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Apolipoprotein B-mediated control of Staphylococcus aureus virulence

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APOLIPOPROTEIN B-MEDIATED CONTROL OF *STAPHYLOCOCCUS AUREUS* VIRULENCE

BY

MEGHAN MICHAL PETERSON

B.S., Microbiology, Brigham Young University, 2003

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy
Biomedical Sciences

The University of New Mexico
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May, 2010

DEDICATION

I dedicate this work to my beloved grandparents Ray and Golda Heal for teaching me that love and service are the secrets to a happy life, and to my future children, that they may have the courage and commitment to follow their dreams.

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ABSTRACT

Staphylococcus aureus is a colonizer of the human skin and mucosa that can cause minor to severe disease. Originally afflicting patients at the extremes of age or those with immunocompromising co-morbidities, *S. aureus* strains have emerged that cause invasive disease in otherwise healthy populations with no health-care associated risk factors. In addition, these community-acquired strains predominately contain antibiotic resistance genes, making them difficult to treat with conventional interventions. Invasive disease in *S. aureus* is partly regulated by a quorum sensing signaling system that relies on the secretion and signaling function of a bacterial peptide pheromone (AIP). *S. aureus* strains that lack this virulence regulator system still cause disease, but it is much less severe and is typified by a colonizing, rather than an invasive, phenotype. Therapeutics or host factors that inhibit virulence instead of viability can limit both invasive disease as well as the development of resistance.

During infection, the inflammatory process causes serum to extravasate into the affected tissue site. Serum has been shown to inhibit virulence signaling in *S. aureus*, though the mechanism is unknown. This dissertation identifies apolipoprotein B (apoB), the major protein component of very low and low density lipoproteins, as a potent inhibitor of virulence signaling in *S. aureus* by binding the bacterial signaling pheromone AIP. Though binding of host lipoproteins to bacterial products has been described in Gram-negative infections, this is the first demonstration of a lipoprotein component having protective effects in a Gram-positive infection. This work further describes the inhibitory action of apoB on virulence signaling and subsequent invasive infection in multiple *S. aureus* strains and identifies the globular amino terminal domain of apoB as the AIP binding site. These findings have important implications for understanding the host-pathogen interaction and for developing therapeutics that can be effective without causing bacterial resistance.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Pathogenicity of *Staphylococcus aureus*

Staphylococcus aureus, a colonizer of the human skin and mucosa, causes mild to severe disease, ranging from localized skin and soft tissue infections to necrotizing pneumonia or sepsis. A Gram-positive coccus, *S. aureus* derives its name from its resemblance to a cluster of grapes when observed under the microscope, and from its golden pigmented colonies. Over one third of the human population is permanently colonized, while 60% are transiently colonized with this organism, predominately in the nares and on the skin (Klevens et al., 2007). Colonization is a risk factor for infection, though not an exclusive determinant of disease development or outcome (Ellis et al., 2004; Wertheim et al., 2005).

S. aureus is a very genetically heterogeneous pathogen and strains are classified by using pulsed field gel electrophoresis (PFGE) typing (Prevost et al., 1991). The increase of resistance to methicillin, a penicillin family member, has led to the distinction of methicillin resistant strains (methicillin resistant *Staphylococcus aureus*, MRSA) from those that are methicillin sensitive (MSSA)(Sakoulas and Moellering, 2008). Of these, PFGE types USA100, USA300, and USA400 are responsible for most MRSA disease in the United States (Klevens et al., 2007). These MRSA isolates are typified by antibiotic resistance, increased virulence factor production, and aggressive invasive disease (Baba et al., 2002; Chambers, 2005). In the United States in 2005, the incidence of invasive MRSA infection was 31.8 out of 100,000: greater than the incidence of all other invasive bacterial infections combined. That same year, invasive MRSA disease caused more deaths in the U.S. than did HIV/AIDS (Klevens et al., 2007).

While *S. aureus* is typically an opportunistic pathogen causing infections in those with chronic health conditions and at extremes of age, community acquired (CA) MRSA infections have been described in otherwise healthy populations, including adolescents, children in daycare centers, prisoners, and athletes (Miller et al., 2005)which have no health care-associated risk factors (Deleo et al., 2010; Miller et al., 2005). CA-MRSA strains do not evolve under antibiotic selection in a hospital setting, but rather among the healthy public. Transmission for the CA-MRSA USA300 and USA400 strains is most frequently skin-to-skin contact or contact with contaminated surfaces, as evidenced by the populations affected, while hospital acquired disease (HA-MRSA) is generally caused by patients' colonizing strains gaining physiological access beyond the skin or mucosal barrier (Wertheim et al., 2004; Wertheim et al., 2005).

Complicated disease from MRSA is of concern not only because of its virulence, but also because of its resistance to standard antibiotic therapy. The development of antibiotic resistance in *S. aureus* began in 1947 with the discovery of infections that were resistant to penicillin treatment (Barber, 1947). Shortly thereafter, in 1961, resistance to the penicillin family member methicillin was described (Barber, 1961). The resistance to methicillin in the CA-MRSA strains seen today is due to the acquisition of a staphylococcal chromosomal cassette SCC*mec*IV which contains the gene *mecA* that encodes for specific resistance to β-lactam antibiotics including methicillin (Baba et al., 2002). Though many infections with CA-MRSA should be treatable by other non-β-lactam antibiotics, the diagnostic window for determining the antibiotic resistance profile of any particular strain is inconsistent with providing timely treatment. Thus, patients are often treated with powerful antibiotic regimens of intravenous vancomycin (Baba et al.,

2002; Deleo et al., 2010). As a result, vancomycin resistant strains have been reported, though rarely, probably due to the acquisition of vancomycin resistance genes from *Enterococcus faecalis* (Chang et al., 2003). Since the standard treatment by antibiotics is limited against MRSA regardless of its source, new treatment options that do not promote the development of resistance need to be investigated and developed. One such option involves understanding MRSA virulence as a potential therapeutic target. Since virulence factors play a role in infection and clinical outcome, but not in bacterial viability, any therapy or host defense against them is not expected to induce the development of bacterial genetic resistance.

Quorum Sensing as a Regulator of Bacterial Virulence

Virulence factor production in *S. aureus* is highly regulated and dependent on the growth phase, bacterial environment, and population density. The regulation of virulence factors by sensing the environment and bacterial density, referred to as quorum sensing, is a molecular mechanism of bacterial communication utilized by several bacterial pathogens of humans (Parker and Sperandio, 2009). Common to these quorum sensing systems is the use of an autoinducer pheromone that is released by the bacteria into the environment; once the bacterial population and thus autoinducer concentration increases, the bacteria respond by changing gene expression which results in a shift in the bacterial life cycle, metabolism, and phenotype (de Kievit and Iglewski, 2000). Four quorum sensing systems have been described so far based on their signaling molecules, receptors and response regulators. Gram negative bacteria such as *Vibrio fisheri* and *Pseudomonas*

aeruginosa synthesize *N*-acyl homoserine lactone (AHL) signaling molecules which (1) diffuse across the bacterial membrane into the environment, (2) accumulate as bacterial population increases, (3) diffuse back into the cell, and (4) bind its receptor, activating the receptor to be a transcription factor for several downstream genes (Williams and Camara, 2009).

A second, similar system relies on the production of a furanone that accumulates in the environment before diffusing back into the periplasmic space of the cell, binding to a transporter, entering the cell, and being phosphorylated. The phosphorylated pheromone is then believed to bind to transcriptional repressors as well as act as a transcription factor for downstream expression events to occur (Taga et al., 2001). This second system has been observed in *Escherichia coli*, *Salmonella* Typhimurium, and *Streptococcus pyogenes* among other both Gram-positive and Gram-negative species (Lyon et al., 2001; Xavier and Bassler, 2003).

A third mechanism of quorum sensing has only been partially described in Gram-negative bacteria including *Shigella, Klebsiella*, and *Salmonella* species as well as non-pathogenic *E. coli* (Walters et al., 2006). This system relies on a third kind of autoinducer, the structure and synthesis of which is unknown, that is recognized by the sensor kinase QseC on the bacterial membrane which then phosphorylates the response regulator QseB, activating it to exert its transcriptional regulation on downstream gene targets (Parker and Sperandio, 2009).

Quorum Sensing in *S. aureus*

The fourth quorum sensing system is found only in Gram-positive bacteria and was originally characterized in *S. aureus* though it has been identified in *Enterococcus faecalis* and *Staphylococcus epidermidis* (Qin et al., 2001; Van Wamel et al., 1998; Yarwood and Schlievert, 2003). Quorum sensing in *S. aureus* is controlled by the accessory gene regulator, or *agr* operon. This bacterial regulator operon has been well characterized and it functions to control the expression of many virulence factors associated with invasive MRSA disease (Cheung et al., 2002; Cheung et al., 2004; George and Muir, 2007; Yarwood and Schlievert, 2003). The *agr* operon is a two-component sensor regulator system that consists of two divergent promoters, P2 and P3. The P2 promoter drives the *agrA,B,C,D* genes and the P3 promoter induces RNAIII, a regulatory RNA. When the P2 promoter is activated, the peptide AgrD is secreted through AgrB as a cyclic thiolactone structure termed the autoinducing peptide (AIP, see Figure 1A). Different *S. aureus* strains may express one of four different AIPs which differ slightly in amino acid composition and overall length (see Figure 1B). The thiolactone ring, however, is a common element and is essential for binding and signaling through the AgrC receptor (Wright et al., 2004). The difference of AIP type allows *S. aureus* strains to be further classified as *agr* types 1, 2, 3, or 4. These different AIPs are mutually antagonistic to one another (Ji et al., 1997). Prevailing CA-MRSA strains USA 300 and USA 400 are *agr* groups 1 and 3, respectively, though all four types are represented in human disease (Jarraud et al., 2002; Klevens et al., 2007).

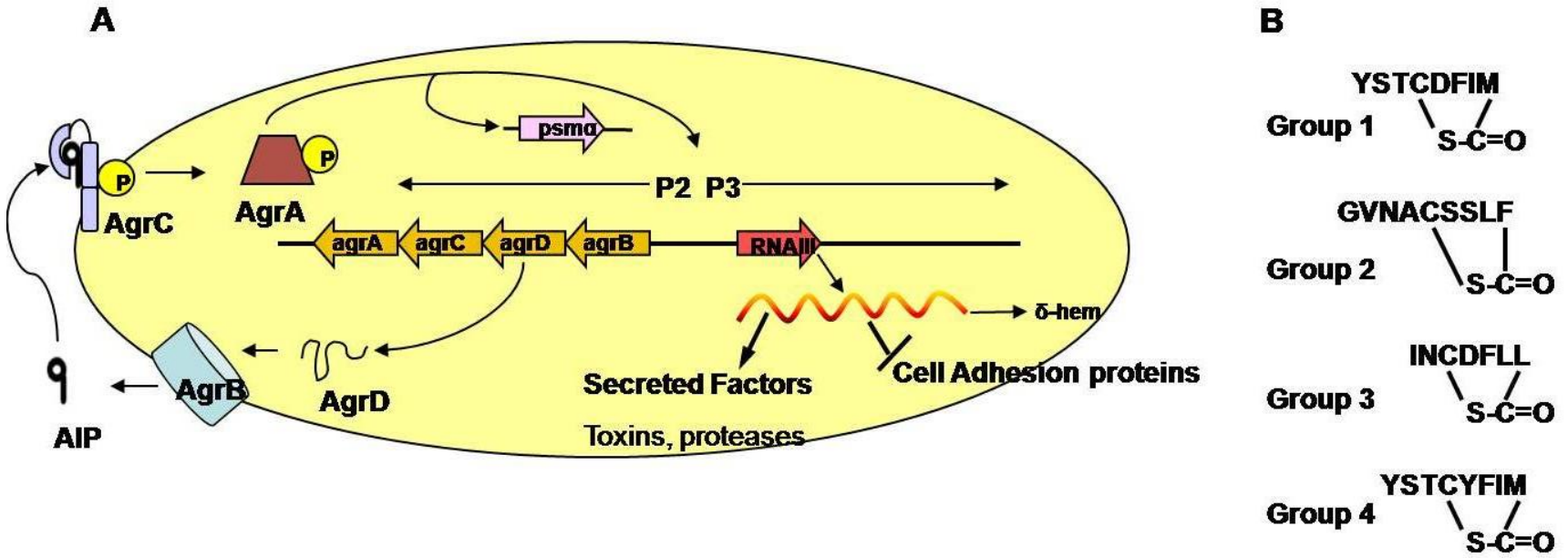

Figure 1. The *agr* Quorum Sensing Operon in *S. aureus*

(A) The P2 promoter encodes the *agr* signaling and response regulator proteins. AgrD is exported through AgrB as the thiolactone AIP. Binding of AIP to AgrC activates the histidine kinase AgrA, which acts on the P2/P3 promoter as well as the promoter for the toxin psmα. The P3 promoter induces transcription of regulatory RNAIII, which acts on the promoters and translation initiation regions of several genes to upregulate secreted proteins important for invasion and to downregulate adhesins. δ-hemolysin is also transcribed from the RNAIII message.

(B) Sequences of the four different AIP structures found among *S. aureus* strains. All share a cyclic thiolactone important for binding to the AgrC receptor. The amino-terminal residues are required for AIP signaling through AgrC. Schematic adapted from George and Muir, 2007

When the AIP binds its receptor AgrC, a histidine kinase, AgrA is phosphorylated which then acts on the P2/P3 promoter site to induce increased expression of the Agr genes and begin expression of RNAIII (Novick, 2003). RNAIII itself is a 514 nucleotide transcript with a secondary structure consisting of 14 hairpin stem loops. It functions primarily as a regulatory RNA, but its 5′-end encodes the gene for δ-hemolysin, a small cytolytic toxin, the expression of which is controlled by the 3′-end of RNAIII (Benito et al., 2000). When autoinducer concentration is high, the resultant RNAIII goes on to act on the promoters of several genes by either annealing to the promoters to expose their transcriptional start sites, or interacting with the mRNA to occlude or expose ribosomal binding sites, ultimately resulting in the downregulation of colonizing bacterial factors and the upregulation of invading factors (George and Muir, 2007).

RNAIII acts as a master regulator to mediate a drastic phenotypic switch from colonizing to invading. The gene targets regulated by RNAIII are classified as colonization or invasion factors. Colonization factors include *S. aureus* protein A (spa) which binds to IgG antibody, fibronectin binding protein (fbp), and clumping factors A and B (clfA and clfB), all of which allow for adherence to tissues and the formation of a biofilm phenotype. Invasion factors serve to disseminate the bacteria throughout the body and include the cytolytic molecules alpha-hemolysin (hla) and Panton-Valentine leukocidin (PVL) and proteases that destroy tissue. Other components of the *S. aureus* arsenal which contribute to disease directly include toxins such as enterotoxins and toxic shock syndrome toxin (TSST) (Yarwood and Schlievert, 2003). AgrA also acts directly on the promoter of the *psmα* gene, which encodes phenol soluble modulin α, a cytolytic peptide known to be highly expressed in CA-MRSA (Figure 1A) (Queck et al., 2008;

Wang et al., 2007). The importance of the *agr* quorum sensing system to virulence is evidenced by the fact that bacterial strains lacking the *agr* operon are less virulent in several animal models of infection. Conversely, the production of RNAIII at the site of infection has been shown to be essential for acute invasive infection at the entry site (Rothfork et al., 2003; Rothfork et al., 2004).

CA-MRSA strains, especially USA300, overproduce virulence factors regulated by the *agr* system: alpha hemolysin, PVL, and psmα, in particular (Deleo et al., 2010). The importance of PVL in CA-MRSA has been under debate, since PVL expression is linked epidemiologically to human necrotizing pneumonia caused by USA300, but experimental murine models showed no difference between wild type and PVL deletion mutant strains (Bubeck Wardenburg et al., 2008; Gillet et al., 2002). Recently, however, it has been shown that murine neutrophils are less sensitive to the PVL toxin, whereas rabbit and human neutrophils lyse to a smiliar extent in response to PVL. In the context of a more appropriate animal model, it is now clear that PVL does contribute to invasive USA300 infection (Diep et al., 2010). USA300 has also acquired the arginine catabolic mobile element (ACME), probably from *S. epidermidis*, which contains the gene for an arginine deiminase, allowing the bacteria to better survive the acidic growth conditions of the skin and thereby have increased fitness for transmission (Baba et al., 2002; Diep et al., 2006). While both colonization and invasion factors are considered to be virulence factors, those associated with invasive disease often cause the most dramatic and damaging outcomes for the patient. The prevention of this phenotypic switch, therefore, is of interest for treating patients with *S. aureus* infection.

Host Immunity to *S. aureus* Infection

Although most adults have antibodies to *S. aureus* (Holtfreter et al., 2010), control and clearance of infection relies on innate immune sensors and effectors rather than the adaptive humoral response. Lipoteichoic acid, capsular polysaccharide, peptidoglycan, and secreted bacterial proteins induce peripheral blood monocytes, epithelial cells, and endothelial cells to produce IL-8, a potent neutrophil recruiting cytokine (Soell et al., 1995; Standiford et al., 1994). Once neutrophils are recruited to the site of infection, they sense bacterial products by Toll-like Receptor 2 (TLR2) (Kanneganti et al., 2007; Takeuchi et al., 2000) or recognize host opsonins such as antibody or complement proteins on the bacteria to induce phagocytosis. Upon phagocytosis, neutrophils destroy the bacteria generally by oxidant-mediated killing (Fournier and Philpott, 2005). The NADPH oxidase is activated upon bacterial phagocytosis and generates high levels of superoxide anion, which quickly forms other reactive oxygen species like hydrogen peroxide, singlet oxygen, and hypochlorous acid (Klebanoff, 1968; Rosen and Klebanoff, 1977). Mice lacking the NADPH oxidase are more susceptible to invasive *S. aureus* infections than wild type controls, demonstrating the vital role this enzyme plays in host defense (Rothfork et al., 2004). Intracellular killing also occurs by fusion of the phagosome with cytoplasmic granules, containing antimicrobial peptides, α-defensins, cathepsins, and other antimicrobial proteins which permeabilize pathogen membranes (Ganz et al., 1985). The complement system also serves as a barrier to infection, causing direct lysis of invading bacteria, opsonization and subsequent uptake by immune effector cells, and recruitment of inflammatory cells to the site (Walport, 2001a; Walport, 2001b).

The adaptive humoral immune system appears to provide limited control of *S. aureus* infection despite increases of serum anti-staphylococcal antibodies (Holtfreter et al., 2010). Antibodies found in the serum of patients reflect a robust response to the staphylococcal antigens responsible for maintaining a colonizing phenotype, and they are specific to the patient's own individual colonizing strain of *S. aureus.* As such, the antibodies have little activity against the invading virulent forms of the bacteria that cause morbidity (Holtfreter et al., 2010). For these reasons, attempts at human vaccination against *S. aureus* have failed despite successful animal vaccine studies (Schaffer and Lee, 2009). Some adaptive immune system components, like Th17 cells, have been shown to enhance innate immune phagocytic killing of *S. aureus,* leading Lin and colleagues to develop a vaccine that specifically elicits a robust Th17 response which can then drive effective neutrophil killing of bacteria in a mouse model of vaccination and infection (Lin et al., 2009). It remains to be seen if this vaccine would be effective in human cases of infection and if this strategy of eliciting a good PMN response by vaccination will prove to be more effective than those focused on inducing humoral immunity.

Bacterial Evasion of Host Immune Effectors

Notwithstanding the various immune mechanisms to clear infection, *S. aureus* has developed numerous evasion methods in order to survive in the host (see Figure 2). Serum immunoglobulin can opsonize the bacteria, but a secreted protease, staphylokinase, activates host plasminogen on the bacterial surface, leading to cleavage of surface-bound IgG as well as complement component C3b by the highly active

protease plasmin (Rooijakkers et al., 2005b). *S. aureus* also produces an abundance of inhibitors of complement activity, such as staphylococcal complement inhibitor (SCIN) which stabilizes the C3 convertase to prevent deposition of C3b on the bacterial surface (Rooijakkers et al., 2005a), and fibrinogen binding protein (Efb) and staphylococcal binding protein (Sbi) which both block C3 accumulation on the cell surface (Burman et al., 2008; Lee et al., 2004). Protein A, which is encoded by virtually all *S. aureus* strains, binds to and immobilizes serum IgG, preventing the Fc portion from interacting with receptors on inflammatory cells thus inhibiting subsequent phagocytosis (Foster, 2005). Other bacterial surface-associated molecules such as capsule (Thakker et al., 1998) and clumping factors A and B (Palmqvist et al., 2002) confer opsonic resistance and inhibit phagocytosis as well. A secreted bacterial protein, chemotaxis inhibitory protein of *S. aureus* (CHIPS), binds to the neutrophil receptors for chemotactic and migration-inducing molecules such as the complement anaphylatoxin C5a and bacterial formyl peptide, slowing down the cellular response to infection (Postma et al., 2004). Reports show that *S. aureus* can survive within the phagosome by preventing fusion with azurophilic granules and escape the phagosome once inside neutrophils and multiply rapidly in the intracellular environment (Gresham et al., 2000). Specialized bacterial superoxide dismutases can prevent damage by the reactive oxidant species created by the neutrophil NADPH oxidase (Liu et al., 2005). Secreted lytic factors are also in abundance: alpha hemolysin, Panton-Valentine leukocidin, and phenol soluble modulins, to name a few, which allow the

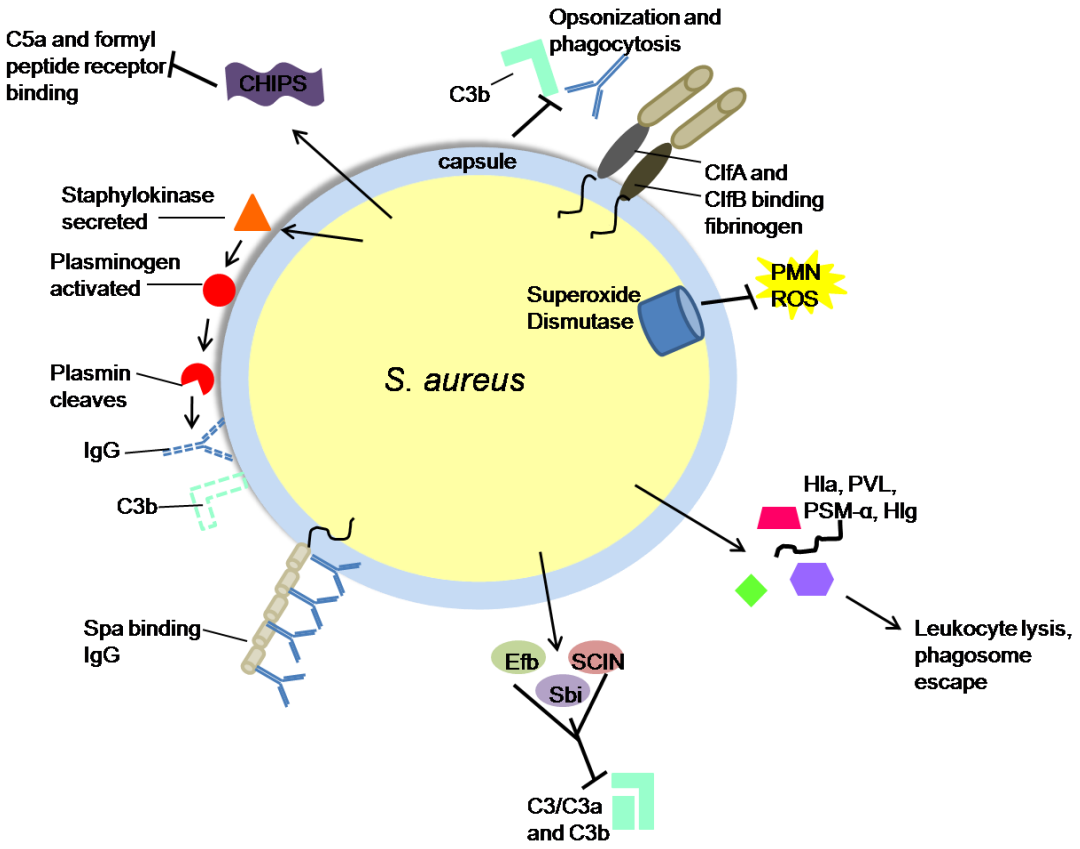

Figure 2. Host evasion by *S. aureus*

S. aureus employs multiple mechanisms to evade host defense molecules, most of them targeted at avoiding opsonization by complement, immunoglobulin, and fibrinogen and phagocytosis by neutrophils. Other molecules are secreted to act directly on host cells. Abbreviations: ClfA,B, Clumping factor A and B; PMN, Polymorphonuclear leukocyte; ROS, reactive oxygen species; Hla, alpha-hemolysin; PVL, Panton-Valentine Leukocidin; PSM-α, phenol soluble modulin α; Hlg, gamma-hemolysin; Efb, fibrinogen bindng protein; Sbi, staphylococcal binding protein; SCIN, staphylococcal complement inhibitor; Spa, staphylococcal protein A; CHIPs, chemotaxis inhibitory protein of *S. aureus*.

Figure modified from Foster, 2005.

bacteria to escape from intracellular compartments and whole host cells alike (Gillet et al., 2002; Li et al., 2009; Menestrina et al., 2003). The host, therefore, must have multiple layers of defense in order to contain infection.

