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Apolipoprotein B containing lipoproteins contribute to host innate immune pulmonary defense against Staphylococcus aureus

Brett Manifold-Wheeler

Brett Manifold-Wheeler

Candidate

Department of Pharmaceutical Sciences

Department

This dissertation is approved, and it is acceptable in quality and form for publication.

Approved by the Dissertation Committee:

Pamela R. Hall

_____

Chairperson

Jon Femling

_____

Pavan Mutill

_____

Kevin Harrod

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APOLIPOPROTEIN B CONTAINING LIPOPROTEINS CONTRIBUTE TO HOST INNATE IMMUNE PULOMARY DEFENSE AGAINST *STAPHYLOCOCCUS AUREUS*

BY

BRETT MANIFOLD-WHEELER

B.S. Genetics, University of California, Davis, 2005

DISSERTATION

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DEDICATION

I would like to dedicate this dissertation to everyone who has made this possible for me. It has been a long road; ten years since I first started graduate school, and none of my accomplishments would be possible without the people who believed in me, including all of my wonderful friends, my family, and my many mentors along the way.

Finally, I would like to dedicate this work to my mother, Cosette Wheeler, for being my protector in life, for showing me love and kindness despite my many faults, and for being a true inspiration as a scientist. I love you very much, and I know you'll always be my candle in the dark.

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ABSTRACT

Circulating lipoproteins are increasingly recognized as innate immune effector molecules. Severe hypolipidemia, which may occur with trauma or critical illness, is clinically associated with bacterial pneumonia. Importantly, pneumonia caused by *Staphylococcus aureus* is a major cause of morbidity and mortality in both hospital- and community-acquired pneumonia cases. Here we report that lipoprotein deficiency impairs early pulmonary innate defense against *S. aureus* quorum-sensing dependent pathogenesis. Specifically, apoB levels in the lung early post-infection are significantly reduced with lipoprotein deficiency, coinciding with impaired host control of *S. aureus* *agr*-signaling and increased *agr*-dependent morbidity and inflammation. Given that lipoproteins also inhibit LTA- and LPS-mediated inflammation, these results suggest that hypolipidemia may broadly impact post-trauma pneumonia susceptibility to both Gram positive and Gram negative pathogens. Together with previous reports demonstrating that hyperlipidemia also impairs lung innate defense, these results suggest that maintenance of normal serum lipoprotein levels is necessary for optimal host innate defense in the lung.

Table of Contents

Contents

List of Figures and Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 1 – Introduction

Methicillin-resistant *Staphylococcus aureus*

S. aureus is a Gram-positive bacteria and a human commensal of the skin and the anterior nares of the upper respiratory tract, with ~20% of the world's population being persistently colonized, and an additional ~60% being transiently colonized [1]. The measure of *S. aureus* as a successful commensal is clearly indicated by its ubiquitous prevalence among the human population. In addition, *S. aureus* is also one of the most important bacterial pathogens world-wide, based on its prevalence in hospital and community acquired infections [2-4], and the particular virulence of more recent emerging antibiotic resistant strains. Since the 1990s, highly virulent community acquired methicillin-resistant *S. aureus* (CA-MRSA) strains have become more prevalent world-wide, leading to significantly increased lengths of hospitalization and healthcare costs [5, 6], though the mechanistic reasons for increased virulence in CA-MRSA strains is still incompletely understood [6]. Importantly, in 2013 MRSA was identified as a "serious threat" organism by the U.S. Centers for Disease Control (CDC), and was the direct cause over 11,000 deaths in 2011 [7]. However, this is likely an underestimation of the true mortality costs of MRSA as cases in which it contributed to death were not included in this study.

MRSA Pneumonia

While *S. aureus* is a leading cause of skin and skin structure infections (SSSI), it can also lead to more severe life-threatening illnesses, such as bacteremia, endocarditis, and pneumonia. Pneumonia caused by *S. aureus* is a major cause of morbidity and mortality, particularly in cases of post-influenza pneumonia [2]. With the rise in MRSA cases over the past two decades, along with increases in prolonged ventilator support in an aging and chronically ill populations, there has been a significant increase in the number of hospital-acquired pneumonia and ventilator-associated pneumonia cases, with MRSA accounting for 20-40% of these [4]. A survey of 59 US hospitals in 2005 revealed that MRSA accounted for a significant portion of community and hospital acquired pneumonia cases, as well as a significant portion of healthcare and ventilator-associated pneumonia cases, with *S. aureus* being the only pathogen independently associated with mortality [3]. With the rise in pneumonia caused by MRSA, There is a need for an enhanced understanding of specific host-pathogen interactions during *S. aureus* infection in the lung.

The accessory gene regulator, virulence, and pneumonia

The dichotomy of *S. aureus* as a ubiquitous commensal and a highly virulent pathogen is, in part, regulated by the accessory gene regulator (*agr*) operon. The *agr* operon encodes a two-component, secretion controlled quorum-sensing (QS) system that mediates a temporal and cell-density-dependent “switch” from a colonizing to an

invasive and virulent phenotype [8] (**Figure 1.1**). This conserved four gene operon consists of *agrA-D*, which is expressed from the P2 promoter [9]. AgrD is a linear peptide that is cyclized into a mature auto inducing peptide (AIP), and secreted through the transmembrane protein, AgrB. Secreted AIP signals in an autocrine and paracrine manner through the transmembrane histidine kinase receptor, AgrC, causing autophosphorylation of this receptor. AgrC, in turn, phosphorylates the transcription factor AgrA, allowing AgrA to activate the P2 and P3 promoters. Activation of the P2 promoter drives expression of the four gene operon, creating a positive feedback loop by increasing *agr* gene transcription. AgrA activation of transcription from the divergent P3 promoter drives expression of RNAIII. RNAIII is a small effector RNA which modulates expression of hundreds of genes, and turns on an arsenal of virulence factors including a number of hemolytic toxins, lipases, proteases, and many other exotoxins necessary for *S. aureus* invasive infection [8, 10].

Among the most studied *agr*-regulated virulence factors shown to play a role in the pathogenesis of *S. aureus* pneumonia are alpha-hemolysin (Hla), Panton-Valentine leukocidin (PVL), and the phenol soluble modulins (PSM). Hla is a hemolytic toxin which is post-transcriptionally regulated by RNAIII [11], and has been shown to be an important contributor to pathogenesis of *S. aureus* pneumonia [12, 13]. Through interaction with the mammalian cell surface receptor ADAM10 [14, 15], Hla forms a heptameric, barrel shaped, cytolytic pore. This in turn causes a loss of cell membrane integrity due to potassium efflux, activating the NLRP3 inflammasome, and eventually leading to cell death [16-18]. PVL is a β-barrel pore forming toxin which is cytotoxic to

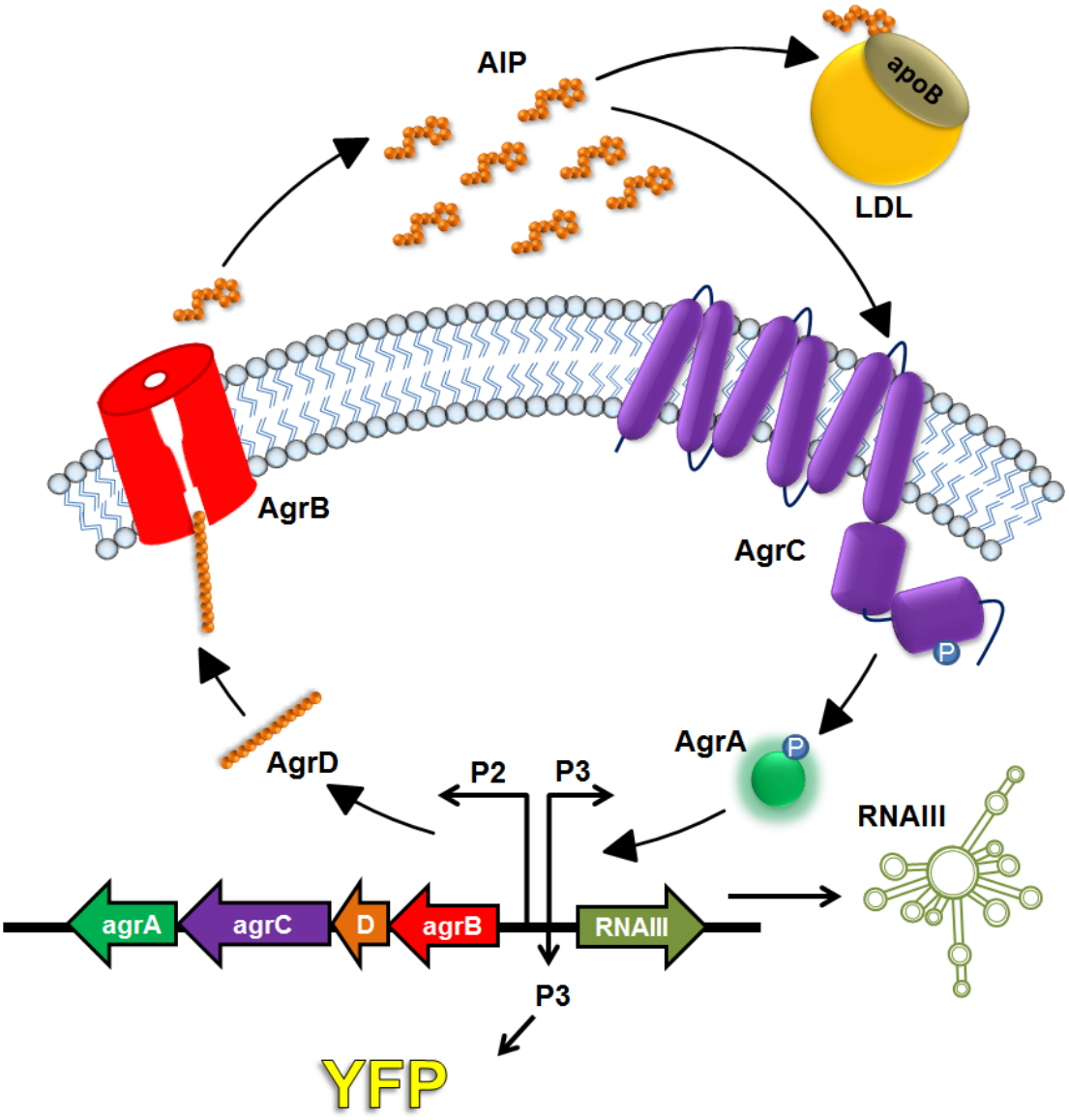

Figure 1.1 - The *S. aureus* accessory gene regulator (*agr*).

The *agr* operon mediates a density dependent phenotype switch from colonizing to invasive via a positive feedback loop. This in turn increases production of the global effector RNA, RNAIII, which regulates the expression of an arsenal of virulence factors. [8].

leukocytes [19]. Although, PVL has been epidemiologically associated with CA-MRSA infection world-wide [20], its contribution to invasive disease, specifically necrotizing pneumonia, has been controversial [20-24]. PSMs are small amphipathic lytic peptides considered major virulence determinants associated with leukocyte destruction [25], and whose expression modulates Hla expression [26]. PSMs have been shown to be important for biofilm structuring and dispersal [27]. Introduction of the PSM expressing locus, *psm-mec*, into non-PSM expressing *S. aureus* strains promoted biofilm formation [28]. Together, these studies suggest that PSMs may contribute to biofilm infections such as those associated with catheters and ventilators. Together, these *agr*-regulated virulence factors which play a role in *S. aureus* pneumonia pathogenesis highlight the importance of understanding host innate immune responses in the lung which target this QS system.

Innate immunity in the lung

The human lung is the largest air to surface exposure site in the human body, with millions of liters of air ventilated throughout the human lifespan [29], and is thus constantly exposed to an array of foreign particles and airborne microbial organisms (**Figure 1.2**). To avoid constant infection, innate immunity in the lung has become highly adapted, using physical and chemical barriers in the form of surfactant, the mucocilliary escalator, antimicrobial peptides (AMP), and lung-specific immune cells.

The lung expresses many AMPs, including lysozyme, α-defensin and β-defensin, lactoferrin, surfactant proteins A and D, and cathelicidin. As part of the first-line of defense, AMPs help to directly prevent infection and limit subsequent, potentially

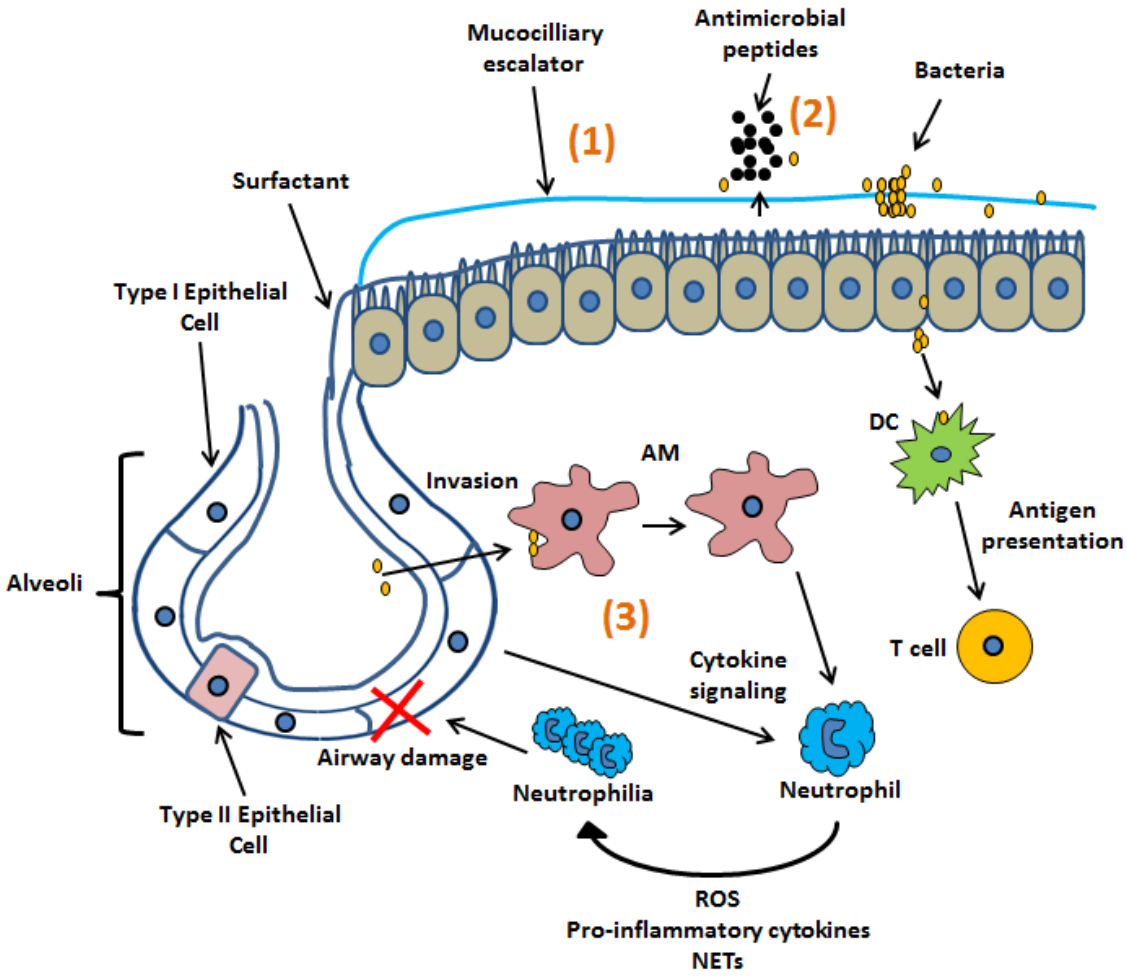

Figure 1.2 - Innate immunity in the lung.

