)** representative histogram and pooled data for E-cadherin expression on human gastric ($n = 5$) and MoDCs ($n = 8$). **(I)** MoDCs were treated with anti-E-cadherin antibody before and during co-culture with HT-29 cells, and the number of adherent DCs was determined. $N = 4$, $^*P \leq 0.05$; unpaired, two-tailed Student's *t*-test.

Neutralization of DC CD103 Does Not Inhibit Adhesion to E-cadherin Expressing Epithelial Cells

To specifically assess the involvement of CD103 in the interactions between DCs and epithelial cells, RA-treated MoDCs were blocked with a CD103 neutralizing antibody prior to adding the cells to the HT-29 monolayer in the presence of Mn^{2+}. To avoid loss of blocking activity due to CD103 internalization, excess antibody was left in the cell culture medium during the adhesion assays. However, no decrease in DC adhesion to the HT-29 cells was seen with a wide range of antibody concentrations (**Figure 7F**).

Homotypic E-cadherin Interactions May be Involved in DC Binding to E-cadherin on Gastrointestinal Epithelial Cells

To form adherens junctions, E-cadherin undergoes homotypic interactions with E-cadherin expressed on other cells (46), and DCs have been shown to express E-cadherin in previous studies (47–49). Our FACS analysis revealed that subsets of both human gastric DCs and MoDCs expressed E-cadherin (**Figures 7G,H**), independent of RA treatment (**Supplemental Figure 2**). Thus, E-cadherin-E-cadherin interactions may contribute to DC-epithelial cell interactions. Indeed, pre-treatment of the MoDCs with an E-cadherin neutralizing antibody significantly decreased DC adhesion to HT-29 monolayers (**Figure 7I**). However, when we compared MoDC adhesion to HT-29 cells with adhesion to AGS cells, which lack E-cadherin expression, we found that a significantly higher number of DCs adhered to the AGS cells than the HT-29 cells (**Supplemental Figure 3**, $P \leq 0.01$). Interestingly, binding to AGS cells decreased when DCs were generated in the presence of RA. Overall, these data suggest that both E-cadherin-dependent and integrin-dependent mechanisms contribute to DC binding to the gastrointestinal epithelium, but that CD103-E-cadherin interactions are only minor contributors.

DISCUSSION

CD103 (αE integrin) is widely used as a maker for DC subsets in humans and mice (29), but the functional role of CD103 for the DCs has attracted little investigative attention. We here sought to elucidate whether CD103 could mediate DC-epithelial cell interactions in the human gastric mucosa. A number of previous reports had speculated that CD103 might mediate adhesion of gastrointestinal DCs to E-cadherin expressed in the epithelial layer (15–17), similar to the mechanism shown for the retention of intraepithelial lymphocytes within the epithelial compartment (22, 50). Studies from our laboratory have shown that DCs in the gastric mucosa are exposed to RA generated by gastric epithelial cells, and that RA induces CD103 expression in human MoDCs (14, 26). Our results from the present study suggest that CD103 is engaged upon binding of primary DCs to gastrointestinal epithelium, but is not a major mediator of adhesion.

One specific consideration when investigating CD103 in human gastric DCs was that <10% of the DCs expressed CD103 on their surface, as we have previously shown (14, 26). However, based on the detection of intracellular CD103 (αE integrin), we hypothesized that these intracellular pools could be recruited to the cell surface for dynamic interactions with their ligands. Thus, integrins α5β1, α6β4, αMβ1, and α4β1 are continuously recycled through endosomal pathways during cell migration (33). However, not all integrins participate in the endocytotic cycle, and some integrins are recycled at only low rates (31). Indeed, we here confirmed that α4 integrin, which, like αE integrin, pairs with β7 integrin, was not expressed intracellularly. Our report is the first to demonstrate that CD103 in human DCs is expressed in endosomal compartments and recirculates through the cell membrane, suggesting that αE integrin recycling occurs in human DCs. It has been proposed that motile cells, such as DCs performing immunosurveillance functions, may require more trafficking of integrins and therefore contain a higher intracellular proportion (51). We demonstrated that 40% of surface CD103 was internalized in <1 h. Moreover, 23–30% of CD103 co-localized with clathrin, consistent with the established role of clathrin in the endocytic recycling of integrin-mediated adhesions (52). A lower percentage of CD103 co-localized with the early endosome antigen 1 (EEA-1, 7%) and Rab11 (17%), a marker for long-loop endosomal recycling. Notably, previous publications have similarly reported co-localization co-efficients between 5 and 30% for integrins and endosomal markers in cells that were not specifically treated to enhance endosomal trafficking. Thus, Ezratty et al. (52) reported that 20–25% of β1 integrin co-localized with Rab5. In a publication by the Goldenring group, a Manders' co-localization co-efficient of >0.2 (>20%) for co-localization of Rab25 with β1 and α5 integrins was considered high (53). Gu et al. (54) from the Brenner lab analyzed co-localization of β3 integrin with endosomal markers and found between 2 and 8% of co-localization with EEA-1, Rab4, 5, and 11 at baseline, with increased co-localization upon PDGF-stimulated micropinocytosis. Khandelwal et al. (55) used a functional endocytosis assay with fluorescently labeled cargo and detected co-localization co-efficients of 10 and 20% for the endosomal cargo with EEA-1 and Rab11, respectively, and Karjalainen et al. (56) analyzed co-localization of α2 integrin with caveolin and detected 5–10% of co-localization at baseline. Therefore, we consider our observed co-localization of CD103 with the endosomal markers to be biologically relevant. Co-localization of CD103/integrin αE with clathrin, EEA1, and Rab11 suggests that integrin αE undergoes canonical trafficking similar to α5β1 integrin (57). However, the fact that CD103 also was co-localized with the late endosomal marker Rab7a may indicate that a proportion of intracellular CD103 is targeted for lysosomal degradation rather than recycling to the cell surface. Notably, when added together, <60% of all CD103 co-localized with any endosomal marker, suggesting that a significant proportion of CD103 is present at sites that are not endosomal compartments. These might represent newly synthesized CD103 molecules in the endoplasmic reticulum or in the Golgi apparatus. Interestingly, RA treatment of the MoDCs led to increased co-localization with clathrin and EEA-1, but decreased co-localization with Rab11 and Rab7a. Thus, RA seems to both upregulate CD103 expression (26, 58) and alter its trafficking. Overall, our results suggest that, even on cells with low surface CD103 expression, CD103 may be recruited from endosomal pools for dynamic binding to epithelial E-cadherin or other ligands.

In support of a role for CD103 in human DC-epithelial interactions, we here showed significantly increased expression of CD103 on human HLA-DR$^+$ DCs that were integrated into the gastric epithelial layer. Moreover, Z-stack analysis of MoDCs bound to E-cadherin-coated glass slides showed significant re-distribution of DC-expressed CD103 to the E-cadherin positive interface, albeit only in RA-treated cells. In addition, *in vitro* adhesion assays to E-cadherin-expressing HT-29 cells revealed a dependence on bivalent cations including manganese, consistent with an integrin-dependent mechanism (45). Conversely, when

MoDCs were treated with a CD103 neutralizing antibody, adhesion to E-cadherin-positive HT-29 cells or to E-cadherin-coated glass slides was unaffected, arguing against a major role of CD103 in mediating DC binding to the gastrointestinal epithelium. Notably, while internalization of CD103 with bound blocking antibody may have decreased the efficiency of the neutralization, this would not be expected to completely abrogate functional activity of the blocking antibody, especially since a high antibody concentration (5 μg/mL) was used and additional antibody was present in the culture medium during the assay. Also, RA treatment, which increases MoDC CD103 expression, did not significantly influence MoDC binding to E-cadherin-expressing HT-29 cells or spreading on E-cadherin-coated glass surface, corroborating the results from the antibody neutralization experiments. Thus, adhesion of human DCs to gastrointestinal epithelia does not appear to be driven by CD103-E-cadherin interactions. Notably, we used high expression of HLA-DR to detect DCs in human gastric tissue sections, since no other more specific general DC marker has been identified for human stomach (2, 28). In the murine gastric mucosa, CX_3CR1^+ $CD103^-$ macrophages, and $CD103^-$ DCs were able to sample *H. pylori* bacteria, whereas bacterial uptake by $CD103^+$ DCs could not be detected (25), which also does not support our original hypothesis that CD103 positions gastric mononuclear cells at the epithelial interface for luminal *H. pylori* uptake. Along the same lines, an earlier report that investigated intraepithelial DCs in murine small intestine, the spatial relationship of murine intestinal DCs with the epithelium was not altered in CD103 knockout mice (59), whereas the number of IELs was significantly reduced in these animals (60). Together, these observations indicate that there are functional differences between T cell and DC-expressed CD103.

Our adhesion experiments did show that antibody inhibition of DC-expressed E-cadherin significantly suppressed DC binding to HT-29 cells, suggesting a role for homotypic E-cadherin-E-cadherin interactions. Thus, our results corroborate previous studies that showed Langerhans cells and other DCs of the skin and female genital tract interact with the epithelium through E-cadherin-E-cadherin binding (47, 61, 62). Notably, although homotypic E-cadherin interactions are calcium dependent, it appears that calcium can be replaced by the bivalent transitional elements manganese (Mn^{2+}) and cadmium (Cd^{2+}) (63), which would explain the observed increase in adhesion in the presence of Mn^{2+}. The significant decrease in MoDC HT-29 adhesion in the presence of EGTA is consistent with either homotypic E-cadherin-E-cadherin interactions, which are calcium-dependent (64), or heterotypic E-cadherin interactions with αE integrin, which is activated by manganese (Mn^{2+}) (65). Since E-cadherin is widely expressed on mucosal epithelial cells, it is a likely candidate for mediating the retention of motile immune cells at the epithelial barrier, and additional heterotypic E-cadherin ligands with expression on DCs including killer cell lectin-like receptor G1 (KLRG1) have been identified (22, 49, 66, 67). Surprisingly, MoDC adhesion to E-cadherin negative AGS cells was significantly higher than adhesion to HT-29 cells. These results suggest that DC adhesion to the gastrointestinal epithelium may involve additional molecular interactions independent of CD103 or E-cadherin, such as tight junction proteins (11, 68) and other integrins. Notably, all myeloid cells including DCs express β2 integrin (CD18). CD18 forms heterodimers with CD11a, CD11b, and CD11c (69) and contributes to cell-cell contact formation by binding to intracellular adhesion molecules (ICAMs), which may be expressed on gastric and intestinal epithelial cells (70, 71). Notably, adhesion of DCs to epithelial cells via β2 integrins would be consistent with our experimental observations that showed increased binding in the presence of Mn^{2+} and decreased binding in the presence of EGTA. Whether β2 integrins are major mediators of DC adhesion to the human gastric epithelium will be a subject of future studies.

If the interactions between DC-expressed CD103 and epithelial cells that we observed in human gastric tissue section do not result in strong adhesion, one might question the relevance of these interactions. However, engagement of CD103 by epithelial E-cadherin may lead to outside-in signaling through the cytoplasmic portion of the integrin (72). Previous studies have shown that in cytotoxic T cells, engagement of E-cadherin by CD103 triggers the phosphorylation of PLCγ1 and ERK1/2 (73), and engagement of CD103 by an anti-CD103 antibody can enhance T cell proliferation (74). Therefore, interactions between DC CD103 and epithelial E-cadherin could regulate certain DC functional characteristics through the activation of intracellular signaling cascades.

In summary, our study has provided novel insights in the regulation and function of CD103 (E integrin) in human DCs. We show that, like other integrins in motile cells, CD103 undergoes endosomal trafficking, which likely enables dynamic interactions between CD103 and its ligands. Our results also corroborate previous reports (59) that CD103 is not essential for the retention of DCs at gastrointestinal epithelial sites. The mechanisms by which CD103 on DCs in the human gastrointestinal tract interact with epithelial cells may be more subtle than simple adhesive interactions and requires further experimental exploration.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the U.S. Department of Health and Human Services' Policy for Protection of Human Research Subjects (45 CFR 46) with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Institutional Review Boards of Montana State University and the University of Alabama at Birmingham.

AUTHOR CONTRIBUTIONS

DB and SS planned and oversaw the experiments. DB, SS, MR, BS, JD, TS, and RV performed the experiments. DB, PS, and LS initiated the project, critically discussed the data and obtained

funding. DB, SS, and MR analyzed the data. MR, SS, and DB wrote the manuscript. All authors provided feedback on the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02989/full#supplementary-material

Supplemental Figure 1 | Coating of glass surfaces with rh E-cadherin. Untreated or poly-L lysine-coated wells of a glass bottom 96 well plate were incubated with recombinant human E-cadherin (1 μg/mL) in PBS for 60 min. Following a blocking step with 10% human serum, and several washes, E-cadherin bound to the plate was detected using an anti-E-cadherin antibody and visualized using an alkaline phosphatase detection system. Left panel: mean absorption (405 nm) ± SEM of duplicate wells. Right panel: plate image.

Supplemental Figure 2 | Retinoic acid significantly increases expression of CD103, but not E-cadherin in human MoDCs. MoDCs were treated with 100 nM RA and surface expression of E-cadherin (left panel, $n = 12$) and CD103 (right panel, $n = 13$) were analyzed with flow cytometry. $^*P \leq 0.05$; unpaired, two-tailed *T*-test.

Supplemental Figure 3 | Strong adhesion of MoDCs to E-cadherin negative AGS cells. Top panel: confocal analysis of an AGS and an HT-29 monolayer shows surface E-cadherin expression (green) by the HT-29, but not by AGS cells. Bottom panel: RA-treated or untreated MoDCs were added to confluent monolayers of AGS or HT-29 cells for 2 h. Non-adherent cells were then removed by gentle washing, the remaining cells were collected by trypsinization, and the number of adherent DCs was determined using counting beads and CD11c-labeling of the DCs. Mean ± SEM of three independent experiments. Data were analyzed by ANOVA with Tukey's *post-hoc* test. a, b: different letters indicate significantly different values ($P \leq 0.05$).