The Acute Phase Response and the Role of Serum Lipoproteins in Infection

An important mechanism the body has to respond to infection, inflammation, and trauma is the acute phase response. Hepatocytes respond to inflammatory mediators and serum cytokines produced by injured tissue and leukocytes during inflammation and infection by increasing the production of numerous serum proteins, including C reactive protein (CRP) and complement proteins, and decreasing others such as albumin and alpha-fetoprotein (Baumann and Gauldie, 1994; Gabay and Kushner, 1999). The most dramatic alteration in serum concentrations of the acute phase proteins occurs 1-2 days following the inflammatory stimulus, begins to resolve within days, and completely returns to normal levels by 4 weeks (Gabay and Kushner, 1999). The presence of these acute phase response proteins in the serum indicates ongoing inflammation and can be useful in the diagnosis and treatment of patients. They also participate in host defense, by opsonizing invading pathogens as is the case with complement proteins, and CRP has several functions including: opsonizing bacteria for clearance by phagocytic cells, stimulating inflammatory cytokine release, and downregulation of neutrophil activity (Gabay and Kushner, 1999). Despite these beneficial functions, a persistant acute phase response can lead to anemia, hypoalbuminemia, and secondary amyloidosis, while an overactive acute phase response can contribute to septic shock (Gabay and Kushner, 1999).

Serum lipids and lipoprotein levels are also altered during the acute phase response, with varied and sometimes conflicting reports of the direction and degree of alteration (Khovidhunkit et al., 2004; Sammalkorpi et al., 1988). Most studies agree that total cholesterol, high density lipoprotein (HDL) and low density lipoprotein (LDL) serum levels decrease during acute infection or inflammation (Khovidhunkit et al., 2004). Plasma triglyceride levels increase during sepsis, but not following trauma or surgery (Alvarez and Ramos, 1986; Carpentier and Scruel, 2002). All these changes are often the opposite in rodent models of infection, making them difficult to study in rodent models of human disease (Sammalkorpi et al., 1988).

Alterations in serum lipoproteins during the acute phase response could have great impact on the outcome of severe infection, since they have been identified as having a role in host defense, especially against Gram negative pathogens, by binding and neutralizing lipopolysaccharide (LPS) (Barcia and Harris, 2005; Feingold et al., 1995; Harris et al., 1990; Harris et al., 1993; Read et al., 1995). The protein components apoA-1 (found on HDL) and apoE (found on LDL, HDL, and chylomicrons) have specifically been shown to inactivate LPS *in vitro* while apoE–deficient mice are more susceptible to Gram negative infections (de Bont et al., 1999; Emancipator et al., 1992; Flegel et al., 1993). The interplay of lipoproteins and Gram positive pathogens, including *S. aureus*, has been less well characterized, though studies indicate that lipoproteins bind lipoteichoic acid in a manner similar to the LPS-binding activity described above (Levels et al., 2003). Importantly, our work included below describes a novel role for apolipoprotein B of VLDL and LDL in host defense against quorum sensing *S. aureus* infections.

In light of these immunity-promoting properties, the decrease of LDL serum concentrations during the acute phase response could have detrimental effects on the host. This decrease is usually due to retention in the tissues by the LDL receptor or the reduced activity of the lipoprotein lipase that converts very low density lipoprotein (VLDL) into LDL (Carpentier and Scruel, 2002; Khovidhunkit et al., 2004). The relative VLDL concentration is therefore increased in serum, tipping the balance even more in favor of high triglycerides and low cholesterol content (Khovidhunkit et al., 2004; Marik, 2006). Further studies concluded that inflammatory cytokines reduce both total LDL and its associated protein apolipoprotein B (apoB) in the serum of treated subjects and also in the supernatant of human hepatic cells in culture (Ettinger et al., 1994; Schectman et al., 1992). The decrease in serum lipoproteins is correlated with poor outcomes in septic patients (Gui et al., 1996; Marik, 2006), leading us to further investigate how lipoproteins may be important in host defense beyond what has previously been described.

Lipoproteins: Synthesis and Function in the Host

Serum lipoproteins are heterogeneous particles made up of a core of triglycerides and cholesterol esters surrounded by a layer of phospholipids and unesterified cholesterol. Protein components are added to the particles either during formation or after secretion from hepatocytes (Mahley et al., 1984; Segrest et al., 2001; Shelness and Sellers, 2001). Different classes of lipoproteins are distinguished by their density, composition, and function. LDL and VLDL transport triglycerides and cholesterol from the liver to the tissues for use in metabolism. HDL is responsible for clearing

triglycerides and cholesterol from the circulation for recycling or excretion in the liver. Apolipoprotein B (apoB) is a large (4536 amino acid), amphipathic lipid binding protein found in the serum associated with LDL and VLDL. The pentapartite structure is composed of a globular amino terminus, two domains of beta pleated sheets, and two alpha helical domains that span the entire surface of the lipoprotein particle (Segrest et al., 2001) (see Figure 3A). Unlike other apoproteins which are nonstructural and exchangeable among lipoprotein particles, apoB is a non-exchangeable, structural component of both LDL and VLDL (Segrest et al., 2001). ApoB synthesis initiates assembly of the very low density lipoproteins in the endoplasmic reticulum, maturing into the particle as lipid droplets fuse into the triglyceride core before being secreted from the hepatocytes. Unlike apoB, other apoproteins do not share this assembly function; rather, they are added to the particles after secretion (Mahley et al., 1984). Following secretion, lipoprotein lipase (Lpl) metabolizes the VLDL, removing some of the lipids to form LDL particles (Figure 3B). The synthesis of VLDL and subsequent metabolism is depicted in Figure 3, the properties of all lipoprotein particles are summarized in Table 1.

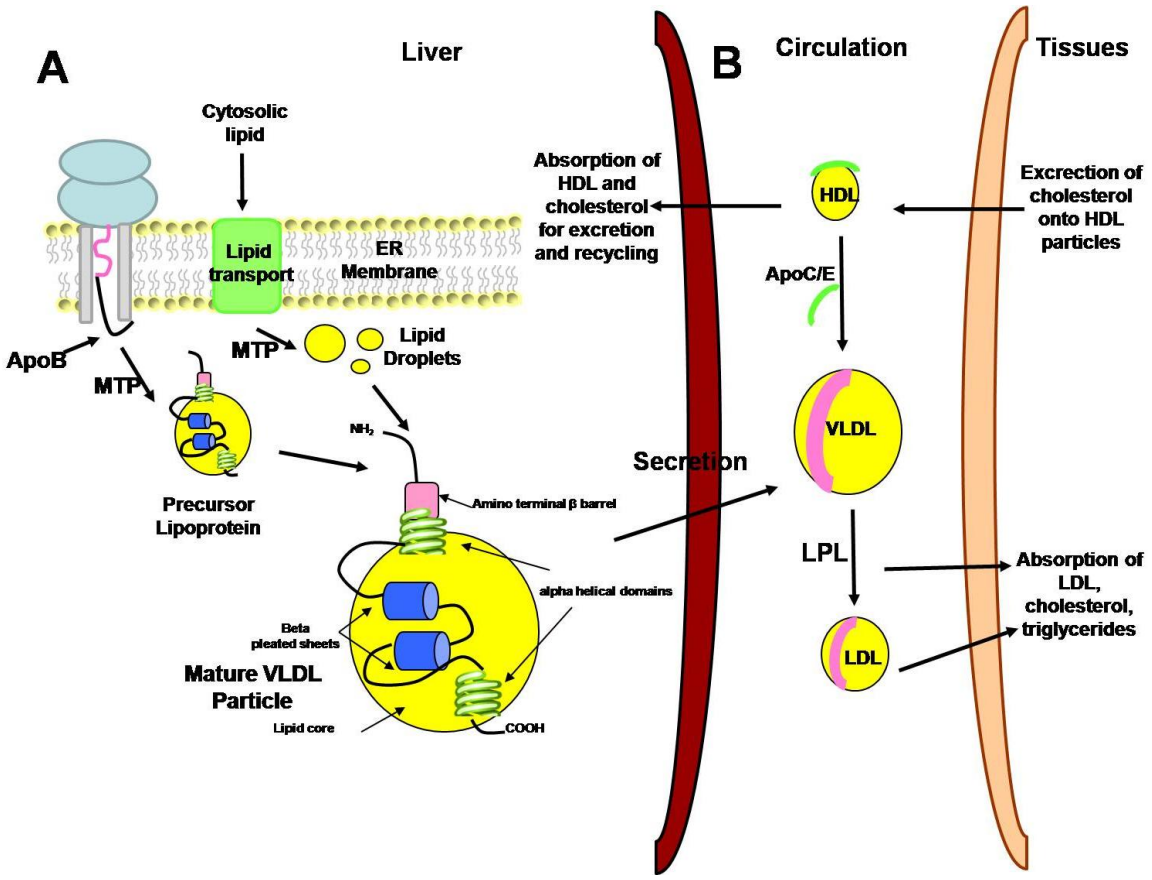

Figure 3. Synthesis, Structure, and Metabolism of Apolipoprotein B-containing Particles

(A) Synthesis of VLDL is initiated by the translation of the apoB message into the lumen of the endoplasmic reticulum. The Microsomal Triglyceride Transfer Protein (MTP) functions to mediate lipid droplet formation from cytosolic lipids and also to transfer lipids to the growing lipoprotein particle. Additional lipid droplets fuse with the precursor particle to form the complete VLDL before it is secreted by the hepatocyte and into the circulation. The pentapartite domain structure of apoB is shown wrapping around the lipid core.

(B) In the circulation, apolipoproteins C and E (green) are transferred from HDL to VLDL which already contains apolipoprotein B (pink). Lipoprotein lipase (LPL) removes lipid components from VLDL to form the smaller, denser LDL particle. LDL and the free lipid components are absorbed by the tissues for use in metabolism. Excreted cholesterol is carried on HDL to the liver for excretion or recycling.

Figure adapted from Shelness and Sellers, 2001 and the American Oil Chemists' Society webpage (http://lipidlibrary.aocs.org/Lipids/lipoprot/index.htm).

Table 1-Properties of Serum Lipoproteins

Particle	Size	Protein composition	Lipid composition	Function
HDL	~8 nm	apoA-I, apoA-II, apoE,	Phosphatidylcholine (40%), choleseryl ester (30%)	Transport cholesterol from tissues to liver
LDL	~20 nm	apoB100	Cholesteryl esters (50%), phospholipids (30%), unesterified cholesterol (10%), triglycerides (10%)	Transport cholesterol and triglycerides to tissues from liver
VLDL	30-90 nm	apoB100, apoC-I, apoC-II, apoC-III, apoE	Triglycerides (55%), phospholipids (20%),unesterified cholesterol (10%) esterified cholesterol (5%)	Transport cholesterol and triglycerides to tissues from liver
Chylomicrons	200-600 nm	apoB48, ApoA-I, apoC-I, apoC-II, apoC-III	Triglycerides (85%), phospholipids (8%),unesterified cholesterol (2%) esterified cholesterol (3%)	Transport cholesterol and triglycerides to tissues from intestine (diet)

Sources: Black, 2007; Morrisett et al., 1975, and the American Oil Chemists' Society webpage (http://lipidlibrary.aocs.org/Lipids/lipoprot/index.htm).

ApoB is necessary for lipoprotein particle binding to the LDL receptor, which is found on all nucleated cells and is necessary to the provision of essential lipids required for normal cell and organ function. LDL also binds to scavenger receptors found on macrophages. These scavenger receptors recognize modified low density lipoproteins such as acetylated LDL (ac-LDL) and oxidized LDL, which are associated with atherosclerosis (Henriksen et al., 1981; Henriksen et al., 1983). Some of these receptors play important roles in host defense. Particularly for *S. aureus*, CD36 is important for both sensing in combination with TLR2 a lipid derivative of this pathogen and directly for phagocytosis of this pathogen. Thus, multiple components of lipoprotein metabolism interact to contribute to host defense against *S. aureus* infections.

Summary and Hypothesis

The rise of antibiotic-resistant strains of *S. aureus* that cause severe invasive infection highlights the need to understand and develop alternative strategies for therapy. One such strategy is to target virulence instead of viability, thereby reducing the chance for development of resistance. The *agr* system of virulence regulation, or quorum sensing, is a good target since *agr* mutants are drastically attenuated in their virulence. A few host defense mechanisms against quorum sensing signaling pheromones have been described in recent years, including oxidant modification of *S. aureus* AIP (Rothfork et al., 2004) and cleavage of the *P. aeruginosa* AHL (Chun et al., 2004; Ozer et al., 2005). Neutrophil-derived oxidants effectively inactivate *agr* type 1 *S. aureus* AIP by oxidizing the C-terminal methionine residue (Rothfork et al., 2004). However, as described above, bacterial neutrophil escape mechanisms are varied and abundant. Work in our group

indicates that normal serum lipoproteins inhibit quorum sensing *in vitro*. Since serum components extravasate into an infected tissue site along with infiltrating leukocytes, we hypothesized that serum lipoproteins serve as an innate barrier to *agr*-dependent *S. aureus* infection. To address this hypothesis, we focused on apolipoprotein B as the main inhibitory component of serum lipoproteins and pursued the following specific aims: 1) to define the role of apolipoprotein B as a specific barrier to quorum sensing signaling in *agr* type 1 strains both *in vitro* and in an experimental animal model of skin infection; 2) to define the role of apoB in control of *agr* signaling from *agr*2 and *agr*3 strains; and 3) to define the role of the amino terminal domain of apoB in control of *agr* signaling. We show that apoB demonstrates protective action against invasive disease by binding and sequestering the AIP1 signaling peptide away from its receptor, thus preventing quorum sensing-mediated virulence. We present preliminary data that this mechanism of control is limited against *agr*3 *S. aureus* strains which predominate as causes of blood stream infections and that several approaches demonstrate that the amino terminal domain of apoB is sufficient to control *agr*1 signaling. These data represent the first demonstration of apoB as a host defense barrier and describe a novel mechanism for control of invasive infections in contrast to colonization.

CHAPTER 2

METHODS

Reagents

Synthetic AIPs were generated as follows and stored in DMSO at -80°C: cyclic AIP1 (YSTC[→M]DFIM[→C]), FITC-AIP1, and biotin-AIP1 were synthesized by Commonwealth Biotechnologies, Inc., Richmond, VA, and cyclic AIP1, cyclic AIP2, cyclic AIP3 FAM-AIP2, and biotin AIP1 were synthesized byb Biopeptide Co., Inc. San Diego, CA using a described method (Otto et al., 1998). Cyclic AIP 2 (GVNAC[→F]SSLF[→C]), AIP4 (YSTC[→M]YFIM[→C]), and AIP3 antagonist (YINC[→L]DFLL[→C]) were generated as described (Otto et al., 1998). A disulfide linked cyclic peptide, C(→C)LLRMKSAC(→C), was used as a control. Linear AIP1 and biotin-AIP1 were generated by alkaline hydrolysis and neutralization. The following reagents and sera were obtained as indicated: purified human VLDL, LDL, HDL, apoB, apoA1, apoC1, and apoE (US Biological, Swampscott, MA); purified VLDL, LDL, Ac-LDL, DiI-Ac-LDL, and DiI-LDL (Biomedical Technologies Inc., Stoughton, MA); pooled human serum, lipoprotein-deficient serum, chicken serum, tributyrin, 4-aminopyrazolo-(3,4-D) pyrimide (4APP), DL-Dithiothreitol, Iodoacetamide, Dimethyl pimelimidate dihydrochloride, Pepstatin, trypsin soybean inhibitor, V8 protease, thrombin and a liposome preparation reagent containing cholesterol, phosphatidyl choline, and stearylamine (Sigma-Aldrich, St. Louis, MO); Pepsin (EMD-Calbiochem, Gibbstown, NJ), 4-12% Tris-HCl Ready gels, Precision Plus Protein Standard, and Silver stain kit (Bio-Rad, Hercules, CA), cholesterol and phosphatidylcholine (Avanti Polar Lipids); polyclonal goat IgG anti-human apolipoprotein B 100 (R&D Systems, Minneapolis, MN); monoclonal murine IgG1 anti-human apoB C1.4 (Santa Cruz

Biotechnology, Santa Cruz, CA); and monoclonal murine IgG1 anti-human apoB 4C11 (Biodesign International, Saco, ME).

Bacterial Strains

S. aureus strains used in these studies were as follows: ALC 1743 (*agr* group 1 RN6390 containing reporter *agr*:P3-*gfp*), ALC 1740 (RN6390 containing reporter *hla-gfp*), ALC 1753 (RN6911 *agr* deletion mutant of RN6390 containing reporter *agr*:P3-*gfp*). RN6390, SH1000 (σB corrected RN6390) , ALC 1225 *geh*- (strain 8325-4 with *geh* deletion mutant with reduced lipase secretion) (Kupferwasser et al., 2003; Rothfork et al., 2004), and USA100 strain 502A (*agr* group 2 clinical isolate). USA300 strain UAMS1378 and its *agr* deletion mutant UAMS1540 were provided by Dr. Mark Smeltzer (University of Arkansas), and USA300 strain LAC and its *agr* deletion mutant were provided by Dr. Michael Otto (NIAID). USA400 strain MW2 and its *agr* deletion mutant were also provided by Dr. Otto. Clinical isolates of CA-MRSA *agr* group 1 USA 300 (NM 300) and *agr* group 3 USA 400 (NM 400) were provided by Dr. Steve Young, Department of Pathology, University of New Mexico, and USA 300 strain FPR 3757 was provided by Dr. Francoise Perdreau-Remington, UCSF.

Preparation of Bacterial Cultures

Primary stocks of all bacterial strains were prepared as followed: 25 ml of Columbia or Trypticase Soy Broth (TSB, prepared with antibiotics as necessary, see below) in a 50 ml Falcon tube was inoculated with either a 1:2500 dilution of original stock or a colony from an original culture, loosely capped, and incubated at 37°C with shaking at 200 rpm overnight. Cultures were centrifuged at 3000 rpm for 4 minutes at 4°C and washed twice

with 10 ml of sterile saline. The pellet was resuspended in 5 ml of broth and two 1:84 dilutions of this culture were prepared in two 50 ml Falcon tubes containing broth and antibiotics as necessary. Cultures were loosely capped and incubated at 37°C with shaking at 200 rpm for 6 hours. Following incubation, the cultures were centrifuged and washed with 5 ml saline per tube as above. The two cultures were combined during a second wash with 10 ml of saline before pelleting a final time. The bacteria were resuspended with 2 ml of broth containing 10% glycerol, placed on ice, and subjected to two bursts of sonication for 5 seconds each. Stock was then aliquoted into vials containing 100 µl each and then stored at -80°C. Strains requiring antibiotic selection and the final concentration of antibiotic needed were: ALC1743 (10 µg/ml Chloramphenicol), ALC 1740 (10 µg/ml Chloramphenicol), ALC1753 (10 µg/ml Chloramphenicol, 4 µg/ml Tetracycline), UAMS1540 (2 µg/ml Minocycline), LAC Δ*agr* (4 µg/ml Tetracycline).

To generate synchronized early exponential phase, nonfluorescent (where applicable) bacteria, frozen stocks were cultured either in Columbia or Trypticase soy broth at a 1:2500 dilution in one 50 ml Falcon tube overnight at 37°C with shaking at 200 rpm. The overnight culture was then washed three times with sterile saline in 20 or 10 ml volumes, diluted 1:1000 in one Falcon tube and grown for 2 hours at 37°C with shaking at 200 rpm. Two hour growths were repeated twice more, with washing at the end of each 2 hour growth period, and cultures diluted first 1:84 into two tubes and finally 1:125 into eight tubes (ALC strains) or four tubes (all other strains). At the end of the three two hour growth periods, the cultures were combined into one tube with saline during the washing and then ultimately resuspended in 2 ml of broth/10% glycerol, sonicated as described above, and aliquoted 100 µl per cryovial and stored at -80°C. The titer of each stock was

determined after the vials had been frozen 20 minutes-24 hours by resuspending the frozen pellets of three vials with sterile saline to a volume of 1 ml each, centrifuging in a microcentrifuge at 4°C for 4 minutes at a speed of 12,500 rpm. After discarding the glycerol-containing supernatant, the pellets were resuspended in fresh saline to a volume of 1 ml each, each vial was sonicated and then serial dilutions of the stock were plated on blood agar.

Bacterial Promoter Activation Assays

Early exponential phase, nonfluorescent reporter bacteria (ALC1743 and ALC1740) (2×10^7/ml) were incubated in 1 ml TSB in polystyrene tubes with shaking (200 rpm, 37°C) for three hours or as indicated in the text. Alternatively, the bacteria were grown in 100 µl TSB in the wells of a 96-well round bottomed plate and placed on a Nutator at 37°C for three hours. Samples included vehicle control, synthetic AIP1 at the concentrations indicated or AIP1 treated with antagonists as referenced in the text. After incubation, bacteria were washed by centrifugation at 3000 rpm for 4 min at 4°C in PBS with 0.1% Triton X-100, sonicated, cultured for CFU, and then fixed with 1% paraformaldehyde containing 25 mM CaCl2 for analysis by flow cytometry (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ or Accuri C6, Accuri Cytometers, Inc., Ann Arbor, MI). Promoter activation was demonstrated as fluorescence induction and measured as the mean channel fluorescence (MCF) of GFP-positive bacteria.

Quantitative RT-PCR

Early exponential phase bacteria (2×10^7/ml, or as indicated in the text) were cultured as above with the indicated AIP stimulation or treatment for the timepoints described in the

figures. Bacterial RNA for RNAIII or psmα was quantified relative to 16S RNA using a probe-based assay as described with minor modifications (Sawires and Gresham, 2008). RNA was isolated and purified using the Qiagen RNAprotect Bacteria Reagent and RNeasy Mini Kit, Protocol 3 with mechanical (sterile 0.1 mm zirconia silica beads, Biospec, Bartlesville, OK) and enzymatic disruption by Proteinase K (Qiagen, Valencia, CA). Contaminating DNA was removed by an on-column DNase treatment. cDNA was generated from purified RNA using a high capacity cDNA RT kit with an RNase inhibitor (Applied Biosystems, Foster City, CA) and a PTC-100 thermocycler (MJ Research Inc., Watertown, MA). Thermal cycling conditions were as follows per the kit instructions: 10 minutes at 25°C, 120 minutes at 37°C, 5 seconds at 85°C, hold at 4°C. Quantitative PCR was done using an ABI7300 or ABI7500 Real-Time PCR system with Taqman Gene Expression master mix, ROX probe/quencher, and appropriate primer sequences (Applied Biosystems). Each experiment was performed in duplicate and samples assayed in triplicate. Relative quantification of *S. aureus* RNA III was determined by the change in gene expression relative to 16S gene expression. As a control, no RNAIII was detected in the *agr* deletion mutant RN6911. Cycling conditions were 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C with 1 minute at 60°C. The primer-probe sequences used were as follows: rnaIII forward primer 5′-AATTAGCAAGTGAGTAACATTTGCTAGT-3′, rnaIII probe 5′-6-FAMAGTTAG-TTTCCTTGGACTCAGTGCTATGTATTTTTCTT-BHQ-2-3′, rnaIII reverse primer 5′-GATGTTGTTTACGATAGCTTACATGC-3′, psmα forward primer 5′-TATCAAAAG-CTTAATCGAACAATTC-3′, psmα probe 5′-6-FAMAAAGACCTCCTTTGTTTGTTA-TGAAATCTTATTTACCAGBHQ-2-3′, psmα reverse primer 5′-CCCCTTCAAATA-

AGATGTTCATATC-3′, 16S forward primer 5′-TGATCCTGGCTCAGGATGA-3′, 16S probe 5′-6-FAMCGCTGGCGGCGTGCCTA-BHQ-2-3′, 16S reverse primer 5′-TTC-GCTCGACTTGCATGTA-3′.

Virulence Factor Assays

Lipase and alpha hemolysin were measured in 0.45 µm filtered cultured supernatants from bacterial strains grown as above in TSB with and without the relevant AIP or AIP treated with various antagonists. Lipase (7 hr of culture) was measured as described using a triglyceride substrate, tributyrin (Smeltzer et al., 1992). The tributyrin emulsion was prepared in sterile lipase buffer (100 mM Tris pH 8.0, 25 mM $CaCl_2$) by adding Tributyrin (Sigma 113026-50G) to a final concentration of 0.5% and then subjected to 3 minutes of sonication at 40W. Separately, a 0.8% solution of low-gelling temperature agarose was prepared in sterile lipase buffer with heating to dissolve and kept at 50°C until use. The tributyrin emulsion and agarose solutions were then combined in equal volumes for use in the assay. Lipase activity was observed by measuring the OD_{450} of the a 1:10 mixture of supernatant:tributyrin substrate every 2 minutes for 12 minutes and then plotted as optical density versus time.

Alpha hemolysin (6.5 hr of culture or as indicated) was measured as described using rabbit erythrocyte lysis (Bernheimer, 1988). Rabbit erythrocytes (CS1081, Colorado Serum Company) were washed in PBS twice by centrifugation at 3200rpm for 10 minutes at 4°C to remove Alsever's preservation buffer. Erythrocytes were then resuspended in PBS to a final concentration of 4% erythrocytes. Serial dilutions of the sample supernatants were then mixed in equal volumes with the erythrocyte suspension in a 96 well round bottom plate and incubated for 1 hour at 37°C. Following incubation,

the plate was centrifuged for 3 minutes at 3800 rpm at 4°C and the supernatants were transferred to a new 96 well plate and then read on a spectrophotometer at a wavelength of OD_{450} nm. The lysis by the individual samples was compared to the total lysis of erythrocytes that resulted from mixing equal volumes of the blood suspension and PBS/0.1% Triton. One unit of hemolytic activity was defined as the amount of bacterial supernatant able to liberate half of the total hemoglobin from the erythrocytes.

AIP Binding Assay

Early-exponential phase *S. aureus* (RN6390 or LAC at 2×10^7 cfu/ml) were incubated in 1 ml TSB in polystyrene tubes without shaking for 1 hour at 37°C. Cultures were incubated in the presence of 1 µM FITC-AIP1 or control FITC-IgG alone or in combination with 2 µg/ml VLDL, 10 µg/ml apoB, 10 µg/ml apoA1, or 1 µM native AIP1. PBS was used as a vehicle control. Following incubation, samples were washed and then fixed in 1% paraformaldehyde/25 mM CaCl2. Binding of FITC-AIP1 to the bacteria was determined by flow cytometry.

