Modulation of Innate Immune Cell Signaling Pathways by *Staphylococcus aureus* and OmniGen-AF®

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Abstract

Staphylococcus aureus causes chronic mastitis in bovines that is difficult to treat with current therapeutics. The goal of this research is to provide information about and improve innate immune responses to infection. Infection can result in host cell apoptosis or programmed cell death. Many pathogens can inhibit apoptosis; thereby acquiring a replicative niche, a reprieve from immune responses, and an escape from treatments. We hypothesize that *S. aureus* inhibits apoptosis in dendritic cells (DC). To investigate our hypothesis, DC were infected with live *S. aureus* (LSA), γ-irradiated *S. aureus* (ISA), or *Streptococcus agalactiae* (*Strep ag*.) for 2 hours. Stimulations of DC included ultraviolet light (UV) and lipoteichoic acid (LTA). Results indicate that γ-irradiated *S. aureus* can inhibit UV-induced apoptosis by upregulating LTA. This research provides information about *S. aureus* infections, but further research is needed to improve responses to this type of infection. One way to improve innate immune responses to infection is by supplementing bovines with OmniGen-AF®, a probiotic that restores neutrophil function during immunosuppression. To determine the mechanism by which OmniGen-AF® functions, wildtype, MyD88 KO, and TLR4 KO mice were fed either normal chow or supplemented with OmniGen-AF® for two weeks. Mice were immunosuppressed with dexamethasone and challenged with LTA. LTA overcame immunosuppression in a TLR4-depenent manner

regardless of supplementation with OmniGen-AF®. Overall this research supplies knowledge about *S. aureus* inhibition of apoptosis in DC and *S. aureus* LTA activation of PMN regardless of immunosuppression or supplementation with OmniGen-AF®.

Dedication

This work is dedicated to my family and boyfriend; Tricia and William Johnson, Emma Johnson, Mary Anne Gresham, Mary Sue and Perry Lee Johnson, Norman and Anne Gresham, and Tyler Lewandowski.

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List of Abbreviations

Full Name	Abbreviation
β-mercaptoethanol	BME
Dendritic Cell	DC
Ethylenediaminetetraacetic acid	EDTA
Fluorescein isothoicyanate	FITC
γ-irradiated *Staphylococcus aureus*	ISA
Granulocyte-Monocyte Colony Stimulating Factor	GMCSF
Interleukin-4	IL-4
Lipoteichoic Acid	LTA
Live *Staphylococcus aureus*	LSA
Peripheral Blood Mononuclear Cell	PBMC
Polymorphonuclear Cell	PMN
Propidium Iodide	PI
Staphylococcus aureus	*S. aureus*
Streptococcus agalactiae	*Strep. ag.*
Toll-like Receptors	TLR
Ultraviolet Light	UV

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Chapter 1. Review of Literature

Staphylococcus aureus and Mastitis

Mastitis, inflammation of the mammary gland, costs the U.S. dairy industry over $2 billion annually (Jones, 2009). Costs to the industry stem from a decrease in milk production, an increase in healthcare costs, and animal replacement (Oliver and Sordillo, 1988, Young et al., 1985). *Staphylococcus aureus* (*S. aureus*) causes problematic mastitis that is difficult to treat with current therapeutics. This bacterium causes serious infections in humans and, in some cases, is resistant to antibiotics (Benner and Morthland, 1967). *S. aureus* is a Gram positive cocci and a classical extracellular pathogen; however, research indicates that this pathogen invades cells and becomes intracellular (Bayles et al., 1998, Hamill et al., 1986, Ogawa et al., 1985, Peacock et al., 1999). The ability of this bacterium to flourish inside cells protects it from antibiotic therapy as well as the host immune response.

Immune responses to infection include direct bacterial killing, the death of infected cells, and development of memory (Cline and Lehrer, 1969, Fettucciari et al., 2006, Vischer, 1967). Immune effectiveness can be diminished by *S. aureus*. For example, abscess-formation increases *S. aureus* avoidance of phagocytic cells and many antibiotic treatments (Lam et al., 1963). *S. aureus* also produces virulence factors that prevent the immune system from being effective (Frost et al., 1977). Some of these virulence factors are anchored to the surface of *S. aureus* by sortase A (Mazmanian et al., 1999). Lipoteichoic acid (LTA) is a surface-associated cell wall component which increases shedding of L-selectin (an adhesion molecule) by polymorphonuclear cells (PMN). Also, LTA increases the expression of pro-inflammatory

cytokines interleukin-8 (IL-8), tumor necrosis factor α (TNF α), and granulocyte colony stimulating factor (G-CSF) (Lotz et al., 2004, Neuber and König, 1992). *S. aureus* cell wall components LTA and peptidoglycan (PGN) both induce the production of IgA and IgG (Immunoglobulins) (Lotz et al., 2004, Neuber and König, 1992). PGN also induces a pro-inflammatory response by upregulating chemokine c_c motif ligand 5 (CCL5) in murine langerhans cells (Matsui K Fau - Wirotesangthong et al., 2009). Fibronectin-binding proteins (FNBPs) are *S. aureus* surface-associated proteins that bind with $\alpha_5\beta_1$ integrins for invasion of host cells (Sinha B Fau - Francois et al., 1999). In some cases, it is beneficial to *S. aureus* to prevent uptake and remain in the environment. *S. aureus* prevents phagocytosis by alternative sigma factor B (ASFB)-regulated production of biofilm, a physical barrier between the bacterium and the environment (Rachid et al., 2000). These surface-associated proteins promote *S. aureus*' virulence, especially when in close contact with host cells.

Secreted proteins from *S. aureus* function to reduce host immune responses. One example of a secreted virulence factor of *S. aureus* is protein A, which binds IgG1, IgG2, and IgG4 in the incorrect orientation, preventing the phagocytosis of *S. aureus* by immune cells (Kronvall and Williams, 1969). Prevention of phagocytosis also occurs by *S. aureus* secretion of auerolysin, which cleaves complement thereby preventing opsonization (Laarman et al., 2011). *S. aureus* secretes α-hemolysin, a toxin that forms pores in the membranes of cells to induce lysis of the cell (Harshman et al., 1989). Other toxins produced by *S. aureus* include toxic shock syndrome toxin 1 (TSST-1), enterotoxin A, and enterotoxin B. TSST-1 induces non-specific lymphocyte proliferation with leads to toxic shock syndrome (Poindexter Nj Fau - Schlievert and PM, 1985). Enterotoxin A and B bind to major histocompatibility complex II (MHCII) and induce T cell proliferation. This leads to massive cytokine production and toxic shock syndrome (Hudson et

al., 1995). The production of these virulence factors prevents the immune system from effectively clearing the pathogen.

Mammary Innate Immunity

Dendritic Cells

Dendritic cells (DC) are the bridge between the innate and adaptive immune responses (Steinman, 2006). Their morphology is distinct from other cells in that they have long, fingerlike projections, called dendrites (Billingham, 1948). They are antigen-presenting cells that induce a specific immune response to the invading pathogen (Banchereau and Steinman, 1998). DC survive from 48 hours to 9 days depending on their location in the body and their subset (Chen et al., 2007a, Kamath et al., 2000, O'Keeffe et al., 2002). Granulocyte-monocyte colony stimulating factor (GM-CSF), a cytokine involved in granulocyte growth, is required for survival of DC whereas IL-10, an immunosuppressive cytokine, and transforming growth factor beta 1 (TGF-β1), an immunomodulatory cytokine, both induce death in DC (Chang et al., 2007, Chen et al., 2007b, Ito et al., 2006). DC are vital in responses to infection as they initiates the development of memory responses.

Intracellular *Staphylococcus aureus*

Intracellular *S. aureus* invades the host immune cells via FNBPs which create a bridge with $\alpha_5\beta_1$ integrins and leads to the induction of phagosomes (Sinha et al., 2000) (Figure 1.1). Subsequent phagocytosis of *S. aureus* yields either death, survival in the phagosome, or escape from the phagosome in an α-toxin dependent mechanism into the cytosol (Bayles et al., 1998, Hamill et al., 1986, Kubica et al., 2008, Lowy et al., 1988, Schröder et al., 2006, Tuchscherr et

al., 2011, Vann and Proctor, 1988) (Figure 1.1). Post-invasion of host cells, *S. aureus* has one of three fates. One result of intracellular invasion is replication of *S. aureus* inside phagosomes until the bacteria escape the vacuole and induce host cell death. In contrast, a second possible result of intracellular infection is inhibition of host cell apoptosis. The third possible result is the recognition of intracellular PGN by nucleotide-binding oligomerization domain-containing protein 2 (NOD2) which results in activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFĸB) and cytokine expression (Kapetanovic et al., 2007). (Figure 1.1) These differing results of intracellular invasion by *S. aureus* may be dependent on strain variation.

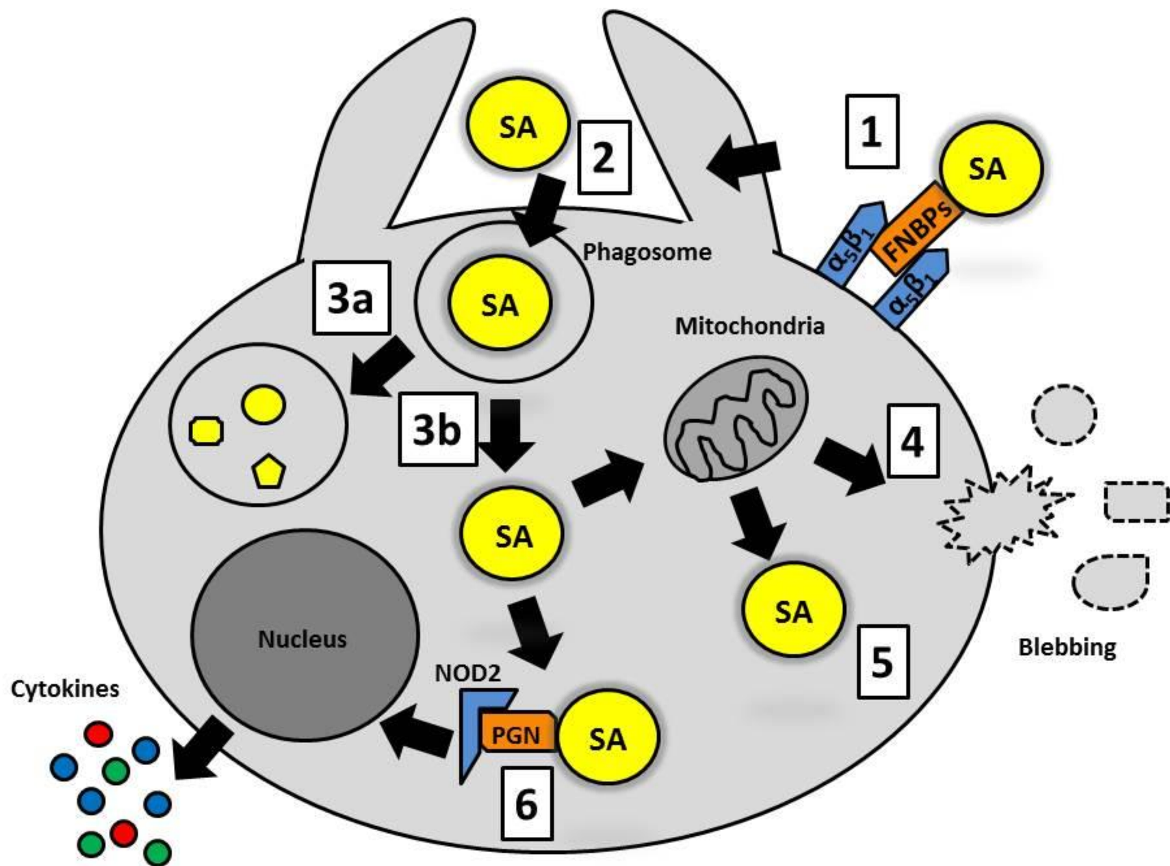

Figure 1.1 Fates of Intracellular S. aureus. 1) *S. aureus* FNBPs create a bridge with host cell $\alpha_5\beta_1$ integrins inducing phagosome formation. 2) Phagocytosis of *S. aureus*. 3a) Degradation of *S. aureus* inside phagolysosomes. 3b) Survival of *S. aureus* and subsequent escape from phagolysosome into the cytosol. 4) Induction of host cell apoptosis. 5) Inhibition of mitochondrial apoptosis and prolonged survival of *S. aureus*. 6) Recognition of PGN by NOD2 resulting in NFĸB activation and subsequent pro-inflammatory cytokine release. (Figure modified from review by Fraunholz and Sinha in 2012. (Fraunholz and Sinha, 2012))

Cell Death Mechanisms in Response to Intracellular Infection

Apoptosis

Apoptosis is the programmed death that a cell undergoes for many reasons, one of which is infection. Apoptosis results in DNA degradation, cellular blebbing, an increase in cell membrane permeability, and phosphatidylserine translocation (Collins et al., 1997, Ormerod MG, 1993, Robertson AM, 1978, Vermes et al., 1995). This form of cell death is very controlled as compared to necrosis, which is the inflammatory death of a cell.

Apoptosis can be induced either intrinsically or extrinsically. Extrinsically induced apoptosis begins a cell signaling cascade via binding of a protein to a receptor on the cell's surface. Fas-Ligand binding to the Fas receptor and TNF-α binding to the TNF receptor can initiate the extrinsic pathway (Wong and Goeddel, 1994). A chain of caspases are then activated. Caspase-8 and 10 are activated downstream of the extrinsic receptors. These caspases lead to the cleavage and subsequent activation of caspase-3 and 7 (Haupt et al., 2003). Eventually the cascade ends in the nucleus and results in degradation of DNA into 180 base pair segments (Kerr, 1972, Peter, 2011). (Figure 1.2).

Intrinsically induced apoptosis begins with inhibition of the anti-apoptotic protein B cell lymphoma 2 (BCL-2) (Cosulich et al., 1997). The release of cytochrome C from the mitochondria follows soon after in response to triggers such as ultraviolet light (UV) (Goldstein et al., 2000). Cytochrome C then binds to apoptotic protease activating factor 1 (Apaf-1), which in turn binds procaspase-9 (Li et al., 1997, Zou et al., 1997). This combination of bound proteins is known as the apoptosome, which binds and activates caspase-9, which in turn activates

caspase-3 and 7 to begin cellular degradation and blebbing (Acehan et al., 2002, Cain et al., 2000, Haupt et al., 2003, Hill et al., 2004, Li et al., 1997, Zou et al., 1999). (Figure 1.2)

Apoptosis is induced by chemical, bacterial, and physical stimulants. Gram negative bacterial cell wall component lipopolysaccharide (LPS) induces apoptosis in some cell types, but induces maturation in DC (De Smedt et al., 1996). UV induces apoptosis in cells via the intrinsic pathway (Wei et al., 2001). Thymol, a chemical antimicrobial, induces apoptosis in DC via the extrinsic pathway and involves the activation of caspase-8 and caspase-3 (Xuan et al., 2010). Apoptotic cells not ingested by phagocytes undergo secondary necrosis, where the cell loses its permeability and acquires the morphology of a necrotic cell (Majno and Joris, 1995). Apoptosis is induced by many stimulants for many different reasons.