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Aspartic Acid Residue 51 of SaeR Is Essential for *Staphylococcus aureus* Virulence

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Staphylococcus aureus is a common Gram-positive bacteria that is a major cause of human morbidity and mortality. The SaeR/S two-component sensory system of *S. aureus* is important for virulence gene transcription and pathogenesis. However, the influence of SaeR phosphorylation on virulence gene transcription is not clear. To determine the importance of potential SaeR phosphorylation sites for *S. aureus* virulence, we generated genomic alanine substitutions at conserved aspartic acid residues in the receiver domain of the SaeR response regulator in clinically significant *S. aureus* pulsed-field gel electrophoresis (PFGE) type USA300. Transcriptional analysis demonstrated a dramatic reduction in the transcript abundance of various toxins, adhesins, and immunomodulatory proteins for SaeR with an aspartic acid to alanine substitution at residue 51. These findings corresponded to a significant decrease in cytotoxicity against human erythrocytes and polymorphonuclear leukocytes, the ability to block human myeloperoxidase activity, and pathogenesis during murine soft-tissue infection. Analysis of SaeR sequences from over 8,000 draft *S. aureus* genomes revealed that aspartic acid residue 51 is 100% conserved. Collectively, these results demonstrate that aspartic acid residue 51 of SaeR is essential for *S. aureus* virulence and underscore a conserved target for novel antimicrobial strategies that treat infection caused by this pathogen.

Keywords: ***Staphylococcus aureus*, *saeR/S*, virulence, pathogenesis, two-components system, neutrophil, toxin, transcription**

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INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a Gram-positive bacterium that can cause a wide range of disease in both humans and animals (Nygaard et al., 2008). The diverse pathogenesis of *S. aureus* can be attributed to the expression of an extensive array virulence factors that are often redundant in function (Guerra et al., 2017). Expression of these virulence genes *in vivo* is thought to be primarily dictated by the concerted influence of two-component sensory systems that recognize environmental signals and alter gene expression accordingly. The *S. aureus* genome encodes 16 two-component systems that have been identified by sequence analysis (Cheung et al., 2004). Of these, the SaeR/S two-component system has been shown to be an important mediator of *S. aureus* virulence by transcriptionally upregulating numerous adhesins, toxins, and immunomodulatory

proteins that interact directly with host components to advance pathogenesis (Giraudo et al., 1997; Goerke et al., 2001; Voyich et al., 2009; Nygaard et al., 2010; Borgogna et al., 2018). The mechanisms used by this two-component system to recognize host-specific cues and alter gene transcription in response are not completely understood.

Typical two-component systems are minimally composed of a transmembrane histidine kinase sensor and cognate intracellular response regulator (Groisman, 2016). Recognition of environmental stimulus activates the histidine kinase sensor, inducing autophosphorylation at a histidine residue on the intracellular domain. This phosphate group is then transferred to an aspartic acid residue within the receiver domain of the cognate response regulator. In general, the phosphorylated response regulator then binds to promoter regions within the bacterial genome to mediate gene transcription and promote survival. However, some response regulators such as RcsB from *Escherichia coli* (Pannen et al., 2016) and SreR from *Xanthomonas campestris* (Wang et al., 2014) can regulate gene transcription without being phosphorylated. For the SaeR/S two-component system, the need to artificially phosphorylate recombinant SaeR for the *in vitro* DNA binding activity of this response regulator has not been consistent between studies (Nygaard et al., 2010; Sun et al., 2010). It has also been suggested that different levels of SaeR phosphorylation correspond to the upregulation of distinct groups of SaeR/S regulated genes (Mainiero et al., 2010).

To clarify the importance of potential SaeR phosphorylation sites for mediating *S. aureus* pathogenesis, we have generated individual single amino acid substitutions at conserved aspartic acid residues of SaeR in the genome of *S. aureus* PFGE-type USA300. In this report, we assess the significance of these residues for mediating the virulence of a clinically relevant MRSA strain using both *in vitro* and *in vivo* models of infection. Results from this study underscore the importance of SaeR aspartic acid residue 51 for virulence gene transcription and demonstrate that the substitution of this single amino acid in the USA300 genome can attenuate pathogenesis of this clinically significant MRSA strain.

MATERIALS AND METHODS

Bacteria Strains and Culture Conditions

Staphylococcus aureus PFGE-type USA300 strain LAC has been described previously (Diep et al., 2006) and the USA300 isogenic deletion mutant of *saeR/S* (USA300Δ*saeR/S*) was generated in previous studies (Nygaard et al., 2010). Bacteria were cultured in an Excella E24 rotary incubator (New Brunswick) at 250 rpm and 37°C. Unless noted otherwise, overnight bacteria cultures grown in 20 mL of tryptic soy broth (TSB; EMD Millipore) were used to start subcultures in 20 mL TSB containing 0.5% glucose (1:100 dilution). Optical density at 600 nm (OD_{600}) was measured using a NanoDrop 2000c Spectrophotometer (ThermoFisher Scientific) and colony forming units (CFUs) were determined by plating diluted samples on tryptic soy agar (TSA; EMD Millipore).

DNA Sequence Alignment

DNA sequence alignment of SaeR homologs was performed using Clustal Omega[1] with protein sequences obtained from the NCBI protein data base using the following accession numbers: ABD22784 for USA300, ATX72322 for USA400, EOR90509 for USA100, WP_076742615.1 for MT-0541, AAW53763 for *Staphylococcus epidermidis* RP62A, Q8CQ17 for *Staphylococcus epidermidis* 12228, WP_049307325 for *Staphylococcus capitis*, WP_029378577 for *Staphylococcus xylosus*, PCQ20359 for *Klebsiella pneumonia*, NP_346930 for *Clostridium acetobutylicum*, ACJ50526 for *Escherichia coli*, and NP_388082 for *Bacillus subtilis*.

Generation of USA300 Genomic Mutations

Allelic exchange with plasmid pKOR1 was used to impart genomic mutations in USA300 as previously described (Bae and Schneewind, 2006). All primers used to generate and sequence USA300 mutants are listed in **Supplementary Table S1**. Primers containing *attb* sites were used to amplify staphylococcal peroxidase inhibitor (*spn*) and saeR from the USA300 genome for BP Clonase II (ThermoFisher Scientific) mediated insertion into pKOR1. Spn-check-fwd and rvs primers were used to verify the loss of *spn*. Site-directed mutagenesis was performed on pKOR1-*saeR* using saeR-D46A, saeR-D51A, or saeR-D61A primers and PfuUltra II Fusion HS DNA polymerase (Agilent Technologies, Inc.) following the manufacture's protocol and as previously performed (Ran et al., 2010). The *saePQRS* operon in generated *saeR* point mutants was PCR amplified using saePQRS_fwd and rvs primers and sequenced (BigDye Terminator v3.1 Cycle Sequencing Kit) using saeR-seq and saePQRS-seq primers. PCR amplification was performed using saeR-EcoRI-fwd and saeR-XhoI-rvs and cloned into pEPSA5 as previously described (Flack et al., 2014) to generate the complementary plasmid that expresses wt SaeR, pEPSA5-*saeR*.

Whole Genome Sequencing and *saeR* Sequence Analysis

Wild-type USA300 and its SaeR mutants were subject to whole genome sequencing. Briefly, genomic DNA was extracted using a Wizard genomic DNA purification kit (Promega, Madison, WI, United States) following by treatment with 20 μg/ml lysostaphin. The DNA library was sequenced on an Illumina NextSeq platform (Illumina, San Diego, CA, United States) with 2 × 150 bp paired-end reads. The reads were mapped against the published NC_007793 (*S. aureus* USA300_FPR3757) genome using BWA (Li and Durbin, 2009) and Samtools (Li et al., 2009), and the SNPs and InDels were examined using freebayes[2]. SNPs and InDels were further annotated using snpEff (Cingolani et al., 2012).

[1]https://www.ebi.ac.uk/Tools/msa/clustalo/
[2]https://github.com/ekg/freebayes

Blast analysis of the USA300 *saeR* (locus_tag SAUSA300_0691) sequence against more than 8,000 *S. aureus* draft genomes downloaded from the NCBI FTP site[3] (dated as June 1, 2018) were extracted, translated into amino acids and aligned using Geneious 11.1.

Relative Quantitative Real Time RT-PCR

Staphylococcus aureus transcription analysis using relative quantitative real time RT-PCR was performed as previously described (Voyich et al., 2005, 2009; Nygaard et al., 2010). Briefly, subcultured strains were harvested at mid-exponential (ME; $OD_{600} = 1.5$) or early stationary (ES; $OD_{600} = 3.0$) growth phases, mechanically disrupted using a FastPrep FP120 cell disrupter (ThermoFisher Scientific), and RNA purified with an RNeasy Kit (Qiagen). TaqMan real-time RT-PCR was performed using previously published primer and probe sets (Nygaard et al., 2010).

Hemolysis Assays

Heparinized venous blood from healthy donors was collected in accordance with the protocol approved by the Institutional Review Board for Human Subjects at Montana State University. All donors provided written informed consent to participate in the study. We adopted protocol described by others (Young et al., 1986) to quantify hemolysis of human blood by *S. aureus* extracellular proteins. Briefly, freshly drawn human blood was washed three times with 10 times the volume of sterile DPBS then resuspended at a final dilution of 1:200 with sterile DPBS. Sterile-filtered (0.22 um) *S. aureus* supernatants from 6-h subcultures grown in TSB were combined with washed diluted blood in individual wells of a 96 well plate on ice at a ratio of 1:1. TSB alone and TSB + 0.5% Triton X-100 were also used as negative and positive controls for hemolysis. Samples were then placed in a SpectraMax 190 microplate reader (Molecular Devices) heated to 37°C and absorbance at 630 nm was measured after 6 min of incubation. Percent hemolysis was determined using the following formula: $\%\text{Hemolysis} = (\text{Absorbance}_{Experimental} - \text{Absorbance}_{TSBcontrol})/(\text{Absorbance}_{\text{Triton X}} - \text{Absorbance}_{TSBcontrol}) \times 100$.

Human Myeloperoxidase Activity Assays

To obtain extracellular proteins from USA300 strains, supernatant from *S. aureus* sub-cultured for 5 h was sterile-filtered (0.22 um) and stored at −80°C. For human myeloperoxidase (MPO) activity assays, 0.5 ug of recombinant human MPO (R&D Systems) in 50 uL of DPBS was mixed with 50 uL of sterile-filtered *S. aureus* supernatant for 30 min at room temperature. The MPO-supernatant solution was then exposed to 150 uL of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate reagent set (BD Biosciences) and incubated at 37°C. The oxidation of TMB catalyzed by human MPO was quantified by measuring the OD_{650} every minute for 30 min using a SpectraMax Paradigm microplate reader (Molecular Devices). The MPO inhibitor sodium azide was used at a concentration of 1 mM.

[3]http://ftp.ncbi.nih.gov

Human PMN Plasma Membrane Integrity Assays

Human polymorphonuclear leukocytes (neutrophils or PMNs) were isolated under endotoxin-free conditions (<25.0 pg/ml) using freshly drawn heparinized venous blood from healthy donors with written informed consent as previously described (Voyich et al., 2005; Nygaard et al., 2013). Cell viability and purity of preparations were assessed using a FACSCalibur Flow cytometer (BD Biosciences) and only preparations containing ≥ 98% viable PMNs were used. Assays intoxicating PMNs with extracellular *S. aureus* proteins were performed as previously described (Nygaard et al., 2012; Flack et al., 2014). Briefly, supernatant from *S. aureus* subcultured for 5 h in TSB was sterile-filtered (0.22 um) and diluted by 1:10 with TSB. To intoxicate PMNs, 20 ul of diluted *S. aureus* supernatant was combined with 100 uL Roswell Park Memorial Institute (RPMI) 1640 Medium (Corning Cellgro) with 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Corning Cellgro) containing $1x10^6$ purified human PMNs in a serum coated well of a 96 well plate. Samples were incubated at 37°C for 90 min then stained with propidium iodide (PI; ThermoFisher Scientific) following the manufactures protocol and then analyzed with a FACSCalibur Flow cytometer. Triton-X 100 (0.5%) was used as a positive control for causing PMN plasma membrane permeability. The following formula was used to determine % propidium iodide^+: $\%\text{Propidium Iodide}^+ = (\text{Mean PI signal}_{Experimental} - \text{Mean PI signal}_{untreated})/(\text{Mean PI signal}_{\text{Triton-X}} - \text{Mean PI signal}_{untreated})$.

Assays measuring human PMN plasma membrane permeability following the phagocytosis of live *S. aureus* were performed as previously described (Voyich et al., 2009; Nygaard et al., 2012; Flack et al., 2014). Briefly, subcultured *S. aureus* was harvested at ME growth by centrifugation (5,000 × g, 5 min, 4°C), washed with DPBS, then opsonized with 50% normal human serum for 15 min at 37°C. Opsonized bacteria were washed with DPBS and then 2×10^7 CFU in 100 uL of DPBS was combined with 100 uL of RPMI/H containing 1×10^6 purified human PMNs in a serum coated well of 96 well plate. Phagocytosis was synchronized by centrifugation (524 × g, 8°C, 8 min) in an Allegra X-15R centrifuge (Beckman Coulter) and samples were incubated at 37°C for 90 min. Following incubation, human PMNs were stained with PI and analyzed using flow cytometry as described above.

Murine Model of Soft-Tissue Infection

All animal studies conformed to National Institute of Health guidelines and were approved by the Animal Care and Use Committee at Montana State University-Bozeman. Female BALB/C mice (8–10 weeks old) with an average weight of 22 g were purchased from Animal Resource Facility at Montana State University (Bozeman, MT, United States). The murine model of soft-tissue infection was performed as previously described (Voyich et al., 2006; Nygaard et al., 2010; Malachowa et al., 2013).

Briefly, 12-week-old BALB/C mice were shaved and hair completely removed with Nair™ treatment. Two days later, shaved mice (5 per group) were subcutaneously inoculated with 1×10^7 CFUs of *S. aureus* in 100 uL DPBS. Mice were weighed and abscess size measured at indicated times post-inoculation. The area of soft-tissue infections was determined using the following formula as previously published (Malachowa et al., 2013): Area = π (Length/2) × Width/2.

RESULTS

The Generation of Point Mutations in the USA300 Genome That Confer Aspartic Acid to Alanine Substitutions Within the Receiver Domain of SaeR

Analysis of the three-dimensional crystal structure of the two-component response regulator PhoB of *Escherichia coli* (Solà et al., 1999) indicates that the homologous *S. aureus* response regulator SaeR is phosphorylated on a conserved aspartic acid at residue 51 (**Figure 1A**). Aspartic acid residue 46 is also highly conserved in the OmpR family of proteins, suggesting this residue might be important for SaeR function. Analysis of the SaeR sequences extracted from over 8,000 draft *S. aureus* genomes revealed that aspartic acid residue 51 is 100% conserved. The biological significance of this conservation is heightened by the identification of 50 unique SaeR protein sequences identified from the draft genomes (**Supplementary Figure S1**).