(1) The lung uses a mucocilliary escalator as a physical barrier that continuously removes foreign particulates, including potentially harmful microbes. **(2)** Additionally, lung epithelial and resident immune cells secrete a myriad of antimicrobial peptides that act to limit the viability and infectivity of invading pathogens. **(3)** Resident immune cells function to phagocytose and remove invading pathogens, and in the case of more severe infection, regulate inflammation to limit pathology from recruited neutrophils.

damaging inflammatory responses [30]. However, these AMPs can also have direct effects on host cell immune cell function. Antimicrobial lung surfactant proteins have been shown to directly act on lung resident alveolar macrophages (AM) in both a pro- and anti-inflammatory manner, dependent on the lung microenvironment [31]. For example, SP-A has also been shown to induce respiratory burst and nitric oxide synthase (NOS) expression in AMs [32]. Invading pathogens first interact with alveolar epithelial cells and AMs, which in turn secrete chemokines including IL-8 and RANTES, to recruit inflammatory cells such as neutrophils, monocytes, and Natural Killer (NK) cells [33-35]. The resident proximity of AMs to the alveolar epithelium and lumen makes them well suited for lower airway innate immune defense [36]. AMs have been shown to be essential for phagocytic clearance of a multitude of airway infecting microbes [37, 38], and the phagocytic abilities of AMs is enhanced during infection and inflammation in the lung [39]. In addition to their role in removing invading pathogens, AMs and lung resident dendritic cells, along with regulatory T cells and the lung epithelium, regulate and minimize potentially damaging inflammation caused by neutrophils [29], via production of anti-inflammatory cytokines such as IL-10 and TGF-β [40].

While neutrophils are critical to host defense against invading pathogens in the airway [41, 42], they are also involved in inflammatory pathology in various disease states [42, 43]. The local inflammatory response during lower airway bacterial infection is a complex and important process, necessary for host immune defense in the lung (**Figure 1.2**). Toll-like receptors (TLR) on neutrophils recognize specific pathogen-associated molecular patterns (PAMP) on invading pathogens, such as LPS and LTA, leading to eventual activation of the transcription factor NF-κB, which in turn upregulates

production of pro-inflammatory cytokines and chemokines. Infiltrating neutrophils also produce proteases, reactive oxygen species (ROS), and reactive nitrogen species (RNS) which induce necrotic cell death, leading to extensive lung injury [42]. In this way, neutrophils present a "double-edged sword" component of innate immunity in the lung, whereby an appropriate neutrophil recruitment response is necessary to limit invasive infection, but over-recruitment can lead to irreparable damage to the lung. Therefore, inflammation in the lung must be tightly regulated in order to minimize damage from invading pathogens, as well as from the host inflammatory response.

Multifunctional role of serum lipoproteins

The term "serum lipoproteins" encompasses a variety of macromolecular lipid-protein complexes which can be divided into different classes based on size, density, and apolipoprotein associations (**Figure 1.3**). Among the different serum lipoproteins are chylomicrons, chylomicron remnants, very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL), which function to store and transport water-insoluble lipids (cholesterol and triglycerides) throughout the body in a water-soluble form.

The exogenous lipoprotein metabolism pathway starts when dietary lipids are incorporated into chylomicrons in the intestine. Processing of chylomicrons by lipoprotein lipase in peripheral tissues forms chylomicron remnants which are taken up by the liver. The liver is the major source of the three primary lipoprotein particles

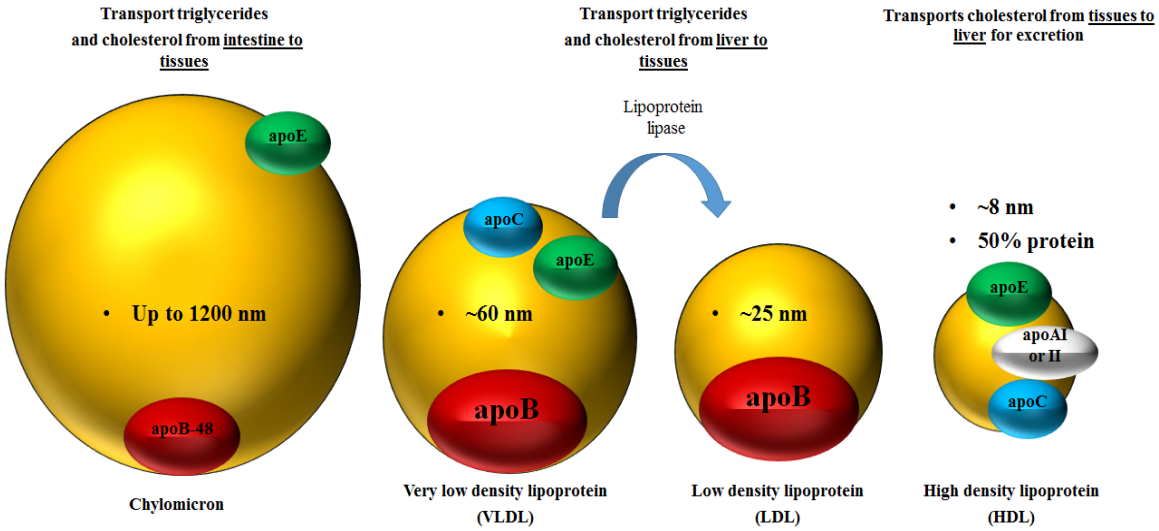

Figure 1.3 - Differing sizes, apoprotein associations, and functions of serum lipoproteins.

Chylomicrons are made in the intestine for transporting dietary lipids to tissues. Very low density lipoprotein (VLDL) is produced in the liver and further metabolized, via lipoprotein lipase, to low density lipoprotein (LDL) which is the primary source of cholesterol transported from the liver to the peripheral tissues. HDL is responsible for reverse cholesterol transport from tissues back to the liver. Adapted from [44].

produced endogenously, which include VLDL, LDL, and HDL. The liver forms VLDL which is processed into LDL by lipoprotein lipase in the peripheral tissues. LDL, often referred to as “bad cholesterol,” contains the highest percentage cholesterol content of all serum lipoproteins, and is responsible for the majority of cholesterol transport from the liver to the tissues for uptake via the LDL receptor. In contrast, the reverse cholesterol transport particle HDL, often referred to as “good cholesterol,” carries cholesterol from the peripheral tissues back to the liver for excretion, and is anti-atherogenic [45]. In the case of hyperlipidemia, VLDL and LDL are known to be important contributors to atherosclerosis and cardiovascular disease [46-49]. In this respect, statins, used to lower LDL cholesterol levels, have become among the most commonly prescribed drugs world-wide, and have had a significant impact on lowering mortality from cardiovascular disease [50]. Statins lower serum cholesterol and triglycerides by inhibiting HMG-CoA reductase, a rate-controlling enzyme in the mevalonate metabolic pathway which controls cholesterol production [51]. Interestingly, statins have also been associated with prevention of morbidity and mortality in sepsis [52]. However, this is likely due to their pleiotropic effects on inflammation and the immune system, including effects occurring at doses below those required to reduce cholesterol production [53, 54].

In addition to cholesterol transport, recent research also supports the role of lipoprotein particles as contributors to host innate immunity [55-66]. For example, apolipoprotein B (apoB) on VLDL and LDL, and apolipoproteins A1 and A2 on HDL, inhibit lipoteichoic acid (LTA)-mediated inflammatory cytokine release [67]. In mice, LDL has also been shown to bind lipopolysaccharide (LPS) from Gram-negative bacteria, reducing its ability to cause endotoxin induced toxicity, morbidity and mortality [68].

Table 1.1 - Apolipoprotein associations with bacterial infection.

Apolipoprotein	Associated Lipid Molecule(s)	Association to infection (Host)	Publication(s)
ApoA1	HDL	Haplotypes protective against sepsis-associated acute lung injury (Human) Anti-microbial against *Aeromonas hydrophila* and immunostimulatory to macrophages (Catfish) Protective against *Trypanosoma brucei brucei* (Human)	[69-71]
ApoA2	HDL	Suppressor of immune activation by LTA from *S. aureus* (Human/Mouse immune cells)	[67]
ApoC1	VLDL	Enhances LPS-induced inflammatory response (Mouse)	[72]
ApoC2	Chylomicrons VLDL HDL	Low levels, a predictive biomarker for septicemia and necrotizing entercolitis (Human infants)	[73]
ApoE	Chylomicrons HDL	Increased susceptibility to *Borrelia burgdorferi* and *Borrelia hispanica* ($ApoE^{-/-}$ mice) Binds to *Legionella pneumophilia* and may impede host cell penetration and intracellular growth, Enhanced *Porphyromonas gingivalis* induced neuronal injury ($ApoE^{-/-}$ mice) Determinant of innate immune response to Toll-like receptor ligands in human sepsis (Human) Protective against *Cryptosporidium parvum* infection (Mouse) Anti-microbial activity against *Pseudomonas aeruginosa* and *S. aureus*	[74-79]
ApoB-100	VLDL LDL	Inhibits *S. aureus* quorum-sensing (Mouse) Suppressor of immune activation by LTA from *S. aureus* (Human/Mouse immune cells) Metabolism of apoB plays a role in acute phase reaction against sepsis (Rat)	[67, 80-82]
ApoB-48	Chylomicrons	Inhibits *S. aureus* quorum-sensing (Mouse)	[83]

These and other findings demonstrate that lipoproteins play a role in host innate immunity against both Gram-positive and Gram-negative pathogens (**Table 1**).

Dyslipidemia and pulmonary innate defense

Despite the multifunctional role of lipoproteins, most research on the consequences of dyslipidemia has been largely focused on hyperlipidemia, with these terms often used interchangeably in the literature, and its impact on cardiovascular disease [66]. In contrast, few studies have investigated the consequences of hyper- or hypolipidemia on the host innate response to bacterial infection. Recent findings suggest that hyperlipidemia results in impaired intrapulmonary host immunity [66, 84], and has also been shown to impact lipid content of lung surfactants [85]. Alterations in lung surfactant lipid levels may impair the innate defense function of antimicrobial surfactant proteins, and have deleterious effects on host defense against bacterial lung infection [86]. Similarly, hypolipidemia has epidemiologically been associated with increased mortality [87], as well as with increased susceptibility to bacterial pneumonia [88-90]. The increased infection risk associated with extremes of dyslipidemia suggests that the role of lipoproteins in host innate immune defense may be under-acknowledged. However, there is critical gap in knowledge as to how hypolipidemia, as seen during the acute phase response during severe illness or traumatic injury [91], may play a role in host innate immune pulmonary defense.

ApoB, the shared structural protein component of chylomicrons, VLDL, and LDL (**Figure 1.3**), was previously shown to protect against *Staphylococcus aureus* skin and

skin structure infection (SSSI) through inhibition of the *S. aureus* QS system [80, 81, 83] (**Figure 1.1**). In addition, LDL has been reported to directly bind and inactivate *S. aureus* Hla [56], and to a certain extent, the *agr*-regulated PSMs [65]. Importantly, uptake of apoB-containing lipoproteins into the lung by the lung capillary endothelium [92], as well as by carriage through binding to leukocytes [93] has been previously demonstrated. Although low levels of serum lipoproteins are also associated with increased susceptibility to bacterial pneumonia [88-90], the contribution of apoB to host defense in the lung has not been thoroughly investigated.

Concluding remarks and hypothesis

As outlined above, the vast majority of research in dyslipidemia has been focused on hyperlipidemia, due to its importance in causing cardiovascular disease, but recent research supports the role of lipoprotein particles as contributors to host innate immunity against infectious disease. Given the increase in pneumonia caused by MRSA, and the association between hypolipidemia and susceptibility to bacterial pneumonia [88-90], we postulated that hypolipidemia, which can result from the acute phase response during severe illness, may impair host innate immune pulmonary defense (**Figure 1.4**).

Specifically of interest to the body of research presented herein, apoB quenches *S. aureus* QS, thereby limiting the bacteria's ability to turn on a myriad of virulence factors necessary for invasive infection. To test the hypothesis that hypolipidemia impairs innate

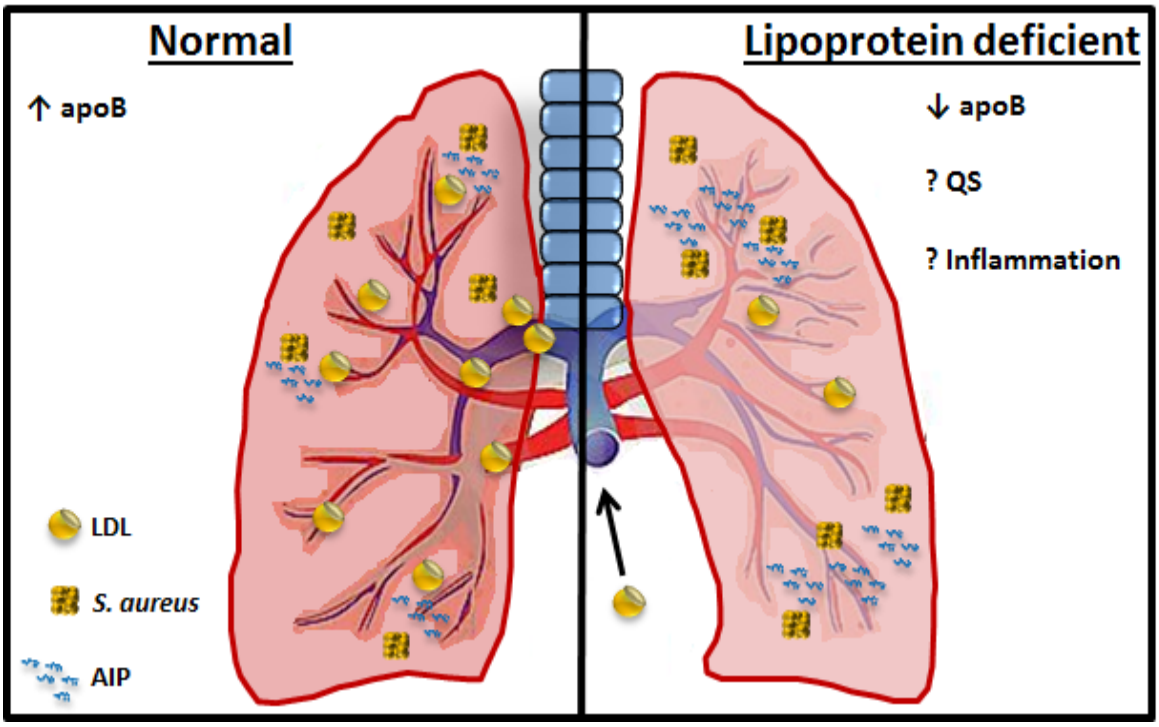

Figure 1.4 - Do serum levels of apoB-containing **lipoproteins contribute to host innate pulmonary immune defense against *S. aureus* infection?**

We hypothesized that in a hypolididemic state (right panel), as seen post trauma and during the acute phase response, lack of apoB-containing lipoproteins in the lung and airway would lead to lack of control of *S. aureus* QS during pulmonary infection, as compared to a normal lipidemic state (left panel).

pulmonary immune defense against *S. aureus agr*-mediated infection (**Figure 1.4**), we used a sub-lethal model of *S. aureus* pneumonia in pharmacologically-induced and genetic mouse models of lipoprotein deficiency. In Chapter 2, we detail optimization of our chosen *S. aureus* pneumonia infection model, the importance of consistent inoculation methodologies for pulmonary infection, procedural considerations and their effects on consistency of bacterial delivery to the lung. More specifically, we discuss different infection methods, the effects of intranasal delivery volume on initial infection, how the choice of anesthetic may effect or confound results, and choosing relevant time points for our model of infection. In Chapter 3, we show that lipoprotein deficiency impairs host control of *S. aureus* QS in the lung, resulting in *agr*-dependent increases in pro-inflammatory cytokine production and neutrophil influx in the lung. Furthermore, we show that apoB inhibits *agr*-dependent inflammatory cytokine expression by human alveolar epithelial cells, supporting a role for apoB in limiting QS-dependent virulence and inflammation during human lung infection. Finally, in Chapter 4, we discuss considerations for and present data from experimental efforts to use apoB-containing serum lipoproteins therapeutically in lipoprotein deficient mice infected with *S. aureus* pneumonia.

