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Liver-dependent protection during pneumonia and sepsis

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LIVER-DEPENDENT PROTECTION DURING PNEUMONIA AND SEPSIS

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"Even though there are no ways of knowing for sure, there are ways of knowing for pretty sure." – Lemony Snicket

DEDICATION

For my parents and my sister Ellen.

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LIVER-DEPENDENT PROTECTION DURING PNEUMONIA AND SEPSIS

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ABSTRACT

Pneumonia and sepsis are distinct but linked public health concerns. Each condition is the leading cause of the other; however, the responses controlling the susceptibility between the two disease processes remain speculative. The acute phase response (APR) is an important component of the host immune response during pneumonia and sepsis, and primarily driven by the activation of hepatocyte transcription factors NF-κB RelA and STAT3.

While the NF-κB pathway is essential for inflammation and hepatocyte function, its inactivation has been associated with hepatotoxicity. Liver injury is an independent risk factor for sepsis morbidity and mortality, suggesting that pathways promoting liver homeostasis may limit the systemic consequences of pneumonia. To identify conditions in which NF-κB RelA is required for liver resilience, we challenged mice lacking hepatocyte RelA (hepRelA$^{\Delta/\Delta}$) and wildtype (WT) controls with *E. coli*, *K. pneumoniae*, *S. pneumoniae*, LPS, or αGalCer to induce pneumonia, sepsis, and/or NKT cell activation. Severe hepatotoxicity was observed in hepRelA$^{\Delta/\Delta}$ mice in all conditions examined in association with apoptosis, which could be prevented by neutralization of TNFα. Lastly, these changes were associated with remodeling of the hepatic transcriptome, likely reflecting both the cause and consequence of hepatoxicity.

We have previously shown that activation of STAT3 in hepatocytes limits pneumonia susceptibility during endotoxemia, but the mechanisms whereby this liver APR provides protection are unknown. Iron sequestration is a defense mechanism against bacterial infections, which require iron for growth. Based on previous observations that alveolar lining fluid is favorable for bacteria in the absence of liver STAT3, we investigated whether liver APR limits pneumonia susceptibility during sepsis by withholding iron to prevent bacterial outgrowth. WT mice or mice lacking hepatocyte STAT3 (hepSTAT3$^{\Delta/\Delta}$) mice were challenged with endotoxemia followed by *E. coli* pneumonia, or cecal ligation and puncture (CLP). Induction of mRNA encoding several essential iron-regulating factors was ablated in hepSTAT3$^{\Delta/\Delta}$ mice after endotoxemia and pneumonia, and post CLP. Additionally, liver STAT3 activation significantly remodeled the pulmonary transcriptome during endotoxemia, which potentially represents other protective mechanisms.

Taken together, these results suggest that hepatic APR is an important immunological interface modulating pneumonia and sepsis interaction and susceptibility.

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LIST OF ABBREVIATIONS

7AAD ..... 7-Aminoactinomycin D

ALT ..... Alanine Transaminase

ANOVA ..... Analysis of Variance

APC ..... Allophycocyanin

APP ..... Acute Phase Protein

APR ..... Acute Phase Response

ARDS ..... Acute Respiratory Distress Syndrome

AST ..... Aspartate Transaminase

BAL ..... Bronchoalveolar Lavage

BCA ..... Bicinchoninic Acid

BUV ..... Brilliant Ultraviolet

BV ..... Brilliant Violet

°C ..... Degree Celsius

CAP ..... Community-Acquired Pneumonia

CCL ..... Chemokine (C-C Motif) Ligand

CD ..... Cluster of Differentiation

CFU ..... Colony Forming Unit

Cl. Casp-3 ..... Cleaved Caspase-3

Cl. Casp-8 ..... Cleaved-Caspase-8

CLP ..... Cecal-Ligation and Puncture

cm ..... Centimeter

CPR ..... C-Reactive Protein

CXCL ..... Chemokine (C-X-C Motif) Ligand

DALY ..... Disability-Adjusted Life Year

DAMP ..... Damage Associated Molecular Pattern

EDTA ..... Ethylenediaminetetraacetic Acid

ELC ..... Enhanced Chemiluminescence

F4/80 ..... Adhesion G protein-Coupled Receptor E1

FACS ..... Fluorescence-Activated Cell Sorter

FAM ..... Fluorescein

FasL ..... Fas Ligand

FBS ..... Fetal Bovine Serum

FDR *q* ..... False Discovery Rate adjusted *p*-value

FITC ..... Fluorescein Isothiocyanate

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

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

H&E ..... Hematoxylin and Eosin

HAMP ..... Hepcidin

hepRelA$^{\Delta/\Delta}$ ..... Hepatocyte RelA-Null

hepSTAT3$^{\Delta/\Delta}$ ..... Hepatocyte STAT3-Null

HAP ..... Hospital-Acquired Pneumonia

HIV ..... Human Immunodeficiency Virus

HMGB1 ..... High-Mobility Group Protein B1

HP ..... Haptoglobin

HPX ..... Hemopexin

HRP ..... Horseradish Peroxidase

IACUC ..... Institutional Animal Care and Use Committee

IFNγ ..... Interferon Gamma

IgG ..... Immunoglobulin G

IL ..... Interleukin

I.P. ..... Intraperitoneal Injection

I.T. ..... Intratracheal Instillation

IU ..... International Unit

I.V. ..... Intravenous Injection

kg ..... Kilogram

LB ..... Lysogeny Broth

LCN2 ..... Lipocalin-2

LPS ..... Lipopolysaccharide

Ly6C ..... Lymphocyte Antigen 6 Complex, Locus C1

MDR ..... Multidrug Resistant

mg ..... Milligram

MHCII ..... Major Histocompatibility Complex Class II

min ..... Minute

mL ..... Milliliter

MLKL ..... Mixed Lineage Kinase Domain-Like

mm ..... Millimeter

mM ..... Millimolar

mRNA ..... Messenger RNA

MRSA ..... Methicillin-Resistant *Staphylococcus aureus*

mtDNA ..... Mitochrondrial DNA

n ..... Quantity

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

NK ..... Natural Killer

NKT ..... Natural Killer T

p ..... Probability Value

PAMP ..... Pathogen Associated Molecular Pattern

PE ..... Phycoerythrin

PE-Cy7 ..... PE Cyanin 7

PBS ..... Phosphate-Buffered Saline

PCV ..... Pneumococcal Conjugate Vaccine

PD-1 ..... Programmed Cell Death Protein 1

PFA ..... Paraformaldehyde

PPSV ..... Pneumococcal Polysaccharide Vaccine

PVDF ..... Immobilon-P Polyvinylidene Fluride

qRT-PCR ..... Quantitative Real-Time Polymerase Chain Reaction

RelA ..... V-Rel Avian Reticuloendotheliosis Viral Oncogene Homolog A

RIPK ..... Receptor-Interacting Protein Kinase

rRNA.....Ribosomal RNA

RNA-Seq..... RNA Sequencing

RPM.....Revolutions per Minute

RSV.....Respiratory Syncytial Virus

ROS.....Reactive Oxygen Species

RPMI.....Rosewell Park Memorial Institute Medium

SAA..... Serum Amyloid A

SEM .....Standard Error of the Mean

SIRS ..... Systemic Inflammatory Response Syndrome

SMAD9 ..... SMAD Family Member 9

SOFA ..... Sequential Organ Failure Assessment

SOX17.....SRY-Related HMG-Box 17

STAT3.....Signal Transducer and Activator of Transcription 3

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

TFR2 ..... Transferrin Receptor 2

TGFβ.....Transforming Growth Factor Beta

TLR.....Toll-Like Receptor

TNFα.....Tumor Necrosis Factor Alpha

TRAIL..... TNF-Related Apoptosis-Inducing Ligand

Treg.....Regulatory T Cell

Tween.....Polyethylene glycol sorbitan monolaurate

uPAR.....Urokinase Receptor

VAP..... Ventilator-Associated Pneumonia

vs. ..... Versus

WNT3A..... Wingless-Type MMTV Integration Site Family, Member 3A

WT .....Wildtype

x g.....Relative Centrifugal Force

αGalCer.....Alpha-Galactosylceramide

µL.....Microgram

µL..... Microliter

µm .....Micrometer

CHAPTER ONE: INTRODUCTION

Pneumonia

Significance and Epidemiology

Pneumonic infections have been recognized throughout human history as a prevalent and severe disease entity, with descriptions of symptoms dating back to ancient civilizations (Abdelnaby, El Deeb et al. 2017). Even Hippocrates (4^{th}-3^{rd} century BC), who bestowed the name "pneumonia" to the most serious infections of the lungs, described the condition as a disease previously "named by the ancients" (Gattarello and Rello 2017). The recognition of microorganisms as causative agents of lung infections occurred roughly 150 years ago. Yet, pneumonia continued to cause widespread death and disability into the late 1800s and early 1900s. William Osler, the father of modern medicine, described pneumonia as "the most fatal of all acute diseases… the 'Captain of Men of Death'", as pneumonia was the leading cause of death due to infectious disease and the third leading cause of death overall (Osler 1898).

Several advancements in medicine and public health interventions in the 1900s were vital in improving pneumonia mortality. The use of antiserum therapy (Robertson and Sia 1924), followed by the discovery of penicillin and other antibiotic therapies drastically reduced mortality by nearly 30% in developed countries (Podolsky 2005). Improvements in modern surgery techniques and intensive care, as well as development of vaccines against bacterial *Haemophilus influenzae* in 1988 and *Streptococcus*

pneumoniae in 1977 and 2000 further improved pneumonia outcomes (Blasi, Aliberti et al. 2007).

However, pneumonia still remains fairly prevalent, even in developed countries. It is responsible for a significant portion of mortality and morbidity worldwide as measured by disability-adjusted life years (DALYs) lost (Mizgerd 2012). Despite the advancements of modern medicine, pneumonia mortality rates have remained relatively stable in the last several decades: approximately 40 deaths per 100,000 persons per year (Armstrong, Conn et al. 1999). Presently, pneumonia is the third leading cause of hospitalizations, the eighth leading cause of mortality (the leading cause of mortality from infection), and the leading cause of sepsis (a tightly linked complication discussed below) (Rider and Frazee 2018). Lung infections affect persons across the socioeconomic and health spectrum; however, incidence and need for hospitalization is higher at extreme age distributions. The hospitalization rate of children under two years of age is approximately 1,000 hospitalizations per 100,000 persons per year, while rates for elderly adults age 85 or greater is over 4,000 hospitalizations per 100,000 persons per year (Griffin, Zhu et al. 2013). Outcomes for hospitalized patients are also poor, with 30-day mortality at 10-12%, readmission rate at 18% (Musher and Thorner 2014).

Causes and Classifications of Pneumonia

Pneumonia is not a single disease but a group of syndromes caused by a heterogeneous group of microorganisms that includes bacteria, virus, and less frequently fungi, and parasites infecting the lung parenchyma (Musher and Thorner 2014).

However, a single cause of pneumonia is identified in less than 10% of emergency patients (Bartlett 2011). When pneumonia-causing organisms are identified, they are numerous and varied by geographic and time setting, vaccine trends, and specific patient subpopulations (DeAntonio, Yarzabal et al. 2016, Wuerth, Bonnewell et al. 2016). A common classification of pneumonia etiology is based on the setting in which the patient acquired the infection. Community-acquired pneumonia (CAP) is any pneumonia that was acquired outside of a hospital, in a community setting (Mandell, Wunderink et al. 2007). A patient who acquired pneumonia 48 hours after being admitted in an inpatient setting, such as a hospital, is classified as having hospital-acquired pneumonia (HAP) (Kalil, Metersky et al. 2016). Further, any pneumonia acquired 48 hours after endotracheal intubation is classified as ventilator-associated pneumonia (VAP) (Kalil, Metersky et al. 2016).

While there is a spectrum and combination of microorganisms, the majority of those detected were viruses (Jain, Self et al. 2015). The most common viruses were rhinovirus, influenza viruses, and respiratory syncytial virus (RSV) (Jain, Self et al. 2015). Although viruses are implicated in pneumonia, the direct cause is often complicated by secondary bacterial infections (Hendaus, Jomha et al. 2015). The most common bacterial pathogen and third most common causative pathogen in CAP detected was *Streptococcus pneumoniae*, followed by *Mycoplasma pneumoniae* and *Staphylococcus aureus* (Jain, Self et al. 2015). Other typical bacteria detected in CAP include *Klebsiella pneumoniae*, *Haemophilus influenza*, *Psudomonas aeruginosa*, and other aerobic and anaerobic gram-negative bacteria (Musher and Thorner 2014, Jain, Self

et al. 2015). Although the study by Jain et al. were empowered to identify causative agents, organisms were not detected in an astounding 62% of patients tested, and further, no single individual organism caused more than approximately 8% of total adult cases. Thus, the diverse etiology emphasizes the importance of understanding the underlying host biology controlling disease susceptibility. Fungal pathogens are implicated in immunocompromised patients with HIV, organ transplants, among others and is also commonly associated with HAP (Hage, Knox et al. 2012). Etiology of HAP in non-ventilated and ventilated patients have many overlaps. Gram-negative bacilli (*Escherichia coli*, *Psudomonas aerugenoisa*, *Acinetobacter*, and *Enterobacter*) and Gram-positive cocci (*Staphlococcus aureus*) are prevalent (Calik, Ari et al. 2018). HAP and VAP patients are of particular risk to a growing prevalence of multidrug-resistant (MDR) bacterial strains (Rider and Frazee 2018). While penicillin was sufficient treatment for *Streptococcus pneumoniae*, penicillin-resistant strains have risen from 18% in 1991 to 35% in 2002 (Doern 2005). Other resistant pneumonia causing strains include macrolide-resistant pneumococcus, carbapenem-resistant *Klebsiella pneumoniae*, and methicillin-resistant *Staphylococcus aureus* (MRSA) (Rider and Frazee 2018).

Clinical Presentation and Pathology

Pneumonia is clinically described as an infection of the lower respiratory tract and alveoli (Mackenzie 2016). The respiratory tract is not sterile and is constantly exposed to commensal and environmental microorganisms (Wu and Segal 2018). Pneumonia occurs when intricate balance between pathogens and host defense is disturbed, leading to

invasion and propagation in lower airways and alveoli (Mizgerd 2017). There are a number of host defenses that work to prevent the proliferation of microorganisms, including the host innate immune response composed of immune cells. Sentient cells, such as alveolar macrophages and epithelial cells, work to recognize pathogen associated molecular patterns (PAMPs) through various receptors to phagocytose and kill proliferating bacteria (Quinton, Walkey et al. 2018). However, when the bacteria overcome the capacity of the host defenses and proliferate, alveolar macrophages initiate the inflammatory response through production of early cytokines such as TNFα, IL-1 and IL-6 (Mizgerd 2017, Quinton, Walkey et al. 2018).

The inflammatory response, though an essential component of the host defense against microorganisms, is also responsible for the clinical and pathological findings observed during the course of pneumonia (van der Poll and Opal 2009). Local inflammatory cytokines and factors recruit additional immune cells (i.e. neutrophils and lymphocytes), humoral defenses, and initiate systemic inflammatory responses (Quinton, Walkey et al. 2018). The alveolar capillaries become leaky at the site of inflammation to allow extravasation of these immune defenses (Quinton, Walkey et al. 2018). However, killing of pathogens by reactive oxygen species and enzymes produced by neutrophils also results in acute lung injury (Brinkmann, Reichard et al. 2004, Quinton, Walkey et al. 2018). The alveolar epithelium becomes damaged and the alveolar space becomes filled with proteinaceous and purulent exudate (Ware, Neyrinck et al. 2012). Exudative congestion leads to decreased lung compliance, shortness of breath, and worsening

hypoxemia, all of which are important clinical signs of severe pneumonia (Brar and Niederman 2011).

Pneumonia is diagnosed through history and physical examination of respiratory infection symptoms, pulse oximetry, and lung sounds. Chest radiographic evaluations are a central method to diagnose pneumonia (Watkins and Lemonovich 2011). When alveolar infiltrates are seen in the pulmonary spaces of chest radiographs, laboratory evaluations are conducted to identify the causative agent and course of treatment (Watkins and Lemonovich 2011).

Treatment, Prognosis, and Prevention

Once pneumonia diagnosis is made, patients are administered treatment as soon as possible (Watkins and Lemonovich 2011, Kalil, Metersky et al. 2016). Antibiotic and antiviral therapies are commonly used, but the specific course of treatment varies by severity, presence of complications, patient risk factors, and causative agents. Some risk factors include young and old age, presence of comorbidities and chronic diseases, immunocompromised states, behavioral factors such as smoking and alcohol consumption, compliance with immunization and therapy, and the likelihood of developing antibiotic-resistant infection (Niederman, Mandell et al. 2001).

Recommended antibiotics include fluoroquinolones, β-lactams, macrolides, and doxycycline (Waterer, Rello et al. 2011). For CAP, the antibiotic regime is typically five to seven days of treatment, except for infections with *S. aureus* and those complicated with bacteremia (Li, Winston et al. 2007, Liu, Bayer et al. 2011). In severe cases of

pneumonia, such as when patients progress to acute respiratory distress syndrome (ARDS), supportive care and ventilation become necessary (Matthay, Ware et al. 2012). HAP often leads to use of broad-spectrum antibiotics due to multi-drug resistant organisms and patient risk factors, and generally covers *S. aureus*, *P. aeruginosa*, and Gram-negative bacilli (Madaras-Kelly, Remington et al. 2012).

Many previously healthy adults typically resolve pneumonia after initiating antibiotic therapy. However, complications can arise from pneumonia leading to respiratory failure, sepsis, metastatic infections, empyema, lung abscesses, multi-organ failure, coagulopathy, exacerbations of comorbidities, and complicated pleural effusion (Mbata, Chukwuka et al. 2013, Sattar and Sharma 2018). Additionally, patients hospitalized with pneumonia suffer from many long-term effects. These patients are at increased risk of developing chronic respiratory diseases and recurrent infections. Mortality rates are 2.5 times higher 1 year after hospital discharge as compared to hospitalized patients who did not have pneumonia (Kaplan, Clermont et al. 2003, Waterer, Kessler et al. 2004). Other long-term consequences associated with pneumonia hospitalizations include cognitive decline, depression, cardiovascular, cerebrovascular, physical limits, and decreased lifespan (Davydow, Hough et al. 2013, Grijalva 2015).

The most important measure to reduce the risk of pneumonia is the cessation of smoking, which interferes with the immune system and lung function (Almirall, Gonzalez et al. 1999). Immunization is also an important preventative measure, particularly for patients with existing pulmonary diseases. Two pneumococcal vaccines are commercially available: the pneumocoocal polysaccharide vaccine (PPSV23) which

is comprised of purified polysaccharides of 23 different serotypes, and the pneumococcal conjugate vaccines (PCV7, PCV13) which are comprised of polysaccharides from serotype 7 or 13 conjugated with a highly immunogenic diphtheria toxoid protein (Pisano and Cifu 2015). However, the PPSV23 does not illicit a strong immunological memory, and is not recommended for children under two years of age (Pletz, Maus et al. 2008, Pisano and Cifu 2015). The PCV vaccines have been shown to reduce pneumonia hospitalizations in children and elderly adults (Assaad, El-Masri et al. 2012, Izu, Solomon et al. 2017). *Haemophilus influenza* type b and influenza vaccines are the only other known vaccines that currently exist for other etiologies of pneumonia (Rider and Frazee 2018). Despite the availability of these vaccines, morbidity and mortality of pneumonia worldwide remains significant.

Sepsis

Clinical Definition and Significance

The first documentation of sepsis occurred in 1600 BC; however, the term "sepsis" came from ancient Greek meaning "decay of organic matter" and was referenced in the Hippocratic corpus (4th century BC) (Kempker and Martin 2016). However, it was not clinically defined until the late 20th century when the discovery of antimicrobials and improvements in supportive care allowed physicians to study it in surviving patients. The earliest modern definition of sepsis occurred in 1989, when Roger Bone and colleagues introduced "sepsis syndrome" which laid the foundation for systemic inflammatory response (SIRS) criteria (Bone, Fisher et al. 1989). SIRS was defined as a complex

immune response to infection or injury, which include changes in respiratory rate, heart rate, temperature, and white blood count (Bone, Balk et al. 1992). The 1991 International Consensus Conference formed the first official definition of sepsis (1992). Sepsis was defined as SIRS with a suspected or proven infection, and severe sepsis described septic patients with organ dysfunction. Septic shock was described as a severe state with an acute circulatory failure characterized by persistent arterial hypotension. The definition was lightly revised in 2001 when it was acknowledged that SIRS was not specific to sepsis and therefore was redefined as the "signs and symptoms of sepsis", which included other clinical symptoms such as altered mental status and hyperglycemia (Levy, Fink et al. 2003). In 2016, the definition of sepsis was updated as a life threatening organ dysfunction caused by a dysregulated host response to infection, while septic shock referred to patients with circulatory and cellular or metabolic abnormalities, identified by the requirement of vasopressors (Singer, Deutschman et al. 2016).

The true incidence rate of sepsis is unknown and is complicated by the changing definition of sepsis, the reporting system, heterogeneity of infecting organisms, and geographical diversity (Kempker and Martin 2016). Most incidence reports are from high-income countries, such as the U.S. While the results vary, the average incidence of sepsis is estimated to range from 300 to 1,031 per 100,000 persons (Gaieski, Edwards et al. 2013). An annual 2.8 million deaths are attributed to sepsis (Adhikari, Fowler et al. 2010), which accounts for approximately a third to half of in-hospital deaths in the U.S (Liu, Escobar et al. 2014). While in-hospital mortality has decreased from 28% to 18% (Martin, Mannino et al. 2003), the incidence of sepsis has elevated over the past 40 years,

with an average annual increase of 13% (Gaieski, Edwards et al. 2013). However, data from low-income countries are scarce and are often extrapolated from respiratory tract infection trends, with 90% of chest infection deaths thought to be due to sepsis (Kempker and Martin 2016).

Causes of Sepsis

Sepsis can originate from any infecting organism, and the primary site of infection also varies among septic patients. However, sepsis is most frequently caused by lung infections, with approximately 40 to 60% of septic patients having causative respiratory infections (Karlsson, Varpula et al. 2007, Vincent, Rello et al. 2009). Other common sites of infection that leads to sepsis is the abdomen (20%), blood (15%), and renal and genitourinary (14%) (Karlsson, Varpula et al. 2007, Vincent, Rello et al. 2009). Bacterial infections are commonly associated with sepsis, although data are limited by the ability to grow and identify organisms. Gram-negative bacteria, such as *Pseudomonas* (62%) and *E. coli* (16%), are most commonly detected in those with positive microbiological cultures (Gotts and Matthay 2016). Gram-positive bacteria are detected in 47% of which *S. aureus* alone accounts for 20%, and fungal species are detected in 19% (Gotts and Matthay 2016). Sepsis mortality is especially high in patients with certain drug-resistant organisms such as MRSA, *Klebsiella*, *Pseudomonas*, and *Acinteobacter* (Hanberger, Walther et al. 2011, Gotts and Matthay 2016).