Surface Plasmon Resonance

Surface plasmon resonance was performed with the Biacore X100 (Biacore Life Sciences, GE Healthcare, Piscatawy, NJ) to analyze the interaction of apoproteins with immobilized AIPs. N-terminal biotinylated AIP1, AIP2, AIP3, or Y-AIP3 in the native or linear conformation as indicated in the figures were immobilized on streptavidin sensor chips according to the manufacturer's protocol. The chips were regenerated with 50 mM NaOH, 1 M NaCl and then washed with immobilization buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 3 mM EDTA). The biotinylated AIPs were pulsed onto the chip at 30

µg/ml for 420 s at a flow rate of 10 µl/min followed by extensive washing. For binding studies, the analytes (apoB, apoA1, apoC1, VLDL and LDL) at 10 nM in running buffer (10 mM Hepes, pH 7.4, 250 mM NaCl, 3 mM EDTA) were applied at a flow rate of 10 µl/min with a contact time of 60 s and a dissociation time of 60 s. Chip platforms could be regenerated to permit additional binding studies after a 60 s wash with 0.5% SDS followed by 120 s stabilization period. For each experiment specific binding was measured as the RU generated by analyte binding to the test surface minus RU generated by analyte binding to the reference surface (streptavidin without biotin-AIP). Kinetic data for apoB binding to biotin-AIP1 were collected at a flow rate of 30 µL/min with association measured at 60 s and dissociation measured over 300 s. The Biacore evaluation software (X100 Version 1.0) was used to analyze the results. For competition studies, 50 nM soluble inhibitor peptides (AIP1, linear AIP1, AIP2, AIP4, YAIP3, oxidized AIP1) in running buffer with 1% DMSO were incubated with 10 nM apoB prior to injection over immobilized AIP1. Binding was measured at a flow rate of 10 µL/min with contact and dissociation times of 60 s. All analyses were performed at 25°C.

Macrophage Uptake of AIP-LDL Complexes

RAW264 murine macrophage cell line (ATCC, Manassas, VA) was maintained in Dulbecco's Modified Eagle Media supplemented with L-glutamine, penicillin/streptomycin (Gibco, Carlsbad, CA), and 5% lipoprotein-deficient fetal calf serum (Sigma-Aldrich Co. St. Louis, MO). Cells (1×10^6) were incubated in suspension in polystyrene tubes in 1 ml of serum-free, phenol red-free DMEM containing either 1 µg/ml DiI-Ac-LDL plus 9 µg/ml Ac-LDL or 10 µg/ml Ac-LDL at 4°C for 30 minutes. Following incubation, 1 µM FITC-AIP1 or FITC-streptavidin was added, mixed gently,

then placed at 37°C for 10 or 30 minutes. For controls, AcLDL was incubated with mAb anti-apoB on ice for 30 min prior to addition to the cells. In addition, 1 μM FITC-AIP1 and 10 μg AcLDL were incubated on ice for 1 hr and then dialyzed prior to addition to the cells. Following treatment, cells were washed twice in buffer, fixed with 3% paraformaldehyde for 10 minutes, rinsed and resuspended in PBS. Cytospins were made using a Shandon Cytospin 3 (Thermo Scientific, Waltham, MA), and coverslipped with Prolong Gold anti-fade. Images were acquired on a Zeiss LSM 510 confocal microscope and the percent co-localization of green and red fluorescence determined using Slidebook software (Intelligent Imaging Innovations, Denver, CO).

Secretion of Human ApoB by Rat Hepatoma Cell Lines

McArdle-RH7777 cells stably transfected with constructs for human apoB-100, apoB-48 and apoB-17 have been provided by Zemin Yao, University of Ottawa, CA. Non-transfected control McArdle-RH7777 cells were purchased from ATCC (Manassas, VA). All cells were maintained in Dulbecco's Modified Eagle Media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (Gibco, Carlsbad, CA) , 10% horse serum (VWR, West Chester, PA), 1%HEPES, and 1% penicillin/streptomycin in tissue culture treated 75 cm^2 flasks (Corning, Corning, NY). Transfected cell lines also received 0.2 mg/ml G418-sulfate (VWR, West Chester, PA). Sub-culture ratios were 1:4; cells were rinsed with 1X PBS and detached with 0.05% Trypsin-EDTA (Gibco, Carlsbad, CA) before removal and division among fresh flasks. To stimulate secretion of apoB, cells were incubated for two days in serum-free media containing 0.2 mg/ml G418-sulfate. Supernatants were collected and concentrated using Amicon Ultra-15 Centrifugal filter

devices (Millipore Billerica, MA), supplemented with an EDTA-free protease inhibitor (Roche, Indianapolis, IN) and stored at 4°C .

Isolation of McArdle Cell-derived ApoB fragments

A murine monoclonal antibody C1.4 (Santa Cruz Biotechnology, Santa Cruz, CA) was immobilized onto Protein G agarose beads (Thermo Scientific, Waltham, MA) following a modified protocol from (Schneider et al., 1982). Beads (500 µl weight/volume) were washed twice in a 0.1 M Sodium Borate solution and then combined with 200 µg of antibody and incubated for 30 minutes at room temperature with gentle shaking. Beads were pelleted by centrifugation and the supernatant removed and reserved. The beads were then washed three times with borate buffer and once with 0.2 M triethanolamine. Beads were then incubated for 45 minutes at room temperature with gentle shaking in a solution of 25 mM Dimethyl pimelimidate dihydrochloride (Sigma, St. Louis, MO) made up in 0.2 M triethanolamine, pH 8.2. Beads were then washed with 0.1 M ethanolamine and then incubated 1 hour at room temperature with gentle shaking in fresh ethanolamine. Following incubation, beads were washed three times in 1X PBS , rinsed with 0.1 M glycine pH 2.5 for 5 minutes at room temperature to remove unlinked antibody, washed once more in PBS, resuspended in PBS and stored at 4°C. To purify apoB fragments from McArdle cell supernatant, these antibody-coated beads were incubated with the supernatant samples overnight at 4°C with gentle rotation. The beads were then washed once with PBS and then incubated for 5 minutes with 0.1 M glycine pH 2.5 and then neutralized with 1 M Tris pH 8. The supernatant was collected and stored at 4°C.

Preparation of Lipoproteins from Pooled Human Sera

Lipoproteins were isolated from whole pooled human sera using the OptiPrep reagent (Sigma, St. Louis, MO) as described (Graham et al., 1996). To remove chylomicrons from the samples, protease inhibitor-treated pooled human sera was placed in 14×89 mm centrifuge tubes, covered with mineral oil, and centrifuged in an ultra centrifuge (Beckman, Brea, CA) at 16°C at 100,000 × *g* for 30 minutes using a TH-641 swinging bucket rotor. Following centrifugation, the mineral oil and white chylomicron layer were removed and discarded and the chylomicron-free serum was combined into one conical tube, mixed 4 parts serum to 1 part OptiPrep. Mixture was divided evenly into Optiseal centrifuge tubes (Beckman, Brea, CA), with 3-4 mls solution in each and then covered with a layer of Hepes-buffered saline solution (0.85% w/v NaCl, 10 mM Hepes-NaOH, pH 7.4) on top to fill the tube up into the neck and tubes were sealed with the black plugs included in the package. Tubes were place in balanced positions in a NVT 90 rotor and secured with brass threaded caps and then centrifuged at 350,000 × *g* for 3 hours at 16°C. Following centrifugation, the tubes were carefully removed and the top transparent layer of the gradient containing VLDL was removed with a 5cc syringe fitted with an 18 gauge needle; the yellow layer containing LDL was removed in a similar fashion. Fractions were immediately dialyzed against sterile PBS in Slide-A-Lyzer dialysis cassettes (Pierce, Rockford, IL) to remove the OptiPrep solution. ApoB protein concentrations of the lipoprotein preps were determined by a human apoB ELISA (Cayman Chemical, Ann Arbor, MI or Alerchek, Portland, ME).

Preparation of AIP-Enriched Supernatant

Bacteria-produced AIP was prepared and concentrated by first growing an overnight culture of *agr*2 strain 502A or *agr*3 strain MW2 in 15 ml of TSB in a 50 ml Falcon tube at 37°C with 200 rpm shaking. The culture was then centrifuged at 4°C at 3000 rpm for 4 minutes, the supernatant was filtered through a 0.22 μm filter, and stored on ice. Centricon YM-10 concentrator tubes (Millipore, Billerica, MA) were rinsed with deionized water according to the manufacturer's instructions by centrifuging in a Sorvall RC2-B centrifuge with fixed angel rotor SS-34 at 1000 × *g* for 30 minutes. Supernatant samples were then loaded onto the centricon tubes and centrifuged at 5000 × *g* for 1-1.5 hours. The resulting filtrate was aliquoted and stored at -80°C.

Mice

The appropriate institutional committees approved all experiments involving animals. C57BL/6 mice (≈ 10 wk, ≈ 22-28 g) from Charles Rivers (Wilmington, MA), NADPH oxidase knock-out on the C57BL/6 background (*gp91*$^{phox-/-}$) (Pollock et al., 1995), B6 x 129, and PCSK9 knock-out (*Pcsk9*$^{-/-}$) (Rashid et al., 2005) on the B6 x 129 background all from Jackson Laboratory (Bar Harbor, ME) were gender- and age-matched. Mice receiving 4APP treatment were injected i.p. 48 hours and 24 hours prior to infection with 100 μl of 5 mg/ml 4APP prepared by dissolving in 1 M HCl at 100 mg/ml and diluted to 5 mg/ml in 0.025 M phosphate buffer (pH 8). The solution was adjusted to pH 4 with 7.5% $NaHCO_3$ immediately before injection. Total cholesterol levels were determined using a kit and standard (Thermo Electron, Louisville, CO) following the manufacturer's instructions. Wild-type levels were 113 ± 2.5 mg/dl, 4APP treatment reduced levels to 56.5 ± 1.9 mg/dl, and PCSK9 deficiency reduced levels to 84.3 ± 13 mg/dl.

Air Pouch Infection Model

Subcutaneous air pouches were created seven days before infection by injection of 5cc air on the backs of healthy mice using a 5cc syringe and a 27 ½ gauge needle. Four days prior to infection, the pouches were re-inflated with 2.5cc air. On the day of infection, early exponential phase bacteria at the dose indicated in the figure legends were injected directly into the pouch and mice were weighed, as previously described (Rothfork et al., 2003; Rothfork et al., 2004). Twenty-five to 28 hours after infection, the mice were weighed and scored for morbidity by the following scale: Appearance: 0-4; natural behavior: 0-3; hydration status (skin pinch test): 0-3; provoked behavior: 0-4. The morbidity score is the sum of the scores in the 4 categories with a maximum of 14 at which point the mice were considered moribund. The animals were then euthanized by isoflurane inhalation and the following samples were collected: blood, pouch lavage, pouch tissue, and spleen. Blood was collected using a 19 gauge needle and a 1 cc syringe to withdraw it from the aorta immediately after euthanasia; blood was placed in sterile glass tubes and placed on ice for 2 hours. After clotting, the blood was centrifuged at maximal speed at 4°C for 10 minutes and then sera was collected and stored at 4°C. The pouch tissue was carefully exposed and rinsed with 5 ml 1X Hanks Balanced Salt Solution containing 0.2% HSA; this lavage fluid was collected with a 10cc syringe and 18 gauge needle and transferred to a tube and stored on ice. Basolateral pouch tissue was surgically removed and fixed in 3% paraformaldehye containing 0.1 mM CaCl2 and 0.1 mM MgCl in PBS for 3 to 4 hours on ice. Spleens were removed and placed in 1 ml of HBSS/HSA in a bead beating tube containg several sterile 2.3 mm beads (Biospec, Bartlesville, OK) and stored on ice. Lavage and spleen samples were processed for bacterial CFU by homogenizing the spleens in a bead beater, diluting all samples 1:10 in

1 ml 1X PBS/0.1%Triton, sonicating the sample, then plating serial dilutions on blood agar, all as described (Rothfork et al., 2004). Cytokine levels in the filtered lavage fluid were determined using a MIP-2 ELISA kit (R&D Systems, Minneapolis, MN).

Tissue Invasion Assay

After pouch tissues were fixed in 3% Paraformaldehyde for 3-4 hours they were rinsed in PBS and were stored overnight in 25% sucrose in PBS, oriented and embedded in OCT (Sakura Finetek, Torrance, CA) in cryomolds, frozen in liquid nitrogen, and stored at -80°C. Cryosections (10 µm) were cut onto slides and fixed and permeabilized with acetone at -20°C for 5 minutes, rehydrated with PBS, blocked for endogenous biotin with a streptavidin-biotin blocking kit (Vector Labs, Burlingame, CA), and blocked with 5% normal rabbit serum in PBS containing 0.1% Tween and 1% BSA for 2 hr at 4°C. The blocked sections were stained with 2µg/ml mouse IgG3 anti-*S. aureus* (GeneTex Inc, San Antonio, Tx) labeled with biotin (FluoReporter Mini¬Biotin-XX, Molecular Probes, Eugene, Or) for 1 hour at 4°C, rinsed and incubated with 0.5 µg/ml streptavidin-rhodamine (Jackson ImmunoResearch, West Grove, PA) for 30 min at 4°C, rinsed, and cover-slipped with ProLong Gold antifade reagent (Molecular Probes, Eugene OR). Uninfected pouch tissue stained as above served as the negative control. Images were acquired on a Zeiss LSM 510 confocal microscope. Bacterial density as a measure of *in vivo* biofilm formation in the epidermis and tissue invasion into the dermis was quantified from LSM images using Slidebook software (Intelligent Imaging Innovations, Denver, CO). Regions of interest were selected using histiologic criteria for the epidermis (~first 100 µm) or dermis (~200-300 µm) and the total area and the portion of that area stained for *S. aureus* quantified in microns^2. Values are displayed as % of epidermis or dermis

stained from a minimum of 19 sections for each experimental condition. Total bacteria were determined by adding green, yellow and red fluorescence for the relevant sections and quorum-sensing bacteria determined by adding green and yellow fluorescence for the relevant sections.

Statistical Evaluation

Data are displayed as the mean ± SEM. *In vitro* data were analyzed by the Student's t test and the *in vivo* results by the Mann-Whitney U test for nonparametrics using StatView for Macintosh or GraphPad Prism 4.0.

CHAPTER 3

APOLIPOPROTEIN B IS AN INNATE BARRIER AGAINST INVASIVE *STAPHYLOCOCCUS AUREUS* INFECTION

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Summary

Staphylococcus aureus is both a colonizer of humans and a cause of severe invasive infections. Although the genetic basis for phenotype switching from colonizing to invasive has received significant study, knowledge of host factors that antagonize the switch is limited. We show that VLDL and LDL lipoproteins interfere with this switch by antagonizing the *S. aureus agr* quorum-sensing system that upregulates genes required for invasive infection. The mechanism of antagonism entails binding of the major structural protein of these lipoproteins, apolipoprotein B, to an *S. aureus* autoinducing pheromone, preventing attachment of this pheromone to the bacteria and subsequent signaling through its receptor, AgrC. Mice deficient in plasma apolipoprotein B, either genetically or pharmacologically, are more susceptible to invasive *agr*+ bacterial infection, but not to infection with an *agr* deletion mutant. Therefore, apolipoprotein B at homeostatic levels in blood is an essential innate defense effector against invasive *S. aureus* infection.

Introduction

The host response to bacteria that both colonize their hosts and cause invasive, lethal infection is complex and requires selective host defense effectors that limit invasive infection while tolerating colonization at epithelial surfaces. *Staphylococcus aureus* colonizes 20%–30% of the population persistently and another ~50% – ~60% transiently, and, while the number of invasive infections is increasing, especially those caused by methicillin-resistant bacteria (MRSA)-like strains of the USA300 PFGE type (Klevens et

al., 2007), they still reflect a small percentage of colonized individuals. This suggests that the majority of normal human hosts, even if colonized, have intact barriers to invasive infection. Moreover, it suggests that failures in these barriers may increase the susceptibility to invasive infection. Therefore, we postulated that specific elements of acute inflammation are likely to discriminate between these two outcomes of *S. aureus*-host interaction.

Virulence factor expression by *S. aureus* is regulated by a complex set of signals that are integrated to induce transcription of genes required for survival within distinct niches in response to sensing the host environment (Cheung et al., 2004; Torres et al., 2007; Yarwood et al., 2002). *agr* is a four-gene operon present in the majority of *S. aureus* clinical isolates that controls, in part, a change in phenotype from adhesive and colonizing to tissue damaging and invasive (George and Muir, 2007). It encodes a quorum-sensing system driven from the transcription of two promoters, P2 and P3; one codes for synthesis and secretion of an autoinducing cyclic thiolactone peptide (AIP) and a two-component regulatory pathway, and the other generates in response to AIP a regulatory RNA transcript, RNAIII, that is the effector of the operon. Four different AIPs are produced by *S. aureus* types that differ in amino acid composition and length but retain the thiolactone structure (George and Muir, 2007) with type 1 AIP predominating in clinical isolates. After RNAIII is produced, it downregulates expression of surface adhesins while upregulating expression of secreted toxins, proteases, lipase, and metabolic pathways (George and Muir, 2007). We postulated that innate immunity and, specifically, elements of acute inflammation would regulate this phenotypic change and thus contribute to maintaining the host-pathogen balance toward a noninvasive outcome.

During acute inflammation, increases in vascular permeability result in plasma leakage into the injured tissue (Bucci et al., 2005). Because AIP interaction with its receptor, AgrC, involves, in part, recognition of the thiolactone ring by a hydrophobic pocket within the receptor (Wright et al., 2004; George and Muir, 2007; Jensen et al., 2008), we hypothesized that lipoproteins secreted by the liver and present in plasma that extravasates to acutely infected tissue would antagonize AIP signaling through AgrC. Here, we show that apolipoprotein B, the major structural protein of very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL), sequesters AIP1 and thus inhibits *agr* signaling and limits invasive infection caused by both laboratory strains and MRSA USA 300 isolates.

Results

Serum Low-Density Lipoproteins Antagonize Pheromone AIP1 Signaling

We hypothesized that constituents of plasma, specifically lipoproteins, could provide a check against quorum-sensing-dependent virulence at sites of *S. aureus* infection by interfering with the interaction of AIP with an essential hydrophobic pocket within its cognate receptor AgrC (Wright et al., 2004; Jensen et al., 2008). Using a reporter strain where activation of the *agr* P3 promoter drives expression of GFP (Rothfork et al., 2004), synthetic AIP1 (100 nM) induced activation of the P3 promoter optimally at 3 hr, and inclusion of dilutions of pooled human serum (PHS), as compared to lipoprotein-deficient pooled human serum (LPDS), inhibited its function (Figure 1A), indicating that serum lipoproteins can antagonize AIP1 signaling without affecting

bacterial growth (CFU) (Figure 1A, inset). Inclusion of mouse, chicken, rabbit, or bovine sera also inhibited activation (data not shown). Culture of the bacteria in 10% PHS also inhibited spontaneous P3 promoter activation during longer culture times as compared to 10% LPDS without affecting bacterial growth, indicating that serum lipoproteins can inhibit endogenous AIP function (Figure S1A, Appendix).

Purified human VLDL and LDL particles, as compared to high-density lipoproteins (HDL) at equivalent cholesterol concentrations (0.4 mM), significantly antagonized AIP1-induced P3 activation (Figure 1B) also with no effect on bacterial CFU (data not shown). Moreover, inclusion of VLDL significantly inhibited AIP1-induced RNAIII transcript production relative to 16S RNA as measured by qRT-PCR in the USA300 clinical isolate UAMS1378 (Figure 1C). These data indicate that the lowest density lipoproteins secreted by the liver into blood significantly and specifically antagonize AIP1-dependent signaling in both laboratory strains and clinical isolates associated with invasive infections.

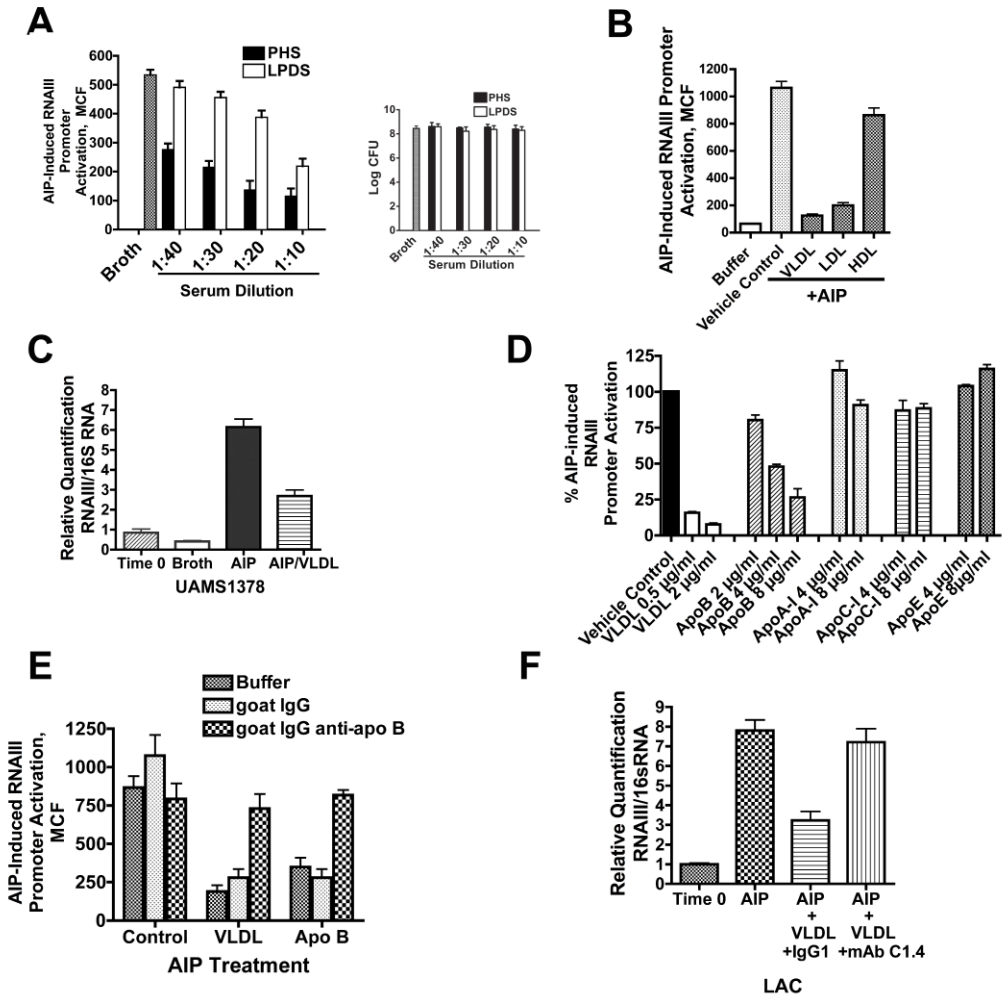

Figure 1. Serum Lipoproteins Antagonize *agr* Signaling

(A) *agr*:P3 promoter activation by 100 nM AIP 1 is inhibited in *S. aureus* (ALC 1743 with *agr*:P3-*gfp*, 2×10^7/ml) during 3 hr of culture with either broth or the indicated dilutions of pooled human serum (PHS) as compared to lipoprotein deficient sera (LPDS). Magnitude of promoter activation was measured as fluorescence induction (mean channel of fluorescence [MCF]). (Inset) Log CFU indicated that bacterial growth was equivalent under both conditions. Data are represented as the mean ± SEM; n = 3 performed in duplicate.

(B) Effect of VLDL, LDL, or HDL at equivalent cholesterol concentration (0.4 mM) on 100 nM AIP1-induced *agr:*P3 activation during 3 hr of culture of ALC1743. Data are represented as the mean ± SEM; n = 3 performed in duplicate.

(C) Relative quantification of RNA III transcript to 16 s RNA produced by USA300 clinical isolate UAMS 1378 cultured at 2×10^7/ml with 100 nM AIP1 with or without 2

mg/ml VLDL for 1 hr. Data are represented as the mean ± SEM; n = 4 performed in triplicate.

(D) Dose-dependent inhibition of AIP1-induced *agr*:P3 activation by purified apolipoprotein B, but not other serum lipoproteins. Concentrations tested: apoB, 2–8 μg/ml (4–16 nM); apoA-I, 4–8 μg/ml (143–288 nM); apoC-1, 4–8 μg/ml (0.61–1.2 mM); and apoE, 4–8 μg/ml (117–234 nM).

(E) Goat IgG anti-apoB (5 μg/ml) versus control goat IgG reversed VLDL (5 μg/ml) and purified apoB (5 μg/ml) inhibition of AIP1-induced *agr*:P3 activation in ALC1743. Data are represented as the mean ± SEM; n = 3 performed in duplicate.

(F) mAB C1.4 IgG1 anti-apoB (5 μg/ml) versus control mouse IgG1 reversed VLDL (5 μg/ml) inhibition of 100 nM AIP1-induced relative RNA III transcript produced by USA300 clinical isolate LAC cultured at 2×10^7/ml for 1 hr. Data are represented as the mean ± SEM; n = 3 performed in duplicate.

