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The functional significance of the lung-liver axis during pneumonia

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THE FUNCTIONAL SIGNIFICANCE OF THE LUNG-LIVER AXIS DURING PNEUMONIA

by

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"All seats provide equal viewing of the universe." -Museum Guide, Hayden Planetarium

DEDICATION

For my parents, whose happiness and unending love and support have inspired me. If I were to become half the person they each are, then I know I will succeed in life.

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THE FUNCTIONAL SIGNIFICANCE OF THE

LUNG-LIVER AXIS DURING PNEUMONIA

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ABSTRACT

The hepatic acute phase response (APR), stimulated by injury or inflammation, is characterized by significant changes in circulating acute phase protein (APP) concentrations. While individual functions of liver-derived APPs are known, the net consequence of APP changes is unclear. Pneumonia and sepsis elicit systemic inflammation and induce a robust APR. Although APR activation is regarded as a hallmark of infection, direct contributions of liver activation to pulmonary defense during pneumonia and sepsis-induced pneumonia remain unclear. Pneumonia causes a pulmonary inflammatory response coordinated largely by alveolar macrophages, and is typified by cytokine production, leukocyte recruitment and plasma extravasation, the latter of can enable delivery of hepatocyte-derived APPs to the infection site. To determine the functional significance of the hepatic APR during pneumonia, we challenged APR-null mice lacking hepatocyte signal transducer and activator of transcription-3 (STAT3) and RelA with 10^6 colony-forming units (CFU) *Escherichia coli* intratracheally. HepSTAT3/RelA$^{-/-}$ mice displayed ablated APP induction, significantly

increased mortality, tumor necrosis factor-dependent hepatotoxicity, and pulmonary bacterial burdens. Following a lower ($4x10^5$ CFU) *E. coli* inoculum, hepSTAT3/RelA$^{-/-}$ mice had decreased APP concentrations with reduced pulmonary inflammation and diminished airspace macrophage activation. Similar results were obtained in the context of endotoxemia and pneumonia. We employed an endotoxemia/pneumonia model, whereby 18 hours of intraperitoneal *E. coli* lipopolysaccharide (5 mg/kg) was followed by intratracheal *E. coli* (10^6 CFU) in mice lacking hepatocyte STAT3 (hepSTAT3$^{-/-}$) or control hepSTAT3$^{+/+}$ mice. Following endotoxemia and pneumonia, hepSTAT3$^{-/-}$ mice, with significantly reduced levels of circulating and airspace APPs, exhibited significantly elevated lung and blood bacterial burdens and mortality. While neither recruited airspace neutrophils nor lung injury were altered in endotoxemic hepSTAT3$^{-/-}$ mice, *in vivo* production of reactive oxygen species in alveolar macrophage was significantly decreased. Additionally, bronchoalveolar lavage fluid from this group of hepSTAT3$^{-/-}$ mice allowed greater bacterial growth *ex vivo.* These results identify a lung-liver axis, whereby the liver response enhances macrophage activation and pulmonary host defense during pneumonia and sepsis-induced pneumonia. Taken together, induction of liver acute phase gene expression programs contributes to countering the deleterious consequences of pneumonia, whether it is alone or in the context of sepsis-induced infection.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

7AAD ..... Live/Dead Stain (7-Aminoactinomycin D)

ALI ..... Acute Lung Injury

ANOVA ..... Analysis of Variance

APC ..... Allophycocyanin

APP ..... Acute Phase Protein

APR ..... Acute Phase Response

ARDS ..... Acute Respiratory Distress Syndrome

BAL ..... Brochoalveolar Lavage

BALF ..... BAL Fluid

BSA ..... Bovine Serum Albumin

C1-9 ..... Complement Components 1-9

CCL ..... Chemokine

CD ..... Cluster of Differentiation

CLP ..... Cecal Ligation and Puncture

CRP ..... C-Reactive Protein

CXCL1 ..... Chemokine

DALYs ..... Disability-Adjusted Life Years Lost

DIC ..... Disseminated Intravascular Coagulation

DNA ..... Deoxyribonucleic Acid

ECL ..... Enhanced Chemiluminescence

EDTA ..... Ethylenediaminetetraacetic Acid

ELISA .....Enzyme-Linked Immunosorbent Assay

FBS .....Fetal Bovine Serum

FITC.....Fluorescein Isothiocyanate

G-CSF .....Granulocyte Colony-Stimulating Factor

GM-CSF.....Granulocyte/Macrophage Colony-Stimulating Factor

h.....Hours

H & E .....Hematoxylin and Eosin

HBSS ..... Hank's Balanced Salt Solution

HEPES ..... 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic Acid

HRP.....Horseradish Peroxidase

i.p. .....Intraperitoneal

i.t. ..... Intratracheal

i.v. .....Intravenous

ICU..... Intensive Care Unit

Ig .....Immunoglobulin

IL.....Interleukin

IL-1R1 .....IL-1 Receptor 1

kg..... Kilogram

LBP .....LPS-Binding Protein

LIF..... Leukemia Inhibitory Factor

LPS.....Lipopolysaccharide

Ly6G ..... Lymphocyte Antigen 6 Complex, Locus G

MFI ..... Mean Fluorescence Intensity

mg ..... Milligram

min ..... Minutes

ml ..... Milliliter

mM ..... Millimolar

MMP ..... Matrix Metalloproteinase

MOPS ..... 3-propansulfonic acid

MPO ..... Myeloperoxidase

mRNA ..... Messenger RNA

NET ..... Neutrophil Extracellular Trap

NF-κB ..... Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells

OSM ..... Oncostatin-M

p-value ..... Probability Value

PAMP ..... Pathogen-Associated Molecular Pattern

PAR ..... Proteinase-Activated Receptor

PBS ..... Phosphate Buffered Saline

PCR ..... Polymerase Chain Reaction

PCV ..... Pneumococcal Conjugate Vaccine

PE ..... Phycoerythrin

Pen-Strep ..... Penicillin-Streptomycin

PFA ..... Paraformaldehyde

pg ..... Picogram

PPSV ..... Pneumococcal Polysaccharide Vaccine

PRR ..... Pattern Recognition Receptor

PVDF ..... Polyvinylidene fluoride

qRT-PCR ..... Quantitative Real-Time PCR

RBC ..... Red Blood Cell

RNA ..... Ribonucleic Acid

ROS ..... Reactive Oxygen Species

RPM ..... Revolutions Per Minute

RPMI ..... Roswell Park Memorial Institute Media

RSV ..... Respiratory Syncytial Virus

RT ..... Room Temperature

SAA ..... Serum Amyloid A

SAP ..... Serum Amyloid P

SDS-PAGE ..... Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SIRS ..... Systemic Inflammatory Response Syndrome

STAT3 ..... Signal Transducer and Activator of Transcription

TBS-T ..... Tris Buffered Saline with Tween

TLR ..... Toll-Like Receptor

TNFR1/2 ..... TNF Receptor 1/2

TNFα ..... Tumor Necrosis Factor-α

U ..... Active Units

V ..... Volts

WHO ..... World Health Organization

WT ..... Wildtype

αTNF ..... Anti-TNF

μg ..... Microgram

μl ..... Microliter

CHAPTER 1: INTRODUCTION

Pneumonia

History, Clinical Significance, and Epidemiology

Pneumonia has long been appreciated throughout the history of modern culture as an acute, but severe illness. In fact, Dr. William Osler, the father of modern medicine, stated that as "the most widespread and fatal of all acute diseases, pneumonia is now the 'Captain of Men of Death'" (Osler, 1905). Before the advent of antibiotics, there was no specific treatment recommended. Dr. Osler wrote that "alcohol may be used with benefit in a majority of cases" (Osler, 1905). Without antibiotics, mortality rates were as high as 30% in city hospitals (Dowling, 1972). Mortality rates due to pneumonia have seen some important trends throughout the 20th century, with the most notable being the 1918 influenza epidemic, which quadrupled mortality rates, peaking around 600,000 deaths per 100,000 people per year. Interestingly, the majority of these deaths were attributable to bacterial superinfections and not the influenza infection itself (Mizgerd, 2012). It wasn't until the mid 20th century with the advent of antibiotic therapies, that mortality rates dropped to around 40 deaths per 100,000 persons per year by 1960 (from around 100 deaths per 100,000 persons per year in 1940) and have remained relatively unchanged since (Armstrong et al., 1999).

Presently, pneumonia accounts for the greatest global burden of disease, as it is responsible for the most disability-adjusted life years (DALYs) lost, a metric put forth by the World Health Organization (WHO), than any other disease including diarrheal diseases, human immunodeficiency virus/acquired immunodeficiency syndrome and

tuberculosis (Mizgerd, 2012). Within the United States, acute lung infections, including both pneumonia and influenza, cause the most infection-related deaths (Mizgerd, 2006), and pneumonia itself is responsible for the most childhood hospitalizations (Yu et al., 2011). Overall, there are about 5 million cases of pneumonia each year, with about one million patients hospitalized annually due to pneumonia (Eddy, 2009). Hospitalization rates for pneumonia are stratified greatly due to age, with the greatest incidence in the elderly and children under two years of age (Griffin et al., 2013). In fact, in elderly adults, age 85 or greater, hospitalization rates due to pneumonia are over 4,000 hospitalizations per 100,000 persons annually. This rate is cut in half in adults 74-85 years old; and for children under two years of age hospitalization rates are just under 1,000 hospitalizations per 100,000 persons (Griffin et al., 2013).

Hospital-acquired or nosocomial pneumonia is a major complication in intensive care units (ICUs) that is associated with high morbidity and mortality (Lynch, 2001). The general incidence of nosocomial pneumonia is 0.5-2.0% of all hospital admissions. It is a common hospital-acquired infection (second to urinary tract infections) that has the highest associated mortality rate of any nosocomial infection (30-70%) (Blasi, 2010; Lynch, 2001; Richards et al., 1999).

Causes of Pneumonia

Pneumonia is caused by a multitude of organisms, including bacterial, viral and fungal agents (Musher and Thorner, 2014). The Gram-positive bacterium, *Streptococcus pneumoniae*, remains the number one causative agent of community-acquired pneumonia

(Garau and Calbo, 2008). There are a number of different estimates as to the number of cases caused by *S. pneumoniae* annually, and reports have determined that anywhere from 10-15% of inpatient cases (Musher and Thorner, 2014) to 30-50% (Garau and Calbo, 2008; Rudan et al., 2008) of all reported cases are due to pneumococcus. There are over 90 immunologically distinct serotypes of pneumococcus, each having a distinct composition of the polysaccharide capsule, which results in large differences in virulence among serotypes (Hammerschmidt et al., 2005; Hausdorff et al., 2005; Kadioglu et al., 2008). Other bacterial species, such as *Haemophilus influenza*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* and other Gram-negative rods, are important, but less frequent causes of community-acquired pneumonia (Garau and Calbo, 2008; Musher and Thorner, 2014; Rudan et al., 2008). On the other hand, hospital-acquired pneumonia is most frequently caused by *S. aureas* and other Gram-negative bacterial species, including *P. aeruginosa*, *K. pneumoniae*, *E. coli*, and other enterobacteria (Blasi, 2010; Jones, 2010; Lynch, 2001; Richards et al., 1999).

It is now becoming more recognized, with the advent of polymerase chain reaction (PCR) used as a diagnostic, that various respiratory viruses are also a major causative agent for pneumonia (Musher and Thorner, 2014). Respiratory syncytial virus (RSV), parainfluenza virus, influenza, and human metapneumovirus are among some of relevant causes of viral pneumonia (Garau and Calbo, 2008; Musher and Thorner, 2014; Rudan et al., 2008). In fact, a recent study has suggested that the incidence of viral pneumonia in children is more prevalent that originally thought, with the majority of the

cases due to RSV infection. These studies, however, should be interpreted cautiously since they were more empowered to detect viral species (Jain et al., 2015).

Pathology and Immune Response

Pneumonia is defined clinically as an acute inflammatory condition in which the alveolar space, the site of gas exchange, is filled with fluid and immune cells (Guyton and Hall, 2000). Once in the lower respiratory tract, recognition of pathogens occurs through pattern-recognition receptors (PRRs) on sentinel cells within the airspaces, which are predominantly alveolar macrophages and epithelial cells (Mizgerd, 2008). This recognition causes an innate immune response that is typified by alveolar inflammation. Proinflammatory cytokines and chemokines are produced, and immune cells, particularly neutrophils, are recruited to the airspaces, resulting in a hostile environment for microbes to survive (Guyton and Hall, 2000; Longo, 2012; Mizgerd, 2008; Society and America, 2005). Pathogen killing, however, generally results in acute lung injury (ALI), which damages the delicate alveolar epithelium and allows for fluid buildup and plasma protein extravasation into the alveolar space (Matthay et al., 2012; Mizgerd, 2008; Ware and Matthay, 2000). Indeed, proteinaceous edema is a hallmark of the acute respiratory distress syndrome (ARDS), which is a complex and deadly syndrome most often caused by pneumonia (Matthay et al., 2012; Ware and Matthay, 2000). The specific innate immune response and the cell types involved are reviewed later in this dissertation.

Pneumonia and ARDS are diagnosed by radiographic confirmation, the majority of time being X-ray, of fluid buildup within the lungs. Instead of normal, clear airspaces,

cloudy infiltrates can be seen within the pulmonary space (Longo, 2012; Niederman et al., 2001; Society and America, 2005). Patient sputum samples are also cultured and Gram-stained to identify the causative bacterial species, or in the case of viral pneumonia, subjected to PCR analysis.

Treatment and Vaccines

Antibiotic therapy is the most common treatment of bacterial pneumonia (Niederman et al., 2001). As a better determinate of the specific antibiotic treatment, patient populations are stratified into four groups based on specific risk factors and their outpatient or inpatient standing. Some risk factors include the presence of cardiopulmonary diseases like congestive heart failure or chronic obstructive pulmonary disease, and the likelihood of developing an antibiotic-resistant infection, which is based on modifying factors such as age, alcoholism, residence in a nursing home, and recent antibiotic therapy (Niederman et al., 2001). Recommended antibiotics include advanced generation macrolides, doxycycline, β-lactams, and antipneumococcal fluoroquinolones given either orally or intravenously, depending on the severity of infection (Niederman et al., 2001; Society and America, 2005). Antibiotic resistance has become an increasing concern, especially in hospital-acquired pneumonia (Moroney et al., 2001). Several risk factors, including length of hospital stay, recent antibiotic therapy, and presence of immunosuppressive disease, are used to identify patients at risk to develop resistant pneumonias. Of all pneumonia etiologies, *P. aeruginosa* is most frequently multidrug-resistant, followed by *Klebsiella* and *Enterobacter* and other Gram-negative species

(Moroney et al., 2001; Society and America, 2005). In severe cases of pneumonia, like those that progress to ARDS, supportive care and ventilation are necessary in addition to antibiotics (Matthay et al., 2012; Ware and Matthay, 2000; Ware and Matthay, 2005).

Two pneumococcal vaccines are commercially available, both worldwide and in the United States: the pneumococcal polysaccharide vaccine (PPSV23 or Pneumovax®) and the pneumococcal conjugate vaccine (PCV or Prevnar®). PPSV23 has been commercially available since 1983, and has been shown to reduce the incidence of invasive pneumococcal disease, but not pneumonia or pneumonia-related mortality rates (Pisano and Cifu, 2015). The PPSV23 vaccine is a combination of purified polysaccharides from the capsules of 23 different serotypes of *S. pneumoniae*, which induce a B-cell-dependent immune response through immunoglobulin M (IgM) (NCIRD, 2013b; Pisano and Cifu, 2015; Pletz et al., 2008). Because this vaccine does not stimulate a robust response, immunological memory is not induced and boosters are needed every 5-6 years (Moberley et al., 2013; Pletz et al., 2008; Rubins et al., 1998). Additionally, PPSV23 is not recommended for children under two years of age (NCIRD, 2013b; Pisano and Cifu, 2015). The first pneumococcal conjugate vaccine, PCV7 or Prevnar7®, was introduced in 2000 and has since been adapted as part of the pediatric vaccine schedule (NCIRD, 2013a; Pisano and Cifu, 2015). PCV7, and its later counterpart PCV13, are comprised of capsular polysaccharides from 7 or 13 serotypes, respectively, of *S. pneumoniae* that are conjugated to a diphtheria toxoid protein that is highly immunogenic. By utilizing this protein as an immunomodulator, these vaccines are able to induce immune memory and provide lasting protection (Pisano and Cifu,

2015; Pletz et al., 2008). The Centers for Disease Control recommend all children under the age of two and adults over 65 receive the PCV13 vaccination (NCIRD, 2013a). After its introduction in 2000, the PCV7 vaccine reduced childhood hospitalization rates by ~550 per 100,000 children per year and those in elderly adults aged 85 and older by ~1,300 per 100,000 cases a year. Even with the advent of the pneumococcal vaccines, however, the mortality and morbidity of pneumonia remains significant, as there are still high levels of hospitalizations and mortality. Worldwide, the WHO recommends vaccination for all children. Even so, vaccination rates are low, as only 25% coverage is reported globally (Organization, 2014; Organization, 2015).

While the PCV and PPSV vaccines have reduced disease associated with *S. pneumoniae*, only one other vaccine exists for other bacterial etiologies – *Haemophilus influenzae* type b (Hib). There are three different Hib vaccines available, and all are conjugate vaccines whereby, similar to the PCV, the capsular polysaccharide of Hib (PRP) is conjugated to immunogenic protein (Adams et al., 1993). One vaccine, PRP-D, utilizes the Hib PRP polysaccharide protein that is conjugated to a diphtheria toxoid conjugate, another similar conjugate used the diphtheria CRM197 protein as the conjugate (called HbOC), while the third is conjugated to a meningococcal protein (referred to as (PRP-OMP) (Adams et al., 1993). Besides the conjugate, they differ in the size of the polysaccharide used and the method of chemical conjugation. All three were approved by the Food and Drug Administration by 1989 and have been integrated into the pediatric vaccination schedule (Adams et al., 1993). Worldwide the Hib vaccines are also recommended for children under five (Organization, 2014). The WHO reports

global vaccination coverage of 52% (Organization, 2015). Currently, there are no other vaccinations available for other etiologies.

Sepsis

Clinical Definition and Significance

Sepsis, which is tightly linked to pneumonia (more below), is a complex and variable syndrome that is defined as a systemic inflammatory response to infection (Angus and van der Poll, 2013; Lagu et al., 2012). Differences in severity delineate the syndrome into four conditions. The systemic inflammatory response syndrome (SIRS) is defined as having two or more of the following symptoms: fever, rapid breathing (tachypnea), high heart rate (tachycardia), and high (leukocytosis) or low (leucopenia) white blood cell counts (Longo, 2012). Sepsis is defined as SIRS with a suspected or proven infection, and severe sepsis includes patients that have sepsis with one or more signs of organ failure. Septic shock, however, requires hypotension to be present (Angus and van der Poll, 2013; Longo, 2012).

Sepsis and its associated conditions are a major cause of mortality and morbidity in the United States and worldwide, as they are responsible for greater than 750,000 cases each year in the United States and an estimated 19 million cases around the globe (Adhikari et al., 2010; Angus and van der Poll, 2013; Cohen, 2002; Hotchkiss and Karl, 2003). This adds up to 2% of all hospital admissions, which is a huge economic burden. In fact, hospital costs for patients with severe sepsis amounted to $24.3 billion in 2007 and was averaged to around $20,000 per case (Lagu et al., 2012). Within the ICUs,

sepsis is to blame for 10% of admissions (Angus et al., 2001; Angus and van der Poll, 2013). Even with antibiotics and modern medicine, mortality rates remain high, hovering between 30 and 50% (Angus et al., 2001; Angus and van der Poll, 2013; Cohen, 2002; Hotchkiss and Karl, 2003).

Etiology

The primary site of infection varies among septic patients, but it is most frequently caused by respiratory infections, as 40-60% of all septic patients have causative respiratory infections (Alberti et al., 2002; Angus et al., 2001; Vincent et al., 2009). Thus, in addition to the important complications of pneumonia alone, its role in predisposing patients to sepsis makes it particularly deadly. Intra-abdominal and urinary tract infections are other major sites of origin (Alberti et al., 2002; Angus et al., 2001; Angus and van der Poll, 2013; Vincent et al., 2009). The bacterial species most commonly associated with sepsis are Gram-negative pathogens, such as *E. coli*, *Pseudomonas* species, and *K. pneumoniae*, which are detected in about 60% of all cases. Gram-positive bacteria were observed in around 40% of cases, with *S. aureus*, *S. pneumoniae*, and *Staphlococcus epidermidis* being the predominant species present (Opal et al., 2003; Vincent et al., 2009).

There are several risk factors associated with sepsis, the primary one being age. The incidence of sepsis increases dramatically with age, with 5.3 per 1,000 cases in adults age 60-64 increasing to 26.2 per 1,000 cases in the elderly over 85 years of age (Angus et al., 2001). The incidence of pneumonia alone in adults 85 years and over is

almost double, with 40 per 1,000 cases, demonstrating the link between the two conditions, as pneumonia is the number one cause of sepsis. Interestingly, gender plays an important role in susceptibility as well, with men having increased incidence and mortality rates than women of the same age (Angus et al., 2001). Underlying conditions and immunosuppression are also notable risk factors (Angus and van der Poll, 2013).

Pathology and Immune Response

The immune response during sepsis is comprised of both pro- and anti-inflammatory mechanisms that can contribute to pathogen clearance, tissue recovery, and risk of secondary infections, but can also lead to severe immunopathology (Angus and van der Poll, 2013). Bacteria and their immunogenic products (predominantly lipopolysaccharide [LPS] in Gram-negative infections) are recognized through PRRs on leukocytes and endothelial cells, and elicit a primary inflammatory response (Angus and van der Poll, 2013; Cohen, 2002). This response is characterized by immune cell activation (specifically of circulating monocytes and neutrophils), release of proinflammatory cytokines, complement and coagulation cascade activation, and eventually cellular necrosis (Angus and van der Poll, 2013; Cohen, 2002). Tissue damage is thought to be a result of proinflammatory processes aimed at clearing pathogens. Coagulation defects, including disseminated intravascular coagulation (DIC), are prominent features of severe sepsis and play an important role in perpetuating the cycle of inflammation. DIC, defined as aberrant intravascular fibrin deposition, is a condition caused predominantly by sepsis. It is brought about by excessive tissue factor

activity (Longo, 2012), which de-represses protein C (a coagulation inhibitor) and stimulates the coagulation cascade, eventually ending in fibrin deposition (Angus and van der Poll, 2013; Levi and van der Poll, 2010). These same proteases that mediate coagulation, also induce inflammation by activating protease-activated receptors (Angus and van der Poll, 2013; Cohen, 2002).

While the inflammatory response of sepsis causes great collateral damage and tissue injury, the secondary anti-inflammatory response needed to control inflammation paradoxically leads to reduced immunoresponsiveness and is thought to be equivalently dangerous. In fact, multiple studies have shown increased apoptosis of immune cells, expansion and increased activity of regulatory cells (such as myeloid-derived suppressor cells and T regulatory lymphocytes), and neuroendocrine regulation of the inflammatory response during the anti-inflammatory phase of sepsis. Together, this decreased immnuoresponsiveness induces an immunosuppressed state (Angus and van der Poll, 2013; Cohen, 2002; Hotchkiss et al., 2009; Hotchkiss et al., 2013; Reddy et al., 2001b). This immunosuppression leads to increased susceptibility to secondary infections, including hospital-acquired pneumonia, which, in turn, causes significant morbidity and mortality (Cohen, 2002; Dellinger et al., 2013; Hotchkiss et al., 2013).

Sepsis-Induced Immunosuppression

There have been multiple reports detailing the functional consequences of sepsis-induced immunosuppression. Post-mortem studies have revealed active opportunistic infections in patients who died from sepsis (Otto et al., 2011; Torgersen et al., 2009), and

peripheral blood mononuclear cells isolated from septic patients showed a dramatic decrease in proinflammatory cytokine production and responsiveness after *ex vivo* stimulation (Ertel et al., 1995; Munoz et al., 1991; van der Poll and Opal, 2008; van Dissel et al., 1998). Additionally, various immune cells, including B cells, both cluster of differentiation-4 (CD4) and CD8 T cells, and dendritic cells, necessary to mount an effective response against opportunistic infections, are more apoptotic in patients with severe sepsis. Moreover, prevention of immune cell apoptosis in mouse models has improved survival of sepsis, implicating cell death as a physiological mechanism associated with the pathogenesis of sepsis-induced immunosuppression (Hotchkiss et al., 2013; Reddy et al., 2001b). Other cellular and humoral antimicrobial functions, such as reactive oxygen species (ROS) generation, phagocytosis, and neutrophil chemotaxis and function are also impaired in septic patients (Angus and van der Poll, 2013; Cohen, 2002; Hotchkiss et al., 2013; Reddy et al., 2001a). Dysfunction of the immune system causes increased susceptibility to secondary, hospital-acquired infections like pneumonia.

Relationship to Pneumonia

Sepsis and pneumonia are both inflammatory diseases and are integrally linked. As stated above, pneumonia is the number one cause of sepsis, with 40% of septic patients having a primary respiratory infection (Angus et al., 2001; Vincent et al., 2009). Furthermore, patients with severe sepsis are more likely to develop pneumonia (Alberti et al., 2002), and ventilator-associated pneumonias develop in 10-30% of mechanically ventilated, septic shock patients (Chastre and Fagon, 2002). Multiple studies have shown

the deleterious effect of sepsis on pneumonia outcomes. Using different mouse models of sepsis, including cecal-ligation and puncture (CLP) and endotoxemia, different groups have shown increased mortality to various pulmonary bacterial challenges (Benjamim et al., 2010; Delano et al., 2010; Jung et al., 2012). Additionally, pulmonary host defense is compromised in these same models and was associated with decreased pulmonary cytokine responses and airway neutrophil recruitment (Benjamim et al., 2010; Cao et al., 2014; Carrick et al., 1997; Frevert et al., 1994; Nelson et al., 1990; Reddy et al., 2001a; Wagner et al., 1999; Wagner et al., 2002; White et al., 1986). Work by other groups using these same mouse models suggested that both interleukin-10 (IL-10) and IL-1 receptor associated kinase M mediate sepsis-induced immunosuppression of lung immunity (Deng et al., 2006; Reddy et al., 2001a; Steinhauser et al., 1999).

Treatments and Therapeutics

The current treatment recommendation for sepsis, septic shock, and severe sepsis is antibiotic therapy to control the infection as well as any supportive care necessary to stabilize the patient (Angus and van der Poll, 2013; Longo, 2012). Presently, there are no Food and Drug Administration approved therapeutics available for the treatment of sepsis. In the past 30 years, there have been multiple clinical trials focusing on severe sepsis treatments, but none have proven fruitful (Angus and van der Poll, 2013). These therapeutics were aimed at countering different arms of septic pathologies. For example, multiple trials were designed to counter the initial inflammatory cascade with either anti-LPS or anti-proinflammatory agents, while others more recently were intended to

modulate the coagulation cascade (Abraham et al., 1995; Angus and van der Poll, 2013). The most recent trial, involving an activated, recombinant protein C (Drotrecogin Alfa [Activated]), was approved after the initial trial showed an improvement in mortality among patients with severe sepsis (Dhainaut et al., 2003; Opal et al., 2003). It wasn't until a recent, second clinical trial in which the efficacy of Drotrecogin Alfa (Activated) was disproven, and the drug was taken off of the market (Angus and van der Poll, 2013; Ranieri et al., 2012). Both intravenous Ig and statins have been associated with improved outcomes of severe sepsis, but neither is currently in clinical trials or part of the standard of care (Dellinger et al., 2013; Laupland et al., 2007; Yende et al., 2011). It is unsurprising, however that one specific treatment or therapeutic has not been successful in improving sepsis outcomes for all patient groups, as sepsis is a very heterogeneous syndrome affecting numerous populations with a multitude of bacterial etiologies. Perhaps the most effective treatments will be tailored to specific population groups and utilize a number of combined therapies.

Innate Immunity During Pneumonia

Alveolar Macrophage Activation and Functions

Alveolar macrophages are considered the sentinel cells in airspaces and are the first line of leukocytic defense against respiratory infections. They are long-lived, phagocytic cells within the lungs that alert other leukocytes to the presence of microbes. Pathogens are recognized through various PRRs on the cell surface that recognize conserved motifs common to pathogens, the most well know being the Toll-like receptors

(TLRs) (Hussell and Bell, 2014; Mizgerd, 2008; Werner and Steele, 2014). There are currently 13 discovered TLRs, nine of which are active in both mice and humans, and each recognizing different pathogen associated molecular patterns such as LPS, flagellin and viral ribonucleic acids (RNAs) (Kawai and Akira, 2010; Takeda and Akira, 2005). Recognition of lower respiratory pathogens by PRRs has been comprehensively reviewed elsewhere (Eddens and Kolls, 2012; Mizgerd, 2008). TLRs are particularly relevant sensors for detecting bacteria in the lungs, as exemplified by ablated defenses in whole body knockout mouse model of MyD88, which is an adaptor protein that is shared amongst this family of receptors (Skerrett et al., 2004). TLR4 recognition of Gram-negative respiratory infections such as *E. coli* occurs primarily through recognition of LPS (Branger et al., 2004), while TLR2 senses Gram-positive *S. pneumoniae* infections through peptidoglycan, a bacterial cell wall component (Dessing et al., 2007; Knapp et al., 2004; Koedel et al., 2003; Lee et al., 2007; Mogensen, 2006). TLR2 can also activate macrophages through recognition of the acute phase protein (APP), serum amyloid A (SAA) (Cheng et al., 2008). Interestingly, pneumolysin, an important pneumococcal virulence factor, causes TLR4 activation, implying a synergistic effect of multiple PRRs (Lee et al., 2007; Malley et al., 2003). In addition to TLRs, macrophage receptor with collagenase structure is also important in initiating responses to pneumococcal infections, as knockout mice have decreased bacterial clearance and killing (Arredouani et al., 2004; van der Poll and Opal, 2009).

Microbe recognition by TLRs initiates a signaling cascade culminating in nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and interferon regulatory

factor activation, leading to the production of a myriad of proinflammatory factors including cytokines and chemokines. The importance of alveolar macrophage NF-κB signaling in initiating the immune response can be seen in a myeloid-specific NF-κB knockout. After a pneumococcal infection, cytokine responses and neutrophil recruitment are delayed (Pittet et al., 2011). Early response cytokines tumor necrosis factor-α (TNFα), IL-1, and interferon-γ, which are induced by NF-κB activation, act in an autocrine and paracrine fashion to initiate the inflammatory cascade in nearby macrophages and epithelial cells. This feed-forward loop results in the secretion of additional proinflammatory cytokines and chemokines such as CXCL1, CXCL2, and CCL2, prompting the recruitment of neutrophils and monocytes into the airspaces (Aggarwal et al., 2014; Herold et al., 2011; Mizgerd, 2008).

Along with recruited neutrophils, macrophages potentiate pathogen clearance. Alveolar macrophages not only coordinate immune responses from other cells, but they also directly eliminate bacteria through phagocytosis and release of noxious free radicals and proteases, such as reactive oxygen species (ROS), reactive nitrogen species, matrix metalloproteinases (MMPs), and lysozyme (Aggarwal et al., 2014; Mizgerd, 2008; Sibille and Reynolds, 1990). Secretion of these toxic substances, however, induces tissue injury and plays an important role in the development of ALI and ARDS (Aggarwal et al., 2014; Sibille and Reynolds, 1990; van der Poll and Opal, 2009). Following bacterial clearance, alveolar macrophages also function to promote tissue resolution and the clearance of apoptotic neutrophils (Bratton and Henson, 2011; Henson and Bratton, 2013; Herold et al., 2011; Sibille and Reynolds, 1990).

Neutrophils and Their Function in Pneumonia

Neutrophil recruitment is a hallmark of pneumonia and occurs early during infection. Both alveolar macrophages and epithelial cells play an important role in their recruitment, and pathogen clearance is not as efficient without them, as antibody depletion of neutrophils results in increased bacterial outgrowth and mortality following infection (Garvy and Harmsen, 1996; Kadioglu et al., 2000; van der Poll and Opal, 2009). Once alveolar macrophages sense pathogens in the airspaces, alveolar epithelial cells are stimulated by early response cytokines to induce additional inflammatory cytokines and chemokines, and these factors cooperate to drive neutrophil emigration into the airspaces (Aggarwal et al., 2014; Quinton and Mizgerd, 2015; van der Poll and Opal, 2009).

Once recruited, neutrophils are responsible for the majority of pathogen clearance from the alveolar space. Similar to macrophages, neutrophils can phagocytose bacteria and release antimicrobial molecules including ROS and proteases. Neutrophils are granulocytic cells that have four types of granules: azurophilic or primary granules, specific or secondary granules, gelatinase or tertiary granules, and secretory vesicles. Primary and secondary granules are released once neutrophils have entered the lungs and contain neutrophil elastase, myeloperoxidase (MPO), defensins, lactoferrin, lysozyme, MMPs and other bactericidal proteins (Borregaard and Cowland, 1997; Grommes and Soehnlein, 2011). While release of antimicrobial molecules is one of the main mechanisms of pathogen clearance, their toxic nature causes collateral tissue damage and

is a major cause of ALI (Grommes and Soehnlein, 2011; Mantovani et al., 2011; Tamakuma et al., 2004).

In addition, neutrophils have adapted a unique form of bacterial killing using neutrophil extracellular traps (NETs). Upon activation, granulocytes release genetic material laced with antimicrobial components, which trap and kill invading microbes (Brinkmann and Zychlinsky, 2007; Brinkmann and Zychlinsky, 2012). ROS activity seems to be necessary for this process and microscopy has confirmed that the release of NETs occurs through cell membrane rupture and eventual cell death, a process coined "NETosis". Moreover, NETs have been shown to bind to both Gram-negative and Gram-positive bacteria (Brinkmann and Zychlinsky, 2007; Brinkmann and Zychlinsky, 2012).

Cytokines/Chemokines

Cytokines and chemokines are the molecular messengers within the airspaces. As mentioned earlier, TNFα and IL-1 are early response cytokines, which act as alarm signals and induce the inflammatory cascade, and their importance during lung infections was shown before using various mouse models, as detailed above (Jones et al., 2005; Mizgerd et al., 2004; Mizgerd et al., 2001; van der Poll et al., 1997a). Additionally, evidence from a clinical trial for rheumatoid arthritis has shown the necessity for TNF and IL-1 to protect against pneumonia in humans, as combining the TNF receptor fusion protein (etanercept) with an IL-1 receptor antagonist (anakinra) significantly increased the risk of lung infection (Genovese et al., 2004). IL-6 is another major cytokine necessary for host defense during lung infections, as knockout mice show defects in host

defense (Jones et al., 2006; van der Poll et al., 1997b). Patients with a unilateral pneumonia are reported to have an increase in IL-6 in the involved lobe compared to uninvolved lobes (Dehoux et al., 1994), and IL-6 is increased in human subjects after instillation of endotoxin into the lungs (O'Grady et al., 2001). Moreover increases in IL-6 were associated with lymphocytosis in the bronchoalveolar lavage fluid (BALF) of patients with idiopathic interstitial pneumonia, suggesting that IL-6 regulates pulmonary inflammation during pneumonia in humans as well as mice (Park et al., 2000). Recently, IL-17 has gained more notoriety with its involvement in lung infections. Patients with Hyper IgE Syndrome have mutations in the transcription factor signal transducer and activator of transcription-3 (STAT3), which leads to an increased occurrence of lung infections in this patient population. These patients have defective T helper 17 cells and decreases in its main effector cytokine, IL-17, suggesting that this cytokine plays an important role in pulmonary resistance to infection (Eddens and Kolls, 2012; Mizgerd, 2003; Quinton and Mizgerd, 2015). Other cytokines dependent on STAT3 activity, leukemia inhibitory factor (LIF) and oncostatin-M (OSM), work upstream to activate STAT3, and are necessary for tissue protection and neutrophil recruitment, respectively, during *E. coli* pneumonia (Quinton et al., 2012b; Traber et al., 2015). Both have been implicated in human pneumonia, as LIF is increased in the BALF of patients with ARDS (Jorens et al., 1996), and OSM is produced by human neutrophils and is increased in the BALF of pneumonic patients (Grenier et al., 2001; Grenier et al., 1999). Multiple other cytokines, including the anti-inflammatory cytokine IL-10 and IL-1 receptor antagonist,

and their roles in acute respiratory infections have been reviewed by others (Eddens and Kolls, 2012; Mizgerd, 2008; Quinton and Mizgerd, 2015).