Risk factors for the development of sepsis is associated with a patient's predisposition to infection. These include young and elderly patients, immunosuppressive

states, and comorbidities, which are reflective of risk factors for the development of pneumonia (Brun-Buisson, Doyon et al. 1995, Angus, Linde-Zwirble et al. 2001, Banta, Joshi et al. 2012). However, factors linked to progression of pneumonia to sepsis and organ dysfunction are less characterized.

Clinical Presentation & Pathology

The clinical presentation of sepsis depends on the site of infection and type of pathogen. Typically, sepsis progresses from a local infection to mild systemic inflammation and organ dysfunction, to septic shock when the cardiovascular system undergoes major alterations (Gotts and Matthay 2016). A patient's state of organ function or rate of failure is determined by the sequential organ failure assessment score (SOFA score), which includes respiratory, cardiovascular, hepatic, coagulation, renal, and neurological assessments (Singer, Deutschman et al. 2016). Organ dysfunction perpetuates and self-reinforces injury during sepsis. For example, endothelial and epithelial changes during sepsis are associated with barrier functions of other organs. In response to injury or inflammatory signals, the endothelium alters leukocyte adhesion and trafficking, coagulant state, vasodilation, and barrier functions (Deutschman and Tracey 2014). More permeable endothelial and epithelial capillaries in the lung lead to edema and hypoxia. When mechanical ventilation becomes necessary for proper lung function, it can lead to further injury and enhanced inflammatory responses (Zampieri and Mazza 2017). Epithelial barrier loss in the intestines increases gut bacterial translocation (Fink 2003). Impaired hepatocytes of the liver are unable perform functions such as clearing

bilirubin or processing translocated gut bacteria, worsening systemic inflammation (Strnad, Tacke et al. 2017).

The inflammatory response during sepsis consists of both pro- and anti-inflammatory states. Proinflammatory responses are initiated by sentient leukocytes (i.e. macrophages, monocytes, granulocytes, natural killer cells, and dendritic cells) which detect pathogens through PAMPs and injured cells through damage associated molecular patterns (DAMPs) (Delano and Ward 2016). PAMPs and DAMPs activate the innate immune system through various cell surface and cytosolic receptors that initiate the transcription of type 1 interferons and proinflammatory cytokines TNFα, IL-1, and IL-6 (Schulte, Bernhagen et al. 2013, Delano and Ward 2016). These early inflammatory cytokines orchestrate events that rapidly control minor and localized infections (Schulte, Bernhagen et al. 2013). However, when the response exceeds a threshold, systemic injury can result. Reactive oxygen species (ROS) generated by neutrophils damage cellular components and mitochondria, leading to cellular metabolic dysfunction (Victor, Espulgues et al. 2009, Leliefeld, Wessels et al. 2016). Complement activation increases ROS and enzyme release by granulocytes and increases endothelial permeability (Ward and Gao 2009). Aberrant activation of coagulation, fibrin deposition, and tissue factor can result in disseminated intravascular coagulation that injures the microvasculature and organs, activating inflammation (Simmons and Pittet 2015).

Compensatory anti-inflammatory response is also activated in early states of sepsis. IL-10 is produced by a variety of leukocytes to suppress the production of IL-6 and stimulate soluble TNFα receptor and IL-1 receptor antagonist (Schulte, Bernhagen et

al. 2013). Cells that undergo apoptosis are engulfed by macrophages which release IL-10, TGFβ, and bioactive lipids that reduce ROS, endothelial permeability, and leukocyte recruitment (Fullerton, O'Brien et al. 2013). Expansion of T regulatory cells (Tregs) also plays an important role in producing anti-inflammatory cytokines and clearance of cytotoxic cells (Fullerton, O'Brien et al. 2013). However, these anti-inflammatory responses contribute to an immune dysfunctional state of immunosuppression, where patients become susceptible to secondary infections such as hospital-acquired pneumonia (van Vught, Klein Klouwenberg et al. 2016). Several anti-inflammatory cytokines have been implicated in immunoparalysis. In particular, high levels of IL-10 stimulated by PD-1 in response to high TNFα has been associated with impairment of T cell proliferation and maintenance of immunoparalysis in patients during sepsis (Said, Dupuy et al. 2010). Anergy of immune cells also contributes to sepsis-related immunosuppression. T cells demonstrate impaired response to antigen and decreased release of cytokines (Heidecke, Hensler et al. 1999). Macrophages and monocytes express less MHCII and co-stimulatory molecules, and are associated with poorer outcomes in sepsis (Saenz, Izura et al. 2001). Other immune functions such as ROS generation, phagocytosis, and chemotaxis are also impaired in septic patients (Hotchkiss, Monneret et al. 2013). Additionally, autopsy studies of patients who died of sepsis show profound immune cell death (Hotchkiss, Monneret et al. 2013). Apoptosis is commonly observed in T cells, B cells, natural killer cells, and follicular dendritic cells in septic patients, and lymphopenia is associated with secondary infection and mortality (Hotchkiss, Monneret et al. 2013, Drewry, Samra et al. 2014). Sepsis reflects the interaction between mechanisms that

increase and decrease inflammation, leading to immune dysfunction with deleterious consequences.

Relationship to Pneumonia

Sepsis and pneumonia are distinct inflammatory diseases in response to infection. However, they are integrally linked with each exacerbating the other. Sepsis arises from pneumonia more than any other cause (as mentioned above). Conversely, sepsis from non-pulmonary infection predisposes patients to pneumonia due, at least in part, to sepsis-induced immunosuppression (Bouras, Asehnoune et al. 2018). Ventilator associated pneumonia occurs in 10-30% of ventilated septic patients (Chastre and Fagon 2002). Several studies in rodent models have shown the causative links between pre-existing sepsis and outcomes of subsequent pneumonia including defective neutrophil recruitment, decreased local and systemic cytokine production, and IL-10 in the impairment of host defenses (Benjamim, Hogaboam et al. 2003, Deng, Cheng et al. 2006, Cao, Xu et al. 2014). However, the mechanisms and signals that prevent pneumonia and sepsis from promoting the other are still not well understood.

Management, Prognosis, and Prevention

There are no approved molecular therapies for sepsis. The current recommendations in the management of sepsis include the early administration of appropriate antibiotic treatment to control infections, restoration of tissue perfusion by fluid resuscitation, and supportive care (Levy, Dellinger et al. 2010). Vasopressors are

recommended, particularly in patients with septic shock (Levy, Dellinger et al. 2010). Treatments to normalize gas exchange, glucose control, and oxygen delivery in septic patients have been ineffective. Therapies aimed at specific molecular targets during sepsis have also been either unsuccessful or harmful. For example, anti-TNFα aimed to decreased inflammation increased mortality in septic patients (Fisher, Agosti et al. 1996). Use of corticosteroids lacked consensus on efficacy after several trials (Schumer 1976). Other targets including other cytokine pathways, bacterial virulence factors, and the coagulation cascade have also proven unsuccessful (Fink and Warren 2014).

It is unsurprising that a single treatment or therapy has not improved sepsis outcomes. Studies attempting to prevent sepsis in animal models using anti-TNFα demonstrated that administration before an *E. coli* challenge could prevent shock and organ failure (Tracey, Fong et al. 1987). However, the effect was lost if administered several hours after infection, demonstrating the dynamic shifting kinetics of a single cytokine. Even beyond the challenges of optimizing drug pharmacokinetics and treatment length, sepsis is a complex heterogeneous syndrome. Heterogeneity derives from biological variability of the patient population and type of infection (etiology and location) (Iskander, Osuchowski et al. 2013). Additionally, sepsis is a complex syndrome that affects multiple molecular cascades and organ dysfunction that is self-reinforcing and linked to other systems, thus blocking one pathway as a possible treatment may not in fact be effective or have unintended consequences.

Sepsis and septic shock are associated with high mortality and substantial morbidity. Nearly 25-30% of septic patients die during hospitalization (Cohen, Vincent et

al. 2015). Early antibiotic treatment and fluid resuscitation has been associated with reduced mortality in clinical trials (Pro, Yealy et al. 2014). However, many patients are admitted to long-term facilities (Kahn, Benson et al. 2010), and readmissions are frequent (Wang, Derhovanessian et al. 2014). Sepsis survivors suffer from long-term morbidity including decreased quality of life, cognitive impairment, and functional disability (Nesseler, Defontaine et al. 2013).

The Liver During Infection

The Hepatic Acute Phase Response (APR)

The liver plays functional roles in metabolism, storage, detoxification, and immunity. The healthy liver is in a constant but regulated state of inflammation due to the exposure of pathogens, toxins, malignant cells, and other host factors within the circulation (Robinson, Harmon et al. 2016). These PAMPs and DAMPs are detected by hepatocytes and a large pool of liver resident immune cells, of which the full spectrum is still uncertain (Robinson, Harmon et al. 2016). Kupffer cells, the resident macrophages which compromise a third of non-parenchymal cells, produce pro- and anti-inflammatory cytokines in response to stimuli (Kojima, Suzuki et al. 2003). Dendritic cells, Myeloid-derived suppressor cells (MDSCs), innate lymphoid cells (NK and NKT cells), lymphocytes, and even parenchymal cells contribute to the hepatic cytokine milieu (Robinson, Harmon et al. 2016). Normal metabolic processes and products such as succinate, triglycerides, and cholesterol promote TLR signaling and inflammasome formation, triggering inflammation (Tannahill, Curtis et al. 2013, Tall and Yvan-Charvet

2015). Despite basal levels of inflammation, the liver is mostly immune tolerogenic (Robinson, Harmon et al. 2016). However, unchecked or overwhelming inflammation drives liver dysfunction and organ injury with systemic consequences.

The liver is also extremely responsive to extra-hepatic inflammation. The hepatic acute phase response (APR) is an innate immune response to inflammation, infection, and injury, which results in changed plasma concentrations of acute phase proteins (APPs) (Gruys, Toussaint et al. 2005, Crispe 2016). Hepatocytes alter transcription and production of APPs in response to major proinflammatory cytokines TNFα, IL-1, IL-6, and IFNγ, among others (Gruys, Toussaint et al. 2005, Crispe 2016). The relevance of these early response cytokines were discovered studying products of leukocytes, which are the first cells attracted to sites of injury (Heinrich, Castell et al. 1990). Indeed, the APR is a quick response with several APPs detectable as early as 4 to 8 hours after LPS administration and elevated 24 to 48 hours post stimuli (Gruys, Toussaint et al. 2005).

Historically, many APPs have been used as biomarkers for the severity of various diseases. For example, C-reactive protein (CRP) has been used as a marker of infection and cardiovascular disease (Liu, Bui et al. 2010, Huang, Gulshan et al. 2017), serum amyloid A (SAA) for various infections and inflammatory states (Eckersall and Bell 2010), and haptoglobin (Hp) for lung cancers (Chang, Lai et al. 2016). Although there are dozens of known liver-derived APPs, the functions have not all been elucidated. Moreover, how changes in many APPs coalesce to have significant biological impact is very poorly understood, and most likely, context-dependent. That said, many individual APPs have been shown to modulate immune responses through various mechanisms.

Several positive APPs, *i.e.*, those with increasing blood concentrations during the APR, are thought to prevent microorganism growth, restore homeostasis, activate complement, aid in opsonophagocytosis, scavenge cell remnants and free radicals, and neutralize proteolytic enzymes (Tillett and Francis 1930, Gruys, Toussaint et al. 2005, Chami, Barrie et al. 2015). Several negative APPs have been shown to reduce blood concentration of zinc, iron, and cortisol-binding globulin, among others (Gruys, Toussaint et al. 2005, Johnson and Wessling-Resnick 2012), likely interfering with the growth and survival of select pathogens.

The Lung-Liver Axis during Pneumonia

The activation and functional role of the APR has been shown to be important for lung defense during infections. Studies have demonstrated that significant remodeling of the liver transcriptome occurs hours after bacterial lung infections (Quinton, Blahna et al. 2012). The regulation of the hepatic APR gene program is reliant on several known transcription factors, of which NF-κB and STAT3 are shown to play significant roles during lung infections (Quinton, Blahna et al. 2012). While over 1,000 hepatic gene transcripts were altered following a *S. pneumoniae* lung infection, simultaneous deletions of NF-κB RelA and STAT3 in hepatocytes essentially eliminated the APR to the pneumonia challenge (Quinton, Blahna et al. 2012). Activation of these transcription factors is initiated by early-response cytokines TNFα, IL-1, and IL-6, as IL-6-null mice or mice lacking all signaling receptors for TNFα and IL-1 have a markedly reduced capacity to activate liver STAT3 and NF-κB RelA, respectively, following a bacterial

pneumonia challenge in association with reduced APP induction (Quinton, Jones et al. 2009). These studies support the central role of a lung-liver axis, in which early and local responses to respiratory infections remodel the hepatic transcriptome and modulate host responses.

The physiological mechanisms through which the lung-liver axis provides protection during pneumonia is unclear, and pursuit of such information is challenged by the large scope of hepatic acute phase changes observed in response to this condition. However, studies have now unveiled the protective function of the APR during respiratory infections. APR-mice lacking hepatocyte RelA and STAT3 have reduced induction of both circulating APPs and those accumulating in the alveolar lining fluid in pneumonic mice (Quinton, Blahna et al. 2012, Hilliard, Allen et al. 2015). Additionally, these APR-null mice exhibit decreased host defense associated with higher bacterial burdens, and increased mortality. Mechanisms implicated in APR-dependent protection during lung infections include diminished airspace macrophage activation, and decreased complement-mediated opsonization (Quinton, Blahna et al. 2012, Hilliard, Allen et al. 2015). Hepatocyte STAT3, alone, has been implicated in altering the lung environment during endotoxemia, leading to substantially increased pneumonia susceptibility (Hilliard, Allen et al. 2015), suggesting that normal liver activity may serve to limit the degree to which sepsis (and/or other systemic challenges) predispose to lung infection. Consistent with the idea of liver-derived lung protection, IL-6 driven induction of APP hepcidin (HAMP) was shown to be important in promoting pulmonary defense and preventing bacterial dissemination through iron sequestration (Michels, Zhang et al.

2017). But APPs represent only a portion of the numerous liver pathways altered during a lung infection (Quinton, Blahna et al. 2012, Weber, Lambeck et al. 2012). While these changes may reflect processes supporting APPs synthesis and/or secretion, activation of "non-APP" pathways may also include homeostatic measures such as metabolism and other forms of adaptation. For instance, *S. pneumoniae* infections reduce liver cholesterol biosynthesis and heighten pneumococcal virulence, possibly by limiting cholesterol-dependent protection against the injurious toxin pneumolysin (Weber, Lambeck et al. 2012). Tissue resilience of the liver itself may also represent part of the APR, as STAT3/RelA-deficient APR-null mice exhibit increased liver injury during an acute lung infection in association with increased mortality (Hilliard, Allen et al. 2015). While the significance of the lung-liver axis is now appreciated, coordinating its protective effects are only beginning to be elucidated.

Liver Injury in Severe Infection

While the liver has a central role in immune regulation, it is a common target of dysregulation during infections. In particular, liver injury has prognostic relevance for sepsis (Minemura, Tajiri et al. 2014). As such, clinical measures of liver dysfunction are a component of the SOFA score (Singer, Deutschman et al. 2016). Liver injury commonly occurs during the course of sepsis, with mean incidence in 30-50% of sepsis patients (Wang, Yin et al. 2014, Yan, Li et al. 2014). Development of liver dysfunction is a strong predictor of morbidity and mortality (Yan, Li et al. 2014, Nesseler, Launey et al. 2016). Incidence rates of sepsis are higher in patients with pre-existing liver dysfunction

or disease (Canabal and Kramer 2008). Even in the setting of pneumonia, modest liver injury was associated with negative patient outcomes (Jinks and Kelly 2004, Xu, Ying et al. 2018).

There are several well-described mechanisms of sepsis-related liver injury. Hypoxic hepatitis, known as shock liver, occurs in approximately 10% of septic patients and is associated with 50% mortality (Kramer, Jordan et al. 2007, Jager, Drolz et al. 2012). Shock liver arises from inadequate oxygen concentration due to reduced blood flow, decreased oxygen carriers, and microthrombi. Elevated serum transaminases, miR-122, and keratins are detected just hours after injury, and are indicative of apoptotic and necrotic cell death (Roderburg, Benz et al. 2015, Ku, Strnad et al. 2016). Patients develop cholesteric hepatic dysfunction resulting from impaired bile formation or decreased bile flow. Experiments in mouse models of sepsis show evidence that proinflammatory cytokines TNFα and IFNγ alter ion transport systems and promote inflammation, leading to impaired bile flow (Spirli, Nathanson et al. 2001). Changes in bile flow contribute to the accumulation of bilirubin, altered glucose and lipid metabolism, and vasodilation, leading to increased oxidative stress and cell membrane permeability (Trauner, Baghdasaryan et al. 2011).

Less studied mechanisms of sepsis-related liver injury include immunotoxicity. Innate leukocytes are known to be sufficient and necessary to promote liver injury (Wang, Yin et al. 2014). Activation of hepatic NKT cells have been linked to increased mortality in association with systemic inflammation in murine models of sepsis (Hu, Venet et al. 2009). Likewise, Kupffer cells have been shown to drive programmed cell

death of hepatic endothelial cells during sepsis (Hutchins, Wang et al. 2013). Several mechanisms of cell death by immune cells and immune mediators have been implicated in the promotion liver injury. Apoptosis of hepatocytes and endocytosis of apoptotic bodies activate Kupffer cells, which upregulate death ligands TNFα, TRAIL, and FasL (Canbay, Feldstein et al. 2003). These death ligands are capable of inducing cell death receptor-mediated apoptosis (Beg, Sha et al. 1995, Tsutsui, Matsui et al. 1997, Ochi, Ohdan et al. 2004), which further promote liver inflammation. Necroptosis, a form of programmed cell death dependent on RIP kinases, is also driven by TNFα and Fas-mediated cell death (Linkermann and Green 2014). Necroptosis and necrosis are accompanied by release of cellular constituents, known to elicit significant inflammatory responses and promote liver injury (Kaczmarek, Vandenabeele et al. 2013, Huang, Tohme et al. 2015, Yamamoto and Tajima 2017).

Aims and Hypothesis

Pneumonia and sepsis are distinct but integrally linked public health concerns that are a common cause of morbidity and mortality. The mechanisms of protection that limit harmful systemic consequences of pneumonia as well as its likelihood in the setting of sepsis are unclear. The hepatic APR is a coordinated response to maintain host homeostasis during infection, inflammation, and tissue injury. Our laboratory has previously revealed the importance of hepatocyte transcription factors STAT3 and NF-κB RelA (p65) for the APR during pneumonia or sepsis. Our prior studies utilizing mice with hepatocyte-specific deletion of both transcription factors revealed elimination of the acute

phase response, impaired host defense, and increased susceptibility to liver injury. Therefore, we propose the central hypothesis that **hepatic APR transcriptional responses are critical for deleterious interactions between pneumonia and sepsis**.

Previous reports highlight liver dysfunction as an important independent risk factor for sepsis mortality (Yan, Li et al. 2014, Nesseler, Launey et al. 2016). Our preliminary data allude to the existence of liver-derived homeostatic signals that prevent hepatotoxicity from becoming a more prominent feature of pneumonia. While hepatocyte transcription factors STAT3 and NF-κB RelA (p65) are essential for acute phase changes, our preliminary studies demonstrate that the absence of RelA alone leads to mortality and liver injury, possibly due to dysregulation of locally-derived death-promoting signals. These findings suggest that RelA activity normally counters pneumonia-induced sepsis by preventing programmed hepatocyte cell death, thereby preserving liver homeostasis. Given these findings, we hypothesize that **RelA-dependent gene programs counter damaging signals from hepatic leukocytes to maintain liver homeostasis and limit the systemic consequences of pneumonia and sepsis**.

Our lab has previously shown that activation of the STAT3 transcription factor in hepatocytes limits pneumonia susceptibility during endotoxemia, a model of sepsis, but the mechanisms whereby this liver APR provides protection are unknown. Sequestering iron is a form of nutritional immunity and a first line of defense against bacterial infections, which require iron for growth. Based on our previous finding that growth conditions in alveolar lining fluid are favorable for bacteria in the absence of liver STAT3, we also propose the hypothesis **that the liver APR limits pneumonia**

susceptibility during sepsis by withholding available iron to prevent bacterial outgrowth.

CHAPTER TWO: MATERIALS AND METHODS

Mouse Models

Mouse experiments were performed using C57BL/6 mice, or mice with hepatocyte-specific deletions of either NF-κB RelA/p65 or STAT3. This well-established system utilizes a Cre-*LoxP* system driven by an albumin promoter, which results in a functional deletion of RelA or STAT3 by 6 weeks of age (Postic and Magnuson 2000). Our lab has previously verified the deletion of RelA and STAT3 in the livers of these mice models (Quinton, Blahna et al. 2012). Mouse experiments were performed at least twice using mice of both sexes, between the ages of 6 and 15 weeks. All animal experiments and protocols were approved by Boston University Institutional Animal Care and Use Committee (IACUC).

C57BL/6 Mice

C57BL/6 mice were purchased directly from Jackson Laboratory, or were from a colony maintained by our laboratory.

Hepatocyte RelA-null (hepRelA$^{\Delta/\Delta}$) Mice

Dr. R.M. Schmid generated and kindly provided our laboratory with RelA-floxed mice (*RelA*$^{LoxP/LoxP}$), which have *LoxP* sites flanking exons 7-10 of the NF-κB *RelA* gene (Algul, Treiber et al. 2007). Homozygous *RelA*-floxed mice were crossed with transgenic mice purchased from Jackson Laboratory containing a Cre-recombinase under transcriptional control of the albumin promoter. The resulting hepRelA$^{\Delta/\Delta}$ (Alb-Cre$^{tg/-}$

/*RelA*$^{LoxP/LoxP}$) mice have hepatocyte-specific deletions of *RelA* exons 7 through 10. The truncated form of RelA prevents nuclear translocation and activation (Carpenter, Schroeder et al. 2012). Results from hepRelA$^{\Delta/\Delta}$ mice were compared to wild type (WT) littermates lacking Cre-recombinase (Cre$^{-/-}$/*RelA*$^{LoxP/LoxP}$).

Hepatocyte STAT3-null (hepSTAT3$^{\Delta/\Delta}$) Mice

Dr. S. Akira generated and kindly provided our laboratory with *STAT3*-floxed mice (*STAT3*$^{LoxP/LoxP}$), which have *LoxP* sites flanking exon 21 of the *STAT3* gene (Akira 2000). Homozygous floxed *STAT3* mice were crossed with transgenic mice purchased from Jackson Laboratory, containing a Cre-recombinase under transcriptional control of the albumin promoter. The resulting hepSTAT3$^{\Delta/\Delta}$ (Alb-Cre$^{tg/-}$/*STAT3*$^{LoxP/LoxP}$) mice have hepatocyte-specific deletions of *STAT3* exon 21. The truncated form of STAT3 lacks a tyrosine residue that is necessary for dimerization and activation (Akira 2000). Results from hepSTAT3$^{\Delta/\Delta}$ mice were compared to WT littermates lacking Cre-recombinase (Cre$^{-/-}$/*STAT3*$^{LoxP/LoxP}$).