Apolipoprotein B Is Sufficient and Necessary to Antagonize AIP1 Signaling and Secretion of Virulence Factors Required for Invasive Infection

Serum lipoproteins are complex particles composed of a neutral core containing triglycerides and cholesterol esters covered by an amphipathic monolayer of phospholipids and unesterified cholesterol. The protein, apo, components bind to the surface of the particles and are either restricted to particular lipoproteins or freely exchangeable across lipoprotein categories (Olofsson and Boren, 2005). We asked whether the major structural nonexchangeable apo protein, apolipoprotein B, of VLDL and LDL was able to inhibit AIP1-induced *agr* P3 promoter activation. ApoB inhibited in a dose-dependent fashion at concentrations (8 μg/ml) less than normal serum values (~80 μg/ml) (Figure 1D) with no effect on bacterial CFU (data not shown). Other apolipoproteins, apoA-1, apoC-1, and apoE, had no effect, indicating that AIP antagonism is not a general property of lipid-binding proteins (Figure 1E). Treatment of either VLDL or apoB with antibody against apoB, but not control IgG, reversed the ability of both to inhibit AIP1-induced P3 activation (Figure 1E), indicating that any associated lipids were not sufficient for antagonism and that antigenic epitopes of apoB were essential. Moreover, VLDL inhibition of AIP1-induced RNAIII transcript production in the USA300 strain LAC (also AIP1) was reversed by a monoclonal antibody against an epitope in the amino terminal domain of apoB (mAb C1.4), but not by an isotype control antibody (Figure 1F). Additionally, treatment of PHS with mAb C1.4 versus an isotype control increased spontaneous P3 promoter activation in ALC1743 and RNAIII transcript production in LAC during culture in the presence of 10% PHS (Figures S1B and S1C). Consistent with these data indicating apoB in the

control of *agr*:P3 promoter activation, liposomes composed of various phospholipids and cholesterol had no effect on AIP signaling (Figure S2A). Inclusion of the triglyceride tributyrin partially reduced AIP signaling (Figure S2B), but it required concentrations approximately five times higher than normally observed in serum. These data demonstrate that apolipoprotein B is both sufficient and necessary for inhibition of AIP1 signaling in both laboratory strains and important clinical isolates.

Activation of *agr* contributes to the regulation of at least 70 genes, including 23 known virulence actors like α hemolysin and lipase. We determined the effect of VLDL and purified apoB on α hemolysin (*hla*) promoter activation and secretion of α hemolysin and lipase by multiple laboratory strains and clinical isolates, including MRSA isolates of the USA 300 and 400 PFGE types that currently contribute to invasive human infection (Klevens et al., 2007). Apolipoprotein B inhibited AIP1-induced *hla* promoter activation (Figure S3A) and both AIP1-induced (Figure S3B) and spontaneous (Figure S3C) secretion of α hemolysin by both a laboratory strain (SH1000) and multiple USA 300 isolates (USA 300 3757, UAMS 1378, and LAC). In addition, apoB and VLDL inhibited AIP1-induced lipase secretion in all strains tested, including USA300 isolates (Figures S3D and S3E). Moreover, apoB inhibited type 3 AIP signaling in a USA 400 strain, suggesting that apolipoprotein B inhibition may not be limited to the type I peptide (Figure S3B).

Apolipoprotein B and Its Associated Lipid Particles Antagonize agr Signaling by Inhibiting AIP1 Binding to Bacteria and Sequestering It for Uptake by Macrophages

ApoB-containing lipoproteins could antagonize activation of the *agr* P3 promoter either by blocking AIP1 binding to the bacteria or by inhibiting signaling consequent to AIP1 binding. To determine whether apoB could block recognition of AIP1 by AgrC, we evaluated binding of AIP1 synthesized with an FITC tag in the amino terminus to wild-type bacteria. Specific FITC-AIP1 binding was inhibited by ~50% by an equal concentration of soluble native AIP1, indicating that FITC-AIP1 and native AIP1 compete equally for binding (Figure 2A). While both VLDL and purified apoB inhibited FITC-AIP1 binding to both RN6390 and the clinical USA300 isolate, LAC (Figures 2A and 2C), apoA-1 did not. Neither VLDL nor apoB inhibited binding of FITC-labeled IgG to the bacteria, indicating that lipoprotein-mediated inhibition was specific to AIP and not the FITC tag (Figure 2B). These data indicate that apoB-containing lipoproteins antagonize *agr* signaling by blocking AIP1 binding.

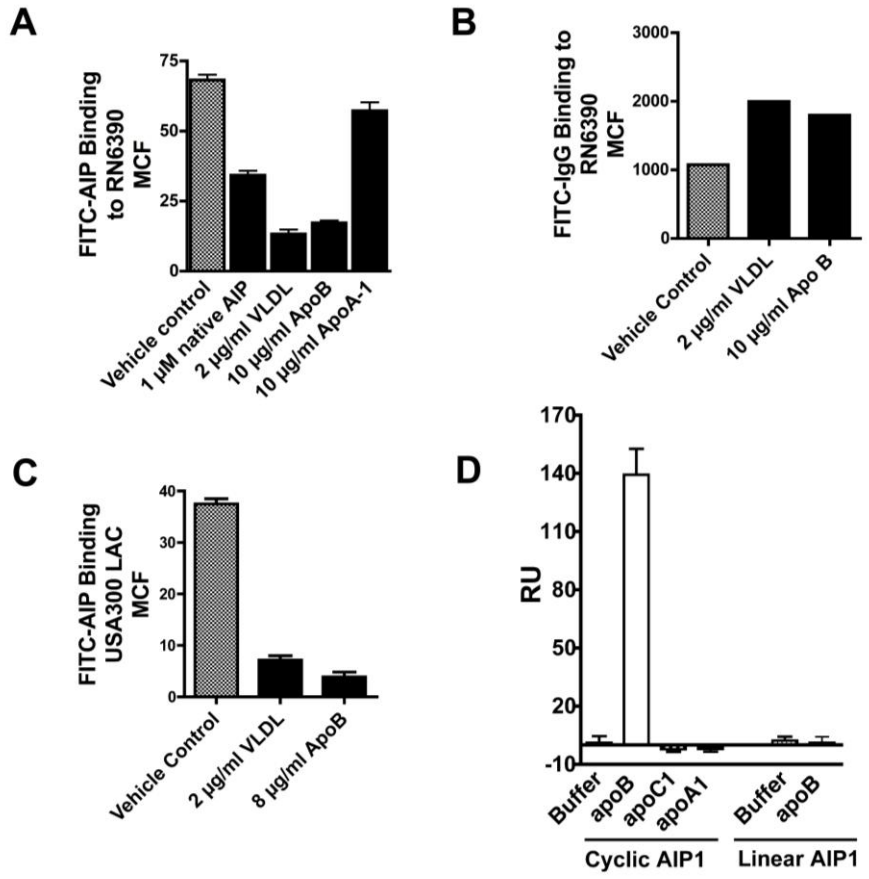

Figure 2. Apolipoprotein B and Its Associated Lipid Particles Inhibit AIP Binding to *S. aureus* and Interact with AIP by Surface Plasmon Resonance

(A) S. aureus (RN 6390, 2×10^7/ml) was incubated with 1 mM FITC-AIP1 alone (vehicle control) or in combination with potential inhibitors including 1 mM native AIP1, VLDL (2 μg/ml), apoB (8 μg/ml), or apoA-1 (8 μg/ml) at 37°C for 3 hr. Specific binding was measured by flow cytometry. Data are represented as the mean ± SEM; n = 4.

(B) One micromolar FITC-IgG binding to *S. aureus* RN6390 was not inhibited by VLDL or apoB. Data are represented as the mean ± SEM; n = 3.

(C) *S. aureus* (USA300 LAC, 2×10^7/ml) was incubated with 1 mM FITC-AIP1 in vehicle control or in combination with VLDL (2 μg/ml) or apoB (8 μg/ml) at 37°C for 3 hr. Specific binding was measured by flow cytometry. Data are represented as the mean ± SEM; n = 2 performed in duplicate.

(D) Biacore X100 analysis in resonance units (RU) of the interaction of 10 nM apoB, apoC1, or apoA1 with either cyclic biotin-AIP1 or linear biotin-AIP1 immobilized on streptavidin chips after 60 s of contact time followed by 60 s of dissociation time at a flow rate of 10 μl/min. Data are represented as mean ± SEM; n = 3–5.

ApoB-containing lipoproteins could inhibit AIP1 binding either by occupying its binding site in its cognate receptor or by sequestering AIP1 away from the bacteria. Unlike LDL binding to Group A Streptococci (Han et al., 2006), we did not detect direct binding of LDL to the *S. aureus* strains we studied under the conditions we examined (data not shown). We used surface plasmon resonance (SPR) to compare direct binding of purified apoB (10 nM) to either native AIP1 or linear AIP1 immobilized at equivalent concentrations via a biotin at the amino terminus to a streptavidin-coated chip (Figure 2D). This binding was dose dependent (Figure S4A) and specific to apoB as equal molar concentrations of apoC1 or apoA1 did not result in detectable binding as measured as resonance units (Figure 2D). Injection of apoB over biologically inactive linear AIP1 did not result in detectable binding even though equivalent amounts of the cyclic and linear forms of AIP1 were bound to the chips (Figure 2D), indicating that an intact thiolactone ring is required for interaction. Therefore, we determined whether other native AIPs and AIPs modified to limit their biologic activity while retaining the thiolactone ring could compete against immobilized AIP1 to prevent apoB binding. As predicted, soluble native AIP1, but not linear AIP1, prevented apoB binding (Figure S4B). Native AIP2 and AIP4 were equivalent if not better than soluble native AIP1 and caused significant ($p < 0.001$) inhibition of apoB binding to immobilized AIP1 (Figure S4B). Interestingly, AIP1 treated with oxidants to create a methionine sulfoxide variant that lacks biologic activity (Rothfork et al., 2004) and AIP3 synthesized with a tyrosine in the amino terminus that is nonfunctional (Otto et al., 1998) prevented apoB binding to immobilized AIP1 (Figure S4B), indicating that chemical modifications that result in loss of function but retain the thiolactone ring permit interaction with apoB. A control cyclic peptide made by disulfide

crosslinking had no effect (Figure S4B). In addition, apoB bound to immobilized AIP1 could be removed by washing with 0.5% SDS and the chip reused to demonstrate new apoB binding, indicating that the interaction of apoB with AIP1 retained the native conformation of AIP1 required for apoB recognition. This suggests that the interaction of the two is reversible. These data indicate that apoB-containing lipoproteins antagonize *agr* signaling by binding AIP through recognition of the thiolactone ring and preventing AIP ligation of the AgrC receptor.

Because macrophages express scavenger receptors that bind apoB-containing LDL modified either by oxidation or acetylation (Moore and Freeman, 2006), we postulated that lipoprotein sequestering of AIP1 could contribute to host defense by facilitating uptake and destruction of AIP1 by macrophages. LDL modified by acetylation was equivalent to native lipoproteins in antagonizing AIP1 signaling, and the inhibition was reversed by an mAb to an amino-terminal epitope in apoB, but not by an mAb of the same isotype against a carboxy-terminal epitope, indicating that chemical modification of LDL does not affect apoB antagonism of AIP1 (Figure 3A). To exclude the possibility of a direct inhibitory effect of AcLDL on the bacteria, we incubated bacteria with AIP1 and AcLDL for 3 hr, washed, and then incubated with either broth or AIP1. These bacteria were fully able to demonstrate spontaneous activation of the *agr*:P3 promoter as well as respond to AIP (Figure 3B). To examine scavenger receptor uptake of AIP1, murine RAW264 macrophages were incubated on ice with acetylated LDL with a red fluorescent label (DiIAc- LDL) and then with FITC-AIP1. After increasing the temperature to 37°C, the percentage of colocalization of the red and green fluorescence was evaluated by confocal microscopy (Figure 3C). At 10 min, ~60% of the FITC-AIP1

colocalized with Ac-LDL (Figure 3D). Controls included using unlabeled Ac-LDL that demonstrated only green fluorescence, using DiI-Ac-LDL without FITC-AIP1 to demonstrate only red fluorescence, and using FITC avidin to demonstrate that the colocalization was specific to AIP1 and not to the FITC tag. To demonstrate that this colocalization was dependent on apoB, an mAb that reversed apoB inhibition of AIP1 signaling (mAb C1.4, Figure 3A) significantly inhibited colocalization of FITC-AIP1 with AcLDL (Figure 3D). Complexes of AcLDL and FITC-AIP1 that were dialyzed prior to addition to the cells demonstrated equivalent levels of colocalization (Figure 3D). Similar results were obtained with primary murine bone marrow macrophages (data not shown). These data demonstrate that modified LDL can specifically bind and sequester AIP1 for uptake and destruction by macrophages present at the site of infection.

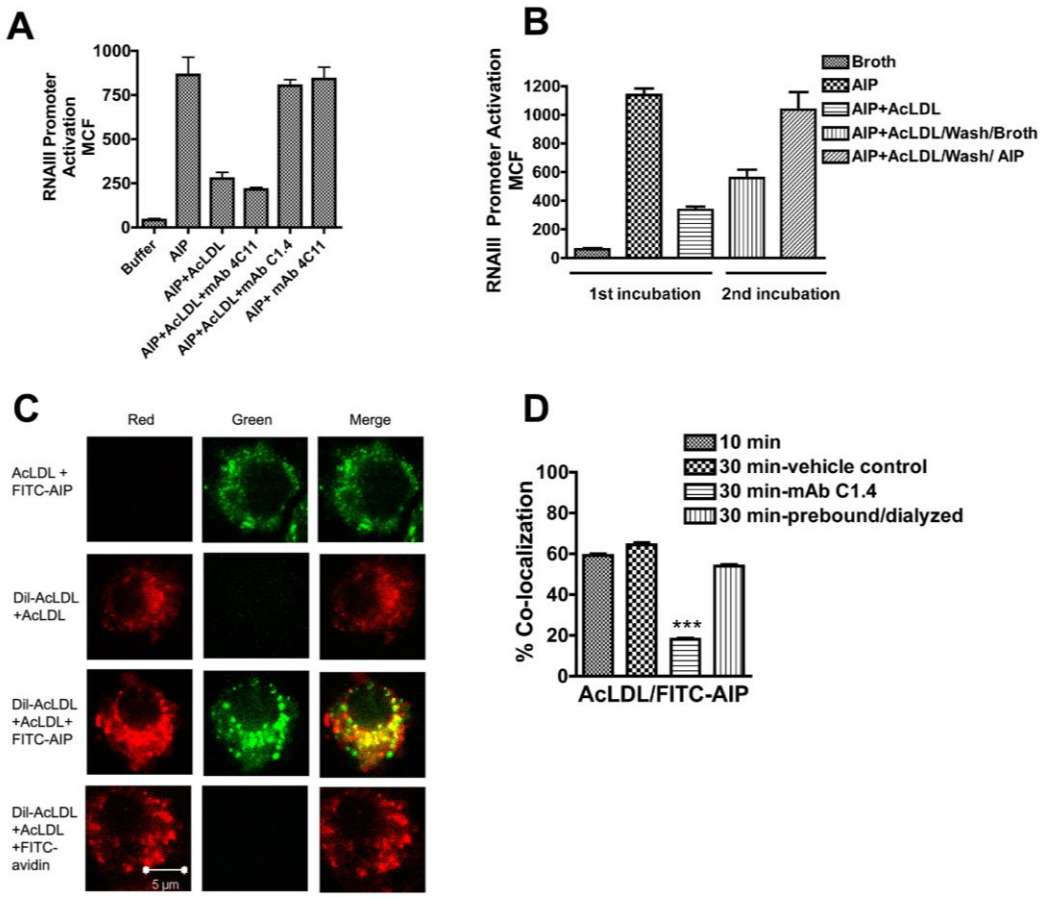

Figure 3. Interaction of AIP with Modified LDL and Uptake by Macrophage Scavenger Receptors

(A) LDL modified by acetylation (5 µg/ml) inhibits AIP1-induced *agr*:P3 promoter activation, and the inhibition is reversed by an mAb to an N-terminal epitope of apoB (mAb C1.4) (5 µg/ml), but not by an mAb against a C-terminal epitope (mAb 4C11) (5 µg/ml). Data are represented as the mean ± SEM; n = 3 performed in duplicate.

(B) Reversibility of AIP1-induced *agr*:P3 promoter activation inhibited by modified LDL. Reporter bacteria incubated with 50 nM AIP1 and 5 µg/ml AcLDL for 3 hr first and then washed and incubated subsequently with either broth or 500 nM AIP1 demonstrated both spontaneous and AIP1-induced *agr*:P3 promoter activation. Data are represented as the mean ± SEM; n = 2 performed in duplicate.

(C) RAW264 murine macrophages were incubated first with mixtures of fluorescently labeled and unlabeled AcLDL on ice and then with FITC-AIP1 for 10 or 30 min at 37° C. Fixed cells were imaged by confocal microscopy. (First row) RAW cells incubated with unlabeled AcLDL (10 µg/ml) and then with FITC-AIP1 (green fluorophore, 1 mM). Confocal images show no bleed through to the red channel. (Second row) RAW cells

incubated with DiI-AcLDL (red fluorophore, 1 μg/ml) and unlabeled AcLDL (9 μg/ml). Confocal images show no bleed through to the green channel. (Third row) RAW cells incubated with DiI-AcLDL (1 μg/ml) and unlabeled AcLDL (9 μg/ml) and then FITC-AIP1 (1 mM). Images show colocalization of LDL and AIP. (Fourth row) RAW cells incubated with DiI-AcLDL (1 μg/ml) and unlabeled AcLDL (9 μg/ml) and then FITC-avidin (1 mM). The FITC tag does not colocalize with Ac-LDL.

(D) Percent colocalization of FITC-AIP1 with AcLDL determined at both early (mean ± SEM; n = 38 cells) and later time points (mean ± SEM; n = 30 cells) using Slidebook as described in Experimental Procedures. As controls, AcLDL was treated with mAb anti-apoB (clone C1.4) (5 μg/ml) before interacting with FITC-AIP1, and the percent colocalization (mean ± SEM; n = 50 cells) was significantly reduced ($p < 0.001$). Also, dialyzing the AcLDL/FITC-AIP1 complex prior to incubation with the macrophages resulted in equivalent colocation (mean ± SEM; n = 36 cells).

Mice with Genetically Low Levels of Plasma Apolipoprotein B Are More Susceptible to agr+ versus Δagr S. aureus Infection

Synthesis of apolipoprotein B by hepatocytes initiates assembly of VLDL particles that after secretion into blood are metabolized into LDL particles that are removed for degradation by the LDL receptor. The absolute blood level of apoB is determined by both liver VLDL secretion and the uptake and degradation of apoB-containing particles by the LDL receptor (Shelness and Sellers, 2001). If apolipoprotein B is essential for defense against quorum-sensing-dependent invasive infection, then mice with low blood levels of apoB should be more susceptible to *agr*+ infection, but not infection with an *agr* deletion mutant. To test this, we used an air pouch model of infection to compare the responses of wild-type mice and mice with diminished blood levels of apoB due to overexpression of the LDL receptor as a consequence of genetic deletion of PCSK9, a subtilisin family proprotein convertase that downregulates LDL receptor expression (Horton et al., 2007). These mice are viable, develop normally, and are otherwise healthy (Rashid et al., 2005), unlike *apoB* knockout mice that have multiple development problems and early embryonic lethality (Farese et al., 1995). Pouches generated by the subcutaneous injection of air become lined with epithelium over 6 days and have been used to study *in vivo* bacterial biofilm formation (Yoshikawa et al., 2004) (Figure 1E), and we have shown that *agr* contributes to invasive infection in this model in a dose-dependent manner (Rothfork et al., 2003, 2004). Infection of *Pcsk9*$^{-/-}$ mice (black bars) with early exponential phase (i.e., nonfluorescent) *agr* P3:*gfp* reporter bacteria at a dose handled easily by wild-type mice (white bars) resulted in significantly greater morbidity, bacterial burden at the site of infection and spleen, *agr* P3 promoter activation

by the bacteria at the site of infection, inflammatory cytokine (MIP-2) concentration at the site of infection, and epidermal biofilm formation and invasion into the dermis (Figures 4A–4D). In contrast, infection with an equivalent CFU of an isogenic *agr* deletion mutant resulted in no significant differences between wild-type and *Pcsk9* knockout mice (Figures 4A, 4B, and 4D). Importantly, *Pcsk9* deficiency at this challenge dose revealed significant differences in morbidity, bacterial burden, and MIP-2 concentration between *agr*+ and *agr*- infected mice (black bars, Figures 4A, 4B, and 4D), demonstrating its requirement to control contributions of *agr* to pathogenesis. Microscopic evaluation of the pouch tissue from either strain of mice infected with the *agr* deletion mutant and stained with anti-*S. aureus* linked to a red fluorophore revealed minimal numbers of detectable bacteria (data not shown). In contrast, confocal microscopy of pouch tissue from the *Pcsk9*$^{-/-}$ mice infected with either early (Figure 4F, right panel) or midexponential phase (Figure 4G right panel) *agr*+(ALC1743) bacteria, as compared to wild-type mice (Figures 4F and 4G, left panels), had significantly greater bacterial density in the epidermis (measure of biofilm formation) and significantly greater numbers of GFP-expressing bacteria (either green or yellow), indicating that they had undergone quorum sensing (quantification shown in Figures 4F and 4G graphs). Moreover, the bacterial density, the magnitude of in vivo quorum sensing, and invasion beyond the epidermis into the dermis and underlying muscle correlated with the morbidity score of the mice, and in all cases, these were significantly greater for the apoB-deficient mice (Figures 4F and 4G, right panels and graph). These data indicate that the reduction of apoB-containing lipoproteins in the plasma of otherwise healthy mice

makes them significantly more susceptible to quorum-sensing-dependent invasive infection, but not to infection by nonquorum-sensing *S. aureus*.

VLDL and LDL Represent an Additional Barrier beyond the NADPH Phagocyte Oxidase for Control of agr+ versus Δagr S. aureus Infection

The phagocyte NADPH oxidase (phox) plays multiple roles in host defense against *S. aureus* infection (Quinn et al., 2006; Kobayashi et al., 2004). We previously demonstrated that its contribution to oxidant inactivation of AIP1 signaling contributes, in part, to control of quorum-sensing-dependent infection (Rothfork et al., 2004). To determine that apoB-containing lipoproteins represent a separate and distinct barrier against invasive quorum-sensing-dependent infection, we used pharmacologic treatment to lower plasma apoB levels of mice genetically deficient in *phox* (*gp91*$^{phox-/-}$) (Pollock et al., 1995). *Phox*-deficient mice treated with a drug 4APP that inhibits low-density lipoprotein secretion by the liver (Mounkes et al., 2001) were significantly more susceptible to MRSA USA300 LAC *agr*+ infection than vehicle control-treated mice. They had significantly greater morbidity, weight loss, and bacterial burden at the site of infection and systemically (Figures 5A–5C). In contrast, 4APP treatment of these mice did not increase susceptibility to infection with the *agr* deletion mutant (Figures 5A–5C). Importantly, as with *Pcsk9* deficiency, 4APP treatment revealed significant differences in morbidity, weight loss, and bacterial burden between *agr*+ and Δ*agr* infected mice (Figures 5A–5C). Microscopic evaluation of the pouch tissue stained with anti-*S. aureus* (red fluorescence) revealed significantly greater bacterial density in the epidermis of 4APP-treated mice infected with *agr*+ bacteria (Figures 5D and 5E). In contrast, 4APP treatment had no significant effect on the pouch tissue of mice infected with the *agr*

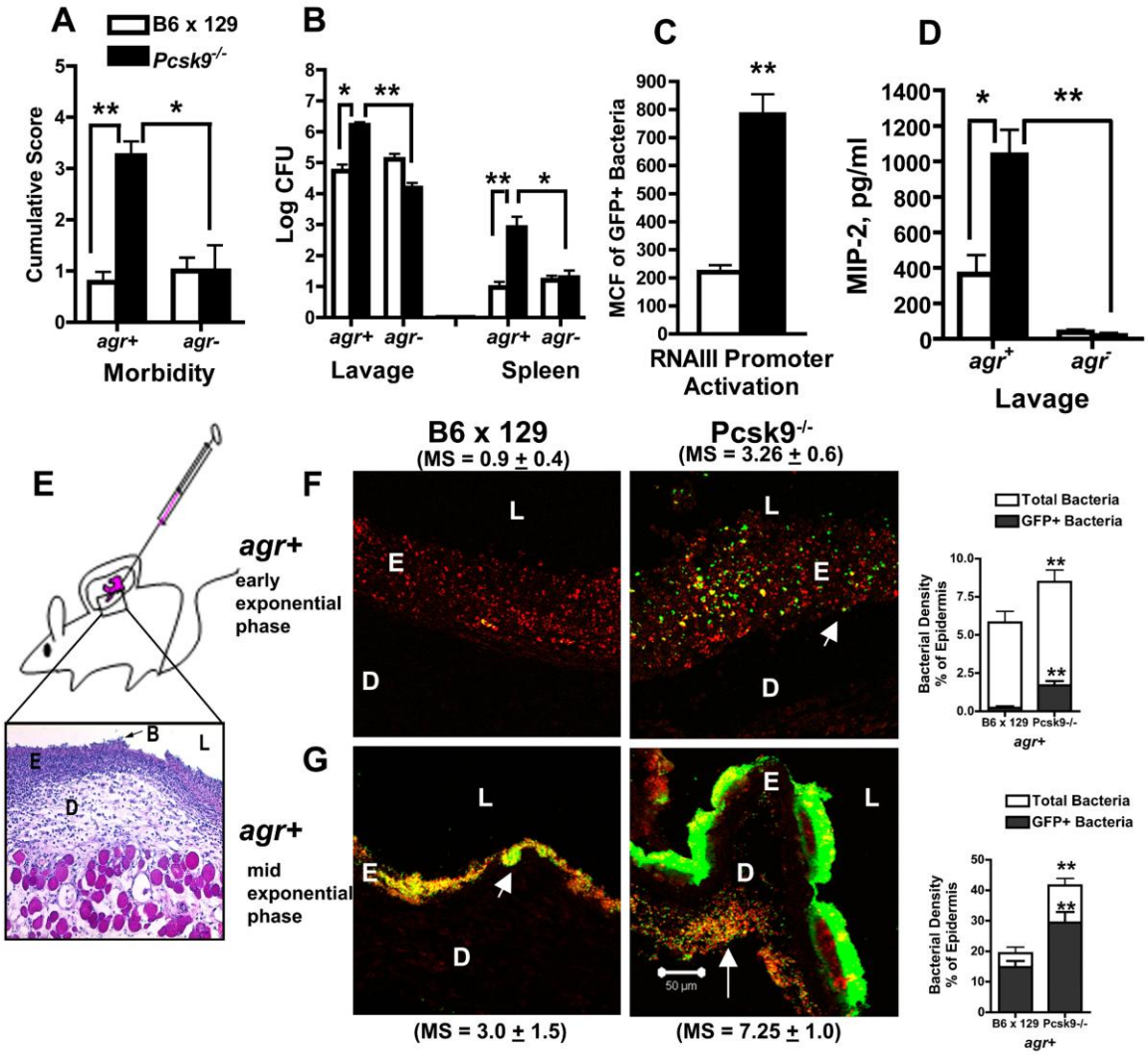

Figure 4. Effect of *agr* on *S. aureus* Infection of Apolipoprotein-Deficient *Pcsk9*$^{-/-}$ Mice as Compared to Wild-Type Mice

Air pouches generated on the backs of B6×129 wild-type or *Pcsk9*$^{-/-}$ mice (n = 8 for each group) were infected with either 3.5×10^7 nonfluorescent early exponential phase *S. aureus agr*:P3-*gfp* ALC 1743 (*agr*+) or 3.2×10^7 *agr*:P3-*gfp* ALC1753 (*agr*-). At 28 hr postinfection, the following parameters were determined and represented as the mean ± SEM:

(A) Morbidity was scored on a 0–14 point scale.