Chemokines are small molecules, similar to cytokines, that are necessary to recruit leukocytes to the site of infection (Standiford et al., 1996). Chemokines are classified based on the spacing of the cysteine residues in the N terminal region, and are denoted as being part of the C, CC, CXC, CX3C chemokine family, where the X denotes amino acids between the two cysteine residues. Both CXCL1 and CXCL2 are two important chemokines in pneumonia, as they are produced by a number of cell types and are the main chemokines responsible for neutrophil recruitment into the airways (Le et al., 2004; Mizgerd, 2008; Quinton and Mizgerd, 2015; Standiford et al., 1996). CXCL5, CCL20 and GM-CSF are alveolar epithelial cell-specific and also play an important role in granulocyte recruitment during bacterial pneumonia (Quinton and Mizgerd, 2015; Yamamoto et al., 2014; Yamamoto et al., 2012). In addition to the chemokines mentioned above, GM-CSF and granulocyte colony-stimulating factor (G-CSF) are growth factors necessary for maximal neutrophil recruitment. Both GM- and G-CSF control the mobilization and maturation of neutrophils from bone marrow and GM-CSF also has a significant effect on monocyte/macrophage recruitment (Quinton and Mizgerd, 2011; Quinton and Mizgerd, 2015; Shi et al., 2006). In fact, G-CSF restores circulating neutrophil counts in patients with neutropenia (Welte et al., 1996) and is increased after endotoxin delivery to the airways in humans (O'Grady et al., 2001). Standiford, *et al.* has reviewed other chemokines and their roles in lung infections (Standiford et al., 1996).

Acute Phase Response

History and Definition

The acute phase response (APR) is a conserved hepatic response that is defined as significant (at least 25%) increases or decreases in circulating APP concentrations. The APR was first observed in 1930 with the discovery of a pneumococcus-reactive serological fraction (fraction "C", later named C-reactive protein [CRP]) in samples from pneumonic patients (Tillett and Francis, 1930). This protein and others would later be coined as acute phase-reactants (Abernethy and Avery, 1941; MaCleod and Avery, 1941; McCarty, 1947). The APR is commonly used as a biomarker, but its functional and physiological role has yet to be elucidated. CRP continues to be utilized as a biomarker for infection and cardiovascular disease (Cals et al., 2009; Du Clos, 2000; Ridker, 2008), and other APPs, including SAA, serum amyloid P (SAP), LPS-binding protein (LBP) and ferritin have been used as biomarkers for diseases including cancer, sepsis and Alzheimer's (Armstrong, 2006; Beard et al., 2006; de Torre et al., 2006; Le, 2005; Sakr et al., 2008; Urieli-Shoval et al., 2000; Verwey et al., 2008). The functional properties of multiple APPs have been identified, including several of which are particularly relevant to immunity and inflammation (see below). But the physiological significance of the APR as a whole has yet to be elucidated, especially in the context of lung infections.

Functions of Select, Relevant Acute Phase Proteins

Historically, there have been over 40 liver-derived APPs identified, each with its own individual function (Gabay and Kushner, 1999; Moshage, 1997; Suffredini et al., 1999). APPs can be categorized based on their function, many of them playing a role in the innate immune response (Table 1). The complement system, one major, well-appreciated group of APPs, is crucial in pathogen detection and in induction of the inflammatory cascade (Bode et al., 2012; Doan, 2013; Gabay and Kushner, 1999; Suffredini et al., 1999). This group consists of opsonins (C3), pathway activators and regulators (mannose-binding lectin, C4, factor B, C4b-binding protein, and C1 inhibitor), and parts of the membrane-attack complex (C9), which is necessary for bacterial lysis (Bode et al., 2012; Suffredini et al., 1999). Another group important for pathogen recognition and inflammation are the secreted pathogen recognition receptors and opsonins. Short pentraxins, like CRP and SAP, as well as LBP are among the most widely recognized (Bode et al., 2012; Du Clos, 2000; Suffredini et al., 1999; Wurfel, 1995). LBP plays a major role in TLR4 activation by bringing LPS together with CD14 and TLR4 to help initiate inflammation (Akira and Takeda, 2004; Chow et al., 1999). Other APPs, namely SAA, have been implicated in leukocyte activation, including that mediated by TLR2 and induce phagocytosis (Anthony et al., 2013; Bode et al., 2012; Cheng et al., 2008; Shah et al., 2006; Suffredini et al., 1999; Uhlar and Whitehead, 1999). Another group of APPs are those necessary for inducing and regulating the coagulation cascade. Prothrombin, fibrinogen, factor VIII, antithrombin, plasminogen, and others are important activators of the clotting response, while proteinase inhibitors such as α_1-

antichymotrypsin, antitrypsin, and α_2-macroglobulin can have regulatory effects. Metal chelating and transport proteins, like haptoglobin, hemopexin, and ferritin are also considered APPs whose function is to sequester important metals necessary for bacterial growth (Bode et al., 2012; Gabay and Kushner, 1999; Moshage, 1997; Suffredini et al., 1999).

Table 1. Functions of select, relevant APPs

APP	Function
Complement (C3, Manose Binding Lectin, C4, Factor B, C1 Inhibitor, C9)	Opsonization of Pathogens, pathway regulators, bacterial lysis
C-reactive protein	Opsonin, complement activation, mediates phagocytosis
Serum Amyloid A	Leukocyte activation (some through TLR2)
Serum Amyloid P	Opsonin, leukocyte activation
LPS-Binding Protein	Recognizes and binds LPS, brings LPS together with CD14 to induce TLR4 activation
Fibrinogen, prothrombin, antithrombin, etc.	Key regulators of the coagulation cascade
Lipocalin2, hemopexin, haptoglobin	Metal chelators, sequester minerals necessary for bacterial growth

Regulation of the Acute Phase Response

The hepatic APR is mainly regulated by two major pathways: those downstream of IL-6 and those initiated by the early response cytokines IL-1 and TNFα. Indeed, knockout mouse models show this to be true, as IL-6 deficient mice challenged with

turpentine or *Listeria monocytogenes* had ablated APP production (Kopf et al., 1994), and a similar APP defect was seen following turpentine or LPS treatment in either IL-1 receptor 1 (IL-1R1) deficient, TNF receptor 1 (TNFR1) and TNFR2 double knockout, or IL-1β deficient mouse models (Leon et al., 1996; Leon et al., 1997; Zheng et al., 1995). IL-6 and early response cytokines predominantly signal through STAT3 and NF-κB RelA (also know as p65), respectively, and the necessity of these two transcription factors in the regulation of APP expression has been shown through multiple promoter analysis and biochemical studies. For instance, deletions of STAT3 or NF-κB promoter sites in SAP and SAA, respectively, rendered these promoters inactive (Betts et al., 1993; Hagihara et al., 2005; Ochrietor et al., 2000), and hepatocytes lacking NF-κB activity show reduced CRP expression (Patel et al., 2007). Additionally, endotoxin-induced APP expression is significantly reduced in conditional STAT3 knockouts (Alonzi et al., 2001), and treatment with TNFα, IL-1 and IL-6 can induce APP expression in mice (Agrawal et al., 2003; Betts et al., 1993; Gabay and Kushner, 1999).

The studies detailed above implicate IL-6 and early response cytokine signaling (through STAT3 and NF-κB, respectively) as the major regulators of the hepatic APR, and thus led to the creation of a double knockout mouse model of APR deficiency. To establish this, STAT3 and RelA were specifically and effectively targeted for deletion in hepatocytes using the Cre-*LoxP* system. Mice bearing homozygous floxed alleles for *Stat3* and *RelA* (Algul et al., 2007; Takeda et al., 1998) were crossed onto a background containing a transgenic Cre-recombinase under the transcriptional control of an albumin promoter (Alb-Cre$^{tg/-}$/ *Stat3*$^{LoxP/LoxP}$/ *RelA*$^{LoxP/LoxP}$) (Quinton et al., 2012a). Consequently,

hepatic APP changes were virtually abolished in response to all stimuli tested, including intrapulmonary bacterial infections, intravenous cytokines, and subcutaneous casein (a sterile irritant known to induce the APR), indicating that hepatocyte STAT3 and RelA function cooperatively to induce the liver APR (Quinton et al., 2012a).

Kinetics of the Acute Phase Response

The induction of the APR happens relatively quickly in response to an inflammatory stimulus, and can vary depending on the type of stimulus itself. During an *E. coli* lung infection, liver STAT3 and NF-κB activation peak by six hours of infection, with activation observed after only two hours of infection. This liver activation was associated with induction of hepatic APP transcripts after only six hours of infection (Quinton et al., 2009). APP induction is occurs more slowly in response to a pneumococcal lung infection, as liver STAT3 and RelA activation peaked around 15 hours of infection. Additionally, liver APP transcript induction after *S. pneumoniae* infection was similarly observed as early as 15 hours post infection (Quinton et al., 2009). APR stimulation also occurs within six hours after endotoxemia, as STAT3 activation is observed 4.5 hours post-i.p. LPS injection with APP induction by six hours (Alonzi et al., 2001). These studies suggest, just as its name does, that the APR occurs quickly and acutely after inflammation. Importantly, the liver responses we have observed are consistent with an earlier release of the required upstream cytokines, TNF,

IL-1, and IL-6, supporting the critical role for these cytokines in eliciting a downstream hepatic APR (Quinton et al., 2009).

Acute Phase Response During Pneumonia: The Lung-Liver Axis

In addition to initiating an APR, IL-6, TNFα, and IL-1 are critical determinants of lung defense. Using triple mutant mice, lacking all signaling receptors for TNFα and IL-1 ($TNFR1^{-/-}/TNFR2^{-/-}/IL\text{-}1R1^{-/-}$), Jones *et al.* showed the necessity of these early response cytokines during pneumonia to induce the inflammatory cascade and drive neutrophil recruitment to the lungs, and other groups have used the same mouse model and a neutralizing TNFα antibody during pneumonia to show similar results (Jones et al., 2005; Mizgerd et al., 2004; Mizgerd et al., 2001; van der Poll et al., 1997a). Moreover, IL-6 deficient mice show defects in bacterial clearance and lung defense after pulmonary infection with either *E. coli* or *S. pneumoniae* (Jones et al., 2006; van der Poll et al., 1997b). The importance of these pulmonary host defense cytokines in the induction of the hepatic APR was elucidated using these same mouse models. Intrapulmonary LPS treatment induced systemic and BAL APP protein and hepatic APP mRNA expression in wildtype mice. This response was IL-6-dependent, as systemic IL-6 neutralization or IL-$6^{-/-}$ mice had diminished hepatic APP expression and plasma APP concentrations (Gamble et al., 2009; Vernooy et al., 2005). Furthermore, APR inhibition during both Gram-negative, *E. coli* and Gram-positive, *S. pneumoniae* pneumonias in IL-$6^{-/-}$ mice was associated with decreased liver STAT3 activation (Quinton et al., 2009), and similarly, triple mutant mice also exhibited defective APP induction after *S. pneumoniae* and *E. coli*

pneumonias that was associated with decreased hepatic NF-κB RelA nuclear translocation (Quinton et al., 2009). In humans with Hyper IgE syndrome, polymorphisms in STAT3 render it transcriptionally inactive (Holland et al., 2007; Minegishi et al., 2007). Because of this, these patients suffer from repeated bacterial pneumonias, mostly due to *S pneumoniae*, *H. influenzae*, and *S. aureus*, suggesting that STAT3 in humans is necessary to mount effective pulmonary defense (Freeman et al., 2007). The ability of these specific host defense cytokines to facilitate both lung defense and hepatic APP expression suggests a role for liver activation in the local immune response.

Presently, there are remarkably few studies that were designed to evaluate the net, functional role of the APR during inflammation of any kind, including that triggered by lung infections. A handful of groups have shown functional consequences of the APR during inflammation. Hepatic STAT3-dependent APPs were protective in response to both systemic LPS and a CLP model of sepsis, as hepatocyte $STAT3^{-/-}$ mice had increased inflammatory cytokine production (Sakamori et al., 2007). Other studies interrogated the role of a turpentine-induced, preexistent APR on pulmonary inflammation and host defense. After pulmonary challenges with *P. aeruginosa* or *A. baumannii*, Renckens *et al.* observed impaired host defense and pulmonary cytokine responses in pretreated mice, suggesting that a preexistent APR dampens local immune responses (Renckens et al., 2006; Renckens et al., 2008). While informative, these specific studies induce an APR through turpentine injection, the offhand affects of which are unknown. By utilizing the above-mentioned APR-null mouse model, Quinton *et al*.

determined the necessity of the APR during pneumococcal pneumonia for survival and systemic host defense. APR-null mice had significantly decreased opsonophagocytosis and bacterial complement deposition in the circulation, leading to increased bacteremia in hepSTAT3/RelA$^{-/-}$ mice lacking STAT3 and RelA in hepatocytes (Quinton et al., 2012a). The data suggest that not only does the APR function to limit bacterial dissemination during pneumonia, but also that key lung defense cytokines (IL-6 and the early response cytokines) activate hepatic STAT3 and RelA to induce the APR, forming a "lung-liver axis" (Figure 1). However, those studies were not specifically empowered to observe effects of the APR on lung inflammation itself due to the highly virulent strain of pneumococcus used.

Other studies have interrogated the role of specific APPs in pneumonia. Infections in LBP deficient mice show its necessity to facilitate bacterial clearance and inflammation after intrapulmonary *K. pneumoniae* and LPS, respectively (Branger, 2004; Brass et al., 2004; Fan et al., 2002; Knapp et al., 2006), while other groups have shown that LBP over-expression reduces mortality and improves respiratory function during Gram-negative pneumonia (Hemmila et al., 2006). Additionally, mice deficient in SAP could not mount an early inflammatory response during Gram-positive *S. pneumoniae* pulmonary infection (Yuste et al., 2007) and, in a mouse model of pulmonary fibrosis, SAP$^{-/-}$ mice had increased pulmonary fibrosis and inflammation (Pilling and Gomer, 2014). Complement has also been determined as a necessary component of pulmonary host defense and inflammation (Czermak et al., 1998; Younger et al., 2003). While these studies are notable, the knockout mouse models used are whole body and not liver-

specific, making it hard to delineate the specific role of liver-derived APPs during lung infections. Additionally, individual APP deletions would affect baseline levels and changes due to the APR itself, and deletion of just one APP does not ablate the entire APR, again making it difficult to elucidate the function of the hepatic APR as a whole. Even with these limitations, these studies suggest that the APR and its individual APPs play a more biologically important role in lung infections than previously appreciated, indicating a need for a deeper understanding of the role of the APR as a whole (in contrast to individual APPs) in lung biology.

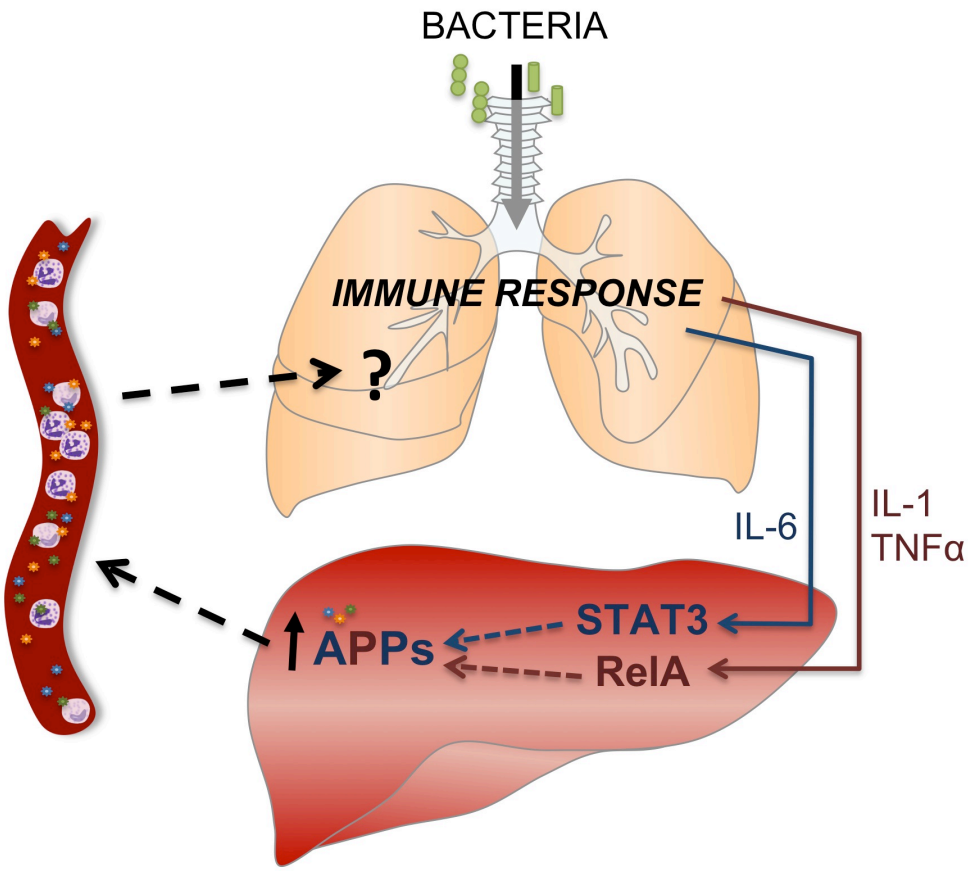

Figure 1. The Lung-Liver Axis.

Upon entrance into the lower respiratory tract, pathogens initiate an immune response that includes cytokine production and leukocyte recruitment. Cytokines critical to pulmonary defense, IL-6, TNFα, and IL-1, then activate key hepatic transcription factors, STAT3 and NK-κB RelA, respectively, to induce the expression and production of APPs, thus initiating the APR. The various APPs then circulate throughout the body, possibly to the original site of infection, where they can gain access to injured airspaces. The function of the APR as a whole during lung infections is poorly understood.

Specific Aims and Hypothesis

As described earlier, the hepatic APR is a virtually ubiquitous response to inflammation or injury, including that caused by pneumonia and/or sepsis. Specific functions of individual APPs have been recognized, but other than its common use as a biomarker, the biological role of the APR has yet to be elucidated, especially in pneumonia. Induction of the APR requires critical pulmonary host defense cytokines to activate key hepatocyte transcription factors, STAT3 and NF-κB RelA, suggesting that pneumonia induces a systemic response in the liver by effectively creating a lung-liver axis. Our laboratory has recently created an APR-null mouse model in which hepatocyte STAT3 and RelA were functionally deleted. This mouse model revealed a systemic necessity for the APR in protecting against bacterial dissemination during pneumococcal pneumonia, but these studies were not empowered to determine the role of the APR on local inflammation and host defense in the lungs themselves. Additionally, other groups have shown the requirement of hepatic STAT3 activation for survival during sepsis, but none have interrogated whether or how this response influences pneumonia biology. Uncovering a functional role of the hepatic APR will provide valuable avenues of research for potential therapeutics, prognostics, or diagnostics during both pneumonia and sepsis, conditions where present treatments are sorely lacking. Furthermore, the liver is a more targetable organ than the lungs and has great potential for small molecule modulation of the APR to help patients with or at risk for pneumonia. Thus, the goal of this research was to understand the functional role of the liver APR in lung biology during pneumonia, along with whether or how such a response can be modified in the

context of sepsis. We propose that circulating plasma proteins, including APPs, extravasate into the injured airspaces during infection and regulate the pulmonary immune response. It is feasible that circulating APPs gain access to the infected airspaces as serum components flood injured lungs. Our rationale is based largely on studies in which the APR is chemically induced prior to pneumonia and is shown to regulate the inflammatory response. Taken together, these findings led us to **hypothesize that the APR plays a functional, protective role in the pulmonary immune response during pneumonia**. This dissertation is divided into two main research aims. In the first, we determine if the APR maintains pulmonary defense and inflammation during *E. coli* pneumonia, and whether this is modulated in a cell-specific manner. In the second, we aim to interrogate the direct influence of a pre-existing, endotoxemia-induced APR on pulmonary host defense in the clinically relevant setting of sepsis followed by pneumonia.

CHAPTER TWO: MATERIALS AND METHODS

Mouse Models

Mouse experiments were performed using hepatocyte-specific functional deletions of either STAT3 alone or STAT3 in combination with RelA using the Cre-*LoxP* system driven by an albumin promoter. This is a well-established system that is specific to hepatocytes and enables Cre-recombinase gene expression (and gene targeting) by 6 weeks of age (Postic and Magnuson, 2000). We have previously verified the deletion of STAT3 and RelA liver protein using this system (Quinton et al., 2012a). Mixed sexes were used between 6 and 12 weeks of age and each experiment was performed at least twice. All animal protocols were approved by the Boston University Institutional Animal Care and Use Committee.

Hepatocyte STAT3$^{-/-}$ Mice

Dr. S. Akira generated and provided our laboratory with floxed STAT3 mice (Stat3$^{LoxP/LoxP}$). Exon 21 of *Stat3*, which contains the tyrosine residue necessary for dimerization and therefore its function, is flanked by *LoxP* insertions (Takeda et al., 1998). Mice with homozygous floxed alleles for *Stat3* were crossed with mice containing a Cre-recombinase transgene under the transcriptional control of an albumin promoter (purchased from Jackson Labs) to create a functional deletion of STAT3 specifically in hepatocytes (Alb-Cre$^{tg/-}$/*Stat3*$^{LoxP/LoxP}$). Results from hepSTAT3$^{-/-}$ mice were compared to littermate controls lacking the Cre-recombinase transgene (Alb-Cre$^{-/-}$/*Stat3*$^{LoxP/LoxP}$).

Hepatocyte STAT3/RelA$^{-/-}$ Mice

Dr. R. Schmid generated and provided our laboratory with floxed *RelA* mice (*RelA$^{LoxP/LoxP}$*), in which exons 7-10 of NF-κB RelA are flanked by *LoxP* insertions. These exons encode the nuclear localization sequence and part of the Rel homology domain required for activation (Carpenter et al., 2012). The floxed *RelA* mice were crossed with both *Stat3* floxed mice and with mice containing the transgenic albumin-driven Cre-recombinase to create two stable colonies: Alb-Cre$^{tg/-}$/ *Stat3$^{LoxP/LoxP}$*/ *RelA$^{LoxP/LoxP}$* and Alb-Cre$^{-/-}$/ *Stat3$^{LoxP/LoxP}$*/ *RelA$^{LoxP/LoxP}$*. The latter genotype was used as control mice, as they do not express Cre-recombinase, and were compared against littermates of the former genotype lacking both STAT3 and RelA in hepatocytes.

Bacterial Stocks

Two bacterial strains were used for all of the infections: *E. coli* serotype 06:K2:H1 (American Type Culture Collection, #19138), which was utilized for the majority of the studies, and *E. coli* Xen14 (Caliper [now Perkin Elmer], #119223), which is a luminescent strain derived from the parental *E. coli* WS2572 strain that contains a stable copy of the entire *lux* operon from *Photorhabdus luminescens* in the bacterial chromosome. Infections were all done with the non-luminescent *E. coli* unless otherwise stated.

Bacterial stocks were made by growing bacteria overnight on sheep blood agar plates (BD Biosciences) or lysogeny broth (LB-also known as Luria broth) agar plates with 30 μg/ml of kanamycin (for Xen14 only) to select for the luminescent colonies.

Sixteen hours later, colonies were picked from the plates using sterile pipette tips and grown to log phase in sterile LB (with kanamycin for the Xen14 strain) at 37°C, shaking at 300 revolutions per minute (rpm). Once broth was cloudy and grown to mid-log phase (for about 4 hours), 700 μl aliquots were mixed with 300 μl of a 50% sterile glycerol solution to make a 1 ml bacterial stock in a final concentration of 16% glycerol. The aliquots were then snap frozen in liquid nitrogen and stored at -80°C.

Mouse Infections

Intratracheal Instillations

Mice were anesthetized by intraperitoneal (i.p.) injection of a mixture of ketamine (50 mg/kg) and xylazine (5 mg/kg). Once unconscious, a single incision was made above the trachea and the tissue was cleared away to expose it. A 24-gauge catheter was then inserted into the trachea, and a 50 μl bolus of saline containing *E. coli* was instilled into the left lobe to create a lobar infection. Two different inoculums of *E. coli* were used in these studies: a milder inoculum of $4x10^5$ CFU or a more severe inoculum of $1x10^6$ CFU. Inoculums were confirmed by culturing serial dilutions of the instillate on blood agar plates overnight at 37°C. Mice were euthanized 0-48 hours post infection by isoflurane overdose.

Endotoxemia/Pneumonia Two-Hit Model

HepSTAT3$^{-/-}$ and hepSTAT3$^{+/+}$ (control) mice were i.p. injected with 5 mg/kg of ultrapure LPS 18 hours before intratracheal (i.t.) instillation of 10^6 CFU of *E. coli*. Mice were then euthanized 0-24 hours after i.t. infection by isoflurane overdose (Figure 2).

Intravenous anti-TNF Treatment

HepSTAT3/RelA$^{-/-}$ or control hepSTAT3/RelA$^{+/+}$ mice were treated with a 100 μl, 5 mg/ml bolus of either a control IgG (Bio X-cell, clone HRPN) or an anti-TNFα antibody (Bio X-cell; clone XT3-11) by tail vein injection immediately prior to i.t. *E. coli*. Mice were then sacrificed 24 hours post-infection by isoflurane overdose.

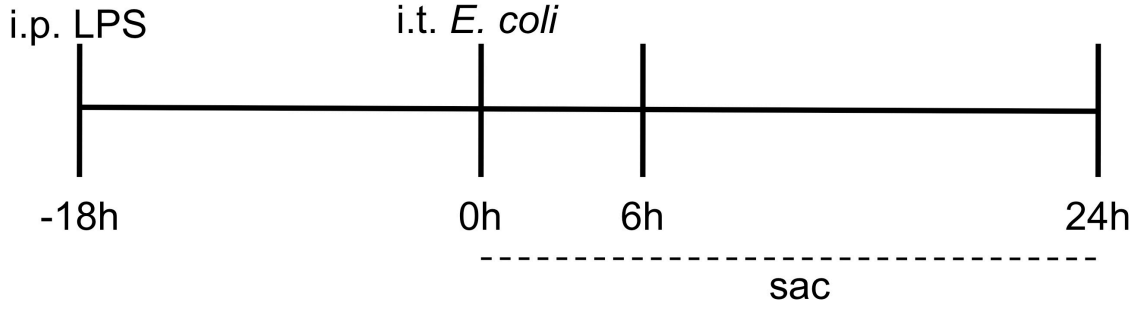

Figure 2. Two-Hit Endotoxemia/Pneumonia Model

Mice were pretreated with 5 mg/kg of LPS intraperitoneally 18 hours before intratracheal infection with 10^6 CFU of *E. coli*. Mice were then sacrificed 0, 6, or 24 hours after *E. coli* infection for various endpoint analyses.

Endpoint Tissue Collection

Brochoalveolar Lavage

Mice were euthanized at the indicated time points and the lungs were removed. The trachea, with the lungs and heart still attached, was slid over a 20-gauge blunted, stainless steel catheter, which was connected to a stop-cock with two different syringes attached—one filled with phosphate buffered saline (PBS) or lavage buffer (indicated in each specific method) used to wash the airspaces and another empty one used to collected the lavage fluid (Figure 3).

Once secured to the catheter with surgical suture (Roboz Surgical), the lungs were lavaged 10 times with 1 ml of PBS or lavage buffer used per wash, with the first lavage collected in a separate 1-ml syringe. All washes were then centrifuged at 300 x g for 5 minutes at 4°C to pellet the cells. The supernatant from the first lavage was aliquoted and stored at -80°C for protein analysis. Pooled cells from all washes were counted using a hemacytometer and differential counts were determined after cytocentrifugation and Diff-Quick (Dade-Behring) staining (Figure 4).

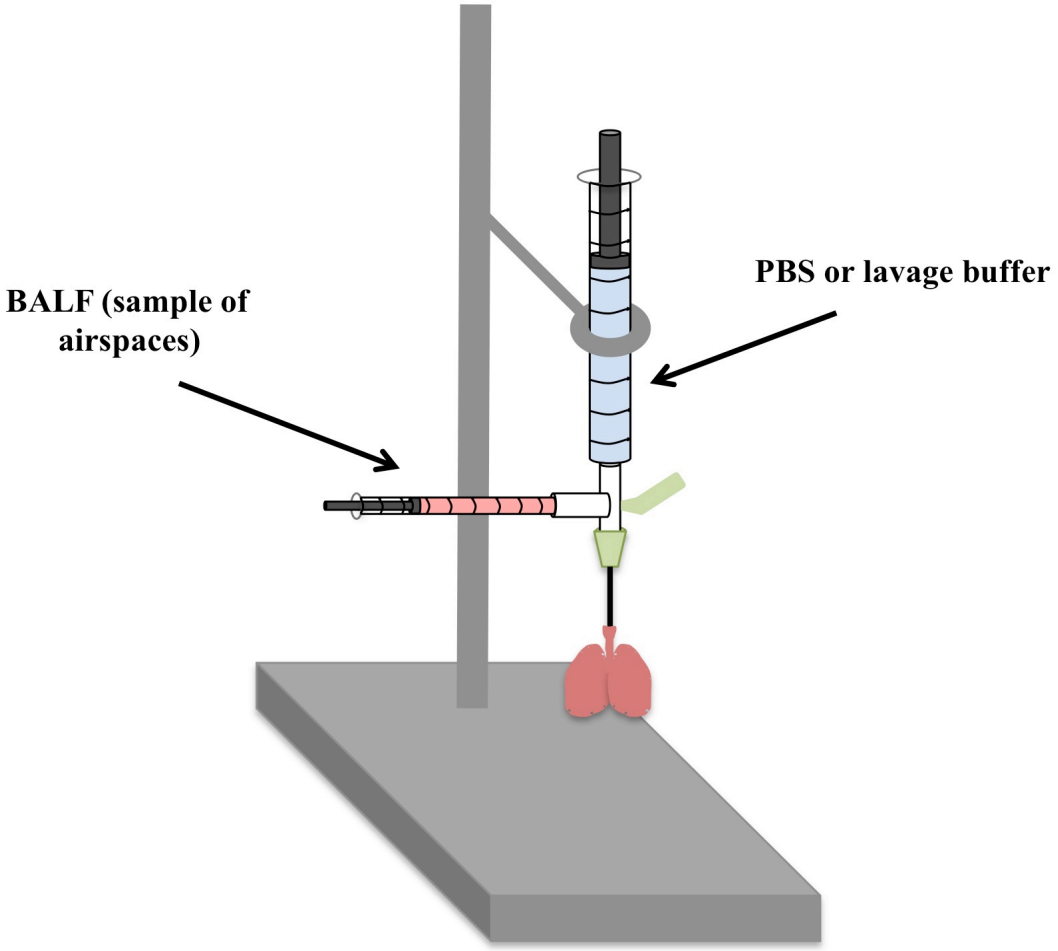

Figure 3. Schematic of BAL setup

The lungs were tethered to a stainless steel catheter that was attached to a stopcock connected to two different syringes. The syringe immediately above the lungs was filled with PBS or lavage buffer, which was pushed into the airspaces on milliliter at a time. The stopcock was then turned to allow the BALF to be pulled back from the lungs into the second syringe on the left.

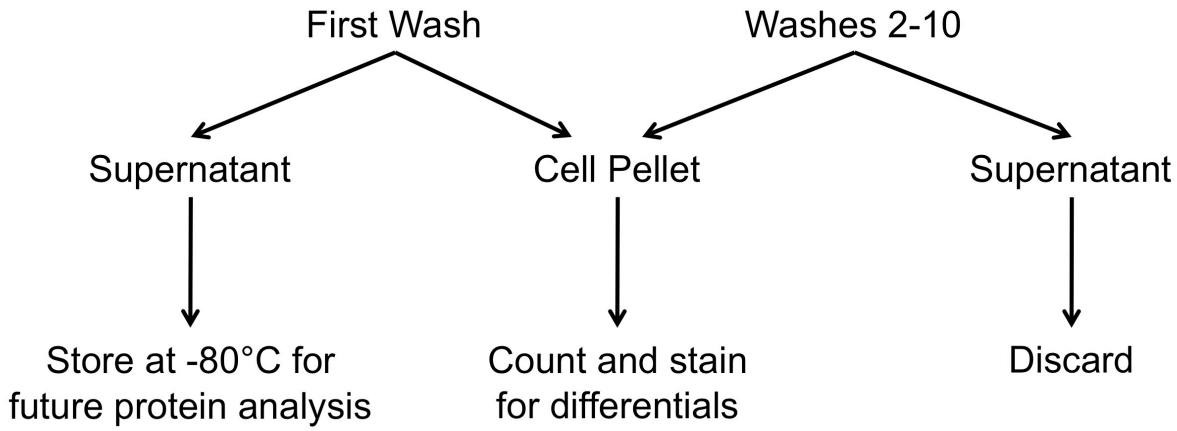

Figure 4. BALF Processing Flow Chart

The first wash of the lungs was collected separately from the other 9 washes. Both sets of washes were then centrifuged at 300 x g for 5 min at 4°C. The cell-free supernatant from the first wash was aliquoted and then stored at -80°C for future analysis, while the supernatant from the remaining washes were discarded. The cells from all washes were pooled, counted, and centrifuged onto a slide using a Cytospin. Following staining, differential numbers of neutrophils and macrophages were determined.

Blood Collection

After euthanasia, blood was collected from the anterior vena cava with a 25-guage needle. After collection, the blood was incubated in MiniCollect Z Serum Separator tubes (Greiner Bio-One) for 30 minutes at room temperature and then centrifuged for 15 min at 1500 x g and 4°C for serum separation. The serum was collected, aliquoted, and stored at -80°C for future protein analysis. For CFU analysis, the needle was heparinized and no serum separation occurred.

Lung and Blood CFU Analysis

At the indicated time points, mice were sacrificed and lung lobes were removed without the connective tissue and put into Bullet Blender 5 ml tubes containing 7-9 3.2-mm diameter stainless steel Bullet Blender beads (Next Advance) and 400 µl of 1x protease inhibitor (Roche) in sterile water. Once homogenized using the Bullet Blender (Next Advance), lung homogenates were brought up to 5 ml with the 1x protease inhibitor solution. Homogenates and heparinized blood were then serially diluted in sterile water and plated on sheep blood agar plates. After an overnight incubation at 37°C, colonies were counted and expressed as total CFU per lung or per milliliter of blood.

RNA Isolation

Lung RNA Isolation

After lavage, the left lobe was isolated and homogenized in buffer RLT (from Qiagen RNeasy kit) and RNA was isolated using the Qiagen RNeasy kit following the manufacturers instructions.

Liver RNA Isolation

The same liver lobe was isolated from each mouse and stored in RNAlater (Qiagen) at -20°C indefinitely. To isolate RNA, livers were homogenized using 7-9 2.0-mm zirconium oxide Bullet Blender beads (Next Advance) in Trizol Reagent (Life Technologies) and the manufacturer's instructions were followed. After isolation, the RNA was cleaned using the RNeasy kit and stored at -80°C.