Together, the work reported here advances our understanding of the negative impact of dyslipidemia on pulmonary host innate defense, and may have broad implications for hypolipidemia in increased susceptibility to post-trauma pneumonia caused by both Gram positive and Gram negative pathogens.

Chapter 2 – *Staphylococcus aureus* pneumonia mouse model dosing strategy

Introduction

Mouse models of infection are an important tool for studying infectious agents and their sequelae. Rapidly reproducing, inbred mouse strains allow for ease of study in genetically identical backgrounds, facilitating investigation of host-pathogen interactions via genetics-based approaches. However, using mouse models also comes with many caveats, including choosing a proper model to elucidate answers to questions about human disease, or about relevant host-pathogen interactions. Discussed here will be different accepted practices for using mice as a model organism for studying bacterial pneumonia and the advantages and pitfalls of different infection methods. We will also describe the approach we employed to optimize the consistency of *S. aureus* intranasal infection looking specifically at the effects of anesthetic on bacterial delivery and *S. aureus* infection outcomes, the importance of choosing observational time points for a mouse model of *S. aureus* pneumonia relevant to the effects of lipoprotein contributions to defense against *S. aureus* in the lung, and intranasal dose delivery volumes.

Inoculum delivery methods for bacterial pneumonia

Aerosol exposure to pathogens mirrors airborne exposure scenarios of infection [94]. Animals are placed in an enclosed chamber, and aerosolized pathogen is pulled into

the chamber. This method has the advantage of requiring no anesthesia, and provides consistent pathogen deposition into the lung across experimental animal groups [95-97]. Concentrations for appropriate deposition of pathogen into the lungs can be determined by adjusting exposure time and aerosol flow into the chamber. However, there are many caveats for aerosol exposure methods. They generally require costly exposure systems, and in the case of more dangerous pathogens, can create costly containment considerations. Additionally, fairly large inoculation volumes and concentrations are required relative to actual pathogen delivery into the lung, creating a limitation on inoculation level in the lung. Whole-body exposure systems come with the added concern that off-target inoculation sites will confound experimental results [95], and while nose-only aerosol exposure systems exist, they are more expensive and require additional costly equipment and time for animal restraint.

Intratracheal infection mouse models of pneumonia mirror lower respiratory tract infections. Two typical methods for this inoculation method include transtracheal injection and peroral intubation [94]. Transtracheal injection requires anesthesia followed by surgical exposure of the trachea, by which a small gauge needle is inserted and the infection inoculum is delivered directly into the trachea before suturing of the surgical wound. Peroral intubation avoids potential complications of surgery by delivering inoculum via blunted needle injection after oral insertion of the needle directly above the tracheal bifurcation. Whereas both these methods allow for larger bolus dose delivery to the lung than aerosol administration (discussed above) or intransal infection (discussed below) [95, 98], the need for prolonged general anesthesia combined with longer delays between inoculation of individual animals can lead to confounding non-inoculum

dependent effects. More specifically challenges include anesthesia/aspiration induced death, and difficulties maintaining consistency of data collection at specific post-infection time points. The latter is particularly important for short, acute infection models such as utilized in Chapter 3.

Finally, intranasal infection is another commonly used practice for infecting mice with a variety of pathogens [13, 99, 100], and may be more relevant in modeling organisms which infect both the upper and lower respiratory tract. The first intranasal *S. aureus* pneumonia model in immunocompetent mice was described by Bubeck-Wardenburg et al. [13]. Since this method was published it has been become standard practice amongst the *S. aureus* pneumonia research community [101-103]. In this approach, mice are anesthetized and the inoculum suspension is introduced into the nares through aspiration. While this method allows for simple and quick introduction of pathogens into the mouse airway, it is the most highly variable method for pathogen delivery to the lung [94], with little control over where pathogens are deposited in the airway or final inoculum delivered to desired site of infection.

Results and Discussion

Choice of anesthetic impacts intranasal administration of *S. aureus*

Since intranasal infection requires aspiration by the animal, anesthetic effects on respiration rates may cause variable levels of inoculum delivery to the lung [104, 105]. Physical effects of particular anesthetics can have profound impact on variation in inoculum delivery and infection results [94]. In a recent study looking at dosing

efficiency of fluorescent *F. tularensis* via whole body imaging, mice anesthetized with ketamine/xylazine maintained a steadier breathing pattern than those anesthetized with short periods of inhaled isoflurane [106]. Despite this, more efficient delivery of fluorescent *F. tularensis* to the lungs was seen in mice anesthetized with isoflurane [106]. Additionally, our own observations described below indicate that anesthetic choice can have profound impacts on weight loss (often used as a measure of morbidity) and infection outcomes. There are a very limited number of studies observing effects of different anesthetics on infection outcomes. Interestingly, despite all these variables, there is a low overall reporting in the literature of actual *S. aureus* inoculum delivery to the lung using intranasal infection [106], and though the anesthetic used for infection is typically reported, the potential impacts of anesthetic choice are not discussed.

In our own studies comparing intranasal inoculation of *S. aureus* following parenteral metamine/xylazine or inhaled isoflurane, we found that ketamine/xylazine led to significant drops in body weight compared to isoflurane (**Figure 2.1A**). Additionally, ketamine/xylazine required petroleum jelly to keep the animals' eyes from drying out during recovery from anesthesia. Together, this appeared to increase overall morbidity scoring (consisting of observational measurements of grooming, natural, and provoked behavior) related to grooming, due to petroleum jelly on and around the eyes, and weight loss, leading to more moribund mice overall as compared to infection using isoflurane (**Figure 2.1B**). We would hypothesize that the longer recovery time from ketamine/xylazine, compared to isoflurane, leads to significant anesthesia induced weight

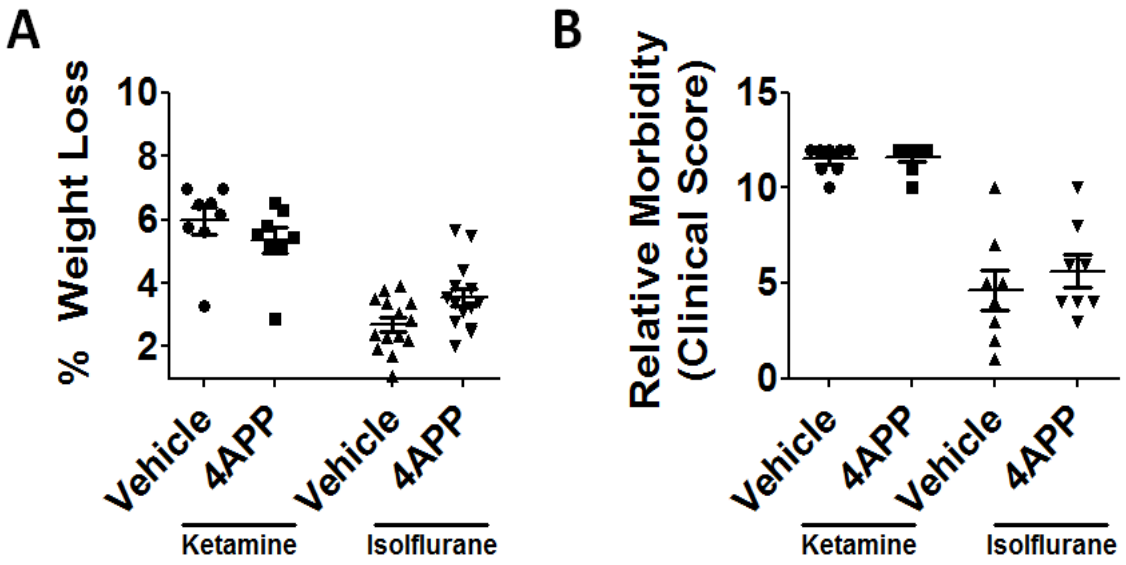

Figure 2.1 - Comparison of weight loss and clinical scores using parenteral ketamine/xylazine or inhaled isoflurane in mice infected intranasally with *S. aureus*. (**A**) Comparison of weight loss at six hours post intranasal infection with *S. aureus*, of vehicle-treated vs. 4-aminopyrizolo[3,4-d]pyrimidine (4APP)-treated mice, anesthetized for infection using ketamine/xylazine or isoflurane. (**B**) Comparison of relative morbidity based on clinical scores at six hours post intranasal infection with *S. aureus*, of vehicle-treated vs. 4APP-treated mice, anesthetized for infection using ketamine/xylazine or isoflurane.

loss during recovery and infection. Additionally, observational morbidity measurements (specifically grooming) seemed to be increased based on lack of grooming in mice that had petroleum jelly still on and around the eyes (data not shown).

Intranasal dose delivery volumes

Intranasal dose delivery volumes have been shown to have dramatic impacts on mouse survival during infection. Profound effects related to the deposition site of the delivered pathogen have also been observed, with lower volumes tending to preferentially deposit in the upper respiratory tract [104-106]. However, there is a surprising lack of literature specifically describing the efficiency of delivery into the lung employing different intranasal dosing volumes for infectious agents. In a study by Miller et al. [84], they found that delivery volumes between 50 µL to 100 µL were more efficient at delivery of fluorescent *Francisella tularensis* into the lung, compared to volumes of 10 µL which resulted in more deposition into the upper respiratory tract. In the case of intranasal instillation of *S. aureus*, delivery volumes between 20 µL to 50 µL are commonly reported [16, 22], however delivery efficiency to the lung at these volumes is seldom provided [13]. Specifically, the range of commonly reported intranasal bacterial dosing volumes (2 µL to 100µL), may have dramatically different effects on aspiration of and general inoculum loss through expulsion by normal breathing.

We observed that delivery volumes above 15 µL required multiple aspiration steps to limit loss through expulsion by normal breathing, and that splitting a 30 µL volume delivered in two 15 µL boluses (one to each nare) had less apparent loss of inhaled volume through expulsion from the airway (data not shown). However, given

previous findings that volumes above 50 µL showed more efficient bacterial delivery to the lung during intranasal infection [106], we implemented a "wash down" of the initial bacterial delivery bolus with two subsequent aspirations of normal saline (15 µL in each nare), for a total delivery volume of 60 µL. Using this method of intranasal infection, we found consistent delivery of bacteria to the lung of ~10-20% of initial inoculum (data not shown).

Choosing observational time points in a mouse model of *S. aureus* pneumonia

Though mice are often used as models of infection, due to the ease of use and wide availability of many potentially clinically relevant genetic knockout mouse models, one must be careful in selecting an appropriate model for testing a specific hypothesis. For example, mouse models of *S. aureus* infection are often criticized because of their lack of clinical relevance, since *S. aureus* is not a regular occurring commensal of mice, and since mice show particular resilience against many of the relevant human virulence factors [107]. Indeed, the titers needed to achieve significant disease in a mouse model of *S. aureus* pneumonia are quite high (2-4 x 10^8 CFU, [107]). For our specific purposes, established protocols for intranasal infection with *S. aureus*, along with two established models of lowered serum lipoproteins in mice (discussed in detail in Chapter 3), have allowed us the ability to model infection during the acute phase response of humans, as related to reductions in serum lipoprotein levels. We found that an increase in a half-log of *S, aureus* inoculum titer can make the difference between 24 hour mortality (data not shown), or an almost three log reduction in CFUs in the lung by 24 hours (**Figure 2.2A**).

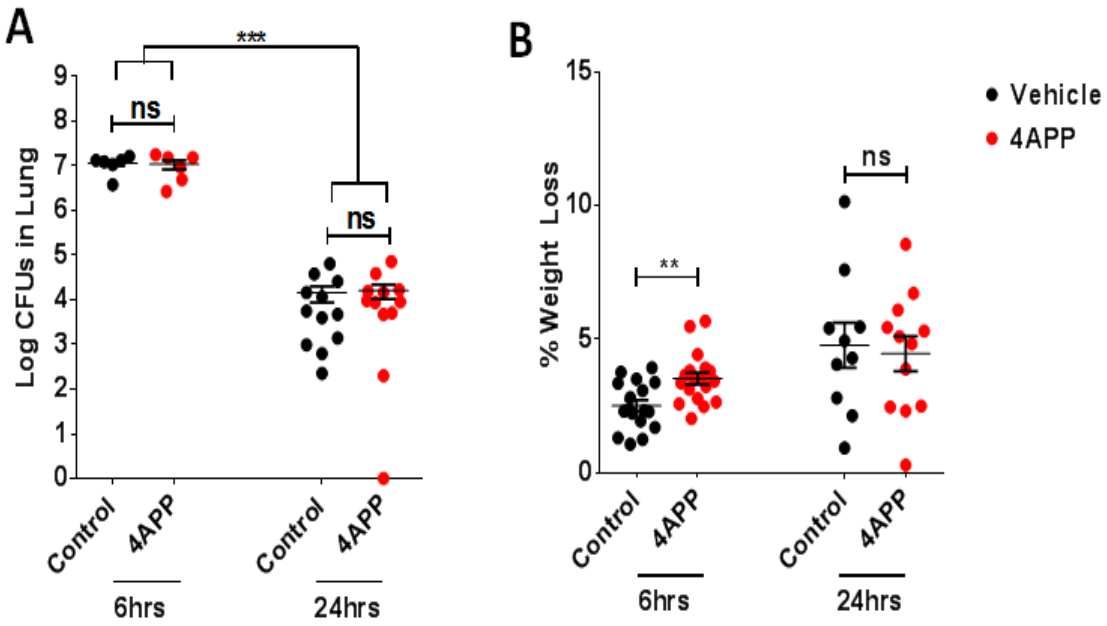

Figure 2.2 - Clearance of *S. aureus* from the lung, and weight loss differences at different time-points of pneumonia.

(**A**) Differences in CFUs present in the lung at 6 and 24 hours post-infection, of lipoprotein deficient and control mice, infected intranasally with LAC. (**B**) Differences in percentage weight loss at 6 and 24 hours, of lipoprotein deficient and control mice, infected intranasally with LAC.

Previous research has also shown that serum lipoprotein levels increase in the lungs of mice during *S. aureus* pulmonary infection at 6 hours post-infection [108], as well as showing increases in inflammatory cytokines (such as IL-1β) in the lung at this early time point of infection. Given this, and our hypothesis that serum lipoproteins play a role in innate immunity in the lung during *S. aureus* infection, a 6 hour model of infection was chosen as the most relevant time point to assess early control of *S. aureus* QS in the lung. Importantly, we observed that at 6 hours post infection, infected mice still had a substantial portion of the initial infection inoculum present in the lung (**Figure 2.2A**), and lipoprotein deficient mice showed increased weight-loss compared to controls (**Figure 2.2B**). Based on this and previous observations about serum lipoproteins in the lung and increases in inflammation at this infection time point, we predicted that the contribution of lipoproteins to pulmonary innate immunity may be best examined at 6 hours post-infection.

Conclusions

Here we emphasize the importance of publishing detailed protocols for pulmonary infection with bacteria. The described comparative studies resulted in the following protocol:

1) Isolate 4 mice into an isoflurane chamber and anesthetize using 3.5 L/min of isoflurane with 2.5 L/min O_2 flow.

2) Remove the first mouse after 5 min in isoflurane chamber, and holding the mouse at a ~45˚ angle with the anterior positioned towards the floor, pipette 15 μl of saline containing ~2-4 x 10^8 CFU per 30 μl, onto the left nare.
3) After the mouse has inhaled the first bolus, repeat this for the right nare.
4) Repeat steps 2 and 4 using sterile saline.
5) After the mouse has aspirated the final saline dose, hold the mouse at a ~45˚ angle with the anterior positioned towards the floor, for an additional 1 min.
6) Place mouse prostrate on the floor of housing cage.
7) Repeat this same process for the remaining mice in the isoflurane chamber.