Death receptors including Fas, TNF receptors, and TNF-related apoptosis inducing ligand (TRAIL) are expressed by DC (Chen et al., 2006, Diehl et al., 2004, Funk et al., 2000). These death receptors induce apoptosis in DC when the appropriate complementary proteins bind to their respective receptors. However, mature DC resist Fas-induced apoptosis (Ashany et al., 1999). Although Fas does not induce apoptosis in DC, Bim, Bax, and Bak, all pro-apoptotic proteins, do induce apoptosis (Chen et al., 2007b, Lindsten et al., 2000, Wei et al., 2001). Apoptosis in DC is regulated by a family of anti-apoptotic proteins, one of which is BCL-2 (Chen et al., 2007a). BCL-xl , another anti-apoptotic protein, is induced by TLR stimulation on the surface of DC, promoting cell survival (Chen et al., 2007b). Apoptosis of DC in response to intracellular *S. aureus* infection has not been investigated.

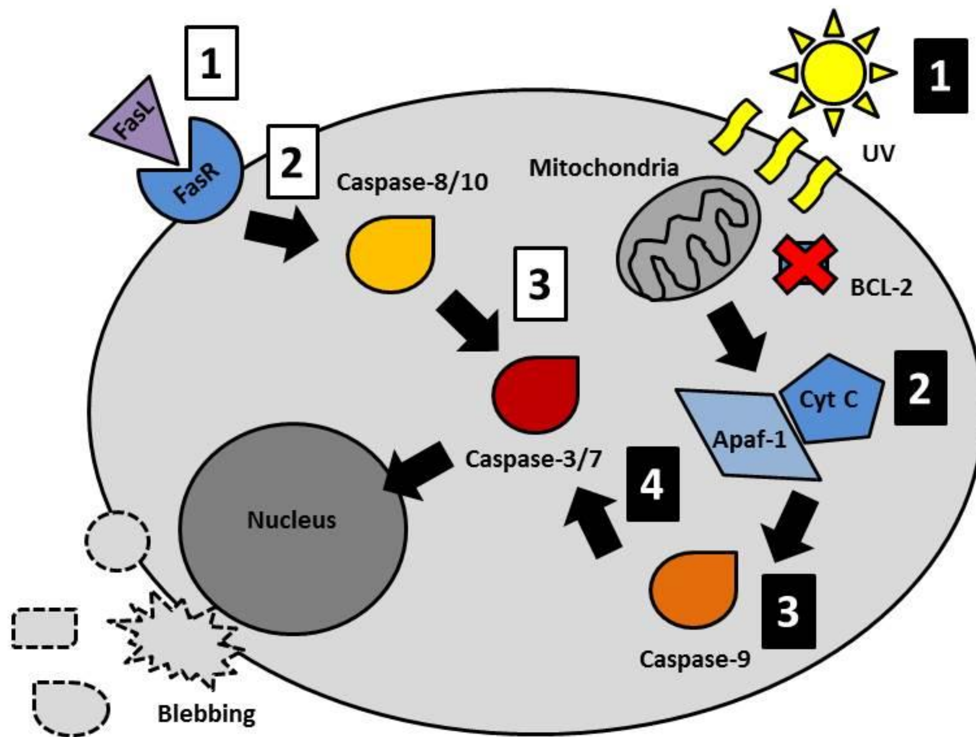

Figure 1.2. Apoptosis Signaling Cascade. White (W) background indicative of extrinsically-induced apoptosis. Black (B) background indicative of intrinsically-induced apoptosis. W1) Fas ligand binding to Fas Receptor to initiate extrinsic apoptosis. W2) Caspase 8 and 10 activation. W3) Caspase 3 and 7 activation. Pathway ends with DNA degradation and blebbing. B1) UV-induced intrinsic apoptosis and inhibition of BCL-2 function. B2) Cytochrome C release from mitochondria and subsequent binding to Apaf-1. B3) Caspase 9 activation by bound cytochrome C and Apaf-1. B4) Caspase 3 and 7 activation. Pathway ends with DNA degradation and blebbing.

Necrosis

Necrosis is an inflammatory form of cell death, involving ruptured membranes and dilation of organelles as well as the release of factors that can cause inflammation (Edinger and Thompson, 2004, Festjens et al., 2006, Kroemer et al., 2005, Zitvogel et al., 2004). Necrosis is a more controlled signaling cascade in contrast to its conventional characterization as unregulated. For example, inhibition of apoptosis and autophagy induces necrosis in mouse kidney epithelial cells (Degenhardt et al., 2006). Also, inhibition of autophagy, a cell survival mechanism, and apoptosis induces necrosis in mouse embryonic fibroblasts (Shimizu et al., 2004). This suggests that necrosis is programmed to occur in the absence of apoptosis or autophagy. Necrotic cell death involves different proteins dependent on cell types. Receptor interacting protein 1 (RIP-1), a kinase recruited to the death-inducing signaling complex (DISC), is required for Fas/TNF-R/TRAIL-R induced necrotic death in Jurkat cells (Chan et al., 2003, Holler et al., 2000). Cyclophilin D is a protein involved in destroying the mitochondrial transmembrane potential. It is required for necrotic cell death (Nakagawa et al., 2005, Schinzel et al., 2005). TNF- α induces apoptosis in many cells, but induces necrosis in a mouse fibrosarcoma cell line (Vercammen et al., 1997). ATP depletion, excess calcium through the plasma membrane, reactive oxygen species, and proteases can all induce necrosis (Bano et al., 2005, Bianchi et al., 2004, Marnett, 2000, McConkey and Orrenius, 1997, Nishimuar, 2001, Wang, 2000, Waring, 2005, Watson et al., 1995, Xu et al., 2001, Yamashima, 2004, Yu et al., 2002). These findings suggest that necrosis is indeed a controlled mechanism in response to many factors.

Staphylococcus aureus Immune Evasion

Intracellular pathogens evade host immune responses via many mechanisms. One example of host cell manipulation by an intracellular pathogen is *Coxiella burnetii*. This pathogen can delay its transit to the phagolysosome by involving autophagic pathways (cell survival mechanism) and a Type IV secretion system (Voth and Heinzen, 2007). Another pathogen capable of modulating host cell signaling pathways is *Listeria monocytogenes*, which lyses the phagolysosome by forming a pore in the membrane of the vacuole and escaping into the cytosol (Goebel and Kuhn, 2000). Migration from a cell membrane-enclosed vacuole occurs in *S. aureus* infections as well. Bayles et al. in 1998 showed that *S. aureus* undergoes endocytosis in epithelial cells, but then escapes the endosome into the cytosol (Bayles et al., 1998). It is suspected that α-hemolysin is required for *S. aureus* to escape the phagolysosome into the nutrient-rich cytosol (Abel et al., 2011). These are just a few of the ways bacteria can avoid destruction.

Staphylococcus aureus and Apoptosis

Many extracellular pathogens induce apoptosis in host cells. *Streptococcus agalactiae* (*Strep. ag.*) induces apoptosis in macrophages in a caspase-independent manner by activating caplain (Fettucciari et al., 2006, Ulett et al., 2005). The extracellular pathogen *Escherichia coli* induces apoptosis in human monocyte-derived macrophages in a time and dose-dependent manner via the Fas/FasLigand pathway (Wang et al., 2011). Induction of apoptosis is the cell's way of preventing the spread of infection to other cells.

Many intracellular pathogens inhibit cellular apoptosis to prolong their survival inside the cell. *Chlamydia*, an intracellular pathogen, can persist inside HeLa cells by three methods. These include degradation of pro-apoptotic proteins (BH3-only proteins of the Bcl-2 family), simple inhibition of apoptosis (upregulation of apoptosis inhibiting proteins), and prevention of necrosis (Rödel et al., 2012). The intracellular pathogen *Mycobacterium tuberculosis* inhibits apoptosis in alveolar macrophages and human mononuclear phagocytes and *Anaplasma phagocytophilum*, another intracellular pathogen, inhibits apoptosis in human neutrophils (Borjesson et al., 2005, Toossi et al., 2012). *Brucella sp.* inhibits cytochrome C release in macrophages, thereby inhibiting apoptosis (He et al., 2006). Bacterial survival is increased by the aforementioned mechanisms.

S. aureus inhibits apoptosis in many different immune cells. In macrophages, *S. aureus* upregulates the anti-apoptotic BCL-2 family proteins and inhibits cytochrome C release from mitochondria (Koziel et al., 2009). Also, the bacteria persist inside macrophage vacuoles for 3-4 days prior to escaping into the cytoplasm and causing cell lysis. This effect is dependent on ASFB, *agr* gene regulator, α-toxin, aureolysin, protein A, and sortase A (Kubica et al., 2008). In monocytes, TSST-1 inhibits apoptosis and increases production of the pro-survival cytokines GMCSF, TNF- α, IL-1β, and activates the transcription factor NFĸB (Bratton et al., 1999). The induction of these cytokines and transcription factor activation indicate an activated cell profile, the opposite of what characterizes an apoptotic cell. In neutrophils, enterotoxin A & B, TSST-1, and phenol-soluble modulin delay apoptosis (Liles et al., 2001, Moulding et al., 1999). Also, adenosine synthase A (AdsA) was required for intracellular survival of *S. aureus* in neutrophils (Thammavongsa et al., 2009). In lymphocytes protein A increases BCL-2 expression (Das et al., 1999). In eosinophils, staphylococcal entertoxins and TSST-1 inhibit Fas-induced apoptosis

(Wedi et al., 2002). In PMNs, LTA acts through cluster of differentiation 14 (CD14) and toll-like receptor 2 (TLR2) to inhibit apoptosis by inducing expression of IL-8, TNF-α, GMCSF, and activation of NFĸB (Lotz et al., 2004). Overall, *S. aureus* increases its survival and ability to cause chronic infections by inhibiting apoptosis in the aforementioned ways.

Neutrophils

Neutrophils are cells of the innate immune system that are often the first responders to invading pathogens. These cells are sometimes referred to as polymorphonuclear cells (PMN) (Hektoen, 1898). Neutrophils produce reactive oxygen species that are vital to pathogen killing and can cause an inflammatory response in the host (Babior, 1984). Neutrophils are capable of phagocytosis and aid in the clearance of apoptotic cells (Kim et al., 2010). They are short-lived cells that undergo apoptosis and are then phagocytized by macrophages for clearance (Brubaker et al., 1977, Greenberg and Grinstein, 2002). Neutrophils express glucocorticoid receptor 1 (GR-1), L-selectin, CCL5, and 60s ribosomal protein ligand 19 (RPL-19) (Menezes-Souza et al., 2012, Peterson et al., 1981, Tedder et al., 1990, Vorachek et al., 2013). GR-1 is involved in signaling responses to glucocorticoids (Murakami et al., 1979). L-selectin acts as an adhesion molecule (Tedder et al., 1989). CCL5 is a chemokine that attracts other immune cells to the site of stimulation or infection (Schall, 1990). Upregulation of these genes are indicative of neutrophil activation. Neutrophil function is decreased in response to glucocorticoids, which implies that during the periparturient period, a cow's neutrophils may be less responsive to assault (Burton et al., 2005).

Innate Pathogen Recognition

Innate cellular responses, including neutrophil responses, to infection rely on pathogen-associated molecular patterns (PAMPs). PAMPs are recognized by receptors on the surface of the cell. One type of receptor that recognizes PAMPs is the TLR (Ozinsky et al., 2000). TLR-2 recognizes LTA, a component of Gram positive bacterial cell walls; whereas TLR-4 recognizes LPS, a component of Gram negative bacterial cell walls (Poltorak et al., 1998, Schwandner et al., 1999). LTA-induced cytokine production in neutrophils occurs in the absence of TLR2 and the presence of CD14, indicating a TLR-independent mechanism for LTA signaling (Hattar, 2006). Apoptosis inhibition occurs in LTA-stimulated neutrophils via CD14 and TLR2 (Lotz et al., 2004). Myeloid differentiation factor 88 (MyD88) is a protein involved in the TLR-2 and TLR-4 signaling cascade. It is downstream of both TLRs. MyD88 activation is crucial for neutrophil recruitment in response to stimuli (Castoldi et al., 2012, Feng et al., 2003, Feng et al., 2008). The TLR signaling pathway is responsible for antigen recognition in innate immune cells.

Immunosuppression during the Periparturient Period

It is common for dairy cows to acquire mammary gland infections during the periparturient period. During this stressful time, the cows are immunosuppressed and are thus more likely to succumb to infection (Cai et al., 1994). Neutrophils decrease expression of L-selectin during the periparturient period. Also, bactericidal activity is decreased in neutrophils during this time (Kimura et al., 1999). Rinaldi et al. in 2008, showed that neutrophils are capable of producing intracellular reactive oxygen species (ROS) during the periparturient period, however extracellular release of superoxide anion and hydrogen peroxide is decreased (Rinaldi et al., 2008). The decrease in production of ROS impairs the ability of neutrophils to kill bacteria.

Immunosuppression occurs as a result of increased circulating glucocorticoids. Dexamethasone, a synthetic glucocorticoid used to induce immunosuppression, increases circulating levels of the immunomodulatory cytokine, IL-10 (Dandona, 1999). The reduction in neutrophil function and increase in circulating immunosuppressive hormones have a direct relationship with increased bovine mastitis during the periparturient period.

Conclusion

In conclusion, the current literature supports the fact that *S. aureus* thrives intracellularly in many cell types. However, the mechanisms by which *S. aureus* causes chronic infections are not all known. We hypothesize that *S. aureus* inhibits apoptosis in a cell type-dependent manner, providing a replicative niche, a reprieve from immune cells, and an escape from treatments. This research is important because it will describe another mechanism by which pathogens may evade treatments. It also provides a base of knowledge about *S. aureus* infections that will aid vaccine and treatment development.

Analyzing the mechanism by which OmniGen-AF® is able to rescue the immune response during the periparturient period is vital because the results will allow for the use of the feed additive in many immunosuppressive conditions and across many species. An increase in neutrophil function is vital for response to infections as these are the first responders to infection.

Overall, this research will provide new knowledge about *S. aureus* infections, the immune responses to this type of infection, and ways to decrease risk of infection. The results from these experiments will aid not only the dairy industry, but also veterinary sciences and human healthcare in the development of vaccines and treatments.