Quantitative Real Time PCR

Quantitative real time PCR (qRT-PCR) was performed on 100 ng of extracted RNA using a CFX96 Real-Time System (Bio-Rad) and TaqMan RNA-to-C_T 1-step kit (Applied Biosystems). Primer and probe sequences for SAA1, SAP, IL-6, TNFα, IL-1β, CXCL1, CXCL2, G-CSF, and 18s are listed in Table 2. Probes were labeled on the 5' end with FAM dye and Black Hole Quencher-1 at the 3' end. Each sample was normalized to 18s ribosomal RNA content and expressed as fold induction compared to uninfected mice, unless otherwise noted.

Table 2: Primer and Probe Sequences for qRT-PCR

Gene	Forward Primer	Reverse Primer	TaqMan Probe
IL-6	AGTTGCCTTCTTGG GACTGATG	CAGGTCTGTTGGGA GTGGTATC	AACCACGGCCTTC CCTACTTCACA
SAA1	GAGGACATGAGGAC ACCATTGC	CCAGAGAGCATCTT CAGTGTTCC	AGGAAGAAGCCCA GACCCCACCCT
SAP	CACACTTTTGTTCCA CACCCAAG	TCTGAAAGAAGGCT GGTGAAGAC	CTGCTGCTGTCATA CCCTGGGCCA
LBP	CTTTGTGATCCTGCC CACCTC	TCAGTCTCACTTGTG CCTTGTC	CCTGTCTTCCGGCT TGGCGTGGTC
TNFα	TCATACCAGGAGAA AGTCAACCTC	TGGAAGACTCCTCC CAGGTATATG	TGCCGTCAAGAGC CCCTGCCCC
IL-1β	AGTTCCCCAACTGG TACATCAG	TCAATTATGTCCTG ACCACTGTTG	ACCTCACAAGCAG AGCACAAGCCT
CXCL1	ACCCAAACCGAAGT CATAGCC	TGGACAATTTTCTG AACCAAGGG	CTTCAGGGTCAAG GCAAGCCTCGC
CXCL2	ATCCAGAGCTTGAG TGTGACG	TTAGCCTTGCCTTTG TTCAGTATC	CCTACTGCGCCCA GACAGAAGTCA
G-CSF	TTCCCCTGGTCACTG TCAGC	CACAGCTTGTAGGT GGCACAC	ACCATCCCTGCCTC TGCC
18s	ATTCGAACGTCTGC CCTATCA	GTCACCCGTGGTCA CCATG	TCGATGGTAGTCG CCGTGCCTACC

Protein Measurements

Single-Plex Enzyme-Linked Immunosorbent Assays (ELISAs)

APP protein measurements were done by single-plex ELISAs. SAA and SAP ELISAs were purchased from Immunology Consultants Laboratory, Inc. Lipocalin-2 (LCN2) and CRP DuoSet kits were purchased from R&D Systems, whereas LBP kits

were purchased from Cell Sciences, Inc. The ELISAs were performed following the manufacturers' instructions.

Multi-Plex Bead Array

Serum and BALF cytokine protein concentrations from each individual mouse were determined using a Bio-plex 200 workstation (Bio-Rad) in conjunction with a Bio-plex cytokine bead array (Bio-Rad). Included in the panel were IL-1β, IL-6, IL-10, IL-17, G-CSF, GM-CSF, CXCL1, LIF, CXCL2 and TNFα. The assay was performed following the manufacturer's protocol.

Bicinchoninic Acid (BCA) Assay

BALF total protein concentrations from each mouse were determined using the BCA assay (Sigma-Aldrich). BSA was used as the protein standard at four different concentrations, 0, 0.2, 0.6, and 0.9 mg/ml, and the manufacturer's instructions were followed to determine total protein concentrations of BALF as an index of pulmonary injury and of liver homogenates for immunoblots.

Alanine Aminotransferase (ALT) Assay

Serum ALT levels from individual mice were determined using Liquid ALT Reagent Set (Pointe Scientific, Inc.). Serum was diluted 10-100 fold in saline and 100 μl was added to 1 ml of pre-warmed reagent. Absorbance at 340 nm was read at minute intervals over 3 minutes and levels were calculated based on the manufacturer's instructions.

Blood Urea Nitrogen (BUN) Assay

BUN was measured in serum using the QuantiChrom Urea Assay Kit from BioAssay Systems following the manufacturers instructions.

Western Blotting

The same liver lobe from each mouse was isolated and homogenized in protein extraction buffer with 7-9 2.0-mm zirconium oxide beads (Next Advance) using the Bullet Blender. Samples were then incubated on ice for 15 min with occasional vortexing to mix, followed by centrifugation for 20 min at 15,000 x g and 4°C to separate cellular debris. The supernatants were aliquoted and stored at -80°C. Total protein concentrations were measured by BCA assay as described above. Immunoblotting was performed using the NuPAGE SDS polyacrylamide gel electrophoresis (PAGE) system (Novex, Life Technologies). Samples were diluted to 20 μg of protein with NuPage lithium dodecyl sulfate sample buffer (Life Technologies), NuPage Reducing Agent (Life Technologies), and ultrapure water. After boiling for 10 min at 70°C, 15 μl of diluted sample and Novex Sharp protein standard (Life Technologies) were loaded onto a 12% Bis-Tris gel (Life Technologies) in 1X 3-propansulfonic acid (MOPS) SDS running buffer and resolved at 200 V for 50 min or until the dye front was at the bottom of the gel. Protein was then transferred onto an Immobilon-P, polyvinylidene fluoride (PVDF) membrane (Millipore) in 1X NuPage transfer buffer (Life Technologies) with 5% methanol at 30 V for one hour. Following transfer, membranes were blocked in blocking

solution for one hour, rocking at room temperature. After washing with TBS-T, the membrane was probed with a primary antibody (see Table 3 for a list of antibodies used, their concentrations and time of incubation). After another wash in TBS-T, primary antibodies were detected using an anti-rabbit-HRP conjugated secondary antibody (Cell Signaling, #7074) at a 1:2000 dilution in blocking buffer. Protein bands were then visualized using the ECLPlus Western Blotting Detection System (GE Healthcare). Membranes were exposed to film (GE Healthcare) for 0.5, 1, or 2 min. Once bands were visualized, membranes were stripped of their antibodies using Re-Blot Plus Mild (Millipore) for 20 min, and then re-probed for loading controls as described above. For cleaved caspase-3, densitometry was performed using ImageJ software (National Institutes of Health). Densitometric values for each sample were normalized to its actin control band. Cleaved-caspase 3:actin ratios were then compared to the HepSTAT3/RelA$^{+/+}$/IgG control group to determine percent control changes in band intensity.

Table 3: Primary Antibodies Used for Western Blotting

Antibody	Species	Company and Catalogue #	Dilution	Incubation Time
Anti-Cleaved Caspase-3	Rabbit Monoclonal	Cell Signaling, Asp175, Clone 5A1E, #9664	1:1000	Overnight at 4°C
Anti-Pan Actin	Rabbit Polyclonal	Cell Signaling, #4968	1:1000	1h at RT

Liver Immunohistochemistry

Liver Hematoxylin and Eosin (H & E) Staining

Livers were isolated and immediately fixed in 10% buffered formalin (EMD Chemicals) indefinitely. Once fixed, tissue sections were put into tissue cassettes and given to the Boston University School of Medicine Experimental Pathology Laboratory Service Core for processing.

Paraffin Embedding for Immunohistochemistry

The same liver lobe was isolated from each mouse, cut into three sections, and immediately incubated in 50-ml conical tubes filled with 4% paraformaldehyde (PFA) in PBS overnight. The next morning the PFA was poured off and livers were incubated in the specified solutions for the indicated times and temperatures in Table 4. Surgipath Paraplast Plus paraffin (Leica Biosystems) was used in all of the embeddings (Fisher Scientific). After the last paraffin incubation in the vacuum, the tissue sections were put into 24 x 24 x 5 mm molds (Fisher Scientific), and cooled overnight at room temperature (RT) and then at 4°C to create the paraffin blocks. Once cool, the blocks were sectioned into 5 μm thick sections using a microtome and adhered to microscope slides using a warm water bath.

Table 4: Paraffin Embedding Protocol

Solution	Incubation Time	Temperature
PBS	30 min	on ice
0.85% NaCl	30 min	on ice
1:1 ethanol: 0.85% saline	30 min	RT
2X 70% ethanol	30 min each	RT
80% ethanol	45 min	RT
90% ethanol	46 min	RT
3X 100% ethanol	30 min each	RT
3X xylene	30 min each	RT
1:1 xylene: paraffin	1.5 h	60°C
2X paraffin	1 h each	60°C vacuum

Ki67 Immunohistochemistry on Liver Sections

Following deparaffinization (see Table 5), liver sections were subjected to antigen retrieval by boiling samples in antigen unmasking solution (Vector) in a microwave for 3 incubations of 5 min each at a low power. After allowing the samples to cool for 30 min at RT in the same solution, slides were washed with PBS for 10 min and endogenous peroxidases were quenched with a 3% hydrogen peroxide solution in methanol (Fisher Scientific) for 15 minutes at RT. Following another wash with PBS for 15 min, sections were blocked in a 1:200 dilution of normal donkey serum (Jackson ImmunoResearch Laboratories, Inc.) in PBS for 45 min at RT. Sections were then stained for Ki67

overnight at 4°C using a primary, rabbit monoclonal, anti-Ki67 antibody (clone SP6, Abcam) at a 1:100 dilution in PBS. The next day, following a 10 min wash in PBS, sections were incubated for 45 min at RT in a biotin-conjugated donkey anti-rabbit IgG secondary (Jackson ImmunoResearch Laboratories, Inc.) at a 1:200 dilution in PBS and then washed for another 10 min in PBS. The secondary was then conjugated to HRP using the Vectastain ABC reagent kit (Vector) for 30 min at RT, washed for 10 min in PBS, and then developed with the DAB enzyme substrate kit (Vector). Development time was kept standard for each sample (1 min). The slides were then counterstained with hematoxylin, dehydrated by following Table 5 in reverse, and mounted with coverslips using Cytoseal XYL (Thermo Scientific). Morphometric analysis was performed on stained sections by determining the number of cells positive for Ki67 per number of cells counted.

Table 5: Deparaffinization Protocol

Solution	Incubation Time
2X Xylene	5 min each
2X 100% Ethanol	2 min each
90% Ethanol	1 min
70% Ethanol	1 min
50% Ethanol	1 min
Distilled Water	5 min

Flow Cytometry

Fluorescence-Activated Cell Sorting (FACS)

BALs were performed with ice-cold lavage buffer and heparinized blood was collected after 15 hours of infection with 4 x 10^5 CFU of *E. coli.* BAL cells were pelleted after centrifugation for 5 min at 300 x g and 4°C followed by resuspension in 100 μl FACS buffer for staining. Each blood sample was aliquoted into 2, 200-μl aliquots for red blood cell (RBC) lysis. 1 ml of 1X lysis buffer (BD Pharm Lyse, BD Biosciences) was added to each sample and incubated at RT for 2 min, with occasional vortexing. Ten ml of PBS was then added to each sample to stop the lysing reaction, cells were pelleted by centrifugation for 5 min at 300 x g and 4°C, and then cells from the same samples were pooled and resuspended in 100 μl of FACS buffer for staining. Surface antigens were stained by adding 20 μl of each diluted antibody (diluted in FACS buffer + FC Block [BD Biosciences]) from Table 6 to each sample. After a 30 min incubation on ice and in the dark, the cells were washed by adding 4 ml of PBS to each tube, followed by pelleting by centrifugation. Blood samples were resuspended in 200 μl of FACS buffer, while BALF samples were resuspended in 300 μl of FACS buffer for analysis. Blood samples were sorted for circulating neutrophils and monocytes, and BAL samples were sorted for airspace neutrophils and airspace macrophages. Surface markers for each cell type are listed in Table 7. Single stained bead controls were utilized for gating and compensation, which was performed using the auto-compensation feature in the FACS Diva program that is used to operate the sorting machine. Cells were sorted using the BD

FACS Aria into PBS containing 1% BSA, centrifuged at 300 x g for 5 min at 4°C and then resuspended in 1 ml of Trizol reagent. RNA was isolated following the manufacturer's protocol and qRT-PCR was performed as described above.

Table 6: Antibodies Used for FACS Analysis

Antibody	Clone	Fluorophore	Dilution	Company
CD45	30-F11	PE-Cy7	1:1000	Biolegend
Ly6G	1A8	APC-Cy7	1:800	Biolegend
F4/80	CI:A3-1	Pacific Blue	1:50	Biolegend
CD11b	M1/70	APC	1:400	eBioscience
CD115	AFS98	PE	1:200	Biolegend
7AAD	N/A	N/A	5 ul /sample	Biolegend

Table 7: Cell Surface Markers Used for FACS Analysis

Cell Type	Surface Antigens
Airspace Macrophage	$CD45^{+}/7AAD^{-}/F4/80^{+}/Ly6G^{-}/Autofluorescence^{hi}$
Airspace Neutrophil	$CD45^{+}/7AAD^{-}/Ly6G^{+}/F4/80^{-}$
Circulating Monocyte	$CD45^{+}/7AAD^{-}/CD115^{+}/CD11b^{+}$
Circulating Neutrophil	$CD45^{+}/7AAD^{-}/Ly6G^{+}/CD11b^{+}$

BALF Neutrophil Live/Dead Gating

Mice were infected for 15 hours with 4 x 10^5 CFU of *E. coli*, the lungs were lavaged with ice-cold lavage buffer, and cells were collected. Cells were then stained for identification of BAL neutrophils as described in Table 6 and flow cytometry was performed as described above using the BD LSRII machine (BD Biosciences). Dead neutrophil percentages were obtained by utilizing FlowJo to gate on $CD45^+/7AAD^+/Ly6G^+$ cells in the BALF.

pHrodo Phagocytosis Assay

BALF cell phagocytosis was measured using red pHrodo *E. coli* bioparticles (Life Technologies), which fluoresce only in low pH environments (such as the phagolysosomal compartment). Lyophilized pHrodo bioparticles were suspended in 250 µl of PBS and sonicated for five minutes to create a single-particle suspension. $HepSTAT3^{-/-}$ and $hepSTAT3^{+/+}$ mice were pretreated with 5 mg/kg of i.p. ultrapure LPS 18 hours before i.t. infection with 10^6 CFU of *E. coli*. Six hours after i.t. *E. coli*, mice were instilled with a 50 µl bolus of the pHrodo bioparticles and, after an hour incubation, the lungs were lavaged with ice-cold lavage buffer. Cells were then stained to identify airspace neutrophils and macrophages as described above and analyzed using the LSRII flow cytometer. Phagocytosis (PE fluorescence) was examined in each cell type using FlowJo software (Figure 5).

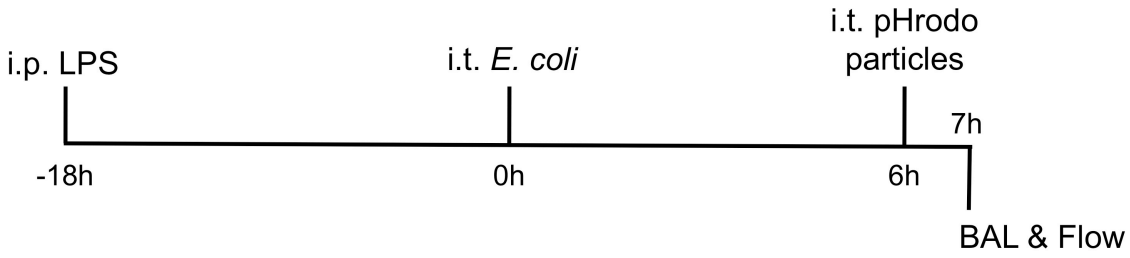

Figure 5. pHrodo Phagocytosis Assay Model

HepSTAT3$^{-/-}$ and control hepSTAT3$^{+/+}$ mice were pretreated with 5 mg/kg of ultrapure LPS intraperitoneally 18 hours prior to i.t. instillation of 10^6 CFU of *E. coli*. After 6 hours of *E. coli* infection, mice were instilled a second time with pHrodo bioparticles. An hour later, lungs were lavaged and phagocytosis was examined using flow cytometry.

ROS Generation Analysis

Mice were treated with i.p. LPS for 18 hours, followed by i.t. *E. coli* (10^6 CFU). Six hours after *E. coli* infection, the lungs were lavaged using ice-cold lavage buffer. Using the CellROX Deep Red Reagent, BAL cells were stained for ROS generation at the same time as surface antigen staining (Life Technologies) with 5 μM of reagent for 30 min on ice. Airspace neutrophils and macrophage ROS generation was analyzed using FlowJo.

BALF Bacterial Growth Assay

Luminescent *E. coli* Xen14 was diluted to $1x10^6$ CFU/ml in PBS. Cell-free BALF from mutant and hepSTAT3$^{+/+}$ mice infected for 0, 6, or 24 hours after i.p. LPS pretreatment was aliquoted into a 96-well plate (90 μl/well) and then incubated with 10 μl of the bacterial suspension, resulting in an initial concentration of $1x10^5$ CFU/ml in each well. Before and after a 5 hour incubation rotating at 37°C, bacterial luminescence (as an indicator of bacterial growth) was measured using a luminometer (Turner BioSystems). Growth was calculated as fold increases based on the starting luminescent values. No viable bacteria were detected from the aliquoted BALF.

Detection of NETs (MPO-DNA ELISA)

A 96-well pate was coated with 5 μg/ml anti-MPO antibody (rabbit polyclonal, catalogue number ab9535, AbCam) overnight at 4°C, washed with PBS, and then blocked for 2 hours at room temperature with 5% BSA in PBS. After washing with PBS, 50 μl of cell-free BALF from hepSTAT3$^{-/-}$ and hepSTAT3$^{+/+}$ mice infected for 0, 6, or 24

hours after LPS injection was added to the plate and incubated while shaking at room temperature for 2 hours. After washing with wash buffer, a peroxidase-labeled anti-DNA monoclonal antibody, diluted 1:100 in 1% BSA in PBS (from Cell Death Detection ELISAPlus Kit, Roche, catalogue number 11774425001), was added and the plate was incubated for another 2 hours. The wells were then washed with wash buffer and 100 µl of ABTS solution (also from Cell Death Detection ELISAPlus Kit) was added for 45 minutes at room temperature in the dark. The optical density of the plate was recorded at a wavelength of 405 nm.

Serum Stimulation Assays

Ex Vivo Serum Treatment Assay

Alveolar macrophages were collected by BAL from uninfected C57BL/6 mice. After lavaging with PBS, macrophages were pelleted after centrifugation for 5 min at 300 x g and 4°C and washed twice in 1 ml of ice-cold, FBS-free RPMI media (with 1% Pen-Strep). Cells were then plated onto a 48-well plate at a concentration of 250,000 cells/well and allowed to adhere for 1 hour at 37°C and 5% CO_2. After macrophages had adhered, RBCs and other non-adherent cells were washed away with 500 µl of complete RPMI media, and the macrophages rested overnight in complete RPMI media at 37°C and 5% CO_2. The next morning, cells were washed with PBS and then stimulated with either 100 ng/ml of ultrapure LPS or 10^5-10^2 CFU/ml of *E. coli* in the presence of 1% serum from either hepSTAT3/RelA$^{+/+}$ or hepSTAT3/RelA$^{-/-}$ mice infected for 15 hours with 4 x 10^5 CFU of *E. coli* in antibiotic- (for *E. coli* stimulation only) and FBS-free

RPMI media. LPS-stimulated cells were incubated for 4 hours. For bacterial stimulations, *E. coli* were washed off with FBS-free RPMI media after 2 hours, and cells were cultured for another 2 hours in media containing the same 1% serum from mice as described above. RNA was then collected by removing the media, aliquoting 1 ml of Trizol directly into each well, and pipetting up and down to release and lyse the cells. RNA isolation was performed according to Trizol's instructions and stored at -80°C. qRT-PCR was performed and expression of IL-6, TNFα, and CXCL1 were analyzed.

Buffer Recipes

Lavage Buffer for BAL Cell Collection

2.7 mM Ethylenediaminetetraacetic acid (EDTA), 20 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic Acid (HEPES), and 100 U/ml Penicillin-Streptomycin (Pen-Strep) in Hanks Balanced Salt Solution (HBSS) –This buffer was used instead of PBS as indicated.

Protein Extraction Buffer

25 mM Tris at pH 7.4, 50 mM Sodium Chloride, 0.5% Sodium Deoxycholate, 2% NP-40, 0.2% Sodium Dodecyl Sulfate (SDS), and 1x Roche Complete Protease Inhibitor in deionized water

Tris Buffered Saline-Tween (TBS-T)

25 mM Tris at pH 8.0, 125 mM Sodium Chloride, 0.1% Tween-20 in deionized water

Blocking Buffer

5% Non-Fat Milk in TBS-T

Fluorescence-Activated Cell Sorting (FACS) Buffer

0.5% Fetal Bovine Serum (FBS) and 2 mM EDTA in PBS – Filter Sterilized

MPO-DNA ELISA Wash Buffer

1% Bovine Serum Albumin (BSA) and 0.05% Tween-20 in PBS

Roswell Park Memorial Institute (RPMI) Complete Media

10% FBS and 1% Pen-Strep in RPMI (with L-glutamine) –Filter Sterilized

Statistical Analysis

All statistical analyses were done using GraphPad Prism 6.0 (GraphPad). CFU data are illustrated as individual values with medians, whereas the remaining data are shown as means ± SEM. Two groups were compared using either a student's *t* test or a Mann-Whitney test, while multiple group comparisons were conducted using either a one- or two-way analysis of variance (ANOVA), followed by specific post hoc tests (Dunn's test for multiple comparisons or the Holm-Sidak test). Data were considered significant if $p \leq 0.05$ for all experiments.

CHAPTER THREE: THE ACUTE PHASE RESPONSE FACILITATES MOUSE SURVIVAL, HEPATOPROTECTION, AND PULMONARY INFLAMMATION DURING *E. COLI* PNEUMONIA

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Rationale

The hepatic APR is stimulated by inflammation or injury, including pneumonia (Gabay and Kushner, 1999; Quinton et al., 2012a; Quinton et al., 2009), and is defined as a significant change in the circulating concentrations of the various APPs. Each APP has a specific function, but the wholesale function of the APR has yet to be elucidated, especially during lung infections (Gabay and Kushner, 1999; Moshage, 1997; Suffredini et al., 1999). Upon entrance into the lungs, microbial killing is mediated by the innate inflammatory response. This complex response is largely mediated by cytokine production and leukocyte recruitment. Proper immune activation required for pathogen clearance, however, can result in ALI by compromising epithelial barrier integrity, thus

allowing for plasma protein (and perhaps APP) extravasation into the airspaces (Mizgerd, 2008).

Previous work from our laboratory has shown that critical pulmonary host defense cytokines, IL-6, TNFα, and IL-1, are necessary for APR induction during bacterial pneumonia, suggesting a lung-liver axis that promotes innate immunity (Quinton et al., 2009). Furthermore, hepatic STAT3 and NF-κB RelA transcription factor activation, which is necessary for APP expression, was dependent on these cytokine pathways (Quinton et al., 2012a; Quinton et al., 2009; Quinton et al., 2008). Based on these findings, our laboratory generated an APR-null mouse model in which hepatocyte STAT3 and RelA were specifically deleted. These mice are phenotypically normal under resting conditions, but after infection display an ablated APR (Quinton et al., 2012a). Previous studies using this model of APR deficiency showed the necessity of the APR for systemic host defense during a Gram-positive pneumonia (Quinton et al., 2012a). While these studies show an important function of the APR as a whole, they were not empowered to determine the role of the APR in lung inflammation due to the high virulence of the pneumococcal strain used (serotype 3). In this aim, we utilized distinct inocula of *E. coli* to precisely and comprehensively determine how the APR affects lung inflammation and host defense during Gram-negative pneumonia.

Results

3.1 The APR is necessary for survival during pneumonia.

To determine the role of liver-specific acute phase changes in response to Gram-negative bacterial lung infections, we used our previously established mouse model in which Cre-recombinase under transcriptional control of an albumin promoter drives the deletion of floxed STAT3 and RelA alleles (Quinton et al., 2012a). This strategy severs the lung-liver axis, ablating gene expression changes in response to all conditions so far tested (Quinton et al., 2012a). HepSTAT3/RelA$^{-/-}$ or control hepSTAT3/RelA$^{+/+}$ mice were infected intratracheally with 10^6 CFU of *E. coli*, strain O6:K2:H1. *E. coli* is amongst several species of Gram-negative enterobacteria that are common and relevant causes of nosocomial pneumonia (Ahmed and Niederman, 2001; El Solh et al., 2007; Jones, 2010). Additionally, *E. coli* is a well-established model of Gram-negative lung infections in mice (Balamayooran et al., 2012; Mei et al., 2010; Quinton et al., 2008; Yamada et al., 2011), and was particularly desirable in our current study due to its ability to be consistently titrated to cause varying degrees of inflammation. *E. coli* causes a self-resolving or severe pneumonia, depending on dose, that causes little to no systemic dissemination and can also elicit significant airspace edema. All of which enable us to effectively study the influence of exudate APPs on localized lung inflammation and host defense without the potentially confounding effects of disseminated bacteria on liver stimulation. Of particular importance to this study is our previous finding that *E. coli* pneumonia induces a robust liver response that occurs independently of dissemination, and is instead dependent on signaling from IL-6, TNFα, and IL-1 (Quinton et al., 2009).

After infection, we observed that hepSTAT3/RelA$^{-/-}$ mice succumbed to the infection at a significantly greater rate than their littermate controls, indicating that hepatic transcription is necessary for survival following what is revealed here as a severe and lethal *E. coli* infection in mutant mice (Figure 6).

3.2 The APR is Dependent on Hepatic STAT3 and RelA Activation

In order to determine the direct relationship between survival and the pneumonia-induced APR, we measured liver mRNA induction (Figure 7) and circulating concentrations (Figure 8) of five representative APPs: SAA, SAP, LBP, CRP, and LCN2. As anticipated, hepSTAT3/RelA$^{+/+}$ mice had a large APP response (liver mRNA and circulating protein) that was completely ablated in hepSTAT3/RelA$^{-/-}$ mice, suggesting that the APR is dependent on hepatocyte STAT3 and RelA activation during *E. coli* pneumonia, consistent with studies already published (Quinton et al., 2012a).

We next sought to determine the ability of APPs to extravasate into infected airspaces where they would be positioned to directly modulate inflammation and host defense, possibly contributing to lethality in hepSTAT3/RelA$^{-/-}$ mice. To this end, BALF protein levels of all APPs measured were markedly elevated in response to infection in control hepSTAT3/RelA$^{+/+}$ mice (Figure 9). As observed in the circulation, SAA protein concentrations were significantly reduced in hepSTAT3/RelA$^{-/-}$ mice (Figure 9A). Lung mRNA induction of SAA1 was unaffected by genotype (Figure 10A), suggesting that differences in BALF protein concentrations were likely due to differences in delivery from the circulation.

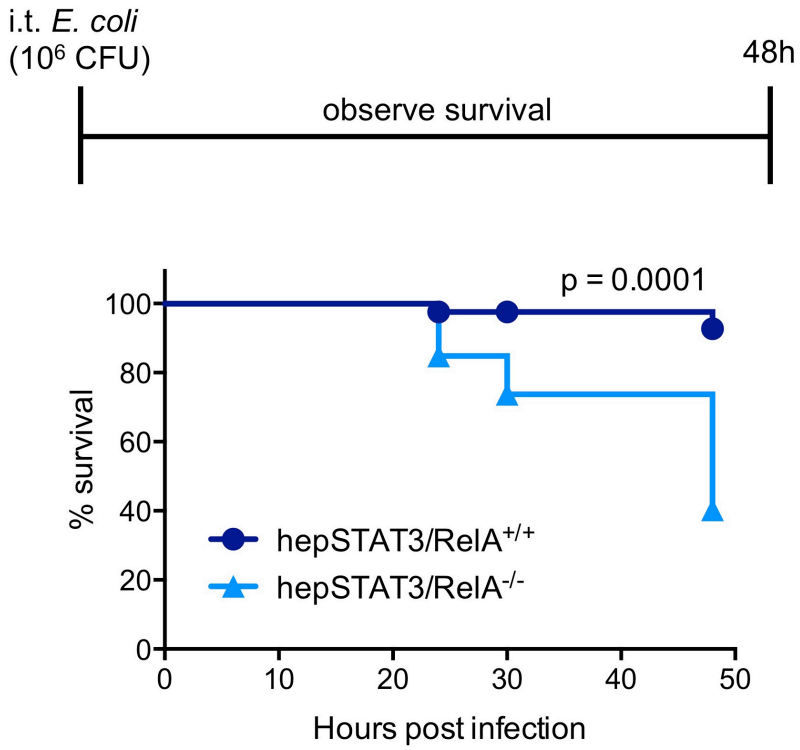


Figure 6. The APR is necessary for survival during a high inoculum *E. coli* pneumonia.

HepSTAT3/RelA$^{-/-}$ and control hepSTAT3/RelA$^{+/+}$ mice were infected intratracheally with 1 x 10^6 CFU of *E. coli*. Survival was observed and significance was assessed with a Mantel-Cox test. The two curves were deemed significant if $p < 0.05$ (n = 33 hepSTAT3$^{+/+}$ and 41 hepSTAT3/RelA$^{-/-}$).

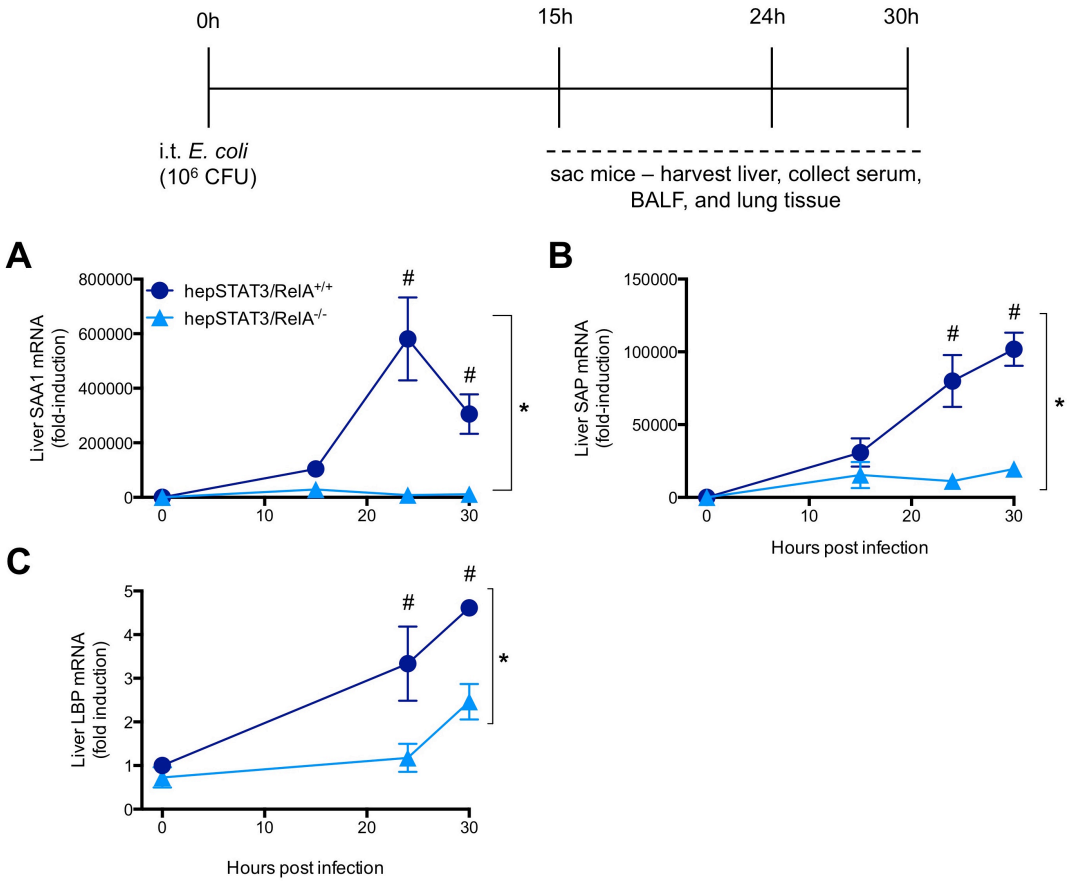


Figure 7. Liver expression of APPs during a high inoculum *E. coli* pneumonia is dependent on hepatic STAT3 and RelA.

Control hepSTAT3/RelA$^{+/+}$ and hepSTAT3/RelA$^{-/-}$ mice were infected intratracheally with 1 x 10^6 CFU of *E. coli*. At the indicated time points, the liver was homogenized and RNA was extracted. Induction of SAA1, SAP, and LBP was analyzed by qRT-PCR. *$p < 0.05$ for overall effect of genotype as determined by 2-way ANOVA. # $p < 0.05$ vs hepSTAT3/RelA$^{+/+}$ mice at the indicated time point as determined by a Holm-Sidak post hoc test (n = 3-8 per group).

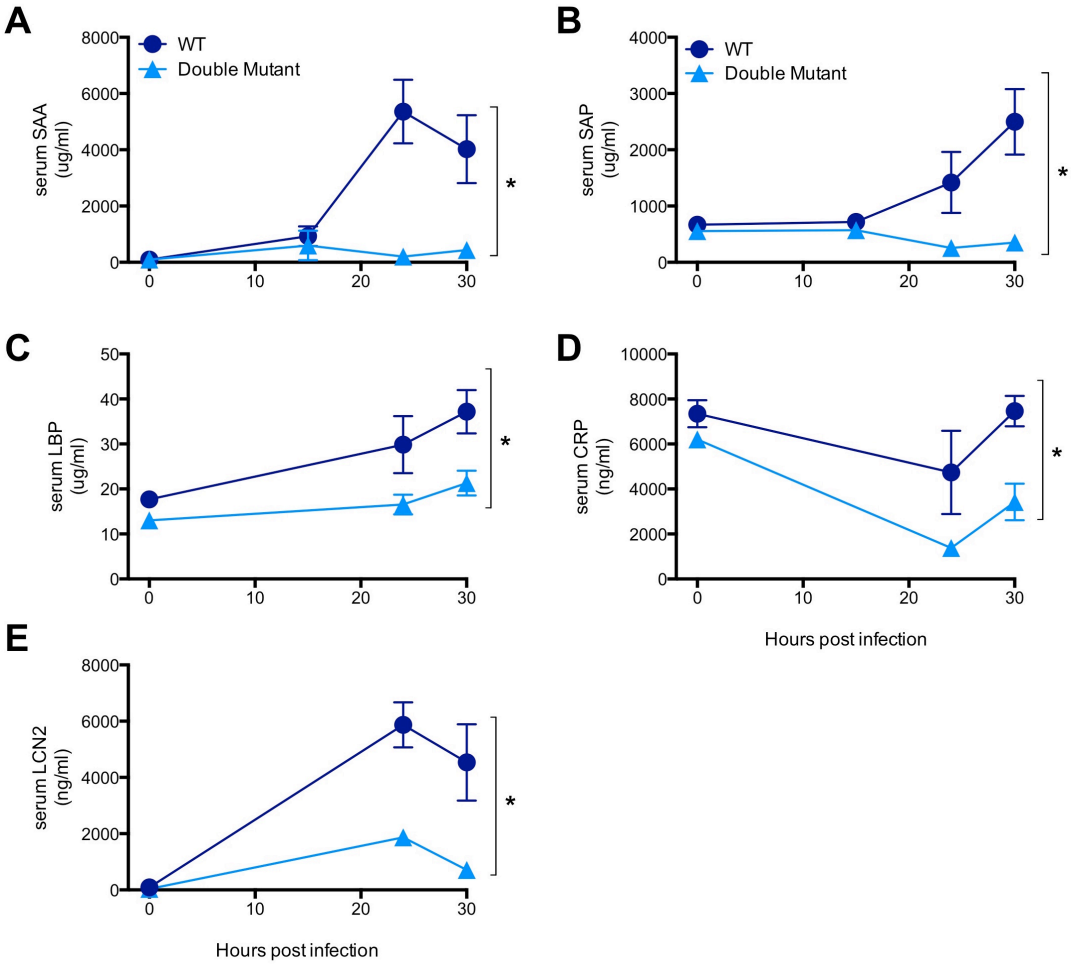


Figure 8. Liver STAT3 and RelA are necessary for induction of circulating APP concentrations during a high inoculum *E. coli* pneumonia.

