Staphylococcus aureus Virulence Factors Dictate Host Signaling Pathways and Immune Responses

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ABSTRACT

Staphylococcus aureus causes nosocomial- and community- acquired infections. This pathogen expresses virulence factors (VF) that enhance establishment of infection and immune evasion. Our research focused on defining the roles of *S. aureus* VF on host immune responses during intracellular or extracellular infections. *Accessory gene regulator* (*agr*) controls VF expression and intracellular survival. Our goal was to determine mammary epithelial cells (MEC) responses to intracellular infection and subsequent polymorphonuclear leukocyte (PMN) responses. Intracellular *S. aureus* increased thrombomodulin expression by MEC and activated protein C (APC) production. APC inhibited PMN chemotaxis. Findings depicted an indirect role for VF on PMN responses, so next we determined signaling and cytokine responses of PMN to *S. aureus* toxins. Live *S. aureus* infections increased activation of stress signaling pathways and highlighted a role for *agr*-regulated genes in MAPK p38 phosphorylation and α-hemolysin in ERK phosphorylation and IL-8 expression in PMN. Although VF such as chemotaxis inhibitory protein of *S. aureus* did not prove to inhibit monocyte chemokine signaling as proposed, activated signaling pathways can reduce PMN function in models such as glucocorticoid treatment. Immunosuppressive effects of glucocorticoids on PMN are restored with OmniGen-AF® supplementation. Glucocorticoid receptor and Toll-like receptor signaling potentially crosstalk to restore PMN function. OmniGen-AF® supplementation restored dexamethasone-induced immunosuppression in a MyD88-dependent manner. Overall, this research focused on characterizing immune responses to *S. aureus* infections and PMN signaling pathways and how it is key to understanding pathogenesis.

Dedication

Dedicated to my family Edgardo, Eneida, Dito, Susanne, Andrés and José Alberto.

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List of abbreviations

Full name	Abbreviation
Accessory gene regulator	*agr*
Autoinducible Protein	AIP
Chemotaxis Inhibitory Protein of *S. aureus*	CHIPS
Complement 5a Receptor	C5aR
complimentary DNA	cDNA
Colony Forming Units	CFU
G-Coupled Protein Receptor	GCPR
Human bronchial epithelial cells	HBE
Interleukin	IL
Lipopolysaccharide	LPS
Minimum Essential media	MEM
Multiplicity of infection	MOI
Nuclear Factor kappa-light-chain-enhancer of activated B cells	NF-κB
Phorbol 12-myristate 13-acetate	PMA
Polymorphonuclear Leukocyte	PMN
Polyvinylidene fluoride	PVDF
Red Blood Cells	RBC
Cystic fibrosis cell line	S9
Small colony variant	SCV
Staphylococcus accessory regulator	*sar*
Staphylococcal Enterotoxin A	SEA
Staphylococcus aureus	*S. aureus*
Staphylokinase	SAK
Staphylococcal Complement Inhibitor	SCIN
Toll-Like Receptors	TLR
Tryptic Soy Broth	TSB

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Chapter 1. Review of Literature

Staphylococcus aureus

S. aureus is a gram positive bacterium, forms grape-like structures, and is cocci in shape. This pathogen readily colonizes the skin and nares of mammals and is a health risk when it becomes an infection. *S. aureus* can lead to fatal nosocomial- and community- acquired infections as well as chronic and subclinical infections. *S. aureus* infections are treated with antibiotics, which create a selective environment for resistant strains and are a growing concern to public health. Also, the acquisition of lysogenic phages enhancing virulent genes has become a severe problem for healthcare and efficient pathogen clearance (32). Therefore, this versatile pathogen has the potential to resist current modes of treatment and the innate immune responses of the host.

Typically, *S. aureus* infections are extracellular, however, intracellular infections have been described (7, 34, 41, 50, 53, 70, 88). Intramammary and lung *S. aureus* infections can progress to chronic infections, which are conducive to intracellular survival of *S. aureus* (2, 41, 53, 70, 88, 89). Survival within host cells provides the pathogen protection from antibiotic treatment and host immune responses.

Small colony variants of *S. aureus*

Colonies that successfully survive within host cells include small colony variants (SCV). Metabolic gene mutations in *S. aureus* colonies can result in formation of SCV (55, 88). These variants coexist with their wildtype parental strains, however wildtype colonies often overpower the population. Though not always more virulent, SCV remain viable inside host cells. Features

of SCV colonies are auxotrophic growth and down-regulation of *accessory gene regulator (agr)*- and α-hemolysin (55, 89). Clinical *S. aureus* isolates can change phenotype and become SCV during chronic intracellular infections of endothelial cells (89). Tuchscherr et al. (2011) reported that the change in bacterial phenotype to SCV was reflected in the down-regulation of inflammatory responses by infected endothelial cells. Therefore, host cells are intracellularly infected with viable *S. aureus*, however pathogen-induced inflammatory responses in host cells are not sufficient to clear infection. *S. aureus* virulence factors may enhance immune evasion mechanisms (28, 40, 77, 79, 93), therefore it is important to understand virulence factor regulation during infection.

Accessory gene regulator (*agr*)

Virulence factor expression and regulation are very important in the progression of *S. aureus* infection. *S. aureus* transcriptional gene regulator, *agr*, controls the transcription of secreted virulence factors. During initial stages of infection, low numbers of bacteria are present and express cell surface virulence factors that enhance immune evasion (protein A, complement inhibitors, and catalase) and adhesion to host cells (microbial surface component recognizing adhesive matrix molecules, fibronectin-binding proteins A and B, collagen-binding protein, and clumping factor A and B) (62, 64, 71, 87, 102). However, when bacteria numbers increase and reach exponential phases of growth, quorum sensing initiates *agr* activity. Activation of *agr* down-regulates *S. aureus* cell surface virulence genes and up-regulates secreted virulence genes. The *agr* locus has two promoters, P2 and P3, which generate RNAII and RNAIII transcripts, respectively. The quorum sensing system, AgrA, -B, -C, and -D, is encoded by P2. The pro-autoinducing peptide (AIP), AgrD, is processed and secreted by AgrB, a transmembrane protein.

The AIP is the quorum sensing signal protein, which accumulates in correlation to cell-density. The AIP binds to AgrC, the transmembrane receptor histidine kinase, which causes autophosphorylation of AgrC. In turn, AgrC phosphorylates AgrA, the DNA-binding response regulator. Phosphorylated AgrA binds to the agr operon intergenic region, the region that separates P2 and P3 promoters. Therefore, AgrA is responsible for transcription and translation of the promoter regions. Activity of P2 continues the transcription of RNAII for the production of AIP, while P3 synthesizes RNAIII, which induces gene transcription and translation of several virulence genes including α- and δ- hemolysins (Figure 1.1). In fact, secretion of α- and δ-hemolysins are a trademark of *agr* activity in *S. aureus* isolates (71, 87). *S. aureus* strains containing the *agr* loci are more virulent and have enhanced intracellular survival compared with strains lacking the *agr* loci (1, 59, 63, 64, 70, 86). Therefore, it is important to identify the virulence factors regulated by *agr* and the host immune responses associated with these virulence factors for efficient diagnoses.

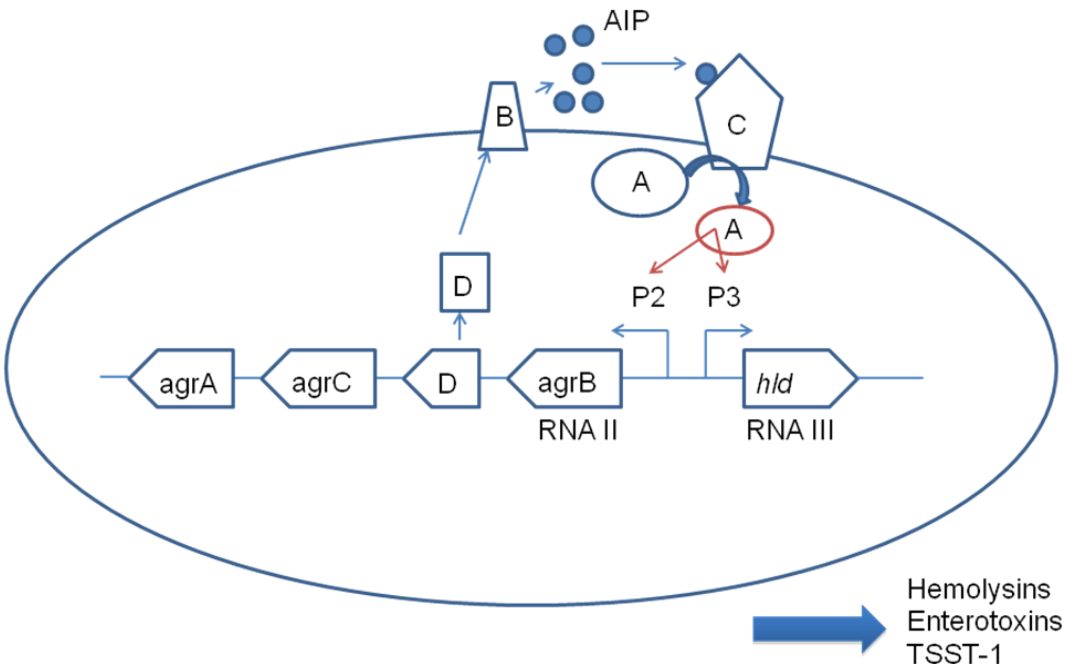

Figure 1.1 *S. aureus* agr regulation. P2 and P3 promoters generate RNAII and RNAIII transcripts, respectively. P2 encodes AgrA, - B, -C, and -D, the quorum sensing system. AgrD is processed and secreted by AgrB, producing autoinducing peptide (AIP). The quorum sensing signal, AIP, accumulates in correlation to cell-density. The AIP binds to AgrC and causes autophosphorylation of AgrC. In turn, AgrC phosphorylates AgrA. Phosphorylated AgrA binds to the agr operon intergenic region, therebytranscribing and translating the promoter regions (P2 and P3). The P2 transcribes RNAII for AIP production and P3 synthesizes RNAIII, which induces gene transcription and translation of several virulence genes including hemolysins, enterotoxins, and toxic shock syndrome toxin-1 (TSST-1).

Hemolysins

S. aureus hemolysins are among the several virulence factors regulated by *agr*. Hemolysins include α, β, δ, and γ. Trademarks of hemolysins are the lytic phenotypes observed in rabbit blood agar plates, however hemolysins do not only encounter red blood cells (RBC) during infection. Leukocytes and host epithelial cells are also sensitive to hemolytic virulence factors and suffer cell damage. Alpha hemolysin is a hollow heptamer, β-protein that forms pores

in cell membranes, thereby inducing lysis (56). Alpha hemolysin induces synthesis of Interleukin-1β (IL-1β) in monocytes (9), Nuclear Factor κ-light-chain-enhancer of activated B cells (NF-κB) -dependent IL-8 expression by monocytic and epithelial cells (24), and murine neutrophil recruitment (65) when introduced in the intraperitoneal cavity.

Beta hemolysin is a Mg^{2+} -dependent sphingomyelinase C (29) that can lyse human monocytes (94). Sphingomyelin is present in the phospholipid bilayer of cell membranes. Beta hemolysin is expressed in the majority of *S. aureus* strains isolated from bovine mastitis cases (49). This virulence factor may promote establishment of infection and enhance pathogen survival in the mammary gland. In murine models, *S. aureus* α- and β- hemolysins along with secreted bacterial lipoproteins induce caspase-1 and inflammatory cytokine production in macrophages (60).

Delta hemolysin permeabilizes target membranes. This hemolysin is the first to be transcribed by RNAIII and may contribute to the expression of some exoprotein genes by *S. aureus* (38). Alpha- and δ-hemolysins enhance intracellular survival of *S. aureus* in host cells (31, 39).

Gamma hemolysin is a two-component toxin, which creates pores in host RBC. This hemolysin has three proteins including HlgA, HLgB, HlgC. From these three proteins only two toxins can be generated: HlgA+HlgB and HlgC+HlgB (27).

Collectively, hemolysins can cause cell damage and induce immune responses in the host, making hemolysins important virulence factors in *S. aureus* pathogenesis. However, particular hemolysins may be necessary to colonize certain species more efficiently than other species as seen with β-hemolysin and bovine mastitis. In addition, human isolates indicate

lysogenic phages can interrupt *S. aureus* hemolysin genes such as β-hemolysin to integrate virulence genes that enhance bacterial evasion of host innate immune responses.

Beta hemolysin converting bacteriophages

S. aureus can acquire bacteriophages through negative and positive lysogenization. Negative lysogenization causes truncation or loss of genes, while positive lysogenization allows for the integration of genes (20) and such is the nature of β-hemolysin converting bacteriophages carried by *S. aureus* pathogenicity island 5, SaPI5 (37, 100).

Integration of β-hemolysin converting bacteriophages truncate the *β-hemolysin* gene, therefore β-hemolysin can no longer be expressed. Genes incorporated by the bacteriophages include *staphylokinase* (*sak*), *enterotoxin A* (*sea*), *chemotaxis inhibitory protein of S. aureus* (*chp*), and *staphylococcal complement inhibitor* (*scn*). Beta hemolysin converting bacteriophages were first described as double converting bacteriophages (phage 13) when determined to negatively convert the *β-hemolysin* gene and integrate *sak* (99). Nevertheless, β-hemolysin converting bacteriophages differ from other well studied bacteriophages such as Serotype A and B. Serotype A phages negatively lysogenize *β-hemolysin* alone (20), whereas Serotype B phages mediate positive lysogenization of *sak*, but *β-hemolysin* remains intact (44).

Winkler et al., (1965) studied positive phage conversion in *S. aureus* strains that express *sak* and *sea* after lysogenization. Their research led to the discovery of a triple converting bacteriophage, where Serotype F strains contained double converting bacteriophage (phage 13) and phage 42 which mediated the integration of *sea* (18, 20). Sequence analysis of bacteriophages and *S. aureus* genome attachment sites indicate a common 14 base pair (bp)

sequence where phages integrate in the 5' end of *β-hemolysin* gene (16). The integration mechanisms for the phages are orientation- and site-specific recombination (15, 16, 18-20, 91).

In addition to *sak* and *sea, chp* and *scn* can also be carried by β-hemolysin converting bacteriophages (91). Chemotaxis inhibitory protein of *S. aureus* (CHIPS) and staphylococcal complement inhibitor (SCIN) are *S. aureus* secreted proteins and potent complement inhibitors (69, 77). Both *chp* and *scn* are located on the 3' end of β-hemolysin converting bacteriophages (91). Researchers, van Wamel et al. (2006), studied six different *S. aureus* research strains carrying bacteriophages incorporated in the β-hemolysin gene. When comparing the bacteriophage sequences, researchers found the first 1000bp at the 5' end (which encode integrase and phage genome) were homologous. Bacteriophages differ in the last 8 kilo bp of the 3' end, which encode *sea*, *sak*, *chp*, and *scn* genes. For example, van Wamel et al. (2006) found phage 13 does not express the *sea* cassette. Also, phage N315 does not express the *sea* cassette, but expressed staphylococcal enterotoxin P (*sep*) cassette instead. In addition to missing *sea* cassettes in some phages, the *chp* cassette is also missing from phages in Mu50, MW2, and MSSA-476 *S. aureus* strains. Not only can all four genes (*sea*, *sak*, *chp*, and *scn*) be found on the bacteriophage, but β-hemolysin converting bacteriophages can have several gene arrangements (20, 47, 91). Researchers, van Wamel et al. (2006), characterized β-hemolysin converting bacteriophage genes in 90 clinical and research isolates and found that the most common gene conjugation is *sak*, *chp*, and *scn*.

Beta hemolysin converting bacteriophages are extremely mobile and are prevalent in clinical *S. aureus* isolates. Sixty to ninety percent of human clinical isolates contain the diverse arrangements of β-hemolysin converting bacteriophages. Approximately 90% of humans are persistent nasal carriers of *S. aureus* strains containing β-hemolysin converting bacteriophages

(91, 93), indicating the importance of characterizing immune responses to these bacteriophages for efficient diagnostic methods and development of antimicrobial therapy.

S. aureus strains carrying β-hemolysin converting bacteriophages invade different human tissues. For example, *S. aureus* Newman was isolated from a human infection in 1952 and since then used extensively in research (4, 5, 30). Bae *et al*. (2006) focused on four prophages found in Newman (including a β-hemolysin converting bacteriophage containing the *sak*, *sea*, *chp*, and *scn* arrangement) and their roles in pathogenesis through mutagenesis. Mice were infected with Newman wildtype or TB1 (Newman mutant lacking β-hemolysin converting bacteriophage). Murine liver and kidney were collected for bacterial load assessment 4 days post infection. There were no significant differences in kidney bacterial loads between the strains. Interestingly, TB1 bacterial load was significantly lower than Newman wildtype in liver samples. These results suggest an important role for β-hemolysin converting bacteriophages in host liver infection. Bacterial colonization of the liver may enhance pathogen access to the host's bloodstream, which may lead to bacteremia or colonization of other tissues.

Antibiotic treatment is the most common remedy for bacterial infections. However, emergence of antibiotic resistant strains threatens this route of treatment. Furthermore, recent studies indicate that antibiotic treatment induces phage induction (33, 76). Goerke et al. (2006) (32) studied the effects of ciprofloxacin on phage induction and expression of phage-induced genes in *S. aureus* strains isolated from cystic fibrosis patients. Only isolates containing β-hemolysin converting bacteriophages were selected (phage 13-like phages (phages only express staphylokinase)). Treating *S. aureus* with ciprofloxacin de-lysogenized these phages from *S. aureus* genomes. Interestingly, antibiotic treatment triggers *recA* transcription in *S. aureus*, which leads to the resumption of the lytic cycle and as a result induced virulence factor

transcription by latent phage promoters. Furthermore, Ubeda et al. (2005) (90) suggested that antibiotic treatment of *S. aureus* induced SOS responses leading to increased dissemination of pathogenicity islands, thus spreading virulence genes. Several factors contribute to phage induction and dissemination within bacterial species, therefore more work needs to be conducted to determine the mechanisms of activation and gene transfer. Overall, research on β-hemolysin converting bacteriophages as a whole during infection is limited. Nevertheless, molecular functions of staphylokinase, enterotoxin A, CHIPS, and SCIN as independent virulence factors have been studied.

Virulence factors targeting innate immune responses contribute to establishment of infection and pathogen survival. In order to understand the impact of *S. aureus* virulence factors on host immune responses, function of innate immunity and pathogen clearance must be understood.

Innate Immunity

Innate immunity comprises several systems that contribute to the host's first line of defense. Innate immune responses include pathogen recognition, chemotaxis to infection site, and pathogen clearance. Host cells recognize and respond to pathogen associated molecular patterns (PAMPs) such as gram-negative lipopolysaccharide (LPS) by Toll-like Receptor (TLR)-4 and gram-positive peptidoglycan and lipoteichoic acid through TLR2 (6, 84). Recognition by TLR plays a role in the different cytokine and chemokine responses during infection. Leukocytes including neutrophils and monocytes respond to chemotactic gradients induced by complement, chemokines, or antibodies and transmigrate to infection sites to clear pathogen. Leukocyte receptors bind complement opsonins and aid in endocytosis of pathogens. Endosomal fusion

with the leukocyte's lysosome induces bacterial destruction through the release of digestive enzymes, synthesis of reactive oxygen species, and increased phagolysosome acidity.

Chemokines play an essential role in monocyte and neutrophil chemotaxis. Chemokines are characterized in two major groups CC and CXC. CC include macrophage chemotactic proteins (MCP-1 to MCP-5), macrophage inflammatory proteins (MIP-α, -β, -γ), and RANTES. CXC such as IL-8 primarily attract and activate neutrophils (43). Importantly, the physiological state of the host may determine glucocorticoid release and circulation, which may affect neutrophil function and response to CXC. During the periparturient period, mammals experience an increase in cortisol in the serum, which reduces glucocorticoid receptor, L-selectin, and CD18 receptor expression by neutrophils (12, 13). This phenomenon highlights the importance of leukocyte responses to proteins in host serum, which may be pro-inflammatory or immunosuppressive.

Complement Cascade:

Complement proteins are liver derived and found in host serum. Complement proteins are cleaved into their active forms upon binding of immune complexes (classical pathway), mannose (lectin pathway), or surface proteins on pathogens (alternative pathway). Although complement activation (pathway) may vary, all three pathways converge at formation of C3 convertase. The activation and cleavage of C3 convertase leads to the generation of anaphylatoxins, C3a and C5a, which enhance phagocyte chemotaxis and pathogen destruction. Other cleaved complement proteins such as C3b and iC3b serve as opsonins and contribute to formation of the membrane attack complex (MAC). The MAC results in pathogen destruction by osmotic lysis.

- **Alternative Pathway**

The alternative complement pathway is activated by microbial cell wall proteins including teichoic acid (68). C3b activation, with the addition of Factor D and substrate, Factor B, yields formation of C5 convertase. The C5 covertase yields C5a and C5b. C5b will continue on to form the MAC, while C5a acts as an opsonin and chemotactic protein. C5a is the ligand for its receptor, C5aR. The receptor is found on the surface of several host epithelial (21), endothelial (83), mesangial (98), and white blood cells (45, 58, 67). C5aR activation results in chemotactic signaling (chemokine production), proteolytic enzyme release, and superoxide production (10), indicating an important role for complement in pathogen clearance and inflammation activation.

- **C5aR signaling**

C5aR is a G-coupled protein receptor (GCPR) and two Gα subunits have been described, $G\alpha_i$ (pertussis toxin sensitive) and $G\alpha_{16}$ (pertussis toxin insensitive). $G\alpha_i$ pathway ultimately activates extracellular signal regulated kinases 1/2 (ERK1/2) through PI3Kγ (8). $G\alpha_{16}$ pathway leads to calcium mobilization and protein kinase C activation for ultimate MAPK p38 activation (54). $G\alpha_{16}$ can activate transcription factors such as NF-κB (101). After GCPR activation and signaling, the receptor is desensitized, internalized for secondary receptor signaling, and recycled or degraded (51). C5aR activation and signaling determine host cell responses through the transcription and production of cytokines (25), chemokines, apoptotic / anti-apoptotic proteins, plasminogen activator inhibitor-1 (42), reactive oxygen species (21), and increased expression of other complement receptors on leukocytes (57). *S. aureus* induces IL-1β, -6, -10, Tumor Necrosis Factor- α (TNF-α), and Interferon- γ (IFN-γ) production by infected macrophages, a response that is reduced by pertussis toxin treatment (GCPR inhibitor), indicating an important

role for GCPR such as C5aR during host responses to infection (26). Consequently, pathogens have evolved to produce and secrete virulence factors that target C5aR expression and downstream responses to enhance their survival in the host.

- **Virulence factors and C5aR**

Microbial PAMPs such as gram negative LPS may induce strong inflammatory responses leading to sepsis (36, 73). LPS stimulations in mice have demonstrated increased IL-6 production, which lead to C5aR up-regulation and increased mouse mortality (35, 72, 74). Researchers speculate that C5aR up-regulation contributes to septic mortality due to an overpowering inflammatory response. Evidently, inflammatory responses to PAMPs regulate C5aR expression and activity.

Microorganisms have developed mechanisms for complement evasion; particularly binding to- and regulating- the expression of complement receptors and inhibiting complement protein activity. *S. aureus* virulence factors secreted in supernatant decrease C5aR expression on neutrophils (3, 75, 92). *S. aureus* can express SCIN (40), SSL-7(48), and CHIPS (69, 100), which inhibit the production of C3 convertase, C5 convertase, and activity of C5aR respectively.

- **Chemotaxis Inhibitory Protein of *S. aureus* (CHIPS)**

Among the repertoire of *S. aureus* virulence factors that inhibit complement activity is CHIPS (23, 28, 40, 80). CHIPS is found in the 8-kilo base pair region of the 3' conserved end of β-hemolysin converting bacteriophages (91). The *chp* gene is regulated by *sae* locus and transcribed during the exponential growth phase of infection (78). Normally, inflammatory cytokines and chemoattractants initiate phagocyte chemotaxis and phagocytic responses, however CHIPS binds host chemotactic complement receptor, C5aR, potentially blocking

phagocyte activity (69). Studies indicate that CHIPS specifically binds to C5aR and prevents internalization of this GCPR (69). Treatment of monocytes and neutrophils with CHIPS decreased chemotaxis towards C5a and decreased calcium mobilization (22). Therefore, CHIPS decreases innate immune responses to *S. aureus* infection through inhibition of C5a responses, impairing chemotaxis, and decreasing pathogen destruction.

Recent literature has elucidated protein residues responsible for CHIPS activity. Through mutagenesis studies, Postma et al. (2005) determined that CHIPS bound to residues 10-18 of C5aR N-terminus, which did not interfere with C5a binding sites on the C-terminus. This data suggests that CHIPS cannot completely inhibit C5aR activation. Similarly, Nikiforovich et al., (2009) (61) used NMR spectrometry to elucidate CHIPS binding sites to C5aR. Researchers confirmed CHIPS bound to the N-terminus and not the transmembrane region of C5aR. Furthermore, Bunschoten et al. (2010) (11) studied CHIPS binding through ITC and NMR and determined that CHIPS binds to the phosphorylated N-terminus of C5aR. Overall, research has focused on understanding CHIPS binding specificity for development of anti-inflammatory remedies as an alternative to autoimmune and antimicrobial treatments. More research is needed to understand CHIPS induced immune modulation during infection. Nevertheless, activated signaling pathways during infection may play a pivotal role pathogen recognition and cytokine production in response to infection.

Mitogen Activated Protein Kinases and -Phosphatases (MAPK and MKP)

Receptor recognition and activation lead to the phosphorylation of kinases. In the case of TLR, myeloid differentiation primary response protein 88 (MyD88) mediates subsequent kinase activation. Depending upon stimuli, the adaptor protein transmits signals to induce immune

responses. Downstream kinases include c-Jun N-terminal kinases (JNK), ERK, mitogen activated protein kinase (MAPK) p38. Kinases are involved in cell stress, inflammation, and protein stability. Mitogen activated protein kinase phosphatase (MKP) is a feedback mechanism for these kinases to reduce excess inflammation. Once kinases are phosphorylated and signaling is carried out, MKP-1 induces JNK, ERK, and MAPK p38 dephosphorylation (52, 81, 95). ERK1/2 has a greater affinity in phosphorylating MKP-1 at the C-terminal Serine 359 and Serine 364 (14, 17). Several studies have focused on TLR signaling and MKP-1 regulation of cytokine production (17, 82, 85, 95, 96). LPS (82) or peptidoglycan (96) stimulation significantly induced cytokine production by MKP-1 knockout mice as compared to wildtype mice, confirming an inhibitory role for MKP-1 in TLR4 and TLR2 signaling pathways.

Kinase activation downstream of TLR has been extensively documented. Wanatabe *et al*., (2007) found that JNK, ERK, and MAPK p38 were phosphorylated (within 30 min) in infected wildtype macrophages. Only JNK was not phosphorylated when TLR2 knockout macrophages were infected with *S. aureus*, suggesting that *S. aureus*-induced JNK phosphorylation in murine macrophages is TLR2-dependent. In the same study researchers found JNK phosphorylation was responsible for decreased superoxide production in macrophages, which may account for increased pathogen survival.

Additional research studied MKP-1 downstream of TLR2 by stimulating macrophages with peptidoglycan. Results showed MKP-1 was present after 45 minutes of peptidoglycan stimulation and coincided with the decreased phosphorylation of ERK, MAPK p38, and JNK (85). The potential pathogen-dependent regulation of these host stress kinases during infection is not well understood, but essential for the comprehension of *S. aureus* pathogenesis. Furthermore, pathogen-evasion of host responses may lead to survival within leukocytes.

Neutrophils and intracellular survival

Neutrophils account for more than 50% of circulating leukocytes and are 'the first line of defense' against infection. As previously discussed, polymorphonuclear leukocytes (PMN) recognize pathogen through TLR or opsonins (complement or antibodies), phagocytize, and clear pathogen. However, *S. aureus* can survive within neutrophils (34, 59). *S. aureus* virulence factor expression may be regulated by the neutrophil immune responses. *S. aureus* virulence cluster *sae* (30) is activated by reactive oxygen species produced by phagocytes in efforts of destroying the pathogen. Other *S. aureus* virulence clusters such as *agr* are activated through quorum sensing, but when activated intracellularly (in the host) enhance pathogen escape from the endosome (70). Gresham *et al*., (2000) studied the effects of yet another *S. aureus* virulence cluster, *sar*. *S. aureus* strains containing the *sar* loci create separate vacuoles in neutrophils and not only survive intracellularly, but are viable to re-infect a vulnerable host (34). Therefore, *S. aureus* can be an intracellular pathogen and regulate the activation of several virulence clusters according to environmental stressors including neutrophil reactive oxygen species.

Macrophages and intracellular survival

Macrophages are important leukocytes in innate immunity and can be 'resident' phagocytes or transmigrate through chemotaxis. As previously mentioned, macrophages express several receptors for pathogen recognition, chemoattractant response, phagocytosis, and cell-mediated immunity. During phagocytosis, macrophages recognize pathogen and ingest through endocytosis. Macrophage phagosomes fuse with lysosomes, forming the phagolysosome. Pathogens are destroyed through enzyme release and reactive oxygen species synthesis. Complement aids in the bactericidal function of macrophage phagosomes by efficient reactive

oxygen species synthesis (21). The undigestible particles left from the destroyed pathogen are then excreted and can be recognized by other host cells, further enhancing the inflammatory and chemotactic response during infection. Macrophages also conduct 'surveillance' in different tissues of the host to prevent the establishment of infection and clear apoptotic neutrophils from infection sites (66).

Pathogens have developed mechanisms to survive within macrophages through manipulation of signaling (97), clearing of apoptotic neutrophils (50), and delaying macrophage apoptosis (46). In a study conducted by Watanabe *et al*., (2007), researchers found that TLR2-positive macrophages infected with *S. aureus* exhibited increased intracellular survival as compared to TLR2-deficient macrophages. TLR2-dependent JNK phosphorylation was the signaling kinase responsible for subsequent low production of reactive oxygen species, therefore enhanced *S. aureus* survival.

Collectively, *S. aureus* virulence factors play a major role on dictating host immune responses to enhance pathogen survival. Virulence factors may target host complement and chemotactic responses, signaling pathways and downstream cytokine production, phagocytic and reactive oxygen species production. Therefore, to understand *S. aureus* pathogenesis, the roles of specific virulence factors on host epithelial and leukocyte immune responses must be characterized.

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Chapter 2. Intracellular *Staphylococcus aureus* induces thrombomodulin expression and modulates polymorphonuclear leukocyte chemotaxis during infection.