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Chapter 2. γ-irradiated *Staphylococcus aureus* Inhibits Dendritic Cell Apoptosis

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ABSTRACT

Staphylococcus aureus causes chronic mastitis in the bovine and is a major health concern in humans. The goal of this study is to elucidate the mechanism by which *S. aureus* infects dendritic cells (DC) and evades immune responses and antibiotic treatments. Inhibiting apoptosis in DC would provide *S. aureus* with a replicative niche, an escape from treatments, and a reprieve from the immune system. We hypothesized that *S. aureus* inhibits apoptosis in DC. Live *S. aureus* (LSA), γ-irradiated *S. aureus* (ISA), live *Streptococcus agalactiae (Strep. ag.*), lipoteichoic acid (LTA), UV, or a combination of treatments was used to infect DC. Apoptosis was measured via Annexin-V/FITC and propidium iodide (PI) staining by flow cytometry at 24 and 48 hrs post-infection. Infection with ISA significantly inhibited UV-induced apoptosis in DC. The supplementation of LSA with LTA significantly decreased apoptosis in DC induced by UV. Overall, these results indicate a novel mechanism of host immune evasion by *S. aureus*.

INTRODUCTION

Staphylococcus aureus is a Gram positive bacterium that causes chronic mastitis in the dairy cow and is a major health concern in humans. There are currently few effective treatments and no successful vaccine against *S. aureus* mastitis in the bovine. The inability of current therapeutics and normal immune function to clear infection indicates a need for more information about *S. aureus* immune evasion. Many intracellular pathogens inhibit cellular apoptosis to avoid immune responses (Kubica et al., 2008, Rödel et al., 2012). Inhibition of apoptosis benefits the pathogen by providing a replicative niche, a reprieve from immune cells, and an escape from treatments. Prevention of this type of cell death allows pathogens to cause chronic, difficult-to-treat infections. We hypothesized that *S. aureus* inhibits apoptosis in DC.

The DC are the bridge between the innate and adaptive branches of the immune system and they function as professional antigen presenting cells (Banchereau and Steinman, 1998, Steinman, 2006). While there is no information about the effects of *S. aureus* infections apoptosis in DC, *S. aureus* inhibits apoptosis in bovine mammary gland lymphocytes (Slama et al., 2009). In monocytes, *staphylococcal* toxic shock syndrome toxin -1 (TSST-1) inhibits apoptosis by increasing the pro-survival cytokines granulocyte-monocyte colony stimulating factor (GMCSF), interleukin-1β (IL-1β), and tumor necrosis factor α (TNF-α) (Bratton et al., 1999, Kubica et al., 2008). In contrast to these findings, *S. aureus* induces apoptosis in bovine mammary epithelial cells and in human monocytes and keratinocytes (Bayles et al., 1998, Kirker et al., 2009, Sladek et al., 2005). The goal of this research is to determine whether or not *S. aureus* inhibits apoptosis in DC.

MATERIALS AND METHODS

Peripheral Blood Mononuclear Cell (PBMC) Isolation

Multiparous cows from the Virginia Tech Dairy were used in this study according to the Virginia Tech IACUC. Two hundred and fifty mL of whole blood was taken by jugular venipuncture into a glass bottle containing 25 mL of Ethylenediaminetetraacetic acid (EDTA, Sigma Aldrich, St. Louis, MO). PBMCs were isolated with a Ficoll-paque gradient according to manufacturer's instruction (GE Healthcare; Wauwatosa, WI). PBMCs were allowed to adhere to 145*20cm petri dishes (Greiner Bio-One, Kremsmuenster, Austria) for 2 hrs before the non-adherent population was washed off. Adherent monocytes were differentiated into DC using bovine recombinant Interleukin-4 (brIL-4) at 200µg/mL and brGMCSF at 100µg/mL. The brIL-4 and brGMCSF were a generous donation from Dr. Mwangi's lab (College Station, TX). RPMI 1640 media (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Thermo Scientific, Waltham, MA), 1% L-glutamine (Thermo Scientific, Waltham, MA), 0.02% β-mercaptoethanol (BME, Sigma Aldrich, St. Louis, MO), and 2% Hepes (Thermo Scientific, Waltham, MA) was changed on day 3. On day 6, DC were lifted with Accutase (Life Technologies, Carlsbad, CA), counted, and seeded into 100mm petri dishes at $1\text{-}2*10^6$ cells/dish depending on cell yield. At this point, DC media was changed to a colorless media using RPMI without phenol red (Life Technologies, Carlsbad, CA) to reduce any background fluorescence. Viability was calculated by trypan blue exclusion.

DC Stimulations

On day 7 of culture, DC were stimulated or infected. LSA, ISA, or *Strep. ag.* was added to DC at a goal multiplicity of infection (MOI) of 10, 300, or 800 and incubated at 37°C for 2 hrs. The bacteria were washed off with hanks balanced salt solution (HBSS) and gentamicin-containing DC media was added to discontinue infection and stimulation of DC. Secondary stimulations included exposure to UV (250nm) with petri dish lid removed for 5 or 15 minutes. In some cases, these stimulations were preceded by infection with LSA, ISA, LTA (20-40 µg/mL) (bacterial cell wall polymer, 3.92% water, 0.24 Alanine/Phosphate ratio, Sigma Aldrich, St. Louis, MO), LSA supplemented with LTA.

Bacterial Culture and Preparation

Clinical *S. aureus* isolated from a cow at the Virginia Tech Dairy was used in all experiments. ATCC 27956 *Strep. ag.* was used to infect DC as well. *S. aureus* was cultured for 6 hrs in 20 or 100 mL trypticase-soy broth (TSB, BD Biosciences, San Jose, CA) at 37°C, in an incubator, at 185 RPM. *Strep. ag.* was cultured for 12 hrs in 400mL TSB in the same conditions. Bacterial cultures were started with either 1 or 2 colonies to yield enough CFU/mL to reach the desired MOI. Briefly, bacterial concentrations were determined by OD readings (600nm) and washed twice in 20mL PBS prior to resuspension in the appropriate media. Actual concentrations were determined by drop-plating serial dilutions of infecting dose.

Flow Cytometry

Twenty-four or forty-eight hrs post-infection/stimulation DC were lifted with Accutase according to manufacturer's instruction. DC (10^4) in 100μL Annexin-binding buffer (1M Hepes, 1M NaCl, 1M CaCl, Ultrapure water) were fluorescently labeled with Annexin-V-FITC (Life Technologies, Carlsbad, CA) and propidium iodide (PI) (Life Technologies, Carlsbad, CA-) and incubated in the dark at room temperature for 15 minutes. Annexin+/PI- DC are apoptotic, Annexin+/PI+ DC are necrotic, and Annexin-/PI- DC are viable. An Accuri™ flow cytometer (BD Biosciences, San Jose, CA) was used to evaluate phosphatidylserine translocation.

Statistical Analysis

GraphPad Prism® (La Jolla, CA) was used to perform all statistical analyses. In most cases, a one-way ANOVA was performed with a Dunnet's post-test. Correlations were determined by simple linear regression. Alpha level was set to 0.05 and a sample size of 3 was required by power analysis to detect statistical significance.

RESULTS

Initial experiments measured the percentage of apoptotic DC in response to infection with LSA or ISA at an MOI of 10. Untreated DC had a baseline level of ~25-30% Annexin+/PI- cells at 24 and 48 hrs post-infection. LSA and ISA infected DC had similar percentages of Annexin+/PI- cells. There was no significant difference in the percentage of Annexin+/PI- cells between infected DC and untreated DC. However, both infected and untreated DC had lower percentages of Annexin+/PI- DC as compared to UV-treated DC (P<0.01). These results were consistent at both 24 hrs (Figure 2.1A) and 48 hrs (Figure 2.1 D) post-infection/stimulation. The

percentage of necrotic (Annexin+/PI+) DC were not significantly different between treatments at either 24 hrs (Figure 2.1B) or 48 hrs (Figure 2.1E) post-infection/stimulation. However, UV-treated DC at both 24 hrs (Figure 2.1C) and 48 hrs (Figure 2.1F) post-infection were decreased in viability (P<0.0001) (Annexin-/PI-). Overall, *S. aureus* at an MOI of 10 failed to induce DC apoptosis.

Subsequent experiments evaluated the percentage of Annexin+/PI- DC infected with either LSA, ISA, or *Strep. ag.* at 48 hrs post-infection. In order for *Strep. ag.* to induce apoptosis, we used a multiplicity of infection (MOI) of 800 (Colino and Snapper, 2003, Fettucciari et al., 2006). To keep our comparisons unaffected by MOI, LSA and ISA MOIs were increased to 800 as well. Even at this much higher MOI, neither LSA nor ISA increased phosphatidylserine translocation as compared to untreated DC (Figure 2.2A). However, *Strep. ag.* increased the percentage of Annexin+/PI- DC as compared to untreated DC ($P<0.05$) (Figure 2.2A). Percentages of necrotic cells were not significantly different at either timepoint (Figure 2.2B). *Strep. ag.* and UV both reduced the viability of DC as compared to untreated DC ($P<0.05$) (Figure 2.2C).

To determine the effects of MOI on DC apoptosis, we performed a linear regression. There was a negative correlation between LSA MOI and the percentage of Annexin+/PI- DC at 24 hrs, ($P<0.05$, $R^2=0.90$) (Figure 2.3A). At both 24 and 48 hrs there was a negative correlation between ISA MOI and the percentage of Annexin+/PI- DC ($P<0.05$, $R^2=0.72$ and 0.22 respectively). Essentially, as MOI increases, the percentage of apoptotic DC decreases in response to infection with LSA and ISA. Infection with *Strep. ag.* at increasing MOI increased DC apoptosis at 48 hrs ($P<0.05$) (Figure 2.3C).

Percentages of apoptotic DC infected with ISA and then treated with UV for 5 min. were lower than UV-treated DC only, indicating that ISA inhibits UV-induced apoptosis in DC ($P<0.05$) (Figure 2.4A). However, in experiments using DC from different cows, ISA failed to inhibit UV-induced apoptosis in DC (Figure 2.5 A & D). Percentage of necrotic DC were not affected by any treatments (Figure 2.4B). Viability was decreased by treatment with UV regardless of pre-treatment ($P<0.01$), however ISA pre-treated DC were more viable than LSA pretreated DC and UV-stimulated DC only ($P<0.05$) (Figure 2.4C).

Treatment of DC with 20µg/mL LTA significantly reduces the percentage of apoptotic DC. Also, supplementation of LSA (MOI 300) with 20µg/mL LTA, mimicking presumed ISA levels of LTA, decreased the percentage of apoptotic DC as compared to LSA-pretreated DC stimulated with UV only ($P<0.05$) (Figure 2.5A and 2.5D). Percentages of apoptotic DC in response to UV stimulation were decreased by LTA-supplemented LSA as compared to only UV-stimulated DC.

DISCUSSION

Inhibition of apoptosis is a technique pathogens use to avoid normal host immune responses. Many intracellular bacteria can prevent this mechanism of cell death. Chlamydia, for example, degrades pro-apoptotic proteins, upregulates anti-apoptotic proteins, and prevents necrotic HeLa cell death (Rödel et al., 2012). *Listeria monocytogenes* increases the survival of macrophages, thereby increasing its own survival intracellularly (Zou et al., 2011). *Brucella* prevents the release of cytochrome C from the mitochondria in murine macrophages which results in inhibition of apoptosis (He et al., 2006). Our results indicate that γ-irradiated, but not live, *S. aureus* inhibits UV-induced (intrinsic) apoptosis in bovine monocyte-derived DC. Koziel

et. al in 2009 showed that LSA upregulates mitochondrial-associated anti-apoptotic proteins B cell lymphoma 2 (BCL-2) and induced myeloid leukemia cell differentiation protein (MCL-1) in macrophages, which in turn prevents the release of cytochrome C from the mitochondria, an event required for intrinsic apoptosis to occur (Koziel et al., 2009). The differences in our results may be due to the responses of DC versus macrophages to varying *S. aureus* strains. In monocytes, the precursor to DC, Bratton *et. al* showed that TSST-1 from *S. aureus* inhibits apoptosis by increasing the production of pro-survival cytokines GMCSF, IL-1β, and TNFα (Bratton et al., 1999). While our LSA failed to inhibit apoptosis, the strain used in our studies should not express TSST-1 until the post-exponential phase of growth (Peng et al., 1988). Our *S. aureus* is grown for 6 hrs to the exponential phase of growth. Also, the varying effects of *S. aureus* on apoptosis of different cell types may explain the lack of apoptosis inhibition by LSA in our DC as compared to the monocytes in Bratton *et al.*'s study.

We found that ISA failed to inhibit UV-induced apoptosis of DC in four cows as compared to the significant inhibition in one cow. This can be explained by the significant correlation found between days in milk (DIM) of the cow and the percentage of apoptotic DC in response to ISA. All four of these cows were later in DIM. As DIM increased, percentage of apoptotic DC in response to ISA stimulation significantly decreased (Supplementary Figure 2.1). This illustrates that stage of lactation in our cows had a significant effect on how their respective DC responded to infection with ISA. The change in DC responses to infection may be the result of physiological changes during pregnancy. In fact, Burton *et al.* in 1995 showed that cortisol, a hormone increased during the periparturient period in cows, decreases the ability of neutrophil to express activation markers L-selectin and CD18 (Burton et al., 1995). Our results indicate that bovine immune responses, specifically DC responses, to infection vary with stage of lactation.

Irradiation of our *S. aureus* cultures occurs at 2.7kGy over a span of 4 hrs. At this dose of γ-irradiation, bacteria can enter a VBNC state (Trudeau et al., 2012). *Enterococcus faecalis*, another Gram positive intracellular pathogen, in this VBNC state has double the expression of LTA as compared to viable, culturable bacteria (Signoretto et al., 2000). We hypothesized that our ISA is in this VBNC state. While in this state, we believe ISA upregulates surface-associated LTA which then interacts with mitochondrial apoptosis signaling pathways to inhibit apoptosis in DC. We showed that at a concentration of 20μg/mL, LTA supplemented-LSA significantly inhibits UV-induced apoptosis in DC (Figure 2.5A and 2.5D). Overall, ISA inhibits intrinsic apoptosis via increased LTA expression and interaction with mitochondrial apoptosis signaling pathways. The ability of LTA-supplemented LSA to inhibit UV-induced apoptosis was dependent on MOI. Inhibition by LTA-supplemented LSA occurs at a MOI of 300, but not a MOI of 800. This can be explained by the significant correlation between DC responses to infection and MOI as illustrated in Figure 2.3.