Control hepSTAT3/RelA$^{+/+}$ and hepSTAT3/RelA$^{-/-}$ mice were infected intratracheally with 1 x 10^6 CFU of *E. coli*. At the indicated time points, serum was collected and APP concentrations were determined by ELISA. *$p < 0.05$ for overall effect of genotype as determined by 2-way ANOVA. # $p < 0.05$ vs hepSTAT3/RelA$^{+/+}$ mice at the indicated time point as determined by a Holm-Sidak post hoc test (n = 3-8 per group).

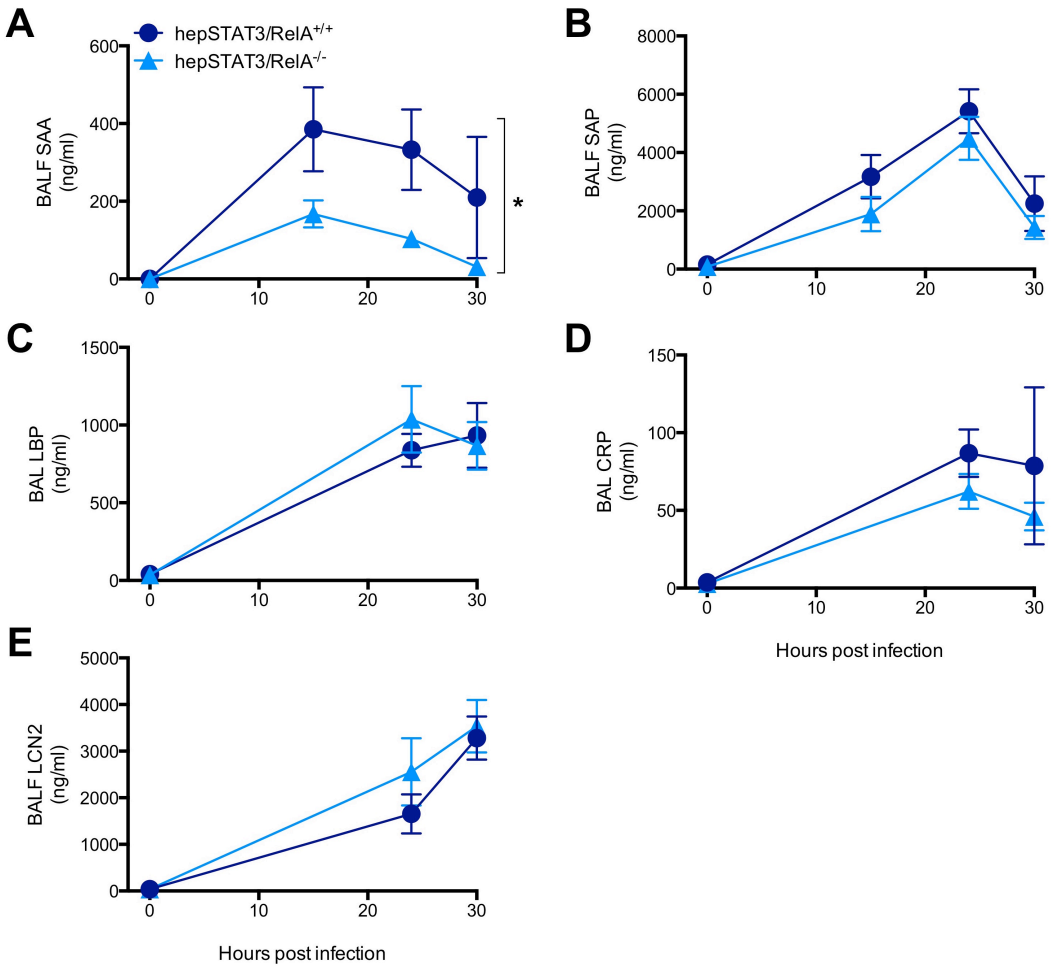


Figure 9. Intrapulmonary APP content is influenced by systemic acute phase changes.

HepSTAT3/RelA$^{-/-}$ and control hepSTAT3/RelA$^{+/+}$ mice were intratracheally inoculated with 1x10^6 CFU of *E. coli*. At the indicated time points, the lungs were lavaged with PBS and BALF was collected. APP concentrations in the BALF were measured by ELISA. * $p < 0.05$ for overall effect of genotype as determined by 2-way ANOVA. # $p < 0.05$ vs hepSTAT3/RelA$^{+/+}$ mice at the indicated time point as determined by a Holm-Sidak post hoc test (n = 3-8 per group).

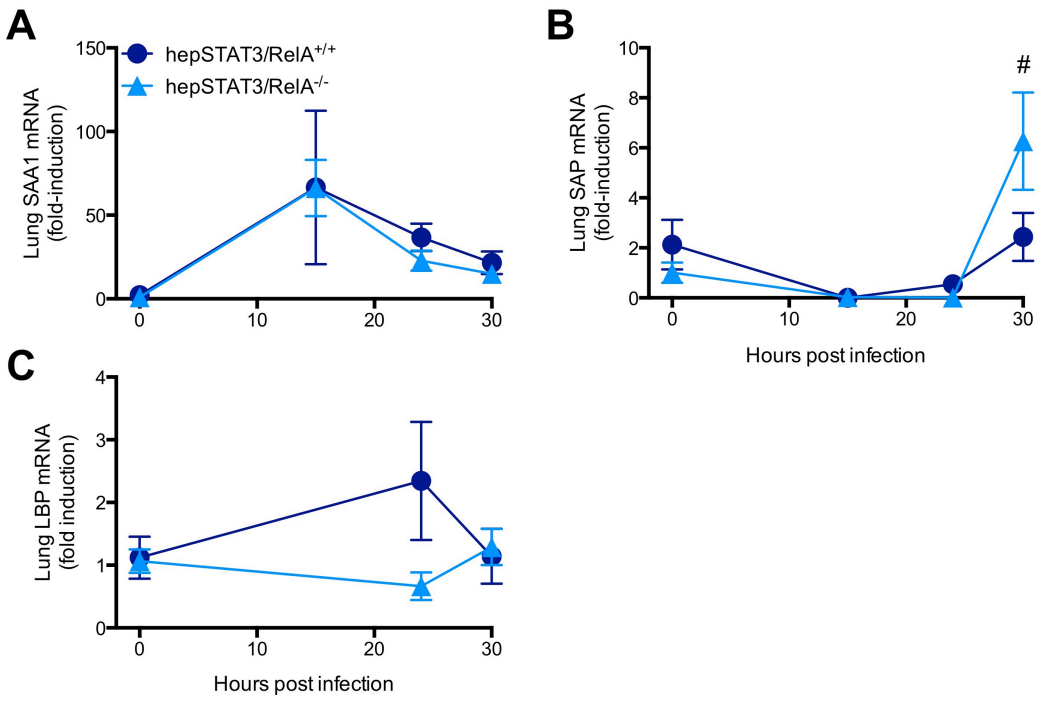


Figure 10. The systemic APR does not mediate pulmonary APP transcript expression.

HepSTAT3/RelA$^{-/-}$ and control hepSTAT3/RelA$^{+/+}$ mice were intratracheally inoculated with 1x10^{6} CFU of *E. coli*, and the infected, lavaged (left) lobe was homogenized. RNA was isolated and APP transcript expression was determined using qRT-PCR (n = 3-8 per group). Significance was assessed by 2-way ANOVA. # $p < 0.05$ vs hepSTAT3/RelA$^{+/+}$ mice at the indicated time point as determined by a Holm-Sidak post hoc test.

This was further evidenced by a direct correlation between BALF and serum SAA concentrations after 30 hours of infection (Figure 11A). The remaining APPs measured (SAP, LBP, CRP, and LCN2), however, showed no difference in airspace protein levels between genotypes (Figure 9B-E), nor was the correlation between BALF and serum SAP, LBP, CRP, or LCN2 concentrations significant (Figure 11B-E), indicating that lung APP content is only selectively dependent on hepatic responses following a high inoculum *E. coli* pneumonia.

3.3 The APR promotes maximal pulmonary bacterial clearance during a lethal E. coli pneumonia.

In order to determine if the APR affects lung inflammation, BALF total protein concentrations (Figure 12A) and recruited neutrophil numbers (Figure 12B) were determined. In both genotypes, the effect of pneumonia was substantial yet equivalent, suggesting that mortality in hepSTAT3/RelA$^{-/-}$ mice is not directly attributable to changes in acute pulmonary inflammation. However, there was a significant increase in pulmonary bacterial burdens in hepSTAT3/RelA$^{-/-}$ mice after 30 hours of infection (Figure 12C). This defect on pulmonary host defense did not translate to systemic defects, as blood bacterial burdens were equivalent in both genotypes (Figure 12D). Due to a potential survival bias, bacterial burdens were not measured at later time points. Nevertheless, increased pulmonary bacterial burdens in hepSTAT3/RelA$^{-/-}$ mice indicate a role for the hepatic APR in promoting host defense during a high inoculum *E. coli* infection.

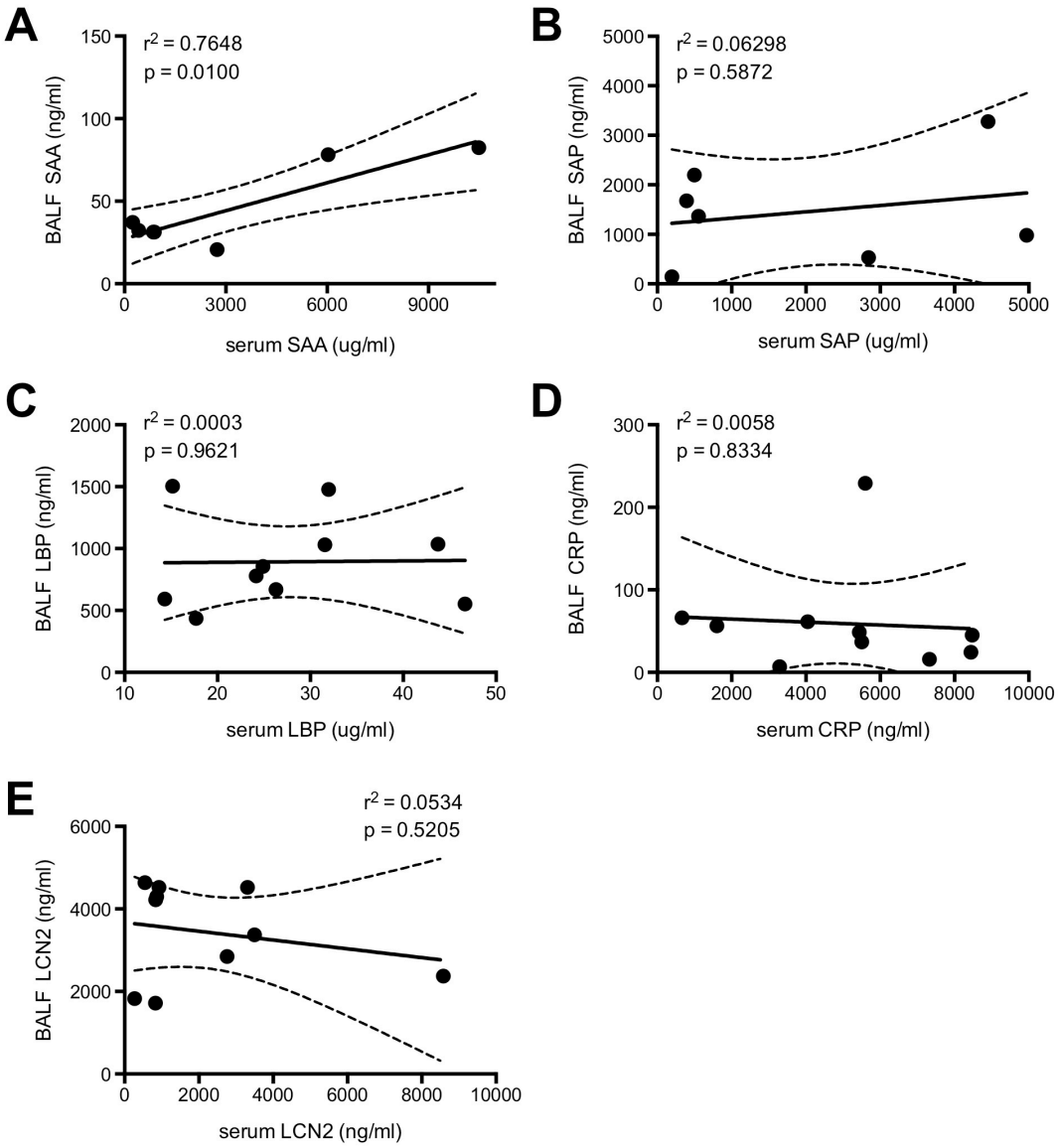


Figure 11. Intrapulmonary concentrations for select APPs significantly correlate with systemic levels.

A correlation with a linear regression was performed comparing serum (from Figure 8) and BALF APP concentrations (from Figure 9). Individual values represent those determined across both genotypes following 30 hours of infection.

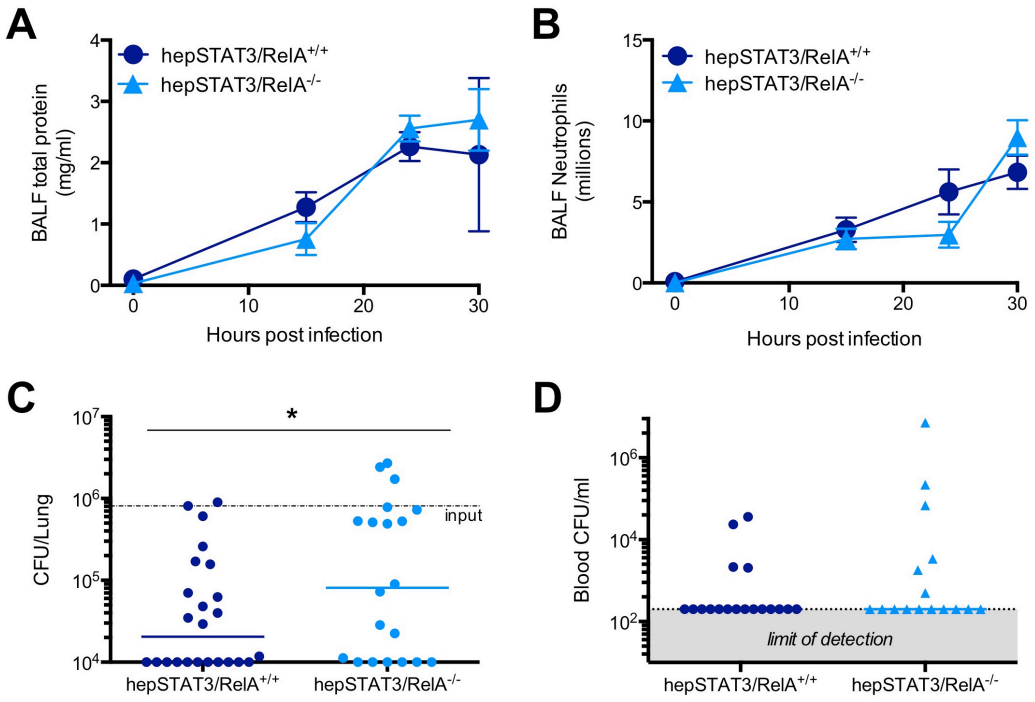


Figure 12. The APR is required for maximal pulmonary host defense.

Mice were infected intratracheally with 1 x 10^6 CFU of *E. coli*. At indicated time points, the lungs were lavaged and BALF was collected. **(A)** Total protein was measured by BCA assay and **(B)** recruited neutrophils were counted as indexes of pulmonary inflammation and injury. Significance was assessed by 2-way ANOVA (n = 4-8 per group). **C, D)** After 30 hours of infection, lung homogenates and heparinized blood were serially diluted and plated on blood agar plates. After an overnight incubation at 37°C, colonies were counted and bacterial burdens were expressed as CFU per lung or milliliter of blood. *$p < 0.05$ vs. control hepSTAT3/RelA$^{+/+}$ mice as determined by a student's *t* test.

3.4 APR deficiency causes liver injury in lethally challenged mice.

Although hepSTAT3/RelA$^{-/-}$ mice have significantly increased lung bacterial burdens, the observed increase is relatively modest, warranting further investigation into the potential cause of death in mice lacking a hepatic APR. It has been shown that NF-κB RelA deficiency results in embryonic lethality due to liver degeneration and hepatotoxicity (Alcamo et al., 2001; Beg and Baltimore, 1996; Beg et al., 1995; Dou et al., 2012; Tanaka et al., 1999). Targeted disruption of RelA in the liver also causes hepatotoxicity in direct response to systemic TNF or LPS, but the importance of inducible RelA (*i.e.* that observed during the APR) has never been explored in the context of lung infections. We hypothesized that pneumonia-induced mortality is attributable, at least in part, to liver injury in hepSTAT3/RelA$^{-/-}$ mice, which lack both RelA and STAT3. To test this, we measured serum ALT levels as a sensitive metric of hepatotoxicity. While pneumonia had no effect on liver injury in hepSTAT3/RelA$^{+/+}$ mice, hepSTAT3/RelA$^{-/-}$ mice had significantly increased levels of serum ALT over the course of infection (Figure 13A). This finding was confirmed by histological evidence of hepatocyte death in the absence of STAT3 and RelA (Figure 14). Interestingly, liver TNFα mRNA induction was significantly, albeit modestly, higher in hepSTAT3/RelA$^{-/-}$ mice (Figure 13B). This finding suggests a cycle whereby both the expression of and response to TNFα is altered in hepSTAT3/RelA$^{-/-}$ mice, resulting in liver damage and toxicity. However, the observed difference in TNFα mRNA was not sufficient to manifest in circulating TNFα protein changes in the same mice (Figure 13C), making the role of differential TNFα mRNA expression unclear at present.

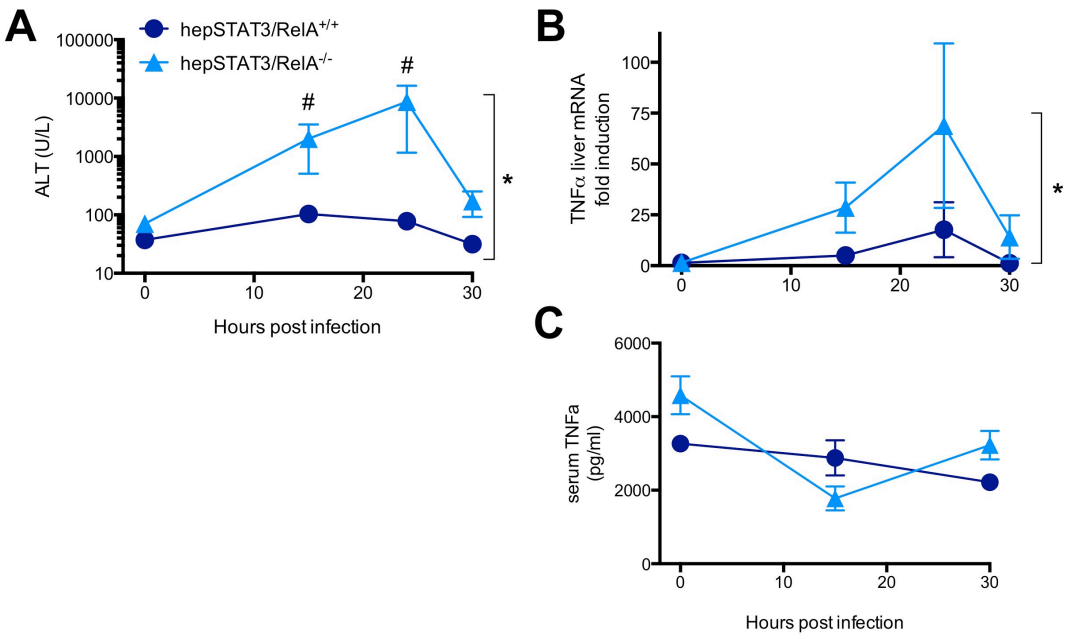


Figure 13. APR-null mice have severe liver injury.

HepSTAT3/RelA$^{-/-}$ and hepSTAT3/RelA$^{+/+}$ mice were infected with 1x10^{6} CFU of *E. coli*. At indicated time points, serum and livers were collected. **A)** Serum ALT levels were measured via colorimetric assay. **B)** Livers were homogenized and RNA was isolated. TNFα mRNA transcript induction was measured by qRT-PCR. **C)** Serum concentrations of TNFα were determined by multiplex bead array. *$p < 0.05$ for overall effect of genotype as determined by 2-way ANOVA. # $p < 0.05$ vs hepSTAT3/RelA$^{+/+}$ mice at the indicated time point as determined by a Holm-Sidak post hoc test (n = 4-9 per group).

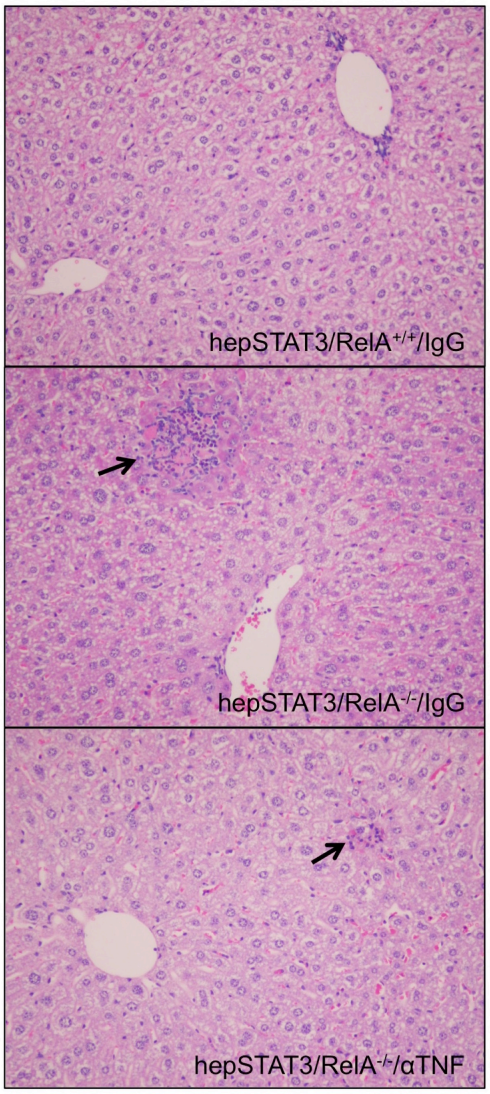


Figure 14. HepSTAT3/RelA$^{-/-}$ mice have liver injury that is TNFα-dependent.

Mice were injected intravenously by tail vein with 5 mg/ml of either a control IgG or anti-TNFα antibody, followed immediately by i.t. *E. coli* (1x10^6 CFU). After 24 hours of infection, histopathology was assessed by H&E stained liver sections. Black arrows indicate areas of acute liver injury. Representative sections were compared between 3 mice per group and were viewed at 20x magnification.

3.5 Liver injury in HepSTAT3/RelA$^{-/-}$ mice is TNFα-dependent.

To determine if this injury was dependent on TNFα, which has previously been linked to hepatotoxicity in absence of RelA (Alcamo et al., 2001; Beg et al., 1995; Ding and Yin, 2004; Doi et al., 1999; Li et al., 1999), we treated mice with 5 mg/ml of either a neutralizing TNFα or IgG control antibody by tail vein injection immediately prior to i.t. *E. coli*. As expected, at 24 hours post infection, hepSTAT3/RelA$^{-/-}$ mice treated with the control IgG antibody had high levels of circulating ALT (Figure 15A), consistent with our initial results (Figure 13A). In contrast, hepSTAT3/RelA$^{-/-}$ mice treated with an anti-TNFα antibody had serum ALT concentrations that were identical to baseline levels observed in hepSTAT3/RelA$^{+/+}$ mice (Figure 15A). Histology also confirmed the protective effect of the neutralizing TNFα antibody, with histopathology far more reflective of hepSTAT3/RelA$^{+/+}$ mice than that of untreated hepSTAT3/RelA$^{-/-}$ littermates (Figure 14). Lung and blood bacterial burdens were also measured at 24 hours post infection to confirm that the anti-TNFα antibody had no adverse effects on host defense, which would confound interpretations of host outcomes (Figure 15B). As expected, the effect of genotype on bacterial killing was similar to that observed at 30 hours post infection (Figure 12C), but this outcome was unaffected by TNFα neutralization (Figure 15B).

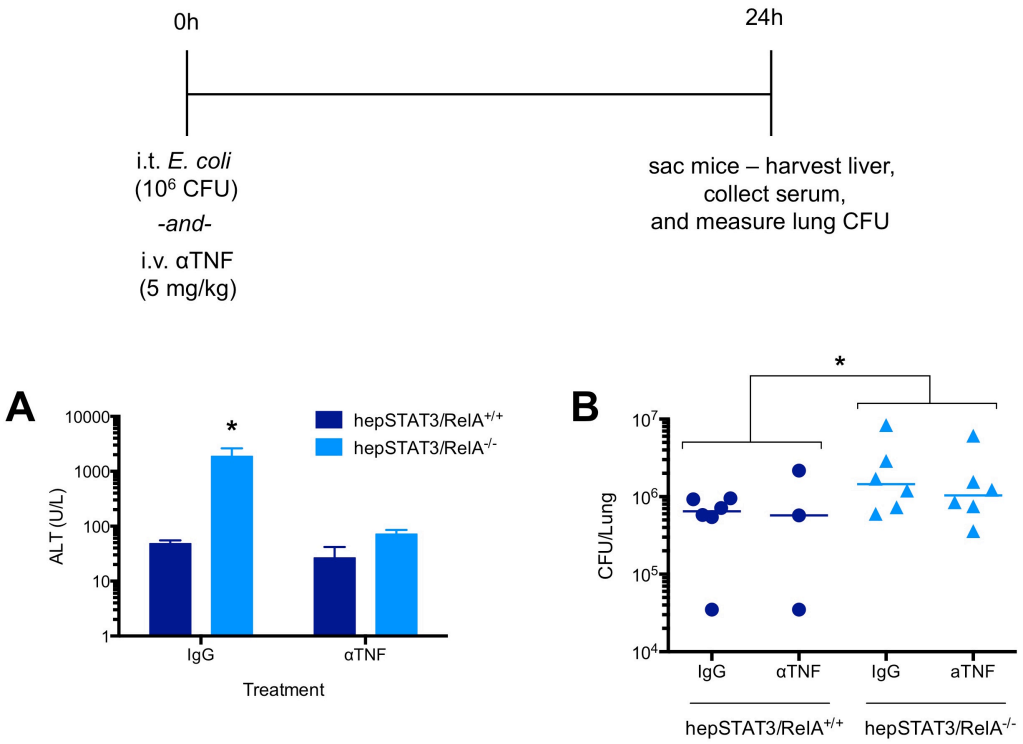


Figure 15. Hepatotoxicity during a high inoculum *E. coli* pneumonia is TNFα-dependent.

In order to determine the influence of TNFα on liver injury, mice were injected intravenously by tail vein with 5 mg/ml of either a control IgG or anti-TNFα antibody, followed immediately by i.t. *E. coli* ($1x10^6$ CFU). **A)** Serum ALT levels were determined 24 hours post infection as an indicator of the amount of liver injury present. $*p < 0.05$ vs. hepSTAT3/RelA$^{+/+}$/IgG control group was determined by a one-way ANOVA followed by a Holm Sidak test (n = 3-13 per group). **B)** Lung homogenates were serially diluted and plated on blood agar plates and bacterial burdens were determined. $*p < 0.05$ for overall effect of genotype as determined by 2-way ANOVA (n = 3-6 per group).

3.6 Liver injury in APR-deficient mice is associated with increased hepatic apoptosis.

To determine whether liver injury was specifically linked to apoptosis in APR-null, hepSTAT3/RelA$^{-/-}$ mice, we determined protein levels of cleaved caspase-3, the effector caspase for the apoptotic pathway, via immunoblot of liver homogenates 24 hours after i.t. *E. coli* (Figure 16A). Densitometric analysis of immunoblots revealed significantly higher levels of cleaved caspase-3 in the absence of hepatocyte STAT3 and RelA that were almost completely reversed with TNFα neutralization (Figure 16B), suggesting that liver injury was at least partly attributable to TNF-induced apoptosis. These data are consistent with previous reports indicating RelA-mediated protection from TNF-induced liver apoptosis (Alcamo et al., 2001).

3.7 Liver cellular repair is not affected by hepSTAT3/RelA deletion.

In addition to hepatocyte loss (*i.e.* injury), hepatocyte replacement could also be compromised in APR-null mice, as supported by studies showing STAT3-dependent liver regeneration (Ding and Yin, 2004; Doi et al., 1999; Li et al., 2002). To address this, we stained liver sections for Ki67, a nuclear antigen used to detect proliferating cells (Figure 17A). As expected, we observed few positively stained cells in the hepSTAT3/RelA$^{+/+}$ control group 24 hours after i.t. *E. coli*, but there was a marked increase in the percentage of Ki67^{+} cells in IgG treated hepSTAT3/RelA$^{-/-}$ mice that was partially reversed upon TNFα neutralization (Figure 17B). Thus, these data do not support a defect in hepatocyte turnover as a cause of liver injury in hepSTAT3/RelA$^{-/-}$ mice. Rather, cell proliferation appeared to be downstream of hepatotoxicity, with the greatest amounts of Ki67 staining in areas of the greatest liver injury.

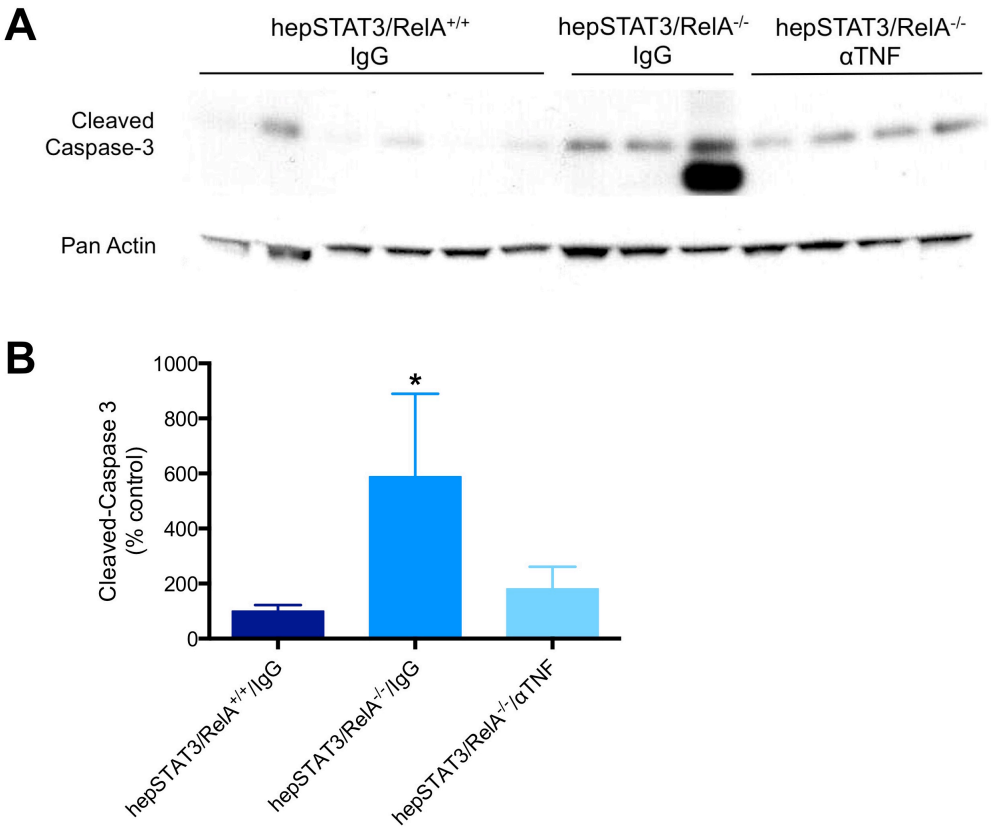


Figure 16. HepSTAT3/RelA$^{-/-}$ mice have increased hepatic apoptosis during a high inoculum *E. coli* pneumonia.

HepSTAT3/RelA$^{-/-}$ and hepSTAT3/RelA$^{+/+}$ mice were injected intravenously by tail vein with 5 mg/ml of either a control IgG or anti-TNFα antibody, followed immediately by i.t. *E. coli* (1x10^{6} CFU). **A)** After 24 hours of infection, protein was extracted from liver homogenates and subjected to immunoblot analysis for cleaved caspase-3 expression. Each band represents data from an individual mouse. One of two representative blots with livers from four independent experiments is shown. **B)** A densitometric analysis of band intensity was performed on the immunoblots. The ratio of cleaved caspase-3/pan actin was compared to the hepSTAT3/RelA$^{+/+}$/IgG control group for each sample to

calculate the percent control changes in band intensity ratios. *$p < 0.05$ vs. hepSTAT3/RelA$^{+/+}$/IgG group was determined by a one-way ANOVA followed by a Holm Sidak test (n = 6-13).

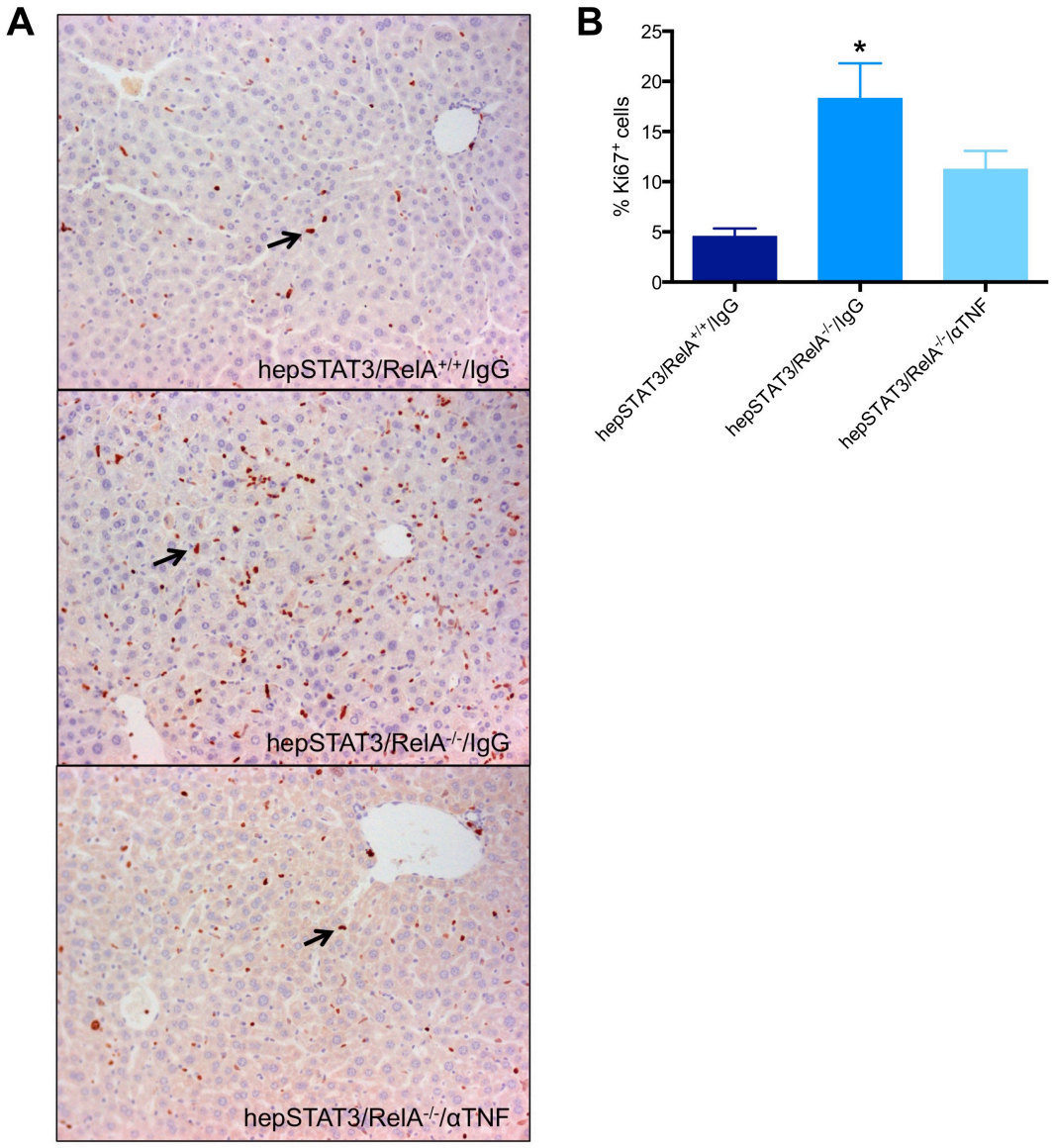


Figure 17. The APR does not affect liver repair during a high inoculum pneumonia.