Running title: Neutrophil modulation by intracellular *S. aureus*

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Abstract

Staphylococcus aureus may become an intracellular pathogen and suppress host immune responses during infection, leading to inefficient pathogen clearance. Accessory gene regulator (*agr*-) controls secretion of α- and δ-hemolysins, which enhance intracellular survival during infection. We hypothesized that intracellular *S. aureus* infections suppress mammary epithelial cell (MEC) immune responses and subsequently impact polymorphonuclear leukocytes (PMN) function. MEC were intracellularly infected (infections) with *S. aureus* wildtype or hemolysin knockout strains to measure host inflammatory and coagulatory responses. Supernatants collected from infected MEC were used to stimulate PMN to determine inflammatory responses. Results indicate that wildtype *S. aureus* infections increased gene expression of thrombomodulin (TM) in MEC ($P<0.01$) as compared with wildtype *S. aureus* supernatant stimulation (stimulations) of MEC. Activated Protein C (APC) production was quantified as a measure of TM activity during infection of MEC and the effect of APC on PMN responses was also analyzed. Wildtype *S. aureus* supernatant suppressed APC production in MEC as compared with no stimulation ($P<0.05$). *S. aureus* agr-KO infections induced APC production in MEC as compared with no stimulation ($P<0.01$) and stimulation with wildtype *S. aureus* ($P<0.001$). Chemotaxis activity of PMN towards interleukin-8 (IL-8) was measured following APC pretreatment. Pretreatment of PMN with 0.5µg or 1.0µg of APC inhibited PMN chemotaxis towards IL-8 ($P<0.01$). Pretreatment of PMN with 2.0µg of APC increased PMN chemotaxis towards IL-8 ($P<0.05$). This study indicates a role for intracellular *S. aureus* in MEC APC production and subsequent inhibition of PMN activity during infection.

Introduction

Staphylococcus aureus is a major cause of chronic and subclinical infections of the mammary gland. Pathogen virulence factors enhance establishment of infection and evasion of host inflammatory and coagulatory responses (10, 24). *S. aureus* virulence cluster, *agr*, regulates the expression and secretion of several virulence factors during infection including hemolysins. Alpha-hemolysin plays a role in mammary epithelial cell (MEC) cytotoxicity (9) and δ-hemolysin contributes to cell damage in mammalian cells (37) during infection. As a result of cell damage, host coagulation is activated to repair mammary tissue and control excessive inflammatory responses. Coagulation proteins such as urokinase- and tissue- plasminogen activators (uPA and tPA, respectively) control the progression of infection (21) and fibrin clot degradation (22).

It is important to address host-pathogen interactions in coagulation regulation because *S. aureus agr*-regulated virulence factors such as staphylokinase, can inhibit plasminogen activation (22), whereas *S. aureus* membrane-associated proteins can induce tissue factor expression (27). Nevertheless, how hemolysins regulate host coagulatory gene expression during infection needs to be determined.

Importantly, *S. aureus* can invade bovine MEC through receptor mediated endocytosis (2) and cause intracellular infections (1-4, 6, 15, 23, 35, 40, 43). Once inside MEC, *S. aureus* is protected from immune responses and can evade endosome destruction through production of *agr*-regulated virulence factors (32, 34, 39). In addition to endosome destruction, both α- and δ-hemolysins enhance *S. aureus* intracellular survival in different cell lines (12, 20). Also, intracellular *S. aureus* suppresses inflammatory gene expression in the host (43), suggesting an

ability to modulate immune responses. The roles of *agr*-regulated hemolysins in modulating MEC gene expression during *S. aureus* intracellular infections have not been determined.

Polymorphonuclear leukocytes (PMN) are the primary immune cell responsible for pathogen clearance in the mammary gland. Infections with *Escherichia coli* induce cytokine and chemokine production and influx of PMN resulting in rapid pathogen clearance (5). However, *S. aureus* infections induce weaker immune responses in the mammary gland as compared to *E. coli* infections (13, 25, 46), resulting in fewer PMN and impaired pathogen clearance. Influx of PMN into the mammary gland can be induced by chemokine production by MEC and complement. Supplementation of inflammatory cytokine, tumor necrosis factor- α (TNF-α), and complement fragment, C5a des-arg, to PMN enhances phagocytic killing of *S. aureus* in the mammary gland (36), suggesting that the bacteria may suppress these host immune responses. Consequently, impaired PMN priming and function during *S. aureus* intramammary infection may be the key to pathogen survival within the host. Therefore, it is important to investigate PMN function during intracellular infection of MEC to identify the mechanisms of immune suppression by *S. aureus*.

We hypothesized that MEC immune responses are suppressed by intracellular *S. aureus* (referred to as infections) as compared to extracellular stimulations with *S. aureus* supernatant (referred to as stimulations). Subsequently, PMN responses to infected MEC may also be suppressed as compared to PMN responses to stimulated MEC.

Materials and Methods

Cell Culture:

MAC-T, bovine mammary epithelial cell line (17), were grown to confluency at 37ºC, 5% CO_2. All tissue culture media ingredients were purchased from Invitrogen (Carlsbad, CA) unless otherwise indicated. MAC-T were grown in Dulbecco's modified Eagle's medium (DMEM) containing heat-inactivated fetal bovine serum (FBS) (5% /vol) (Atlanta Biologicals Lawrenceville, GA), HEPES (2%/vol), L-glutamine (1%/vol), and antibiotic- antimycotic (1%/vol).

Bacterial Strains:

S. aureus strains were obtained from Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) unless otherwise noted. Strains included RN6390B (wildtype, *agr* positive) and RN4220 (referred to as agr-KO in text, is an *agr*A mutant which fails to translate α- and δ-hemolysin (42)). RN6390B hemolysin knockout strains were a generous gift from Dr. Phil Hill (University of Nottingham, UK). Hemolysin knockout strains include RN6390B_hla (hemolysin α-knockout), RN6390B_hld (hemolysin δ-knockout), RN6390B_hlahld (hemolysin α and δ-knockout) (Supplementary Figure 2.1). Knockout strains were created by allele replacement with an erythromycin cassette.

Frozen (-80°C) bacterial stocks were used to streak for isolation (single colony forming units (CFU)) on bovine blood agar plates. Bacterial CFU were allowed to grow on agar plates for 24h at 37°C. One colony from the agar plate was inoculated into 20mL of Tryptic Soy Broth (TSB) and allowed to grow overnight at 37°C, 180 revolutions / min, 5% CO_2 (12400 Incubator

shaker, New Brunswick Scientific, Edison, NJ). Cultures were centrifuged at 850 x g, 4°C, for 10 min. Supernatants were discarded and pellets resuspended in 20mL of Dulbecco's Phosphate Buffered Saline (PBS) (Invitrogen, Carlsbad, CA). Suspensions were centrifuged at 850 x g, 4°C, for 10 min. One mL aliquots of overnight culture supernatants were filtered with a 0.2μm syringe filter and diluted in 9mL of TSB (1:10 dilution). This suspension was used to stimulate MAC-T cells. Bacterial pellets (from centrifugation) were resuspended in PBS to 10^8 CFU / mL in antibiotic free MAC-T medium.

Intracellular Infection:

MAC-T cells were seeded in 60 x 15mm petri dishes (VWR, Suwanee, GA) at $1x10^5$ cells / dish one day before infection. On the day of infection, media was replaced with antibiotic free medium and MAC-T were infected with 10^8 CFU for 2h at 37ºC, 5% CO_2. All experiments included MAC-T unstimulated or stimulated with *S. aureus* supernatant as extracellular controls. After 2h infection, lysostaphin (100 μg/ml; Sigma, St. Louis, MO) was added for 7 min at 37°C to terminate the infection. Cell monolayers were washed in PBS and incubated in gentamicin (50μg/ml; Invitrogen, Carlsbad, CA) -containing MAC-T medium for up to 24h post infection. At time of collection, MAC-T supernatant media was collected, filtered to eliminate bacterial debris, and frozen at -20ºC for use in PMN stimulations. Intracellular infection samples were washed with PBS and incubated in 0.5mL PBS solution containing Triton-X100 (0.05%) (Sigma Aldrich, St. Louis, MO) and EDTA (5mM) (VWR, Suwanee, GA) to lyse cells. Samples were sonicated (Cole Parmer Ultrasonic) for 5 min, 4°C for intracellular CFU confirmation or lysed with RNeasy® lysis buffer (Qiagen, Valencia, CA) for RNA isolation.

16s RNA standard curve:

The 16s ribosomal RNA was used to quantify bacterial CFU during infection using quantitative Real-Time PCR (11). A standard curve was developed by washing and resuspending an overnight culture of *S. aureus* RN6390B strain in PBS to 10^{10} CFU / mL (Supplementary Figure 2.2). This suspension was diluted 10 fold. RNA was collected from each suspension using TriZol (Invitrogen; Carlsbad, CA). MAC-T were seeded in 6-well plates ($1x10^5$ / well) and lysed with 1mL of TriZol. Cell lysates were combined with bacterial lysates and RNA was isolated from samples according to manufacturer's instructions (Invitrogen; Carlsbad, CA). Complimentary DNA (cDNA) was synthesized from sample mRNA (2.2µg) by heating each sample at 70°C for 10min with 250ng of random primers (Invitrogen, Carlsbad, CA). Samples were cooled to 4°C and centrifuged at 350 x g for 30 sec, 4°C. A solution containing 20.5%/vol Buffer, 10%/vol Dithiothreitol (DTT), 5%/vol deoxyribonucleotides (DNTPs) (VWR, Suwanee, GA) , and 2.5%/vol Superscipt II (Reverse transcriptase) were added to each sample. All reagents were purchased from Invitrogen (Carlsbad, CA) unless indicated otherwise. Samples were incubated at 42°C for 50 min to allow replication. Samples were incubated at 70°C for 15 min to de-activate the reverse transcriptase. Samples were diluted to yield 10ng/µL of cDNA with DNase/RNase free water (Qiagen; Valencia, CA).These samples were analyzed using quantitative Real Time-PCR for 16s RNA expression, which correlated to a known concentration of bacteria.

Reverse Transcriptase Real-Time -PCR for mRNA Quantification (qRT-PCR):

Total RNA was isolated from samples using RNeasy® Mini Kit (Qiagen; Valencia, CA). cDNA was synthesized for each sample as indicated above. Primer Express 3 software (Applied

Biosystems; Foster City, CA) was used to design qRT-PCR primer sets spanning exon junctions and synthesize a TaqMan® Probe sequence (See Table 2.1). The TaqMan® Universal PCR Master Mix (Applied Biosystems; Foster City, CA) and the ABI 7300 Real-Time PCR System (Applied Biosystems; Foster City, CA) were used for qRT-PCR analysis. The final volume for qRT-PCR analysis was 25µL containing 300nM of both forward and reverse primers (Integrated DNA Technologies; Coralville, IA), 100nM of 6-carboxyfluorescein (FAM) probe (Biosearch Technologies Inc.; Novato, CA), 2X TaqMan® Universal PCR Master Mix (Applied Biosystems; Foster City, CA), and 50ng cDNA. Samples were heated at 50°C for 2 min once, 95°C for 10 min once, 95°C for 15 sec for 40 cycles, and cooled at 60°C for 1 min. A compiled study of all sample cycle threshold (Ct) values were exported for analysis.

Data was analyzed and graphed using GraphPad Prism Version 4.03 (La Jolla, CA). MAC-T mRNA expression was compiled from at least three separate experiments. Sample Ct values were compared to the Ct value of a stable housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (as determined in preliminary studies for gene consistency). The difference in Ct values (ΔCt) were compared to unstimulated MAC-T ΔCt values (ΔΔCt). This difference was calculated for fold induction ($2^{-\Delta\Delta Ct}$). Statistical analysis was conducted on samples ΔCt values. Significance was determined using one-way ANOVA with a Tukey's post test.

Bovine PMN isolation and stimulation:

All procedures were approved and carried out in accordance with the Institutional Animal Care and Use Committee of Virginia Tech. Blood was collected from the jugular vein of multiparous, non-mastitic, mid-lactation cattle of the Virginia Tech Dairy herd into 10% / vol

PBS containing 40mM EDTA (PBSE) (pH 7.4). Primary PMN were isolated from whole blood as previously described (29). In brief, whole blood was centrifuged at 350 x g for 30 min, 15°C to separate plasma from the lymphocyte layer, red blood cells (RBC) and PMN layer. Plasma and lymphocytes were discarded. RBC were lysed with water. Minimum Essential Media (MEM) (3X, added 33%/vol) (Invitrogen; Carlsbad, CA) was added to adjust the osmolarity, and samples were brought up to 45mL with PBSE. Samples were centrifuged at 350 x g for 5 min, 15°C. Supernatants were discarded and pellets were resuspended in PBSE and centrifuged at 350 x g for 5 min, 15°C. Supernatants were discarded and if RBC were still present in the pellet, a second lysis was applied. Primary PMN were resuspended to $2x10^7$ / mL in MAC-T medium.

For stimulations with MAC-T supernatants, 3mL from intracellular infection or extracellular stimulation supernatants were used to stimulate PMN for 2h at 37ºC, 5% CO_2. Primary PMN were collected in 2mL Eppendorf tubes and centrifuged at 170 x *g* for 5 min at 15ºC. The PMN pellet was resuspended in TriZol for RNA isolation and qRT-PCR analysis. Data was analyzed as indicated above, however PMN Ct values were compared to the Ct value of stable housekeeping gene, β-actin.

Thrombomodulin Activity:

Thrombomodulin activity was measured using an activated protein C (APC) assay. MAC-T cells were seeded in 48-well plates (Falcon, Benton Dickinson, San Jose, CA) and infected or stimulated as indicated above. At 24h post infection, the wells were washed three times with 1mL of APC buffer (50mM Tris-HCl pH 7.5, containing 2mM $CaCl_2$ and 0.1% BSA). Bovine protein C (5µg/ml; Enzyme Research Laboratories, Inc., South Bend, IN) and thrombin (0.12U/well; Enzyme Research Laboratories, Inc., South Bend, IN) were added to the wells with

APC buffer for a total of 100µl volume. After 1h incubation at 37ºC, 5% CO_2, the 48-well plate was centrifuged at 2800 x *g* for 10 min at room temperature. Sample supernatants (30µl) were added to a 96-well plate (Greiner Bio-one, VWR, Suwanee, GA). Generation of APC was measured by the color change induced by the cleavage of APC substrate, S-2366 (Diapharma, West Chester, OH). Hirudin (2U/well; Sigma, St. Louis, MO) was added to inhibit non specific S-2366 cleavage of thrombin (31). After a 2h and 30 min incubation at 37ºC, 5%CO_2, optical density (OD) was read on a 96-well plate reader (µQuant; VWR, Suwanee, GA) at 405nm. Sample OD were compared to an APC standard curve OD. MAC-T APC production was analyzed using a one-way ANOVA and with a Tukey's post test. Samples were run in triplicate for three individual experiments.

Chemotaxis Assay:

Chemotaxis plates (5µm filter; Neuroprobe, Inc., Gaithersburg, MD) were filled with 30µl of PMN media (10% FBS, 1% antibiotic/antimycotic, phenol red free DMEM) containing 100ng of recombinant bovine IL-8 (Fisher Scientific, Waltham, MA). Primary PMN were pretreated with 0.5µg, 1.0µg, 1.5µg, or 2.0µg recombinant bovine APC (Enzyme Research Laboratories, Inc., South Bend, IN) for 10 min at room temperature. Pretreated PMN were added to the top of the filter and allowed to migrate for 30 min at 37ºC, 5% CO_2. After 30 min migration incubation, non-migrating PMN remaining on the top of filter were eliminated using a kimwipe, per manufacturer's instructions. To remove migrated PMN that had attached to bottom of filter membrane, ice-cold 2mM EDTA PBS (pH 7.4) solution was added to the top of the filter and incubated for 30 min at 4ºC. After the 30 min incubation, remaining PBS solution on top of the filter was eliminated using a kimwipe. The chemotaxis plate was centrifuged at 400 x g at

4ºC for 10 min to obtain remaining PMN in the filter. The filter membrane was removed and a funnel plate was used to transfer chemotaxis plate contents to an ELISA 96-well plate by centrifuging at 400 x g for 10 min at 4ºC. Once in 96 well plate, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (60µg) was added to all sample wells of ELISA plate. After incubating for 5h at 37ºC with constant agitation (ThermoRocker by Lab Line, Fisher Scientific, Waltham, MA), 100µl of extraction buffer (50% N,N-dimethylformamide, 20% SDS) was added to wells and OD read at 595nm. Sample OD were compared to PMN migration towards bovine IL-8 (100ng) alone. Chemotaxis of PMN towards bovine IL-8 alone was set to 100% migration. Migration of PMN was calculated as percent difference from IL-8 alone samples. All samples were run in triplicate and experiments were repeated three times with different cows.

Results

MEC inflammatory and coagulatory gene expression during intracellular *S. aureus* infection.

MEC were infected with *S. aureus* wildtype for 2h to establish an intracellular infection or stimulated with bacterial-supernatant to simulate an extracellular infection. MEC inflammatory and coagulatory gene expression profiles were analyzed at 2, 4, 6, 8, 12, 16, 18, and 24h post infection (Figure 2.1). Negative controls consisted of unstimulated MEC lysates and positive controls consisted of *E. coli* infected MEC lysates (data not shown). *S. aureus* stimulations significantly increased MEC inflammatory gene, interleukin-6 (IL-6), expression at 2, 4, 6, 18, and 24h post stimulation as compared to unstimulated MEC (Figure 2.1A). Similarly, *S. aureus* stimulations significantly increased TNF- α gene expression in MEC at 2, 4, and 12h post stimulation as compared to unstimulated MEC (Figure 2.1B). *S. aureus* infections did not increase IL-6 or TNF-α gene expression in MEC. As hypothesized, *S. aureus* stimulations or infections differentially regulate inflammatory genes in MEC.

S. aureus stimulations increased coagulatory genes, tissue factor (TF) and plasminogen activator inhibitor-1(PAI-1) expression in MEC as compared to unstimulated MEC. However, *S. aureus* stimulations significantly decreased thrombomodulin (TM) gene expression as compared to unstimulated MEC at 24h post stimulation. Interestingly, infected MEC significantly increased TM gene expression as compared to stimulated MEC (Figure 2.1C), suggesting that *S. aureus* infections can regulate MEC coagulatory responses during infection.

In addition to MEC coagulatory responses, stimulations or infections altered TF expression at different time points (Figure 2.1D). At 6h post infection, both stimulated and infected MEC as compared to unstimulated MEC decreased TF gene expression. Stimulations

also decreased TF gene expression in MEC at 16h post stimulation. However, stimulations increased TF gene expression at 18h as compared with unstimulated MEC. In addition to TF regulation, stimulations increased PAI-1 gene expression in MEC at 18 and 24h post stimulation (Figure 2.1E). Changes in TF and PAI-1 gene expression profiles may be due to cell damage induced by sustained inflammatory responses or secreted pathogen virulence factors. Nevertheless, stimulations did not significantly increase uPA or tPA gene expression in MEC as compared to unstimulated (Figure 2.1F and 2.1G). This data suggests that *S. aureus* wildtype can regulate MEC coagulatory gene expression during infections and stimulations. Suppression of TF during infections may indicate a role for secreted virulence factors in coagulation induction, an advantageous strategy for pathogen dissemination in the host.

The variation in the data was hard to overcome for analysis. *S. aureus* infections seemed to increase IL-6 and TNF-α gene expression in MEC at 2 and 6h post infection, but the statistical analysis did not detect significant differences due to data variation. The same circumstances can be applied to TM gene expression at 2 and 4h post infection, tPA gene expression at 18h post infections and uPA gene expression at 2 and 12h post infection. This variation suggests an effect of day of infection and potentially an effect of actual infecting dose.

MEC immune responses to *S. aureus* wildtype and virulence knockout strains.

To address *S. aureus* virulence factors involved in MEC immune responses during infection, we infected or stimulated MEC with *S. aureus* strains lacking virulence cluster, *agr*, or specific hemolysins. Strains included *S. aureus* wildtype, *agr* knockout (agr-KO), hemolysin α-knockout (α-KO), hemolysin δ-knockout (δ-KO), and hemolysins α- and δ-knockout (α/δ-KO). Cell lysates for mRNA gene profiles were collected 24h post infection. Figure 2.2A confirms *S.*

aureus wildtype stimulations significantly increased IL-6 gene expression in MEC. *S. aureus* agr-KO stimulations also significantly increased IL-6 gene expression in MEC as compared to unstimulated MEC. However, *S. aureus* wildtype or agr-KO infections did not increase IL-6 gene expression. Similarly, infections or stimulations with *S. aureus* α-KO, δ-KO, or α/δ-KO did not increase IL-6 gene expression in MEC. These findings suggest a role for *S. aureus* hemolysins in regulating IL-6 expression in MEC.

Stimulation with *S. aureus* wildtype decreased TM gene expression in MEC, confirming our previous experiment (Figure 2.1C). Notably, stimulation with agr-KO also decreased TM gene expression in MEC, suggesting that *agr*-regulated virulence genes do not suppress TM expression in MEC. However, infections or stimulations with α-KO, δ-KO, or α/δ-KO did not increase TM gene expression in MEC, which may indicate a role for hemolysins in TM regulation.

Since inflammation and coagulation affect chemotaxis, interleukin-8 (IL-8) was also measured during *S. aureus* infection or stimulation (Figure 2.2C). As compared to unstimulated MEC, stimulations with *S. aureus* wildtype or agr-KO or infections with α-KO increased IL-8 gene expression in MEC. However, infections with *S. aureus* wildtype or agr-KO, stimulation with α-KO, or infections or stimulations with δ-KO or α/δ-KO did not impact IL-8 expression in MEC. These results suggest that secreted virulence factors in both *S. aureus* wildtype and agr-KO can regulate IL-6 and IL-8 gene expression. In addition to virulence factor regulation of host genes, α-hemolysin may regulate host chemotaxis since α-KO infection increased IL-8 expression in MEC. Overall, these data suggests that *S. aureus* virulence factors regulate MEC expression of inflammatory, coagulatory, and chemotaxis genes.

PMN immune response to *S. aureus* stimulated MEC supernatants.

Our findings indicate that *S. aureus* virulence factors and α-KO infections induce IL-8 gene expression in MEC (Figure 2.2). Therefore, to understand the role of live *S. aureus* or secreted virulence factors on subsequent leukocyte responses, bovine PMN gene expression profiles when stimulated with *S. aureus* infected or stimulated MEC supernatants were compared (Figure 2.3). Interestingly, supernatants from MEC infections induced expression of inflammatory genes, IL-6 and TNF-α, in PMN. As compared to unstimulated PMN, MEC supernatants from infections with *S. aureus* wildtype, α-KO, δ-KO, α/δ-KO significantly induced PMN IL-6 expression (Figure 2.3A). Similarly, supernatants from MEC *S. aureus* wildtype, α-KO, and α/δ-KO infections, but not with agr-KO or δ-KO, induced PMN TNF-α expression (Figure 2.3B). In addition, MEC supernatants from *S. aureus* α-KO stimulations induced PMN TNF-α expression. Similar to PMN IL-6 gene expression profile, MEC supernatants collected from infections induced PMN TF expression (Figure 2.3D). These data suggests that a MEC secreted protein in response to *S. aureus* infections can subsequently induce PMN inflammatory and coagulatory responses.

MEC supernatants collected from infection or stimulation with agr-KO significantly induced PMN IL-8 as compared to unstimulated PMN (Figure 2.3C). MEC supernatants from infections with *S. aureus* α-KO or δ-KO induced PMN IL-8 gene expression. Supernatants collected from MEC infection or stimulation with α/δ-KO significantly induced PMN IL-8 gene expression. Interestingly, MEC supernatants collected from *S. aureus* wildtype infections or stimulations failed to induce PMN IL-8 gene expression, suggesting a role for *S. aureus* virulence factors in subsequent PMN chemotaxis.

Only MEC supernatants collected from α/δ-KO infections induced PMN L-selectin gene expression (Figure 2.3E). This result suggests a role for both α- and δ-hemolysins in suppression of PMN adherence protein expression.

The variation in this data was hard to overcome for analysis. *S. aureus* wildtype or knockout infections or stimulations seemed to increase TNF-α, IL-8, or TF gene expression in PMN, but the statistical analysis did not detect significant differences due to data variation. This variation suggests an effect of animal. Primary PMN were isolated from different cows and, particularly, primary cells are more sensitive to stimuli than cell lines. Also, supernatants from MEC infections or stimulations may not have been uniform between treatments. Three milliliters of supernatants were used to stimulate the primary PMN and not a set amount of proteins.

MEC production of APC during *S. aureus* intracellular infection.

The PMN gene expression profiles (Figure 2.3) suggested a response to infected MEC secreted proteins. Our findings indicated *S. aureus* infections significantly increased TM gene expression in MEC at 12h post infection (Figure 2.1). To quantify MEC TM during *S. aureus* infections, MEC production of APC during *S. aureus* infection and stimulation were measured. Since TM gene expression was greater at 12h post infection, APC production was measured at 24h to allow for protein expression. MEC were infected or stimulated with *S. aureus* wildtype, agr-KO, α-KO, δ-KO, α/δ-KO and APC measured 24h later.

Stimulation with *S. aureus* wildtype significantly decreased MEC production of APC as compared with APC production of unstimulated MEC (Figure 2.4A). Infection with *S. aureus* agr-KO significantly increased MEC APC production as compared with APC production of both unstimulated and stimulated MEC (Figure 2.4B). This data suggests that *S. aureus* wildtype

secreted virulence factors may suppress APC production in an agr-dependent manner. Therefore, *agr*-regulated virulence factors may be responsible for enhanced immune suppression during infections due to significant increase in APC production.

To determine if α- or δ-hemolysins played a role in MEC production of APC, MEC were infected or stimulated with α-KO, δ-KO, or α/δ-KO *S. aureus* strains. However, α-KO, δ-KO, or α/δ-KO infections or stimulations did not impact APC production in MEC. Overall, these data suggest that *S. aureus agr*- regulated virulence genes may play an important role on coagulation responses during infections.

PMN chemotaxis in the presence of APC.

APC production during *S. aureus* infections may be the inducing factor of inflammatory responses in PMN (Supplementary Figure 2.3) and subsequently affect PMN activity. Therefore, we tested PMN chemotaxis after APC pretreatment. As shown in Figure 2.5, APC pretreatment (0.5-1.0μg/mL) significantly reduced PMN chemotaxis towards IL-8 as compared to chemotaxis towards IL-8 alone ($P<0.01$). However, pretreatment of PMN with 2.0μg/mL APC showed a significant increase in PMN chemotaxis towards IL-8 ($P<0.05$). This data indicates an inhibiting role for APC in PMN chemotaxis that is concentration-dependent. The exposure of PMN to APC may have an effective range where 2.0μg/mL may induce chemotaxis, but lower concentrations inhibit chemotaxis.

Discussion

Intracellular infections may impart to *S. aureus* protection from host immune responses, thereby propagating subclinical symptoms and establishment of chronic infections (44). *S. aureus* can survive intracellularly in human neutrophils, endothelial cells, pulmonary cells, mouse macrophages, and bovine MEC (1, 4, 14, 23, 26, 43). Intracellular *S. aureus* strains can change phenotypically and genotypically from parental wildtype strains to small colony variants (SCV) resulting in modified immune activation (28, 38). During intracellular infection of human endothelial cells, *S. aureus* induces CCL5, CXCL11, and ICAM-1 gene expression. However after 28 days of intracellular infection, neither host inflammatory genes nor *S. aureus* toxins such as α-hemolysin are measurable (43, 44). These findings suggest that intracellular *S. aureus* can survive within the host without inducing inflammatory responses. Also, as *S. aureus* becomes an intracellular pathogen, lack of virulence factor induction may contribute to survival within the host. Overcoming immune responses by virulence factors during intracellular infections may be the key to initiating successful pathogen clearance.

Coagulation is seldom researched in regard to intramammary infections. Nevertheless, it is well accepted that inflammation and coagulation work together to clear pathogens and repair tissue (8, 30, 33). During *S. aureus* intramammary infection, tissue damage suggests activation of coagulation, which may result in abscess formation (41). Abscesses allow pathogens to survive within the mammary gland, proliferate, evade antibiotics, and secrete virulence factors. Previous research indicated uPA up-regulation in bovine MEC during *S. aureus* M60 infection (47). Compared to our findings, *S. aureus* stimulations only increased TF and PAI-1 gene expression in MEC. Contrasting results to Zavizion et al. (1997) may be due to differences in *S. aureus* strains used and differential expression of virulence factors. Nevertheless, in our study *S.*

aureus stimulation increased inflammatory genes, IL-6 and TNF-α, expression which may cause the subsequent generation of coagulation genes (30). Haslinger-Löffler et al., (2007), found that recombinant TNF-α stimulation significantly induced PAI-1 production as compared to *S. aureus* supernatant stimulation of human mononuclear cells. Our data shows *S. aureus* stimulation significantly increased TNF-α gene expression in MEC. Together these findings suggest that the TNF-α produced by *S. aureus* stimulated MEC may be responsible for PAI-1 induction. Extended cell exposure to inflammatory cytokines may cause cell injury, which also leads to induction of coagulation (16, 33, 45), thereby emphasizing notable interactions between inflammation and coagulation.

Existing data focuses on how secreted virulence factors play an important role in establishment of infection and host immune evasion. For example, α-hemolysin increases *S. aureus* adherence to host cells (10) and the expression of agr by *S. aureus* is required to escape the endosome of mammary epithelial (MAC-T) (34, 39), cystic fibrosis epithelial (CFT-1) (20), and hepatic cell lines (19). Furthermore, intracellular *S. aureus* expresses α- and β-hemolysin transcripts at 3-5h post invasion which contribute to endosomal degradation (39), emphasizing an important role for hemolysins in endosomal escape to the cytoplasm.

Little information exists on the role of *agr*-regulated hemolysins in immune responses during intracellular survival. Our data suggests that *agr*-regulated hemolysins may enhance survival within the host through manipulation of coagulation genes. As seen in murine models, TM induction may enhance host cell viability and reduce pathogen induced cell damage (7). Our indicator of TM expression during infection was APC production by MEC. Our data indicated that *S. aureus* wildtype supernatant suppressed APC production in MEC as compared with unstimulated and *S. aureus* agr-KO intracellular infections induced APC production in MEC as

compared to unstimulated and stimulated MEC. Together, *S. aureus* virulence factors may suppress APC production, but during intracellular infection enhance host cell viability through TM expression. Also, our data indicated that APC inhibited PMN chemotaxis (Figure 2.6). Previous research indicates that *S. aureus* intramammary infections significantly prolong activated partial thromboplastin time and lower platelet and leukocyte counts in cows (18). Together, these findings confirm that *S. aureus* infections increase expression of coagulation proteins, which inhibit PMN chemotactic responses and may be detected as low leukocyte counts in the host.

Similar to our findings, APC exhibits an anti-inflammatory effect on human gastric epithelial cells infected with *Helicobacter pylori* (31). Our intracellular *S. aureus* model shows a lack of inflammatory responses in infected MEC, however future research needs to determine how APC contributes to this effect. Also, more experiments need to be conducted in order to conclude that APC is the protein that increased PMN inflammatory gene expression during *S. aureus* intracellular infections. Although APC seemed to induce inflammatory responses in PMN (Supplementary Figure 2.3), inflammatory gene fold inductions observed in Figure 2.3 were much greater, suggesting that proteins other than APC may play a role on this response.

It was necessary to analyze PMN activity in the presence of APC to better understand the immune responses in our model. Activity of PMN is greatly defined by chemotaxis ability, therefore it was necessary to analyze PMN chemotaxis in the presence of APC to better understand the immune responses in our model. Similar findings to the inhibitory effects of APC on bovine neutrophil chemotaxis towards IL-8 have been reported using a human model (31). However, more experiments need to be conducted to specifically indicate that this effects is APC-induced and not induced by any other protein found in bovine serum.

Overall, our findings indicate that *S. aureus* intracellular infections regulate host inflammatory and coagulatory gene responses and these regulatory mechanisms are impacted by virulence factor expression. In addition, modulation of MEC genes has subsequent effects on leukocyte activity and influx. Collectively, *S. aureus* intracellular infections may be successful because of suppressed inflammatory responses that decrease initial leukocyte influx and enhance coagulation.