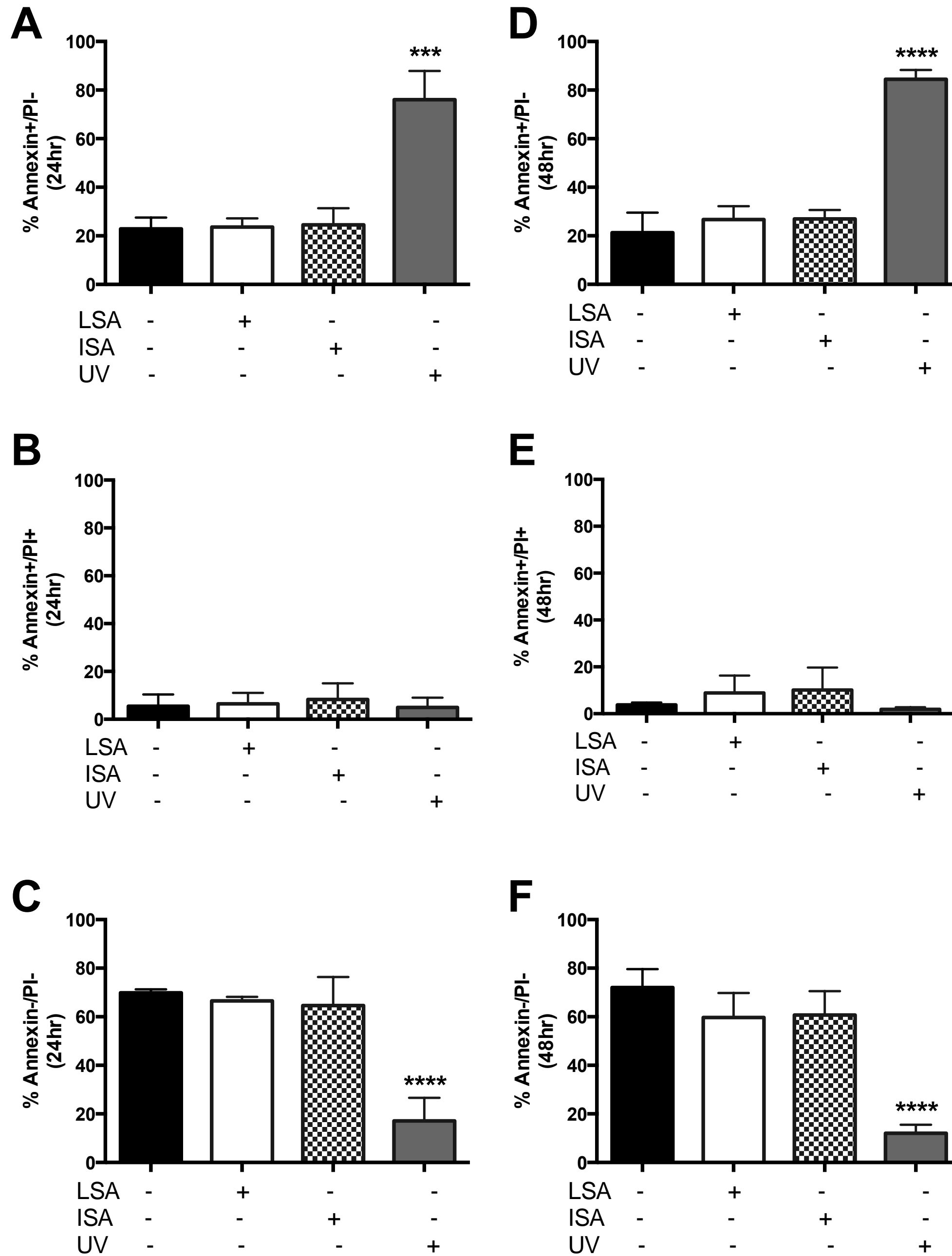
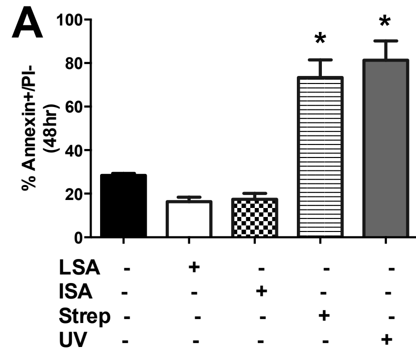
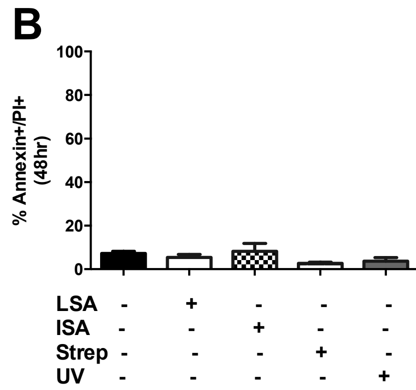


Figure 2.1 Percentage of apoptotic, necrotic, and viable DC in response to LSA and ISA at a MOI of 10. DC were infected for 2 hrs at a MOI of 10 or stimulated with UV for 15 min. at 250nm. Apoptosis (A&D), necrosis (B&E), and viability (C&F) were measured at 24 hrs (A-C) and 48 hrs (D-F). Significance was determined by a one-way ANOVA and a Dunnet's post-test (compared to untreated). n=3. ***$P<0.001$, ****$P<0.0001$.





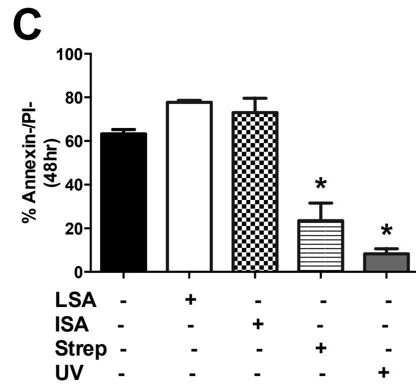


Figure 2.2 Percentage of apoptotic, necrotic, and viable DC in response to LSA, ISA, or Strep. ag. at a MOI of 800. DC were infected for 2 hrs at a MOI of 800 or stimulated with UV for 15 min. at 250nm. Apoptosis (A), necrosis (B), and viability (C) were measured 48 hrs post-infection/stimulation. Significance was determined by a one-way ANOVA and a Dunnet's post-test (compared to untreated). n=3. $^*P<0.05$.

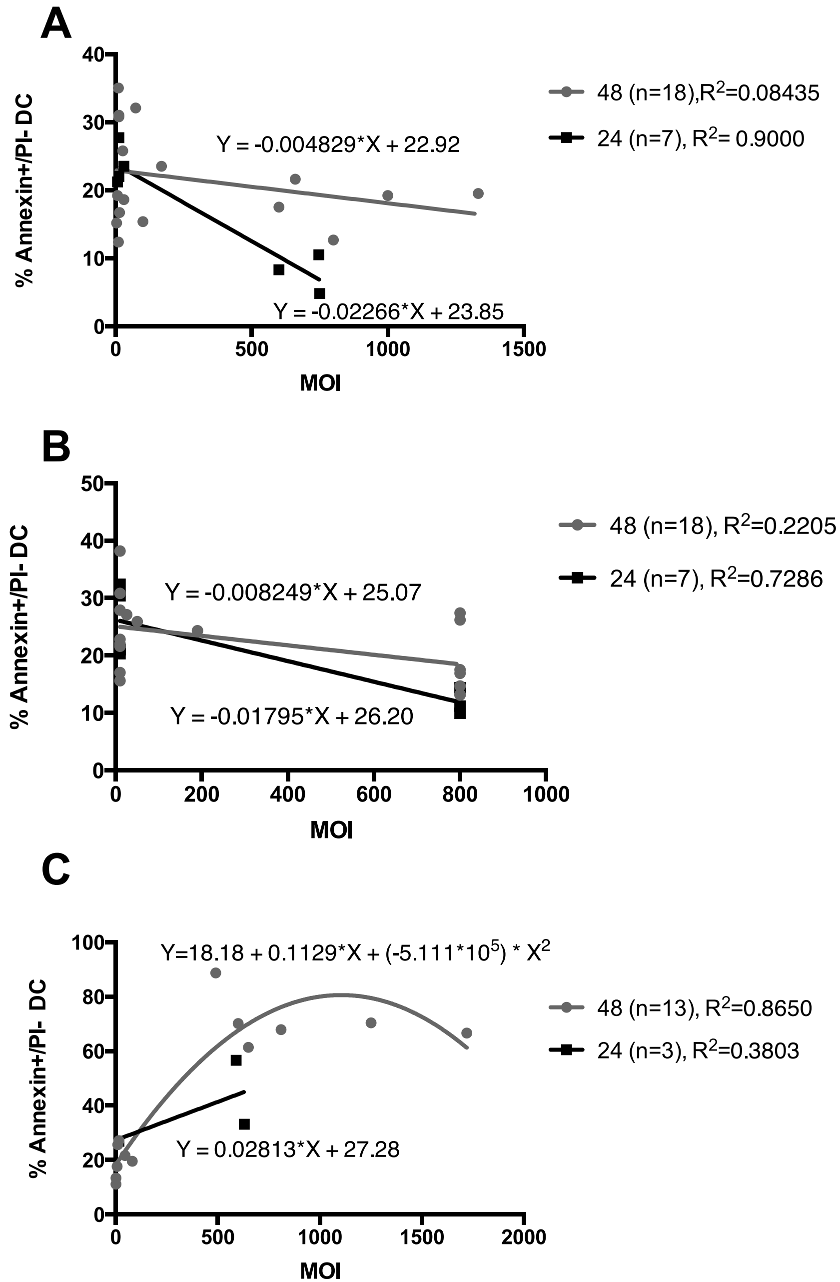


Figure 2.3 Correlation between MOI and percentage of apoptotic DC in response to infection. Linear regression showed a significant negative correlation between LSA-treated DC (A) and MOI at 24hrs. ISA-treated DC (B) were significantly negatively correlated to MOI at 24 and 48 hrs. **P*<0.05. *Strep ag.* (C) MOI and apoptotic DC were significantly positively correlated at 24 and 48 hrs. **P*<0.05.

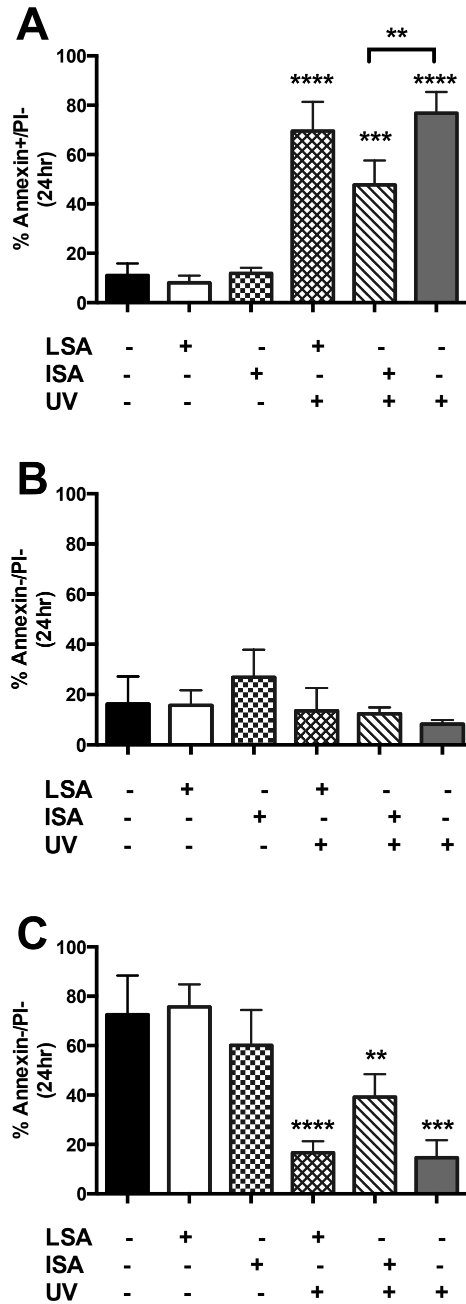


Figure 2.4 Percentage of apoptotic, necrotic, and viable LSA or ISA-treated DC secondarily stimulated with UV. DC were pretreated for 2 hrs with LSA or ISA and were stimulated with UV at 250nm for 5 min. Apoptosis (A), necrosis (B), and viability (C) were measured at 24 hrs. Significance was determined with a one-way ANOVA and a Dunnet's post-test (* compared to untreated; bars compare 2 treatments). n=3. ***P*<0.01, ****P*<0.001, *****P*<0.0001.