Mice were injected intravenously by tail vein with 5 mg/ml of either a control IgG or anti-TNFα antibody, followed immediately by i.t. *E. coli* ($1x10^6$ CFU). **A)** After 24 hours of infection, cell turnover, as a marker of liver repair, was assessed by immunohistochemical staining for Ki67. Black arrows indicate areas of acute liver injury

(top panels) or a $Ki67^+$ cell (bottom panels). Representative images are shown for livers collected from at least 3 individual mice per group at 20X magnification. **B)** A blinded morphometric analysis was performed on liver sections stained for Ki67 from panel A. Data are expressed as the percentage of all cells that stained positively for Ki67. $*p < 0.05$ vs. hepSTAT3/$RelA^{+/+}$/IgG control group was determined by a one-way ANOVA followed by a Holm Sidak test (n = sections from 3 mice per group, with at least 6 fields of view analyzed).

3.8 Hepatotoxicity is the not sole cause of mortality in APR-deficient mice during E. coli pneumonia.

To determine whether liver injury was the sole cause of mortality in hepSTAT3/RelA$^{-/-}$ mice, we measured survival in APR-null mice treated with either the control or αTNF antibody, the latter of which reversed all metrics of hepatotoxicity. Surprisingly, hepSTAT3/RelA$^{-/-}$ mice treated with the neutralizing TNFα antibody, which had normal liver function and baseline levels of hepatic injury, exhibited no improvement in survival (Figure 18A), suggesting that the physiological significance of the APR extends beyond inducible hepatoprotection. As another insight into the potential cause of mortality in the APR-null mice, we measured BUN levels as an indicator of kidney function, and there were no differences between genotypes (Figure 18B). The mechanism by which the APR prevents mortality during a severe pneumonia remains unknown.

3. 9 Liver-derived proteins are necessary to promote pulmonary inflammation during a low inoculum (non-lethal) E. coli pneumonia.

Due to lethality differences and a potential survival bias introduced to the results described above, we inoculated mice with a milder dose of *E. coli* (4 x 10^5 CFU) to more carefully determine the impact of liver acute phase changes on lung inflammation and host defense. At this dose, we observed only rare mortality with no significant difference between genotypes.

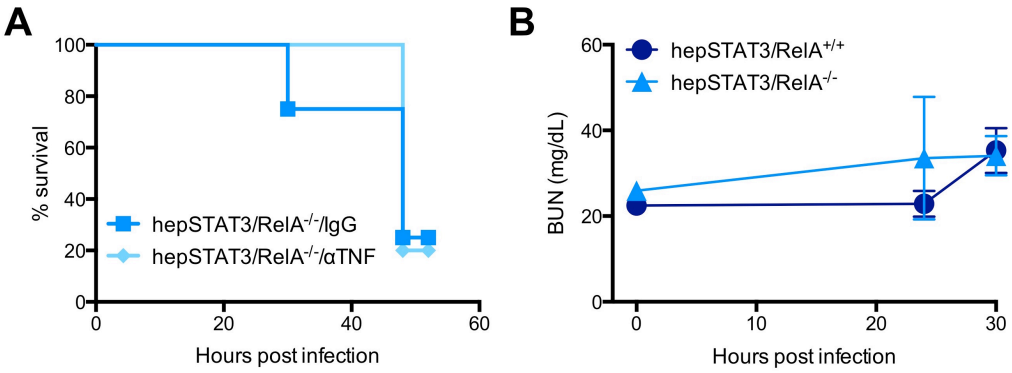


Figure 18. Reversal of liver injury does not improve survival in APR-deficient, hepSTAT3/RelA$^{-/-}$ mice during a high inoculum *E. coli* pneumonia.

A) HepSTAT3/RelA$^{-/-}$ mice were infected intratracheally with 1x10^{6} CFU of *E. coli* immediately following i.v. injection of 5 mg/ml of a control IgG or neutralizing TNFα antibody. Survival was observed through 48 hours of infection. A Mantel-Cox test was used to determine significance (n = 4-5 per group). **B)** HepSTAT3/RelA$^{+/+}$ and hepSTAT3/RelA$^{-/-}$ mice were infected with i.t. *E. coli* (1 x 10^{6} CFU). At the indicated time points, blood was collected and serum was isolated. BUN was determined using a colorimetric assay as a determinate of kidney function. Significance was assessed by 2-way ANOVA (n = 4-9 per group).

The magnitude of the APR itself, as determined by systemic SAA and SAP expression (liver mRNA and circulating protein content), was much milder in response to the non-lethal challenge compared to that described above, and remained blunted in hepSTAT3/RelA$^{-/-}$ mice (Figures 19A-D). We also observed an increase in airspace SAA and SAP concentrations in hepSTAT3/RelA$^{+/+}$ mice that was completely absent in hepSTAT3/RelA$^{-/-}$ mice (Figure 20A, B). Again, this disparity between genotypes was not due to differential local expression of these proteins, as there was no significant decrease in lung mRNA induction between genotypes (Figure 20C, D).

In association with decreased APP levels, we also observed significantly reduced total protein concentrations and a trend towards less neutrophil recruitment in the BALF of hepSTAT3/RelA$^{-/-}$ mice (Figures 21A, B). This possible decrease in airspace neutrophil numbers was not due to greater cell death, as 7AAD+ (dead) neutrophil percentages were unchanged in BALF from hepSTAT3/RelA$^{-/-}$ mice 15h after i.t. *E. coli* (Figure 21C). Lung bacteria were virtually cleared by 30 hours of infection in response to this milder inoculum, with no difference observed between genotypes (Figure 22A). Additionally, there was no observable bacteremia through 30 hours for either group (Figure 22B). In accordance with decreased pulmonary inflammation, the majority of cytokines measured (IL-6, G-CSF, CXCL1, TNFα, CXCL2, IL-10 and LIF) were significantly decreased in hepSTAT3/RelA$^{-/-}$ mice compared to hepSTAT3/RelA$^{+/+}$ mice after 15 hours of infection (Figure 23A).

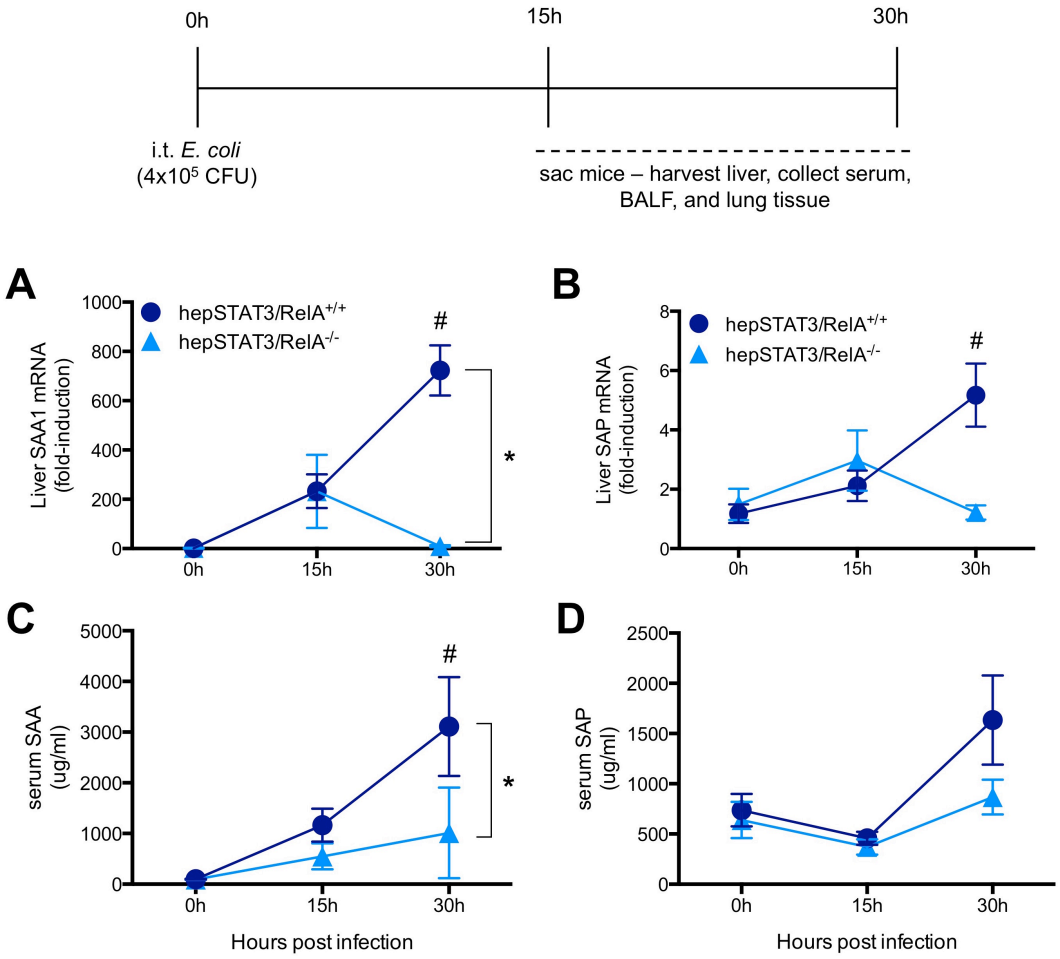


Figure 19. Low inoculum infected mice have an impaired APR.

HepSTAT3/RelA$^{+/+}$ or hepSTAT3/RelA$^{-/-}$ mice were instilled intratracheally with 4x10^5 CFU of *E. coli.* **A, B)** At the indicated time points, liver mRNA was extracted and SAA1 and SAP transcript fold induction was determined using qRT-PCR. **C, D)** Serum was collected and concentrations of SAA and SAP were determined by ELISA. *$p < 0.05$ for overall effect of genotype over the indicated time course as determined by 2-way ANOVA. # $p < 0.05$ vs hepSTAT3/RelA$^{+/+}$ mice at the indicated time point as determined by a Holm-Sidak post hoc test (n = 5-11 per group).

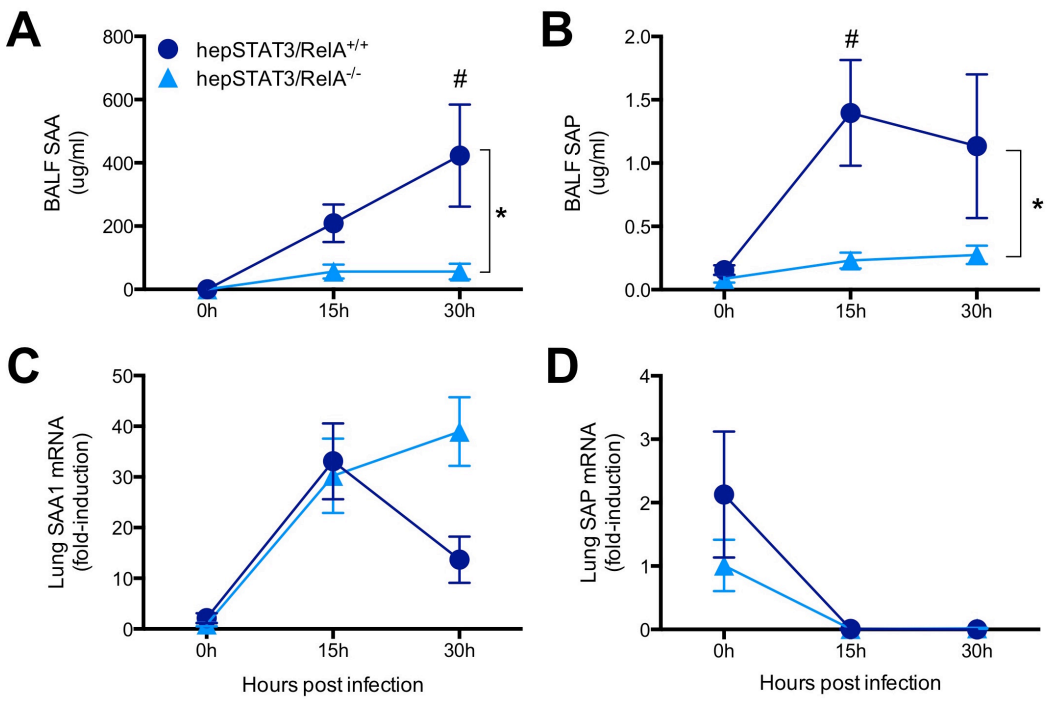


Figure 20. Intrapulmonary APP concentrations are decreased after a low inoculum *E. coli* pneumonia.

HepSTAT3/RelA$^{-/-}$ and hepSTAT3/RelA$^{+/+}$ mice were infected with 4x10^5 CFU of *E. coli* and lungs were lavaged at the indicated time points with PBS. **A, B)** BALF SAA and SAP concentrations were determined by ELISA. **C, D)** Lavaged, infected (left) lobes were homogenized and RNA was extracted. SAA1 and SAP transcript fold inductions were determined using qRT-PCR. *$p < 0.05$ for overall effect of genotype as determined by 2-way ANOVA. # $p < 0.05$ vs hepSTAT3/RelA$^{+/+}$ mice at the indicated time point as determined by a Holm-Sidak post hoc test (n = 5-11 per group).

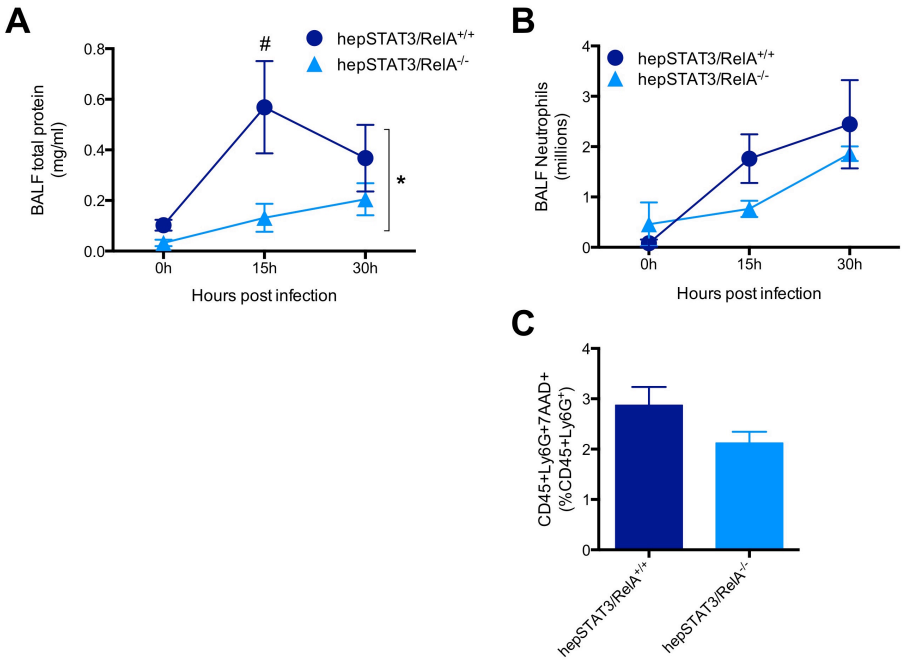


Figure 21. The APR facilitates pulmonary inflammation during a low inoculum *E. coli* pneumonia.

HepSTAT3/RelA$^{-/-}$ and hepSTAT3/RelA$^{+/+}$ mice were infected with 4x10^5 CFU of *E. coli* and lungs were lavaged at the indicated time points with PBS. **(A)** BALF total protein concentrations were measured by BCA assay and **(B)** total BALF neutrophils were enumerated. *$p < 0.05$ for overall effect of genotype as determined by 2-way ANOVA. # $p < 0.05$ vs hepSTAT3/RelA$^{+/+}$ mice at the indicated time point as determined by a Holm-Sidak post hoc test (n = 5-11 per group). **C)** After 15 hours of infection with 4 x 10^5 CUF of *E. coli*, cells from the BALF were collected and stained for total (CD45$^+$/Ly6G$^+$) or dead (CD45$^+$/7AAD$^+$/Ly6G$^+$) neutrophils. Percentages of dead neutrophils are calculated from total numbers of neutrophils. Significance vs. hepSTAT3/RelA$^{+/+}$ group was assessed by Student's *t* test (n = 3-5 per group).

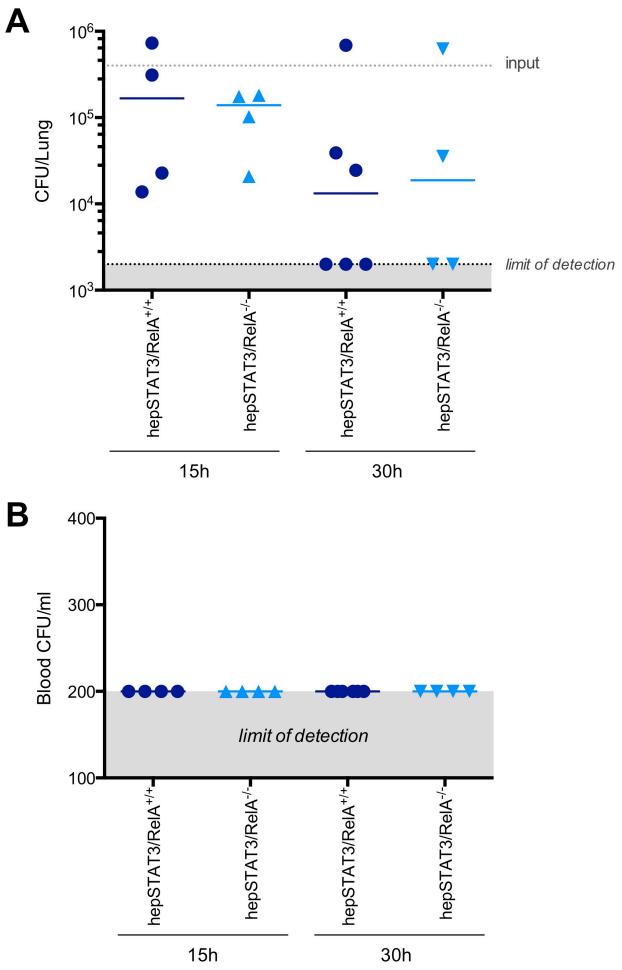


Figure 22. Pulmonary bacterial clearance is not impaired in hepSTAT3/RelA$^{-/-}$ mice during a low inoculum *E. coli* infection.

HepSTAT3/RelA$^{-/-}$ and hepSTAT3/RelA$^{+/+}$ mice were infected intratracheally with 4x10^5 CFU of *E. coli*. After 15 or 30 hours of infection, lung homogenates and heparinized blood were serially diluted and plated on blood agar plates. The lowest dilution utilized is indicated as the limit of detection in each panel. After an overnight incubation at 37°C, colonies were counted, and bacterial burdens were expressed as CFU per lung or milliliter of blood. Significance was assessed by 2-way ANOVA (n = 4-6).

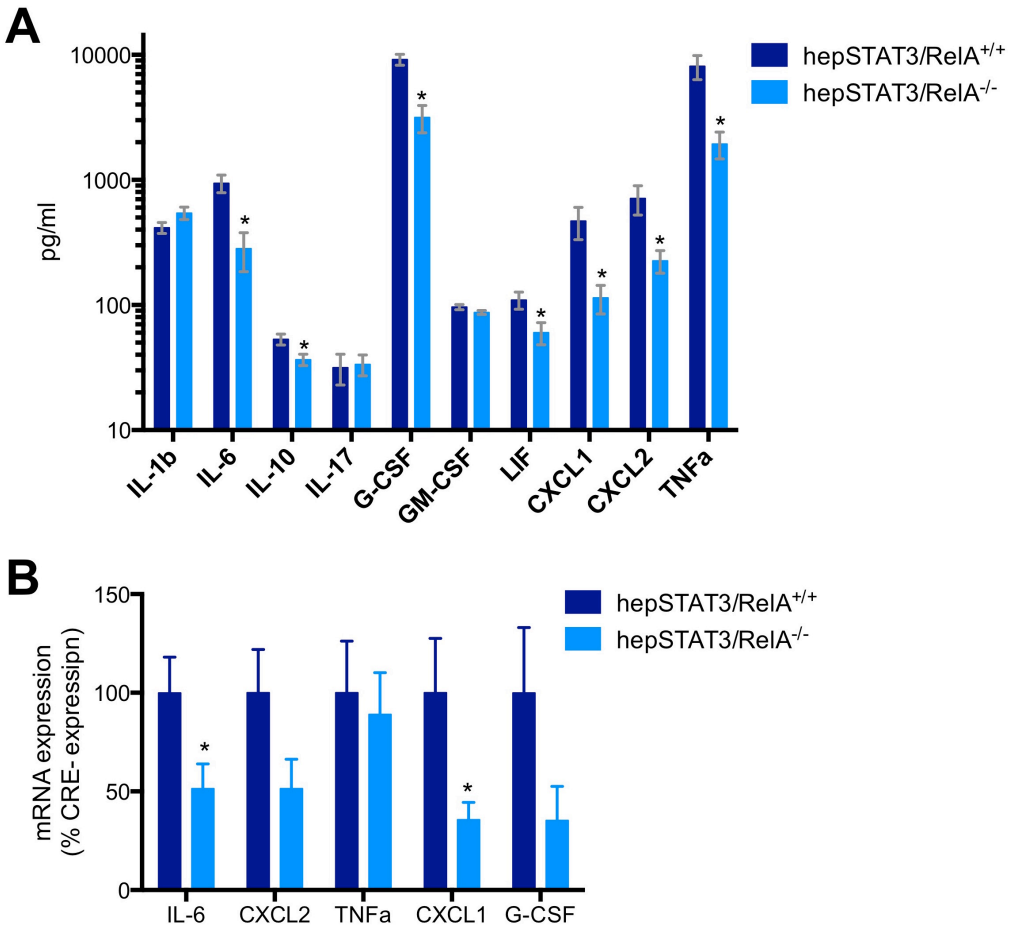


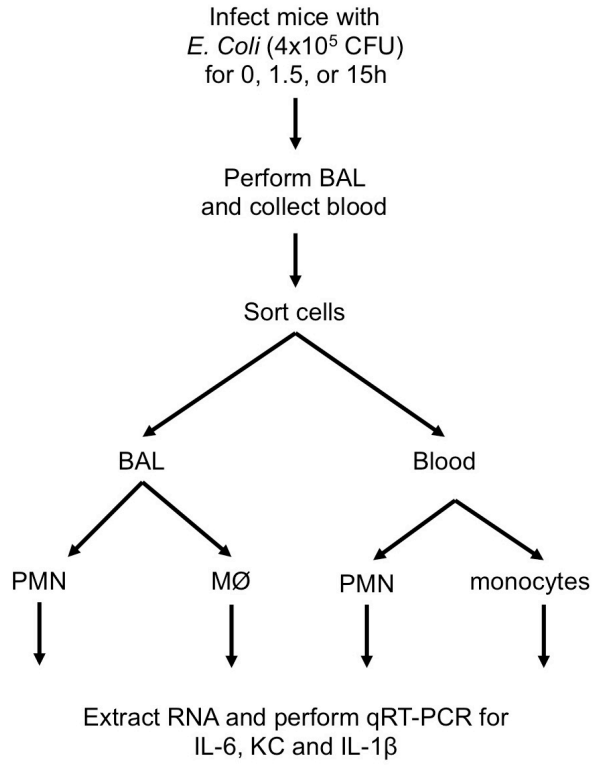
Figure 23. Pulmonary cytokine production during a low inoculum *E. coli* pneumonia is mediated by the hepatic APR.

HepSTAT3/RelA$^{-/-}$ and hepSTAT3/RelA$^{+/+}$ mice were infected intratracheally with 4x10^5 CFU of *E. coli*. **A)** At 15 hours post infection, BALF cytokine protein concentrations were measured by a multiplex bead array (Luminex). **B)** At the same time point, lavaged, infected (left) lobes were homogenized, RNA was extracted, and cytokine mRNA induction was measured by qRT-PCR. *$p < 0.05$ vs. hepSTAT3/RelA$^{+/+}$ mice, assessed by Student *t* test (n = 10-11 per group).

Specific inflammatory cytokines were decreased by at least 50%, some greater, indicating that the APR induces a biologically relevant change in pulmonary inflammation. mRNA induction of IL-6, TNFα, CXCL2, CXCL1 and G-CSF were also measured in lung homogenates (Figure 23B). Despite the fact that only IL-6 and CXCL1 were significantly decreased in hepSTAT3/RelA$^{-/-}$ mice, CXCL2 and G-CSF also trended toward decreased in hepSTAT3/RelA$^{-/-}$ mice, corroborating airspace protein levels. While cytokines known to be downstream of IL-17 during pneumonia (*e.g.* CXCL1/2 and G-CSF) (Ye et al., 2001) were decreased in mutant mice, IL-17 itself remained unchanged (Figure 23A), supporting the observed phenotype as a Th17-independent phenomenon. These data indicate that the hepatic transcriptional responses facilitate acute pulmonary inflammation, as revealed by a milder, nonlethal pneumonia.

3.10 The liver APR facilitates cytokine expression through airspace macrophages.

The data above suggest that liver-derived APPs enhance immune responsiveness, demanding a more complete understanding of where this process occurs. For instance, it is plausible that products downstream of the liver APR act upon circulating cells in the blood, recruited and/or resident cells in the infected airspaces, or perhaps both. In order to determine the effect of the APR on cytokine expression in circulating, recruited, and lung-resident myeloid-derived leukocytes, we used FACS to isolate cells from blood and BALF, and determined cytokine induction using qRT-PCR (Figure 24).



Cell Type	Surface Marker
Monocyte	$CD11b^{hi}$ $CD115^{hi}$
Macrophage	$F4/80^{+}$Autofluorescence^{hi}
Neutrophil	$CD11b^{+}$ $Ly6G^{+}$

Figure 24. Airspace and circulating cell sorting workflow.

HepSTAT3/RelA$^{-/-}$ and hepSTAT3/RelA$^{+/+}$ mice were infected for 0, 1.5, or 15 hours with 4x10^5 CFU of *E. coli*. Lungs were lavaged, blood was collected, and RBCs were lysed. Cells were collected and stained for FACS using the cell surface markers shown above. After individual cell type separation, RNA was isolated and cytokine induction was determined by qRT-PCR.

Both circulating monocytes ($CD45^+/7AAD^-/CD115^+/CD11b^+$) (Hanna et al., 2011) and neutrophils ($CD45^+/7AAD^-/Ly6G^+/CD11b^+$) showed no differences in cytokine induction between genotypes at 15 hours post infection (Figure 25B, C). In these cells, cytokine expression varied from undetectable (monocyte CXCL1), to increased (monocyte IL-6 and IL-1β; neutrophil CXCL1), to decreased (neutrophil IL-1β). In contrast, induction of IL-6 and CXCL1 in airspace macrophages ($CD45^+/7AAD^-/F4/80^+/Ly6G^-$) from hepSTAT3/$RelA^{-/-}$ mice was significantly decreased after 15 hours of infection compared to macrophages from hepSTAT3/$RelA^{+/+}$ mice (Figure 26B). Induction of IL-1β was unaffected, which was consistent with unchanged BALF protein concentrations (Figures 26B and 23A). This cytokine induction phenotype was unique to airspace macrophages, as airspace neutrophils ($CD45^+/7AAD^-/Ly6G^+/F4/80^-$) showed no differences in gene expression for the cytokines measured (Figure 26C). To determine if the changes in cytokine induction required APP delivery into the airspaces (via serum extravasation), we measured cytokine induction at 1.5 hours post infection, a time preceding loss of alveolar barrier integrity, and thus serum protein (and APP) extravasation. Cytokine mRNA was induced, but was equivalent between genotypes at this early point of infection (Figure 26B), suggesting that changes in cytokines are not intrinsic to local cells, but rather, are due to differences in APP exposure from extravasated serum. Taken together, the data imply a role for airspace macrophage cytokine induction by the APR to induce pulmonary inflammation during pneumonia.

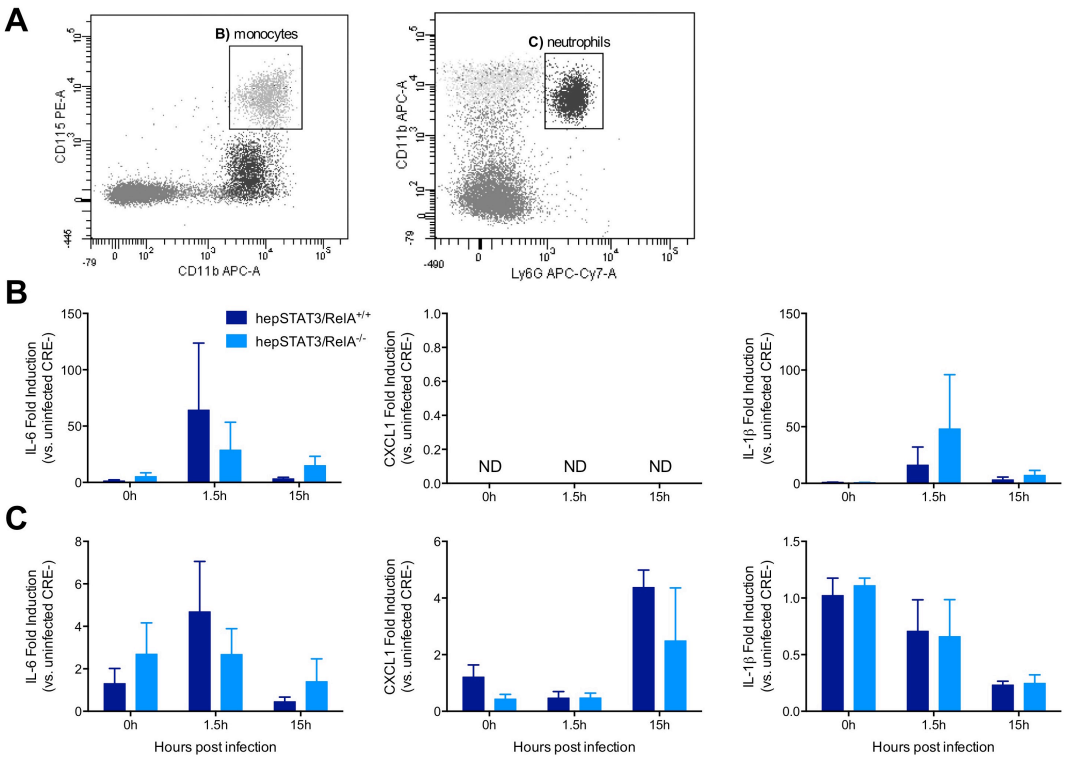


Figure 25. Cytokine induction in circulating cells is not affected by the APR.

HepSTAT3/RelA$^{-/-}$ and hepSTAT3/RelA$^{+/+}$ mice were infected for 0, 1.5, or 15 hours with 4x10^5 CFU of *E. coli*. Blood was collected, RBCs were lysed, and cells were stained for FACS. **A)** Representative dot plot are shown. Circulating monocytes were gated on CD45$^+$/7AAD$^-$/CD115$^+$/CD11b$^+$ and circulating neutrophils were gated on CD45$^+$/7AAD$^-$/Ly6G$^+$/ CD11b$^+$. RNA was extracted and qRT-PCR was performed to determine induction of IL-6, CXCL1 and IL-1β in both circulating monocytes **(B)** and neutrophils **(C)**. The overall effect of genotype was determined by 2-way ANOVA (n = 3-6 per group).

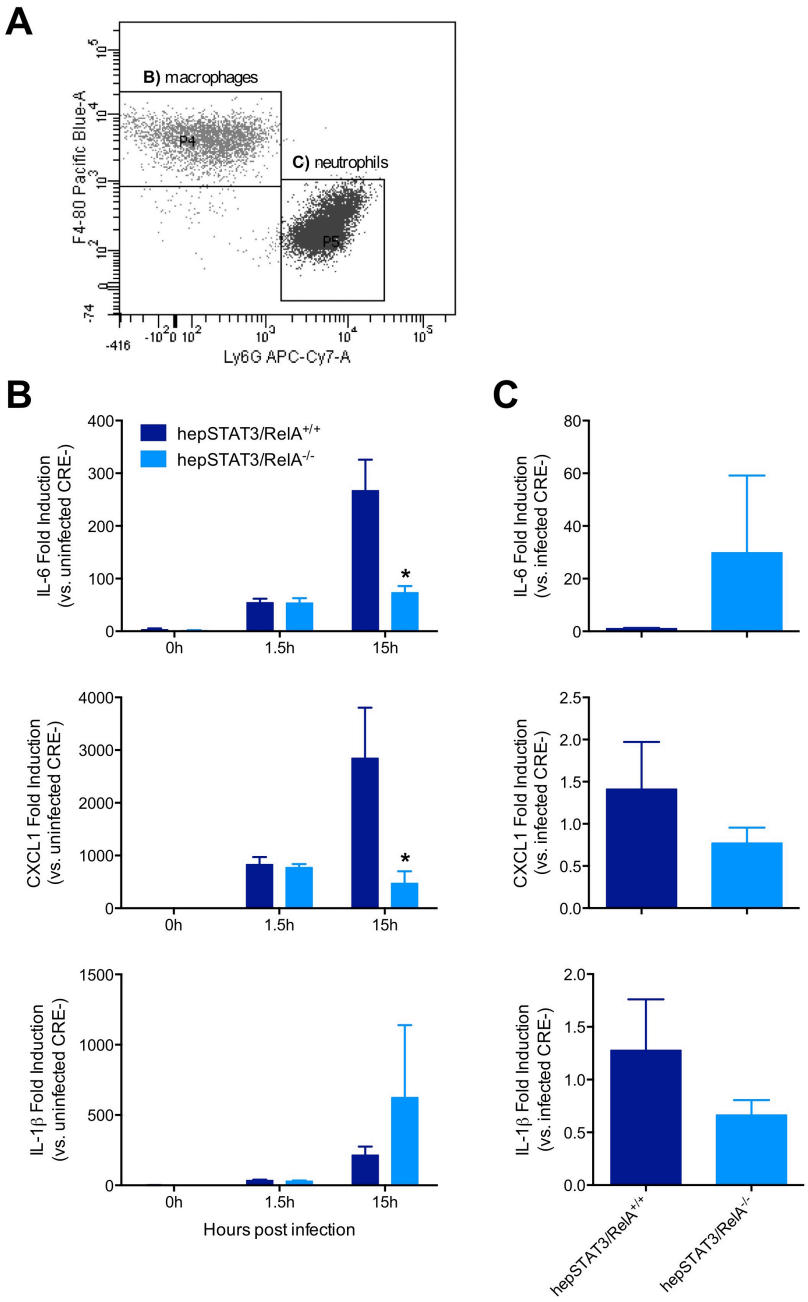


Figure 26. Airspace macrophages are responsible for APP-induced cytokine changes in low inoculum infected mice.