This protocol resulted in a very consistent bacterial delivery to lung (~10-20%) of initial bacterial suspension titer across all groups and experiments.

Chapter 3 – Serum Lipoproteins are Critical for Pulmonary Innate Defense against *Staphylococcus aureus* Quorum-sensing

Serum Lipoproteins are Critical for Pulmonary Innate Defense against *Staphylococcus aureus* Quorum-sensing

Brett C. Manifold-Wheeler[*], Bradley O. Elmore[*], Kathleen D. Triplett[*], Moriah J. Castleman[*], Michael Otto† and Pamela R. Hall[*]

[*]Department of Pharmaceutical Sciences, University of New Mexico College of Pharmacy, Albuquerque, New Mexico, USA, 87131; †Pathogen Molecular Genetics Section, Laboratory of Bacteriology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA 20892

Short Title: Hypolipidemia impairs pulmonary innate defense

Corresponding author: Pamela R. Hall, Phone: 505-272-0915, Fax: 505-925-4549, Email: phall@salud.unm.edu

Abstract

Hyperlipidemia has been extensively studied in the context of atherosclerosis, whereas the potential health consequences of the opposite extreme, hypolipidemia, remain largely uninvestigated. Circulating lipoproteins are essential carriers of insoluble lipid molecules and are increasingly recognized as innate immune effectors. Importantly, severe hypolipidemia, which may occur with trauma or critical illness, is clinically associated with bacterial pneumonia. To test the hypothesis that circulating lipoproteins are essential for optimal host innate defense in the lung, we used lipoprotein deficient mice and a mouse model of *Staphylococcus aureus* pneumonia in which invasive infection requires virulence factor expression controlled by the accessory gene regulator (*agr*) operon. Activation of *agr* and subsequent virulence factor expression is inhibited by apolipoprotein B, the structural protein of low density lipoprotein, which binds and sequesters the secreted *agr*-signaling peptide (AIP). Here we report that lipoprotein deficiency impairs early pulmonary innate defense against *S. aureus* quorum-sensing dependent pathogenesis. Specifically, apoB levels in the lung early post-infection are significantly reduced with lipoprotein deficiency, coinciding with impaired host control of *S. aureus agr*-signaling and increased *agr*-dependent morbidity (weight loss) and inflammation. Given that lipoproteins also inhibit LTA- and LPS-mediated inflammation, these results suggest that hypolipidemia may broadly impact post-trauma pneumonia susceptibility to both Gram positive and Gram negative pathogens. Together with previous reports demonstrating that hyperlipidemia also impairs lung innate defense,

these results suggest that maintenance of normal serum lipoprotein levels is necessary for optimal host innate defense in the lung.

Introduction

Hyperlipidemia is a clinical syndrome with high circulating levels of cholesterol, triglycerides and the lipoprotein particles which carry them, including very low and low density lipoproteins (VLDL, LDL) [87]. Hyperlipidemia is a risk factor for cardiovascular disease [109, 110], yet serum lipoproteins also contribute to host innate defense against infection [57, 111]. Interestingly, hyperlipidemia results in impaired intrapulmonary host immunity [84], suggesting that maintenance of normal circulating cholesterol and lipoprotein levels is crucial for optimal host innate defense in the lung. Although severe hypolipidemia, which often accompanies the acute phase response (APR) following surgery or trauma (reviewed in [112-114]), has been associated with bacterial pneumonia [88-90], a critical gap in knowledge remains regarding the impact of extremely low serum lipoprotein levels on host innate defense in the lung.

Staphylococcus aureus, and methicillin-resistant *S. aureus* (MRSA) in particular, accounts for 20% to 40% of hospital-acquired pneumonia (HAP) cases in the USA, as well as a growing number of cases of community-acquired pneumonia (CAP) [115-117]. Invasive pulmonary infection caused by *S. aureus* requires the expression of virulence factors controlled by the accessory gene regulator (*agr*) operon [13, 118-120], which encodes a two-component quorum-sensing (QS) system for bacterial communication and coordinated gene expression (reviewed in [8, 121]). QS is facilitated by secretion of a cyclic autoinducing peptide (AIP), which binds to and activates its cognate surface receptor AgrC. This in turn leads to expression of over 200 virulence factors [122], many of which are pre- and post-transcriptionally regulated by a small RNA molecule, called

RNAIII, produced by transcription from the *agr* P3 promoter [8, 121]. Importantly, apolipoprotein B (apoB), the sole protein component of LDL lipoprotein particles, and not other serum apoproteins or associated lipids, binds and sequesters AIP, thereby inhibiting *agr*-signaling and limiting pathogenesis during *S. aureus* skin infection [80, 83, 123]. However, the impact of apoB deficiency and hypolipidemia on host innate defense in the lung, and against *S. aureus* pneumonia in particular, has not been investigated. We hypothesized that serum lipoproteins would contribute to pulmonary host defense against *S. aureus* QS and *agr*-mediated inflammation.

Here we used a sub-lethal model of *S. aureus* pneumonia to demonstrate that severe hypolipidemia impairs the early host innate defense response to lung infection. Specifically, lipoprotein deficiency impairs host control of *S. aureus* QS in the lung, resulting in *agr*-dependent increases in pulmonary pro-inflammatory cytokine production and neutrophil influx. Furthermore, apoB inhibits *agr*-dependent inflammatory cytokine expression by human alveolar epithelial cells, supporting a role for apoB in limiting QS-dependent virulence and inflammation during human lung infection. Given that serum lipoproteins also limit inflammation via sequestration of lipotechoic acid (LTA) and lipopolysaccharide (LPS) [124-126], these studies may have broad implications for hypolipidemia in increased susceptibility to post-trauma pneumonia caused by both Gram positive and Gram negative pathogens.

Materials and Methods

Bacterial strains and growth conditions

USA300 LAC and its isogenic *agr* deletion mutant (USA300 LACΔ*agr*) were provided by Dr. Frank DeLeo (Rocky Mountain Laboratories, NIAID/NIH, Hamilton, MT) and Dr. Michael Otto (NIAID/NIH, Bethesda, MD), LACΔ*hla* was provided by Dr. Juliane Bubeck-Wardenburg (University of Chicago, Department of Microbiology, Chicago, IL) and AH1677 *agr*::P3-*yfp* (USA300 LAC*yfp*) was provided by Dr. Alex Horswill (University of Iowa, Department of Microbiology, Carver College of Medicine). Bacteria were grown in trypticase soy broth (TSB) and early exponential phase frozen stocks were prepared as previously described [127]. CFU of frozen stocks were determined by plating of serial dilutions on blood agar (BD Biosciences, Franklin Lakes, NJ).

Mouse model of S. aureus pneumonia

Animal work was carried out at the AAALAC accredited Animal Research Facility of the University of New Mexico Health Sciences Center in accordance with recommendations in the Eighth Edition of *The Guide for the Care and Use of Laboratory Animals* and the USA Animal Welfare Act. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of New Mexico. Eight to twelve week old male mice (C57BL/6, *Pcsk9*$^{-/-}$ on the B6 x 129 background, and B6 x 129 wild-type) were purchased from Jackson Laboratories (Bar Harbor, ME). Mice receiving 4-aminopyrazole-(3,4-D)pyrimidine (4APP) treatment were injected i.p. with 100 µl of 5.15 mg/ml 4APP (Sigma-Aldrich, St. Louis, MO) prepared as previously described [80,

123], or buffer control at 48 and 24 hours before infection, as well as at the time of infection. Reductions in serum cholesterol were determined using Infinity Cholesterol Liquid Stable Reagent (Thermo Scientific, Middletown, VA) according to manufacturer's directions.

The mouse model of *S. aureus* pneumonia was performed as previously described [13]. Briefly, mice were anesthetized by isoflurane inhalation and 30 μl of sterile saline containing ~4 x 10^8 CFU of *S. aureus* was administered intranasally, followed by an additional 30 μl of saline alone. Mice were weighed at the time of infection and again prior to sacrifice. At 6 hours post-infection, mice were sacrificed by CO_2 asphyxiation and blood and tissues collected. For CFU determinations, right lungs were collected in bead-beating tubes containing 2.3-mm Zirconia/Silica beads (BioSpec Products, Bartlesville, OK) in 1 ml $HBSS^-$ (Life Technologies, Grand Island, NY) with 0.2% human serum albumin (Sigma-Aldrich, St. Louis, MO), and lung tissue disrupted for 1 min using a Mini-Bead Beater-24 (Biospec). Homogenates were diluted 1:10 in PBS with 0.1% Triton X-100, sonicated, and serial dilutions plated on blood agar (BD Biosciences, Franklin Lakes, NJ). For *agr*::P3-YFP promoter activation assays, bacteria from diluted homogenates were pelleted by centrifugation and fixed for 10 min with 1% paraformaldehyde containing 25mM $CaCl_2$. Promoter activation was determined by measuring mean channel fluorescence (MCF) by flow cytometry (Accuri C6, BD Accuri Cytometers, Ann Arbor, MI). For cytokine and Western blot analyses, lung homogenates were clarified by centrifugation at 12,500 x *g* and supernatant was stored at -80 °C until use as described below. For transcription analyses, left lungs were harvested,

immediately placed into 1.8 mL of RNAlater (Qiagen, Valencia, CA), and frozen at -80°C.

A549 cell exposure to bacterial supernatant

For A549 assays, bacteria were cultured in TSB (5 mL with 10:1 air to volume ratio) for 6 hours at 37 °C with shaking and with or without 100 nM human LDL (hLDL). Bacteria were then pelleted by centrifugation and supernatants filter sterilized by passage through 0.2 µm filters, aliquoted, and frozen at -80 °C until use. A549 human alveolar epithelial cells were grown to 80-90% confluence in 12-well plates in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated Fetal Bovine Serum (HI-FBS) and penicillin/streptomycin. Twenty-four hours prior to use, cell culture medium was changed to DMEM with 10% human lipoprotein deficient serum with penicillin/streptomycin. Sterile bacterial supernatants were thawed, diluted to working concentrations in TSB, and added to A549 cells at a 1:10 dilution in cell culture media. Post-culture hLDL was added to respective supernatants at 100 nM concentration prior to addition to A549 cells. Cells were incubated at 37 °C for 4 hours. At 4 hours post-exposure, cell culture supernatants were collected and frozen at -80 °C for future analysis. To collect RNA, cells were immediately lysed with Qiagen RLT buffer with 1% β-mercaptoethanol and QIAshredder spin columns (Qiagen, Valencia, CA). RNA was processed using an RNeasy Mini kit, according to the manufacturer's Animal Cell Spin protocol (Qiagen, Valencia, CA).

Quantification of cytokines and myeloperoxidase ELISA

Cytokines were measured in cell culture supernatant or clarified supernatant from mouse lung homogenates using custom designed Milliplex Cytokine Magnetic kits according to the manufacturer's specifications (Millipore, Billerica, MA). Cell culture supernatants were pooled from identically treated triplicates. Myeloperoxidase was also measured in clarified supernatants using the Mouse Myeloperoxidase DuoSet ELISA (R&D Systems, Minneapolis, MN) according to manufacturer's directions.

Western blot analyses

Clarified lung homogenate supernatants were quick thawed (37°C) and protein concentrations determined by A_{280} absorption (Nanodrop 100 Spectrophotometer, Thermo Fisher Scientific, Wilmington, DE). For apoB Western blots, equivalent amounts of total protein were separated by SDS-PAGE on 3-8% Tris-Acetate gels (Novex Life Technologies, Grand Island, NY) prior to transfer to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% non-fat milk in TBS (20mM Tris pH 7.5, 150 mM NaCl) containing 0.1% Tween-20 (TBST), then probed with rabbit anti-apoB antibody (Abcam, Cambridge, MA). Unbound antibody was removed by triplicate 5 min washes with TBST, and membranes developed using goat anti-rabbit IgG-alkaline phosphatase-conjugated secondary antibody (KPL, Gaithersburg, MD) and 1-Step nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Thermo Scientific).

For Western blot analysis of secreted Hla in bacterial supernatants applied to A549 cells (above), equivalent amounts of total protein were separated by SDS-PAGE on 4-12%

Bolt gels (Novex Life Technologies, Grand Island, NY) prior to transfer to polyvinylidene fluoride (PVDF) membranes and overnight blocking. Membranes were probed with mouse anti-Hla mAb (Integrated BioTherapeutics, Gaithersburg, MD), followed by goat anti-rabbit IgG poly-horseradish peroxidase (Thermo Fisher Scientific). Blots were developed using Thermo Pierce SuperSignal West Pico Chemiluminescent substrate (Thermo Fisher Scientific). All blots were imaged using a ProteinSimple FluorChem R instrument and quantified with AlphaView software (Protein Simple, Santa Clara, CA).

Rabbit erythrocyte lysis assay

Sterile bacterial culture supernatants prepared as described above and used for A549 cytokine induction assays were tested for hemolytic activity as previously described [128]. Briefly, a 4% solution of rabbit RBCs in PBS was incubated at 37 °C for 1 h with 2-fold serial dilutions of sterile-filtered bacterial supernatant. Lysis was determined by the A_{450} of the resulting RBC supernatant. Data were analyzed by nonlinear regression and are shown as the HA_{50} which equals 1/ the log of the dilution needed for 50% complete lysis.

Quantitative RT-PCR

For bacterial RNA isolation, bronchial alveolar lavage fluid (BALF) was collected as described below but in a 10-fold volume of RNAlater (Qiagen) and frozen at -80 °C for later processing. After thawing, bacteria were pelleted by centrifugation, RNAlater was aspirated, and pellets were incubated with 100 µL Tris-EDTA (TE) buffer, with 20

µL/mL proteinase K (Qiagen) and 20 µg/mL of lysostaphin for 10 min. Thawed lungs were removed from RNAlater and RNA extracted using Qiazol according to manufacturer's directions (Qiagen). RNA isolation from cell culture was performed as described above. An RNAeasy Mini kit (Qiagen) was used for RNA purification and cDNA was generated using a high-capacity cDNA RT kit with RNAse inhibitor together with random hexamer primers (Applied Biosystems, Foster City, CA). Taqman Gene Expression master mix (Applied Biosystems) and an ABI7000 Real-Timer PCR system were used for quantitative PCR. Prime Time Predesigned qPCR primers and probes (Integrated DNA Technologies, Coralville, IA) were used for transcriptional analyses of mouse *hprt*, *il-1b*, *mip-2* and *il-6,* and for human *hrpt* and *il-8*. Primers and probes for quantification of *S. aureus 16S, RNAIII* and *lukS* were previously described [129]. Gene expression was quantified using SDS RQ Manager Version 1.2.2 software (Applied Biosystems) relative to mouse or human *hprt*, and *S. aureus 16S*, as appropriate.

Cell influx analysis of bronchial alveolar lavage fluid

BALF was collected from sacrificed mice by intratracheal lavage, using three sequential washes (700 µl each) with ice cold Dulbecco's Phosphate-Buffered Saline (2.67 mM KCl, 1.47 mM KH_2PO_4, 138 mM NaCl, 8.1 mM Na_2HPO_4) (Corning, Corning, NY). Total cell count and live/dead counts were determined by trypan blue staining using a TC 20 Automated Cell Counter System (Biorad, Hercules, CA). For immunostaining, cells were centrifuged at 800 x *g* for 3 min then pelleted cells resuspended in cold PBS with 0.5% BSA and 0.075% sodium azide. Cells were blocked for 30 min with 2% BSA, followed by a 1-h, 4°C incubation with anti-mouse Ly6G (neutrophils) (BioXCell, West

Lebanon, NH) conjugated to AlexFluor 488 (Protein labeling kit, Molecular Probes Inc., Eugene, OR), anti-mouse CD11c-PE (alveolar macrophages) (Biolegend, San Diego, CA), or isotype controls. Cells were washed with PBS prior to analysis by flow cytometry (Accuri C6, BD Accuri Cytometers).