Bacteria and Stimuli

Generation of Bacterial Stocks

Frozen stocks were prepared by growing bacteria overnight on 5% sheep blood agar plates (BD Biosciences). Colonies were picked and grown to mid-log phase (approximately 4 hours) in sterile lysogeny broth (LB) broth at 37°C and shaking at 300

rpm. Bacterial aliquots were mixed with sterile glycerol (final concentration = 16%). Aliquots were snap frozen in liquid nitrogen and stored at -80°C until use.

Preparation of Bacteria for In Vivo Experiments

Bacteria were streaked on 5% blood agar plates and incubated 12 to 15 hours in 37°C and 5% CO_2. Colonies were picked off the plate and suspended in sterile saline. Colony forming unit (CFU) inputs were estimated by optical density and confirmed by serial dilution on agar plates. Heat-killed bacteria were prepared by incubating suspended bacteria in a 55°C water bath for 45 minutes.

Bacteria and Other Stimuli

Bacterial strains were purchased from ATCC: *Escherichia coli* (serotype O6:K2:H1, 19138), *Klebsiella pneumoniae* (serotype 2, 43816), and *Streptococcus pneumoniae* (serotype 3, 6303). Alpha-Galactosylceramide (αGalCer) was purchased from Enzo Life Sciences (BML-SL232) and lipopolysaccharide (LPS-EB from *E. coli* O111:B4) was purchased from InvivoGen (tlrl-eblps).

Infection Models

Intraperitoneal Injections (I.P.)

Mice were administered intraperitoneal injections (i.p.) in the lower right quadrant of the abdomen using a 25-gauge needle. Anesthesia for the following *in vivo* procedures and LPS stimulation (0.1 mg/kg diluted in sterile saline) were administered i.p.

Intratracheal Instillations (I.T.)

Mice were anesthetized by i.p. injection of ketamine (50 mg/kg) and xylazine (5 mg/kg) in sterile saline. A small incision was made, and the trachea was exposed and cannulated with a 24-gauge angiocatheter directed into the left bronchus. A 50 µL bolus of bacteria (10^6 CFU of *E. coli*, 10^3 CFU of *K. pneumoniae*) suspended in sterile saline was instilled using a micropipetter. The inoculum was confirmed by plating serial dilutions of the bacterial suspension on agar plates and incubating overnight in 37°C.

Intravenous Injections (I.V.)

Mice were anesthetized by an i.p. injection of ketamine (50 mg/kg) and xylazine (5 mg/kg) in sterile saline. A 100 µL bolus of bacteria or stimuli (10^6 CFU of *E. coli*, 10^6 CFU of live *S. pneumoniae*, 10^7 of heat-killed *S. pneumoniae*, 2 ug/mouse of αGalCer) suspended in sterile saline was administered by tail vein injection using a 27-gauge needle.

Endotoxemia-Pneumonia Two-Hit-Model

HepSTAT3$^{\Delta/\Delta}$ and WT mice were administered i.p. 1 mg/kg of LPS 18 hours prior to intratracheal instillation of 10^6 CFU of *E. coli*.

Cecal-Ligation and Puncture Model (CLP)

Mice were anesthetized by inhalation of 2 to 5% isoflurane in 100% oxygen. A small incision was made through the skin and linea alba. The cecum was exposed, ligated with a silk suture, and perforated twice with a 23-guage needle with stool extruded. The cecum was replaced in the original position and the incision was closed with sutures and skin adhesive glue (Nexaband Liquid, Abbott Laboratories). In Sham mice, the cecum was replaced without ligation or puncture. Mice were administered 1 mL of warm saline (37°C) by subcutaneous injection. 2 hours after surgery, and every 12 hours thereafter, mice were administered subcutaneous injection of buprenorphine (0.05 mg/kg) suspended in 1 mL of lactated Ringer's solution for pain management and imipenem (25 mg/kg) for antibiotic treatment. CLP was performed by Dr. J. Kim in the laboratory of Dr. D.G. Remick at Boston University School of Medicine.

Neutralization, Blocking, and Depletion Strategies

To interrogate the role of NKT cells, mice received i.v. injections of either anti-Cd1d (Biolegend 123515) or control IgG (Biolegend RTK4530) at a dose of 0.1 mg/mouse. TNFα neutralization was achieved through i.v. administration of 100 μl saline containing 0.5 mg anti-TNFα (BioXCell BE0058) or control IgG (BioXCell BE0088) at the time of challenge. To investigate the role of recruited monocytes, mice received i.p. injection of 300 μg/mouse of either anti-Ccl2 (BioXCell BE0185) or control IgG (BioXCell BE0091) 24 hours prior to and at the time of challenge. To deplete macrophages, mice received i.v. 0.1 mL/10 g body weight of either clodronate- or PBS-

containing liposomes (Liposoma BV) 24 hours prior to challenges as described by manufacturers. To investigate the role of necroptosis, mice received i.v. injections of either Nec-1s (Biovision Inc. 2263) at dose of 6.25 mg/kg or vehicle 15 min prior to challenge.

Endpoint Collections

Blood and Tissue Collection

Mice were euthanized at indicated time points by isoflurane inhalation overdose. After euthanasia, blood was collected from the inferior vena cava with a 25-gauge needle. Blood was deposited into MiniCollect Tube Z Serum separator tubes (Greiner BioOne) and incubated at room temperature for 30 min for serum, or collected with a heparinized needle and kept on ice for plasma. Blood samples were centrifuged at 1500 x g for 15 min at 4°C. Serum or plasma was collected, aliquoted, and stored at -80°C until future analysis.

The medial lobe of the liver of each mouse was collected after euthanasia, snap frozen in liquid nitrogen, and stored at -80°C until future analysis. Likewise, the left lobe of the lung was snap frozen and stored at -80°C after bronchoalveolar lavage.

Bronchoalveolar Lavage (BAL)

Mice were euthanized at indicated time points by isoflurane inhalation overdose. The inferior vena cava was cut to exsanguinate or blood was collected from the inferior vena cava. The thoracic cavity was exposed and the trachea, lungs, and heart were

removed *en bloc*. The trachea was cannulated with a 20-gauge blunt stainless steel catheter and secured with a silk suture. Lungs were injected with 1 mL of ice-cold PBS, lavage fluid was withdrawn and collected. Lungs were lavaged 10 times, with the first wash collected separately. Washes were centrifuged at 300 x g for 5 min at 4°C to separate supernatants and cell pellets. The supernatant from the first lavage was aliquoted and stored at -80°C until analysis. For select studies, BAL fluid was sent to the laboratory of Marianne Wessling-Resnick at Harvard School of Public Health for iron concentration measurements.

Bacteriology

Blood samples were serially diluted in sterile water, plated on 5% sheep blood agar plates, and incubated at 37°C overnight. Colonies were counted and calculated as total CFU per mL of blood.

Aminotransferase Assays

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations were measured using AST and ALT Reagent Set (Point Scientific Inc.). 100 µL of 10 to 100-fold diluted serum was added to 1 mL of pre-warmed reagent, and absorbance was read at one min intervals over 3 min. Concentrations were calculated according to manufacturer's instructions.

Immunohistochemistry

Liver Fixation and Hematoxylin and Eosin (H&E) Staining

The left lateral lobe of each mouse was isolated, cut into three sections, and fixed in 4% paraformaldehyde (PFA) overnight at 4°C. Tissue sections were placed in tissue cassettes and sent to Boston University School of Medicine Experimental Pathology Laboratory Service Core for processing and H&E staining.

Lung Fixation and Hematoxylin and Eosin (H&E) Staining

After euthanasia, the thoracic cavity was opened and inferior vena cava cut to exsanguinate. 5 mL of ice-cold PBS was injected into the right ventricle of the beating heart with a needle to perfuse the lungs. The heart was tied off with a suture to prevent backflow of blood. The trachea, lungs, and heart was removed *en bloc*. The trachea was cannulated and secured to a 20-gauge catheter. The lungs were filled with 4% PFA at a 22 cm-height pressure. The trachea was tied off with a suture and lungs were immersed in 4% PFA overnight at 4°C. The left lobe was cut into three sections, which were placed in tissue cassettes, and sent to Boston University School of Medicine Experimental Pathology Laboratory Service Core for processing and H&E staining.

Hypoxyprobe-1 Immunohistochemistry Staining

Mice were administered 60 mg/kg Hypoxyprobe-1 (hypoxyprobe.com) in sterile saline by i.p 1 hour prior to euthanasia. The left lateral lobe of each mouse was isolated, cut into three sections, and fixed in 4% PFA overnight at 4°C. Livers were incubated in

specified solutions for indicated times and temperatures (**Table 1**). Tissues were embedded in Surgipath Paraplast Plus paraffin (Leica Biosystems) within 24 x 24 x 5 mm molds (Fisher Scientific) and cooled overnight at room temperature. Paraffin blocks were cooled to 4°C and sectioned into 5 μm thick sections by microtome and placed onto microscope slides.

Sectioned liver tissues were de-paraffinized using the indicated protocol (**Table 2**), and antigen retrieval was performed by low power microwave (5 min, three times) in Antigen Unmasking Solution (Vector Laboratories). Slides were cooled for 30 min, washed in PBS for 10 min, and endogenous peroxidase was quenched using 3% hydrogen peroxide in PBS for 15 min at room temperature. Slides were washed in PBS for 15 min and tissue sections were blocked with Mouse on Mouse Blocking Reagent (Vector Laboratories) for 60 min following manufacturer's instructions. Sections were stained with Hyproxyprobe-1 MAb1 (hyproxyprobe.com) primary antibody diluted 1:50 in PBS for 1 hour at room temperature, and washed in PBS for 10 min. Tissue sections were incubated with secondary antibody using the Vectastain ABC HRP Kit, Peroxidase (Vector Laboratories) for 30 min at room temperature, washed in PBS for 10 min, and developed using DAB peroxidase HRP Substrate Kit (Vector Laboratories) for 1 min. Sections were counterstained with hematoxylin, dehydrated (reverse of **Table 2**), and mounted with a coverslip.

Solution	Incubation Time	Temperature
PBS	30 min	On ice
0.85% NaCl	30 min	On ice
1:1 Ethanol : 0.85% NaCl	30 min	Room temperature
70% Ethanol (2 times)	30 min each	Room temperature
80% Ethanol	45 min	Room temperature
90% Ethanol	45 min	Room temperature
100% Ethanol (3 times)	30 min each	Room temperature
Xylene (3 times)	30 min each	Room temperature
1:1 Xylene : paraffin	1.5 hr	60°C
Paraffin (2 times)	1 hr each	60°C, in vacuumn

Table 1. Paraffin Embedding Protocol.

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

Table 2. Deparaffinization Protocol.

Aminotransferase Assays

Liver RNA Isolation

To isolate RNA from livers, a portion of snap frozen livers were homogenized using 7 to 9 2.0-mm zirconium oxide Bullet Blender beads (Next Advance) in TRIzol (Life Technologies) using manufacturer's instructions. RNA was cleaned using the RNeasy Kit (Qiagen), and RNA samples were stored at -80°C until analysis.

Lung RNA Isolation

To isolate RNA from the left lobe of the lungs, snap frozen lungs were homogenized using 7 to 9 3.2-mm stainless steel Bullet Blender beads (Next Advance) in

buffer RLT (from Qiagen's RNeasy Kit) using manufacturer's instructions. RNA was isolated using the RNeasy Kit (Qiagen), and RNA samples were stored at -80°C until analysis.

RNA Isolation from Sorted Cells

To isolated RNA from sorted cells, cells stored in RNAprotect Cell Reagent (Qiagen) were thawed to room temperature, disrupted with RLT buffer (Qiagen) and homogenized by centrifugation through a QIAshredder (Qiagen) following manufacturer's instructions. RNA was isolated using the RNeasy Kit (Qiagen), and RNA samples were stored at -80°C until analysis.

Quantitative Real-Time PCR (qRT-PCR)

Quantitative real-time PCR (qRT-PCR) was performed with 10 ng of isolated RNA using the StepOne Plus Real-Time PCR System (Thermo Fisher Scientific) and the TaqMan RNA-to-CT 1-Step Kit (Thermo Fisher Scientific), following manufacturer's suggested protocol. RNA was probed with primers and FAM-labeled probes for *TNF* (Applied Biosystems Mm00443258_m1), *CCL2* (Mm00441242_m1), and *CXCL10* (Mm00445235_m1), *HAMP* (Mm04231240_s1), *HP* (Mm00516884_m1), *HPX* (Mm00457510_m1), *TFR2* (Mm00443703_m1), *WNT3A* (Mm00437337_m1), *SMAD9* (Mm00649885_m1), *SOX17* (Mm00488363_m1). Primer and probe sequences for *LCN2*, *GCSF*, *GMCSF*, *CXCL11*, and *CXCL10* are listed in **Table 3**. Probes were labeled with FAM at the 5' end and Black Hole Quencher-1 at the 3' end. Each sample was also

probed with VIC-labeled eukaryotic 18S rRNA Endogenous Control (Life Technologies 4319413E). Sample expression values were calculated and presented as fold induction as normalized to 18S rRNA, and expressed as compared to control groups.

Gene	Forward Primer	Reverse Primer	TaqMan Probe
LCN2	ATATGCACAGGTA TCCTC	AAACGTTCCTTCA GTTCA	CCACCACGGACTA CAACA
GCSF	TTCCCCTGGTCACT GTCAGC	CACAGCTTGTAGG TGGCACAC	ACCATCCTTGCCT CTGCCCCGAAG
GMCSF	ATTTACTTTTCCTG GGCATTGTGG	CAGGAGGTTCAGG GCTTCTTTG	TACAGCCTCTCAG CACCCACCCGC
CXCl1	CAGAGATCGAGAA AGCTTCTGTA	TCCTGGCACAGAG TTCTT ATT	CGAGTAACGGCTG CGACA

Table 3. List of qRT-PCR Primer and Probe Sequences.

Microarray

Total liver RNA quality of each sample was determined using the Agilent Bioanalyzer, followed by microarrays using Affymetrix GeneChip Mouse Gene 2.0 ST Arrays. Analysis and microarrays were performed at the Boston University Microarray and Sequencing Resource. After data normalization, filtering, and processing, fold changes were calculated between experimental groups. Gene expression differences between the indicated groups were considered statistically significant when False Discovery Rate (FDR) $q < 0.05$. Ingenuity Pathway Analysis (Qiagen Bioinformatics) were used to identify molecular, cellular, or functional pathways.

RNA Sequencing (RNA-Seq)

Total lung RNA quality of each sample was determined using the Agilent Bioanalyzer, and the Illumina NextSeq 500 system for whole transcriptome RNA sequencing was performed. A high output of 400M single, 75-base paired end length reads were generated. RNA amplification, library preparation, and sequencing were performed at the Boston University Microarray and Sequencing Resource. After data normalization, filtering, and processing, fold changes were calculated between experimental groups by the Microarray and Sequencing Resource and Dr. M.R. Jones. Gene expression differences between the indicated groups were considered statistically significant when FDR $q < 0.05$.

Protein Isolation and Measurements

Protein Isolation

Snap frozen liver lobes were homogenized using 7 to 9 2.0-mm zirconium oxide Bullet Blender beads (Next Advance) in protein extraction buffer. Homogenates were incubated on ice for 15 min with occasional vortexing. After centrifugation for 20 min at 15,000 x g and 4°C, supernatants were aliquoted and stored in -80°C until analysis.

Bicinchoninic Acid (BCA) Assay

Concentrations of total protein isolated from liver homogenates were determined by a BCA assay (Sigma-Aldrich) using bichinchoninic acid and copper (II) sulfate

solution. Total protein concentration were calculated using manufacturer's instructions by comparing sample to BSA protein standards at 0, 0.2, 0.6, and 0.9 mg/mL concentrations.

Immunoblot

Protein samples were diluted to 20 µg in NuPage lithium dodecyl sulfate sample buffer (Life Technologies), NuPage Reducing Agent (Life Technologies), and ultrapure water, and heated for 10 min at 70°C. Immunoblots were performed using the NuPAGE Bis-Tris Gel system and tank.15 µL of samples and protein standards (Novex Sharp, Life Technologies) were loaded onto a NuPAGE 4-12% Bis-Tris gel (Novex by Life Technologies). 1x MOPS SDS running buffer was added and the gel was run for 50 min at 200V. Protein was transferred onto an immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore) in 1x NuPage transfer buffer (Life Technologies) with 10% methanol at 30V for 1 hour. Membranes were blocked in 5% non-fat dry milk in tris-buffered saline with Tween 20 (TBS-T) for 1 hour at room temperature with agitation, followed by three 10-min washes in TBS-T. Membranes were probed using primary antibodies from Cell Signaling Technology (**Table 4**) diluted in 10 mL blocking buffer with agitation. After three washes, anti-rabbit HRP-conjugated secondary antibody (Cell Signaling 7074) diluted 1:2000 in 10 mL blocking buffer was added to membranes for 1 hour at room temperature with agitation. Membranes were visualized using the ECL Prime Western Blotting Detection System (Sigma-Aldrich) on film (GE Healthcare) following manufacturer's instructions. Membranes were stripped with Re-Blot Plus Mild antibody stripping solution(Millipore) before blocking and probing of the next primary

antibody. Densitometry was performed using Image Processing and Analysis in Java (ImageJ, NIH).

Antibody	Species, Clone	Dilution	Incubation Time	Company (Cat. No)
Cleaved Caspase-3	Rabbit monocolonal, Asp175, 5A1E	1:000	Overnight at 4°C	Cell Signaling Technologies (9664)
Cleaved Caspase-8	Rabbit monocolonal, Asp387, D5B2	1:000	Overnight at 4°C	Cell Signaling Technologies (8592)
Pan-Actin	Rabbit Polyclonal	1:000	1 hr at room temperature	Cell Signaling Technologies (4968)

Table 4. List of Immunoblot Antibodies.

Multi-Plex Bead Array

Serum protein concentrations of CCL2, CCL3, CCL4, CCL11, CXCL1, CXCL2, CXCL5, CXCL10, IL-1α, IL-1β, IL-6, IL-10, IL-17a, IL-23p19, G-CSF, IFNγ, and TNFα were measured by using a Mouse Magnetic Luminex Assay (R&D Systems LXSAMSM) on the Bio-Plex 200 multiplexing analyzer system following manufacturer's instructions.

Plasma and lung homogenate concentrations of CCL2, CCL19, CXCL1, CXCL2, G-CSF, GM-CSF, IL-1α, IL-6, IL-10, osteopontin, podocalyxin, and uPAR were measured using the Mouse Magnetic Luminex Assay system (R&D Systems LXSAMSM).

Flow Cytometry and Cell Sorting

Cells were resuspended in 100 µL FACS buffer with Fc block (TruStain FcX antibody, Biolegend). Cell surface markers were stained with 20 µL of diluted antibodies (**Table 5**), incubated on ice for 30 min, covered from light. Cells were washed with 4 mL of PBS, pelleted by centrifugation at 300 x g, 4°C for 5 min, and resuspended in 400 µL FACS buffer. Cells were kept on ice until flow cytometry analysis on the BD LSRII or cell sorting by an operator on the FACSAria II SORP at Boston University Flow Core Facility. NKT cells, NK cells, Kupffer cells, and monocytes were identified by surface markers in **Table 6**. Single stained cells or bead controls (UltraComp eBeads, Thermofisher) were utilized for compensation and gating in the FACS Diva program. Flow cytometry analysis was performed on FlowJo (Version 10). Sorted cells were pelleted and resuspended in 300 µL RNAprotect Cell Reagent (Qiagen) and stored at -80°C until RNA isolation.

Antibody	Clone	Fluorophore	Dilution	Company (Cat. No.)
CD45	30-F11	PE-Cy7	1:1000	Biolegend (103113)
CD3	17A2	APC	1:50	Biolegend (100236)
NK1.1	PK136	FITC	1:100	Biolegend (108705)
NKp46	29A1.4	BV605	1:50	Biolegend (137619)
CD1d-loaded tetramer	PBS-57	PE	1:50	NIH Tetramer Core Facility (20670)
F4/80	RTK2758	APC-Fire750	1:100	Biolegend (123151)
CD11b	M1/70	BUV395	1:100	BD Horizon (563553)
Ly6C	HK1.4	BV785	1:100	Biolegend (128041)
7AAD	N/A	N/A	5 µL / sample	Biolegend (420404)

Table 5. List of Flow Cytometry Antibodies.

Cell Type	Surface Markers
Kupffer Cells	$7AAD^{-}/CD45^{+}/F4/80^{+}$
Monocytes	$7AAD^{-}/CD45^{+}/CD11b^{+}/Ly6C^{+}$
NK Cells (C57/Bl6)	$7AAD^{-}/CD45^{+}/CD3^{-}/NK1.1^{+}$
NK Cells ($hepRelA^{\Delta/\Delta}$)	$7AAD^{-}/CD45^{+}/CD3^{-}/NKp46^{+}$
NKT Cells (C57/Bl6)	$7AAD^{-}/CD45^{+}/CD3^{+}/NK1.1^{+}$
NK Cells ($hepRelA^{\Delta/\Delta}$)	$7AAD^{-}/CD45^{+}/CD3^{-}$/CD1d-loaded $Tetramer^{+}$

Table 6. Cell Populations and Surface Markers for Flow Cytometry.

Reagents and Buffer Recipes

- *Phosphate-buffered saline* (*PBS*, Gibco, ThermoFisher Scientific)

- *Hank's balanced salt solution* (*HBSS*, ThermoFisher Scientific)

- *Protein extraction buffer*: 2 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

- *Methanol* (Sigma-Aldrich)

- *Tris-buffered saline-Tween-20* (*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 dry milk in TBS-T

- *NuPAGE MOPS SDS running buffer* (Invitrogen, ThermoFisher Scientific)

- *NuPAGE Transfer buffer* (Invitrogen, ThermoFisher Scientific)

- *4% paraformaldehyde (PFA)*: 10 mL 16% PFA (Ted Pella, Inc), 4 mL 10x PBS, 26 mL distilled water

- *Sodium Chloride* (*0.9% NaCl, Saline,* Baxter Healthcare Corporation)

- *Ethyl alcohol* (Fisher Chemical): 90-50% ethanol diluted in distilled water

- *Xylenes* (Fisher Chemical)

- *Hematoxylin stain* (Ricca Chemical Company)

- *Roswell Park Memorial Institute 1640 Media* (*RPMI1640*, Gibco, ThermoFisher Scientific)

- *Collagenase solution*: 0.05% collagenase/dispase (Sigma-Adrich), 0.01% trypsin inhibitor (soybean, Gibco, ThermoFisher Scientific) in RMPI1640; 15 mL per sample

- *Normo-osmotic Percoll solution*: 37 mL Percoll Plus (GE Healthcare, Sigma-Aldrich), 2.88 mL 10x HBSS, 480 µL sodium bicarbonate solution (7.5%, Gibco, ThermoFisher Scientific); for 6 samples

- *33% Percoll solution*: 33 mL normo-osmotic Percoll solution, 66 mL RPMI1640, 1 mL heparin (1000 U/mL); for 6 samples

- *Red Blood Cell Lysing Buffer Hybri-Max* (Sigma-Aldrich)

- *Fluorescence-Activated Cell Sorting (FACS) buffer*: 0.5% fetal bovine serum (FBS), 2 mM EDTA in PBS; Sterilized by filter

Statistics

Statistical analyses were performed using GraphPad Prism 8.0. All data are presented as mean ± SEM. Two-group comparisons were made using a student's t test. Multiple group comparisons were made using one- or two-way analysis of variance (ANOVA) followed by a Tukey multiple comparisons test. All data were tested for normality (Shapiro-Wilk test) and equal variance (F-test or Spearman's test). Data that were not normally distributed were log transformed before statistical analysis. Results were considered significant if $p < 0.05$.