HepSTAT3/RelA$^{-/-}$ and hepSTAT3/RelA$^{+/+}$ mice were infected for 0, 1.5, or 15 hours with 4x10^5 CFU of *E. coli*. Lungs were lavaged, and cells were collected and stained for FACS. **A)** Airspace macrophages were gated on CD45$^+$/7AAD$^-$/F4/80$^+$/Ly6G$^-$ and airspace neutrophils were gated on CD45$^+$/7AAD$^-$/Ly6G$^+$/F4/80$^-$. RNA was extracted and qRT-PCR was performed to determine induction of IL-6, CXCL1 and IL-1β in airspace macrophages **(B)** and determine expression of IL-6, CXCL1 and IL-1β relative hepSTAT3/RelA$^{+/+}$ controls in airspace neutrophils **(C)**, as there is no recruitment of neutrophils to the airspaces at baseline or 1.5 hours after infection. For panel B, **p* < 0.05 for overall effect of genotype or vs. control mice in the same group as determined by 2-way ANOVA followed by Holm Sidak test (n = 3-6 per group). For panel C, significance was assessed by student *t* test (n = 10-11 per group).

3.11 Serum components do not directly modulate cytokine expression ex vivo.

The above data suggest that the hepatic APR promotes cytokine induction, specifically in airspace macrophages. We sought to develop an *ex vivo* system to test if serum components, including APPs, could directly modulate cytokine production in primary alveolar macrophages after *ex vivo* stimulation with LPS (Figure 27). In order to determine the dose of LPS most effective in activating the cells and inducing cytokine expression, we stimulated primary alveolar macrophages from C57BL/6 mice with varying doses of LPS for four hours. After stimulation, RNA was isolated and IL-6 expression was determined using qRT-PCR (Figure 28A). IL-6 production was greatest at the lowest concentration of LPS (100 ng/ml). Macrophages were then stimulated in the presence of varying concentrations of serum from hepSTAT3/RelA$^{+/+}$ mice infected for 15 hours with 4 x 10^5 CFU of *E. coli*. Mouse serum had a generally repressive effect, as the higher percentages of serum used resulted in the lowest cytokine expression compared to cells stimulated in the presence of FBS (Figure 28B). Because of this, we used 1% serum in all stimulations so that changes in cytokine expression could be observed. Following protocol optimization, we stimulated primary alveolar macrophages with 100 ng/ml of LPS in the presence of serum from uninfected or 15 hour, mildly infected hepSTAT3/RelA$^{-/-}$ or hepSTAT3/RelA$^{+/+}$ mice. Expression of both IL-6 and TNFα were unchanged due to serum source, as neither infection status nor genotype of the serum was able to significantly affect cytokine induction (Figure 28C, D).

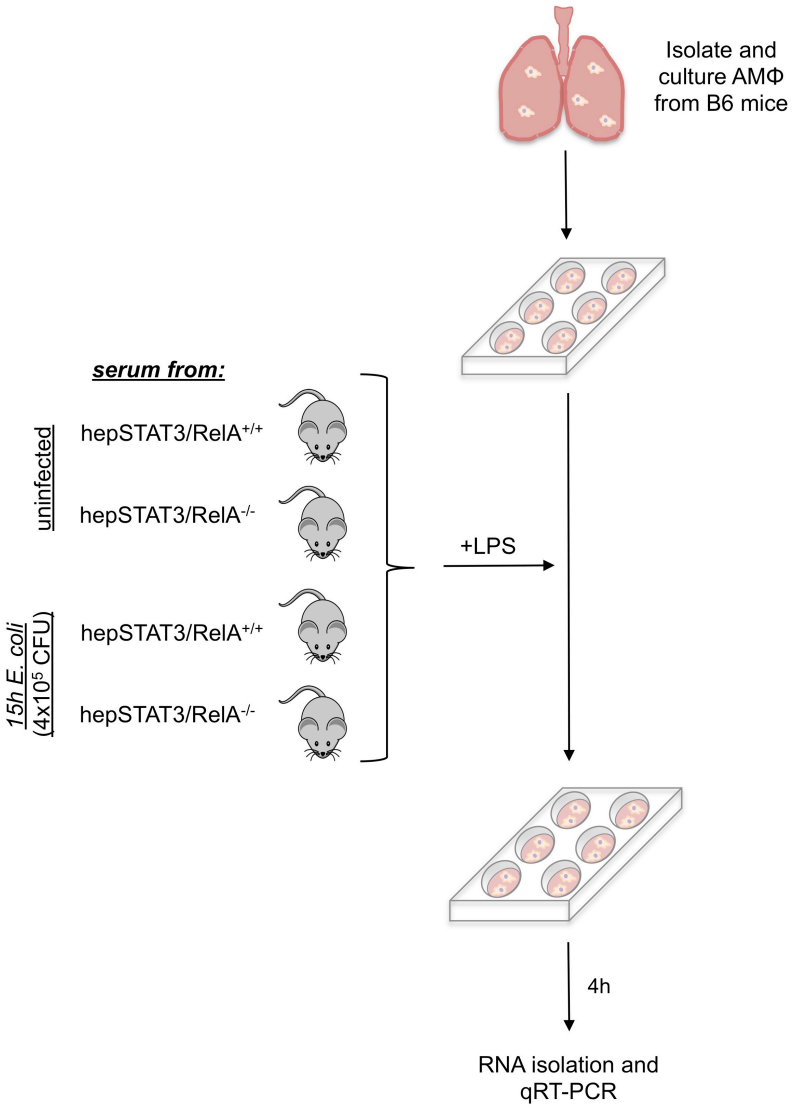


Figure 27. *Ex vivo* LPS and serum stimulation assay.

Primary alveolar macrophages were isolated from C57BL/6 mice. Once adhered to the culture dish and rested overnight, cells were stimulated with 100 ng/ml of LPS in the presence of serum from hepSTAT3/RelA$^{-/-}$ or hepSTAT3/RelA$^{+/+}$ mice, uninfected or infected for 15 hours with 4 x 10^5 CFU of *E. coli*. After 4 hours of stimulation, RNA was isolated using Trizol and cytokine expression was determined using qRT-PCR.

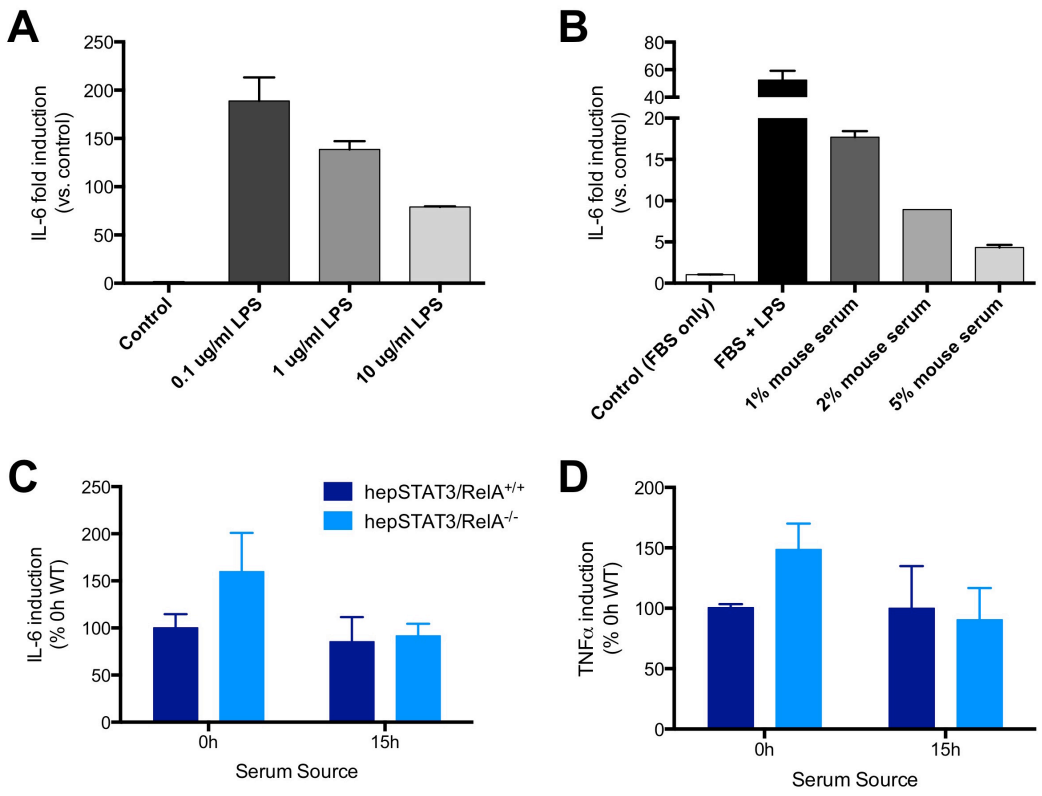


Figure 28. Serum components do not directly modulate *ex vivo* cytokine production in primary alveolar macrophages during LPS stimulation.

A) Primary alveolar macrophages from C57BL/6 mice were stimulated with varying concentrations of LPS for 4 hours. RNA was isolated using Trizol and IL-6 expression was determined using qRT-PCR. **B)** Primary alveolar macrophages from C57BL/6 mice were stimulated with 100 ng/ml of LPS in the presence of either FBS or varying concentrations of serum from hepSTAT3/RelA$^{+/+}$ mice infected for 15 hours with 4 x 10^5 CFU of *E. coli*. After 4 hours of stimulation, RNA was isolated using Trizol and IL-6 expression was determined using qRT-PCR. **C, D)** Primary alveolar macrophages from C57BL/6 mice were stimulated with 100 ng/ml of LPS in the presence of serum from

hepSTAT3/RelA$^{-/-}$ or hepSTAT3/RelA$^{+/+}$ mice, uninfected or infected for 15 hours with $4x10^5$ CFU of *E. coli*. After 4 hours of stimulation, RNA was isolated using Trizol and cytokine expression was determined using qRT-PCR. Significance was assessed using a 2-way ANOVA (n = 3).

While this assay is aimed at determining the effect of serum components on macrophage activation, the type of stimulation may impact the outcome, as LPS stimulation is TLR4 specific and may not mimic the complexities involved in an *in vivo* infection model with live *E. coli*. Thus, we optimized our *ex vivo* system and stimulated primary alveolar macrophages with live *E. coli* (Figure 29) at varying concentrations and for increasing durations to determine the optimal dose and infection length at which to study APR-dependent changes in cytokine expression (Figure 30A, B). High concentrations of *E. coli*, along with longer stimulation times, resulted in cell death and lower IL-6 expression (Figure 30A, B). Therefore, we performed *ex vivo* assays using two different bacterial concentrations, one low (10^2 CFU/ml) and another high (10^5 CFU/ml), for four hours to better determine if APR-dependent serum components can modulate macrophage cytokine production. After stimulation with 10^5 CFU/ml of *E. coli* for four hours in the presence of serum from hepSTAT3/RelA$^{-/-}$ or hepSTAT3/RelA$^{+/+}$ mice, uninfected or infected for 15 hours with 4 x 10^5 CFU of *E. coli*, we observed no changes in cytokine expression due to either genotype or infection (Figure 30C). Stimulation with 10^2 CFU/ml of *E. coli* resulted in low-level inductions of IL-6 and no induction of TNFα, suggesting that this concentration of bacteria was not an effective dose to stimulate macrophages *ex vivo* (Figure 30D). Taken together, the data above suggest that APR-dependent serum components cannot directly modulate alveolar macrophage cytokine production *ex vivo*.

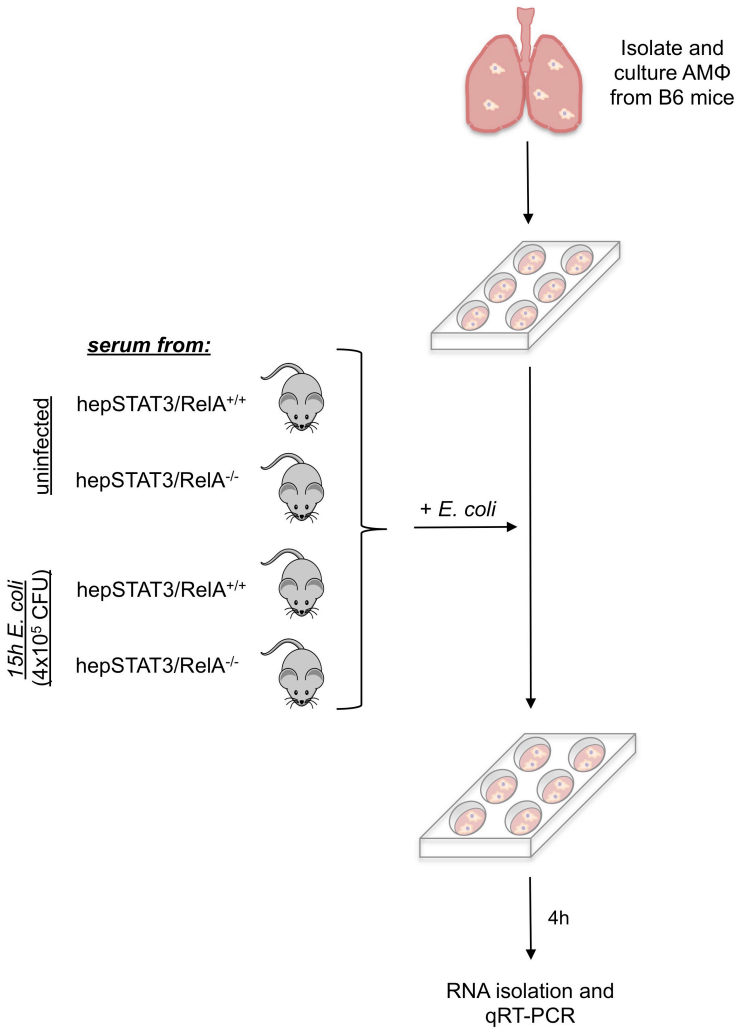


Figure 29. *Ex vivo E. coli* and serum stimulation assay.

Primary alveolar macrophages from C57BL/6 mice were stimulated with 10^5 CFU/ml of *E. coli* in the presence of 1% serum from hepSTAT3/RelA$^{-/-}$ or hepSTAT3/RelA$^{+/+}$ mice, uninfected or infected for 15 hours with 4 x 10^5 CFU of *E. coli*. After 2 hours, cells were washed and then incubated for another 2 hours in media containing the same type of mouse serum. RNA was isolated using Trizol and cytokine expression was determined using qRT-PCR.

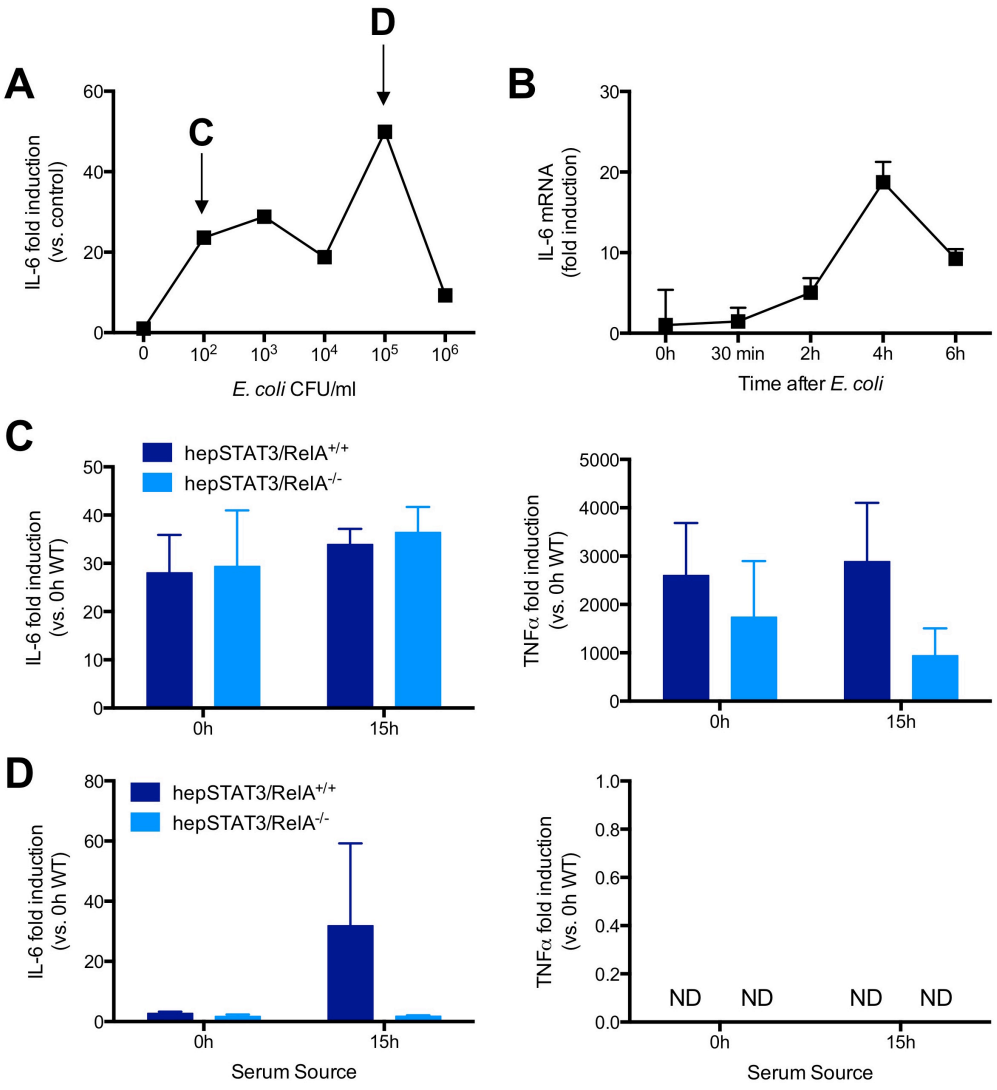


Figure 30. Serum components do not directly modulate *ex vivo* cytokine production in primary alveolar macrophages during *E. coli* stimulation.

A) Primary alveolar macrophages from C57BL/6 mice were stimulated with varying concentrations of log phase *E. coli* for 2 hours. After 2 hours, the bacteria were washed off and cells were incubated for another 2 hours in complete media. RNA was isolated using Trizol and IL-6 expression was determined using qRT-PCR (Duplicate values were

determined; n = 1). **B)** Primary alveolar macrophages from C57BL/6 mice were stimulated with 10^5 CFU/ml of *E. coli* for varying durations. For stimulations over 2 hours, bacteria were washed off at 2 hours and cells were incubated for the remaining time in complete media. RNA was isolated using Trizol and IL-6 expression was determined using qRT-PCR (Duplicate values were determined; n = 1). **C)** Primary alveolar macrophages from C57BL/6 mice were stimulated with 10^5 CFU/ml of *E. coli* in the presence of 1% serum from hepSTAT3/RelA$^{-/-}$ or hepSTAT3/RelA$^{+/+}$ mice, uninfected or infected for 15 hours with 4 x 10^5 CFU of *E. coli*. After 2 hours, cells were washed and then incubated for another 2 hours in media containing the same type of mouse serum. RNA was isolated using Trizol and cytokine expression was determined using qRT-PCR. Significance was assessed by 2-way ANOVA (n = 3). **D)** Primary alveolar macrophages from C57BL/6 mice were stimulated with 10^2 CFU/ml of *E. coli* in the presence of serum from hepSTAT3/RelA$^{-/-}$ or hepSTAT3/RelA$^{+/+}$ mice, uninfected or infected for 15 hours with 4 x 10^5 CFU of *E. coli*. After 2 hours, cells were washed and incubated for another 2 hours in media containing the same type of mouse serum. RNA was isolated using Trizol and cytokine expression was determined using qRT-PCR. Significance was assessed using a 2-way ANOVA (n = 3).

Discussion

Our current results are the first, to our knowledge, to indicate a direct and significant influence of hepatic responses on pulmonary inflammation and host outcome during pneumonia. Following a higher inoculum of *E. coli*, hepatocyte STAT3 and RelA are necessary for survival, maximal bacterial clearance, and liver protection. We have shown that this response, likely via RelA, is required to counter TNF-dependent toxicity in the liver itself. On the other hand, our data following a milder, non-lethal inoculum also suggest an extra-hepatic role of the APR, whereby liver-derived products promote macrophage cytokine expression and innate immunity in the lungs.

To date, there have been limited studies investigating the functional role of the hepatic APR during inflammation, yet most suggest an anti-inflammatory role (Renckens et al., 2006; Renckens et al., 2008; Sakamori et al., 2007). Sakamori *et al*. showed that inflammatory cytokine production was amplified in hepatocyte $STAT3^{-/-}$ mice after intraperitoneal LPS injection, suggesting that STAT3-dependent APPs limit excessive inflammation during sepsis (Sakamori et al., 2007). Renckens *et al.* demonstrated that a pre-existing APR reduces subsequent immune responses to pulmonary challenges with *P. aeruginosa* or *A. baumannii* (Renckens et al., 2006; Renckens et al., 2008). While this is seemingly contradictory to the present results in which the APR supports cytokine synthesis and inflammation, it is notable that the models involve very different scenarios. Renckens *et al.* induced a premature APR by injection of turpentine prior to lung infection, whereas we interrogated the influence of hepatic responses elicited by the pneumonia itself. Moreover, the APR being studied here is exclusively being induced in

hepatocytes downstream of STAT3 and RelA, as opposed to any and all cells responding to systemic turpentine administration. Recently, our laboratory has also shown that the APR is necessary for survival during a Gram-positive, *S. pneumoniae* infection (Quinton et al., 2012a). Indeed, these studies and others have now revealed over 1000 gene changes and numerous biological processes in the liver that are altered during pneumonia, any or all of which could influence disease outcome (Ahyi et al., 2013; Quinton et al., 2012a; Weber et al., 2012). In those studies, hepSTAT3/RelA$^{-/-}$ mice showed decreased complement deposition and opsonophagocytosis of pneumococcus, leading to increased bacteremia, and suggesting that one function of the APR is to limit dissemination of infection. However, those studies were not specifically empowered to observe effects of the APR in the lungs themselves due to the highly virulent strain of pneumococcus (Quinton et al., 2012a). Using phenotypically distinct inocula of *E. coli* in our current study has enabled us to more precisely identify novel functions of the APR both inside and outside of the lungs during pneumonia.

HepSTAT3/RelA$^{+/+}$ mice infected with a high dose of *E. coli* had a robust APR and high concentrations of circulating APPs, and this response was ablated in the absence of hepatocyte STAT3 and RelA. Interestingly, airspace concentrations of SAA in hepSTAT3/RelA$^{+/+}$ mice were significantly increased and reflected those in the circulation, but there was no change from baseline in hepSTAT3/RelA$^{-/-}$ mice. There was also no difference in mRNA induction in whole lung homogenates between genotypes, suggesting that SAA in the airspaces resulted from plasma extravasation, which is also evident by a significant correlation between serum and BALF SAA levels. Other APPs

tested (SAP, LBP, LCN2, and CRP), however, showed no changes in BALF concentrations between genotypes. This is possibly due to the kinetics of circulating APP changes versus lung barrier integrity. For instance, accumulation of SAP in the airspaces through 24 hours may solely represent equivalent extravasation in both genotypes of serum SAP, which remains mostly unchanged until 30 hours, a point at which injury and protein influx may be receding. SAA, on the other hand, increases more rapidly in the circulation of hepSTAT3/RelA$^{+/+}$ mice, perhaps allowing for earlier representation of this difference in the airspaces. Alternatively, different APPs may have diverse abilities to extravasate into the lungs during infection. There have been multiple reports about active uptake of various serum proteins by alveolar epithelial cells (including albumin and ferritin) to transport them into the alveolar space, and SAA could be another serum protein whose BALF concentrations are aided by such transport mechanisms (John et al., 2001; Kim and Malik, 2003; Williams, 1984a; Williams, 1984b; Wright et al., 1987). While SAP expression is restricted to the liver, LBP and LCN2 are produced in the lungs by alveolar epithelial cells and neutrophils (Chan et al., 2009; Cowland and Borregaard, 1997; Dentener et al., 2000; Kjeldsen et al., 2000; Klein et al., 1998), which may contribute to BALF concentrations. Overall, the relationship between circulating and lung APP content appears to be complex and selective, with multiple factors dictating protein distribution. It is important to note, however, that our model is engineered to specifically indicate the consequences of liver-dependent changes, and these hepatic changes are ultimately responsible for the observed phenotypes.

Following a higher inoculum of *E. coli*, hepatocyte STAT3 and RelA are necessary for liver protection. We have shown that this response, likely via RelA, is required to counter TNF-dependent toxicity in the liver itself. HepSTAT3/RelA$^{-/-}$ mice infected with a severe *E. coli* pneumonia also had a greater mortality rate than littermate controls, despite equivalent lung injury and inflammation between genotypes. It has long been recognized that mice with complete RelA deficiency are embryonic lethal due to TNFα-dependent liver injury (Alcamo et al., 2001; Beg and Baltimore, 1996; Beg et al., 1995; Ding and Yin, 2004; Doi et al., 1999; Li et al., 1999). There have also been reports linking STAT3 and liver injury in various models (Haga et al., 2003; Klein et al., 2005; Kovalovich et al., 2000; StreetZ et al., 2003). In our own studies, mice lacking both transcription factors suffered from TNFα-dependent liver injury, likely due to dysregulated apoptosis that was reversed by TNFα neutralization. However, liver regeneration, which has specifically been shown to rely on STAT3 (Li et al., 2002), does not appear to be defective in hepSTAT3/RelA$^{-/-}$ mice during pneumonia since Ki67 staining was actually increased in the absence of this transcription factor (along with RelA). Interestingly, liver injury was not responsible for the increase in mortality, as hepatoprotection via TNFα blockade was insufficient to reduce mortality in hepSTAT3/RelA$^{-/-}$ mice. Additionally, kidney function was normal in both groups; thus, the cause of mortality in hepSTAT3/RelA$^{-/-}$ mice remains elusive. One possibility is that combined changes in both local lung innate immunity and liver injury are together contributing to mortality, whereas neither alone is great enough to impair survival. The

degree to which these outcomes and/or other manifestations in the absence of an intact liver response contribute to mortality during pneumonia remains to be determined.

Our lower inoculum infection protocol revealed that the APR promotes lung inflammation. HepSTAT3/RelA$^{-/-}$ mice lacking the APR have a significant reduction in BALF total protein and cytokine concentrations. These findings were directly associated with marked decreases in BALF APP concentrations, without such differences in lung APP mRNA. This, along with the hepatocyte-specific nature of our mouse model, strongly suggests that liver APP expression is a primary determinant of their concentration in injured airspaces. One curious observation to note, however, is that significant differences in lung APP content (15h) precede what appear to be little (SAA) to no (SAP) changes in blood protein. This discrepancy between circulating and lung APP content could be related to the timing of the measurements, which were taken after 15h. Circulating APP concentrations could be significantly different between genotypes at earlier time points. Alternatively, early changes in airspace APP concentrations could have been secondary to reduced inflammation in hepSTAT3/RelA$^{-/-}$ mice, such that reduced levels of SAA and SAP resulted from a global decrease in protein flux. Nonetheless, pulmonary APP content was inherently hepatocyte STAT3- and RelA-dependent, as the presence of these two transcription factors was the only difference between genotypes, suggesting that liver-derived APPs are necessary for maximal lung inflammation.

We aimed to identify cell types in the lungs directly targeted by acute phase changes in the liver by measuring cell-specific cytokine induction in airspace

macrophages and neutrophils, both of which represent important cytokine sources in the lungs (Cassatella, 1995; Mosser and Edwards, 2008; Sibille and Reynolds, 1990). Excitingly, we found an altered cytokine profile in airspace macrophages, but not in circulating monocytes or neutrophils from any site. The macrophage mRNA changes were consistent with the results observed in whole lung measurements. To more conclusively determine whether plasma, and hence APP, extravasation was required for APR-dependent cytokine synthesis in macrophages, we also measured this outcome at 1.5 hours of infection, a time sufficient to detect increased cytokine responses but preceding alveolar edema. At this early time point, macrophage responses were identical between genotypes, supporting the conclusion that extravasated proteins facilitate macrophage cytokine production in an APR-dependent manner. Interestingly, no such effect was observed in circulating cells, suggesting that the reprogramming of macrophages by acute phase serum occurs strictly in the alveolar compartment. The specific soluble mediators responsible for linking the hepatic APR to macrophage cytokine responses remain unknown. Multiple APPs can bind and activate macrophages, including, but not limited to, SAA (Cheng et al., 2008; Niemi et al., 2011; Shah et al., 2006), SAP (Zhang et al., 2011), LBP (Tobias et al., 1992) and CRP (Barna et al., 1984; Mold and Du Clos, 2006). It is also possible that the net effect of acute phase changes is determined by a combination of relatively modest influences from numerous factors. Thus, it is possible, if not likely, that our findings cannot be traced to a single protein. Additionally, we show that APR-dependent serum components cannot directly modulate macrophage cytokine responses *ex vivo*. Whether or not these results recapitulate an *in*

vivo setting, remains to be determined. Regardless, the data provide evidence for a cell-specific, functional role of the APR in facilitating macrophage activation during pneumonia.

Our data are the first to show that APR signals intrinsic to the liver are hepatoprotective, while extra-hepatic consequences of the APR are necessary to promote host defense and pulmonary inflammation during pneumonia. While promoting inflammation can be detrimental in the context of acute lung injury, the liver response to pneumonia is also pro-defense, and importantly, pro-survival. This protective nature of the liver is consistent with our own studies (Quinton et al., 2012a) and others' during infection (Sander et al., 2010; Weber et al., 2012). For instance, Weber *et al.* showed that liver-derived cholesterol attenuates the pathogenesis of a common streptococcus virulence factor, pneumolysin (Weber et al., 2012). Sander *et al*. observed that hepatic STAT3-dependent signals (SAA in particular) could attenuate mortality during polymicrobial sepsis, perhaps through mobilization of myeloid derived suppressor cells (Sander et al., 2010). While neither of these particular examples is likely to explain our own results with Gram-negative pneumonia, they importantly put forth additional evidence that liver function is indispensable for modulating the pathogenesis of acute infection. Overall, our results indicate that APPs serve critical roles extending beyond their common use as biomarkers. A better understanding of the lung-liver axis will provide valuable insights into potential therapeutic or diagnostic targets for clinical intervention in patients with or at risk for pneumonia.

CHAPTER FOUR: ACTIVATION OF HEPATIC STAT3 MAINTAINS PULMONARY DEFENSE DURING ENDOTOXEMIA.

Rationale

Sepsis is a complex immunopathological syndrome defined by the systemic inflammatory response to infection, and is a leading contributor to morbidity and mortality in intensive care units as evidenced by approximately 750,000 cases per year (2% of all hospital admissions) (Angus et al., 2001; Angus and van der Poll, 2013; Lagu et al., 2012). This multifaceted, systemic inflammatory response can be further complicated by organ dysfunction (severe sepsis) and hypotension (septic shock), all which lead to a complex, variable syndrome with mortality rates between 30 and 50% (Bosmann and Ward, 2013). While pneumonia is the leading cause of sepsis, with about half of all sepsis cases originating as respiratory infections (Angus and van der Poll, 2013), sepsis also greatly increases a patient's subsequent susceptibility to bacterial pneumonia (Alberti et al., 2002). In fact, 10 - 30% of mechanically ventilated, septic shock patients develop ventilator-associated pneumonia (Chastre and Fagon, 2002). This positive association extends beyond ventilator-related circumstances, and has been corroborated experimentally by multiple studies demonstrating deleterious effects of sepsis and/or endotoxemia on pneumonia outcomes (Benjamim et al., 2010; Delano et al., 2010; Deng et al., 2006; Frevert et al., 1994; Jung et al., 2012; Nelson et al., 1990; Steinhauser et al., 1999; Wagner et al., 1999; White et al., 1986). With the rapid increase in prevalence of drug resistant pathogens and limited treatment options available, there is

a growing need to develop novel pharmaceutical interventions and to elucidate our understanding of the inflammatory processes involved in both pathologies.

A shared and prominent feature of sepsis, pneumonia, and other inflammatory conditions is the hepatic APR (Gabay and Kushner, 1999; Gamble et al., 2009; Quinton et al., 2009; Weber et al., 2012). While it is well appreciated that sepsis can cause pulmonary immunosuppression and pneumonia susceptibility (Alberti et al., 2002; Benjamim et al., 2010; Delano et al., 2010; Deng et al., 2006; Frevert et al., 1994; Jung et al., 2012; Nelson et al., 1990; Steinhauser et al., 1999; Wagner et al., 1999; White et al., 1986), it is unclear whether or how pre-existing liver activation (*i.e.* sepsis-induced APR) modulates subsequent responses to local lung infections.

As detailed above, STAT3 is one of two transcription factors (along with RelA) required for induction of a strong hepatic APR during pneumonia (Ahyi et al., 2013; Hilliard et al., 2015; Quinton et al., 2012a). In Chapter 3 (Hilliard et al., 2015), we show that this lung-liver axis, enabled by both transcription factors, is required for maximal protection during pneumonia alone, but the distinct roles of STAT3 (vs. RelA) in this process remain unclear. Others have linked hepatic STAT3 activity to the APR in models of sepsis (Alonzi et al., 2001; Sakamori et al., 2007). Given the close association between pneumonia, sepsis, STAT3, and the APR, we sought to determine the direct influence of systemic STAT3-dependent liver activity on subsequent pneumonia outcomes.

Results

4.1 The APR is dependent on liver STAT3 during endotoxemia followed by pneumonia.

In order to determine the effect of STAT3-dependent liver activation in the context of sepsis and pneumonia, we used the Cre-*LoxP* system to obtain a mouse model of hepatocyte specific, functional STAT3 deletion (hepSTAT3$^{-/-}$). To model aspects of the clinical circumstances of sepsis preceding pneumonia, we employed a dual challenge of endotoxemia followed by a bacterial lung challenge (Figure 31). HepSTAT3$^{-/-}$ and hepSTAT3$^{+/+}$ mice were administered an i.p. injection of either 5 mg/kg of LPS or vehicle (saline). After 18 hours, 1x10^{6} CFU of *E. coli*, strain O6:K2:H1, was intratracheally instilled into left lung lobes for an additional 0, 6, or 24 hours. As stated previously, Gram negative infections, including *E. coli* pneumonias, are a major cause of nosocomial pneumonia (Ahmed and Niederman, 2001; Jones, 2010), which are particularly relevant during sepsis as septic patients have a much greater risk of developing enterobacterial, hospital-acquired pneumonias (Alberti et al., 2002; Chastre and Fagon, 2002). As such, *E. coli* pneumonia was utilized in this model for sepsis-induced pneumonia because of its specific relevance to septic patients. Because liver STAT3 activation is required for maximal APR induction (Ahyi et al., 2013; Alonzi et al., 2001; Quinton et al., 2012a; Sakamori et al., 2007), we measured concentrations of two representative, circulating APPs –SAA and SAP. In hepSTAT3$^{+/+}$ mice, serum concentrations of both SAA and SAP were induced dramatically above baseline with LPS pretreatment alone (Figure 31A, B– 0h).