To determine if these aspartic acid residues in the receiver domain of SaeR are important for *S. aureus* pathogenesis, we used allelic recombination to induce substitutions of specific nucleic acids within the *S. aureus* genome as previously described by others (Bae and Schneewind, 2006; Villaruz et al., 2009; Mairpady Shambat et al., 2016). Using this technique, we generated USA300 strains with an aspartic acid to alanine substitution at the conserved SaeR residue 51 (USA300Δ*saeR*-D51A) or the proximal conserved residue 46 (USA300Δ*saeR*-D46A). In addition, we generated an aspartic acid to alanine substitution at the less maintained residue 61 of SaeR (USA300Δ*saeR*-D61A) to serve as a control for this study. Primary DNA sequencing analysis and additional whole genome sequencing analysis verified the targeted amino acid substitutions that were generated in the three mutants and showed that there were no additional SNPs or InDels when compared to the wild-type strain. As with previously generated isogenic deletion mutants of *saeR/S* in *S. aureus* (Voyich et al., 2009; Nygaard et al., 2010),

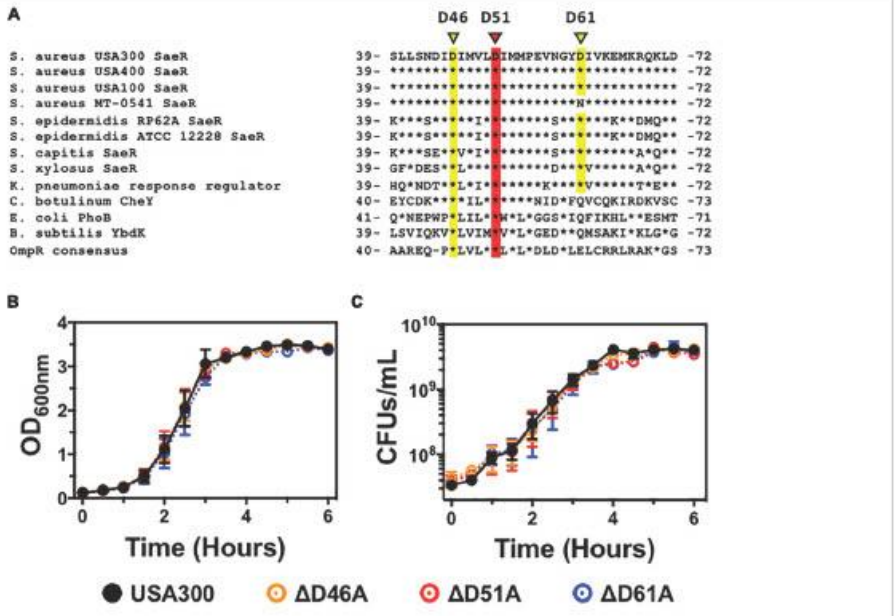

FIGURE 1 | The generation of USA300 genomic point mutations in specific aspartic acid residues of *saeR*. **(A)** DNA sequence alignment of *saeR* homologues from different bacteria highlighting the putative phosphorylation site at conserved aspartic acid residue 51 (red) as well as proximal aspartic acid residues 46 and 61 (yellow). *In vitro* growth as measured by **(B)** absorbance at 600 nm, and **(C)** colony forming units (CFU) per mL of USA300, USA300 with an aspartic acid to alanine point mutation at SaeR residue 46 (ΔD46A), at residue 51 (ΔD51A), or at residue 61 (ΔD61A). Data in panels **(B,C)** are presented as the mean ± SEM of three independent experiments.

no differences during *in vitro* growth could be detected in these SaeR point mutants relative to the parental USA300 strain (**Figures 1B,C**).

Substitution of SaeR Aspartic Acid Residue 51 With Alanine Substantially Decreases the Transcript Abundance of USA300 Virulence Genes

The SaeR/S two-component system plays an essential role during pathogenesis by upregulating *S. aureus* virulence gene transcription (Giraudo et al., 1997; Goerke et al., 2001; Voyich et al., 2009; Nygaard et al., 2010). It is thought that this process requires the activation of SaeR via phosphorylation at an aspartic acid residue within the receiver domain of this response regulator. Indeed, others have indicated that aspartic acid residue 51 of SaeR is necessary for the transcription of α-hemolysin (Hla) using an artificial plasmid overexpression system in *S. aureus* strain Newman lacking wild-type *saeR/S* (Mainiero et al., 2010). However, the transcription of *saeR* that is under a positive feedback loop by the SaeR/S two-component system was actually enhanced in the absence of aspartic acid 51 while no difference in SaeR/S regulated coagulase A transcription could be demonstrated (Mainiero et al., 2010). This study suggested that different levels of SaeR phosphorylation corresponds to the up-regulation of specific sets of virulence genes, prompting us to also examine the influence of the highly conserved SaeR aspartic acid residue 46 and partially conserved aspartic acid residue 61 on SaeR/S-mediated virulence gene transcription.

To resolve the importance of these SaeR aspartic acid residues for virulence gene expression in clinically relevant MRSA, we examined the transcript abundance of numerous adhesins, toxins, and immunomodulatory proteins in the USA300 aspartic acid point mutant strains using relative quantitative real time RT-PCR (**Figure 2**). Compared to USA300, we observed substantial decreases in the abundance of transcripts encoding various adhesins, toxins, and immunomodulatory proteins in a USA300 isogenic deletion mutant of *saeR/S* (USA300Δ*saeR/S*) relative to the USA300 wt (**Figures 2A,B**). The virulence gene transcription profile for USA300Δ*saeR*-D51A was almost identical to that of USA300Δ*saeR/S*. In contrast, the transcript abundance of virulence genes in USA300Δ*saeR*-D46A and USA300Δ*saeR*-D61A was comparable to the USA300Δ parental wt (**Figures 2A,B**). Reintroduction of wild-type SaeR to USA300Δ*saeR*-D51A rescued defects in gene transcription (**Supplementary Figure S2**), supporting results indicating aspartic acid residue 51 of SaeR is required for

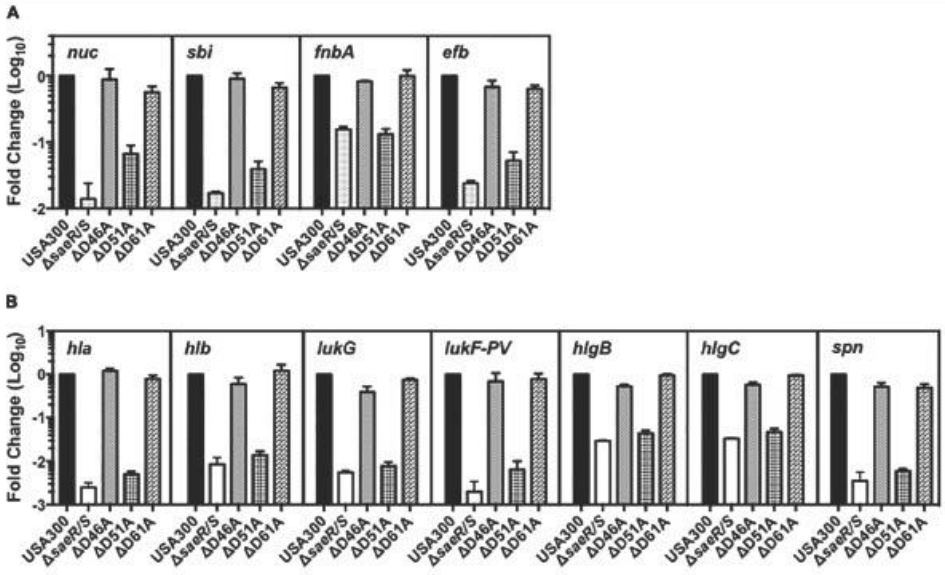

FIGURE 2 | Aspartic acid residue 51 of SaeR is important for the transcription of numerous virulence genes in USA300. Taqman® RT-PCR analysis of USA300, an isogenic deletion mutant of *saeR/S* in USA300 (USA300Δ*saeR/S*), and USA300 genomic point mutants that confer aspartic acid to alanine substitutions at SaeR residue 46 (ΔD46A), 51 (ΔD51A), or 61 (ΔD61A) during growth *in vitro*. Transcriptional analysis was performed at **(A)** mid-exponential growth for nuclease (*nuc*), the second binder of IgG (*sbi*), fibronectin-binding protein A (*fnbA*), and the extracellular fibrinogen-binding protein (*efb*) or at **(B)** early-stationary growth for γ-hemolysin (*hla*), γ-hemolysin (*hlb*), leukocidin subunit G (*lukG*), the Panton-Valentine leukocidin subunit F (*lukF-PV*), γ-hemolysin component B (*hlgB*), γ-hemolysin component C (*hlgC*), and the staphylococcal peroxidase inhibitor (*spn*). All panels show the mean ± SEM of at least two separate experiments and are presented as fold change relative to USA300 wt.

the upregulation of virulence gene expression by this response regulator.

Aspartic Acid Residue 51 of SaeR Is Essential for the SaeR/S Mediated Hemolysis of Human Erythrocytes by USA300

Staphylococcus aureus expresses numerous hemolysins under direct transcriptional regulation by the SaeR/S two-component system (Voyich et al., 2009; Nygaard et al., 2010) that lyse mammalian erythrocytes including Hla, Hlb, and HlgA/B (Salgado-Pabón et al., 2014; Spaan et al., 2015). Real time RT-PCR analysis indicated aspartic acid residue 51 of SaeR is essential for the transcriptional upregulation of these hemolysins (**Figure 2**). To determine the importance of this putative SaeR phosphorylation site for hemolysis caused by *S. aureus*, we first qualitatively assessed the ability of USA300, USA300Δ*saeR/S*, USA300Δ*saeR*-D46A, USA300Δ*saeR*-D51A, and USA300Δ*saeR*-D61A to lyse human red blood cells during growth on agar (**Figure 3A**). The zone of hemolysis generated by USA300Δ*saeR/S* on agar was decreased relative to USA300 (**Figure 3A**), corresponding to observations in this study (**Figure 2**) and in previously published reports (Voyich et al., 2009; Nygaard et al., 2010) that demonstrate a decrease in transcript abundance of various *S. aureus* hemolysins when this two-component system is absent. A reduced hemolysis of human erythrocytes during growth on agar of USA300Δ*saeR*-D51A paralleled that of USA300Δ*saeR/S*, supporting the notion that aspartic acid residue 51 is essential for SaeR/S activity. In contrast, the zone of hemolysis for USA300Δ*saeR*-D46A and USA300Δ*saeR*-D61A was indistinguishable from that caused by USA300.

To quantify the hemolysis caused by aspartic acid point mutants of SaeR, we measured the turbidity of human erythrocytes in solution after being combined with extracellular proteins produced by these strains. Corresponding to hemolysis during growth on agar, significantly less human red blood cells were lysed by filtered supernatants from USA300Δ*saeR/S* or USA300Δ*saeR*-D51A relative to the USA300 parental strain (**Figure 3B**). In addition, extracellular proteins produced by USA300Δ*saeR*-D46A generated significantly less hemolysis than USA300. In support of these findings, the reintroduction of wild-type SaeR to USA300Δ*saeR*-D46A or USA300Δ*saeR*-D51A

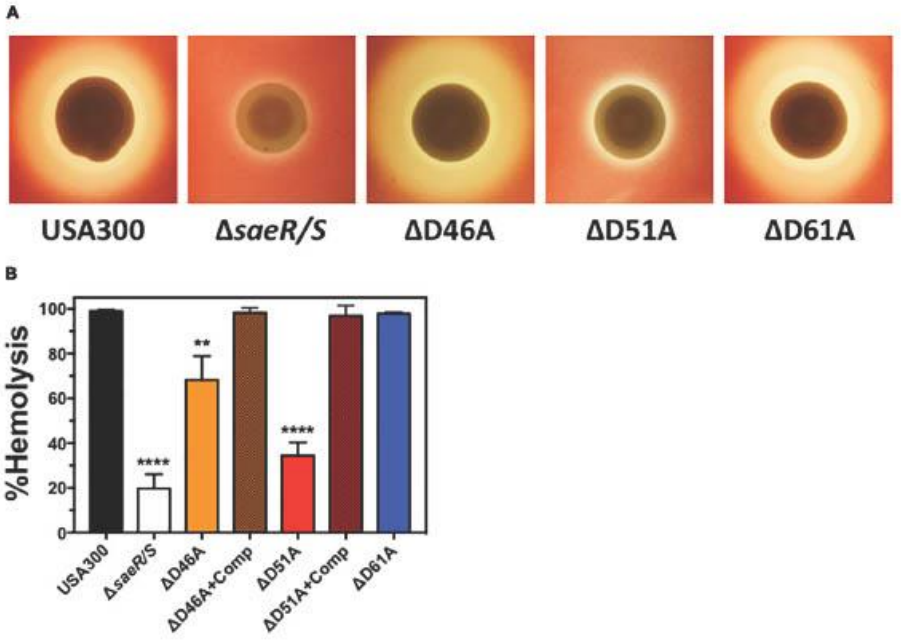

FIGURE 3 | Aspartic acid residues 46 and 51 of SaeR are important for the hemolysis of human erythrocytes by USA300. **(A)** Zone of hemolysis caused by USA300, an isogenic deletion mutant of *saeR/S* in USA300 (USA300Δ*saeR/S*), or USA300 expressing SaeR with aspartic acid to alanine substitutions at residue 46 (ΔD46A), 51 (ΔD51A), or 61 (ΔD61A) during growth on agar containing 2.5% human red blood cells. **(B)** Percent hemolysis of human red blood cells in solution exposed to 6 h supernatants from USA300, USA300Δ*saeR/S*, ΔD46A, ΔD51A, ΔD61A or ΔD46A and ΔD51A complemented with a plasmid expressing wt SaeR (ΔD46A+Comp or ΔD51A+comp, respectively). All panels represent at least 3 separate experiments. Panels **(B,C)** show the mean ± SEM with $^{**}P \leq 0.01$ and $^{****}P \leq 0.0001$ relative to USA300 as determined by one-way ANOVA with Dunnett's multiple comparison test.

rescued the hemolytic activity of supernatants from these strains to levels observed for USA300. Taken together, these findings show that aspartic acid residue 51 of SaeR is necessary for causing the hemolysis of human erythrocytes that is facilitated by the SaeR/S two-component system. In addition, SaeR aspartic acid residue 46 appears to play a significant but less important role than residue 51 mediating hemolysis caused by USA300.