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Table 2.1 Bovine primer sequences used for quantitative Real Time PCR (5'→3')

Table 2.1. Bovine primer sequences used for quantitative Real Time- PCR (5'→3')		
Gene Name	**Primer**	**Sequence**
Glyceraldehyde-3-Phosphate Dehydrogenase	Forward	GACCCCTTCATTGACCTTCA
Glyceraldehyde-3-Phosphate Dehydrogenase	Reverse	GATCTCGCTCCTGGAAGATG
Glyceraldehyde-3-Phosphate Dehydrogenase	Probe	TTCCAGTATGATTCCACCCACGGCA
β-actin	Forward	CAGCAAGCAGGAGTACGATGAGT
β-actin	Reverse	AAGGGTGTAACGCAGCTAACAGT
β-actin	Probe	TCCATCGTCCACCGCAAATGCTTCTA
Interleukin-6	Forward	CCAGAGAAAACCGAAGCTCTCA
Interleukin-6	Reverse	CTCATCATTCTTCTCACATATCTCCTTT
Interleukin-6	Probe	AGCGCATGGTCGACAAAATCTCTGC
Tumor Necrosis Factor-α	Forward	TCTCCTTCCTCCTGGTTGCA
Tumor Necrosis Factor-α	Reverse	GTTTGAACCAGAGGGCTGTTG
Tumor Necrosis Factor-α	Probe	CCCAGAGGGAAGAGCAGTCCCCA
Thrombomodulin	Forward	CCCGGCAAGCTGGTTAGA
Thrombomodulin	Reverse	CGTTGGCAGCTGTCTGGAA
Thrombomodulin	Probe	AGGCGCCATCCCACCTACTGCAG
Tissue Factor	Forward	TGAGAGAGACATATTTGGCGAGAGT
Tissue Factor	Reverse	AAGGTTTGTCTCTAGGTAGGGTGTGA
Tissue Factor	Probe	CCTACCCCGCAGACACTAGCAGTTCCA
Plasminogen Activator Inhibitor-1	Forward	CCGCAACGTGGTTTTCTCA
Plasminogen Activator Inhibitor-1	Reverse	GGCCATGCCCTTCTCTTCA
Plasminogen Activator Inhibitor-1	Probe	AGGAGAAACCCGCCAGCAGATCCA
Tissue Plasminogen Activator	Forward	TGCCCGTCAGAAGTTGCA
Tissue Plasminogen Activator	Reverse	CGGTAGCATCTATTTCACAGAGCTT
Tissue Plasminogen Activator	Probe	TGTCGGCAGGCCCTCTATTCTTCAGA
Urokinase Plasminogen Activator	Forward	GTAGCCGCGGAGCTTCTG
Urokinase Plasminogen Activator	Reverse	CAGACAGCCACAGTTCGATTCA
Urokinase Plasminogen Activator	Probe	CAGCCTTGCCACCATGAGGGTCCT
Interleukin-8	Forward	GAGTGGGCCACACTGTGAAA
Interleukin-8	Reverse	TGCTTCTCAGCTCTCTTCACAAA
Interleukin-8	Probe	AAACGAGGTCTGCTTAAACCCCAAGGAA
L-Selectin	Forward	ACCAACCTGTCGAGTGATTCAA
L-Selectin	Reverse	GGTGCATGTGGAGGAAAAGC
L-Selectin	Probe	TGGACTGTAATCACCCCCTTGTCGACTTT

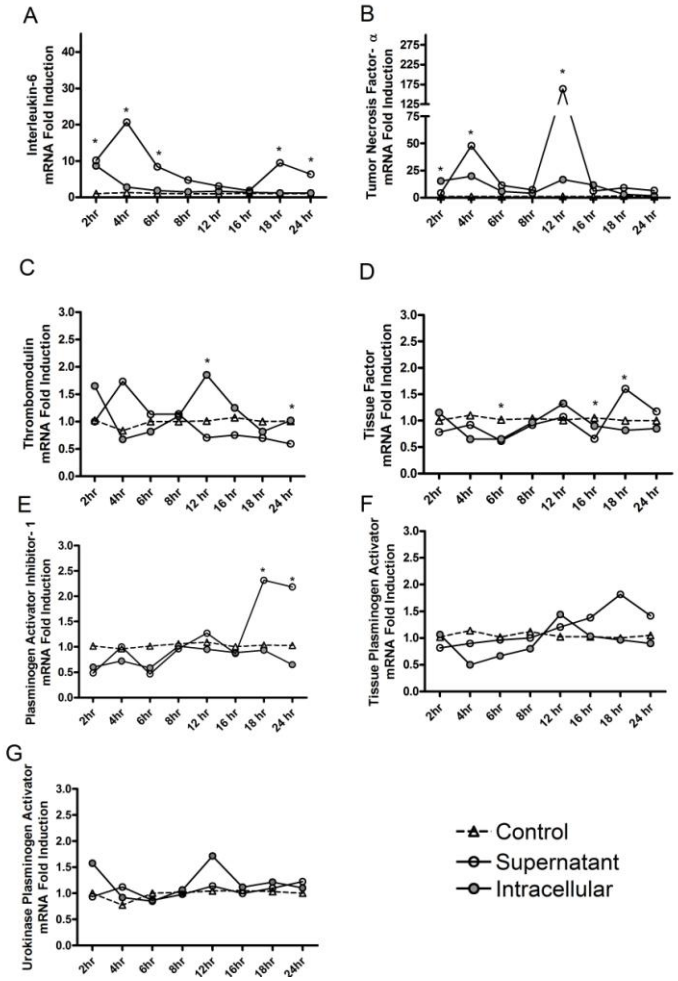


Figure 2.1. Coagulation and inflammatory gene expression by MAC-T treated with *S. aureus* supernatant or intracellularly infected with *S. aureus*. MAC-T were infected with *S. aureus* strain, RN6390B, or stimulated with respective supernatant for 2h. Cell lysates were collected for gene expression profiles of Interleukin-6 (A), Tumor necrosis factor –α (B), Thrombomodulin (C), Tissue factor (D), Plasminogen activator inhibitor-1 (E), Tissue plasminogen activator (F), Urokinase plasminogen activator (G). Data represents gene expression profiles throughout time for unstimulated controls (-Δ-), *S. aureus* supernatant stimulated MAC-T (-o-), or intracellularly infected MAC-T with *S. aureus* (grey circles). Experiments were run in duplicate three times (n=3). Data was analyzed using a one-way ANOVA with a Tukey's post test and significance isindicated by *P<0.01.

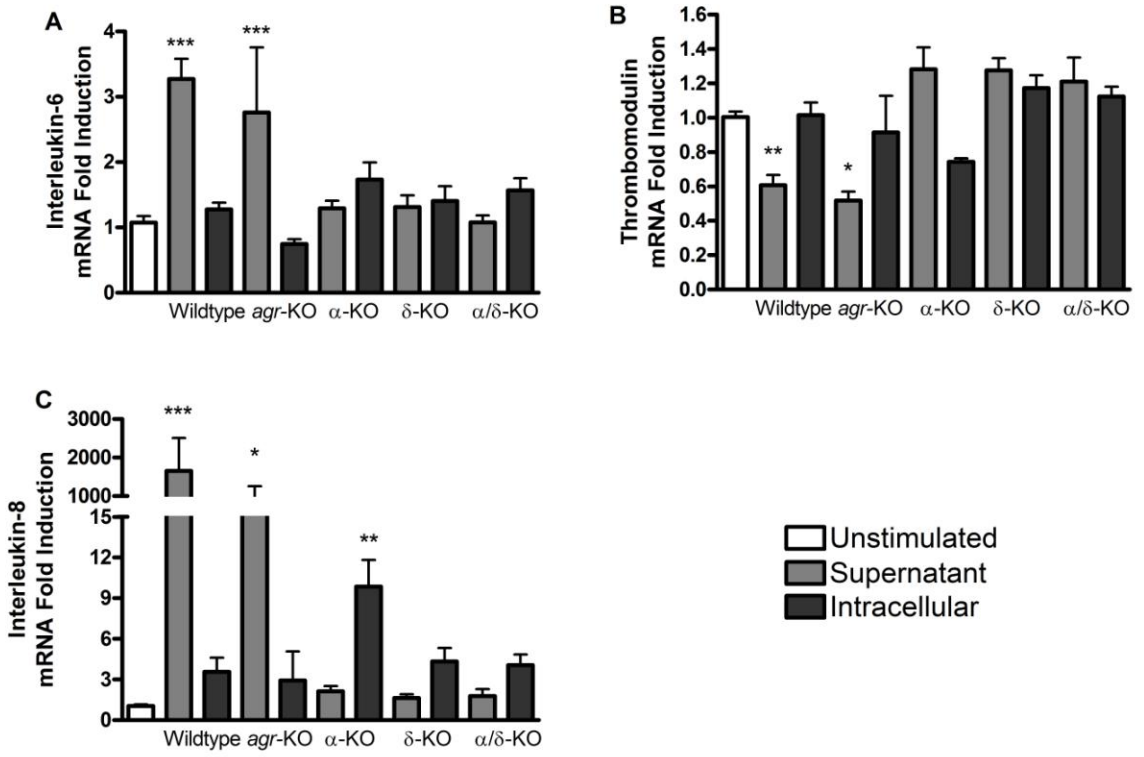


Figure 2.2. MAC-T immune responses to *S. aureus* wildtype and virulence knockout strains. MAC-T were intracellularly infected or supernatant stimulated with *S. aureus* wildtype, *agr*- knockout (agr-KO), α-hemolysin knockout (α -KO), δ-hemolysin knockout (δ -KO), or α / δ -hemolysin knockout (α / δ -KO) strains for 24h. Cells were lysed for gene profiling of Interleukin-6 (A), Interleukin-8 (B), and Thrombomodulin (C). Data represents unstimulated controls (white bars), stimulated MEC with *S. aureus* supernatant (grey bars), or intracellularly infected MEC with *S. aureus* strains (black bars). Experiments were run in duplicate three times (n=3). Data was analyzed using a one-way ANOVA with a Dunnett's post test. Significance as compared to unstimulated controls are indicated by *P<0.01, **P<0.05, ***P<0.001.

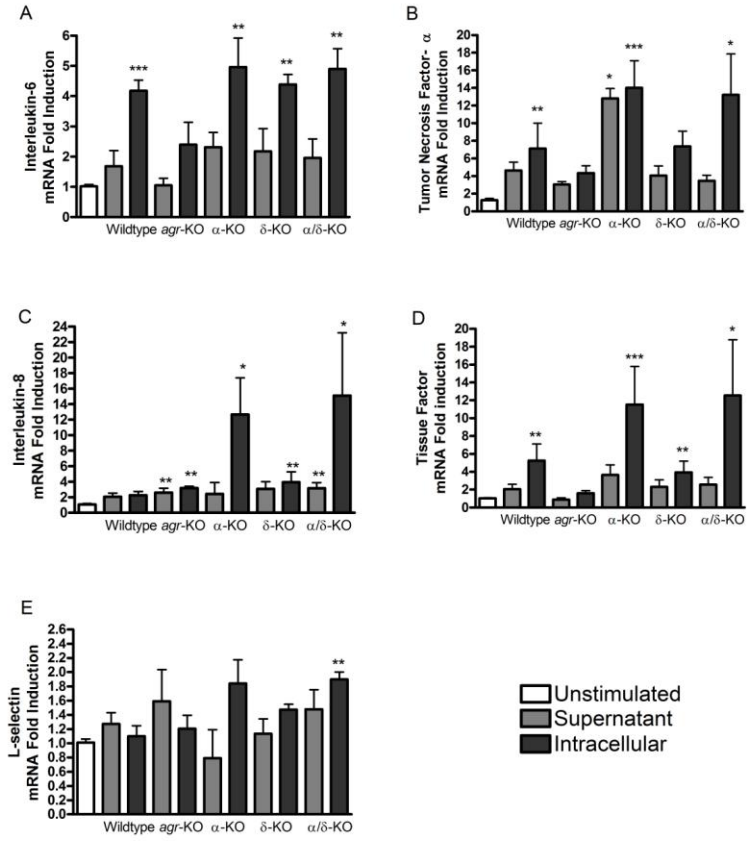


Figure 2.3. Bovine PMN stimulation with *S. aureus* infection supernatants.

Polymorphonuclear leukocytes (PMN) were incubated with supernatants collected from *S. aureus* infections or stimulations of MAC-T cells. After 2h stimulation at 37°C, PMN lysates were collected for gene expression profiles of Interleukin-6 (A), Tumor necrosis factor-α (B), Interleukin-8 (C), Tissue factor (D), L-selectin (E). Data represents unstimulated controls (white bars), stimulated MEC with *S. aureus* supernatant (grey bars), or intracellularly infected MEC with *S. aureus* strains (black bars). Samples were run in duplicate and data represents three separate experiments (n=3). Data was analyzed using a one-way ANOVA with a Dunnett's post test. Significance as compared to unstimulated controls are indicated by *P<0.01, **P<0.05, ***P<0.001.

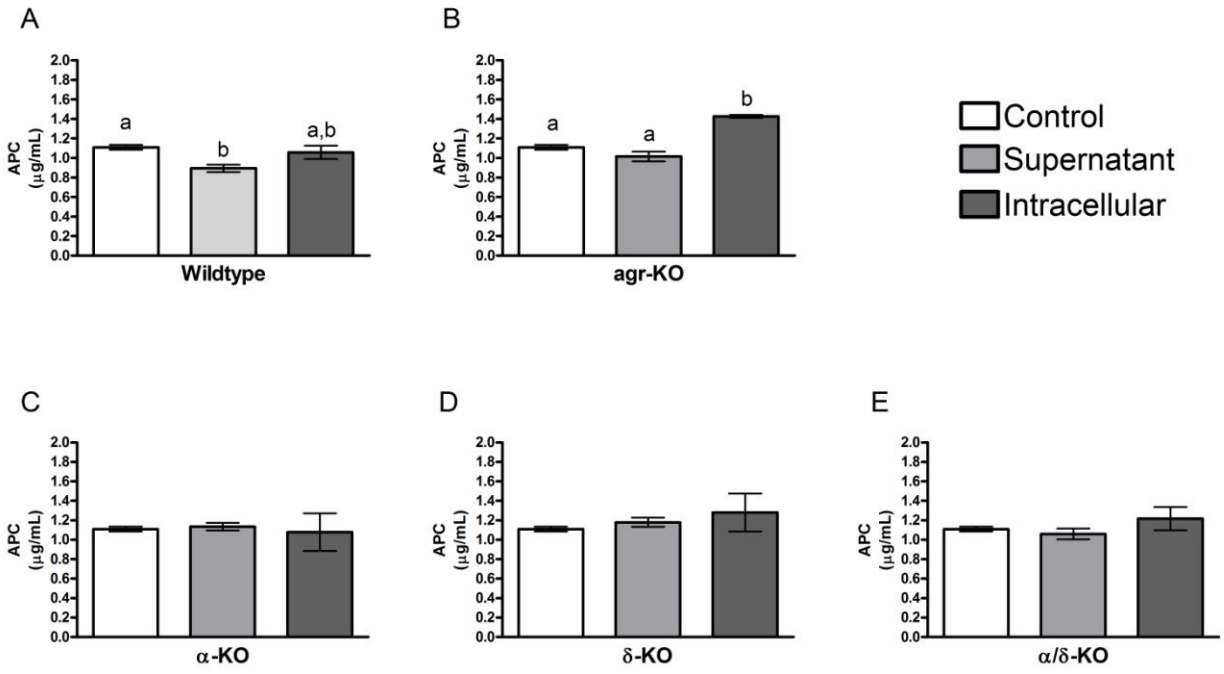


Figure 2.4. Activated protein C (APC) production in *S. aureus* supernatant stimulated and intracellularly infected MAC-T cells. MAC-T cells were stimulated with *S. aureus* supernatant or intracellularly infected with *S. aureus* Wildtype (A), agr-KO (B), α-KO (C), δ -KO (D), α / δ -KO (E). After 24h post infection incubation, cells were washed and stimulated with bovine protein C and thrombin to induce production of APC. Results were measured with an ELISA plate reader and quantified with a standard curve. Data represents unstimulated controls (white bars), stimulated MEC with *S. aureus* supernatant (grey bars), or intracellularly infected MEC with *S. aureus* strains (black bars). Samples were run in triplicate and experiments conducted three times (n=3). Significance was determined at *P<0.01 using a one-way ANOVA with a Tukey's post test. Different letters (a and b) indicate significant difference between treatments.

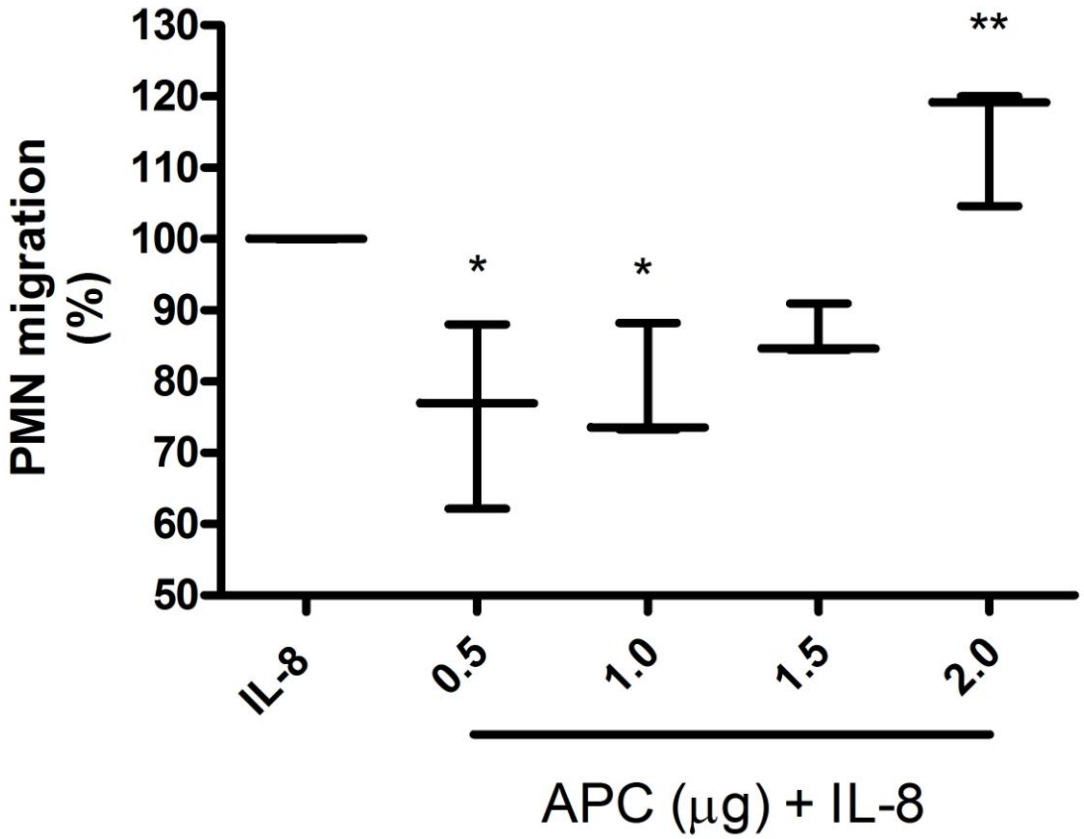
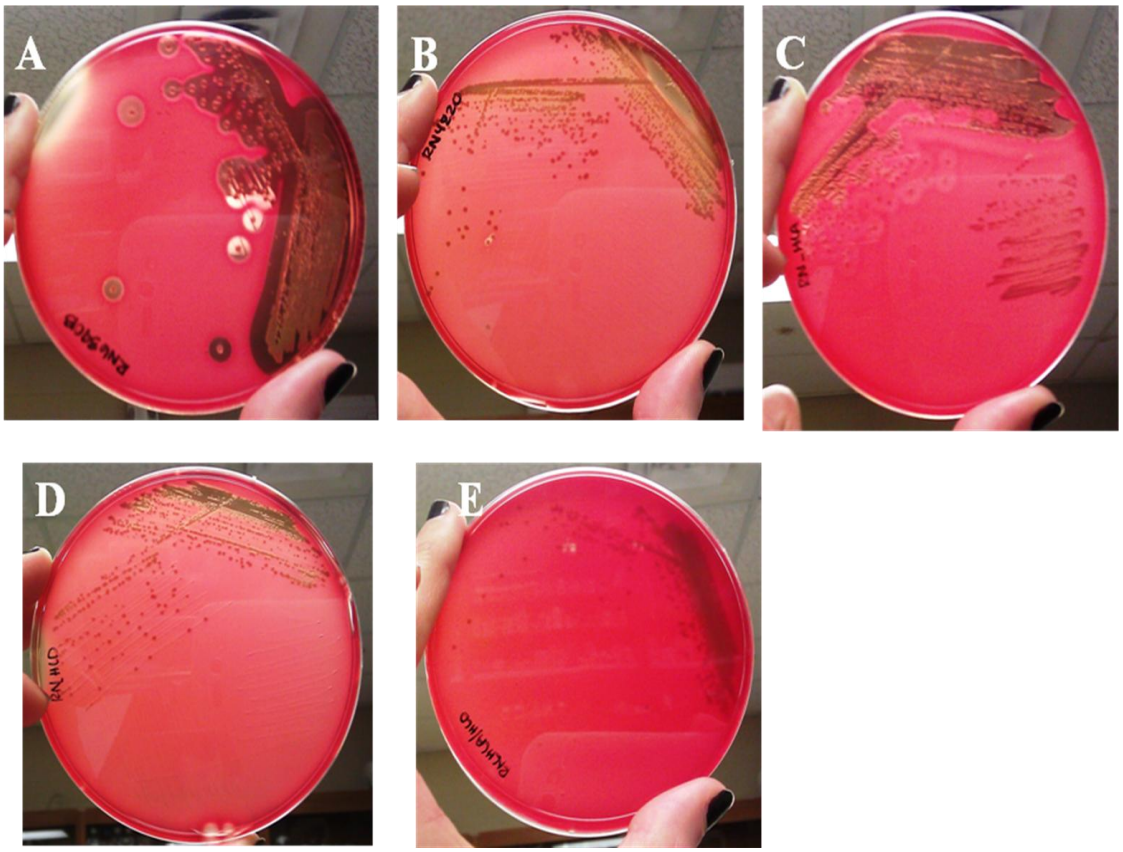
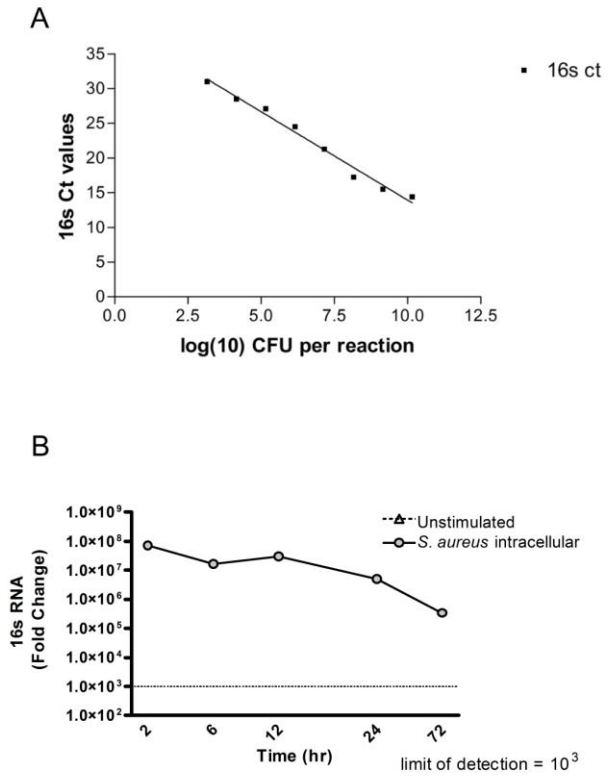


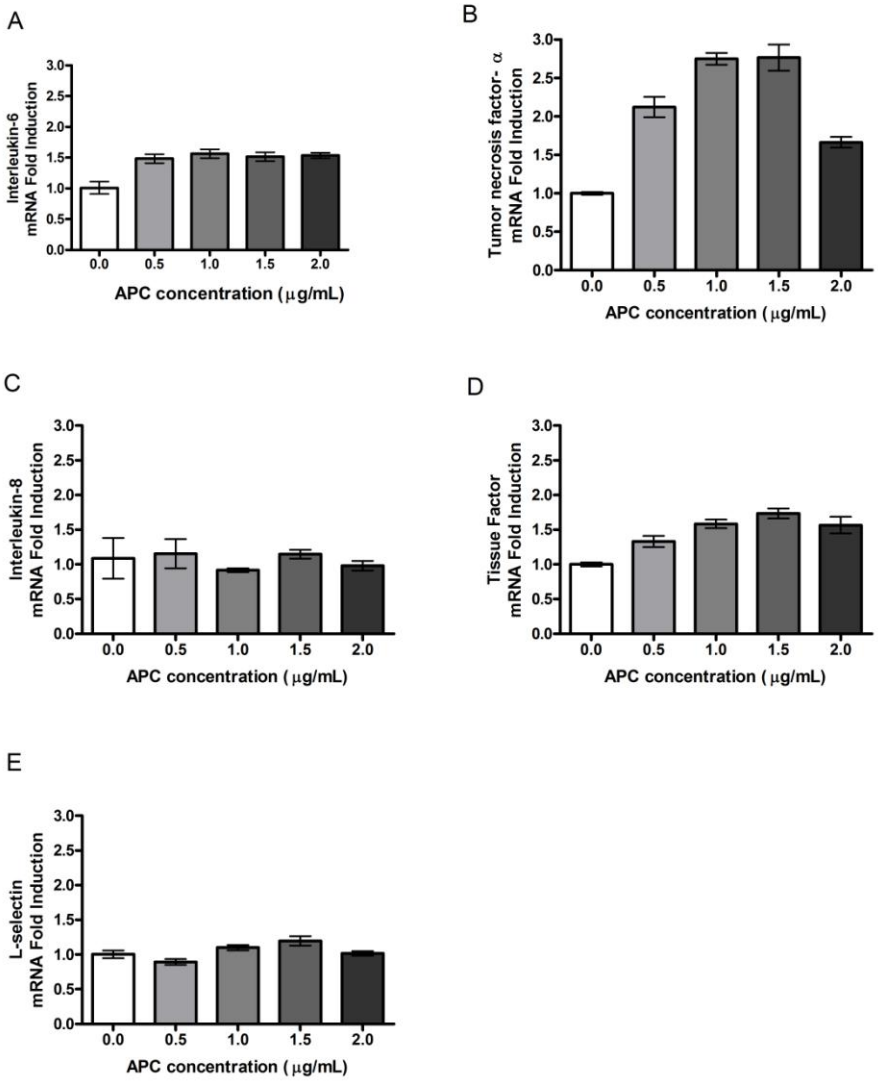
Figure 2.5. PMN APC pretreatment and chemotaxis. Polymorphonuclear leukocytes (PMN) were pretreated with increasing amounts of activated protein C (APC) (0.5μg-2.0μg). After 30 min incubation, chemotaxis towards bovine Interleukin-8 (IL-8) (100ng) gradient was measured. Data represents chemotaxis towards IL-8 alone and chemotaxis towards IL-8 following APC pretreatment. Samples were run in triplicate and data represents three separate experiments (n=3). Mean values from each separate experiment were normalized to IL-8 PMN migration, assuming IL-8 is 100% migration. Results were analyzed using a one-way ANOVA with a Dunnett's post test to compare treatments to IL-8 alone. Significance is indicated by **P<0.05, *P<0.01 as compared to IL-8 only.



Supplementary Figure 2.1. *S. aureus* strain hemolytic phenotypes. Knockout strains were created by allele replacement in *S. aureus* wildtype, RN6390B, using 2kb up- and down-stream of the gene to be knockout out and replacing the gene with an erythromycin cassette. The following pictures illustrate the hemolytic phenotype of *S. aureus* wildtype (A), agr-KO (B), α-KO (C), δ-KO (D), α/δ-KO (E). Wildtype expresses all hemolysins, especially indicated by α-hemolysin (clear hemolysis) phenotype (A). Strain, agr-KO, is an agrA mutant that cannot translate α- or δ- hemolysins, therefore expresses no hemolysin phenotype (B). α-KO expresses β-hemolysin (partial hemolysis) (C). δ-KO and α/δ-KO do not express hemolysin phenotype (D and E, respectively).



Supplementary Figure 2.2. 16s ribosomal RNA standard curve and stable indicator of CFU in infection model. 16s ribosomal RNA was used to indicate bacterial CFU during intracellular infection. Standard curve was developed by collecting RNA from overnight bacterial suspensions and cell lysates. Samples were analyzed using quantitative Real Time PCR (qRT-PCR) for 16s RNA expression, which correlated to a known concentration of bacteria in our samples (A). Mammary epithelial cells (MEC) were intracellularly infected with *S. aureus* (MOI 1000) and lysates were collected for 16s RNA gene expression as an indicator of intracellular *S. aureus* CFU for up to 72h post infection (B). Data represents fold induction of 16s RNA gene expression (normalized to MEC housekeeping gene GAPDH). Unstimulated controls indicated the limit of detection (dotted line) and 16s RNA induction in samples is represented by grey circles (B). Data indicates 16s RNA is a stable indicator of CFU quantity in our sample and confirms CFU detection throughout our time course.



Supplementary Figure 2.3. APC stimulation of bovine PMN and resulting gene expression profiles. Polymorphonuclear leukocytes (PMN) were stimulated with increasing concentrations of bovine activated protein C (APC). After 2h incubation, PMN lysates were collected for gene expression profiles of Interleukin-6 (A), Tumor necrosis factor- α (B), Interleukin-8 (C), Tissue factor (D), and L-selectin (E). Data represents unstimulated controls (white bars), stimulated PMN with APC (shades of grey bars). Experiments were run in triplicate (n=1). Data sugggests that APC stimulates PMN gene expression.

Chapter 3. *Staphylococcus aureus* hemolysins dictate bovine polymorphonuclear leukocyte signaling pathways and immune responses

Running title: Neutrophil modulation by *S. aureus* hemolysins

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Abstract:

Staphylococcus aureus is a major cause of subclinical and chronic mastitis. *S. aureus* virulence factors such as hemolysins may impair host inflammatory responses and contribute to enhanced pathogen survival through immune evasion. Polymorphonuclear leukocytes (PMN) are the host's primary defense in the mammary gland during infection. The objective of this study was to determine the role of *S. aureus* hemolysins in PMN immune responses during infection. We hypothesized that *S. aureus agr*- regulated virulence factors including α- and δ-hemolysins induce inflammatory signaling and chemotactic responses in PMN. Bovine PMN were infected with live or irradiated *S. aureus* wildtype, agr-knockout (–KO), α-KO, δ-KO, and α/δ-KO. Lysates were collected for western blot analysis of signaling proteins and quantitative Real Time PCR analysis of chemokine gene expression. Results indicate that live *S. aureus* strains inducephosphorylation of PMN signaling proteins whereas irradiated strains do not, suggesting that secreted toxins and not bacterial-membrane proteins induce signaling activity in PMN. Live *S. aureus* wildtype or δ-KO induced JNK, ERK, MAPK p38 phosphorylation in infected PMN. Live α-KO did not induceERK phosphorylation and suppressed IL-8 gene expression in infected PMN. Live agr-KO or α/δ-KO did not induce MAPK p38 phosphorylation in infected PMN. Data suggests that expressed *agr*-regulated hemolysins dictate PMN signaling activity and impact chemokine production during *S. aureus* infections. Also, suggests a role for *S. aureus* α -hemolysin in activating the ERK signaling pathway and IL-8 gene expression. Future research will identify the transcription factors downstream of ERK in infected PMN.