Statistical analyses

All data were analyzed using Prism 5.0 software for Windows (GraphPad Software Inc., La Jolla, CA). Parametric data were analyzed using the two-tailed Student's t-test or ANOVA with either Dunnett's post-hoc test or Bonferroni's multiple comparison test, and are displayed as the mean ± SEM. Non-parametric data were analyzed by the Mann-Whitney U test and displayed as the median plus 5^{th}-95^{th} percentiles.

Results

Lipoprotein deficiency impairs host control of *S. aureus* QS in the lung

Since lipoproteins are detectable in lungs shortly after intranasal infection with *S. aureus* [108] and apoB on LDL binds *S. aureus* AIP and inhibits *agr*-activation [80, 83, 123], we predicted that decreased circulating lipoprotein levels would result in impaired control of *S. aureus* QS in the lung. To address this, we compared *S. aureus agr*-activation in the lungs of mice with reduced apoB levels versus controls. Given that *APOB* deletion is embryonic lethal [130], we used pharmacological treatment with 4-aminopyrazole-(3,4-D)pyrimidine (4APP), which inhibits lipoprotein secretion from the liver [131], and *Pcsk9*$^{-/-}$ mice, which have reduced circulating LDL levels due to overexpression of the LDL receptor (LDLR) [132], as models of apoB deficiency. Both models have an approximately 50% reduction in apoB measured in the serum (**Figure 3.1A,B**), consistent with those of trauma patients at risk of infection [114]. Using a sub-lethal challenge model [13], we intranasally infected mice with a reporter strain of the *agr*+ clinical isolate LAC, which expresses YFP under the control of the *agr*::P3 promoter [133]. We measured *agr*-activation at six hours post-infection, at which time *agr* activation is detectable in the lung, as are differences in lung pathogenesis and bacterial burden between mice infected with *agr*$^{+}$ versus *Δagr* isolates [118, 134]. As expected, *agr*::P3 promoter activation was significantly increased in the lungs of both 4APP-treated and *Pcsk9*$^{-/-}$ mice compared to controls (**Figure 3.2A,D**). In addition, bacteria in BALF collected from LAC infected 4APP-treated mice showed increased

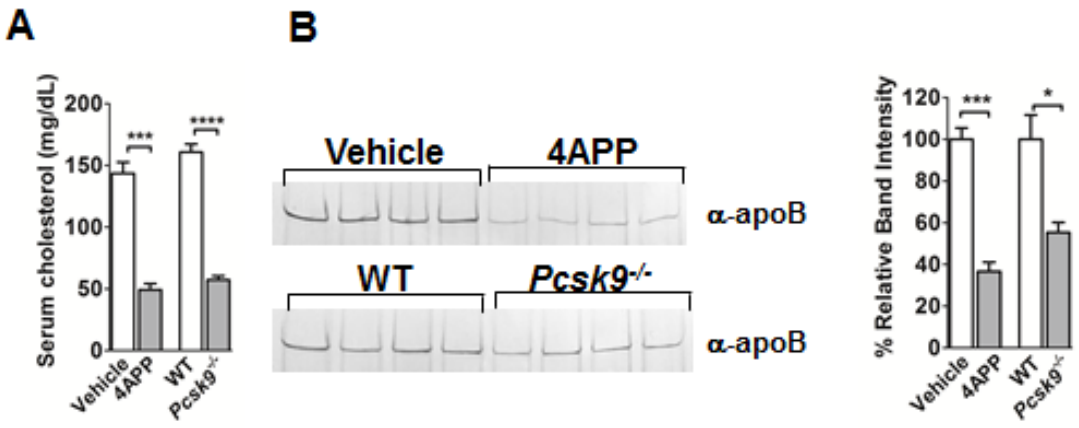

Figure 3.1 - 4APP-treated and *Pcsk9*$^{-/-}$ mice show similar reductions in serum cholesterol and apoB.

(**A**) Quantification of mouse serum cholesterol. (**B**) Western blot for serum apoB (left) and apoB quantification by relative band intensity versus control (right). Data shown as mean ± SEM. N= four mice per group. *, p≤0.05; ***, p≤0.001; ****, p≤0.0001, Student's t-test.

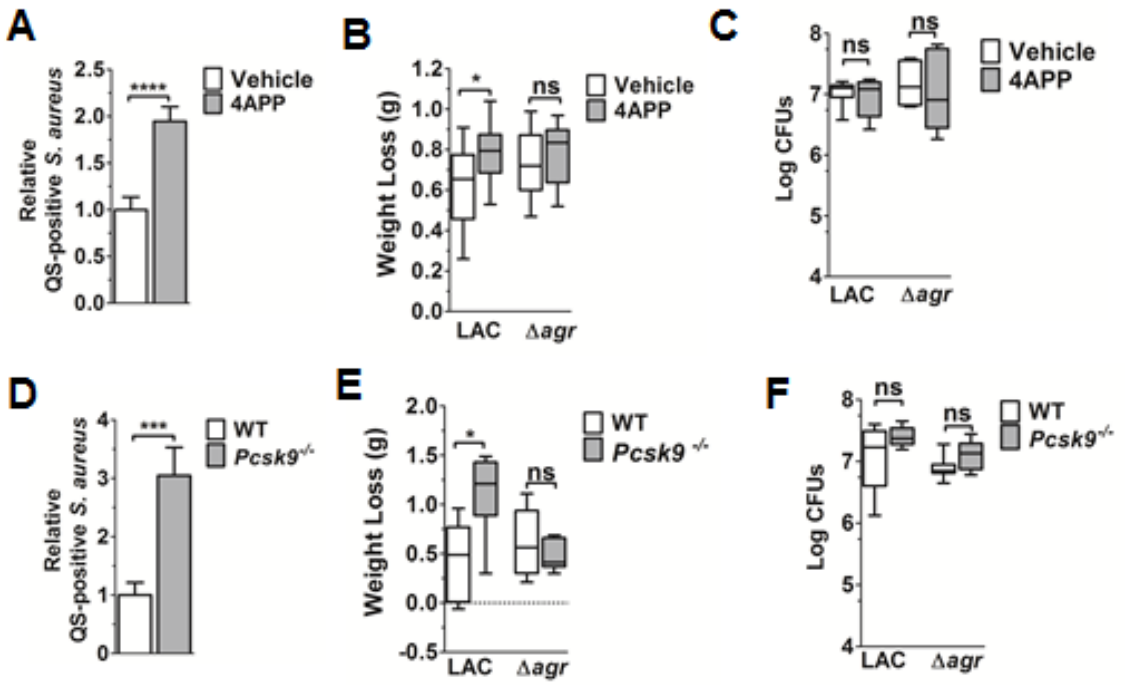

Figure 3.2 - Reduction of circulating lipoproteins increases pulmonary *agr*-signaling and weight loss.

Vehicle- or 4APP-treated (**A-C**, white and gray bars, respectively) and B6 x 129 (WT) or *Pcsk9*$^{-/-}$ mice (**D-F**, white and gray bars, respectively) were intranasally infected with *agr*::P3-YFP LAC or LAC*Δagr* (4 x 10^8 CFU) and analyses performed at 6 hours post-infection. (**A, D**) Flow cytometric quantification of *S. aureus agr*::P3 promoter activation in the lungs of lipoprotein deficient mice relative to controls (N=6 mice per group). Data shown as mean ± SEM. ***, p≤0.001; ****, p≤0.0001, Student's t-test. (**B, E**) Weight loss (g) of infected lipoprotein deficient mice versus controls (N ≥ 8 mice per group from at least two independent experiments). (**C, F**) Bacterial burden (Log CFU) in the lung (N=8 mice per group from two independent experiments). (**B-C, E-F**) Data shown are median plus 5th-95th percentiles. ns, not significant; *, p≤0.05, **, p≤0.01, Mann-Whitney test.

transcription of *agr*-regulated RNAIII and *lukS-PV*, the latter encoding the Panton-Valentine leukocidin (PVL) (**Supplemental Figure 3.1**). Furthermore, 4APP-treated and *Pcsk9*$^{-/-}$ mice infected with LAC, but not with an isogenic *agr* mutant (LAC∆*agr*), showed increased weight loss, used as a measure of morbidity, compared to vehicle-treated and WT controls, respectively (**Figure 3.2B,E**), suggesting increased virulence in the lipoprotein deficient mice due to an inability to control *agr*-signaling. Importantly, differences in QS and weight loss at this time point were not the result of increased bacterial burden, as the number of CFUs in the lungs of lipoprotein deficient mice did not differ from that of controls (**Figure 3.2C,F**). Therefore, these results suggest that serum lipoproteins contribute to host control of *S. aureus* QS-dependent virulence in the lungs and that this contribution is independent of host control of bacterial burden.

Serum lipoprotein deficiency impacts post-infection lung apoB concentrations

Increased *agr*-signaling in the lungs of *S. aureus* infected lipoprotein deficient mice (**Figure 3.2A,D**) suggested that these mice would have decreased apoB present in the lungs compared to lipoprotein sufficient controls. As suggested, LAC-infected *Pcsk9*$^{-/-}$ mice showed significantly decreased apoB in the lungs at six hours post-infection compared to wild-type controls (**Figure 3.3A,B**). Similarly, compared to vehicle-treated mice, BALF from mice treated with 4APP and infected with LAC also showed significant reductions in apoB at the six hour time point (**Figure 3.3C,D**). In contrast, differences in apoB levels in BALF from mice infected with LAC∆*agr* did not reach

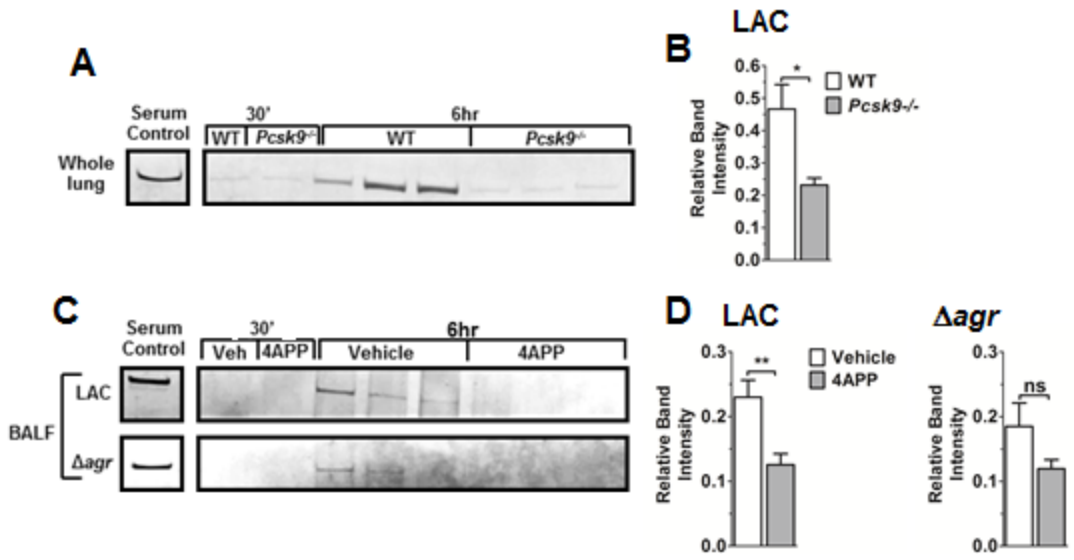

Figure 3.3 - Reduction of serum lipoproteins decreases post-infection apoB levels in the lung.

(**A**) Western blot analysis of apoB and (**B**) relative quantification of apoB versus serum control in whole lung homogenates of B6 x 129 (WT) or *Pcsk9*$^{-/-}$ mice 6 hours following intranasal infection with LAC (N=6 mice per group). (**C**) Western blot analysis and (**D**) relative quantification of apoB in BALF from vehicle- or 4APP-treated mice 6 hours following intranasal infection with LAC or LAC*Δagr* (N=6 and 3 mice per group, respectively). Data shown are mean ± SEM. ns, not significant; *, $p \leq 0.05$, **, $p \leq 0.01$, Student's t-test.

statistical significance. Together, these data suggest that the presence of apoB in the lung during *S. aureus* infection, and thus its ability to control *agr*-signaling in the lung, is dependent upon circulating lipoprotein concentrations.

Lipoproteins regulate both *agr*-dependent and *agr*-independent induction of pro-inflammatory cytokines in the lung

S. aureus stimulates the release of a variety of cytokines and chemokines in the lungs of infected mice, including cytokines produced in response to *agr*-regulated virulence factors [13, 18, 101, 108, 119, 135, 136]. In particular, expression of the *agr*-regulated virulence factor alpha-hemolysin (Hla), a pore-forming toxin, induces pulmonary release of the pro-inflammatory cytokine IL-1β [18, 101], suggesting there would be an *agr*- and Hla-dependent increase in IL-1β in the lungs of lipoprotein deficient mice compared to controls. As predicted, 4APP-treated mice infected with LAC, but not with LACΔ*agr* or LACΔ*hla*, had significantly increased pulmonary IL-1β levels at six hours post-infection compared to vehicle-treated mice (**Figure 3.4A**), consistent with increased morbidity (weight loss) relative to vehicle controls only with LAC infection (**Figure 3.2B, 3.4B**). Also, as seen for LAC and LACΔ*agr* infected mice, bacterial burden did not differ between 4APP- and vehicle-treated mice infected with LACΔ*hla* (**Figure 3.4C**). Interestingly, IL-1β expression was reduced in lipoprotein deficient mice infected with LACΔ*agr* (**Figure 3.4A**), although the mechanism driving this response remains unclear. Therefore, although Hla expression in lung homogenate of

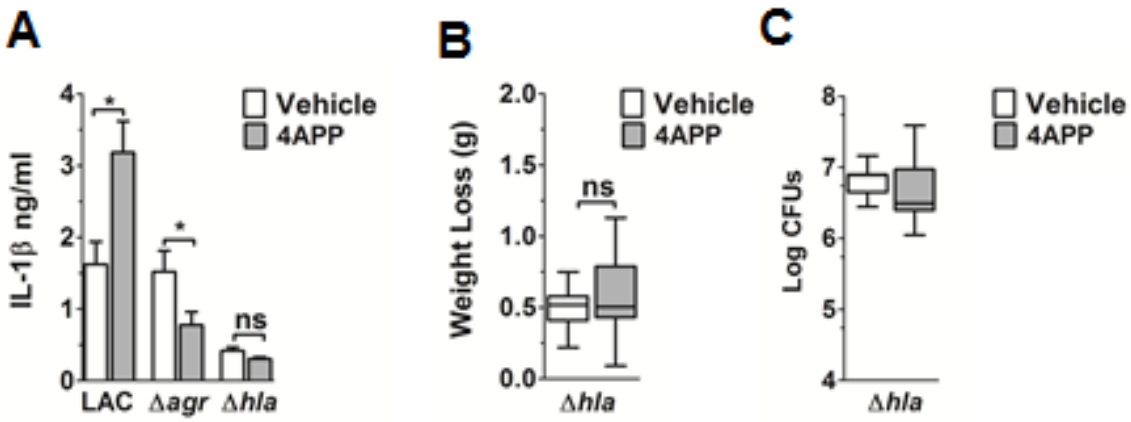

Figure 3.4- Lipoproteins limit Hla-dependent pulmonary IL-1β production.

(**A**) IL-1β levels in whole lung homogenate from vehicle- and 4APP-treated mice (white and gray bars, respectively) 6 hours following intranasal infection with the indicated isolate. Data shown are mean ± SEM. ns, not significant; *, p≤0.05, Student's t-test. (**B**) Weight loss (g) of infected lipoprotein deficient mice versus controls (N=8 mice per group from two independent experiments. (**C**) Bacterial burden (Log CFU) in the lung. N=8 mice per group. (**B-C**) Data shown are median plus 5th-95th percentiles. ns, not significant, Mann-Whitney test.