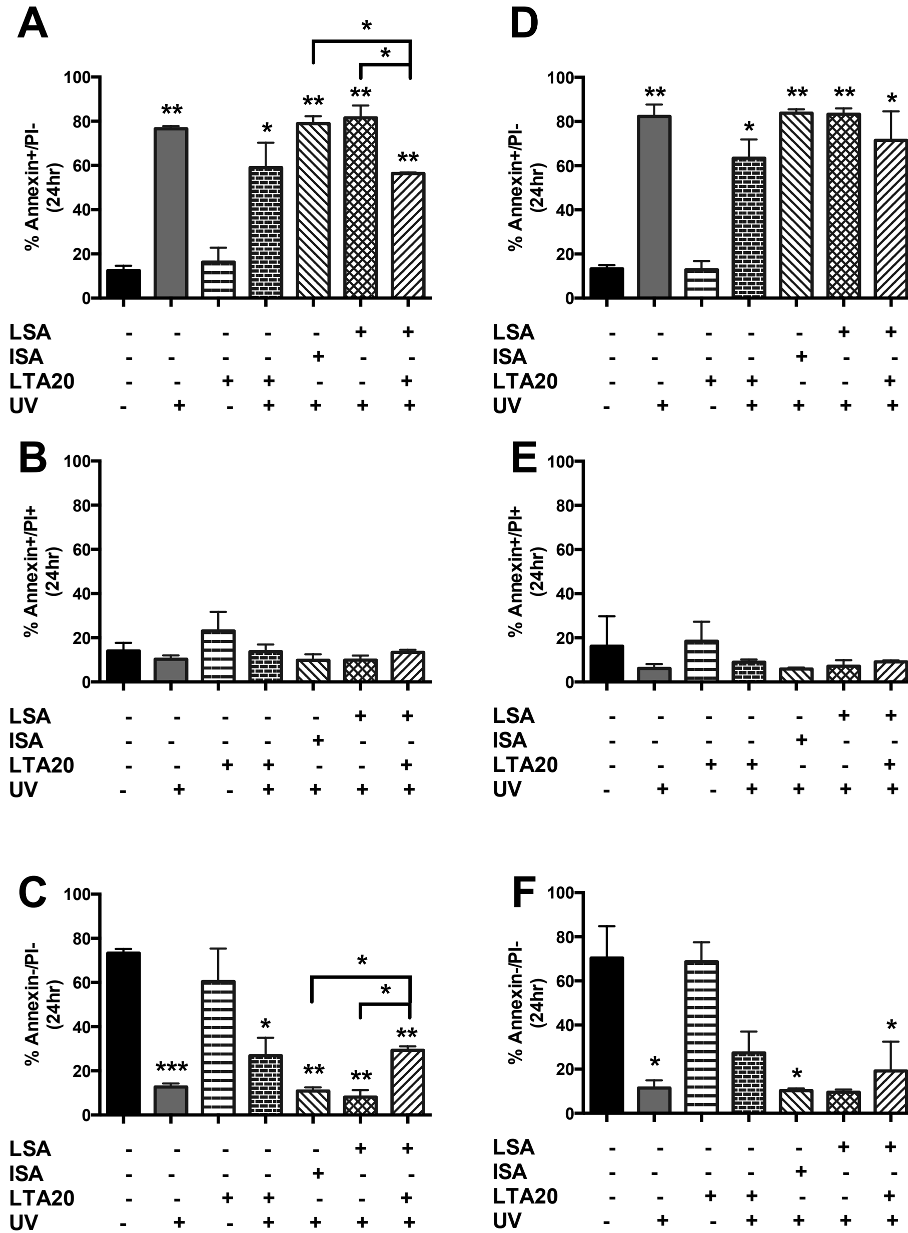
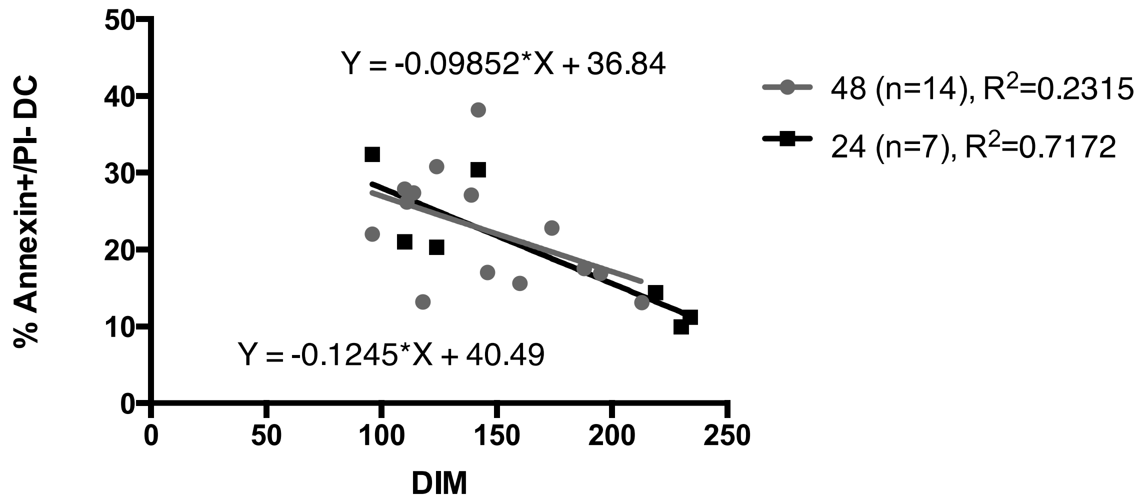


Figure 2.5 Percentage of apoptotic, necrotic, and viable DC in response to infection or stimulation with LSA, ISA, LTA, or UV. DC were pretreated for 2 hrs at MOI 300 (A-C) or MOI 800 (D-F) and supplemented with 20μg/mL LTA. Apoptosis (A & D), necrosis (B & E), and viability (C & F) were measured at 24 hrs. DC were stimulated at 250nm UV for 5 min. Significance was determined with a one-way ANOVA and a Dunnet's post-test (* compared to untreated; bars compare 2 treatments). n=3. *P<0.05, **P<0.01, ***P<0.001.



Supplementary Figure 2.1 Percentage of apoptotic DC in response to ISA correlated to DIM.

Percentage of apoptotic DC at 24 and 48 hrs post-infection with ISA were correlated to DIM. Significance was determined by a simple linear regression. *P<0.05.

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Chapter 3. LTA-Induced Expression of PMN Activation Markers is Unaffected by Immunosuppression or Supplementation with a Feed Supplement

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ABSTRACT

Incidences of bovine mastitis increase during the periparturient period as a result of stress-induced immunosuppression. The first immune cells to respond to infection are neutrophils which recognize pathogen-associated molecular patterns (PAMPs) via toll-like receptors (TLRs). TLRs initiate a signaling pathway involving myeloid-differentiation factor 88 (MyD88) that results in an inflammatory response. This inflammatory reaction is decreased in bovine neutrophils in response to stress. Stress-induced immunosuppression can be counteracted by OmniGen-AF®, a probiotic supplement used to enhance performance in dairy cows (Wang et al., 2009). This probiotic immunomodulator increases neutrophil function during the periparturient period; however, the mechanism by which this occurs is unknown (Wang et al., 2007). We hypothesize that dietary supplementation with the probiotic immunomodulator restores neutrophil function during immunosuppressive conditions in a MyD88-dependent fashion by interacting with TLR-signaling pathways. MyD88 knock-out mice and wildtype (WT) mice were fed normal chow or probiotic immunomodulator-supplemented chow for two weeks, immunosuppressed with dexamethasone, and challenged with lipoteichoic acid (LTA). PMNs were isolated from intraperitoneal lavage and gene expression was evaluated. LTA challenge yielded activated neutrophil gene expression profiles in WT and MyD88 KO mice. MyD88 was not required for neutrophil responses to LTA and supplementation had no effect on neutrophil gene expression profiles in response to LTA. However, TLR4 was required for LTA to induce PMN expression of L-selectin, glucocorticoid receptor 1 (GR-1), and chemokine c_c motif ligand 5 (CCL5). Overall, LTA elicits pro-inflammatory gene expression in a TLR4-dependent, mechanism in PMN.

INTRODUCTION

Mastitis causes major economic hardships for the dairy industry. Incidences of bovine infectious mastitis increase during the periparturient period due to an increase in stress hormones and reduced function of neutrophils (Burton et al., 1995, Burton et al., 2005, Cai et al., 1994). Circulating cortisol, a stress hormone, is significantly increased in cows on the day of calving (Jacob et al., 2001). This increase in cortisol suppresses the normal function of the immune system; specifically, decreases in adhesion factor and chemokine expression are observed during the periparturient period (Burton et al., 1995). Combating this immunosuppression would decrease rates of infection during the periparturient period. OmniGen-AF® is a probiotic immunomodulatory supplement shown to counteract this immunosuppression. Previous studies have shown that mice fed a supplemented diet for two weeks prior to infections with *Streptococcus uberis, S. aureus,* and *Escherichia coli* have significantly less bacterial DNA in the mammary tissue than mice fed un-supplemented chow (Rowson et al., 2011). This is indicative of an increased immune clearance of pathogens in the mammary gland. Increased immune function is characterized by an increase in pro-inflammatory cytokines. One such cytokine, interleukin 1β (IL-1β), may be upregulated by immunomodulatory supplementation due to an elevation in interleukin-1β converting enzyme (ICE) (Wang et al., 2009). Wang et. al showed that in sheep, supplementation with the probiotic immunomodulator during immunosuppressive conditions increases expression of L-selectin on neutrophils, indicating a restorative effect of OmniGen-AF® on neutrophil migration (Wang et al., 2007). In the bovine model, dexamethasone decreases the expression of L-selectin and cluster of differentiation 18 (CD18) (chemokine) in neutrophils (Burton et al., 1995). It is clear that the probiotic

immunomodulator combats immunosuppression by restoring neutrophil L-selectin expression; however the mechanism by which neutrophil L-selectin expression is restored is unknown.

Neutrophils are the first responders to infection. Pathogen-associated molecular patterns (PAMPs) are recognized by toll-like receptors (TLRs) on the surface of neutrophils (Ozinsky et al., 2000). TLR2 recognizes LTA from *S. aureus* (Schwandner et al., 1999). An intracellular protein downstream of TLR2 activation via LTA-binding is myeloid-differentiation factor 88 (MyD88), which is crucial for neutrophil function (Feng et al., 2008). Immunosuppression of host cells occurs by the binding of glucocorticoids to their receptor, glucocorticoid receptor 1 (GR-1) (Vacca et al., 1992). Ogawa et al. in 2005 demonstrated crosstalk between glucocorticoid signaling pathways and TLR signaling pathways (Ogawa et al., 2005). The goal of this research was to determine the mechanism by which the probiotic immunomodulator restores neutrophil function. We hypothesize that supplementation with the probiotic immunomodulator restores neutrophil function in immunosuppressed mice in a MyD88-dependent fashion as illustrated in Figure 3.1.

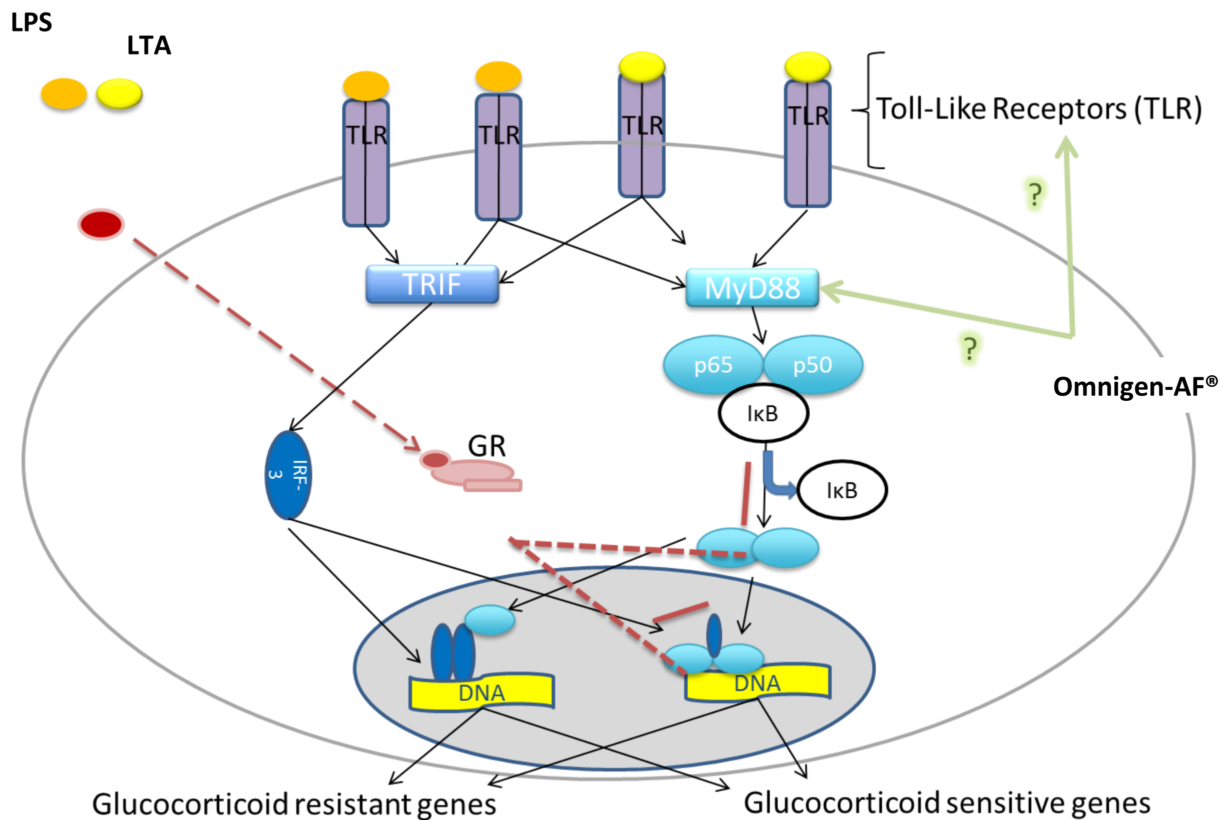


Figure 3.1 Hypothesized mechanism of OmniGen-AF® inhibition of glucocorticoid immunosuppression via GR-1 signaling. Crosstalk between glucocorticoid signaling pathways and TLR signaling pathways has been illustrated previously in macrophages (Ogawa et al., 2005). We hypothesize that supplementation with the probiotic immunomodulator acts in a MyD88-dependent fashion to prevent glucocorticoid-induced immunosuppression. (Figure courtesy of Dr. Isis Kanevsky-Mullarky).

MATERIALS AND METHODS

Mice

C57BL/6J wildtype (WT) and Myd88 knock-out (MyD88 KO) mice were purchased from Jackson Labs at 5-10 weeks of age. All murine experiments were performed according to Virginia Tech IACUC approval. Mice were housed in a biosafety level 1 (BSL-1) room at the Corporate Research Center vivarium. WT mice were age-matched to the MyD88 KO mice. Mice were allowed one week to adjust to the environment of the vivarium before supplementation for 2-3 weeks with either normal chow or OmniGen-AF® supplemented chow. OmniGen-AF® was provided by Prince Agri Products and Harlan Labs (Harlan Laboratories, Inc.; Madison, WI) combined the supplement into a pelleted feed (5g/kg OmniGen-AF®, 995g/kg, 2018 Teklad Global, 18% Protein Rodent Diet). Mouse weights were monitored over the 2-week period.

Immunosuppression and LTA Challenge

All injectable materials were purchased from Sigma-Aldrich, St. Louis, MO, unless otherwise noted. Injectable dexamethasone was diluted in 100% ethanol at 5mg/mL and stored at -20°C in aliquots. Doses were prepared at 3mg/kg body weight (unpublished data from our lab) and diluted in cell-culture grade sterile PBS (Fischer Scientific; Suwanee, GA) prior to use. Dexamethasone was injected into the intraperiotoneal (i.p.) cavity of mice 24 hrs prior to harvest (unpublished data from our lab). Aged (at least one month) 3% thioglycollate broth (1 mL, Sigma Aldrich; St. Louis, MO) was injected i.p. 12 hrs prior to harvest (unpublished data from out lab). *S. aureus* lipoteichoic acid (LTA) (250µg, bacterial cell wall polymer, 3.92% water content, Alanine/Phosphate ratio of 0.024, Sigma Aldrich; St. Louis, MO) or

(Lipopolysaccharide (LPS) (100 µg, 055:B5, Sigma Aldrich; St. Louis, MO) diluted in PBS was injected i.p. 2-4 hrs prior to harvest.

Intraperitoneal Lavage

Mice were euthanized with CO_2 and sprayed with 70% ethanol to clean the area. Scissors were used to cut into the epidermis and separate the peritoneal sac from the skin. Five mL ice-cold 1X Dubellco's PBS (DPBS, Invitrogen; Carlsbad, CA) with 10mM EDTA (Calbiochem; San Diego, SA) was injected into the intraperitoneal cavity. Mice were gently "swished" to begin the lavage and the 5mL PBSE was aspirated from the i.p. cavity. Lavages were expelled into 15mL conical tubes and kept on ice until polymorphonuclear cell (PMN) isolation.