Inhibition of Human MPO Activity by USA300 Extracellular Proteins Requires Aspartic Acid Residue 51 of SaeR

Recently published findings demonstrate that *S. aureus* prevents the generation of reactive oxygen species by human polymorphonuclear leukocytes (neutrophils or PMNs) via SPIN, a protein under strong SaeR/S regulation that binds directly to the active site of human MPO and inhibits peroxidase activity of this enzyme (Guerra et al., 2016; de Jong et al., 2017). To determine if the putative phosphorylation site at aspartic acid residue 51 of SaeR is required for the inhibition of MPO activity by USA300, we assessed the activity of human MPO in the presence of extracellular proteins produced by USA300, an isogenic deletion mutant of SPIN in USA300 (USA300Δ*spn*), USA300Δ*saeR/S*, USA300Δ*saeR*-D46A, USA300Δ*saeR*-D51A, and USA300Δ*saeR*-D61A (**Figure 4**). Congruent with previously published findings (de Jong et al., 2017), a strong inhibition of human MPO activity was observed in the presence of the MPO inhibitor azide or extracellular proteins produced by USA300 but not extracellular proteins produced by USA300Δ*saeR/S* or by USA300Δ*spn* (**Figures 4A,B**). As with USA300Δ*saeR/S* and USA300Δ*spn*, extracellular proteins produced by USA300Δ*saeR*-D51A did not inhibit human MPO activity (**Figures 4A,B**). Reintroduction of wild-type SaeR to USA300Δ*saeR*-D51A rescued the inhibition of human MPO

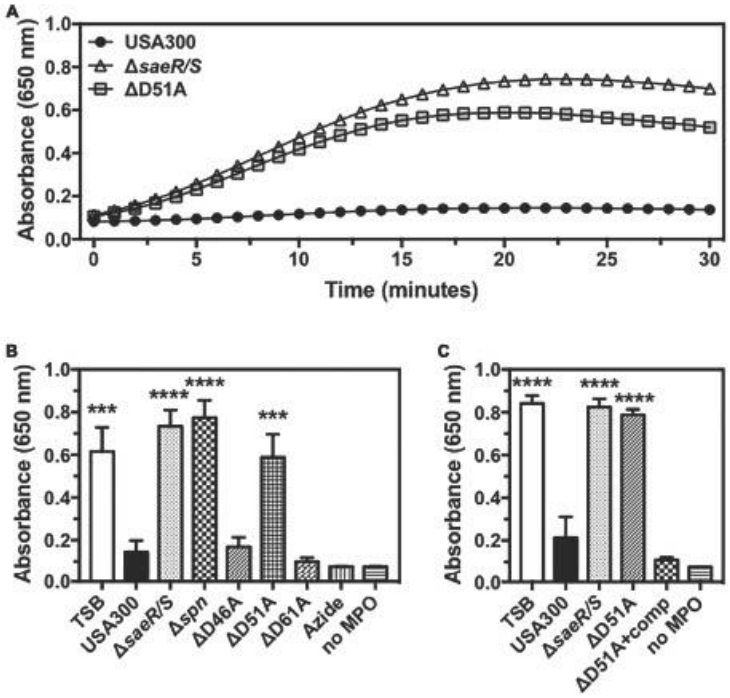

FIGURE 4 | Aspartic acid residue 51 of SaeR is essential for the inhibition of human myeloperoxidase activity by USA300. **(A)** Human myeloperoxidase (MPO) activity in the presence of extracellular proteins produced by USA300, an isogenic deletion mutant of *saeR/S* in USA300 (Δ*saeR/S*), or an aspartic acid to alanine substitution at residue 51 of SaeR in USA300 (ΔD51A) as measured by absorbance at 650 nm following exposure to 3,3′,5,5′-Tetramethylbenzidine (TMB) and hydrogen peroxide. **(B)** The activity of human MPO in the presence of extracellular proteins produced by USA300, Δ*saeR/S*, an isogenic deletion mutant of SPIN in USA300 (Δ*spn*), ΔD51A, or USA300 with an aspartic acid to alanine substitution at residue 46 (ΔD46A) or 61 (ΔD61A) of SaeR. Peroxidase activity in the presence of the MPO inhibitor sodium azide (Azide) or samples lacking MPO (no MPO) were included as negative controls. **(C)** Experiments in panel **(B)** were repeated using a D51A point mutant of *saeR* complemented with a plasmid expressing wt SaeR (ΔD51A+comp). All panels show the mean ± SEM of at least three independent experiments. For panels **(B,C)**, absorbance is shown at 20 min with ***$P < 0.001$ and ****$P < 0.0001$ relative to USA300 as determined by one-way ANOVA with Dunnett's multiple comparisons test.

activity by extracellular proteins produced from this strain (**Figure 4C**). In contrast, supernatant from USA300Δ*saeR*-D46A and USA300Δ*saeR*-D61A blocked the activity of human MPO.

Human PMN Plasma Membrane Permeability Caused by USA300 Requires Aspartic Acid at Residue 51 of SaeR

Previous studies have demonstrated that the SaeR/S two-component system upregulates *S. aureus* toxins that target human PMNs (Gauduchon et al., 2001; Voyich et al., 2009; Nygaard et al., 2010; Ventura et al., 2010; DuMont et al., 2011; Malachowa et al., 2011). To assess the importance of aspartic acid residues within the receiver domain of SaeR for mediating toxicity against PMNs, we first examined the ability of extracellular proteins produced by D46A, D51A, and D61A point mutants of SaeR to cause human PMN plasma membrane permeability as measured by propidium iodide staining (**Figure 5A**). No plasma membrane permeability was observed for PMNs exposed to extracellular proteins produced by USA300Δ*saeR*-D51A, indicating aspartic acid residue 51 of SaeR is necessary for the toxicity of USA300 against human PMNs. A significant reduction in PMN plasma membrane permeability was also observed for extracellular proteins produced by USA300Δ*saeR*-D46A, though this difference was less than that observed for USA300Δ*saeR*-D51A. In support of these findings, complementation of USA300Δ*saeR*-D46A, or USA300Δ*saeR*-D51A with wild-type SaeR rescued the lytic capacity of extracellular proteins produced by these strains against human PMNs. Corresponding to the toxicity of extracellular proteins produced by these strains, significantly less PMN plasma membrane permeability was observed following phagocytosis of live USA300Δ*saeR/S*, USA300Δ*saeR*-D51A, and USA300Δ*saeR*-D46A relative to USA300 (**Figure 5B**). These results demonstrate that SaeR aspartic acid residue 51 and, to a lesser extent, aspartic acid residue 46 are important for activity of this two-component system.

Aspartic Acid Residue 51 of SaeR Is Essential for the Pathogenesis of USA300 During Murine Soft-Tissue Infections

Previously published findings demonstrate the upregulation of virulence gene transcription *in vivo* by the SaeR/S two-component system is essential for the pathogenesis of *S. aureus* during both systemic and localized infection (Voyich et al., 2009; Nygaard et al., 2010). Transcriptional analysis of USA300Δ*saeR*-D51A in this report (**Figure 2**) indicates aspartic acid residue 51 of SaeR is required for upregulating *S. aureus* virulence gene transcription mediated by SaeR/S *in vitro*. To determine if potential SaeR phosphorylation sites are important for pathogenesis *in vivo*, we assessed the virulence of USA300, USA300Δ*saeR/S*, USA300Δ*saeR*-D46A, USA300Δ*saeR*-D51A, and USA300Δ*saeR*-D61A during a murine model of soft-tissue infection (**Figure 6**). In a manner indistinguishable from USA300Δ*saeR/S*, soft-tissue infections caused by USA300Δ*saeR*-D51A were significantly smaller than infections caused by USA300 (**Figure 6A**) and did not exhibit the open dermonecrotic lesions characteristic of USA300 pathogenesis (**Figure 6B**) that is attributed to the high expression of Hla by this strain (Kennedy et al., 2010). In addition, a significant decrease in the weight of mice following infection with USA300 was not observed following inoculation with USA300Δ*saeR/S* or USA300Δ*saeR*-D51A (**Figure 6C**). These findings could not be explained by differences in inoculum

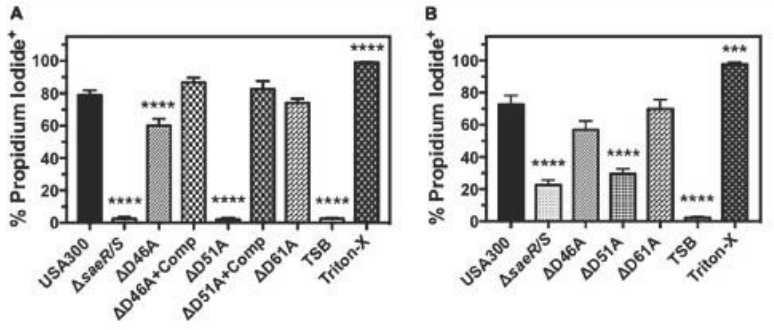

FIGURE 5 | Human PMN plasma membrane permeability caused by USA300 is significantly influenced by aspartic acid residues 46 and 51 of SaeR. **(A)** Flow cytometry was used to assess the percentage of purified human PMNs permeable to propidium iodide 90 min after exposure to 5 h supernatants from USA300, an isogenic deletion mutant of *saeR/S* in USA300 (USA300Δ*saeR/S*), or USA300 expressing SaeR with aspartic acid to alanine substitutions at residue 46 (ΔD46A), 51 (ΔD51A), 61 (ΔD61A), and ΔD46A or ΔD51A complemented with plasmids expressing wt SaeR (ΔD46A+Comp or ΔD51A+comp, respectively). **(B)** The percentage of propidium iodide positive human PMNs 90 min after the phagocytosis of live *Staphylococcus aureus* at a ratio of 20 colony forming units per PMN. For these experiments, PMNs were also treated with tryptic soy broth (TSB) alone or TSB with 0.5% Triton X-100 (Triton-X). All panels show the mean ± SEM of at least 5 independent experiments. ***$P \leq 0.001$ and ****$P \leq 0.0001$ relative to USA300 as determined by one-way ANOVA with Dunnett's multiple comparison test.

concentration given to mice for each *S. aureus* strain tested (**Figure 6D**). As opposed to USA300Δ*saeR*-D51A, no differences could be distinguished between soft-tissue infections caused by USA300Δ*saeR*-D46A or USA300Δ*saeR*-D61A relative to USA300 (**Figures 6A–C**). These results demonstrate that USA300 pathogenesis during murine soft-tissue infection is dependent upon aspartic acid residue 51 of SaeR while aspartic acid residues 46 and 61 are not essential for causing disease.

DISCUSSION

Bacterial pathogens must recognize environmental cues and respond appropriately to cause disease. For *S. aureus*, the SaeR/S two-component system plays an important role during pathogenesis by sensing host-specific signals and up-regulating virulence gene expression in response (Geiger et al., 2008; Zurek et al., 2014). In this report, we show that the substitution of the putative SaeR phosphorylation site at aspartic acid residue 51 completely ameliorates SaeR/S-mediated virulence. Specifically, we observed a dramatic decrease in the transcript abundance of various hemolysins and leukocidins in USA300 lacking aspartic acid residue 51 of SaeR. These findings correspond to a decrease in the ability of this strain to lyse human erythrocytes and PMNs. SaeR aspartic acid residue 51 was also shown to be important for the transcription of the human MPO inhibitor SPIN and USA300 lacking SaeR aspartic acid residue 51 did not inhibit human MPO activity. Moreover, we show that the virulence of USA300Δ*saeR*-D51A during murine models of soft-tissue infection paralleled the transcript abundance of Hla in this strain, consistent with previous findings demonstrating this toxin is a major *S. aureus* virulence determinant in this model (Kennedy et al., 2010). Collectively, these findings indicate that SaeR residue 51 is essential for *S. aureus* evasion of innate immunity and support other studies that suggest only phosphorylated SaeR has DNA binding activity (Sun et al., 2010).

We also observed a more subtle decrease in the toxicity of USA300 against human erythrocytes and PMNs in the absence of the conserved aspartic acid residue 46 of SaeR. It is not clear if substitution of this residue simply perturbs the receiver domain structure of SaeR to diminish activity or if aspartic acid residue 46 plays a more direct role in the phosphorylation state of this response regulator. Regardless, a reduction in the lytic capacity of USA300Δ*saeR*-D46A suggests this conserved residue also influences SaeR function and will be further examined in future studies.

This study demonstrates that the substitution of a single amino acid residue in the SaeR response regulator of USA300 renders this highly pathogenic strain avirulent, highlighting the critical importance of two-component sensory systems for bacterial pathogenesis. Indeed, others have shown that point mutations in the dimerization interface of the histidine kinase sensor AgrC (Mairpady Shambat et al., 2016) or in the P2 promoter region of the Agr two-component system (Villaruz et al., 2009) have a profound effect on the lytic capacity and colonization potential of *S. aureus* while a point mutation in the histidine kinase sensor LiaS of *Streptococcus pyogenes* contributes to the carrier phenotype of this bacterium (Flores et al., 2017). Interestingly, no natural mutations of aspartic acid residue 51 could be identified in over 8,000 *S. aureus* genomes, suggesting this putative phosphorylation site is imperative for the fitness of this pathogen. Taken together, these studies indicate that novel therapeutic strategies that inhibit the activation of this highly conserved response

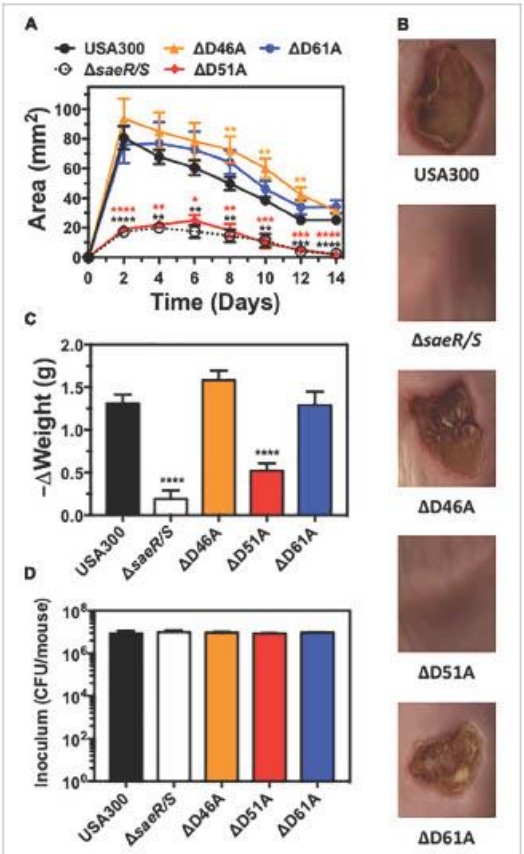

FIGURE 6 | Aspartic acid residue 51 of SaeR significantly influences the pathogenesis of murine soft-tissue infections caused by USA300. **(A)** Area of soft-tissue infection caused by USA300, an isogenic deletion mutant of *saeR/S* in USA300 (Δ*saeR/S*), or USA300 with an aspartic acid to alanine substitution at SaeR residue 46 (ΔD46A), 51 (ΔD51A) or 61 (ΔD61A). **(B)** Representative images of soft-tissue infections from experiments in panel **(A)** on day 6 post-inoculation. **(C)** Change in the weight of mice during experiments in panel **(A)** between days 0 and 2 post-inoculation. **(D)** Inoculum of each strain given to mice in panels **(A-C)**. Panels **(A)**, **(C)**, and **(D)** show the mean ± SEM of two independent experiments. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ and $^{****}P \leq 0.0001$ relative to USA300 as determined by one-way ANOVA with Dunnett's multiple comparison test.

regulator would impede the expression of multiple virulence factors and effectively block different aspects of pathogenesis resulting in clearance of *S. aureus* by innate immune mechanisms.