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CHAPTER THREE: HEPATIC NF-KAPPAB RELA ACTIVATION IS REQUIRED FOR HEPATOPROTECTION DURING PNEUMONIA AND SEPSIS

Rationale

Sepsis is a complex disorder that arises as a dysregulated host response to infection, and is associated with acute organ dysfunction (Singer, Deutschman et al. 2016). This condition remains a common and deadly public health concern with lasting morbidity and considerable economic consequences (Paoli, Reynolds et al. 2018). Sepsis is the leading cause of mortality in hospitalized patients, with annual rates of incidence steadily increasing worldwide (Walkey, Lagu et al. 2015). Respiratory infections, which are the most common cause of sepsis (Mayr, Yende et al. 2014), represent the greatest disease burden worldwide, and account for the most infection-related deaths (Quinton, Walkey et al. 2018). Sepsis can occur when bacteria disseminate from the lungs and compromise non-pulmonary tissue resilience and vascular homeostasis (Wunderink and Walley 2014). While pneumonia and sepsis are intimately linked, it remains unclear whether and how extra-pulmonary signals actively fortify tissue protection during lung infections as a means to limit the progression of the former towards the latter.

The hepatic acute phase response (APR) is a hallmark of sepsis, pneumonia, and other inflammatory conditions (Gabay and Kushner 1999). This event is typified by changes in circulating acute phase proteins (APPs) such as C-reactive protein (Gabay and Kushner 1999), which are clinically relevant disease biomarkers. However, most hepatic gene programs altered during pneumonia do not, in fact, encode APPs (Quinton, Blahna

et al. 2012), implicating other biological pathways as an important aspect of the APR. Our previous studies in APR-null mice, lacking both the NF-κB RelA and STAT3 transcription factors in hepatocytes, revealed immune defects and mortality in the settings of pneumonia and sepsis (Quinton, Jones et al. 2009, Quinton, Blahna et al. 2012, Hilliard, Allen et al. 2015). Interestingly, liver injury was also observed in APR-null mice during pneumonia, suggesting that hepatoprotection is an inducible process that curbs the systemic consequences of lung infections (Hilliard, Allen et al. 2015). But whether hepatocytes launch countermeasures against immunotoxic signals during infection remains unclear.

Both infection and organ dysfunction are defining characteristics of sepsis (Singer, Deutschman et al. 2016), and the liver is one of several such threatened organs (Wang, Yin et al. 2014). Indeed, liver injury is a significant risk factor of sepsis morbidity and mortality (Yan, Li et al. 2014, Nesseler, Launey et al. 2016), and its underlying causes likely extend beyond hypoxic hepatitis and cholestasis to include less-known triggers such as immunotoxicity and dysregulated cell death (Hu, Venet et al. 2009, Hutchins, Wang et al. 2013). Meanwhile, host pathways that limit hepatotoxicity in response to infection represent a major knowledge gap. Given prior evidence of NF-κB RelA in curbing hepatocyte injury (Beg, Sha et al. 1995, Geisler, Algul et al. 2007), we hypothesized that RelA-dependent acute phase changes fortify liver tissue resilience in the settings of pneumonia and sepsis.

Results

3.1 Hepatoprotection during pneumonia is ReA-dependent

Prior investigations in mice lacking both STAT3 and RelA in hepatocytes indicated that the combined presence of these transcription factors is required for liver acute phase changes during pneumonia (Quinton, Jones et al. 2009, Quinton, Blahna et al. 2012, Hilliard, Allen et al. 2015, Hilliard, Allen et al. 2015). Additionally, these APR-null mice exhibited signs of severe hepatotoxicity (Hilliard, Allen et al. 2015), demanding a better understanding of the signals that actively fortify liver tissue integrity during infection. To distinguish the functional contributions of STAT3 *vs.* RelA with regards to hepatoprotection during pneumonia, we compared results from mice lacking one or the other in hepatocytes. Littermate control (WT) or mutant (hepSTAT3$^{\Delta/\Delta}$ or hepRelA$^{\Delta/\Delta}$) mice were infected i.t. with 10^6 CFU of *E. coli*. Despite the acute (24-hour infection) design and typically non-lethal dose of *E. coli* selected for this study, we observed significant mortality in mutant mice lacking hepatocyte RelA (**Figure 1A**). The surviving hepRelA$^{\Delta/\Delta}$ mice exhibited significant liver injury based on elevated concentrations of serum ALT 24 hours after the pneumonia challenge (**Figure 1A**). In contrast, no evidence of liver injury or mortality was observed in hepSTAT3$^{\Delta/\Delta}$ mice under the same conditions (**Figure 1B**). Although both transcription factors are necessary components to induce the hepatic APR, these results suggest that RelA is primarily responsible for hepatoprotective signals elicited by pneumonia. Interestingly, RelA targeting had no effect on bacteremia **(Figure 2A)**. Also, liver injury was not significantly elevated in hepRelA$^{\Delta/\Delta}$ mice without detectable bacteremia, despite a

noticeable trend (**Figure 2B**). However, the effect of genotype was far more robust in mice with positive blood cultures (**Figure 2B**). These results suggest that pneumonia-induced liver injury following i.t. *E. coli* may be largely attributable to extrapulmonary dissemination.

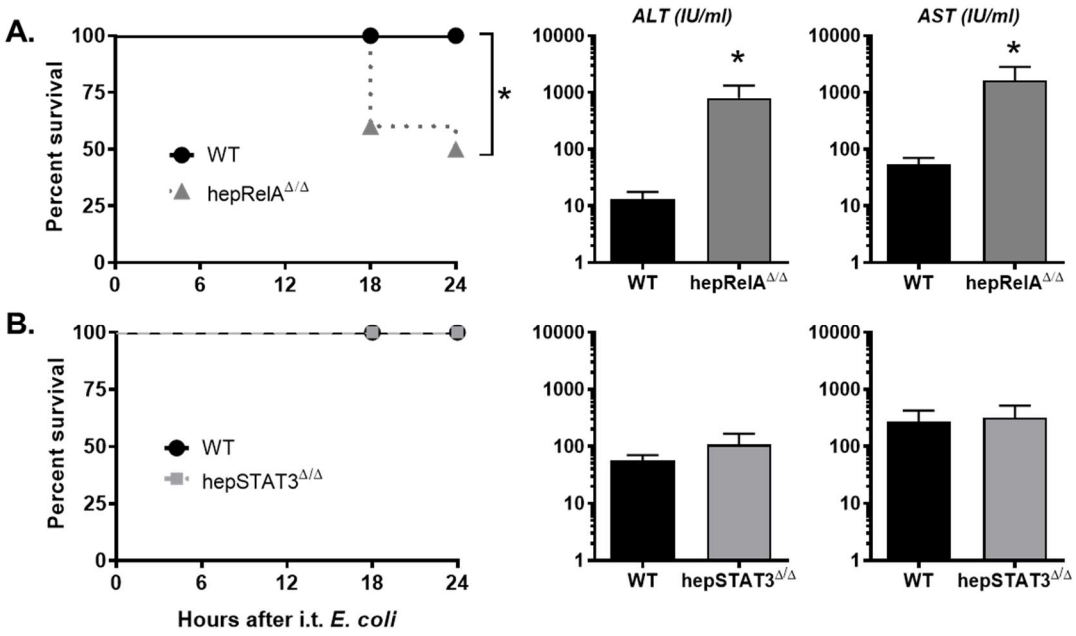

Figure 1. Hepatoprotection during pneumonia is RelA-dependent.

10^6 CFU of *E. coli* was instilled i.t. into 15 week-old mice lacking either **(A)** NF-κB RelA (hepRelA$^{\Delta/\Delta}$) or **(B)** STAT3 (hepSTAT3$^{\Delta/\Delta}$) in hepatocytes, and results were compared to littermate controls (WT). Survival is illustrated over a 24 hour period. Liver injury was assessed by serum ALT and AST concentrations 24 hours after i.t. *E. coli*. * $p < 0.05$ *vs.* WT; $n = 4$ to 6 per group.

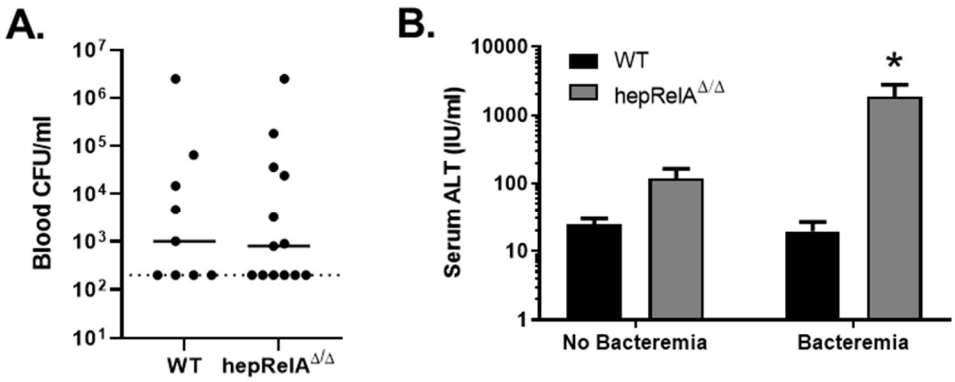

Figure 2. Bacteremia following pneumonia increases liver injury in the absence of RelA.

(A) Blood bacterial burdens were quantified in WT or hepRelA$^{\Delta/\Delta}$ mice 24 hours after i.t. *E. coli*. The dotted line represents the limit of detection. **(B)** Serum ALT levels were compared in WT or hepRelA$^{\Delta/\Delta}$ mice with and without detectable bacteremia 24 hours after i.t. *E. coli*. * $p < 0.05$ *vs.* WT; $n = 3$ to 4 per group.

3.2 RelA-dependent hepatoprotection extends beyond pneumonia

To determine whether the aforementioned effect of hepatocyte RelA targeting was specific to pneumonia induced by *E. coli* infection, we challenged mice intratracheally (i.t.) or intravenously (i.v.) with additional stimuli (**Figure 3**). Intratracheal infections with live *K. pneumoniae* (10^3 CFU) or *S. pneumoniae* (10^6 CFU) were sufficient to elicit severe hepatotoxicity in hepRelA$^{\Delta/\Delta}$ mice but not WT controls (**Figure 3A, 3B**). Similar outcomes were observed in hepRelA$^{\Delta/\Delta}$ mice following i.v. *E. coli* (10^6 CFU) or *S. pneumoniae* (10^6 CFU) (**Figure 3C, 3D**), as well as non-infectious challenges with heat killed *S. pneumoniae* (10^7 CFU i.v.) or lipopolysaccharide (LPS) (0.1 mg/kg i.p.) (**Figure**

3E, 3F). Following the i.v. *E. coli* challenge, liver injury was also confirmed histologically **(Figure 4)**. Together, these results indicate that hepatocyte RelA is indispensable for hepatoprotection in settings of infection or inflammation, and that liver injury is not restricted to that induced by live bacteria.

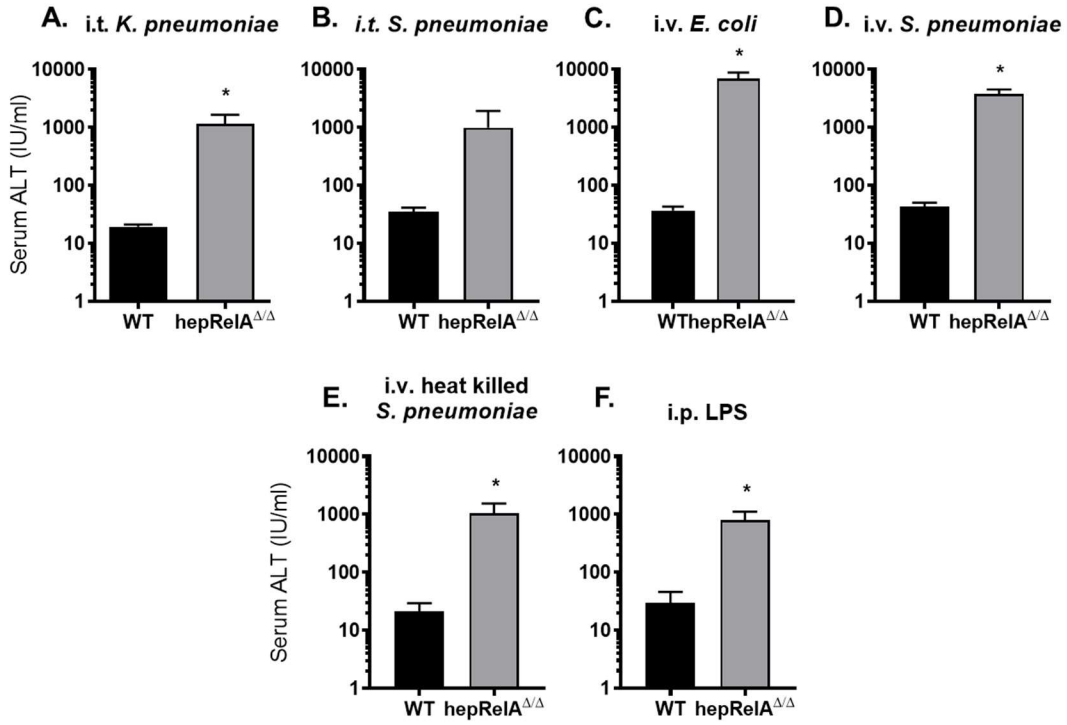

Figure 3. RelA-dependent hepatoprotection extends beyond pneumonia.

Serum ALT concentrations were measured in hepRelA$^{\Delta/\Delta}$ or WT mice challenged with **(A)** i.t. *K. pneumoniae* (10^3 CFU), **(B)** i.t. *S. pneumoniae* (10^6 CFU), **(C)** i.v. *E. coli* (10^6 CFU), **(D)** i.v. *S. pneumoniae* (10^6 CFU), **(E)** i.p. lipopolysaccharide (0.1 mg/kg), or **(F)** i.v. heat killed *S. pneumoniae* (10^7 CFU). * $p < 0.05$ *vs.* WT; $n = 3$ to 12 per group.

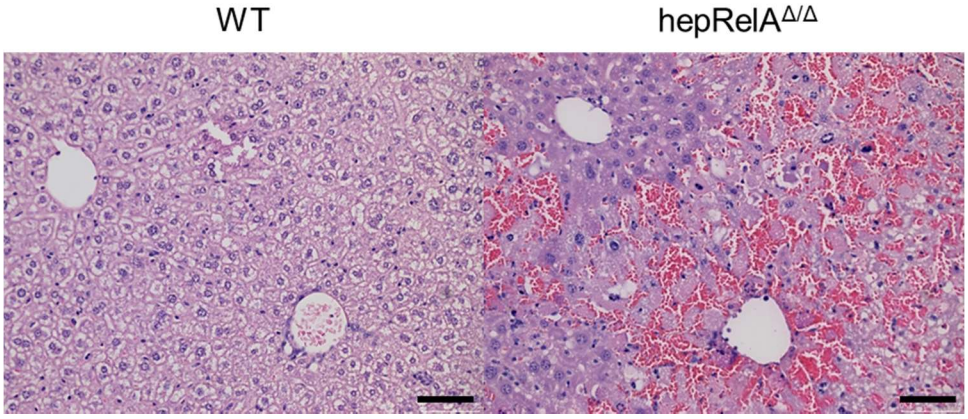

Figure 4. Bacteremia induces liver injury in the absence of RelA.

Livers were collected 24 hours after i.v. *E. coli* and processed for histological analysis by hematoxylin and eosin (H&E) staining. Representative images are shown for each genotype. Scale bar: 75 μm.

3.3 Aged livers are more vulnerable to liver injury

As shown above substantial liver injury occurred in hepRelA$^{\Delta/\Delta}$ mice following i.v. *E. coli*, and curiously, the degree of injury was associated with age. 10-18 week old hepRelA$^{\Delta/\Delta}$ mice exhibited significantly more intense liver injury as measured by serum ALT, and bacteremia (**Figure 5**). Thus, while we do not detect an overall effect of liver genotype on bacteremia when combining mice of all ages used (7-18 weeks), these data support the concept that aging livers have a greater demand for hepatoprotection and systemic defense.

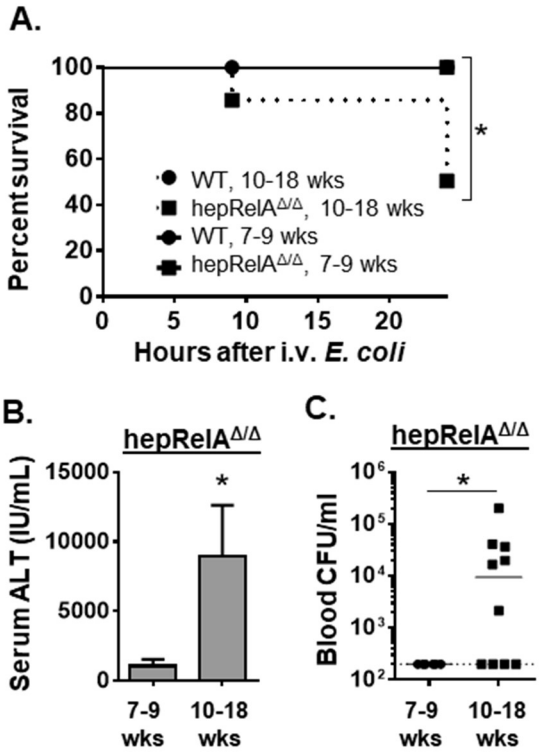

Figure 5. Hepatotoxicity and bacteremia are exacerbated in older mice in the absence of hepatocyte RelA.

WT and hepRelA$^{\Delta/\Delta}$ mice were challenged with i.v. 10^6 CFU of *E. coli* for 24 hours. **(A)** Survival, **(B)** serum ALT, and **(C)** blood bacterial burdens were compared between 7-9 and 10-18 week old mice. The dotted line represents the limit of detection. *$p < 0.05$ compared to 7-9 week old mice; $n = 10$ to 24 per group.

3.4 Liver injury during pneumonia and sepsis is associated with increased apoptosis

Studies using RelA knockout mice demonstrated TNFα-dependent liver failure and death during gestation (Beg, Sha et al. 1995). Furthermore, hepatocyte-specific deletion of RelA can render mice vulnerable to liver injury by administration of TNFα (Geisler, Algul et al. 2007), which can induce extrinsic apoptosis through death receptor

signaling. To determine the relationship between liver injury and apoptosis in hepRelA$^{\Delta/\Delta}$ mice in the setting of live infection, we measured active caspase levels in liver specimens collected during pneumonia and/or bacteremia. We observed substantially increased levels of cleaved caspase-3 (a pan-marker of apoptosis) and cleaved caspase-8 (a marker of extrinsic apoptosis) in the livers of hepRelA$^{\Delta/\Delta}$ mice compared to WT counterparts 16 hours after i.t. *E. coli* (**Figure 6A**). Similar results were observed in livers from hepRelA$^{\Delta/\Delta}$ mice following i.t. *K. pneumoniae* and i.v. *S. pneumoniae* (**Figure 6B, 6C**), implicating dysregulated extrinsic apoptosis as a mechanism promoting hepatotoxicity in the absence of hepRelA.

Our ability to measure high levels of serum aminotransferases in pneumonic and bacteremic hepRelA$^{\Delta/\Delta}$ mice suggests a loss of plasma membrane integrity, which is consistent with necrotic cell death. Additionally, ligands such as TNFα promoting extrinsic apoptosis can also induce programmed necrosis (necroptosis) through the interaction of RIPK1, RIPK3, and MLKL (Eguchi, Wree et al. 2014). To determine whether necroptosis contributes to hepatotoxicity, we treated hepRelA$^{\Delta/\Delta}$ mice with Nec-1s, a selective inhibitor of RIPK1 (Filliol, Piquet-Pellorce et al. 2016), prior to an i.v. *S. pneumoniae* (**Figure 7A**). Nec-1s did not reduce serum ALT concentrations in hepRelA$^{\Delta/\Delta}$ mice.

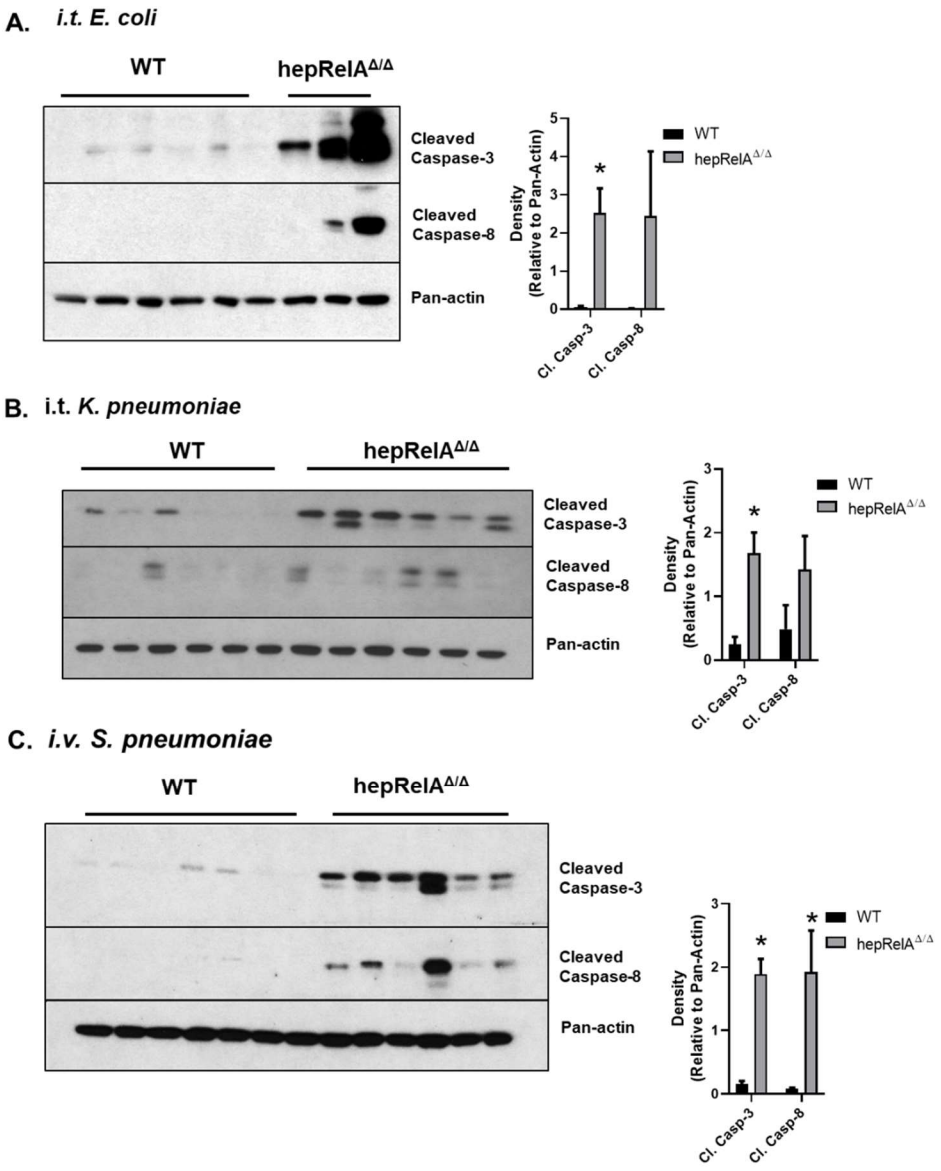

Figure 6. Liver injury in hepRelA$^{\Delta/\Delta}$ mice is associated with apoptosis.