PMN Isolation

PMN were isolated as follows. Volumes of lavage tubes were brought to 10mL with ice-cold PBSE in some experiments. In subsequent isolations, volumes were not adjusted and supernatants were stored for future experiments. In either case, tubes were centrifuged for 10 minutes, 4°C, 100g. Supernatants were either discarded or stored at -80°C for cytokine detection and PMN were washed in 5mL ice-cold PBSE prior to centrifugation. Red blood cell (RBC) lysis buffer (0.8% NH_4Cl, 0.01M EDTA, pH 7.4) was used to lyse any red blood cells present. Pellets were resuspended in 5mL RBC lysis buffer, vortexed, and placed on ice for 10 minutes prior to centrifugation. Supernatants were discarded and pellets were resuspended in 600µL RLT buffer (Qiagen, Valencia, CA) with 10µL β-mercaptoethanol (BME, Sigma Aldrich; St. Louis, MO) /mL RLT buffer. Samples were stored at -80°C until further use.

RNA Isolation, Making cDNA, RT-PCR

Qiagen RNeasy Kits (Qiagen, Valencia; CA) were used according to manufacturer's instruction to isolate RNA from PMN samples. cDNA was made by first heating the RNA (2.2µg) with 250ng random hex primers (Invitrogen; Carlsbad, CA) at 70°C for 10 minutes. Samples were placed on wet ice for 2 minutes prior to centrifugation at 350g, 30 seconds, 4°C. A mixture of 5X Buffer (20.5%/volume), Dithiothreitol (ddTs, 10%/volume), deoxyribonucleotides (dNTPs, 5%/volume, 10mM, VWR, Suwanee, GA), and Superscript II (Reverse transcriptase, 2.5%/volume) was added to the samples. Unless otherwise noted, all aforementioned reagents were purchased from Invitrogen (Carlsbad, CA). Samples were incubated first at 25°C for 10 minutes, second at 42°C for 50 minutes, and third at 70°C for 15 minutes. RNAse-free water (Qiagen; Valencia, CA) was used to bring the cDNA samples to the same concentration (10ng/ µL).

Primer/Probe Mixes were purchased from Applied Biosystems (Foster City, CA). Primer/Probe mixes for chemokine C_C motif ligand 5 (CCL5), L-Selectin, glucocorticoid receptor 1 (GR-1), and ribosomal protein ligand 19 (RPL19) are described in Table 1. Twenty microliters of a mixture consisting of TaqMan® Universal PCR Master Mix (2X, Applied Biosystems; Foster City, CA), forward and reverse primers (300nM, Integrated DNA Technologies; Coralville, IA), and 6-carboxyfluorescein (FAM) probe (100nM, Applied Biosystems; Foster City, CA) was added to 50ng cDNA. The ABI 7300 Real-Time PCR System (Applied Biosystems; Foster City, CA) was used for gene expression detection. Samples were heated in the following cycles: once at 50°C for 2 min, once at 95°C for 10 min, 40 cycles at 95°C for 15 seconds, and finally once at 60°C for one min. Cycle threshold values were used for analysis.

Statistics

GraphPad Prism® was used to perform statistics. Alpha level was set to 0.05. Gene of interest cycle threshold was compared to RPL-19, a housekeeping gene. Differences in cycle threshold (ΔCt) were determined by comparison to the housekeeping gene. A one-way ANOVA and Tukey's post-test were used to determine statistical differences on ΔCt values. ΔCt values were compared to PBS-stimulated PMN to yield ΔΔCt values. Fold-induction was calculated ($2^{-\Delta\Delta Ct}$).

RESULTS

LTA overcomes immunosuppression independent of OmniGen-AF® and requires TLR4, but not MyD88 for CCL5, L-selectin, and GR-1 expression.

In WT mice, LTA induced the expression of L-selectin (Figure 3.2) and GR-1 (Figure 3.3) in all treatment groups as compared to PBS-treated mice ($p<0.05$). Expression of CCL5 in PMN was only increased in WT mice stimulated with LTA ($p<0.05$) (Figure 3.4). Immunosuppression with dexamethasone was unable to significantly decrease the expression of L-Selectin, GR-1, and CCL5 in response to LTA in WT mice (Figures 3.2, 3.2, 3.4). Supplementation with the probiotic immunomodulator had no significant effect on the expression of L-selectin, GR-1, or CCL5 in response to LTA (Figure 3.2, 3.3, 3.4).

In MyD88 KO mice, LTA induced expression of L-selectin in comparison to PBS-treated WT mice ($p<0.05$) (Figure 3.2). Expression of GR-1 was increased in MyD88 KO mice stimulated with LTA ($p<0.05$). Also MyD88 KO mice supplemented with the probiotic immunomodulator and stimulated with LTA upregulated GR-1 ($p<0.001$). Dexamethasone-

treated, LTA-stimulated MyD88KO mice had an increase in GR-1 mRNA fold induction as compared to WT PBS-treated mice ($p<0.001$) (Figure 3.3).

Stimulation with LTA had no effect on CCL5 in MyD88 KO mice (Figure 3.4). Immunosuppression with dexamethasone was unable to prevent the expression of L-selectin or GR-1 in response to stimulation with LTA (Figure 3.2, 3.3). Supplementation with the probiotic immunomodulator did not significantly alter the effects of LTA on MyD88 KO mice PMN gene expression (Figure 3.2, 3.3, 3.4).

Evaluation of TLR4 involvement in the probiotic immunomodulator function showed that in TLR4 KO mice, LTA did not induce the expression of L-selectin (Figure 3.5), GR-1 (Figure 3.6), or CCL5 (Figure 3.7) in PMN. Neither immunosuppression nor probiotic immunomodulator supplementation had any effect on the expression, or lack thereof, of L-selectin, GR-1, or CCL5 in PMN (Figures 3.5, 3.6, 3.7).

DISCUSSION

Immunosuppression that occurs during the periparturient period increases the incidence of infectious mastitis due to a decrease in neutrophil function (Cai et al., 1994). Combating infection during this time could prevent costs to the dairy industry in the form of decreased milk production and healthcare costs (Young et al., 1985). Previous, unpublished work from our lab indicates that MyD88 is required for the probiotic immunomodulator to increase L-selectin, GR-1, and CCL5 expression on neutrophils in response to LPS during dexamethasone-induced immunosuppression. However, we showed that the probiotic immunomodulator has no

significant effect on LTA-induced PMN gene expression profiles regardless of immunosuppression, TLR4 KO, or MyD88 KO. This may indicate that LTA at a dose of 250μg/mouse overcomes immunosuppression without any aid from the probiotic immunomodulator and induces a pro-inflammatory response via neutrophil activation. In fact, coliform, not *S. aureus*, mastitis is most prevalent during the periparturient period (Eberhart, 1977, Matthews et al., 1992). The fact that immunosuppression during the periparturient period has no effect on *S. aureus* infections is supported by our findings.

To our knowledge, this is the first study to propose that LTA induces PMN L-selectin, GR-1, and CCL5 expression in a TLR4-dependent manner. Hattar et al. in 2006 showed that LTA increased the production of tumor necrosis factor-α (TNFα) and IL-1β independent of TLR2 and TLR4, but dependent on CD14. This affect was abrogated by the immunosuppressive cytokine IL-10 (Hattar, 2006). These experiments were performed in isolated human neutrophils as compared to our studies *in vivo* in the murine model. The results of the human *in vitro* studies may not be as biologically relevant as a whole-system model such as the murine.

We also showed that LTA requires both TLR4 and MyD88 to induce the expression of CCL5 in PMN. This agrees with results from Yang et al. in 2001 who determined that LTA functions via TLR4 to induce monocyte expression of the chemokine IL-8 (Yang et al., 2001). These are the first experiments to our knowledge to describe the involvement of TLR4 and MyD88 in LTA induction of PMN CCL5.

Preliminary experiments showed that dexamethasone suppressed the ability of LTA to induce L-selectin, GR-1, and CCL5 expression in murine neutrophils (Supplementary Figures 3.1, 3.2, 3.3). Probiotic-supplemented, immunosuppressed, LTA-stimulated WT mice have

similar levels of L-selectin, GR-1, and CCL5 gene expression as compared to WT mice stimulated with LTA only (Figure 3.2, 3.3, 3.4). This data would suggest that the probiotic immunomodulator restores neutrophil function in response to LTA stimulation. This agrees with previous unpublished data from our lab that illustrates probiotic immunomodulator restoration of neutrophil L-selectin, GR-1, and CCL5 expression in response to LPS stimulation. Our lab also showed (unpublished work) a requirement for MyD88 in the restoration of L-selectin, GR-1, and CCL5 expression in immunosuppressed PMN in response to LPS. Our results demonstrated that MyD88 is not a requirement for the probiotic immunomodulator to maintain LTA-stimulated levels of L-selectin, GR-1, or CCL5 in immunosuppressed mice (Figure 3.2, 3.3, 3.4). Therefore, we can conclude that the probiotic immunomodulator maintains neutrophil expression of L-selectin, GR-1, and CCL5 independent of MyD88.

CONCLUSION

Our results describe a TLR4, MyD88-dependent mechanism by which LTA induces the expression of the chemokine CCL5 in murine PMNs. Also, we show that LTA requires TLR4 to induce PMN expression of the adhesion marker L-selectin and the surface marker GR-1. Neither WT, MyD88 KO, nor TLR4 KO mice remained immunosuppressed by dexamethasone injections after stimulation with LTA. These results indicate that LTA can overcome immunosuppression and we showed that this effect was without the aid of a probiotic immunomodulating supplement that restores neutrophil function in response to LPS stimulation (unpublished data from our lab, Dr. Ortiz-Marty). Overall, we describe a novel signaling mechanism by which LTA overcomes dexamethasone-induced immunosuppression to induce PMN activation.

Table 2.1 Primer/Probe mixes used to detect neutrophil gene expression of activation markers. (Applied Biosystems)	
Gene Name	**Reference Number**
RPL19	Mm02601633_g1
L-selectin	Mm00441291_m1
GR-1	Mm00433832_m1
CCL5	Mm01302427_m1

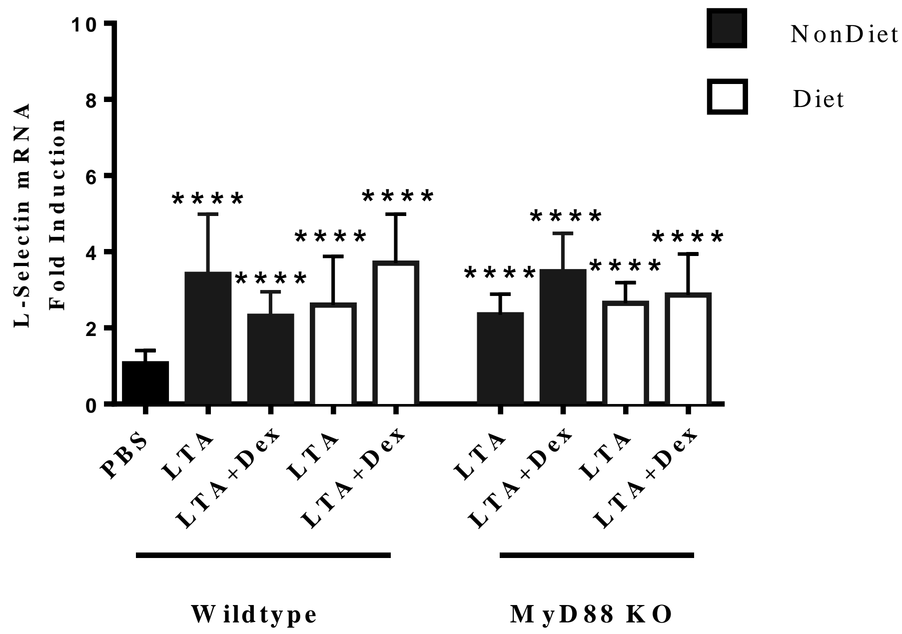


Figure 3.2 L-Selectin mRNA Fold-Induction in PMN from WT and MyD88 KO mice. PMN from immunosuppressed (Dex), LTA-challenged WT and MyD88 KO mice were evaluated for L-selectin expression via RT-PCR. Treatment groups are compared to PBS-treated WT mice. Significance was determined by a one-way ANOVA and a Tukey post test. n=3. ****$P<0.0001$.

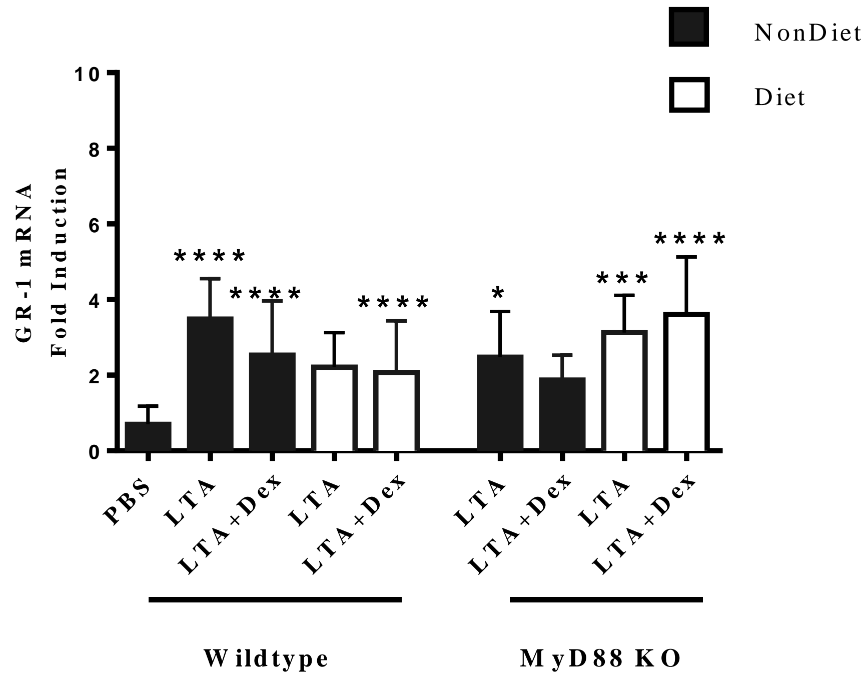


Figure 3.3 GR-1 mRNA Fold-Induction in PMN from WT and MyD88 KO mice. PMN from immunosuppressed (Dex), LTA-challenged WT and MyD88 KO mice were evaluated for GR-1 expression via RT-PCR. Treatment groups are compared to PBS-treated WT mice. Significance was determined by a one-way ANOVA and a Tukey post test. n=3. *$P<0.05$, ***$P<0.001$, ****$P<0.0001$.