AUTHOR CONTRIBUTIONS

TN and JV contributed to the conception and design of this study. TN, TB, ES, FG, JD, MC, KP, LC, and BK performed the experiments and data analysis. TN and JV wrote and prepared the manuscript for submission. All authors read and approved this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.03085/full#supplementary-material

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The Accessory Gene *saeP* of the SaeR/S Two-Component Gene Regulatory System Impacts *Staphylococcus aureus* Virulence During Neutrophil Interaction

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Staphylococcus aureus (*S. aureus*) causes a range of diseases ranging from superficial skin and soft-tissue infections to invasive and life-threatening conditions (Klevens et al., 2007; Kobayashi et al., 2015). *S. aureus* utilizes the Sae sensory system to adapt to neutrophil challenge. Although the roles of the SaeR response regulator and its cognate sensor kinase SaeS have been demonstrated to be critical for surviving neutrophil interaction and for causing infection, the roles for the accessory proteins SaeP and SaeQ remain incompletely defined. To characterize the functional role of these proteins during innate immune interaction, we generated isogenic deletion mutants lacking these accessory genes in USA300 (USA300Δ*saeP* and USA300Δ*saeQ*). *S. aureus* survival was increased following phagocytosis of USA300Δ*saeP* compared to USA300 by neutrophils. Additionally, secreted extracellular proteins produced by USA300Δ*saeP* cells caused significantly more plasma membrane damage to human neutrophils than extracellular proteins produced by USA300 cells. Deletion of *saeQ* resulted in a similar phenotype, but effects did not reach significance during neutrophil interaction. The enhanced cytotoxicity of USA300Δ*saeP* cells toward human neutrophils correlated with an increased expression of bi-component leukocidins known to target these immune cells. A *saeP* and *saeQ* double mutant (USA300Δ*saePQ*) showed a significant increase in survival following neutrophil phagocytosis that was comparable to the USA300Δ*saeP* single mutant and increased the virulence of USA300 during murine bacteremia. These data provide evidence that SaeP modulates the Sae-mediated response of *S. aureus* against human neutrophils and suggest that *saeP* and *saeQ* together impact pathogenesis *in vivo*.

Keywords: sae, *Staphylococcus aureus*, neutrophil, gene regulation, virulence

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INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a highly-adaptable pathogen able to infect various tissues. Common manifestations of *S. aureus* infections range from mild skin and soft-tissue infections to invasive disease. Additionally, this pathogen has gained resistance against many anti-microbial drugs, leaving healthcare providers with few options for the treatment of infections (Chambers, 2001; Chambers and Deleo, 2009). In the past, antimicrobial-resistant *S. aureus* infections were mostly associated with a recent hospital stay, but the rise in community-associated infections has steadily increased since the late 1990s (Chambers, 2001; King et al., 2006). Drug-resistance combined with the lack of understanding of protective immunity to *S. aureus* has delayed the development of new therapeutics to treat this ubiquitous opportunistic pathogen.

The human neutrophil is essential for resolution of *S. aureus* infections, as individuals suffering from defects in neutrophil function are more susceptible to *S. aureus* infection (Lekstrom-Himes and Gallin, 2000). *S. aureus* has evolved many mechanisms to circumvent killing by these potent innate immune cells. The production of secreted virulence factors during pathogenesis is primarily controlled by the combined influence of two-component systems (TCSs) that sense the host environment and respond accordingly. Of these, the Sae TCS has been shown to be essential for evasion of human neutrophil killing (Voyich et al., 2009; Guerra et al., 2016). SaeR/S is immediately up-regulated following neutrophil phagocytosis and the histidine kinase, SaeS, is thought to specifically recognize neutrophil components (Voyich et al., 2005, 2009; Geiger et al., 2008; Mainiero et al., 2010; Cho et al., 2015; Zurek et al., 2015). The response regulator, SaeR, is activated following phosphorylation by SaeS and subsequently alters gene transcription by directly binding to a specific recognition sequence in the promoter region of numerous virulence genes including *nuc* and the bi-component leukotoxins *lukF* (PVL), *lukGH* (*lukAB*), and *hlgBC* that target human neutrophils (Nygaard et al., 2010; Olson et al., 2013; Liu et al., 2016). The upregulation of these genes facilitates *S. aureus* survival following neutrophil phagocytosis (Voyich et al., 2009; Flack et al., 2014). However, the *sae* locus also includes two accessory genes, *saeP* and *saeQ*, whose gene products are not entirely understood. Previously published *in vitro* studies suggest these proteins form a complex with SaeS that deactivates SaeR (Jeong et al., 2012). It has also been shown that increased expression of *saeP* impacts biofilm formation by increasing retention of high molecular weight DNA on the biofilm surface (Kavanaugh et al., 2019). The same study also demonstrated increases in *saeP* gene expression correlated with decreases in nuclease activity during biofilm development (Kavanaugh et al., 2019). Considering the importance of Sae during neutrophil interactions, we investigated the importance of *saeP* and *saeQ* during challenge with human neutrophils and *in vivo* using murine models of invasive disease and skin and soft-tissue infection. For these studies, we used *S. aureus* strain LAC, a USA300 isolate, as USA300 is the dominant clone causing community-associated methicillin resistant *S. aureus* (CA-MRSA) disease in the United States (David and Daum, 2010). Results demonstrate that deletion of *saeP* increased *S. aureus* cytotoxicity against neutrophils *ex vivo*. Moreover, the deletion of both *saeP* and *saeQ* markedly increased both nuclease expression in kidneys and overall mortality following intravenous infection.

RESULTS

Generation of USA300 Isogenic Mutants Deficient in Either *saeP* or *saeQ*

The Sae TCS is composed of four genes: *saeP*, *saeQ*, *saeR*, and *saeS* (**Figure 1A**). Although *saeS* and *saeR*, encoding the sensory kinase and response regulator respectively, have been demonstrated to be essential in *S. aureus* virulence and pathogenesis (Voyich et al., 2009; Nygaard et al., 2010, 2018; Flack et al., 2014; Liu et al., 2015, 2016; Zurek et al., 2015; Guerra et al., 2016), surprisingly little research has been performed on the two accessory genes of the Sae system, *saeP* and *saeQ*. Published data indicate that *saeP* encodes a lipoprotein anchored to the exterior surface of the plasma membrane, whereas *saeQ* encodes a transmembrane protein (Jeong et al., 2012; Kavanaugh et al., 2019). To investigate the roles of these genes, we utilized a *saeP* deletion mutant (USA300Δ*saeP*) generated previously in Kavanaugh et al. (2019), and deleted the first third of *saeQ* (164 bp) in USA300 LAC using allelic replacement to create an isogenic *saeQ* deletion mutant (USA300Δ*saeQ*) that preserves the P3 promoter (Bae and Schneewind, 2006; Jeong et al., 2011). Absence of *saeP* and *saeQ* genes were verified by PCR (**Figure 1A**). Importantly, deletion of *saeP* or *saeQ* did not substantially impact *saeR* and *saeS* gene expression. TaqMan® real-time RT-PCR analyses indicated a slight increase in *saeR* transcript levels in the USA300Δ*saeP* mutant at both mid-exponential (ME) and early stationary (ES) phases of growth relative to USA300. This trend was also established for the expression of *saeS* in USA300Δ*saeP* at ME phase. Expression of *saeR* and *saeS* were essentially unchanged in USA300Δ*saeQ* relative to USA300 at ME but were decreased during ES (**Figure 1B**). Basal expression from the P3 promoter is likely unaffected by deletion of *saeQ* since the deletion ends 108 base pairs upstream of the P3 promoter (Jeong et al., 2011) and ME expression is similar to USA300. When the Sae TCS is activated, transcription from the P1 promoter increases (Novick and Jiang, 2003; Steinhuber et al., 2003). Therefore, when the system activates in ES (Flack et al., 2014), mRNA transcripts containing the *saeQ* deletion may have reduced stability since the mRNA secondary structure has recently been shown to be important (Marincola and Wolz, 2017). Nevertheless, this is not expected to affect the activation of SaeR/S target genes, as overexpression of *saeRS* does not alter the expression profile of the Sae-regulon (Mainiero et al., 2010; Liu et al., 2016). Additionally, USA300Δ*saeP* and USA300Δ*saeQ* strains showed no significant growth defects compared to USA300 (**Figure 1C**).

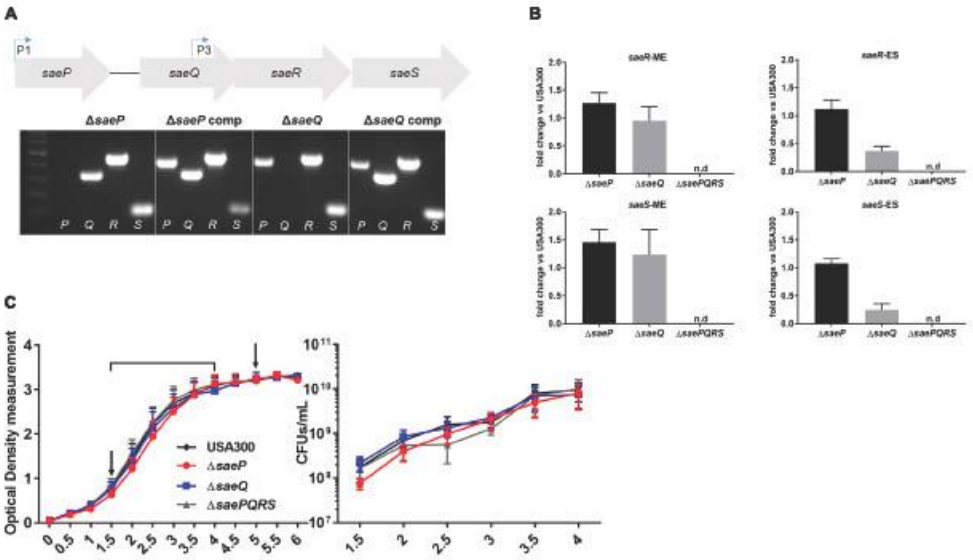

FIGURE 1 | Generation of an isogenic *saeQ* mutant. **(A)** (Above) Schematic of the four genes of the *sae* locus. Bent arrows indicate transcriptional start sites described in Novick and Jiang (2003), Steinhuber et al. (2003), and Jeong et al. (2011). (Below) Results of agarose gel electrophoresis showing USA300 mutants lacking *saeP* (from Kavanaugh et al., 2019), *saeQ*, and respective complemented strains. Letters at the bottom of the gel indicate the *sae* gene targeted by PCR analysis. Primer sequences are included in **Table 1**. **(B)** TaqMan® RT-PCR analysis of *saeS* and *saeR* transcript levels in USA300Δ*saeP* and USA300Δ*saeQ* relative to USA300 at mid-exponential phase (ME) and early stationary phase (ES). Transcript levels in samples were analyzed in triplicate and results are from two independent experiments. **(C)** *In vitro* growth of USA300, USA300Δ*saeP*, USA300Δ*saeQ*, and USA300Δ*saePQRS* (Flack et al., 2014) measured by optical density at 600 nm (OD_{600}) (left) and colony forming units (CFUs) (right). Timepoints are indicated by the bracket on the OD_{600} plot. Arrows indicate time points for RNA harvest (used in **B**). Data are presented as the mean ± SEM of seven independent experiments. n.d., not detected.

Deletion of *saeP* Increases Bacterial Survival Following Neutrophil Phagocytosis

SaeS has been shown to be activated by neutrophil phagocytosis and associated components including alpha-defensin and hydrogen peroxide (Voyich et al., 2005; Geiger et al., 2008; Zurek et al., 2014). Moreover, deletion of *saeR/S* has been shown to significantly decrease *S. aureus* survival and cytolytic capacity following neutrophil phagocytosis (Voyich et al., 2009; Flack et al., 2014). However, nothing is known about how SaeP and SaeQ contribute to staphylococcal neutrophil evasion. To determine the role of these accessory proteins during interaction with human neutrophils, we initially evaluated phagocytosis and killing of USA300Δ*saeP*, USA300Δ*saeQ*, USA300Δ*saePQRS* (Flack et al., 2014), or USA300. Importantly, there were no significant differences in the uptake of these strains by human neutrophils (**Figures 2A,B**), consistent with previous observations using Δ*saeR/S* strains that have shown the SaeR/S system has no significant impact on neutrophil phagocytosis (Voyich et al., 2009; Guerra et al., 2016). Next, we assessed *S. aureus* survival after neutrophil phagocytosis. After 30 min, we measured very modest increases in the survival of the *saeP* and *saeQ* mutant strains relative to the parental wild-type strain. However, deletion of *saeP* significantly increased bacterial survival compared to USA300 5-h after phagocytosis. There was also a noticeable although not statistically significant increase in survival of USA300Δ*saeQ* compared to USA300 at 5 h post-neutrophil exposure. We measured a significant reduction in survival of USA300Δ*saePQRS* strain at both timepoints, confirming the importance of *saeR/S* for *S. aureus* survival following neutrophil phagocytosis as previously observed (Voyich et al., 2009; **Figure 2C**).