Introduction:

Staphylococcus aureus is a major cause of nosocomial- and community- acquired infections including subclinical and chronic mastitis. This gram positive pathogen expresses a repertoire of virulence factors that contribute to enhanced pathogen survival through immune evasion and tissue destruction in the host. *S. aureus* hemolysins and additional secreted virulence factors are regulated in part by the *accessory gene regulator* (*agr*). Virulence genes regulated by *agr* are expressed during the exponential stage of growth of *S. aureus* (16). During the exponential growth phase, this quorum sensing mechanism is initiated and causes the up-regulation of secreted virulence factors and the down-regulation of cell-membrane virulence factors (22, 24). Secreted virulence factors such as hemolysins induce immune responses in human monocytes (8) and murine neutrophils (17). Therefore, hemolysins may be responsible for inducing inflammatory responses in leukocytes.

Polymorphonuclear leukocytes (PMN) are the host's primary defense in the mammary gland during infection. However, the role of hemolysins on PMN immune responses during mastitis has not been documented. *S. aureus* hemolysins may contribute to the establishment of infection by impairing host inflammatory responses during infection. We hypothesized that *S. aureus agr*- regulated virulence factors such as α- and δ-hemolysins induce PMN inflammatory signaling and chemotactic responses. This research will contribute to the understanding of host inflammation responses to *S. aureus* infections and identify hemolysin- induced signaling pathways in the host.

Materials and methods

Bacterial strains:

S. aureus strains were obtained from Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) unless otherwise noted. Strains included RN6390B (wildtype, *agr* positive) and RN4220 (referred to as agr-KO in text, is an *agr*A mutant which fails to translate α- and δ-hemolysin (21)). RN6390B hemolysin knockout strains were a generous gift from Dr. Phil Hill (University of Nottingham, UK). Hemolysin knockout strains include RN6390B_hla (hemolysin α-knockout), RN6390B_hld (hemolysin δ-knockout), RN6390B_hlahld (hemolysin α and δ-knockout) (Supplementary Figure 3.1). Knockout strains were created by allele replacement with an erythromycin cassette.

Frozen (-80°C) bacterial stocks were used to streak for isolation (single colony forming units (CFU)) on bovine blood agar plates. Bacterial CFU were allowed to grow on agar plates for 24h at 37°C. One colony from the agar plate was inoculated into 20mL of Tryptic Soy Broth (TSB) and allowed to grow overnight at 37°C, 180 revolutions / min, 5% CO_2 (12400 Incubator shaker, New Brunswick Scientific, Edison, NJ). Cultures were centrifuged at 850 x g, 4°C, for 10 min. Supernatants were discarded and pellets resuspended in 20mL of Dulbecco's Phosphate Buffered Saline (PBS) (Invitrogen, Carlsbad, CA). Suspensions were centrifuged at 850 x g, 4°C, for 10 min. Supernatants were discarded and pellets resuspended in PBS for 10^9 CFU / mL. Cultures were drop plated to confirm CFU / mL for infection the next day. Aliquots of bacterial suspensions were taken for Cobalt 60 irradiation for 4h (1400 R/ min) (JL Shepherd Model 109 Irradiator, Alta Loma, CA). Bacterial cultures were kept in the fridge overnight.

FITC staining bacterial strains:

Live and irradiated bacterial cultures (10^9 CFU / mL) were resuspended in carbonate bicarbonate buffer (Sigma Aldrich, St. Louis, MO) and FITC was added to the culture (100µg / mL) and allowed to incubate at room temperature for 1h. Cultures was centrifuged at 850 x g for 10 min, 4°C and washed in PBS containing 5mM EDTA (PBSE) (pH 7.4) (VWR, Suwanee, GA). Samples were centrifuged at 850 x g for 10 min, 4°C and pellet was resuspended in PBSE containing 10%/vol glycerol. Aliquots were frozen in -20°C.

Bovine PMN isolation:

All procedures were approved and carried out in accordance with the Institutional Animal Care and Use Committee of Virginia Tech. Blood was collected from the jugular vein of multiparous, non-mastitic, mid-lactation cattle of the Virginia Tech Dairy herd into 10%/vol PBS containing 40mM EDTA (pH 7.4) (VWR, Suwanee, GA). PMN were isolated from whole blood as previously described (15).). In brief, whole blood was centrifuged at 350 x g for 30 min, 15°C to separate plasma from the lymphocyte layer and red blood cells (RBC) and PMN layer. Plasma and lymphocytes were discarded. RBC were lysed with water. Minimum Essential Media (MEM) (3X, added 33%/vol) (Invitrogen; Carlsbad, CA) was added t adjust the osmolarity, and samples were brought up to 45mL with PBSE. Samples were centrifuged at 350 x g for 5 min, 15°C. Supernatants were discarded and pellets were resuspended in PBSE and centrifuged at 350 x g for 5 min, 15°C. Supernatants were discarded and if RBC were still present in the pellet, a second lysis was applied. PMN were resuspended to $2x10^7$ / mL in phenol red free Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen; Carlsbad, CA) containing 10% FBS (Atlanta Biologicals Lawrenceville, GA) and 1% L-glutamine (Invitrogen; Carlsbad, CA).

Infection and stimulation:

One milliliter of bacterial suspension ($2x10^8$ CFU / mL) was added to 1 mL PMN suspensions ($2x10^7$ PMN / mL) (multiplicity of infection (MOI) 10) with live or irradiated *S. aureus* wildtype, agr-KO, α- KO, δ-KO, or α/δ- KO for 30 min at 37°C, 5% CO_2. Infection samples were centrifuged at 100 x g for 2 min, 4°C. Supernatants were collected for Nitrite/Nitrate concentration analysis and pellets were resuspended in ice-cold PBS to wash the pellets. Infection samples were centrifuged at 100 x g for 2 min, 4°C. PBS supernatant was discarded and pellets were lysed with western lysis buffer or TriZol (Invitrogen; Carlsbad, CA).

The PMN responses to recombinant *S. aureus* α-hemolysin (List Biological Laboratories, Inc., Campbell, CA) were first tested by stimulating PMN with increasing concentrations of recombinant α-hemolysin (0.03μg-2μg) for 30 min at 37°C, 5% CO_2. Lysates were collected for western blot analysis of phosphorylated- and total ERK (Supplementary Figure 3.2). For α-hemolysin add-back stimulations, PMN were infected (MOI 10) with *S. aureus* wildtype, α- KO, α-hemolysin (0.5μg) alone, or α- KO with α-hemolysin (0.5μg) for 30 min at 37°C, 5% CO_2. Lysates were collected western blot analysis.

Western blot analysis:

Supernatants were discarded and pellets were resuspended in ice-cold PBS solution containing 1mM sodium fluoride (NaF, VWR, Suwanee, GA) and 10μM sodium orthovanadate (Na_3VO_4, Sigma Aldrich, St. Louis, MO). Cells were lysed in western lysis buffer (1M Tris-HCl (VWR, Suwanee, GA), 0.5M EDTA (VWR, Suwanee, GA), IM NaCl (Fisher Scientific, Suwanee, GA), NP-40 (1%/vol; USB Corporation, Cleveland, OH), deoxycholate (0.5%/vol; Sigma Aldrich, St. Louis, MO), glycerol (5%/vol; Fisher Scientific, Suwanee, GA), and

protease/phosphatase inhibitor cocktail (1%/vol; Fisher Scientific, Suwanee, GA). Bradford analyses were conducted to quantify protein concentration per µL of sample. Standard curves were designed using bovine serum albumin (BSA) (starting with 6µg/µL) and the lysis buffer as diluent. Five microliters of standard or sample were added to wells in 96-well plate (Greiner Bio-one, VWR, Suwanee, GA). Two hundred microliters of Coomasie Protein Assay Reagent (Fisher Scientific, Suwanee, GA) were added to each standard or unknown sample, mixed, and incubated at 37°C with constant agitation for 30min. Standard and sample optical density was read on plate reader ,µQuant (VWR, Suwanee, GA), at 562nm. Concentrations (µg / µL) of unknown samples were determined using the standard curve.

Thirty micrograms of sample with 1x laemmli buffer (40%/vol glycerol (Fisher Scientific, Suwanee, GA), 12%/vol SDS (Fisher Scientific, Suwanee, GA), 250nM Tris (VWR, Suwanee, GA), 1% β-mercaptoethanol (Sigma Aldrich, St. Louis, MO), 0.01% bromophenol blue (Sigma Aldrich, St. Louis, MO), pH 6.8) were boiled for 5 minutes at 95°C. Samples were loaded on an SDS-PAGE gradient gel (Invitrogen; Carlsbad, CA) at 125 volts for 90 min. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane for 90 min at 30 volts. Membranes were blocked in 3% BSA-TBST for 1h. Membranes were incubated with primary antibody overnight at 4°C. (Primary antibodies included phospho- and total MAPK p38 (Cell Signaling, Danvers, MA), phospho- and total ERK (Cell Signaling, Danvers, MA), phospho- and total JNK (Cell Signaling, Danvers, MA), and phospho- MKP-1 (Cell Signaling, Danvers, MA)). Secondary antibodies were incubated with membranes for 1h at room temperature. (Secondary antibodies included anti-rabbit (Fisher Scientific, Suwanee, GA) and anti-mouse (Santa Cruz Biotechnology, Santa Cruz, CA) conjugated antibodies with horse radish peroxidase). Membranes were developed using ECL (General Electric Healthcare, UK) as indicated by

manufacturer. ChemiDoc XRS (BioRad, Hercules, CA) was set to chemi hi-sensitivity and allowed to develop for 10 min, capturing images every 30 sec. Images were processed by Quantity One 4.6.7 (BioRad, Hercules, CA).

Reverse Transcriptase Real-Time -PCR for mRNA Quantification (qRT-PCR):

Total RNA was isolated from samples using TriZol Reagent as indicated by manufacturer (Invitrogen; Carlsbad, CA). Complimentary DNA (cDNA) was synthesized from sample mRNA (2.2µg) by heating sample at 70°C for 10min with 250ng of random primers (Invitrogen, Carlsbad, CA). Samples were cooled to 4°C and centrifuged at 350 x g for 30 sec, 4°C. Samples were brought to 19.5µL with a solution containing 20.5%/vol Buffer, 10%/vol Dithiothreitol (DTT), 5%/vol deoxyribonucleotides (DNTPs) (VWR, Suwanee, GA) , and 2.5%/vol Superscipt II (Reverse transcriptase). All reagents were purchased from Invitrogen (Carlsbad, CA) unless indicated otherwise. Samples were incubated at 42°C for 50 min. Samples were incubated at 70°C for 15 min to de-activate the reverse transcriptase. Samples were brought up to 10ng/µL of cDNA with DNase/RNase free water (Qiagen; Valencia, CA).

Primers were purchased from Applied Biosystems (Table 3.2). The TaqMan® Universal PCR Master Mix (Applied Biosystems; Foster City, CA) and the ABI 7300 Real-Time PCR System (Applied Biosystems; Foster City, CA) were used for qRT-PCR analysis. The final volume for qRT-PCR analysis was 25µL containing 300nM of both forward and reverse primers (Integrated DNA Technologies; Coralville, IA), 100nM of 6-carboxyfluorescein (FAM) probe (Biosearch Technologies Inc.; Novato, CA), 2X TaqMan® Universal PCR Master Mix (Applied Biosystems; Foster City, CA), and 50ng cDNA. Samples were heated at 50°C for 2 min once,

95°C for 10min once, 95°C for 15sec for 40 cycles, and cooled at 60°C for 1 min. A compiled study of all sample cycle threshold (Ct) values were exported for analysis.

Flow cytometry and quenching of FITC by trypan blue:

Cells were infected as indicated above, but with Fluorescein isothiocyanate (FITC) (Sigma Aldrich, St. Louis, MO) - labeled live and irradiated bacterial strains. At time of collection, samples were centrifuged at100 x g, 4°C, for 2 min. Supernatants were discarded and PMN pellets were resuspended in 1mL of Hank's Balanced Salt Solution (HBSS) (Atlanta Biologicals Lawrenceville, GA). Samples were centrifuged at100 x g, 4°C, for 2 min. Supernatants were discarded and pellets were resuspended in HBSS. Aliquot of infection suspension containing $1x10^6$ PMN was stained with 5µl of propidium iodide (PI) (Invitrogen, Carlsbad, CA) and incubated at room temperature for 5 min. Samples were analyzed by Accuri C6 Flow cytometer (Benton Dickinson, San Jose, CA) to detect FITC and PI. After the first reading, trypan blue (1X, added 50%/vol) (Sigma, St. Louis, MO) was added to each sample to quench extracellular FITC- *S. aureus* (Supplementary Figure 3.1).

Nitrite / Nitrate concentration assay:

Supernatants collected from PMN infections with live and irradiated *S. aureus* were frozen at -20°C until sample analysis. Samples were analyzed using Nitrate/nitrite assay kit colorimetric (Sigma, St. Louis, MO) as instructed by manufacturer. In brief, standard curves were developed using $NaNO_2$ or $NaNO_3$ standards and buffer solution provided by the company. Eighty microliters of sample were added to 96-well plate (Greiner Bio-one, VWR, Suwanee, GA) and brought up to 100 µL with buffer solution (supplied by manufacturer). For $NaNO_2$

analysis, samples were incubated for 5 min with 50 μL of Griess Reagent A. Then incubated for 10 min with 50 μL of Griess Reagent B and developed. For $NaNO_3$ analysis, samples were incubated with 10 μL of Nitrate reductase and 10 μL of Enzyme Co-factors (supplied by manufacturer) and incubated at room temperature for 2h. Samples were incubated for 5 min with 50 μL of Griess Reagent A. Then incubated for 10 min with 50 μL of Griess Reagent B and developed.

Standard and sample optical density was read on plate reader, μQuant (VWR, Suwanee, GA), at 555nm (as determined by preliminary studies, data not shown). Sample $NaNO_2$ or $NaNO_3$ concentrations were calculated using standard curves.

Statistical analysis:

Data was analyzed and graphed using GraphPad Prism Version 4.03 (La Jolla, CA). PMN gene expression from qRT-PCR analysis was compiled from four experiments. Sample Ct values were compared to the Ct value of a stable housekeeping gene, β-actin, as determined by preliminary studies (data not shown). The difference in Ct values (ΔCt) were compared to unstimulated PMN ΔCt values (ΔΔCt) of those sample groups. This difference was calculated for fold induction ($2^{-\Delta\Delta Ct}$). Statistical analysis was conducted on samples ΔΔCt values. Significance was determined using one-way ANOVA with a Tukey's post test. Significance was indicated at $P<0.01$.

Pathogen uptake by PMN data (flow cytometry) data was processed by FlowJo (Tree Star, Inc.) and population percentages were analyzed using one-way ANOVA with a Tukey's post test.

Results

S. aureus secreted virulence factors induce PMN stress signaling phosphorylation.

Primary PMN were isolated and infected at MOI 10 for 30 min with live or irradiated *S. aureus* strains. Results indicate that live *S. aureus* strains induce JNK, ERK, and MAPK p38 phosphorylation by PMN, whereas irradiated *S. aureus* strains do not (Table 3.3). This data suggests PMN signaling responses are due to virulence factor expression and not bacterial-membrane bound proteins. Live *S. aureus* wildtype and δ-KO induce JNK, ERK, and MAPK p38 phosphorylation by PMN (Table 3.3). Live agr-KO strains induced JNK phophorylation by PMN, but to a lesser extent ERK and MAPK p38. Infections with α-KO induced JNK and MAPK p38 phosphorylation by PMN. Interestingly, live α-KO strains did not induce ERK phosphorylation by PMN (Table 3.3), suggesting a role for α-hemolysin in activating ERK signaling pathways in the host during *S. aureus* infections. α/δ-KO infections induced JNK and ERK phosphorylation, but to a lesser extent MAPK p38 phosphorylation by PMN (Table 3.3). Collectively, these results suggest a role for *agr*-regulated virulence factors in phosphorylating host signaling pathways during infection.

S. aureus secreted virulence factors differentially regulate chemokine gene expression by PMN.

Signaling protein phosphorylation leads to transcription factor activation and cytokine and chemokine transcription. Investigating downstream activity of these signaling proteins is necessary to define PMN immune responses to *S. aureus* virulence factors. Primary PMN were infected for 30 min with live or irradiated *S. aureus* strains. Lysates were collected for gene

expression analysis of IL-8, IL-6, and TNF-α (Figure 3.1). Only IL-8 gene expression by PMN indicated differential regulation between the strains (Figure 3.1A). Live wildtype *S. aureus* increased expression of IL-8 by PMN significantly more than irradiated wildtype ($P<0.05$). Live α-KO significantly suppressed IL-8 gene expression in PMN as compared to all treatments ($P<0.01$). Live agr-KO seemed to induce IL-8 expression in PMN as compared to irradiated agr-KO, however due to variation in the data and potential animal effect statistical differences could not be determined. Collectively, this data suggests a role for secreted virulence factors in PMN responses. Importantly, live α-KO suppressed IL-8 expression by infected PMN as compared to all live and irradiated strains, suggesting an essential role for α-hemolysin in increasing expression of IL-8 by infected PMN. Live or irradiated δ-KO or α/δ-KO did not indicate significant differences.

***S. aureus* can survive intracellularly in PMN.**

Measuring phagocytosis capability in our model is an indicator of PMN responses to *S. aureus* virulence factors. PMN were infected with FITC-labeled live or irradiated *S. aureus* strains for 30 min and samples were collected for pathogen uptake analysis. Because bacteria can adhere to surface of PMN during infection and be read by the flow cytometer as uptaken bacteria, trypan blue was added to each sample to quench extracellular bacteria and only account for intracellular-fluorescent bacteria (Supplementary Figure 3.1). Results comparing only intracellular-fluorescent bacteria indicated that all strains could intracellularly survive within PMN. Live and irradiated agr-KO *S. aureus* seemed to be phagocytized less by PMN as compared to live or irradiated wildtype, irradiated α-KO, and δ-KO infections, but due to the variation in our data statistical analyses did not indicate differences (Figure 3.2).

***S. aureus* induces nitrite/nitrate production by infected PMN.**

The phagocytic process induces the fusion of the host phagosome and lysosome, which incorporates digestive enzymes and reactive oxygen species to destroy pathogen. Since Figure 3.2 indicated pathogen uptake by PMN, the presence of reactive oxygen species such as nitric oxide (NO) are an indicator of efficient pathogen clearance responses by PMN. Therefore, supernatants collected from PMN infections with live or irradiated *S. aureus* wildtype, agr-KO, α-KO, δ-KO, and α/δ-KO strains were analyzed for nitrite and nitrate concentrations (Figure 3.3). There was a lot of variation in our data and limited reagents to run the assay. Result trends suggest that PMN produced more nitrate than nitrite during infections. Irradiated α/δ-KO infections seemed to induce greater nitrate and nitrite production than our standard curves, therefore we could not assess the concentration of this sample. Similarly, live α-KO or irradiated δ-KO infections induced greater nitrate production as compared to our standard and we could not assess the concentrations of these samples.

***S. aureus* α- hemolysin induces ERK phosphorylation by PMN.**

Though there were no reportable differences in nitrite/ nitrate concentrations between infected PMN with *S. aureus*, Table 3.3 and Figure 3.1 indicated a potential role for α-hemolysin in ERK phosphorylation and IL-8 induction by infected PMN. Stimulation of PMN with α-hemolysin (0.5μg) for 30 min induced ERK phosphorylation in PMN (Supplementary Figure 3.2). Therefore, to test if ERK phosphorylation would be restored with the addition of α-hemolysin during infection with *S. aureus* α-KO, PMN were infected with live wildtype, α-KO, α-hemolysin (0.5μg), and α-KO with α-hemolysin (0.5μg) (Figure 3.4A) for 30 min. Lysates were collected and ERK phosphorylation analyzed. Alpha-hemolysin alone did not induce ERK phosphorylation by stimulated PMN (Figure 3.4A). However α-hemolysin (0.5μg) and α-KO

infection induceERK phosphorylation in PMN (Figure 3.4A and C). This effect may be animal-dependent due to our results from Figure 3.4B. PMN isolated from a different cow were infected with α-KO and stimulated with α-hemolysin (0.5μg), but ERK was not phosphorylated in PMN. Therefore, more experiments need to be conducted in order to conclude that α-hemolysin activates the ERK signaling pathway during *S. aureus* infection.

Discussion

S. aureus virulence factors play an important role in establishment of infection and innate immune evasion. This study focused on defining the roles of *S. aureus* hemolysins in PMN signaling pathways, chemokine gene expression, and pathogen uptake. Our data indicated a role for secreted virulence factors on activation of PMN stress signaling pathways. This was evident by the lack of signaling protein phosphorylation by PMN infected with irradiated *S. aureus* strains (Table 3.2). Research has focused on the effect of *S. aureus* hemolysins on pulmonary epithelial cell and monocyte signaling pathways (3, 8, 19). Wildtype *S. aureus* induces calcium production by pulmonary epithelial cells, which induce ERK and MAPK p38 phosphorylation, activate NF-κB, and increase IL-8 production (19). However, when the same infections are conducted with agr-KO *S. aureus* strains, this immune response is lost. This data agrees with our findings (Table 3.3) which indicate that live wildtype *S. aureus* induces phosphorylation of JNK, ERK, and MAPK p38. When infections were conducted with agr-KO, infection-induced ERK and MAPK p38 phosphorylation was not consistent in PMN, suggesting a role for *agr*-regulated virulence factors in host signaling responses. Also, live α-KO did not induce ERK phosphorylation in PMN, indicating a role for α-hemolysin in PMN immune responses to infection.

Specific hemolysins may be determinants of immune responses during infection. For example, two different pulmonary epithelial cells lines (cystic fibrosis cell line (S9) and human bronchial epithelial cells (HBE)) stimulated with recombinant α- or β-hemolysin induced IL-8 production by S9 epithelial cells in an ERK-dependent manner. Interestingly, this effect was only detected in HBE cells stimulated with β-hemolysin, indicating that immune responses to hemolysins such as α- and β-hemolysins may depend on the cell type and stimulant. Dragneva et

al. (2001) indicated that α-hemolysin induced IL-8 production by human monocytes in a NF-κB-dependent manner. These data agree with our findings that *S. aureus* hemolysins are responsible for activation of signaling pathways in the host. Also, that α-hemolysin is responsible for ERK phosphorylation and IL-8 gene expression in leukocytes including our bovine PMN model (Table 3.3 and Figure 3.1A).

S. aureus agr-regulated virulence factors may be important in pathogen uptake and cytokine production by PMN. Leukocytes, such as macrophages, can phagocytize unopsinized *S. aureus* through induction of signaling processes (7). Furthermore, MAPK p38 is important in leukocyte phagocystosis of heat-killed *S. aureus* and subsequent TNF responses (13). When investigating the uptake of *S. aureus* strains by bovine PMN, our data indicated that our strains (unopsonized) were phagocytized by PMN (Figure 3.3). Together with our results from Table 3.3, agr-KO and α/δ-KO infections did not induce MAPK p38 phosphorylation by PMN, a mechanism that may inhibit PMN phagocytic and cytokine responses (13). Although there was no statistical significance, agr-KO uptake by PMN seemed to be less than the other strains and the lack of MAPK p38 activity may help explain this phenomenon. Also, due to a mutation in the agr-KO, α- and δ- hemolysins cannot be transcribed (11, 21), therefore the lack of MAPK p38 signaling for both agr-KO and α/δ-KO helps confirm the importance of *agr*-regulated genes in the MAPK p38 signaling pathway. Importantly, *S. aureus* strains expressing *agr*-regulated virulence factors can evade phagosome destruction by PMN, a mechanism reported in cystic fibrosis cell lines (CFT-1) (12), hepatic cell lines (9), and mammary epithelial cells lines (18). Our data also indicated that irradiated *S. aureus* strains were still detected in infected PMN under the same infection conditions, which may suggest an important role for membrane-expressed virulence factors on inhibiting pathogen destruction by PMN (10).

Our model suggested a potential role for α-hemolysin in induction of ERK phosphorylation and IL-8 gene expression by PMN. The specificity of immune responses induced by α-hemolysin has been described in several other cell models (3, 8). Therefore, future research needs to focus on defining the signaling pathways, transcription factor activation, and cytokine production by bovine PMN in response to α-hemolysin stimulation. Determining the unique roles of virulence factors on immune responses may give insight to diagnosis (2) and potential outcome of infection. ERK and MAPK p38 signaling pathways have several upstream activators, however their downstream activities are very specific. Due to the complexity of signaling pathways and the roles ERK and MAPK p38 play in several stress and metabolic responses in mammalian cells (20), therapeutic targeting for inhibition of these pathways is not an ideal route for treatment. Instead, identifying the immune responses downstream of the signaling pathways would be more promising. MAPK p38 activity has been associated with TNF and IL-1β gene expression (1). ERK1/2 activity not only can induce NF-κB-dependent IL-8 transcription (8), but also has specificity to phosphorylate MKP-1 which can inhibit the activity of several other pathways (4-6, 14, 23).

Overall, this research confirms the importance of secreted virulence factors in PMN responses and the importance of *agr*-regulated virulence factors in evading immune responses. Due to subtle immune responses associated with *S. aureus* infections, it is imperative to characterize the immune responses to particular virulence factors for efficient therapeutic remedies.

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Tables and Figures

Table 3.1 *S. aureus* hemolysin-mutant strains used in experiments.		
Strain name	**Genotype**	**Source**
Wildtype (RN6390B)	agr +	NARSA
agr KO (RN4220)	agr A mutant	NARSA, (21)
α- KO (RN6390B_hla)	Hemolysin-α knockout	Dr. Hill, UK (unpublished data)
δ-KO (RN6390B_hld)	Hemolysin-δ knockout	Dr. Hill, UK (unpublished data)
α/δ- KO (RN6390B_hlahld)	Hemolysins-α and -δ knockout	Dr. Hill, UK (unpublished data)

Table 3.2. Bovine primer sequences used for quantitative Real Time PCR (5'→3')

Gene Name	Primer	Sequence
β-actin	Forward	CAGCAAGCAGGAGTACGATGAGT
	Reverse	AAGGGTGTAACGCAGCTAACAGT
	Probe	TCCATCGTCCACCGCAAATGCTTCTA
Interleukin-6	Forward	CCAGAGAAAACCGAAGCTCTCA
	Reverse	CTCATCATTCTTCTCACATATCTCCTTT
	Probe	AGCGCATGGTCGACAAAATCTCTGC
Tumor Necrosis Factor-α	Forward	TCTCCTTCCTCCTGGTTGCA
	Reverse	GTTTGAACCAGAGGGCTGTTG
	Probe	CCCAGAGGGAAGAGCAGTCCCCA
Interleukin-8	Forward	GAGTGGGCCACACTGTGAAA
	Reverse	TGCTTCTCAGCTCTCTTCACAAA
	Probe	AAACGAGGTCTGCTTAAACCCCAAGGAAA

Strain		p-JNK	p-ERK	p-p38
Wildtype	Live	+++++++	+++++ - +	0 ++++++
	Irradiated	- - - - - - -	- - - - ++ -	0 - - - - + -
agr-KO	Live	++ - - +++	+ - + - + + -	0 + - - - + -
	Irradiated	- - - - - - -	- - - - - - -	0 + - - - + -
α- KO	Live	+++++++	- - - + - - -	0 ++++ - -
	Irradiated	- - ++ - - -	- - - +++ -	0 + - + - - -
δ-KO	Live	+++++++	+++++++	0 ++ - - ++
	Irradiated	- - ++ - - -	- - + - + - -	0 - + - + - -
α/δ-KO	Live	0 ++++++	0 + - + + + +	0 + - - + - -
	Irradiated	0 - + - - + - -	0 - - - + - -	0 + - - + - -

Table 3.3. Phosphorylated signaling pathways by PMN infected with live or irradiated *S. aureus* strains. Bovine PMN were infected with live or irradiated *S. aureus* wildtype, agr-KO, α-KO, δ-KO, and α/δ-KO strains for 30 min (MOI 10). Lysates were collected for western blot analysis of signaling protein phosphorylation of JNK, ERK, or MAPK p38. Data is representative of seven experiments in individual cows (n=7). All experiment treatments were run in triplicate. Each plus sign (+) indicates an experiment that indicated phosphorylation of the signaling protein. Each minus sign (-) indicates an experiment that indicated no phosphorylation of the signaling protein. Each zero (0) means that strain or protein was not run for that experiment.

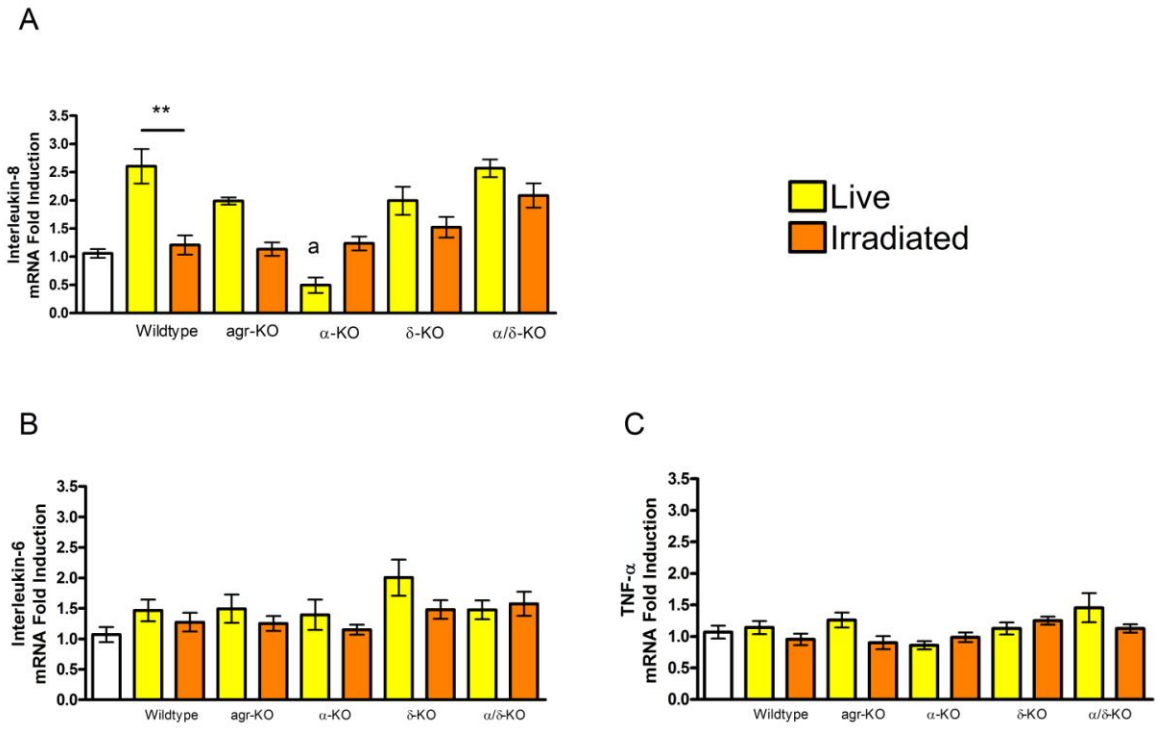

Figure 3.1. PMN gene expression profiles in response to live or irradiated *S. aureus* infections. PMN were infected with live or irradiated *S. aureus* wildtype, agr-KO, α-KO, δ-KO, and α/δ-KO strains for 30 min (MOI10). Lysates were collected for gene expression analysis of (A) Interlekin-8 (IL-8), (B) Interleukin-6 (IL-6), and (C) Tumor necrosis factor –α (TNF-α) through quantitative Real Time-PCR. All samples were normalized to housekeeping gene, β-actin. Live *S. aureus* induction of genes by PMN (yellow bars) was compared to irradiated *S. aureus* induction of genes by PMN (orange bars). Data represents four separate experiments on different cows (n=4). Data was analyzed using a one-way ANOVA with a Tukey's post test. Significance is indicated at **P<0.05 and letter (a) indicates significance as compared to all treatments. Due to an animal effect and variation in our data, only significant differences in IL-8 gene expression were reported for wildtype *S. aureus* (live vs. irradiated) and α-KO (compared to all treatments). IL-6 (B) and TNF-α (C) did not indicate significance between treatments.