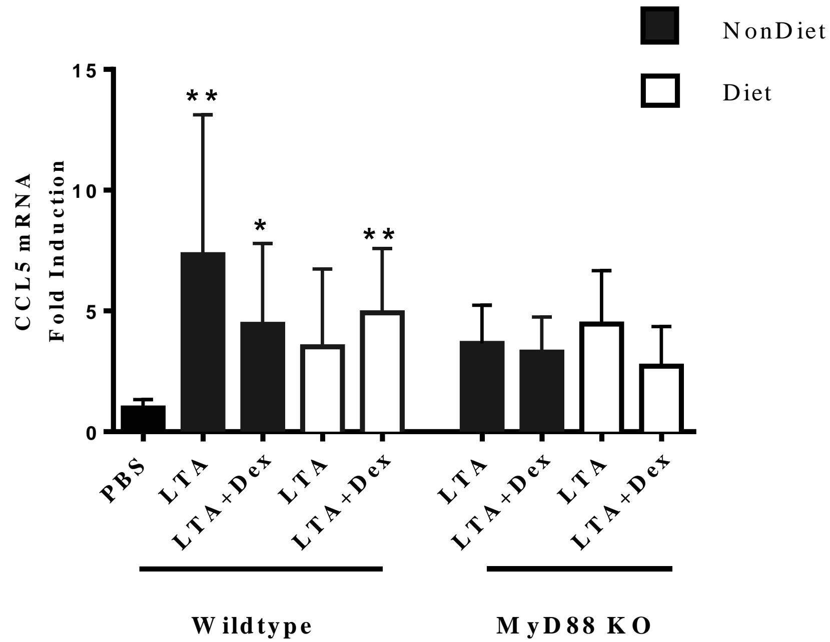


Figure 3.4 CCL5 mRNA Fold-Induction in PMN from WT and MyD88 KO mice. PMN from immunosuppressed (Dex), LTA-challenged WT and MyD88 KO mice were evaluated for CCL5 expression via RT-PCR. Treatment groups are compared to PBS-treated WT mice. Significance was determined by a one-way ANOVA and a Tukey post test. n=3. **P*<0.05, ***P*<0.05.

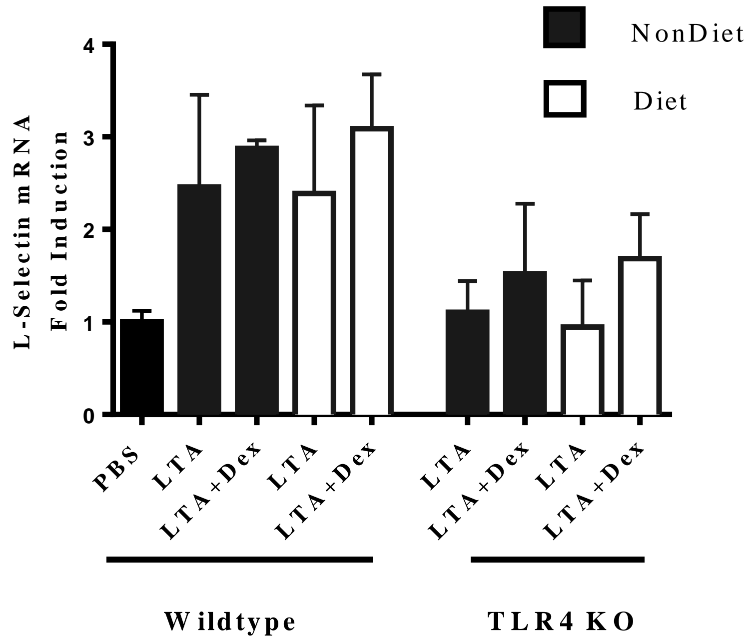


Figure 3.5 L-selectin mRNA Fold-Induction in PMN from WT and TLR4 KO mice. PMN from immunosuppressed (Dex), LTA-challenged WT and TLR4 KO mice were evaluated for L-selectin expression via RT-PCR. n=1.

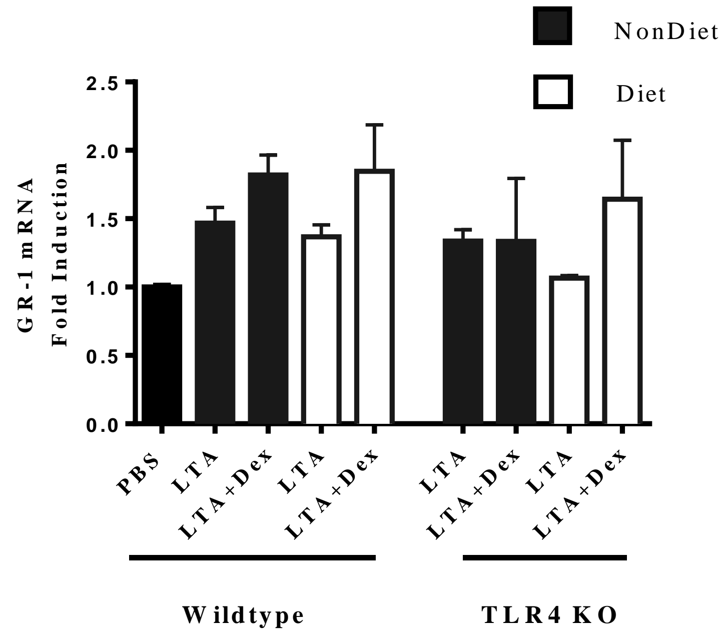


Figure 3.6 GR-1 mRNA Fold-Induction in PMN from WT and TLR4 KO mice. PMN from immunosuppressed (Dex), LTA-challenged WT and TLR4 KO mice were evaluated for GR-1 expression via RT-PCR. n=1.

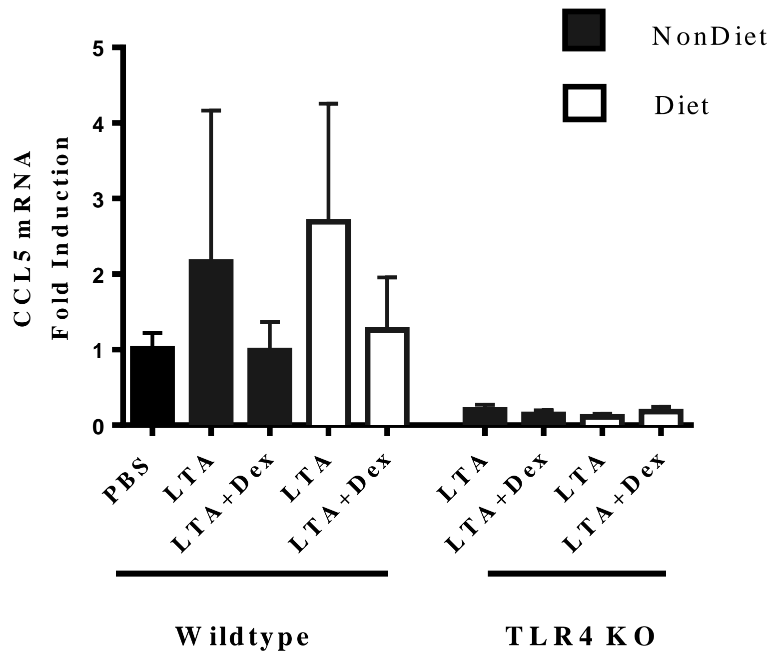
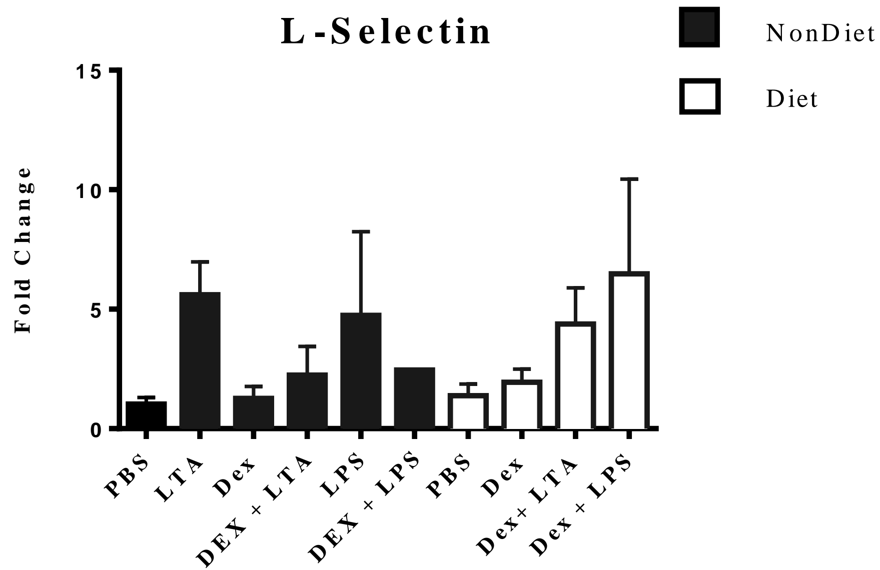
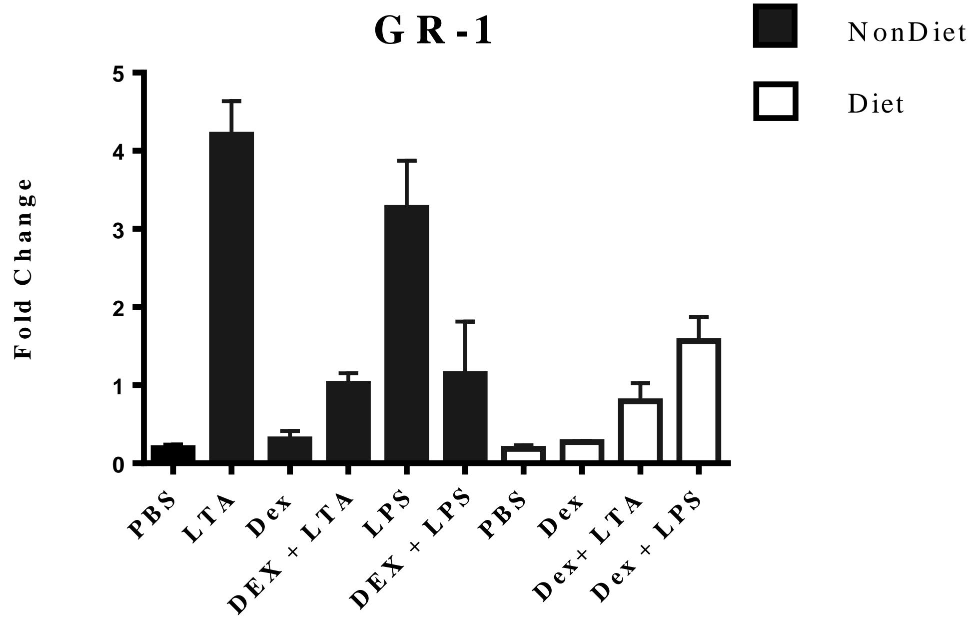


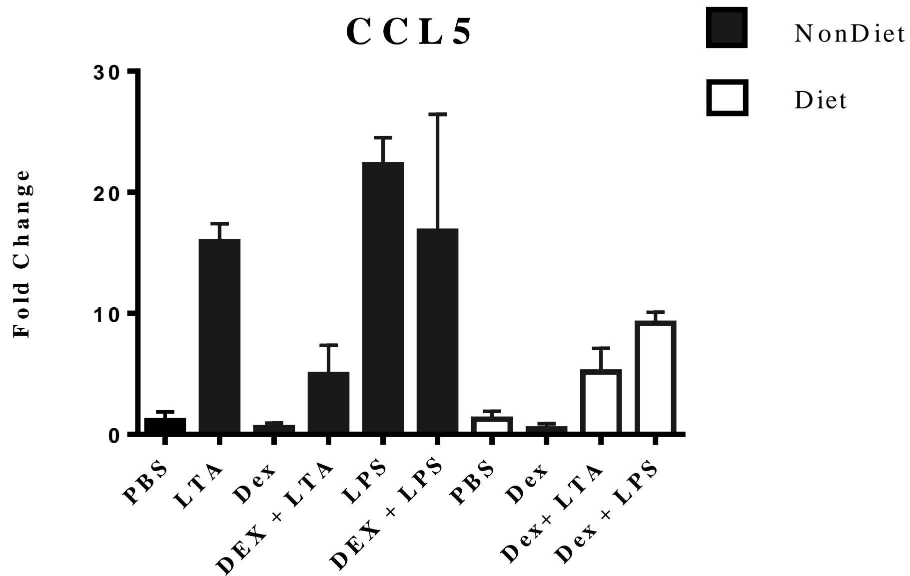
Figure 3.7 CCL5 mRNA Fold-Induction in PMN from WT and TLR4 KO mice. PMN from immunosuppressed (Dex), LTA-challenged WT and TLR4 KO mice were evaluated for CCL5 expression via RT-PCR. n=1.



Supplementary Figure 3.1. L-selectin expression in WT murine PMN in response to LPS, LTA, dexamethasone and combinations of treatments. WT mice were supplemented for 2 weeks with OmniGen-AF prior to immunosuppression (Dex) and challenge with LPS or LTA. n=1.



Supplementary Figure 3.2. GR-1 expression in WT murine PMN in response to LPS, LTA, dexamethasone and combinations of treatments. WT mice were supplemented for 2 weeks with OmniGen-AF prior to immunosuppression (Dex) and challenge with LPS or LTA. n=1.



Supplementary Figure 3.3. CCL5 expression in WT murine PMN in response to LPS, LTA, dexamethasone and combinations of treatments. WT mice were supplemented for 2 weeks with OmniGen-AF prior to immunosuppression (Dex) and challenge with LPS or LTA. n=1.

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Chapter 4. Conclusion and Future Research

S. aureus is a major health concern in both veterinary medicine and in human healthcare as it causes chronic, difficult to treat infections. Increasing our understanding of how *S. aureus* causes chronic infections is important in the development of effective treatments, preventative measures, and vaccines. The goal of the research presented in this thesis was to provide information about the effects of *S. aureus* infections on innate immunity and to evaluate the mechanism by which OmniGen-AF® restores immune function during immunosuppressive conditions.

We found that intracellular γ-irradiated *S. aureus* inhibits intrinsically induced apoptosis in bovine monocyte-derived DC. It is presumed that ISA is in a VBNC state. In order to determine if this VBNC state occurs *in vivo*, future research should determine if other immune cells can damage *S. aureus* in the same way that γ-irradiation does. This would confirm the importance of apoptosis inhibition.

We found that LTA is responsible for ISA inhibition of apoptosis in DC. Overexpression of LTA by ISA in a VBNC state could be responsible for the inhibition of DC apoptosis by ISA. Measurement of LTA on the surfaces of both LSA and ISA would confirm this overexpression. Also, blocking of LTA on ISA would further confirm these results.

We showed this inhibition of apoptosis occurs *in vitro*. These experiments should be performed *in vivo* to confirm their biological relevance. Tracking apoptotic DC in a live bovine model will confirm this inhibition of apoptosis during an infection.