Deletion of *saeP* Increases Production of Neutrophil Cytolytic Factors

SaeR/S up-regulates the transcription of numerous secreted virulence factors including the bi-component leukotoxins LukG/H, PVL, and HlgB/C that specifically target and disrupt the neutrophil plasma membrane (Voyich et al., 2009; Nygaard et al., 2010; Sun et al., 2010; Ventura et al., 2010; Flack et al., 2014; Zurek et al., 2014). Since USA300Δ*saeP* cells demonstrated increased survival following phagocytosis, we hypothesized that *saeP* might influence the production of secreted cytolytic factors (i.e., ability to permeabilize neutrophils). Indeed, neutrophils exposed to filtered supernatants taken from ES cultures of the USA300Δ*saeP* mutant exhibited significantly

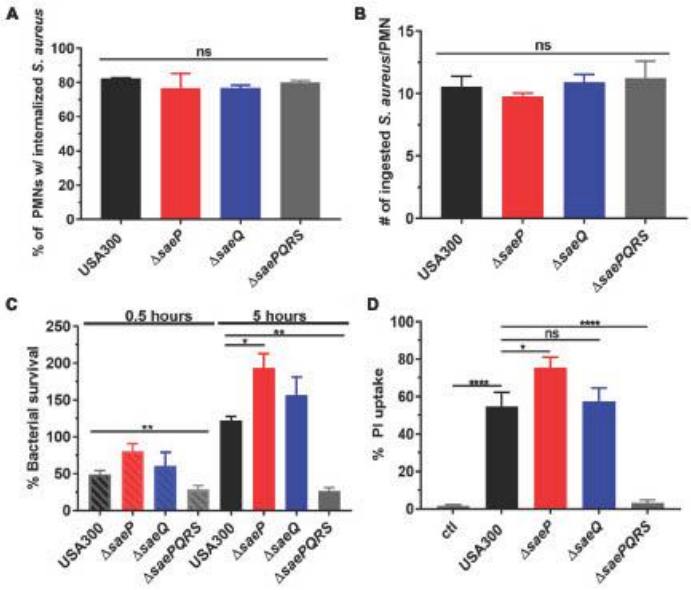

FIGURE 2 | Deletion of *saeP* significantly increases *S. aureus* survival and toxicity during neutrophil interaction. **(A)** Percent *S. aureus* ingested by neutrophils. Samples were collected on an Image Stream® Imaging Flow Cytometer and *S. aureus* internalization was analyzed using the IDEAS software® as described in Materials and Methods for three independent experiments. **(B)** Numbers of ingested *S. aureus* per PMN are shown. The average number of *S. aureus* detected to be ingested per PMN is ~ 10 for all strains. Data are presented as the mean ± SEM of three independent experiments. **(C)** Percent survival for the indicated strains and timepoints is shown. Bacterial survival is significantly increased in USA300Δ*saeP* compared to USA300 at 5 h following phagocytosis. Survival was calculated with the following equation: (CFU + PMN at time$_h$/CFU + PMN at time$_0$) × 100 (as in Voyich et al., 2005). Data are presented as the mean ± SEM of six independent experiments. At 0.5 h, USA300Δ*saePQRS* was significantly different from USA300 $^{**}P \leq 0.001$ using one-way ANOVA with Tukey's post-test. At 5 h, $^{*}P \leq 0.05$ and $^{**}P \leq 0.001$ relative to USA300 using one-way ANOVA and Tukey's post-test. **(D)** Supernatants from USA300Δ*saeP* cultures cause significantly more neutrophil plasma membrane damage compared to supernatants from USA300 cultures. *S. aureus* strains were grown to early stationary phase, supernatants harvested as described in Materials and Methods (diluted 1:10), and incubated with neutrophils for 1 h. Propidium iodide (PI) uptake was assessed by flow cytometry. Data are presented as the mean ± SEM of five independent experiments. $^{*}P \leq 0.05$, $^{****}P \leq 0.0001$ using one-way ANOVA with Tukey's post-test. ctl, neutrophil-only control. ns, not significant.

more plasma membrane damage than neutrophils exposed to supernatants taken from cultures of USA300 as determined by propidium iodide uptake (**Figure 2D**). Supernatants from USA300Δ*saeQ* showed no significant differences in cytolytic activity compared with USA300. Confirming previous observations with Δ*saeR/S* strains, neutrophils exposed to the supernatants from USA300Δ*saePQRS* cultures showed significantly reduced plasma membrane damage compared to results from exposure to culture supernatants from all other *S. aureus* strains tested and similar to that of neutrophils not exposed to *S. aureus* supernatants (Voyich et al., 2009; Flack et al., 2014). USA300Δ*saeP* complemented with *saeP* in trans reduced the cytotoxicity of this strain to levels that paralleled USA300 (**Supplementary Figure S1A**).

Deletion of *saeP* Increases Transcript Abundance of Several Leukotoxins

The SaeR/S system is essential for transcriptional regulation of virulence factors known to impact neutrophil function (Voyich et al., 2009; Mainiero et al., 2010; Nygaard et al., 2010; Flack et al., 2014; Zurek et al., 2014; Guerra et al., 2016). Since the USA300Δ*saeP* strain demonstrated increased survival following phagocytosis by neutrophils, and supernatants from USA300Δ*saeP* had increased cytolytic activity toward neutrophils, we profiled the transcript abundance of select SaeR/S-regulated virulence factors known to impact neutrophil viability. During mid-exponential phase (ME) we measured subtle increases in *lukG* transcript abundance in both USA300Δ*saeP* and USA300Δ*saeQ* mutant strains, as well as a subtle increase in *lukF*-PVL transcript abundance levels in USA300Δ*saeP* (**Figure 3A**). Importantly, we measured more pronounced increases in *lukG*, *lukF-PV*, *hlgA*, *hlgB*, and *hlg*C transcript abundance in USA300Δ*saeP* relative to USA300 during early stationary (ES) phase. Transcript abundance of all select virulence genes was reduced in the USA300Δ*saePQRS* mutant in accordance with previous observations examining the influence of Sae on virulence gene transcription (Voyich et al., 2009; Nygaard et al., 2010; Flack

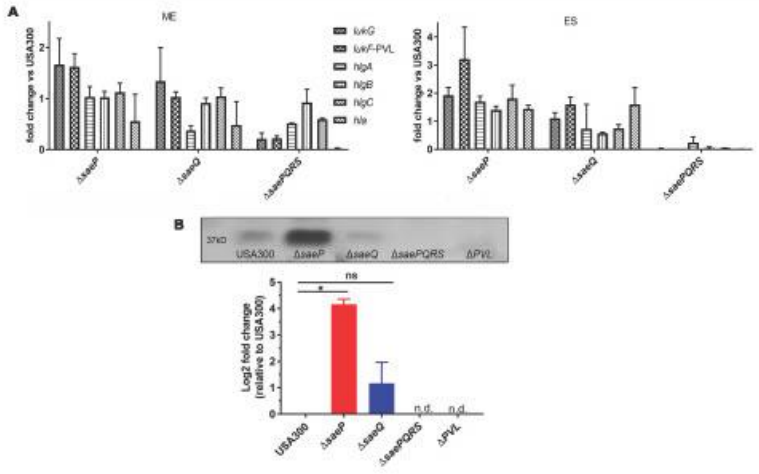

FIGURE 3 | USA300Δ*saeP* demonstrates increased transcript abundance of several known SaeR/S-virulence factors. **(A)** Mean fold-change of known SaeR/S-regulated virulence genes in USA300Δ*saeP* and USA300Δ*saeQ* relative to USA300 is shown. Transcript abundance was measured using TaqMan® RT-PCR at mid-exponential (ME; left) and early stationary (ES; right) phases of *in vitro* growth. Transcripts were normalized to *gyrB* and calibrated to transcript abundance in USA300. Data are from at least two independent experiments. **(B)** The USA300Δ*saeP* strain of *S. aureus* produces significantly more Panton Valentine Leukocidin (PVL) compared to USA300. (Top) Representative Western blot of PVL protein in supernatants from *S. aureus* grown overnight. (Bottom) Quantification of PVL was calculated using densitometry as described in Materials and Methods. Data shown are amounts relative to USA300 and presented as the mean ± SEM of three independent experiments. *$P \leq 0.05$ One-Way ANOVA with Tukey's post-test. n.d., not detected.

et al., 2014; Zurek et al., 2014). Expression of *saeP* in trans in the USA300Δ*saeP* strain reduced transcript abundance and cytotoxicity to levels at or below those measured in USA300 (**Supplementary Figures S1B,C**).

Supporting transcript analysis of *lukF-PV*, secreted PVL in overnight culture supernatants was significantly increased in the USA300Δ*saeP* strain compared to USA300 (**Figure 3B**). As anticipated, PVL was essentially undetectable in culture supernatants from the Δ*saePQRS* mutant, demonstrating a strong dependency on Sae for production of PVL (**Figure 3B**).

Deletion of *saeQ* Attenuates Mortality in Mice Following Intravenous Infection

To investigate the individual roles of *saeP* and *saeQ* during staphylococcal disease, we used a well-established model of acute bacteremia (Voyich et al., 2009; Nygaard et al., 2010). Mice (groups of 10) were infected intravenously with 1×10^7 CFUs of either *S. aureus* USA300, USA300Δ*saeP*, USA300Δ*saeQ*, or USA300 Δ*saePQRS*. Consistent with previous studies (Nygaard et al., 2010), ~65% of the mice infected with USA300 died within 48 h, and on average fewer than 10% of the mice survived 72 h post-infection. Although there were no significant differences in the mortality of mice challenged with USA300Δ*saeP* compared with USA300, nearly all mice infected with USA300Δ*saeQ* survived 72 h post-infection (**Figure 4**). All mice challenged with Δ*saePQRS* survived, congruent with previous studies (Voyich et al., 2009; Nygaard et al., 2010) and demonstrating the critical role of the Sae system following bloodstream infection (**Figure 4**).

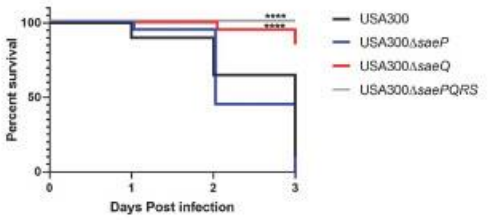

FIGURE 4 | *saeQ* contributes to staphylococcal disease during bacteremia. Groups of 10 C57BL/6 mice were infected intravenously via the tail vein with 1×10^7 CFU of the indicated *S. aureus* strains. Survival curves are from two independent experiments ****$P < 0.0001$, log-rank (Mantel-Cox) test.

SaeR/S is also critical for *S. aureus* pathogenesis during murine skin and soft-tissue infection (SSTI) (Voyich et al., 2009; Nygaard et al., 2010, 2018). To investigate the importance of SaeP and SaeQ in SSTI, BALB/c and C57BL/6 mice were infected subcutaneously with 1×10^7 CFUs of either USA300 or our isogenic mutants, and abscess area was monitored for 10 days. While we measured a significant decrease in the abscess area of mice infected with the USA300Δ*saePQRS* mutant compared to USA300, we detected no significant differences in abscess size or incidence of dermonecrosis when either *saeP* or *saeQ* were deleted (**Supplementary Figures S2A,B**). Taken together, although Sae TCS activity is required for full virulence in both

bacteremia and SSTI, our data indicate SaeP and SaeQ are dispensable during SSTI, but SaeQ appears to be important during bacteremia.

USA300Δ*saePQ* Mimics the USA300Δ*saeP* Phenotype During Neutrophil Interaction, but Significantly Increases Mortality Following Intravenous Infection in Mice

Due to our observations that USA300Δ*saeP* demonstrates increased ability to survive neutrophil phagocytosis and that USA300Δ*saeQ* had increased (although not significant) survival following neutrophil phagocytosis, we wondered whether a double mutant deficient in both *saeP* and *saeQ* might exhibit an enhanced phenotype in the aforementioned neutrophil assays. To test this, we first deleted the entire *sae* locus in LAC using allelic exchange and then introduced *saeRS* driven by their native P3 promoter into the chromosome at the *geh* locus using the single copy integration plasmid pCL55 (Liu et al., 2015). The resulting strain (hereafter referred to as USA300 Δ*saePQ*) exhibited reduced expression of *saeR/S* during exponential growth *in vitro* (**Supplementary Figures S3A,B**). This could be due to the absence of transcriptional readthrough from the stronger P1 promoter. Regardless, the USA300 Δ*saePQ* double mutant is still capable of inducing SaeR/S-mediated virulence gene expression in response to human neutrophil peptide-1 (HNP-1) exposure (**Supplementary Figure S3C**). Compared to USA300, USA300 Δ*saePQ* exhibited a significant increase in both bacterial survival following phagocytosis as well as increased cytotoxicity toward neutrophils. This increase in virulence was consistent with observations made with USA300Δ*saeP* in both neutrophil survival and plasma membrane damage (**Figure 5** compared to **Figure 2**). We measured similar fold-changes in the expression of Sae-dependent virulence genes in USA300Δ*saePQ* (also similar to those observed in USA300Δ*saeP*) during ES compared to USA300 (compare *lukG* and *lukF-PVL* in **Figure 5** and **Figure 2**). As we saw in the SSTI model following challenge with the USA300Δ*saeP* or USA300Δ*saeQ* mutants, mice challenged with the USA300Δ*saePQ* mutant showed no significant differences in abscess area compared to USA300 (**Figure 5D**). However, we found that intravenous infection with the USA300Δ*saePQ* double mutant led to a significant increase in mortality compared to infection with USA300 in the bacteremia model (**Figure 6**).

To investigate if the increased mortality in mice challenged with the USA300 Δ*saePQ* mutant was due to increased expression of SaeR/S-dependent virulence factors, we infected mice with either USA300 or USA300Δ*saePQ* mutant carrying an integrated *nuc-gfp* translational fusion (Behera et al., 2019). Three days post-infection, groups of three-eight C57BL/6 mice were euthanized. Kidneys were harvested, fixed and embedded, and examined using confocal microscopy. Consistent with previous

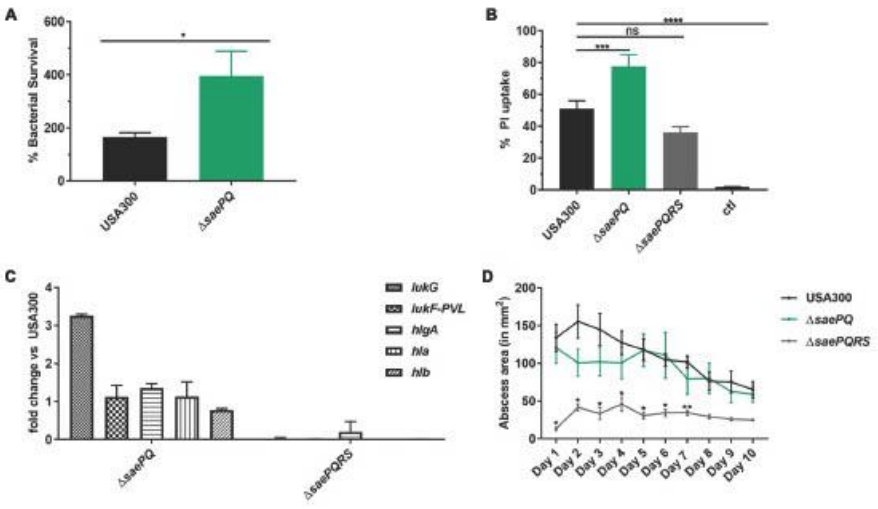

FIGURE 5 | USA300Δ*saePQ* demonstrates enhanced survival following neutrophil phagocytosis. **(A)** USA300Δ*saePQ* survives neutrophil killing significantly better than USA300 after 5 h incubation. Data are presented as the mean ± SEM of eight independent experiments. **(B)** PMN plasma membrane damage was significantly increased in neutrophils infected with USA300Δ*saePQ* compared to infection with USA300 (neutrophil: bacteria ratio of 1:5). Propidium iodide uptake was assessed at 3 h post-infection by flow cytometry and indicate USA300Δ*saePQ* produces significantly more cytolytic proteins in the supernatants than USA300. Data are presented as the mean ± SEM of five independent experiments. **(C)** Transcript abundance of *lukG* is increased in USA300Δ*saePQ* relative to expression in USA300 at early stationary phase. Gene transcripts were normalized to *gyrB* and calibrated to the expression levels of USA300. **(D)** Deletion of *saePQ* did not significantly impact abscess area. C57BL/6 mice were infected subcutaneously with 1×10^7 CFU of *S. aureus*. Abscess area was measured daily and results shown are the average of five mice per group. $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$ via one-way ANOVA Tukey's post-test. Data are presented as the mean ± SEM.