HepRelA$^{\Delta/\Delta}$ or WT mice were challenged with: **(A)** i.t. *E. coli* (10^6 CFU) for 16 hours; **(B)** i.t. *K. pneumoniae* (10^3 CFU) for 48 hours intravenously; or **(C)** i.v. *S. pneumoniae* (10^6 CFU) for 24 hours. Livers were collected and active caspase -3 and -8 were determined in liver tissue homogenates by immunoblot. Densitometric analyses are shown as caspase/pan-actin ratios. * $p < 0.05$ *vs.* WT.

Hypoxia is associated with hepatocyte necrosis during sepsis (Bantel and Schulze-Osthoff 2009), and hepatocytes have been shown to produce increased levels of TNFα following hypoxia in settings such as bacteremia (Loftis, Johanns et al. 2000, Spencer, Zhou et al. 2013). To determine whether livers of hepRelA$^{\Delta/\Delta}$ mice undergo hypoxic stress during infection, we probed for Hypoxyprobe-1 adducts in livers of mice challenged with i.v. *E. coli* (**Figure 7B**). Differences in hypoxia levels were not detected histologically. Together, these data suggest that liver injury is not a result of exaggerated necroptosis or hypoxia.

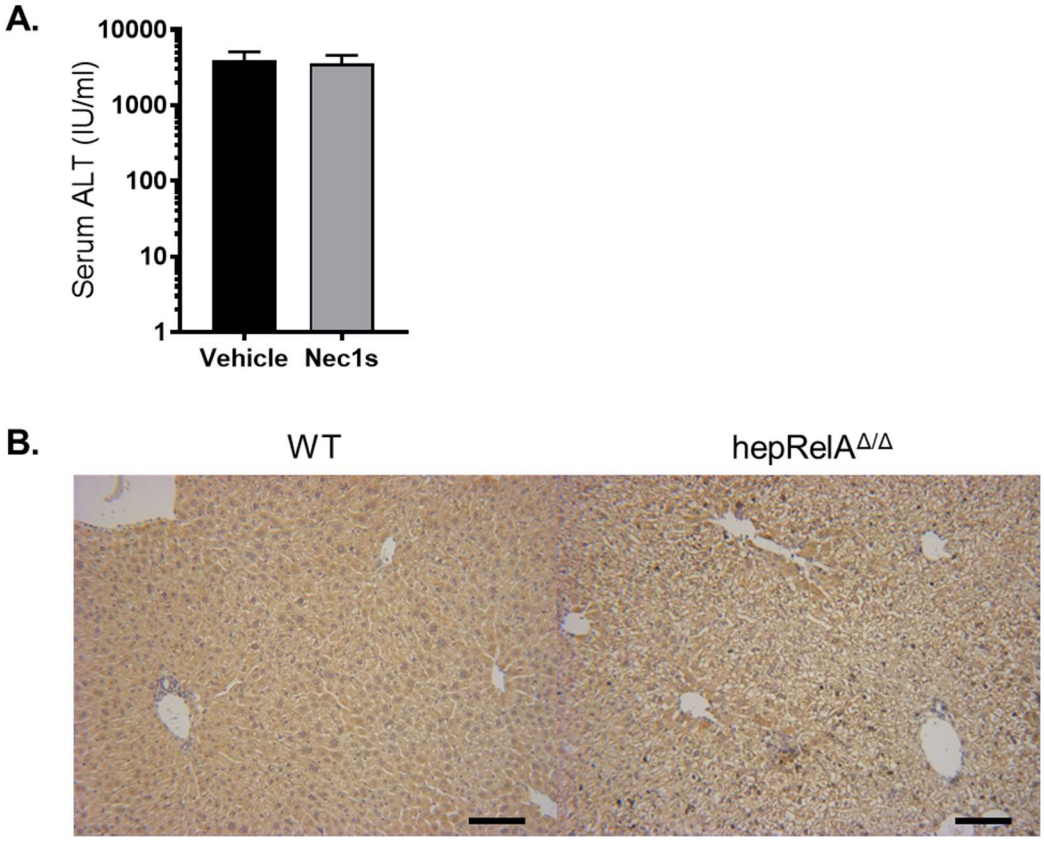

Figure 7. Liver injury in hepRelA$^{\Delta/\Delta}$ mice is not associated with necroptosis or hypoxia.

(A) Serum ALT concentrations were measured in hepRelA$^{\Delta/\Delta}$ mice pre-treated with Nec-1s or vehicle and challenged with i.v. heat killed *S. pneumoniae* for 24 hours (n = 3 per group). **(B)** Livers were collected 24 hours after i.v. *E. coli* and 1 hour after i.p. Hypoxyprobe-1. Livers were processed and probed for signs of hypoxia. Representative images are shown for each genotype. Scale bar: 75 μm.

3.5 Liver apoptosis is the absence of RelA is TNF-dependent

Serum levels of TNFα have been linked to hepatotoxicity (Pryhuber, Huyck et al. 2005, Manco, Marcellini et al. 2007), and this cytokine can be produced by multiple cells

in the liver, including NKT cells, Kupffer cells, and monocytes (Liaskou, Wilson et al. 2012). Our observation of enhanced liver apoptosis in our current study combined with previous studies in RelA-deficient mouse models (Beg, Sha et al. 1995, Geisler, Algul et al. 2007) supports a pathological role for TNFα and/or related pro-apoptotic ligands during pneumonia and sepsis that is countered by RelA-dependent gene programs in the liver. To determine the direct influence of TNFα on liver injury during infection, hepRelA$^{\Delta/\Delta}$ mice and WT controls were challenged i.v. with *S. pneumoniae* in the presence of a neutralizing TNFα antibody or control IgG (**Figure 8A**). As expected, *S. pneumoniae* caused severe liver injury in hepRelA$^{\Delta/\Delta}$ mice treated with isotype control antibody. However, hepatotoxicity was eliminated in hepRelA$^{\Delta/\Delta}$ mice treated with TNFα-neutralizing antibody (**Figure 8A**). Additionally, following the *S. pneumoniae* challenge, cleaved caspase-3 and -8 were concomitantly decreased in livers from hepRelA$^{\Delta/\Delta}$ mice treated with TNFα-neutralizing antibody (**Figure 8B**). Together, these findings suggest that TNFα is a critical upstream driver of hepatotoxicity in the absence of RelA.

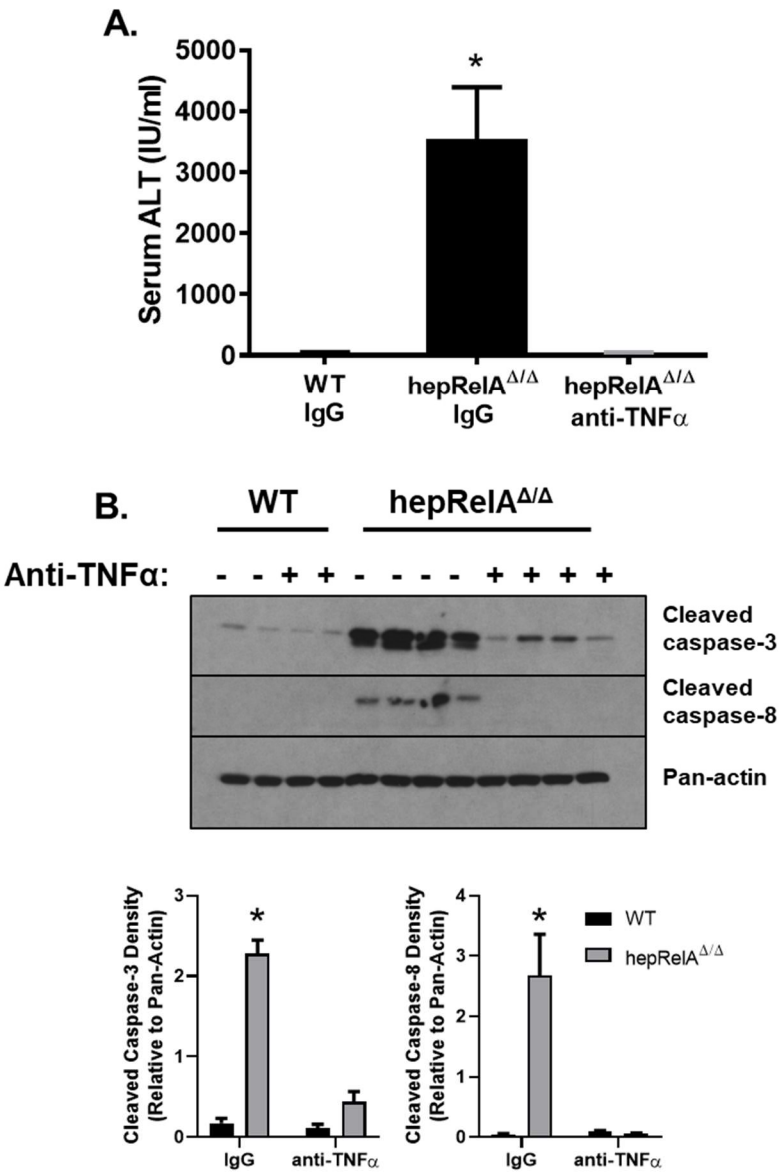

Figure 8. Liver injury TNFα-dependent in the absence of RelA.

(A) Serum ALT was measured in hepRelA$^{\Delta/\Delta}$ or WT mice challenged i.v. for 24 hours with 10^6 CFU of *S. pneumoniae* in the presence of an isotype antibody or TNF-α neutralizing antibody. n = 3 to 4 **(B)** Livers were collected 24 hours after i.v. *S. pneumoniae*, and the presence of activated caspase -3/-8 in liver homogenates was measured by immunoblot. Densitometric analyses are shown as caspase/pan-actin ratios. * $p < 0.05$ *vs.* WT.

3.6 Liver TNF is increased after infection in the absence of hepatocyte RelA

In order to investigate the liver as a potential source of TNFα, we measured TNFα mRNA in WT and hepRelA$^{\Delta/\Delta}$ mice 0, 6, and 24 hours after i.v. *S. pneumoniae*. Liver TNFα mRNA induction was significantly elevated following i.v. *S. pneumoniae*, and this response was further exaggerated in livers of hepRelA$^{\Delta/\Delta}$ mice compared to WT 24 hours after *S. pneumoniae* challenge (**Figure 9A**). In order to identify potential hepatic cellular sources of TNFα, we challenged WT mice with i.v. *S. pneumoniae* or saline for 24 hours, isolated mononuclear cells from whole liver tissue enzymatic digests, and sorted several candidate cell populations by flow cytometry for mRNA analysis: NKT cells (CD45^{+}/CD3^{+}/NK1.1^{+}), NK cells (CD45^{+}/CD3^{-}/NK1.1^{+}), Kupffer cells (CD45^{+}/F4/80^{Hi}/CD11b^{+}), and monocytes (CD45^{+}/F4/80^{Lo}/CD11b^{Hi}/Ly6C^{Hi}). However, TNFα was unaffected by bacteremia in our selected panel of leukocytes in WT mice at this time point (**Figure 9B**), suggesting alternative cell types as the primary source(s) of liver TNFα.

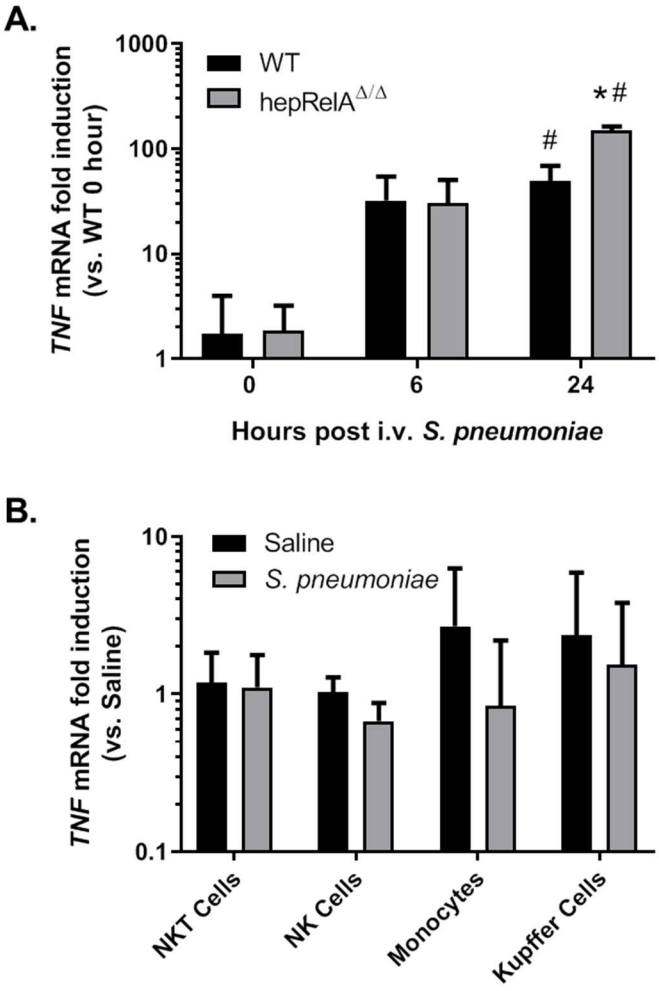

Figure 9. *TNF* transcripts are increased in livers after infection.

TNFα mRNA induction was measured by qRT-PCR in **(A)** whole liver tissues of hepRelA$^{\Delta/\Delta}$ or WT mice 0, 6, and 24 hours after i.v. *S. pneumoniae* or **(B)** NKT cells (CD45^{+} CD3^{+} NK1.1^{+}), NK cells (CD45^{+}, CD3^{-}, NK1.1^{+}), Kupffer cells (CD45^{+} F4/80^{Hi} CD11b^{+}), and monocytes (CD45^{+} F4/80 ^{Lo} CD11b^{Hi}, Ly6C^{Hi}) sorted from liver tissues of WT mice 24 hours after i.v. *S. pneumoniae*. * $p < 0.05$ *vs*. WT, $^{\#}$ $p < 0.05$ *vs.* 0 hour, $n = 3$ to 5 per group.

3.7 NKT cell activity is sufficient for hepatotoxicity

As previous studies have identified NKT cells as a potential source of liver injury during sepsis (Hu, Venet et al. 2009), we next determined the influence of this cell type on liver injury in our vulnerable hepRelA$^{\Delta/\Delta}$ mice. I.v. *E. coli* was sufficient to significantly induce activation of liver NKT cells (CD45^{+}/CD3^{+}/αGalCer-loaded CD1d tetramer^{+}) in WT mice based on CD69 surface expression (**Figure 10A**).

To determine whether NKT cell activity is sufficient to induce liver injury in the absence of RelA, WT and hepRelA$^{\Delta/\Delta}$ mice were challenged with αGalCer, which is a well-established specific activator of NKT cells. As with our other experimental systems **(Figure 3)**, NKT cell stimulation caused substantial liver injury in hepRelA$^{\Delta/\Delta}$ mice **(Figure 10B)**. Moreover, this effect was completely abrogated by TNFα neutralization **(Figure 10C)**, consistent with our observations following i.v. *S. pneumoniae* **(Figure 8).**

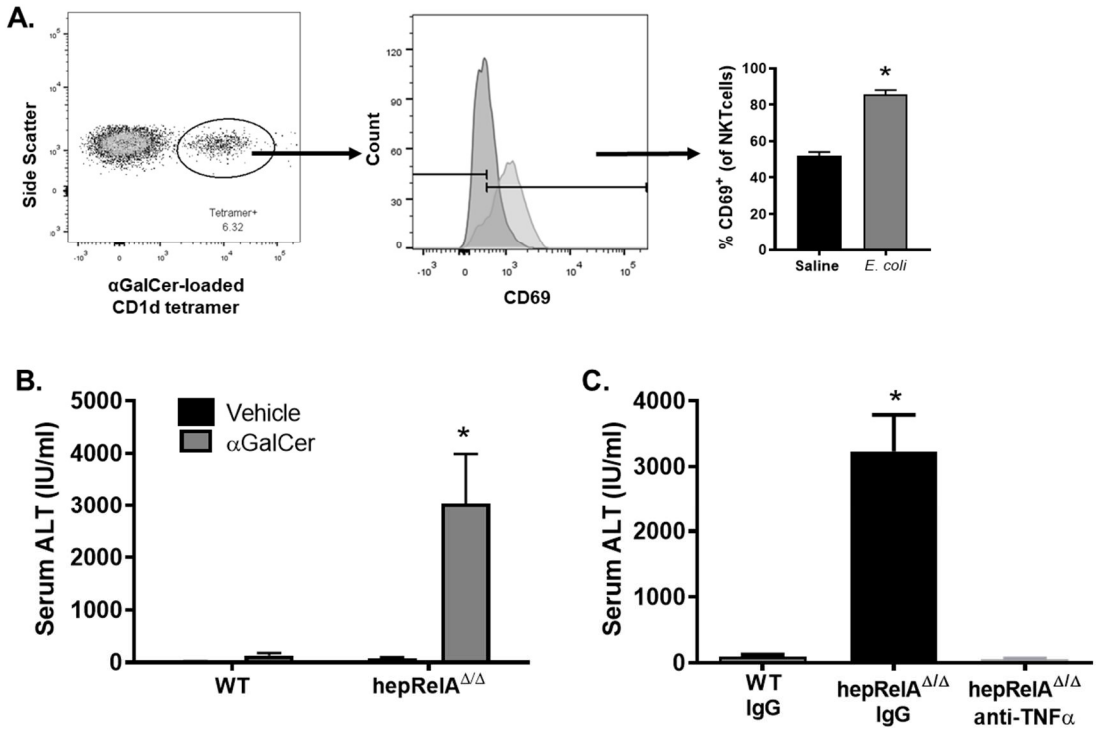

Figure 10. NKT cell activity is sufficient to cause hepatotoxicity in hepRelA$^{\Delta/\Delta}$ mice.

(A) Mice were challenged i.v. with 10^6 CFU of *E. coli* for 24 hours. Single cell suspensions of mononuclear cells were generated from whole liver tissues. NKT cells ($CD45^+/CD3^+$/ α-galactoceramide (αGalCer)-loaded CD1d $tetramer^+$) were identified by flow cytometry and CD69+ surface expression was measured as an activation marker. * $p < 0.05$ *vs*. saline, $n = 2$ to 3 per group. Serum ALT was measured **(B)** in hepRelA$^{\Delta/\Delta}$ or WT mice challenged i.v. for 24 hours with 2 μg α-GalCer or vehicle (*$p < 0.05$ *vs.* vehicle hepRelA$^{\Delta/\Delta}$, $n = 3$ to 6 per group), and **(C)** in αGalCer-treated mice co-instilled with control IgG or anti-TNFα neutralizing antibody (* $p < 0.05$ *vs*. anti-TNFα hepRelA$^{\Delta/\Delta}$, $n = 3$ to 6 per group).

3.8 NKT cell activity is not necessary for hepatoprotection

To investigate contributions of NKT cell activity to immunopathology during infection, anti-CD1d, a well-established means to pharmacologically block NKT cell

activity *in vivo* (Rhee, Carlton et al. 2003), was co-instilled i.v. with 10^6 CFU of *E. coli* in WT and hepRelA$^{\Delta/\Delta}$ mice. However, blocking NKT cell activation with anti-CD1d during bacteremia significantly compromised anti-bacterial defense, as evidenced by greater bacterial burdens in the blood of anti-CD1d-treated mice compared to isotype-treated controls (**Figure 11A**). While NKT cells have been identified as an important mediator of defense in other settings (Liaskou, Wilson et al. 2012), this is the first evidence to our knowledge that this cell type is required for systemic defense against *E. coli*. In order to circumvent the confounding effects of differential bacterial loads after NKT cell blockade, we repeated this experiment utilizing non-infectious heat killed *S. pneumoniae*, which is equally effective at causing liver injury in hepRelA$^{\Delta/\Delta}$ mice **(Figure 11B)**. Under these conditions, NKT cell blockade had no protective effect on liver injury in hepRelA$^{\Delta/\Delta}$ mice (**Figure 11B**). Instead, anti-CD1d increased serum ALT levels, strongly arguing against NKT cell activity as the sole source of injury under these conditions. These data suggest that NKT cell activity is sufficient but not necessary for liver injury in the absence of RelA-dependent hepatoprotection.

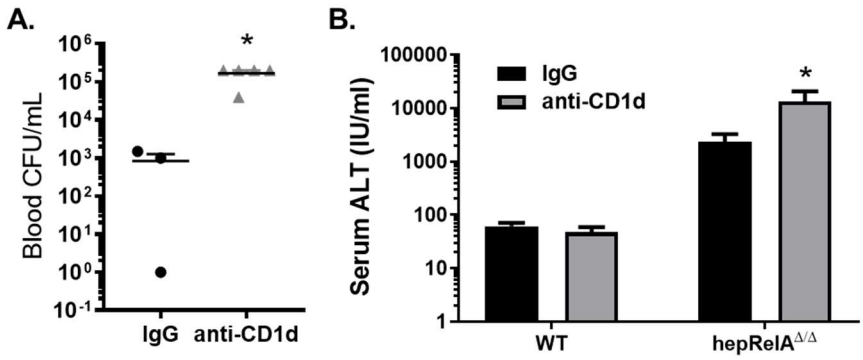

Figure 11. NKT cell activity is not necessary for hepatotoxicity in hepRelA$^{\Delta/\Delta}$ mice.

(A) Blood bacterial burdens were measured 7 hours after i.v. 10^6 CFU of *E. coli* in wild type mice treated with control IgG or an anti-CD1d antibody blocking NKT cell activity. * $p < 0.05$ *vs*. IgG. **(B)** Serum ALT was measured in hepRelA$^{\Delta/\Delta}$ or WT mice challenged with i.v. 10^7 CFU of heat killed *S. pneumoniae* for 24h hours. * $p < 0.05$ *vs*. WT, $n = 3$ to 6 per group.