While we showed statistically significant differences in our experiments, most repeats were performed with cells isolated from the same cow and all experiments were performed with the same strain of *S. aureus.* It would be interesting to evaluate the effects of animal and strain variation by performing these experiments with multiple cows and bacterial strains. It is possible that breed, days in milk, lactation number, and previous exposure to *S. aureus* could have an effect on these studies. Also, we would benefit from genotyping our *S. aureus* strains prior to performing these studies and evaluating the ability of each strain to inhibit intrinsically-induced apoptosis. Strain and cow differences could yield different results and discovering those differences would add to the current knowledge about *S. aureus* infections that would benefit the development of treatments and vaccines.

Our results indicate that late-lactation and pregnant cows respond differently to infection. We found a significant correlation between days in milk (DIM) and apoptosis of DC in response to ISA. Future work should evaluate the responses of DC to Gram negative infections to confirm the effects are due to the ability of DC to respond to infection and are not a result of *S. aureus* infections specifically. Determining the effects of glucocorticoids in circulation on the function of DC would provide valuable knowledge concerning immune function during later stages of lactation.

While treatment and vaccine development is still in the distant future, the probiotic supplement OmniGen-AF® is already on the market and available for use. Previous unpublished data from out lab found a MyD88-dependent mechanism by which OmniGen-AF® restores neutrophil function in response to LPS stimulations. My study evaluating the effects of this supplement on murine PMN function during immunosuppressive conditions found that OmniGen-AF® has no significant effect on PMN function in response to LTA stimulation. We

described a novel mechanism of LTA activation of PMN. However, dexamethasone stocks were frozen and thawed more than once and we believe that this possibly had an effect on immunosuppression. Future experiments will use fresh dexamethasone stocks each time to induce immunosuppression.

Our murine model was beneficial as the technology for gene knock-outs is much more prevalent in the mice as compared to bovines. However, mice are not ruminants and therefore differences in responses to infection are possible. Future work should perform this research in the bovine to confirm the effects of OmniGen-AF® during the periparturient period when immunosuppression occurs as a result of circulating cortisol. Our work with LTA is a good way to mimic infection; however live bacteria should be used to determine whether OmniGen-AF® can still function to maintain neutrophil function in the presence of the virulence factors and toxins produced by viable *S. aureus*.

Overall, this research provides a mechanism by which *S. aureus* causes chronic infections and a way to prevent these types of infection during immunosuppressive conditions. However, there are still many questions that need to be answered before treatment and vaccine development can successfully proceed.

Appendix A: Supporting Data

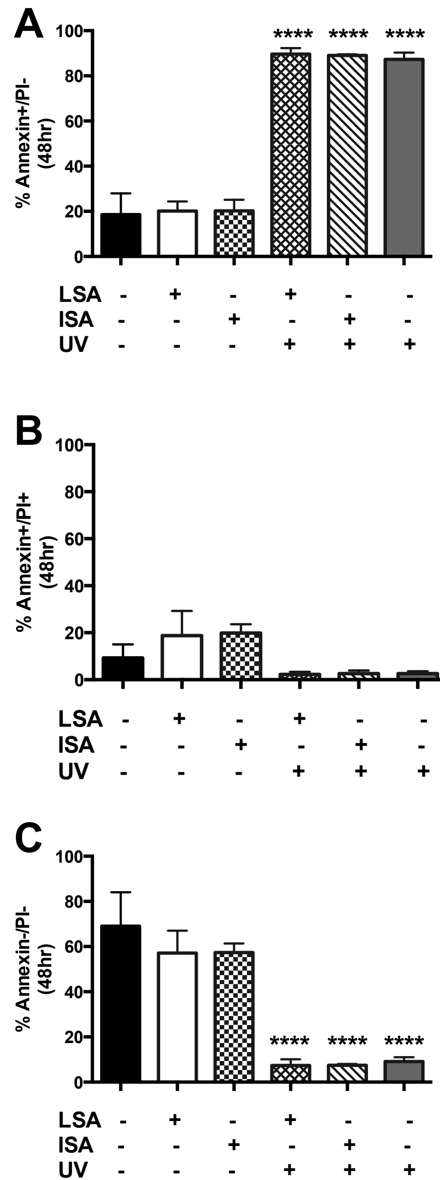

Figure A.1 Percentage of apoptotic, necrotic, and viable DC infected with LSA or ISA and secondarily stimulated with UV. DC were pretreated for 2 hrs with LSA or ISA and were stimulated with UV at 250nm for 15 min. Apoptosis (A), necrosis (B), and viability (C) were measured at 48 hrs. Significance was determined with a one-way ANOVA and a Dunnet's post-test (compared to untreated). n=3. ****P<0.0001.

Appendix B: Detailed Protocols

A.1 Isolation of Mononuclear Cells from Bovine Whole Blood

Reagents:

EDTA

HBSS or PBS

(For Porcine Lymphocytes use 0.3% EDTA in PBS -PBSE)

Ficoll-paque (Amersham, 17-1440-03)

Sterile ddH_2O

2 X RPMI or 2 X MEM, pH 7.4

Procedure:

1. Collect 50-60 ml blood using EDTA (10% of 2x citrate) or 10% of 40 mM EDTA. Transfer blood into 50 ml centrifuge tubes.

2. Spin at 2000 RPM for 30 min at 15°C with brake turned off.

3. Remove 5 ml buffy coat layer using 10 ml pipette in the smallest possible volume with the least amount of RBC's and transfer into a 50 ml centrifuge tube containing 20ml HBSS/PBS and EDTA. Then layer over 12.5 ml ficoll-paque in a 50 ml centrifuge tube.

   -pick up 25 ml of cells in 25 ml pipette

-tip tube so that ficoll-paque almost reaches front edge of the tube, and carefully place one drop of cells just in front of the ficoll. Tip the tube so the cells run onto the ficoll, then slowly add the remaining cells into the tube. As you add more cells, bring the tube back to a near vertical position.

4. Centrifuge for 45 min at 1300-1350 RPM (Beckman CS-6R centrifuge), 25°C with brake off.

5. Take off mononuclear cell band and add to 20 ml HBSS in a 50 ml centrifuge tube with a 10 ml pipette. Try not to pick up much ficoll.

6. Resuspend in HBSS to 45 ml, and spin 10 min at 1,000 RPM, 15°C.

*7a. If RBC's are present in pellet: Resuspend cells in 5 ml HBSS. Add 10 ml ddH_2O, pipette for 18 sec., then add 10 ml 2 X MEM and mix.

*7b. Top off tube with HBSS, then spin 10 min. at 1000 RPM, 4°C. May repeat if necessary.

8. Resuspend cells in 10 ml HBSS, count while cells are pelleting 5 min. 1000 RPM 4°C. Bring volume to 45 mL for centrifuge.

9. Resuspend to use concentration in appropriate medium (approx. 10 mL medium).

* Only if RBC's are present

A.2 Derivation of DC from Monocytes

1. Resuspend the pelleted cells to approximately 8 x 10^7 cells/mL in DC Media for culture in 145*20cm petri dishes.
2. Add GMCSF (100ng/mL) and IL-4 (200ng/mL).
3. Place plate into humidified incubator at 37° C.
4. Culture cells for 3 days.
    a. Cells must be 'fed' new medium on day 3.
    b. Remove plate from incubator to biosafety cabinet.
    c. Carefully remove media from each dish, care is required to avoid disturbing the cells.
    d. Centrifuge media at 1000 RPMs, 15°C, 10 minutes.
    e. Add half the total volume of pre-warmed medium to each well.
    f. Add fresh cytokines to dishes in concentrations mentioned above.
    g. Add the other half of the volume from the spun down old media tubes.
    h. Re-culture the DC for required time up to 7 days.

A.3 Culturing S. aureus

Reagents:

Staphylococcus aureus cultures

Disposable cuvettes

Spectrophotometer

Phosphate Buffered Saline (PBS)

Procedure:

1. Inoculate 100 ml TSB using one colony of *Staphylococcus aureus* from overnight culture on TSA (see protocol Culturing *Staphylococcus aureus* strains).
2. Place TSB in shaker at 37°C overnight (6-18 hrs depending on protocol and target growth phase).
3. Prepare serial dilutions of overnight culture in 15 ml sterile conical tubes.
4. Take OD readings at 600nm of serial dilutions to determine concentrations of overnight stock culture of *Staphylococcus aureus* based on Standard Curve.
5. Transfer 50 ml of overnight TSB culture into 50 ml conical tube.
6. Centrifuge at 3000 rpm for 10 min at 4°C.
7. Remove supernatant into 20% Lysol container and resuspend bacterial pellet in 10 ml PBS. Bring volume up to 50 ml.
8. Centrifuge at 3000 rpm for 10 min at 4°C.
9. Repeat wash.

10. Resuspend bacterial pellet at appropriate concentration (infectious dose). (PBS for infection of mice, RPMI or carbonate buffer for infection of cell cultures (see appropriate protocol)).
11. Confirm all doses by drop plating (SOP 2.5).

A.4 γ-Irradiation of *S. aureus* cultures

Purpose: Irradiation of *S. aureus* to stop toxin protein release.

1. Irradiate bacteria in a Model 109 research cobalt irradiator (JL, Shepherd and Associates, San Fernando, CA) for 4 h.

2. Streak the colonies in EBA plate and incubate overnight at 37ºC to ensure irradiation.

A.5 Annexin/PI Staining for Flow Cytometry

1. Resuspend DC at 10^5 DC/mL in Annexin-Binding Buffer.

2. Add 100µL (10^4) DC to each well of a 96-well flow plate.

3. Add 0.5µL Annexin-V/FITC and Propidium Iodide to appropriate wells.

4. Cover and vortex.

5. Incubate in the dark at room temperature for 15 minutes.

6. Remove from dark, remove cover, add 100 µL Annexin-Binding buffer to each well and mix.

7. Read samples on Accuri Flow Cytometer.

A.6 Intraperitoneal Injection:

1. Hold mouse by tail.
2. Push on shoulders of mouse and move fingers up to just behind the ears.
3. Pull back as much scruff as possible making sure the mouse can still easily breathe.
4. Tuck tail between pinkie and ring finger to expose the peritoneal cavity.
5. Wipe area with 70% ethanol and inject just slightly off-center into the cavity (further in than subcutaneous, but far enough away from organs to be just inside the peritoneal sac.
6. Remove needle quickly and replace mouse in cage.

A.7 Murine Euthanasia

1. Place mouse/mice in cage.

2. Cover cage with lid attached to CO_2 hose.

3. Turn CO_2 tank to about 1 liter per minute.

4. Watch mice carefully to ensure they have stopped breathing.

5. Turn off CO_2, remove mice, and check for vital signs (pinch ears, poke eyes).

A.8 Intraperitoneal Lavage

Reagents:

PBSE (1mM) = 0.146 g EDTA to 500mL sterile PBS.

1. Spray mouse with 70% ethanol.
2. Use tweezers to pull the skin away from cavity.
3. Use scissors to cut a small hole in the skin. Insert closed scissors, then open carefully to widen the hole.
4. Pull skin up to the diaphragm with tweezers, exposing the peritoneal sac.
5. Using a 5mL syringe and 25g 5/8 inch needle, inject 5mL ice-cold 1mM PBSE into cavity. If there are leaks, clamp off the holes with hemostats.
6. Carefully "swish" mouse to get cells into suspension, keeping needle in cavity so as not to allow leaking.
7. Gently remove as much liquid as possible, keeping bevel of needle up, and being careful not to clog needle with any organs. Most likely, you will not get full 5mL volume back.
8. Remove needle, eject fluid into a labeled 15mL conical tube and keep on ice for PMN isolation.

A.9 PMN Isolation from IP Lavage

Reagents:

RBC Lysis Buffer = 0.8% NH_4Cl (0.802g) + 10mM EDTA (0.372g) + 100mL ultrapure water. pH 7.4. Filter sterilize.

1. Bring volumes up to 10mL with ice-cold PBSE if not saving supernatants. If saving supernatants for cytokine evaluation, do not alter volumes prior to centrifugation.
2. Centrifuge samples at 100 G, 4 °C, 10min.
3. Decant or save supernatants. Resuspend in 5mL ice-cold PBSE.
4. Centrifuge samples at 100 G, 4 °C, 10min.
5. Decant supernatants. Resuspend in 5mL ice-cold RBC Lysis buffer.
6. Vortex and let sit on ice for 10min.
7. Centrifuge samples at 100 G, 4 °C, 10min.
8. Decant supernatants. Resuspend pellet in 600µL RLT buffer with BME (10µL/mL).
9. Store at -80 °C.

A.10 RNA Isolation from Qiagen Kit

<u>Reagents</u>:

Qiagen RNA Isolation Kit

B-mercaptoethanol

<u>Procedure</u>:

1. Add 600 µl lysis buffer (10µl B-ME/ml Lysis Buffer) to cells.
2. Store in -80°C.
3. Proceed with RNA isolation.
    a. Spin down tubes for 3 min at 3000rpm, brake off.
    b. Add 600 µl 70% ETOH to RNA in 1.7 ml tubes
        i. Mix 5-6x
    c. Transfer onto column
        i. Centrifuge 14000 rpm 20 sec
        ii. Dump flo-through
    d. Add remaining sample to column
        i. Centrifuge
        ii. Dump flo-through
    e. Pipet 350 µl Buffer RW1 onto column
        i. Centrifuge 15 seconds at 14000 rpm
        ii. Dump flo through

f. Premix 10 µl DNASE stock 1 (2.7Kunitz units/µl) with 70 µl buffer RDD per sample

g. Add 80 µl DNASE I mix onto column

h. Incubate 30 min RT

    i. Add 350 µl Buffer RWL onto column

    i. Centrifuge 15 sec

    ii. Dump flo-through

j. Transfer column to new collection tube

k. Add 350 µl RPE Buffer

    i. Centrifuge

    ii. Dump flo-thru

    iii. Repeat wash

l. Spin dry 2 min 14000 rpm

m. Transfer column to new 1.7 ml collection tube

    i. Add 30 µl DEPC H_2O

    ii. Centrifuge 8000 g for 2 min

4. O.D. Reading of RNA

    a. Using NanoDrop, determine OD of RNA samples.

A.11 Making cDNA from RNA

1. In 1.7 ml tube mix

   1 µl Hex Primers (125 ng/µl) + 11 µl RNA (2.2 µg total)

2. Heat 70°C for 10 min
3. Make up buffer mix (per sample)

5x buffer	4.0 µl
0.1mM DTT	2.0 µl
10mM DNTP	1.0 µl
Superscript	0.5 µl

4. Place samples on wet ice for 2 min
5. Centrifuge 4°C for 30 sec, 8000 RPM.
6. Add 7.5 µl buffer mix per sample on wet ice
7. Incubate 25°C for 10 min
8. Incubate 42°C for 50 min
9. Incubate 70°C for 15 min
10. Add appropriate volume H_2O per sample to get 10ng/µl cDNA
11. Store at -20°C.