LAC infected mice was below the limit of detection at six hours post-infection (Western blot data not shown), these results suggest that lipoproteins inhibit *agr*-dependent Hla production and associated virulence in the lungs early during *S. aureus* infection.

In addition to inhibiting *agr*-signaling, apoB on LDL also binds lipotechoic acid (LTA) from *S. aureus* and limits LTA-induced expression of IL-6 [126]. To distinguish between *agr*-dependent and *agr*-independent effects of lipoprotein deficiency on pulmonary inflammatory cytokine production, we measured lung cytokine levels in WT and *Pcsk9*$^{-/-}$ mice intranasally infected with LAC or LACΔ*agr*. In addition to IL-1β and IL-6, we measured production of macrophage inflammatory protein 2 (MIP-2), a neutrophil chemotactic protein, which has also been shown to be driven by *agr*-regulated Hla [137]. At six hours post-infection, IL-1β and MIP-2 levels were significantly increased in the lungs of *Pcsk9*$^{-/-}$ mice infected with LAC, but not with LACΔ*agr* (**Figure 3.5A,B**), consistent with impaired host control of QS in the lungs of lipoprotein deficient mice. In contrast, compared to WT mice, IL-6 was increased in the lungs of both LAC and LACΔ*agr* infected *Pcsk9*$^{-/-}$ mice (**Figure 3.5C**), further supporting a role for apoB in limiting LTA-induced expression of this inflammatory cytokine [126]. Therefore, these data demonstrate that apoB limits *S. aureus*-induced inflammatory cytokine production in the lung in both an *agr*-dependent and an *agr*-independent manner.

Lipoproteins regulate *agr*-dependent neutrophil influx in the lung

Appropriate recruitment of neutrophils during *S. aureus* infection is critical for bacterial clearance [138]; however, excessive neutrophil recruitment can result in severe injury to sensitive lung tissues (reviewed in [42, 139]). Given that MIP-2 levels were

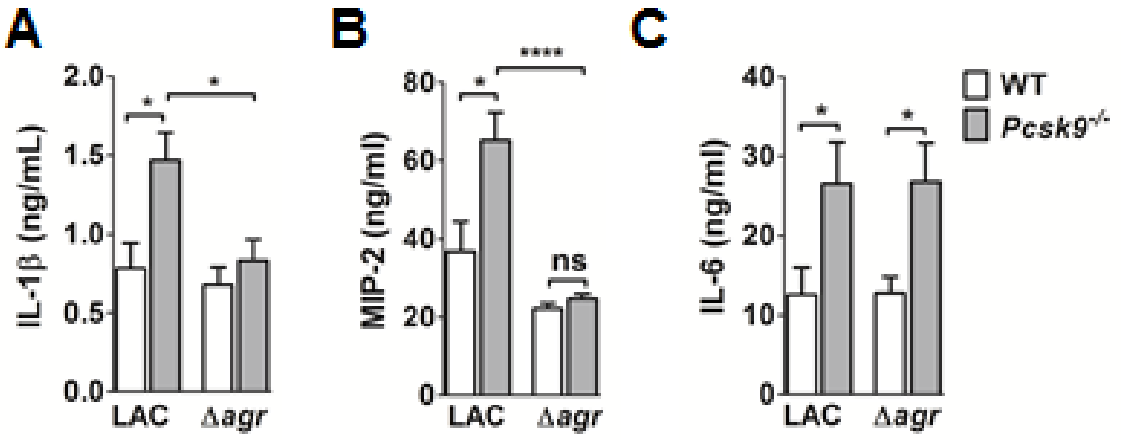

Figure 3.5 - Lipoproteins limit *agr*-dependent and *agr*-independent pulmonary cytokine expression.

(**A**) IL-1β (ANOVA p=0.0035), (**B**) MIP-2 (ANOVA, p<0.0001) and (**C**) IL-6 levels in clarified whole lung homogenate of WT and *Pcsk9*$^{-/-}$ mice (**A-C**, white and gray boxes, respectively) at 6 hours following intranasal infection with the indicated isolate (N=8 mice per group). Data shown are mean ± SEM. (**A**-**B**) ns, not significant; *, p≤0.05, ****, p≤0.0001, ANOVA with Dunnett's multiple comparison analysis. (**C**) *, p≤0.05, Student's t-test.

increased in the lungs of lipoprotein deficient mice infected with LAC, but not with LACΔ*agr*, we compared myeloperoxidase (MPO) levels, a surrogate marker of neutrophil influx, in the lungs of LAC-infected *Pcsk9*$^{-/-}$ mice versus controls. MPO levels were significantly higher in the lungs of lipoprotein deficient mice (**Figure 3.6A**), and this paralleled an increase in Ly6G^{+} cells in BALF from 4APP- versus vehicle-treated mice also infected with LAC (**Figure 3.6B**). In contrast, neutrophil influx and MPO levels did not differ between 4APP- and vehicle-treated mice infected with LACΔ*agr* (**Figure 3.6B**) or LACΔ*hla* (**Supplemental Figure 3.2**), respectively. Furthermore, although macrophage levels overall were increased in BALF from LAC-infected versus LACΔ*agr*-infected mice (**Figure 3.6C**), macrophage presence was independent of circulating lipoprotein levels. Together, these data indicate that lipoproteins are important for early host control of *agr*-dependent neutrophil influx during *S. aureus* lung infection.

ApoB limits *agr*-dependent cytokine expression by human alveolar epithelial cells

Murine MIP-2 is a functional homologue of human IL-8, and Hla strongly induces IL-8 release by human alveolar epithelial cells at toxin concentrations below those causing cell lysis [140]. To demonstrate that lipoproteins protect against early inflammatory cytokine production in the lung via inhibition of *agr* activation, rather than by direct effects on lung cells or sequestration of *agr*-regulated virulence factors [65, 126, 141], we measured IL-8 expression by human alveolar epithelial cells exposed to sterile bacterial supernatant from LAC cultured in the presence versus the absence of LDL. Consistent with previous reports [80, 123], LDL limited accumulation of Hla in LAC

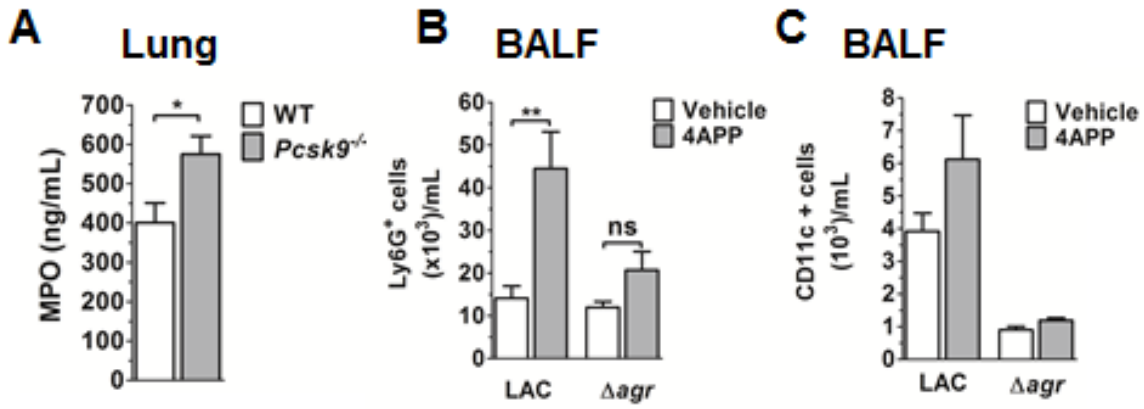

Figure 3.6 - Lipoproteins limit *agr*-dependent neutrophil influx.

(**A**) MPO levels in whole lung homogenate of WT and *Pcsk9*$^{-/-}$ at 6 hours following intranasal infection with the indicated isolate (N=8 mice per group). (**B**) Ly6G+ and (**C**) CD11c+ cells in BALF from vehicle- and 4APP-treated mice at 6 hours post-infection. N=6 (LAC) and 3 (LACΔ*agr*) mice per group. Data shown are mean ± SEM. ns, not significant; *, p≤0.05. **, p≤0.01, Student's t-test.

culture supernatant (**Figure 3.7A**), and thus hemolytic activity (**Figure 3.7B**), whereas LDL incubated with sterile supernatant post-culture, and either used immediately or after a one hour co-incubation, did not inhibit hemolysis. Using A549 cells as a model for human alveolar epithelial cells, LAC supernatant induced *il-8* transcription in a dose-dependent manner (**Figure 3.7C**). Furthermore, compared to IL-8 release following exposure to LAC supernatant, IL-8 production by A549 cells was significantly reduced when exposed to supernatant from LAC cultured in the presence of LDL (**Figure 3.7D**). This reduction in IL-8 was not due to direct effects of LDL on the A549 cells as LDL incubated with cells for one hour prior to the addition of LAC supernatant did not inhibit IL-8 production. Also, while there was a small reduction in IL-8 levels when LDL was co-incubated with LAC supernatant for one hour prior to addition to the A549 cells, this reduction did not reach statistical significance. This suggests that the impact of LDL on IL-8 expression in these assays is mainly independent of sequestration of virulence factors or pathogen associated molecular patterns (PAMPs) within the supernatant [65, 126, 141]. Importantly, IL-8 production did not significantly differ between cells exposed to supernatant from LAC cultured with LDL and supernatant from cultures of LACΔ*agr* or LACΔ*hla*. Therefore, these results support a mechanism whereby apoB limits *S. aureus* QS in the lung and protects the host against excessive inflammatory cytokine production in response to *agr*-regulated virulence factors.

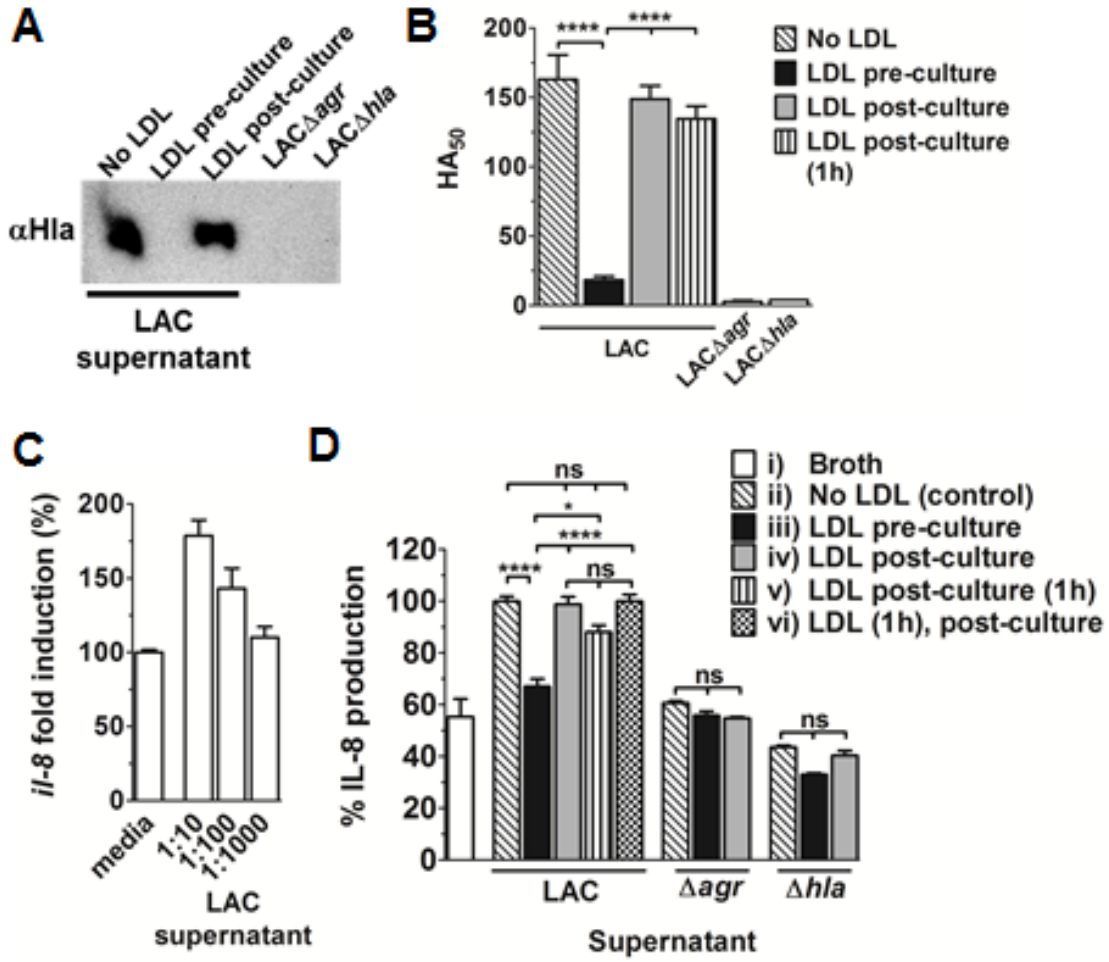

Figure 3.7 - Serum lipoproteins limit *agr*-dependent cytokine expression by human alveolar epithelial cells.

(**A**) Western blot analysis and (**B**) functional assay of Hla in 6 hour sterile supernatant from LAC cultured ± LDL (100 nM), LAC supernatant with LDL (100nM) added post-culture, post-culture supernatant incubated with LDL (100nM) for 1h, or supernatant from LACΔ*agr* or LACΔ*hla*. Data shown are mean ± SEM. ANOVA ($p<0.0001$) with Dunnett's multiple comparison post-hoc analysis. ****, $p\leq0.0001$. (**C**) Transcription of *il-8* relative to *hprt* by A549 cells following 4 hours incubation with LAC sterile supernatant at the indicated dilutions, and (**D**) IL-8 release after 4 hours exposure to 1:10

dilutions of the following: i) broth control, ii) supernatant from LAC cultured in the absence of LDL, iii) LAC cultured in the presence of LDL (100nM), iv) LAC supernatant with LDL added post-culture, v) post-culture supernatant incubated with LDL for 1h or vi) LDL incubated with cells for 1h followed by addition of post-culture supernatant. Data shown are mean percent (± SEM) of IL-8 production relative to the no LDL (ii) control. ANOVA ($p<0.0001$) with Bonferroni's multiple comparison post-hoc analysis. ns, not significant; *, $p\leq0.05$; ****, $p\leq0.0001$.

Discussion

Considerable attention has been given to the role of hyperlipidemia in cardiovascular disease, such that the terms dyslipidemia and hyperlipidemia are often used synonymously [66]. This illustrates the limited consideration given to the potential health consequences of the other dyslipidemia, severe hypolipidemia and lipoprotein deficiency, often experienced post-trauma and by other critically ill patients [112-114]. Since hypolipidemia has been clinically associated with bacterial pneumonia [88-90], we sought to determine the impact of extremely low circulating lipoprotein levels on host innate defense in the lung. Using a sub-lethal mouse model of *S. aureus* pneumonia, we demonstrate that lipoprotein deficiency impairs early pulmonary innate defense against bacterial pathogenesis. Specifically, apoB is present in the lung early post-infection and its levels decrease significantly with lipoprotein deficiency. This decrease coincides with impaired host control of *S. aureus agr*-signaling, as well as increased *agr*-dependent morbidity and inflammation, in alignment with the role of apoB in controlling *S. aureus* QS-dependent virulence in the skin [80, 83, 123]. In addition, lipoprotein deficiency results in an *agr*-independent increase in pulmonary IL-6 expression, consistent with the ability of apoB to bind LTA and limit LTA-mediated inflammation [126]. Given that hyperlipidemia also impairs lung innate defense mechanisms [84], these results strongly suggest that maintenance of normal serum cholesterol and lipoprotein levels is necessary for optimal host innate defense in the lung.