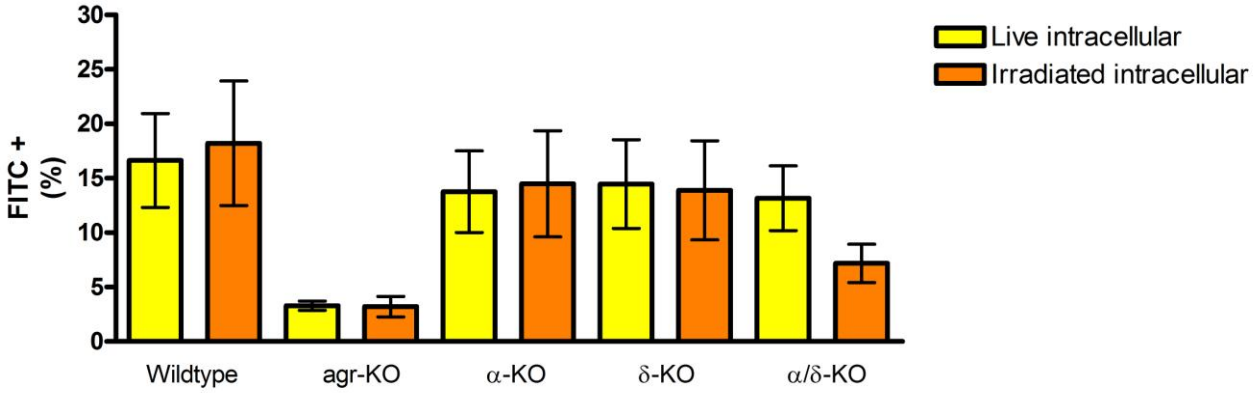

Figure 3.2. Intracellular survival of *S. aureus* strains in PMN during infection. Bovine PMN were infected with FITC-labeled live or irradiated *S. aureus* wildtype, agr-KO, α-KO, δ-KO, and α/δ-KO strains for 30min and collected for flow cytometry analysis of bacteria uptake by PMN. To differentiate between viable extracellular and intracellular bacteria, trypan blue was added to samples to quench FITC-labeled bacterial signal (See Supplementary Figure 3.1). Percentage of live FITC-labeled bacteria uptaken by PMN (yellow bars) was compared to percentage of irradiated FITC-labeled bacteria uptaken by PMN (orange bars). Data represents percentage of FITC-labeled bacteria present in our sample. Data is representative of three individual experiments conducted on PMN isolated from different cows (n=3). Data was analyzed using a one-way ANOVA with a Tukey's post test. No significant differences were reported because of variation in the data due to an effect of day and animal.

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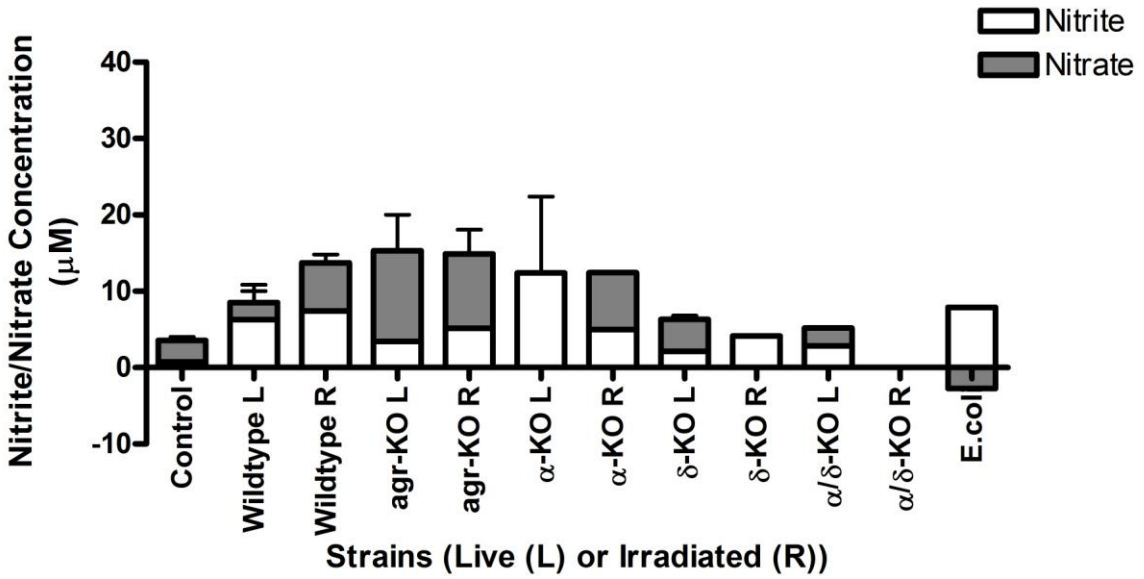


Figure 3.3. Nitrite/nitrate concentrations in infection supernatants. PMN were infected with live or irradiated *S. aureus* wildtype, agr-KO, α-KO, δ-KO, or α/δ-KO strains for 30 min (MOI 10). Samples were centrifuged (100 x g, 2 min, 4°C) to separate cells from supernatants. Supernatants were collected for nitrite/nitrate concentration analysis as a determinant of digestion of *S. aureus* by PMN. Using standard curves, nitrite (white bars) and nitrate (grey bars) concentrations (μM) were calculated. Experiments were run on supernatants collected from two separate PMN infection experiments (n=2). Due to variation in the data and limited reagents for the assay, only two experiments were conducted and no statistical analysis could be performed.

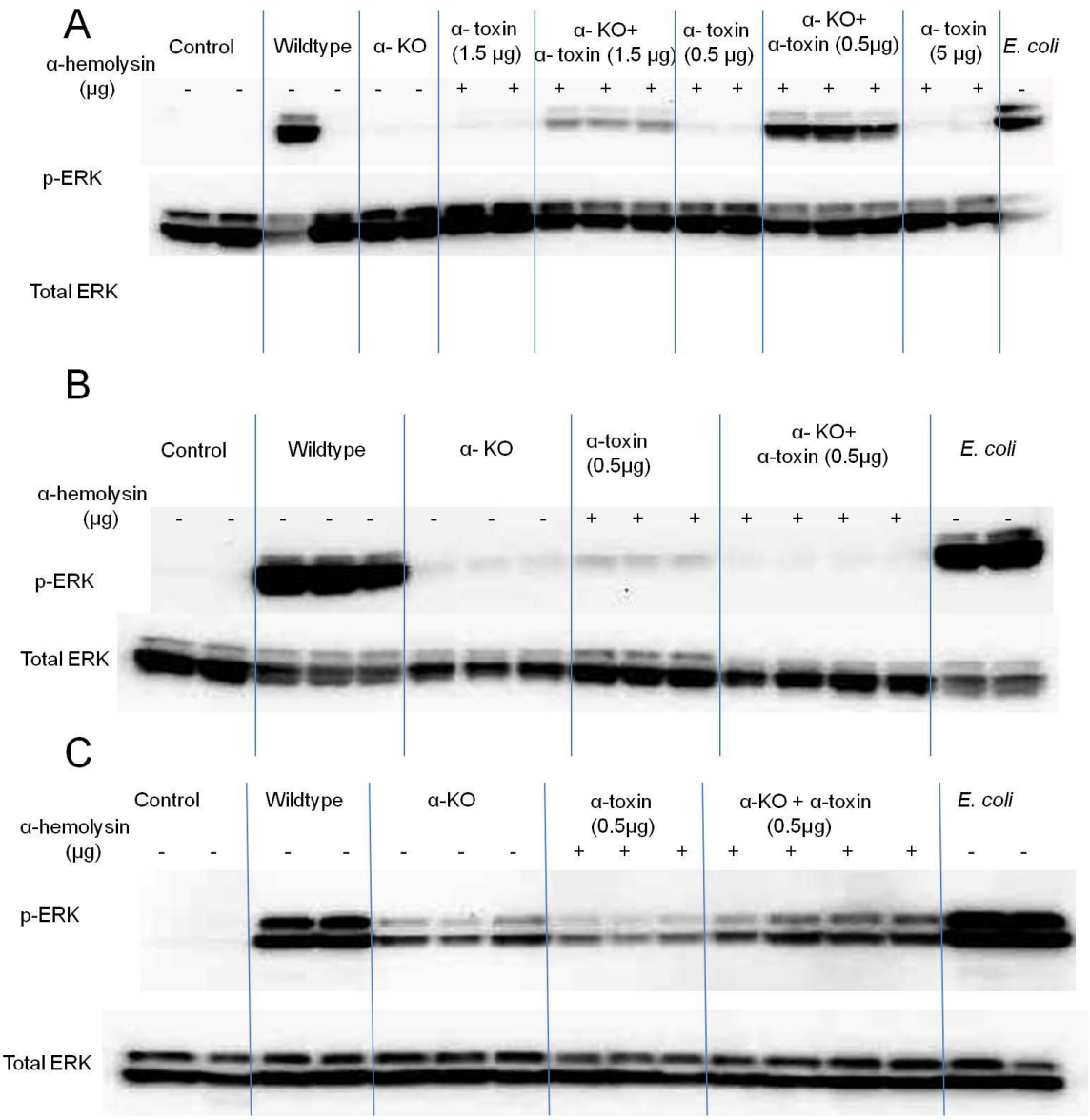
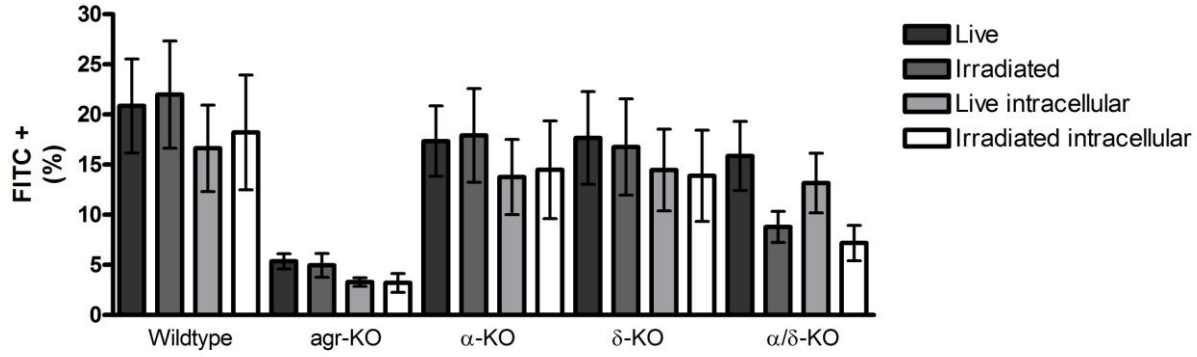
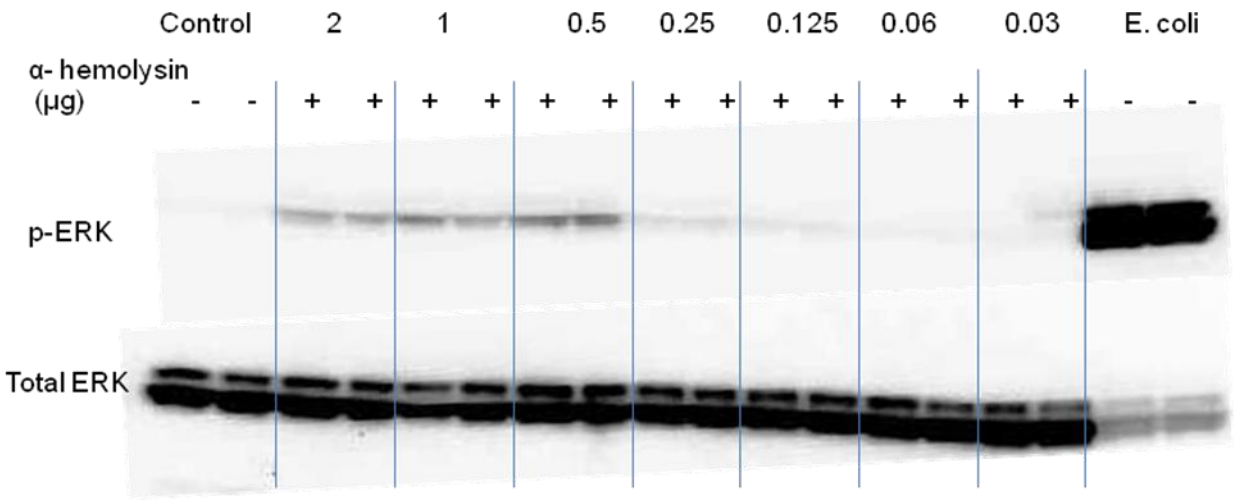


Figure 3.4. Restoration of ERK phosphorylation in PMN during *S. aureus* infections. PMN were infected with live wildtype, α-KO, α-KO including α-hemolysin stimulation (1.5-0.5μg), and stimulated with α-hemolysin alone (1.5-0.5μg) for 30 min. Lysates were collected for western blot analysis of ERK phosphorylation. Membranes were blotted with phosphorylated ERK and total ERK antibodies. Data represents ERK phosphorylation when infected with *S. aureus* wildtype, α-KO, α-hemolysin alone (1.5-0.5μg), α-KO including α-hemolysin stimulation

(1.5-0.5μg), and *E. coli* (A). Data also represents a repetition of assay on PMN isolated from a different cow and infected with *S. aureus* wildtype, α-KO, α-hemolysin alone (0.5μg), α-KO including α-hemolysin stimulation (0.5μg), and *E. coli* (B). Data also represents a repetition of assay on PMN isolated from a different cow and infected with *S. aureus* wildtype, α-KO, α-hemolysin alone (0.5μg), α-KO including α-hemolysin stimulation (0.5μg), and *E. coli* (C).



Supplementary Figure 3.1. Intracellular survival of *S. aureus* strains in PMN during infection. PMN were infected with FITC-labeled live or irradiated *S. aureus* strains for 30min and collected for flow cytometry analysis of bacteria uptake by PMN. To differentiate between viable extracellular and intracellular bacteria, trypan blue was added to samples to quench FITC-labeled bacterial signal. Data represents percentage of FITC-labeled bacteria in our sample. Live (black bars) and irradiated (light grey bars) represent samples prior to trypan blue addition and Live intracellular (grey bars) and Irradiated intracellular (white bars) represent samples that received trypan blue treatment. Data is representative of three individual experiments conducted on PMN isolated from different cows (n=3). Data was analyzed by one-way ANOVA with a Tukey's post test ($P<0.01$).



Supplementary Figure 3.2. ERK phosphorylation induced in stimulated PMN with increasing concentrations of α-hemolysin. To determine the optimal concentration of α-hemolysin in inducing ERK signaling by stimulated PMN, PMN were stimulated with increasing concentrations (0.03μg-2μg) of recombinant *S. aureus* α-hemolysin for 30 min. Additional samples included unstimulated (negative control) and *E. coli* infected PMN (positive control). All treatments were run in duplicates. Lysates were collected to analyze ERK phosphorylation and total ERK through western blotting.

Chapter 4. Chemotaxis Inhibitory Protein of *Staphylococcus aureus* does not inhibit C5a signaling in THP-1 cells.

Running title: CHIPS and C5a signaling

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Abstract:

S. aureus is a major cause of nosocomial- and community- acquired infections. Genetic analyses of *S. aureus* clinical isolates indicate a prevalence of β-hemolysin converting bacteriophages. These lysogenic phages integrate human specific virulence factors that target host innate immune responses during infection. Chemotaxis inhibitory protein of *S. aureus* (CHIPS) targets the human complement response. CHIPS inhibits neutrophil and monocyte chemotactic responses to host anaphylatoxin, C5a, by binding the C5a receptor, C5aR. C5aR signaling leads to phosphorylation of ERK stress signaling pathways that result in inflammatory cytokine production, enhanced chemotaxis and pathogen destruction. We hypothesized that *S. aureus* strains expressing CHIPS suppressed host stress signaling and subsequent cytokine production during infection. To test our hypothesis, we stimulated THP-1 monocytes with recombinant C5a alone or pretreated with recombinant CHIPS and stimulated with C5a. Lysates were collected for western blot analysis of ERK phosphorylation or for quantitative Real Time PCR analysis of inflammatory gene expression. Results indicated that CHIPS did not inhibit C5a-induced ERK phosphorylation or induce inflammatory cytokine production by monocytes. Our data agrees with recent literature indicating that C5aR expresses two binding sites for C5a. CHIPS is specific for one binding site, leaving the second available for C5a binding. Therefore, C5a can still activate C5aR, induce stress signaling and cytokine production by monocytes. Future research needs to define the mechanism by which CHIPS inhibit monocyte chemotaxis towards C5a.

Introduction:

S. aureus is a major cause of severe nosocomial- and community- acquired infections. Approximately 60-90% of *S. aureus* clinical isolates carry β-hemolysin converting bacteriophages in their genome (26). These prophages interrupt the *β-hemolysin* gene and integrate four human specific virulence factors that target host innate immune responses (1, 4, 6, 7, 14, 25). *S. aureus* strains lacking β-hemolysin can survive in murine and bovine mammary epithelial cells more efficiently than strains containing β-hemolysin (16). Therefore, *S. aureus* strains containing β-hemolysin converting bacteriophages may be more virulent due to enhanced intracellular survival in host cells and expression of virulence factors such as staphylokinase, enterotoxin A, chemotaxis inhibitory protein of *S. aureus* (CHIPS), and staphylococcal complement inhibitor.

CHIPS specifically targets human complement C5a receptor, C5aR (3, 21). Anaphylatoxin C5a is responsible for leukocyte chemotaxis, downstream signaling of stress proteins for cytokine and reactive oxygen species production, and pathogen destruction (2, 9, 22, 23, 29). *S. aureus* secretion of CHIPS suppresses monocyte and neutrophil chemotactic responses (10). However, impact of CHIPS on leukocyte cell signaling pathways and cytokine production is unknown. We hypothesize that CHIPS suppresses host cellular stress signaling and subsequently suppresses cytokine production during infection, thereby enhancing pathogen survival in human monocytes.

Materials and methods:

Cell Culture:

THP-1(human monocytic cell line, ATCC #TIB-202) were grown to confluency at 37ºC, 5% CO_2. THP-1 were grown in RPMI 1640 (Sigma Aldrich, St. Louis, MO) containing heat-inactivated fetal bovine serum (FBS) (10% /vol) (Atlanta Biologicals Lawrenceville, GA), L-glutamine (1%/vol) (Invitrogen, Carlsbad, CA), antibiotic- antimycotic (1%/vol) (Invitrogen, Carlsbad, CA), and 50μM β-mercaptoethanol (Sigma Aldrich, St. Louis, MO).

For stimulations, THP-1 were seeded in 6-well tissue culture plates (Fisher Scientific, Suwanee, GA) at $1.5x10^6$ cells / well. THP-1 cells were differentiated to macrophages in THP-1 medium containing 100nM phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich, St. Louis, MO) for 24h. Cells were washed in RPMI 1640 and incubated in non-PMA containing THP-1 medium for 48 hours at 37C, 5% CO_2.

Bacterial strains:

Frozen (-80°C) bacterial stocks were used to streak for isolation (single colony forming units (CFU)) on tryptic soy agar plates. CFU were allowed to grow for 24h at 37°C. By taking one colony from the agar plate, 20mL of Tryptic Soy Broth (TSB) were inoculated and allowed to grow for 6h at 37°C, 180 RPM, 5% CO_2 (12400 Incubator shaker, New Brunswick Scientific, Edison, NJ). Cultures were centrifuged at 850 x g, 4°C, for 10 min. Supernatants were discarded and pellets resuspended in 20mL of Dulbecco's Phosphate Buffered Saline (PBS) (Invitrogen, Carlsbad, CA). Suspensions were centrifuged at 850 x g, 4°C, for 10 min. Supernatants were discarded and pellets resuspended in PBS for 10^8 CFU / mL. Cultures were drop plated to

confirm CFU / mL for infection the next day. DNA was isolated from all strains to confirm the presence or deletion of *hlb* and *chp* through PCR (data not shown).

Infection:

THP-1 monocytes were resuspended to $1.5x10^6$ cells / mL in antibiotic free cell culture medium. *S. aureus* Newman, TB1, or chp KO cultures were resuspended to $1.5x10^7$ CFU / mL and added to THP-1 suspension for 1h (MOI 10). For intracellular survival assays, after 1h infection, lysostaphin (100 μg/ml; Sigma, St. Louis, MO) was added for 7 min, 37°C to terminate the infection. Cells were washed in PBS and incubated in THP-1 medium containing gentamicin (50μg/ml; Invitrogen, Carlsbad, CA) for 2, 4, 6, 26, or 48h post infection. At time of collection, samples were rinsed with PBS and incubated in 0.5mL PBS solution containing Triton-X100 (0.05%) (Sigma Aldrich, St. Louis, MO) and EDTA (5mM) to lyse cells. Samples were sonicated for 5 min for intracellular CFU retrieval and drop plated.

Cell stimulation:

THP-1 were resuspended to $1.5x10^6$ cells / mL in cell culture medium containing 1% FBS. Samples were pretreated with recombinant CHIPS (0.1μg/mL; Hycult Biotech, Plymouth Meeting, PA) for 15 min at 37°C, or stimulated with recombinant human C5a (25ng, 50ng, or 100ng/mL; R&D Systems, Minneapolis, MN), or infected with live *S. aureus* Newman, TB1, chp KO, 8325-4, or DU5719 strains (MOI 10). At 5 min, 30 min, or 1h stimulated or infected THP-1 were lysed for signaling protein expression analysis through western blotting or for gene expression analysis through quantitative Real-Time PCR (qRT-PCR).

Western blot analysis:

For western blot analysis assays, after 1h infection cells were centrifuged at 100 x g, 2 min, 4°C. Supernatants were discarded and pellets were resuspended in ice-cold PBS solution

containing 1mM sodium fluoride (NaF, VWR, Suwanee, GA) and 10µM sodium orthovanadate (Na_3VO_4, Sigma Aldrich, St. Louis, MO). Cells were lysed in western lysis buffer (1M Tris-HCl (VWR, Suwanee, GA), 0.5M EDTA (VWR, Suwanee, GA), IM NaCl (Fisher Scientific, Suwanee, GA), NP-40 (1%/vol; USB Corporation, Cleveland, OH), deoxycholate (0.5%/vol; Sigma Aldrich, St. Louis, MO), glycerol (5%/vol; Fisher Scientific, Suwanee, GA), and protease / phosphatase inhibitor cocktail (1%/vol; Fisher Scientific, Suwanee, GA). Bradford analyses were conducted to quantify protein concentration per µL of sample. Standard curves were designed using bovine serum albumin (BSA) (starting with 8µg/µL) and the lysis buffer as diluent. Five microliters of standard or sample were added to wells in 96-well plate (Greiner Bio-one, VWR, Suwanee, GA). Two hundred microliters of Coomasie Protein Assay Reagent (Fisher Scientific, Suwanee, GA) were added to each standard or unknown sample, mixed, and incubated at 37°C with constant agitation for 30 min. Standard and sample optical density was read on plate reader ,µQuant (VWR, Suwanee, GA), at 562nm. Concentrations (µg / µL) of unknown samples were determined using the standard curve.

Thirty micrograms of sample with 1x laemmli buffer (40%/vol glycerol (Fisher Scientific, Suwanee, GA), 12%/vol SDS (Fisher Scientific, Suwanee, GA), 250nM Tris (VWR, Suwanee, GA), 1% β-mercaptoethanol, 0.01% bromophenol blue (Sigma Aldrich, St. Louis, MO), pH 6.8) were boiled for 5 min at 95°C. Samples were loaded on an SDS-PAGE gradient gel (Invitrogen; Carlsbad, CA) at 125 volts for 90 min. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane for 90 min at 30 volts. Membranes were blocked in 3% BSA-TBST for 1h. Membranes were incubated with primary antibody overnight at 4°C. Primary antibodies included phospho- and total MAPK p38 (Cell Signaling, Danvers, MA), phospho- and total ERK (Cell Signaling, Danvers, MA), phospho- and total JNK (Cell Signaling,

Danvers, MA), and phospho- MKP-1 (Cell Signaling, Danvers, MA). Secondary antibodies were incubated with membranes for 1h at room temperature. Secondary antibodies included anti-rabbit (Fisher Scientific, Suwanee, GA) and anti-mouse (Santa Cruz Biotechnology, Santa Cruz, CA) conjugated antibodies with horse radish peroxidase. Membranes were developed using ECL (General Electric Healthcare, UK) as indicated by manufacturer. ChemiDoc XRS (BioRad, Hercules, CA) was set to chemi hi-sensitivity and allowed to develop for 10 min, capturing images every 30 sec. Images were processed by Quantity One 4.6.7 (BioRad, Hercules, CA).

Reverse Transcriptase Real-Time -PCR for mRNA Quantification (qRT-PCR):

Total RNA was isolated from samples using RNeasy® Mini Kit (Qiagen, Valencia, CA) as indicated by manufacturer. Complimentary DNA (cDNA) was synthesized from sample mRNA (2.2µg) by heating sample at 70°C for 10 min with 250ng of random primers (Invitrogen ,Carlsbad, CA). Samples were cooled to 4°C and centrifuged at 350 x g for 30 sec, 4°C. Samples were brought to 19.5µL with a solution containing 20.5%/vol Buffer, 10%/ vol Dithiothreitol (DTT), 5%/vol deoxyribonucleotides (DNTPs) (VWR, Suwanee, GA) , and 2.5%/vol Superscipt II (Reverse transcriptase). All reagents were purchased from Invitrogen (Carlsbad, CA) unless indicated otherwise. Samples were incubated at 42°C for 50 min. Samples were incubated at 70°C for 15 min to de-activate the reverse transcriptase. Samples were brought up to 10ng/µL of cDNA with DNase/RNase free water (Qiagen; Valencia, CA).

Primers were purchased from Applied Biosystems (Table 4.2). The TaqMan® Universal PCR Master Mix (Applied Biosystems; Foster City, CA) and the ABI 7300 Real-Time PCR System (Applied Biosystems; Foster City, CA) were used for analysis. The final volume for qRT-PCR analysis was 25µL containing 300nM of both forward and reverse primers (Integrated DNA Technologies; Coralville, IA), 100nM of 6-carboxyfluorescein (FAM) probe (Biosearch

Technologies Inc.; Novato, CA), 2X TaqMan® Universal PCR Master Mix (Applied Biosystems; Foster City, CA), and 50ng cDNA. Samples were heated at 50°C for 2 min once, 95°C for 10 min once, 95°C for 15 sec for 40 cycles, and cooled at 60°C for 1 min. A compiled study of all sample cycle threshold (Ct) values were exported for analysis.

Staistical Analysis:

Data was analyzed and graphed using GraphPad Prism Version 4.03 (La Jolla, CA). Monocytic THP-1 mRNA expression was compiled from two stimulations. Sample Ct values were compared to the Ct value of a stable housekeeping genes, β-actin and ribosomal protein ligand 17 (RPL17) (15). The difference in Ct values (ΔCt) were compared to unstimulated THP-1 ΔCt values (ΔΔCt). This difference was calculated for fold induction ($2^{-\Delta\Delta Ct}$). Comparisons were conducted on samples ΔCt values.

Results:

Intracellular survival of *S. aureus* strains in THP-1 monocytes.

Monocytic THP-1 were intracellularly infected with *S. aureus* Newman, TB1, or *chp* KO strains for 1h. Aliquots of THP-1 samples were collected for viability analysis and remaining sample volume was collected for CFU retrieval. Results indicate that intracellular infection was established for all strains (Figure 4.1), suggesting that *S. aureus* Newman or knockout strains can survive intracellularly in THP-1 monocytes. However, there was variation in CFU retrieval, preventing the analysis of which strains survive more efficiently in THP-1 monocytes.

THP-1 monocyte viability was much lower when infected with chp KO as compared to THP-1 monocyte viability when infected with Newman or TB1. Due to the differences in THP-1 viability when infected with different *S. aureus* strains, data analyses were not possible. Observational records of infection indicate that chp KO infections activate THP-1 cells within 15 minutes of infection (as determined by monocyte binding to bottom of sample wells). Therefore, to increase consistency of THP-1 viability and CFU retrieval we may try to differentiate THP-1 to macrophages as to assure initial uniform stage of differentiation and run proteomic assays on chp KO supernatants to determine if gene deletion causes overproduction of another virulence factor, which may induce THP-1 monocyte differentiation.

Western analysis of infected THP-1 monocytes.

S. aureus Newman strain and Newman knockout strains (TB1 and chp KO) were used to study the roles of the β- hemolysin converting bacteriophage and CHIPS on ERK and MAPK p38 phosphorylation by THP-1. As a control for signaling induction due to phages, we also infected THP-1 with strain 8325-4, which lacks prophages (Table 4.1). Prior to 30 min and 1h

infections, THP-1 samples were stimulated with C5a, or pretreated with CHIPS, or pretreated with CHIPS and stimulated with C5a. *S. aureus* strains induce phosphorylation of ERK and MAPK p38 in infected THP-1, however replicates were not consistent and unstimulated controls showed signaling phosphorylation activity as well (Figure 4.2A and 4.2B). Therefore, different signaling phosphorylation patterns induced by strains could not be analyzed. Also, differences in signaling patterns could not be attributed to the presence or absence of CHIPS.

Infected THP-1 monocyte gene expression.

Due to the presence of β- hemolysin in strain 8325-4, we decided a better control for our model would be 8325-4 knockout strain, DU5719, which lacks β- hemolysin and prophages. Thus, to analyze inflammatory gene expression by THP-1 in the presence of CHIPS, THP-1 were infected with Newman, TB1, *chp* KO, or DU5719 *S. aureus* strains or pretreated with CHIPS (0.1µg) prior to infections. Lysates were collected for total RNA (Figure 4.3). Results indicated no inductions in IL-1β, IL-8, TNF-α, or RANTES gene expression by infected THP-1 monocytes. Also, there was no difference in IL-1β, IL-8, TNF-α, or RANTES gene expression by THP-1 pretreated with CHIPS and infected. Taken together with our western data analysis, if unstimulated controls were initially activated, *S. aureus* strain-induced inflammatory responses by THP-1 monocytes cannot be compared either.

Since we could not detect differences in inflammatory responses induced by *S. aureus* strains, we decided to focus on the role of CHIPS in inhibiting THP-1 monocyte responses to C5a. THP-1 were stimulated with C5a, CHIPS, or C5a following CHIPS pretreatment. Lysates were collected for inflammatory cytokine gene expression profiles after 30 min stimulations (Figure 4.4) and 1h stimulations (Figure 4.5). Experiments were only repeated twice to confirm that CHIPS pretreatment indicated an inhibitory trend in C5a-induced cytokine production in

THP-1 monocytes. Results indicated that C5a stimulation alone did not induce IL-1β, IL-8, TNF-α, or RANTES by stimulated THP-1. Also, CHIPS pretreatment and C5a stimulation did not depict a pattern of inhibition. C5a stimulation is documented to induce IL-8 and TNF-α in host cells (5, 24, 28), therefore these data indicated a problem in our model.