(B) Bacterial burden (Log CFU) of pouch lavage and spleen.

(C) Quantification of *agr*:P3-*gfp* promoter activation by flow cytometry of bacteria isolated from the pouch lavage.

(D) MIP-2, a murine inflammatory cytokine, was measured in the pouch lavage.

(E) H & E stain of the basal section of pouch tissue demonstrating bacteria at the surface of the lumen, the epidermis and dermis with infiltrating leukocytes, and the skeletal muscle.

(F) Representative pouch tissue from B6×129 mice (left panel) versus *Pcsk9*$^{-/-}$ mice (right panel) infected with early exponential phase *S. aureus* (*agr*:P3-*gfp* ALC1743). Pouch tissue was fixed and sectioned, and sagittal sections were stained with anti-*S. aureus* antibody (red fluorophore). Quorum-sensing *S. aureus* were visible by production of GFP in the pouch. Graph represents the relative density of both total and GFP+ bacteria in the epidermis from at least 19 representative areas.

(G) Representative pouch tissue from B6×129 mice (left panel) versus *Pcsk9*$^{-/-}$ mice (right panel) infected with midexponential phase *S. aureus* (*agr*:P3-*gfp* ALC1743) and stained with anti-*S. aureus* (red fluorophore) demonstrated significantly more quorum sensing, biofilm formation, and invasion into the dermis at 28 hr postinfection in *Pcsk9*$^{-/-}$ mice. Graph represents the relative density of both total and GFP+ bacteria in the epidermis from at least 19 representative areas. *$p < 0.02$; **$p < 0.005$. L = lumen, D = dermis, E = epidermis, and MS = morbidity score. Arrows indicate point of greatest penetration by quorum-sensing bacteria. Scale bar, 50 μm.

deletion mutant. Importantly, 4APP-treated mice infected with wild-type LAC had significantly increased density of bacteria that had invaded deep into the dermis and muscle underlying the air pouch with bacteria surrounding the muscle fibers (Figures 5F and 5G). Equivalent results were obtained with the *agr*+ and *agr*- RN6390 strains (Figure S5) and with the USA300 UAMS1378 strain and its *agr* deletion mutant (UAMS1540) (Figure S6). These data indicate that secreted low-density lipoprotein represents an innate barrier against quorum-sensing-dependent invasive *S. aureus* infection caused by *agr* type 1 laboratory strains or MRSA clinical isolates associated with invasive skin infections.

Discussion

Staphylococcus aureus, a colonizer of mucosal and epithelial surfaces and a pathogen capable of invading beyond these barriers, uses a complex network to regulate gene transcription essential for survival at these different sites. One mechanism by which the innate immune system could provide defense against invasive infection is not just by providing effectors that lead to bacterial killing but also by providing effectors that target virulence systems that permit survival beyond the epithelial barrier. In this work, we demonstrate that the major structural protein of VLDL and LDL, apolipoprotein B, antagonizes the *agr* quorum-sensing operon and selectively limits invasive infection of *agr* group 1 bacteria to epidermal spaces without any direct bactericidal effect. Importantly, we were able to show a role for these lipoproteins in control of invasive MRSA USA300 infections that are increasing in number and severity (Klevens et al., 2007).

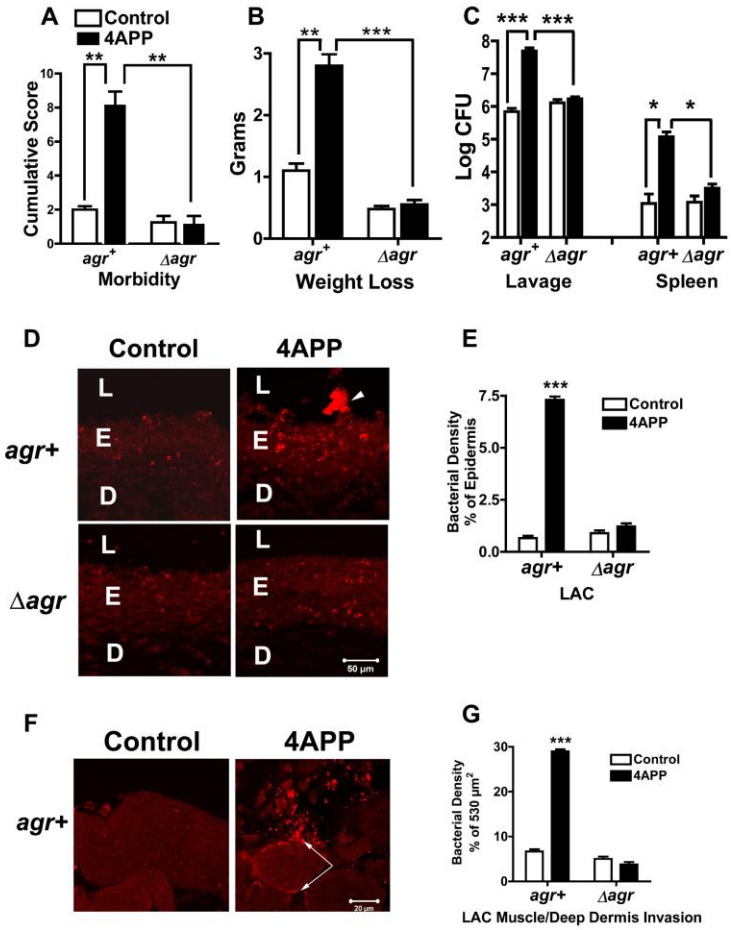

Figure 5. Effect of *agr* on USA300 (LAC) *S. aureus* Infection of NADPH Oxidase Knockout (*gp91 $^{phox-/-}$*) Mice Treated with 4APP to Inhibit Liver Secretion of Lipoproteins

Mice were treated with 100 μl of 5 mg/ml 4APP or vehicle control (0.025 M phosphate buffer) (n = 4 for each group) i.p. 48 and 24 hr prior to infection of air pouches with either 2.5×10^7 CFU of USA300 LAC or LAC *Δ agr*. At 25 hr postinfection, the following parameters were determined and the data represented as the mean ± SEM:

(A) Morbidity was scored on a 0–14 point scale.

(B) Weight loss in grams.

(C) Bacterial burden (Log CFU) in pouch lavage and spleen.

(D) Representative pouch tissue from control and 4APP-treated mice stained with anti-*S. aureus* antibody (red fluorophore), demonstrating biofilm-like aggregates (arrowhead) and increased numbers of bacteria in the epidermis in 4APP-treated mice infected with wild-type LAC. Scale bar, 50 μm.

These data extend our previous observation that reactive oxygen and nitrogen intermediates generated by the phagocyte oxidase inactivate the type 1 autoinducing pheromone of the *agr* operon (Rothfork et al., 2004), demonstrating that multiple effectors of innate immunity provide defense by targeting virulence gene regulation by *S. aureus*. However, in contrast to the current work, the phagocyte oxidase provides defense by multiple mechanisms, including both intracellular and extracellular killing of this pathogen (Ahluwalia et al., 2004; Fuchs et al., 2007; Quinn et al., 2006). Another example of a mammalian defense effector that targets quorum-sensing signaling without affecting bacterial viability is the paraoxonase family of lactonases that inactivate the acyl homoserine lactone pheromones of *Pseudomonas aeruginosa* (Ozer et al., 2005; Yang et al., 2005).

Apolipoprotein B is a large (4536 amino acids) amphipathic lipid-binding protein with a pentapartite structure composed of a globular amino terminus, two domains of β pleated sheets, and two α-helical domains that span the entire surface of the lipoprotein particle (Segrest et al., 2001; Olofsson and Boren, 2005). While it never exists in blood independently of the associated lipid particle, our data indicate that this structural protein is responsible for VLDL and LDL inhibition of AIP1 binding and signaling because (1) the individual lipid constituents of the lipoprotein particles at normal serum concentration did not inhibit AIP1 function, (2) antibody against apoB reversed VLDL, LDL, and AcLDL inhibition, (3) apoB purified away from its associated lipid by ether extraction and size chromatography was sufficient for inhibition, and (4) apoB bound directly to immobilized native AIP1, but not linear AIP1, by SPR. While direct binding of LDL via apoB to prions (Safar et al., 2006) and group A streptococci (Han et al., 2006) has been

demonstrated, whether this contributes to host defense and/or to virulence is unknown. We did not detect direct binding of LDL to the strains we studied. Therefore, we believe that the mechanism of antagonism involves sequestering of AIP1 by apoB within the lipoprotein particle. While the domain or site involved is presently not known, a monoclonal antibody to an epitope in the amino terminus reversed apoB interaction with AIP1, suggesting that the amino-terminal domain that initiates lipoprotein assembly in the liver may be involved. In addition, analysis of the SPR kinetic data revealed that either a one-to-one or a two-site interaction model could explain our results. Therefore, isolation of the domain or domains involved will be required for definitive data on the affinity of binding and the relevant stoichiometry. Moreover, because alterations in the lipid composition of VLDL and LDL are known to alter the conformation of apoB (Wang et al., 2000) and apoB within VLDL appeared more potent than purified apoB in our assays, the conformation of apoB as modified by associated lipid may contribute to AIP antagonism.

While the majority of our data were demonstrated with apoB interaction with AIP1 that predominates in clinical isolates, incubation of AIP2 and AIP4 with apoB prevented its binding to solid phase AIP1 and apoB inhibited AIP3-induced hemolysin production, indicating that apoB may be involved in regulating quorum sensing in all four *S. aureus* types. Because all four AIPs have different amino acid sequences but retain the thiolactone ring (George and Muir, 2007) and apoB did not bind to linear solid phase AIP1, we suspect that antagonism requires recognition of a thiolactone-dependent conformation that is essential for binding to the cognate AgrC receptor (Wright et al., 2004; Jensen et al., 2008). Once bound via apoB to the lipoprotein particle, AIP could be

degraded following uptake by macrophage scavenger receptors (Moore and Freeman, 2006). Intriguingly, mice deficient in one of these, CD 36, are more susceptible to *S. aureus* infection (Hoebe et al., 2005; Stuart et al., 2005), and whether this involves impaired control of quorum sensing is a point of speculation. Finally, because neat lipoprotein-deficient serum retained some ability to inhibit AIP1 signaling, other in vivo antagonists of AIP function are likely to contribute to control of quorum sensing.

The *agr* operon is only one of many global regulators that act subsequent to specific sensing to alter expression of various virulence factors by *S. aureus* (Cheung et al., 2004; Torres et al., 2007). However, the majority of clinical isolates are *agr*+, and *agr* deletion mutants are attenuated particularly in skin infection models (Rothfork et al., 2003, 2004; Wright et al., 2005), suggesting that, even though it shares targets with other regulators, it has a unique role in the pathogenesis of *S. aureus* infections. In our model, Δ*agr* bacteria are confined to the ~100 μm of the epidermis at the base of the air pouch, indicating that *agr*-mediated repression of adhesins that retain bacteria in the epidermis and upregulation of tissue-degrading enzymes are required for bacterial invasion into the dermis and possibly across the endothelium into the blood stream. In addition, our data demonstrate that the NADPH oxidase of phagocytes and VLDL and LDL in plasma play nonredundant roles in limiting AIP1-dependent activation of *agr* at this site. However, this has not been confirmed for all AIP types. In acute inflammation, leukocyte emigration across the endothelium into injured tissue occurs early with subsequent vascular leakage and plasma extravasation (Bucci et al., 2005) and, thus, would provide temporal control of *agr* activation. Moreover, sites of acute infection can become rapidly hypoxic (Peyssonnaux et al., 2005), which would limit oxidase control of AIP function,

and extravasated lipoprotein-mediated inhibition would predominate. If this barrier fails, the bacteria would gain access via the blood stream to virtually every tissue site. In the vasculature, *agr* may have little if any role (Yarwood et al., 2002), and recent data indicate that hemoglobin released from lysing erythrocytes or present within reticuloendothelial tissues actively suppresses production of the *agr* effector, RNAIII (Schlievert et al., 2007), possibly through the newly described heme sensor system HssRS (Torres et al., 2007), leading to reduced toxin production and attenuation. This could benefit the bacterium by permitting re-expression of adhesins required for attachment to endothelium to aid in escape from the neutrophil-rich blood stream into other tissues. In the blood stream, quorum sensing would be limited to bacteria able to escape antagonism or repression of *agr* either by clumping to prevent inactivation of the AIP (Rothfork et al., 2003) or by internalization into cells. Thus, multiple cycles of *agr* activation and repression regulated by both the pathogen and the host may occur during pathogenesis of this infection.

Lipoprotein particle levels and their constituents are extensively altered by acute inflammation and infection (Gabay and Kushner, 1999). In fact, hypocholesterolemia has been consistently correlated with worse outcomes in critically ill patients (Gordon et al., 2001; Gui et al., 1996; Marik, 2006; Windler et al., 1994) The molecular mechanisms underlying this are poorly understood, but measurements of various lipid parameters in patients with severe sepsis or septic shock indicate that apoB lipoprotein levels can be very low (Kitchens et al., 2003;Vermont et al., 2005; van Leeuwen et al., 2003). Because our data indicate that homeostatic levels of apoB-expressing lipoproteins are required to provide adequate defense against quorum-sensing invasive *S. aureus* infections, clinical

scenarios that result in extremely low apoB-containing lipoproteins may increase the risk of invasive *S. aureus* infection, including MRSA infections. In this regard, no detailed analyses of lipoprotein metabolism have been performed on patients with limited versus invasive *S. aureus* infection. Moreover, expression of truncated variants (Shelness et al., 2003) and/or known polymorphisms in apoB (Chiodini et al., 2003) may affect its ability to antagonize *agr* signaling depending on the site or domain within apoB that binds AIP. Therefore, variations in either the quality or amount of apoB in plasma may alter innate defense against quorum sensing by this pathogen.

Our data do not address whether excess apoB-containing lipoproteins as would occur in hyperlipidemia can prevent infection. In fact, high circulating levels of oxidized LDL contribute to impaired Th1 immunity and increased susceptibility to experimental infection with some pathogens (Shamshiev et al., 2007), suggesting, in conjunction with the present work, that dyslipidemia alters multiple parameters required for host defense (Marik, 2006). While the majority of work published to date on the role of lipoproteins in host defense has focused on neutralization of lipopolysaccharide (Kitchens et al., 2003), our work defines a previously unrecognized role of lipoproteins in interfering with bacterial communication.

Experimental Procedures

Reagents

Synthetic AIPs were generated as follows and stored in DMSO at -80°C: cyclic AIP1 (YSTC[→M]DFIM[→C]), FITC-AIP1, and biotin-AIP1 were synthesized by Commonwealth Biotechnologies, Inc., Richmond, VA, using a described method (Otto et al., 1998). Cyclic AIP 2 (GVNAC[→F]SSLF[→C]), AIP4 (YSTC[→M]YFIM[→C]), and AIP3 antagonist (YINC[→L]DFLL[→C]) were generated as described (Otto et al., 1998). A disulfide linked cyclic peptide, C(→C)LLRMKSAC(→C), was used as a control. Linear AIP1 and biotin-AIP1 were generated by alkaline hydrolysis and neutralization, and a methionine sulfoxide variant of AIP1 was produced by incubation with hydrogen peroxide and myeloperoxidase as described (Rothfork et al., 2004). Both were checked for loss of functional activity and structure by mass spectroscopy as described (Rothfork et al., 2004). The following reagents and sera were obtained as indicated: purified human VLDL, LDL, HDL, apoB, apoA1, apoC1, and apoE (US Biological, Swampscott, MA); purified Ac-LDL, DiI-Ac-LDL, and DiI-LDL (Biomedical Technologies Inc., Stoughton, MA); pooled human serum, lipoprotein-deficient serum, chicken serum, tributyrin, 4-aminopyrazolo-(3,4-D) pyrimide (4APP), and a liposome preparation reagent containing cholesterol, phosphatidyl choline, and stearylamine (Sigma-Aldrich, St. Louis, MO); cholesterol and phosphatidylcholine (Avanti Polar Lipids); polyclonal goat IgG anti-human apolipoprotein B 100 (R&D Systems, Minneapolis, MN); monoclonal murine IgG1 anti-human apoB C1.4 (Santa Cruz Biotechnology, Santa Cruz, CA); and monoclonal murine IgG1 anti-human apoB 4C11 (Biodesign International, Saco, ME).

Bacterial Strains and Culture

The bacterial strains used in this study were as follows: ALC 1743 (*agr* group 1 RN6390 containing reporter *agr*:P3-*gfp*), ALC 1740 (RN6390 containing reporter *hla-gfp*), ALC 1753 (RN6911 *agr* deletion mutant of RN6390 containing reporter *agr*:P3-*gfp*), RN6390, SH1000 (σ B corrected RN6390), and ALC 1225 *geh*-(strain 8325-4 with *geh* deletion mutant with reduced lipase secretion) (Kupferwasser et al., 2003; Rothfork et al., 2004). USA300 strain UAMS1378 and its *agr* deletion mutant UAMS1540 were provided by Dr. Mark Smeltzer (University of Arkansas), and USA300 strain LAC and its *agr* deletion mutant were prepared as described (Wang et al., 2007). Clinical isolates of CA-MRSA *agr* group 1 USA 300 (NM 300) and *agr* group 3 USA 400 (NM 400) were provided by Dr. Steve Young, Department of Pathology, University of New Mexico, and USA 300 strain FPR 3757 was provided by Dr. Francoise Perdreau-Remington, UCSF. To generate synchronized early exponential phase, nonfluorescent bacteria, frozen stocks were cultured either in Columbia broth or Trypticase soy broth (TSB) (Becton Dickinson, Franklin Lakes, NJ) as described (Rothfork et al., 2003). CFU were determined after washing and sonication to disrupt clumps by plating serial dilutions on blood agar (Becton Dickinson, Franklin Lakes, NJ).

Bacterial Promoter Activation

Early exponential phase, nonfluorescent reporter bacteria (ALC1743 and ALC1740) (2×10^7/ml) were incubated in 1 ml TSB in polystyrene tubes with shaking (200 rpm, 37°C) for the indicated times with either broth or vehicle control, synthetic AIP1 (100 nM), or AIP1 treated with antagonists including sera, lipoprotein particles, apo protein, or other lipids. For inhibition of spontaneous activation of *agr*:P3, AlC1743 was incubated with

90% TSB with either 10% PHS, 10% LPDS, 10% PHS preincubated on ice for 30 min with 10 μg/ml murine IgG1, or 10% PHS preincubated on ice for 30 min with mAB C1.4 for the times indicated in the figure legend. After incubation, bacteria were washed by centrifugation at 3000 rpm for 4 min at 4°C in PBS with 0.1% Triton X-100, sonicated, cultured for CFU, and then fixed with 1% paraformaldehyde containing 25 mM CaCl2 for analysis by flow cytometry (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ). Promoter activation was demonstrated as fluorescence induction and measured as the mean channel fluorescence (MCF) of GFP-positive bacteria.

Quantitative RT-PCR

Early exponential phase USA300 strains UAMS1378 or LAC (2×10^7/ml) were cultured as above with either broth, 100 nM AIP1, or AIP1 with 2 μg/ml VLDL for 1 hr. For strain LAC, the VLDL was preincubated with either IgG1 anti-apoB (mAb C1.4) or isotype control (both 5 μg/ml) on ice for 30 min prior to addition to the assay. In addition, LAC was incubated with either 10% PHS treated with murine IgG1 or with 10% PHS treated with mAb C1.4 anti-apoB and cultured as indicated in the figure legends. RNAIII was quantified relative to 16S RNA using a probe-based assay as described with minor modifications (Sawires and Gresham, 2008) as detailed in Chapter 2.

Surface Plasmon Resonance

Surface plasmon resonance was performed with the Biacore X100 (Biacore Life Sciences, GE Healthcare) to analyze the interaction of apoproteins with immobilized AIP1. N-terminal biotinylated AIP1 in both the native and linear conformation were immobilized on streptavidin sensor chips according to the manufacturer's protocol. The

details of the conditions of running the assays and their evaluation are given in Chapter 2 and the figure legends.

Additional experimental details of virulence factor assays, FITC-AIP1 binding to the bacteria, macrophage colocalization experiments, and the air pouch model of infection and its evaluation are given in Chapter 2.

Statistical Evaluation

Data are displayed as the mean ± SEM. In vitro data were analyzed by the Student's t test and the in vivo results by the Mann-Whitney U test for nonparametrics using StatView for Macintosh.

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CHAPTER 4

APOLIPOPROTEIN B DIFFERENTIALLY PROTECTS AGAINST INVASIVE *STAPHYLOCOCCUS AUREUS* INFECTION BASED ON *AGR* TYPE

Summary

Staphylococcus aureus is a colonizer of the human skin and mucosa that can also cause severe invasive disease. The phenotypic switch from colonization to invasion is mediated by bacterial pheromone peptide activation of the *S. aureus agr* quorum-sensing system that upregulates virulence genes necessary for invasive infection. As an innate host defense mechanism, apolipoprotein B binds to the bacterial quorum sensing peptides and antagonizes virulence gene expression. Here we show preliminary data which suggests that the effectiveness of apoB as a barrier to invasive infection is dependent on the type of *agr* peptide made and secreted by the bacteria. Initial experiments suggested that mice with pharmacologically lowered serum apoB are more susceptible to *agr* type 2, but not type 3 strains, compared to controls. This may explain why *agr*3 strains cause a high incidence of bacteremic infections that are poorly controlled by the host.

Introduction

Host defense mechanisms against organisms that can both colonize and cause infection must necessarily be multi-faceted and complex. *Staphylococcus aureus* is one such organism: 20%-30% of humans are persistently colonized, while another 60% experiences transient colonization. While primarily considered an opportunistic pathogen causing infection in individuals with chronic medical conditions and at the extremes of age, the emergence of community acquired methicillin resistant *S. aureus* (CA-MRSA) infections in health individuals with no underlying health problems (Miller et al., 2005) has focused interest on CA-MRSA infection as a major public health threat. In this

regard, research from the Centers for Disease Control confirmed that in 2005 more deaths occurred in the USA from MRSA infection than HIV infection and MRSA was responsible for more cases of invasive bacterial infections than all other pathogens combined (Klevens et al., 2007). Importantly, very little is known about host factors that regulate susceptibility to CA-MRSA infection (Lowy, 2007)

Numerous innate mechanisms contribute to control *S. aureus* infection, including neutrophil recruitment to the site of infection, oxidant-mediated killing, antibacterial molecules and peptides, and neutralizing antibodies (Fournier and Philpott, 2005; Ganz et al., 1985; Holtfreter et al., 2010). Previously, we identified serum apolipoprotein B (apoB) found on low and very low density lipoproteins (LDL, VLDL) as an innate barrier to *S. aureus* virulence gene expression and invasive infection (Peterson et al., 2008). ApoB interacts with a bacterial quorum sensing signaling peptide (autoinducing peptide, AIP) such that the peptide is sequestered away from its bacterial receptor AgrC. AIP is a cyclic thiolactone peptide and its biologic function requires the cyclic form of the peptide and the presence of carboxy terminal hydrophobic amino acids (Novick, 2003). ApoB binds to the cyclic biologically active form of the peptide but not to the linear form. By this mechanism, apoB inhibits a phenotypic switch in this pathogen preventing an upregulation of virulence factors required for invasive infection. This switch is mediated in part by a quorum sensing operon *agr* which contains two divergent promoters: the P2 promoter drives synthesis of AIP, its transporter AgrB, its receptor (AgrC), and the response regulator protein AgrA; the P3 promoter responds to activated AgrA by driving expression of a regulatory RNA, RNAIII, which induces gene expression of virulence factors important for invasion and repression of colonizing factors (Novick, 2003). The

P2 promoter is also activated by AgrA, thereby perpetuating an AIP-dependent autoinducing loop. AgrA also directly activates the promoter for a cytolytic peptide, PSM alpha, which is a known virulence factor in CA-MRSA strains and causes neutrophil lysis at the site of infection (Wang et al., 2007).

S. aureus strains are divided into one of four *agr* types depending on the sequence of the AIP produced and sequence variations in the cognate AgrC receptor. The MRSA strains causing the majority of community-acquired disease, PFGE types USA300 and USA400, are *agr* types 1 and 3, respectively. Although the greater part of CA-MRSA disease is attributed to the USA300 strain, those cases caused by an *agr*3 USA400 strain often lead to severe persistent bacteremia (Tenover et al., 2006; Xiong et al., 2009). USA100, which is found mostly in hospital-acquired cases, is an *agr* type 2 strain. Whether apoB binds directly to all AIP types is unknown. Because bloodstream *S. aureus* infections are predominantly caused by *agr* type 3 strains, we hypothesized that apoB is primarily important for control of *agr*1 and *agr*2 infections and not for infections caused by *agr*3 strains (Xiong et al., 2009).