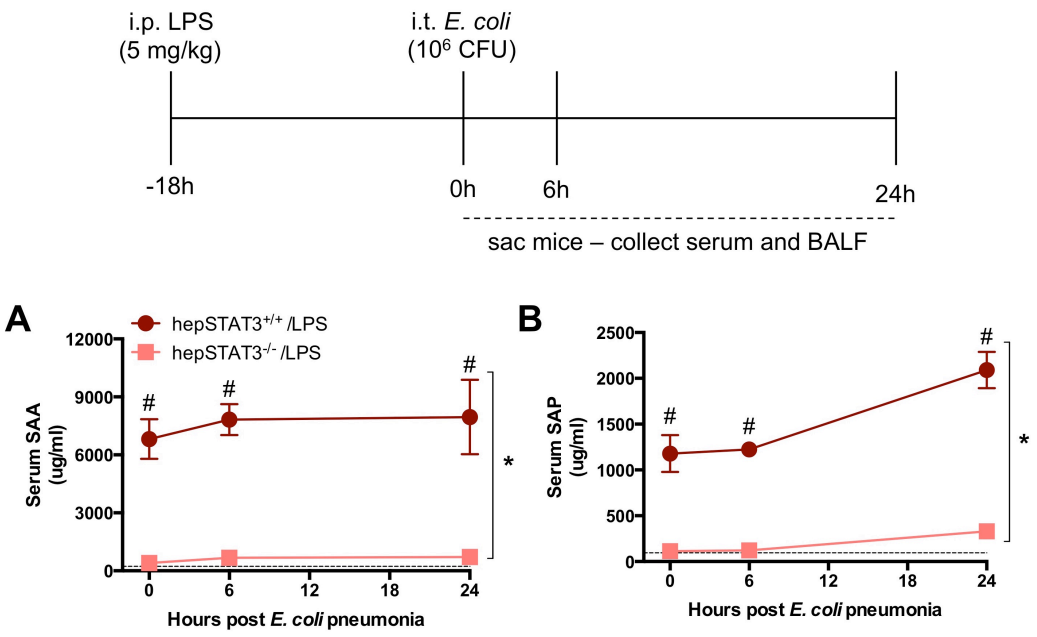


Figure 31. The APR is dependent on liver STAT3 activation during endotoxemia followed by pneumonia.

HepSTAT3$^{+/+}$ and hepSTAT3$^{-/-}$ mice were pretreated with an intraperitoneal injection of 5 mg/kg LPS. After 18 hours, mice were intratracheally infected with 1x10^6 CFU of *E. coli*. Mice were euthanized at 0, 6, or 24 hours post *E. coli* infection. At the indicated time points, serum was collected, and SAA and SAP acute phase protein concentrations were measured using an ELISA. Dashed lines indicate baseline concentrations in vehicle-treated, hepSTAT3$^{+/+}$ mice without pneumonia. *$p < 0.05$ for overall effect of genotype as determined by 2-way ANOVA. # $p < 0.05$ vs hepSTAT3$^{+/+}$ mice at the indicated time point as determined by a Holm-Sidak post hoc test (n = 3-9 per group).

Unlike SAA, SAP serum concentrations were further increased by *E. coli* infection, as there was a significant effect of infection only for SAP serum levels (Figure 31B). Independent of treatment (LPS and/or *E. coli* pneumonia) APP concentrations remained unchanged in hepSTAT3$^{-/-}$ mice but were significantly different from hepSTAT3$^{+/+}$ mice, indicating that hepatic STAT3 function is necessary for a maximal APR.

In order to determine whether an endotoxin-induced (STAT3-dependent) APR could affect the local lung environment, we sampled the protein and cellular content of the airspaces by BAL. LPS pretreatment alone was insufficient to alter baseline concentrations of airspace SAA and SAP in either mouse genotype (Figure 32 – 0h). However, intrapulmonary infection with *E. coli* markedly increased concentrations of both APPs in the BALF of hepSTAT3$^{+/+}$ mice. SAP increases in BALF were significantly blunted in hepSTAT3$^{-/-}$ mice, with a similar trend observed for SAA (Figure 32). These changes resembled those in the blood compartment, suggesting that airspace APP content is a function of plasma extravasation into pneumonic lungs, especially in the case of SAP. This is further evidenced by significant correlations between serum and BALF concentrations of both APPs after 24 hours of infection (Figure 33).

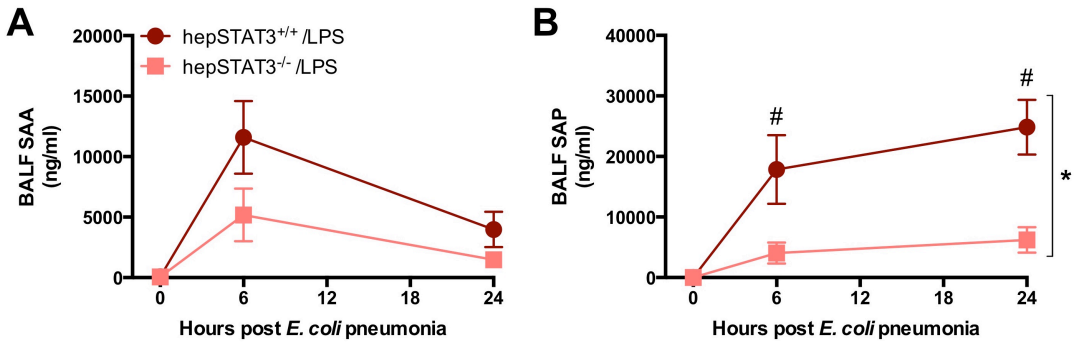


Figure 32. During endotoxemia and pneumonia, intrapulmonary APPs are reflective of serum APP content.

HepSTAT3$^{+/+}$ and hepSTAT3$^{-/-}$ mice were pretreated with an intraperitoneal injection of 5 mg/kg LPS. After 18 hours, mice were intratracheally infected with 1x10^{6} CFU of *E. coli*. Mice were euthanized at 0, 6, or 24 hours post *E. coli* infection. BALF was collected, and SAA and SAP concentrations were measured using an ELISA. *$p < 0.05$ for overall effect of genotype as determined by 2-way ANOVA. #$p < 0.05$ vs hepSTAT3$^{+/+}$ mice at the indicated time point as determined by a Holm-Sidak post hoc test (n = 3-9 per group).

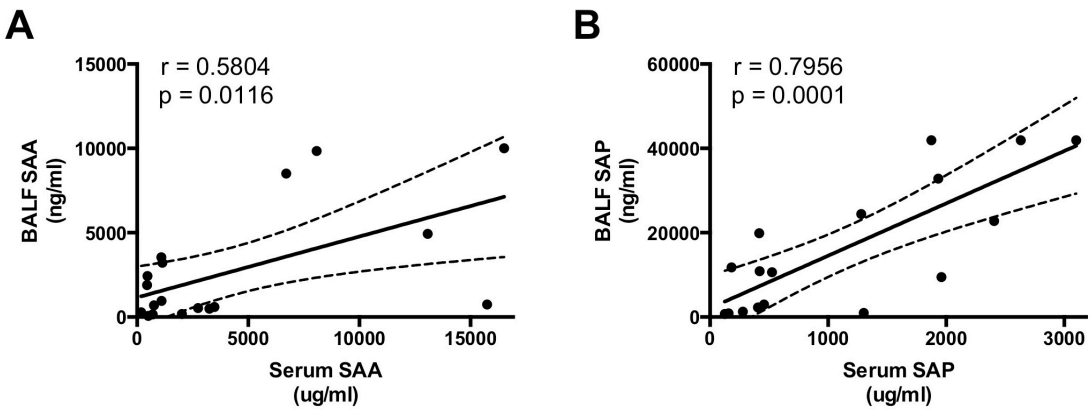


Figure 33. Circulating levels of APPs significantly correlate with airspace concentrations after endotoxemia followed by pneumonia.

(A) SAA and **(B)** SAP concentrations from 24-hour serum and BALF (from Figures 27 and 28) were compared, and a correlation with a linear regression was performed. Each individual point represents a single mouse (both hepSTAT3$^{+/+}$ and hepSTAT3$^{-/-}$). Pearson *r* values and *p* values for each correlation and shown (n = 19).

4.2 Host defense during endotoxemia and pneumonia is compromised by lack of hepatic STAT3.

In order to determine if an endotoxemia-induced hepatic APR affects pulmonary host defense and/or inflammation during pneumonia, we measured 24-hour lung and blood bacterial burdens in both genotypes of mice pretreated with either LPS or vehicle. While antibacterial defense was equivalent in 3 of 4 groups, hepSTAT3$^{-/-}$ mice pretreated with LPS had significantly greater lung bacterial burdens (Figure 34A), suggesting that STAT3-dependent liver activity is required for local defense in response to a pre-existing endotoxemia. HepSTAT3$^{-/-}$ mice pretreated with LPS also had significantly increased bacteremia, possibly due to differences in dissemination and/or systemic clearance (Figure 34B). Interestingly, 24 hours after *E. coli* in endotoxemic hepSTAT3$^{-/-}$ mice, impaired antibacterial defense was associated with increased mortality (Figure 34C). Given the results detailed in Chapter 3 showing the necessity for hepatic STAT3 *and* RelA in pulmonary host defense during pneumonia alone (Hilliard et al., 2015), we aimed to determine if the hepatic APR induced by both transcription factors played a similar role in lung defense during sepsis-induced pneumonia as observed with single STAT3 mutant mice. After just 15 hours of i.p. LPS treatment, endotoxemic hepSTAT3/RelA$^{-/-}$ mice had significantly greater mortality than all other groups tested (Figure 34D). This result is unsurprising given that liver RelA activation is necessary for hepatoprotection during pneumonia. Intraperitoneal LPS may have caused a rapid liver response preceding liver injury, which is likely attributable to the mortality observed here.

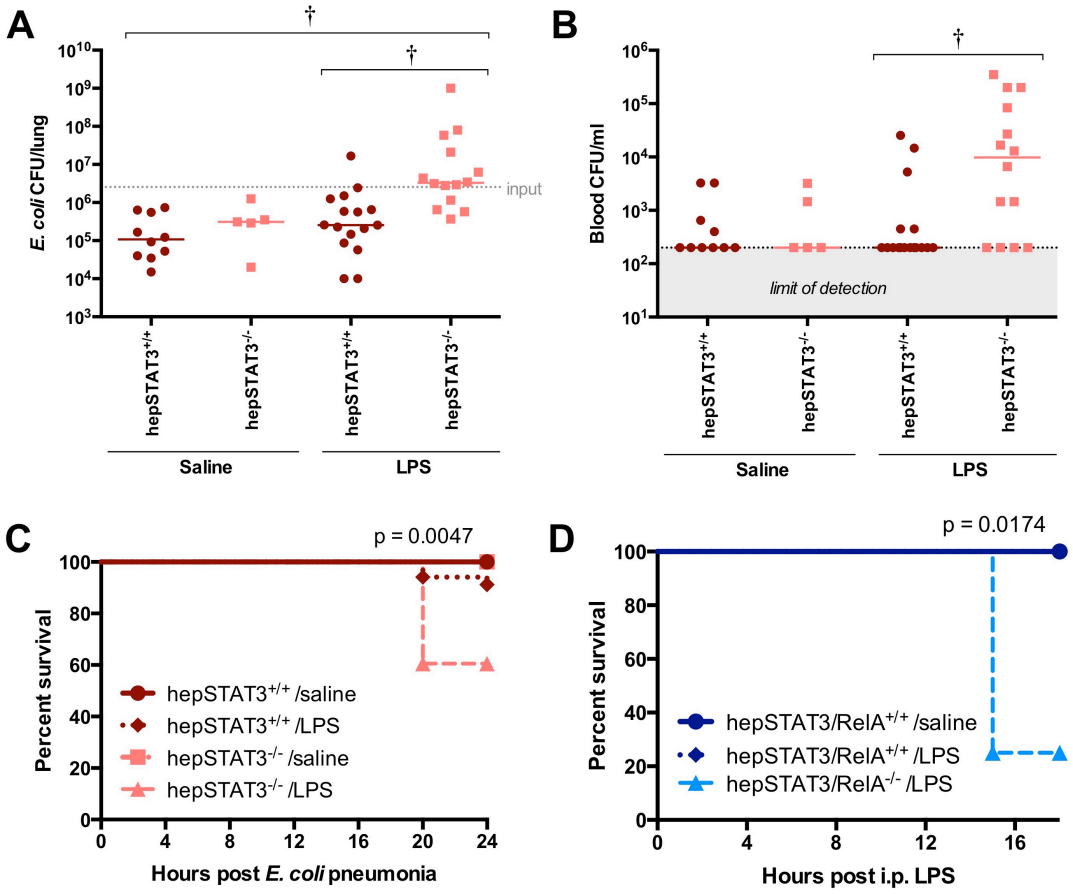


Figure 34. Host defense during endotoxemia and pneumonia is compromised by lack of hepatic STAT3.

HepSTAT3$^{+/+}$ and hepSTAT3$^{-/-}$ mice were treated for 18 hours with intraperitoneal LPS or saline followed by intratracheal *E. coli*. After 24 hours of *E. coli* infection, **(A)** lungs were homogenized and **(B)** blood was processed for quantification of viable bacteria. † $p < 0.05$ between the denoted groups based on a Kruskal-Wallis test followed by Dunn's multiple comparison test (n = 5-16 per group). **C)** Survival was observed through 24 hours of infection. A Mantel-Cox text was used to determine significance (n = 4-38 per group). **D)** HepSTAT3/RelA$^{-/-}$ and hepSTAT3/RelA$^{+/+}$ mice were treated with

intraperitoneal LPS. Survival was observed through 18 hours. A Mantel-Cox text was used to determine significance (n = 4-6 per group).

While future experiments are needed to confirm hepatotoxicity as the cause of mortality in APR-null, hepSTAT3/RelA$^{-/-}$ mice, this finding was sufficient to preclude the efficacy of this mouse model for studies employing endotoxemia prior to pneumonia.

Bacterial killing in the lungs relies on innate immunity, including that provided by recruited neutrophils and other extravasated plasma constituents during inflammation (Quinton and Mizgerd, 2015). In order to determine whether local inflammation was compromised by STAT3 deficiency, we measured BALF neutrophil recruitment and total protein concentrations (Figure 35A, B). We observed an influx of neutrophils at 24 hours post *E. coli* in both hepSTAT3$^{+/+}$ and hepSTAT3$^{-/-}$ mice, consistent with an acute pneumonia (Figure 35A). Additionally, there were significantly greater numbers of neutrophils recruited to the airspaces in hepSTAT3$^{-/-}$ mice compared to hepSTAT3$^{+/+}$ mice at 24 hours post *E. coli*, which was likely secondary to increased bacterial loads. BALF total protein concentrations were also increased due to infection, but no differences were observed between genotypes (Figure 35B). These data suggest that STAT3-dependent liver responses are protective in the setting of sepsis followed by pneumonia. This response, however, does not appear to be mediated through alveolar neutrophil recruitment.

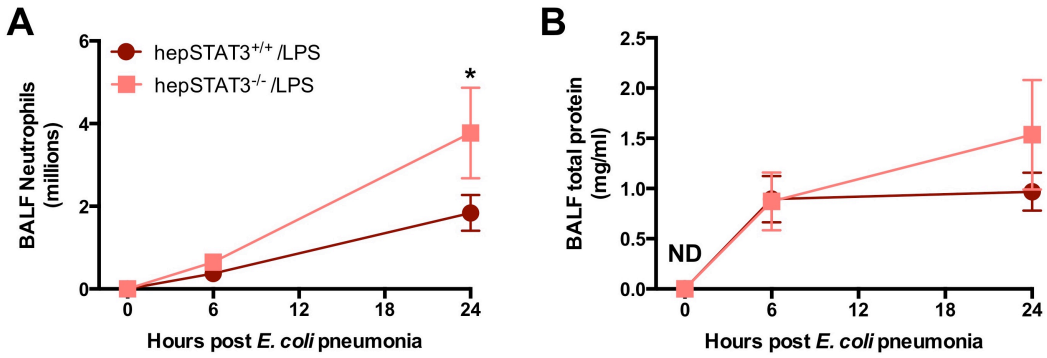


Figure 35. Neutrophil recruitment is not dependent on hepatic STAT3 activation during endotoxemia followed by pneumonia.

HepSTAT3$^{+/+}$ and hepSTAT3$^{-/-}$ mice were treated for 18 hours with intraperitoneal LPS followed by intratracheal *E. coli.* At the indicated time points, BALF was harvested for determination of **(A)** recruited neutrophils numbers and **(B)** total protein concentrations. * $p < 0.05$ vs hepSTAT3$^{+/+}$ mice at the indicated time point as determined by a two-way ANOVA followed by a Holm-Sidak test (n = 3-9 per group). ND = not detected.

4.3 Pulmonary and systemic cytokine induction is not reliant on STAT3-dependent acute phase changes.

As another index of lung and systemic inflammation, BALF and serum cytokine protein concentrations were measured (Figures 36 and 37). We utilized a multiplex bead array to determine concentrations of 10 cytokines, all of which are relevant to pneumonia and/or lung injury (Quinton and Mizgerd, 2011; Quinton and Mizgerd, 2015) — IL-1β, IL-6, IL-10, IL-17, G-CSF, GM-CSF, CXCL1, TNFα, LIF, and CXCL2. We observed several patterns of cytokine kinetics in the airspaces, ranging from increases due to *E. coli* infection to no change at all, but there were no changes in BALF cytokine concentrations due to genotype (Figure 36). Serum cytokine changes were also variable across targets, however, unlike in the BALF, three serum cytokines were significantly changed due to the absence of liver STAT3 —IL-1β, IL-17 and TNFα (Figure 37). Concentrations of both IL-17 and TNFα were significantly greater in hepSTAT3$^{-/-}$ mice, consistent with increased bacteremia. Interestingly, IL-1β was significantly decreased in hepSTAT3$^{-/-}$ mice after LPS pretreatment (0h post-*E. coli*), but not during the course of infection, potentially indicating a small defect in systemic innate immunity. Whether or how this genotype-dependent decrease in IL-1β contributes to the phenotype of this group remains unclear.

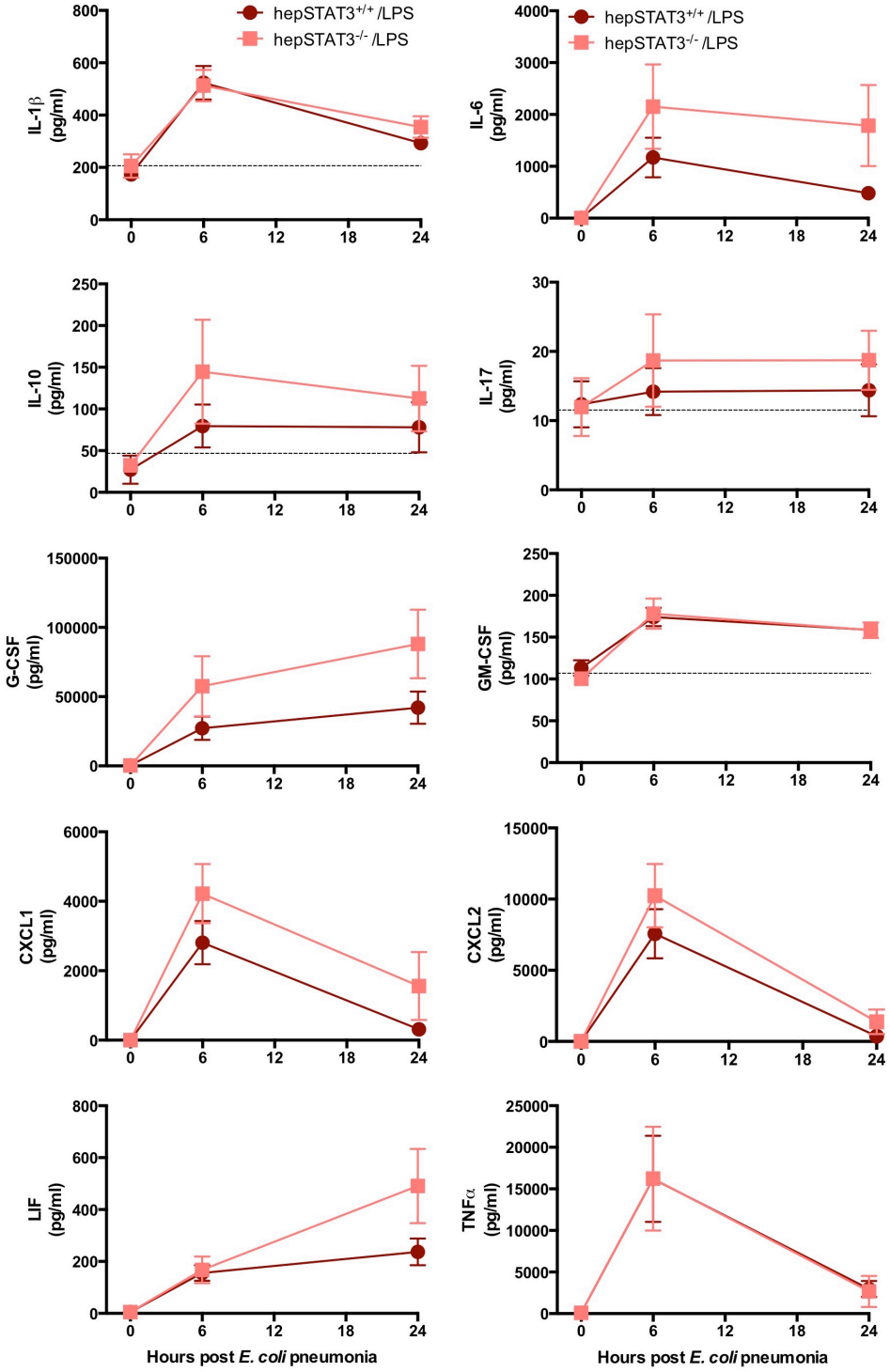


Figure 36. Pulmonary cytokine induction is unaffected by hepatic STAT3 deletion.

HepSTAT3$^{+/+}$ and hepSTAT3$^{-/-}$ mice were treated for 18 hours with intraperitoneal LPS followed by intratracheal *E. coli*. At the indicated time points after *E. coli* infection, lungs were lavaged and BALF cytokine protein concentrations were determined using a multiplex bead array. Dashed lines (some of which overlap with the X-axis) indicate baseline concentrations in vehicle-treated, hepSTAT3$^{+/+}$ mice without pneumonia. There was no significant overall effect of genotype observed. Significance was assessed using a two-way ANOVA (n = 3-9 per group).

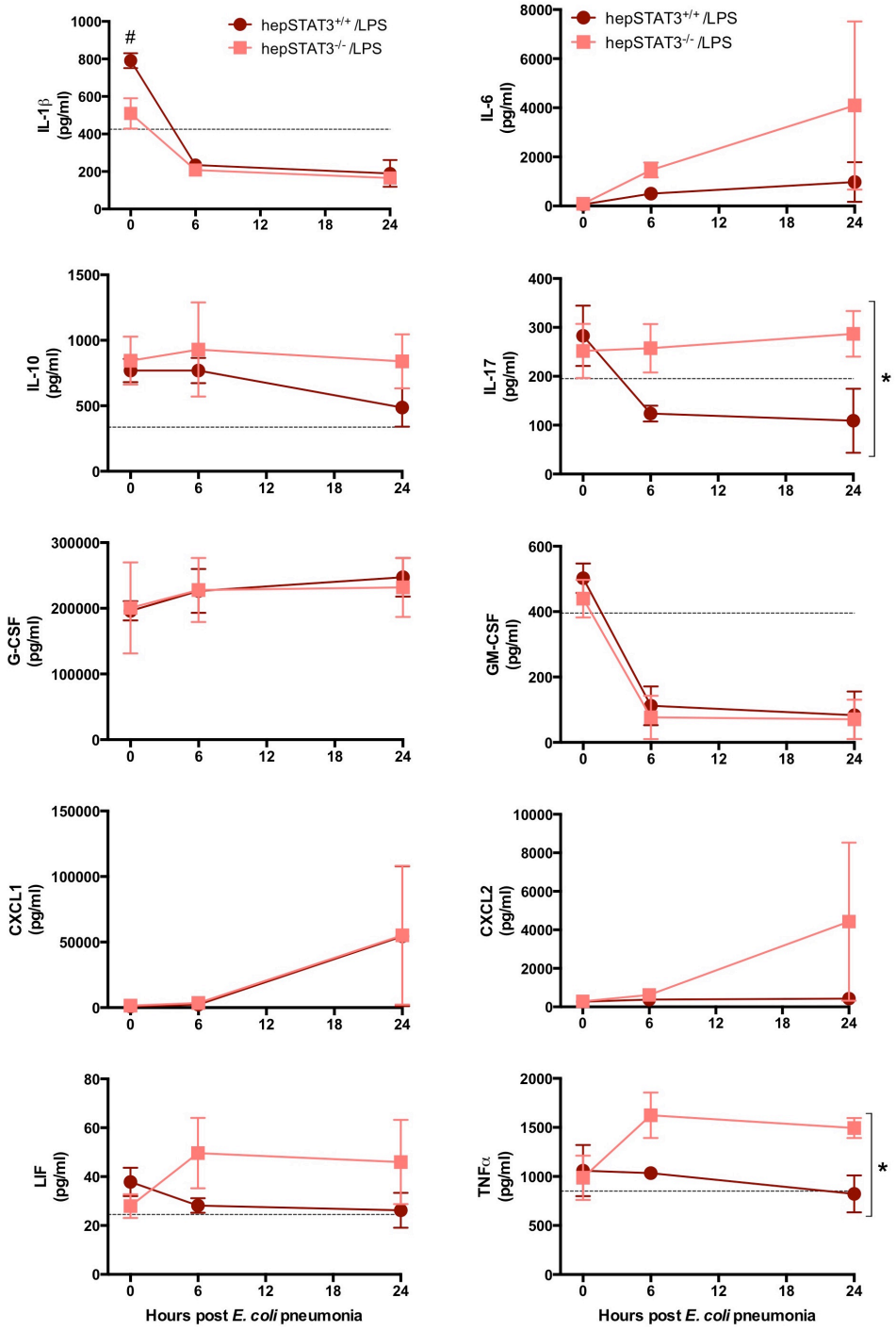


Figure 37. Hepatic STAT3 activation has a minimal effect on circulating cytokine concentrations.

HepSTAT3$^{+/+}$ and hepSTAT3$^{-/-}$ mice were treated for 18 hours with intraperitoneal LPS followed by intratracheal *E. coli*. At the indicated time points after *E. coli* infection, serum was collected and cytokine concentrations were measured with a multiplex bead array. Dashed lines (some of which overlap with the X-axis) indicate baseline concentrations in vehicle-treated, hepSTAT3$^{+/+}$ mice without pneumonia. For IL-1ß, *$p < 0.05$ vs. hepSTAT3$^{+/+}$ mice at that time point as determined by a two-way ANOVA followed by a Holm-Sidak test. For IL-17 and TNFα *$p < 0.05$ for overall affect of genotype as determined by two-way ANOVA. n = 3-9 per group.

4.4 The hepatic APR does not modulate phagocytosis in airspace cells during endotoxemia and pneumonia.

Hepatic STAT3$^{-/-}$ mice with a pre-existent endotoxemia have increased bacterial burdens both systemically and locally during pneumonia. Neutrophil recruitment and other inflammatory mediators (*i.e.* cytokines) were either unchanged or increased in hepSTAT3$^{-/-}$ mice, suggesting that these aspects of host defense are uncompromised in hepSTAT3$^{-/-}$ mice. To determine if endotoxin-induced liver STAT3 activation affects cellular defenses during pneumonia, we measured phagocytosis in airspace macrophages and neutrophils using pHrodo *E. coli* bioparticles. These bioparticles are conjugated to a phycoerythrin (PE) fluorophore that fluoresces only in low pH environments, characteristic of the phagolysosomal compartment. This system has been validated by multiple laboratories as an effective strategy for discriminating between surface bound and internalized particles (Berger et al., 2010; Deriy et al., 2009; Neaga et al., 2013). After 18 hours of intraperitoneal LPS, hepSTAT3$^{-/-}$ and hepSTAT3$^{+/+}$ mice were i.t. infected with *E. coli* for 6 hours followed by a second i.t. instillation with pHrodo *E. coli* bioparticles. After one hour, the lungs were lavaged and cells were analyzed by flow cytometry (Figure 38). Airspace macrophages and neutrophils included cells positive for phagocytosis of pHrodo bioparticles (Figure 38A). Neither macrophages nor neutrophils in the airspaces, however, exhibited genotype-dependent differences in the frequency (Figure 38B) or magnitude (Figure 38C) of phagocytosis.

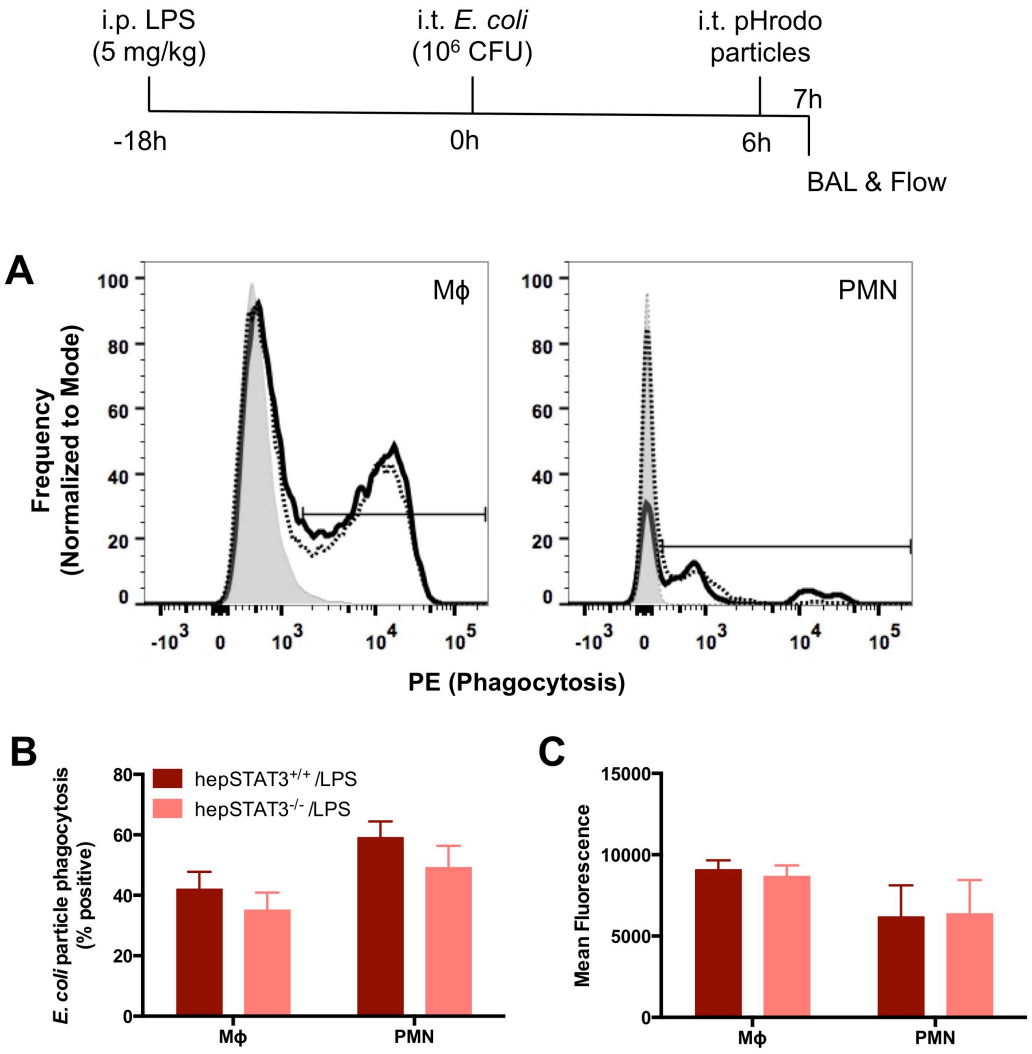


Figure 38. The hepatic APR does not modulate phagocytosis in airspace cells during endotoxemia and pneumonia.

HepSTAT3$^{+/+}$ and hepSTAT3$^{-/-}$ mice were treated with intraperitoneal LPS for 18 hours. Afterwards, *E. coli* was instilled intratracheally, followed six-hours later by a second instillation of *E. coli* pHrodo particles. Lungs were lavaged an hour later and cells were stained as follows for flow cytometry: neutrophils (CD45^{+}/7AAD^{-}/Ly6G^{+}/F4/80^{-}) and alveolar macrophages (CD45^{+}/7AAD^{-}/F4/80^{+}/Ly6G^{-}/Autofluorescence^{hi}). **A)**

Representative histograms illustrate the percentages of cells positive for pHrodo particle phagocytosis in hepSTAT3$^{+/+}$ (black line) and hepSTAT3$^{-/-}$ (dashed line) mice. Filled curves (gray) represent cells not exposed to pHrodo particles. Summarized data for all mice studied were calculated to determine the **(B)** frequency and **(C)** magnitude of particle ingestion, as determined by the percentage of positive cells and mean fluorescence intensity, respectively. No significant changes between genotypes were detected, as assessed by a student's *t* test (n = 5-6 per group).

These data suggest that bacterial uptake and phagolysosomal fusion are unlikely to be responsible for impaired bacterial killing in the absence of hepatocyte STAT3 during endotoxemia and pneumonia.

4.5 Maximal ROS generation in airspace macrophages is dependent on hepatic STAT3 activation.

As an alternative contributor to cellular host defense, ROS generation was measured in airspace cells from both genotypes following 6 hours of pneumonia in endotoxemic mice. Total cells were stained for surface antigens to identify macrophages and neutrophils as described above, and ROS production was measured using the CellROX Deep Red Reagent from Life Technologies (Figure 39A). Interestingly, airspace macrophages from hepSTAT3$^{-/-}$ mice had significantly less ROS production than those from hepSTAT3$^{+/+}$ mice (Figure 39B). A similar trend was apparent with neutrophils, but this did not reach statistical significance (Figure 39B). These data connect compromised macrophage ROS production to impaired pulmonary host defense in endotoxemic hepSTAT3$^{-/-}$ mice.

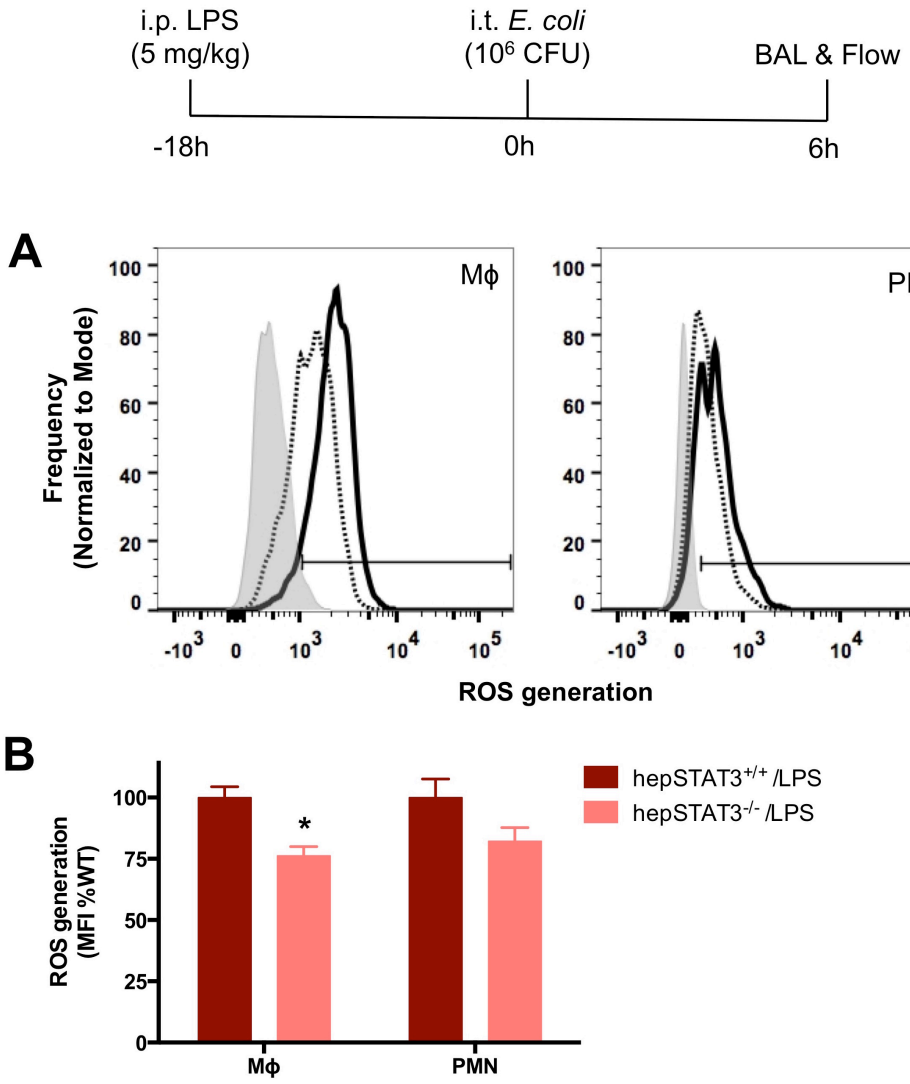


Figure 39. Alveolar macrophage ROS production is dependent on hepatic STAT3 activation.