PMA differentiation of THP-1 and 1% FBS THP-1 medium.

In attempts to optimize our model, THP-1 stage of differentiation and FBS concentrations were considered. Activated THP-1 monocytes express C5aR (19), which led us to activate THP-1 with PMA. PMA stimulation differentiates THP-1 into macrophage-like phenotypes of the monocytes (8). Differentiated THP-1 express more C5aR (11) and may be more responsive than monocytes to our C5a model. Also, high FBS concentrations may be responsible for the lack of THP-1 responses to C5a stimulations due to greater amounts of antibodies binding or interfering with recombinant proteins, C5a and CHIPS. Tests were conducted to determine the optimal FBS (0%-10%) concentrations in the media that would allow efficient THP-1 responses to C5a (data not shown). Additional tests were conducted to determine optimal PMA concentrations (10ng or 50ng) for THP-1 differentiation and their signaling responses to recombinant C5a (Figure 4.6). PMA-differentiated THP-1 with 10ng of PMA successfully induced ERK and MAPK p38 phosphorylation in response to C5a, as expected (Figure 4.6A). In contrast, THP-1 differentiated with 50ng of PMA induced ERK phosphorylation in unstimulated controls, indicating PMA concentration may be too high and THP-1 are responding to PMA stimuli. Also, C5a stimulations did not induce MAPK p38 phosphorylation by PMA (50ng) differentiated THP-1 (Figure 4.6B), indicating that 10ng of PMA for THP-1 differentiation was optimal for our model.

PMA differentiated THP-1 and C5a stimulations.

After optimizing our model to measure THP-1 responses to C5a, the role of CHIPS in inhibiting C5a signaling was tested. THP-1 cells were differentiated with 10ng of PMA for 24h. After a 48h recovery period, cells were stimulated with increasing concentrations of C5a (50ng to 200ng) for 30 min or pretreated with CHIPS for 15 min and then stimulated with increasing concentrations of C5a for 30 min (Figure 4.7A). Only samples pretreated with CHIPS and stimulated with 50ng of C5a induced phosphorylation of MAPK p38 in THP-1(data not shown). C5a (100ng) induced phosphorylation of ERK by stimulated THP-1(Figure 4.7A). Interestingly, CHIPS pretreatment and C5a (50ng to 200ng) stimulation induced ERK phosphorylation. However, results were not reproducible. The experiment was repeated (Figure 4.7B) and C5a stimulation alone or prior CHIPS pretreatment did not induce MAPK p38 phosphorylation in THP-1(data not shown). C5a stimulation did not induce phosphorylation of ERK in THP-1. Again, CHIPS pretreatment followed by C5a stimulation induced ERK phosphorylation by THP-1.

Due to the lack of C5a-induced signaling phosphorylation, our time frame was speculated to be too long, which may allow MKP-1, a feedback mechanism (27), to phosphorylate and down-regulate ERK signaling in our model. Therefore, western membranes were probed for phosphorylated MKP-1. C5a stimulation induced phosphorylation of MKP-1 by THP-1 (Figure 4.7B). Also, CHIPS pretreatment followed by C5a stimulation only phosphorylated MKP-1 in samples stimulated with 50ng or 100ng of C5a (Figure 4.7B). These results suggested that C5a stimulation may induce phosphorylation of stress signaling proteins prior to 30 minutes. Therefore, differentiated THP-1 were stimulated with 25ng or 50ng of C5a or pretreated with CHIPS followed by C5a stimulation for 5 or 30 min (Figure 4.8). When analyzing ERK

phosphorylation by THP-1, results indicated no differences in ERK phosphorylation among C5a stimulated or CHIPS pretreated and C5a stimulated THP-1. Unfortunately, when analyzing MKP-1 phosphorylation, there did not seem to be a difference between THP-1 cells stimulated with 25ng or 50ng of C5a for 5 or 30 min or CHIPS pretreated under the same conditions (data not shown).

Discussion:

Previous research has reported that CHIPS inhibits chemotactic responses in human neutrophils and monocytes (10). However, in our THP-1 monocytic model we observed several inconsistencies. Chemotaxis inhibition of monocytes due to CHIPS pretreatment was reported on U937 monocytic cells, not THP-1. U937 cells may express more C5aR on their membranes than THP-1, making U937 a better model for studying the role of CHIPS on monocyte immune responses.

THP-1 monocytes express C5aR at the gene level (19), however at the protein level, THP-1 monocytes express low levels of C5aR as compared to PMA differentiated THP-1 (11). O'Barr et al. (2000) researched THP-1 cytokine responses to C5a stimulation and reported that pretreatment with amyloid-β was necessary for significant IL-1β and IL-6 gene expression and production by monocytes. Therefore, THP-1, as a cell line, may need prior activation in order to respond to the C5a stimulation, where as primary monocytes might not. Their report was lacking quantification of C5aR before and after amyloid-β stimulation, leaving room to speculate if the amyloid-β stimulation enhances expression of C5aR on monocytes, thereby enhancing THP-1 responses to C5a. This data agrees with our findings (Figure 4.4 and 4.5) because our stimulation with C5a did not induce inflammatory cytokine expression by THP-1 monocytes and may be due to the low levels of C5aR expression.

After optimization of our model, C5a stimulation inconsistently induced ERK phosphorylation by differentiated THP-1, an effect that was not inhibited by CHIPS pretreatment. In fact, CHIPS pretreatment followed by C5a stimulation induced ERK phosphorylation in PMA-differentiated THP-1 (Figure 4.7A-B). This data agrees with recent

findings, that C5aR expressed two binding sites for C5a. CHIPS binds specifically to residues 10-18 of the phosphorylated N-terminus of the C5aR (3, 20) and does not compete with C5a fragment 59-74, which binds to the second binding site (17). Although CHIPS is present, C5a can still activate the receptor and induce stress signaling pathways such as ERK. Overall, our data does not explain how CHIPS inhibits chemotaxis towards C5a because our model did not indicate CHIPS inhibition of C5a induced stress signaling. Future research needs to focus on the mechanism of CHIPS inhibition of neutrophil and monocyte chemotaxis towards C5a and whether or not this effect is time sensitive, as our data indicated MKP-1 activity.

Interestingly, our data indicated that CHIPS alone did not induce ERK phosphorylation in THP-1, however CHIPS pretreatment and C5a stimulation did induce ERK phosphorylation in THP-1. This information suggests a possible CHIPS:C5a complex, which may be responsible for the signaling induction seen in Figure 4.7 and 4.8. It is our understanding that no prior research has investigated the possibility of a CHIPS:C5a complex or the potential advantage for the pathogen to secrete a virulence factor that can bind host chemotactic proteins and still induce host immune responses. Previous research focuses on the binding specificity of CHIPS to host C5aR (3, 10, 12, 13, 17, 20, 21), therefore future research may also investigate CHIPS binding to C5a. Overall, our research does not corroborate with our hypothesis that CHIPS inhibits leukocyte responses to C5a through signaling, but does broaden the spectrum of questions associated with the CHIPS binding specificity.

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Tables and Figures:

Table 4.1 Names and genotypes of *S. aureus* mutant strains used for THP-1 infections.

Each minus sign (-) means the strain is deficient of the gene and each plus sign (+) means the strain expresses the gene.

***S. aureus* strain name**	**Genotype**	**Source**	**Reference**
Newman	β- hemolysin – chp + Four prophages	Olaf Schneewind	**(1)**
TB1	β- hemolysin + chp – Three prophages	Olaf Schneewind	**(1)**
chp KO	β- hemolysin – chp – Four prophages	Jos A.G. van Strijp	**(10)**
8325-4	β- hemolysin + cured of prophages	Tim Foster	**(18)**
DU5719	β- hemolysin – cured of prophages	Tim Foster	**(18)**

Table 4.2. Primer/Probe Sets for inflammatory human cytokines gene expression analysis.

Primer/ Probe Set	**Reference Number**	**Company**
Human RANTES	Hs99999048_m1	Applied Biosystems, Foster City, CA
Human Tumor Necrosis Factor-α	Hs00174128_m1	Applied Biosystems, Foster City, CA
Human RPL37	Hs02340038_g1	Applied Biosystems, Foster City, CA
Human β-actin	Hs03023943_g1	Applied Biosystems, Foster City, CA
Human IL-8	Hs00174103_m1	Applied Biosystems, Foster City, CA
Human IL-1β	Hs001742097_m1	Applied Biosystems, Foster City, CA

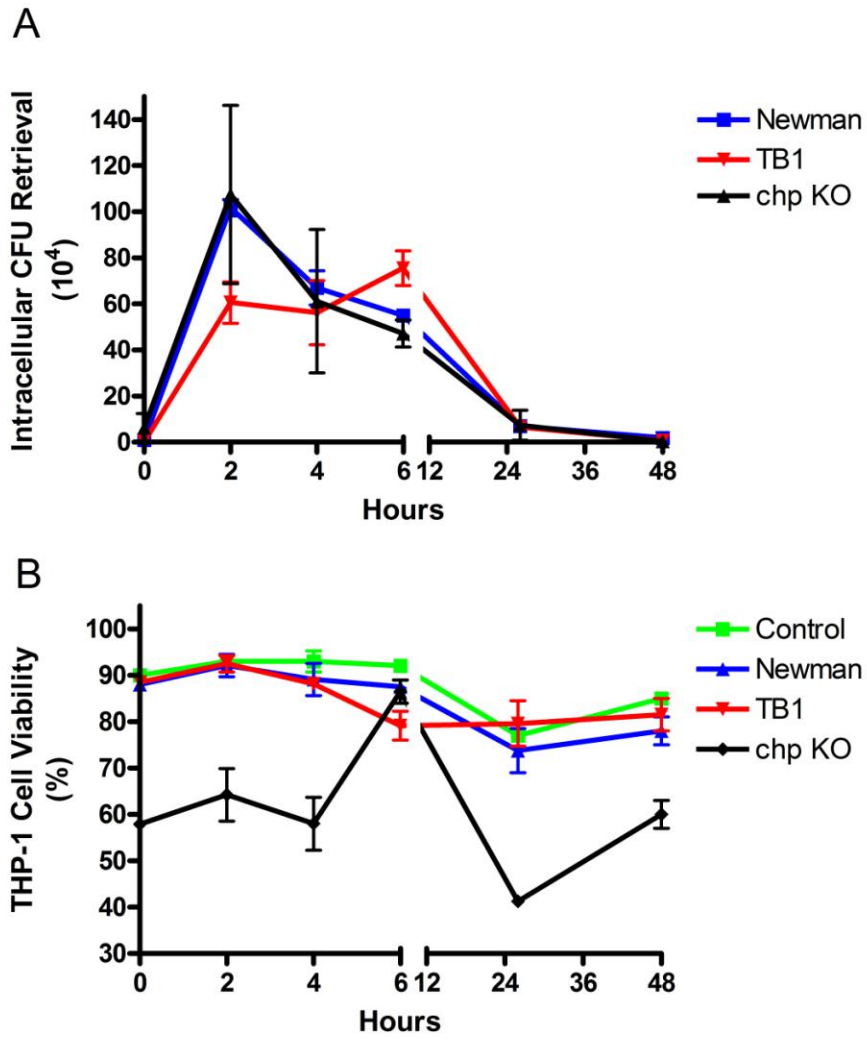

Figure 4.1. *S. aureus* Newman, TB1, or chp KO intracellular survival in THP-1 monocytes. To determine if *S. aureus* strains expressing CHIPS survived more efficiently in THP-1 monocytes, THP-1 were infected for 1h (MOI 10) with *S. aureus* Newman, TB1, or chp KO. Cells were treated with lysostaphin to terminate infection and incubated in gentamicin-containing culture media for 2, 4, 6, 12, 26, and 48h post infection. Cells were lysed for CFU retrieval as an indicator of intracellular survival (A). Prior to cell lysis for CFU retrieval, aliquots of THP-1were collected in PBS and THP-1 viability (B) was analyzed through trypan blue

exclusion. Data represents Newman (blue lines), TB1 (red lines), and chp KO (black lines) CFU retrieval from infected THP-1 and viability of infected THP-1 throughout time. Samples were run in duplicate and repeated twice (n=2). Due to the variation in our results, especially between Newman and chp KO strains (A) and (B), experiments were only repeated twice..

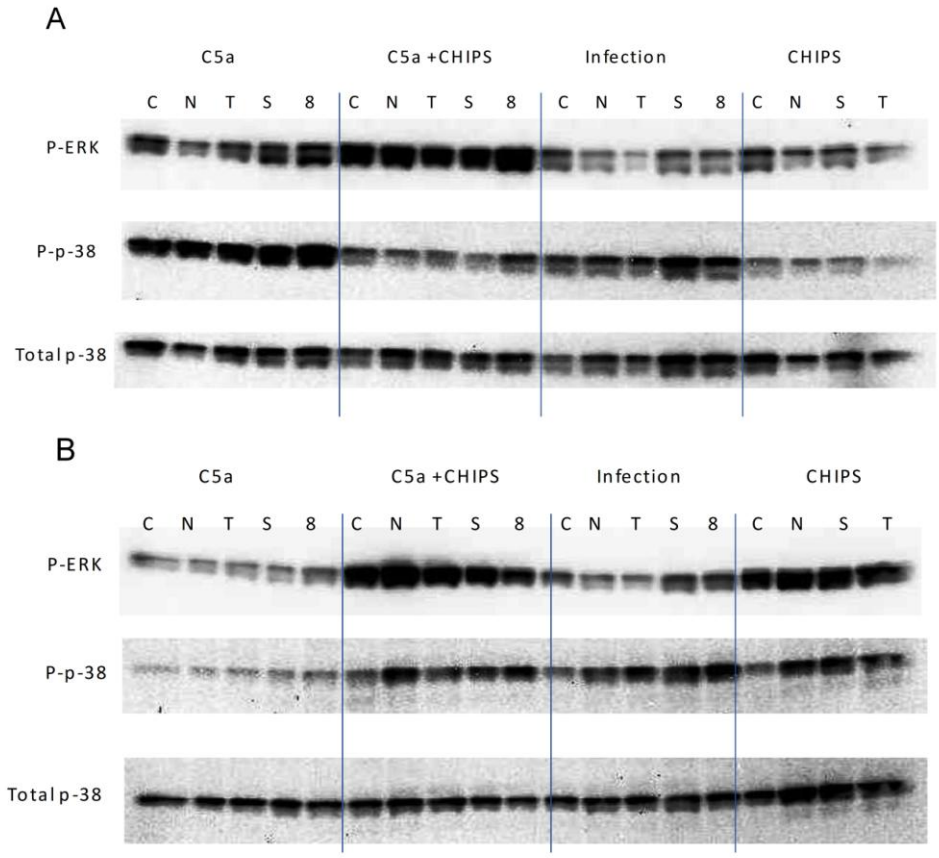

Figure 4.2. Western analysis of stress signaling protein phosphorylation during THP-1 infections with *S. aureus* strains. To determine if C5a enhanced signaling responses of infected THP-1 or if CHIPS inhibited THP-1 responses to infection, THP-1 monocytes were unstimulated (C) or infected with Newman (N), TB1 (T), chp KO (S), or 8325-4 (8) at MOI 10 for 30 min (A) or 1h (B). THP-1 monocytes were treated with C5a (100ng), CHIPS (0.1μg), or pretreated with CHIPS for 15 min, 37°C and stimulated with C5a for 30 min or 1h. Samples were lysed for western blotting analysis of phosphorylated ERK or MAPK p38. Experiments were conducted twice (n=2). The phosphorylation in unstimulated controls indicated a problem with our infection model and additional experiments were not conducted.

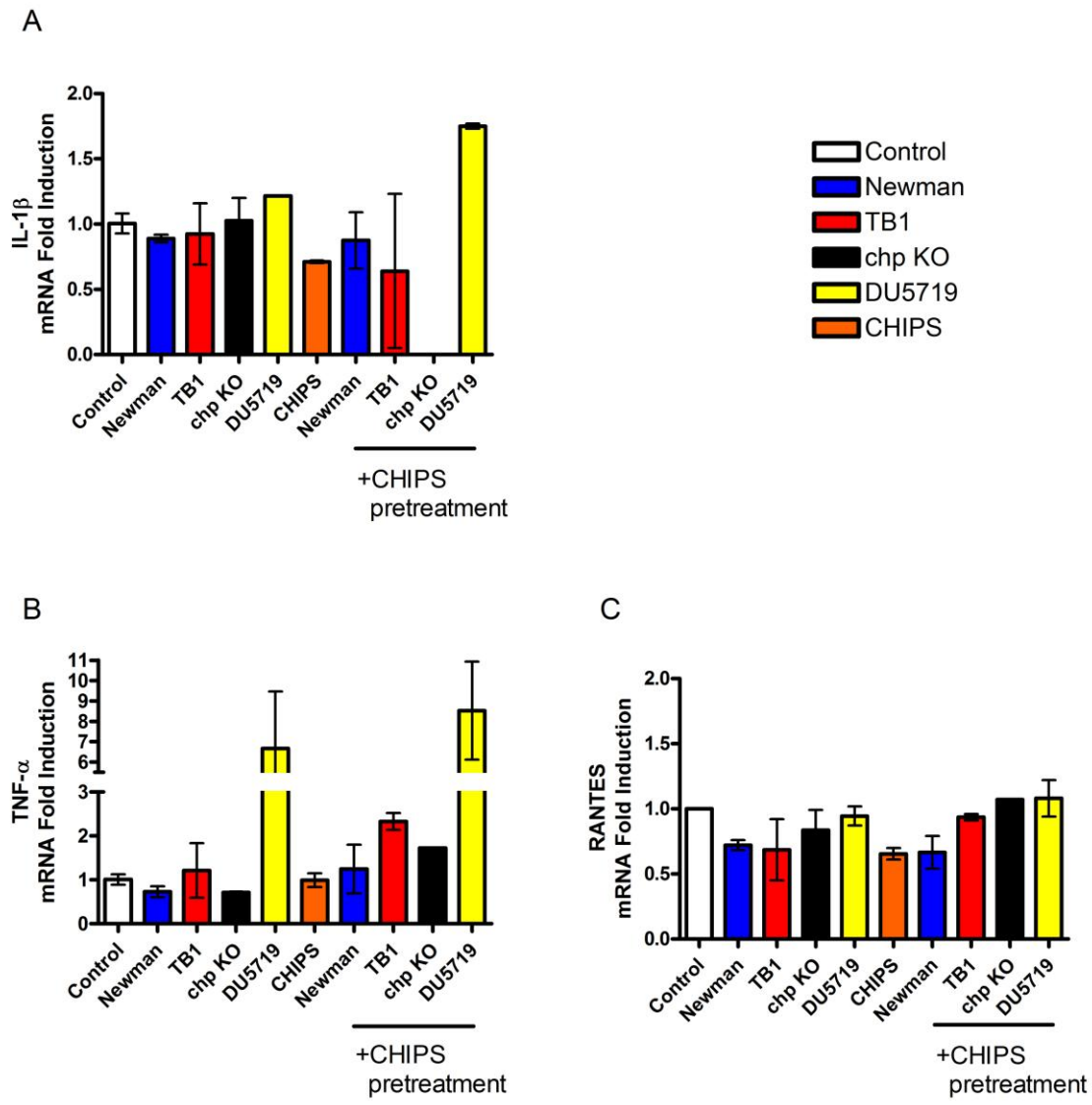

Figure 4.3. Gene expression profiles by THP-1 during *S. aureus* Newman, TB1, chp KO, or DU5719 infections with or without CHIPS pretreatment. To determine if CHIPS inhibited THP-1 monocyte inflammatory expression induced by infections, THP-1 monocytes were infected with Newman, TB1, chp KO, or DU5719 *S. aureus* strains at MOI 10 for 1h or pretreated with CHIPS (0.1μg) for 15 min, 37°C prior to infections. Lysates were collected for gene expression analysis through quantitative Real Time PCR of IL-1β (A), TNF-α (B), and RANTES (C). Data represents gene expression profiles by infected THP-1 monocytes with *S.*

aureus Newman (blue bars), TB1 (red bars), chp KO (black bars), DU5719 (yellow bars), and CHIPS alone (orange bars). Data represents two separate experiments (n=2). Due to the variation in our results and speculated activation of unstimulated control, additional experiments were not conducted.

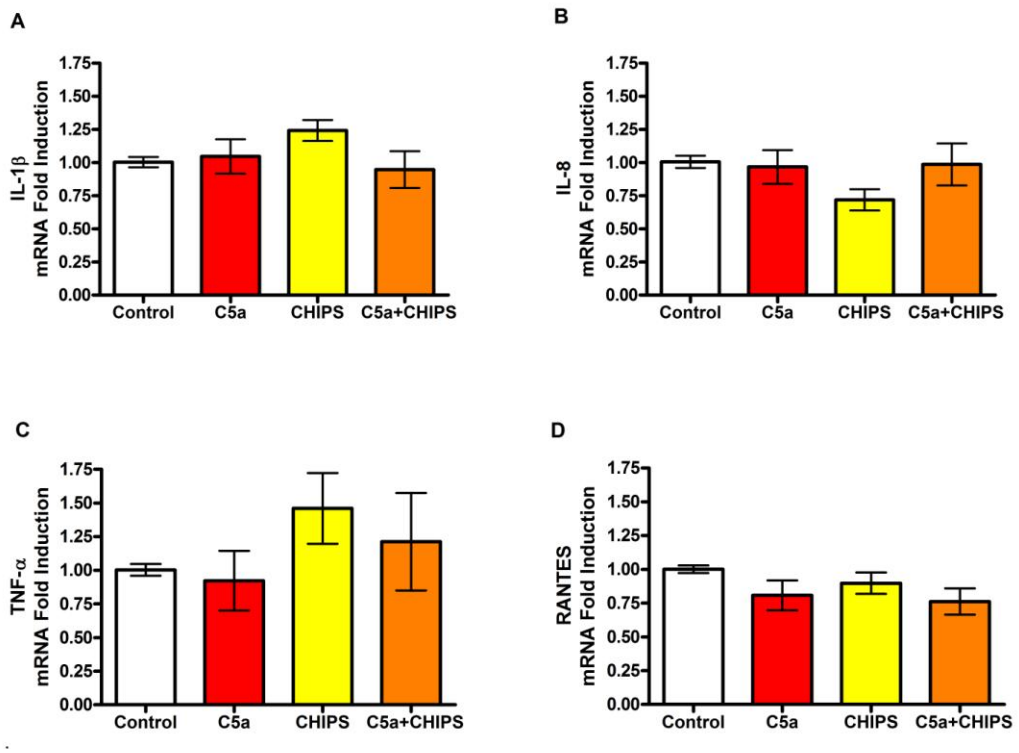

Figure 4.4. Gene expression profiles by THP-1 stimulations with C5a, CHIPS, or C5a and CHIPS for 30min. To determine if CHIPS inhibited THP-1 monocyte inflammatory responses to C5a, THP-1 monocytes were unstimulated (control), stimulated with C5a (100ng) alone, CHIPS (0.1μg) alone, or pretreated with CHIPS for 15 min, 37°C and stimulated with C5a for 30 min. Cells were lysed for gene expression analysis of IL-1β (A), IL-8 (B), TNF-α (C), and RANTES (D). Data represents gene expression profiles by stimulated THP-1 monocytes with C5a (red bars), CHIPS (yellow bars), and CHIPS pretreatment following by C5a stimulation (orange bars). Data represents two separate experiments (n=2). Due to the variation in our results and speculated activation of unstimulated control, additional experiments were not conducted.

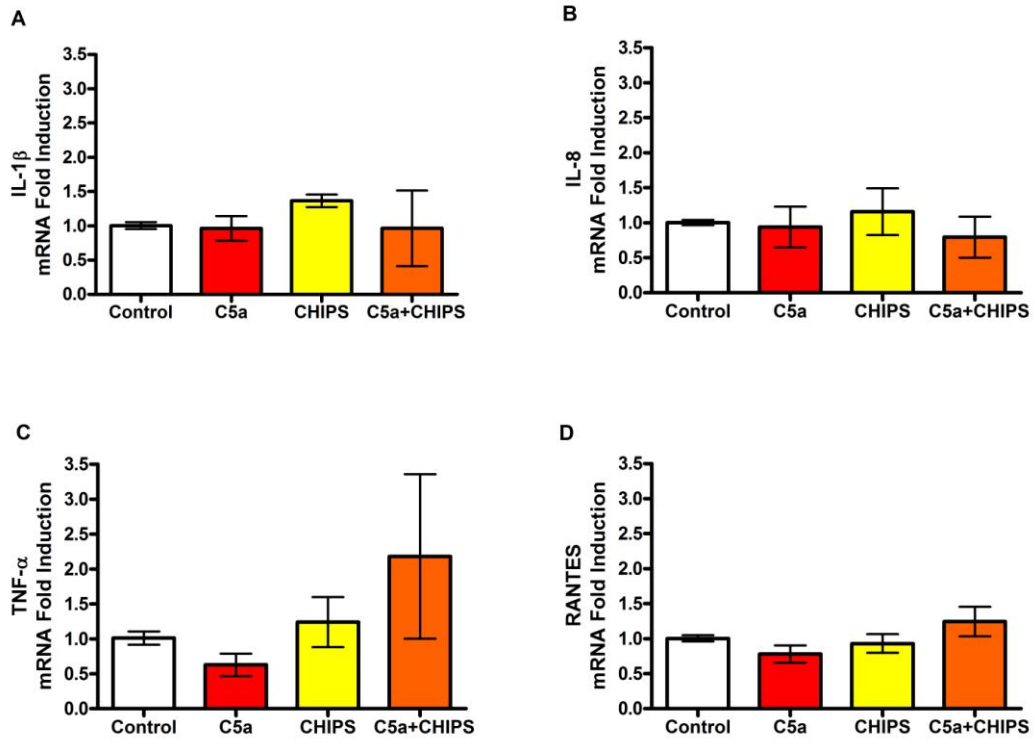

Figure 4.5. Gene expression profiles by THP-1 stimulations with C5a, CHIPS, or C5a and CHIPS for 1h. To determine if CHIPS inhibited THP-1 monocyte inflammatory responses to C5a, THP-1 monocytes were unstimulated (control), stimulated with C5a (100ng) alone, CHIPS (0.1μg) alone, or pretreated with CHIPS for 15 min, 37°C and stimulated with C5a for 1h. Cells were lysed for gene expression analysis of IL-1β (A), IL-8 (B), TNF-α(C), and RANTES (D). Data represents gene expression profiles by stimulated THP-1 monocytes with C5a (red bars), CHIPS (yellow bars), and CHIPS pretreatment following by C5a stimulation (orange bars). Data represents two separate experiments (n=2). Due to the variation in our results and speculated activation of unstimulated controls, additional experiments were not conducted

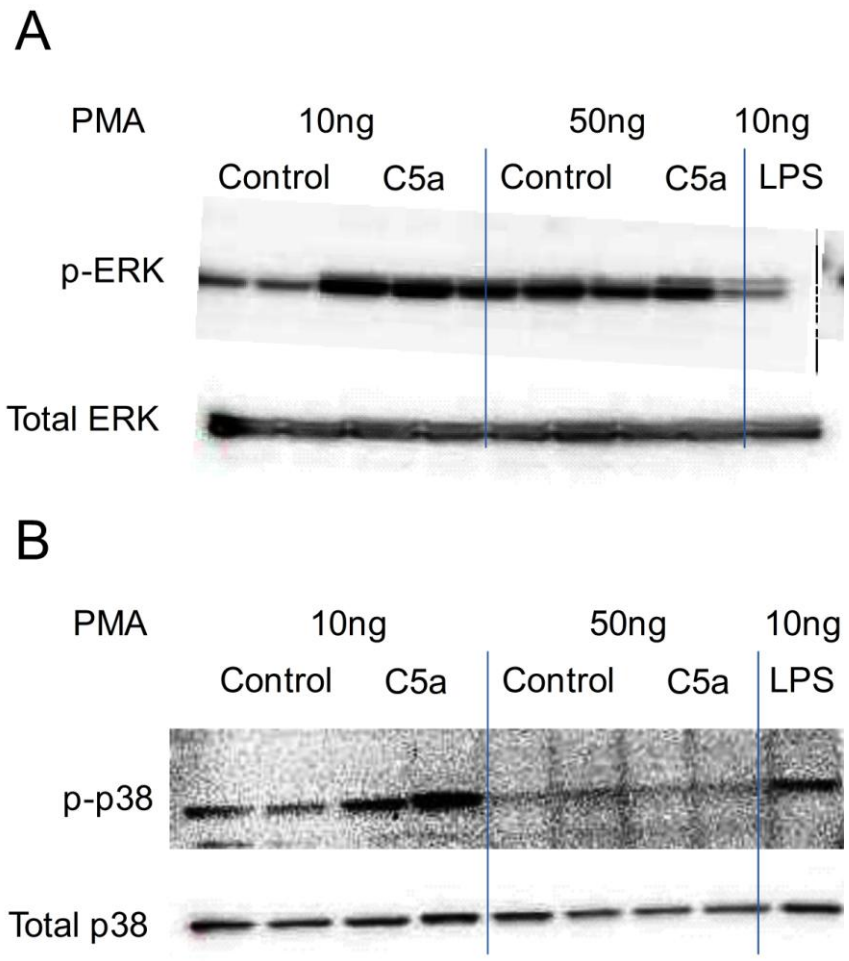

Figure 4.6. PMA differentiation of THP-1 monocytes and subsequent signaling responses to human C5a. To determine PMA concentration for differentiation of THP-1, THP-1 monocytes were incubated for 24h with media containing 10ng or 50ng of PMA to allow for monocyte differentiation into macrophages. Cells recovered from stimulation for 48h in non-PMA containing media. Differentiated cells were unstimulated (control), stimulated with C5a (100ng) alone, or LPS (1μg) as a positive control for 30 min. Cells lysates were collected for western blot analysis of ERK phosphorylation (A) and MAPK p38 phosphorylation (B).