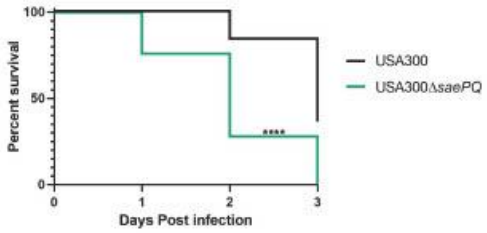

FIGURE 6 | Deletion of *saePQ* enhances the virulence in *S. aureus* in a mouse model of systemic infection. Survival curves for C57BL/6 mice (n = 13/group) challenged with either 1×10^7 CFU *S. aureus* or USA300 or USA300Δ*saePQ* via tail vein injection. Data shown are death from infection and euthanasia (for exceeding scores on the welfare rubric) during the 72 h endpoint. Data are from two independent experiments. ****$P < 0.0001$, log-rank (Mantel-Cox) test.

results, USA300 formed clearly-defined abscesses containing a nidus of staphylococci also referred to as a staphylococcal abscess community (SAC). These bacteria exhibit spatial regulation of *nuc-gfp* as we previously reported (Behera et al., 2019). That is, we detected strong expression of *nuc-gfp* in the core of the SAC and muted expression on the periphery of the SAC. On the other hand, the USA300Δ*saePQ* mutant failed to form discrete, well-formed abscesses. Instead, widespread infiltration of the renal tissues was apparent, and the *nuc-gfp* reporter was highly expressed in bacteria on the periphery of the lesions (**Supplementary Figure S4**). Taken together, these data indicate that the *saePQ* mutant phenocopies a *saeP* mutant during human neutrophil interaction, and lack of both gene products impacts pathogenesis and virulence expression during bacteremia.

DISCUSSION

The Sae TCS of *S. aureus* contributes to the expression and production of virulence and immunomodulatory factors that are essential for *S. aureus* neutrophil evasion and pathogenesis (Voyich et al., 2009; Nygaard et al., 2010; Sun et al., 2010; Flack et al., 2014; Zurek et al., 2014). Much is known about the molecular genetics of activation of the SaeS bifunctional kinase/phosphatase and the SaeR response regulator. However, despite its discovery over two decades ago, relatively little is known about how the auxiliary proteins SaeP and SaeQ contribute to Sae TCS activity and staphylococcal disease. Herein, we utilized *saeP* and *saeQ* single and double mutant strains to characterize the role of these accessory proteins using both *ex vivo* human neutrophil assays and *in vivo* mouse models of infection. Our results suggest that SaeP acts as a regulator of SaeR/S-dependent virulence during challenge with human neutrophils. Although no statistically significant phenotype could be established for USA300Δ*saeQ* following interactions with human neutrophils, we did note an increase in the survival of mice following intravenous infection with this strain, suggesting that SaeQ contributes to SaeR/S signaling during bacteremia.

Our findings support previously-published *in vitro* data (Jeong et al., 2012; Kavanaugh et al., 2019) suggesting SaeP may regulate SaeR/S-dependent effectors during human neutrophil encounters. The observation that the USA300Δ*saeP* strain is more cytotoxic against neutrophils and exhibits increased bacterial survival following phagocytosis suggest that the impact of SaeP on SaeR-target genes is specific and likely dependent on environmental cues. The observation that there was no additive effect of enhanced survival following neutrophil phagocytosis of USA300Δ*saePQ* compared to USA300Δ*saeP* also supports a specific role for *saeP* during interaction with human neutrophils. Future studies will continue to characterize the role of SaeQ.

S. aureus uses secreted nuclease (Nuc) along with secreted adenosine synthase (AdsA) to escape neutrophil extracellular traps (NETs) (Thammavongsa et al., 2013). Increasing Nuc expression and production in kidney tissues may increase the production of deoxyadenosine, and this could trigger caspase-3-mediated immune cell death (Thammavongsa et al., 2013). In USA300 LAC, SaeP down-regulated *nuc-gfp* gene expression via SaeR/S (Kavanaugh et al., 2019). Thus, the hypervirulent phenotype of the *saePQ* double mutant during acute bacteremia is not unexpected. However, it is unclear why the *saeP* and *saeQ* single mutants behave differently. Since we used a purified diet for the *in vivo nuc-gfp* mouse reporter studies, we cannot exclude the possibility that this diet amplifies mild phenotypes observed in mice challenged with the *saeP* and *saeQ* mutant strains. It would be interesting to examine the impact of SaeP and SaeQ during chronic kidney infections in mice. The observation that the *saeQ* mutant is highly attenuated was unexpected. Given the known protein-protein interactions between SaeQ and SaeS, it is possible that the stability and/or activity of SaeS is compromised in this mutant. Indeed, SaeQ is required for hyperactive SaeS ($SaeS^{L18P}$) stability in strain Newman (Jeong et al., 2011, 2012). Clearly there is still much to learn about the function of these proteins; only a few studies investigate SaeP and/or SaeQ.

Recently, Kavanaugh et al. (2019) confirmed the cellular localization of SaeP on the cell surface as a lipoprotein, and that its C-terminal domain is facing the extracellular matrix. SaeQ is predicted to be a membrane protein with three membrane-spanning domains, and forms a complex with SaeP and SaeS in the membrane (Jeong et al., 2012). A conserved domain search revealed that the C-terminal portion of SaeP looks like a member of the DM13 superfamily of proteins. Because of its association with the DOMON domain, is thought DM13 proteins might be involved in electron transfer. SaeQ is predicted to be a member of the DoxX family of proteins similar to *Bacillus subtilis* putative oxidoreductases MhqP and CatD, and *Escherichia coli* inner membrane proteins YphA and YqjF (Iyer et al., 2007). The Sae system is responsive to cellular respiratory status but the mechanism is unclear. One model posits that inhibition of respiration by oxygen depletion or chemical disruption of the electron transport chain by reactive oxygen species or nitrosative species could lead to a block in the respiratory chain and a buildup of reduced quinones in the membrane, activating Sae activity (Mashruwala et al., 2017). It is conceivable SaePQ sense this perturbation, go inactive, and promote SaeS kinase activity. Alternatively, SaeP possesses a pI of ~8 and is capable

of binding negatively charged eDNA in acidic environments (Kavanaugh et al., 2019). It is tempting to speculate that a physical interaction with neutrophil NET DNA induces some conformational change in SaeP that hinders its ability to stimulate phosphatase activity of SaeS in the staphylococci nearest the neutrophil cuff. Either repressive mechanism could explain the apparent increased *nuc-gfp* expression in the periphery of lesions formed by the *saePQ* double mutant (**Supplementary Figure S4**, compare panels D vs. A). Increased nuclease expression may result in increased virulence during immune cell encounter and could explain why no discrete abscesses could be found when mice were infected with the *saePQ* double mutant (**Supplementary Figure 4**).

In vivo observations made with USA300Δ*saePQ* suggest that neither *saeP* nor *saeQ* influence virulence factors that contribute to murine skin and soft-tissue abscess severity. Inasmuch as alpha-toxin (Hla) is known to play a key role in dermonecrosis caused by USA300 during murine skin and soft tissue infection (Kennedy et al., 2010), results from the skin infection model are in agreement with our gene expression data that demonstrate *sae*P and *sae*Q do not influence *hla* transcript abundance (**Supplementary Figures S2A,B**). However, the observation that USA300Δ*saePQ* is hypervirulent in the bacteremia model is more difficult to explain with our current data. Potentially, different host niches have varying levels of different activating cues and levels of Sae TCS activity. Clearly, additional studies are needed to precisely determine the importance and impact of SaeP and SaeQ at these sites *in vivo* and to identify conditions that influence their expression and function.

MATERIALS AND METHODS

Bacterial Strains and Culture

All *S. aureus* strains used in this study are derivatives of the clinically-relevant CA-MRSA strain USA300 (LAC) that was previously cured of the plasmid encoding erythromycin resistance (Boles et al., 2010). Unless otherwise indicated, overnight and subculture media consisted of tryptic soy broth (TSB) (EMD Millipore; Darmstadt, Germany) supplemented with 0.5% (w/v) glucose. When needed, antibiotics were included in the medium at the following concentrations: ampicillin (Amp), 50 $\mu g\ ml^{-1}$; chloramphenicol (Cm), 5 $\mu g\ ml^{-1}$; and erythromycin (Erm), 5 $\mu g\ ml^{-1}$. Subcultures were created using 1:100 dilution of the overnight culture. For the growth curves, OD_{600} readings were collected every 0.5 h using a Nanodrop 2000C UV-Vis Spectrophotometer (ThermoFisher Scientific; Wilmington, DE, United States) or an Amersham Ultraspec 2100 pro UV-visible spectrophotometer and colony forming units (CFUs) were enumerated after incubation overnight at 37°C with 5% CO_2 as described (Voyich et al., 2005).

Generation of Mutant Strains

Construction of the isogenic *saeQ* deletion mutant was performed using allelic exchange and pJB38 plasmid (Bae and Schneewind, 2006; Bose et al., 2013). The *saeP* mutant was constructed previously (Kavanaugh et al., 2019). To construct the *saePQ* strain, we first deleted the entire *sae* operon using pKOR1-sae (Bae and Schneewind, 2006). Next, we transduced the strain to chloramphenicol resistance, moving in the P_{saeP3}-*saeRS* construct (*saeRS* under the control of their native promoter, cloned into pCL55 and integrated into the *geh* locus, Cm^R). Then, we integrated the *nuc-gfp* reporter as described by allelic exchange (Behera et al., 2019). Briefly, DNA fragments upstream and downstream of the gene or gene fragment of interest were amplified using primers listed in **Table 1**, purified by agarose gel electrophoresis, then combined in a two-step overlap PCR reaction and cloned into pJB38 (Flack et al., 2014). Δ*saePQRS* was made previously in Flack et al. (2014). The resulting plasmid was transformed sequentially into *Escherichia coli* (*E. coli*) strain ER2566 (New England Biolabs), then *S. aureus* strain RN4220, and the final background USA300 LAC (Flack, 2014). Final mutants were verified by PCR amplification of the chromosomal region of interest and DNA sequencing. Lack of *saeP* and *saeQ* in the mutant strains were verified by PCR and agarose gel electrophoresis.

For complementation strains, the *saeP* and *saeQ* genes were cloned into the pEPSA5 plasmid (Forsyth et al., 2002) using restriction enzymes (EcoR1 and BamH1) and primers listed in **Table 1**. The resulting plasmids (pEPSA5-*saeP*comp and pEPSA5-*sae*Qcomp) drive expression of the *sae* genes from the xylose-inducible P_{xyl} promoter. To induce expression, the medium was supplemented with 2% (w/v) xylose in experiments involving these strains as indicated (Forsyth et al., 2002). These plasmids were transformed into electrocompetent *E. coli* GM2163 (New England Biolabs), then directly into the respective mutant *S. aureus* strain (USA300Δ*saeP* and USA300Δ*saeQ*) via *E. coli* strain IM08B (Monk and Foster, 2012) and called pEPSA5-*saeP*comp and pEPSA5-*saeQ*comp. The resulting strains were confirmed using PCR amplification and agarose gel electrophoresis, and presence of transcript abundance verified by TaqMan RT-PCR as done previously (Voyich et al., 2009; Nygaard et al., 2010; Flack et al., 2014).

Neutrophil Isolation

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. Human neutrophils [polymorphonuclear leukocytes (PMNs)] were isolated under endotoxin-free conditions ($<25pg\ ml^{-1}$) as previously described (Voyich et al., 2005, 2009). Purity (<1% PBMC contamination) and viability (<2% propidium iodide positivity) of neutrophil preparations were assessed by flow cytometry on a FACS Calibur instrument and BD Biosciences Cell Quest Pro software (version 0.3.3f1b).

Image Stream Phagocytosis Assay

Neutrophil phagocytosis was determined using a fluorescence-based flow cytometry/microscopy method described previously (Ploppa et al., 2011). Briefly, *S. aureus*

TABLE 1 | Primers used to generate *S. aureus* mutant strains, respective complemented strains, and TaqMan® primer and probe sequences.