3.9 Kupffer cells and recruited monocytes are not required for liver injury in hepRelA$^{\Delta/\Delta}$ mice

To determine other immunological effects of liver RelA deficiency during bacteremia, we measured serum cytokine and chemokine concentrations utilizing a multiplex bead array for 17 inflammation-relevant cytokines: CCL2, CCL3, CCL4, CCL11, CXCL1 CXCL2, CXCL5, CXCL10, IL-1α, IL-1β, IL-6, IL-10, IL-17a, IL-23p19, G-CSF, IFNγ, and TNFα. CCL2 and CXCL10 were the only two significantly elevated in mutant mice as compared to their WT counterparts 24 hours after i.v. *S. pneumoniae* (10^6 CFU) (**Figure 12A**). Concomitantly, both chemokines were also increased at the mRNA level in liver tissues collected from mice (**Figure 12B, 12C**).

Both CCL2 and CXCL10 contribute to the recruitment of monocytes to sites of infection (Deshmane, Kremlev et al. 2009, Petrovic-Djergovic, Popovic et al. 2015), prompting us to consider the roles of both resident (Kupffer cells) and recruited macrophages and monocytes in the context of hepatic RelA deficiency.

To determine the impact of monocyte recruitment on hepatotoxicity, WT and hepRelA$^{\Delta/\Delta}$ mice were administered neutralizing CCL2 antibody and heat killed *S. pneumoniae* for 6 hours. CCL2 neutralization, which is sufficient to block liver monocyte recruitment (Teng, Han et al. 2017), had no significant effect on liver injury in hepRelA$^{\Delta/\Delta}$ mice (**Figure 12D**). Meanwhile, we also tested the effect of i.v. clodronate-encapsulated liposomes, a well-established approach for depleting Kupffer cells (Van Rooijen and Sanders 1996). Similar to the results observed following anti-CCL2, clodronate administration had no effect on *S. pneumoniae*-induced bacteremia (**Figure 12E**). Together, these results suggest that neither Kupffer cells nor recruited monocytes are required for hepatotoxicity in bacteremic hepRelA$^{\Delta/\Delta}$ mice.

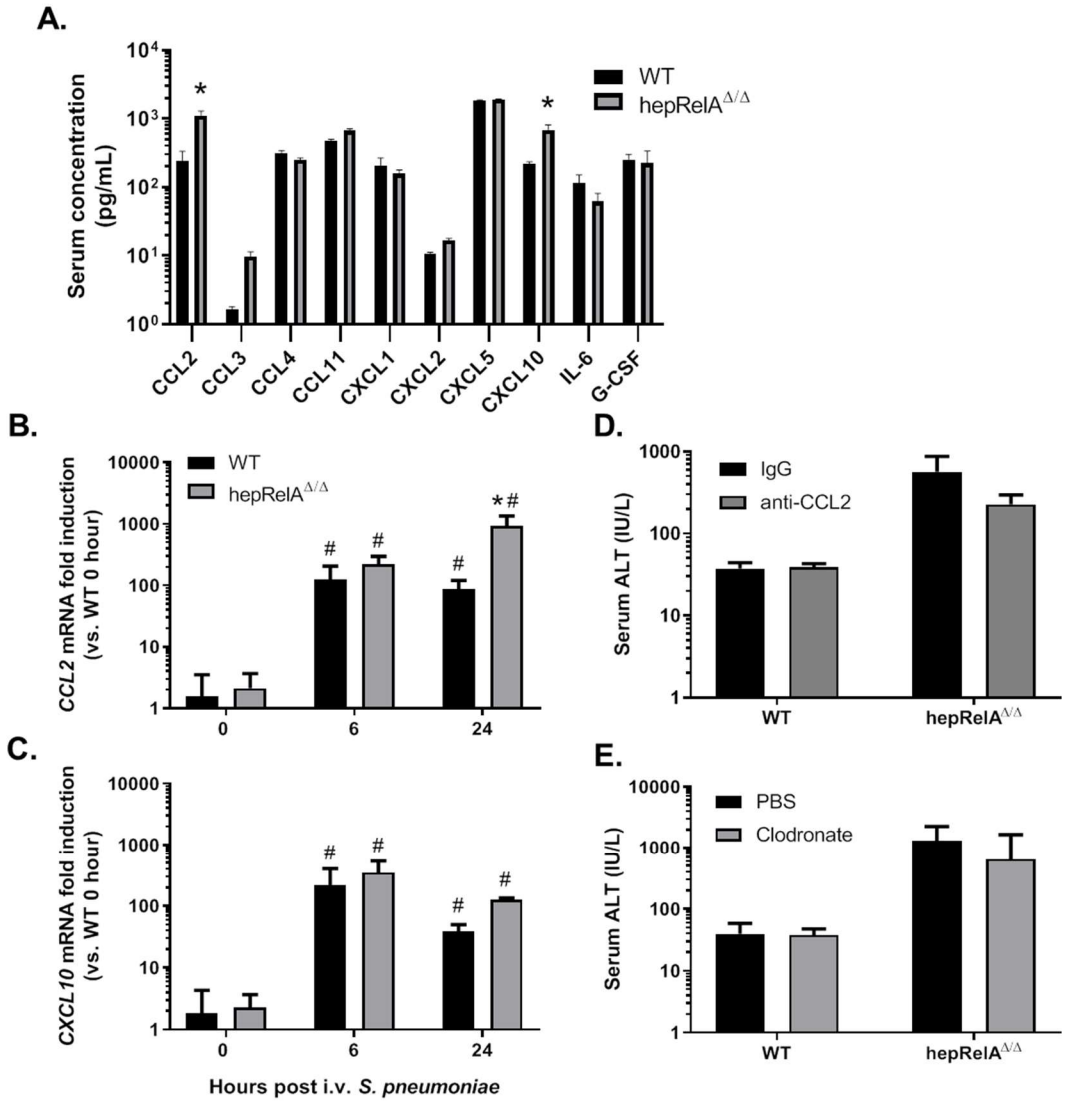

Figure 12. Kupffer cells and recruited monocytes are dispensable for hepatotoxicity in hepRelA$^{\Delta/\Delta}$ mice.

(A) Serum concentrations of the indicated cytokines and chemokines were measured in serum collected from hepRelA$^{\Delta/\Delta}$ or WT mice 24 hours after i.v. *S. pneumoniae* (10^6 CFU). (n = 10 per group). mRNA levels of **(B)** *CCL2* or **(C)** *CXCL10* were measured by qRT-PCR in whole liver tissues of hepRelA$^{\Delta/\Delta}$ or WT mice 0, 6, and 24 hours after i.v. *S. pneumoniae*. (n = 3 to 5 per group) Serum ALT was measured in hepRelA$^{\Delta/\Delta}$ or WT mice **(D)** co-instilled i.v. with IgG control or anti-CCL2 neutralizing antibody (n = 3 to

5 per group) or **(E)** pre-treated i.v. with clodronate- or PBS-liposomes 24 hours before i.v. heat killed *S. pneumoniae* (10^7 CFU) for 6 hours (n = 6 to 8 per group). * $p < 0.05$ *vs*. WT, $^{\#}$ $p < 0.05$ *vs*. 0 hour.

3.10 Transcriptional remodeling coincides with liver injury in hepRelA$^{\Delta/\Delta}$ mice

To comprehensively evaluate RelA-dependent gene networks associated with liver injury during infection, we performed genome-wide expression analyses on liver RNA collected from WT and hepRelA$^{\Delta/\Delta}$ mice 0, 6, and 24 hours after i.v. *S. pneumoniae*. Bacteremia had a substantial effect on the liver transcriptome, and this response was remarkably exaggerated in RelA-deficient livers (**Figure 13A-B**). Indeed, by 24 hours, thousands of bacteremia-induced gene changes (FDR $q < 0.05$) were restricted to hepRelA$^{\Delta/\Delta}$ mice. Principal component analysis illustrated marked divergence in overall gene expression patterns amongst the experimental groups due to infection and/or genotype (**Figure 13C**). Most notably, the 24 hour WT samples closely resemble samples from uninfected mice, whereas the 24 hour hepRelA$^{\Delta/\Delta}$ samples comprise a highly distinct cluster. This analysis suggests that during the course of infection, the transcriptional landscape of WT livers returns to homeostasis, whereas that of hepRelA$^{\Delta/\Delta}$ livers has a divergent fate.

In order to begin dissecting gene programs associated with hepatotoxicity in hepRelA$^{\Delta/\Delta}$ mice, we used Ingenuity Pathway Analysis to align our data with curated data sets, including toxicological functions, potential upstream regulators, and biological functions **(Table 7)**. Based on data acquired from differentially expressed genes (WT *vs.* hepRelA$^{\Delta/\Delta}$; FDR $q < 0.05$; changes ≥ 2-fold), we unsurprisingly found that the top 5

toxicological functions all involved liver injury, whereas biological functions included several events both up- and downstream of immunotoxicity, such as immune cell trafficking and cell death. Top upstream regulators are led by TNFα, which is highly consistent with our neutralization studies **(Figures 7-8, 10-11)**; further, additional candidates were revealed which may also have important roles in liver homeostasis. Lastly, the most up- and downregulated genes include transcripts related to cell death/inflammatory injury (*e.g. Cidec* and *Mmp12*) (Cursio, Mari et al. 2002, Xu, Cai et al. 2015) and typical liver functions such as metabolism/acute phase proteins (*e.g. Cyp8b1* and *Saa3*) (Zhang and Chiang 2001, Gruys, Toussaint et al. 2005) (**Table 8**). Together, these data reveal the scope of biological changes resulting from RelA deficiency, reinforce TNFα as a source of hepatotoxicity, and highlight additional factors that are likely involved with liver failure during infection.

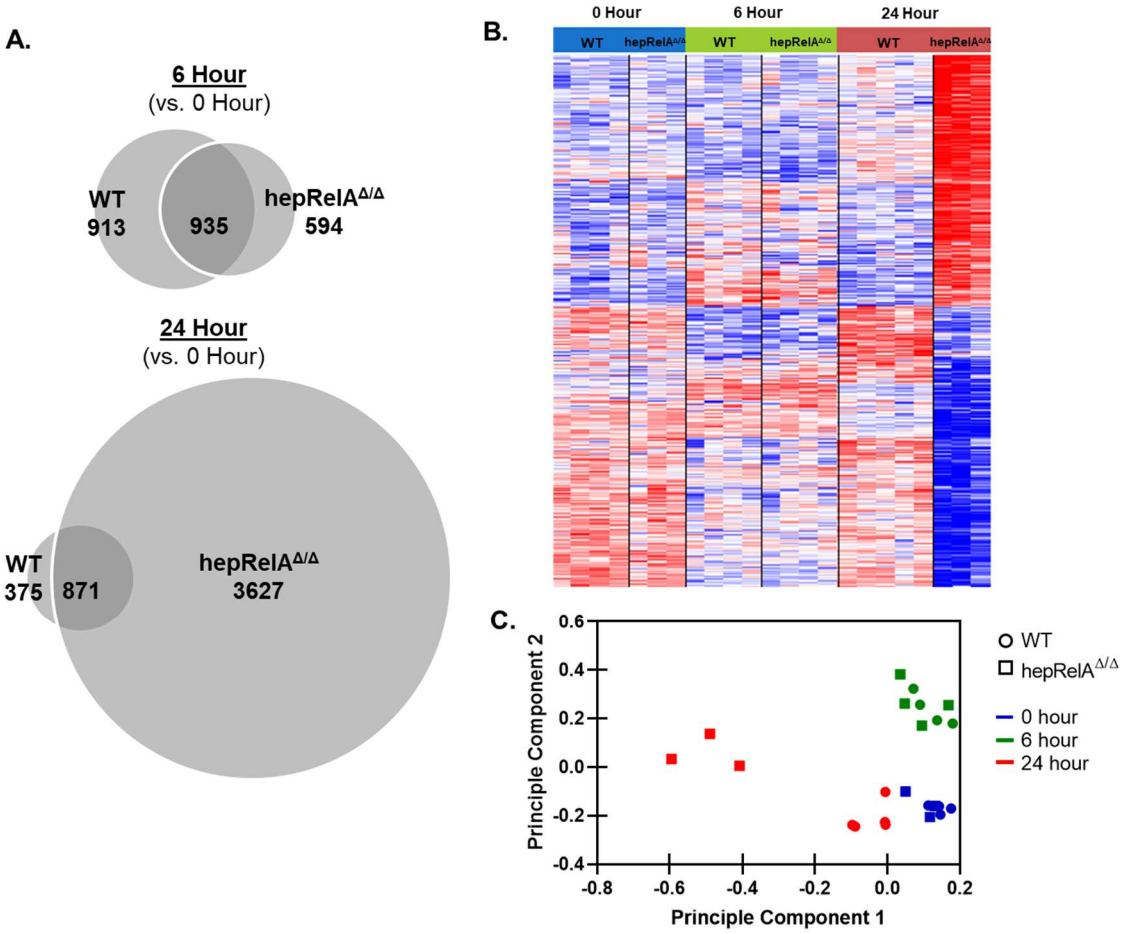

Figure 13. The transcriptional response to bacteremia is remodeled in RelA-deficient livers.

WT and hepRelA$^{\Delta/\Delta}$ mice were challenged with i.v. *S. pneumoniae* (10^6) and microarray analysis was performed on liver RNA. **(A)** Venn diagrams illustrate the number of significantly altered genes due to bacteremia. False discovery rate (FDR) $q < 0.05$ after expression filtering. **(B)** Heatmap of genes from a two-way ANOVA (time point and genotype interaction, FDR $q < 0.05$), arbitrarily broken up into clusters of similar patterns of differential expression. Columns correspond to samples and rows correspond to a single gene. Red, white, and blue assigned genes indicate z-score-normalized expression $z \leq -2$, 0, and ≥ 2, respectively. **(C)** Principal Component Analysis was performed to compare the overall variance amongst

experimental groups (WT or hepRelA$^{\Delta/\Delta}$ at 0, 6, or 24 hours post *i.v. S. pneumoniae*) across all detected transcripts.

Top Predicted Upstream Regulators	
Regulator	***p*-Value**
TNF	8.92E-50
IL1B	5.40E-37
IFNG	5.77E-34
ACOX1	1.35E-30
TGFB1	2.17E-29

Top Toxicology Functions		
Category	***p*-Value**	**Gene No.**
Liver Steatosis	1.93E-16 - 2.53E-01	53
Liver Inflammation / Hepatitis	2.71E-16 - 3.78E-01	56
Liver Damage	5.94E-15 - 3.78E-01	51
Liver Necrosis / Cell Death	3.28E-13 - 7.04E-02	41
Liver Proliferation	1.84E-12 - 1.04E-01	35

Top Disease & Biological Functions		
Category	***p*-Value**	**Gene No.**
Cellular Movement	9.27E-35 - 1.06E-07	269
Immune Cell Trafficking	3.01E-34 - 1.06E-07	177
Hematological System Development and Function	7.11E-29 - 1.06E-07	270
Organismal Development	1.95E-28 - 7.35E-08	298
Cell Death and Survival	5.52E-27 - 1.04E-07	331

Table 7. Top predicted upstream regulators and functions of hepRelA$^{\Delta/\Delta}$-specific gene changes.

Candidate upstream regulators, toxicology, and biological functions identified by Ingenuity Pathway Analysis of genes significantly (FDR $q < 0.05$, Fold change ≥ 2) changed in hepRelA$^{\Delta/\Delta}$ 24 hours post i.v. *S. pneumoniae* as compared hepRelA$^{\Delta/\Delta}$ at 0 hours.

Top Upregulated Genes			
Symbol	**Name**	**FDR *q***	**Fold Change**
Ly6d	Lymphocyte antigen 6 complex, locus D	1.20E-05	↑ 37.009
Cidec	Cell death-inducing DFFA-like effector c	4.11E-05	↑ 30.656
Mmp12	Matrix metallopeptidase 12	2.36E-06	↑ 30.397
A130040M12Rik	RIKEN cDNA A130040M12 gene	4.64E-06	↑ 19.591
Tnfrsf12a	Tumor necrosis factor receptor superfamily, member 12a	3.87E-06	↑ 16.412
Fam83a	Family with sequence similarity 83, member A	1.21E-06	↑ 13.131
Slc25a30	Solute carrier family 25, member 30	7.33E-05	↑ 12.393
Nrg1	Neuregulin 1	1.76E-05	↑ 11.336
Il1rn	Interleukin 1 receptor antagonist	8.71E-05	↑ 10.632
Egr1	Early growth response 1	7.18E-05	↑ 10.334
Top Downregulated Genes			
Symbol	**Name**	**FDR *q***	**Fold Change**
Hsd3b5	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 5	4.01E-02	↓ -27.694
Upp2	Uridine phosphorylase 2	1.12E-04	↓ -19.709
Keg1	Kidney expressed gene 1	4.92E-04	↓ -11.621
Saa3	Serum amyloid A 3	3.47E-04	↓ -11.559
Cyp8b1	Cytochrome P450, family 8, subfamily b, polypeptide 1	2.04E-04	↓ -9.852
Hamp2	Hepcidin antimicrobial peptide 2	1.44E-02	↓ -9.681
Abca8a	ATP-binding cassette, sub-family A (ABC1), member 8a	2.04E-04	↓ -8.420
Cyp3a25	Cytochrome P450, family 3, subfamily a, polypeptide 25	7.99E-05	↓ -8.152
C730036E19Rik	RIKEN cDNA C730036E19 gene	5.07E-05	↓ -8.041
Ppm1k	Protein phosphatase 1K (PP2C domain containing)	2.16E-05	↓ -7.989

Table 8. Top 10 induced and reduced genes between WT and hepRelA$^{\Delta/\Delta}$ livers during bacteremia.

Transcriptional profiling was performed on whole liver RNA 24 hours post i.v. *S. pneumoniae*. Fold change of differentially expressed genes (WT *vs.* hepRelA$^{\Delta/\Delta}$, FDR $q < 0.05$) were ranked. The top 10 most upregulated and downregulated genes are shown.

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CHAPTER FOUR: HEPATIC STAT3 ACTIVATION MODULATES THE PULMONARY MICROENVIRONMENT DURING ENDOTOXEMIA

Rationale

Sepsis is a syndrome defined by life-threatening organ dysfunction caused by a dysregulated host response to infection, and is a leading cause of mortality amongst critically ill patients (Walkey, Lagu et al. 2015, Singer 2016). Sepsis commonly arises from pneumonia (Mayr, Yende et al. 2014); however, non-pulmonary sepsis increases the susceptibility of patients to subsequent bacterial pneumonia (Alberti, Brun-Buisson et al. 2002). A significant proportion of septic patients exhibit sepsis-induced immunosuppression or immune dysfunction, leading to complications of secondary infections and delayed recovery (Hotchkiss, Monneret et al. 2013). However, it is unclear how a pre-existing immune response to sepsis modulates responses to lung infections.

The hepatic acute phase response (APR) to infection and injury robustly modifies the liver transcriptome, altering gene programs including but not limited to those encoding acute phase proteins (APPs) (Gabay and Kushner 1999, Quinton, Blahna et al. 2012). While APPs have long been appreciated as biomarkers for disease severity, their regulation and physiological significance *in vivo* are poorly understood (Gabay and Kushner 1999). It has been previously shown that transcription factors STAT3 and NF-κB RelA (p65) are together required for activation of hepatic APR during pneumonia (Quinton, Jones et al. 2009, Quinton, Blahna et al. 2012, Hilliard, Allen et al. 2015). Hepatic STAT3 activation, in particular, has been shown to be important for limiting pneumonia susceptibility during endotoxemia (Hilliard, Allen et al. 2015).

Nutritional immunity is a process by which a host sequesters minerals during infections (Hood and Skaar 2012). Bacteria require iron among other transition metals for survival and modulation of iron availability is essential in hosts to prevent bacterial outgrowth and pathogenesis (Ratledge and Dover 2000). Defects in iron-regulating proteins in mouse models have been shown to worsen pneumonia and sepsis outcomes (Bachman, Lenio et al. 2012, Zeng, Chen et al. 2015), and patients with hemochromatosis are highly susceptible to infections (Khan, Fisher et al. 2007). While iron-regulating proteins can be produced in various tissues, the hepatic APR includes gene products, such as hepcidin, that are known to modulate iron availability (Johnson and Wessling-Resnick 2012). However, neither the regulation nor the importance of liver-derived iron handling is understood in the context of pneumonia susceptibility. Therefore, we hypothesize that hepatic STAT3 activation is required for the amplification of iron-regulating factors during sepsis, and that this response is linked to reduced pneumonia susceptibility and mortality.

Results

4.1 Iron status is linked to liver activation during endotoxemia and sepsis

Prior studies from our laboratory in mice lacking STAT3 in hepatocytes demonstrated that STAT3-dependent gene expression during an endotoxemia is essential for limiting severity of subsequent pneumonia challenges (Hilliard, Allen et al. 2015). In these studies, hepSTAT3$^{\Delta/\Delta}$ mice exhibited increased mortality, and higher bacterial loads in the blood and lungs during pneumonia (Hilliard, Allen et al. 2015). However,

differences in inflammatory cytokines, pulmonary edema, and pulmonary cellularity were not observed between WT and hepSTAT3$^{\Delta/\Delta}$ mice (Hilliard, Allen et al. 2015), suggesting impairment of other mechanisms distinct from liver-dependent acute inflammation in mutant mice. Interestingly, cell- and bacteria-free BALF from hepSTAT3$^{\Delta/\Delta}$ mice was supportive of bacterial growth *ex vivo* (Hilliard, Allen et al. 2015), suggesting the presence of humoral components derived from liver STAT3-dependent changes confers pulmonary protection.

Because iron metabolism is an essential feature of bacterial growth and host defense during infections (Hood and Skaar 2012), we performed a study to examine the influence of STAT3 on liver mRNA induction of several iron-regulating genes during endotoxemia and subsequent *E. coli* pneumonia challenge. We focused on several representative iron regulating genes (hepcidin, hemopexin, haptoglobin, lipocalin, and transferrin receptor 2) which alter free iron availability by modulating absorption, release and transport of iron (Hood and Skaar 2012). In WT mice, endotoxemia induced transcription of all five genes, which were sustained 6 and 24 hours following the pneumonia challenge (**Figure 14**). In comparison, induction of these transcripts in hepSTAT3$^{\Delta/\Delta}$ mice remained at baseline (**Figure 14**). These findings were consistent in mice challenged in a cecal ligation and puncture (CLP) model, in which hepSTAT3$^{\Delta/\Delta}$ mice had significantly reduced induction of the select iron-regulating genes as compared to WT mice (**Figure 15**).

However, to further understand whether iron status in the lungs is dependent on liver STAT3 activation, we collected BALF from mice treated with LPS and measured

concentrations of iron by mass spectrometry. While the sample size is limited, the results trended towards increased iron in BALF of hepSTAT3$^{\Delta/\Delta}$ mice (**Figure 16**). Together, these results suggest iron-regulating proteins as an important intermediate between liver STAT3 activation and pulmonary defense during sepsis.