HepSTAT3$^{+/+}$ and hepSTAT3$^{-/-}$ mice were treated for 18 hours with intraperitoneal LPS followed by an intratracheal instillation of *E. coli.* 6 hours later, the lungs were lavaged and recovered cells were stained using the CellROX Deep Red Reagent to determine ROS generation in neutrophils (CD45^{+}/7AAD^{-}/Ly6G^{+}/F4/80^{-}) and alveolar macrophages

($CD45^{+}/7AAD^{-}/F4/80^{+}/Ly6G^{-}/Autofluorescence^{hi}$). **A)** Representative histograms illustrate the mean fluorescence intensity (MFI) for the populations positive for ROS generation in $hepSTAT3^{+/+}$ (black line) and $hepSTAT3^{-/-}$ (dashed line) mice. Filled curves (gray) represent cells not exposed to CellROX Reagent. **B)** ROS generation was quantified in each cell type and data are illustrated as the percentage of ROS generation observed in $hepSTAT3^{+/+}$ mice. § $p < 0.05$ vs $hepSTAT3^{+/+}$ as determined by a student's *t* test (n = 5-6 per group).

4.6 Soluble host defense mediators within the airspaces are dependent on the hepatic APR.

Neutrophils are immediately recruited to the alveolar compartment during early stages of infection to aid in pathogen clearance (Mizgerd, 2008). As an innate defense, in addition to phagocytosis, neutrophils are equipped to release endogenous genomic DNA laced with antimicrobial proteins to effectively trap and lyse invading microbes. These NETs are studded with granulocytic proteins, including MPO (Brinkmann and Zychlinsky, 2012). As a way to determine whether NET release is affected by the APR, we measured relative concentrations of NETs in BALF from hepSTAT3$^{+/+}$ and hepSTAT3$^{-/-}$ mice after endotoxemia and pneumonia using a previously described MPO-DNA ELISA (Figure 40A) (Caudrillier et al., 2012). As anticipated, we observed an overall increase in NET release due to pneumonia, and while there is a trend towards decreased NET release in hepSTAT3$^{-/-}$ mice, this difference did not reach statistical significance (Figure 40B).

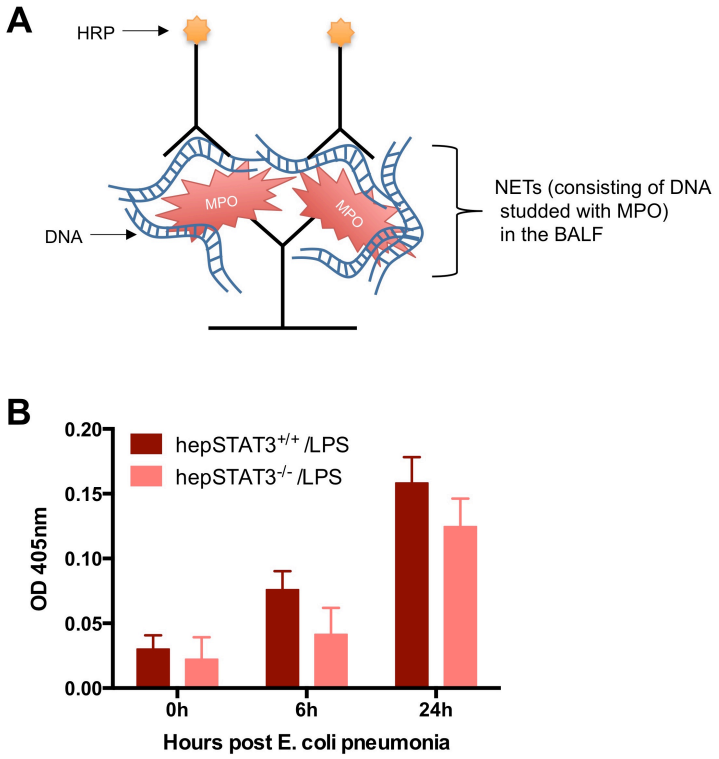


Figure 40. Neutrophil extracellular trap release is unchanged in BALF from hepSTAT3$^{-/-}$ mice during endotoxemia and pneumonia.

A) NETs in the BALF were quantified by a MPO-DNA ELISA. Briefly, a 96-well plate coated with an anti-MPO capture antibody was incubated with the BALF. MPO-DNA complexes (indicative of NETs) bound to the anti-MPO capture antibody were detected using a peroxidase-labeled anti-DNA detection antibody. **B)** BALF was collected from hepSTAT3$^{+/+}$ and hepSTAT3$^{-/-}$ mice pretreated with LPS for 18 hours followed by intrapulmonary infection with *E. coli* for the indicated time periods, and NETs were quantified using the MPO-DNA ELISA as described above. Significance was assessed by a two-way ANOVA followed by a Holm-Sidak test (n = 3-9 per group).

In order to determine whether extracellular products other than NETs may contribute to differential bacterial resistance in the alveolar lining fluid, we developed an assay in which we incubated luminescent *E. coli* (strain Xen14) in cell- and bacteria-free BALF from endotoxemic and pneumonic hepSTAT3$^{+/+}$ or hepSTAT3$^{-/-}$ mice (Figure 41A). Bacterial growth was calculated as fold increases in luminescence compared to the starting values for each sample. Interestingly, BALF from hepSTAT3$^{-/-}$ mice supported bacterial growth significantly more than that from hepSTAT3$^{+/+}$ mice (Figure 41B), suggesting that the airspace milieu of hepSTAT3$^{-/-}$ mice is less resistant to bacterial growth. Whether and how this change in bacterial resistance in the airspaces relies on differences in the antimicrobial proteome or nutrient availability remains uncertain.

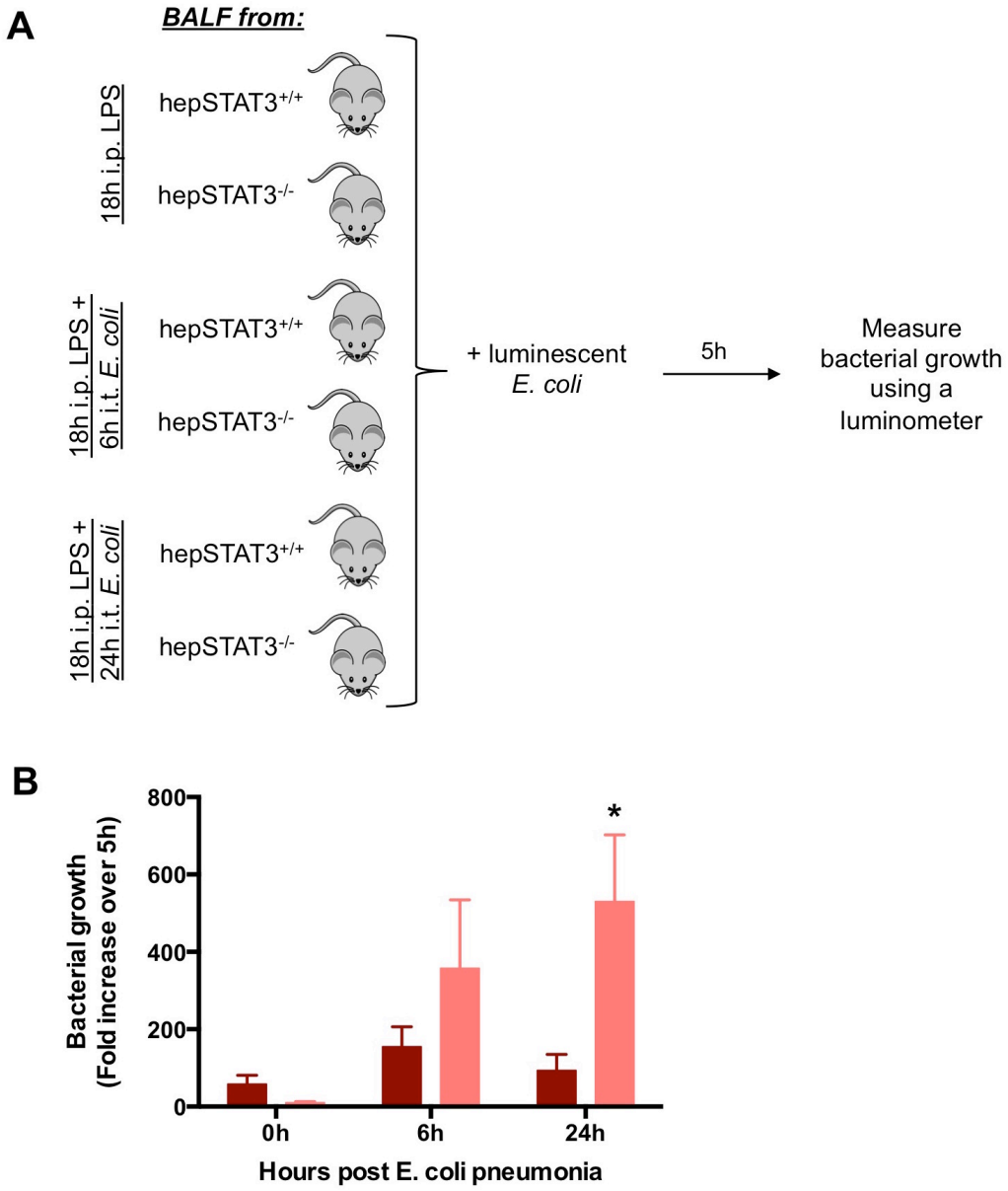


Figure 41. *Ex vivo* bacterial growth assay

A) BALF was collected from hepSTAT3$^{+/+}$ and hepSTAT3$^{-/-}$ mice pretreated with LPS for 18 hours followed by intrapulmonary infection with *E. coli* for the indicated time periods. The cell- and bacteria-free BALF was incubated with log-phase, luminescent *E.*

coli, rotating at 37°C for five hours. Luminescence, as a function of bacterial growth, was measured using a luminometer. **B)** Bacterial growth was calculated as fold increases in luminescence compared to the starting values for each sample. * $p < 0.05$ vs hepSTAT3$^{+/+}$ at the specified time point as assessed by two-way ANOVA followed by a Holm-Sidak test (n = 3-4 per group).

Discussion

The results of this study demonstrate a novel role for the STAT3-dependent liver acute phase response in driving innate host defenses during pneumonia in endotoxemic animals. Using a two-hit model of endotoxemia and intrapulmonary *E. coli*, we observed impaired antibacterial defense and higher mortality in mice that were deficient in hepatic STAT3. While several indices of inflammation (*e.g.* neutrophilia, edema, and cytokine induction) were largely unaffected by interruption of hepatic activation, others (*e.g.* macrophage ROS generation and airway lining fluid content) were dependent on hepatic STAT3.

Physiologic and molecular mechanisms by which hepatic innate responses mediate host defense during sepsis and pneumonia have never been elucidated. Several studies, however, have implicated important roles for hepatic STAT3 activation during either sepsis or pneumonia alone. Alonzi *et al.* described the necessity of STAT3 activation during endotoxemia for induction of the APR (Alonzi et al., 2001). Additionally, Sakamori *et al.* used a hepatocyte-specific STAT3 knockout mouse to show the importance of this signaling pathway in controlling excessive inflammation during polymicrobial sepsis induced by CLP (Sakamori et al., 2007). In fact, their results in mutant mice during sepsis alone were consistent with our own, with decreased survival, as well as increases in circulating cytokines; although, they did not detect changes in blood bacterial burdens. Similarly, Sander *et al.* demonstrated that liver STAT3-dependent signaling was crucial to attenuate mortality, but not host defense, in response to CLP through a process facilitated by SAA-dependent mobilization of myeloid-derived

suppressor cells (Sander et al., 2010). The latter two studies described above, while notable, were not designed to determine the degree to which sepsis-induced liver activation (via STAT3) calibrates subsequent responses to pneumonia, which is a highly distinct and clinically relevant scenario.

It is well established that in both septic patients and animal models, sepsis results in immunosuppression (Reddy et al., 2001b), which is thought to promote secondary infections such as those causing pneumonia (Delano et al., 2010; Hotchkiss et al., 2013). A multitude of studies have revealed detrimental consequences of sepsis-induced immunosuppression on critical pneumonia outcomes, including antibacterial defense, alveolar macrophage function, alveolar neutrophil recruitment, and cytokine production (Benjamim et al., 2010; Carrick et al., 1997; Chen et al., 2000; Frevert et al., 1994; Jung et al., 2012; Nelson et al., 1990; Reddy et al., 2001a; Song et al., 2014; Steinhauser et al., 1999; Wagner et al., 1999; Wagner et al., 2002; White et al., 1986). Our own protocol of endotoxemia followed by pneumonia, however, was not sufficient to recapitulate the circumstances of sepsis-induced immunosuppression. We observed no effect of endotoxemia alone on pulmonary defense in hepSTAT3$^{+/+}$ mice, but rather, endotoxemia compromised bacterial clearance only in mice lacking hepatic STAT3. There are many possible explanations for this. First, the dose of LPS (5 mg/kg) and/or its type (an ultrapure, TLR4 agonist) may not be sufficient to induce immunosuppression in the setting of our pneumonia protocol. Additionally, the timing of LPS pretreatment (18 hours before *E. coli* infection) and/or the genetic background of our hepSTAT3$^{-/-}$ mouse strain could also be factors. The lack of observable LPS-induced immunosuppression in

hepSTAT3$^{+/+}$ mice, however, empowered us to more precisely examine the roles of endotoxin-induced hepatic STAT3 activation on a subsequent lung infection, and this opportunity may have been diminished by overwhelming immunosuppression due to LPS alone.

Independently, our laboratory and others have reported a functional role for the APR in pneumonia alone. We have shown, using an APR-null mouse model (lacking both hepatic STAT3 and RelA), that liver activation is required for survival, hepatoprotection, and maximal pulmonary inflammation during an *E. coli* pneumonia (detailed in Chapter 3), as well as systemic defense and opsonophagocytosis during pneumococcal pneumonia (Quinton et al., 2012a). The common clinical observation that sepsis is frequently followed by pneumonia (Alberti et al., 2002; Chastre and Fagon, 2002; Delano et al., 2010) raises the question of whether or how a pre-existing liver response alters pneumonia susceptibility, for better or for worse. Renckens *et al.* determined that a preexisting APR induced by turpentine impairs the pulmonary inflammatory response to *P. aeruginosa* and *A. baumannii* (Renckens et al., 2006; Renckens et al., 2008). The model of inducing a preexisting APR via turpentine injection is very different from our method of inducing the APR through endotoxemia. Additionally, turpentine's effects are unlikely to be limited to liver activation. Using our hepatocyte-specific STAT3-null mouse in our model of endotoxemia followed by pneumonia allowed us, for the first time, to interrogate the role of pre-existing liver-specific acute phase changes on pneumonia susceptibility.

In association with impaired APP induction, hepSTAT3$^{-/-}$ mice pretreated with LPS had significantly greater bacterial loads in the lungs and blood during pneumonia, implying that in the absence of an intact liver response, local pulmonary defenses are particularly affected during endotoxemia. Increased mortality was also observed in this group, suggesting this defect in host defense as a potential cause of mortality. These outcomes were also associated with an increase in serum TNFα that is likely due to greater amounts of circulating bacteria and could also contribute to death in hepSTAT3$^{-/-}$ mice, as TNFα can cause septic shock (Tracey et al., 1987). In trying to determine which aspects of host defense are mediated by the sepsis-induced APR, we measured pulmonary inflammation and injury. We observed no decrease in neutrophil recruitment, pulmonary cytokine concentrations, or proteinaceous edema between genotypes, suggesting that these characteristic measures of inflammation were unlikely to contribute to host defense differences in endotoxemic hepSTAT3$^{-/-}$ mice. Moreover, phagocytosis and NET production were also equivalent between groups. Regarding the former, however, we acknowledge the fact that pHrodo *E. coli* bioparticles (our method of quantifying phagocytosis) may not perfectly replicate interactions between living *E. coli* and the inflammatory milieu (including opsonins such as extravasated APPs). Yet, we observed extremely efficient uptake using this system (around 40-60%) in both cell types analyzed, supporting a sufficient environment for comparing phagocytic function. Interestingly, ROS generation was significantly attenuated in alveolar macrophages from hepSTAT3$^{-/-}$ mice, suggesting that the endotoxemia-induced hepatic APR facilitates at least one fundamental aspect of cell-mediated antimicrobial defense. Results from the previous

chapter have implicated the APR in activation of airspace macrophages during pneumonia and a multitude of APPs have been shown to activate macrophages (Cheng et al., 2008; Mold and Du Clos, 2006; Tobias et al., 1992; Zhang et al., 2011).

We also assessed the capacity of alveolar lining fluid to influence bacterial growth after endotoxemia in mice with and without STAT3-dependent liver responses. Indeed, the composition of soluble mediators in this niche could have large implications on pathogen resistance at the air-liquid interface. In an effort to understand whether the balance of growth-promoting and growth-inhibiting soluble factors were dependent on the APR, we incubated luminescent *E. coli* with cell- and bacteria-free BALF from hepSTAT3$^{-/-}$ and hepSTAT3$^{+/+}$ mice collected at different time points following endotoxemia and *E. coli* infection. Interestingly, BALF from hepSTAT3$^{-/-}$ mice supported *E. coli* growth significantly more than that from hepSTAT3$^{+/+}$ mice, suggesting that products downstream of hepatic STAT3 activation create a less favorable environment for infection in the airspaces. There have been multiple reports of antimicrobial polypeptides, including lactoferrin, lysozyme, lipocalins and beta-defensins, which are produced in the liver and/or lungs (Bachman et al., 2011; Chan et al., 2009; Ganz, 2002; Li et al., 2014) and could be modulated by the APR either directly or indirectly. Alternatively, increased growth in the BALF from hepSTAT3$^{-/-}$ mice could be attributable to an altered nutrient pool that is more supportive of bacterial growth. While our assay does not allow us to discriminate between the bactericidal and bacteriostatic components of BALF, this finding, combined with the defect in macrophage ROS generation and increased bacterial burdens strongly support the concept

that the sepsis-induced hepatic APR is a required component for maintaining pulmonary defense. Future insight into the mechanisms by which sepsis-mediated liver activation is protective during subsequent lung infections will provide valuable, alternative avenues for the treatment and prevention of sepsis and pneumonia.

CHAPTER FIVE: CONCLUDING REMARKS

Summary of Results

As a classical response, the APR has been employed as a biomarker for a number of diseases (Abernethy and Avery, 1941; Armstrong, 2006; Beard et al., 2006; de Torre et al., 2006; Le, 2005; MaCleod and Avery, 1941; McCarty, 1947; Sakr et al., 2008; Urieli-Shoval et al., 2000; Verwey et al., 2008). As discussed above, each APP has its own individual function, ranging from opsonization to anti-proteases activity and coagulation mediators (Gabay and Kushner, 1999; Suffredini et al., 1999), yet their collective function, especially during lung infections, has yet to be elucidated. Previous work from our laboratory has shown the necessity of critical pulmonary host defense cytokines to activate key hepatocyte transcription factors, STAT3 and NF-κB RelA, to induce this response (Quinton et al., 2009), suggesting that its induction during pneumonia plays an important, biological role.

Using our mouse model of APR deficiency, we show, that the APR functions to facilitate survival, pulmonary bacterial clearance, and hepatoprotection during a high inoculum *E. coli* pneumonia. Infection with a lower inoculum revealed that pulmonary cytokine expression was also mediated by the APR. This effect on cytokine production was orchestrated largely through airspaces macrophages, as neither circulating cells (monocytes and neutrophils) nor airspace neutrophils had differences in cytokine induction due to APR deficiency. This function of the APR during pneumonia was further evidenced by work previously published by our laboratory (Quinton et al., 2012a),

which in accordance with the results detailed in this dissertation, showed the necessity of the APR for survival and systemic host defense during pneumococcal pneumonia.

Not only does the hepatic APR play a functional role during pneumonia, but systemic STAT3-dependent liver activation also appears to modulate subsequent responses to pneumonia. After our two-hit model of endotoxemia followed by bacterial lung infection, we observed greater pulmonary bacterial burdens in endotoxemic hepSTAT3$^{-/-}$ mice, suggesting a functional role of liver STAT3-dependent acute phase changes in maintaining pulmonary defense during endotoxemia. Specifically, macrophage ROS generation and resistance of alveolar lining fluid to bacterial growth were compromised after endotoxemia in hepSTAT3$^{-/-}$ mice, implicating that both cellular and humoral defenses are reliant on liver STAT3-dependent acute phase activity. Taken together, our results suggest that pulmonary and hepatic responses are connected via a "lung-liver axis," which protects the host through multiple mechanisms. Importantly, our findings support that the hepatic APR functions in a biologically relevant manner that far exceeds its usefulness as a biomarker.

Putting it all together: The role of sentinel cells within the lungs

While this dissertation is comprised of two results chapters investigating the role of the APR in two different yet clinically relevant scenarios, the overarching role of the hepatic APR is becoming increasingly clear. Both pneumonia and sepsis are inflammatory conditions by nature and have similar inflammatory pathologies. Thus it is unsurprising that the functions of the APR are similar in both pneumonia alone and

pneumonia preceded by sepsis. Interestingly, a maximal hepatic APR was necessary for survival of both insults and pulmonary host defense is compromised in its absence. While it may seem that there are unique functions of the APR during pneumonia alone (*e.g.* induction of airspace macrophage cytokine production) or endotoxemia followed by pneumonia (*e.g.* macrophage ROS production and alveolar bacterial resistance), these findings appear to revolve around a central participant—the alveolar macrophage. Evidence from both pneumonia and endotoxemia followed by pneumonia suggest that the function of this key sentinel airspace cell is strongly dependent on liver activation (Figure 42). This is consistent with the ability of alveolar macrophages to coordinate inflammatory responses, even after an extra-pulmonary insult (Aggarwal et al., 2014; Eddens and Kolls, 2012; Hussell and Bell, 2014; Mizgerd, 2008; van der Poll and Opal, 2008; Werner and Steele, 2014). Future lines of investigation include understanding the extent of the APR's ability to modulate macrophage functions, and the mechanism by which this occurs, whether it is through a direct or indirect manner.

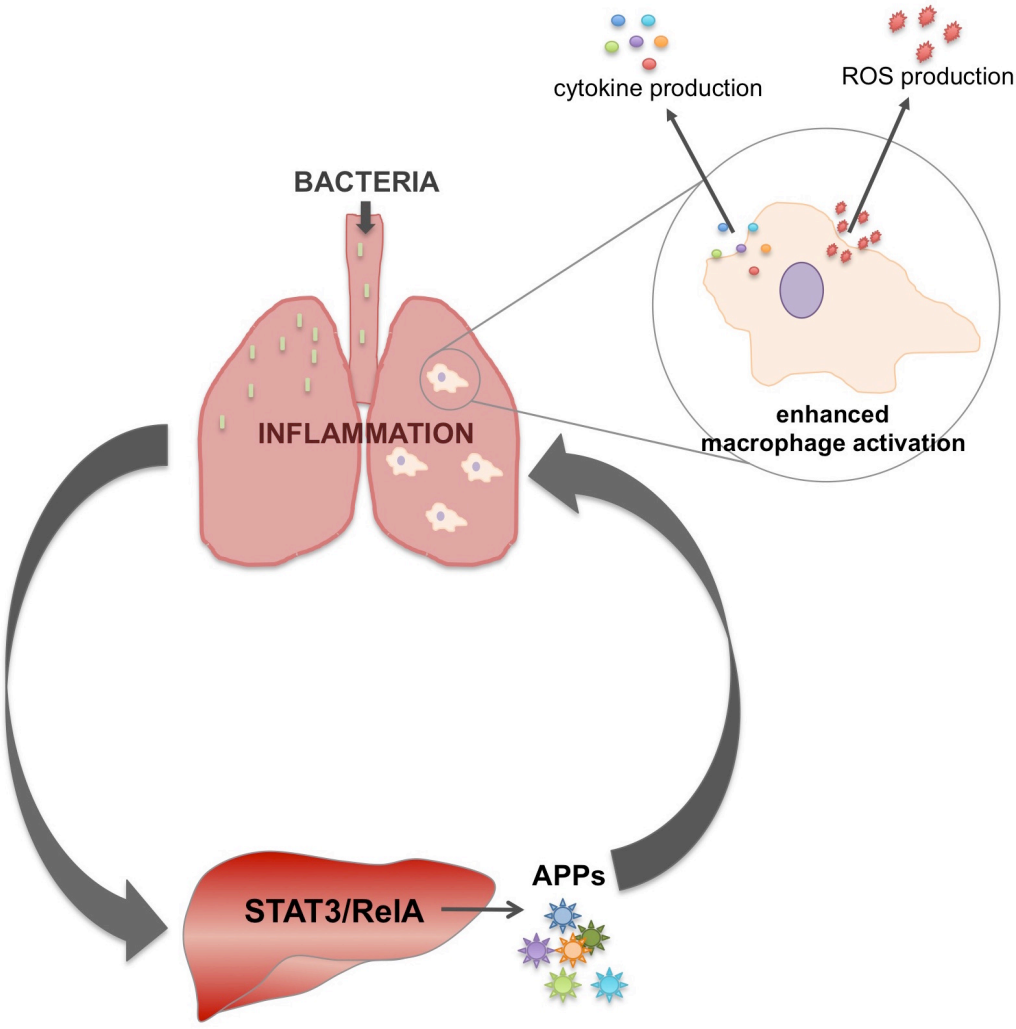


Figure 42. Model: The hepatic APR induced during pulmonary inflammation maintains pulmonary host defense and augments alveolar macrophage activation.

Pulmonary inflammation caused by pneumonia alone or in the context of endotoxemia activates hepatic STAT3 and RelA to induce the APR, leading to production of circulating APPs. Circulating APPs gain access to the airspaces by way of the injured alveolar epithelium and maintain pulmonary host defense by enhancing macrophage activation.

Long-term effects of the APR and pneumonia susceptibility

This study characterizes the role of the hepatic APR during an acute pneumonia, with outcomes measured no longer than 48 hours post infection. Various APPs are used as biomarkers and prognostic indicators for a number of chronic diseases, including arthritis and atherosclerosis (Liuzzo et al., 1994; Mallya et al., 1982; Plant et al., 2000; Yudkin et al., 1999). These chronic inflammatory conditions are associated with increases in circulating APP concentrations (Chambers et al., 1983; Cunnane et al., 2000; Liuzzo et al., 1994; Mallya et al., 1982; Yudkin et al., 1999). The effect of these increases over long periods of time has yet to be examined in any setting, but our work here can point us in the right path in understanding the long-term effects of the APR during chronic inflammatory diseases. For instance, it is possible that repeated exposure of myeloid cells to APPs could potentially induce tolerance of these cells to immune activation perhaps making patients more susceptible to secondary infections. More likely however (and more supported by our data) is that a long-term increase in circulating APP concentrations actually protects the host, balancing the deleterious effects of the chronic inflammation itself. For example, rheumatoid arthritis increases patients' risk for secondary infections, like pneumonia, as well as increase APP levels, while the induced APR is necessary to promote survival and host defense during pneumonia (Doran et al., 2002). In this scenario, the APR can potentially act as a balance, protecting patients with chronic diseases from developing pneumonia. Specific studies, however, need to be performed to understand the functions of the APR in long-term, chronic, inflammatory disorders.

Model Limitations

Our results implicate the lung-liver axis in facilitating host defense and survival during *E. coli* pneumonia and endotoxemia followed by *E. coli* pneumonia. While *E. coli* is an etiology of pneumonia that is clinically relevant, our specific work outlined here does not expand to other bacterial or viral causes of pneumonia and sepsis-induced pneumonia. Our laboratory has previously published evidence that the APR has important functions during a Gram-positive, pneumococcal pneumonia to facilitate survival and prevent invasive disease (Quinton et al., 2012a). This observation could certainly extend to other types of pneumonia and/or systemic infections. In fact, multiple APPs and the APR as a whole have been implicated in antibacterial defenses in response to a variety of pulmonary and systemic insults, including *S. pneumoniae*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, *E. coli*, *A. baumannii, S. pyogenes,* and LPS (Ahyi et al., 2013; Alonzi et al., 2001; Bachman et al., 2011; Branger, 2004; Chan et al., 2009; Hamann et al., 2005; Klein et al., 2007; Lamping et al., 1998; Mold and Du Clos, 2006; Noursadeghi et al., 2002; Quinton et al., 2012a; Quinton et al., 2009; Renckens et al., 2006; Renckens et al., 2008; Sakamori et al., 2007; Sander et al., 2010; Shah et al., 2006; Vernooy et al., 2005; Yuste et al., 2007). These studies put forth multiple lines of evidence that bring the APR to the center of multiple inflammatory conditions, like pneumonia and sepsis, and link them through a common hepatic response. These studies, and our work with two different incoulums of *E. coli* also suggest that the magnitude of the response is dependent on the dose and pathogen. Organisms less likely to mount an immune response (for their own benefit) would be less likely to induce the hepatic APR, as the

APR is stimulated by the immune response itself and not directly by the pathogen. Our own studies, however, are the first to show a functional role for the hepatic APR as a whole (not just individual APPs) induced by the inflammatory response in facilitating survival and maintaining pulmonary host defense in multiple contexts of *E. coli* pneumonia. Future studies, however, are necessary to determine the holistic role of the APR in other types of inflammatory conditions.

The efficacy of mouse models has recently been debated, especially their usefulness in understanding various inflammatory conditions. It has long been understood that mice can tolerate greater quantities of endotoxin that humans. In fact, mice require 250 times more endotoxin than humans to cause the same level of inflammation (Copeland et al., 2005). Copeland *et al*. also show that while this response must be stimulated by a much greater insult, it is very similar to that induced in humans (Copeland et al., 2005). Additionally other groups have reported on the usefulness of mouse models. Two reports, published back to back, used the same data set to come to opposite conclusions about the similarity of the inflammatory response in mice and humans (Seok et al., 2013; Takao and Miyakawa, 2015). By comparing the transcriptional response of total blood leukocytes to trauma, burn, or endotoxin in humans and mice, Seok, *et al*. concluded that mouse models poorly mimicked human patients of the same condition, whereas *Takao et al*. determined the opposite (Seok et al., 2013; Takao and Miyakawa, 2015). Disparate results could be a result of the differential white blood counts in mice and humans. Moreover, human patients received hospital supportive care, while mice did not. Therefore, it is unsurprising that comparing the

responses of different cells, under different circumstances would lead to disparate conclusions. By utilizing our novel APR-null mouse model, we are able to specifically determine the necessity of hepatic STAT3 and RelA during lung infections, something that could not have been done with cell culture or patient samples alone. Additionally, by providing a better understanding of a conserved response that is already utilized as a biomarker in humans, we will be better able to develop therapeutics that modulate the hepatic APR to treat patients with or at risk for pneumonia and other inflammatory conditions.

Future Directions

Our studies revealed a crucial role for the APR in maintaining pulmonary host defense and inflammation via alveolar macrophages during pneumonia. Future directions should focus on specifically how the ARP does this and whether it is through direct or indirect mechanisms. Our *ex vivo* serum stimulation assays suggest that serum components themselves cannot directly modulate macrophage activation. Negative data, however is hard to interpret, as the experimental design could be hampering our ability to see APR-dependent changes in macrophage cytokine production *ex vivo*. Other studies, including microarrays on macrophage RNA from uninfected or infected APR-null or control mice would reveal genome wide, APR-dependent changes that could indicate specific signaling pathways or receptors involved. Additionally, *in vivo* gain of function studies in which serum from hepSTAT3/RelA$^{+/+}$ mice is instilled into APR-null, hepSTAT3/RelA$^{-/-}$ mice during a low inoculum pneumonia to rescue pulmonary

inflammation would again indicate whether APR-dependent serum components can directly influence macrophage activation. It is important to note, however, that the role of the APR in pulmonary inflammation and host defense is most likely not attributable to just one APP, but rather is likely a combined effect of a multitude of APPs. Thus, narrowing down the direct mechanism may be difficult.

While our findings point to macrophages in regulating the APR-dependent inflammatory response, we have not determined if other cell types, namely alveolar epithelial cells, are modulated by the APR as well. Our lab has developed a novel lung digest protocol to isolate intact alveolar epithelial cells from infected lungs. Using this protocol, we can determine if the APR facilitates pulmonary inflammation and host defense through alveolar epithelial cells in addition to macrophages. These cells have recently gained more notoriety in having specific antibacterial and immune stimulating functions separate from their classic role as barrier cells (Mizgerd, 2008; Quinton and Mizgerd, 2011; Quinton and Mizgerd, 2015), and are the first cells to encounter exudate APPs as they enter the injured lungs; thus it would be unsurprising if they were involved.

As described earlier, many APPs are mediators of the coagulation cascade, an important pathway in promoting both resolution and repair in the lungs after an acute pneumonia. Future studies should include those to determine the role of the hepatic APR in this process. While differences in survival with an *E. coli* pneumonia may make this difficult, other model organisms or agonists could be particularly helpful. Intratracheal administration of LPS is used as a classic inducer of acute lung injury, resulting in a robust repair process (Mizgerd and Skerrett, 2008). Additionally, LPS is a sterile insult,

thus removing the confounding effect of any potential host defense differences in our APR-null mouse model. Understanding how and if the hepatic APR modulates this process can reveal yet another functional role of the APR during pneumonia and further help patients with or at risk for pneumonia.

Clinical Implications

Treatments for both sepsis and pneumonia are severely limited and are generally reliant on antibiotic therapy and supportive care. Unfortunately, antibiotic resistant infections, including both pneumonia and sepsis, are becoming more prevalent (van der Poll and Opal, 2009). Because of this, the need for traditional antibacterial compounds has decreased, while the necessity of novel immunomodulators has greatened. As such, we have shown that the liver-derived APR modulates the immune response in two very important clinical scenarios. Additionally, the liver is an easily targetable organ because of its highly vascularized nature (Bhadra et al., 2005; Mishra et al., 2013; Wang et al., 2014), and the APR could be easily regulated with different small molecule agonists and antagonist for STAT3 and/or RelA, some of which are already undergoing the patenting process (Yu et al., 2013). As observed in both experimental scenarios tested, the APR was effective in controlling bacterial outgrowth, without compromising tissue integrity, thus the potential for boosting host defense, without contributing to antibacterial resistance is evident. More work needs to be done, however, to determine the efficacy of such exogenous manipulation.

Additionally, this work will expand the usefulness of the hepatic APR as a prognostic or diagnostic tool during a multitude of inflammatory disorders. Both pneumonia and sepsis are heterogeneous conditions that affect a multitude of patients with various pre-existing conditions and backgrounds. Moreover, differences in infection etiologies only complicate patient stratifications more. Hepatic APR activation occurs in response to a variety of different pathogens, including both Gram negative and positive infections, and is modulated based on the severity of infection. Thus, liver activation status can help to stratify patients and delineate subgroups, which are better suited to respond to certain drugs or treatments than other therapeutics. A better understanding of the causal link between the APR as a biomarker and its role in the outcome of disease will guide treatments and therapeutics used.

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