Primer	Sequence	Description	References
Construction of USA300Δ*saeP* and USA300Δ*saeQ*			
Forward	5′-GTTGTTGAATTCACCTGATACATTACAGACC-3′	600 bp upstream of *saeP*	Kavanaugh et al., 2019
Reverse	5′-CAGAAATTGAGTACTAGATCTGTATTCATGCTAACTCCTCATTTC-3′	Upstream of *saeP* plus overlap	Kavanaugh et al., 2019
Forward	5′-GAATACAGATCTAGTACTCAATTTCTGAGTTAAACTTTTATTTACAAC-3′	Downstream of *saeP* plus overlap	Flack, 2014
Reverse	5′-GTTGTTGGTACCAAGAAACTAGCAGCATATGC-3′	600 bp downstream of *saeP*	Flack, 2014
Forward	5′-GTTGTTGAATTCCCTAACAGGTACATTCAGTTC-3′	EcoR1, 600 bp upstream of *saeQ*	Flack, 2014
Reverse	5′-GCGAGTACTAGATCTCATTCTTTCTATTATTGTGTGTAATTTATAT-3′	Upstream of *saeQ* plus overlap	Flack, 2014
Forward	5′-AGAATGAGATCTAGTACTCGCAAATATAGTTGCACATAC-3′	165 bp into *saeQ* plus overlap	Flack, 2014
Reverse	5′-GTTGTTGGTACCGATGGTATATGTTGTAAAGCTCTC-3′	*Kpn*I, 900 bp downstream of *saeQ*	This work
Forward	5′-TAATTTAGCGCCGCCGAAGA-3′	*saeP*	This work
Reverse	5′-TTTTTAGCAGCTGGTGCTGT-3′	*saeP*	This work
Forward	5′- CTCTGTTCTTACGACCTCTAAAGTAAT-3′	*saeQ*	This work
Reverse	5′-GTTTAGTACCAGTCATCGCTAACA-3′	*saeQ*	This work
Forward	5′-GGTGGTGAATTCTTAACTTATCAAATTGAAGAAATGAGGAGTTAGC-3′	pEPSA5-*saeP*-EcoR1	This work
Reverse	5′-ACCACCGGATCCAATTGATTATTTTAATTTAGCGCCGCC-3′	pEPSA5-*saeP*-*Bam*HI	This work
Forward	5′-GGTGGTGAATTCTTATATAAATTACACACAATAAATAGAAAGAATGTGAACATC-3′	pEPSA5-*saeQ*-EcoR1	This work
Reverse	5′-GGTGGTGGATCCTGTTCATCATCCACGATCAGTAAGT-3′	pEPSA5-*saeQ*-*Bam*HI	This work
Taqman® primer/probe sequences			
Forward	5′-CACCTAACAGGTACATTCAGTTCTA-3′	*saeP* primer	This work
Reverse	5′-GGTAGACGTATAAATCTGGACCTTT-3′	*saeP* primer	This work
Probe	5′-ACGGTGAAACTGTTGAAGGTAAAGCTGA-3′	*saeP* probe	This work
Forward	5′-CACCAGAGTGGTATAAGTGGTT-3′	*saeQ* primer	This work
Reverse	5′-CAAAGCCTCCAAAGAAACTAGC-3′	*saeQ* primer	This work
Probe	5′-TTGTTGTCCCACTCGGAGAGATTGC-3′	*saeQ* probe	This work

was grown to mid-exponential phase, opsonized with 50% (vol/vol) normal human serum and labeled with 750 μL fluorescein isothiocyanate (FITC) at a final concentration of 0.002 mg mL^{-1}. *S. aureus* strains were combined with neutrophils at a multiplicity of infection (MOI) of 10:1 (bacteria: neutrophils) in 96-well plates coated with human serum coated (20% v/v). Phagocytosis was synchronized by centrifugation as described (Voyich et al., 2005) and incubated at 37°C with 5% CO_2 for 30 min. Cells were fixed in 2% (v/v) Periodate-Lysine-Paraformaldehyde (PLP) for 10 min at room temperature (Pieri et al., 2002). PLP was then washed away and antibodies/stains were applied: mouse anti-human CD11b antibody-PE (BD; Franklin Lakes, NJ, United States) and nuclear stain DRAQ5™ (ThermoFisher Scientific; Wilmington, DE, United States). Cells were washed and suspended in 50 μL sterile Dulbecco's phosphate buffered saline (DPBS) and analyzed by an ImageStream®X Mark II Imaging Flow Cytometer (Millipore Sigma) the following day. Phagocytosis was analyzed using IDEAS® software (AMNIS®, Millipore Sigma, Darmstadt, Germany) where cell images were gated to include neutrophils that were both in focus and singlets. Of these cells, images fluorescing both neutrophil and *S. aureus* membrane dyes were analyzed using the AMNIS internalization wizard (Ploppa et al., 2011).

Bacterial Survival Assay

Bacterial survival was assessed following synchronized phagocytosis as previously described (Voyich et al., 2005). Briefly, *S. aureus* strains were grown to mid-exponential phase, opsonized in 50% (v/v) normal human serum, and combined with neutrophils in 96-well plates coated with 20% (v/v) human serum (MOI of 10:1) and incubated at 37°C with 5% CO_2. At indicated times, 11 μL of 2% (w/v) saponin solution was added to each well and incubated for 15 min on ice. Samples were sheared using a 1 mL syringe with a blunt needle and bacteria were enumerated by dilution on tryptic soy agar (TSA) following overnight incubation at 37°C with 5% CO_2.

Plasma Membrane Damage

Propidium iodide (PI) uptake was used as a measure of plasma membrane permeability to assess damage of neutrophils by secreted *S. aureus* proteins as described (Nygaard et al., 2012, 2018; Flack et al., 2014). Briefly, bacterial strains were cultured at 37°C for 5 h with shaking (250 RPM) in TSB. After, 1×10^9 CFUs of bacteria were collected and centrifuged for 5 min at 8,000 × g. Supernatants were sterile-filtered and diluted (as indicated in figure legends) with DPBS and exposed to neutrophils for 1 h at 37°C with 5% CO_2. After incubation, cells were stained with 0.5 μL PI (1 mg mL^{-1} Life Technologies) and analyzed by flow cytometry on a FACS Calibur flow cytometer (BD Biosciences; Franklin Lakes, New Jersey). Neutrophil membrane damage was also assessed by flow cytometry using whole bacteria. For these experiments, neutrophils were exposed to live bacteria (MOI 5:1) and incubated for 3 h at 37°C with 5% CO_2.

Transcriptional Analysis of Target Genes

TaqMan® gene expression experiments were performed as previously described (Voyich et al., 2005, 2009; Nygaard et al., 2010). Relative quantification of *S. aureus* target genes was determined by the change in expression of target transcripts normalized to that of the housekeeping gene [gyrase B (*gyrB*)] and relative to USA300 LAC transcript levels. Primer/probe sequences are described in **Table 1** (Voyich et al., 2009; Nygaard et al., 2010). Where indicated, transcript abundance was also measured using SYBR Green chemistry and the absolute transcript abundance method as indicated and as described in Mlynek et al. (2018).

Western Blot Analysis

Supernatants from overnight cultures in TSB without supplemented glucose were harvested as described above, total protein was measured (Pierce BCA Protein Assay) and adjusted to 500 $\mu g\ mL^{-1}$. Samples (14 μL) were resolved using 12% SDS-PAGE gels, (100 V for 45 min) and transferred onto nitrocellulose (at 10 mAmps overnight). Membranes were washed and blocked in DPBS containing 5% (w/v) milk solution for 1 h followed by incubation with rabbit anti-LukS-PV primary antibody (abcam; Cambridge, MA, United States) at a concentration of 0.6 $\mu g\ mL^{-1}$ (4 h at 4°C). PVL was detected after 1 h incubation with goat anti-rabbit IgG coupled to horseradish peroxidase (at 1:10,000 dilution) (Jackson ImmunoResearch; West Grove, PA, United States) and developed using 5 mL 3,3′,5,5′-tetramethylbenzidine (TMB) substrate. Images were taken with an Gel Doc Imager (ProteinSimple; San Jose, CA, United States) and analyzed by ImageJ densitometry software (Schindelin et al., 2012).

GFP Reporter Assays With HNP-1

Bacteria were grown to exponential phase (OD_{600} ~ 0.6–0.8) as described previously (Waters et al., 2016) in 250 ml DeLong flasks containing dilute Luria broth (Geiger et al., 2008) (5:1 flask:medium ratio) with vigorous shaking (280 RPM) at 37°C in a water bath. Cultures were diluted to a starting OD_{600} ~ 0.1 in fresh medium and aliquoted into individual wells of a 96 well plate (cultures of 200 μl each) and incubated in a computer controlled Tecan F200 plate reader at 37°C. The optical density at 600 nm (OD_{600}) and GFP fluorescence (485 nm excitation, 535 nm emission) values were read every 15 min after shaking (15 s, 2 mm amplitude). When OD_{600} values reached ~0.4, the plate was removed, and the indicated wells were spiked with 5 $\mu g\ ml^{-1}$ of the human neutrophil peptide-1 (HNP-1) or vehicle and returned to the plate reader. Data acquisition continued for an additional 12 h. Mean ± SEM relative fluorescence units (RFUs; GFP fluorescence/OD_{600}) from three independent experiments are reported.

Mouse Infection Models

Skin and Soft-Tissue Infection (SSTI)

All studies conformed to NIH guidelines and were approved by the Institutional Animal Care and Use Committee at Montana State University. Female C57BL/6 mice (12 weeks old) were purchased from Charles River Laboratories and maintained at the Animal Resources Center at Montana State University. Female and male BALB/C mice were purchased from the Animal Resources Center at Montana State University. *S. aureus* strains: USA300 LAC, and isogenic mutants USA300Δ*saeP*, USA300Δ*saeQ*, USA300Δ*saePQRS*, USA300Δ*saePQ* strains were grown to mid-exponential phase, washed twice with sterile DPBS and resuspended in DPBS at a concentration of 1×10^7 cells per 50 μL. The dose was confirmed by plating serial dilutions on TSA plates For the abscess model, mice (groups of five) were shaved and inoculated with *S. aureus* subcutaneously into the lower back (Voyich et al., 2009; Nygaard et al., 2010). Infected area was measured using the formula: (l × w).

Bacteremia Model

Experiments were performed following a protocol approved by the Animal Care and Use Committee at Georgetown University (GUACUC). *S. aureus* strains were grown to exponential phase in 250 ml DeLong shake flasks (5:1 flask: medium ratio), harvested at OD_{600} ~0.4–0.6, washed twice in sterile phosphate buffer saline (PBS), and resuspended to an appropriate optical density equivalent to 1×10^8 colony forming units (CFUs) ml^{-1}. Groups of female C57BL/6 mice (6–8-weeks old, purchased from Charles River Laboratories) were infected intravenously via the tail vein with ~1×10^7 cells in 100 μl of sterile PBS. The dose was confirmed by plating serial dilutions on TSA plates. Animals were monitored twice daily and evaluated following a GUACUC-approved scoring rubric. Infections were allowed to progress for 72 h or until humane endpoints were reached.

To analyze *nuc-gfp* expression in tissues, infections were performed as described for USA300 LAC and the Δ*saePQ* double mutant essentially described above (and specifically in Behera et al., 2019); notably, animals were fed AIN-93 purified diet (Reeves et al., 1993). Briefly, mice were euthanized 72 h post-infection and kidneys were harvested, fixed with 10% (v/v) buffered formalin, embedded in Sub Xero clear tissue freezing medium (Mercedes Medical), and sectioned into 10 μm slices. Sections were mounted with 4′,6-diamidino-2-phenylindole (DAPI) stain and imaged using laser confocal scanning microscopy. Images were processed using ImageJ (Schindelin et al., 2012). Excitation wavelengths for the fluorescence channels are as follows: DAPI, 405 nm; GFP, 488 nm. Emitted fluorescence data were collected over the following ranges of wavelengths: DAPI, 419–481 nm, GFP, 505–551 nm.

Statistics

Statistical analyses were performed using GraphPad Prism version 8.0 (GraphPad Software, La Jolla, CA, United States) with *t*-tests and ANOVA as indicated. Error bars represent the standard error of the mean (SEM).

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board for Human Subjects at Montana State University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MC, JV, RB, and SB contributed to the conception and design of this study. MC, RB, TE, OB, BC, WP, CF, KP, FG, TN, and JD performed the experiments and data analysis. MC, JV, and SB wrote and prepared the manuscript for submission. All authors read and approved this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00561/full#supplementary-material

FIGURE S1 | Complementation of *saeP* and *saeQ* restores USA300 phenotype. Gene complementation with *saeP* and *saeQ* on the xylose-inducible pEPSA5 plasmid restores USA300 phenotype. Promoter expression induced with 2% (w/v) xylose in the medium. **(A)** Gene expression of *saeP* and *saeQ* is rescued in *S. aureus* mutant strains complemented with *saeP or saeQ* expressed in trans. **(B)** Complementation of *saeP* restores transcript of select *S. aureus* genes to levels observed in USA300 (or higher). Transcript abundance is relative to *gyrB* and calibrated to expression in USA300 (n = 2/gene). **(C)** Complementation of *saeP* and *saeQ* in trans reduces secreted cytolytic factors that target neutrophil plasma membrane damage to levels secreted by USA300. Data are presented as the mean ± SEM of four independent experiments. Stats: One-Way ANOVA with Tukey's post-test; ns, not significant.

FIGURE S2 | Deletion of *saeP* or *saeQ* did not significantly impact abscess area. Deletion of *saeP* or *saeQ* did not significantly impact abscess area. **(A)** BALB/C mice (5 per group) and **(B)** C57BL/6 mice (5 per group) were infected subcutaneously with 1×10^7 CFUs of each *S. aureus* strain and abscess area was subsequently monitored for 10 days. Abscess area was measured daily and results shown are the average area per strain. Representative images are from C57BL/6 mice on day 2. Graphs represent data from two biological replicates for BALB/c and one for C57BL/6. Unpaired t-test relative to USA300, $^*p$-value ≤ 0.05, $^{**}p$-value ≤ 0.01. Data are presented as the mean ± SEM.

FIGURE S3 | The Δ*saePQ* mutant has reduced *saeR/S* transcript levels but induces Sae-dependent genes normally. **(A,B)** Normalized transcript abundance is shown for USA300 and the indicated mutant strains of *S. aureus* grown to exponential phase using SYBR green qRT-PCR as described in Materials and Methods and as described in Mlynek et al. (2018). Data indicate the mean ± SEM from three independent experiments. Statistical analysis: ANOVA (ordinary one-way), Dunnett's multiple comparison test, asterisks indicate the level of significance compared to the USA300 ($P \leq 0.05$). For USA300Δ*saePQRS*, the transcript level is below the limit of detection. **(C)** The indicated strains carrying a *nuc-gfp* reporter fusion were grown to exponential phase ($OD_{600} \sim 0.4$) in dilute Luria broth, at which time bacteria were exposed to either vehicle (water) or HNP-1 for 12 h. The data shown are the mean relative fluorescence units (RFUs; fluorescence/OD_{600}) ±SEM for three independent experiments performed in technical triplicate; statistical significance was assessed by paired t-test ($^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$). ND, not detected: signal was below the limit of detection.

FIGURE S4 | Deletion of *saePQ* influences lesion structure and *nuc* expression in infected kidneys. **(A–F)** Representative confocal micrographs of staphylococcal lesions in kidney produced by USA300 and USA300Δ*saePQ*. Channels: *nuc-sGFP* **(A,D)**, DAPI **(B,E)**, and merge **(C,F)** (scale bar is 25 μm). The fluorescence image acquisition parameters used are as follows; excitation for DAPI (blue) and GFP (green) are 405 and 488 nm respectively; emission ranges are 419–481 nm (DAPI) and 505–551 nm (GFP).

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