Results

ApoB-containing Lipoproteins Bind to AIPs of Multiple agr Types

The overall structure of AIP across *agr* types of *S. aureus* is very similar, each containing a thiolactone ring which is important for binding to the cognate bacterial receptor AgrC (Figure 1A) (Jensen et al., 2008; Wright et al., 2004). However, key differences among the peptides, such as peptide length and composition, distinguish them one from another and from their receptors, such that a peptide from one *agr* type can

inhibit receptor activation from another type (Ji et al., 1997). Based on our previous observation that apoB binds cyclic but not linear AIP1 (Peterson et al., 2008), we hypothesized that apoB and apoB-containing lipoproteins would also bind to AIP2 and AIP3, since all AIPs share a hydrophobic cyclic structure. We used surface plasmon resonance (SPR) to analyze binding of purified apoB, LDL, and VLDL to the different AIPs (Figure 1B). AIP was N-terminally biotinylated to allow immobilization to a streptavidin-coated chip and apoB, LDL and VLDL were used at equivalent apoB molar concentrations as determined by ELISA (Cayman Chemical, Ann Arbor, MI or Alerchek, Portland, ME). Binding between each of the analytes and AIPs was observed, with binding to AIP2 resulting in the greatest response overall as measured by resonance units (RU), with intermediate binding to AIP1 and the least binding to AIP3. These results suggest that in this system the length of peptide correlated with the magnitude of binding. To address this, we measured binding to modified AIP3 synthesized with an amino-terminal tyrosine (Y-AIP3). Addition of the N-terminal tyrosine residue to AIP3 resulted in increased binding by the apoB-containing analytes to a level comparable to that bound to AIP1 indicating that the length of the amino acid tail also contributes to recognition of AIP by apoB. Interestingly, VLDL binding of each AIP type was greater than binding by either LDL or apoB, between which resulting RU were comparable. This observation suggests that the conformation of apoB within VLDL particles may enhance its interaction with AIP. Alternatively, the lipids within VLDL may play a more direct role in recognition of AIP. Differentiation of these possibilities will require further investigation. These data demonstrate that apoB either as a purified protein or within a

lipoprotein particle binds AIP2 > AIP1 = Y-AIP3 > AIP3, suggesting that apoB may have the least control of *agr* type 3 infections.

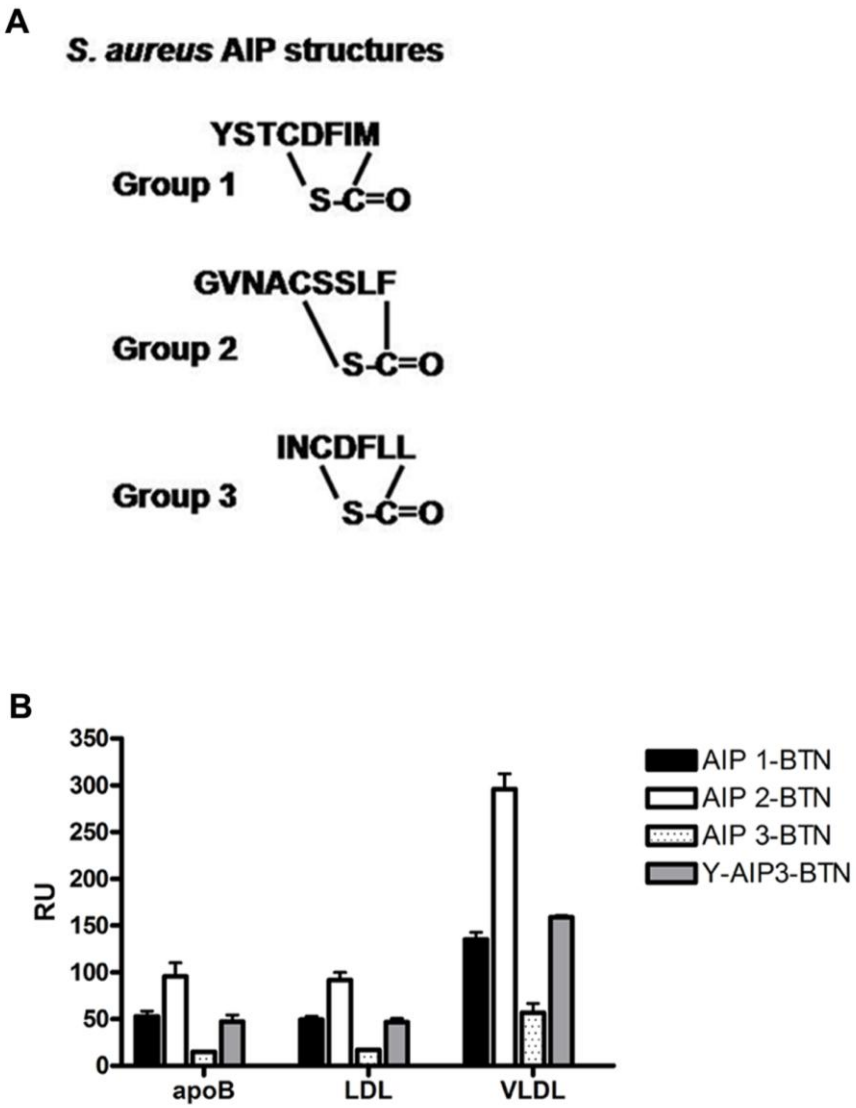

Figure 1. Structure and Binding of AIPs to apoB-containing Particles by Surface Plasmon Resonance

(A) Peptide sequences of the signaling pheromone peptide AIP from *agr* groups 1, 2, and 3. A conserved central cysteine residue forms a thiolactone ring structure essential for AIP signaling through the bacterial AgrC receptor.

(B) Biacore X100 analysis in resonance units (RU) of the interaction of 20 nM apoB, LDL, or VLDL with biotin-AIP1, biotin-AIP2, biotin-AIP3, or biotin-Y-AIP3 immobilized on streptavidin chips after 60 s of contact time followed by 60 s of dissociation time at a flow rate of 10 µl/min. Data are represented as mean ± SEM; n = 2-4.

Apolipoprotein B Inhibits AIP-induced Virulence Factor Production

Based on the results of apoB binding to all AIP types and our previous demonstration of apoB antagonism of AIP1-mediated quorum sensing dependent virulence (Peterson et al., 2008), we asked whether apoB-containing lipoproteins can antagonize AIP2 and AIP3-mediated quorum sensing and virulence factor upregulation *in vitro*. To address this, we used qRT-PCR to measure RNAIII expression in the *agr*2 strain 502A (Blair and Tull, 1969; Light et al., 1967) following exogenous stimulation with bacterial-derived AIP2. Specifically, early-exponential phase 502A were grown for 2h at 37°C with and without AIP2 derived from concentrated overnight supernatant in the presence or absence of VLDL or apoB at equimolar apoB concentrations. As shown in Figure 2A, RNAIII expression was significantly inhibited by VLDL and, to a slightly lesser extent, by apoB. We next examined the ability of apoB-containing lipoproteins to antagonize production of a key virulence factor, the toxin alpha hemolysin. Alpha hemolysin is a virulence factor upregulated by RNAIII following stimulation with AIP. As shown in Figure 2B, the *agr*2 strain 502A produces abundant α-hemolysin, as measured by rabbit erythrocyte lysis, in response to synthetic AIP2 which is attenuated by the addition of VLDL or apoB to the culture. These data demonstrate that apoB-containing lipoproteins antagonize AIP2-mediated upregulation of virulence factors *in vitro.*

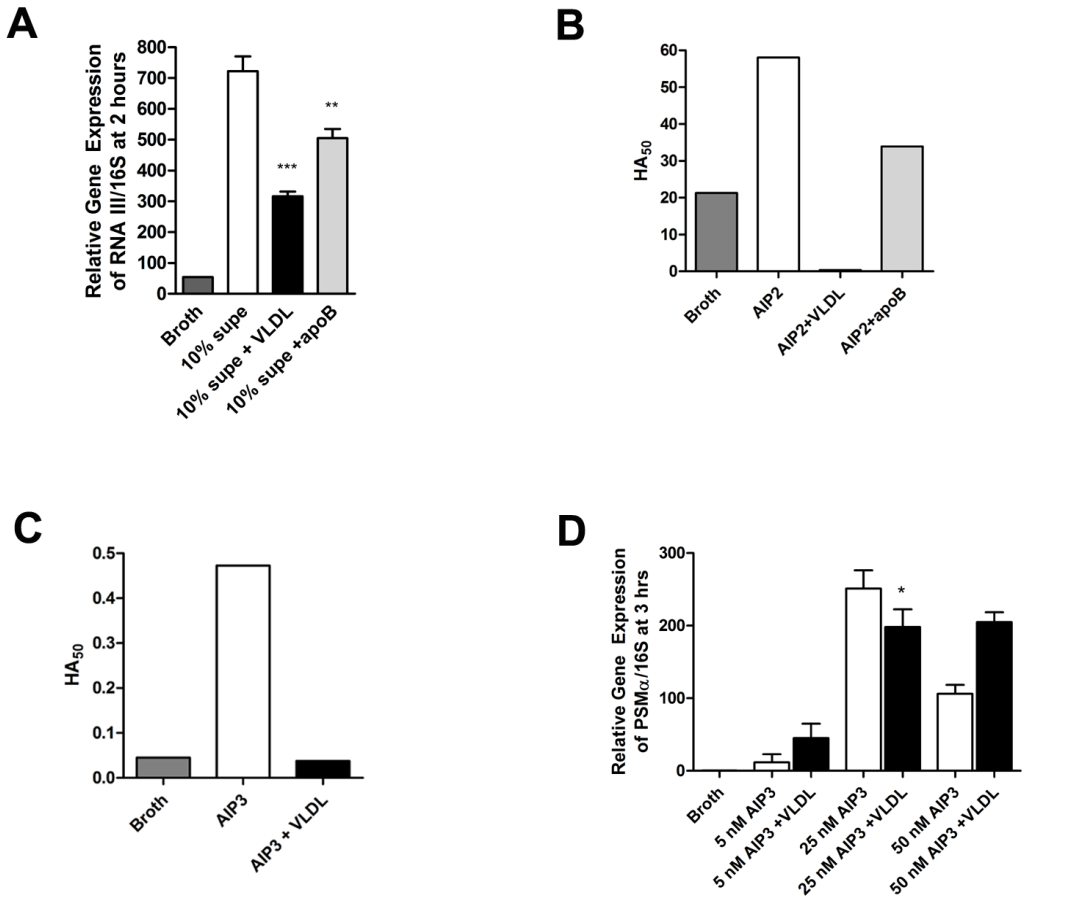

Figure 2. Apolipoprotein B-containing Particles Antagonize *agr*2 and *agr*3 Signaling and Virulence Factor Production

(A) Gene expression of RNAIII as measured by qRT-PCR in *agr*2 strain 502A (2×10^8/ml) after exogenous stimulation with 10% overnight supernatant pre-incubated with or without VLDL or apoB (2 μg/ml apoB each) for 30 minutes at 37°C and then grown for 2 hours.

(B) Alpha hemolysin in supernatants from cultures of *agr*2 strain 502A (1×10^8/ml) stimulated with 100 nM AIP2 and grown with or without VLDL or apoB (2 μg/ml apoB each) for 6 hours as measured by a hemolytic assay.

(C) Alpha hemolysin in supernatants from cultures of *agr*3 USA400 strain MW2 (4×10^7/ml) stimulated with 100 nM AIP3 and grown with or without VLDL (5 μg/ml) for 5 hours as measured by a hemolytic assay.

(D) Gene expression of *psmα* as measured by qRT-PCR in *agr*3 USA400 strain MW2 (1×10^7/ml) after exogenous stimulation with indicated AIP3 concentrations pre-incubated with or without VLDL (5 μg/ml apoB) for 30 minutes at 37°C and then grown for 3 hours.

Data are represented as mean ± SEM; n = 2; * $p < 0.1$ ** $p < 0.05$ *** $p < 0.02$ as compared to AIP-induced expression

We next examined the ability of apoB-containing lipoproteins to antagonize quorum sensing-mediated virulence factor expression in the clinically-relevant CA-MRSA USAA400 *agr*3 strain MW2 (Baba et al., 2002). We first measured the ability of VLDL to inhibit the production of α-hemolysin by MW2. Although in vitro MW2 does not make appreciable amounts of α-hemolysin in response to synthetic AIP3, VLDL inhibited α-hemolysin to the level of the broth control (Figure 2C). Next, we used qRT-PCR to assess the ability of VLDL to inhibit expression of *psmα,* a gene target of activated AgrA (Queck et al., 2008) and a direct readout of AgrA activation. As expected, MW2 expressed *psmα* in response to exogenous synthetic AIP3. Message for *psmα* peaked at 25 nM AIP3 but decreased at 50 nM, demonstrating that upregulation of *psmα* in MW2 *in vitro* is mediated in a biphasic dose-response mechanism (Figure 2D). Interestingly, it was only at the peak of *psmα* expression (at 25 nM AIP3) that VLDL inhibition was observed and this inhibition, although significant, was moderate in relation to the control of AIP1-induced RNAIII production we reported previously (Peterson et al., 2008). These data suggest that apoB is minimally effective at antagonism of AIP3-mediated virulence gene expression *in vitro,* and this is supported by the decreased binding of AIP3 by apoB-containing lipoproteins demonstrated by SPR. Based on these preliminary findings, apoB is able to interact with AIP2 to inhibit virulence in an *agr*2 strain of *S. aureus in vitro* whereas its control of AIP3 stimulated *agr*3 activation in strain MW2 is greatly reduced.

Mice with Pharmacologically-depleted Serum apoB are More Susceptible to agr2 but not agr3 S. aureus Infection

Apolipoprotein B is secreted from hepatocytes into the blood as a major structural protein of VLDL particles and remains associated with the metabolized LDL particles that circulate to the tissues for uptake by the LDL receptor (Shelness and Sellers, 2001). Mice treated with the drug 4-aminopyrazolo-(3, 4-D) pyrimidine (4APP), have decreased serum cholesterol, VLDL, and LDL levels due to inhibition of VLDL secretion from the liver (Mounkes et al., 2001). To test whether apolipoprotein B is a mediator of host defense against invasive *S. aureus* infections of the *agr*2 and 3 types, we used an air pouch model of invasive infection to compare the responses of vehicle or 4APP-treated C57Bl/6 mice. Subcutaneous injections of air create a pouch that becomes lined with epithelium over 6 days, a model that has been used for both biofilm (Yoshikawa et al., 2004) and invasive infection studies (Peterson et al., 2008; Rothfork et al., 2003; Rothfork et al., 2004). In preliminary experiments, infection of 4APP-treated mice (black bars) with early exponential phase *agr*2 bacteria 502A (Blair and Tull, 1969; Light et al., 1967) at a dose easily tolerated by vehicle control mice (white bars) resulted in increased morbidity, weight loss, and bacterial burden in the pouch lavage and spleen samples (Figures 2A-C). Although only the morbidity score showed significant difference between groups, the other parameters suggest a trend that low serum apoB levels contribute to an exacerbated infection, and repeated experiments must be performed to verify this conclusion. In contrast to mice exposed to *agr*2 strain 502A, mice receiving an equivalent dose of MW2 (an *agr* type 3 strain (Baba et al., 2002)) showed no significant difference in morbidity, weight loss or bacterial burden in the pouch lavage

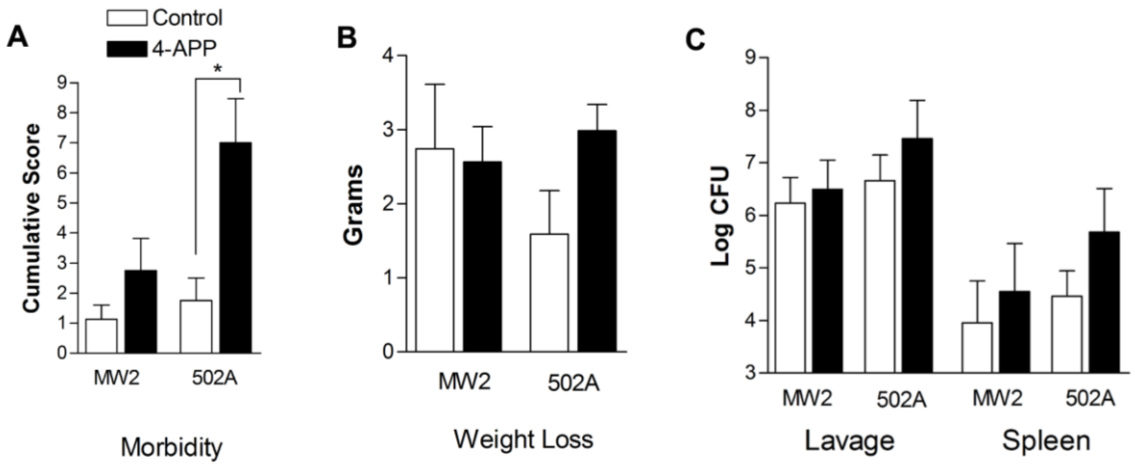

Figure 3. Effect of Depletion of Serum apoB by 4APP on *agr*2 502A infection and *agr*3 MW2 (USA400) Infection

Mice were treated with 100 µl of 5 mg/ml 4APP or vehicle control (0.025M phosphate buffer) (n=8 for each group for MW2 data, n=4 for each group for 502A data) i.p. 48 and 24 hours prior to infection of air pouches with either 7×10^7 MW2 USA400 or 8×10^7 502A. At 28 hours hrs post infection, the following parameters were determined and the data represented as the mean ± SEM:

(A) Morbidity score 0-14.

(B) Weight loss

(C) Bacterial burden (Log CFU) in pouch lavage and spleen.

$p < 0.05$

and spleen between 4APP and vehicle control treatment. These preliminary data suggest that apoB-containing lipoproteins are important for controlling invasive infection in this mouse model of infection an *agr*2 strain, but not an *agr*3 strain.

Discussion

Staphylococcus aureus has numerous mechanisms to resist host defense measures as well as antibiotic treatment. The development of difficult-to-treat MRSA strains emphasizes the need to develop better therapies that do not drive bacterial resistance. One such method is to target virulence factors that contribute to disease but are not required for bacterial survival (Alksne and Projan, 2000; Garcia-Lara et al., 2005). We have previously shown that nature already utilizes this approach as demonstrated by apolipoprotein B-mediated antagonism of *S. aureus* quorum sensing-dependent virulence of *agr*1 strains (Peterson et al., 2008). Here we show preliminary data indicating that virulence signaling in *agr*2 strains is also inhibited by apoB.

Apolipoprotein B specifically antagonizes one of the major *S. aureus* virulence regulatory systems, *agr*, by binding to the signaling peptide AIP. In this work we present preliminary data suggesting that this inhibitory action applies not only to *agr* type 1 strains, but to *agr*2 strains as well. ApoB binds to AIPs 1, 2, and 3, with maximal binding between AIP2 and VLDL. *In vitro* studies show that apoB can greatly inhibit AIP2 downstream signaling events and is less effective against AIP3. Mice with reduced serum apoB are less capable of controlling an *agr*2 infection, indicating the importance of serum apoB for host defense against this strain. However, both vehicle and 4APP treated mice infected with an *agr*3 strain were equally susceptible. The basis for this differential protective effect of apoB is most likely due to the differences in AIP length. AIP3 is two

amino acids shorter in length than AIP2, which seems to impact its ability to bind or remain bound to apoB. These findings taken together may explain why *agr*3 strains cause persistent bacteremia in patients more frequently than other *agr* types (Xiong et al., 2009). When serum lipoproteins are unable to inhibit the induction of an invasive phenotype, the bacteria can rapidly disseminate through the bloodstream. The robust binding of apoB-containing particles to AIP2 and the subsequent apoB-mediated control of type 2 invasive infection could explain the absence of *agr*2 USA100-caused disease in the community setting (Limbago et al., 2009). Homeostatic levels of apoB, along with an intact immune response, are sufficient for control of this type of *S. aureus* that normally is found in hospital-acquired cases where the patient is already immunocompromised and experiencing low levels of serum lipoproteins (Gui et al., 1996; Limbago et al., 2009; Marik, 2006). This work highlights the phenotypic diversity among strains of *S. aureus* which must be considered when developing or analyzing a potential clinical therapy: even the host's innate barriers have limits to their effectiveness and range of utility. Apolipoprotein B is an important antagonist of *S. aureus* quorum sensing insofar as it can bind and sequester the signaling peptide, as is the case in *agr* types 1 and 2 infections. The shorter *agr*3 peptide is not well regulated by apoB-containing particles and as a result is able to induce invasive infection that leads to systemic disease.

CHAPTER 5

THE AMINO-TERMINAL DOMAIN OF APOLIPOPROTEIN B BINDS *STAPHYLOCOCCUS AUREUS* AUTOINDUCING PEPTIDE TO ANTAGONIZE INVASIVE INFECTION

Introduction

Apolipoprotein B (apoB) is the large structural protein of serum low density and very low density lipoproteins (LDL and VLDL, respectively); it initiates synthesis of VLDL in the liver, interacts with the LDL receptor on cell surfaces to allow for uptake, and recently was shown to antagonize virulence signaling in *Staphylococcus aureus* (Mahley et al., 1984; Peterson et al., 2008). ApoB binds to a *S. aureus* signaling pheromone peptide, AIP1, and sequesters it away from the bacterial receptor AgrC, preventing virulence signaling. This function of apoB represents a unique barrier to invasive infection that otherwise would result from AIP1 signaling. Polymorphisms in apoB or truncated variants of the protein are known to exist among the human population (Hooper et al., 2005; Tybjaerg-Hansen, 1995) and may affect the efficacy of this innate barrier. Therefore, investigating the nature of the apoB-AIP1 complex and identifying the domain of apoB that mediates this interaction is important for understanding human susceptibility to invasive *S. aureus* infection.

Based on our previous *in vitro* observations that a monoclonal antibody directed to the amino-terminus of apoB reversed apoB-mediated inhibition of *S.aureus* virulence signaling as well as AIP1 binding to modified LDL particles (See chapter 3, Figures 1F, 3A, and 3D), we hypothesized that the N-terminal domain of apoB is responsible for binding AIP1. This work aims to isolate a fragment of apoB that retains inhibitory function that can then be characterized in terms of its structure, conformation, and binding properties to AIP1.

Results

Limited proteolysis of LDL generates fragments that retain inhibitory activity

Apolipoprotein B is a large (515 kDa) protein that is always associated with a lipoprotein particle *in vivo* (Segrest et al., 2001). The pentapartite domain structure consists of amphipathic alpha helices and beta sheets that span the bulk of the lipid portion of the particle. Our first strategy to identify the inhibitory domain was to treat apoB with proteases to generate fragments that still inhibited AIP-induced P3 promoter activation and then to purify these fragments and characterize them. Both LDL and VLDL are cleaved by several proteases into smaller fragments, and VLDL is more resistant to proteolysis compared to LDL (Chen et al., 1989). In the context of either lipid particle, the globular structure of apoB has three identified thrombin cleavage sites (Figure 1A), as well as sites of cleavage for trypsin, *S. aureus* V8 protease, and pepsin. The epitope for the monoclonal antibody C1.4, which reverses apoB-mediated antagonism of AIP1 function, is located within the T4 thrombin cleavage fragment.

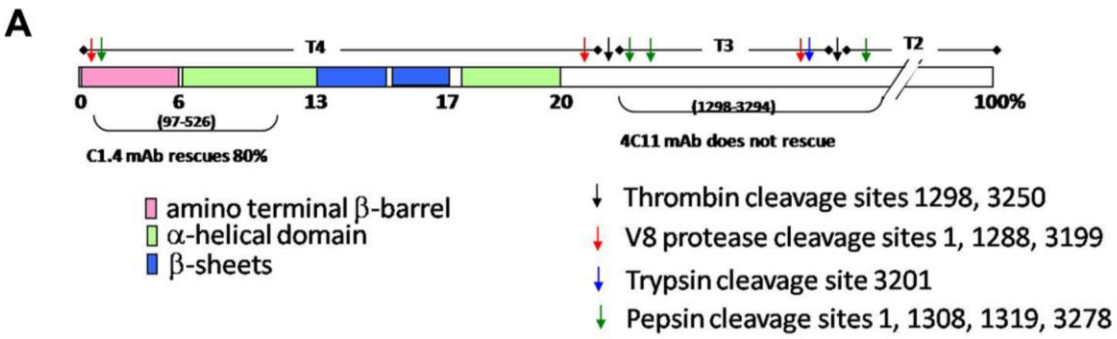

Figure 1. Linear Protein Map of Apolipoprotein B

(A) A linear representation of ApoB 100. Percentage of length is shown below the map, as well as the antigenic epitopes recognized by antibodies used in a *S. aureus* promoter assay to rescue apoB-mediated inhibition of AIP1-induced P3 promoter activation. Peptide sequences that later form secondary structures are color coded as indicated; arrows above the map indicate cleavage sites confirmed by Chen and colleagues (Chen et al., 1989), though numerous additional cleavage sites are predicted by the sequence.

Adapted from Pam Hall and Chen et al., 1989

We chose to utilize proteolysis to address two objectives: 1) to generate proteolytic fragments sufficient for apoB antagonism of AIP1 function to allow for purification and characterization and 2) to more definitively demonstrate that the lipid constituents of VLDL and LDL plays purely a supporting role in AIP1 antagonism by maintaining the conformation of apoB. We should be able to reverse the antagonism by digesting apoB into sufficiently small fragments. We began by treating LDL with thrombin, trypsin, *S. aureus* V8 protease, and pepsin, proteases whose cleavage of apoB has been well documented to generate N-terminal fragments of varying lengths (Chen et al., 1989). Following treatment and confirmation of cleavage by SDS-PAGE, the LDL was incubated with early exponential phase cultures of the ALC1743 *agr*:P3-*gfp* reporter strain, allowing us to analyze the degree of P3 promoter activation or inhibition by measuring GFP fluorescence by flow cytometry. As expected, thrombin treatment did not affect the ability of the LDL to inhibit P3 promoter activation (Figure 2A). Likewise, LDL treated with trypsin, V8 protease, or pepsin also retained inhibitory activity. However, pepsin treated LDL was slightly less effective at antagonizing AIP1 function. As pepsin is expected to generate the shortest N-terminal fragment of apoB, it is possible that the lack of significance of this single experiment is due to a mixed population resulting from incomplete digestion. Further experiments using LDL digested with increasing amounts of pepsin or for an extended time are warranted.