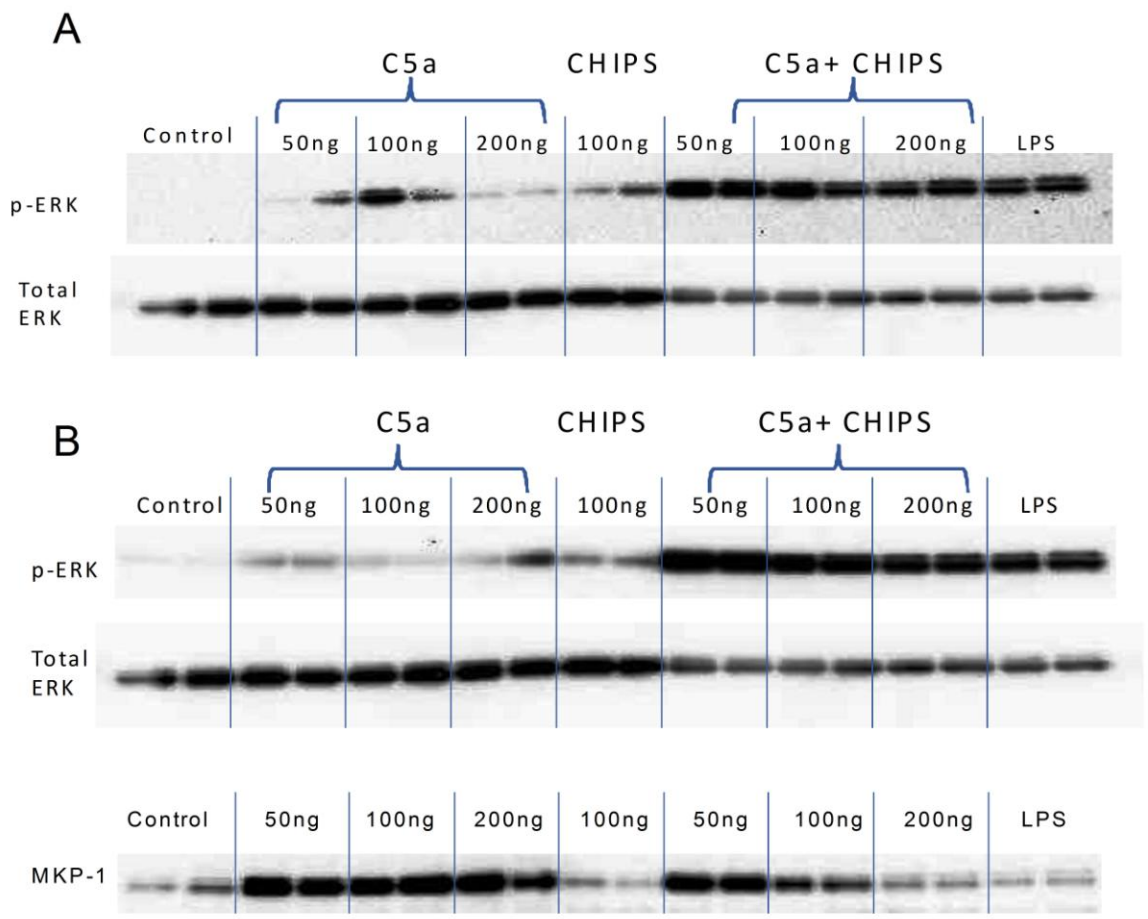

Figure 4.7. PMA differentiated THP-1 signaling responses to C5a alone or C5a stimulation following CHIPS pretreatment. THP-1 were differentiated with 10ng PMA for 24h and allowed to recover for 48h. THP-1 were unstimulated, stimulated with increasing concentrations of C5a (50ng-200ng) alone, or pretreated with CHIPS (0.1µg) for 15 min, 37°C and stimulated with increasing concentrations of C5a (50ng-200ng), or stimulated with LPS (1µg) as a positive control for 30 min to test the inhibitory role of CHIPS on host responses to C5a . Lysates were analyzed for phosphorylated- and total ERK alone (A) or phosphorylated- and total ERK and MKP-1 phosphorylation (B).

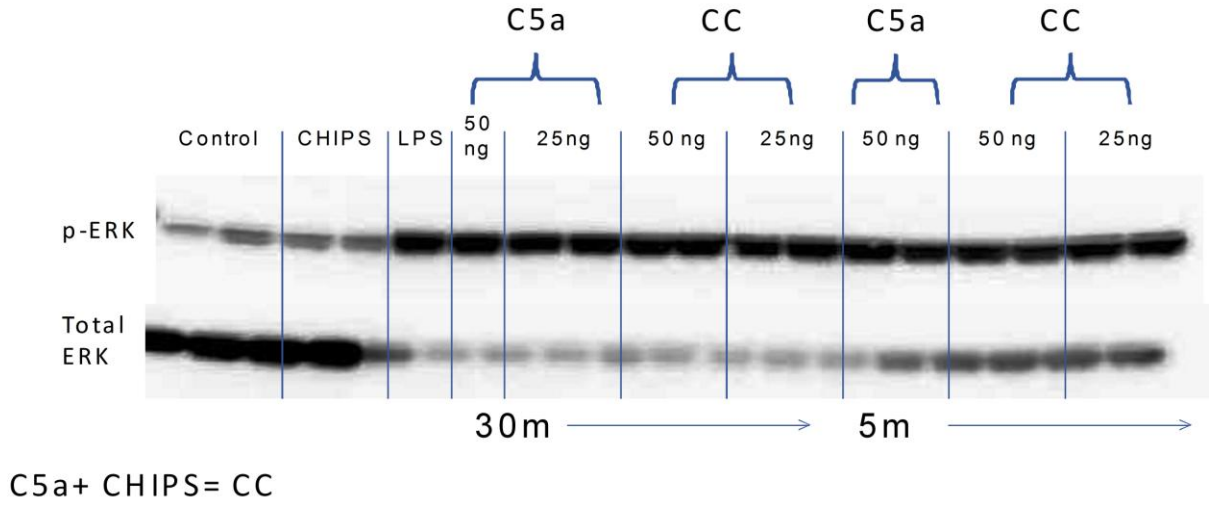

Figure 4.8. PMA differentiated THP-1 signaling responses to C5a alone and CHIPS pretreatments followed by C5a stimulation. To test shorter stimulation times, differentiated THP-1 were unstimulated, stimulated CHIPS (0.1µg) alone, LPS (1µg) as a positive control, increasing concentrations of C5a (25ng-50ng), or pretreated with CHIPS (0.1µg) for 15 min, 37°C and stimulated with increasing concentrations of C5a (25ng-50ng) for 30 min or 5 min. Lysates were collected for western blotting analysis of phosphorylated- and total ERK analysis.

Chapter 5. Omnigen-AF® restores GR-1, L-selectin, and RANTES expression by immunosuppressed murine PMN challenged with lipopolysaccharide in a MyD88-dependent manner.

Running title: Neutrophil function restoration by OmniGen-AF®

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Abstract

Bovine mastitis costs the dairy industry billions of dollars every year and presents a health challenge in dairy facilities. Immunosuppressive effects of the periparturient period present a challenge for dairy men in reducing the incidence of mastitis. During this time, cattle experience an increase in circulating cortisol, which reduces PMN function and ability to clear infection. OmniGen-AF® is a feed additive that reduces the rates of mastitis by restoring PMN function. Signaling pathways involved in restoring PMN function are undetermined. We hypothesized that OmniGen-AF® restores PMN function through cross-regulation of TLR signaling. To test our hypothesis, wildtype or MyD88 knockout (KO) mice were unsupplemented or supplemented with OmniGen-AF® in the diet, challenged with LPS, immunosuppressed with dexamethasone, or immunosuppressed with dexamethasone and challenged with LPS. PMN were isolated through intraperitoneal lavages and analyzed for gene expression profiles. Results indicated that LPS significantly upregulated GR-1, L-selectin, and RANTES (genes) in PMN isolated from wildtype mice as compared to immunosuppressed mice. Interestingly, LPS did not upregulate genes in PMN from supplemented wildtype mice as compared to supplemented, immunosuppressed wildtype mice. Stimulation with LPS differentially regulated genes in PMN from MyD88 KO mice as compared to PMN from supplemented MyD88 KO mice. These results suggest that OmniGen-AF® supplementation restores responses of immunsuppressed PMN to LPS challenge and that the restorative role may be MyD88-dependent. Future research needs to determine the specific TLR, transcription factors, and biochemical properties of OmniGen-AF® that restore gene expression in immunosuppresed PMN.

Introduction

Bovine infectious mastitis is an economic challenge for the dairy industry. During the periparturient period, glucocorticoids circulate at high levels in the host and suppress polymorphonuclear (PMN) function by reducing adhesion factor and chemokine receptor expression (2, 3, 8, 9), thereby reducing the host's ability to clear infection. On the day of parturition, there is an increase in circulating cortisol in the serum, which correlates to suppressed expression of glucocorticoid receptor (GR)- α, pro- and anti-apoptotic, CD18 and L-selectin gene expression in bovine blood neutrophils (2, 3, 8). Following parturition, circulating cortisol levels return to their regular level, but the suppressive effects on bovine neutrophils are still evident (3, 8). OmniGen-AF® feed additive increases PMN function and lowers the rates of intramammary infections during the periparturient period (12). However, the signaling pathways involved in the restoration of PMN functions are undetermined.

Mammalian cell membranes express Toll-like receptors (TLR), which recognize pathogen associated membrane patters (PAMPs) to induce inflammatory immune responses in order to clear pathogens. Signaling pathways are also induced by glucocorticoids binding to their GR. In fact, there are several transcription factors downstream of TLR and GR that potentially cross-regulate immune responses in PMN (5, 7). We proposed to identify the cellular pathways affected by OmniGen-AF® that restore PMN signaling pathways and reduce intramammary infections in the host. We hypothesized that OmniGen-AF® restored PMN function through inhibiting the regulation of GR signaling on TLR signaling (Figure 5.1).

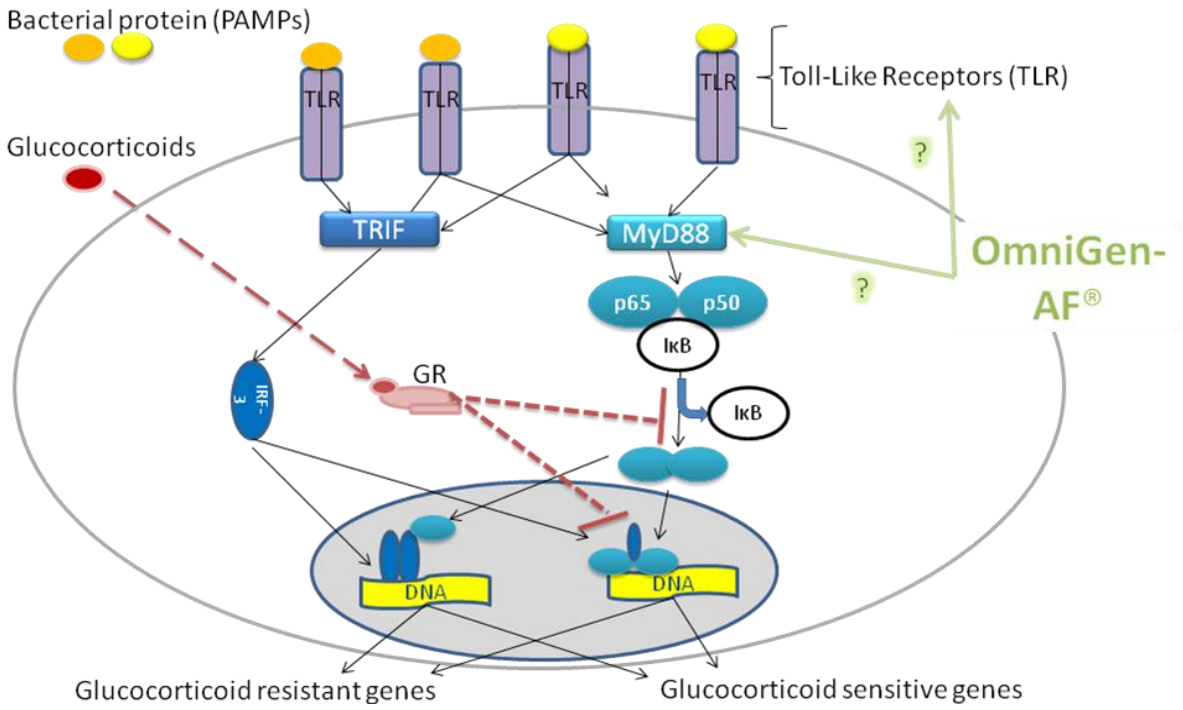

Figure 5.1. Potential role for OmniGen-AF® restoration of Toll-like Receptor (TLR) signaling through inhibition of Glucocorticoid Receptor (GR) signaling. Immune genes can be sensitive to glucocorticoid modification depending on activated TLR signaling pathways (MyD88 or TRIF) (7). We propose that OmniGen-AF® may counter-regulate glucocorticoid sensitive gene expression by modulation of TLR activities (Figure provided by I.K.Mullarky, Ph.D.).

Through understanding the mechanisms by which OmniGen-AF® counteracts the suppressive impacts of glucocorticoids and enhances PMN function, we can identify new infections and diseases that may be treated with OmniGen-AF® supplementation.

Materials and methods

Mice and OmniGen-AF® supplementation:

C57BL/6 wildtype and MyD88 knockout (MyD88 KO), pathogen free mice were obtained from Jackson Laboratories and housed in the Corporate Research Center Vivarium (ABSL-2 facility), Virginia Tech campus. Mice were 5 weeks of age when received and harvested at 8 weeks of age. Mice were housed in groups of five and fed a commercial chow (Harlan Laboratories, Inc., Madison, WI) ad lib with unlimited access to water for the first week. Mice were either unsupplemented or supplemented with OmniGen-AF® for two weeks until time of harvest.

Prince Agri Products supplied the OmniGen-AF® feed additive. Our laboratory sent the feed to get pelleted by Teklad Lab Animal Diets (Harlan Laboratories, Inc., Madison, WI) at a concentration of 5g/kg of 2018 Harlan Chow. Mice were feed regular chow or supplemented chow ad lib. All procedures were approved and carried out in accordance with the Institutional Animal Care and Use Committee of Virginia Tech.

Immunosuppression and LPS challenge:

All mice were injected with 1mL of aged 3% thioglycollate (Sigma Aldrich, St. Louis, MO) solution into the peritoneal cavity 18-24h prior to euthanization for maximal PMN extraction (Supplementary Figure 5.1). Following thioglycollate injection, mice were euthanized at 4, 12, or 18h to collect PMN through intraperitoneal lavages. PMN and macrophage populations were compared with murine CD14 and GR-1 densities through flow cytometry. CD14 populations were considered macrophages and GR-1 populations were considered PMN. The 18-24h incubation time allowed for efficient PMN recruitment and enough time for PMN to respond to dexamethasone treatment.

Mice undergoing immunosuppression were injected with 3mg/kg of dexamethasone (Sigma Aldrich, St. Louis, MO) in to the peritoneal cavity 12h prior to euthanization (dose was determined by preliminary studies, Supplementary Figure 5.2). Mice undergoing LPS challenge were injected with 100μg of LPS (Sigma Aldrich, St. Louis, MO) into the peritoneal cavity 2h prior to euthanization.

Intraperitoneal Lavages:

At time of euthanization, mice were placed in CO_2 chamber for 1-2 min and checked for vital signs to assure death (as indicated by IACUC). Mice were laid on their backs to expose chest and belly. Using tweezers and scissors, fur and skin were removed to expose the intraperitoneal cavity. Ice-cold PBS (Fisher Scientific, Suwanee, GA) containing 1mM EDTA (VWR, Suwanee, GA) was injected into cavity to resuspend PMN. PMN were extracted and put into sterile 15mL tubes. All samples were kept on ice til PMN isolation.

PMN isolation:

Samples were centrifuged at 100 x g, 4°C for 10 min. Supernatants were discarded and pellets were resuspended in ice-cold PBSE to wash the pellet. Samples were centrifuged at 100 x g, 4°C for 10 min again. Supernatants were discarded and pellets were resuspended in ice-cold red blood cell lysis buffer (8% NH_4Cl, 0.01M EDTA (pH 7.4). Samples were vortexed and let sit on ice for 10 min. Samples were centrifuged at 100 x g, 4°C for 10 min again. Supernatants were discarded and pellets were resuspended in RNeasy® Mini Kit (Qiagen; Valencia, CA) lysis buffer containing 10μL/mL of β-mercaptoethanol (Sigma Aldrich, St. Louis, MO) for RNA isolation.

Reverse Transcriptase Real-Time -PCR for mRNA Quantification (qRT-PCR):

Total RNA was isolated from samples using RNeasy® Mini Kit (Qiagen, Valencia, CA) as indicated by manufacturer. Complimentary DNA (cDNA) was synthesized from sample mRNA (2.2µg) by heating sample at 70°C for 10min with 250ng of random primers (Invitrogen ,Carlsbad, CA). Samples were cooled to 4°C and centrifuged at 350 x g for 30 sec, 4°C. Samples were brought to 19.5µL with a solution containing 20.5%/vol Buffer, 10%/ vol Dithiothreitol (DTT), 5%/vol deoxyribonucleotides (DNTPs) (VWR, Suwanee, GA) , and 2.5%/vol Superscipt II (Reverse transcriptase). All reagents were purchased from Invitrogen (Carlsbad, CA) unless indicated otherwise. Samples were incubated at 42°C for 50 min. Samples were incubated at 70°C for 15 min to de-activate the reverse transcriptase. Samples were brought up to 10ng/µL of cDNA with DNase/RNase free water (Qiagen; Valencia, CA).

Primers were purchased from Applied Biosystems (Table 5.1). The TaqMan® Universal PCR Master Mix (Applied Biosystems; Foster City, CA) and the ABI 7300 Real-Time PCR System (Applied Biosystems; Foster City, CA) were used for analysis. The final volume for analysis was 25µL containing 300nM of both forward and reverse primers (Integrated DNA Technologies; Coralville, IA), 100nM of 6-carboxyfluorescein (FAM) probe (Biosearch Technologies Inc.; Novato, CA), 2X TaqMan® Universal PCR Master Mix (Applied Biosystems; Foster City, CA), and 50ng cDNA. Samples were heated at 50°C for 2 min once, 95°C for 10min once, 95°C for 15 sec for 40 cycles, and cooled at 60°C for 1 min. A compiled study of all sample cycle threshold (Ct) values were exported for analysis.

Statistical analysis

Data was analyzed and graphed using GraphPad Prism Version 4.03 (La Jolla, CA). Murine PMN mRNA expression was compiled from two separate experiments, containing 5 mice per treatment, per experiment. Sample Ct values were compared to the Ct value of stable housekeeping genes, RPL19 (ribosomal protein ligan 19) and YWHAZ (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide) (as determined by preliminary studies for gene consistency). The difference in Ct values (ΔCt) were compared to unstimulated PMN ΔCt values (ΔΔCt). This difference was calculated for fold induction ($2^{-\Delta\Delta Ct}$). Statistical analysis was conducted on samples ΔCt values. Significance was determined using one-way ANOVA with a Tukey's post test.

Results

OmniGen-AF® restores dexamethasone-induced immunosuppression through MyD88 pathway.

Murine PMN were isolated from wildtype or MyD88 KO mice unsupplemented or supplemented with OmniGen-AF® in their diet for two weeks. Select mice were injected with dexamethasone to induce immunosuppression and all mice were challenged with LPS, except for unstimulated controls (Figure 5.2). LPS challenge significantly upregulated GR-1 expression in wildtype PMN as compared to immunosuppressed wildtype PMN (Figure 5.2A). LPS challenge did not significantly increase GR-1 expression in supplemented wildtype PMN as compared to immunosuppressed wildtype PMN; suggesting OmniGen-AF® supplementation restored the immunosuppressive effects of dexamethasone in PMN responses. LPS challenge did not significantly increase GR-1 expression in MyD88 KO PMN as compared to immunosuppressed MyD88 KO PMN (Figure 5.2A). Also, LPS did not significantly increase GR-1 expression in supplemented MyD88 KO PMN as compared to supplemented immunosuppressed MyD88 KO PMN. These data suggest a role for MyD88 in LPS induction of GR-1 in PMN.

LPS challenge significantly upregulated GR-1 expression in unsupplemented MyD88 KO PMN as compared to supplemented MyD88 KO PMN. Also, LPS significantly upregulatedGR-1 expression in unsupplemented immunosuppressed MyD88 KO PMN as compared to supplemented MyD88 KO PMN. Interestingly, LPS significantly induced GR-1 expression in supplemented wildtype PMN (both untreated or treated with dexamethasone) as compared to supplemented MyD88 KO PMN (Figure 5.2A).

LPS challenge significantly upregulated L-selectin expression in wildtype PMN as compared to immunosuppressed wildtype PMN (Figure 5.2B). LPS challenge did not

significantly increase L-selectin expression in supplemented wildtype PMN as compared to supplemented immunosuppressed wildtype PMN, suggesting a role for diet in restoring the immunosuppressive effects of dexamethasone treatment. LPS challenge significantly upregulated L-selectin expression in supplemented immunosuppressed wildtype PMN as compared to immunosuppressed wildtype PMN. LPS did not significantly increase L-selectin expression in MyD88 KO PMN as compared to immunosuppressed MyD88 KO PMN. LPS did not significantlyincrease L-selectin expression in supplemented MyD88 KO PMN as compared to supplemented immunosuppressed MyD88 KO PMN. LPS significantly upregulated L-selectin expression in MyD88 KO PMN as compared to supplemented immunosuppressed MyD88 KO PMN (Figure 5.2B).

LPS challenge significantly upregulated CCL5 (RANTES) expression in wildtype PMN as compared to immunosuppressed wildtype PMN (Figure 5.2C). LPS did not significantly increase CCL5 expression in supplemented wildtype PMN as compared to immunosuppressed wildtype PMN. LPS did not significantly increase CCL5 expression in MyD88 KO PMN as compared to LPS immunosuppressed MyD88 KO PMN. LPS significantly upregulated CCL5 expression in supplemented MyD88 KO PMN as compared to supplemented immunosuppressed MyD88 KO PMN. This data suggests that the restorative effect of OmniGen-AF® on dexamethasone-induced immunosuppression may be MyD88-dependent.

Discussion

During the periparturient period, dairy cattle are susceptible to infections due to the immunosuppressive effects of circulating cortisol on circulating leukocytes (3, 8). The inhibitory effects of glucocorticoids on neutrophil function of periparturient cattle impact membrane receptors, GR, and apoptotic genes (2, 3). OmniGen-AF® feed additive has been reported to reduce the rates of mastitis in periparturient cattle (11, 12), however the signaling pathways involved in OmniGen-AF® restoration of PMN function is undetermined. We are the first to introduce the GR and TLR crosstalk model to describe a potential mechanism for glucocorticoid-induced suppression of inflammatory genes in PMN during the periparturient period and restoration of responses by OmniGen-AF® feed additive.

Our data indicates that OmniGen-AF® restores LPS-induced gene expression of GR-1, L-selectin, and RANTES in immunosuppressed wildtype PMN in a MyD88-dependent manner. Our data agrees with Ogawa et al. (2005), which described dexamethasone-induced suppression of LPS-dependent inflammatory gene responses in murine macrophages, suggesting a role for GR cross regulation of TLR-dependent genes. In fact, Ogawa et al. (2005) found that GR-suppression of LPS-induced genes in murine macrophages was IRF3-dependent; inhibiting p65 and IRF3 interaction. TLR4 can induce IRF3 activation through TRIF- or MyD88-dependent pathways, but TLR4-induction of NF-κB is solely through MyD88 pathways (7). Therefore, using MyD88 KO mice, researchers confirmed that GR-suppression of LPS-induced genes was IRF3-dependent. Together with our model, LPS significantly upregulated GR-1 and L-selectin expression in wildtype PMN as compared to immunosuppressed wildtype PMN (Figure 5.2A-B). However, LPS did not significantly increase GR-1 and L-selectin expression in MyD88 KO PMN as compared to immunosuppressed MyD88 KO PMN, suggesting that LPS-dependent

increases of GR-1 and L-selectin expression in PMN may be MyD88-dependent. Therefore, future research needs to determine if IRF3 is the transcription factor that inhibits TLR-induced gene expression during dexamethasone treatment.

Our data also indicated that OmniGen-AF® supplementation in the diet of wildtype mice restored the LPS-dependent upregulation of GR-1, L-selectin, and RANTES gene expression in PMN (Figure 5.2A-C). This data agrees with Wang et al. (2004) (13) findings, which indicate that OmniGen-AF® restores L-selectin and IL-1β expression in *Aspergillus fumigatus* challenged sheep. Our concern with their model is that *A. fumigatus* is mainly recognized by TLR2 (6), which activates NF-κB to induce inflammatory responses, where as Ogawa et al. (2005) determined that GR-dependent suppression of TLR-induced genes is mainly through transcription factor IRF3. Their findings indicate the potential for OmniGen-AF® to regulate more transcription factors other than GR- regulated IRF3 and be applied to gram-positive pathogen infections.

We conclude that MyD88-dependent signaling pathways play a role in OmniGen-AF® restoration of the immunosuppressive effects of dexamethasone. Our model investigated TLR4 MyD88-dependent responses in PMN, indicating that our data can be applied to gram-negative pathogen infections. However, as determined by Ogawa et al. (2005), GR regulation of TLR-induced transcriptional activation is by disrupting IRF complexes, which are required for TLR4 and TLR9 downstream signaling. CpG-containing DNA is the ligand for TLR9 (1) and TLR9 agonists have been well studied to develop adjuvants for vaccines (4, 10). Therefore, when developing vaccines against opportunistic pathogens, we may consider supplementing the host with OmniGen-AF® to counteract the immunosuppressive effects of glucocorticoids and restore

TLR9 activity. Taken together with Wang et al., (2004), this concept may be applied to several, if not all, TLR.

Nevertheless, other studies conducted on the effects of OmniGen-AF® in dairy cattle reported no significant differences in L-selectin, IL-8 receptor, and IL-1 converting enzyme by cows fed the additive as compared to unsupplemented cows (11). A potential reason for the lack of differences between unsupplemented and supplemented cows may be the lack of pathogenic challenge. During the optimization of our model, we noticed that dexamethasone-induced immunosuppression was not statistically significant between our diet treatments (regardless of mouse genotype (in wildtype or knockout mice)) without being challenged (data not shown). Another potential reason for lack of differences between unsupplemented and supplemented cows may be the origin of PMN studied. Our samples only encompass PMN which have already migrated to the intraperitoneal cavity. However, peripheral blood PMN and PMN collected from intraperitoneal lavages may express different gene expression profiles in response to LPS challenges and dexamethasone-induced immunosuppression. For example, PMN collected from intraperitoneal lavages transmigrated to the cavity by chemotactic responses to the 3% thioglycollate injections. However, peripheral blood PMN affected by circulating dexamethasone may not transmigrate towards the thioglycollate due to the down-regulation of adhesion factors (3) and may express different gene expression profiles in response to LPS challenge.

Future research needs to determine which TLR are involved in OmniGen-AF® restoration of immune responses, which transcription factors are involved downstream of different pathogen challenges and the biochemical properties of OmniGen-AF® additive that restores gene expression, and determine the use of this additive treat more diseases ailing immunodeficient patients.

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Table 5.1. Primer/Probe Sets for inflammatory murine receptors and cytokines gene expression analysis.

Primer/ Probe Set	Reference Number	Company
RPL19	Mm02601633_g1	Applied Biosystems, Foster City, CA
YWHAZ	Mm01158416_g1	Applied Biosystems, Foster City, CA
GR-1	Mm00433832_m1	Applied Biosystems, Foster City, CA
L-selectin	Mm00441291_m1	Applied Biosystems, Foster City, CA
CCL5 (RANTES)	Mm01302427_m1	Applied Biosystems, Foster City, CA
IL-1β	Mm01336189_m1	Applied Biosystems, Foster City, CA
CCL2 (MCP-1)	Mm00441242_m1	Applied Biosystems, Foster City, CA
IL-8α Receptor	Mm007311329_s1	Applied Biosystems, Foster City, CA
ITGB2 (CD18)	Mm00434513_m1	Applied Biosystems, Foster City, CA
LTBR (CD18)	Mm00440235_m1	Applied Biosystems, Foster City, CA

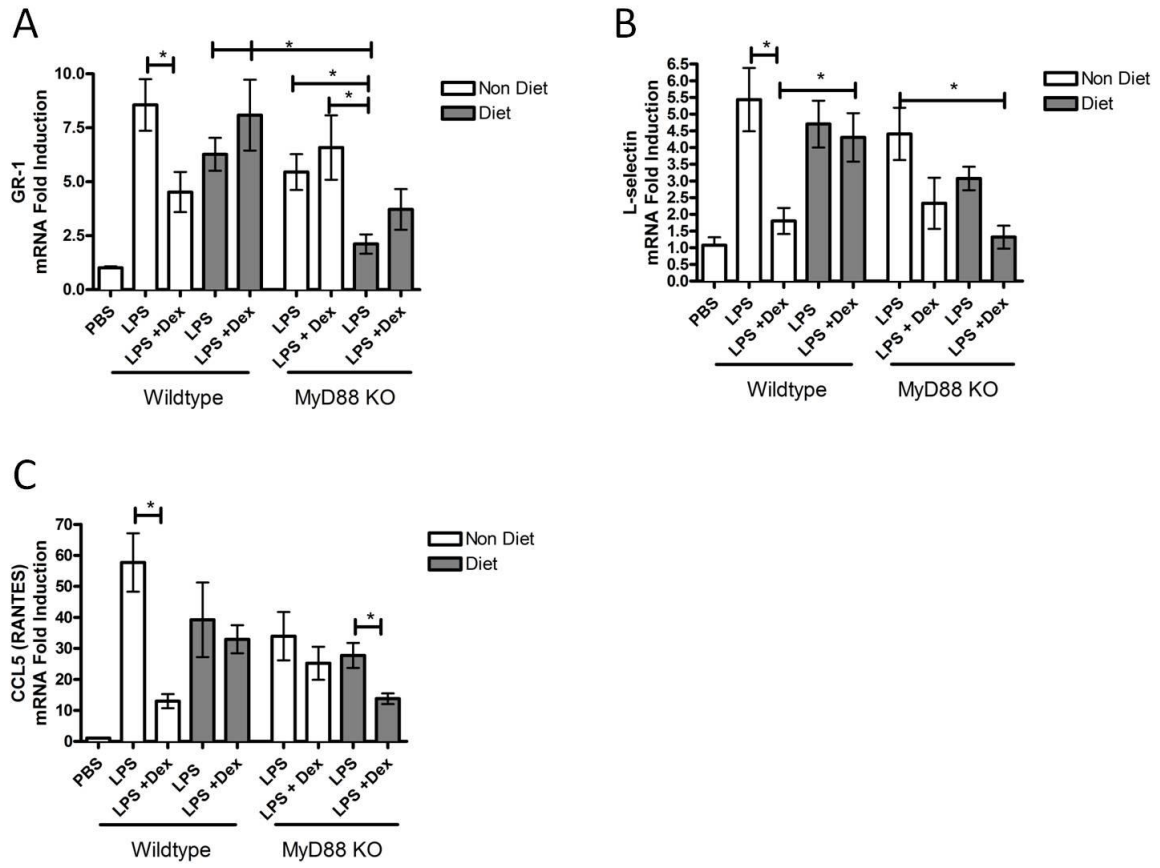
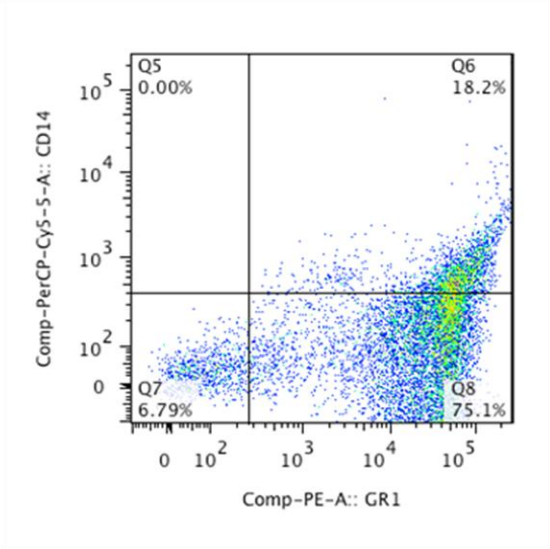


Figure 5.2. Gene expression profiles on wildtype and MyD88 KO PMN responses to LPS with or without OmniGen-AF® supplementation. Mice were fed unsupplemented or supplemented feed for two weeks. Twelve hours before euthanization, mice were injected with 3mg/kg of PBS or dexamethasome in the intraperitoneal cavity. Two hours before euthanization, mice were injected with PBS or 100μg of LPS in the intraperitoneal cavity. PMN were collected through intraperitoneal lavage for gene expression analysis of glucocorticoid receptor (GR-1) (A), L-selectin (B), CCL5 (RANTES) (C). Data represents gene expression profiles of PMN from mice fed regular diet (white bars) and OmniGen-AF® supplemented diet (grey bars). Analysis was conducted on 5 mice per treatment (n=5). Data was analyzed through one-way ANOVA with a Tukey's post test (within mice type). Significance was considered at $P<0.01$.