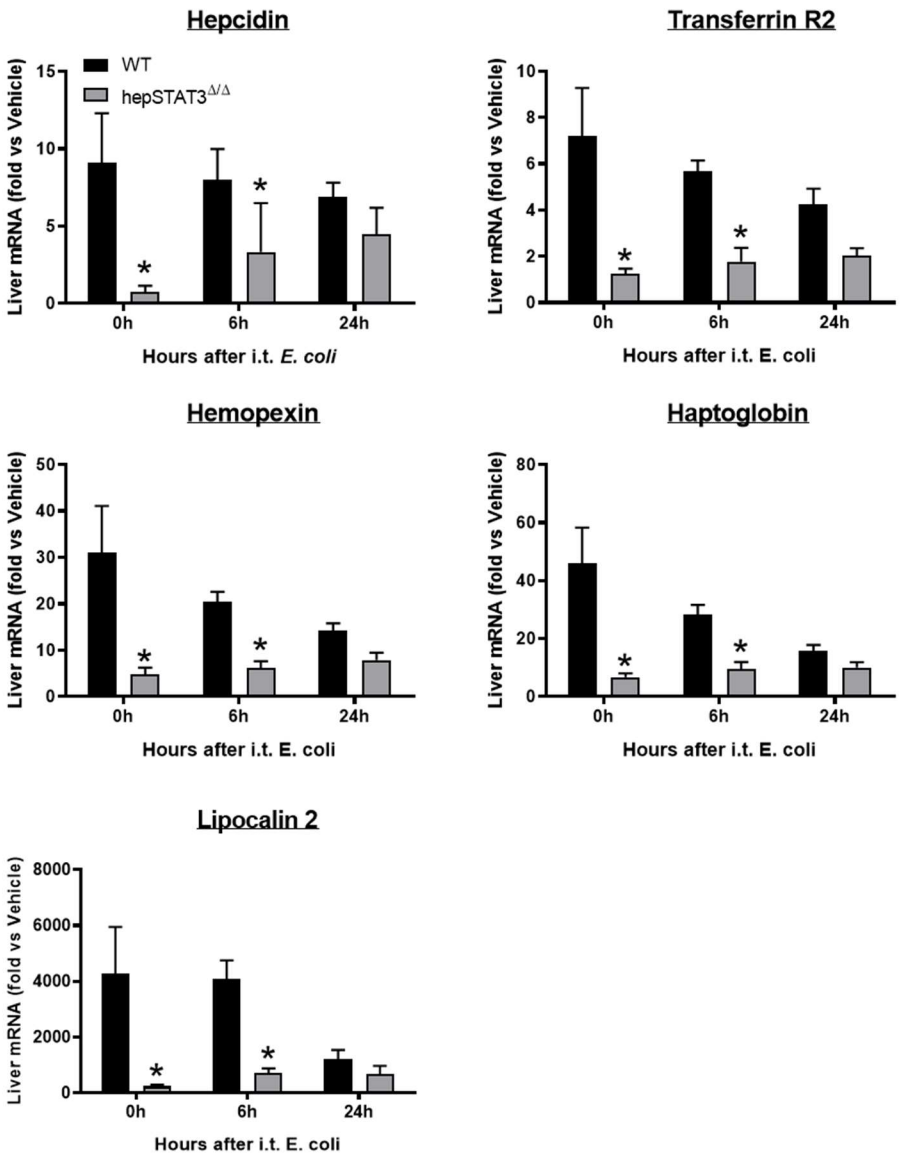


Figure 14. STAT3 is required for the expression of iron-handling factors during endotoxemia followed by pneumonia.

WT and hepSTAT3$^{\Delta/\Delta}$ mice were challenged with i.p. LPS (5 mg/kg) or vehicle (saline) for 18 hours followed by i.t. of 10^6 CFU *E. coli*. Liver tissue was harvested at the indicated times after i.t. *E. coli*, and mRNA induction of representative iron-regulating factors was quantified by qRT-PCR. * $p < 0.05$ vs. WT; $n = 3$ to 6 per group.

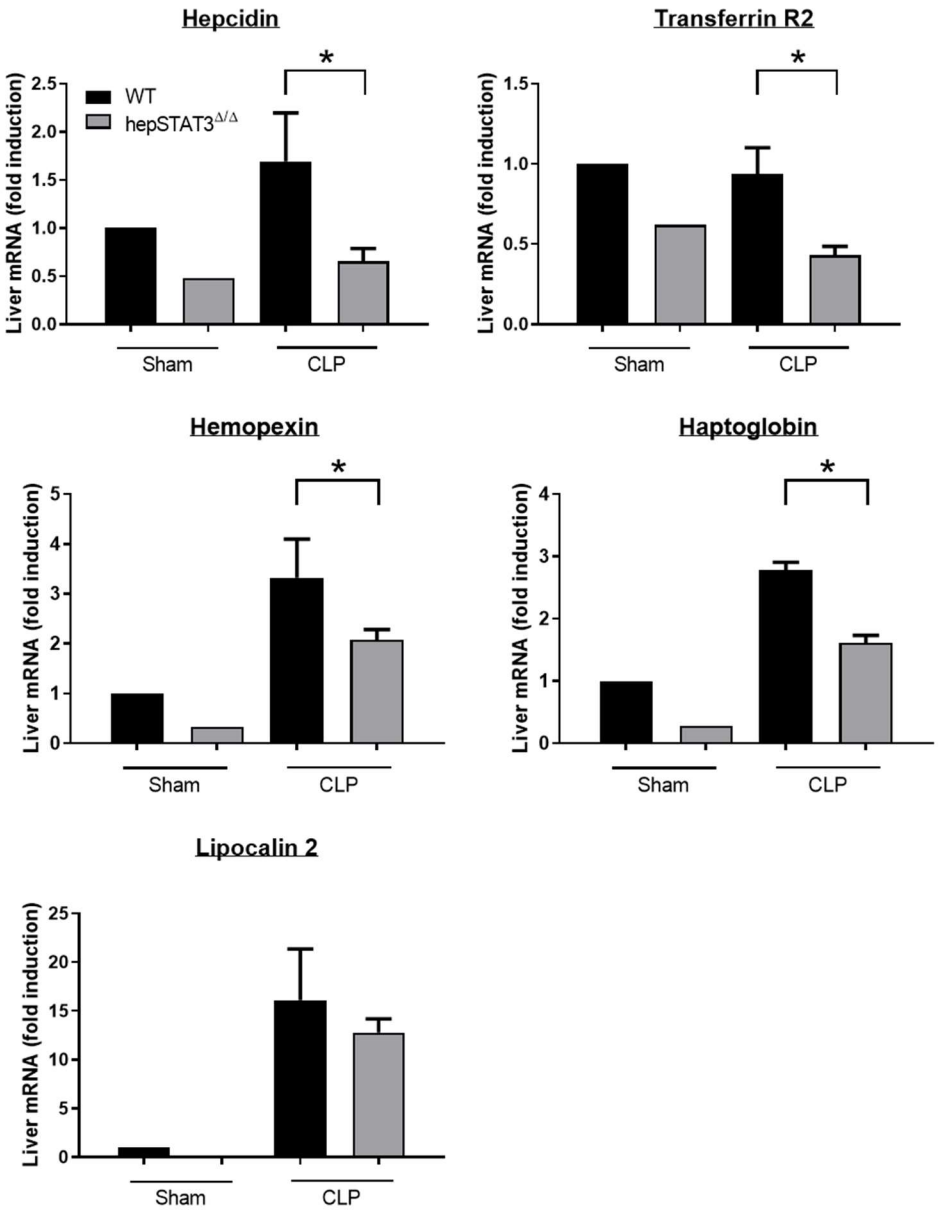


Figure 15. STAT3 is required for the expression of iron-handling factors during polymicrobial sepsis.

WT and hepSTAT3$^{\Delta/\Delta}$ mice underwent cecal ligation and puncture (CLP) or sham procedures. Mice were sacrificed 24 hours after surgery, and liver mRNA induction was determined for representative iron-regulating factors using qRT-PCR. * $p < 0.05$ vs WT; $n = 4$ to 5 per group.

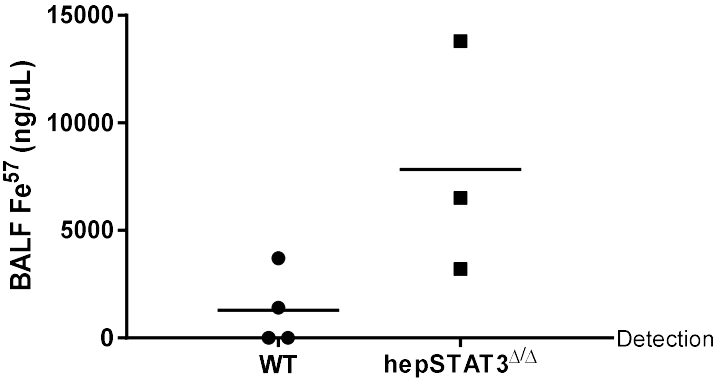


Figure 16. Alveolar iron content may be linked to hepatic STAT3 activity.

WT and hepSTAT3$^{\Delta/\Delta}$ mice were challenged with i.p. LPS (5 mg/kg) or vehicle (saline) for 18 hours followed by i.t. of 10^6 CFU *E.* coli. BALF was collected 24 hours after i.t. *E. coli* for the assessment of free iron concentrations by mass spectrometry. Individual points represent a single sample.

4.2 Sepsis-induced liver activation remodels lung transcription

While we hypothesize that iron-regulating APPs are an intermediate between liver STAT3 activation and pulmonary defense, we only investigated the induction of a select panel of iron-regulating genes. It is possible that other iron-handling APPs or other liver-derived components can directly and indirectly influence the lung environment. For example, macrophage ROS production was decreased in hepSTAT3$^{\Delta/\Delta}$ mice after an endotoxemia challenge (Hilliard, Allen et al. 2015). In order to assess transcriptional changes due to STAT3-dependent liver activation, we performed RNA sequencing on whole lung tissue in WT and hepSTAT3$^{\Delta/\Delta}$ mice challenged with endotoxin and *E. coli* pneumonia (**Figure 17**). Due to substantial mortality in hepSTAT3$^{\Delta/\Delta}$ mice after 5 mg/kg

LPS challenge, the dose used in our prior study (Hilliard, Allen et al. 2015), the LPS dose was lowered to 1 mg/kg for these studies in order to ensure 100% survival.

To compare the influence of endotoxin on lung transcription, WT mice treated with vehicle were compared to WT mice treated with LPS. To compare the influence of pneumonia, mice challenged with LPS alone were compared to mice challenged with LPS followed by pneumonia. And, to compare the effect of liver STAT3 activation, both WT and hepSTAT3$^{\Delta/\Delta}$ mice were included in the LPS alone group and the LPS and pneumonia group. The effect of endotoxemia (WT vehicle *vs.* WT LPS; **Figure 18A**) yielded 7,791 significant gene changes in the lungs. While 1,859 significant (FDR $q <$ 0.05) gene changes were observed between genotypes during endotoxemia (WT *vs*. hepSTAT3$^{\Delta/\Delta}$ LPS; **Figure 18B**), there were no significant gene differences between genotypes after LPS and pneumonia (WT *vs*. hepSTAT3$^{\Delta/\Delta}$ LPS + *E. coli*; **Figure 18C**). Several upregulated (i.e. *GCSF*, *GMCSF*), unchanged (*CXCL10*, *LIFR*), and downregulated (*WNT3a*, *SOX17*) genes in hepSTAT3$^{\Delta/\Delta}$ following endotoxemia were validated by qRT-PCR (**Figure 19**). These data suggests that bacteria are encountering different lung environments influenced by liver STAT3 activation. Further analysis of differences in transcription due to endotoxemia or genotype may reveal potential liver-dependent signals that confer pulmonary protection.

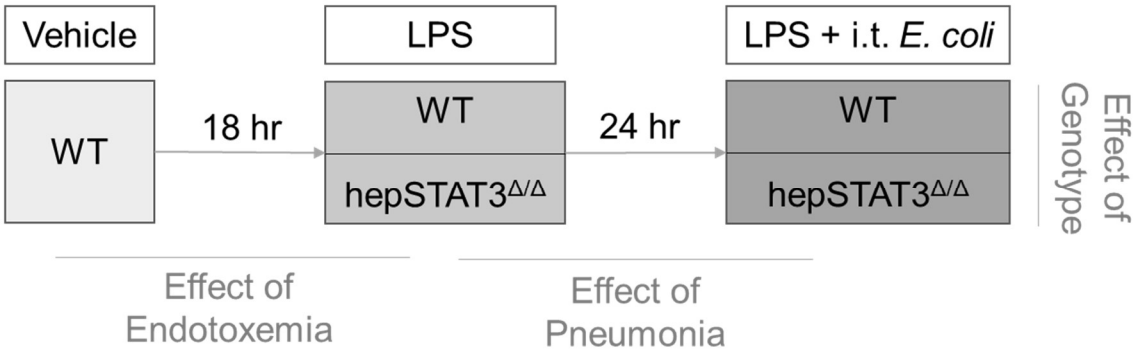
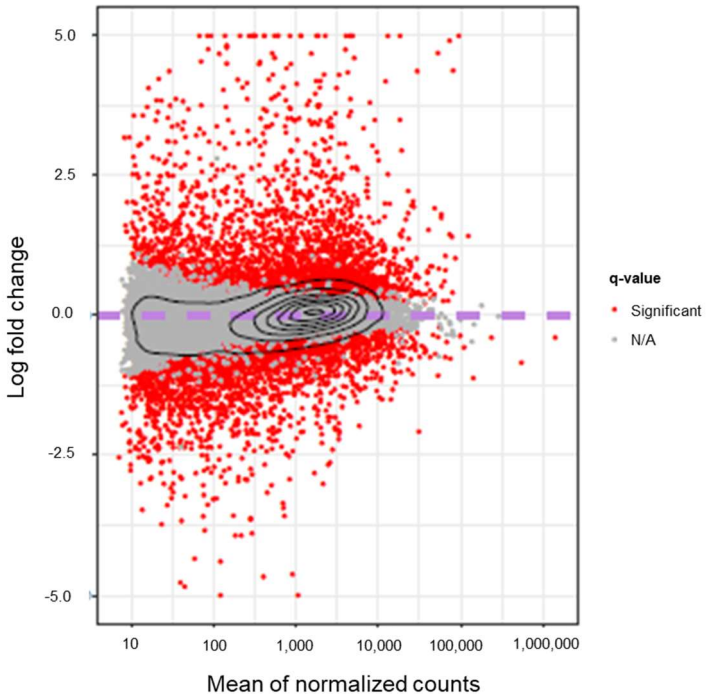


Figure 17. Groups for RNA-sequencing of whole lung tissues.

WT and hepSTAT3$^{\Delta/\Delta}$ mice were challenged with i.p. LPS (1 mg/kg) or vehicle (saline) for 18 hours followed by i.t. of 10^6 CFU *E. coli*. Mice were sacrificed 0 hours after i.p. vehicle, 18 hours post i.p. LPS, or 24 hours post i.t. *E. coli*. RNA from left lung lobes were isolated from the indicated groups for RNA sequencing analysis and comparison.

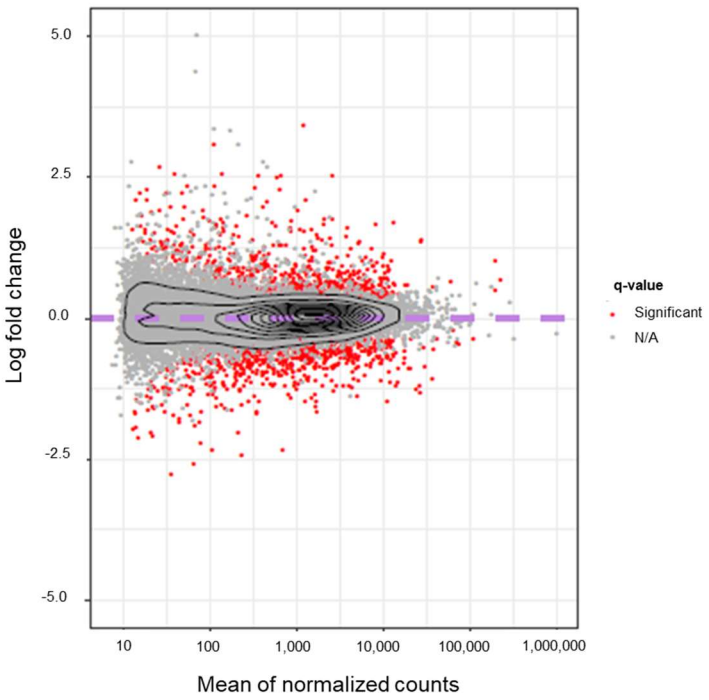
A. **WT (saline) vs. WT (LPS)**

7791 significant gene changes



B. **WT (LPS) vs. hepSTAT3$^{\Delta/\Delta}$ (LPS)**

1859 significant gene changes



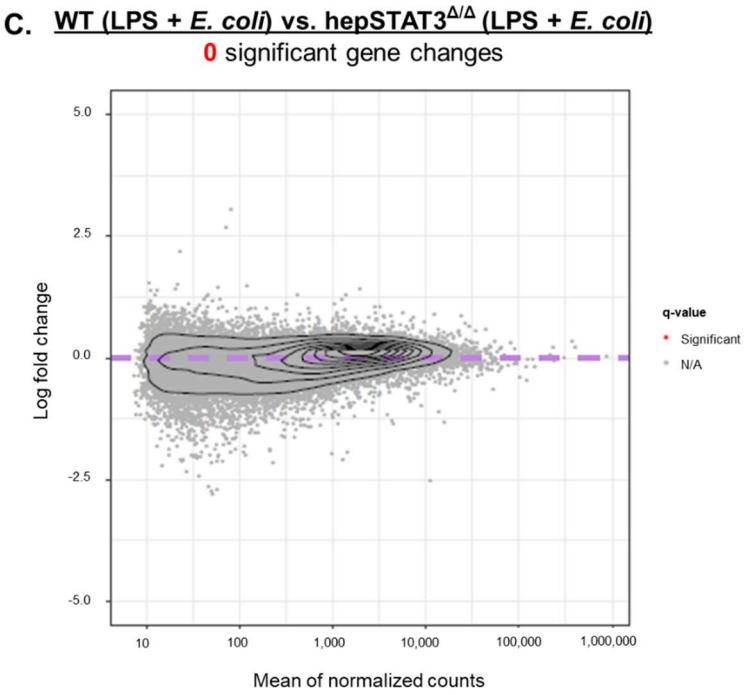


Figure 18. The lung transcriptome is changed due to pre-existing STAT3-dependent liver activation.

WT and hepSTAT3$^{\Delta/\Delta}$ mice were challenged with i.p. LPS or vehicle, and followed by i.t. *E. coli* (10^6) and RNA-sequencing analysis was performed on lung RNA. MA-plots illustrate the 18,000 differential genes according to log2 fold change and the mean of normalized counts between **(A)** WT vehicle *vs.* WT LPS, **(B)** WT LPS *vs.* hepSTAT3$^{\Delta/\Delta}$, **(C)** WT LPS + *E. coli vs.* hepSTAT3$^{\Delta/\Delta}$ LPS + *E. coli* groups. Points colored red represent significantly altered genes (FDR $q < 0.05$) and points colored gray represent non-significantly altered genes.

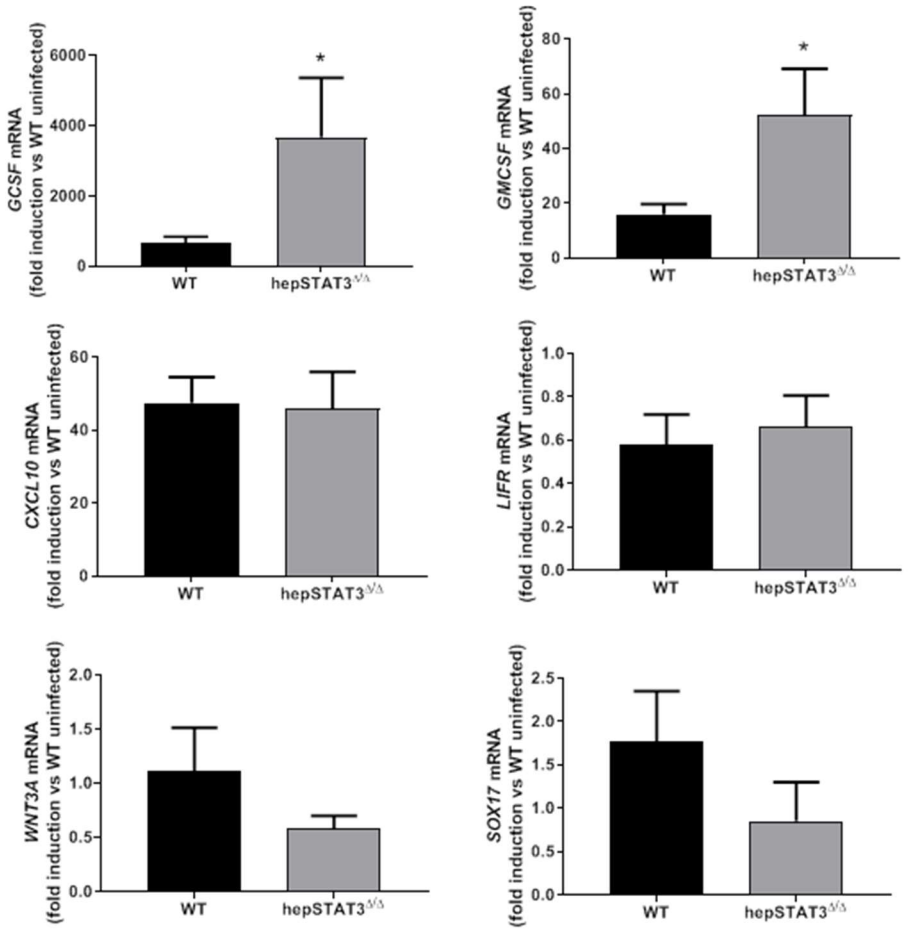


Figure 19. Validation of select RNA sequencing findings by qRT-PCR.

WT and hepSTAT3$^{\Delta/\Delta}$ mice were challenged with i.p. LPS (1 mg/kg) for 18 hours. Liver tissue was harvested and mRNA induction of representative significantly altered genes by RNA-seq (FDR $q < 0.05$) was quantified by qRT-PCR. * $p < 0.05$ vs. WT; $n = 6$ to 7 per group.

4.3 Liver STAT3 alters milieu of cytokines and chemokines in lungs after endotoxemia

To further confirm the results of our RNA sequencing analysis, we measured protein concentrations of several candidate genes changed between WT and hepSTAT3$^{\Delta/\Delta}$ mice challenged with endotoxin. Genes were selected based on association with pulmonary defense during pneumonia (**Table 9**). For example, several selected genes include cytokines and chemokines that are important recruitment of leukocytes, leukocyte activity, and lung remodeling (Wiersinga, Kager et al. 2010, van der Windt, Hoogendijk et al. 2011, Debruin, Hughes et al. 2014, Quinton, Walkey et al. 2018). GM-CSF protein concentration in whole lung homogenates (**Figure 20A**) was reflective of both RNA-sequencing and qRT-PCR validation results. Additionally, some targets that were significantly increased in lung homogenates of hepSTAT3$^{\Delta/\Delta}$ mice were also represented in plasma (**Figure 20B**), suggestive of protein or cellular extravasation into the lungs after endotoxemia. While increases in chemokines in the lungs of mutant mice is suggestive of leukocyte recruitment and inflammation, the lung architecture and cellularity, as analyzed by histology, was unaffected between WT and hepSTAT3$^{\Delta/\Delta}$ mice. How these increases in cytokines during endotoxemia alter lung environment or contributes to susceptibility to pneumonia is still unclear.