As the N-terminal region of apoB contains numerous disulfide bonds (Shelness and Thornburg, 1996), we used the reducing agent dithiothreitol (DTT) in an effort to expose additional protease cleavage sites otherwise protected in the globular structure.

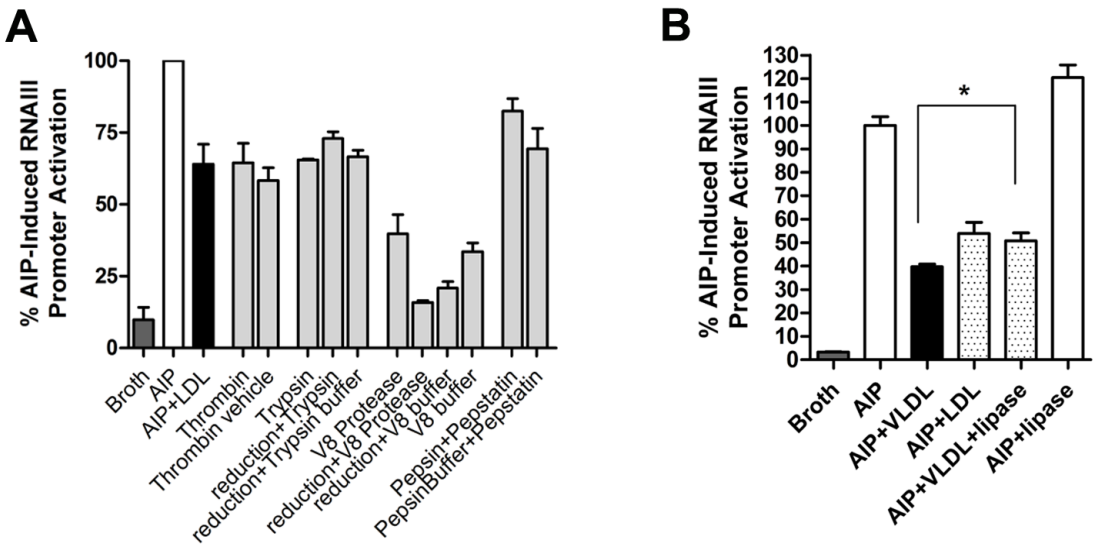

Figure 2. Effect of Protease and Lipase Treatment of Lipoproteins on ApoB-mediated Inhibition of AIP1-induced Virulence Signaling

(A) *agr*:P3 promoter activation by 10 nM AIP 1 is inhibited in *S. aureus* (ALC 1743 with *agr*:P3-*gfp*, 2×10^7/ml) during 3 hr of culture with LDL (2 μg/ml) or LDL treated with proteases as follows: Thrombin (E:S 1:2) or thrombin vehicle for 2 hours at 37°C; Trypsin (E:S 1:6) or buffer with or without reducing treatment (DTT for 1 hr followed by Iodoacetamide for 3 hours at room temperature followed by buffer exchange) for 2 hours at 37°C; V8 protease (E:S 3:1) or buffer with or without reducing treatment (DTT for 1 hr followed by Iodoacetamide for 1 hour at room temperature followed by buffer exchange) for 3 hours at 37°C; Pepsin (E:S 1:300) or buffer for 1.5 hours at 37°C followed by addition of pepstatin (equal molarity as pepsin) before inclusion in the assay. Data are represented as percent activation of the P3 promoter as compared to AIP1 stimulation, and as the mean ± SEM; n = 3.

(B) VLDL inhibition of *agr*:P3 promoter activation induced by 10 nM AIP 1 in *S. aureus* (ALC 1743 with *agr*:P3-*gfp*, 2×10^7/ml) during 3 hr of culture is rescued following treatment of VLDL with lipoprotein lipase. VLDL (2 μg/ml) was incubated with (0.15 μg/ml) bovine lipoprotein lipase for 15 minutes at 37°C before added to cultures. LDL(2 μg/ml) treatment is included as a control. Data are represented as percent activation of the P3 promoter as compared to AIP1 stimulation, and as the mean ± SEM; n = 3

Combining reduction with trypsin digestion did not result in a significant change in the ability of LDL to antagonize AIP1 function as measured by GFP fluorescence. Surprisingly, the buffer and incubation conditions of the V8 protease treatment seemed to enhance LDL inhibition of promoter activation, alter the bacterial response, or quench the fluorescence of the GFP. These experiments may be enhanced by a thorough exchange of the digest product into an inert buffer following digestion to avoid introducing artifacts to the reporter assay. In addition, the reduction and digestion approach will be expanded to include use of the pepsin protease as described above. Attempts to isolate active proteolytic fragments via Protein G-agarose beads covalently coated with the C1.4 monoclonal antibody have been initially unsuccessful (data not shown). Future efforts to isolate these active proteolytic fragments will include both ion exchange and size exclusion chromatography.

As mentioned in Chapter 4, we previously noted that apoB, in the context of the larger lipid particle VLDL, showed improved binding and functional antagonism of AIP1 compared to apoB in LDL. The conformation of apoB on VLDL differs slightly from the conformation on LDL (Tsao et al., 1982). In our assays, LDL appears to bind and functionally antagonize AIP1 similar to commercially available apoB purified from the intact lipoprotein by ether extraction (US Biological, Swampscott, MA). The amount and type of lipids remaining with the purified protein, if any, have not been determined, but it is reasonable to expect that some lipids may remain since completely delipidated apoB is reported to be insoluble (Fless et al., 1990). Based on these data and as an extension of the objective to further address the role of the lipid particle as having a supporting role, we treated VLDL with bovine lipoprotein lipase (LPL). Removal of lipids from the larger

VLDL particle by LPL should reduce the diameter of the particle and result in a change in apoB conformation similar to the apoB conformation in an LDL particle. Assessment of VLDL before and after LPL treatment in the P3:GFP reporter assay resulted in the treated VLDL particle being more "LDL-like" in its inhibitory capacity (Figure 2B). Although this does not exclude a direct role for the lipoprotein lipids in AIP1 antagonism, these data support the notion that the size of the lipid particle which carries apoB contributes to the ability of apoB to antagonize AIP1.

Hepatocyte-derived amino terminal fragments of apoB retain inhibitory activity

As another approach to isolating amino-terminal fragments of apoB, we obtained transfected rat hepatoma cells (McArdle- RH7777) that stably express and secrete full-length or truncated forms of human apolipoprotein B in the context of an intact lipoparticle (gift of Dr. Zemin Yao, Ontario) (White et al., 1992). This strategy ensures that the protein is properly folded and associated with lipids in a physiologically-appropriate conformation, unlike recombinant protein fragments alone (data not shown) or artificial lipoprotein "particles" made by incubation of apoB with lipid emulsions (Jiang et al., 2006).

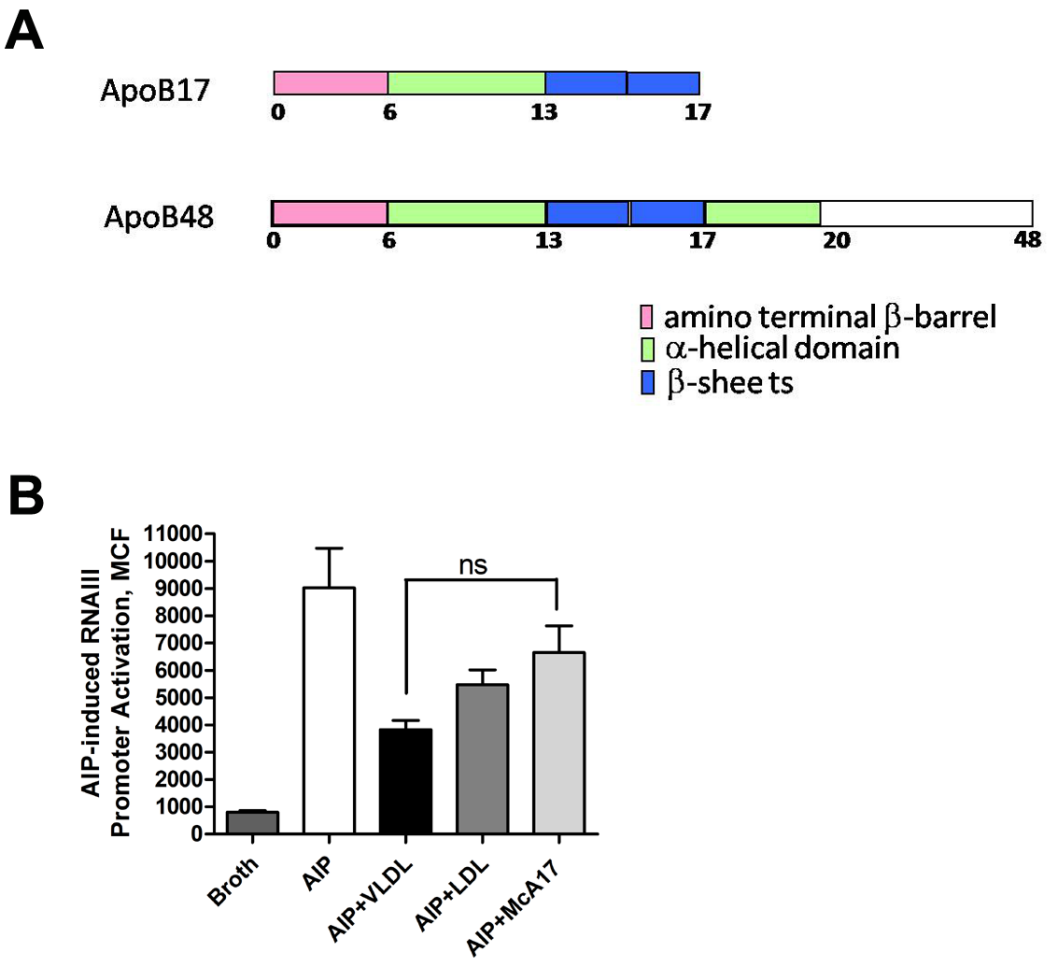

Figure 3. Fragments of Apolipoprotein B Produced by Stably Transfected McArdle-RH7777 cells

(A) Linear protein map depicting the lengths of the ApoB fragments secreted by transfected McArdle-RH7777 rat hepatoma cells. Peptide sequences that form secondary structures are color coded as indicated.

(B) *agr*:P3 promoter activation by 10 nM AIP 1 is inhibited in *S. aureus* (ALC 1743 with *agr*:P3-*gfp*, 2×10^7/ml) during 3 hr of culture with VLDL, LDL, or the McArdle apoB17 fragment (2 μg/ml apoB content each). Data are represented as the mean channel fluorescence (MCF) of total *S. aureus* by flow cytometry, and as the mean ± SEM; n = 3

We used McArdle cells expressing either the first 17% of the apoB protein (apoB17) or the first 48% of the protein (apoB48, see figure 3A) to generate fragments to use in our inhibition assays. Supernatants from the cells grown under serum-free conditions for two days were collected, concentrated, selected for human apoB fragments by pulldown with Protein G agarose beads covalently liked to monoclonal antibody C1.4, and analyzed by SDS-PAGE before inclusion in the biological tests. The fragment isolated from the apoB17 expressing cells antagonized the activation of the *S. aureus* P3 promoter as measured by flow cytometry (figure 3B). Further work must be done to isolate fragment preparations of greater purity and to compare the inhibitory ability of the different sized fragments.

The apoB-AIP1 interaction is pH dependent

Variations in interactions between binding partners in different pH environments is to be expected as the surface charge of both molecules can greatly affect attraction or repulsion at the protein:protein interface. This can be of importance in a physiologic setting such as the low pH environment of a phagolysosome. In addition, binding analyses performed under variable pH conditions has the potentially to reveal weaker, secondary or alternate binding sites. To further investigate the nature of the binding between apoB and AIP1, SPR was used to measure the binding of VLDL or purified apoB to AIP1 at under different pH conditions. Binding as determined by the number of RU generated, increased as pH was lowered from 7.4 to 5.8 (Figures 4A and B). These data suggest that the site(s) of apoB binding to AIP1 contain amino acids whose charge can be readily modified based on pH and, potentially, that the interaction between AIP1 and apoB would be enhanced in the acidic environment of the lysosome. In addition,

since cyclic AIP can become linear at highly basic pH, it is possible that the cyclic AIP structure is more stable at lower pH, thus contributing to enhance binding by apoB. Clearly, these results require further investigation. We aim to continue this work by kinetic evaluation of the AIP1-VLDL interaction under different pH conditions using fluorescence polarization, a technique with which we have had recent success.

Discussion

The inhibition of AIP-induced virulence in *S. aureus* by apolipoprotein B represents a newly described host innate barrier to invasive disease. The mechanism by which apoB antagonizes AIP is only partially understood. Here we began investigations to identify and characterize the AIP1 binding site within apoB. In these preliminary studies, we demonstrate that apoB fragments generated here by proteolysis retain their inhibitory activity, showing promise for fractionation and isolation of the specific functional fragment. The ability of LPL to convert VLDL to an LDL-like inhibition profile suggests a possible direct role for lipids in the lipoparticle-AIP1 interaction, and also supports the notion that lipid induced conformational changes in apoB affect the accessibility of the AIP1 binding site. Specifically, the conformation of the protein in which it binds best to AIP may be dictated by its association with the lipid core. Finally, our demonstration of enhanced binding of VLDL to AIP1 at lower pH suggests that the binding site(s) contain residues whose charge is readily altered by changes in pH. Together, these observations provide some insight into how apoB interacts with AIP1, and will serve as the basis of future, more definitive experiments.

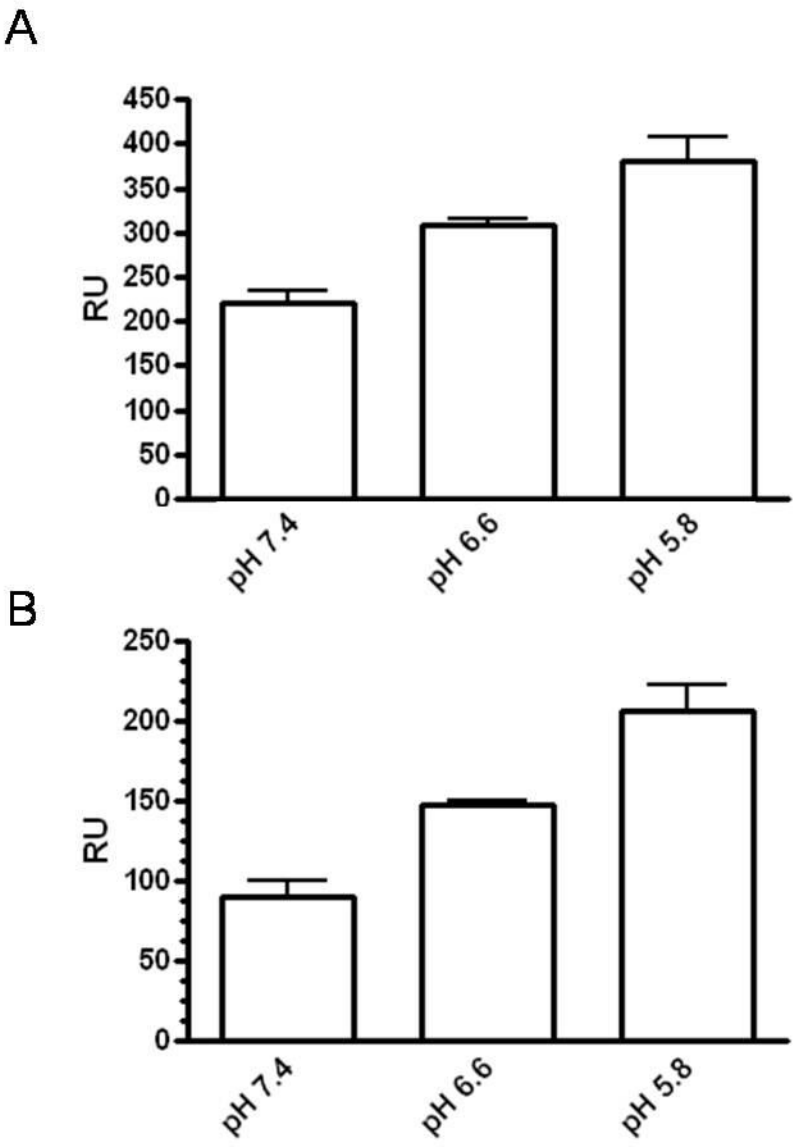

Figure 4. pH Dependence of Apolipoprotein B-AIP1 interaction

(A) Surface Plasmon resonance analysis of whole VLDL particles binding to immobilized AIP1 under different pH conditions shows greater binding response at lower pH.

(B) SPR analysis binding between purified apoB and immobilized AIP1 under different pH conditions. Greater binding response is observed at low pH.

The ability of proteolytically cleaved apoB and supernatants of McRH7777 cells expressing human apoB-17, although confounded by the presence of rat apoB, to functionally antagonize AIP1 in our reporter assay may suggest that patients who have truncated forms of apoB in their sera (such as those with familial hypobetalipoproteinemia) may be at increased risk for invasive infection not because of the truncated apoB, but because of their low apoB levels in general. Alternatively, these patients may be more susceptible to invasive *S. aureus* infection due to the inability of their truncated apoB-containing lipoproteins to be cleared by the LDL receptor (LDLR) or other receptors that require the C-terminus of apoB for binding (Hooper et al., 2005; Linton et al., 1993). This hypothesis will require more direct testing using suitable animal models lacking specific receptors for clearance of apoB.

Further work that focuses on improved purification, measuring biological activity, and describing the binding properties of generated fragments must be performed before identification of the minimum inhibitory domain of apoB can be complete. The future experiments most vital to understanding the mechanism of apoB binding to AIP are those that aim to purify homogenous preparations of fragments so they can be compared in their ability to bind AIP and inhibit downstream virulence signaling. Using more sensitive techniques like ion exchange and size exclusion chromatography will greatly improve the ability to not only interpret results but also to perform the right experiments with appropriate controls. Besides identifying a minimum inhibitory protein fragment, it will also be important to see if there is a minimum lipid component that is required for inhibition, either by providing proper structure scaffolding for the protein, or by directly interacting with the AIP after it is bound to the protein. In addition, if a lipid component

is necessary for proper AIP-lipoprotein interaction, it will be necessary to identify the type of lipid (triglyceride, phospholipid, cholesterol, or cholesterol esters) required for the interaction. Our previous work showed that high concentrations of a triglyceride emulsion could inhibit the ability of AIP to activate the quorum sensing promoter P3 (See Appendix, Figure S2B), indicating that the triglyceride components of lipoprotein particles may have some binding activity. Liposomes consisting of mixtures of phospholipids and cholesterol, however, did not inhibit AIP-induced promoter activation (See Appendix, Figure S2A). Based on these observations in addition to knowing that VLDL has the greatest quantity of triglyceride content than any other lipoprotein, and it elicits both the strongest AIP-binding and inhibiting responses as compared to LDL and purified apoB, the triglyceride component may be important for the AIP1-VLDL interaction. The role of lipids being important for a host defense mechanism of lipoproteins has already been described in the context of HDL binding to LPS; however, in the case of HDL, studies indicate that the phospholipid components of HDL are responsible for this interaction that can provide protection from endotoxemia (Parker et al., 1995). A similar approach to that taken by Parker and colleagues may be needed to identify the type of lipid, namely, comparing AIP1 binding and inhibitory responses elicited from particles formed from apoB combined with just one type of lipid. Combining a number of different approaches as described here that address both the protein and lipid aspects of apoB-containing particles will be necessary to fully define the nature of the binding between apoB and AIP.

CHAPTER 6

DISCUSSION AND CONCLUSIONS

Summary and Future Directions

This work describes a role for the serum lipoprotein component apoB in providing innate protection against invasive *S. aureus* infection. Apolipoprotein B antagonizes the phenotypic switch in a *S. aureus* population from colonizing to invading by binding and sequestering the quorum sensing peptide AIP away from its receptor AgrC. The absence of apoB leads to invasive infection from both *agr*1 and *agr*2 strains in an animal model of skin infection. The globular amino-terminal domain of apoB is responsible for binding AIP via hydrophobic interactions with the thiolactone ring of the peptide. These observations expand current knowledge regarding host defense against invasive bacterial infection and the functions of lipoproteins beyond metabolism.

S. aureus has evolved numerous mechanisms to offset host defense. During the immune response to *S. aureus* infection, neutrophils are recruited to the site and proceed to phagocytose the bacteria for subsequent killing by antimicrobial peptides and reactive oxygen species. Besides their antibacterial function, the ROS produced by neutrophils also oxidize the bacterial signaling peptide AIP so it cannot function (Rothfork et al., 2004). The mechanism of apoB antagonism of quorum sensing described in this thesis acts as an additional barrier to AIP signaling that does not rely on the presence of neutrophils or a functional NADPH oxidase. This barrier is also unaffected by the many immune-evasion tactics employed by *S. aureus* to escape detection and killing which target neutrophils' mechanisms of host defense (see Chapter 1 figure 2). Although bacterial proteases and lipases could alter host lipoprotein particles, expression of these enzymes is regulated by the quorum sensing operon *agr*, so the action of apoB against quorum sensing signaling could prevent its own degradation by bacterial factors.

Several aspects of this apoB-mediated control of *S. aureus* virulence remain to be elucidated. For example, the fate of the AIP-apoB complex is unknown. Specifically, it is unclear whether sequestration of AIP by apoB is sufficient to maintain a protective phenotype or if receptor-mediated clearance of the complex is required. In this regard, scavenger receptors on macrophages could play a crucial role. Studies show that mice deficient in the macrophage scavenger receptor CD36 are more susceptible to *S. aureus* infection, though it is unknown if this is related to quorum sensing (Hoebe et al., 2005; Stuart et al., 2005). Besides its normal metabolic function of removing modified LDL particles from circulation, CD36 also recognizes bacterial diacylglycerides present in cell wall components and can be a co-receptor with Toll-like Receptor 2 to sense microbial pathogens. However, it may also act more directly to inhibit infection by removing AIP-bound LDL during a quorum sensing-dependent infection. If this is the case, then CD36 knockout mice should be more susceptible to an *agr*+ infection but not an *agr*- infection as compared to wild type mice. This subject is part of an ongoing investigation in our laboratory.

Hypocholesterolemia is known to correlate with severity of illness due to sepsis from multiple bacterial pathogens (Marik, 2006). One expansion of this work would be to investigate the role of apoB in other bacterial infections, especially those that have similar quorum sensing systems such as *Enterococcus faecalis* and *S. epidermidis*. In the environment of the gut, apoB48 on chylomicrons may help to control quorum sensing mediated by the cyclic *E. faecalis* pheromone GBAP (Nakayama et al., 2006). Like MRSA, vancomycin resistant enterococcus infection is a growing problem, especially in hospitalized patients (Chavers et al., 2003) and any understanding of host molecules that

antagonize virulence could lead to important developments in treatment and therapeutic options. ApoB may therefore have a role in host defense against other bacterial pathogens beyond antagonizing *S. aureus* virulence signaling.

This work focuses on serum apoB as a specific barrier to *S. aureus* invasive infection; however, other blood proteins exist that are already known to have antibacterial or anti-virulence properties including complement, antibodies, C reactive protein, and even hemoglobin, as has been recently described (Schlievert et al., 2007). From our own experiments, even lipoprotein deficient serum has some activity against virulence signaling, although the amount of apoB remaining has not been determined. Therefore, other serum components must also be at work to limit invasive infection. Some candidates we would suspect include HDL and albumin, since our own assays have been influenced by the presence of these molecules in the experimental conditions. HDL is known to bind both LPS and lipoteichoic acid, so it may be able to bind other bacterial products as well. This may further support the idea that downregulation of HDL during the acute phase response of infection is a good indicator of complication. Albumin is the most abundant protein in the serum, so even if its anti-virulence properties are weak, its sheer abundance could contribute to host control of bacterial virulence. Therefore, a thorough investigation of the role of each of these serum components in antagonism of *S. aureus* quorum sensing-mediated virulence is warranted.

Therapeutic application of this work is another possibility for development. Providing lipid-rich nutritional support does not contribute to resolution of the acute phase response or tolerance of invasive *S. aureus* infection (Marik, 2006; Hattie Gresham and Jessica Mack, unpublished observation), but it is unknown if treatment with apoB or

an apoB mimetic yet to be defined would be beneficial in models of local or invasive infection or human cases. A pilot study using de-identified patient samples from the New Mexico Veterans Affairs Health Sciences Center indicates that patients with invasive MRSA infection have lower serum apoB levels compared to patients with localized MRSA infections (Dr. Jon Femling, personal communication). These data indicate that the protective effect of apoB seen in mice may apply to human infection as well; therefore, treatment with exogenous apoB or apoB-containing particles may limit infection and prevent dissemination. Animal studies that determine the best route of administration, dosing, and time course will be necessary to demonstrate the feasibility of the therapy before it can be proposed for human treatment.

An additional animal model of infection that is relevant to the abscess-forming skin infections predominately seen in patients with MRSA is a dermonecrotic skin abscess mouse model that is widely used in *S. aureus* virulence factor studies (Bubeck Wardenburg et al., 2008; Voyich et al., 2006). This model would be very useful to examine how well apoB extravasates with plasma to the site of infection. Interestingly, the measurement of apoB in the skin is used as an indicator of coronary artery disease in patient studies (De Graeve et al., 1984; Douste-Blazy et al., 1985), indicating that measurable apoB would be present at the skin to limit *agr*-mediated virulence signaling, allowing for innate immune effectors to clear the infection. This model would also be a good method for the therapeutic development of apoB described above, since administration of the apoB or apoB mimetic could be given directly into the infected site.