The contribution of host lipoproteins to pulmonary innate immunity is not surprising considering their previously described host defense contributions, together

with their demonstrated uptake by lung capillary endothelium [92] and carriage on infiltrating leukocytes [93]. For example, circulating lipoproteins, including LDL and high density lipoprotein (HDL), non-specifically bind and sequester LPS, thus limiting endotoxin induced toxicity and lethality [124, 125, 142]. In addition, apoB from LDL and apolipoproteins A1 and A2 from HDL bind soluble LTA and inhibit LTA-mediated cytokine release from both human and murine cells [126]. The most pronounced inhibition comes from LDL which dose-dependently inhibits LTA-mediated IL-6 expression by human PBMCs. In support of this finding, here we show that lipoprotein deficiency results in significantly increased IL-6 levels in the lungs of *S. aureus* infected mice independent of *agr* status. Although *in vivo* validation of the role of lipoproteins in controlling LTA-mediated inflammation in the lung will require comparison of lipoprotein-sufficient versus -deficient mice following pulmonary administration of LTA or infection with LTA-deficient *S. aureus* mutants [143, 144], our data and that of others suggests that hypolipidemia may broadly impact post-trauma pneumonia susceptibility to both Gram positive and Gram negative pathogens.

Along with sequestration of AIP, lipoproteins have other roles in host defense against *S. aureus* pathogenesis. Specifically, LDL has been reported to bind and partially inactivate Hla [141], while the phenol soluble modulins (PSMs) [122, 145, 146], which attract and lyse neutrophils, are also bound by lipoproteins, in particular by HDL [65]. In this regard, the data reported here indicate that LDL limits IL-8 production by human alveolar epithelial cells by inhibiting expression and secretion of *agr*-regulated virulence factors. This is supported by our findings that (i) A549 cells produce IL-8 when exposed to LAC sterile supernatant, but not when exposed to sterile supernatant from LAC∆*agr* or

LAC∆*hla*, (ii) that inclusion of LDL during LAC culture limits both Hla accumulation in the supernatant [80, 123] and the ability of the resulting supernatant to induce IL-8 release by A549 cells and (iii) that the addition of LDL to directly to LAC supernatant does not inhibit IL-8 release by A549 cells. Although these data suggest that LDL primarily functions by prevention of virulence factor expression in this system, lipoproteins may also directly inhibit virulence factor function in the lung. Here, pulmonary administration of LAC supernatant, purified Hla or PSMs to serum lipoprotein-sufficient versus -deficient mice would further clarify this issue. Regardless, our findings and those of others [65, 66, 84, 124-126, 141] strongly suggest that lipoproteins likely contribute in a variety of both direct and indirect means to pulmonary host innate defense.

In addition to the negative effects of lipoprotein deficiency on pulmonary innate immunity reported here, severe hypolipidemia may also impair the innate defense function of lung surfactant (reviewed in [86]). Lung surfactant is comprised of a mixture of lipids, phospholipids and proteins, and maintenance of the appropriate ratios of these components is required for functional surfactant self-assembly [85, 147, 148]. Of particular importance to surfactant-mediated host innate defense are the surfactant-associated proteins, SP-A and SP-D, which act as opsonins to promote bacterial clearance [149-153]. Since lipoproteins contribute to the regulation of surfactant cholesterol metabolism [154], it is unclear whether severe hypolipidemia results in impaired surfactant assembly and negatively impacts bacterial clearance by altering the distribution or otherwise impairing the antibacterial functions of SP-A and SP-D. While we saw no evidence of impaired pulmonary bacterial clearance in lipoprotein deficient mice at the

early post-infection time point investigated here, the potential impact of severe hypolipidemia on pulmonary bacterial clearance warrants further investigation.

By focusing on early control of *S. aureus* QS in the lung, we have demonstrated one mechanism by which severe hypolipidemia impairs pulmonary host innate defense. Although a significant gap in knowledge remains regarding the full impact of hypolipidemia on infection susceptibility, disease progression and survival during pneumonia caused by *S. aureus* and other microbial pathogens, the work reported here regarding hypolipidemia, together with the work of others focused on hyperlipidemia [84], represent important advances in understanding the impact of both extremes of dyslipidemia on pulmonary host innate defense. Furthermore, these studies point to the potential clinical impact of severe hypolipidemia on pulmonary innate defense in critically ill patients.

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Disclosures

The authors have no financial conflicts of interest.

Footnotes

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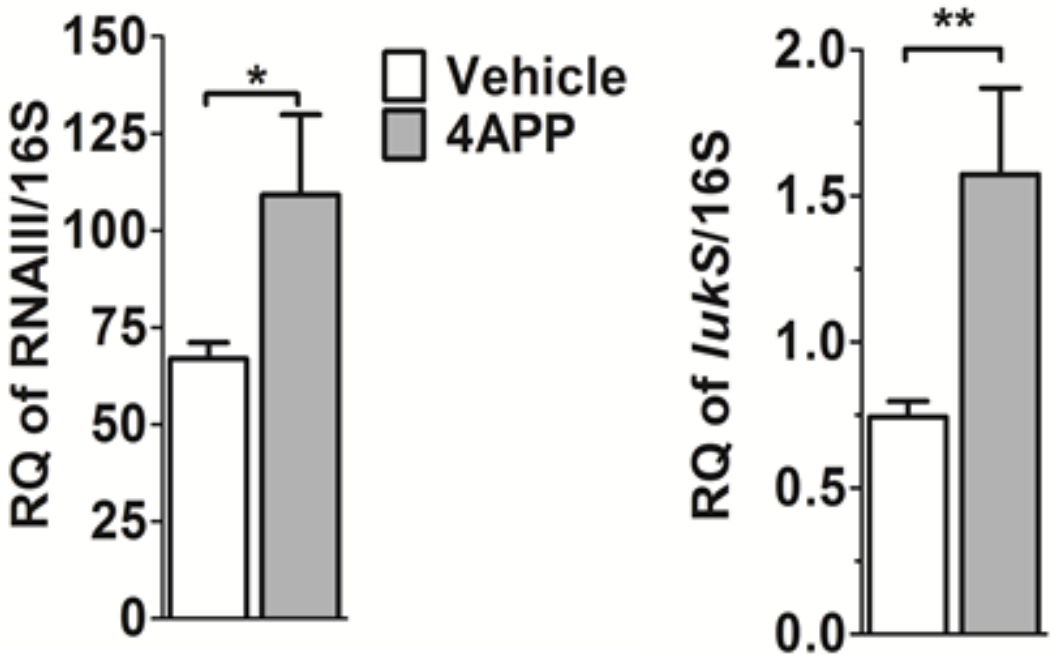
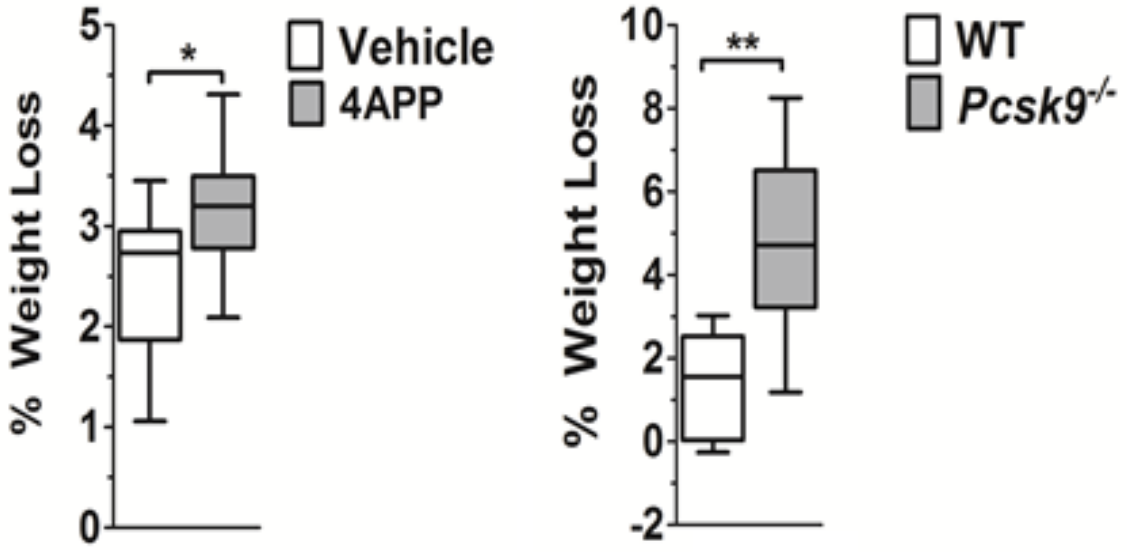
Corresponding author: Pamela Hall, email: phall@salud.unm.edu, phone: (505) 272-0915, fax: (505) 925-4549, mailing address: University of New Mexico COP, 1 University of New Mexico, MSC09-5360, Albuquerque, NM 87131

Supplemental Information



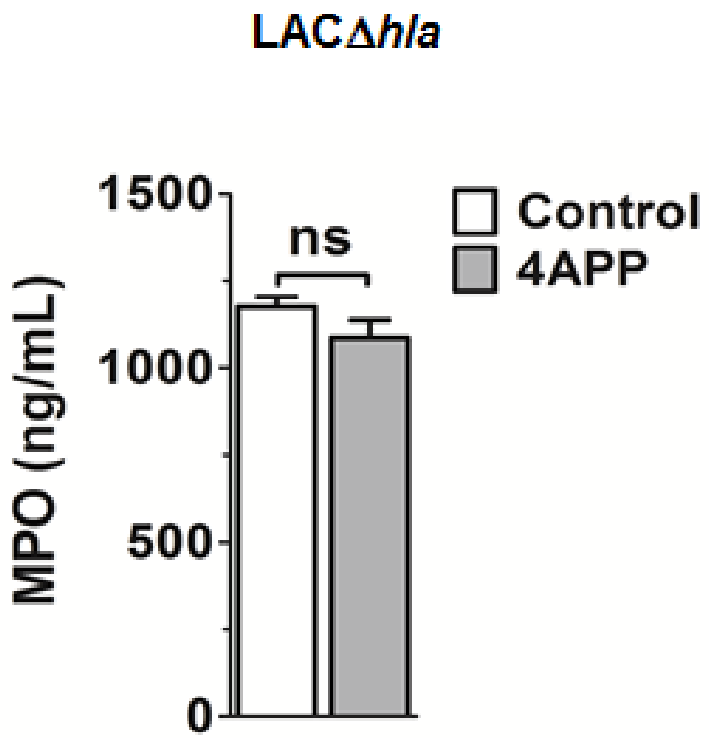
Supplimental Figure 3.8 - *agr*-regulated transcription is increased in LAC collected by BAL from lipoprotein deficient mice.

Bacteria in BALF was collected at six hours following intranasal infection of vehicle- or 4APP-treated mice. Relative quantification (RQ) of RNAIII and *lukS-PV* transcription relative to 16S was determined by qRT-PCR. Data shown as mean ± SEM. N= 8 mice per group. *, $p \leq 0.05$; **, $p \leq 0.01$, Student's t-test.



Supplimental Figure 3.9 - Percent weight loss is increased in LAC infected lipoprotein deficient mice.

Vehicle- or 4APP-treated (left) and B6 x 129 (WT) or *Pcsk9*$^{-/-}$ mice (right) were intranasally infected with *agr::*P3-YFP LAC (4 x 10^8 CFU). Mice were weighed at the time of infection and at 6 hours post-infection. Data shown as median and 5^{th}-95^{th} percentile. N= 7-8 mice per group. *, $p \leq 0.05$; **, $p \leq 0.01$, Mann-Whitney test.



Supplimental Figure 3.10 - MPO levels in lung homogenate from LACΔ*hla* infected mice.

MPO in whole lung homogenate of vehicle- and 4APP-treated mice at 6 hours following intranasal infection with LACΔ*hla*. Data shown as mean ± SEM. N= 12 mice per group. ns, not significant, Student's t-test.

Chapter 4 – Discussion

There is a growing body of evidence in the literature for lipoprotein particles as contributors to host innate immunity against a multitude of pathogens [55-66] (**Table 1**). However, aside from data presented in Chapter 3, there is no direct evidence showing how hypolipidemia affects pulmonary innate immunity. Investigation of the contributions of lipoproteins to pulmonary innate immunity presents a unique opportunity to understand the potentially complicated interplay of lipoproteins, the immune system, and related host-pathogen interactions.

Given that most research on the consequences of dyslipidemia has been largely focused on hyperlipidemia, due to its impact on cardiovascular disease [46-49], there is a critical gap in knowledge as to what role hypolipidemia may play in pulmonary host innate immune defense. The potential negative effects and associations of hypolipidemia include impacts on plasma membrane composition and fluidity, increased risk of cerebral hemorrhage, adrenal failure, and mortality during sepsis and other critically ill states, such as extreme trauma or pneumonia [155-161]. In Chapter 3, we presented evidence that very low apoB containing serum lipoprotein levels during *S. aureus* pneumonia leads to increased QS, resulting in increased inflammation and neutrophil recruitment to the lung at early time points of infection (**Figure 4.1**). To our knowledge, this is the first indication that reduced serum lipoproteins impair innate immune defense during pulmonary infection.

Despite the growing body of evidence that lipoproteins play an important role in innate immunity, there are still many gaps in knowledge to address, including i) what are

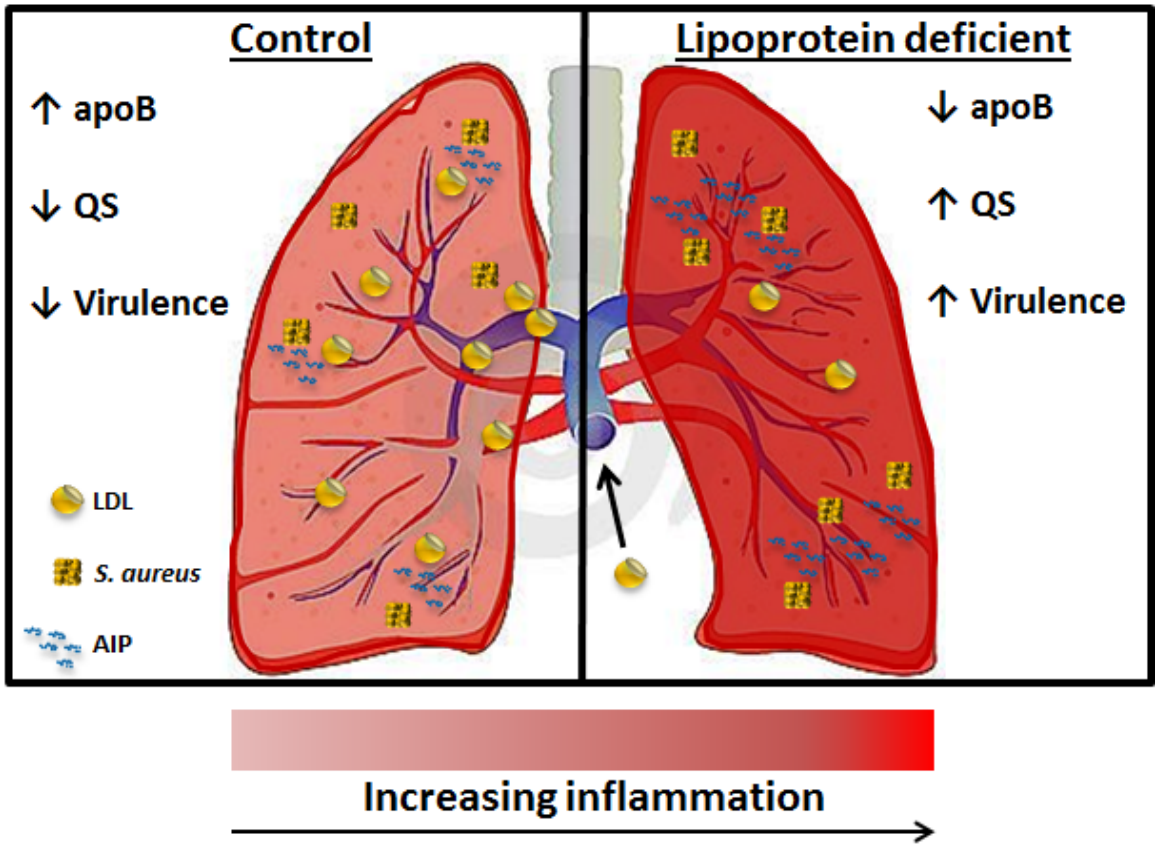


Figure 4.1 – ApoB in pulmonary defense against *S. aureus.*

Lowered apoB-containing serum lipoprotein levels during *S. aureus* pneumonia leads to increased QS, resulting in increased inflammation and neutrophil recruitment to the lung at early time points of infection.

effects of hypolipidemia on lung surfactant structure and function in innate immunity? ii) what are the effects of hypolipidemia directly on pulmonary immune cells and inflammation? and iii) how do cholesterol lowering dependent and independent effects of drugs, such as statins, contribute to pulmonary infection outcomes? Herein we address these gaps in knowledge based on the available literature, as well as discuss how hypolipidemia may affect infection with other lung pathogens, some of the pros and cons of hyper- and hypolipidemia in infection and immunity, and highlight the concept that either extreme of dyslipidemia can have negative consequences in pulmonary infection and immunity.

i) Serum lipoprotein and pulmonary surfactant structure and function

Pulmonary surfactants are primarily composed of lipids and cholesterol and function to lower surface tension at the air-to-surface interface in the lung, as well as help serve as a physical barrier for removal of particulates from the lung [162]. Abnormal levels of cholesterol carrying serum lipoproteins, high or low, have been shown to have detrimental impacts on pulmonary surfactant structure and ability to function as a surface tension regulator in the lung [85, 147], consistent with evidence that dyslipidemia in either direction has negative health effects. The effects of lipoproteins on surfactant composition, structure and function, as well anti-microbial surfactant proteins, merits further investigation.