Symbol	Name	FDR	Fold Change
Ccl19	C-C motif chemokine ligand 19	0.00977	↑ 1.151
Cxcl1	C-X-C motif chemokine 11	3.78 E-05	↑ 1.416
Csf3	Granulocyte-colony stimulator factor (G-CSF)	2.05 E-10	↑ 3.413
Cxcl2	C-X-C motif chemokine 2	1.9E-11	↑ 1.403
Il1a	Interleukin 1 alpha	0.000137	↑ 1.113
Csf2	Granulocyte-macrophage colony-stimulating factor (GM-CSF)	3.90E-22	↑ 2.533
Il10	Interleukin 10	0.004724	↑ 1.002
Plaur	Urokinase receptor (uPAR)	5.08E-05	↑ 0.554
Ccl2	C-C motif chemokine ligand 2	0.331637	↑ 0.496
Il6	Interleukin 6	0.413948	↑ 1.168
Spp1	Osteopontin	0.010267	↓ -0.534
Cd34	Podocalyxin	0.451132	↓ -0.113

Table 9. Candidate genes changed between WT and hepSTAT3$^{\Delta/\Delta}$ lungs during endotoxemia.

RNA-sequencing analysis was performed on lung RNA of WT and hepSTAT3$^{\Delta/\Delta}$ mice challenged with LPS (1 mg/kg) for 18 hours. Genes were selected based on the ability to measure of equivalent proteins by commercially available multiplex bead array.

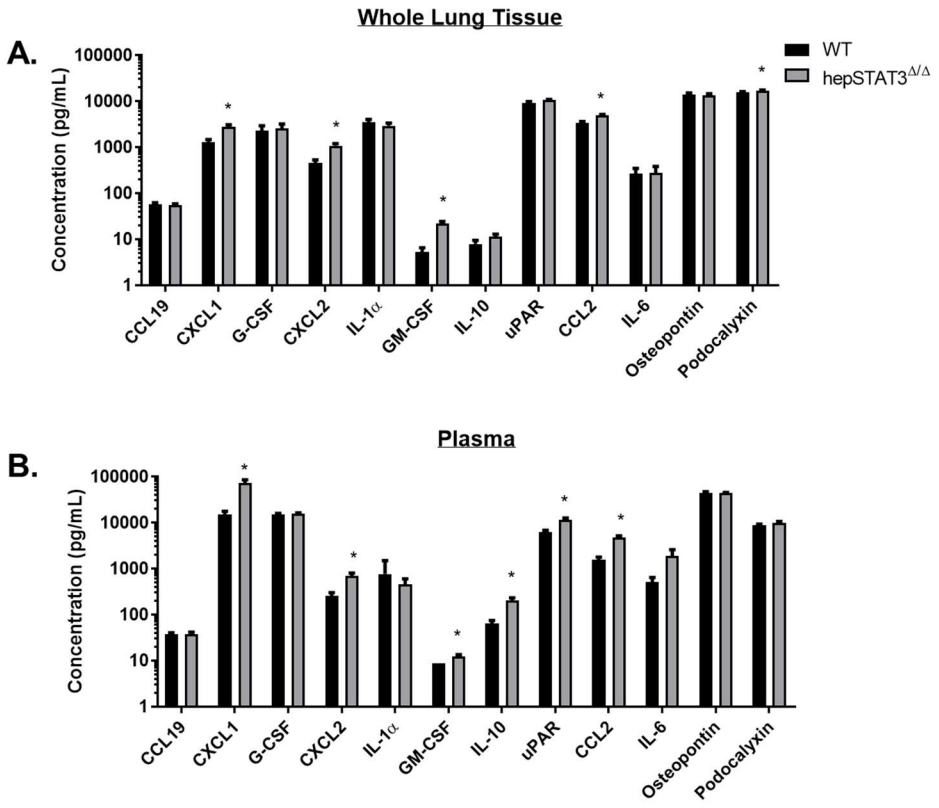


Figure 20. Lung and plasma cytokine changes are associated with lung transcriptional changes during endotoxemia.

Plasma from hepSTAT3$^{\Delta/\Delta}$ or WT mice 18 hours after i.p. LPS (1 mg/kg) were measured for concentrations of the indicated cytokines and chemokines by multiplex bead array * $p < 0.05$ vs. WT; $n = 7$ to 11 per group.

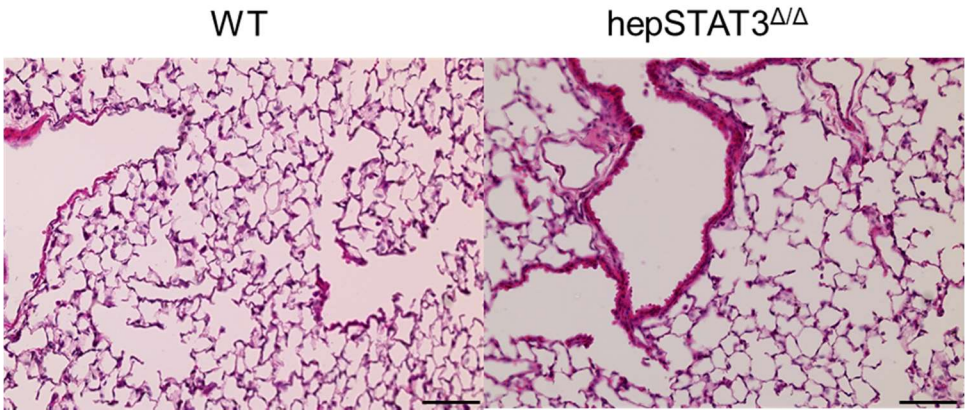


Figure 21. Lung architecture and cellularity is not altered after endotoxemia in hepSTAT3$^{\Delta/\Delta}$ mice.

Lungs were collected 24 hours after i.p. LPS (1 mg/kg) for 18 hours, and processed for histological analysis by hematoxylin and eosin (H&E) staining. Representative images are shown for each genotype. Scale bar: 75 μm.

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CHAPTER FIVE: DISCUSSION

Role of RelA-dependent APR activation in hepatoprotection

The results of this study are the first to indicate a requirement for RelA-driven inducible hepatoprotection during pneumonia and bacteremia. Across several pneumonia and sepsis models, the absence of hepatocyte RelA resulted in TNFα-driven hepatotoxicity associated with exaggerated extrinsic apoptosis. These changes were also associated with substantial remodeling of the hepatic transcriptome, likely reflecting processes that underlie both cause and consequence of liver pathology and laying the foundation for future investigations. Although we were unable to identify a prominent cell type required for infection-induced liver failure in mutant mice, our results support a role for hepatocyte RelA in countering hepatotoxicity driven by NKT cell activity. As the liver represents an important reservoir of NKT cells (Liaskou, Wilson et al. 2012), this finding likely has important implications for our understanding of liver toxicity, including but not limited to that triggered by infection.

The liver represents the first line of defense during sepsis, but it is also a common target of dysregulation during infections (Minemura, Tajiri et al. 2014). The mean incidence of liver dysfunction is nearly 40% in patients with sepsis, and development of liver dysfunction is associated with high mortality rates (Wang, Yin et al. 2014, Yan, Li et al. 2014, Nesseler, Launey et al. 2016). Studies have shown that processes leading to liver dysfunction include aggressive systemic inflammatory reactions to bacterial toxins (Csak, Ganz et al. 2011), immune cells and mediators (Hutchins, Wang et al. 2013,

Wang, Yin et al. 2014), and changes in organ perfusion (Canabal and Kramer 2008). As the leading cause of sepsis (Mayr, Yende et al. 2014), pneumonia represents a critical threat to the liver. Independently of sepsis *per se*, pneumonia is associated with liver failure, and even modest changes in liver function tests may be predictive of pneumonia outcome (Jinks and Kelly 2004, Xu, Ying et al. 2018). However, mechanisms of hepatoprotection in the face of these damaging stimuli are not well understood. We posit that hepatoprotective measures of the APR represent an important means through which the liver limits the systemic consequences of infection, likely compartmentalizing inflammatory responses to locally infected tissues such as the lungs.

The liver has many functions, including its critical impact on both metabolic and immunological homeostasis (Strnad, Tacke et al. 2017). The APR, most quintessentially defined by the release of classic acute phase proteins such as CRP (Gabay and Kushner 1999), is one such function; yet, its net physiological significance remains speculative. Here we propose the induction of tissue resilience pathways as a critical and active liver function during pneumonia and bacteremia, extending the definition of the APR to include cell and organ self-preservation. Our previous investigation demonstrated that ablation of the hepatic APR (via combined deletion of RelA and STAT3) compromises immunity, survival, and liver integrity during pneumonia (Hilliard, Allen et al. 2015). However, the experimental circumstances of that study, which were limited to a single challenge (Gram-negative pneumonia), were such that the distinct roles of STAT3 and RelA could not be distinguished. Here we show that while both transcription factors are ultimately critical for pneumonia outcome (Quinton, Jones et al. 2009, Quinton, Blahna et

al. 2012, Hilliard, Allen et al. 2015), hepatoprotection is exclusively attributable to RelA. Furthermore, the importance of RelA-driven liver protection extends beyond pneumonia, possibly limiting the degree to which systemic infection (with or without pneumonia) progresses to organ injury and sepsis.

Unlike RelA, deletion of STAT3 alone did not affect liver injury or mortality in our *E. coli* pneumonia model, contrasting previous reports of STAT3-dependent hepatoprotection. However, many of these studies are in non-infectious settings such as liver tumorigenesis (Wang, Lafdil et al. 2011), Fas-driven injury (Haga, Terui et al. 2003), and chemical toxicity (Feng, Wang et al. 2014, Hafez, Al-Harbi et al. 2015). On the other hand, a connection between RelA and liver protection has been previously established. RelA knockout mice exhibit TNFα-dependent liver failure and death during gestation (Beg, Sha et al. 1995). Additional studies have revealed RelA as a driver of anti-apoptotic and cell survival genes (D'Souza, Edelstein et al. 2004), as well as its potential role in subverting TNFα-induced immunotoxicity in both embryonic (Rosenfeld, Prichard et al. 2000) and adult livers (Geisler, Algul et al. 2007). Deletion of IKKβ, a different component of the NF-κB pathway, also resulted in liver injury and apoptosis following ConA or LPS stimulation, and ischemia-reperfusion injury (Maeda, Chang et al. 2003, Luedde, Assmus et al. 2005). Remarkably little is currently understood regarding RelA-driven hepatocyte protection during infection. Clinically, RelA expression has been associated with a lower degree of apoptosis and cirrhosis in livers of patients with hepatitis C (Boya, Larrea et al. 2001). Moreover, decreased expression of RelA was noted in pregnant women infected with hepatitis E, and liver RelA mRNA

levels were inversely associated with severe liver damage and mortality (Prusty, Hedau et al. 2007). Our current study extends the hepatoprotective and anti-apoptotic roles of hepatocyte RelA to include local and systemic bacterial infections (i.e. pneumonia and bacteremia), as well as non-infectious stimuli such as αGalCer and heat-killed bacteria.

While the molecular mechanisms of RelA-dependent liver resilience are only beginning to be understood, our results suggest that these events counteract cell death driven by exaggerated apoptosis. Livers of hepRelA$^{\Delta/\Delta}$ mice contained higher levels of activated caspase-3 and -8 in several models tested. Caspase-8 cleavage is consistent with extrinsic apoptosis, typically driven by TNFα and related ligands through FADD (Eguchi, Wree et al. 2014). Not only was TNFα the top candidate upstream regulator of gene expression changes in hepRelA$^{\Delta/\Delta}$ livers after i.v. *S. pneumoniae* **(Table 7)**, but, neutralization of this ligand reversed both liver injury and apoptosis. The necroptosis inhibitor Nec-1s, however, had no effect on liver injury in hepRelA$^{\Delta/\Delta}$ mice. Necroptosis has been associated with liver injury in certain settings (Cho, Challa et al. 2009, Jouan-Lanhouet, Arshad et al. 2012), and both apoptosis and necroptosis have been attributed to embryonic lethality in RelA knockout mice (Xu, Wu et al. 2018). Based on our results, we speculate that if necroptosis is, in fact, related to liver injury during infection, such pathology was overwhelmed by TNFα-driven apoptosis. Thus, we speculate that our observed phenotype may be largely due to secondary necrosis (Silva 2010). Paradoxically, TNFα-induced NF-κB is required for initiating the hepatic APR (Quinton, Jones et al. 2009), which involves NF-κB-dependent protection from TNFα itself. We posit that in the absence of RelA, TNFα signaling favors the cell death pathway, whilst

protective NF-κB-driven anti-apoptotic signals, such as cFLIP (Kondylis, Kumari et al. 2017), remain dormant. Furthermore, we observed a positive association between liver TNFα expression and injury in hepRelA$^{\Delta/\Delta}$ mice, suggesting that TNFα is both up- and downstream of hepatotoxicity without the protection normally afforded by RelA.

Transcriptional profiling revealed that the consequences of liver RelA deletion are vast, including but not limited to changes in gene networks related to cell-survival **(Table 8)**. A limitation to this study, and this outcome in particular, is the difficulty in distinguishing cause from effect. Major transcriptional remodeling was not observed until 24 hours of bacteremia, a time by which liver injury was severe **(Figure 13)**. This phenotype is almost certainly attributable to some of the identified gene changes, but others are likely altered in response to the injury itself. Similarly, other gene changes may or may not reflect differences in liver cellularity, both up- and downstream of injury. Indeed, immune cell trafficking genes are highly represented amongst those differentially expressed based on our bioinformatic analyses **(Table 8)**. Ultimately, these results provide important avenues for future investigation.

While the results unequivocally reveal TNFα as a prominent cause of immunopathology in the absence of hepatocyte RelA, the cellular sources of this cytokine remain unknown under the conditions studied. We focused on populations of leukocytes that have been implicated in liver injury and aging (as suggested by **Figure 5**) (Mehal, Azzaroli et al. 2001, Hilmer, Cogger et al. 2007). NKT and Kupffer cells, in particular, are sufficient to promote liver injury through production of cytokines and other inflammatory mediators (Hu, Venet et al. 2009, Hutchins, Wang et al. 2013). Our results

utilizing αGalCer to specifically activate NKT cells demonstrate that these cells are sufficient to drive TNFα-dependent hepatotoxicity in the absence of hepatocyte RelA, implicating them as a source of this cytokine. However, blocking NKT cell activity was inconsequential with regards to liver injury, suggesting alternative TNFα sources. Notably, inactivation of NKT cells during a live *E. coli* infection markedly impaired defense, which is reflective of other infection models (Brigl, Tatituri et al. 2011) and consistent with the notion that NKT cells are essential in this setting. Similarly, we did not observe changes in liver injury in hepRelA$^{\Delta/\Delta}$ mice after depletion of Kupffer cells (using clodronate liposomes), nor was there an effect of blocking monocyte recruitment (using a CCL2-neutralizing Ab). We also isolated liver NK cells, NKT cells, Kupffer cells, and recruited monocytes following bacteremia in an effort to identify their respective capacities to synthesize TNFα. However, we did not observe significant TNFα mRNA induction in any case, suggesting other potential sources of this cytokine. One possible explanation for our results is that our leukocyte blockade/depletion strategies were insufficient. However, we find this unlikely given the routine nature of these procedures and/or other coinciding outcomes (*e.g.* increased bacterial burdens following anti-CD1d **(Figure 11A)**). Perhaps the more likely alternative is that other cell types such as lymphocytes, endothelial cells, hepatic stellate cells, neutrophils, and/or injured hepatocytes themselves are more prominently connected to hepatotoxicity (Strnad, Tacke et al. 2017). These include extra-hepatic cells that may serve as distant sources of injurious mediators.

Our present study is the first to investigate the direct role of hepatocyte RelA in response to pneumonia and bacteremia. Based on these findings, RelA-dependent gene programs are a critical component of the hepatic APR, fortifying liver homeostasis and survival by shielding against TNFα-dependent immunotoxicity. Future studies designed to reveal signaling pathways mechanistically linked to RelA-dependent protection may help identify or treat individuals with or at risk for pneumonia and sepsis.

Role of STAT3-dependent APR activation on lung defense

The results of this study implicate a mechanism by which STAT3-dependent activation of the hepatic acute phase response promotes iron withdrawal and lung defenses to counter sepsis-induced pneumonia. We observed the induction of several iron-regulating genes in mice following a two-hit model of endotoxemia followed by *E. coli* pneumonia. Similar results were also observed following a cecal ligation and puncture challenge. However, induction was impaired in mice lacking hepatocyte STAT3. We also discovered that sepsis-induced liver activation remodels the lung transcriptome, providing potential signals and mechanisms of liver-dependent protection for future studies.

Previous studies from our group have demonstrated that in the absence of hepatocyte STAT3, mice exhibited heightened susceptibility to pneumonia during endotoxemia, as evidenced by increased bacterial burdens and mortality (Hilliard, Allen et al. 2015). While it is established that sepsis has immunosuppressive effects that promote pneumonia susceptibility (Benjamim, Hogaboam et al. 2003, Jung, Perrone et

al. 2012, Sundar and Sires 2013), very little is known about liver contributions to this process. Several studies support a role for hepatocyte STAT3 in activation of the liver APR during endotoxemia (Alonzi, Maritano et al. 2001, Delano, Thayer et al. 2011), including its importance for survival and cytokine regulation during CLP (Sakamori, Takehara et al. 2007, Sander, Sackett et al. 2010). Furthermore, our own prior study implicates STAT3-dependent acute phase responses in modulating the constituents of airspace lining fluid, providing humoral protection against bacterial outgrowth (Hilliard, Allen et al. 2015). However, the mechanisms directing lung immunity during sepsis is less clear.

While the direct influence of liver-derived iron regulatory factors has never been determined in the context of sepsis-induced pneumonia susceptibility, our studies show that hepatic STAT3 activation is essential for the induction of several iron-regulating proteins during endotoxemia and polymicrobial sepsis. Trends towards increased iron concentrations in the airspace lining fluid implicate a link between liver activation and changes in lung environment. Based on the above results, it is plausible that STAT3-dependent liver activation dispatches iron handling proteins to the circulation and/or the pneumonic airspaces to limit systemic and local iron availability for bacteria. Similar results have shown IL-6 dependent induction of hepcidin from the liver was important for preventing bacteremia following respiratory *K. pneumoniae* challenge (Michels, Zhang et al. 2017).

Our current investigation is limited to only a select group of iron-handling APPs. To complement our initial studies focused on iron-handling factors, we performed RNA-

sequencing to comprehensively evaluate the degree to which lungs are transcriptionally remodeled by endotoxemia in mice lacking hepatocyte STAT3. Surprisingly, the near 2,000 significant gene changes in the lung due to STAT3-activation by LPS, were lost following the onset of pneumonia. We speculate that the combined effects of both endotoxemia and *E. coli*-induced pneumonia, which resulted in over 13,000 significant lung gene changes *vs*. unchallenged mice (data not shown), simply overwhelmed the biological capacity of the system to detect further changes due to liver genotype. Importantly, however, our data reveal that STAT3-dependent liver activity drastically alters the transcriptional landscape of the lungs during endotoxemia, and future investigations to determine how this renders lungs vulnerable to subsequent infections represent a high priority for our research program. Corresponding with lung transcriptional profiles, the systemic LPS challenge significantly altered several cytokines in both whole lung tissues and plasma, including GM-CSF, CXCL1, and CXCL2. However, in studies employing a similar experimental design, albeit with a higher dose of i.p. LPS (5 mg/kg *vs.* 1 mg/kg), we previously showed only modest changes in the BALF and plasma of few common cytokines (Hilliard, Allen et al. 2015). We speculate that this is mostly due to what can be significant effects of lowering LPS concentrations (Monguio-Tortajada, Franquesa et al. 2018), along with the identity of the examined specimen (BALF *vs.* lung tissue homogenate).

These early studies demonstrate liver STAT3-dependent iron sequestration and lung remodeling as potential means of improving protection against respiratory infections during sepsis. Ongoing studies will further determine the impact of liver STAT3 activity

on iron-regulating factors and pulmonary signals, and whether these responses are mechanistically linked to pneumonia susceptibility.

Future Directions

Our studies reveal a direct and critical role of hepatocyte RelA during pneumonia and sepsis in countering TNFα-driven programmed cell death. Future studies are required to determine cells and signals both up- and downstream of liver injury in RelA-deficient mice. This information is important for dissecting the physiological pathways that normally serve to limit liver injury during infection. For instance, additional experiments are needed to follow up on those above to determine the cellular requirements of liver injury in vulnerable (RelA-deficient) mice. Outside of those discussed, others include, but are not limited to NK cells (Tian, Chen et al. 2013), damaged liver sinusoidal endothelial cells (LSECs) (Ni, Li et al. 2017), dendritic cells (Dou, Ono et al. 2018), and neutrophils which accumulate in the liver during injury (Xu, Huang et al. 2014).

Interrogating the mechanisms of interaction between cells and hepatocytes leading to cell death is an important avenue of future studies. The sources of TNFα during these severe infections are still unknown. In the contexts of pneumonia and bacteremia, the sources of TNFα could be intra-hepatic, extra-hepatic, or both. Additionally, TNFα is only one of several known death receptor ligands, including TRAIL and FasL, which contribute to hepatocyte death (Tsutsui, Matsui et al. 1997, Ochi, Ohdan et al. 2004). Significant liver injury and serum transaminase levels following pneumonia and sepsis suggest injured hepatocytes as a potential and prominent

source of danger associated molecular patterns (DAMPs). DAMPs such as high-mobility group protein B1 (HMGB1) and mitrochondrial DNA (mtDNA) are implicated in sepsis severity and acute liver failure (Yang, Ochani et al. 2004, Evankovich, Cho et al. 2010, Zhang, Raoof et al. 2010). DAMPs may promote and sustain liver injury when protective mechanisms go awry.

Signals downstream of hepatocyte RelA required to counter programmed cell death or promoting homeostasis are also unclear. Analysis of differentially expressed genes from our microarray dataset may reveal anti-apoptotic or other protective pathways controlled by RelA. This will be complemented by a more targeted approach to interrogate whether select RelA-dependent factors are sufficient or necessary to prevent hepatotoxicity,

Our other studies implicate a mechanism by which STAT3-dependent liver activity promotes iron withdrawal and lung defenses to counter sepsis-induced pneumonia. However, the studies did not mechanistically link iron regulation, *per se*, to protection, nor did they reveal which of the numerous affected lung pathways are genuinely responsible for immunity and survival. Future studies should confirm changes in iron and iron-handling proteins, as well as the degree to which iron manipulation can recapitulate or prevent the observed phenotype of STAT3-deficient mice during endotoxemia.

The goal of the present studies was to better understand the integrated responses influencing susceptibility during pneumonia and sepsis. Improvement in our understanding of how changes in the hepatic acute phase response influence liver

homeostasis, sepsis susceptibility, and mortality during pneumonia and sepsis may reveal novel signaling pathways that can be targeted for clinical intervention in patients with or at risk for these conditions.

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