Another extension of this work would focus not on the host, but on elucidating the relative resistance to apoB inhibition of quorum sensing in *S. aureus agr*3 strains. Based

on genetic typing studies, it is theorized that *agr* groups began diverging from one another about 55 million years ago to select for greater specificity in the *agr* locus, not as a response to environmental pressures that could come from the host (Wright et al., 2005). Since this *agr* diversification serves to isolate different groups one from another instead of expanding virulence across sequence types, and the type 3 peptide seems to be the most poorly bound by apoB, it appears that resistance of *agr*3 strains to apoB is an evolutionary coincidence. The higher rate of bloodstream and menstrual toxic shock diseases in *agr*3 infections as compared to *agr*1 and 2, could indicate another bacterial mechanism of host evasion, a mechanism that is based simply on the length of the bacterial AIP3 peptide. To address this hypothesis experimentally, a modified AIP3 peptide could be synthesized with two extra amino acids on the amino-terminal end, thus making the peptide the same length as AIP2. The elongated AIP3 could then used in the binding studies as measured by SPR and fluorescence polarization to determine if binding to apoB-containing lipoproteins is improved over that of native AIP3. If the longer peptide binds with greater affinity than the native peptide equal to that of AIP2, then it can be concluded that peptide length determines the ability of apoB to control AIP-mediated virulence, and by extension, why apoB cannot control bloodstream infections with bacteria of the *agr*3 type.

In summary, much of the preliminary work described in this dissertation must be expanded upon to reach definitive conclusions regarding the mechanism of apoB inhibition of AIP signaling. First, the AIP binding of apoB must be defined. With this information we can look at the polymorphisms of apoB found in the human population to determine if any of those variations may directly or indirectly contribute to binding and

proper conformation of apoB such that AIP antagonism is reduced or enhanced. These polymorphisms may explain in part why some patients experience severe and complicated infections where others have nothing more than a minor skin irritation. It may also clarify why so many individuals can be colonized with *S. aureus* and not succumb to invasive infection. Second, experimental determination of a K_d describing the affinity of the interaction between apoB, either by itself or in the context of lipoprotein particles, and the different AIPs should be accomplished. This would provide additional information regarding the potential effectiveness of apoB as an innate protector against invasive infection by *S. aureus* of different *agr* types. One method of generating this data is fluorescence polarization, whereby a small fluorescent molecule initially depolarizes light until it is immobilized by a larger binding partner, allowing the light to remain polarized. Fluorescence polarization may be a better tool to measure binding affinity than SPR because it does not rely on immobilization of one of the binding partners, should be unaffected by the tendency of lipoproteins to adhere to surfaces, and will not be affected by the differences in size between whole lipoprotein particles and apoB alone. In addition, it may be more beneficial for examining the function of the amino terminal tail relative to the thiolactone ring for AIP interaction with apoB. Finally, a prospective study of serum apoB levels and apoB polymorphisms in patients at risk for *S. aureus* infection will be needed to ascertain whether what we have demonstrated in our mouse model of invasive infection has a clinically relevant human correlate.

Conclusions

The data and interpretations presented in this manuscript describe the role of apolipoprotein B as a specific inhibitor of *S. aureus* virulence, the efficacy of this

inhibition among different *agr* types of *S. aureus*, and the mechanism by which the inhibition is mediated. As the incidence of CA-MRSA infections continues to increase, it will become more vital to understand host factors that are important for limiting infection, including factors that may be lacking in populations of otherwise healthy individuals. The demonstration of apolipoprotein B-mediated control of *S. aureus* virulence in this dissertation is one example of a host defense mechanism that is important for control of CA-MRSA infections. The data herein specifically show that apoB is effective against *agr* type1 strain USA300, a CA-MRSA clone that has spread globally, replacing endemic CA-MRSA strains (Diep and Otto, 2008). Though this strain produces a number of virulence factors in abundance, its rapid dissemination is attributed to its fitness for transmission in the human population. Historical and current strategies for treating *S. aureus* and other bacterial diseases have emphasized limiting growth, which has promoted the development of bacterial resistance. New methods that still curb severe disease without contributing to drug resistance are necessary to effectively manage cases of *S. aureus* infection. As we continue to study the role of apoB in limiting invasive infection, therapeutic measures can be developed to combat USA300 disease by enhancing the body's own anti-virulence methods. Apolipoprotein B-mediated control of invasive *S. aureus* infection provides a new way of looking at the management of bacterial disease by focusing on inhibiting virulence rather than growth.

APPENDIX

Supplemental Figures to Chapter 3

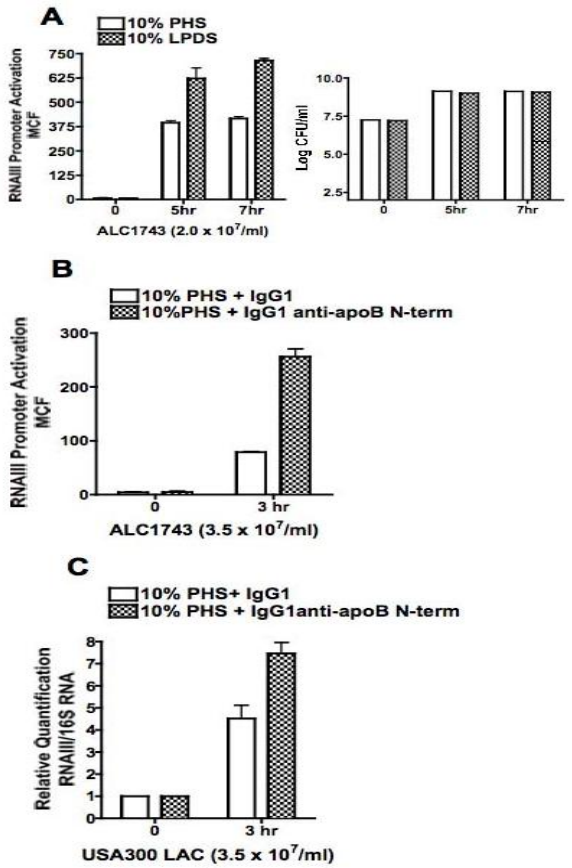

Figure S1. Effect of Serum Lipoproteins on Spontaneous *agr*:P3 Promoter Activation by Serum

(A) ALC1743 (non-fluorescent, early exponential phase, 2.0×10^7/ml)) were cultured in 90% TSB and 10% either pooled human serum (PHS) or 10% lipoprotein deficient pooled human serum (PHS) at 37°C with shaking. To prevent clumping, the samples were sonicated on ice at 30 min intervals. At the indicated times, the bacteria were plated for CFU determination and washed and fixed for flow cytometry to determine GFP induction. Spontaneous *agr*:P3 promoter activation is significantly enhanced in the presence of LPDS vs PHS after 5 and 7 hrs of culture whereas bacterial CFU is unaffected. Data are represented as the mean ± SEM, n=2 performed in duplicate.

(B) ALC1743 (non-fluorescent, early exponential phase, 3.5×10^7/ml) were cultured in 90% TSB and 10% PHS (pretreated with either 10 µg/ml murine IgG1 or murine IgG1 anti-apoB N-term mAb C1.4 on ice for 30 min) for 3 hr at 37°C with shaking. To prevent clumping, the samples were sonicated on ice at 30 min intervals. At the indicated times, the bacteria were washed and fixed for flow cytometry to determine GFP induction. Spontaneous *agr*:P3 promoter activation is significantly enhanced in the presence of anti-

apoB treated PHS vs. the isotype control treated PHS after 3 hrs of culture. Data are represented as the mean ± SEM, n=2 performed in duplicate.

(C) USA300 LAC wild-type (early exponential phase, 3.5×10^7/ml) were cultured in 90% TSB and 10% PHS (pretreated with either 10 µg/ml murine IgG1 or murine IgG1 anti-apoB N-term mAb C1.4 on ice for 30 min) for 3 hr at 37°C with shaking. To prevent clumping, the samples were sonicated on ice at 30 min intervals. At the indicated times, RNA was extracted and RNAIII quantified relevant to 16S RNA by qRT-PCR as described in the Materials and Methods.

RNAIII transcript levels are significantly increased in the presence of anti-apoB treated PHS vs the isotype control treated PHS. Data are represented as the mean ± SEM, n=2 performed in triplicate.

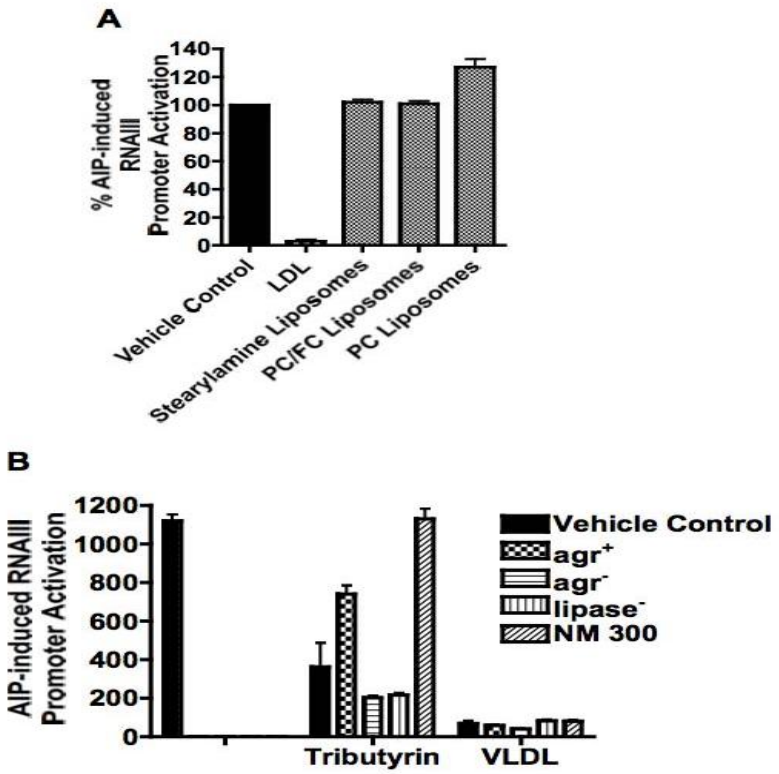

Figure S2. Effect of Liposomes and Triglycerides on AIP-Dependent *agr*:P3 Promoter Activation

(A) Incubation of AIP1 with liposomes containing stearylamine (10 mM phosphatidylcholine, 1.5 mM cholesterol, 3 mM stearylamine), phosphatidylcholine (PC, 10 mM), or phosphatidylcholine/free cholesterol (PC/FC, 7.1mM/2.9mM) did not inhibit AIP1-dependent *agr*:P3 promoter activation as compared to LDL. Data are represented as the mean ± SEM, n=2 performed in triplicate.

(B) Supernatants from overnight cultures of lipase-producing (*agr*+ and NM300) or lipase-nonproducing (*agr*- and *lipase*-) strains were incubated with a 0.5% solution of the triglyceride tributyrin (5x normal serum levels) prior to incubation with AIP1 to test in the *agr*:P3-*gfp* reporter assay. Tributyrin inhibition could be reversed by lipase whereas VLDL inhibition was not reversed by bacterial lipase. Data are represented as the mean ± SEM, n=2 performed in duplicate.

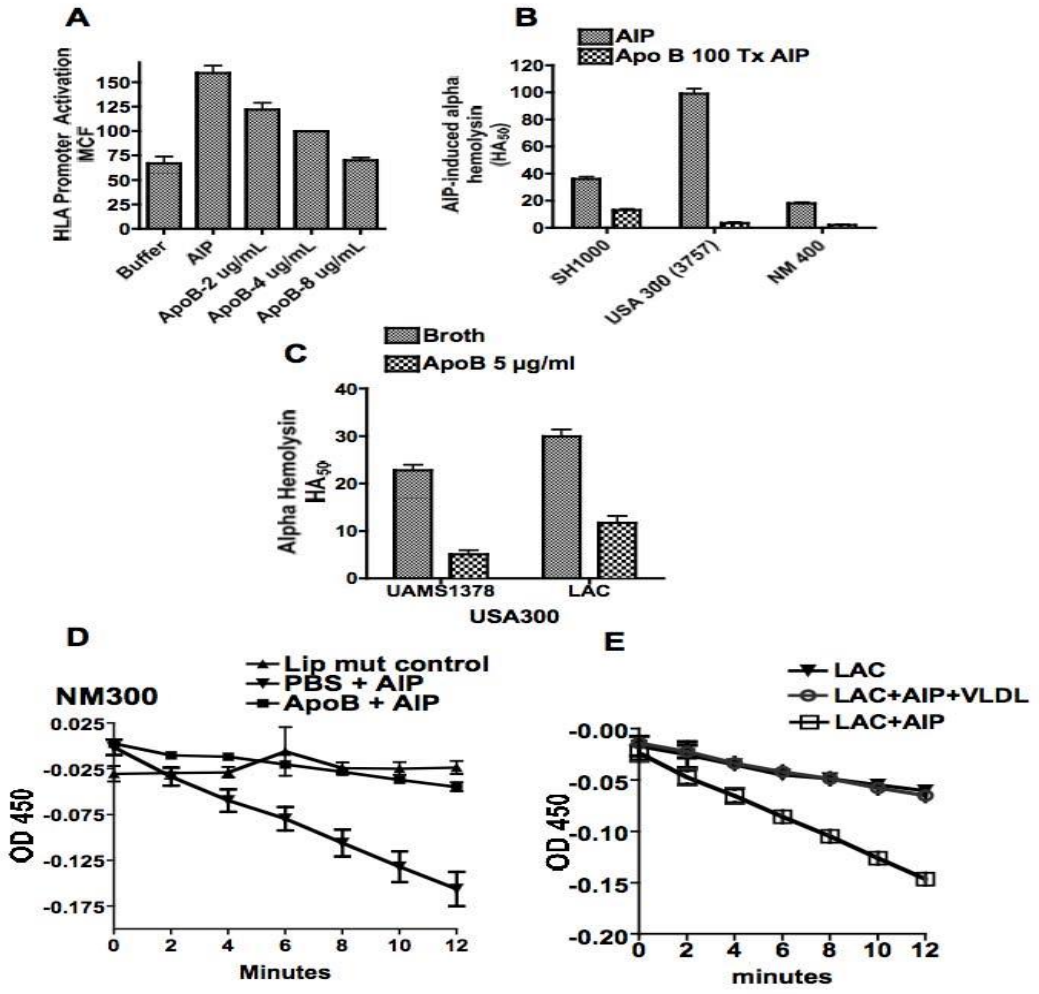

Figure S3. Virulence Factor Production Suppressed by Lipoproteins

(A) Apolipoprotein B inhibited in a dose-dependent fashion AIP1-induced *hla* promoter activation. *S. aureus* ALC 1740 containing an *hla-gfp* plasmid was cultured with 100 nM AIP1 with or without increasing concentrations of purified apoB. After 6.5 hours, promoter activation was measured by flow cytometry. Data are represented as the mean ± SEM, n=3.

(B) Alpha hemolysin in culture supernatants generated by growing bacteria in the presence of 100 nM AIP1 (synthetic or partially purified from culture supernatant by size exclusion) with or without 8 µg/ml apoB for 6.5 hr was measured in a hemolytic assay. SH1000 is a sigma B corrected variant of RN6390, FPR 3757 is a MRSA USA 300 PFGE clinical isolate, and NM 400 is an *agr* group 3 MRSA USA 400 PFGE clinical isolate. Data are represented as the mean ± SEM, n=3.

(C) Inhibition of spontaneous alpha hemolysin production by two MRSA USA300 strains, UAMS1378 and LAC, after 6.5 hr of culture in the presence and/or absence of purified apoB. Data are represented as the mean ± SEM, n=2 performed in duplicate.

(D) Lipase in culture supernatants generated by culturing NM300 (MRSA USA 300 clinical isolate) with 100 nM AIP1 with or without apoB (5 µg/ml) for 7 hr was measured by the rate of cleavage of a triglyceride substrate (tributyrin) assessed at OD450 nm. Values are compared to the supernatant of a lipase deficient strain. Data are represented as the mean ± SEM of duplicates and are representative of at least 3 separate experiments.

(E) Lipase in culture supernatants from MRSA USA300 LAC cultured in the presence of 100 nM AIP1 with or without VLDL (2 µg/ml) was measured as in (D). Data are represented as the mean ± SEM of duplicates and representative of two separate experiments.

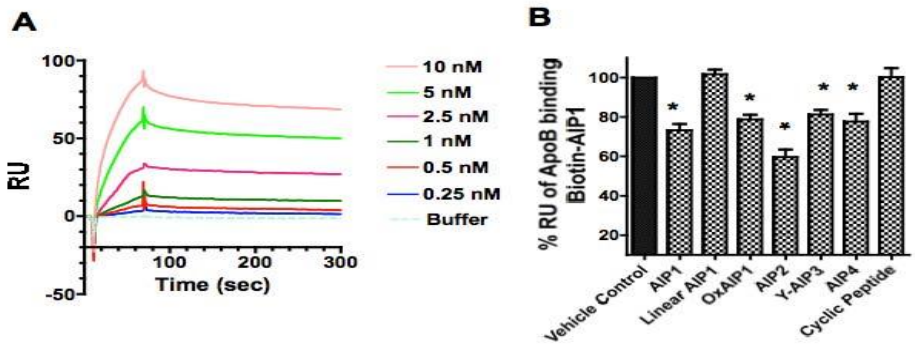

Figure S4.Binding of Apolipoprotein B and AIP1 by Surface Plasmon Resonance

(A) Biacore X100 sensorgram of the interaction of apoB (0.25-10 nM) with biotin-AIP1 immobilized on a streptavidin chip following the manufacturer's protocol. Kinetic data were collected at a flow rate of 30 µl/min in running buffer with a contact time of 60 s and dissociation over 300 s. Data are representative of 3 separate experiments.

(B) Native AIPs (1, 2, &4), modified AIPs (linear AIP1, oxidized AIP1, and Y-AIP3), or a control cyclic peptide made by disulfide cross linking (all at 50 nM) were mixed with 10 nM apoB prior to injection onto immobilized biotin-AIP1 at a flow rate of 10 µl/min with a contact time of 60 s followed by a 60 s dissociation time. The resulting resonance units were analyzed as a percentage of the resonance units of apoB binding in vehicle control. Data are represented as the mean ± SEM, n=3-4. *$p<0.001$.

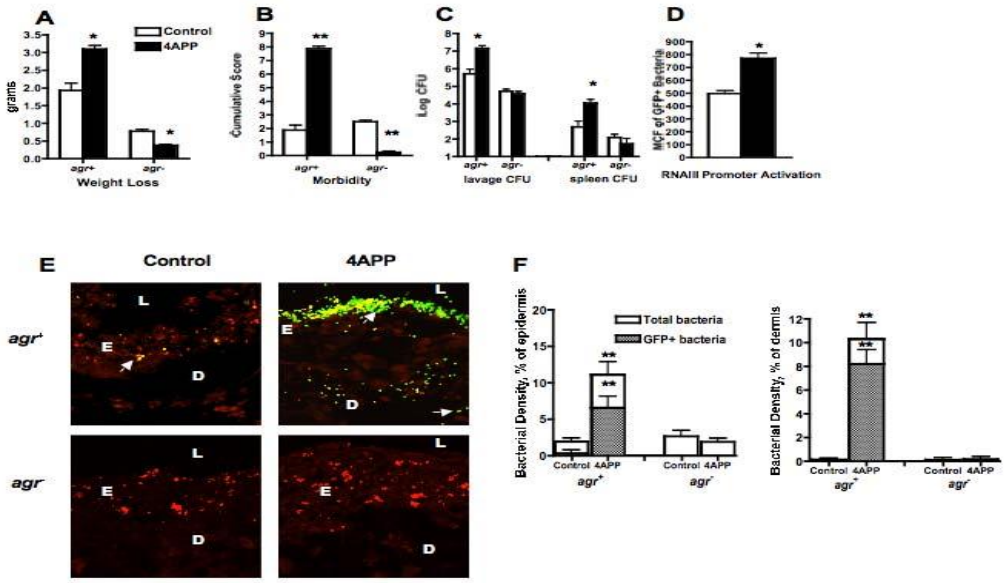

Figure S5. Effect of *agr* on *S. aureus* Infection of NADPH Oxidase Knockout (*gp91*$^{phox-/-}$) Mice Treated with 4APP

Mice were treated with 100 µl of 5 mg/ml 4APP or vehicle control (0.025M phosphate buffer) (n=8 for each group) i.p. 48 and 24 hours prior to infection of air pouches with either 7×10^6 (*agr*:P3-*gfp*, ALC 1743, or *Δagr*:P3-*gfp*, ALC 1753). At 28 hours hrs post infection, the following parameters were determined and the data represented as the mean ± SEM:

(A) Weight loss.

(B) Morbidity score 0-14.

(C) Bacterial burden (Log CFU) in pouch lavage and spleen.

(D) Quantification of *agr*:P3-*gfp* promoter activation by flow cytometry of bacteria isolated from the pouch lavage.

(E) Representative pouch tissue from the mice stained with anti-*S. aureus* antibody (red fluorophore). Quorum-sensing *S. aureus* are visible by production of GFP in the pouch.

(F) Quantification of bacterial density in epidermis and dermis of pouch tissue demonstrating significantly greater bacterial density of *agr*+ bacteria but not *agr*- bacteria and greater quorum sensing in 4APP-treated mice from at least 19 representative areas. L = lumen D = dermis E = epidermis

Arrows indicate point of greatest penetration by quorum sensing bacteria

* $p < 0.05$ ** $p < 0.01$

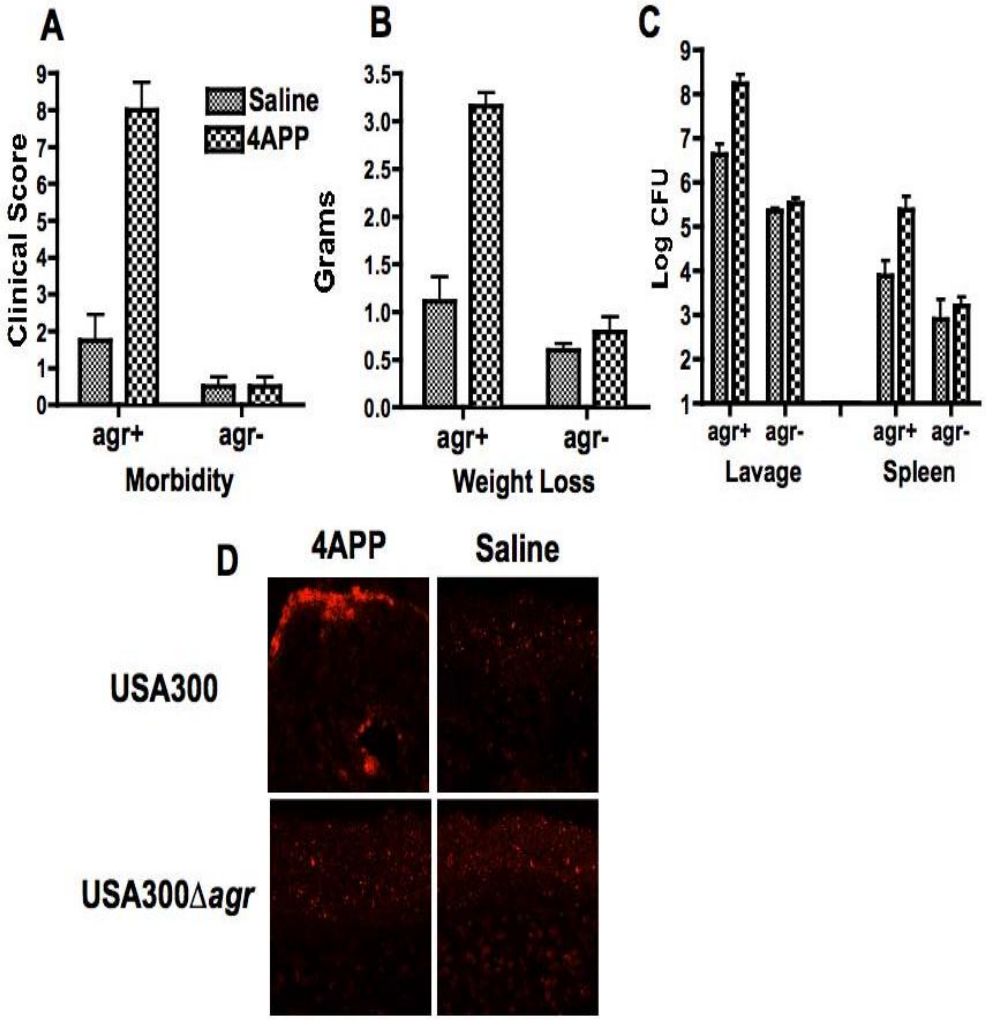

Figure S6. Effect of *agr* on MRSA USA300 *S. aureus* Infection of NADPH Oxidase Knockout (*gp91*$^{phox-/-}$) Mice Treated with 4APP

Mice were treated with 100 µl of 5 mg/ml 4APP or vehicle control (0.025M phosphate buffer) (n=8 for each group) i.p. 48 and 24 hours prior to infection of air pouches with either 2.5×10^7 USA300 UAMS1378 or *Δagr* UAMS1540). At 28 hours hrs post infection, the following parameters were determined and the data represented as the mean ± SEM:

(A) Morbidity score 0-14.

(B) Weight loss.

(C) Bacterial burden (Log CFU) in pouch lavage and spleen.

(D) Representative pouch tissue from the mice stained with anti-*S. aureus* antibody (red fluorophore).

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