The antimicrobial surfactant proteins SP-A and SP-D interact with a wide variety of pathogens, including *S. aureus, S. pneumoniae,* and group A *Streptococcus* [163-167],

bind LPS [168], and opsonize bacteria for phagocytosis by alveolar macrophages (AM) [152]. Interestingly, serum proteins can inhibit surfactant surface tension lowering activity [169, 170], and SP-A reduces this inhibition [171], suggesting a potential role for serum proteins in regulating normal lung function during infection. Hypolipidemic states may contribute to dysregulation of the interplay of lung specific factors, such as SP-A and SP-D, leading to worse clinical outcomes in patient populations that are more susceptible to pulmonary infection with a multitude of pathogens.

Despite this, the effects of hypolipidemia on SP-A and SP-D function and potential consequences for how it might affect gene regulation or protein expression during pulmonary infection, has not been investigated. Given that we know lipoproteins can have detrimental effects on lung surfactants, and that serum lipoprotein levels effect lipoprotein levels in the lung, we hypothesize that SP-A and SP-D function and regulation may be affected by serum lipoprotein levels, leading to different outcomes during pulmonary infection in an *in vivo* model. Include collected lung samples for qRT-PCR of SP-A/SP-D To test this, we would first consider the effects of the addition of serum lipoproteins on SP-A and SP-D expression and secretion from A549 cells [172].Additionally, regulation of SP-A and SP-D in our hypolipidemic mouse models (detailed in Chapter 3), compared to controls would represent an important next evaluation. The results of these experiments could have further implications in the contributions of serum lipoproteins in pulmonary innate immunity against a variety of pathogens.

ii) Serum lipoproteins modulate the inflammatory response

In addition to the potential effects of lipoproteins on physical immune barriers such as surfactant and associated surfactant proteins, we know from studies of atherosclerosis that serum lipoproteins can also modulate inflammatory responses in many different cell types [173]. For example, exposure of macrophages to modified LDL increases expression of the oxidized LDL (oxLDL) scavenger receptor, CD36, which contributes to development of foam cells in auto-immune development of atherosclerotic lesions [174]. OxLDL can also induce monocyte chemoattractant protein-1 (MCP-1) expression from endothelial cells, inhibit macrophage migration, and induce proinflammatory cytokine expression from macrophages [173]. SP-A has also been shown to induce respiratory burst and nitric oxide synthase (NOS) expression in AMs [32]. Furthermore, uptake of LDL by the LDLR on neutrophils transiently increases their oxidative burst [175], which is important in auto-immune development of atherosclerosis via oxidative modification of LDL, as well as important in microbial killing. Unsurprisingly, systemic oxidation of lipoproteins occurs during the acute phase response to LPS induced septic shock [176]. Myeloperoxidase (MPO) produced by neutrophils during infection is important in oxidative killing of microbes via production of cell generated hypochlorous acid, which can also oxidize lipoproteins [177]. Additionally, HDL has also been shown to mediate anti-inflammatory reprogramming of macrophages through reduced expression of TLR-induced cytokines [178], to attenuate neutrophil activation via reduction of lipid raft abundance associated with CD11b activation [179], and limit the oxidative burst of neutrophils by oxLDL [180]. Based on these studies, it is

clear that lipoproteins play an important role in helping activate inflammatory responses, are modified by ROS produced by activated leukocytes, and that these modified lipoproteins can further modulate inflammation. However, since most atherosclerosis research is done in hyperlipidemic models, there is a need to address how extreme hypolipidemia may affect innate immune cell signaling and function, particularly in the context of pulmonary infection.

Despite the wealth of research on lipoproteins in atherosclerosis, there is conflicting literature regarding the effects lipoproteins and cholesterol can have both detrimental and beneficial immunomodulatory and inflammatory effects during infections. For example, exposure of guinea pigs to *Mycobacterium tuberculosis* increases expression of CD36 on AMs, thus increasing uptake of oxLDL by AMs resulting in increased intracellular bacterial survival [181], likely due to use of cholesterol as a carbon source by *M. tuberculosis*. Additionally, deletion of the ATP-binding cassette transporter A1 (ABCA1), which plays a role in efflux of intracellular free cholesterol, has been shown to increase resistance to infection via increases in the proinflammatory state of macrophages, in a mouse model of intraperitoneal *Listeria monocytogenes* [182]. The deletion of ABCA1 also resulted in higher systemic concentrations of MCP-1, MIP-2, and IL-6. Given our findings showing association of elevated MIP-2 and neutrophil recruitment with worse disease outcomes in the lung in lipoprotein deficient mice, one may expect that a mutation which causes dysfunction in ABCA1, and the associated lipid metabolism differences could have different effects in a pulmonary disease model. Interestingly, cholesterol crystals have been shown to prime neutrophils to release neutrophil extracellular traps (NETs), prime macrophages to

release IL-1β, and activate T helper 17 cells which increase immune cell recruitment [183]. It has also been hypothesized that oxidation-specific epitopes on oxLDL that are similar to epitopes on pathogens, serve to maintain natural antibodies against those pathogens [184]. Together, these varied studies indicate the complex interplay of lipoproteins and the immune system and the importance of both extremes of dyslipidemia to negative disease states or infection outcomes. Given this, and the increased presence of serum lipoproteins in the lung during infection, a role for lipoproteins in directly modulating inflammation during pulmonary infection may be indicated.

iii) Statins in innate immunity and pleotropic effects on inflammation

Statins as treatments for lowering cholesterol can dramatically reduce instances of cardiovascular disease [185, 186]. In contrast to the idea that severe reduction in cholesterol levels could knockdown serum lipoproteins as an innate immune barrier [87], statins appear to have some beneficial effects in severe infectious disease outcomes. For example, in a clinical study of 575 patients, looking at pre-hospitalization use of statins, beneficial effects were found in lowering rates of sepsis and pneumonia in humans during early critical illness [187]. A conflicting clinical study's results showed no benefits to 90-day mortality rates in community acquired pneumonia (CAP) patients, and no significant elevation in plasma markers of severe sepsis and CAP, such as inflammatory cytokines and leukocyte cell surface protein expression markers [188]. However, the authors of this study still suggest that the trends favored statin usage over no statin usage, and suggest (while not statistically significant) a potentially clinically meaningful benefit of statin

usage in CAP cases. While prophylactic usage of statins appeared to have beneficial effects in these severe disease outcomes, post-hospitalization treatment with statins appeared to have no effect in either study [187, 188].

The suggestion that statins have a clinically beneficial role in severe disease outcomes merits further investigation into the mechanisms behind this benefit. Laboratory investigations found that simvastatin may play a role in inhibiting the actin dynamics in human endothelial cells required for *S. aureus* endocytosis and invasion [189], inhibit LTA induced inflammation in human AMs through inhibition of NF-κB activation [190], and enhance extracellular trap formation by human and murine leukocytes via inhibition of sterol production [191]. Another study found that simvastatin and pravastatin (at sub-cholesterol lowering concentrations) were able to limit the cytotoxicity of Hla and pneumolysin, a major pore forming toxin of *S. pneumoniae* , against human airway epithelial cells [192], and that the mechanism for this was independent of the cholesterol-lowering mevalonate pathways. These studies indicate that statins have a pleotropic role in lowering damaging inflammatory responses via mechanisms independent of their targeted use against cardiovascular disease. This further indicates the complicated and sometimes paradoxical interplay of the pulmonary immune milieu in clearing infection, through phagocytosis and killing of bacterial pathogens, while simultaneously limiting potentially damaging inflammation. These findings present interesting avenues for research looking at these effected pathways for lowering initial inflammatory responses in severe infection cases.

Despite evidence suggesting that statin usage may be beneficial in severe sepsis and pulmonary disease outcomes, there are no reports looking at statin usage and the state

of hypolipidemia during severe pulmonary infection. The evidence showing sub-cholesterol lowering, anti-inflammatory effects of statins may indicate confounding variables in statin using patients in extreme hypolipidemic states and merits further investigation. We propose that sub-cholesterol lowering concentrations of statins could have beneficial immunomodulatory effects on inflammatory signaling, and detrimental effects during infection at higher concentrations (relevant to CVD treatment) via further knockdown of lipoproteins as innate immune barriers. Given the wide usage of statins, perhaps this could be elucidated by doing a database search of clinical outcomes in hypolipidemic patient populations (such as in the case of severe trauma), comparing statin and non-statin users. We would also suggest experiments to test the effects of simvastatin treatment at previously reported sub-cholesterol reduction levels (10 nM to 1000 nM) [192] in a lipoprotein deficient mouse model of *S. aureus* pulmonary infection, compared to control mice. We would predict that pre-infection statin treatment would have beneficial effects in control mice at sub-cholesterol reducing levels, but increase QS and resulting inflammation and disease in lipoprotein deficient mice. The results of these studies could point to sub-cholesterol reducing levels of statins as potential treatments in critically ill patient populations.

Closing comments

Lipoprotein research has been primarily focused in the area of hyperlipidemia, largely because of its known association with increased CVD and atherosclerosis. Through this, the scientific community's understanding of lipoproteins as immune system modulators and causes of immune dysfunction in these disease states, is vast. Perhaps naturally arising from this large body of research is more recent research showing that hyperlipidemia has important consequences in infectious disease outcomes (Madenspacher et al., 2010, From Paper), and that infectious agents can also enhance disease progression in CVD and atherosclerosis (Dutta et al. 2009, Repeated Systemic). However, there is comparatively very little research on the effects of hypolipidemic states and their contributions to infectious disease.

Given the importance of CVD and atherosclerosis, statins have become some of the most prescribed drugs world-wide (Herbert et al. 1997, Cholesterol Lowering, Gotto 1997, Cholesterol Management). However, because of the limited research done on the effects of hypolipidemia during trauma and outcomes in infectious disease, the consequences of statins as reducers of lipoproteins as an innate immune barrier in these populations has not been accurately assessed. Furthermore, whether the potentially beneficial effects of statins that are unrelated to their ability to lower cholesterol are outweighed by further knockdown of lipoproteins as agents of innate immunity has not been thoroughly investigated in hypolipidemic patient populations.

One of the hallmarks of susceptibility to infection is a compromised immune system. Perhaps not coincidentally, individuals recovering from traumatic insult are

likely to have lowered serum levels of circulating apoB containing lipoproteins, and are more susceptible to infection [91]. Addressing the gaps in knowledge described above may have implications in early prediction of pneumonia susceptibility in trauma patients. Perhaps lipoproteins as potential future treatment options could ultimately help contribute to better outcomes in hypolipidemic patients who are more susceptible to infection. Additionally, the studies discussed herein suggest that lipoproteins could serve as an innate immune barrier during early infection in the lung against a multitude of pathogens.

The lung is particularly sensitive to damage caused by infectious disease and inflammation. Due to the lungs indispensability as an organ, pneumonia in particular is an important and severe disease state, resulting in overall high levels of morbidity and mortality. Though the lung has become highly adapted with physical barriers, and unique antimicrobial agents and resident immune cells, likely because of these very reasons, it also presents a unique disease microenvironment where lipoproteins may have even further pleotropic effects beyond direct innate immune agents and inflammatory signaling modulators. Based on the presented research, as well as our own findings, dyslipidemia in either direction appears to have detrimental effects in infectious disease outcomes in pulmonary infection. Both over activation of inflammatory signaling and lack of infection control may be particularly detrimental in the lung, with over activation of inflammatory signaling leading to increased inflammation induced damage. Perhaps when dyslipidemia in either direction can cause dysregulation of inflammatory signaling, or deficiencies in innate immunity and ultimately lack of pathogen control, leading to increased toxin induced inflammation and tissue damage.

Appendix A: Abbreviations

4APP	4-aminopyrizolo[3,4-d]pyrimidine
agr	Accessory gene regulator
AIP	Auto-inducing peptide
AM	Alveolar macrophage
AMP	Antimicrobial peptide
ApoA1	Apolipoprotein A1
ApoA2	Apolipoprotein A2
ApoB	Apolipoprotein B
ApoB-48	N-terminal 48% of apolipoprotein B
ApoC1	Apolipoprotein C1
ApoC2	Apolipoprotein C2
ApoE	Apolipoprotein E
APR	Acute phase response
BALF	Bronchial alveolar lavage fluid
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BSA	Bovine serum albumin
CA-MRSA	Community acquired methicillin-resistant Staphylococcus aureus
CAP	Community acquired pneumonia
CDC	Centers for Disease Control
cDNA	Complimentary deoxyribonucleic acid
CFU	Colony forming unit

CVD	Cardiovascular disease
DC	Dendritic cell
HAP	Hospital acquired pneumonia
HDL	High density lipoprotein
Hla	Alpha-hemolysin
hLDL	Human low density lipoprotein
i.n.	Intranasal
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
mAb	Mouse antibody
MCF	Mean channel fluorescence
MPO	myeloperoxidase
MRSA	Methicillin-resistant Staphylococcus aureus
NBT	Nitroblue tetrazolium
NET	Neutrophil extracellular trap
NK	Natural killer cell
NOS	Nitric oxide synthase
ns	Not significant
oxLDL	Oxidized low density lipoprotein
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern

PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PSM	Phenol soluble modulins
PVDF	Polyvinylidene fluoride
PVL	Panton-Valentine leucocidin
qRT-PCR	Quantitative real-time polymerase chain reaction
QS	Quorum sensing
RBC	Red blood cell
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RQ	Relative quantification
RT	Reverse transcriptase
SEM	Standard error of the mean
SSSI	Skin and skin structure infection
TBS	Tris buffered saline
TLR	Toll-like receptor
TSB	Tryptic Soy Broth
VLDL	Very low density lipoprotein
WT	Wild-type
YFP	Yellow fluorescent protein

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