A



B

		PMN	Mac	PMN : MAC
Thio	4 hr	5.2E+04	1.7E+04	3
	12 hr	3.1E+06	7.8E+05	4
	18 hr	2.7E+05	9.6E+04	3

Supplementary Figure 5.1. Compare murine PMN with macrophage populations for optimal isolation time. Mice were injected with 3% thioglycollate at 4h, 12h, or 18h and primary murine PMN were extracted through intraperitoneal lavage and purified ex vivo. Populations were analyzed for CD14 (high concentrations indicate macrophage population) and GR-1 expression (indicator of PMN) (A) at different time points (B). (A) is an example of our results from 18h time point.

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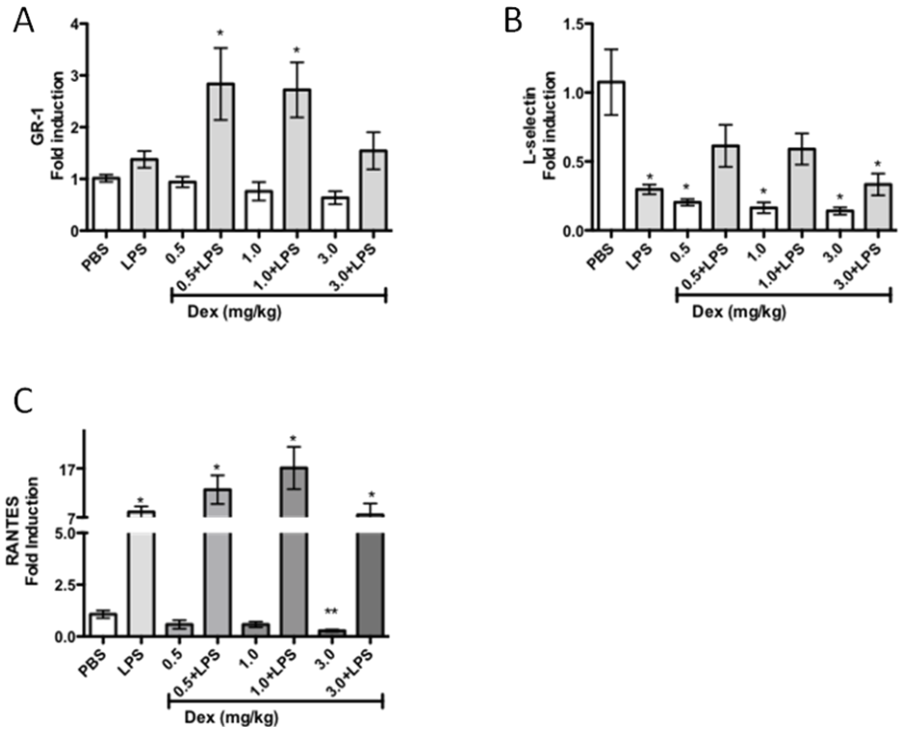

Supplementary Figure 5.2. Dexamethasone titration for optimal PMN immunosuppression to LPS challenge. Wildtype mice were injected in the intraperitoneal cavity with 3% thioglycollate 24h prior to euthanization, increasing concentrations of dexamethasone (Dex) (0.5-3mg/kg) 12h prior to euthanization, and challenged with 100µg of LPS 2h prior to euthanization. Primary PMN were extracted through intraperitoneal lavage and expression of glucocorticoid receptor (GR-1) (A), L-selectin (B), and RANTES (C) was determined. Data represents PBS control (white bars), LPS (light grey bar), dex treatment alone or dex treatments with LPS challenge (shades of grey bars). Each treatment was conducted on five mice (n=5). Data was analyzed through a one-way ANOVA with a Dunnett's post test. Significance was assessed at $P<0.01$.

Chapter 6. Conclusion and Future Research

S. aureus is a major concern for public health due to the increasing number of nosocomial- and community- acquired infections. Regulation of virulence factor expression is pivotal to *S. aureus* establishment of infection and immune evasion mechanisms. Understanding how such a virulent pathogen can colonize the host and induce subclinical symptoms is of particular interest to our laboratory. The focus of this dissertation was to characterize immune responses to *S. aureus* infections and activated polymorphonuclear leukocytes (PMN) signaling pathways in response to virulence factors. A novel finding was the role for APC during *S. aureus* intracellular infections of mammary epithelial cells (MEC) and their subsequent effects on PMN. We can attribute these reported effects to the subclinical symptoms associated with *S. aureus* mastitis. A concern with our results would be the control environments imposed by these models. We had to intracellularly or extracellularly infect MEC with *S. aureus* and analyze immune responses separately. However, these immune responses would not be exclusive to treatment during an *in vivo* scenario. Since our findings indicate potential interactions between inflammatory and coagulatory responses, it would be interesting to determine if our data holds true when cells experience intracellular infection and are stimulated extracellularly with irradiated or live toxin-secreting bacteria. This model will determine if MEC respond to the extracellular stimuli in the same manner when intracellularly infected at the same time and if suppression of cytokines during intracellular infection affect host responses to extracellular stimuli.

Another concern with our model is the variation associated with our animal model. The bovine species provide an excellent research model for human pathogens because this species is

outbred, exposed to- and vulnerable to- human pathogens, and provides a great amount of sample without euthanizing the animal. The concern with animal variation only adds to how good the bovine model is because it reflects the obstacles research needs to overcome to develop human treatments and vaccines. Developing a vaccine to *S. aureus* will also need to overcome individual variation. A potential way to overcome bovine variation in our sample is to eliminate certain variables. Our laboratory did not have control over the necessary procedures conducted by the dairy farm, including the administration of hormones or exact date of pregnancy. Since our studies involved peripheral blood neutrophils, both of these variables could impact our neutrophil responses to infection due to prior exposure to circulating glucocorticoids. Also, our time of blood collection consistently ranged between 8-9:00AM. However, these studies took months to complete, so another variable we need to consider is the physiological effects of the time of year and the specimen's exposure to sunlight. Applying this information to our model and controlling the environment of these cows may provide more consistent data. Nevertheless, using the bovine model as a research model also proves very expensive and limiting in available antibodies for research. Just considering the housing, the number of animals needed, and the reagents to conduct all the assays would be very expensive.

Nevertheless, the bovine research is model is very promising for developing treatment and vaccines against pathogens such as *S. aureus*. Cattle are naturally susceptible to this pathogen and understanding their immune responses to this pathogen and how they develop immunological memory against it may contribute to the discovery of a vaccine. Some vaccines are designed to trigger pathogen toxins that cause disease. However, for a pathogen like *S. aureus* we believe a vaccine targeting a preserved membrane protein would be more efficient. *S. aureus* strains expressing β-hemolysin are more prevalent in the bovine species than in human.

Also, clinical *S. aureus* isolates express a repertoire of different virulence factors and difficult to predict virulence factor regulation *in vivo*.

Virulence factors targeting innate immune responses make *S. aureus* a very successful, opportunistic pathogen. Humans are natural carriers of *S. aureus* strains containing β-hemolysin converting bacteriophages. These phages can express human-specific virulence factors that enhance pathogen survival in the host. Our goal of determining if chemotaxis inihibitory protein of *S. aureus* (CHIPS) enhanced pathogen survival in the host was not completed due to flaws in the model. I regret not focusing enough on justifying our model and giving too much focus to signaling pathways associated with C5a receptor. However, all the time spent reading literature about cell signaling pathways benefited my understanding of the OmniGen-AF® project.

Immunosuppression as a result to glucocorticoid exposure or treatment is well documented. The periparturient period is a natural physiological state for mammals that causes immunosuppression. Since these immunosuppressive effects are observable in PMN function, a major leukocyte in innate immune responses, determining how OmniGen-AF® restores PMN function may be vital to developing therapeutic remedies to restore immune function in diseases. It is important to know which receptors and cytokines are being restored and up-regulated due to OmniGen-AF®, but future research should also consider understanding the specific transcription factors and feedback mechanisms responsible for immunosuppression and how OmniGen-AF® acts on the already established specific signaling pathways. For example, previous literature associates glucocorticoid-induce immunosuppression with Mitogen activated protein kinase phosphatase-1 (MKP-1) activation (2). MKP-1 is downstream of Toll-like receptors (TLR) signaling as well (1). Therefore, integrating potential roles for MKP-1 to the model may enhance our knowledge of OmniGen-AF® in restoring PMN function and signaling. Future research can

investigate the role of OmniGen-AF® in restoring signaling through feedback mechanisms such as MKP-1.

Overall, research leaves us with more questions than answers. These chapters encompass three very different projects, mainly focusing on the roles of *S. aureus* virulence factors on host immune responses and PMN signaling pathways in response to virulence factors or immunosuppression. Conducting these projects in unison has provided much understanding of host-pathogen interactions and innate immune responses.

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Appendix: Detailed Protocols

A.1 *Staphylococcus aureus* intracellular infections

Purpose: Establish an intracellular infections *in vitro.*

Reagents:

60 x 15mm petri dishes (VWR, Suwanee, GA)

Lysostaphin (100 μg/ml; Sigma, St. Louis, MO)

Gentamicin (50μg/ml; Invitrogen, Carlsbad, CA)

Triton-X100 (0.05%)

PBS + 5mM EDTA (PBSE) pH7.4

RNeasy® lysis buffer (Qiagen, Valencia, CA)

1.7mL Eppendorf tubes

Procedure:

1. Seed in 60 x 15mm petri dishes 24h prior to infections if monolayer cells or the day of infection if suspension cells.
2. For monolayer cells, replace antibiotic-containing media with antibiotic free medium on day of infection.
3. For cells in suspension, seed cells in antibiotic free media.
4. Infect cells with corresponding CFU for MOI. Infect for 1- 2h (depending on infection) at 37ºC, 5% CO_2
5. Include in assay unstimulated controls, stimulated samples with *S. aureus* supernatant as extracellular infection controls, and *E. coli* infected cells for positive controls.
6. Add lysostaphin (100 μg/ml) for 7 min to terminate infection.

7. Wash cells with PBS and incubate in medium containing gentamicin (50µg/ml) for hours post infection.
8. At time of collection, collect infection supernatant media, filter-sterilize to eliminate debris, and freeze at -20ºC.
9. Rinse intracellular infection samples with PBS and incubate with 0.5mL PBS solution containing Triton-X100 (0.05%) and EDTA (5mM) to lyse cells.
10. Collect lysates in 1.7 mL Eppendorf tubes and sonicate for 5 min at 4°C.
11. For determination of intracellular CFU, drop plate from samples.
12. For RNA isolation: lyse samples with RLT lysis buffer (Qiagen).

A.2 Bovine Polymorphonuclear leukocyte isolation from whole blood

Purpose: To isolate neutrophils from whole blood samples for use in functional assays.

Reagents:

Citrate anticoagulant, 2x

PBSE (PBS + 5 mM EDTA, pH 7.4)

Sterile ddH_2O (no Ca^+ or Mg^+)

3 X Minimum Essential Medium (3 X MEM, from 10 X MEM, Sigma M0275) (pH 7.4)

Procedure:

1. Start with blood collected with 40mM EDTA.
1. Transfer blood to 50 ml tubes.
2. Centrifuge for 30 min, 2000 RPM, 15°C (no brake).
3. Discard plasma and buffy coat layer.
4. Resuspend blood and neutrophils by pipetting up and down.
5. Rinse pipette for cells.
6. Add no more than 7 ml of blood to 20 ml sterile ddH_2O in a 50 ml centrifuge tube. Lyse red blood cells by pipetting up and down 4 X for a total of 30 sec.
7. Add 10 ml 3 X MEM to the tubes.
8. Bring up the volume to 45mLwith PBSE.
9. Centrifuge for 5 min, 2000 RPM, 15°C. Pour off supernatant.
10. Rinse cells with 35mL of PBSE.
11. Centrifuge at 2000RPM, 5 min, 15°C.
12. Discard supernatant.

13. Add 10 ml sterile ddH_2O. Pipette up and down 4 X (30-40sec).
14. Add 5 ml 3 X MEM, and mix well. Bring volume up to 40mL with PBSE.
15. Centrifuge for 5 min, 2000 RPM, 15°C. Pour off supernatant and discard.
16. Rinse each tube with 5 mL of media then rinse with other 15 mL.
17. Centrifuge for 5 min, 2000 RPM, 15°C.
18. Pour off supernatant and resuspend cells in 20mL of media. (Total volume should be 20 mL).
19. Neutrophils are ready to count.

A.3 Thrombomodulin Activity Assay

Purpose: Measure APC activity

Reagents:

APC buffer:

(50mM Tris-HCl pH 7.5, containing 2mM $CaCl_2$ and 0.1% BSA).

Bovine protein C (5µg/ml; Enzyme Research Laboratories, Inc., South Bend, IN)

Thrombin (0.12U/well; Enzyme Research Laboratories, Inc., South Bend, IN)

APC substrate, S-2366 (Diapharma, West Chester, OH).

Hirudin (2U/well; Sigma, St. Louis, MO)

Procedure:

1. Wash infected cells three times with APC buffer.
2. Add 95µL of APC buffer to each well.
3. Add 112.5µLof APC buffer to Bovine protein C aliquot from -80°C, vortex, and add 5µL of suspension per well.
4. Add 250µL of APC buffer to Thrombin aliquot from -80°C, vortex, and add 5µL of suspension per well.

   HENCE, 100µL final volume per well.
5. Incubate on plate shaker at 37°C for 1h (Covered in foil!)
6. Centrifuge at 2800 x g for 10 min at room temperature.

Mean time, prepare standard curve:

- Make 10µg/mL recombinant APC solution in 500µL of APC buffer (aliquots in -80°C. Do 1:2 dilutions.

7. End of 1 hour, add 30µl of sample supernatants and standard curve to 96-well plate.

8. Add 50 µL of APC substrate, S-2366 (1mmol), to measure APC generation.
9. Add 13.75 µL of APC buffer, to brin volume up 93.75 µL.
10. Add 6.25 µL of hirudin (total volume 100 µL) to inhibit non specific S-2366 cleavage of thrombin.
11. Incubate for 2 h and 30 min at 37 ºC, 5%CO2,
12. Read optical density (OD) on ELISA plate reader (µQuant) at 405nm.
13. Sample OD are compared to APC standard curve OD.

A.4 Chemotaxis Assay Protocol

Purpose: Measure PMN migration towards IL-8 gradient.

Reagents:

Chemotaxis plates (5μm filter; Neuroprobe, Inc., Gaithersburg, MD)

PMN media:

10%FBS, 1% antibiotic/antimycotic, colorless DMEM

Recombinant bovine IL-8 (Thermo Fischer Scientific, Waltham, MA

Recombinant bovine APC (Enzyme Research Laboratories, Inc., South Bend, IN)

Ice cold 2mM EDTA PBS (pH 7.4)

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (60μg)

Extraction buffer:

50% N,N-dimethylformamide, 20% SDS

Procedure:

1. Make APC dilutions for PMN pretreatment.
    a. Tube A
        i. (1.62 μg/μl) (x) = 2μg
        ii. X=1.24 x4 7.5 = 58.6 μl of APC +2316 μl of media
    b. Tube B
        i. Tube A is at (0.04 μg/μl) (x) = 1.5 μg
        ii. X=37.5 x 38=1425 μl of Tube A + 492 μl of media
    c. Tube C
        i. Tube B is at (0.03 μg/μl) (x) =1 μg
        ii. X=33.3 x 29=967 μl of Tube B + 459 μl of media

d. Tube D

i. Tube C is at (0.02 µg/µl) (x) =0.5 µg

ii. X=25 x 19= 475µl of Tube C + 475µl of media

2. IL-8 calculations for 100ng/ 30µl:

a. Stock is at 330ng/µl

b. Example: Need 34 wells, so total volume of 1020µl

i. X=0.3µl x 34 = 10.2µl of IL-8 + 1010µl of media=1020µl

3. Isolated PMN need to be resuspended to $4x10^5$ PMN/mL so that every 50µl contain $2x10^4$ PMN.

4. Take 950µl of cell suspension and spun at 1500 RPM for 2min. Resuspended pellet in 950µl of media or APC treatment media.

5. Pretreatment time: while loading wells with 30µl of PMN media or media containing 100ng of recombinant bovine IL-8 (approximately 10 min).

6. Put filter lid on well plate.

7. Add PMN to the top of the filter and allow to migrate for 30 min at 37ºC, 5% CO_2.

8. After 30 min migration incubation, eliminate non-migrating PMN remaining on top of filter using a kimwipe (swipe quickly over plate) (as indicated by manufacturers).

9. Add ice cold 2mM EDTA PBS (pH 7.4) solution to remove migrated PMN that had attached to bottom of filter membrane and incubate for 30 min at 4ºC.

10. After the 30 min incubation, eliminate remaining PBS solution on top of the filter using a kimwipe.

11. Centrifuge the chemotaxis plate at 400 x g at 4ºC for 10 min to obtain remaining PMN in the filter.

12. Remove the filter membrane and use a funnel plate to transfer chemotaxis plate contents to an ELISA 96-well plate by centrifuging at 400 x g for 10 min at 4ºC.
13. Once in 96 well plate, add MTT to all sample wells of ELISA plate.
14. After incubating for 5 h at 37ºC with constant agitation, add 100µl of extraction buffer to wells and OD read at 595nm.
15. Sample OD is compared to PMN migration towards bovine IL-8 alone.
16. PMN chemotaxis towards bovine IL-8 alone is set to 100% migration. APC pretreated PMN migration is calculated as percent difference from IL-8 alone samples.

A.5 Alpha toxin add-back protocol

Purpose: Determine the role of α-hemolysin in host cell signaling and gene expression during infection.

Reagents:

Sterile 2mL and 500 μL Eppendorf tubes

Ice-cold PBS (pH 7.4)

Ice bucket

Tissue culture tubes

Recombinant α-hemolysin

Trizol

Western Lysis Buffer

PMN media: 10%FBS DMEM + 1% L-glutamine

Procedure:

1. Once PMN are isolated following SOP, count cells and resuspend pellet to $2x10^7$ PMN/ mL in media.
2. Calculate infecting doses (MOI 10) for all strains.
3. Calculate CFU/mL for the bacteria you washed yesterday:

(average of CFU) x 40 x $10^{\text{(dilution from plate)}}$ = CFU / mL

4. Calculate infecting doses:

*What you want/what you have= mL to add

Example:

($2x10^8$ CFU/ mL) / CFU per mL from washed bacteria = y x 5mL= (mL to add from bacterial culture) + (media to add to bring up to 5mL)

5. Make dilutions for recombinant alpha toxin.

a. Aliquots in tin can in refrigerator.

    a. Each aliquot is at 0.5µg/µl. Need stimulation media to be at 0.5µg/mL. (Example: Add 5 µl of alpha toxin to 5mL of media).

6. Once bacterial dilutions and alpha toxin solutions are made, put 1mL of PMN into labeled tissue culture tubes.
7. Add 1mL of bacterial dilutions to pertaining sample tubes.
8. Carefully vortex and place into incubator for 30 min.
9. While incubating, prepare 50mL PBS solution with 50 µl of NaF and 10 µl of Na_3VO_4.
10. Prepare western lysis buffer.

    Stock in the refrigerator.

    Calculate 200 µl of lysis buffer per sample and add 10 µl/mL of protease inhibitor to lysis buffer.

    (Example: If 3mL of lysis buffer, you need 30 µl of protease inhibitor).

11. Label set of 2mL tubes for saving supernatants and 2 sets of 500 µl tubes to collect lysates for western analysis.
12. Once 30min are over. Centrifuge samples at 1000 RPM, 2 min, 4°C with brake.
13. Decant supernatants into labeled 2mL tubes (Store at -20°C).
14. Resuspend pellets in approx. 1mL of PBS solution.
15. Centrifuge.
16. Keep samples on ice at ALLTIMES!!!!
17. Decant supernatants (careful pellet does not go with supernatant!!!!).
18. Add 200 µl of lysis buffer at 4°C.
19. Let sit on ice for 15 min (Vortex every 5 min).

20. At 15 min, collect lysates from tissue culture tubes and put into one set of labeled 500 µl tubes.
21. Centrifuge at 3500 RPM, 4°C, 10 min.
22. Add supernatants from centrifuged tubes to second set of 500 µl tubes.
23. Store at -20°C for bradford assay.

A.6 Bradford Assay

Purpose: Quantify protein in samples for loading consistency in SDS-PAGE.

Reagents:

96-well plate

Coomasie Blue

BSA solution

Western lysis Buffer

Procedure:

1. Prepare 1.7mL Eppendorf tubes with the following dilution:

Tube	Diluent (Lysis Buffer) (µL)	BSA (6mg/ml) (µL)	Concentration (mg/mL)
A	0.0	150.0	6.0
B	62.5	187.5	4.5
C	162.5	162.5	3.0
D	87.5	87.5 from tube B	2.3
E	162.5	162.5 from tube C	1.5
F	162.5	162.5 from tube E	0.8
G	162.5	162.5 from tube F	0.4
H	200.0	50.0 from tube G	0.1
I	200.0	0.0	0.0

2. If starting with different concentration BSA, calculate concentrations using the following equation:

   (ul of BSA)(your concentration of BSA ug/ul)/total volume ul =concentration ug/ul

   Example: Tube B

   (187.5µl)(6µg/µl)/250ul= 4.5µg/µl

3. Vortex dilutions.

4. Add 5 µl of dilution to corresponding well.
5. Add 5 µl of sample to corresponding well.
6. Add 200µl of Coomasie stain to each well.
7. Cover with aluminum foil and place in the plate rocker in tissue culture room at 37°C for 30 minutes with constant rocking.
8. Take to Dr. Akers lab and read in Quant plate reader at 562nm (Open Becky Bradford protocol).
9. Export file to excel.
10. Organize data to match standard curve concentrations to OD values.
11. Subtract blank (Tube I) from ALL samples.
12. Make a scatter plot graph and determine your R^2 value. (Make sure as close to 1 as possible).
13. From slope equation, determine unknown sample concentrations.
14. Calculate quantity of sample you will need to add 30µg/well and 1 X Laemmli buffer for western.

A.7 Western lysis buffer recipe

Purpose: Prepare stock for future experiments.

Reagents:

Quantity to add (total 50mL)	Reagents	Final Concentration
2.5 mL	TrisHCl (1M)	50mM
0.2 mL	EDTA (0.5M)	2mM
7.5mL	NaCl (1M)	150mM
0.5mL	NP-40	1%
250 mg	Deoxycholate	0.5%
2.5mL	Glycerol	5%
36.8mL	Distilled water	

Procedure:

1. When everything is in-solution, filter sterilize.
2. Keep in the refrigerator and keep sterile.
3. When using add 10μL of protease/phosphatase inhibitor cocktail / mL of lysis buffer.
    a. Protease/phosphatase inhibitor cocktail must be cold at all times and kept away from the light!

A.8 Western blot analysis

Purpose: Demonstrate protein expression of infected cells.

Reagents:

Ice bucket

6 X Laemmli Buffer (40% glycerol, 12% SDS, 250nM Tris, 1% β-mercaptoethanol, 0.01% bromophenol blue, pH 6.8)

Thermocycler at 95°C

Microcentrifuge at 4°C

Running Buffer

Transfer Buffer

TBS (10X: 22g ofTris Base, 80g NaCl, bring up 1L, pH7.6)

TBST (1XTBS + 1%Tween20)

Bovine Serum Albumin (BSA)

Procedure:

1. Samples need to be on ice at all times.
2. Label 1.7mL Eppendorf tubes with sample you will add.
3. Add the corresponding amount of sample need to have 30μg of protein and 1 X Laemmli (as calculated from Bradford Assay) and place on ice until all samples are ready.
4. Boil samples in thermocycler (95°C) for 5 minutes.
5. Place samples on ice and centrifuge at 3000 RPM, 4°C, 30 seconds.
6. Place on ice. Samples are ready to be loaded in western gels.

7. Load samples in an SDS-PAGE gradient gel (Invitrogen; Carlsbad, CA) and run at 125 volts for 90 minutes.
8. Transfer proteins onto PVDF membrane for 90 minutes at 30 volts.
9. Block nonspecific binding on membranes using 3% BSA-TBST for 1 hour.
10. Incubate membranes with primary antibody overnight at 4°C.
    a. Primary antibodies include phospho- and total MAPK p38 (Cell Signaling, Danvers, MA), phospho- and total ERK (Cell Signaling, Danvers, MA), phospho- and total JNK (Cell Signaling, Danvers, MA), and phospho- MKP-1 (Cell Signaling, Danvers, MA).
11. Incubate membranes with secondary antibodies 1h at room temperature.
    a. Secondary antibodies included anti-rabbit (Fisher Scientific, Suwanee, GA) and anti-mouse (Santa Cruz Biotechnology, Santa Cruz, CA) conjugated antibodies with horse radish peroxidase).
12. Develop membranes using ECL (General Electric Healthcare, UK) as indicated by manufacturer.
13. Develop membranes in Chemidoc (BioRad, Hercules, CA) and calculate band densities.

A.9 Reverse Transcriptase Real -Time PCR

Purpose: Quantify gene expression.

Reagents:

RNeasy® Mini Kit (Qiagen, Valencia, CA)

Superscript II (Invitrogen; Carlsbad, CA)

TaqMan® Universal PCR Master Mix (Applied Biosystems; Foster City, CA)

ABI 7300 Real-Time PCR System

Procedure:

1. Isolate total RNA from samples using RNeasy® Mini Kit (Qiagen; Valencia, CA).
2. Synthesize complimentary DNA from sample mRNA using reverse transcriptase and bring samples up to 10ng/μL with DNase/RNase free water.
3. For quantitative Real Time PCR analysis, use TaqMan® Universal PCR Master Mix and the ABI 7300 Real-Time PCR System.
4. Add 300nM of both forward and reverse primers, 100nM of 6-carboxyfluorescein (FAM) probe, 2X TaqMan® Universal PCR Master Mix, and 50ng cDNA per well for a total of 25μL.
5. Compare cycle threshold (Ct) values of a stable housekeeping gene to Ct values of desired gene by subtracting values (ΔCt).
6. Compare the difference in Ct values (ΔCt) to the unstimulated cell ΔCt values (ΔΔCt) by subtracting.
7. This difference is then calculated for fold induction ($2^{-\Delta\Delta Ct}$).

A.10 Flow Cytometry for determination of intracellular FITC-labeled *S. aureus*

Purpose: Quantify intracellular *S. aureus* in infected PMN and infected, apoptotic PMN

Reagents:

Fluorescein isothiocyanate (FITC)-labeled bacteria (Sigma, St. Louis, MO)

Propidium iodide (PI) (Invitrogen, Carlsbad, CA)

Trypan blue (Sigma, St. Louis, MO)

PMN media

10% FBS, 1% L-glutamine, colorless DMEM

HBSS (Atlanta Biologicals Lawrenceville, GA)

Flow cytometer tube

Accuri C6 Flow cytometer (Benton Dickinson, San Jose, CA)

Procedure:

1. Conduct infection.
2. Collect samples by centrifuging 100 x g, 4°C, for 2 min.
3. Discard supernatants. Resuspend pellets in 1mL of HBSS.
4. Re-centrifuged samples.
5. Discard supernatants. Resuspend pellets in 1mL of HBSS.
6. Add cell suspension containing $1x10^6$ PMN to flow cytometer tube.
7. Add 5µl of PI and let sit at room temperature for 5 min.
8. Analyze samples using Accuri C6 Flow cytometer to detect FITC and PI.
9. After the first reading, add 50%/vol of trypan blue to each sample to quench extracellular FITC- *S. aureus* and only account for intracellular FITC.

A.11 Griess Assay Kit Colometric (Sigma Aldrich #23479)

Purpose: Measure Nitric Oxide in supernatants collected from PMN infections

Reagents:

$NaNO_2$ standard solution

$NaNO_3$ standard solution

Buffer solution (20mM, pH 7.6)

Nitrate reductase

Enzyme Co-factors

Griess Reagent A

Griess Reagent B

Procedure:

1. Prepare nitrate reductase and enzyme co-factors by adding 1.2mL of buffer solution to each lyophilize sample.
2. Make standard curves for $NaNO_2$ (nitrite) and $NaNO_3$ (nitrate+ nitrite) in 96-well plate.

$NaNO_2$ or $NaNO_3$ (μM)	Dilutions	Buffer solution (μL)
(A) 0.00	0 μL	100μL
(B) 0.31	50 μL of C	100μL
(C) 0.90	50 μL of D	100μL
(D)2.80	50 μL of E	100μL
(E) 8.30	50 μL of F	100 μL
(F) 25.00	20μL of standard (100μM)	130 μL

3. After dilutions are made, add 80 μL of unknown samples to 96 well plate.
4. For $NaNO_3$ samples, add 10μL of nitrate reductase and 10μL of enzyme co-factors and incubate for 2h at room temperature.
5. For $NaNO_2$ samples add 20μL of buffer solution and continue on to developing.
6. After incubation, add 50μL of Griess Reagent A and mix.

7. Wait 5 min then add 50µL of Griess Reagent B and mix.
8. Wait 10 min at room temperature and measure absorbance at 555nm.
9. Plot the standard curves and determine sample concentration.

A.12 Murine Intraperitoneal Lavages and PMN isolation

Purpose: Isolate primary murine PMN for gene expression analysis

Reagents:

3% Thioglycollate (aged for atleast 1 month) (Sigma Aldrich, St. Louis, MO)

Dexamethasone (Sigma Aldrich, St. Louis, MO)

LPS (Sigma Aldrich, St. Louis, MO)

3mL and 5mL syringes

Ice-cold 1mM EDTA + PBS (pH 7.4)

C57BL/6 mice (CRC vivarium)

Red blood cell (RBC) lysis buffer:

8% NH_4Cl, 0.01M EDTA (pH 7.4)

Procedure:

1. Inject 1mL of aged (atleast 1 month) 3% thioglycollate into peritoneal cavity of all mice 24h prior to harvest.
2. Inject 3mg/kg of dexamethasone in to the peritoneal cavity of select mice or sterile PBS into the peritoneal cavity of control mice 18h prior to harvest.
3. Inject 100µg of LPS into the peritoneal cavity of select mice 2 hours prior to harvest.
4. Harvest:
    a. Place mice in CO_2 chamber for 1-2 min.
    b. Check for vital signs (feel heart rate, poke eyes, or pinch paws) and make sure mouse is dead.
    c. Use tweezers and scissors to cut skin/fur and expose chest and peritoneal cavity.

d. With 5mL syringe, add 10mL of ice-cold PBSE. Massage the peritoneal cavity to rinse all organs and get as much PMN suspension as possible.
e. Extract PBSE + PMN solution and place on ice until reach lab.

5. In the lab, bring sample volume up to 15mL with ice-cold PBSE.
6. Centrifuge samples at 100 x g, 10 min, 4°C, without brake.
7. Discard supernatant and resuspend pellet with 5mL of ice-cold PBSE.
8. Centrifuge samples at 100 x g, 10 min, 4°C, without brake.
9. Discard supernatant and resuspend in ice-cold RBC lysis buffer.
10. Vortex and let sit on ice for 10 min.
11. Centrifuge samples at 100 x g, 10 min, 4°C, without brake.
12. Discard supernatant and resuspend pellet in 600µL of Qiagen RLT buffer + β-ME (10µL/mL of RLT).
13. Store samples in -80°C until RNA isolation.